

**Key Mechanisms Leading to the Contrasting Nitrogen  
Deficiency Response in a Pair of Potato Cultivars**

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## Key Mechanisms Leading to the Contrasting Nitrogen Deficiency Response in a Pair of Potato Cultivars

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- (I) **Jozefowicz A. M.**, Hartmann A., Matros A., Schum A., Mock H.-P., *Nitrogen deficiency induced alterations in the root proteome of a pair of potato (*Solanum tuberosum* L.) varieties contrasting for their response to low N*. Proteomics 2017, 17(23-24). \*
- (II) **Jozefowicz A. M.**, Matros A., Witzel K., Mock H.-P., *Mini-scale isolation and preparation of plasma membrane proteins from potato roots for LC/MS analysis*, Methods Mol Biol. 2018;1696:195-204. \*
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- (V) **Jozefowicz A. M.**, Kaspar-Schoenefeld S., Schum A., Matros A., Mock H.-P., *Nitrogen withdrawal leads to distinct changes in the phosphoproteome of two potato cultivars*, manuscript in preparation

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Additional papers related to the subject, which are not the part of this thesis.

Meise P., **Jozefowicz A. M.**, Uptmoor R., Mock H.-P., Ordon F., Schum A., *Comparative shoot proteome analysis of two potato (*Solanum tuberosum* L.) genotypes contrasting in nitrogen deficiency responses in vitro.* J Proteomics 2017, 166:68-82.

Bündig C., **Jozefowicz A. M.**, Mock H.-P., Winkelmann T., *Proteomic analysis of two divergently responding potato genotypes (*Solanum tuberosum* L.) following osmotic stress treatment in vitro.* J Proteomics 2016, 143; 227:241.

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## 1. Abbreviations

|         |   |
|---------|---|
| 2D-PAGE | two-dimensional gel electrophoresis           |
| AMT     | ammonium transporters                         |
| C       | carbon  |
| cv.     | cultivar                                      |
| DAPs    | differentially abundant proteins              |
| GABA    | $\gamma$ -aminobutyric acid                   |
| GAPDH   | glyceraldehyde 3-phosphate dehydrogenase      |
| GDH     | glutamine dehydrogenase                       |
| GOGAT   | glutamate synthase                            |
| GS      | glutamine synthetase                          |
| HAT     | high affinity transporter                     |
| HPLC    | high-performance liquid chromatography        |
| LAT     | low affinity transporter                      |
| LC      | liquid chromatography                         |
| LHT1    | lysine histidine transporter                  |
| MFVS    | membership function value of stress tolerance |
| MRM     | multiple reaction monitoring                  |
| MS      | mass spectrometry                             |
| MS/MS   | tandem mass spectrometry                      |
| N       | nitrogen                                      |
| NiR     | nitrite reductase                             |
| NR      | nitrate reductase                             |
| NRT     | nitrate transporters                          |
| PEPC    | phosphoenolpyruvate carboxylase               |
| PIP     | aquaporins                                    |
| PM      | plasma membrane                               |
| PTMs    | post-translational modifications              |
| Q       | quadrupole                                    |
| TCA     | tricarboxylic acid cycle                      |
| TOF     | time-of-flight                                |

## 2. Summary

Nitrogen deficiency is known as one of the major limitations in crop productivity and the yield losses caused by inadequate N supply are far greater than those caused by a deficiency in other macronutrients. To provide food for a constantly growing population, there is a demand for an increase in crop plant productivity, but excessive fertilization is not only very expensive but also leads to strong environmental pollution. In the case of potato, due to its low capability of N retraction from the soil, there is a higher demand for fertilization and therefore a higher negative impact on the environment. The aim of the presented thesis was to elucidate key mechanisms, metabolic pathways, and candidate proteins determining the contrasting responses of the two potato cultivars ‘Lambada’ (sensitive) and ‘Topas’(tolerant) to nitrogen deficiency.

The nitrogen deficiency experiments were conducted in an *in vitro* based system, to exclude the impact of external environmental factors. The potato proteome was analyzed using mass spectrometry-based approaches, in respect to the crude root proteome, with an additional focus on the proteins of the plasma membrane and on the changes in the phosphorylation pattern. The commonly used methodology was adapted to the small amounts of root tissue. The most promising candidate proteins were further validated by using enzymatic assays, Western blotting and by using *Arabidopsis thaliana* mutant plants.

In response to nitrogen deficiency, the sensitive cv. ‘Lambada’ showed a dramatic reduction in the biomass production. The tolerant cultivar ‘Topas’ showed higher accumulation of proteins participating in nitrogen assimilation, carbon metabolism as well as in protein synthesis and degradation, together with a stable growth and higher nitrogen content under N limiting conditions. The study of the plasma membrane composition revealed a higher abundance of the nitrate transporter NRT2.1 as well as two isoforms of aquaporins. The relevance of aquaporins in the response to N deficiency was confirmed by experiments with *Arabidopsis* mutants, which showed significantly reduced nitrogen content in the leaves. The main conclusion of the study was, that under non-limiting conditions, the sensitive cultivar used all the applied nitrogen to produce biomass, whereas the tolerant cultivar produced N-containing compounds and more proteins. When nitrogen is limited, the sensitive cultivar’s ability to grow rapidly decreases. In contrast, the tolerant cultivar uses the NRT2.1 as well as aquaporins for more efficient N uptake but it also consumes the accumulated resources to adapt to the lower nitrogen conditions.



### 3. Zusammenfassung

Stickstoffmangel gilt als eine der größten Beschränkungen für die Produktivität von Kulturpflanzen. Die Ertragsverluste, die durch eine unzureichende N-Versorgung verursacht werden, sind weitaus größer als durch den Mangel an anderen Nährstoffen. Um die ständig wachsende Bevölkerung mit Nahrungsmitteln zu versorgen, besteht der Bedarf nach einer Steigerung der Produktivität der Kulturpflanzen. Übermäßige Düngung ist nicht nur sehr teuer, sondern führt auch zu einer starken Umweltverschmutzung. Im Fall von Kartoffeln besteht aufgrund der geringen N-Aufnahmefähigkeit ein höherer Bedarf an Düngung und damit eine höhere negative Auswirkung auf die Umwelt.

Ziel der vorliegenden Arbeit war es, Unterschiede zwischen den Kartoffelsorten ‚Topas‘ (tolerant) und ‚Lambada‘ (empfindlich) zu erkennen. Damit sollte gezeigt werden, welche Proteine und Stoffwechselwege zur Stickstoffmangeltoleranz beitragen. Die Stickstoffmangelerperimente wurden in *In-vitro*-Systemen durchgeführt, um den Einfluss von Umweltfaktoren auszuschließen. Das Wurzelproteom von Kartoffeln wurde mit massenspektrometrischen Ansätzen analysiert, mit dem Fokus auf den Proteinen der Plasmamembran und den Veränderungen im Phosphorylierungsmuster. Die allgemein verwendete Methodik wurde an die geringen Mengen an Wurzelgewebe angepasst. Die vielversprechendsten Kandidatenproteine wurden mit enzymatischen Assays, Western Blotting und *Arabidopsis thaliana* Mutanten weiter validiert.

In Reaktion auf Stickstoffmangel zeigte das empfindliche Kultivar ‚Lambada‘ eine Reduzierung der Biomasseproduktion. Zusammen mit dem stabilen Wachstum und dem höheren Stickstoffgehalt unter N-limitierenden Bedingungen zeigte die tolerante Sorte ‚Topas‘ eine höhere Abundanz von Proteinen, die an der Stickstoffassimilation, dem Kohlenstoffmetabolismus sowie der Proteinsynthese und dem Proteinabbau beteiligt sind. Die Untersuchung der Plasmamembran ergab eine höhere Abundanz des Nitrattransporters NRT2.1 sowie von zwei Isoformen von Aquaporinen. Die Relevanz von Aquaporinen in der Stickstoffmangelreaktion wurde durch Versuche mit *Arabidopsis*-Mutanten bestätigt. Die wichtigste Schlussfolgerung der Studie war, dass unter nicht limitierenden Bedingungen die empfindliche Sorte ‚Lambada‘ den gesamten Stickstoff zur Erzeugung von Biomasse nutzt, während die tolerante Sorte ‚Topas‘ N-haltige Moleküle und Proteine produziert. Wenn das Stickstoffniveau begrenzt ist, verringert die empfindliche Sorte schnell ihr Wachstum. Im Gegensatz dazu verwendet die tolerante Sorte den Nitrattransporter NRT2.1 sowie Aquaporine für eine effizientere N-Aufnahme, verbraucht jedoch auch die angesammelten Ressourcen, um sich an die niedrigeren Stickstoffbedingungen anzupassen.



## 4. Introduction

### 4.1 Nitrogen is essential for plant growth and development

Five main elements, indispensable for sustaining life, are nitrogen (N), carbon (C), phosphorus, oxygen, and sulfur. Nitrogen is a component of proteins, nucleic acids, primary and secondary metabolites as well as many of cofactors and is therefore required in high concentrations to ensure optimal development and yield of plants [1]. Nitrogen availability is one of the most critical and limiting factors in crop plant production. Although it is the most abundant gas in the atmosphere, hydrosphere, and biosphere, nitrogen is the least available for the uptake by living organisms. It is because it is almost entirely in the form of molecular N<sub>2</sub>, which is not absorbed by most of the organisms. A significant amount of energy in the form of high temperature or processing by few specialized N-fixing microbes is required to break a triple bond holding the two atoms of N together [2].

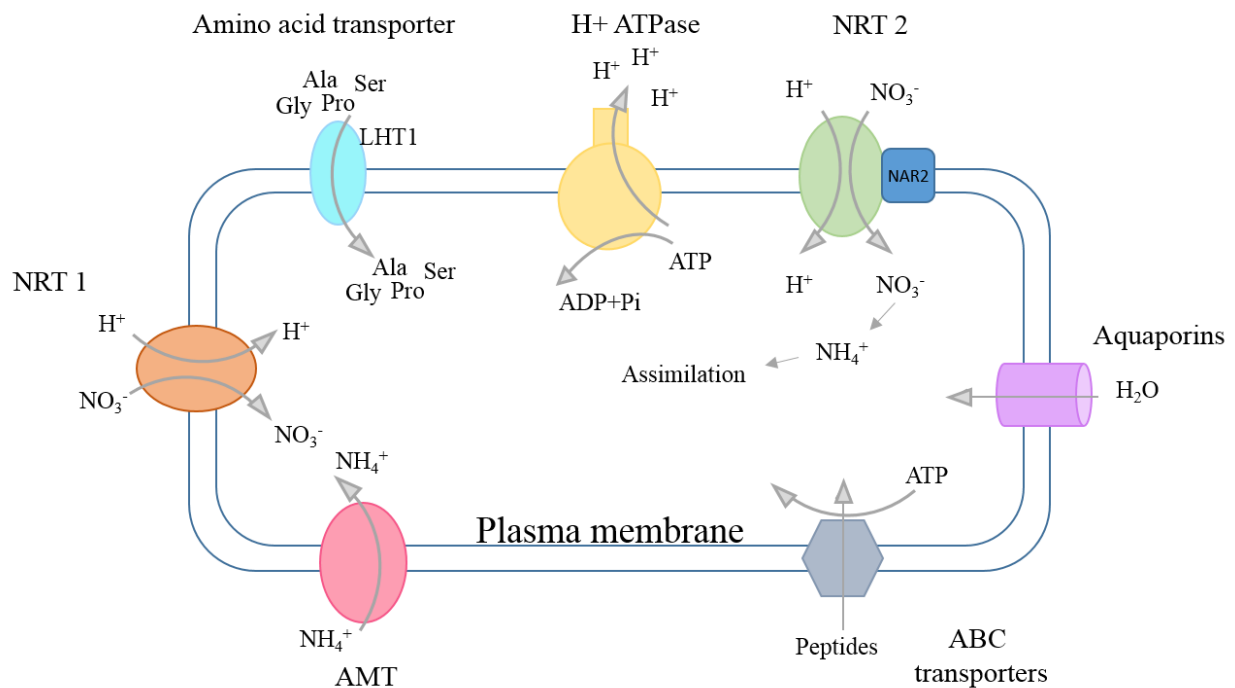
The limitation in N supply inhibits plant growth. Depending on the duration of the deficiency, plants are stunted and pale, or if the deficiency develops during the growth phase, N is translocated from older to the youngest leaves. The old ones become pale, or if the deficiency is severe, brown and abscise. Most of the N in leaves is stored in the chloroplasts in the form of proteins. When N is limited, the protein levels decrease and this results in the degradation of chloroplast structure, causing, e.g. a reduction in the number of grana [3]. Plants affected by N deficiency develop larger root systems with more extensive branching but the lateral root formation is suppressed [4]. As a result of N deficiency, carbohydrates, particularly starch, but also secondary metabolites including phenylpropanoids (flavonoids, anthocyanins) are accumulated in the leaves [5, 6]. As an example, low N regime caused redirection of biomass allocation to roots versus shoots and an accumulation of soluble carbohydrate in tobacco (*Nicotiana tabacum*) [7], whereas in barley (*Hordeum vulgare*) and pea (*Pisum sativum*), it caused a limitation in the growth, respiration, and utilization of carbohydrates [8].

### 4.2 Root plasma membrane proteins participating in N uptake

The major forms of N present in the soil are nitrate and ammonium and most plants have the capacity to use both as an N source. Both nitrate and ammonium are incorporated into the same metabolic pathways and share common features. To maintain the N homeostasis, plants use a set of transporters and channels located in the plasma membrane (PM, Figure 1). The transport of ammonium is actively maintained by the proteins of the AMT family. In *Arabidopsis*, there are six genes encoding AMTs, all of which are high affinity transporters [9]. The *AtAMT1.1*, *AtAMT1.3* and *AtAMT1.5* are localized in the root tip and in the epidermal cells, thereby they are responsible for the uptake directly from the soil and the *AtAMT1.2* is localized in the endodermis and cortex, thereby it is responsible for the uptake of apoplastic ammonium to the cell [9, 10].

For the nitrate, plants have two types of the transport system: high- and low affinity (HATS and LATS, respectively). The LATs operate when the concentration of nitrate is above 0.5 mM and HATs between 0.5 and 0.2 mM [11]. Four families of proteins are responsible for the uptake of nitrate, namely NPF (nitrate transporter 1/ peptide transporter family), NRT2, *CLC* (chloride channels), and *SLAC1/SLAH* (slow anion channel-associated). Except for NRT1.1, which shows dual-affinity transport and sensing functions [12], all of the other NPF members belong to the LAT group. NRT2 mediate a high affinity transport, with no other substrate than nitrate known. There are seven NRT2 genes in *Arabidopsis*, and four of them (*AtNRT2.1*, *AtNRT2.2*, *AtNRT2.4* and *AtNRT2.5*) are expressed in roots [13]. They are responsible for 95% of the high affinity nitrate uptake, and NRT2.1 is the major contributor to the process [14]. NRT2.1 is localized in the PM of epidermal and cortical cells of mature roots, where the majority of the nutrient transport occurs [15]. The properties of the *AtNRT2.2* are similar to NRT2.1, but it is only weakly expressed, whereas NRT2.4 is rapidly expressed under nitrogen starvation and contributes to the uptake when the concentration of nitrate is very low [16]. To be expressed and addressed to the PM, the NRT2 transporters are dependent on the second protein NAR2.1 [17].

Other proteins involved in the transport of nutrients, such as ammonium and nitrate are  $H^+$  ATPases. They are one of the most abundant proteins of the PM, constituting several percent of its total protein content. The PM  $H^+$  ATPases maintain the membrane potential and generate a proton gradient, which is required for the transport of many ions [18]. The transport of nitrate occurs via symport with the  $H^+$  ions in the stoichiometry 1:2 [19].



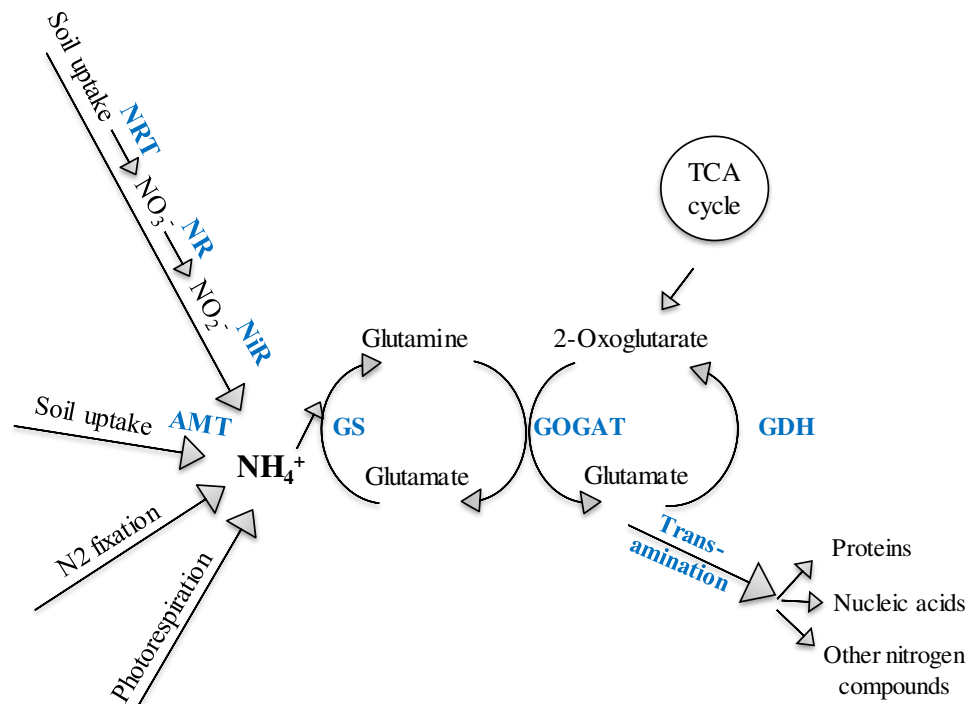
**Figure 1 PM Transporters and channels taking part in the N uptake.**

Other very abundant proteins in the PM are water channels (aquaporins, PIP), which constitute more than 20% of the PM proteins. Water uptake can increase within minutes after N-supplementation, which suggests that the activity of PIPs is regulated by nutrient availability around the roots [20]. Recently it was shown that the *Atnrt2.1* mutant has decreased root hydraulic conductivity and PIP expression, which suggests that nitrate supply is correlated with aquaporin activity [21]. Moreover, some members of the aquaporin subfamilies transport ammonia and urea [22, 23].

Additionally to the inorganic ions also amino acids and peptides are translocated through the PM. One of the best characterized is a root lysine histidine transporter 1 (LHT1), which is active in the transport of several amino acids from the soil [24]. The peptides can be transported to the cell through the members of the ABC transporter family and peptide transporter proteins [25].

### **4.3 Nitrogen assimilation in roots**

Nitrate can be either assimilated directly in the roots or translocated to the green parts of the plant, where it is reduced or can serve as osmotic regulator in the vacuole [26]. The first step of nitrate assimilation involves reduction by nitrate reductase and nitrite reductase to ammonium, which is then incorporated into an organic form [27, 28]. Ammonium is toxic to plants already at low concentrations. Biosynthesis of amino acids and amides is the way to detoxify ammonium taken up by the roots or derived from nitrate reduction. The key enzymes of ammonium assimilation are glutamine synthetase (GS) and glutamate synthase (GOGAT) as shown in Figure 2. Two major isoforms of GS have been identified in plants, the cytosolic GS1 and the chloroplastic/mitochondrial GS2 [29]. The main task of GS1 is the assimilation of primary  $\text{NH}_4^+$  in roots and reassimilation of ammonium generated from protein turnover in the leaves. GS2 functions mainly in the reassimilation of  $\text{NH}_4^+$  formed in the photorespiratory pathway in the chloroplasts and assimilation of ammonium deriving from  $\text{NO}_3^-$  reduction in the plastids [30]. Glutamate and glutamine formed in the GS/GOGAT cycle can be further translocated to sink tissues [31, 32]. Integration of ammonium into amino acids requires a large quantity of carbon skeletons provided by tricarboxylic acid cycle (TCA), which have to be replenished by increased activity of phosphoenolpyruvate carboxylase (PEPC). To minimize the carbon costs for root to shoot transport, the bulk of N assimilated in the roots is transported in the form of N-rich compounds such as glutamine, asparagine, arginine or allantoin. The organically bound N can be subsequently used for synthesis of other amides, amino acids, amines, peptides, and proteins [1].



**Figure 2** Nitrogen assimilation pathways in plants, as described in . The steps include the following proteins: nitrate transporters (NRT), nitrate reductase (NR), nitrite reductase (NiR), ammonium transporters (AMT), glutamine synthetase (GS), glutamate synthase (GOGAT), glutamine dehydrogenase (GDH).

#### 4.4 Regulation of N transporters and assimilatory proteins by phosphorylation

Plants as sessile organisms need to respond quickly to the changes in the internal N status, but also to the constantly changing concentration of nitrate in the soil. N uptake must be tightly regulated in response to the demands of the plant and to its availability. Nitrate acts as a signal that coordinates its uptake with plant growth and development. Protein phosphorylation, one of the most important post-translational modifications (PTM), which allows for rapid adaptation to the new environmental conditions through changes in protein activity, localization, protein-protein interactions and protein degradation [33], was proposed to participate in nitrate signaling.

As presented in Figure 3, in *Arabidopsis*, the NRT1.1 works as a dual-affinity transporter and sensor, which elicits changes in the expression of genes related to nitrate assimilation [12]. Its affinity depends on the phosphorylation status of Thr101 [34]. Under low nitrate conditions, it is phosphorylated by CBL-Interacting Protein Kinase 23 to function as a high-affinity transporter and it is dephosphorylated when the level of nitrate is high [12]. Changes in the phosphorylation status of NRT1.1 subsequently trigger a signal transduction cascade. By the action of phospholipase C, calcium influx is activated, acting as a secondary messenger. This cascade regulates the expression of transcription factors such as TGA1 and genes involved in nitrate transport (NRT2.1, NRT2.2, NRT3.1) and assimilation (NIA1, NiR), as reviewed by Undurraga et al. [35].

Phosphorylation regulates number of other proteins taking part in N uptake and assimilation. Wirth et al. suggested that NRT2.1 is regulated on the post-translational level, as its protein abundance

did not correlate with its activity [15]. Ammonium transport is regulated by the phosphorylation as well. Through the phosphorylation of Thr460, AMT1.1 is deactivated [36], however unlike NRT1.1, it is not involved in ammonium sensing [37]. A similar regulation was proposed for AMT1.3 and this protein was also shown to elicit signaling responses [37]. Moreover, nitrate reductase (NR) is inactivated by phosphorylation at serine (phosphorylation site depends on the species) in the diurnal way [38]. In darkness, the phosphorylated region of NR is a binding place for an inhibitory 14-3-3 protein. Light induces dephosphorylation and thereby activation of NR [39].

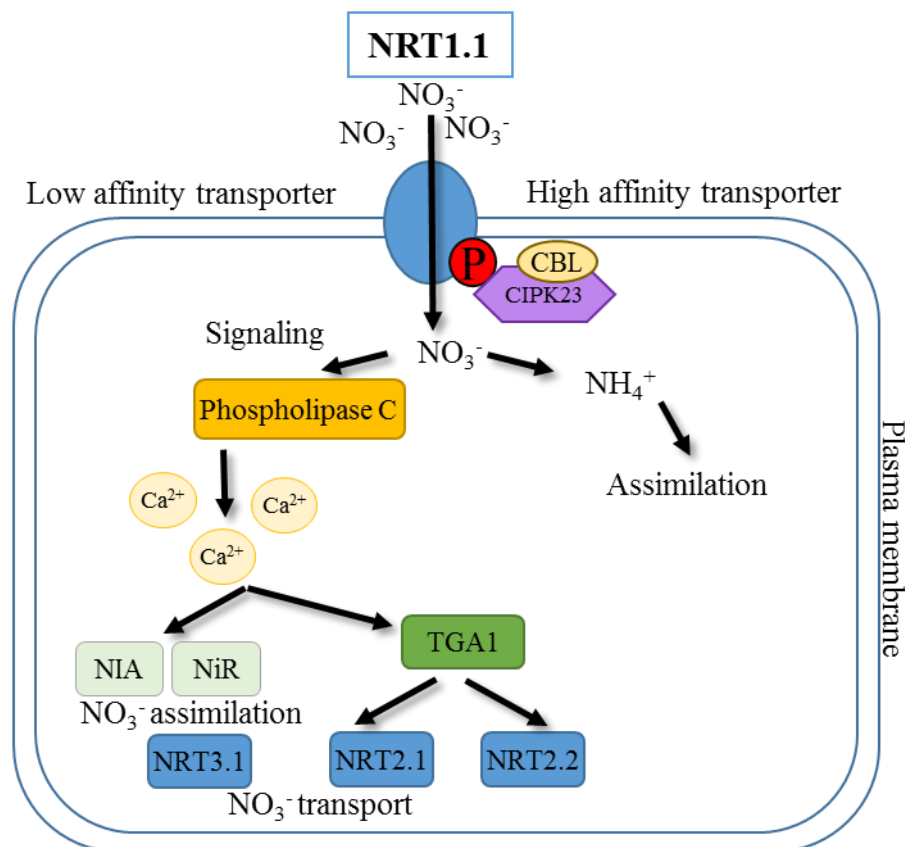


Figure 3 Nitrate signaling through phosphorylation of NRT1.1, adopted from [35].

#### 4.5 Nitrogen management in potato

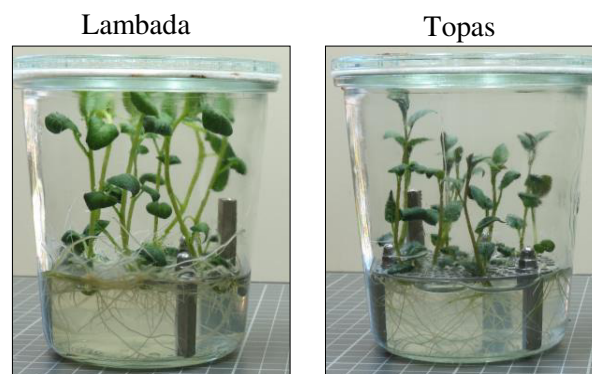
Potato (*Solanum tuberosum* L.) is one of the major food crops in both developed and developing countries, following only rice, maize, and wheat. It contains energy-rich carbohydrates, high-value proteins and is an important source of vitamins, minerals as well as antioxidants. Additionally, it has a higher dry mass and protein content per unit growing area than cereals [40, 41]. The world potato production was estimated on 376 million tons in 2016 and is constantly increasing, especially in Asia where the consumption of potato is nearly 50% of the global production [42].

Potato is especially sensitive to changes in the N level. In the early season, during the development of canopy, it requires a modest amount of N. Excessive N level at this point can delay linear tuber

growth and reduce the yield. High and stable supply is needed when the tubers are formed, at the beginning of the bulking phase. Inadequate amount of N in this phase results in reduced canopy growth, pre-mature senescence and reduced yield. However, when the content of N is too high, vegetative growth is preferred instead of tuber bulking. Imbalance in the N level causes the development of internal and external malformations in the tuber, such as brown centers or a knobby form. In the maturity phase, the level of N in the plant should diminish to maximize transport of carbohydrates to the tuber and enhance skin formation [43, 44].

Nitrogen recovery from the soil is especially low for potato and estimated at 45% of applied N [45]. This is explained by the shallow root system of this crop [46]. Additionally, potato is often cultivated on sandy soils, which are more susceptible to nitrate leaching [47]. The modern, high-yielding potato varieties require as much as 2.5-5.9 kg/ha N per ton of tuber yield [48]. This excessive N fertilization seriously contributes to the environmental pollution and loss of biodiversity [49]. More than 60% of the applied N is lost through leaching, surface run-off, denitrification, volatilization, and microbial consumption [50]. Reactive N compounds are one of the main causes of the global climate change as they are sources of nitrous oxide, which is released from agricultural soils and responsible for about 7.5% of the calculated greenhouse effect caused by human activity [51]. Moreover, the production of N fertilizers by the Haber–Bosh process is energy intensive and by this, very expensive [52]. The high nutrient demand of potato leads to higher nutrient inputs, nutrient losses, and pollution than in other arable crops. Therefore, there is a need for selection of potato varieties with improved nitrogen use efficiency.

Nowadays, the cultivated potato genotypes represent only a small fraction of the genetic diversity, present in South American cultivars and wild species. When comparing 205 cultivars with 1220 genotypes of wild potato relatives, large variability was found in all examined traits, including dry matter, starch, resistant starch, starch granule diameter, and protein content [53]. This, together with the already known potato genome gives an opportunity to improve potato yield under more unfavorable conditions including insufficient nitrogen supply.



**Figure 4** Cultivars Lambada (left) and Topas (right) grown under N sufficient conditions in the in vitro cultivation system.



Detailed physiological characterization of various potato genotypes cultivated *in vitro* was performed under nitrogen deficient conditions [54-56]. In a recent study, the Membership Function Value of Stress tolerance (MFVS) [57] for selected traits was calculated from the data already published as described in chapter I. This allowed differentiation between cultivars more tolerant and more sensitive to nutrient deficiency and selection of the cultivars ‘Lambada’ and ‘Topas’ (Figure 4) for further investigations. Cultivar ‘Lambada’ is characterized by a high biomass under N sufficient conditions and a strong decrease in growth when N is limited. In contrast, cv. Topas produces lower biomass under a high N regime and has a tolerant response to N deficiency in the form of stable biomass. Cultivars differed also in the efficiency of N withdrawal from the N sufficient medium. In contrast to Lambada, which withdrew about 95% of the N after three weeks of culture, cv. ‘Topas’ took only 71 %. Further traits such as stimulation of root growth under N deficiency, crude protein and chlorophyll content were also affected differently in the two cultivars [55].

### **4.6 Identification of proteins with mass spectrometry**

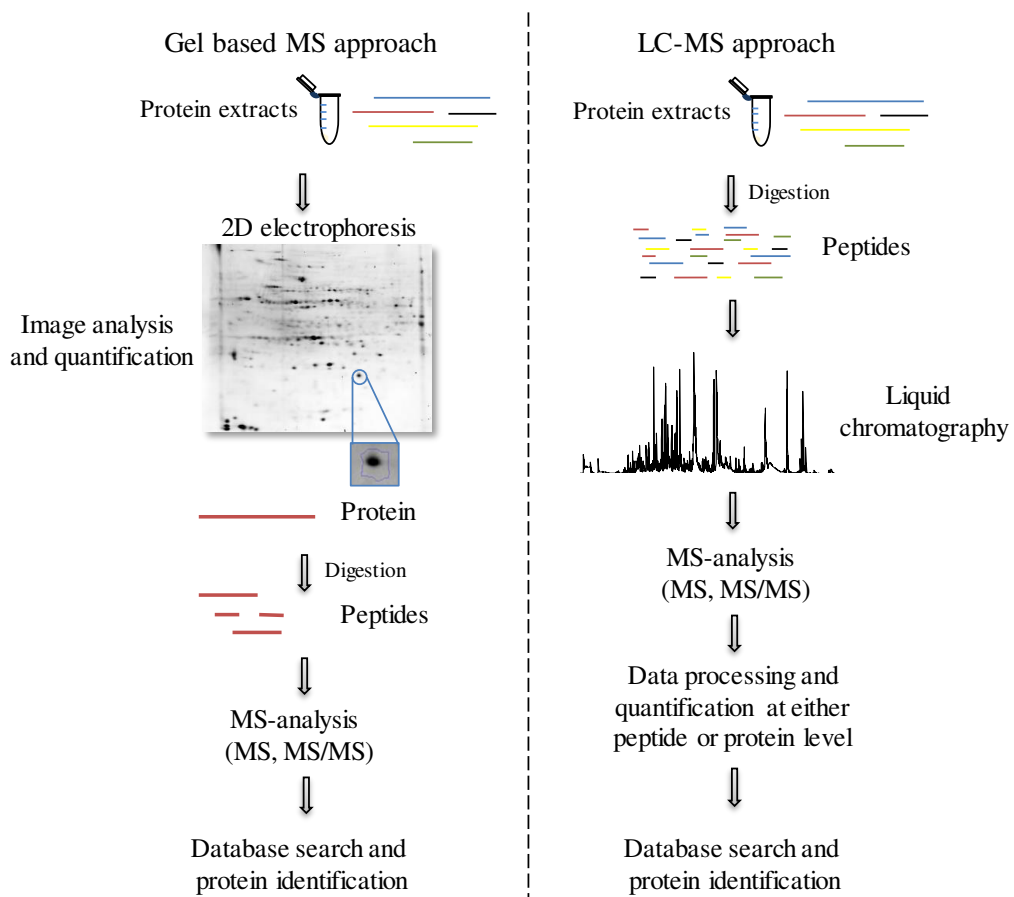
The study of all proteins encoded by the genome is called proteomics. The proteome is dynamic and changes in the response to environmental stimuli, but also due to processes like splicing, post-translational modification and protein degradation, which makes proteomics far more technically challenging than genomics. Identification of proteins and their modifications can supply important information on biological activities. Over the past decades mass spectrometry (MS) has become the method of choice for protein identification from complex samples. There are several MS technologies used, often combined with other analytical methods. In most cases, protein identifications are based on the analysis of peptides created by proteolytic digestion. The most commonly used protease is trypsin, which hydrolyzes protein molecules on the C-terminal side of lysine and arginine, unless the subsequent amino acid in the sequence is a proline [58]. The complexity of proteomic mixtures is the reason that a separation method prior to analysis by mass spectrometry is required. There are two most commonly used methods, namely two-dimensional gel electrophoresis (2D-PAGE) and liquid chromatography (LC). The first approach includes the separation of proteins by their isoelectric point in one dimension and by molecular weight in the second [59]. This method however has some disadvantages including large amount of sample needed, limited dynamic range, and difficulties to resolve low abundant and hydrophobic proteins as well as those with extreme pI and molecular weight [60]. Gel-free proteomic approaches were developed to overcome some of the limitations. The basis of the bottom-up proteomic is the digestion of a protein mixture into peptides. The peptides are further separated by reversed-phase high-performance liquid chromatography (HPLC) or by strong cation exchange chromatography followed by a reversed-phase HPLC and eluted into a tandem mass spectrometer. Mass spectrometers used in proteomic approaches use either matrix-assisted laser desorption/ionization

(MALDI) [61] or electrospray ionization techniques [62]. Four types of mass analyzers, namely quadrupole (Q), ion trap, time-of-flight (TOF) and Fourier-transform ion cyclotron resonance mass analyzer, varying in the physical principles and analytical performance, are commonly used in MS. The capabilities of different mass analyzers are often combined, for instance, Q-TOF or TOF-TOF [63]. The standard workflows of both gel-free and 2D-PAGE based approaches are presented in Figure 5. One of the major differences between both techniques is the way of quantification. The abundance of proteins during 2-DE is based on the pixel intensities of the stained gel spots and the quantification of the bottom-up approach is performed on the peptide level based on mass spectra data. Proteomic approaches have two main goals. They are designed to map all detectable proteins of the organism of interest or to identify proteins showing changes in their abundance pattern because of various biotic and abiotic stresses as well as developmental changes, tissue types or organelles. The analysis of subproteomes often requires additional enrichment method, as the specific proteins are underrepresented in the common LC-MS approaches. For instance, the aqueous two-phase partitioning method was developed and improved over decades for efficient isolation of plasma membrane (PM) vesicles [64]. An important advantage of the LC-MS approaches is the possibility to identify posttranslational modifications (PTMs) of peptides, e.g. acetylation, phosphorylation, ubiquitination, and glycosylation. Protein phosphorylation is one of the most important and diverse PTMs. It can influence proteins characteristics, including enzymatic activity, subcellular localization, protein-protein interactions and half-life [33]. Due to their low abundance and stability, the analysis of phosphopeptides is challenging. The phosphopeptide enrichment must be combined with the protection of the phosphoryl groups to enable a successful analysis. The complete genome sequence is essential to make use of the complex proteomic data sets. The capabilities are improving every year, with an increasing number of plants species being sequenced. The potato genome was sequenced in the year 2011 by the Potato Genome Consortium [65].

### **4.7 Proteomic analyses of the potato**

Thus far, many attempts have been made to investigate the biological processes such as wound-healing, tuber dormancy release, cold-induces tuber sweetening at the proteomic level [66-68]. The protein profiling of potato leaves proteome was performed with regards to cell wall and cytoplasmic fractions [69]. The potato stress responses were investigated when plants were subjected to cold and salinity stress, herbivorous attack as well as osmotic stress [70-72].

Recently, the nitrogen deficiency response of the cultivars ‘Lambada’ and ‘Topas’ was compared on the level of the shoot proteome [73]. The results showed that more proteins were changed in their abundance in the sensitive cultivar ‘Lambada’. The most important differences between cultivars were found with respect to chlorophyll synthesis, photosynthesis, carbon fixation, and metabolism. Consistently with the decrease in the chlorophyll content in cv. ‘Lambada’ there was a decrease in the abundance of proteins participating in chlorophyll synthesis, whereas in the cv. ‘Topas’ enzymes that increased chlorophyll metabolism and stability were enhanced. Additionally, proteins that enhanced the photosynthetic carbon fixation such as RuBisCO activase or carbonic anhydrase increased their abundance in cv. ‘Topas’ under low N regime. Moreover, the light harvesting complexes decreased their abundance in cv. Lambada only [73]. The results presented in the thesis are the first attempts to also resolve the potato root proteome.



**Figure 5 Schematic workflows for the analysis of proteins using 2D-PAGE and liquid chromatography separation coupled with mass spectrometry.**

## 5. Aim of the study

The aim of the presented thesis was to elucidate key mechanisms, metabolic pathways and candidate proteins determining the contrasting response of the two potato cultivars ‘Lambada’ (sensitive) and ‘Topas’ (tolerant) to nitrogen deficiency.

The response of plants to N deficiency is a complex process that depends on the activation and regulation of specific genes, leading to physiological and biochemical changes. The analysis of the proteome allows monitoring the final adaptation processes in the cell, which are reflected by the changes in the composition of proteins and their enzymatic functions. In addition, signaling and regulation of enzyme activities is frequently based on posttranslational modifications such as phosphorylation. The analysis of the shoot proteome is the most common approach when comparing the differences between genotypes contrasting in the N deficiency tolerance [74-76]. The hypothesis of this study was, however, that root proteins are crucial for the N deficiency tolerance, as they directly participate in nitrogen sensing, uptake, and assimilation. To follow this hypothesis:

- The differences in the key metabolic pathways and processes related to N deficiency response were elucidated, by the comparison of the whole root proteome.
- The differences in the abundance of transporters and channels participating in the N uptake were revealed by the comparison of the plasma membrane proteome.
- The very early reactions of the roots towards stress stimuli were compared by means of phosphoproteome, proteome and metabolome analysis.

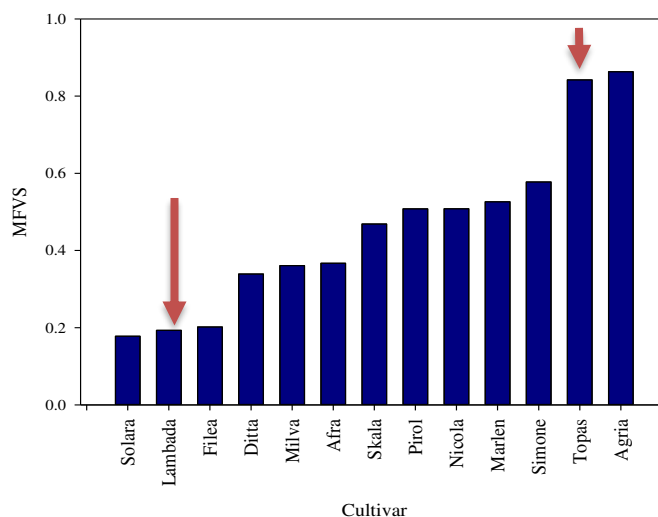
Selected candidate proteins, which supposed to play a role in the N deficiency tolerance were used for targeted analysis to broaden the knowledge about their functional contribution to the trait.

## 6. General discussion

Each chapter (I-V) comprised detailed discussion of the results. In this section, results are discussed in a comprehensive way to highlight the most important findings of the study.

### 6.1 Selection of cultivars contrasting in nitrogen deficiency

The *in vitro* based screening was the basis for selection of cultivars with contrasting nitrogen deficiency response. It allowed for monitoring plant growth, controlling nutrient status, and excluding the impact of the soil, water, pathogens, and climate at the same time. The 13 table potato cultivars were characterized for traits linked with nitrogen uptake and utilization [55]. According to the previously published data, the membership function value of stress tolerance (MFVS) [57] for the traits including fresh and dry matter of shoots, roots and plants, SPAD values, crude protein content of shoots, crude protein yield of plants, dry matter and crude protein yield per unit of available nitrogen was calculated in chapter I (Figure 6).



**Figure 6** The selection of cultivars with contrasting response to N deficiency. Adopted from [77].

This allowed to select cultivar ‘Lambada’ as more sensitive and ‘Topas’ as more tolerant to decreasing N content. The cv. ‘Lambada’ was characterized by high biomass when the N level was sufficient and a drop in the root and shoot weight in the N deficient conditions. Cultivar ‘Topas’ produced less biomass under N sufficient conditions but reacted stable to the low N regime [55, 56]. Similarly, in chapter IV of the study, the biomass of cv. ‘Lambada’ decreased to the 65% of the control after 72 hours of N withdrawal, and the effect was less drastic for cv. ‘Topas’ (90 %).

## 6.2 Selection of LC-MS technique as a basis for identification and quantification of protein related to N deficiency

To answer the question what the differences are between the two potato genotypes in their response to nitrogen deficiency, three main approaches were proposed, namely the analysis of the whole proteome, of the plasma membrane proteome, and of the phosphoproteome (Figure 7). The analysis of the whole proteome provides as much as hundreds of differentially abundant proteins. This, however, includes usually only highly abundant proteins participating in primary metabolism; low abundant proteins, such as signaling proteins, remain poorly covered. Mass spectrometry is also the method of choice for the analysis of PTMs. Low stoichiometry and low ionization efficiency belong to the main challenges in the detection of PTMs and an enrichment method is necessary to reduce sample complexity prior to the MS analysis. One of the most important PTMs is phosphorylation and about 30% of proteins undergo this modification [78]. Adaptation of commonly used protocols for PM (chapter II and III) and for phosphoprotein enrichment (chapter V) for the potato root tissue was the basis for an investigation of differences in the response of potato cultivars to N deficiency. As a gel-based approach is not suitable for the separation of hydrophobic membrane proteins, liquid chromatography coupled with ESI-QTOF mass spectrometer was used for the analysis of proteins in chapters I-V.

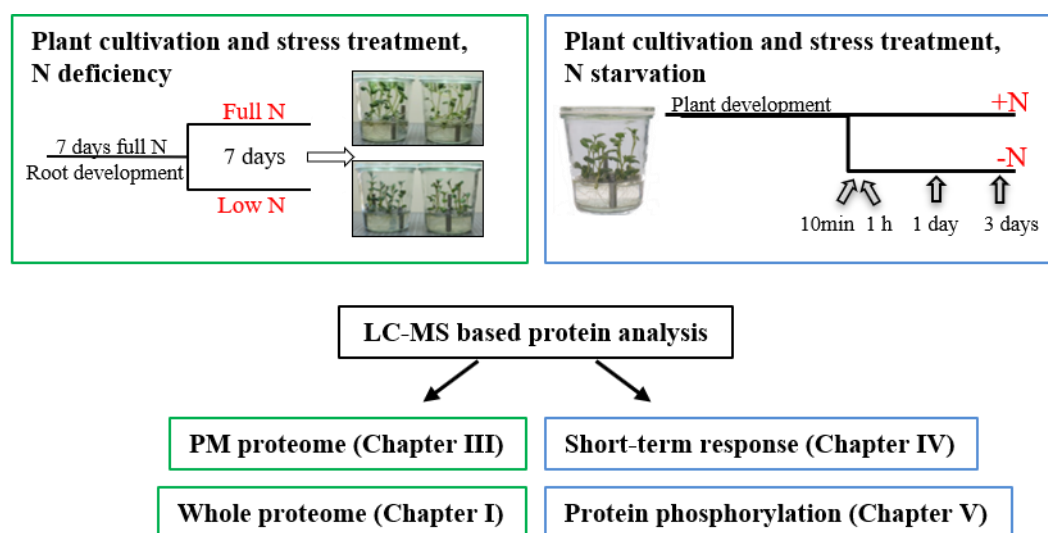


Figure 7 Schematic representation of the workflow presented in the thesis

## 6.3 Influence of N deficiency on the whole proteome of potato roots

Chapter I comprised the analysis of the whole root proteome of the potato cultivars ‘Lambada’ and ‘Topas’. In the study, plants were cultivated for one week on a medium containing a sufficient level of N. When the roots were fully developed, half of the plants were moved to N deficient medium and the other half to a fresh N sufficient medium. 103 proteins were differentially changed in the response to nitrogen deficiency in both cultivars. Among those, the majority of proteins were related to the metabolic, energetic and disease/defense related processes. The cultivars differed in the

number of central metabolic pathways including the components of the GS/GOGAT cycle. GS, which is essential for the synthesis of all N-containing compounds, was selected as one of the key candidate proteins. It was identified with two isoforms, one more abundant in cv. 'Lambada' during stress, and the other decreased in this cultivar but strongly induced in cv. 'Topas'. The enzymatic assay, however, showed only marginally higher activity of GS activity. The enzymes involved in the glycolysis/gluconeogenesis and TCA cycle were more abundant in the tolerant cultivar. Additional differences were associated with protein catabolism, amino acid synthesis and defense mechanisms. The main conclusion was that under nitrogen deficiency, the tolerant cultivar is able to better regulate key metabolic pathways.

#### **6.4 How to identify and quantify plasma membrane proteins?**

Plasma membrane proteins constitute only about 5-20% of all membrane proteins and remain underrepresented in proteomic studies [79]. Chapter II describes the method adapted for the plasma membrane enrichment of the potato roots obtained in the *in vitro* culture system. The two-phase partitioning with Dextran T500/PEG 3350 was applied for many different animal and plant tissues to enrich the plasma membrane. However, the typical quantities of material used in the enrichment protocols greatly exceed the tissue yields of the *in vitro* culture system. Additionally, roots are characterized by a low protein concentration. The protocol established in the book chapter is adjusted to the amounts of the root material as low as several grams and allows studying PM proteome by an LC-MS approach.

#### **6.5 PM proteins involved in the differential response toward N deficiency**

In chapter III the method described in chapter II was used to enrich the plasma membrane from the roots of potato cultivars. In the study, 65 proteins were differentially affected in response to N deficiency.

As described in the introduction, NRT2 is involved in the high-affinity transport of nitrate, with NRT2.1 as the major contributor [80]. In chapter III, NRT2.1 increased in the tolerant cultivar and decreased in the sensitive one. Decrease in the nitrate concentration was described as the reason for an increase in the NRT2.1 abundance in cv. 'Topas'. As it was previously described, cv. 'Lambada' absorbed all available N faster than cv. 'Topas' [56], so the decrease in NRT2.1 is probably caused by severe N deficiency. NRT2.1 is regulated by phosphorylation [48] but the corresponding phosphopeptide was not identified by phosphoproteomics (chapter V). Nitrate transport mediated by NRT2.1 depends on the activity of PM H<sup>+</sup>-ATPase [11, 81]. Three isoforms of H<sup>+</sup>-ATPase were among the inducible proteins and for all of them strong positive correlation between their abundance and amount of NRT2.1 was found. Two isoforms of aquaporins, the proteins which mediate water transport (PIP1;1 and PIP1;3), were found to be more abundant in the tolerant cultivar after N deficiency treatment. The indication is therefore that some of the aquaporins are involved in the more tolerant variety's ability to better cope with N deficiency. The examination of T-DNA

insertion mutants of *Arabidopsis* confirmed the role of PIP1;1 and PIP1;3 in the nitrogen uptake under nitrogen limiting conditions. The *Atpip1;1* and *Atpip1;3* mutants had a significantly lower nitrogen content in the leaves than the wild type plants. In a yeast growth assay, it was shown that both potato aquaporins isoforms do not possess the ability to transport ammonia. Therefore their part in the nitrogen limiting conditions is rather to promote the convection flow of nutrients from the soil to the root surface, increasing the N acquisition, as described before [82]. The hypothesis was that under N limiting conditions cv. ‘Topas’ has a more efficient N transport system.

### **6.6 Is the reaction of the two cultivars different at the very early stage of N deficiency?**

The differences in the temporal response to N starvation are described in chapter IV. The proteomic changes in roots were investigated after 10 min, 1 hour, 24 hours, and 72 hours of N deficiency. To induce a rapid response, the N sufficient medium was replaced by the nutrient solution lacking any source of N. The proteomic data were complemented by the analysis of amino acids and other primary metabolites to obtain a broad view of changes in the roots.

Only a limited number of proteins was differentially changed following the early N deficiency imposition (10 min, 1 hour), indicating that the plant needs more time to adapt its proteome and metabolome towards new environmental conditions. More proteins were affected in the sensitive genotype than in the tolerant ones at later time points. The abundance of proteins and the level of metabolites participating in the N uptake, assimilation, and reassimilation was one of the most pronounced differences observed between the cultivars ‘Lambada’ and ‘Topas’. The Fd-dependent GOGAT, GS, GDH and NiR were all more abundant in the tolerant cultivar ‘Topas’ under both stress and control conditions. Glutamate was almost two times higher in cv. ‘Topas’ and there was a strong positive correlation between the abundance of GOGAT and GDH and the concentration of glutamate. Similarly, the enzymes involved in C metabolism and protein synthesis and degradation were more abundant in the cv. ‘Topas’ roots. A higher abundance of C metabolism-related enzymes in cv. ‘Topas’ was probably connected to a higher representation of enzymes participating in the GS/GOGAT/GDH cycle to maintain the metabolic flux but also to provide adequate energy supply. An efficient system of protein accumulation and recycling, due to the higher abundance of protein degradation enzymes, could explain the better performance of cv. ‘Topas’ under N limitation but also higher abundance of N assimilatory/re-assimilatory enzymes.

The conclusion was that under non-limiting N conditions, cv. ‘Lambada’ used the provided N to produce high biomass, whereas cv. ‘Topas’ allocated more resources into protein production. Under N limiting conditions, the biomass production dramatically dropped in cv. ‘Lambada’ but not in cv. ‘Topas’ that used its resources to respond to N deficiency.

### **6.7 Regulation of the proteins taking part on N deficiency response by phosphorylation**

Phosphorylation does not require *de novo* synthesis of protein and therefore allows for faster adaptation to new environmental conditions. The analysis of the phosphoproteome was therefore



chosen to compare very early reactions of the potato to N deficiency in chapter V. The highest number of peptides significantly changed in the phosphorylation pattern was found after 10 min of treatment, confirming that this PTM is required for rapid response to changes in the environment. The affected proteins were involved in the transcriptional regulation processes, signaling and in C metabolism, especially glycolysis. Importantly, in the tolerant cultivar ‘Topas’ several kinases (e.g. serine-threonine kinases) were phosphorylated to a higher degree than in cv. ‘Lambada’. Additionally, the MAPKKK and MAPKK, which participate in transduction of the environmental signals to the cell through the mitogen-activated protein kinase (MAPK) cascade, were both phosphorylated in cv. ‘Topas’ by N starvation.

Additionally, the lysine histidine transporter, which was proposed to be involved in the uptake of amino acids into root [83] was changed in the phosphorylation level over the time of the treatment suggesting that there are differences in the transport efficiency of amino acids in the two cultivars. The importance of phosphorylation diminished within the time of the experiment, as the number of peptides with a change in the phosphorylation pattern decreased. At the later time points, the differential changes in the phosphorylation pattern of ubiquitination proteins were observed including 26S proteasome regulatory subunit and E3 ubiquitin-protein ligase and ubiquitin-conjugating enzyme E2, supporting the results of chapter IV, where proteolysis was proposed as one of the aspects of a better tolerance of cultivar Topas to N deficiency.

## **6.8 Which metabolic processes are influenced in potato during N deficiency?**

As nitrogen is a constituent of proteins, nucleic acids, metabolites and hormones, insufficient amounts of this nutrient influence many processes and metabolic pathways. In this section, the most prominent changes in the metabolic pathways of potato roots are discussed and compared to the responses of other plant species.

### **6.8.1 Nitrogen uptake and assimilation**

As expected, nitrogen limitation had a very high impact on the transporter and enzymes participating in its uptake and assimilation. Ammonium and nitrate are transported by several types of transporters, as described in the introduction. In the potato, the NRT2.1 transporter increased due to the low N in the tolerant potato cultivar. In *Arabidopsis* this transporter was found to be dephosphorylated 3 min after nitrate resupply to the starved seedlings, suggesting its regulation by the phosphorylation. Corresponding regulatory peptide was however not identified in chapter V. In the cell, nitrate is reduced to ammonium via nitrate and nitrite reductases (NiR). In rapeseed, NR and NiR decreased in their protein abundance in N limited plants [84], whereas in barley, NiR increased in the roots under long term low N [85]. In contrast, in potato NiR was constantly more abundant in cv. ‘Topas’ but not responsive to N deficiency. The glutamine synthetase fixes ammonium and glutamate to form glutamine. Glutamine reacts with 2-oxoglutarate to form 2 molecules of glutamate in the reaction catalyzed by GOGAT [26]. The abundance of GS1 was

shown to increase in barley plants exposed to a sub-optimal N level for an extended period [85], at the early stages of N deficiency in *Arabidopsis* [86] and after N resupply to maize [87]. In this study, abundance of GS1 isoforms was affected by N availability in a cultivar-dependent manner.

### 6.8.2 Carbon metabolism

N and C metabolism are tightly connected. The source of N is necessary to allow utilization of carbohydrates for growth and photosynthesis. In non-photosynthesizing tissues, carbohydrates are used to deliver reducing equivalents, ATP and carbon skeleton for assimilation of N into amino acids and nucleotides [88]. Physiological and biochemical studies showed that in N deficient plants photosynthesis is also negatively affected and similarly when increasing C supply, N uptake and assimilation is promoted [89]. It is hence conceivable that many proteins and compounds related to the C metabolism were responsive to low N treatment in the studies presented in chapters I, IV, and V.

TCA derived organic acids are precursors of amino acids and also act as acceptors for reduced carbon and as a counter anions to replace nitrate and maintain pH balance during nitrate assimilation [90]. Decrease in their level is expected when N is deficient and was already observed for *Arabidopsis* and maize [91-93]. In agreement, the level of malate, succinate, citrate, and fumarate decreased similarly in both cultivars in the early N starvation in both genotypes. The activity of the enzymes of TCA cycle decreased in shoot of maize; the conclusion was that this reflected a reduced allocation of assimilated carbon into amino acids [91, 92]. In rice roots, however, N deficiency increased abundance of the succinyl-CoA ligase [94], while in barley aconitase increased by a prolonged period of N starvation [85]. The higher abundance of TCA enzymes in cv. 'Topas' in chapter IV and their increase in chapter I is associated with a higher representation of enzymes participating in the N metabolism, needed to maintain metabolic flux and to provide adequate energy supply for adaptation to the new environmental conditions.

### 6.8.3 Amino acid metabolism

Glutamine is a primary amino acid, which serves as a donor of the amino group for synthesis of other amino acids. The pool of amino acid varies between the species, cell types, and physiological state of the plant [91]. The general decrease in the content of amino acids is expected during N deficiency and was already reported for *Arabidopsis*, wheat, tomato, and tobacco [91, 93, 95, 96]. However, the response of single amino acids seems to be individual and dependent on the plant and experimental conditions. For example only lysine, arginine, and histidine increased in *Arabidopsis*, whereas in tomato the increase in lysine and leucine was reported [95]. In the present study, a number of amino acids including aspartate, alanine, serine, and valine decreased in both potato cultivars, others followed a cultivar specific trend. The concentration of lysine, cysteine, and tyrosine increased only in cv. 'Lambada' and the amino acids derived from aspartate biosynthetic pathway decreased only in cv. 'Topas'. The changes in amino acids levels in the N deficient plants

might be driven by the perturbation of the N management but also by the abiotic stress. Individual amino acids can have a specific function, as for example, leucine and arginine were proposed to be regulatory molecules [97].

### **6.8.4 Protein synthesis and degradation**

The adaptation to stress requires involvement of regulated protein synthesis and proteolysis. Proteins are reservoirs of amino acids that can be recycled for the synthesis of specific proteins required under N deficiency. Transcripts for components of protein synthesis were previously shown to increase in N-replete *Arabidopsis* [98]. Protease activity is influenced by N supply [87, 99]. Several proteins related to protein synthesis (ribosomal proteins, elongations factors) as well as catabolism were affected in cv. 'Topas', but their abundance was not affected in cv. 'Lambada'. The regulation of the components of the ubiquitin/26S proteasome system (using ubiquitin as a tag for a selective protein breakdown) was an important difference between both cultivars across chapters I, IV, and V. The abundance of a proteasome subunits strongly decreased in cv. 'Topas' in chapter I; those proteins were however more abundant in cv. 'Topas' than in cv. 'Lambada' in chapter IV. Furthermore, there was a different, time dependent phosphorylation profile of E2 and E3 enzymes involved in ubiquitination. The increase in proteins participating in the ubiquitin/26S proteasome system was also reported for *Arabidopsis* and in rice constitutive expression of an E3 ligase gene has been suggested as a way to obtain plants more tolerant to N deficiency [100]. A more efficient protein catabolism is probably a basis for the more effective N reassimilation system in the tolerant cultivar.

### **6.9 Diseases and defense related proteins**

Stress conditions, such as nutrient deficiency leads to the enhanced generation of reactive oxygen species (ROS). It was shown that ROS increased in roots of *Arabidopsis* when nitrogen was limited [101]. N deficiency stimulated production of reactive nitrogen species and the overproduction of those molecules must be controlled by the antioxidants [102]. Defense and antioxidation processes are required for plant survival and especially detoxification seems to be important in a better performance during nitrogen shortages. Several proteins, which were assigned to defense responses, were differentially abundant across chapters III, IV, and V in both genotypes. Among them, glutathione-S-transferase protein species were reacting to insufficient N treatment in both cultivars (chapter I and IV). These enzymes link glutathione to certain xenobiotics or toxic substances generated during oxidative stress to mediate their metabolism and removal [103]. In the N deficiency study, the protective enzyme catalase accumulated more strongly in cv. 'Lambada' and PR10 protein, which is involved in the general defense mechanisms [104] was more abundant in cv. 'Topas'. In conclusion, changes in the abundance of proteins participating in defense are important for plant survival under stress conditions but the responses are cultivar specific.

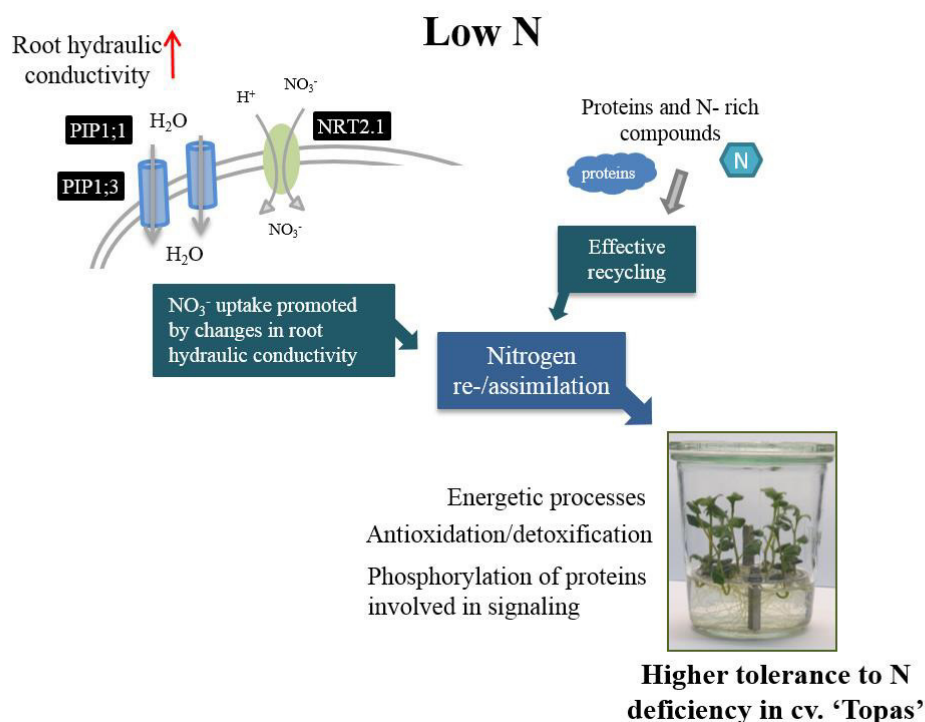
### **6.10 Which key candidate potato proteins are related to the differential performance of plants under N deficiency?**

For reducing the fertilization rates there has been an increased focus on the search for crops with better NUE. Studies on plant physiology, molecular genetics, and agronomy were undertaken in the last decades to identify key components of NUE [105]. The proteins identified in this study as differentially abundant between the two cultivars are possible candidate proteins for increasing a nitrogen deficiency tolerance in potato. One of the promising candidates is a glutamine synthetase. This protein was already studied in other plant species for the effect of overexpression on the plant performance. In tobacco, the overexpression of the cytosolic glutamine synthetase from alfalfa caused an increase in photosynthesis and growth under a low N regime [106]. Transgenic pea (*Pisum sativum*) plants, which overexpressed the soybean *gs15* gene showed increased biomass and N content [107]. Recently, barley which expressed an extra copy of native *HvGS1.1* were shown to have a higher grain yield and NUE than the WT plants [108]. Other interesting proteins, which were constantly more abundant in cv. Topas under both control and stress conditions and might be considered for further investigations were GOGAT and GDH. When the bacterial enzyme glutamate dehydrogenase was constitutively overexpressed in tobacco, biomass production increased by about 10-15 % [109], whereas overexpression of *OsGOGAT1* gene in rice resulted in the increase of spikelet weight up to 80% [110].

The overexpression of aquaporins was shown to increase salt and osmotic stress tolerance in barley and *Arabidopsis*, but also drought stress tolerance in tobacco [111, 112]. The results of the thesis suggest that the potato aquaporins PIP1;1 and PIP1;3 are important for a better tolerance to N deficiency. This is supported by the significantly lower N content in the leaves of *Arabidopsis* lacking these proteins as shown in chapter III. Therefore, PIP1;1 and PIP1;3 are novel candidates for increasing plant N deficiency tolerance and their function will be investigated in further studies. Although it was shown that it is possible to improve N metabolism and increase the yield of crop plants by genetic manipulations, this area remained untouched in the case of potato. This study provides a range of candidate proteins, which can be further studied in potato to increase its performance under low N supply.

### **6.11 Summary and future directions**

Nitrogen deficiency is well known to reduce plant growth and development as well as to reduce yield. In this thesis, the attempts were made to explain the difference in the performance of potato cultivars ‘Lambada’ and ‘Topas’ contrasting in their N deficiency response on the biochemical level. The results of my study suggest that under non-limited N conditions cv. ‘Lambada’ use the provided nitrogen to produce a high biomass, while cv. ‘Topas’ allocate more resources into protein production and synthesis of N-containing compounds. When nitrogen is deficient, the biomass



**Figure 8 Processes involved in the better tolerance of potato cultivar 'Topas' to N deficiency.**

production dramatically drops in cv. 'Lambada'. Under low N conditions, cv. 'Topas' uses the PM aquaporins PIP1;1 and PIP1;3 to promote the water flow to the surface of the root and use the NRT2.1 for the high affinity nitrate uptake. It also uses its stored resources for a stable growth. Figure 8 summarizes the most important findings of the study, which were discussed in the thesis. This PhD study identifies proteins which are likely involved in the better tolerance of the 'Topas' potato variety toward N deficiency. The results presented in the thesis open new possibilities for further research. The follow-up investigations to strengthen the conclusions drawn in the presented studies involve:

- ❖ Screening of a wider set of cultivars for the abundance of candidate proteins. The PhD study was conducted with a pair of cultivars with contrasting N deficiency response. Screening of a wider set of potato genotypes was already conducted identifying new candidate genotypes with more tolerant and sensitive reaction to N deficiency. The targeted proteomic approach using the candidate proteins of this study, in particular aquaporins, NRT2.1 and GS could be performed on other genotypes. This would allow selecting common mechanism leading to the higher N deficiency tolerance in potato. For the proteins where the antibodies are available it might be achieved by using the Western Blot approach. Another possibility is the targeted mass spectrometry approach called MRM (multiple reaction monitoring). It allows for targeted quantification of proteins, based on their unique peptides.

- ❖ The involvement of the aquaporins in the nitrogen uptake and transport should be addressed in the future. In this thesis the role of PIP1;1 and PIP1;3 in the N uptake was confirmed in *Arabidopsis*. Similar investigations should be performed in potato. To achieve this, the targeted genome editing technologies such as CRISPR/Cas could be used. Additionally, future experiment should address the question if the overexpression of aquaporins would positively influence the N deficiency performance of potato.
- ❖ The phosphoproteome analysis revealed novel phosphorylation sites. This includes proteins which have not yet been described as regulated by phosphorylation, such as proteins participating in glycolysis, but also the enzymes participating in the ubiquitination. Validation of the phosphorylation sites could be performed using specific antibodies raised against the phosphorylated form of the peptide. The biological importance of the phosphorylation sites might be further investigated using site-directed mutation with the serine, threonine, or tyrosine mutated to non-phosphorylatable amino acid, such as alanine. The changes in the plant phenotype would confirm the importance of the phosphorylation event.

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## 9. Affirmation

Hereby, I declare that the present dissertation was composed by me independently (apart from the advices of my supervisor), without assistance from external parties and without use of other resources than those indicated in the references. The ideas directly or indirectly from external sources are duly acknowledged in the text. The thesis, either in wholly or partially, has not been previously submitted for grading at this or any other academic institution and academic degree has never been withdrawn. The thesis has been prepared according to the Rules of Good Scientific Practice of the German Research Foundation.

Gatersleben,

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Date

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Signature

# Chapter I



# Nitrogen Deficiency Induced Alterations in the Root Proteome of a Pair of Potato (*Solanum tuberosum* L.) Varieties Contrasting for their Response to Low N

Anna Maria Jozefowicz, Anja Hartmann, Andrea Matros, Annegret Schum, and Hans-Peter Mock\*

Improving crop nitrogen use efficiency is important both from the economic and the environmental viewpoint. Here, the aim is to highlight differences between the proteomic response of the roots of two potato cultivars contrasting in their response to nitrogen (N) deficiency, in an effort to understand which proteins and metabolic pathways contribute to the tolerance of N deprivation. The two cultivars “Topas” (tolerant) and “Lambada” (sensitive) are grown under both an N sufficient and an N deficient regime, using an in vitro-based cultivation system. Responsive proteins are identified and quantified using label-free quantitative shotgun proteomics. The contrasting cultivars differed with respect to components of the glutamine synthetase/glutamine oxoglutarate aminotransferase pathway, tricarboxylic acid cycle, the glycolysis/gluconeogenesis pathway as well as protein and amino acid synthesis machinery. Additional differences are associated with protein catabolism and defense mechanisms.

## 1. Introduction

Nitrogen (N) deficiency is recognized as one of the major constraints over crop productivity. The consequences of an insufficient N supply are increased root to shoot ratio, suppression of lateral root growth, decreased in photosynthesis rate, increase in the C/N ratio, early leaf senescence as well as decreased yield.<sup>[1–6]</sup> In order to meet a growing population there is a demand to increase crop plants productivity. Excessive applications of N fertilizer are, however, not just cost ineffective, but also environmentally damaging.<sup>[7]</sup> Crops are able to utilize only about 30–40% of applied chemical N fertilizers. The rest is lost from the soil through leaching, surface run-off, denitrification, volatilization, and microbial consumption negatively affecting the diversity and functioning of the non-agricultural ecosystems, as well as on the composition of the atmosphere.<sup>[8–10]</sup> Additionally,

according to the estimates a 1% increase in the nitrogen use efficiency could possibly save 1.1 billion USD annually as N fertilizers are typically the highest input cost of crop plant production.<sup>[10]</sup> As a result, there has been an increasing focus on searching and exploiting variation for nitrogen use efficiency in a number of crops.

Plants have developed a variety of strategies to deal with nitrogen deficiency, including increasing their capacity to take up N, remobilizing N already fixed within the plants, suppressing growth and photosynthesis, and boosting the accumulation of anthocyanin.<sup>[11–14]</sup> Most plants have the capacity to assimilate N from the soil in the form of nitrate and ammonium with both forms being incorporated via the same metabolic pathways.<sup>[15]</sup> Active uptake occurs via several high affinity transport systems, two of which target nitrate (inducible and constitutively expressed) and one ammonium. The *Arabidopsis thaliana* genome harbors seven genes encoding transporters involved in nitrate and five in ammonium uptake.<sup>[16]</sup> The first step related to nitrate assimilation involves its reduction by nitrate reductase and nitrite reductase to ammonium.<sup>[17,18]</sup> Subsequently, ammonium is converted to either glutamate or glutamine through the activity of the glutamine synthetase/glutamate synthase (GS/GOGAT) pathway, in which form the N that is translocated to sink tissues.<sup>[19,20]</sup> Two major variants of GS have been identified, one active in the cytosol (GS1) and the other in the chloroplasts/mitochondria (GS2).<sup>[21]</sup> The main role of GS1 is to assimilate primary ammonium in the root and to re-assimilate ammonium generated as a result of protein turnover in the leaf. GS2 functions mainly to re-assimilate ammonium formed as a result of photorespiration and to assimilate the products of nitrate reduction in plastids.<sup>[22]</sup> GS has been identified to play a key role in the control of nitrogen use efficiency.<sup>[23]</sup>

Over the years many transcriptomic studies have been conducted to understand plant response to high and low concentration of N. They showed that various biological processes, such as amino and nucleic acid synthesis, protein folding, RNA processing, and hormone biosynthesis are rapidly affected when

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## Significance of the study

Potato is one of the most important crops worldwide and an improvement of the nitrogen use efficiency is crucial for providing high yield but also for reducing environmental pollution arising from excessive fertilization. In this study we investigated influence of limited nitrogen level on proteome of two cultivars contrasting in tolerance to nitrogen deficiency. The differentially expressed proteins may contribute to the knowledge of mechanisms leading to better tolerance for lower nitrogen levels.

nitrate is depleted or resupplied.<sup>[24–26]</sup> Recently, the role of genes taking part in amino acid metabolism was emphasized by transcriptomic study in potato.<sup>[27]</sup> However, as yet, few equivalent studies at the proteomic level have been reported. A unique feature of proteomic analyses is that, unlike transcriptomics-based ones, they can recognize the presence and influence of posttranslational modifications.<sup>[28]</sup> A characterization of the barley root and shoot proteome in plants exposed to either sustained or short-term N deficiency has revealed that the responses to the treatments are distinct from one another, even though some of the enzymes can participate in both situations.<sup>[29]</sup>

Potato is an important starch crop of temperate agriculture. Understanding the molecular basis for the adaptation of potato to a limited N supply is therefore of particular importance in the context of the rising demand for food as well as renewable raw materials and the need to protect the quality of the rural environment. Variation for the ability to tolerate N deficiency including N utilization rates and chlorophyll content has been demonstrated in potato cultivars previously. Positive relationship between “tolerance to N-shortage” and “N use efficiency” has been determined.<sup>[30–33]</sup> The present research has focused on the proteomic response of the pair of contrasting cvs. “Lambada” (sensitive) and “Topas” (tolerant) in order to identify which proteins underlie their differential tolerance.

## 2. Experimental Section

### 2.1. Plant Material and Culture

The genotypes were selected according to the results of response to N deficiency at the morphological and physiological level under in vitro conditions among a set of table potato cultivars. From the data already published<sup>[30–32]</sup> the Membership Function Value of Stress tolerance (MFVS) was calculated for selected traits as described previously.<sup>[34]</sup> Following formula was applied:

$$U_{ij} = (SC_{ij} - SC_{jmin}) / (SC_{jmax} - SC_{jmin}) \quad (1)$$

$U_{ij}$  = membership function value of stress tolerance for the trait (j) for the cultivar (i);  $SC_{ij}$  = stress tolerance coefficient of the trait (j) for the cultivar (i) evaluated under stress (s) and control conditions (c):  $X_{ijs} / X_{ijc}$ ;  $SC_{jmax}$  = maximum value of the stress tolerance coefficients for the trait (j) in the set of

cultivars;  $SC_{jmin}$  = minimum value of the stress tolerance coefficients for the trait (j) in the set of cultivars.

A ranking of cultivars was performed by calculating the mean value of the MFVS of 11 traits determined after 21 days of culture at an N-level of 7.5 mmol L<sup>-1</sup> as compared to an ample N-supply of 60 mmol L<sup>-1</sup>, taking into account fresh and dry matter of shoots, roots and plants, SPAD values, crude protein content of shoots, crude protein yield of plants, dry matter, and crude protein yield per unit of available nitrogen. This allowed differentiation between cultivars reacting more tolerant and more sensitive, respectively, to nutrient deficiency (Supporting Information, Figure S1).

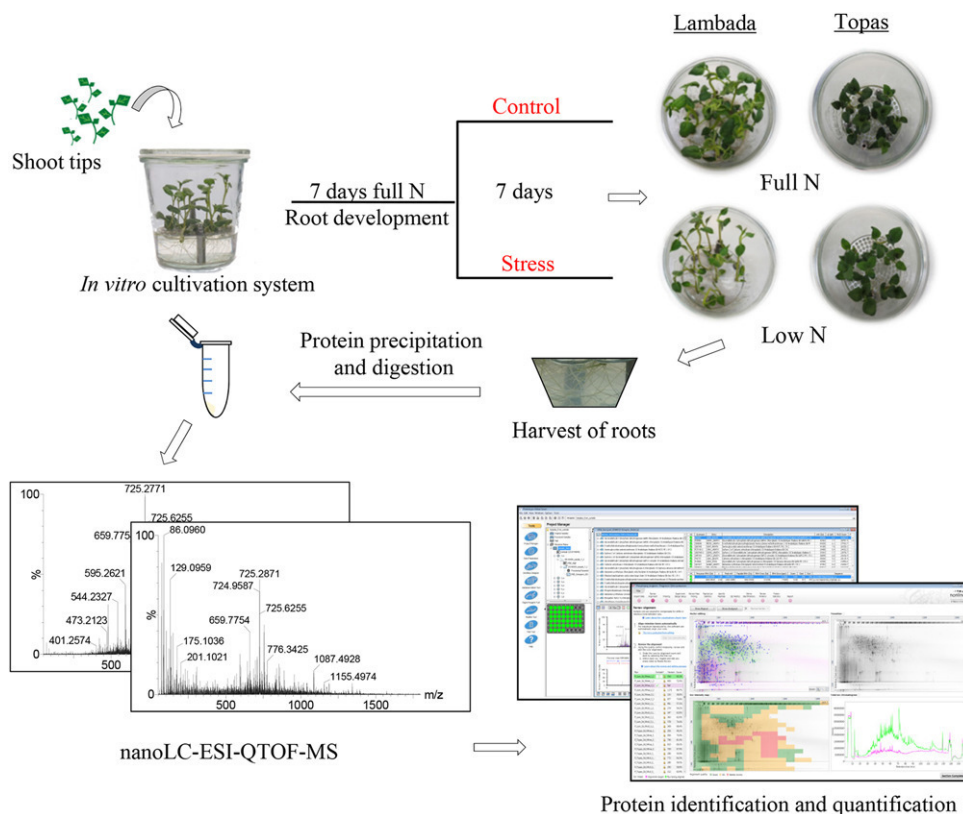
The in vitro cultivation and stress treatments imposed on cv. “Lambada” and cv. “Topas” plants were conducted as described,<sup>[30]</sup> with only minor modifications. Eight shoot tips were mounted on a perforated stainless steel plate fitted within a 500 mL glass vessel containing 140 mL Murashige and Skoog liquid medium containing inorganic nitrogen sources (NH<sub>4</sub>NO<sub>3</sub> and KNO<sub>3</sub>).<sup>[35]</sup> The vessels were held at 21 °C and illuminated to give a 16 h photoperiod. Following the development of roots (one week), half of the plants were moved to a fresh batch of medium supplemented with 60 mmol L<sup>-1</sup> N and the other half to a medium containing only 3.75 mmol L<sup>-1</sup> (Figure 1). The difference in potassium content of the two media was made up by including the relevant amount of KCl in the low N medium. After 7 days, before the symptoms of deficiency occurred, the roots were harvested, snap-frozen in liquid N<sub>2</sub> and stored at -80 °C prior to protein extraction. Plant experiment was performed in triplicate with six vessels pro condition and cultivar in each experiment.

### 2.2. Protein Extraction and Digestion

The harvested roots were homogenized in 50 mM MOPS (pH 7.8), 330 mM sucrose, 10% (v/v) glycerol, 5 mM EDTA, 5 mM ascorbate, 0.6% (w/v) PVP, 5 mM DTT, and complete EDTA-free protease inhibitor cocktail (Roche, Mannheim, Germany). The homogenate was filtered through two layers of Miracloth and centrifuged (10 000 × g, 15 min, 4 °C), after which the proteins were precipitated from the supernatant using the methanol/chloroform method.<sup>[36]</sup> Harvested pellets were suspended in 100 μL of 7 M urea, 2 M thiourea, 2% (w/v) CHAPS, and 5 mM DTT (pH 8.0). The concentration of dissolved protein was estimated using a 2D Quant Kit (GE Healthcare, Munich, Germany) with BSA used as the reference. A 10 μg aliquot of the protein was sampled for filter-based digestion, following Distler et al.<sup>[37]</sup>: this involved an overnight digestion at 37 °C in 200 ng of Sequencing Grade Modified Trypsin (Promega, Mannheim, Germany). Peptides were recovered by centrifugation, dried in vacuum centrifuge, and suspended in 50 μL of 1% ACN, 0.1% formic acid.

### 2.3. Label-Free Quantification of Proteins and Data Analysis

LC and mass spectrometric analysis were performed using a nano LC-ESI-Q-TOF-MS/MS device (Waters Corporation, Eschborn, Germany). The digested proteins were separated using



**Figure 1.** Workflow for the proteome analysis of the two cultivars “Lambada” (sensitive) and “Topas” (tolerant) differing in the tolerance to nitrogen deficiency.

a NanoAcquity system equipped with a 20 mm × 180 μm Symmetry C18 pre-column and a 150 mm × 75 μm BEH (1.7 μm) C18 column (Waters Corporation). Peptides in the amount of 500 ng were separated through a 3–35% acetonitrile gradient over 90 min applying a flow rate of 300 nL min<sup>-1</sup>. MS and MS/MS detection was performed using a Q-TOF Premiere mass spectrometer (Waters Corporation). As a lock mass, a 500 fmol μL<sup>-1</sup> solution of [Glu1]-Fibrinopeptide B (Sigma Aldrich, Taufkirchen, Germany) was supplied at a flow rate of 600 nL min<sup>-1</sup>. MS detection was performed in positive V-nanoelectrospray mode. Accurate mass data were collected in alternating low and elevated energy mode with a spectral acquisition time of 0.9 s and a 0.1 s interscan delay. In the low energy mode, the applied constant collision energy was set to 4 eV, while the collision energy in the elevated energy mode was ramped from 18 to 35 eV during each 1 s data collection cycle. The *m/z* data were acquired in the range of 200–1500 for MS and 50–1500 for MS/MS. Two technical repetitions were run for the three independent harvests. LC-MS data were processed using an algorithm implemented in the ProteinLynx Global SERVER v2.5.3 (Waters Corporation).

Protein identification was performed by searching in a potato protein database based on the sequences from *Solanum tuberosum* group Phureja DM1-3 (PGSC\_DM.v3.4\_pep\_representative, 39,031 entries)<sup>[38]</sup> annotated by matching against available NCBI entries with Blast2GO software (09.2014)<sup>[39]</sup> and merged with the sequences of human keratin and trypsin. The following search

parameters were applied: automatic peptide and fragment mass tolerance, minimum three fragment ion matches per peptide and seven per protein, minimum two peptide matches per protein, one missed cleavage, variable modification: oxidation (Met), carbamidomethyl (Cys), and a 4% false positive rate, as recommended for small databases. For comparative quantification, the protein data were processed using Progenesis LC-MS software (Nonlinear Dynamics, Newcastle, UK) to enable mass correction, alignment, normalization, peak picking, quantification, and statistics. Additionally, 20 ppm peptide mass error threshold was applied in Progenesis. Proteins with a *p*-value below 0.05, a fold change above 1.5 and which were identified with a minimum two unique peptides were considered as being differentially abundant. Quantification was performed by summing abundances of peptides. Additional annotation based on the UniProt server (www.uniprot.org) was performed for selected proteins (Supporting Information, Table S2) to update the annotations and gain basic information on the proteins and their functions. Functional classification of proteins was performed as described.<sup>[38]</sup> Prediction of cellular localization was performed for several metabolic enzymes using Target P 1.1 Server (<http://www.cbs.dtu.dk/services/TargetP/>). Principal component analysis (PCA), Z-score normalization and hierarchical clustering based on the Euclidean distance method were carried out using the Perseus Framework (www.perseus-framework.org). The visualization of protein profiles integrated in metabolic pathways was performed using

the VANTED framework<sup>[39,40]</sup> based on metabolic pathways from the MetaCrop database.<sup>[41]</sup> The LC-MS data are deposited using the e!DAL system of the IPK Gatersleben<sup>[42]</sup> and available at: <https://doi.org/10.5447/IPK/2017/22>.

### 2.3. GS Assay and Immunodetection

Specific GS activity was determined as described elsewhere.<sup>[43]</sup> As negative controls, both a heat deactivated extract and a reaction lacking glutamate were analyzed. Specific GS activity was determined from the difference between samples measured with and without glutamate. For immunodetection, a 5 µg aliquot of the extracted protein was separated by 15% SDS-PAGE,<sup>[44]</sup> and subjected to Western blotting as described elsewhere.<sup>[45]</sup> The blots were probed with anti GS1/GS2 antibody (Agrisera, Vannas, Sweden). Polyclonal anti-rabbit antibodies derived from goat conjugated with IRDye 800CW were used to enable infrared fluorescence detection using a LICOR scanner (LICOR Bioscience GmbH, Bad Homburg, Germany). The relative quantification of signals was carried out by applying Odyssey 3.0 software (LICOR Bioscience).

### 2.4. Quantitative RT-PCR

RNA was extracted from 100 mg potato roots using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), followed by a DNase treatment to remove contaminating genomic DNA. A 2 µg aliquot of RNA represented the template for synthesizing cDNA using Maxima RT (Thermo Fisher Scientific, Dreieich, Germany). The two reference genes used in the subsequent qRT-PCR encoded adenine phosphoribosyl transferase (*aprt*) and the L2 cytoplasmic ribosomal protein, as suggested by Nicot et al.<sup>[46]</sup> Primers directed at *GS* sequences were designed using the NCBI primer design tool ([www.ncbi.nlm.nih.gov/tools/primer-blast](http://www.ncbi.nlm.nih.gov/tools/primer-blast), Supporting Information, Table S1). Each qRT-PCR contained 2 µL cDNA, 30 pmol of each primer, and 5 µL of SsoAdvanced Universal SYBR Green Supermix (BIO-RAD, Munich, Germany). The amplification was performed as previously described.<sup>[47]</sup> All three harvests were measured in triplicate. Quantification of the amplicons and the related statistical analyses were performed using routines implemented in qBase<sup>+</sup> software v.2.3 (Biogazelle, Zwijnaarde, Belgium).

## 3. Results

### 3.1. Differential Profiling of Proteins Using Label-Free LC-MS Approach

The potato cultivars were selected according to MFVS values calculated, as explained<sup>[34]</sup> for previously published data (Supporting Information, Figure S1).<sup>[30–32]</sup> In result cv. “Lambada” was differentiated as reacting more sensitive and cv. “Topas” more tolerant to nutrient deficiency. Proteome profiling was performed 7 days after N withdrawal, before the symptoms occurred, to exclude the secondary stress effects, such as senescence.

The crude protein yield was higher in cv. “Topas” and remained at the level of 90%, whereas in cv. “Lambada” it decreased to 75% of initial value by low N regime. Across the study we found 395 peptides, which were assigned to 103 proteins differing significantly between the high and low N treatments with respect to their abundance (Table 1; Supporting Information, Tables S2, S3). The application of PCA demonstrated that the replicates (both biological and technical) were clustered (Figure 2A) with the first principal component accounted for 72.3% of the explained variance and the second component for 12.9%. The two cultivars behaved similarly under the high N treatment, but dissimilarly under the low N treatment where first principal component separates cv. “Lambada” stress from cv. “Topas” stress. The loadings plot showed that nine proteins, namely, alcohol dehydrogenase I, GS, PR10 protein, cinnamyl alcohol dehydrogenase (CAD), fructokinase (FK), triosephosphate isomerase, fructose-bisphosphate aldolase, catalase, and peroxidase played a major role in the separation along principal components (Supporting Information, Figure S2). The set of differentially abundant proteins is summarized in Table 1 and further details are provided in the Supporting Information, Table S2. When the differentially abundant proteins were assembled into 12 functional classes,<sup>[38]</sup> the majority appeared to be associated with “disease/defense” (21.4%), “metabolism” (17.5%), “energy” (17.5%), and “protein synthesis” (13.6%). Other processes “signal transduction” (6.8%), “transport” (5.8%), “transcription” (3.9%), “secondary metabolism” (2.9%), and “protein destination and storage” (2.9%) were represented by a smaller number of proteins (Figure 2B).

A hierarchical clustering resulted in the recognition of eight groups within each of the members was regulated in a similar fashion (Figure 2C). Cluster I members were accumulated to a similar extent in both cultivars, but to a greater degree under the N deficient regime. Of the four members, two were related to glutamate metabolism (glutamate decarboxylase and dehydrogenase) while the other two comprised an isoform of peroxidase and phospho-2-dehydro-3-deoxyheptonate aldolase. Cluster II members were more abundant in cv. “Lambada” under N deficient regime. The five members were catalase, aspartic protease, formate dehydrogenase, peroxidase, and phosphoserine aminotransferase. Cluster III members were more abundant in cv. “Topas” than in cv. “Lambada” under both N regimes. This harbored three proteins, comprising two isoforms of glycine rich protein 2 and lactoylglutathione lyase. Two proteins showed clearly distinct profiles from the others and were assigned to separate clusters. Putative universal stress protein A-like was abundant in cv. “Lambada” under N sufficient regime and in cv. “Topas” in N deficient (Cluster IV) and ribulose biphosphate carboxylase large chain (Cluster VI) showed a decrease in abundance due to N deficiency only in cv. “Topas”. The members of Cluster V showed a marked increase in abundance under the N deficient regime only in cv. “Topas”. Cluster V captured about half of the set of differentially abundant proteins. A preponderance of these proteins was involved in energy generation, including succinyl-CoA ligase, citrate synthase, malate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, and triosephosphate isomerase. Those categorized as being associated with “metabolism” were involved in either N metabolism (GS, serine hydroxymethyltransferase) or sugar metabolism

**Table 1.** The label-free quantification of the 103 proteins showing a significantly altered abundance in the roots of potato plants exposed to N deficiency. The proteins have been grouped (clusters I through VIII) using a hierarchical clustering technique, based on their abundance in the two cultivars and the two N treatments..

| Accession   | UniProt Blast description                                    | Functional classification       | Ratio<br>LamS/LamC | Ratio<br>TopS/ TopC | Ratio<br>LamC/TopC | Ratio<br>LamS/TopS |
|-------------|--|---------------------------------|--------------------|---------------------|--------------------|--------------------|
| CLUSTER I   |  |                                 |                    |                     |                    |                    |
| 400021553   | Glutamate dehydrogenase                                      | Metabolism                      | 1.42               | 1.76                | 1.06               | 0.86               |
| 400034693   | Glutamate decarboxylase                                      | Metabolism                      | 1.55               | 1.79                | 1.07               | 0.93               |
| 400012995   | Peroxidase   | Disease/defence                 | 2.93               | 2.41                | 1.03               | 1.25               |
| 400079161   | Phospho-2-dehydro-3-deoxyheptonate aldolase 1, chloroplastic | Metabolism                      | 1.51               | 1.58                | 1.11               | 1.06               |
| CLUSTER II  |  |                                 |                    |                     |                    |                    |
| 400040062   | Peroxidase   | Disease/defence                 | 1.18               | 1.55                | 1.45               | 1.11               |
| 400003869   | Phosphoserine aminotransferase                               | Metabolism                      | 1.38               | 1.09                | 1.21               | 1.53               |
| 400038856   | Aspartic protease  | Protein destination and storage | 1.59               | 1.16                | 1.14               | 1.56               |
| 400001303   | Formate dehydrogenase, mitochondrial                         | Energy                          | 1.80               | 1.09                | 1.15               | 1.89               |
| 400075611   | Catalase   | Disease/defence                 | 1.30               | 1.14                | 1.35               | 1.54               |
| CLUSTER III |  |                                 |                    |                     |                    |                    |
| 400004030   | Glycine-rich protein 2                                       | Transcription                   | 0.97               | 1.22                | 0.59               | 0.47               |
| 400004028   | Glycine-rich protein 2                                       | Transcription                   | 1.01               | 0.96                | 0.61               | 0.64               |
| 400042417   | Lactoylglutathione lyase                                     | Metabolism                      | 0.94               | 0.94                | 0.69               | 0.68               |
| CLUSTER IV  |  |                                 |                    |                     |                    |                    |
| 400007287   | Putative universal stress protein A-like                     | Disease/defence                 | 0.76               | 1.55                | 1.29               | 0.63               |
| CLUSTER V   |  |                                 |                    |                     |                    |                    |
| 400074559   | Citrate synthase   | Energy                          | 1.21               | 1.47                | 0.95               | 0.78               |
| 400003774   | PR10 protein   | Disease/defence                 | 1.47               | 2.14                | 0.96               | 0.66               |
| 400064553   | Peroxidase   | Disease/defence                 | 1.37               | 2.04                | 0.96               | 0.64               |
| 400029242   | Glyceraldehyde-3-phosphate dehydrogenase                     | Energy                          | 1.21               | 1.72                | 0.91               | 0.64               |
| 400064554   | Peroxidase   | Disease/defence                 | 1.51               | 2.20                | 0.80               | 0.55               |
| 400072226   | Peroxidase   | Disease/defence                 | 1.14               | 1.50                | 0.95               | 0.73               |
| 400051918   | Succinyl-CoA ligase subunit beta                             | Energy                          | 1.25               | 1.76                | 0.98               | 0.69               |
| 400078520   | Fructose-bisphosphate aldolase, chloroplastic                | Energy                          | 1.29               | 1.68                | 1.09               | 0.84               |
| 400059161   | Purple acid phosphatase                                      | Signal transduction             | 1.51               | 1.91                | 1.06               | 0.84               |
| 400082447   | Glutamate-1-semialdehyde 2.1-aminomutase, chloroplastic      | Secondary metabolism            | 1.14               | 1.23                | 0.82               | 0.76               |
| 400078507   | ATP synthase subunit beta                                    | Energy                          | 1.09               | 1.29                | 0.86               | 0.72               |
| 400037918   | Glutathione S-transferase                                    | Disease/defence                 | 1.22               | 1.20                | 0.75               | 0.76               |
| 400032266   | Malate dehydrogenase   | Energy                          | 1.11               | 1.40                | 0.82               | 0.65               |
| 400013704   | Putative cinnamyl alcohol dehydrogenase                      | Secondary metabolism            | 0.92               | 1.87                | 0.51               | 0.25               |
| 400058435   | Calmodulin-5/6/7/8   | Signal transduction             | 1.17               | 1.47                | 0.60               | 0.48               |
| 400040717   | Serine hydroxymethyltransferase                              | Metabolism                      | 1.15               | 1.36                | 0.81               | 0.68               |
| 400050141   | Copper chaperone   | Transporters                    | 1.18               | 1.24                | 0.57               | 0.55               |
| 400005571   | Probable glutathione S-transferase                           | Disease/defence                 | 1.10               | 1.57                | 0.62               | 0.43               |
| 400063543   | Luminal-binding protein 5                                    | Protein destination and storage | 0.97               | 1.26                | 0.78               | 0.60               |
| 400037996   | Probable glutathione S-transferase                           | Disease/defence                 | 0.92               | 1.37                | 0.74               | 0.50               |
| 400015118   | Putative 40S ribosomal protein S11-like                      | Protein synthesis               | 0.97               | 1.34                | 0.88               | 0.63               |
| 400038488   | 40S ribosomal protein S6                                     | Protein synthesis               | 0.91               | 1.28                | 0.88               | 0.62               |
| 400079071   | Alcohol dehydrogenase 1                                      | Metabolism                      | 0.81               | 1.44                | 1.02               | 0.57               |
| 400060733   | Glutamine synthetase   | Metabolism                      | 0.77               | 1.82                | 1.11               | 0.47               |
| 400002454   | 2.3-bisphosphoglycerate-independent phosphoglycerate mutase  | Metabolism                      | 0.82               | 1.32                | 1.02               | 0.64               |

(Continued)



Table 1. Continued.

| Accession    | UniProt Blast description  | Functional classification          | Ratio<br>LamS/LamC | Ratio<br>TopS/ TopC | Ratio<br>LamC/TopC | Ratio<br>LamS/TopS |
|--------------|--|------------------------------------|--------------------|---------------------|--------------------|--------------------|
| 40001058     | Heat shock protein 70–3  | Disease/defence                    | 1.28               | 1.73                | 0.85               | 0.63               |
| 400075336    | Eukaryotic initiation factor 4A-2  | Transcription                      | 1.14               | 1.41                | 0.87               | 0.71               |
| 400042238    | Acetyl-CoA carboxylase   | Metabolism                         | 1.15               | 1.40                | 0.92               | 0.76               |
| 400068704    | Vacuolar ATPase subunit B  | Transporters                       | 1.17               | 1.55                | 0.97               | 0.73               |
| 400011624    | Adenosylhomocysteinase   | Metabolism                         | 1.08               | 1.53                | 1.05               | 0.74               |
| 400039449    | Glyceraldehyde-3-phosphate dehydrogenase   | Energy                             | 1.08               | 1.74                | 1.02               | 0.63               |
| 400013094    | PR1 protein  | Disease/defence                    | 0.93               | 1.55                | 1.04               | 0.62               |
| 400030830    | Ferredoxin–NADP reductase  | Energy                             | 1.21               | 2.28                | 0.91               | 0.48               |
| 400025323    | Elongation factor 1-delta-like isoform 2   | Protein synthesis                  | 1.16               | 1.64                | 1.02               | 0.72               |
| 400037336    | Heat shock cognate protein 80  | Disease/defence                    | 1.19               | 1.54                | 0.99               | 0.77               |
| 400026743    | Glutathione S-transferase family protein isoform 1   | Disease/defence                    | 0.97               | 1.52                | 1.10               | 0.70               |
| 400065617    | Putative elongation factor 1-gamma-like  | Protein synthesis                  | 1.03               | 1.52                | 1.06               | 0.72               |
| 400036856    | Heat shock protein 70  | Disease/defence                    | 0.95               | 1.54                | 1.03               | 0.63               |
| 400007506    | Sucrose synthase   | Metabolism                         | 1.06               | 1.76                | 1.00               | 0.61               |
| 400069198    | Fructokinase-like  | Metabolism                         | 0.98               | 1.38                | 0.90               | 0.64               |
| 400001879    | Putative glycine-rich RNA binding protein-like   | Unclassified                       | 1.10               | 1.79                | 0.75               | 0.46               |
| 400073713    | Heat shock 70 kDa protein  | Disease/defence                    | 1.16               | 1.44                | 0.88               | 0.71               |
| 400009623    | Thioredoxin H-type 1   | Energy                             | 1.00               | 1.74                | 0.90               | 0.52               |
| 400084266    | Putative glycine-rich RNA-binding protein GRP1A-like   | Unclassified                       | 0.92               | 1.56                | 0.98               | 0.57               |
| 400016576    | Ran protein/TC4 protein-like   | Intracellular traffic              | 1.18               | 1.65                | 0.88               | 0.63               |
| 400063997    | Cytochrome c   | Energy                             | 1.08               | 1.66                | 0.86               | 0.56               |
| 400064031    | Stromal 70 kDa heat shock-related protein,<br>chloroplastic                                    | Disease/defence                    | 1.18               | 1.47                | 0.90               | 0.72               |
| 400071330    | Triosephosphate isomerase, cytosolic   | Energy                             | 1.01               | 1.63                | 0.94               | 0.58               |
| 400060214    | 60S acidic ribosomal protein P0  | Protein synthesis                  | 1.14               | 1.68                | 0.94               | 0.64               |
| 400039842    | Polyadenylate-binding protein  | Transcription                      | 1.08               | 1.49                | 0.95               | 0.69               |
| 400079172    | 14-3-3 protein   | Signal transduction                | 1.13               | 1.44                | 0.93               | 0.73               |
| 400042326    | Succinyl-CoA ligase alpha 1 subunit-like   | Energy                             | 1.08               | 1.61                | 0.91               | 0.61               |
| 400069575    | 40S ribosomal protein S3a  | Protein synthesis                  | 1.16               | 1.61                | 0.92               | 0.66               |
| CLUSTER VI   |  |                                    |                    |                     |                    |                    |
| 400083063    | Ribulose biphosphate carboxylase large chain   | Energy                             | 0.98               | 0.55                | 0.64               | 1.13               |
| CLUSTER VII  |  |                                    |                    |                     |                    |                    |
| 400064270    | Peroxidase   | Disease/defence                    | 0.72               | 0.56                | 1.02               | 1.30               |
| 400076546    | Ribosomal protein L3-like  | Protein synthesis                  | 0.70               | 0.47                | 1.08               | 1.59               |
| 400007177    | Calreticulin   | Protein destination and<br>storage | 0.84               | 0.66                | 0.95               | 1.22               |
| 400063101    | Putative 40S ribosomal protein S19-like  | Protein synthesis                  | 0.87               | 0.56                | 0.96               | 1.50               |
| 400049256    | Chloroplast RuBisCo activase   | Signal transduction                | 0.72               | 0.05                | 0.70               | 9.56               |
| 400010497    | Plasma membrane ATPase   | Transporters                       | 0.44               | 0.06                | 1.97               | 13.83              |
| 400076601    | Molecular chaperone Hsp90-1  | Disease/defence                    | 0.42               | 0.39                | 1.84               | 1.97               |
| CLUSTER VIII |  |                                    |                    |                     |                    |                    |
| 400012159    | Uncharacterized protein  | Unclassified                       | 1.15               | 0.26                | 1.97               | 8.82               |
| 400050235    | Malate dehydrogenase, chloroplastic  | Energy                             | 1.08               | 0.62                | 1.24               | 2.16               |
| 400076175    | 3-ketoacyl CoA thiolase 2  | Metabolism                         | 1.10               | 0.61                | 1.19               | 2.14               |
| 400013068    | Thioredoxin peroxidase 1   | Disease/defence                    | 1.13               | 0.59                | 1.36               | 2.62               |
| 400071776    | Bifunctional aspartate aminotransferase and<br>glutamate/aspartate-prephenate aminotransferase | Metabolism                         | 1.27               | 0.97                | 1.34               | 1.74               |
| 400039788    | Malate dehydrogenase   | Energy                             | 0.95               | 1.06                | 2.50               | 2.24               |
| 400028714    | Glutathione S-transferase L3   | Disease/defence                    | 0.87               | 1.22                | 2.35               | 1.68               |

(Continued)

Table 1. Continued.

| Accession | UniProt Blast description  | Functional classification          | Ratio<br>LamS/LamC | Ratio<br>TopS/ TopC | Ratio<br>LamC/TopC | Ratio<br>LamS/TopS |
|-----------|--|------------------------------------|--------------------|---------------------|--------------------|--------------------|
| 400056871 | Phosphoglycerate kinase, cytosolic   | Energy                             | 0.81               | 0.50                | 1.32               | 2.14               |
| 400034421 | Glutamine synthetase   | Metabolism                         | 1.08               | 0.49                | 1.16               | 2.59               |
| 400041810 | 14-3-3-like protein 16R  | Signal transduction                | 0.98               | 0.81                | 1.23               | 1.49               |
| 400047481 | Actin-101  | Cell structure                     | 0.94               | 0.55                | 1.28               | 2.20               |
| 400022467 | 5-methyltetrahydro-<br>pteroyltriglutamate-homocysteine<br>methyltransferase | Metabolism                         | 0.94               | 0.76                | 1.21               | 1.51               |
| 400074197 | Vacuolar H <sup>+</sup> -ATPase A1 subunit isoform                           | Transporters                       | 0.96               | 0.82                | 1.30               | 1.52               |
| 400073336 | Proline-tRNA ligase  | Protein synthesis                  | 0.94               | 0.54                | 1.20               | 2.09               |
| 400081472 | ADP/ATP carrier protein, mitochondrial                                       | Transporters                       | 1.01               | 0.51                | 1.20               | 2.38               |
| 400043456 | Probable voltage-gated potassium channel subunit<br>beta                     | Transporters                       | 0.86               | 0.56                | 1.43               | 2.17               |
| 400052308 | Putative CBS domain-containing protein CBSX3,<br>mitochondrial               | Unclassified                       | 0.80               | 0.65                | 0.96               | 1.18               |
| 400020969 | Elongation factor 1-alpha  | Protein synthesis                  | 0.95               | 0.52                | 1.03               | 1.88               |
| 400024982 | Proteasome subunit alpha type  | Protein destination and<br>storage | 0.89               | 0.17                | 1.15               | 5.90               |
| 400063205 | Betaine aldehyde dehydrogenase   | Metabolism                         | 0.95               | 0.46                | 1.05               | 2.15               |
| 400069333 | 40S ribosomal protein SA   | Protein synthesis                  | 0.94               | 0.25                | 0.96               | 3.65               |
| 400083968 | 40S ribosomal protein S3a  | Protein synthesis                  | 0.77               | 0.16                | 1.37               | 6.50               |
| 400032862 | Succinate dehydrogenase [ubiquinone] iron-sulfur<br>subunit, mitochondrial   | Energy                             | 0.86               | 0.57                | 1.12               | 1.67               |
| 400012205 | Uncharacterized protein  | Unclassified                       | 0.99               | 0.42                | 1.14               | 2.66               |
| 400080547 | Adenosine kinase 2   | Signal transduction                | 0.91               | 0.58                | 1.09               | 1.72               |
| 400055490 | Adenosine kinase isoform 1T-like protein                                     | Signal transduction                | 0.81               | 0.46                | 1.09               | 1.92               |
| 400073707 | 40S ribosomal protein S12  | Protein synthesis                  | 0.74               | 0.39                | 1.13               | 2.12               |
| 400051218 | Thaliana 60S ribosomal protein L7  | Protein synthesis                  | 0.76               | 0.34                | 1.18               | 2.67               |
| 400030676 | Hyoscyamine 6-beta-hydroxylase-like protein                                  | Secondary metabolism               | 0.84               | 0.53                | 1.22               | 1.92               |

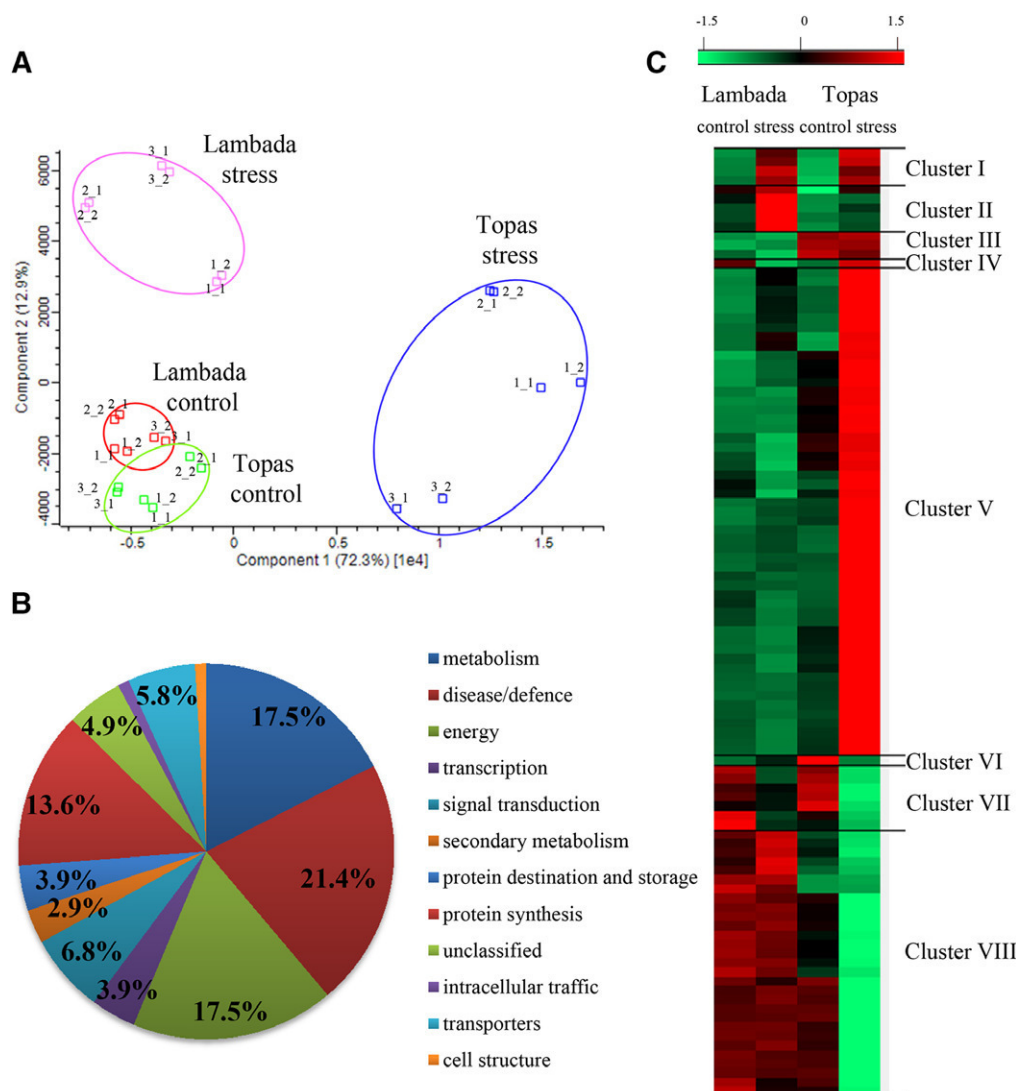
Further details related to protein identification and quantification are provided in Supporting Information, Table S2. Accession numbers are given without the PGSC003DMT prefix. Lam, lambda; Top, topas; C, control; S, stress.

(FK, sucrose synthase). The cluster also harbored proteins involved in the abiotic or biotic stress response, including three peroxidases, thioredoxin, several heat shock proteins, and glutathione S-transferases. Other members were associated with “protein synthesis” (elongation factors, 40S ribosomal proteins). A small proportion of proteins were involved in “secondary metabolism”, “signal transduction”, or “transcription”. Cluster VII contained proteins which were decreased in abundance in both cultivars as a result of N deficiency; namely calreticulin, peroxidase, ribosomal proteins, RuBisO activase, and a plasma membrane ATPase. Cluster VIII featured proteins which were strongly decreased in abundance in cv. “Topas” under the low N regime. It was the second largest cluster, in which 29 proteins were grouped. Prominent among these were proteins involved in “metabolism” (GS, betaine aldehyde dehydrogenase, a bifunctional aspartate aminotransferase/glutamate/aspartate-prephenate aminotransferase, 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase), “energy” (malate dehydrogenase, MDH), phosphoglycerate kinase and succinate dehydrogenase, “protein synthesis” (ribosomal proteins), “signal transduction”

(adenosine kinase, 14-3-3 protein), and three transporters (vacuolar H<sup>+</sup>-ATPase, voltage-gated potassium channel, ADP/ATP carrier protein).

### 3.2. Metabolic Pathways Affected by Nitrogen Deficiency

The metabolic processes affected by N deficiency were visualized in both cultivars by applying VANTED software (Figure 3 and Supporting Information, Figure S1). The highest number of differentially abundant proteins was assigned to the tricarboxylic acid cycle (TCA) and glycolysis/gluconeogenesis. The TCA enzymes malate dehydrogenase, succinyl-CoA ligase, and citrate synthase were more abundant in cv. “Topas” than in cv. “Lambada”, although succinate dehydrogenase was not. We identified three forms of MDH, two of them were predicted to have a mitochondrial localization. Interestingly, one of them was highly abundant in cv. “Topas” under N deficiency and the second was twofold higher in cv. “Lambada” under both stress and control conditions. The third form was predicted to be localized



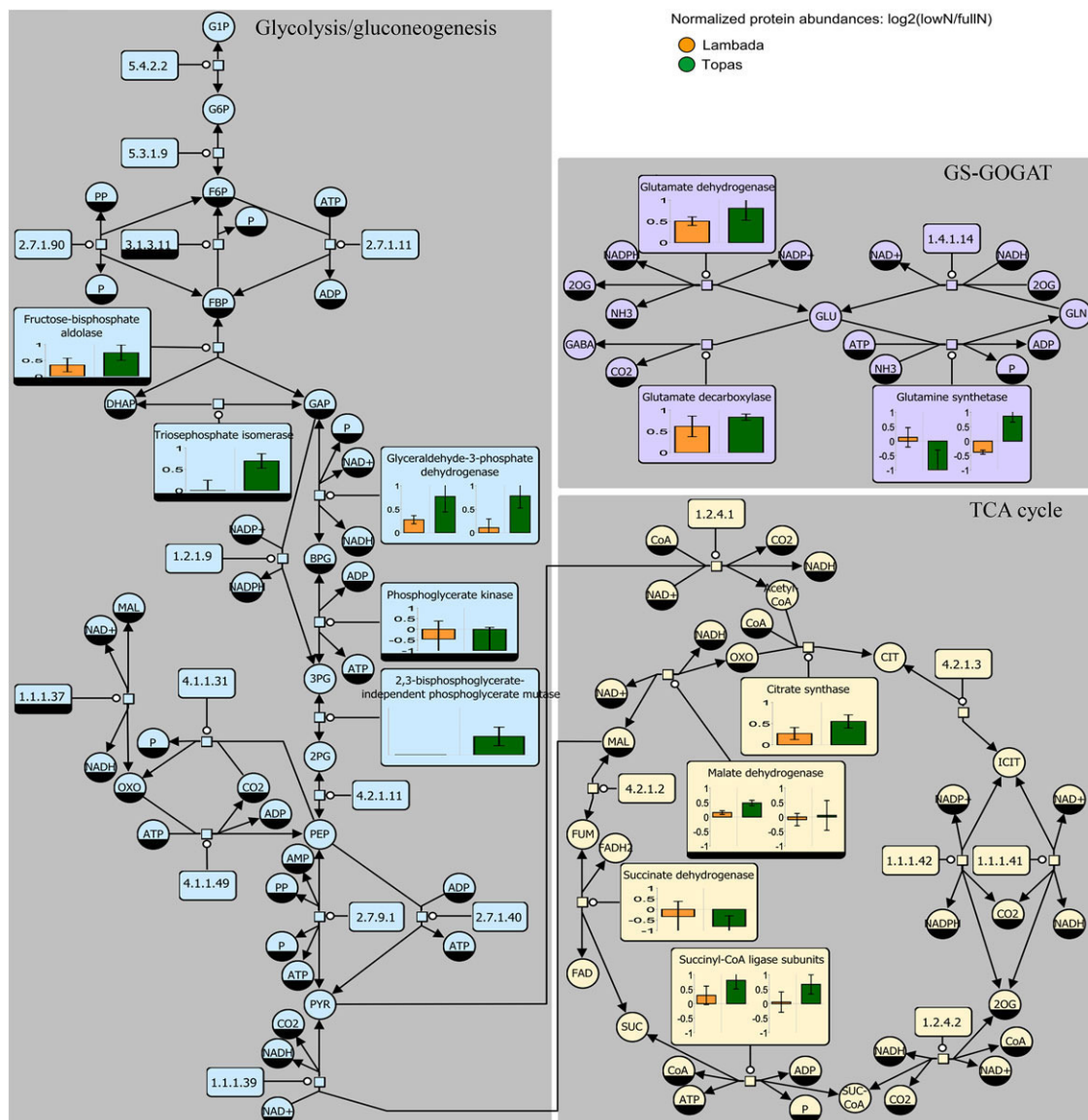
**Figure 2.** Differentially abundant proteins. A) LC-MS based protein profiling of the two cultivars under sufficient and deficient N as analyzed by PCA. Each symbol represents one analysis (two technical replicates of three independent harvests, marked by sample ID). Each biological treatment is denoted by different color. B) Functional classification of the set of differentially abundant proteins. C) The protein abundance profiles of N deficiency responsive proteins in cv. “Lambada” and cv. “Topas”. Eight clusters (I–VIII) were defined. Relative abundances are represented by a color-based scale (–1.5 to 1.5-fold) obtained after Z-score normalization. The listing of the proteins is the same as that presented in Table 1.

in plastids/chloroplasts and was also accumulated in cv. ‘Lambada’. Four glycolysis/gluconeogenesis enzymes were identified: three (triosephosphate isomerase, 2,3-bisphosphoglycerate-independent phosphoglycerate mutase, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH)) were highly abundant by N deficiency in cv. ‘Topas’, whereas phosphoglycerate kinase was less abundant in both cultivars.

In addition, two enzymes were assigned to the Calvin cycle (Supporting Information, Figure S3). Plastidic fructose biphosphate aldolase was more abundant in both cultivars by N deficiency but to the higher extent in cv. ‘Topas’ and ribulose biphosphate carboxylase (RuBisCo) large chain was highly abundant only in cv. ‘Topas’ by N sufficient conditions.

Other differentially abundant proteins were associated with amino acid synthesis, protein synthesis, sucrose breakdown and the GS/GOGAT pathway (Figure 3 and Supporting Information, Figure S3). Two GS isozymes exhibited a rather different pattern of accumulation (see subsequent paragraph). The content of glutamate dehydrogenase increased in response to N deficiency in both cultivars, but to a greater extent in cv. ‘Topas’. Further differentially expressed proteins were linked to amino acid synthesis. A bifunctional aspartate and glutamate/aspartate-prephenate aminotransferase was accumulated to a greater degree in cv. ‘Lambada’ than in cv. ‘Topas’, in which its abundance remained unaffected by the treatment. A similar situation applied to phosphoserine aminotransferase. Serine hydroxymethyltransferase,





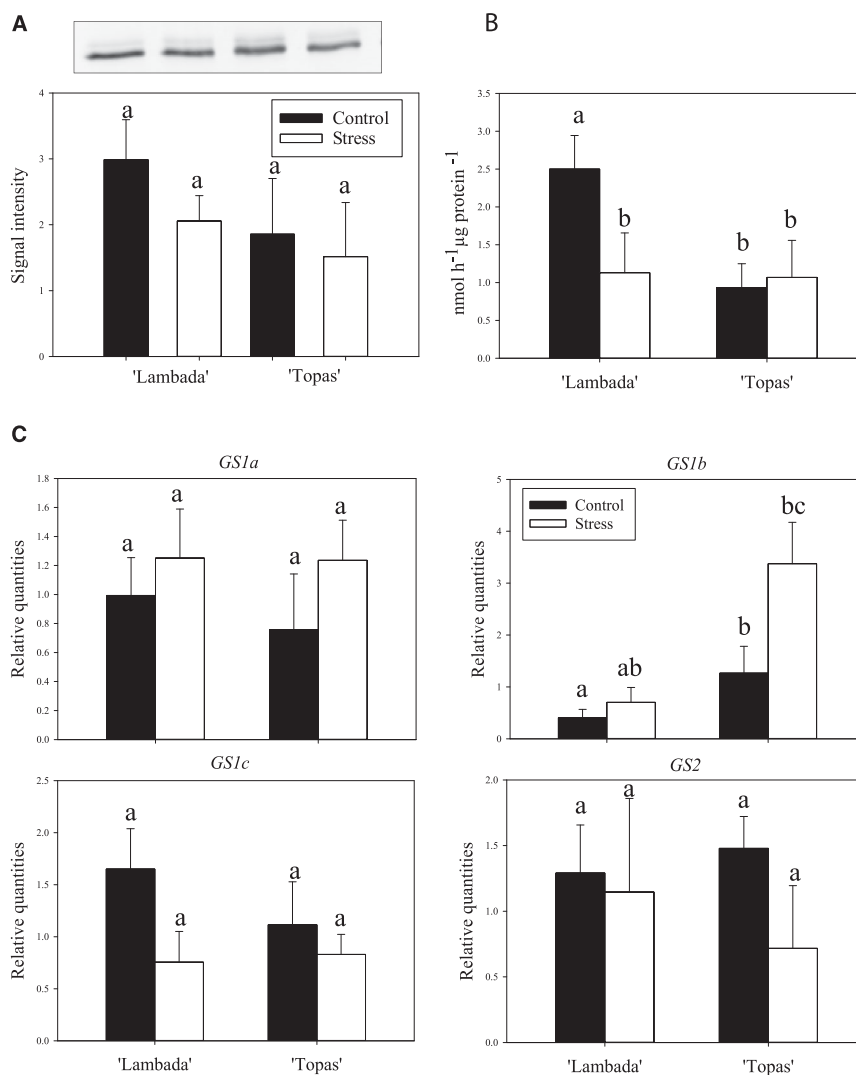
**Figure 3.** Integrated visualization of metabolic processes related to nitrogen deficiency in a metabolic network represented in Systems Biology Graphical Notation (from MetaCrap database). Reactions are catalyzed by enzymes (represented by macromolecules and labeled with EC numbers as unique identifiers, rectangles) converting metabolites (represented by the simple chemicals, circles). Normalized protein abundances ( $\log_2(\text{control}/\text{stress})$ ) are presented in bar charts (cv. “Lambada” orange, cv. “Topas” green). The most important pathways which were observed to differ between both genotypes are presented.

which catalyzes the conversion of glycine to serine, was also more abundant in cv. “Topas” in the low N regime.

### 3.3. Glutamine Synthetase Differs in Abundance in Tolerant and Sensitive Genotype

The potato genome harbors three genes encoding GS1, namely PGSC0003DMT400034421, 400037822, and 400060733; here, the three isoforms have been labeled GS1a, GS1b, and GS1c, respectively, and one encoding GS2. Both GS1a (cluster VIII) and GS1c (cluster V) differed in abundance between the two

cultivars in response to N deficient growing conditions: while GS1a was more abundant in cv. ‘Lambada’ during stress, GS1c was decreased in this cultivar but strongly induced in cv. ‘Topas’. The protein abundance pattern of GS isoforms clearly differed between cultivars pointing toward different mechanisms of response to N limitation. Additional experiments were performed to validate this observation. An immunoblotting experiment revealed that N deficiency significantly reduced GS abundance in cv. “Lambada” but not in cv. “Topas”, while the overall level of GS present in cv. “Lambada” under N sufficiency was higher than in cv. “Topas”; this latter difference disappeared under N deficiency conditions (Figure 4A). GS activity confirmed that “Lambada” had low activity, while cv. “Topas” was unaffected by the



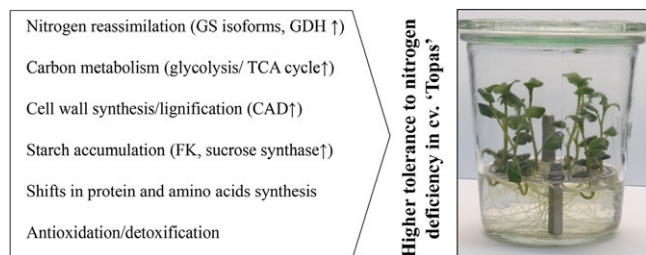
**Figure 4.** Glutamine synthetase in nitrogen deficient roots. A) The abundance of GS in the roots of plants exposed to N deficiency. Immunodetection of GS revealed two bands in the size of about 40 kDa (upper one, GS2; lower one, GS1 isoform). Quantification of fluorescent signal coming from lower band (GS1) is presented below the picture. B) The effect of N availability on GS activity. Different letters above the bars indicate values which differed significantly from one another ( $p < 0.05$ ). C) The effect of N availability on the transcript abundance of the three GS1 isoforms and GS2. Bars marked with a different letter indicate abundances which differed from one another significantly ( $p < 0.05$ ).

N regime (Figure 4B). Their similar molecular weight (about 40 kDa) meant that the two GS1 isoforms were indistinguishable based on their electrophoretic separation. Thus a qRT-PCR assay was performed to reveal the behavior of the two isoforms (Figure 4C). Despite a high standard deviation between the biological replicates, a clear difference in the response was observed as a result of N deficiency. The experiment showed that in N deficient medium, the GS1a transcript was higher in the roots of both cultivars than in N sufficient medium. In contrast, the level of GS1c transcripts was higher in the roots of both cultivars (but especially in cv. "Lambada") in N sufficiency. Meanwhile the level of GS1b transcription increased only marginally in the roots of cv. "Lambada" in response to N deficiency, whereas it experienced a ~2.5 fold induction in the roots of cv. "Topas". GS2 transcription was largely unaffected by N deficiency

in cv. "Lambada", but was down-regulated by ~1.5 fold in cv. "Topas".

#### 4. Discussion

The availability of N has long been known to exert a major influence on the expression of crop yield potential, but as yet rather little attention has been focused on the use of proteomic profiling to reveal the key proteins determining ability of a crop to tolerate N deficiency. Here, a proteomic contrast was drawn between two potato cultivars known to differ with respect to their ability to thrive under N limiting conditions. An in vitro cultivation system was adopted to remove the extraneous influences of soil, water, and climate, an approach which has been recently



**Figure 5.** Summary of the processes proposed to be involved in the adaptation of tolerant potato cv. “Topas” to a reduced supply of N. Proposed plant response is based on the proteome changes summarized in Table 1 and Figure 3.

proposed as a means of evaluating the response to variation in the N supply.<sup>[30,47]</sup> Two potato cultivars “Lambada” and “Topas” were selected exhibiting contrasting response to nitrogen deficiency according to the MFVS values calculated for previously published data (Supporting Information, Figure S1).<sup>[30–32]</sup> When cvs. “Lambada” and “Topas” were included in a respective in vitro screening of 14 starch potato cultivars both varieties consistently differed in their response to N-deficiency as expressed by the MFVS, however at reduced extent.<sup>[33]</sup> Here, samples were collected before the symptoms of the deficiency appeared to avoid secondary stress effects; however, after prolonged cultivation symptoms were observed and became more pronounced with increasing culture duration.<sup>[30–33]</sup> Evidence was assembled to show that not only do these two cultivars differ at the physiological level,<sup>[31]</sup> but also with respect to their biochemical response to N deficiency. The conclusion was that the more tolerant is better able to regulate several key metabolic pathways (**Figure 5**). Despite the gaps in the metabolic pathways unfilled by protein abundance data, it is possible to generate testable hypotheses regarding the nature of the molecular mechanisms which underpin the adaptation of potato to soils lacking adequate N.

#### 4.1. Metabolic Pathways Affected by N Deficiency

##### 4.1.1. The GS/GOGAT Pathway

Plants assimilate ammonium mainly through the action of the GS/GOGAT pathway, with a possible contribution of glutamate dehydrogenase.<sup>[48]</sup> Our study shows evidences that abundance of GS1 isoforms (GS1a and GS1c) was affected by N availability in a cultivar-dependent manner (Figure 3). GS assimilates ammonium into glutamine, which is essential for synthesis of all N containing compounds.<sup>[49]</sup> In barley, the abundance of GS1 has been shown to rise in plants exposed to sub-optimal levels of N for an extended period,<sup>[29]</sup> as also is the case during the early stages of N deprivation in *A. thaliana*<sup>[50]</sup> and after the resupply of N to N-starved maize.<sup>[51]</sup> Evidence for the evolution of distinct functions for GS1 isoforms has been obtained for a range of plant species.<sup>[52,53]</sup> In rice, for example, GS1;2 is responsible for the primary assimilation of N, whereas GS1;1 acts to remobilize it from senescing tissue.<sup>[54]</sup> At the level of transcript abundance, in addition to the predicted differences with respect to GS1a and

GS1c, it was also possible to detect a differential effect for GS1b, which was not reflected at the protein level, perhaps because of its low abundance. We could not find correlation between RNA level and protein abundance. This can be however explained by transcriptional regulation as well as protein degradation processes as described before.<sup>[55,56]</sup>

Glutamate dehydrogenase is involved in production of 2-oxo-glutarate from glutamate during a stress episode and senescence,<sup>[57,58]</sup> but it may also provide an alternative to the GS/GOGAT pathway to assimilate ammonium during a stress episode.<sup>[59]</sup> Here, the abundance of glutamate dehydrogenase was found to have increased in the roots of plants exposed to N deficiency (in cv. “Lambada” by some 1.5 fold and in cv. “Topas” by about twofold). This finding suggests that after 7 days of treatment both genotypes are similarly suffering from malnutrition with effects on the nitrogen assimilation process.

##### 4.1.2. Carbon Metabolism

Carbon compounds act as a source of energy and provides skeleton for assimilation of ammonium in the form of amino acids. Furthermore, the carbon metabolism of plants is strongly affected by N deficiency.<sup>[43]</sup> Four enzymes involved in glycolysis/gluconeogenesis were differentially abundant (as they all catalyze a reversible reaction, it was not possible to distinguish between these two pathways). Triosephosphate isomerase, 2,3-bisphosphoglycerate-independent phosphoglycerate mutase and GAPDH showed increased abundance in roots of N-stressed cv. “Topas”, while phosphoglycerate kinase was reduced in both cultivars. Phosphoglycerate kinase and GAPDH were both reduced in the leaves of cv. “Lambada” by N deficiency.<sup>[60]</sup> GAPDH levels increased in the leaves of cv. “Topas” by N-deficiency<sup>[60]</sup> and also in an N deficiency tolerant rice variety.<sup>[61]</sup> At the transcriptional level, N deficiency in the maize leaf causes low abundance of the genes encoding several glycolytic enzymes,<sup>[7]</sup> while in the roots, phosphoglycerate mutase exhibited increase abundance once N starvation has been relieved.<sup>[51]</sup>

Carbon skeletons in form of ketoacids are required for the assimilation of inorganic N.<sup>[62]</sup> Here, the abundance of three enzymes of the TCA cycle (MDH, succinyl-CoA ligase, and citrate synthase) increased in cv. “Topas” in response to the N stress. Alike, MDH was found to decrease in leaves of cv. “Topas” in N deficiency treatment.<sup>[60]</sup> Interestingly, in cv. “Lambada” another MDH species showed higher abundance under both N regimes. MDH is not only involved in the oxidation of malate in the TCA cycle but also in the reverse reaction during the conversion of glycine to serine for providing the glycine decarboxylase with  $\text{NAD}^+$ .<sup>[63]</sup> Prior analyses in maize have shown that in the shoot, N deficiency suppresses the activity of several TCA enzymes and the accumulation of organic acids; the conclusion was that this reflected a reduced allocation of assimilated carbon into amino acids.<sup>[7,64]</sup> In the rice root, however, N deficiency enhances the content of succinyl-CoA ligase,<sup>[65]</sup> while in barley, aconitase activity has been shown to be increased by a prolonged period of N starvation.<sup>[29]</sup> The apparent role of the TCA cycle in sustaining root metabolism under N deficient conditions is probably re-

lated to its involvement in energy transduction and ATP generation, but a contribution may also derive from the increased demand for carbon skeletons required for the synthesis of phenolic compounds.

#### 4.1.3. Amino Acid Metabolism

Decrease in the amino acids level is expected in N-deficient plants. However, the response of single amino acids seems to be quite individual depending on plant and experimental conditions.<sup>[7,66–68]</sup> The abundance of several enzymes involved in amino acid metabolism was altered by N deficiency in both potato cultivars, but there was no general decreasing tendency. Adenosylhomocysteinase, involved in synthesis of homocysteine (the precursor of methionine and cysteine), was accumulated in the cv. “Topas” root, as it is in the root of N-starved barley.<sup>[29]</sup> At the same time there was a decrease in the abundance of 5-methyltetrahydropteroyltriglutamate–homocysteine that synthesizes methionine from homocysteine. The abundance of this enzyme is reduced by N-stress in the wheat leaf.<sup>[69]</sup> The root content of phosphoserine aminotransferase was raised more substantially by N deficiency in the roots of cv. “Lambada” than in those of cv. “Topas”; this enzyme, which is involved in the synthesis of L-serine from 3-phospho-D-glycerate, is highly abundant in rapidly proliferating tissue such as the root tips of both *A. thaliana* and soybean.<sup>[70,71]</sup> Increase in abundance of the bifunctional enzyme aspartate aminotransferase/glutamate/aspartate-prephenate aminotransferase was observed in cv. “Topas”. Some plant aspartate aminotransferases also display prephenate aminotransferase activity, which catalyzes reversible transamination between glutamate or aspartate and prephenate. As a result, oxoglutarate or oxaloacetate and arogenate are formed, representing a key step during aromatic amino acid synthesis in the plastid.<sup>[72]</sup>

#### 4.1.4. Other Proteins Differentially Abundant During Nitrogen Limitation

N deficiency has far-reaching consequences for the machinery of protein synthesis and degradation. Here, ten different cytosolic ribosomal protein species and three elongation factors were spread through the clusters V, VII, and VIII. Generally, they were rather stable in cv. “Lambada” and decreased/increased in cv. “Topas”. Numerous studies on ribosomal proteins revealed the presence of multigene families with members showing both overlapping and differential pattern of regulation.<sup>[73]</sup> Transcripts for components of protein synthesis were previously shown to increase in N-replete *Arabidopsis*.<sup>[24]</sup> Protease activity is documented to be influenced by the N supply.<sup>[51,74]</sup> The abundance of the component of ubiquitin/26S proteasome system (using ubiquitin as a tag for selective protein breakdown) strongly decreased in cv. “Topas”. Protein degradation increased in cv. “Lambada”, through the involvement of aspartic protease. This is in agreement with the crude protein yield, which decreased in cv. “Lambada” and was rather stable in cv. “Topas”. In previous study several proteases were reduced in the leaves of cv. “Lambada” and leucine aminopeptidase increased strongly in cv. “Topas”.<sup>[60]</sup>

A major group of differentially abundant proteins not assigned to a defined metabolic pathway comprised various defense and antioxidation/detoxification related enzymes. Several isoforms of glutathione-S-transferase, an enzyme which links glutathione to certain toxic substances generated during oxidative stress, were more abundant in the roots of N-deficient cv. “Topas”. A number of peroxidase isoforms was assigned to Clusters I, II, V, and VII. Both glutathione-S-transferase and peroxidases likewise accumulated strongly in the leaf of rice plants exposed to N deficiency.<sup>[75]</sup> The protective enzyme catalase accumulated more strongly in cv. “Lambada”. PR10 protein, which participate in general defense mechanisms<sup>[76]</sup> was highly abundant in cv. “Topas”. Catalase, peroxidase, and PR10 were among proteins with the highest contribution to the separation along principal components. The conclusion is that defense and antioxidation form part of the plant survival machinery and that detoxification in particular has importance in the response to N deficiency.

An increase in the content of phospho-2-dehydro-3-deoxyheptonate aldolase, the first enzyme in the shikimate pathway, was recorded. The shikimate pathway might be a source of precursors for various phenolics, which represent a sink for excess carbon present during an episode of N deficiency.<sup>[77]</sup>

Two isoforms of adenosine kinase, enzyme which converts adenosine into adenosine monophosphate using ATP, decreased in cv. “Topas”. This was also reported for rice.<sup>[65]</sup>

Enzymes participating in starch metabolism, sucrose synthase, and FK were highly abundant in the roots of cv. “Topas” than in cv. “Lambada” by N deficiency. In both photosynthetic and sink tissues, sucrose is first cleaved by sucrose synthase or invertase to form a pool of hexose. Fructose is subsequently phosphorylated to fructose-6-phosphate by FK and used as the substrate for respiration and biosynthesis of starch and other complex carbohydrates.<sup>[78]</sup> In *Arabidopsis* low N initiates pathways directing accumulation of sugars and starch in shoots and translocation of sucrose to the root.<sup>[79]</sup> CAD was one of the proteins having the highest impact on the separation along principal components. This is a key enzyme of lignin biosynthesis, which proceeds via the phenylpropanoid pathway. In barley, CAD has been shown to accumulate strongly in response to a short episode of N-starvation.<sup>[29]</sup> The indications are that the lignification of the potato root may facilitate the adaptation to a limited level of N.

Accumulation of RuBisCo and chloroplastic enzymes in leaves is well known. However, the detection of these enzymes in roots was surprising, because it is a non-photosynthetic organ. RuBisCo was also detected in the roots of *Arabidopsis* and this was explained by its possible role in CO<sub>2</sub> scavenging.<sup>[80,81]</sup> In our study, the identification of RuBisCo is probably due to the influence of experimental conditions. In the in vitro system, roots despite of an additional coverage are imposed on residual light, which cause formation of chloroplasts as described elsewhere.<sup>[82,83]</sup>

In summary, N deficiency differentially modified the root proteome of the two potato cultivars leading to some distinct metabolic responses. A number of candidate proteins and processes relevant to the response of potato to N deficiency have been identified. Considering the proteins with the highest contribution to the variance between cvs. “Topas” and “Lambada” by low N regime suggests that N reassimilation, shifts in the C:N



balance mediated by the increase in the glycolysis and TCA cycle, changes in the cell wall, accumulation of starch as well as antioxidation and detoxification mechanisms leading to the better tolerance of cv. "Topas" to N deficiency (Figure 5). Reassimilation of N provides nutrient for the N compounds synthesis in newly proliferating tissues that might be a key factor for the stable biomass production. Growth of potato is dependent on the form of mineral N applied.<sup>[84]</sup> Thus, it will be interesting to study the proteomic response of cultivars to ammonium and nitrate deficiency independently. Additional studies including enzyme activity assays, transcriptomics, and metabolomics as well as consideration of further genotypes and field trials are necessary to validate the results and to gain deeper knowledge about biochemical and physiological mechanisms of N efficiency.

## Abbreviations

CAD, cinnamyl alcohol dehydrogenase; cv, cultivar; FK, fructokinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GS, glutamine synthetase; GS/GOGAT, glutamine synthetase/glutamate synthase; MDH, malate dehydrogenase; MFVS, membership function value of stress tolerance; N, nitrogen; PCA, principal component analysis; TCA, tricarboxylic acid cycle

## Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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## Keywords

in vitro; label-free LC-MS; mass spectrometry; metabolic pathways nitrogen use efficiency

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# Chapter II

# Chapter 13

## Mini-Scale Isolation and Preparation of Plasma Membrane Proteins from Potato Roots for LC/MS Analysis

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### Abstract

Plasma membrane (PM) proteins are of special interest due to their function in exchanging material and information with the external environment as well as their role in cellular regulation. In quantitative proteomic studies PM proteins are underrepresented mostly because they constitute only small percent of all membrane proteins. Strong demand is placed on plasma membrane enrichment methods. For decades two-phase partitioning Dextran T500/PEG 3350 isolation protocols were applied for many different animal and plant species and also a variety of tissue types. The typical quantity of material used in the enrichment protocols is 10–30 g of fresh weight. The main difficulty of working with in vitro cultivated plants is the low amount of material, especially when roots are examined. In addition, roots are frequently characterized by low protein concentrations. Our protocol established for roots of in vitro cultivated potato plants is adjusted to amounts of fresh weight not exceeding 7.5 g and allows studying the plasma membrane proteome by LC-MS.

**Key words** Plasma membrane, Two-phase partitioning, Plants, Potato, Roots, Enrichment, Digestion, Mass spectrometry

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### 1 Introduction

The plasma membrane has two very important roles, first it acts as a barrier separating cell from its environment and second, it is a sensor for the environmental conditions, which transduces the signal to the inside of the cell [1, 2]. Plasma membrane proteins fulfill many different cellular functions including metabolite and ion transport, endocytosis and cell differentiation [3]. The plasma membrane is likely to be the most diverse cell membrane as its composition is not only organ and tissue specific but also strongly depends on developmental stage and environmental conditions [4]. In plants, signals related to biotic and abiotic stresses are processed in the plasma membrane causing significant alteration in protein composition and abundance. Better understanding of changes



occurring in the proteomic profile of the PM in response to environmental stimuli will help to derive novel concepts not only to generate more resistant plants but also to develop stress and disease biomarkers [5, 6].

In quantitative proteomic studies PM proteins are underrepresented mostly because the PM constitutes only 5–20% of the total membranes [7]. The aqueous two-phase partitioning method, which separates membranes according to their affinity to two immiscible polymers, was developed and improved over decades for efficient isolation of plasma membrane vesicles [8]. Together with the Brij-58 treatment this method results in the highly enriched plasma membrane fraction free of contaminating soluble and loosely bound proteins [9]. The majority of the current available protocols require large quantities of starting material ranging from 30 to 125 g to obtain a decent yield [7, 9–11]. This is especially difficult to obtain when working with plants cultivated *in vitro*. In our protocol established for potato roots steps were adjusted to allow working with amounts not exceeding 7.5 g.

Successful identification of plasma membrane proteins requires not only efficient isolation method. The difficulty of solubilization and mass spectrometry analysis lies in the nature of integral membrane proteins containing transmembrane domains composed of highly hydrophobic amino acids [10]. Commonly used in the gel-based approaches detergents that allow overcoming insolubility problem interfere with LC-MS by suppressing spectral signal and lowering chromatographic performance [12]. In our study proteins are solubilized in the buffer commonly used for the 2-D electrophoresis composed of urea, thiourea, and CHAPS. Quantification is performed using modified version of 2-D Quant Kit protocol, where the amount of protein is reduced to a minimum. Proteins are further digested using the in-filter-based method [13] allowing removal of undesirable compounds prior to trypsination. Peptides are further analyzed using nanoflow liquid chromatography coupled to a tandem mass spectrometer.

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## 2 Materials

### 2.1 *Enrichment of Plasma Membrane*

1. Homogenization Buffer: 50 mM MOPS, 5 mM EDTA, 330 mM sucrose, 10% (w/v) glycerol, 5 mM DTT, 5 mM ascorbate, 0.6% (w/v) PVPP, cOmplete Protease Inhibitor Cocktail Tablets (Roche Diagnostics, Germany), adjust to pH 7.5 with KOH. Prepare the buffer 1 day before and cool it overnight in the fridge. PVPP, DTT, ascorbate, and protease inhibitors should be added right before homogenization.
2. Plasma membrane Buffer: 330 mM sucrose, 0.1 mM DTT, 0.1 mM EDTA, 5 mM potassium phosphate buffer pH 7.8.

**Table 1**  
**Two-phase system composed of 6.2% Dextran T500, PEG 3350, 8 mM KCl, 5 mM potassium phosphate and 300 mM sucrose**

| Component               | Stock concentration | Mixture     |
|-------------------------|---------------------|-------------|
| Dextran T500            | 20% (w/w)           | 3.72 g      |
| PEG 3350                | 40% (w/w)           | 1.86 g      |
| Phosphate buffer pH 7.8 | 200 mM              | 300 $\mu$ l |
| KCl                     | 2 M                 | 48 $\mu$ l  |
| Sucrose                 | 1.6 M               | 2.25 ml     |
| Water                   |                     | To 9 g      |

Prepare aliquots and store it at  $-20^{\circ}\text{C}$ . Add DTT and EDTA before use.

- 20% (w/w) Dextran T500 stock solution (Pharmacosmos; Denmark). Prepare aliquots and store it at  $-20^{\circ}\text{C}$  (*see Note 1*).
- 40% (w/w) PEG 3350. Prepare aliquots and store it at  $-20^{\circ}\text{C}$ .
- 1.6 M sucrose.
- 2 M KCl.
- 200 mM potassium phosphate buffer pH 7.8.
- Two-phase system: 6.2% Dextran T500, 6.2% PEG 3350, 300 mM sucrose, 8 mM KCl, 5 mM potassium phosphate buffer pH 7.8. For one sample four systems 9 g each should be prepared. One day before PM enrichment mix all components in a glass beaker on a balance according to Table 1 and stir it for 5 min. You can mix each system separately but more reproducible results are obtained if a batch is prepared. To prevent phase separation divide the mixture into falcon tubes (9 g each) under constant stirring. Store the two-phase system overnight in the fridge (*see Note 2*).
- Brij-58 Buffer: 330 mM sucrose, 200 mM KCl, 5 mM phosphate buffer pH 7.8, 0.2% (w/v) Brij-58. Prepare 2% stock solution of Brij-58 and 2 M KCl. Mix the components shortly before use.

## 2.2 Protein Precipitation

- Ice-cold methanol.
- Ice-cold chloroform.
- Ultrapure water,  $4^{\circ}\text{C}$ .

### 2.3 Protein Resuspension and Quantification

1. Resuspension Buffer: 7 M urea, 2 M thiourea, 5 mM DTT, 2% CHAPS, adjust to pH 8.0.
2. 2-D Quant Kit (GE Healthcare).

### 2.4 Solutions for Protein Digestion

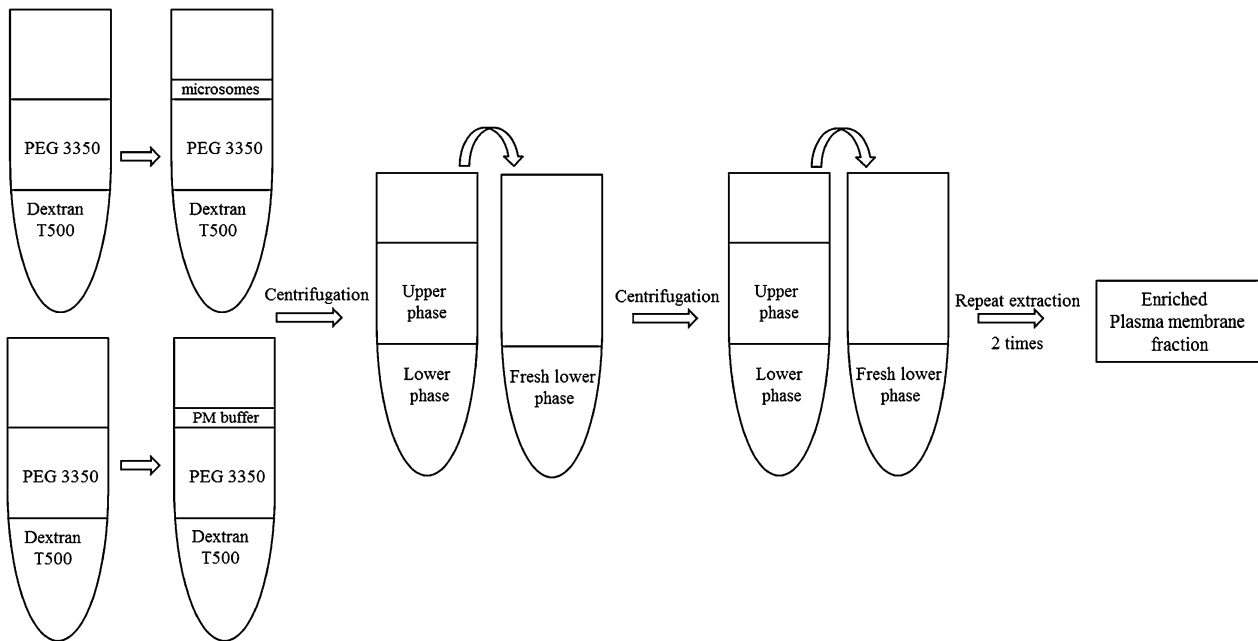
1. Microcon-10 kDa Centrifugal Filter Unit with Ultracel-10 membrane (Merck Millipore; USA).
2. Urea Buffer: 7 M urea, 100 mM Tris-HCl, 5 mM EDTA, adjust to pH 8.0. Buffer can be stored at room temperature.
3. Solution 1: 20 mM DTT in urea buffer. Prepare 1 M stock solutions, aliquot and store at  $-20^{\circ}\text{C}$ .
4. Solution 2: 80 mM Iodoacetamide in urea buffer. Prepare 1 M stock solutions, aliquot and store at  $-20^{\circ}\text{C}$ . Iodoacetamide is light sensitive and should be kept in the dark.
5. Solution 3: 50 mM ammonium bicarbonate.
6. Trypsin Gold, Mass Spectrometry Grade (Promega, USA). Prepare 200 ng/ $\mu\text{l}$  stock solution, aliquot and store at  $-20^{\circ}\text{C}$ .

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## 3 Methods

### 3.1 Enrichment of the Plasma Membrane from Potato Roots

1. Homogenize 5–7.5 g of roots with a kitchen blender in 100 ml of ice-cold homogenization buffer. Use 20 s pulses to avoid overheating of sample (*see Note 3*).
2. Filter the slurry through two layers of Miracloth and centrifuge it for 15 min at  $10,000 \times g$  at  $4^{\circ}\text{C}$ .
3. Ultracentrifuge the supernatant for 50 min at  $50,000 \times g$  at  $4^{\circ}\text{C}$  (e.g., Beckman, Fixed angle rotor type 70 Ti).
4. Resuspend the pellet with a glass tissue homogenizer in about 2.8 ml of plasma membrane buffer. The end mass of the microsomal fraction should not exceed 3 g. The membrane homogenate can be directly loaded onto the two-phase system or frozen in liquid  $\text{N}_2$  and stored at  $-80^{\circ}\text{C}$ .
5. Load exactly 3 g of the microsomal suspension onto the top of the Dextran T500/PEG 3350 two-phase system prepared the day before (Fig. 1). Mix by inversion 30 times (Do not vortex!). Mix the second two-phase system with 3 g of plasma membrane buffer (*see Note 4*).
6. Spin both the tubes at  $1,000 \times g$ ,  $4^{\circ}\text{C}$  for 7 min.
7. On a balance, remove about 90% of upper phase from the second system and replace it with exactly the same amount of upper phase one. Mix it by inversion 30 times. Pretreat a third prepared two-phase system with 3 g of plasma membrane buffer.
8. Spin both the tubes at  $1,000 \times g$ ,  $4^{\circ}\text{C}$  for 7 min.



**Fig. 1** Plasma membrane enrichment workflow

9. Repeat these steps with the third and fourth systems. The final fourth upper phase contains the enriched plasma membrane fraction.
10. Dilute the PM fraction at least 1:4 with plasma membrane buffer. For recovering the endomembranes dilute the first lower phase 1:10 with plasma membrane buffer.
11. Spin it at  $100,000 \times g$ ,  $4^\circ\text{C}$  for 1 h (Beckman, Fixed angle rotor type 70 Ti).
12. Discard the supernatant. In order to remove contaminating soluble and loosely bound proteins resuspend the pellet with a glass tissue homogenizer in 8 ml Brij-58 buffer and incubate it for 10 min at RT.
13. Spin the tube at  $100,000 \times g$ ,  $4^\circ\text{C}$  for 1.5 h (e.g., Beckman, Fixed angle rotor type 70.1 Ti).
14. Resuspend the pellet in 150  $\mu\text{l}$  of plasma membrane buffer and freeze it in liquid  $\text{N}_2$  (*see Note 5*).

### 3.2 Delipidation of Membrane Proteins

1. Mix 100  $\mu\text{l}$  of sample with 200  $\mu\text{l}$  of ice-cold chloroform.
2. Add 400  $\mu\text{l}$  of ice-cold methanol and vortex shortly.
3. Add 1000  $\mu\text{l}$  of cold water, vortex and incubate on ice for 5 min.
4. Centrifuge at  $9,000 \times g$ ,  $4^\circ\text{C}$ , for 2 min.
5. Remove the upper phase carefully without disturbing the middle phase.

**Table 2**  
**Preparation of standard curve**

| Tube number                               | 0         | 1            | 2           | 3            | 4          | 5            |
|---|-----------|--------------|-------------|--------------|------------|--------------|
| Volume of BSA standard solution (2 mg/ml) | 0 $\mu$ l | 1.25 $\mu$ l | 2.5 $\mu$ l | 3.75 $\mu$ l | 5 $\mu$ l  | 6.25 $\mu$ l |
| Protein quantity                          | 0 $\mu$ g | 2.5 $\mu$ g  | 5 $\mu$ g   | 7.5 $\mu$ g  | 10 $\mu$ g | 12.5 $\mu$ g |

Note that each sample is prepared in duplicate

6. Add 500  $\mu$ l of methanol and vortex.
7. Centrifuge at maximal speed, 4 °C, for 5 min.
8. Discard the supernatant and dry the pellet on ice for 15 min (*see Note 6*).
9. Resuspend the pellet in 50  $\mu$ l of resuspension buffer, vortex and sonicate it for 5 min.
10. Incubate the samples at 30 °C with gentle shaking for 1 h.
11. Centrifuge the sample for 15 min at maximal speed and move the supernatant to a new reaction tube.

### 3.3 Protein Quantification with 2-D Quant Kit

1. Prepare the Color Reagent by mixing 100 parts of Color reagent A and one part of Color reagent B. For each single measurement 250  $\mu$ l is needed. Each sample should be measured in duplicate.
2. Use the bovine serum albumin (BSA) 2 mg/ml solution provided by the manufacturer for the standard curve. Set up 1.5 ml reaction tubes and pipette solution according to Table 2 (*see Note 7*).
3. Prepare tubes containing 1–2.5  $\mu$ l of sample. A test assay for any new sample type is necessary to ensure that the protein amount is sufficient to fall within the range of the standard curve.
4. Add 125  $\mu$ l of precipitant to each tube. Use a multipipette to assure accuracy of the assay. Vortex briefly and incubate at RT for 2–3 min.
5. Add 125  $\mu$ l of co-precipitant to each tube. Vortex shortly and centrifuge for 5 min at maximal speed.
6. To avoid resuspension of the pellet rapidly aspire the supernatant (*see Note 8*).
7. Add 25  $\mu$ l of cooper solution and 100  $\mu$ l of water. Vortex each sample about 10–20 s (*see Note 9*).
8. Centrifuge briefly to collect all liquid at the bottom of the tube.
9. Add 250  $\mu$ l of Color reagent to each tube, mix it by inversion and centrifuge shortly.

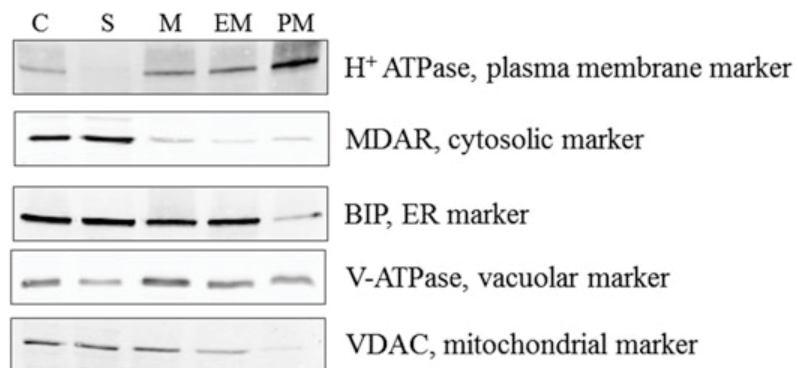
10. Incubate at room temperature for 20 min.
11. Shortly before the end of incubation time move 200  $\mu\text{l}$  of each sample to a 96-well plate.
12. Read the absorbance at 480 nm using water as a blank.

### **3.4 In-Filter-Based Digestion of Proteins**

1. Fill the YM-10 filter with 200  $\mu\text{l}$  of ultrapure water and centrifuge it at  $14,000 \times g$ , 5 min, at RT. Carefully remove remaining water (*see Note 10*).
2. Mix 5–10  $\mu\text{g}$  of PM protein with resuspension buffer to obtain 200  $\mu\text{l}$ . Fill the filter with protein solution.
3. Centrifuge until there is no more than 10–15  $\mu\text{l}$  of sample on the filter (about 45–60 min) (*see Note 11*).
4. Apply 100  $\mu\text{l}$  of urea buffer and centrifuge it for about 20–30 min. Repeat this step three times to remove detergents from the sample.
5. Fill the filter with 200  $\mu\text{l}$  of Solution 1. Incubate for 60 min with gentle shaking at 60 °C.
6. Cool down the sample at RT. Add 100  $\mu\text{l}$  of Solution 2. Incubate at 37 °C, 30 min in the darkness with gentle shaking (*see Note 12*).
7. Centrifuge until no liquid remains (about 40 min).
8. Apply 200  $\mu\text{l}$  of Solution 3 on the filter and centrifuge it for about 20 min. Repeat this step three times (*see Note 13*).
9. Mix 200  $\mu\text{l}$  of Solution 3 with Trypsin stock solution. The recommended ratio of Trypsin to protein is 1:50, so for 10  $\mu\text{g}$  of protein 1  $\mu\text{l}$  of Trypsin stock should be used.
10. Fill the filter with Trypsin solution and incubate it overnight at 37 °C (without shaking) (*see Note 14*).
11. Elute peptides into fresh Eppendorf tube by centrifuging for about 30 min.
12. Apply 50  $\mu\text{l}$  of Solution 3 on the filter and centrifuge it for about 10 min. Repeat this step three times to elute remaining peptides.
13. Dry the sample in the vacuum centrifuge for about 2 h.
14. Prior to mass spectrometric analysis resuspend the pellet in 1% Acetonitrile/0.1% formic acid/water (LC/MS grade) to obtain a concentration suitable for loading onto a reverse-phase column. Vortex and centrifuge for 5 min with maximal speed. Peptides can be analyzed using a nanoflow liquid chromatography coupled to a tandem mass spectrometer.

## 4 Notes

1. Dextran T500 contains 5–10% water, so the end concentration of your stock might not be 20%. By drying or lyophilizing small amounts (10–20 mg) of the stock solution in several replicates the exact concentration can be determined. For reproducible results use the same stock solutions for all the samples.
2. Given concentrations of PEG 3350, Dextran T500, and KCl were optimized for in vitro cultivated potato root tissue. In the literature concentrations used for various tissues and species vary from 6.2% to 6.5% Dextran and PEG and from 2 to 8 mM KCl. A series of test experiments covering concentration range of polymer from 6.0% to 6.5% and KCl from 1 to 8 mM is advised to provide appropriate yield and purity of the PM for specific tissues.
3. All the steps should be performed at 4 °C. All the beakers and centrifugal tubes should be pre-cooled in the fridge before starting the experiment.
4. Phase separation is strongly dependent on temperature. Keep all two-phase systems on ice. Do not overheat the samples during mixing. Every ten inversion cool the tubes down on ice or work in the cold room.
5. Efficiency of the plasma membrane enrichment can be tested by Western blot with marker antibodies for cellular compartments (for example H<sup>+</sup>-ATPase as PM marker, Fig. 2). Due to the low concentration of proteins in obtained membrane fraction a highly sensitive fluorescent detection method is advised.
6. The pellet can be dried in the vacuum centrifuge, but the time should be kept to a minimum. An overdried pellet is hard to resuspend and the protein yield is lower.



**Fig. 2** Purity of plasma membrane fraction was evaluated using Western blot with antibodies against compartment markers. *C* crude, *S* soluble, *M* microsomal, *EM* endomembrane, *PM* plasma membrane fraction



7. To assure better accuracy of the assay and reproducible results using Microman (Gilson) pipettes is recommended.
8. A peristaltic pump with a cut gel loading tip attached to the end of the tubing can be used to remove the supernatant. There should be no remaining liquid in the tube.
9. As the colorimetric reagent reacts with unbound cupric ions it is very important to add exactly the same amount of copper solution to each tube. Use a Microman (Gilson) pipette if available.
10. All steps requiring centrifugation should be performed at maximal  $14,000 \times g$ . High speed might destroy the filter and lead to samples loss. As the solubility of urea drops with temperature do not centrifuge at  $4^\circ\text{C}$ .
11. In the protocol putative times of centrifugation are given. It is recommended to check the level of liquid every 10–15 min. Avoid overdrying of the filter.
12. Iodoacetamide is light sensitive. Brownish coloration of IAA indicates decomposition and such solutions should not be used. To provide proper conditions for sample alkylation cover your Thermomixer with aluminum foil.
13. Urea in high concentration is known to suppress ionization in the ESI source. It is important to remove it from the sample prior to trypsination.
14. Trypsin is usually first choice enzyme for protein digestion. Positively charged amino acids arginine and lysine are under-represented and not equally distributed in the transmembrane helices [14]. To improve membrane protein analysis unspecific proteases like elastase can be additionally used.

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# Chapter III

**Plasma membrane proteome analysis of two divergently responding potato (*Solanum tuberosum* L.) cultivars reveals the role of aquaporins in the low N tolerance**

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## **Abstract**

While nitrogen deficiency is one of the major constraint for crop production, the excessive application of nitrogenous fertilizer is responsible for serious environmental pollution.

The potato cultivars Lambada and Topas are known to contrast with respect to their N deficiency response *in vitro*. The aim of the study was to elucidate differences in the abundance of transporters and channels taking part in the N uptake, by comparing the plasma membrane (PM) proteome of the contrasting cultivars under N deficiency. An aqueous two-phase partitioning method was applied to target the root PM proteome of plants grown *in vitro* under N deficient conditions. The resulting proteins were identified and quantified using label-free liquid chromatography-based mass spectrometry. Overall, 65 proteins were shown to be differentially abundant in the two varieties in response to N deficiency. Amongst those, considered to be involved in N deficiency tolerance, were a nitrate high-affinity transporter, a plasma membrane H<sup>+</sup>-ATPase and two members of the aquaporin family, AtPIP1;1 and AtPIP1;3. The knock out mutants of *AtPIP1;1* and *AtPIP1;3* showed a reduced nitrogen content and accumulation of phenylpropanoids in *Arabidopsis* under N deficiency. We conclude that aquaporins contributed to ability of the tolerant cultivar to grow under N deficiency by increasing root nitrogen acquisition.

**Key words:** aquaporins, aqueous two-phase partitioning, label-free LC-MS, mass spectrometry, nitrogen use efficiency, NRT2.1, plasma membrane H<sup>+</sup>-ATPase, proteomics.

## Introduction

The productivity of most field crops is sensitive to the availability of nitrogen (N) in the soil. Globally around 85-90 metric tons of nitrogenous fertilizer is added to arable soils each year, an amount predicted to more than double by 2050 [1]. The production of nitrogenous fertilizer is demanding of energy, and thus its use represents a significant cost component in modern crop production systems [2]. Mineral N sources, due to microbial transformations, are likely to be oxidized to nitrate, the result is the environmentally damaging leaching of nitrate into the ground water [3, 4]. Furthermore, ammonium salts and urea both release nitrous oxide gas, thought to be responsible for 7.5% of the calculated anthropogenic greenhouse effect [5]. Due to nitrate leaching, denitrification and ammonia volatilization it has been estimated that only 30-50% of fertilizer-supplied N is taken up by crop plants, a range which reflects the combined influence of soil type, environment and plant species [6]. N utilization efficiency is especially poor in the potato (*Solanum tuberosum* L.), on account of its shallow root system [7] exacerbated by the crop being frequently grown on highly leachable sandy soils [8]. Improving nitrogen use efficiency (NUE) has become important in plant breeding research [9-11]. The progress is being made towards this goal in sugarcane, maize and rice [12-14], but not in potato.

The plasma membrane (PM) acts both as a barrier between the cell and the external environment and as a sensor for the prevailing environmental conditions [15]. It controls cellular processes such as transport of metabolites and ions, cell differentiation and proliferation as well as endocytosis. The multiplicity of functions requires structural diversity but also various degrees of association. Some PM proteins are embedded in the membrane's lipid core, while others are located in its periphery reversibly interacting with the membrane [16]. Many of the proteins responsible for N uptake are localized within the root PM. Four distinct nitrate transporter families have been described, namely NRT1/PTR (nitrate 1/peptide transporters), NRT2 (nitrate transporter 2 family), CLC (chloride channels) and SLAC1/SLAH (slow anion channel-associated 1 homologs). Ammonium transport and uptake is conducted by the members of the AMT family (ammonium transporter) [17]. The ability of plants to exploit several sources of N has resulted in the elaboration of a complex network of signaling and regulatory proteins.

In plants, the interaction with various biotic and abiotic stress agents induces alterations in the protein composition of the PM [18]. A better understanding of the response of the PM proteome to various environmental stimuli – and in particular to N deficiency – is seen as providing a route to the development of stress tolerant plants [19, 20]. This is especially important in case of potato, which is an important source of food and industrial starch. When grown under *in vitro* conditions, contrasting responses to N deficiency of the two potato cultivars ‘Lambada’ (sensitive) and ‘Topas’ (tolerant), both at the physiological [21-23] and at the proteome [24, 25] level were observed. Here, we report on the effect of nitrogen deficiency on the root PM proteome composition of the two

potato cultivars and present candidate proteins playing a role in the adaptation of the tolerant cultivar to low N level.

## **Materials and Methods**

### *Plant material-potato*

Plant cultivation and stress treatments of two genotypes contrasting in their nitrogen deficiency response, namely ‘Lambada’ (sensitive) and ‘Topas’ (tolerant), were performed exactly as described [25]. At the time of root appearance, after one week of cultivation, the nutrient solution was exchanged. Control plants were moved to a fresh medium containing the original 60 mM nitrogen and the plants for deficiency treatment were transferred to a medium with the 1/16 level (3.75 mM). The loss of K (as a result of withholding KNO<sub>3</sub>) was compensated by the addition of KCl. After seven days, the roots were harvested, snap-frozen in liquid N<sub>2</sub> and stored at -80°C. The experiment was performed in triplicate with six vessels per condition and cultivar.

### *Plant material-Arabidopsis thaliana*

Experiments on *A. thaliana* were performed using the wild type (WT) ecotype Col-0 and the two T-DNA insertion lines GABI\_437B11 (*Atp1l;1*) [26] and SALK\_051107 (*Atp1l;3*) [27] (mutants supplied by the Nottingham Arabidopsis Stock Centre, Nottingham, United Kingdom). Homozygous mutants were selected by PCR with specific primers listed in Supplementary Table 1. Homozygote lines were selected based on the absence of a wild type amplicon and the presence of the T-DNA insertion (Supplementary Fig. S2). For the cultivation of plants on soil, ‘Fruhstorfer Nullerde’ (white-peat volcanic clay mixture) was used as a substrate and fertilized as described in Pommerenig et al. 2018 with slight modifications to induce N deficiency [28]. The soil was fertilized at the beginning of each experiment with a modified Hoagland solution containing: 60 mM NH<sub>4</sub>NO<sub>3</sub>, 23.5 mM KH<sub>2</sub>PO<sub>4</sub>, 2.8 mM K<sub>2</sub>SO<sub>4</sub>, 13.3 mM MgSO<sub>4</sub>, 420 μM CuSO<sub>4</sub>, 360 μM ZnSO<sub>4</sub>, 2.5 mM MnCl<sub>2</sub>, 3.3 μM NaMoO<sub>4</sub>, 152 μM NaFeEDTA and 226 μM H<sub>3</sub>BO<sub>3</sub>. Nitrogen deficiency was achieved by a reduction of the N concentration to 30 mM (Richardo F. Giehl, personal communication). Plants (8 replicates per condition) were harvested after 4 weeks of growth, snap-frozen in liquid N<sub>2</sub> and stored at -80°C. The nitrogen and carbon contents of the leaves were measured using a Euro EA elemental analyser (HEKAtech, Wegberg, Germany). Statistical analysis was performed using SigmaPlot v14 (Systat Software, Erkrath, Germany) utilizing a two way analysis of variance (ANOVA) and Holm-Sidak post hoc test.

### *Enrichment of plasma membrane*

For the plasma membrane enrichment, all six vessels obtained from one independent biological experiment were pooled. The PM enrichment protocol described by Hynek, Svensson [29] was modified to treat potato roots as described recently [30]. Protein quantification was performed

according to Bradford [31]. Proteins (5 µg per fraction) were separated by electrophoresis through 11.25% sodium dodecyl sulphate polyacrylamide gels [32] and subsequent Western blot analyses performed as described elsewhere [33]. The following antisera were used: anti-H<sup>+</sup>-ATPase, anti-V-ATPase, anti-VDAC (voltage dependent anion channel), anti-BiP (luminal binding protein) (Agrisera, Vannas, Sweden) and self-raised anti-MDAR (monodehydroascorbate reductase). The universal secondary antibody was goat polyclonal anti-rabbit conjugated with IRDye 800CW, which enabled infrared fluorescence detection via a Licor Bioscience scanner (Bad Homburg, Germany). Relative quantification of the fluorescence signal was performed using Odyssey 3.0 software and mean values were compared using the Student's *t*-test.

#### *Protein extraction and digestion*

Protein extraction and digestion was performed according to the protocol of Jozefowicz et al. 2018 [30]. Prior to the processing by liquid chromatography-mass spectrometry (LC-MS) peptides were suspended in 1% acetonitrile with 0.1% formic acid.

#### *Label-free quantification of proteins and data analysis*

The LC-MS analysis of peptides was performed using a Q-TOF Premiere MS device coupled to a NanoAcquity system device (Waters Corporation, Eschborn, Germany) as described elsewhere [25]. Protein identification was performed using ProteinLynx Global SERVER v2.5.3 software (Waters Corp.). The following parameters were used: automatic mass tolerance and fragment mass tolerance, one missed cleavage and variable modification: oxidation (Met), carbamidomethyl (Cys). MS data were searched against a potato database, based on the sequences from *Solanum tuberosum* group Phureja DM1-3. (PGSC\_DM\_v3.4\_pep\_representative, 39,031 entries) [34] annotated by means of the Blast2GO software (09.2014) [35] and supplemented with sequences of human keratin and trypsin. Additional annotation using UniProt server (<http://www.uniprot.org/>) was performed for the proteins of interest. Protein quantification was carried out using Progenesis LC-MS software (Nonlinear Dynamics, Newcastle, UK). Only proteins with more than one unique peptide were included in the analysis and those with ANOVA *p*-values < 0.05 and a fold change > 1.5 were considered as showing a significant degree of variation. Transmembrane regions were predicted by reference to the TMHMM Server v.2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>) [36]. Principal component analysis (PCA), *Z*-score normalization and hierarchical clustering based on the Euclidean distance method were carried out using the Perseus Framework [37].

#### *Extraction and UPLC-PDA profiling of phenylpropanoids*

Extracts were prepared from 100 mg of ground, frozen leaf material using 400 µl of 100% methanol and incubated overnight at 4°C. Samples were centrifuged for 10 min at 4 °C and 28000 *g*. Supernatants were transferred to the fresh tube. Pellets were re-extracted by adding 400 µl of 100% methanol, followed by 2-hour incubation at 4°C and centrifugation. After re-extraction, both

supernatants were combined. Prior to UPLC analysis, extract aliquots were diluted 1:5 with 0.5% (v/v) formic acid and centrifuged 10 min at 4°C and 28000 g. Phenylpropanoid profiling was performed using an Acquity UPLC system (Waters, Eschborn, Germany) equipped with an Acquity UPLC photodiode array (PDA) eLambda detector, coupled to an ESI-QTOF-MS (maXis Impact, Bruker Daltonik GmbH, Bremen, Germany). Sample injection volumes of 5  $\mu$ L per sample were employed for UPLC analysis, using a partial-loop with needle-overfill injection mode. Compound separation was performed in a reverse phase Acquity UPLC BEH phenyl column (130Å, 2.1 x 100 mm, 1.7  $\mu$ m, Waters) combined with an Acquity UPLC BEH phenyl VanGuard pre-column (130 Å, 2.1 x 5 mm, 1.7  $\mu$ m, Waters) using the following gradient: from 0 to 4.4 min 17.6% of solvent B, 5.8 min (22% B), 7.3 min (97% B), and an isocratic hold from 7.3 to 9 min to clean the column. Solvent A was LC-MS grade water/0.5% (v/v) formic acid, and solvent B was LC-MS acetonitrile/0.5% (v/v) formic acid. The column temperature was maintained at 35°C and the flow was set to 500  $\mu$ L min<sup>-1</sup>. PDA-detection of phenylpropanoids was performed in a range between 210 and 800 nm, at a resolution of 1.2 nm and a sampling rate of 20 points s<sup>-1</sup>. Spectra for phenylpropanoids and anthocyanins were recorded at 280 and 520 nm, respectively. A total phenylpropanoids and anthocyanins content was quantified as a total peak area of the 280 and 520 nm spectra. Data analysis was performed using the software packages Compass Data Analysis V4.4, and QuantAnalysis V4.4 (Bruker Daltonik GmbH, Bremen, Germany). Statistical analysis was performed using SigmaPlot v14 with a two way ANOVA and Holm-Sidak post hoc test.

#### *Yeast strains and growth assay*

*StPIP1;1* and *StPIP1;3* were cloned from cv. ‘Topas’ root cDNA using primers listed in Supplementary table S2. PCR products were cloned in the USER-yeast expression vector pYeDP60u [38] and pRS426-pTPIu using an uracil excision-based improved high-throughput USER cloning technique [39]. A *S. cerevisiae* deletion mutant strain ( $\Delta$ *mep1-3* (-ura/-leu) (Anna Maria Marini's lab collection)) was co-transformed with either the two empty vectors pRS426-pTPIu and pYeDP60u (negative control), *hAQP8* (in pYeDP60u) and an empty vector (pRS425-pTPIu) (positive control), *StPIP1;1* or *StPIP1;3* in pYeDP60u or in pRS426-pTPI:N-terminal-GFP and an empty vector (pRS425-pTPIu) or *ZmPIP2;5<sup>opt</sup><sub>H199K</sub>* in pRS425-pTPIu [40]. Transformed yeast was selected on synthetic medium containing 2% glucose, 50 mM succinic acid/Tris base, pH 6.5, 0.7% yeast nitrogen base without amino acids (Difco) and 0.2% proline. For the ammonium transport assay, yeast cells were spotted on synthetic medium containing 2% galactose 50 mM succinic acid/Tris base, pH 6.5, 0.7% yeast nitrogen base without amino acids (Difco) and different concentrations of ammonium (2, 4, 5, 10, 20 mM ammonium sulfate) or 0.2% proline as positive control. Yeast cells were diluted to different OD<sub>600</sub> values (1, 0.01, and 0.0001). Growth was documented after 8-10 days at 30°C.



## Results

### *The effect of N deficiency on the growth of potato in vitro*

The potato cultivars ‘Lambada’ (sensitive) and ‘Topas’ (tolerant) were differentiated from a set of table potato cultivars according to their Membership Function Value of stress tolerance calculated for previously published data as described previously [25]. Here, proteome profiling was performed after seven days of the stress treatment, when the differences between the genotypes were only marginal. This allowed us to exclude secondary effects of nitrogen deficiency, such as senescence and to analyze mechanisms leading to the plant adaptation towards low N.

### *Enrichment of plasma membranes from potato roots*

Plasma membrane enrichment was critical for the subsequent membrane proteome analysis. The effectiveness of the two-phase partitioning PM preparation method was evaluated immunologically with compartment-specific antibody markers (Fig. 1A). The PM marker H<sup>+</sup>-ATPase was ~13 fold more abundant in the PM fraction than it was in the original crude extract and three fold more than in the microsomal (M) fraction (Supplementary Fig. S1). In contrast, both cytosolic marker MDAR (highly abundant in the soluble (S)) and mitochondrial marker VDAC (highly abundant in the microsomal and the lower phase (M and EM)), were barely detectable in the upper phase (PM). BiP was represented in the PM fraction, but at an intensity three fold lower than in the M fraction. V-type ATPase was present at a comparable level in M, EM and PM.

### *Identification of nitrogen responsive PM proteins*

When the PM fraction was subjected to tryptic digestion and the resulting peptide mixtures characterized by label-free LC-MS 1512 unique peptides were identified, corresponding to 215 proteins. Principal component analysis demonstrated that the replicates were clustered, with the first principal component responsible for the 75.3% of the explained variance and the second for 14.4%. The two cultivars had similar protein profiles under sufficient N conditions but dissimilar when N were limited (Supplementary Figure S3A). About 33% of these were predicted to be integral membrane proteins (Fig. 1B), of which 39 harbored at least two transmembrane domains. N deficiency significantly induced an alteration in abundance (fold change >1.5, ANOVA p-value <0.05) for 65 of the proteins (Table 1).

K-means clustering assembled these proteins into seven groups differing in abundance pattern (Figure 2). Single protein, namely defensin was assigned to the cluster I. It showed the lowest abundance in the sensitive cultivar by high N regime. Cluster II comprised two proteins decreasing in abundance in the cv. ‘Lambada’ during low N treatment. Cluster III grouped two proteins with decrease of abundance in the tolerant cultivar and stable, low abundance in sensitive cultivar. Cluster IV captured proteins (22 members), which increased in the abundance in because of N deficiency in cv. ‘Topas’ but no in cv. ‘Lambada’. The overrepresented functional assignments in

this cluster were ‘transporters’ (11 proteins) and ‘protein destination and storage’ (three proteins). Two proteins grouped in the cluster V were higher abundant in cv. ‘Lambada’ under both regimes. Eight proteins in the cluster VI decreased in the abundance in tolerant cultivar. Finally cluster VII included 28 proteins, which decreased in both cultivars, but to a higher extend in cv. ‘Topas’. Hereto belonged proteins assigned to categories ‘transporters’ (five proteins), ‘signal transduction’ (three proteins), ‘cell structure’ (three proteins) and ‘energy’ (three proteins).

#### *Putative candidate proteins involved in the adaptation of potato to N deficiency*

Proteins harboring at least one transmembrane helix, showing the highest impact on the separation along the principal component (Supplementary Fig. 3) and showing increased abundance in the tolerant genotype ‘Topas’ under N limitation were considered as PM proteins likely being involved in potato N deficiency tolerance. A protein identified as high affinity nitrate transporter (NRT2.1, Cluster IV) increased about eight fold in cv. ‘Topas’ under low N regime and decreased in cv. ‘Lambada’. Three isoforms of H<sup>+</sup>-ATPase increased between two and five fold in cv. ‘Topas’, while in cv. ‘Lambada’ their abundance remained unchanged. Four other proteins behaved in a similar manner: one was a sugar carrier protein and three were ABC transporters. Two proteins belonging to the water channel proteins family were identified. The aquaporin isoform PIP1;1 was four times higher in the N deficient ‘Topas’ plants and PIP1;3 about 2.5 times, and remained unchanged in cv. ‘Lambada’ (Figure 2, marked with the asterisks). As the function of aquaporins is crucial for maintaining plant hydro-osmotic homeostasis, this class of proteins is likely involved in the responses to variable nutrient availability. We thus further investigated the involvement of aquaporin isoforms in nitrogen deficiency tolerance.

#### *Functional analysis of aquaporin isoforms in Arabidopsis thaliana T-DNA insertion lines*

At the amino acid level, AtPIP1;3 shares 87.2% and the AtPIP1;1 85.9% sequence identity with the potato proteins StPIP1;3 and StPIP1;1, respectively. Therefore the *A. thaliana* T-DNA insertion mutants *Atpip1;1* and *Atpip1;3* were used to elucidate the functional properties and potential significance of particular aquaporins in the nitrogen deficiency response. The wild type (Col-0) and the T-DNA insertion lines were cultivated on nitrogen-limited soil over four weeks. Under N sufficient conditions the shoot fresh weight of *Atpip1;1* was slightly higher than that of the WT, while that of *Atpip1;3* was comparable to the WT. When the WT and the *pip* mutants were grown under N deficient conditions, reduction in the biomass production was observed in all plants (Figure 3). The nitrogen content of the leaves was comparable in the examined plants when N was supplied in the sufficient amounts (Figure 4A). When the level of nitrogen in the soil was reduced, the WT plants contained about 3.2% of N in the leaf dry weight. The accumulation of N was significantly lower in both mutants, where the N content was as low as 2.2%. Under N sufficient conditions,

carbon content of the leaves was higher in the *Atpip1;3* than in Col-0. When N was deficient the carbon content increased in all plants, but this increase was more pronounced in the two mutants.

As the *pip* mutants (but not the WT) showed a purple coloration of the leaves when the plants were cultivated on nitrogen deficient conditions, we performed an UPLC-PDA profiling of phenylpropanoids. The phenylpropanoids content of the leaves was similar in all plants when nitrogen was sufficient. As shown in the Figure 4B, under nitrogen deficiency, the *Atpip1;1* and *Atpip1;3* mutants, but not the WT, showed significantly higher accumulation of the total phenylpropanoids. The same trend was observed when the level of total anthocyanins was measured with about 4.4-fold increase of the content in the mutants and only 1.7-fold increase in the WT.

#### *Analysis of potato aquaporin isoforms StPIP1;1 and StPIP1;3*

When examining the Arabidopsis mutants we could clearly see decreased N content under nitrogen deficiency in comparison to WT. To determine if StPIP1;1 and StPIP1;3 membrane channels are acting as N facilitators, by mediating the transport of  $\text{NH}_4^+/\text{NH}_3$ , we expressed the two *StPIPs* singly or co-expressed with *ZmPIP2;5* in a *S. cerevisiae* deletion mutant strain ( $\Delta mep1-3$  (-ura/-leu)). This mutant has a deletion in all *mep* transporter genes and is therefore unable to grow on medium containing lower than 5 mM ammonium [41]. An additional source of N (e.g. in form of proline) is essential to ensure its growth. To investigate protein localization in *S. cerevisiae* we used GFP-PIP-fusion proteins of the respective aquaporin expressed in yeast cells. The yeast transformed with single PIPs showed a GFP signal in the non-defined internal structures, whereas co-expressed with *ZmPIP2;5* GFP was clearly fluorescent in the plasma membrane (data not shown). Neither yeast cells expressing StPIP1;1 or StPIP1;3 alone, nor in combination with *ZmPIP2;5* were able to complement the growth (Figure 5). Whereas the expression of the positive control hAQP8 complemented the mutant and allowed growth on the medium with ammonium concentration below 5 mM.

## **Discussion**

Two N deficiency response contrasting cultivars ('Lambada'-sensitive, and 'Topas'-tolerant) formed the basis of the present effort to identify plasma membrane proteins associated with N deficiency tolerance. They were selected out of thirteen cultivars, characterized for traits linked with nitrogen uptake and utilization at an early growth stage [21, 25]. Here, we compared the PM proteome of plants, which were cultivated seven days under nitrogen sufficient and limiting conditions. At this timepoint plants showed only minor signs of nitrogen deficiency. This allowed us to elucidate candidate PM proteins, which play a role in the early adaptation of the tolerant potato variety towards low N.

#### *N deficiency induced variation in the PM proteome of potato cultivars*

In the presented study, we used plasma membrane enrichment of potato root proteins for a subsequent analysis of PM proteins. About a third of the 215 identified PM proteins harbored at least one transmembrane domain, a proportion similar to that obtained in *A. thaliana* [42]. Parts of the non-transmembrane ones, including metabolic enzymes, were considered as contaminations from the soluble protein fraction. Several of them, however, were likewise identified in other PM studies. Ribosomal proteins, which were identified here, were already speculated to originate from polysomes which are attached to the plasma membrane via actin [43]. Some identified proteins lacking a transmembrane domain are closely related to the PM. Over-represented group of proteins, were the Ras superfamily members. These small GTP-binding proteins play a crucial role in signaling pathways, vesicular traffic and targeting to PM [44]. Among the 215 quantified proteins, 65 proteins were differentially abundant in the two potato varieties. A number of differentially expressed proteins lacked any prior functional classification, which reflects the generally poor resolution of the PM proteome. About 30% of the assigned proteins fell into the category of ‘transporters’, with a few others associated variously with ‘signal transduction’, ‘disease/defense’, ‘metabolism’ and ‘protein destination and storage’.

#### *PM proteins involved in the tolerance of potato to N deficiency*

The abundance of the majority of the transporter proteins was largely unaffected by N deficiency in cv. ‘Lambada’, while increasing in cv. ‘Topas’. The most responsive of these was the high affinity nitrate transporter NRT2.1, a key component of the high affinity transport system operating under low levels of external nitrate [45]. In *A. thaliana* N deficiency induced expression of NRT2.1 in the cortex cells of the older part of primary and lateral roots. It was observed that the abundance of NRT2.1 is higher when plants are grown on 0.3 or 1 mM  $\text{NO}_3^-$  in comparison to 5 mM  $\text{NO}_3^-$  [46]. A decrease in the nitrate concentration might be linked to an increase in the NRT2.1 abundance in cv. ‘Topas’. As it was previously described cv. ‘Lambada’ absorbs all available in culturing medium N faster than cv. ‘Topas’ [23], so the decrease in NRT2.1 abundance might be caused by severe N deficiency.

The PM  $\text{H}^+$ -ATPase uses ATP as energy source to pump  $\text{H}^+$  across the plasma membranes into the apoplast. It generates a  $\text{H}^+$  electrochemical gradient, which is a driving force for the active transport of ions and metabolites across the plasma membrane [47]. Nitrate transport mediated by NRT2.1 depends on the activity of PM  $\text{H}^+$ -ATPase [48, 49]. Here, three isoforms of  $\text{H}^+$ -ATPase were among the inducible proteins, and for all of them a strong positive correlation between their abundance and abundance of NRT2.1 (Pearson  $r > 0.9$ , data not shown) was found.

Three proteins harboring an ATP-binding cassette were identified here; two (both putative ABC transporters) were present in significantly higher amounts in cv. ‘Topas’ plants exposed to N deficiency, while the third (a homolog of a multidrug resistance protein) were increased in the

sensitive and decreased in the tolerant genotype. ABC transporters are generally associated with detoxification processes, development and stress responses but also in plant nutrition [50], examples are known for them to act as transmembrane transporters of small peptides [51], which may well be relevant in the context of the N deficiency response. Certain *A. thaliana* receptor-like kinases (RLKs) become phosphorylated upon the re-supply of nitrate/ammonium after an episode of N starvation [52]. Here, the abundance of three putative RLKs (Cluster VII members) was reduced in the N deficiency in both varieties. Calmodulin was more abundant in cv. ‘Topas’ than in cv. ‘Lambada’ in N deficient plants; this protein acts to raise the affinity of the PM Ca<sup>2+</sup> pump to Ca<sup>2+</sup> [53] and is known to be involved in membrane transport regulation during episodes of abiotic stress [54, 55]. Two isoforms of the 14-3-3 protein were among the set of N deficiency-regulated proteins: one (belonging to Cluster VII) decreased in abundance in both varieties, while the other (in Cluster VI) only in cv. ‘Topas’. Certain 14-3-3 proteins can inactivate phosphorylated nitrate reductases [56], while the phosphorylation of the H<sup>+</sup>-ATPase C-terminus can result in the binding of 14-3-3 proteins and displacement of an autoinhibitory domain thereby activating the protein [57]. 14-3-3 proteins are also known to regulate PM and vacuolar K<sup>+</sup> channels [58]. There is a certain likelihood that the identified 14-3-3 isoforms associated with the potato N deficiency response are involved in the regulation of PM transporters.

Another protein, which strongly increased in cv. ‘Topas’ under N deficiency were two aquaporins; they were designated as PIP1;1 and PIP1;3, following the study of Venkatesh, Yu [59]. PIPs are channel proteins deposited both in the PM and other intracellular membranes; they function in promoting the transport of water and small neutral solutes [60]. They regulate plant-water relations by facilitating the transport of water across biological membranes and regulating osmotic water potential and hydraulic conductivity. Aquaporins (mainly TIPs and NIPs) are involved in many key nitrogen related processes such as transport (ammonia, urea), mobilization, and detoxification [61, 62]. The role of aquaporins and in particular single isoforms in determining nitrogen deficiency tolerance remains unknown.

#### *PIP1;1 and PIP1;3 are indirectly involved in the nitrogen uptake*

To further study the function of PIPs, we used the *A. thaliana* T-DNA insertion lines. The loss of function of either PIP1;1 or PIP1;3 resulted in a significant decrease in the nitrogen and increase in the carbon content in the leaves, when nitrogen in the soil was limited. Additionally, the *Atpip* mutants showed typical sign of N deficiency, which is accumulation of phenylpropanoids (e.g anthocyanins). The role of phenylpropanoids under nitrogen deficiency is to be a sink for carbon, or the protection against high light-induced oxidative damage, which is more pronounced when chlorophyll and photosynthetic membranes are disrupted [63, 64]. The lower N content and N deficiency related phenotype of the mutants indicates that both PIP1;1 and PIP1;3 play an important role in nitrogen uptake. Previously it was shown that members of aquaporin family are able to

facilitate the transport of ammonia [65]. Using a yeast complementation assay, we tested if StPIP1;1 and StPIP1;3 are able to mediate the transport of  $\text{NH}_4^+/\text{NH}_3$ . The expression of StPIP1;1 and StPIP1;3 did not increase the growth of yeast mutants defective in their native ammonium uptake system under low nitrogen supply, suggesting that these isoforms do not facilitate ammonium transport. This points towards an indirect role of aquaporins in the nitrogen uptake, probably by increasing the acquisition of nitrogen by regulating the water flux towards and along the root. Previous studies in other plant species showed that the root's hydraulic conductivity is compromised when key nutritional elements are limiting [66-68] and that it can be recovered very rapidly following the supply of N to a N-starved plant [69]. This suggests a mechanism for increasing the acquisition of nitrogen, but the underlying physiological processes are not yet fully understood. Changes to a root's hydraulic conductivity can reflect the regulation or modification of aquaporin activity [69]. Recently it was shown that nitrate is a main signal driving root hydraulic conductivity, with NRT2.1 playing the most important role. In the *Atnrt2.1* mutant the overall abundance of PIP1s proteins decreased when N was limited [70]. Here, in the N deficiency tolerant cultivar we observed an increase in the abundance of both NRT2.1 and PIPs proteins. The relationship between expressional alterations of PIP isoforms and nitrate transport via NRT2;1 in *Arabidopsis* remains to be investigated.

In conclusion, N limitation has been shown to modify the potato root PM proteome, and clear differences have been identified between the two varieties contrasting in their response to N deficiency. It has been possible to identify several candidate proteins underlying nitrogen deficiency tolerance. The data suggest that the enhanced abundance of certain transporter and signaling proteins underpins the superior response of 'Topas' to N deficiency. Nonetheless, further verification of the results in field studies as well as examination of higher numbers of genotypes are initiated to assess the significance of the nitrogen responsive candidate proteins described in this study.

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## Figure legends

**Figure 1.** Enrichment for PM proteins. A-Western blot of the PM fraction obtained from ‘Topas’ plants grown under N-sufficient conditions. The marker proteins investigated were H<sup>+</sup>-ATPase (PM marker), VDAC (mitochondrial marker), BiP (ER marker), MDAR (cytosolic marker), V-ATPase (vacuolar marker). Abbreviations are used as follows, C: crude extract, S: soluble fraction, M: microsomal fraction, EM: endomembrane fraction, PM: plasma membrane fraction. B- The number of transmembrane domains (TMH) predicted for the identified proteins is shown.

**Figure 2.** Clustering of proteins based on their abundance in the two potato varieties, grown under either N-sufficient or -deficient conditions. According to the K-means algorithm, the proteins formed seven clusters. Relative abundance has been color-coded following Z-score normalization. Each column represents one treatment. Abbreviation used LamC- Lambada control, LamS- Lambada stress, TopC- Topas control, TopS- Topas stress. The aquaporin proteins are marked with asterisks.

**Figure 3.** Effect of Nitrogen deficiency on growth of *Arabidopsis thaliana* pip mutants. Plants were grown in the presence of either 60 mM or 30 mM ammonium nitrate. Rosette fresh weight was determined after 4 weeks of cultivation. The data present the mean of 7 plants per treatment. Letters above the histogram columns indicate significant differences in mean values, as predicted by a two way ANOVA (p-value <0.05), the standard error is shown as error bars.

**Figure 4.** The response of *A. thaliana* WT (Col-0) and *pip* mutants to N deficiency. A- Nitrogen and carbon content of the leaves. B- The content of phenylpropanoids and anthocyanins. The data present the mean of 7 plants per treatment. Letters above the histogram columns indicate significant differences in mean values, as predicted by a two way ANOVA (p-value <0.05), the standard error is shown as error bars.

**Figure 5.** The ammonium transport ability of the PIP1;1 and PIP1;3 studied in yeast by the growth complementation assay. The growth of the transformants was recorded after 8-10 days. Displayed image represent the group of images assembled from different growth plates and conditions.

## Supplementary Data

Supplementary Table 1: Primers used for identification of mutation homozygotes among the progeny of *A. thaliana* T-DNA insertion *pip* mutant lines.

Supplementary Table 2. Primers used for cloning of potato aquaporins.

Supplementary Table 3. Proteins identified in the LC-MS (detailed table).

Supplementary Fig. 1. Quantification of Western blot signals used for the determination of PM purity.

Supplementary Fig. 2. Identification of mutation homozygotes among the progeny of *A. thaliana* T-DNA insertion *pip* mutant lines.

Supplementary Fig 3. LC-MS based plasma membrane protein profiling of the two cultivars N deficiency and control conditions analyzed by principal component analysis. Each symbol represents one analysis (two technical replicates of three independent harvests). Each group of samples is denoted by different color. Proteins having the highest impact on the separation along principal component are presented on the loading plot.

## Tables

**Table 1.** Label-free quantification of the set of 70 proteins showing significantly altered abundance in the potato root PM induced by N deficiency treatment. The proteins have been clustered according to their expression profile into five groups (see Fig. 4). The number of peptides used for identification and number used for quantification (in parentheses) are shown. Their functional classification (where known) and their predicted content of transmembrane helices are given.

| Accession | Description  | Function                        | TMH | LamS/LamC | TopS/TopC | TopC/LamC | TopS/LamS |
|-----------|--|---------------------------------|-----|-----------|-----------|-----------|-----------|
| Cluster 1 |  |                                 |     |           |           |           |           |
| 400039130 | Defensin-like protein                                    | disease/defence                 | 1   | 1.56      | 0.86      | 1.56      | 0.85      |
| Cluster 2 |  |                                 |     |           |           |           |           |
| 400079443 | Hemoglobin   | unclassified                    | 0   | 0.34      | 0.91      | 1.07      | 2.89      |
| 400083124 | Uncharacterized protein                                  | unclassified                    | 1   | 0.52      | 0.90      | 0.98      | 1.69      |
| Cluster 3 |  |                                 |     |           |           |           |           |
| 400005571 | Glutathione S-transferase-like protein                   | disease/defence                 | 0   | 0.89      | 0.29      | 5.11      | 1.67      |
| 400040518 | Putative cinnamyl alcohol dehydrogenase                  | secondary metabolism            | 0   | 0.73      | 0.44      | 2.04      | 1.23      |
| Cluster 4 |  |                                 |     |           |           |           |           |
| 400035262 | Sucrose synthase   | metabolism                      | 0   | 0.81      | 1.29      | 1.15      | 1.85      |
| 400047710 | Luminal-binding protein 5                                | protein destination and storage | 1   | 0.80      | 1.75      | 1.00      | 2.17      |
| 400035380 | ADP,ATP carrier protein                                  | transporters                    | 3   | 0.54      | 2.55      | 0.92      | 4.34      |
| 400018820 | Putative ABC transporter family protein                  | transporters                    | 9   | 1.18      | 2.69      | 1.46      | 3.32      |
| 400025670 | U- box domain containing protein 44 like                 | protein destination and storage | 0   | 0.08      | 13.91     | 0.79      | 139.26    |
| 400083041 | Plasma membrane ATPase                                   | transporters                    | 8   | 0.93      | 3.54      | 1.11      | 4.26      |
| 400019462 | Uncharacterized protein                                  | unclassified                    | 0   | 0.48      | 11.49     | 0.68      | 16.27     |
| 400036847 | Pyrophosphate- energized vacuolar membrane proton pump 1 | transporters                    | 13  | 0.00      | 63.08     | 12.91     | Infinity  |
| 400031304 | High-affinity nitrate transporter 2.1                    | transporters                    | 11  | 0.49      | 8.23      | 0.56      | 9.33      |
| 400005236 | Hypersensitive induced response protein 2 like           | disease/defence                 | 0   | 0.50      | 2.44      | 0.85      | 4.18      |
| 400001904 | Ras-related protein RABG3f like                          | intracellular traffic           | 0   | 0.65      | 1.94      | 0.76      | 2.26      |
| 400057709 | Hexose transporter 1                                     | transporters                    | 12  | 1.12      | 2.00      | 0.82      | 1.46      |
| 400029027 | Tetraspanin-8  | disease/defence                 | 4   | 1.05      | 1.66      | 0.91      | 1.44      |
| 400027156 | Ubiquitin  | protein destination and storage | 0   | 1.28      | 1.55      | 1.14      | 1.38      |
| 400046727 | ABC transporter A family member 2                        | transporters                    | 6   | 1.31      | 1.91      | 1.21      | 1.76      |
| 400046278 | Calmodulin-2/4   | signal transduction             | 0   | 1.36      | 1.94      | 1.19      | 1.71      |
| 400032134 | Aquaporin PIP1;3   | transporters                    | 6   | 1.32      | 2.52      | 1.77      | 3.39      |
| 400065168 | Plasma membrane ATPase                                   | transporters                    | 8   | 1.03      | 1.91      | 1.23      | 2.28      |
| 400036643 | Transmembrane protein                                    | unclassified                    | 6   | 1.32      | 2.70      | 1.39      | 2.83      |

|           |   |                                 |    |      |      |      |      |
|-----------|---|---------------------------------|----|------|------|------|------|
| 400012780 | Aquaporin PIP1;1  | transporters                    | 6  | 1.42 | 4.01 | 1.55 | 4.37 |
| 400010497 | Plasma membrane ATPase                                    | transporters                    | 8  | 1.07 | 4.10 | 1.28 | 4.92 |
| 400005889 | Putative membrane protein C24H6.13                        | unclassified                    | 10 | 1.07 | 1.73 | 1.12 | 1.82 |
| Cluster 5 |   |                                 |    |      |      |      |      |
| 400004106 | 1-phosphatidylinositol phosphodiesterase                  | metabolism                      | 0  | 1.32 | 0.56 | 0.63 | 0.27 |
| 400051789 | Uncharacterized protein                                   | unclassified                    | 0  | 1.14 | 0.54 | 0.89 | 0.42 |
| Cluster 6 |   |                                 |    |      |      |      |      |
| 400073713 | 70-kD heat shock protein                                  | disease/defence                 | 0  | 0.98 | 0.49 | 0.89 | 0.44 |
| 400076528 | Putative GTP-binding nuclear protein Ran2                 | intracellular traffic           | 0  | 0.99 | 0.35 | 1.01 | 0.36 |
| 400016422 | 14-3-3 protein 7  | unclassified                    | 0  | 0.98 | 0.54 | 1.06 | 0.58 |
| 400075291 | 40S ribosomal protein S6                                  | protein synthesis               | 0  | 0.87 | 0.37 | 1.26 | 0.54 |
| 400014945 | Putative vacuolar proton ATPase subunit E                 | transporters                    | 0  | 1.01 | 0.41 | 1.23 | 0.50 |
| 400068704 | Vacuolar ATPase subunit B                                 | transporters                    | 0  | 0.93 | 0.49 | 1.12 | 0.58 |
| 400004125 | Peptidyl-prolyl cis-trans isomerase                       | protein destination and storage | 0  | 0.92 | 0.58 | 1.11 | 0.70 |
| 400033202 | Uncharacterized protein                                   | unclassified                    | 0  | 1.00 | 0.63 | 1.17 | 0.74 |
| Cluster 7 |   |                                 |    |      |      |      |      |
| 400047481 | Actin   | cell structure                  | 0  | 0.45 | 0.90 | 0.50 | 1.02 |
| 400058342 | CASP-like protein   | unclassified                    | 4  | 0.90 | 1.16 | 0.48 | 0.62 |
| 400024318 | Uncharacterized protein                                   | unclassified                    | 0  | 0.89 | 0.82 | 0.65 | 0.60 |
| 400062832 | Uncharacterized protein                                   | unclassified                    | 0  | 0.91 | 0.89 | 0.70 | 0.68 |
| 400022574 | Alpha-tubulin   | cell structure                  | 0  | 0.92 | 0.76 | 0.80 | 0.67 |
| 400070407 | ATP synthase D chain                                      | transporters                    | 0  | 0.90 | 0.53 | 0.80 | 0.47 |
| 400035497 | Uncharacterized protein                                   | unclassified                    | 0  | 0.92 | 0.69 | 0.86 | 0.64 |
| 400078507 | ATP synthase subunit beta                                 | transporters                    | 0  | 0.87 | 0.76 | 0.80 | 0.70 |
| 400023732 | Putative leucine-rich repeat receptor-like protein kinase | signal transduction             | 2  | 0.79 | 0.46 | 0.78 | 0.46 |
| 400010174 | Actin 1   | cell structure                  | 0  | 0.74 | 0.25 | 0.80 | 0.28 |
| 400058377 | Pollen-specific lysine-rich protein SBgLR                 | unclassified                    | 0  | 0.54 | 0.64 | 0.21 | 0.25 |
| 400039213 | Probable inactive receptor kinase at2g26730 like          | signal transduction             | 2  | 0.69 | 0.69 | 0.67 | 0.66 |
| 400073467 | Phospholipase D   | metabolism                      | 0  | 0.72 | 0.68 | 0.74 | 0.70 |
| 400045607 | Somatic embryogenesis receptor kinase 3A                  | signal transduction             | 1  | 0.74 | 0.70 | 0.73 | 0.69 |
| 400041634 | 14-3-3 protein  | unclassified                    | 0  | 0.69 | 0.60 | 0.70 | 0.62 |
| 400007182 | Dihydrolipoamide Dehydrogenase                            | energy                          | 0  | 0.65 | 0.75 | 0.60 | 0.69 |
| 400079310 | 60S ribosomal protein L13                                 | protein synthesis               | 0  | 0.30 | 0.39 | 0.62 | 0.79 |
| 400012216 | Cupredoxin superfamily protein isoform 1                  | unclassified                    | 1  | 0.70 | 0.68 | 0.82 | 0.80 |
| 400057418 | Glycerophosphodiesterase-like protein                     | metabolism                      | 1  | 0.65 | 0.63 | 0.74 | 0.72 |
| 400008687 | 60S ribosomal protein L12                                 | protein synthesis               | 0  | 0.57 | 0.40 | 0.88 | 0.62 |
| 400061841 | Xyloglucan endotransglucosylase/hydrolase                 | cell growth/division            | 0  | 0.74 | 0.71 | 0.87 | 0.83 |
| 400036347 | Uncharacterized protein                                   | unclassified                    | 1  | 0.48 | 0.35 | 0.80 | 0.58 |
| 400006894 | Fructose-bisphosphate aldolase                            | energy                          | 0  | 0.62 | 0.44 | 0.90 | 0.64 |

|           |  |              |   |      |      |      |      |
|-----------|--|--------------|---|------|------|------|------|
| 400010366 | V-type proton ATPase subunit E2                    | transporters | 0 | 0.68 | 0.36 | 0.91 | 0.48 |
| 400013297 | Uncharacterized protein                            | unclassified | 0 | 0.79 | 0.62 | 0.92 | 0.73 |
| 400008800 | V-type proton ATPase subunit B2                    | transporters | 0 | 0.74 | 0.37 | 0.94 | 0.47 |
| 400044217 | Malate dehydrogenase                               | energy       | 0 | 0.73 | 0.31 | 0.97 | 0.42 |
| 400074197 | Vacuolar H <sup>+</sup> -ATPase A1 subunit isoform | transporters | 0 | 0.77 | 0.49 | 0.96 | 0.60 |



Figure 1.

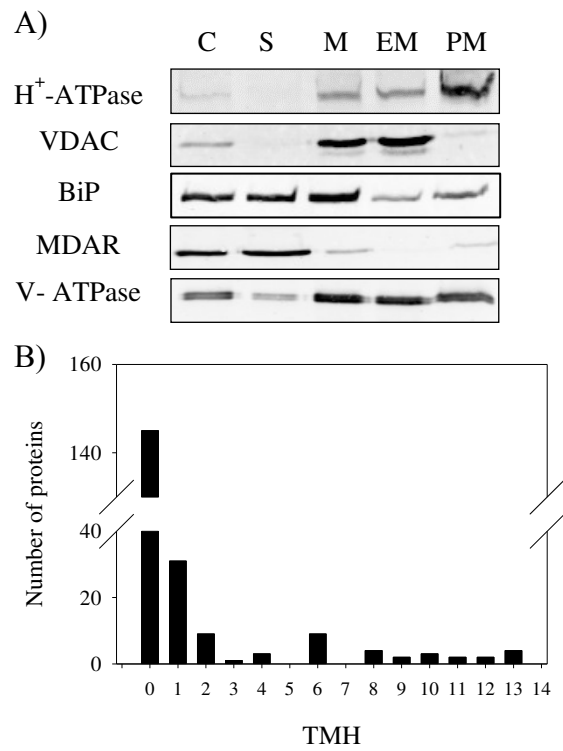


Figure 2.

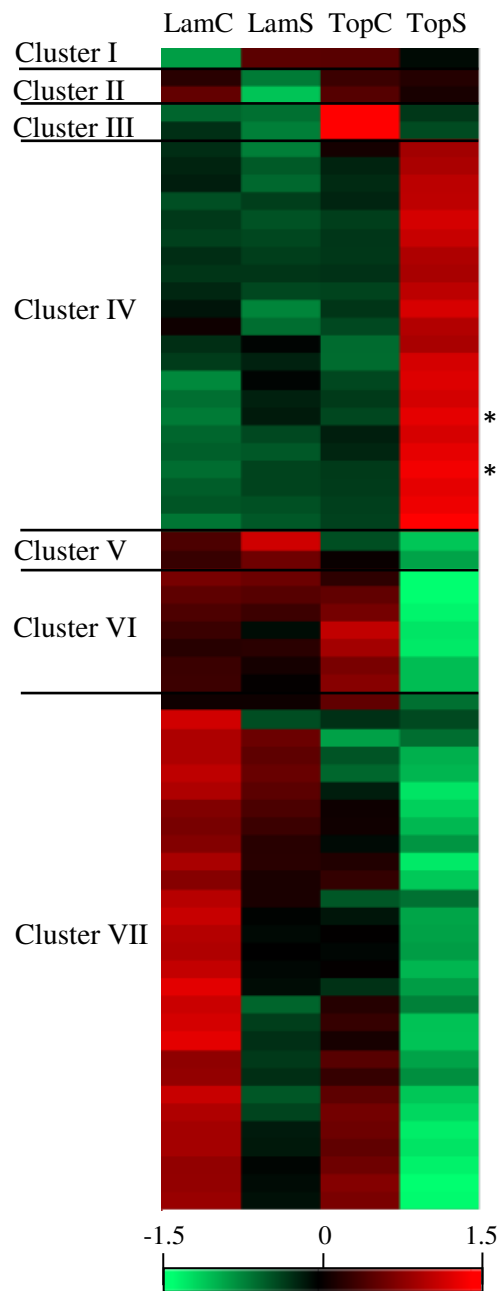


Figure 3.

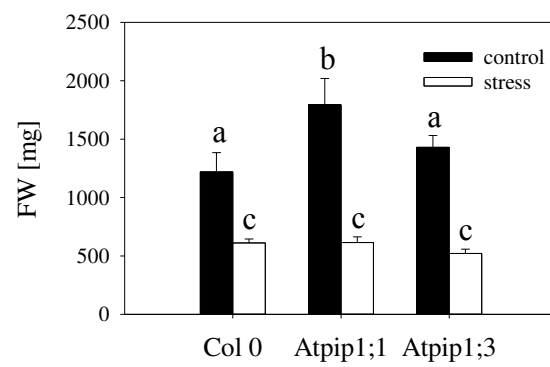
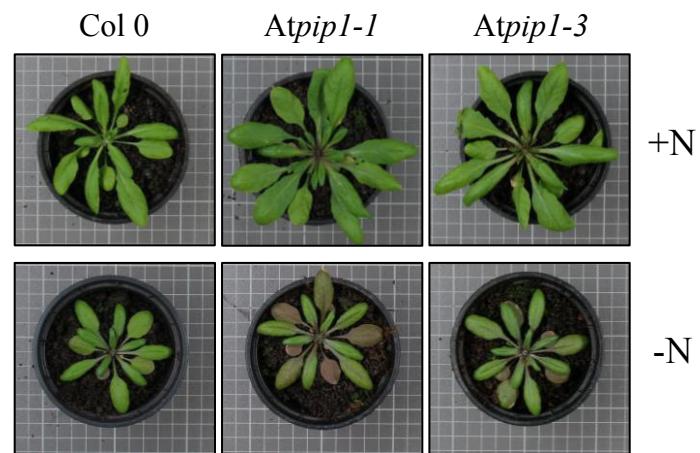


Figure 4.

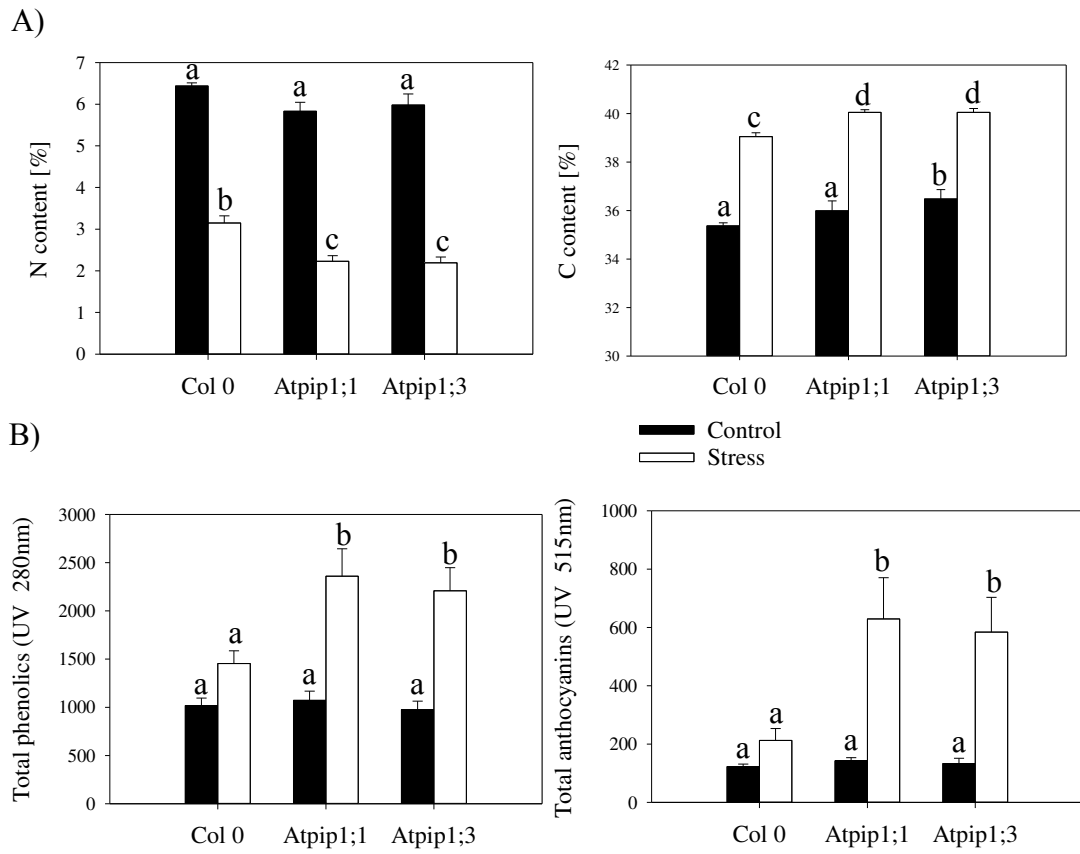
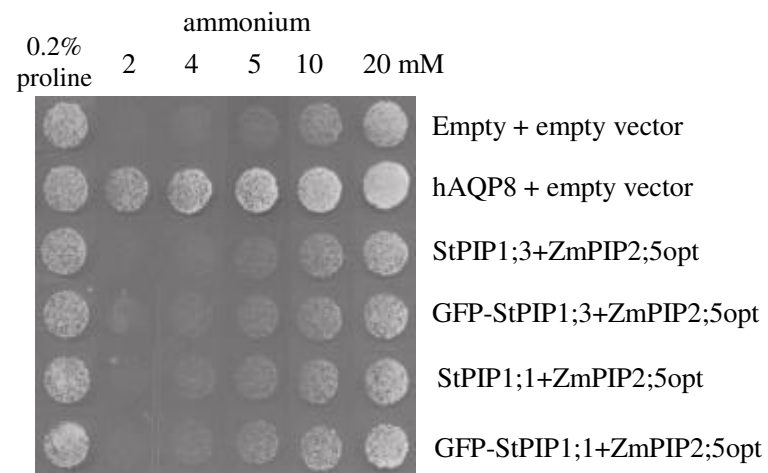


Figure 5.



## Supplementary

Figure S1

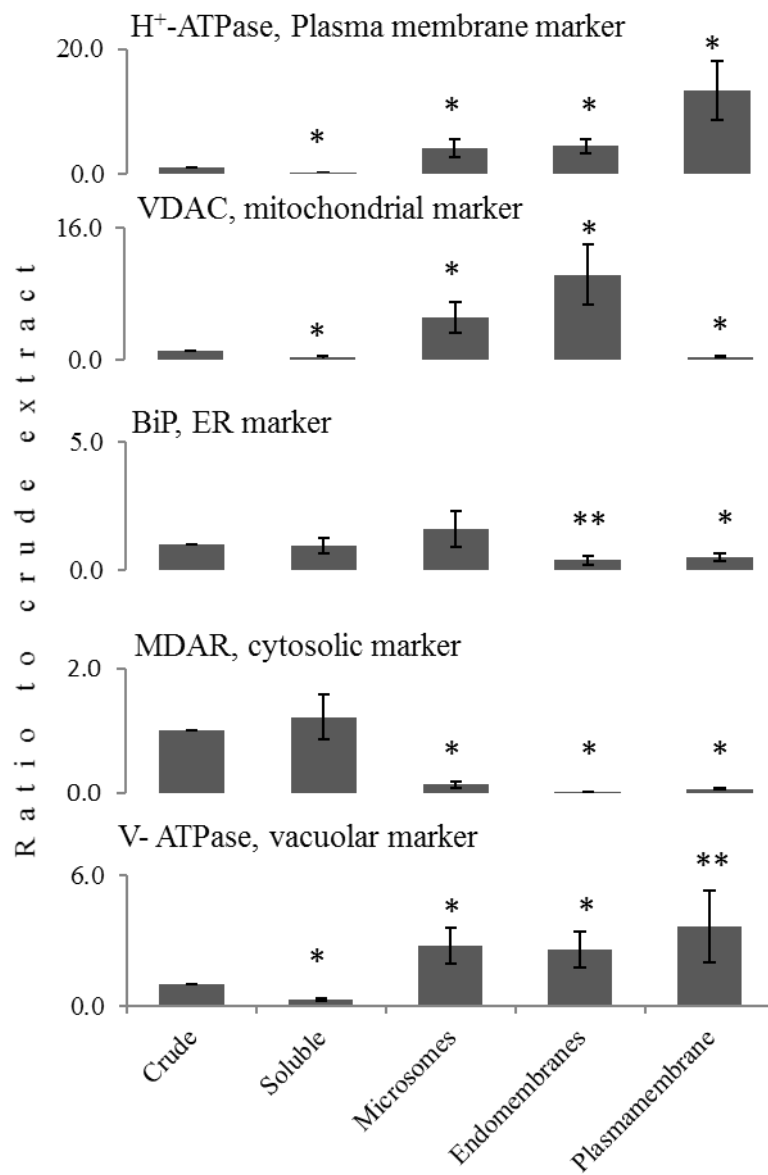


Figure S2

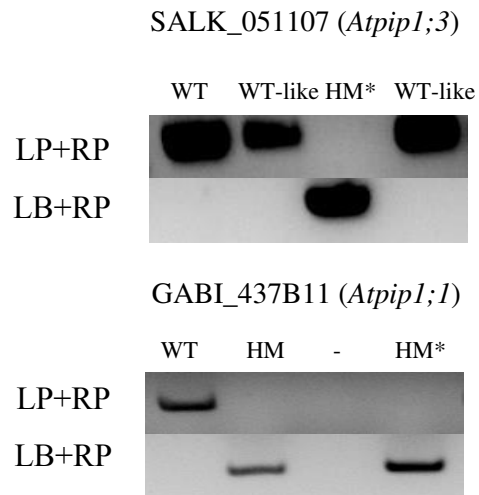
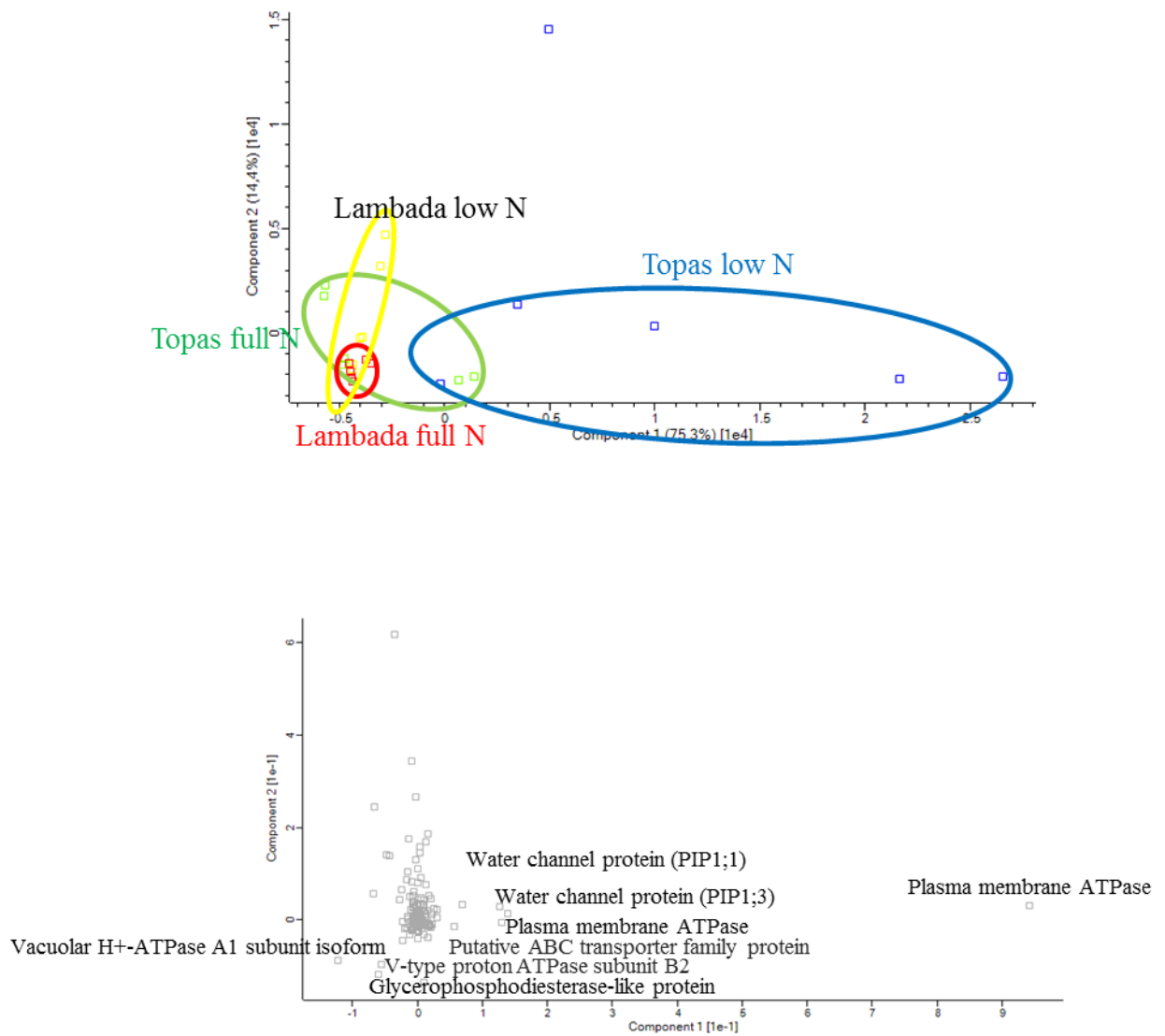


Figure S3





# Chapter IV

## **Multi-level analysis of the temporal responses of two potato cultivars differing in the tolerance to nitrogen deficiency**

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## **Abstract**

Improving the nitrogen use efficiency of potato is a priority, not only to optimize its productivity but also to reduce the level of environmental pollution arising from excessive fertilizer application. The cultivars ‘Lambada’ and ‘Topas’ are known to contrast for their tolerance of nitrogen deficiency *in vitro*. Here, the early responses of the root proteome and metabolome were compared at initial time point as well as 10 min, 1 h, 24 h and 72 h of N deficiency. The differentially changed proteins and metabolites were analysed using an ‘omic’-based approaches. The roots of the more tolerant cultivar ‘Topas’ showed higher accumulation of proteins involved in the nitrogen assimilation and re-assimilation, as well as in carbon metabolism, proteolysis and lignification. The sensitive cultivar ‘Lambada’ accumulated proteins related to defence mechanisms. Cultivars differed also in the capacity to accumulate free amino acids and in the temporal changes in their concentration. Presented data indicates that the tolerant cultivar accumulates more N-containing molecules, which are used for better adaption to N limiting conditions.

**Keywords:** amino acids, label-free protein quantification, metabolites profiling, nitrogen use efficiency, nitrogen starvation

## Introduction

Nitrogen (N) is essential for plant growth, as it is key component of amino acids, proteins, nucleic acids, chlorophyll, hormones and numerous secondary metabolites. Yield losses induced by an inadequate supply of N are far more serious than those induced by deficiency in any of the other macronutrients. A sub-optimal supply of N compromises plant growth, development, architecture, flowering, senescence and photosynthesis [1]. Providing additional N, largely in the form of nitrate and/or ammonium, has had a major positive effect on crop productivity. However, excessive levels of fertilizer application are responsible for serious environmental damage [2], because crops are unable to take up more than about 40% of the applied N fertilizer; the remainder is lost as a result of leaching, surface run-off, denitrification, volatilization and microbial consumption. It has been estimated that annually, 100 Mt of N, largely in the form of N<sub>2</sub>O, is generated from crop production activity [3-5].

The fraction of soil N absorbed and utilized by a plant is referred to as its nitrogen use efficiency (NUE) [6]. Genetic variation for this trait has been identified in a range of crop species, but is characteristically low for the potato (*Solanum tuberosum* L.), reflecting its rather shallow root system [7] and its cultivation on sandy soils in conjunction with high rates of fertilizer application and irrigation [8]. Many years of breeding for performance in a N non-limiting environment have generated cultivars which do not necessarily respond well to N-deficient conditions [9]. In the current socio-economic climate, the focus of breeding has moved to seeking improvements in NUE. A body of research, spanning whole plant physiology, molecular genetics and agronomy has been directed at understanding the physiological and molecular basis of variation in this trait [6]. One of the strategies, which has been explored to raise NUE is to over-express genes known to participate in N metabolism. For example, in tobacco, the constitutive expression of an alfalfa gene encoding a cytosolic glutamine synthetase (GS) has been shown to enhance photosynthesis and growth under conditions of N deficiency [10] and similarly, the effect of constitutively expressing a bacterial gene encoding glutamate dehydrogenase has been demonstrated to boost biomass production [11]. In oilseed rape, the constitutive expression of a barley gene encoding alanine aminotransferase raises biomass accumulation and seed yield under a low N regime [12].

Potato represents a significant source of food and industrial starch. Understanding the molecular basis for the adaptation of potato to a sub-optimal N regime is important, both in the context of the continuing global rise in demand for food and renewable raw materials and as a means of protecting the quality of the rural environment. When grown under *in vitro* conditions, different responses to N deficiency of the two potato cultivars ‘Lambada’ (sensitive) and ‘Topas’ (tolerant), both at the physiological [13-15] and at the proteome [16, 17] level were observed. A positive correlation between plant performance under *in vitro* conditions and NUE in soil-grown plants has been established for a number of potato cultivars [18]. The biochemical differences (especially at the

proteome level) between cultivars differing in their tolerance to N deficiency have not yet been well characterized. Here the difference between cultivars ‘Lambada’ and ‘Topas’ with respect to both their root proteome and metabolome during early N deficiency was investigated. As far as can be ascertained, this study represents the first attempt to compare contrasting cultivars using an ‘omics’-based approach, with a particular focus on the temporal behaviour of the roots biochemistry. The outcomes of the research have allowed for novel insights into both the early and the more long-term response to N deficiency, revealing candidate pathways to achieve improvement in the NUE of potato.

## **Material and methods**

### *In vitro cultivation of potato*

Plants of the two cultivars ‘Topas’ and ‘Lambada’ were raised as described elsewhere [17]. After 11 days, half of the plants were moved into a fresh batch of medium Murashige&Skoog [19] supplemented with 60 mM N and the other half into the same medium lacking any additional N. The difference in the potassium content between the two media was made up by adding the relevant amount of KCl to the N-free medium. Roots were first harvested before the treatment (T0), and subsequently after T0+10 min, T0+1 h, T0+24 h and T0+72 h of the treatment (Fig. 1) after which they were snap-frozen in liquid nitrogen and stored at -80°C. Each treatment comprised four vessels with eight plants for each cultivar and the whole experiment was run in triplicate. The N and carbon contents of the root samples were measured using a Euro EA elemental analyser (HEKAtech, Wegberg, Germany). Soluble protein content was measured according to Bradford [20].

### *Protein extraction and digestion*

Root proteins were extracted using a trichloroacetic acid/acetone protocol [21]. Resulting dried pellets were re-suspended in 100 µl 7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 5 mM DTT (pH 8.0). The concentration of protein in solution was estimated using a 2D Quant Kit (GE Healthcare, Munich, Germany) using BSA as the reference. Aliquots containing 30 µg of proteins were subjected to filter-based digestion, following Distler *et al.* [22], which consisted of an overnight digestion at 37°C in a 1:50 dilution of sequencing grade modified trypsin (Promega, Mannheim, Germany). Peptides to be subjected to LC/MS analysis were recovered by centrifugation, dried by vacuum centrifugation and suspended in 300 µl 1% acetonitrile (ACN), 0.1% formic acid (FA) (v/v).

### *Analysis of peptides*

Peptides were analysed by LC/MS, using Dionex UltiMate™ 3000 RSLCnano System (Thermo Fisher Scientific, Dreieich, Germany) coupled to an Impact II (Bruker Daltonics, Bremen, Germany).

700 ng of the digested proteins were separated using a Nano Trap Column (Acclaim PepMap100 C18, 5 µm, 100 Å) and 50 cm x 75 µm analytical column (Acclaim PepMap RSLC C18, Thermo Fisher Scientific) through a 120 min long linear 2-40% acetonitrile, gradient (buffer A: 0.1% FA; buffer B 0.1% FA in ACN) applying a flow rate of 300 nl/min. Because of loading and washing steps, the total time for an LC-MS/MS run was prolonged to 160 min.

The CaptiveSpray ion source with a nanoBooster was used to connect the LC system to the MS device. The source was operated in positive ion mode at 150°C dry temperature, 1300 V capillary voltage, 0.2 bar nanoBooster and a dry gas flow of 0.3 l/min. For the MS and MS/MS acquisition the predefined 'Instant Expertise' method was used (Compass 1.9, Bruker). Briefly, the *m/z* data were acquired in the range of 150 to 2200 and the fixed total cycle time was set to 3.0 sec. The instrument settings were as follows: hexapole radio frequency (RF) voltage of 350 V peak-to-peak (Vpp), a funnel 1 RF of 400 Vpp, a funnel 2 RF of 600 Vpp, a prepulse storage time of 10 µs, a transfer time of 90 µs and a collision cell RF of 2000 Vpp. For the MS spectra the acquisition speed was 2 Hz with the collision energy of 7 eV. For the MS/MS the acquisition speed was dependent from the precursor signal intensities and was set to 4 Hz for lower (2500 cts) and 16 Hz for higher (25000 cts) intensities with linear adjustment for the precursors between low and high. The collision energy was adjusted between 23–65 eV as a function of the *m/z* value. The instrument was calibrated using 10 mM sodium formate. Two technical repetitions were run for the three independent harvests.

The acquired spectra were analysed for label-free quantifications using Progenesis QI software for proteomics (Version 3.0, Nonlinear Dynamics, Newcastle upon Tyne, U.K.) as recommended by the manufacturer, thereby enabling mass correction, alignment, normalization, peak picking, quantification and statistics. MS/MS spectra were exported from the Progenesis QI software as Mascot generic files and used for peptide identification with Mascot v2.5.1. The potato database based on the sequences from *Solanum tuberosum* group Phureja DM1-3 (PGSC\_DM\_v3.4\_pep\_representative, 39,031 entries) [23] annotated by matching against available NCBI entries with Blast2GO software (09.2014) [24] and merged with the sequences of human keratin and trypsin was used. The search parameters applied were as follows: 15 ppm peptide mass tolerance, 0.05 Da fragment mass tolerance, one missed cleavage allowed, carbamidomethylation as fixed modification, and methionine oxidation as variable modification. A Mascot integrated peptide decoy database search was performed and searches were processed with the Percolator machine-learning algorithm [25]. The false discovery rate was <1% and ion score cut-off 15 and significance threshold  $p < 0.05$ . For subsequent analysis, the set of identified

sequences was re-imported into Progenesis QI. For quantification purposes, all unique peptides were included; the presence of at least two unique peptides, together with  $p$  value below 0.05 and a threshold of 1.5 fold was set as the determinant of significant differential abundance. Additional annotation for selected proteins was sought by referring to the UniProt server ([www.uniprot.org](http://www.uniprot.org)). Proteins were functionally classified as described elsewhere [26]. Z-score normalization and hierarchical clustering based on the Euclidean distance method were carried out using the Perseus Framework ([www.perseus-framework.org](http://www.perseus-framework.org)). The abundances and sequences of peptides used for the identification of proteins discussed in the study are listed in the supplementary Tables S1 and S2. The visualization of protein profiles integrated in metabolic pathways was performed using the VANTED framework [27, 28] based on metabolic pathways from the MetaCrap database [29]. A full listing of the differentially expressed proteins has been archived, together with all of the raw data, in the IPK Gatersleben system e!DAL [30], available at:

<https://doi.ipk-gatersleben.de/DOI/0ec29fc0-7860-4ec7-9f19-83a5ee25f96f/7249892b-374f-4c8c-87c3-27deae1f9006/2/1847940088>

#### *Metabolite analysis*

Metabolites were extracted from a 100 mg sample (fresh weight) of the roots, derivatized and detected using gas chromatography/mass spectrometry (GC/MS). The extraction and detection followed the methods described elsewhere [31]. The annotation of compounds was performed using the NIST database implemented in MassLynx 4.1 (Waters, Milford, MA, USA); retention time indices were compared to those documented in the Golm Metabolome Database (<http://gmd.mpimp-golm.mpg.de/>). The masses used for quantification, together with each compound-specific retention time index are listed in Table S3. All analyses were performed in duplicate. QuantLynx tool implemented in MassLynx 4.1 software was used for data processing and peak integration. The intensity value of each metabolite was normalized to the signal of the internal standard  $^{13}\text{C}_6/\text{D}_7$  glucose (Sigma-Aldrich, Munich, Germany). Statistical analysis was performed using SigmaPlot v13 (Systat Software, Erkrath, Germany) utilizing a one way analysis of variance (ANOVA) and Tukey post hoc test. Each time point was analysed separately.

#### *Amino acid analysis*

Around 20 mg of homogenized root material was suspended in 0.5 ml methanol and held for 30 min at 70°C with shaking, after which 0.25 ml chloroform and 0.5 ml water were added and the mixture centrifuged (18,000 g, 15 min, 4°C). A 100  $\mu\text{l}$  aliquot of the aqueous methanol phase was vacuum-dried and re-dissolved in 10  $\mu\text{l}$  water. Samples were derivatized using an AccQ Fluor kit (Waters, Eschborn, Germany) following the manufacturer's suggested protocol. To enable quantification, serial dilutions of a standard mixture of amino acids were derivatized in the same way. A 1  $\mu\text{l}$  sample was injected into a Waters Acquity UPLC system and separated by passing

through an AccQ-Tag Ultra 1.7  $\mu\text{m}$ , 2.1x100 mm UPLC column (Waters) using a flow rate of 0.7 ml/min. The gradient was from 0 to 0.5 min (99.9% A), 5.7 min (98% A), 7.5 min (91% A), 8.7 min (90.3% A), 9.2 min (87% A), 12 min (86% A), 12.2 – 12.7 min 40% A, 13-14 min (99.9% A) with a Waters Eluent A AccQ Tag and Waters Eluent B AccQ Tag. Derivatized amino acids were detected with ACQUITY fluorescence detector (Waters) with  $\lambda_{\text{Ex}}$  set to 266 nm and  $\lambda_{\text{Em}}$  set to 473 nm. Identification and quantification was performed using Empower 3 software (Waters). Statistical analyses were performed using SigmaPlot v13 (Systat Software) applying a one way ANOVA and Tukey post hoc test. Each time point was analysed separately.

## Results

### *Growth retardation is stronger for the sensitive cultivar 'Lambada'*

The root biomass accumulated by the two cultivars exposed to N deficiency for up to 72 h is presented in Table 1 and Figure S1. Whether or not N was deficient, the root biomass accumulated by the more sensitive cv. 'Lambada' plants exceeded that achieved by cv. 'Topas' plants. After the T0+72 h period of exposure to stress, the root mass of cv. 'Lambada' plants was 65% of that achieved under stress-free conditions, but the inhibitory effect was less drastic (90%) for cv. 'Topas' plants. Other phenotypic symptoms of N deficiency were not visible at the time of treatment. The N content of the cv 'Topas' was about 10% higher than that of cv. 'Lambada' roots at each sampling point, under both N-sufficient and -deficient conditions. It fell to about 85% of the control level by T0+24 h and to 77% by T0+72 h, and their carbon content remained unaffected by the stress. The soluble protein content of cv. 'Topas' roots was about 20% greater than that of cv. 'Lambada' roots, and was not significantly influenced by N deficiency.

### *N deficiency affects the root proteome in a cultivar-specific manner*

Changes in protein profiles during N deficiency were monitored to find out the processes leading to the different behaviour of the cultivars after N limitation. The label-free LC/MS analysis generated a set of 570 differentially abundant proteins (DAPs), identified on the basis of 2,248 peptides (their abundance clustered according to the time point specificity is presented in Figure S2).

At T0, a total of 59 proteins were assessed as being more abundant in the roots of cv. 'Lambada' than in those of cv. 'Topas' and *vice versa* for 199 proteins. Among the latter set were a number of enzymes associated with N uptake or assimilation, namely nitrite reductase, glutamine synthetase (GS), ferredoxin-dependent glutamate synthase (Fd-GOGAT), glutamate dehydrogenase (GDH), glutamate decarboxylase and aspartate aminotransferase. The abundance of all of these enzymes was 1.5-2 fold higher in cv. 'Topas' under both N regimes at each sampling point. The same applied with respect to a number of enzymes active in the tricarboxylic acid (TCA) cycle, in glycolysis, in



lignin synthesis and in protein synthesis and degradation. The set of enzymes, which were more abundant in cv. 'Lambada' included peroxidases, subtilisin-like proteases and phosphoglycerate mutase. The abundance of selected proteins is presented in the Figure 2.

A detailed analysis of the cultivars' differential protein abundance at each time point is shown in the Table 2. At T0+10 min and T0+1 h, there was no discernible alteration in the root proteome. By T0+24 h, 36 DAPs increased in cv. 'Lambada', but none was changed in cv. 'Topas'. All of those 36 DAPs, except of one lipoxygenase, had been identified as being more abundant in cv. 'Topas' than in cv. 'Lambada' roots. Exposure to N deficiency resulted in their abundance either becoming comparable, or remaining higher in cv. 'Topas' roots. The DAPs induced by the stress in cv. 'Lambada' fell into a number of functional categories, namely 'metabolism' (nine proteins), 'energy' (seven proteins), 'disease/defence' (four proteins), 'protein synthesis' (three proteins), 'secondary metabolism (three proteins) and 'protein destination and storage' (three proteins). The nine DAPs associated with 'metabolism' featured proteins involved in lipid synthesis (glyoxysomal fatty acid  $\beta$ -oxidation multifunctional protein, stearyl-(acyl-carrier-protein) 9-desaturase and lipoxygenase), N assimilation (GS) and sugar metabolism (mannose-1-phosphate guanylyltransferase and rhamnose synthetic enzyme); the seven assigned to the 'energy' category were a transketolase, an NADP-dependent malic enzyme, 2,3-bisphosphoglycerate-independent phosphoglycerate mutase, phosphoenolpyruvate carboxylase (PEPC), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and two pyruvate kinases; those associated with 'protein synthesis' were two elongation factors and a ribosomal protein, and those with 'protein destination and storage' were a subunit of 26S proteasome, a T-complex protein and an aminopeptidase.

At T0+72 h, 20 DAPs were identified in cv. 'Lambada' (16 were more abundant than in the control and four less abundant) and eight in cv. 'Topas' (five more abundant and three less). Six of these proteins were differentially abundant in both cultivars' roots: four (protein yippee-like, universal stress protein A-like, scopoletin glucosyltransferase and polyamine oxidase) were more abundant than in control and two (signal recognition particle 54 kDa protein and hyoscyamine 6-dioxygenase-like) were less abundant. The largest group of DAPs identified at T0+72 h fell into the functional category 'secondary metabolism'; three of these (carboxylesterase, caffeic acid 3-O-methyltransferase and acetylajmalan esterase) increased in abundance only in cv. 'Lambada' roots, while the other three proteins responded similarly to the stress in both cultivars. The second largest group belonged to the category 'disease/defence', and included a glutathione S-transferase, a heat shock cognate 70 kDa protein and a protein DJ-1 homolog B, all of which were more abundant in cv. 'Lambada' roots, along with a universal stress protein A, which was more abundant in both cultivars. The proteins taking part in metabolic and energetic processes such as transketolase, phosphoenolpyruvate carboxylase, caffeic acid 3-O-methyltransferase-like and phosphoribosylformylglycinamide synthase represented similar abundance profile as after 24 h treatment in both cultivars.

*Apart from oxoglutarate, the content of TCA-derived organic acids is decreased by N deficiency*

Changed C/N ratios influence the metabolic fluxes in the primary and secondary metabolisms. Additionally we have found differences in the abundance of proteins taking part in the TCA cycle therefore metabolite profiling by GC-MS was performed. Polar extracts of the roots of both cultivars contained an average of 65 identifiable metabolites, of which 40 were quantified. Amino acids detected by GC/MS have not been included in the description which follows as their quantification was documented using the targeted analysis which provides higher dynamic range as described elsewhere [32].

The compounds that differed most notably between the two cultivars and/or responded most clearly to the stress treatment are shown in Figure 3, while the less prominently responding ones are illustrated in Figure S3. Inter-cultivar differences in the content of several metabolites were detected at T0. The content of a number of organic acids, including those associated with the TCA cycle, certain sugars and putrescine was greater in cv. 'Lambada' than in cv. 'Topas' roots; the only one which was greater in cv. 'Topas' roots was ononitol. Exposure to N stress induced a fall in the production of TCA cycle-associated organic acids. A particularly rapid response, already detectable at T0+10 min, was observed for succinate in cv. 'Lambada' roots. The content of both citrate and fumarate fell marginally in both cultivars during the early part of the stress exposure, as did the content of malate in cv. 'Lambada'. After further exposure to the stress, the root content of citrate in cv. 'Lambada' and that of succinate in both cultivars began to fall. The content of both malate and fumarate decreased, but the changes were not statistically significant. By T0+24 h, oxoglutarate had begun to accumulate in cv. 'Topas', and the same was the case for cv. 'Lambada' after a longer exposure to the stress.

The content of quinic acid was depressed by the stress treatment in both cultivars, but the effect was noted earlier in cv. 'Lambada' than in cv. 'Topas' (T0+24 h vs T0+72 h). By T0+72 h, the content of shikimate in cv. 'Topas' was already declining, whereas in cv. 'Lambada', the extent of the fall was only marginal. The roots of both cultivars responded to the stress by accumulating hydroxycinnamic acid.

The level of carbohydrates in the roots did not significantly vary during the experiment, while that of sedoheptulose remained unchanged over time, maintained at about three times the level in cv. 'Lambada' compared to cv. 'Topas' under both stressed and non-stressed conditions. The content of sugar-derived organic acids was slightly lower at T0+10 min than in control, while that of both threonic and gluconic acid was clearly decreased as the N deprivation period was extended; that of glucaric acid fell in cv. 'Lambada', but rose in cv. 'Topas'. A fall in the content of uracil was noted in cv. 'Lambada' roots at T0+24 h and in cv. 'Topas' at T0+72 h. The content of ononitol, octopamine, inositol and inositol-2-phosphate was quite stable over time in cv. 'Lambada' but was lower at T0+72 h in cv. 'Topas'. The content of putrescine was lower at T0+72 h in cv. 'Topas', but did not change in cv. 'Lambada'.

### *Amino acids accumulated differentially by the two potato cultivars*

Different abundance of enzymes taking part in the GS/GOGAT cycle but also reduced total N content suggested that changes in the amino acid level might also occur. A targeted amino acid approach confirmed that the cultivars' stress responses were distinct from one another (Table 3). When subjected to N stress, both cultivars experienced a decline in the total content of free amino acids. By T0+24 h, the content of free amino acids had fallen markedly in cv. 'Topas' roots but had not changed in those of cv. 'Lambada'. By T0+72 h, the content had dropped to 64% of the control in cv. 'Lambada' and to 71% in cv. 'Topas'. The amino acid content was however about 20% higher in cv. 'Topas' when compared to cv. 'Lambada'.

The contents of  $\gamma$ -aminobutyric acid (GABA), glycine and cysteine were all higher in cv. 'Lambada' than in cv. 'Topas' roots, whereas those of aspartate, glutamate, leucine, methionine, serine, phenylalanine, threonine, tryptophan and valine were all higher in cv. 'Topas'. The content of glutamine, the most abundant amino acid in the roots of both cultivars, fell by T0+24 h to 70% of the control in cv. 'Topas' and to 50% by T0+72 h in both cultivars. The glutamic acid content of cv. 'Topas' roots was about double that in cv. 'Lambada' roots and hardly varied during the course of the experiment. The aspartic acid content was reduced by a prolonged exposure to N deficiency, but remained higher in cv. 'Topas'. The asparagine content of the cv. 'Lambada' roots was higher at T0+24 h, and was lower at T0+72 h, whereas in the cv. 'Topas' roots, its variation was statistically insignificant.

The content of GABA in cv. 'Lambada' roots decreased by T0+10 min. As the stress period was prolonged, the GABA content of cv. 'Lambada' roots increased, while that of cv. 'Topas' roots remained stable: by T0+72 h, the GABA concentration in cv. 'Topas' was roughly double that in cv. 'Lambada'. The content of glycine also fell initially in cv. 'Lambada', while that in cv. 'Topas' did not change. Only minor fluctuations in the content of alanine, serine and valine were observed in the initial stages of the stress, but that of both alanine and serine did fall later in both cultivars, whereas that of valine fell only in cv. 'Topas'. The prolonged exposure to N deficiency induced an increase in the content of both cysteine and lysine in cv. 'Lambada', but not in cv. 'Topas' roots. Histidine was more abundant in cv. 'Topas' than in cv. 'Lambada', but fell to the cv. 'Lambada' level by T0+72 h. The abundance of leucine, methionine and threonine was lower at T0+72 h than in control in cv. 'Topas', but not in cv. 'Lambada'. Both cultivars accumulated more tyrosine as a result of the stress, but there was little effect on the content of arginine, isoleucine, phenylalanine, proline or tryptophan. In most cases, the concentration of those individual amino acids which fell in cv. 'Topas' as a result of N deprivation remained higher than in cv. 'Lambada'. A metabolic map, generated to highlight the relationships between the changes induced in the content of the various metabolites and proteins assayed is presented in Supplementary Figure S4.

## Discussion

It is well recognized that N deficiency inhibits plant growth. In potato, N deficiency for a period of up to 21 days revealed a clear difference between the cultivars ‘Topas’ and ‘Lambada’ with respect to their ability to accumulate biomass under *in vitro* conditions [13-15]. The present experiments using *in vitro* cultivated plants showed that this difference was already detectable after an even shorter period of N stress. From the data we gathered it seems that under non-limiting N conditions cv. ‘Lambada’ use the provided nutrients to produce high biomass, whereas cv. ‘Topas’ allocate more resources into protein production. Under N limiting conditions, the biomass production dramatically drops in cv. ‘Lambada’, but not in cv. ‘Topas’ that use its resources to survive N deficiency. Figure 4 summarizes the most important findings and shows the dissimilar responses of the potato cultivars on the biochemical level. The pathways and candidate proteins taking part in the better growth of cv. ‘Topas’ in N deficient conditions are described below.

### *N re-assimilation is more efficient in cv. ‘Topas’*

In higher plants about 95% of available ammonium is assimilated via the GS/GOGAT pathway [33]. A higher capacity to accumulate proteins and metabolites participating in N assimilation and re-assimilation was a key difference between cv. ‘Topas’ and ‘Lambada’ the two potato cultivars. Glutamine is the major output of the GS/GOGAT pathway. It serves as a translocator of N from source to sink tissue and decrease in its abundance reflects a low N environment in the root [34]. The more rapid decrease in its content in the cv. ‘Topas’ roots would have had the effect of an earlier reduction in the total amino acid content. The fall in the glutamine content in the roots of cv. ‘Topas’ is presumed to reflect the synthesis of proteins which act to support the plant’s growth in a N- deficient medium. The slower fall in cv. ‘Lambada’ roots could be explained by the increased abundance of GS observed at T0+24 h. A similar differential temporal behaviour was observed for asparagine, which is a N-rich amino acid used as both a storage and a transport compound [35]. The root content of glutamate, a further product of the GS/GOGAT cycle, was stable over the course of the whole experiment in both cultivars, as has been similarly described in experiments carried out using other plant species [36, 37]. Its content in the roots of cv. ‘Topas’ was roughly double that in the roots of cv. ‘Lambada’ and positively correlated with the abundance of both GOGAT and GDH (Pearson  $r > 0.83$ , data not shown). An increased abundance of GDH is needed to maintain a homeostasis of glutamate originating from GS/GOGAT cycle. The abundance of enzymes involved in N assimilation remained stable in cv. ‘Topas’ throughout the experiment, despite the fall in the content of both glutamine and N. It is possible that cv. ‘Topas’ possesses more effective mechanisms to re-assimilate ammonium from protein breakdown products, as a number of the DAPs were either proteases or belonged to the proteasome 26S protein ubiquitination pathway.

Thus the effective re-assimilation of N may well make a significant contribution to sustaining plant growth under N-limiting conditions.

#### *C metabolism is affected by N starvation*

Faced with N deficiency, photosynthesis is compromised, while a plentiful supply of photoassimilate promotes both the uptake and assimilation of N [38]. Similarly to potato cultivars, experiments in maize have shown that when N is limiting, the output of organic acids from TCA cycle activity is reduced [39, 40]. These organic acids represent the precursor compounds required for the synthesis of amino acids, act as acceptors for reduced carbon, provide a source of counter anions to replace nitrate and maintain pH balance during nitrate assimilation [41]. As was also the case for the proteins associated with N assimilation, the enzymes involved in C metabolism were more abundant in the cv. 'Topas' roots (Fig. 2 and S4). Much of this difference was accounted for by the contrasting abundance of 2-oxoglutarate, a compound required to maintain the cellular content of glutamate.

The content of phosphoenolpyruvate carboxylase (PEPC), NADP-dependent malic enzyme increased in the roots of cv. 'Lambada' at T0+24 h, reaching the level present in cv. 'Topas'. The role of PEPC is to provide oxaloacetate, which can be reduced to malate to replenish carbon skeletons diverted from TCA cycle to enable N assimilation [42]. This function, together with the action of malic enzyme, represents an important interface between carbon and N metabolism [43]. In agreement, the content of amino acids derived from oxaloacetate (asparagine and lysine) was increased in cv. 'Lambada' and in 'Topas' they had a higher initial concentration. A greater abundance of carbon metabolism-related enzymes in cv. 'Topas' is probably connected to a higher representation of enzymes taking part in the GS/GOGAT/GDH cycle to maintain the metabolic flux, but also to provide adequate energy supply.

#### *Protein recycling leads to the better tolerance for N deficiency*

The adaptation to stress involves numerous alterations in protein synthesis, but also protein degradation is crucial for plant survival [44]. Proteins are reservoirs of amino acids that can be recycled for the synthesis of specific proteins required under N deficiency. A higher level of several ribosomal proteins in cv. 'Topas' was responsible for its overall higher protein content. A potentially important difference between the cultivars was higher abundance of proteins assigned to the 26S proteasome in cv. 'Topas' than in cv. 'Lambada'. Furthermore, we observed a different, time dependent phosphorylation profile of the ubiquitination involved E2 and E3 enzymes in potato cultivars (Jozefowicz & Mock, unpublished results). A constitutive expression in rice of a gene encoding E3 ligase has been suggested as a means to obtain plants more tolerant of N deficiency [45]. An efficient system of protein accumulation and recycling, due to the higher abundance of

protein degradation enzymes, could explain the better performance of cv. 'Topas' under N limitation but also higher abundance of N assimilatory/re-assimilatory enzymes.

*The root GABA content of cv. 'Lambada' is higher than cv. 'Topas'*

The final step of the GABA shunt delivers succinate and NADH to the respiratory chain, so the degradation of GABA can limit the accumulation of toxic reactive oxygen species when plants are exposed to oxidative stress [46]. The operation of this mechanism was suggested by the rapid fall observed in the GABA and succinate content of the cv. 'Lambada' roots at T0+10 min. The constant increase of GABA during the time of experiment in cv. 'Lambada' might be related to its general worse performance under N deficiency. GABA accumulates mainly through either the activation of glutamate decarboxylase or the inhibition of GABA transaminase. The abundance of the former enzyme remained constant, however, that of the latter enzyme fell in response to the stress to a similar extent in both cultivars, but its abundance was only negatively correlated with that of GABA ( $r = -0.93$ ) in cv. 'Lambada'. GABA can also be synthesized from polyamines, putrescine and spermidine [47] and the pool of putrescine was higher in cv. 'Lambada'. The abundance of polyamine oxidase, which likely is involved in GABA synthesis, rose as the stress continued in cv. 'Lambada' and in cv. 'Topas' only at T0+72 h. The comparable abundance of enzymes participating in GABA metabolism suggests that they are regulated through post-translational modification. GABA has been associated with a range of activity in plants, including the control of cytosolic pH, protection against insect attack, osmoregulation and signalling [46]. It accumulates in plants exposed to a range of both biotic and abiotic stress [48] and it increased in the roots of *A. thaliana* and in green algae exposed to N deficiency [49, 50].

*Lignification is more pronounced in cv. 'Topas' than in cv. 'Lambada'*

As the period of exposure to N deficiency was prolonged, many pathways and processes other than strictly related to N metabolism were affected. For example, the level of shikimate differed between the two cultivars: it was relatively stable in cv. 'Lambada', but decreased over time in cv. 'Topas'. Although shikimate is a known precursor of the aromatic amino acids [51], the level of these compounds was unaffected. A reduced level of quinic acid implied that lignin synthesis was compromised by N deficiency, as has also been described elsewhere [52]. The level of three key lignin synthesis enzymes was consistently higher in cv. 'Topas' but the abundance of caffeic acid 3-O-methyltransferase increased as a result of 24 and 72 h N deficiency in cv. 'Lambada'. The presence of the cinnamyl alcohol dehydrogenase in cv. 'Topas' has previously been shown to be promoted by a prolonged period of N deficiency [17], as it is also in barley roots and tobacco leaves [52, 53]. Lignin synthesis has been shown to be triggered by exposure to various biotic and abiotic stresses [54]. Here it might be related to the differential cell wall composition of the two cultivars or be the sink for the excess of carbon.

### *Stress related proteins are higher abundant in cv. 'Lambada'*

The number of proteins responsive to N starvation in cv. 'Lambada' 24 and 72 h was related to stress defence mechanisms. One of those was formate dehydrogenase, which is known to respond to abiotic stresses [55, 56]. In potato, high levels of this enzyme have been detected in non-photosynthetic tissues, but not in the roots [57]. Environmental stresses, including N deficiency, have been shown to stimulate the production of reactive N species and the overproduction of those molecules must be controlled by antioxidants [58]. Here, a number of proteins identified as peroxidases were found to be more abundant in cv. 'Lambada'. This suggest that detoxification and defence mechanisms play important role in the response to N deficiency. Additionally, two isoforms of a subtilisin-like protease were more abundant in cv. 'Lambada' than in cv. 'Topas'. This protease contributes to general protein turnover, although some evidence suggests its specific involvement in both the plant–pathogen interaction [59] and apoptosis [60].

Combining data acquired from a range of 'omics' platforms allows for the identification of many potentially significant differences between cultivars. The most pronounced difference was related to the abundance of certain enzymes of central metabolism and proteins synthesis and catabolism, which was higher in the roots of cv. 'Topas', thereby potentially representing a major cause of this cultivar's better tolerance towards N deficiency. Although the abundance of a number of proteins important for adaptation to a dynamic N regime did increase in the roots of cv. 'Lambada' in response to N deprivation, their level was higher in cv. 'Topas', implying that the latter cultivar is better equipped to deal with N deficiency. The content of the majority of metabolites and proteins remained constant during the initial hours following the removal of N, which can be interpreted by recognizing that the plants needed more time to adjust their metabolism to the stress. Validating candidate proteins and pathways identified will require the testing of additional cultivars and/or the construction of specific transgenic mutants, but also trials under field conditions.

### **Author contributions**

AMJ designed the experiments, AMJ, TPP, MP performed the experiments, AMJ analysed the data, AH visualized the data, AM and AS established the methods and participated in the discussion of results, AMJ and HP wrote the paper

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**Table 1.** The effect of N deficiency on the accumulation of plant biomass, the N and carbon content, the C/N ratio and the content of protein and free amino acids in the two potato cultivars ‘Lambada’ and ‘Topas’. Letter suffixes have been used to indicate significant differences in mean values, as predicted by a one way ANOVA (p-value <0.05). FW: fresh weight.

|          |    | Root FW [g]             | Shoot FW [g]            | N %                     | C %                     | C/N ratio               | Soluble protein [mg/g FW] | Amino acids content [pmol/g FW] |
|----------|----|-------------------------|-------------------------|-------------------------|-------------------------|-------------------------|---------------------------|---------------------------------|
| 10 min   |    |                         |                         |                         |                         |                         |                           |                                 |
| Lambada  | N+ | 0.53±0.16 <sup>a</sup>  | 1.51±0.23 <sup>a</sup>  | 5.60±0.14 <sup>a</sup>  | 40.52±0.34 <sup>a</sup> | 7.23±0.13 <sup>a</sup>  | 3.78±0.13 <sup>a</sup>    | 24.45±3.97 <sup>a</sup>         |
|          | N- | 0.50±0.17 <sup>a</sup>  | 1.54±0.35 <sup>a</sup>  | 5.42±0.18 <sup>ac</sup> | 40.93±0.57 <sup>a</sup> | 7.56±0.23 <sup>b</sup>  | 3.81±0.29 <sup>a</sup>    | 23.09±4.43 <sup>a</sup>         |
| Topas    | N+ | 0.25±0.06 <sup>b</sup>  | 0.98±0.06 <sup>b</sup>  | 5.98±0.03 <sup>ab</sup> | 40.58±0.35 <sup>a</sup> | 6.79±0.04 <sup>c</sup>  | 4.70±0.25 <sup>b</sup>    | 26.21±6.01 <sup>a</sup>         |
|          | N- | 0.27±0.06 <sup>b</sup>  | 1.03±0.09 <sup>b</sup>  | 5.93±0.05 <sup>bc</sup> | 41.35±0.54 <sup>a</sup> | 6.97±0.03 <sup>cd</sup> | 4.79±0.31 <sup>b</sup>    | 24.42±3.33 <sup>a</sup>         |
| 1 hour   |    |                         |                         |                         |                         |                         |                           |                                 |
| Lambada  | N+ | 0.58±0.15 <sup>a</sup>  | 1.47±0.26 <sup>a</sup>  | 5.79±0.14 <sup>ac</sup> | 40.85±0.21 <sup>a</sup> | 7.06±0.17 <sup>ad</sup> | 3.99±0.08 <sup>a</sup>    | 23.63±4.81 <sup>a</sup>         |
|          | N- | 0.52±0.11 <sup>a</sup>  | 1.51±0.25 <sup>a</sup>  | 5.56±0.12 <sup>b</sup>  | 41.25±0.29 <sup>a</sup> | 7.42±0.18 <sup>b</sup>  | 4.01±0.28 <sup>a</sup>    | 23.98±6.48 <sup>a</sup>         |
| Topas    | N+ | 0.27±0.04 <sup>b</sup>  | 1.12±0.08 <sup>ab</sup> | 6.10±0.15 <sup>c</sup>  | 40.88±0.34 <sup>a</sup> | 6.71±0.14 <sup>c</sup>  | 4.90±0.38 <sup>b</sup>    | 25.19±5.57 <sup>a</sup>         |
|          | N- | 0.28±0.07 <sup>b</sup>  | 1.15±0.06 <sup>a</sup>  | 5.91±0.07 <sup>c</sup>  | 40.89±0.43 <sup>a</sup> | 6.92±0.04 <sup>cd</sup> | 4.53±0.28 <sup>b</sup>    | 23.87±4.11 <sup>a</sup>         |
| 24 hours |    |                         |                         |                         |                         |                         |                           |                                 |
| Lambada  | N+ | 0.55±0.14 <sup>a</sup>  | 1.85±0.33 <sup>ab</sup> | 5.88±0.20 <sup>a</sup>  | 41.16±0.36 <sup>a</sup> | 7.00±0.19 <sup>a</sup>  | 4.19±0.17 <sup>a</sup>    | 25.70±4.40 <sup>a</sup>         |
|          | N- | 0.54±0.11 <sup>a</sup>  | 1.93±0.29 <sup>a</sup>  | 4.99±0.15 <sup>b</sup>  | 41.43±0.50 <sup>a</sup> | 8.31±0.17 <sup>b</sup>  | 4.15±0.55 <sup>a</sup>    | 24.71±4.18 <sup>a</sup>         |
| Topas    | N+ | 0.29±0.04 <sup>b</sup>  | 1.34±0.17 <sup>ab</sup> | 6.24±0.13 <sup>c</sup>  | 40.48±0.56 <sup>a</sup> | 6.48±0.09 <sup>c</sup>  | 4.49±0.46 <sup>a</sup>    | 26.60±5.70 <sup>a</sup>         |
|          | N- | 0.27±0.05 <sup>b</sup>  | 1.22±0.12 <sup>b</sup>  | 5.38±0.17 <sup>d</sup>  | 41.24±0.68 <sup>a</sup> | 7.67±0.13 <sup>d</sup>  | 4.80±0.12 <sup>a</sup>    | 21.16±3.56 <sup>a</sup>         |
| 72 hours |    |                         |                         |                         |                         |                         |                           |                                 |
| Lambada  | N+ | 0.74±0.14 <sup>a</sup>  | 2.38±0.57 <sup>a</sup>  | 5.45±0.16 <sup>a</sup>  | 40.95±0.50 <sup>a</sup> | 7.49±0.17 <sup>a</sup>  | 3.44±0.34 <sup>a</sup>    | 26.14±3.45 <sup>a</sup>         |
|          | N- | 0.49±0.09 <sup>ab</sup> | 1.61±0.30 <sup>ab</sup> | 4.14±0.09 <sup>b</sup>  | 41.09±0.50 <sup>a</sup> | 9.91±0.18 <sup>b</sup>  | 3.60±0.70 <sup>ac</sup>   | 16.82±3.07 <sup>b</sup>         |
| Topas    | N+ | 0.33±0.03 <sup>ab</sup> | 1.76±0.21 <sup>ab</sup> | 6.10±0.10 <sup>c</sup>  | 40.65±0.44 <sup>a</sup> | 6.67±0.09 <sup>c</sup>  | 4.59±0.35 <sup>b</sup>    | 28.70±5.80 <sup>a</sup>         |
|          | N- | 0.29±0.03 <sup>b</sup>  | 1.56±0.13 <sup>b</sup>  | 4.72±0.06 <sup>d</sup>  | 41.10±0.60 <sup>a</sup> | 8.70±0.13 <sup>d</sup>  | 4.32±0.24 <sup>bc</sup>   | 20.63±4.33 <sup>b</sup>         |

**Table 2.** Differentially abundant proteins identified in roots exposed to either 24 h or 72 h of N deficiency. Further details relating to protein identity and the quantification of individual proteins are provided in Table S1. Accession numbers are given without their PGSC003DMT prefix. FC: fold change, FC N+: ratio cv. ‘Topas’ control to cv. ‘Lambada’ control, FC N-: ratio cv. ‘Topas’ stress to cv. ‘Lambada’ stress.

| Accession          | Description   | Function         | FC Lambada | FC Topas | FC N+ | FC N- |
|--------------------|---|------------------|------------|----------|-------|-------|
| 24 hours treatment |   |                  |            |          |       |       |
| <u>cv. Lambada</u> |   |                  |            |          |       |       |
| 400001303          | Formate dehydrogenase   | diseases/defence | 1.50       | 1.14     | 1.54  | 1.17  |
| 400042483          | L-ascorbate oxidase homolog                                   | diseases/defence | 1.67       | 1.28     | 1.29  | 0.99  |
| 400080580          | Heat shock 70 kDa protein 15-like                             | diseases/defence | 1.64       | 1.14     | 2.29  | 1.59  |
| 400080588          | Heat shock 70 kDa protein 15-like                             | diseases/defence | 1.66       | 1.15     | 2.16  | 1.50  |
| 400002454          | 2.3-bisphosphoglycerate-independent phosphoglycerate mutase   | energy           | 1.50       | 1.05     | 1.73  | 1.22  |
| 400018062          | Transketolase   | energy           | 1.64       | 1.11     | 1.71  | 1.16  |
| 400028308          | Pyruvate kinase, cytosolic                                    | energy           | 1.70       | 1.21     | 1.38  | 0.99  |
| 400030050          | Glyceraldehyde-3-phosphate dehydrogenase A                    | energy           | 1.78       | 1.24     | 3.63  | 2.54  |
| 400054791          | Phosphoenolpyruvate carboxylase                               | energy           | 1.80       | 1.28     | 1.33  | 0.94  |
| 400072411          | Pyruvate kinase 1, cytosolic                                  | energy           | 1.72       | 1.23     | 1.75  | 1.25  |
| 400084201          | malic enzyme  | energy           | 1.58       | 1.33     | 1.58  | 1.33  |
| 400026512          | Probable phosphoribosylformyl-glycinamide synthase            | metabolism       | 1.53       | 1.04     | 1.47  | 1.01  |
| 400006337          | Alpha-1.4 glucan phosphorylase L-1 isozyme                    | metabolism       | 1.84       | 1.56     | 2.41  | 2.05  |
| 400011624          | Adenosylhomocysteinase  | metabolism       | 1.96       | 1.34     | 1.64  | 1.12  |
| 400011792          | Glyoxysomal fatty acid beta-oxidation multifunctional protein | metabolism       | 1.68       | 1.52     | 2.01  | 1.82  |
| 400040409          | Mannose-1-phosphate guanylyltransferase 1                     | metabolism       | 1.62       | 1.10     | 1.76  | 1.20  |
| 400054145          | Lipoxygenase  | metabolism       | 1.84       | 1.29     | 0.78  | 0.55  |
| 400054753          | Rhamnose biosynthetic enzyme 1                                | metabolism       | 1.60       | 1.13     | 1.84  | 1.30  |

|           |   |                                 |      |      |      |      |
|-----------|---|---------------------------------|------|------|------|------|
| 400060733 | Glutamine synthetase cytosolic isozyme 1-1                  | metabolism                      | 1.54 | 1.14 | 1.29 | 0.96 |
| 400063205 | Betaine aldehyde dehydrogenase                              | metabolism                      | 2.01 | 1.39 | 1.52 | 1.06 |
| 400083137 | Putative delta-1-pyrroline-5-carboxylate dehydrogenase 12A1 | metabolism                      | 1.67 | 1.20 | 2.61 | 1.88 |
| 400041874 | Stearoyl-[acyl-carrier-protein] 9-desaturase                | metabolism                      | 1.74 | 1.29 | 1.65 | 1.22 |
| 400042238 | Biotin carboxylase 1  | metabolism                      | 1.51 | 1.17 | 1.61 | 1.24 |
| 400031796 | Puromycin-sensitive aminopeptidase isoform X1               | protein destination and storage | 1.59 | 1.21 | 1.60 | 1.21 |
| 400050534 | T-complex protein 1 subunit beta                            | protein destination and storage | 1.59 | 1.35 | 2.01 | 1.70 |
| 400053010 | 26S protease regulatory subunit 6B homolog                  | protein destination and storage | 1.71 | 1.24 | 1.86 | 1.34 |
| 400009804 | Elongation factor Tu  | Protein synthesis               | 1.82 | 1.09 | 2.15 | 1.29 |
| 400020969 | Elongation factor 1-alpha                                   | Protein synthesis               | 1.69 | 1.15 | 2.33 | 1.58 |
| 400040683 | Elongation factor 1-gamma 2-like                            | Protein synthesis               | 1.82 | 1.35 | 1.68 | 1.25 |
| 400063697 | 40S ribosomal protein S10-1-like                            | protein synthesis               | 1.69 | 1.19 | 2.33 | 1.65 |
| 400029318 | Caffeic acid 3-O-methyltransferase-like                     | secondary metabolism            | 1.71 | 1.12 | 1.58 | 1.03 |
| 400048685 | Polyphenol oxidase precursor                                | secondary metabolism            | 1.65 | 1.30 | 2.57 | 2.02 |
| 400066574 | Scopoletin glucosyltransferase                              | secondary metabolism            | 2.16 | 1.40 | 1.83 | 1.19 |
| 400010497 | Plasma membrane atpase 4                                    | transporter                     | 1.65 | 1.12 | 1.97 | 1.34 |
| 400035380 | ADP.ATP carrier protein. mitochondrial                      | transporter                     | 1.58 | 1.26 | 1.86 | 1.49 |

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72 hours treatment

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cv. Lambada

|           |   |                  |      |      |      |      |
|-----------|---|------------------|------|------|------|------|
| 400047047 | Glutathione S-transferase zeta class-like | diseases/defence | 1.54 | 1.33 | 1.42 | 1.22 |
| 400001180 | Heat shock cognate 70 kda protein         | diseases/defence | 1.59 | 1.30 | 2.16 | 1.77 |
| 400032252 | Protein DJ-1 homolog B-like               | diseases/defence | 1.71 | 1.30 | 1.65 | 1.25 |
| 400018062 | Transketolase                             | energy           | 1.52 | 1.14 | 2.22 | 1.67 |

|   |   |                                 |      |      |      |      |
|---|---|---------------------------------|------|------|------|------|
| 400054791                               | Phosphoenolpyruvate carboxylase                       | energy                          | 1.96 | 1.33 | 1.53 | 1.04 |
| 400026512                               | Probable phosphoribosylformylglycinamide synthase     | metabolism                      | 1.49 | 1.14 | 1.60 | 1.22 |
| 400078045                               | Probable pyridoxal 5'-phosphate synthase subunit PDX1 | metabolism                      | 1.57 | 1.28 | 1.19 | 0.97 |
| 400036974                               | Serine carboxypeptidase-like 45                       | protein destination and storage | 1.50 | 1.38 | 0.78 | 0.72 |
| 400027594                               | Putative methyltransferase DDB_G0268948               | Protein destination and storage | 1.53 | 1.33 | 0.85 | 0.74 |
| 400066954                               | T-complex protein 1 subunit alpha                     | protein destination and storage | 0.58 | 0.79 | 0.73 | 0.99 |
| 400055712                               | Eukaryotic translation initiation factor 3 subunit H  | protein synthesis               | 1.50 | 1.28 | 1.34 | 1.14 |
| 400054431                               | Probable carboxylesterase 15                          | secondary metabolism            | 1.54 | 1.33 | 1.18 | 1.02 |
| 400029318                               | Caffeic acid 3-O-methyltransferase-like               | secondary metabolism            | 1.55 | 1.07 | 1.70 | 1.17 |
| 400058377                               | Nucleolar protein 58-like                             | unclassified                    | 0.64 | 0.92 | 0.38 | 0.55 |
| <hr/> <u>cv. Topas</u>                  |   |                                 |      |      |      |      |
| 400067456                               | Expansin-like B1                                      | cell structure                  | 1.39 | 1.56 | 0.87 | 0.98 |
| 400075261                               | Uncharacterized protein                               | unclassified                    | 0.86 | 0.67 | 1.74 | 1.36 |
| <hr/> <u>Both cv. Lambada and Topas</u> |   |                                 |      |      |      |      |
| 400047644                               | Protein yippee-like At4g27740                         | cell growth/division            | 2.24 | 1.99 | 1.54 | 1.37 |
| 400007287                               | Universal stress protein A-like protein isoform X2    | diseases/defence                | 1.65 | 1.63 | 0.64 | 0.63 |
| 400001674                               | Signal recognition particle 54 kda protein 2          | protein destination and storage | 0.62 | 0.56 | 0.86 | 0.77 |
| 400066574                               | Scopoletin glucosyltransferase                        | secondary metabolism            | 1.93 | 1.54 | 2.38 | 1.90 |
| 400030676                               | Hyoscyamine 6-dioxygenase-like                        | secondary metabolism            | 0.66 | 0.60 | 0.78 | 0.71 |
| 400041150                               | Probable polyamine oxidase 2                          | secondary metabolism            | 1.52 | 1.88 | 1.22 | 1.50 |

**Table 3.** Variation in the content of free amino acids in the roots of plants grown under either N-sufficient or -deficient conditions. Values are given in the form nmol per mg fresh weight. Letter suffixes are used to indicate significant differences in mean concentrations, as predicted by a one way ANOVA (p-value <0.05). Statistically significant increases are highlighted in green and decreases in red.

| Amino acid<br>[nmol/mg<br>FW] | T0            |              | 10 min              |                     |                     |                     | 1 hour              |                     |                     |                     | 24 hours            |                     |                     |                     | 72 hours            |                     |                     |                     |
|-------------------------------|---------------|--------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|
|                               | <u>Lamba.</u> | <u>Topas</u> | <u>Lambada</u>      |                     | <u>Topas</u>        |                     | <u>Lambada</u>      |                     | <u>Topas</u>        |                     | <u>Lambada</u>      |                     | <u>Topas</u>        |                     | <u>Lambada</u>      |                     | <u>Topas</u>        |                     |
|                               |               |              | N+                  | N-                  | N+                  | N-                  | N+                  | N-                  | N+                  | N-                  | N+                  | N-                  | N+                  | N-                  | N+                  | N-                  | N+                  | N-                  |
| Glutamine                     | 13.887        | 12.672       | 12.385 <sup>a</sup> | 11.394 <sup>a</sup> | 12.564 <sup>a</sup> | 11.425 <sup>a</sup> | 12.032 <sup>a</sup> | 10.777 <sup>a</sup> | 12.476 <sup>a</sup> | 11.418 <sup>a</sup> | 12.422 <sup>a</sup> | 11.670 <sup>a</sup> | 12.845 <sup>a</sup> | 8.955 <sup>b</sup>  | 12.534 <sup>a</sup> | 5.675 <sup>b</sup>  | 14.044 <sup>a</sup> | 7.169 <sup>b</sup>  |
| Glutamic acid                 | 1.674         | 2.969        | 1.980 <sup>a</sup>  | 1.896 <sup>a</sup>  | 3.184 <sup>b</sup>  | 3.315 <sup>b</sup>  | 1.724 <sup>a</sup>  | 1.952 <sup>a</sup>  | 2.798 <sup>b</sup>  | 2.932 <sup>b</sup>  | 2.121 <sup>a</sup>  | 1.937 <sup>a</sup>  | 3.145 <sup>b</sup>  | 2.996 <sup>b</sup>  | 1.441 <sup>a</sup>  | 1.267 <sup>a</sup>  | 3.383 <sup>b</sup>  | 3.467 <sup>b</sup>  |
| Proline                       | 1.094         | 0.778        | 0.907 <sup>ab</sup> | 0.992 <sup>a</sup>  | 0.690 <sup>b</sup>  | 0.770 <sup>b</sup>  | 1.188 <sup>a</sup>  | 0.983 <sup>a</sup>  | 0.638 <sup>b</sup>  | 0.638 <sup>b</sup>  | 0.923 <sup>a</sup>  | 0.909 <sup>a</sup>  | 0.665 <sup>b</sup>  | 0.604 <sup>b</sup>  | 1.137 <sup>a</sup>  | 0.928 <sup>a</sup>  | 0.859 <sup>a</sup>  | 1.070 <sup>a</sup>  |
| Arginine                      | 0.110         | 0.093        | 0.068 <sup>a</sup>  | 0.074 <sup>a</sup>  | 0.124 <sup>a</sup>  | 0.065 <sup>a</sup>  | 0.093 <sup>a</sup>  | 0.100 <sup>a</sup>  | 0.073 <sup>a</sup>  | 0.094 <sup>a</sup>  | 0.062 <sup>a</sup>  | 0.078 <sup>a</sup>  | 0.086 <sup>a</sup>  | 0.110 <sup>a</sup>  | 0.143 <sup>a</sup>  | 0.116 <sup>a</sup>  | 0.115 <sup>a</sup>  | 0.111 <sup>a</sup>  |
| Histidine                     | 0.375         | 0.406        | 0.298 <sup>a</sup>  | 0.295 <sup>a</sup>  | 0.424 <sup>ab</sup> | 0.358 <sup>b</sup>  | 0.332 <sup>ab</sup> | 0.310 <sup>a</sup>  | 0.417 <sup>b</sup>  | 0.387 <sup>ab</sup> | 0.311 <sup>a</sup>  | 0.271 <sup>a</sup>  | 0.421 <sup>b</sup>  | 0.305 <sup>a</sup>  | 0.359 <sup>a</sup>  | 0.268 <sup>a</sup>  | 0.494 <sup>b</sup>  | 0.302 <sup>ab</sup> |
| Alanine                       | 2.365         | 1.518        | 1.423 <sup>a</sup>  | 1.193 <sup>a</sup>  | 1.414 <sup>a</sup>  | 1.337 <sup>a</sup>  | 0.952 <sup>a</sup>  | 1.015 <sup>a</sup>  | 1.073 <sup>a</sup>  | 1.030 <sup>a</sup>  | 2.180 <sup>a</sup>  | 1.718 <sup>b</sup>  | 1.378 <sup>bc</sup> | 1.105 <sup>c</sup>  | 2.181 <sup>a</sup>  | 1.593 <sup>b</sup>  | 1.905 <sup>a</sup>  | 1.429 <sup>b</sup>  |
| Asparagine                    | 5.237         | 4.114        | 4.416 <sup>ab</sup> | 4.581 <sup>a</sup>  | 4.107 <sup>ab</sup> | 3.367 <sup>b</sup>  | 4.686 <sup>a</sup>  | 4.571 <sup>ab</sup> | 3.951 <sup>ab</sup> | 3.499 <sup>b</sup>  | 4.589 <sup>a</sup>  | 4.908 <sup>b</sup>  | 4.063 <sup>ac</sup> | 3.660 <sup>c</sup>  | 5.258 <sup>a</sup>  | 3.891 <sup>b</sup>  | 4.906 <sup>ab</sup> | 4.271 <sup>ab</sup> |
| Aspartic acid                 | 0.392         | 0.638        | 0.586 <sup>a</sup>  | 0.535 <sup>a</sup>  | 0.844 <sup>b</sup>  | 0.873 <sup>b</sup>  | 0.654 <sup>a</sup>  | 0.712 <sup>ab</sup> | 0.966 <sup>b</sup>  | 1.001 <sup>b</sup>  | 0.485 <sup>a</sup>  | 0.351 <sup>a</sup>  | 0.848 <sup>b</sup>  | 0.547 <sup>b</sup>  | 0.358 <sup>a</sup>  | 0.281 <sup>ab</sup> | 0.828 <sup>b</sup>  | 0.539 <sup>c</sup>  |
| Isoleucine                    | 0.203         | 0.236        | 0.183 <sup>a</sup>  | 0.182 <sup>a</sup>  | 0.241 <sup>b</sup>  | 0.231 <sup>b</sup>  | 0.166 <sup>a</sup>  | 0.166 <sup>a</sup>  | 0.224 <sup>b</sup>  | 0.212 <sup>ab</sup> | 0.157 <sup>a</sup>  | 0.173 <sup>a</sup>  | 0.184 <sup>a</sup>  | 0.183 <sup>a</sup>  | 0.169 <sup>a</sup>  | 0.189 <sup>a</sup>  | 0.204 <sup>a</sup>  | 0.181 <sup>a</sup>  |
| Lysine                        | 0.157         | 0.174        | 0.121 <sup>a</sup>  | 0.117 <sup>a</sup>  | 0.178 <sup>b</sup>  | 0.147 <sup>ab</sup> | 0.110 <sup>a</sup>  | 0.108 <sup>a</sup>  | 0.149 <sup>b</sup>  | 0.144 <sup>ab</sup> | 0.108 <sup>a</sup>  | 0.149 <sup>b</sup>  | 0.152 <sup>ab</sup> | 0.146 <sup>ab</sup> | 0.126 <sup>a</sup>  | 0.188 <sup>a</sup>  | 0.144 <sup>a</sup>  | 0.144 <sup>a</sup>  |
| Methionine                    | 0.039         | 0.052        | 0.035 <sup>a</sup>  | 0.032 <sup>a</sup>  | 0.055 <sup>b</sup>  | 0.050 <sup>b</sup>  | 0.037 <sup>a</sup>  | 0.043 <sup>a</sup>  | 0.056 <sup>b</sup>  | 0.057 <sup>b</sup>  | 0.032 <sup>a</sup>  | 0.033 <sup>a</sup>  | 0.059 <sup>b</sup>  | 0.053 <sup>b</sup>  | 0.033 <sup>ab</sup> | 0.031 <sup>ab</sup> | 0.051 <sup>a</sup>  | 0.039 <sup>b</sup>  |
| Threonine                     | 0.318         | 0.386        | 0.293 <sup>a</sup>  | 0.273 <sup>a</sup>  | 0.401 <sup>b</sup>  | 0.377 <sup>b</sup>  | 0.267 <sup>a</sup>  | 0.285 <sup>a</sup>  | 0.381 <sup>b</sup>  | 0.372 <sup>b</sup>  | 0.268 <sup>a</sup>  | 0.275 <sup>ab</sup> | 0.368 <sup>b</sup>  | 0.307 <sup>ab</sup> | 0.279 <sup>a</sup>  | 0.277 <sup>a</sup>  | 0.385 <sup>b</sup>  | 0.321 <sup>a</sup>  |
| Leucine                       | 0.243         | 0.292        | 0.214 <sup>a</sup>  | 0.209 <sup>a</sup>  | 0.290 <sup>b</sup>  | 0.279 <sup>b</sup>  | 0.174 <sup>a</sup>  | 0.182 <sup>a</sup>  | 0.266 <sup>b</sup>  | 0.248 <sup>b</sup>  | 0.188 <sup>a</sup>  | 0.185 <sup>a</sup>  | 0.252 <sup>a</sup>  | 0.207 <sup>a</sup>  | 0.196 <sup>ab</sup> | 0.209 <sup>ab</sup> | 0.246 <sup>a</sup>  | 0.173 <sup>b</sup>  |
| Valine                        | 0.577         | 0.725        | 0.512 <sup>a</sup>  | 0.466 <sup>ac</sup> | 0.695 <sup>b</sup>  | 0.623 <sup>bc</sup> | 0.448 <sup>a</sup>  | 0.430 <sup>a</sup>  | 0.659 <sup>b</sup>  | 0.603 <sup>ab</sup> | 0.505 <sup>a</sup>  | 0.437 <sup>ac</sup> | 0.662 <sup>b</sup>  | 0.484 <sup>c</sup>  | 0.468 <sup>a</sup>  | 0.378 <sup>a</sup>  | 0.735 <sup>b</sup>  | 0.491 <sup>a</sup>  |
| Glycine                       | 0.609         | 0.354        | 0.528 <sup>a</sup>  | 0.406 <sup>b</sup>  | 0.323 <sup>b</sup>  | 0.338 <sup>b</sup>  | 0.361 <sup>a</sup>  | 0.438 <sup>a</sup>  | 0.355 <sup>a</sup>  | 0.358 <sup>a</sup>  | 0.614 <sup>a</sup>  | 0.572 <sup>a</sup>  | 0.390 <sup>b</sup>  | 0.380 <sup>b</sup>  | 0.871 <sup>a</sup>  | 0.474 <sup>ab</sup> | 0.311 <sup>b</sup>  | 0.324 <sup>b</sup>  |
| Cysteine                      | 0.056         | 0.035        | 0.056 <sup>a</sup>  | 0.048 <sup>ab</sup> | 0.036 <sup>b</sup>  | 0.037 <sup>b</sup>  | 0.048 <sup>a</sup>  | 0.052 <sup>a</sup>  | 0.037 <sup>b</sup>  | 0.034 <sup>b</sup>  | 0.060 <sup>a</sup>  | 0.077 <sup>b</sup>  | 0.028 <sup>c</sup>  | 0.030 <sup>c</sup>  | 0.068 <sup>a</sup>  | 0.099 <sup>ab</sup> | 0.041 <sup>b</sup>  | 0.047 <sup>b</sup>  |
| Serine                        | 0.827         | 1.071        | 0.775 <sup>a</sup>  | 0.669 <sup>a</sup>  | 1.089 <sup>b</sup>  | 1.030 <sup>b</sup>  | 0.684 <sup>a</sup>  | 0.717 <sup>a</sup>  | 1.062 <sup>b</sup>  | 1.025 <sup>b</sup>  | 0.918 <sup>ac</sup> | 0.783 <sup>a</sup>  | 1.179 <sup>b</sup>  | 0.987 <sup>c</sup>  | 0.740 <sup>a</sup>  | 0.581 <sup>b</sup>  | 1.130 <sup>c</sup>  | 0.893 <sup>d</sup>  |
| GABA                          | 4.528         | 2.040        | 3.762 <sup>a</sup>  | 2.924 <sup>b</sup>  | 2.069 <sup>c</sup>  | 2.281 <sup>c</sup>  | 2.732 <sup>ab</sup> | 2.935 <sup>a</sup>  | 2.179 <sup>b</sup>  | 2.028 <sup>b</sup>  | 3.457 <sup>a</sup>  | 4.415 <sup>a</sup>  | 1.581 <sup>b</sup>  | 1.662 <sup>b</sup>  | 4.159 <sup>a</sup>  | 5.745 <sup>a</sup>  | 2.562 <sup>b</sup>  | 2.531 <sup>b</sup>  |
| Phenylalanine                 | 0.054         | 0.077        | 0.058 <sup>a</sup>  | 0.053 <sup>a</sup>  | 0.077 <sup>b</sup>  | 0.075 <sup>b</sup>  | 0.049 <sup>a</sup>  | 0.054 <sup>ab</sup> | 0.074 <sup>b</sup>  | 0.072 <sup>b</sup>  | 0.046 <sup>a</sup>  | 0.053 <sup>ab</sup> | 0.067 <sup>b</sup>  | 0.060 <sup>b</sup>  | 0.066 <sup>a</sup>  | 0.081 <sup>a</sup>  | 0.068 <sup>a</sup>  | 0.078 <sup>a</sup>  |
| Tryptophan                    | 0.151         | 0.307        | 0.148 <sup>a</sup>  | 0.206 <sup>a</sup>  | 0.310 <sup>b</sup>  | 0.330 <sup>b</sup>  | 0.160 <sup>a</sup>  | 0.172 <sup>a</sup>  | 0.302 <sup>b</sup>  | 0.296 <sup>ab</sup> | 0.131 <sup>a</sup>  | 0.085 <sup>a</sup>  | 0.261 <sup>b</sup>  | 0.250 <sup>b</sup>  | 0.150 <sup>a</sup>  | 0.190 <sup>a</sup>  | 0.200 <sup>a</sup>  | 0.175 <sup>a</sup>  |
| Tyrosine                      | 0.441         | 0.293        | 0.443 <sup>a</sup>  | 0.425 <sup>a</sup>  | 0.269 <sup>a</sup>  | 0.476 <sup>a</sup>  | 0.138 <sup>a</sup>  | 0.611 <sup>b</sup>  | 0.289 <sup>b</sup>  | 0.516 <sup>b</sup>  | 0.675 <sup>a</sup>  | 1.010 <sup>a</sup>  | 0.617 <sup>a</sup>  | 0.841 <sup>a</sup>  | 0.683 <sup>a</sup>  | 0.934 <sup>a</sup>  | 0.559 <sup>a</sup>  | 0.551 <sup>a</sup>  |



## Figure legend

**Fig. 1.** Experimental design. Plants were grown for 11 days under high N conditions. Subsequently half of the plants were moved into a fresh batch of medium supplemented with high N and the other half into the same medium lacking any N source. Roots were harvested before the treatment (T0), and after T0+10 min, T0+1 h, T0+24 h and T0+72 h of the treatment.

**Fig. 2.** The response to N deprivation of proteins involved in carbon and N metabolism, in lignification and in protein catabolism. Relative abundances are represented by a colour-based scale (-2 to 3 fold) obtained after Z-score normalization. Abbreviations: SGAT: serine-glyoxylate aminotransferase; AST: aspartate aminotransferase; MDH malate dehydrogenase; ME malic enzyme; PDH pyruvate dehydrogenase; SDH succinate dehydrogenase; PK: pyruvate kinase; IDH isocitrate dehydrogenase; PFP: pyrophosphate-fructose 6-phosphate 1-phosphotransferase; CS: citrate synthase; PGK: phosphoglycerate kinase; PFK: 6-phosphofructokinase; SCP: serine carboxypeptidase; RP: ribosomal protein; SBT: subtilisin-like protease; RS: regulatory subunit; FTSH ATP-dependent zinc metalloprotease; CAOMT: caffeic acid 3-O-methyltransferase. Where several isoforms are shown, the accession number (without its PGSC003DMT4000 prefix) within the potato database is given to distinguish between them.

**Fig. 3.** The effect of N deficiency over time on the content of selected metabolites. The main components of the TCA cycle, along with metabolites showing the strongest response to N deficiency are shown. The content of the full set of metabolites is given in Figure S3. Green frames refer to metabolites, which responded positively to N stress, red frames to those which responded negatively and blue frames to those which responded in a cultivar-dependent manner. Letters shown above the histogram columns indicate significant differences in mean values, as predicted by a one way ANOVA (p-value <0.05).

**Fig. 4.** The reaction of the two cultivars to N deficiency. The processes, proteins and metabolites, which contribute to the differential cultivar response are shown within a rectangle. Processes not responsive to the time of exposure to the stress, but featuring an altered metabolite or protein level are shown within a rectangle. Colour coding: red=increase, green=decrease, white=cultivar-dependent. The main difference between the cultivars was a higher abundance of proteins taking part in N re-assimilation, carbon metabolism, protein degradation and lignification in cv. ‘Topas’ and of defence in cv. ‘Lambada’. The content of both glutamine and quinate decreased in response to stress unevenly over time, whereas that of GABA increased in cv. ‘Lambada’.

## Supplementary data

Table S1. The abundance of peptides used for identification of proteins responsive to the N deficiency at the T0+24h and T0+72h.

Table S2. The abundance of peptides used for identification of proteins taking part in the C and N metabolism, protein synthesis and catabolism as well as in lignin synthesis pathway.

Table S3. The specific masses of the quantified fragment ion (m/z) along with retention time index of the 65 metabolites.

Fig. S1. The effect of N deficiency on the biomass of the two potato cultivars, shown in the form of a ratio between the performance of plants grown under N-sufficient and -deficient conditions. Asterisks indicate means, which differed from one another at  $p < 0.05$  (Student's T-test).

Fig. S2. The abundance of all 570 differentially abundant proteins over time. Relative abundances are represented by a colour-based scale (-2 to 2.5 fold) obtained after Z-score normalization.

Fig. S3. The effect of N deficiency over time on the content of all metabolites. (A) Organic acids involved in the TCA cycle, (B) organic acids related to the secondary metabolism, (C) sugar-derived organic acids, (D) other organic acids, (E) sugars, (F) sugar alcohols, (G) phosphorylated sugars and sugar alcohols, (H) miscellaneous metabolites. Green frames refer to metabolites which responded negatively to N stress, red frames to those which responded positively, blue frames to those which responded in a cultivar-dependent manner and yellow frames to those which were non-responsive. Letters shown above the histogram columns indicate significant differences in mean values, as predicted by a one way ANOVA ( $p$ -value  $< 0.05$ ).

Figure S4. An integrated view of the metabolic processes related to N and carbon metabolism. Metabolites identified in these experiments are shown within a circle and proteins within a rectangle. The normalized abundances of proteins and metabolites are presented in form of  $\log_2$  of the fold change (N-/N+), (Topas N+/LambadaN+) and (Topas N-/LambadaN-) are shown in individual heat maps, corresponding to the four sample time points.

Figure 1.

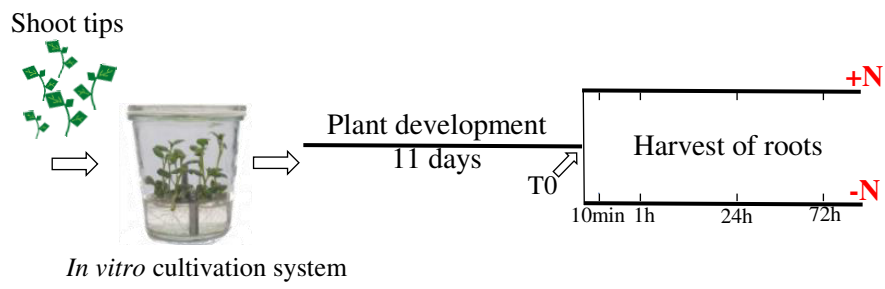


Figure 2.

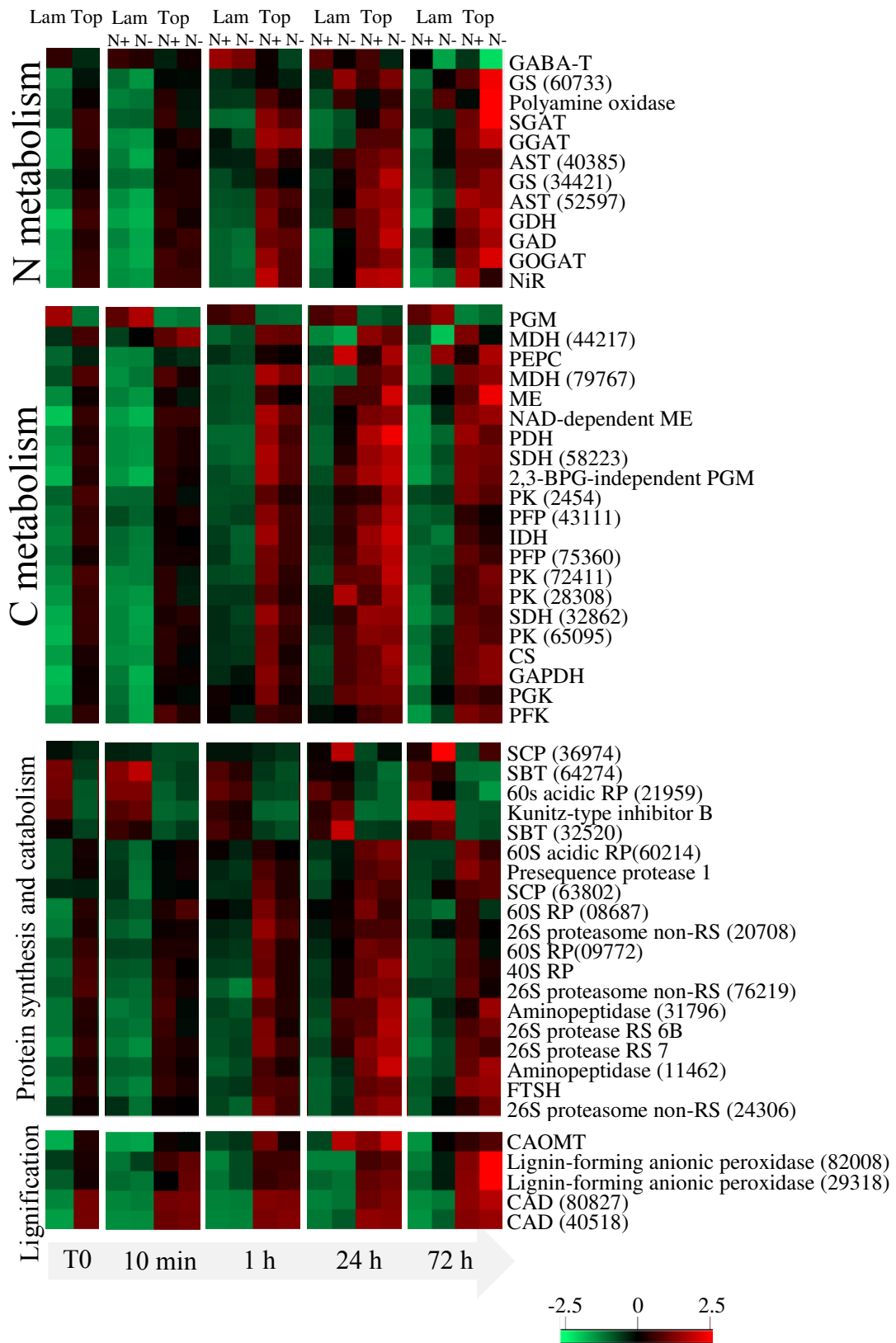


Figure 3.

TCA derived organic acids

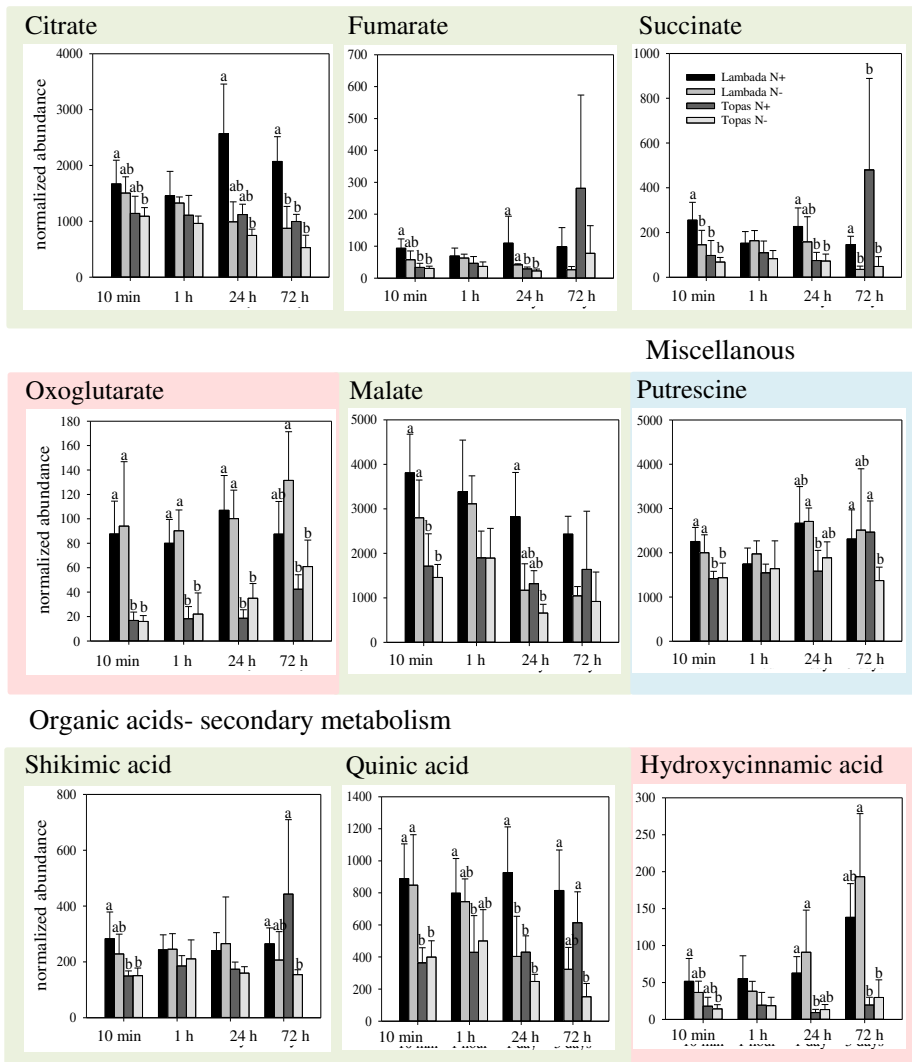


Figure 4.

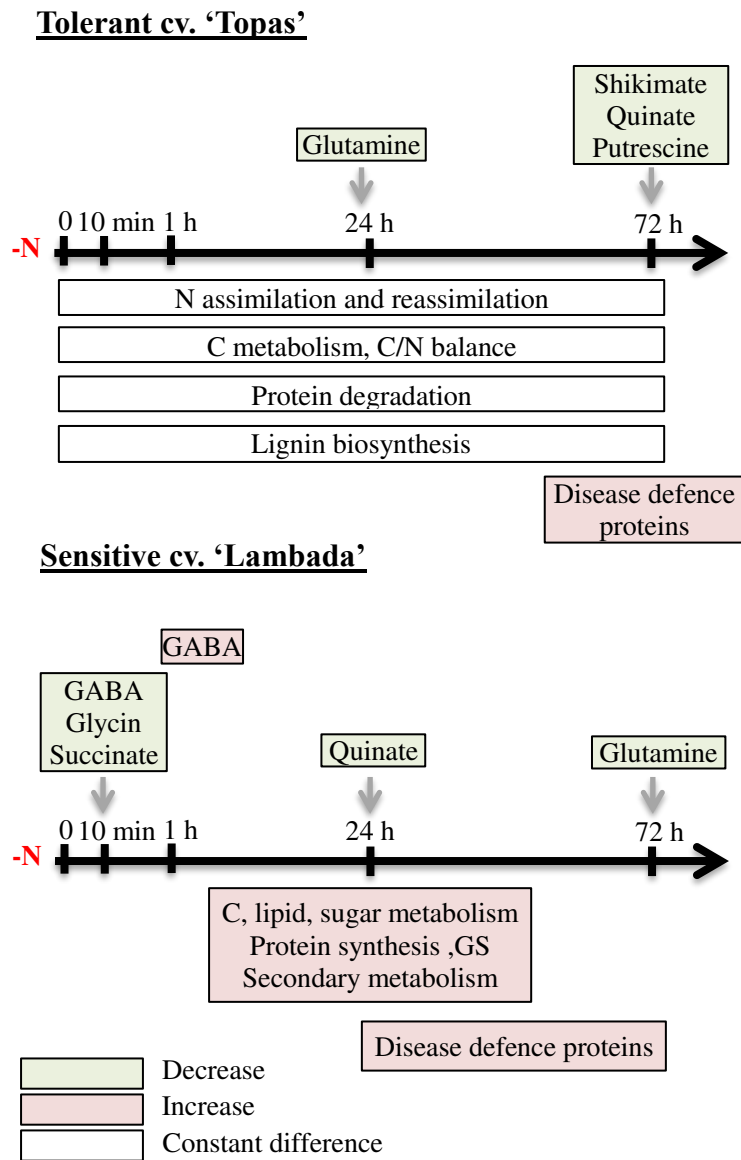


Figure S1.

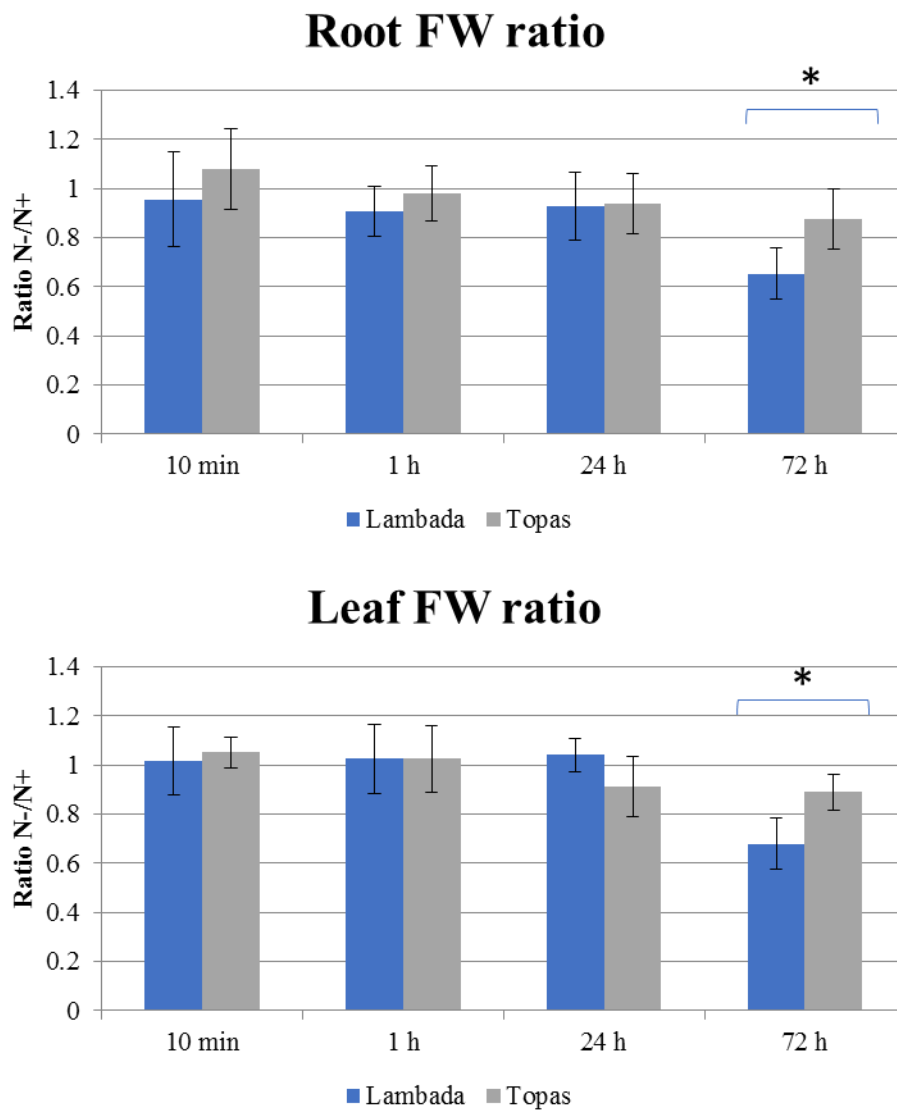
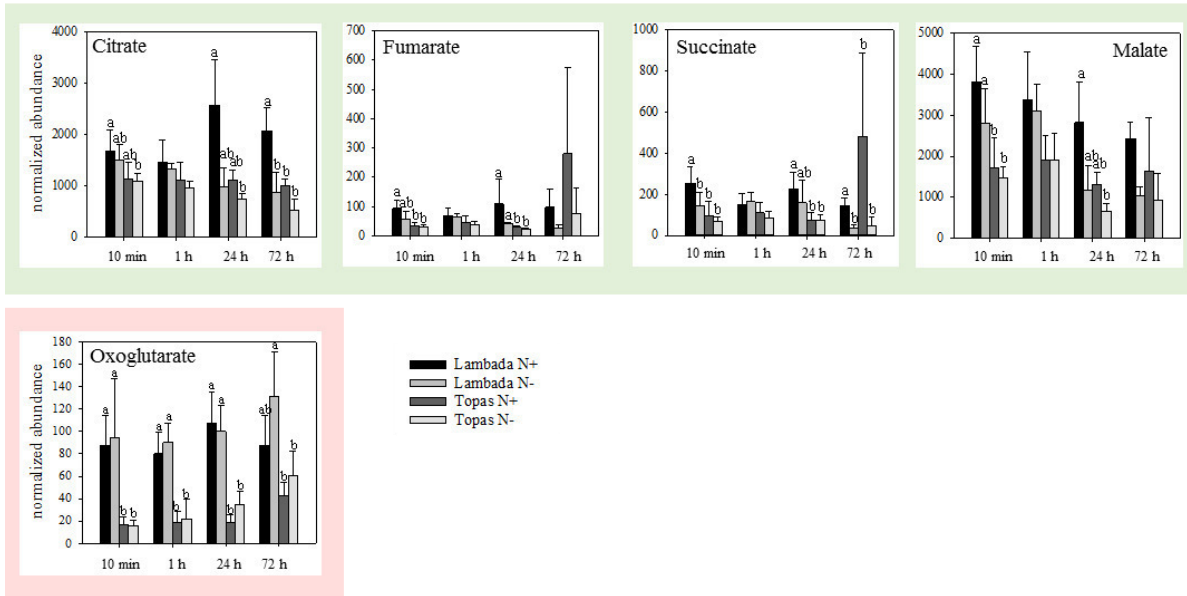




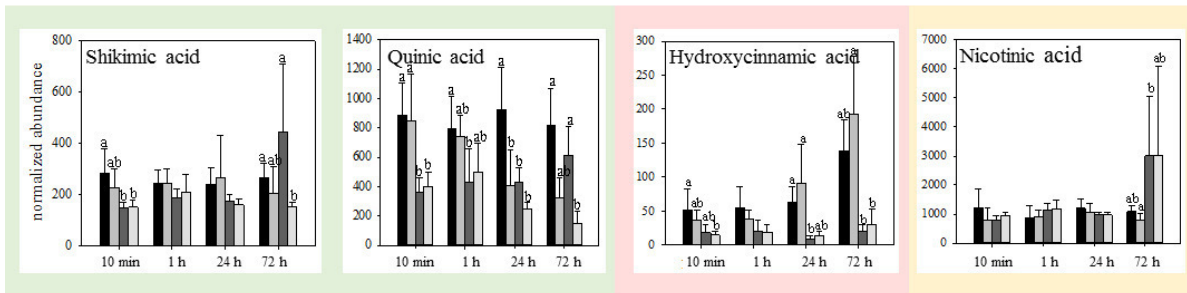


Figure S3.

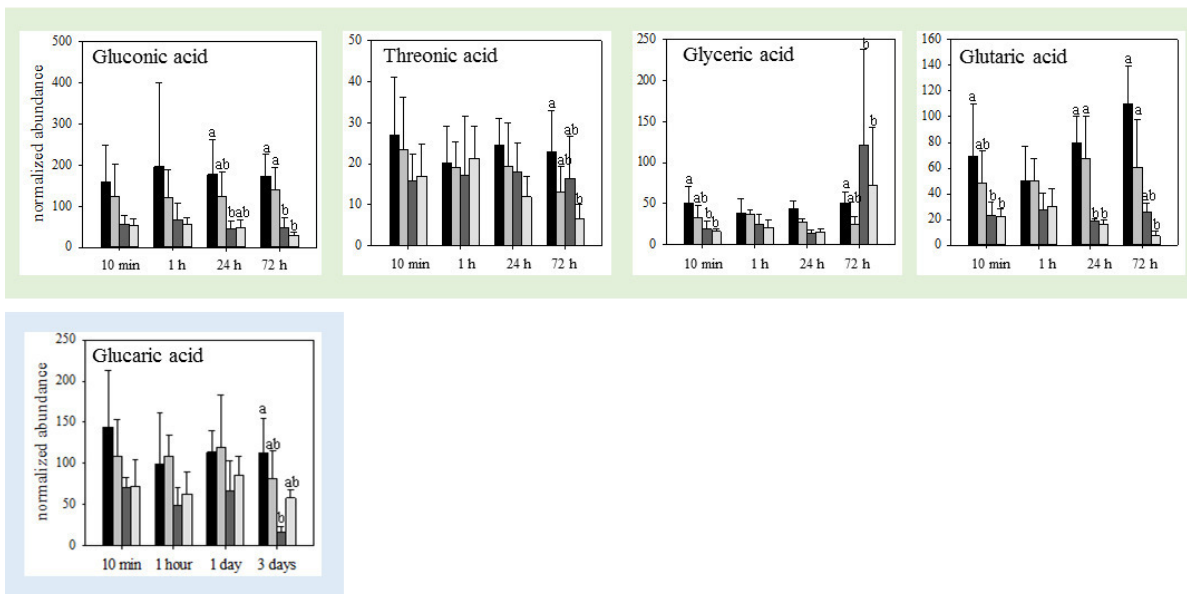
A) TCA derived organic acids



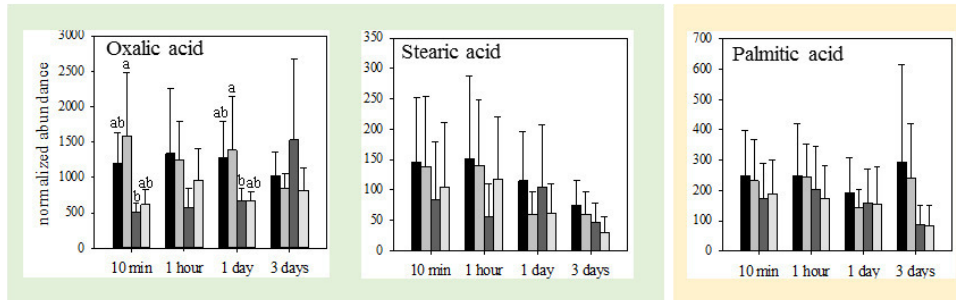
B) Organic acids- secondary metabolism



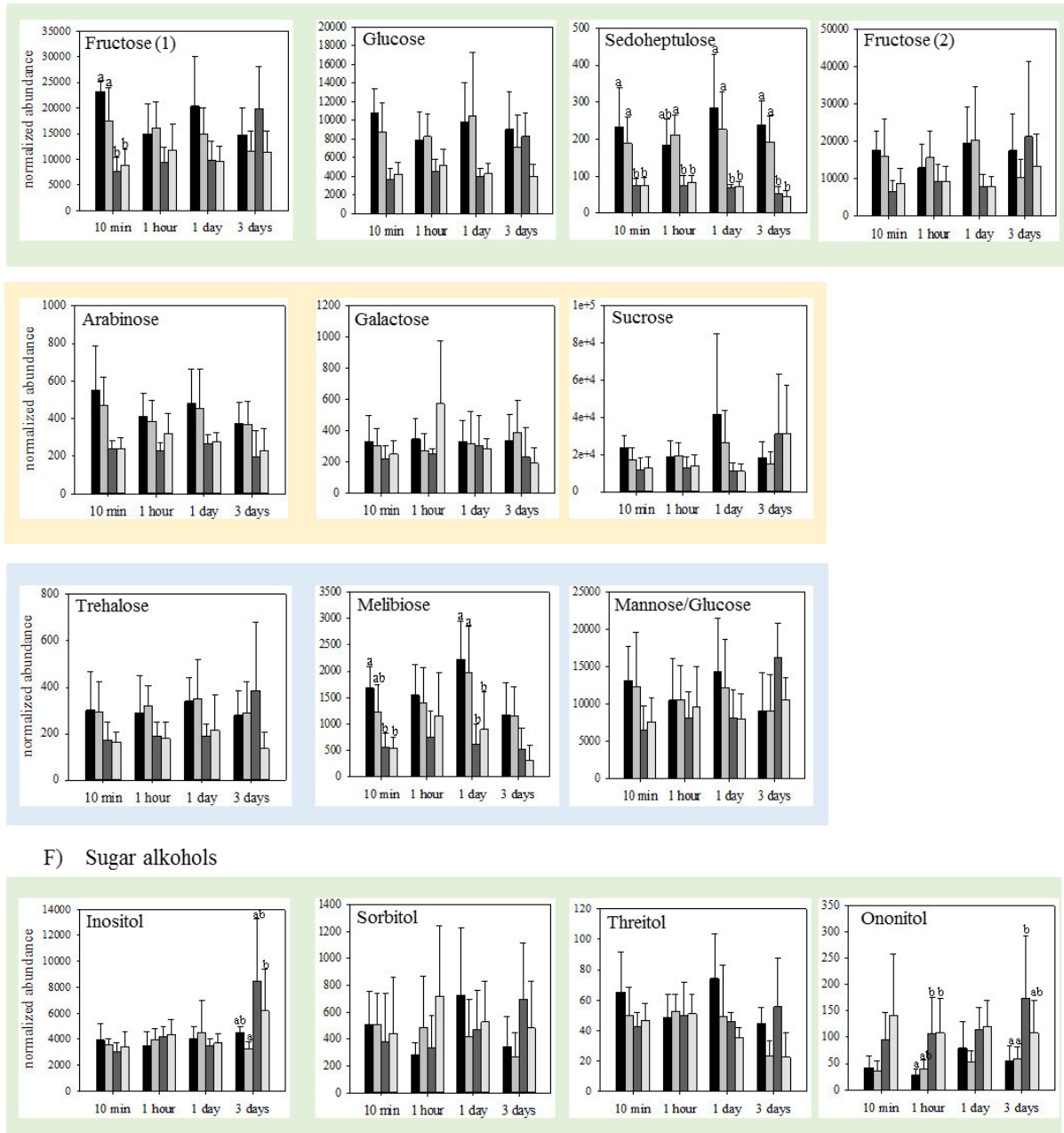
C) Sugar acids



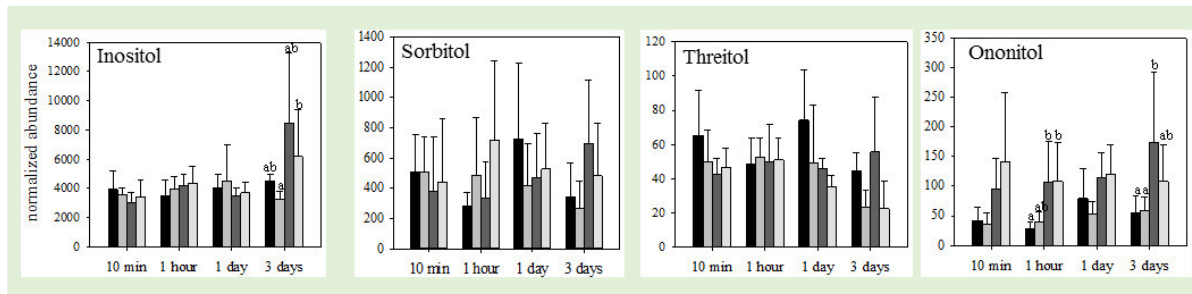
D) Miscellaneous organic acids



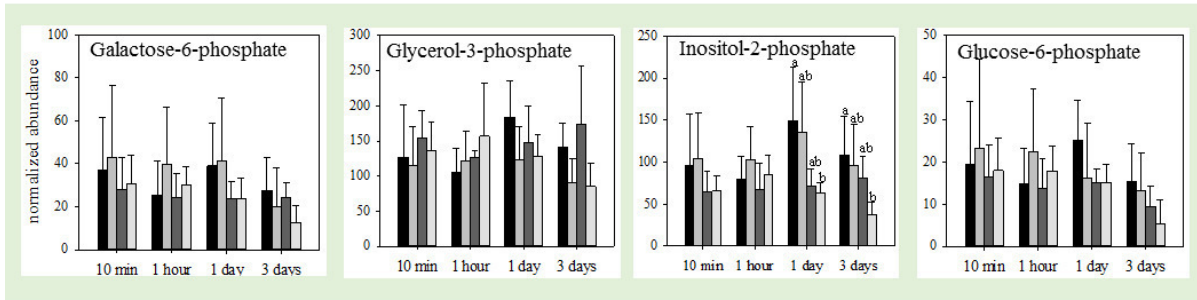
E) Sugars



F) Sugar alcohols



G) Phosphorylated sugars and sugar alcohols



H) Miscellaneous

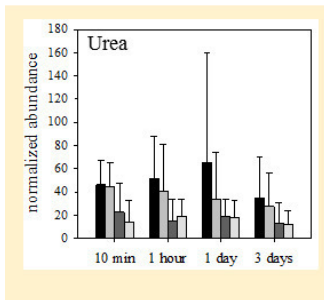
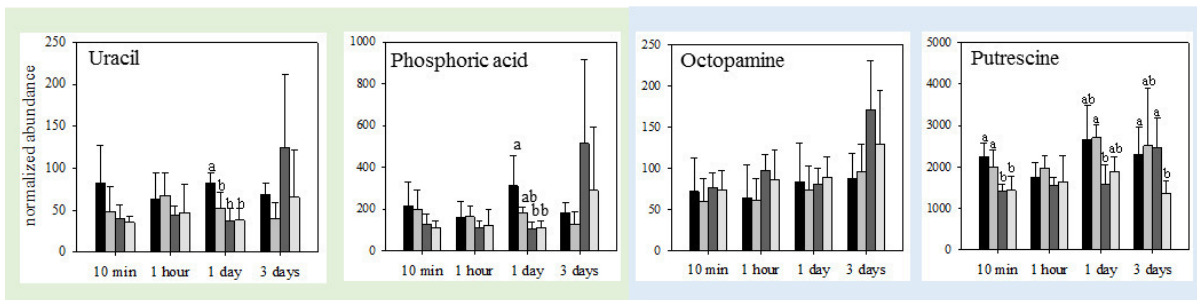
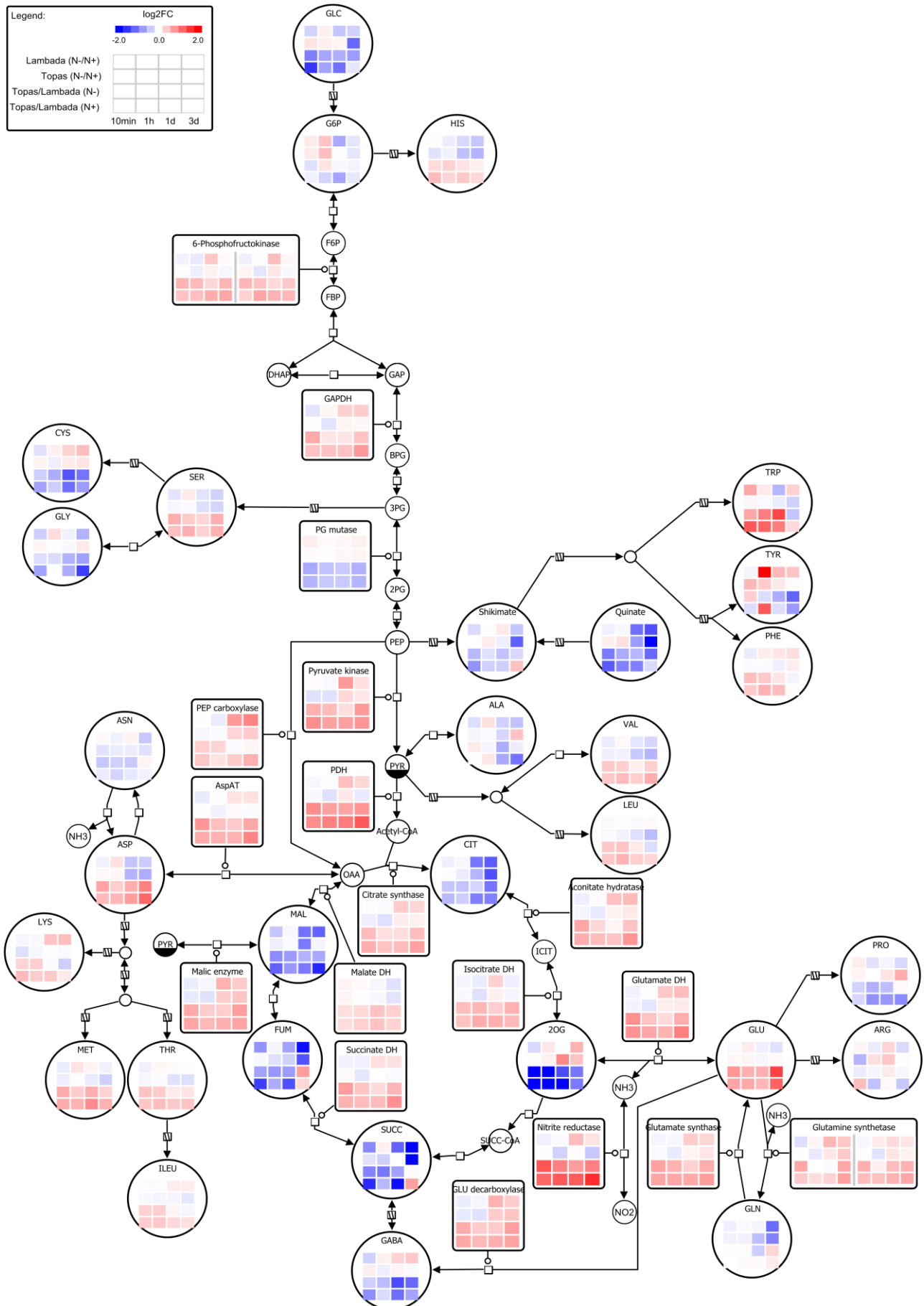


Figure S4.



# Chapter V

**Nitrogen withdrawal leads to distinct changes in the phosphoproteome of two potato (*Solanum tuberosum* L.) cultivars.**

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## **Abstract**

Nitrate concentration in the soil is constantly changing due to its high mobility. Nitrate starvation induces very quick changes in the expression of genes related to N assimilation, but also phosphorylation of proteins play an important role in the fast adaptation of plants to the changing environment. We have previously shown that potato cultivars ‘Lambada’ and ‘Topas’ differ in their response to nitrogen deficiency. Here, we compared the time dependent alterations in the phosphoproteome with an aim to elucidate very early differences in the reaction of the two potato cultivars subjected to nitrogen starvation. We identified 121, 28, 46, and 52 phosphopeptides differentially changed by 10 min, 1 h, 24 h, and 72 h treatment, respectively. The response of the potato to short-term starvation affected predominantly the phosphorylation of proteins involved in the regulation of transcriptional processes, signal transduction but also number of proteins taking part in C metabolism, especially glycolysis. In the tolerant cultivar ‘Topas’ several kinases were phosphorylated to a higher degree than in the cv. ‘Lambada’ after 10 min of treatment. Prolonged 24 and 72 h resulted mainly in the dephosphorylation of proteins taking part in signaling, metabolic processes, and in the proteins of ubiquitination machinery.

## Introduction

Growth and development of plants rely on the source of nitrogen (N), as it is a major component of proteins, nucleic acids and several classes of secondary metabolites. N is one of the most limiting factors in crop plant production and its inadequate supply affects growth, development, architecture, senescence, and photosynthesis [1]. Nitrate is the most abundant source of N in the agricultural soils, with an average concentration between 1 and 5 mM [2]. This concentration is, however, unstable due to the high mobility of nitrate in the soil [3]. To cope with variable  $\text{NO}_3^-$  levels, plants developed multiple mechanisms of increasing capacity for nutrient acquisition. These includes changes in the plant morphology and metabolisms, for instance by increasing root to shoot ratio.  $\text{NO}_3^-$  starvation and resupply induce very fast changes in gene expression, detectable within minutes [4]. To the nitrate responsive genes belong those involved in the N acquisition and assimilation, production of reducing equivalents for N metabolisms, as well as those related to C metabolisms [5].

Initially, the transcriptional regulation of nitrogen transporters was thought to control N uptake, however, there are indications that also posttranslational modifications (PTM) participate in the process [6]. Phosphorylation plays an important role under conditions that require rapid adaptation to sudden changes in the plant environment [7]. It regulates many key components of N transport and assimilation, such as  $\text{NO}_3^-$  transport across the plasma membrane [8]. The phosphorylational status of the nitrate transporter NRT1.1 decides about the affinity to  $\text{NO}_3^-$ . When the level of  $\text{NO}_3^-$  is low, it is phosphorylated by CBL-Interacting Protein Kinase 23 to function as a high-affinity transporter, and it is dephosphorylated when the level of nitrate is high. A mutation which eliminates the T101 phosphorylation site abolishes its dual-affinity transport activity [8]. To the other proteins participating in the nitrogen uptake and regulated by phosphorylation belongs NRT2.1 [9]. Changes in the phosphorylation status of the ammonium transporters AMT1.1 and AMT1.3 were triggered when  $\text{NO}_3^-$  and  $\text{NH}_4^+$  were resupplied to *Arabidopsis* [10]. The activity of nitrate reductase is also controlled by phosphorylation that is necessary for the interaction with 14-3-3 proteins and subsequent inactivation of the enzyme (for a review see [5]).

The potato is a significant food and starch- delivering crop of the temperate agriculture. Understanding the molecular basis for the adaptation of potato to a limited N regime is important in the context of the rising demand for food and the need to protect the quality of the rural environment. The potato cultivars ‘Topas’ and ‘Lambada’ were previously described to differ in their response to nitrogen limitation [11-14]. In our previous studies, we analyzed protein composition of the plasma membrane and identified number of proteins, in particular the NRT2.1 and the members of the aquaporin families, differentially abundant in the two potato cultivars under nitrogen deficiency (Jozefowicz et al, in preparation). These proteins were described as regulated by phosphorylation [9, 15]. The information about protein regulation is needed to understand the



processes undergoing in plants, but it was not resolved in our shotgun proteomic approach. Recently time-dependent proteomic and metabolomic analysis was performed focusing on the early changes roots of the two potato genotypes under N starvation (Jozefowicz et al, submitted). The study showed that the level of few metabolites including GABA and succinate was rapidly reduced within 10 minutes of N limitation. The abundances of corresponding enzymes were, however, stable. At the later time points the level of many amino acids e.g. glutamine, alanine, serine decreased, but the abundance of enzymes taking part in the metabolic pathways consuming this amino acids remained constant. Possible explanation is the additional regulation of the protein activity, for instant by a posttranslational modification. Additionally, the signaling cascades leading to the changes in the expression of genes participating in nitrate transport and assimilation are induced by phosphorylation of protein, for instance by phosphorylation of NRT1.1 [5].

Hence we choose a phosphoproteomic approach to find out and explain the differences in the response of the roots of the two potato cultivars at the very early and later stages of the nitrogen deficiency on the phosphoproteome level. The information gathered in this analysis can help to explain the regulatory mechanisms of nitrogen deficiency response.

## **Material and methods**

### *In vitro cultivation of potato*

Initial cultivation of potato cultivars ‘Topas’ and ‘Lambada’ was performed as described earlier [11]. After 11 days of cultivation, half of the plants were moved to a fresh batch of medium supplemented with 60 mmol/l N and the other half to a medium without any N source. The difference in potassium content of the two media was made up by including the relevant amount of KCl in the N free medium. The roots were harvested before medium exchange (T0) as well as after 10 min, 1 h, 24 h, and 72 h respectively, snap-frozen in liquid N<sub>2</sub> and stored at -80°C prior to protein extraction. The experiments were conducted in triplicate with four vessels per condition and cultivar in each experiment. The time points of harvest were chosen based on two-dimensional SDS-PAGE and ProQ Diamond staining of phosphopeptides (data not published). At this time points the number of spots changed in their abundance was the highest, corresponding to the highest changes in the proteins phosphorylation.

### *Protein extraction and digestion*

The extraction of potato root proteins was performed following the trichloroacetic acid/acetone protocol [16]. Resulting dried pellets were resuspended in 100 µl of buffer (7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 5 mM DTT, pH 8.0). The concentration of dissolved proteins was estimated using the 2D Quant Kit (GE Healthcare, Munich, Germany) with BSA as a reference. 500 µg of the proteins was sampled for filter-based digestion, following [17]; this involved an overnight digestion

at 37°C with Sequencing Grade Modified Trypsin (in ratio 1:50, Promega, Mannheim, Germany). Peptides for LC/MS analysis were recovered by centrifugation, dried and resuspended in 300 µl of 1% ACN, 0.1% formic acid.

#### *Enrichment of phosphopeptides*

Enrichment of phosphopeptides was performed as described elsewhere [18], with only minor modifications. Aliquots containing 500 µg of peptides were used and the most efficient amount of TiO<sub>2</sub> beads was determined as 2 mg. Prior to LC/MS analysis phosphopeptides were resuspended in 15 µl of 1% ACN, 0.1% formic acid.

#### *Analysis of peptides and phosphopeptides with nanoLC-ESI-QTOF-MS*

For the LC/MS analysis nanoLC Dionex UltiMate™ 3000 RSLCnano (Thermo Fisher Scientific, Inc., Waltham, MA, USA) coupled to an Impact II (Bruker Daltonics, Bremen, Germany) was used. Sample analysis was performed according to Jozefowicz et al (submitted).

The acquired spectra were analyzed for label-free quantifications using Progenesis QI software for proteomics (Version 3.0, Nonlinear Dynamics, Newcastle upon Tyne, U.K.) as recommended by the manufacturer to enable mass correction, alignment, normalization, peak picking, quantification and statistic. MS/MS spectra were exported from the Progenesis QI software as Mascot generic files and used for peptide identification with Mascot (Version 2.5.1) using the potato database ([solanaceae.plantbiology.msu.edu/pgsc\\_download.shtml](http://solanaceae.plantbiology.msu.edu/pgsc_download.shtml)) [19] blasted with Blast2GO software [20] and merged with the sequences of human keratin and trypsin. Search parameters used were 15 ppm peptide mass tolerance, 0.05 Da fragment mass tolerance, one missed cleavage allowed, carbamidomethylation as fixed modification, and methionine oxidation as well as phosphorylation on serine, threonine and tyrosine as variable modification. The Mascot integrated peptide decoy database search was performed and searches were processed with the Percolator machine-learning algorithm [21]. The false discovery rate was <1%, the ion score cut-off was set to 15 and the significance threshold  $p < 0.05$ . The identified peptide sequences were re-imported to the Progenesis for further quantification. ANOVA values of  $p < 0.05$  and additionally regulation of 1.5 fold were regarded as significant for all further results. Peptides without phosphorylation were excluded from further analysis. Phosphorylation site localization probability was calculated using Scaffold PTM Version 3.1. Only phosphorylation sites with a probability above 95% were accepted. Additional annotation based on the UniProt server ([www.uniprot.org](http://www.uniprot.org)) was performed for selected phosphoproteins. Functional classification of proteins was performed as described [22]. Z-score normalization and hierarchical clustering based on the Euclidean distance method were carried out using the Perseus Framework [23]. The comparison of cultivar specific peptides was visualized using Venny v.2.1 (<http://bioinfo.gp.cnb.csic.es/tools/venny/>). The gene ontology (GO) term annotation was performed using Blast2GO software [20].

## Results

For the determination of changes in the potato phosphoproteome in response to N limitation, plants were grown for 10 min, 1 h, 24 h, and 72 h under N limited conditions. Characterization of plant performance was described in our previous work (Jozefowicz et al, submitted). Based on the criteria described in Material & Methods 121, 28, 46, and 52 phosphopeptides were significantly changed after 10 min, 1 h, 24 h, and 72 h of N starvation, respectively (Table S1). The majority of significantly altered phosphopeptides were singly phosphorylated and only two peptides possessed more than 1 phosphorylation sites. About 85% of peptides were phosphorylated on serine and the rest on threonine. Generally, the phosphorylation dominated as a result of the 10 min treatment with 74 peptides phosphorylated in cv. ‘Lambada’ and 100 in cv. ‘Topas’ (Figure 1). After 1 h and 24 h of treatment 18 and 24 peptides were dephosphorylated in cv. ‘Topas’, respectively, whereas after 72 hours 9 peptides were dephosphorylated and 4 phosphorylated. In cv. ‘Lambada’ only a few peptides were changed following the 1 h and 24 h treatment. Stronger reaction was observed after 72 h of treatment, where 29 peptides were dephosphorylated.

K-means clustering was performed to group peptides with similar phosphorylation pattern to get an overview about processes regulated in similar way in the two cultivars under N deficiency. It was performed within each time point separately. This was necessary in order to include a control for each time point and to exclude influence of plant developmental stage and mechanical stress caused by medium exchange on the phosphoproteome profile. Additional analyses (data not presented) showed that for cv. ‘Topas’ 99 and for cv. ‘Lambada’ 25 peptides were differentially phosphorylated between T0 and 72 hours, when only N sufficient conditions were compared. Moreover, the overlap of phosphopeptides significantly changed by N starvation in the four time points was low. Not more than 10 phosphopeptides were common, when comparing single time points.

Clustering divided the phosphopeptides into groups with differential or comparable abundance in the two cultivars. In the 10 min treatment six phosphorylation profiles were isolated (Table S2, Figure 2 A). The peptides in clusters I a to I d shows differential and in I e and I f similar profile in the two cultivars. In details, in the cluster I a the phosphorylation level was higher in cv. ‘Lambada’. I b and I c harbored peptides highly phosphorylated in cv. ‘Topas’ and dephosphorylated (I b) or slightly phosphorylated (I c) in cv. ‘Lambada’. In cluster I d peptides are slightly higher phosphorylated in cv. ‘Topas’. Clusters I e and I f comprise peptides with similar degree of phosphorylation in both genotypes. Peptides in cluster I f were, however, phosphorylated to a higher extent. Figure 2 B shows the degree of changes in phosphorylation profile in the clusters visualized as a ratio of abundances under N deficient to N sufficient conditions.

Differentially changed phosphopeptides responsive into 1 h treatment were grouped into five clusters (Figure 2 C). In the cluster II a peptides were phosphorylated in cv. 'Lambada' but not in cv. 'Topas'. Clusters II b, II c and II d grouped peptides dephosphorylated in cv. 'Topas' and with response varied between no reaction to slight phosphorylation in cv. 'Lambada'. The peptide of Cluster II e was dephosphorylated in both cultivars.

Four phosphorylation profiles were elucidated within 24 h treatment (Figure 3 A). Clusters III a comprised peptides with dephosphorylation maxima in cv. 'Topas'. In III b peptides were dephosphorylated in cv. 'Topas' and only marginally changed in cv. 'Lambada'. In cluster III c peptides were phosphorylated in cv. 'Lambada' and dephosphorylated in cv. 'Topas'. In III d peptides were stable by N treatment, but higher phosphorylated in cv. 'Topas' at both N regimes. The phosphopeptides responsive to 72 h treatment were grouped into five clusters (Figure 3 B). Cluster IV a comprised only one phosphopeptide which was highly phosphorylated in both cultivars. The cluster IV b harbored peptides phosphorylated in cv. 'Topas' and with no reaction in cv. 'Lambada'. In IV c and IV d the peptides were dephosphorylated in cv. 'Lambada' and in cv. 'Topas' there was no reaction (IV d) or the peptides were only slightly phosphorylated (IV c). Peptides within the cluster IV e were dephosphorylated to a similar extent in both cultivars.

#### *10 min treatment responsive proteins*

Among the 121 phosphopeptides identified after 10 min of N deficiency 60 was responsive in both cultivars, whereas 10 was specific for the cv. 'Lambada' and 26 for cv. 'Topas' (Figure 4 A). The other 25 phosphopeptides were not responsive to N deficiency, but significantly higher abundant in one of the cultivars under both N sufficient and deficient conditions.

The 121 phosphopeptides were assigned to the 104 proteins. All identified phosphoproteins responsive to the 10 min treatment were classified using gene ontology annotation according to their cellular component (Figure 4 B). The most represented GO terms were related to nucleus (29%), cytosol (27%) and plasma membrane (20%) localization. Other important categories were plastids (10%), vacuole (8%) and Golgi apparatus (6%).

The proteins regulated by phosphorylation were also classified according to their molecular functions to get an overview about processes affected by phosphorylation. Among the 26 peptides phosphorylated only in cv. 'Topas' nine were assigned to the proteins without known function, five to transcriptional processes, three were 'signal transduction' proteins, two transporters and two proteins participating in intracellular trafficking. In cv. 'Lambada' among the 10 specific proteins were two participating in transcriptional processes, two in metabolism and one in defence processes (Figure 4 C).

The majority of phosphopeptides identified in all treatments were assigned to proteins without known yet function. The second largest group were proteins functioning in transcriptomic processes (Figure 5). Zinc finger CCCH domain- containing proteins (seven proteins), AT-hook motif DNA

binding protein family members (four proteins) and several transcription factors (four proteins) were classified here. Proteins participating in the transcriptional processes were distributed in all the clusters, except of I f. It means that they were all phosphorylated in response to N starvation but to a different degree in both potato genotypes. To the second largest group, counted the proteins participating in the ‘signal transduction’, such as kinases. Serine/threonine protein kinases, including mitogen-activated protein kinases family members MAPKKK and MAPKK and calcium-dependent protein kinases were affected. The proteins participating in signal ‘transduction’ were grouped into clusters I b and I c. Most of them were phosphorylated in both cultivars, except of MAPK, MAPKK and two serine/threonine kinases which were phosphorylated only in cv. ‘Topas’. Proteins related to ‘transport’ were tonoplast intrinsic protein (I c), vacuolar amino acid transporter 1 (I d), plasma membrane (PM) ATPase (I d) and lysine histidine transporter- like 8 (I c). ‘Metabolism’ related proteins regulated by the phosphorylation participated in glycolysis (fructose-bisphosphate aldolase and phosphoglycerate mutase, I c and d) and sugar metabolism (glucose-1-phosphate uridylyltransferase (I b), alkaline/neutral invertase D (I d) and alpha-1,4-glucan-protein synthase 2, I d), as well as in lignification (cinnamyl alcohol dehydrogenase, I c). Category ‘diseases and defence’ was represented by heat stress transcription factor, heat shock 70 kDa protein, Cbs domain containing protein, as well as autophagy related protein.

#### *1 h treatment responsive proteins*

Only 27 proteins were significantly changed in the phosphorylation status as a result of 1 h starvation. Six proteins were associated with transcriptional processes and three were transporter proteins. Oppositely to the 10 min treatment, proteins taking part in transcription were dephosphorylated in result of N deficiency. The sodium/hydrogen exchanger was dephosphorylated in cv. ‘Topas’, but not in cv. ‘Lambada’. The lysine histidine transporter (II c) was also dephosphorylated in cv. ‘Topas’ but phosphorylated in cv. ‘Lambada’. To the other proteins phosphorylated by 1 h treatment belonged pyrophosphate--fructose 6-phosphate 1-phosphotransferase and alkaline/neutral invertase D (II d).

#### *24 h responsive proteins*

Among the 45 proteins changed in the phosphorylation status by 24 h of stress, 10 were assigned to the category ‘transcription’. These proteins were all dephosphorylated in cv. ‘Topas’ and differed in the phosphorylation status in cv. ‘Lambada’. Four proteins participated in protein turnover (leucine aminopeptidase 2 (III c), 26S proteasome regulatory subunit (III c), E3 ubiquitin-protein ligase ATL6-like (III d) and epsin-3-like isoform (III c)) had a different phosphorylation profiles in the two potato cultivars. The abundance of the corresponding phosphopeptides was about two times higher in cv. ‘Topas’ although they remained unchanged by the stress. Additionally, 60S ribosomal protein (III e) was dephosphorylated in the tolerant cultivar, but not in the sensitive one. Two

proteins, which are a part of the signal transduction cascades (MKS-1 proteins and MAPKK kinase YODA-like, III e), were also both dephosphorylated in cv. 'Topas'.

#### *72 h responsive proteins*

As a result of the 72 h treatment, 48 proteins were changed in their phosphorylation status. Six proteins were assigned to the category 'transcription' and six to 'signal transduction'. The protein kinases (calcium dependent kinase and serine/threonine kinases, IV d and e) were oppositely, to those found in the 10 minutes treatment, dephosphorylated by stress. Another signaling protein, MKS1 protein (IV c) was phosphorylated in the tolerant cultivar. Several proteins participating in the metabolic processes were regulated by phosphorylation after 72 h treatment. Phosphoenolpyruvate carboxykinase (IV a) was phosphorylated in both cultivars but to a higher extent in cv. 'Topas'. Pyrophosphate--fructose 6-phosphate 1-phosphotransferase subunit beta (IV c) increased in phosphorylation status only in cv. 'Topas'. The abundance of cinnamyl alcohol dehydrogenase phosphopeptide (IV c) increased in both genotypes but generally was higher phosphorylated in cv. 'Topas'. Proteins involved in ubiquitination, ubiquitin-conjugating enzyme E2 and E3 ubiquitin-protein ligase ATL6-like (IV e) were dephosphorylated in cv. 'Lambada', whereas in cv. 'Topas' they were both two times higher phosphorylated in the non-stressed plants and remained phosphorylated under stress conditions.

#### *Processes involved in rapid vs. prolonged N deficiency*

The summary of the identified proteins which undergo regulation by phosphorylation as a result of N deficiency is presented in Figure 6. The general trend observed in both cultivars after short term 10 min N deficiency was an increase in the phosphorylation of proteins taking part in transcriptional as well as in energetic and metabolic processes. We could also detect an increase in the phosphorylational status of proteins taking part in the signal transduction in the tolerant cultivar. Longer (24 h and 72 h) N deficiency led to a general increase in the phosphorylation of proteins participating in the energetic and metabolic processes. The proteins regulated by phosphorylation were however different than those regulated during 10 min treatment. The proteins of transcription and signaling were oppositely to those regulated after short treatment dephosphorylated in both cultivars. Additionally, the proteins participating in the diseases and defence processes were higher phosphorylated in the sensitive cultivar.

## **Discussion**

The potato cultivars 'Lambada' and 'Topas' show contrasting reaction to N deficiency. Performance of the plants under N deficiency was already described in our previous work (Jozefowicz et al, submitted). Briefly, cv. 'Lambada' was characterized by a drop in the biomass production after 72 h treatment, whereas cv. 'Topas' presented stable growth. Additionally, the

protein and N content were both higher in the tolerant genotype. The time-dependent response of the two potato cultivars were studied on the proteomic and metabolomic level, but limited correlation was found between the protein abundances and metabolite levels. This suggested the involvement of further regulatory mechanisms. Recent studies suggest that number of proteins participating in the N acquisition and metabolisms are regulated by phosphorylation as well [6]. In the presented study, we observed that the tolerant cultivar reacts rapidly by an increase in the phosphorylation status of proteins involved in the signaling cascades, but also other metabolic pathways are regulated by in the dissimilar way. After prolonged N starvation, there was an increase in the phosphorylation status of proteins participating in energetic and metabolic processes but decrease in the phosphorylation of signaling and proteins regulating transcription.

*Adaptation to the new environmental conditions is strongly apparent in the 10 min treatment in both cultivars*

The highest number of peptides significantly changed in their phosphorylation status was found after 10 min of treatment. Proteins involved in the processes that allow for adaptation to the new environmental conditions were highly affected and showed similar trend in both cultivars.

The most responsive group of proteins were transcriptional regulators. Phosphorylation and dephosphorylation of these proteins are a common mechanism of controlling gene expression [24, 25]. Seven proteins of CCCH zinc finger proteins family were phosphorylated in both cultivars. In *Arabidopsis* CCCH zinc finger proteins were reported to affect gibberellin- and sugar-mediated growth and stress response [26], to be involved in ABA, jasmonate and oxidative stress response [27, 28], to mediate pathogen-associated molecular pattern triggered immune response [29] and to be involved in salt stress response [30]. So far, there is only little information about regulation of this protein in plants by phosphorylation. *Arabidopsis* tandem CCCH zinc finger 9 was reported to be a target of MAPK 3 and 6 [29]. Transcription factors belonging to the zinc finger group were phosphorylated under nitrate resupply [10]. The AT-hook motif containing proteins were affected as well. They were phosphorylated in both cultivars but to the higher extent in cv. 'Topas'. These proteins play a role in regulation of transcription by influencing association of transcription factors with chromatin [31].

The knowledge about phosphorylation events involved in the response to nutrient deficiency is limited and the kinases involved in the regulation of nitrogen uptake and metabolism remain largely unknown. Here, we found a number of proteins belonging to the broad family of serine-threonine kinases which were phosphorylated to the higher degree in cv. 'Topas' than in cv. 'Lambada'. Two proteins were identified as calcium-dependent protein kinases (CDPKs) and were phosphorylated in both cultivars by N starvation. CDPKs embrace a large family of Ca<sup>2+</sup> regulated kinases [32] that have a domain similar to calmodulin and bind Ca<sup>2+</sup> directly. In the model where nitrate is sensed by NRT1.1, Ca<sup>2+</sup> is described as a second messenger for a signal transduction, which triggers

changes in the gene expression. Changes in the cytosolic  $\text{Ca}^{2+}$  are detected by  $\text{Ca}^{2+}$  sensor proteins such as CDPKs and the signal is transduced to the downstream signaling cascade [33].

*Differences in the phosphorylation response of potato cultivars by 10 min and 1 h treatment*

Kinases that were differentially phosphorylated after N starvation belonged to MAPK family. MAPKKK and MAPKK were both higher phosphorylated in cv. 'Topas' by N starvation. Environmental signals are transduced to the inside of the cell through the MAPK cascade and plasma membrane receptors are stimulated to activate MAPKKKs or MAPKKKKs. Sequential phosphorylation succeed as MAPKKKs activate downstream MAPKKs that in turn activate MAPKs [34]. Induction of MAPK cascade was observed for plants subjected to touch, cold, salinity, genotoxic agents, UV irradiation, ozone, and oxidative stress [35]. Some studies suggest the role of MAPKs in N sensing and metabolism. For instance, nitrate reductase was shown to be a target of MAPK7 in *Arabidopsis* [36].

Several proteins participating in metabolic and energetic processes were differentially regulated in both cultivars. The glycolytic enzymes fructose bisphosphate aldolase (containing two phosphorylation sites) and phosphoglycerate mutase were phosphorylated in cv. 'Topas' and only marginally in cv. 'Lambada'. The peptide GILAADESTGpTIGKR of the first one was identified as conserved phosphorylation site in plants [37] and the phosphopeptide of the latter protein has been identified in tobacco [18]. The knowledge about the regulation of fructose bisphosphate aldolase and phosphoglycerate mutase through phosphorylation is so far limited. Additionally, the regulatory subunit alpha of pyrophosphate: fructose-6-phosphate 1-phosphotransferase (PFP) was dephosphorylated in cv. 'Topas' after 1 h. PFP uses pyrophosphate as an alternative phosphoryl donor in place of ATP during the phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate and this consequently provides an energy advantage to the plants [38].

Several membrane proteins, predicted to be localized in PM, plastids and vacuoles, were phosphorylated in response to N starvation. PM ATPase was phosphorylated in cv. 'Topas' but not in cv. 'Lambada'. The identified peptide is associated with activation of the proton pump [39]. Rapid dephosphorylation of PM ATPase was observed in *Arabidopsis* upon ammonium resupply to N starved seedlings [10]. Two phosphorylation sites of vacuolar amino acid transporter were identified in our study. The peptide SSHpTLLPPLPSR was phosphorylated in cv. 'Lambada' and LGpSSFLASSLTR in cv. 'Topas'. It was shown that in potato tuber concentration of amino acids in vacuole is similar to that in cytosol [40], it is however not known for the roots. Unclear is if the phosphorylation activates the transporter to release the pool of amino acids to cytosol or blocks the transport to vacuole. Considering that GABA and glycine might be stored in the vacuole, their release to cytosol would explain the rapid decrease in their concentration in the 10 min treatment in cv. 'Lambada' (Jozefowicz et al., submitted). Amino acid transporters were also found to be phosphorylated in rice [41].



Cinnamyl alcohol dehydrogenase, a key enzyme of lignin biosynthesis, was higher abundant in cv. ‘Topas’ in result of prolonged N deficiency in our previous studies [11]. Here, this enzyme was phosphorylated by N starvation in cv. ‘Topas’ and only marginally in cv. ‘Lambada’. The lignification might provide a mechanism for protecting the root while facing abiotic stress but also be the sink for excessive carbon during N limitation. The role of phosphorylation in that process remains to be investigated.

The abundance of the transcripts of leucine aminopeptidase was previously described to increase in reaction to wounding, biotic stress but also in drought and salinity stress in tomato [42]. Here, this protein was rapidly phosphorylated in the tolerant genotype, but not in the sensitive one. After 1 h treatment, this peptide was dephosphorylated in both cultivars. Recently the function as molecular chaperon responding in the defense of solanaceous plants against stress was proposed [11].

#### *Differences in the phosphorylation response of potato cultivars after 24 and 72 hours of treatment*

After prolonged treatment, the number of altered phosphopeptides was clearly lower than after 10 min stress (46 after 24 h and 52 after 72 h, respectively). In addition most of the phosphopeptides decreased in their abundance in comparison to the control. This suggests that protein phosphorylation plays a significant role in the redirection of the metabolism following the imposition to N starvation but only minor in the longer N starvation periods.

Prolonged starvation caused a decrease in the phosphorylation status of protein kinases. The MAPKKK YODA like was dephosphorylated in the cv. ‘Topas’ after 24 h, pointing towards downregulation of the signaling cascades. In *Arabidopsis*, YODA was shown to play a role in the root development through an auxin-dependent mechanism regulating cell division [43]. After 72 h phosphorylation of MAP kinase substrate MKS1 was observed in the cv. ‘Topas’. In *Arabidopsis*, MAPK4 functions via its nuclear substrate MKS1 which regulates the activity of the WRKY33 transcription factor [44]. WRKY33 controls the production of the anti-microbial phytoalexins [45]. 72 h starvation resulted in 2.5 and 5 times increase in the phosphorylation status of phosphoenolpyruvate carboxykinase (PEPCK) in cv. ‘Lambada’ and ‘Topas’, respectively. This enzyme catalyzes the conversion of oxaloacetate to pyruvate and it is a rate-limiting step in the gluconeogenesis pathway in plants [46]. Recently, the light related regulation of PEPCK by phosphorylation was suggested in maize [47]. PEPCK may be involved in the conversion of fatty acids to sugars during seed germination [48, 49] and in the accumulation of soluble sugars and dissimilation of organic acids during fruit ripening [50, 51]. PEPCK is induced by some N containing compounds, for example by asparagine in developing seeds. It was suggested that is involved in the metabolism of oxaloacetate originating from asparagine in the tissues that import or recycle this amino acid [52]. In our previous study asparagine level decreased in cv. ‘Lambada’ after 72 h treatment, but only marginally in cv. ‘Topas’ (Jozefowicz, et al., submitted).

The PM ATPase that was rapidly phosphorylated after 10 min was also altered after 24 h treatment, but different phosphorylation site was affected. In cv. ‘Lambada’ a 2.5 increase and in cv. ‘Topas’ about a 2 times decrease was observed. Reduced activity of ATPase is expected by N starvation, as nitrate influx is proton- coupled and depends on the activity of PM H<sup>+</sup>-ATPase [53, 54]. Recent studies indicate however the presence of multiple phosphorylation sites with different responses to various stimuli, with activating but also deactivating role [55]. The phosphorylation on the Ser551 is novel and additional studies are needed to predict its effect on the activity and its function upon N starvation.

The phosphorylation of the  $\gamma$ -glutamylcyclotransferase, catalyzing the degradation of glutathione [56] decreased about three times in cv. ‘Lambada’. This enzyme might play a role in the degradation of glutathione upon prolonged starvation enabling re-usage of the constituent amino acids for metabolic processes.

The protein identified as lysine-histidine transporter was changed in the phosphorylation level over the whole time of treatment. The peptide TASVAQTSPILSLPIpTPR was phosphorylated rapidly in both genotypes after 10 min but dephosphorylated in cv. ‘Topas’ after 1 h. 24 h stress resulted in the phosphorylation of peptide FITPLGpSPLR in cv. ‘Lambada’ and 72 h it the dephosphorylation in both genotypes. The lysine histidine transporter was proposed to be involved in the uptake of amino acids into the root. The LHT1 mutants of *Arabidopsis* had a reduced amino acids uptake activity and restricted growth [57].

Ubiquitin- conjugating enzyme E2 and ubiquitin ligase E3 ATL6-like were dephosphorylated in cv. ‘Lambada’. In cv. ‘Topas’ they remained unchanged but still higher phosphorylated than in ‘Lambada’ at both N levels. Protein ubiquitination is a posttranslational modification, which plays an important role in the diverse processes such as cell cycle control, transcription factor activation, degradation of proteins and metabolic regulation [58]. In *Arabidopsis*, the ATL6 is a close homolog of ATL31 ligase. It was shown that both can have an ubiquitination activity to the 14-3-3 protein [59] and the 14-3-3 is degraded in the proteasome dependent manner [60]. Overexpression of 14-3-3 protein leads to the higher sensitivity of plants to the C/N conditions, especially enhanced by the lack of ATL31 and ATL6 activity. Since the 14-3-3 interacts with the main enzymes of carbon and nitrogen metabolism [61, 62] it was suggested that the proper C/N stress response is controlled via degradation of 14-3-3 protein by 26S Proteasome and this is regulated by the ATL31 protein. Recently it was shown that phosphorylation of ATL31 is necessary for its interaction with 14-3-3 protein [63]. If the phosphorylation site identified in the presented study is a part of 14-3-3 binding site, remains to be investigated.

Proteins which are differentially regulated between the sensitive and tolerant potato genotype are considered to be new candidate phosphoproteins that are important during the adaptation of plants to low N conditions. Further studies are necessary to understand the role which plays

phosphorylation on their activity. Confirmation of the phosphorylation sites could be achieved using specific antibodies against the phosphorylated form of the peptides. Additionally, further analysis of the MAPK cascade might be performed to investigate their role in nitrogen sensing. This includes identification of MAPK phosphorylation substrates using protein microarrays. The higher abundance of the 26S Proteasome protein subunits in cv. ‘Topas’ (Jozefowicz et al, submitted) and differential phosphorylation profiles of the ubiquitin conjugating enzyme and ubiquitin ligase makes this a potential candidate process for exploring its role in N deficiency tolerance. Another valuable candidate proteins are the amino acid transporters (lysine-histidine transporter and vacuolar amino acid transporter). The biological importance of their phosphorylation sites might be further investigated using *Arabidopsis thaliana* plants and site-directed mutation with the serine, threonine, or tyrosine changed to non-phosphorylatable amino acid, such as alanine. The alterations in the plant phenotype would confirm the importance of the phosphorylation site.

In conclusion, we showed that there are a time dependent and cultivar specific changes in the phosphoproteome following N deficiency treatment. We observed that phosphorylation of proteins participating in transcription is a common reaction for both the tolerant and the sensitive potato cultivar. The phosphorylation of the proteins participating in the signal transduction (MAPKK, MAPKKK) was, however specific to tolerant cultivar. It remains unclear if the response of the sensitive cv. ‘Lambada’ is different or just shifted in time. We showed that the protein phosphorylation following stress imposition is rapidly observed, consistent with an adaptation of plants to new environmental conditions. Dephosphorylation was, however, more pronounced after prolonged N deficiency. We also found new phosphorylation sites for proteins which are possibly involved in the response to low N.

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## Legend

**Figure 1.** The changes in the number of phosphopeptides with decreased and increased phosphorylation status in both genotypes over the time of experiment.

**Figure 2.** A- Heat map representing the 10 min treatment responsive phosphopeptides presented on the graphs in the form of log<sub>2</sub> of ratio (N-/N+). K- mean clustering using a Euclidean distance matrix was used to elucidate six distinct clusters. Phosphopeptides are presented in the same order as given in the table S2. B- Visualization of the phosphopeptide abundance profiles elucidated for the 10 min treatment. The treatment responsive phosphopeptides presented in form of abundance ratio (N-/N+). C- Heat map representing the 1 hour treatment responsive phosphopeptides presented on the as described above. B- Visualization of the phosphopeptide abundance profiles elucidated for the 1 hour treatment. The treatment responsive phosphopeptides presented in form of abundance ratio (N-/N+).

**Figure 3.** A- Heat map representing the 24 hours treatment responsive phosphopeptides presented on the graphs in the form of log<sub>2</sub> of ratio (N-/N+). K- mean clustering using a Euclidean distance matrix was used to elucidate four distinct clusters. Visualization of the phosphopeptide abundance profiles elucidated for the 24 h treatment. The treatment responsive phosphopeptides presented in form of abundance ratio (N-/N+). B- Heat map representing the 72 hours treatment responsive phosphopeptides presented on the as described above.

**Figure 4.** Phosphopeptides and proteins responsive to 10 min treatment. A- The number of cultivar specific and common phosphopeptides. B- The 10 min responsive proteins classified according to their cellular localization. C- The cultivar specific proteins grouped according to their molecular function.

**Figure 5.** Phosphoproteins responsive to N deficiency assigned to the functional categories according to Bevan et al.

**Figure 6.** Summary of the results. The most important changes in the phosphorylation profiles of proteins belonging to the categories 'transport', 'energy and metabolism', 'defence', 'signaling' and 'transcription' are displayed. A- 10 min response; B- 24 and 72 h response. The color-coding is following green- proteins higher phosphorylated in cv. 'Lambada', blue- proteins higher phosphorylated in cv. 'Topas', yellow- proteins dephosphorylated in cv. 'Lambada', grey- proteins higher phosphorylated in cv. 'Topas', white- similar response in both cultivars. The major tendency of the changes in the phosphorylation pattern is indicated by an arrow.

## Supplementary data

Table S1. Peptides changed in the phosphorylation status in the response to N starvation.

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Figure 1.

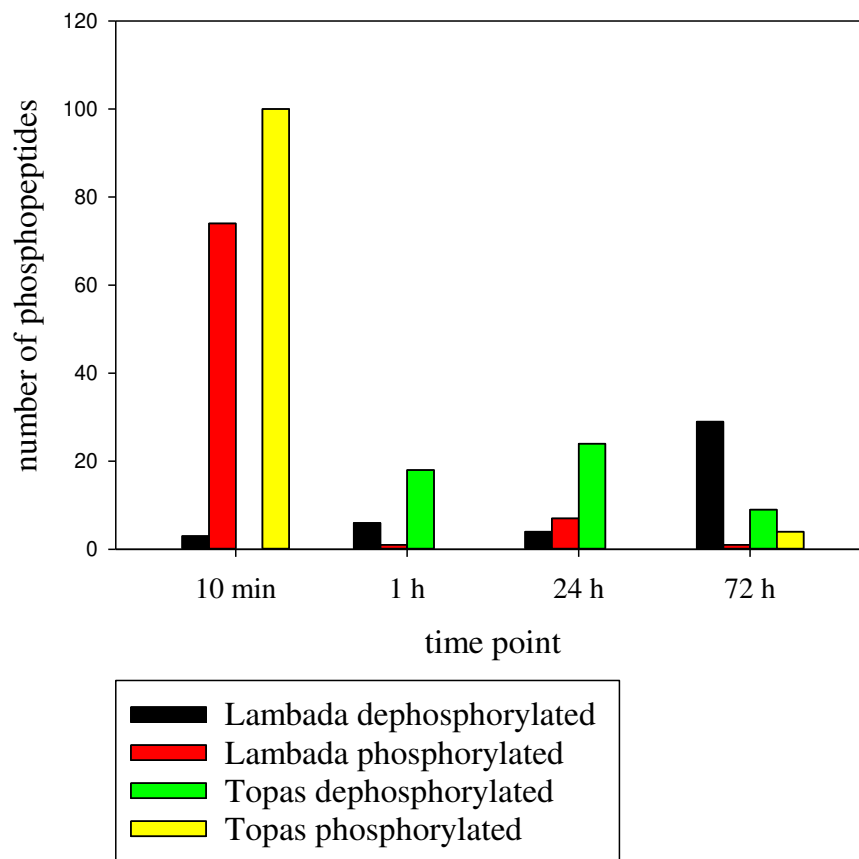


Figure 2.

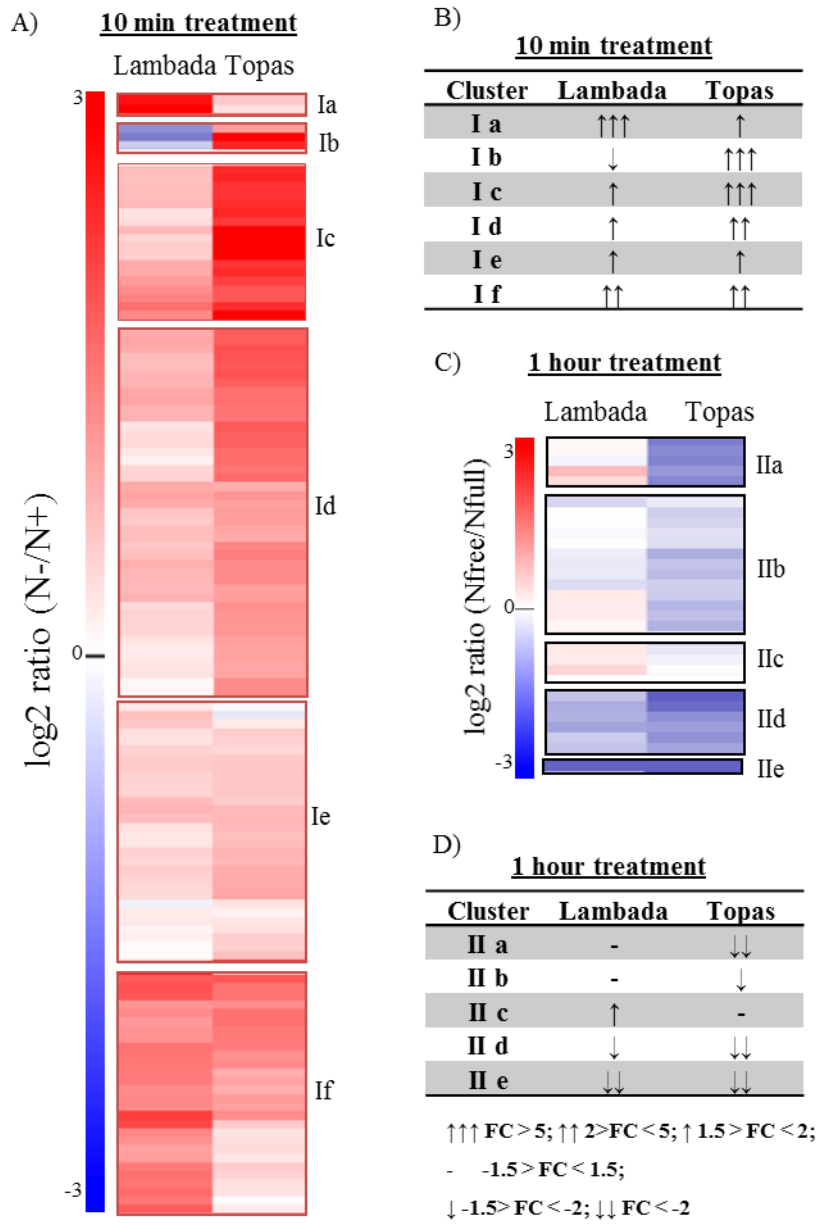


Figure 3.

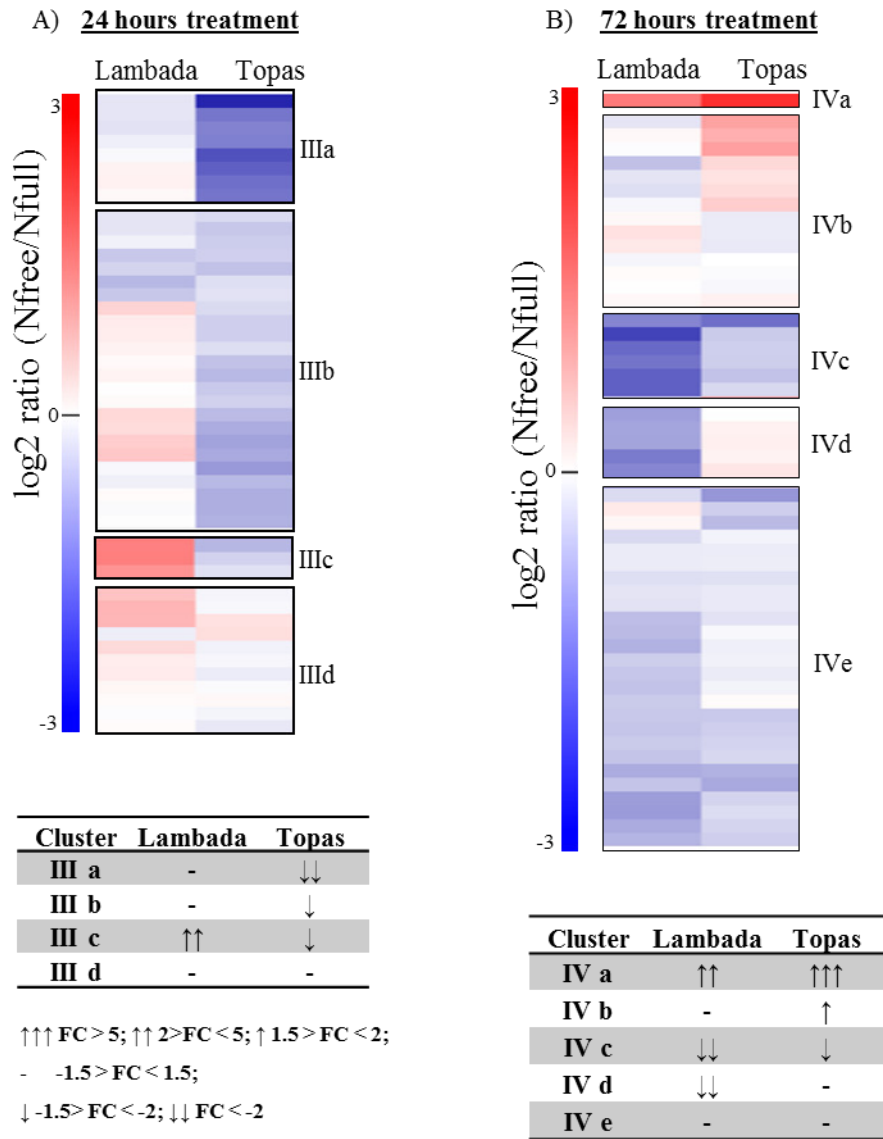


Figure 4.

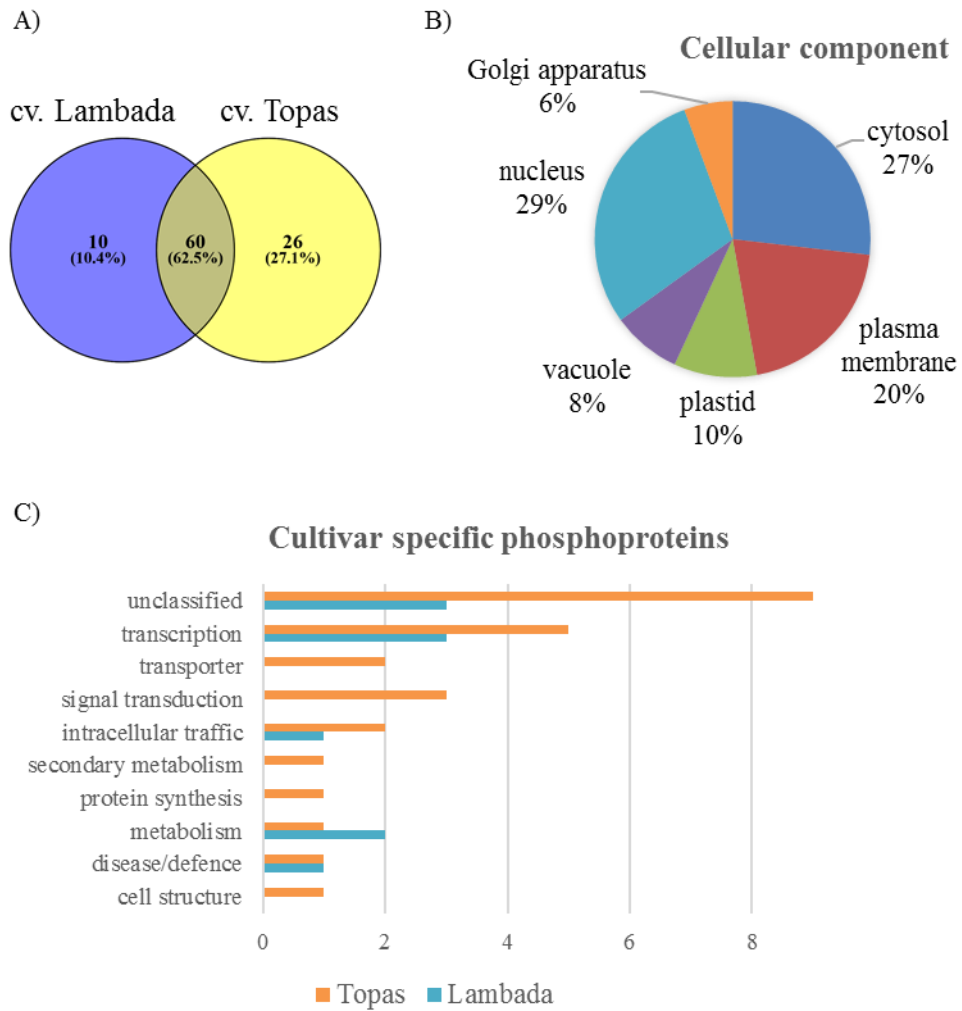


Figure 5.

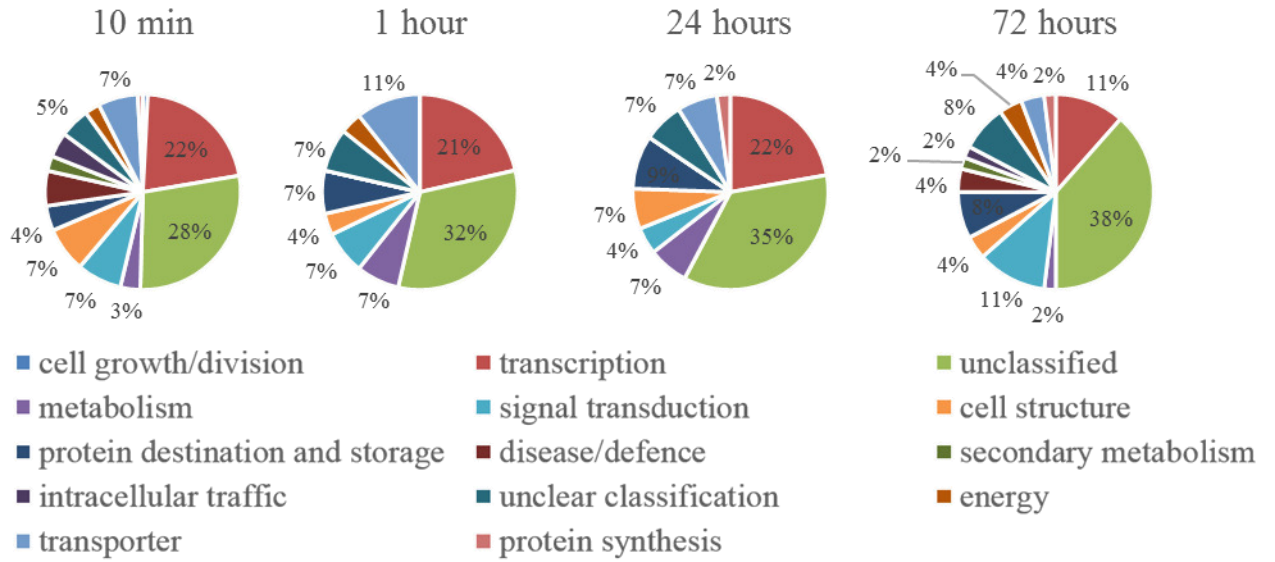
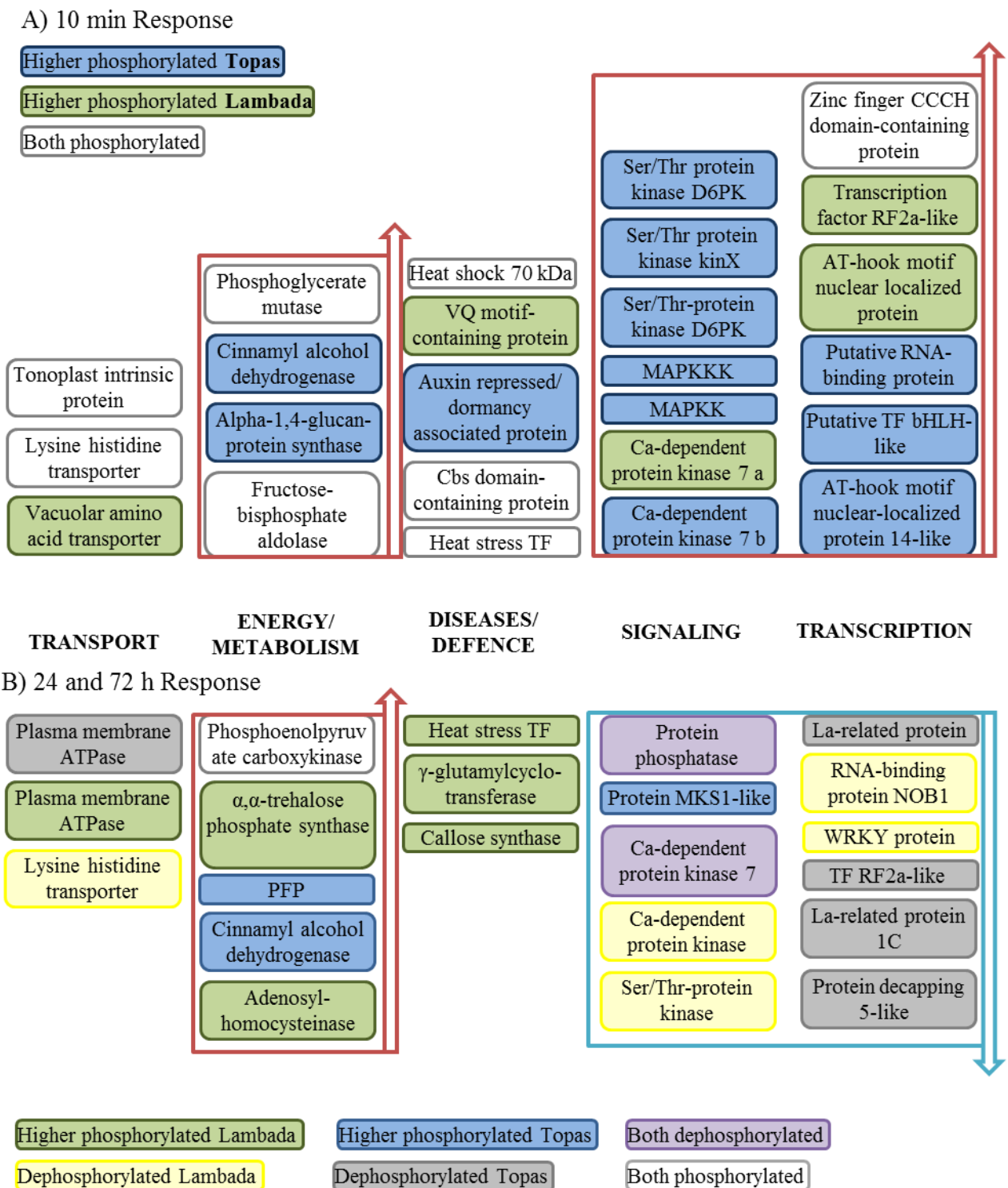


Figure 6.



## Appendices

**Appendix Table 1.** Primers used for identification of mutation homozygotes among the progeny of *A. thaliana* T-DNA insertion *pip* mutant lines, Supplementary Table S1, chapter III.

**Appendix Table 2.** Primers used for cloning of potato aquaporins, Supplementary Table S2, chapter III.

**Appendix Table 3.** Proteins identified in the plasma membrane study presented detailed view, Supplementary Table S3, chapter III.

**Appendix Table 4.** The abundance of proteins responsive to the 24 h and 72 h treatment, Supplementary Table S1, chapter IV.

**Appendix 5.** The abundance of proteins participating in in the C and N metabolism, protein synthesis and catabolism as well as in lignin synthesis pathway, Supplementary table S2, chapter IV.

**Appendix 6.** The specific masses of the quantified fragment ion (m/z) along with retention time index of the 65 metabolites, Supplementary Table S3, chapter IV.

**Appendix 7.** Peptides changed in the phosphorylation status in the response to N starvation, Supplementary Table S1, chapter V.



**Appendix Table 1.** Primers used for identification of mutation homozygotes among the progeny of *A. thaliana* T-DNA insertion *pip* mutant lines, Supplementary Table S1, chapter III.

| Line        | Target gene         | Primers  | Prod. [bp] | BP+RP [bp] |
|-------------|---------------------|--|------------|------------|
| SALK_051107 | PIP1;3<br>AT1G01620 | LP: TAACGTGGCCCATAAAGAGTG<br>RP: AATTGGTCTTTTGTGTCATGC<br>LB : ATTTTGCCGATTTCCGGAAC              | 1214       | 526-826    |
| GABI_437B11 | PIP1;1<br>At3g61430 | LP: CTCAGAGTGACAAGGACTACAAGG<br>RP: TATACAAGAACGAATGTGCCAATG<br>LB: ATAATAACGCTGCGGACATCTACATTTT | 1240       | 583-883    |

**Appendix Table 2.** Primers used for cloning of potato aquaporins, Supplementary Table S2, chapter III).

| Name   | Accession            | Primer Sequence  |
|--------|----------------------|--|
|        |                      | F:   |
| PIP1;1 | PGSC0003DMG400004980 | GGCTTAAUATGGCAGAGAACAAGAAGAAG<br>R: GGTTTAAUTCAAGACCTGCTGCTCTTG  |
|        |                      | F:   |
| PIP1;3 | PGSC0003DMG400012337 | GGCTTAAUATGGCAGAAAACAAGGAAGAAG<br>R: GGTTTAAUTTAAGACCTGCGGAATGGC |

**Appendix Table 3.** Proteins identified in the plasma membrane study presented detailed view, Supplementary Table S3, chapter III.

| Accession        | Description                     | Pep | Uniq | Confide | Anova  | Max  | Normalized abundance |        |        |        | Standard deviation |        |        |        | Peptide information   | RT  | Cham/z                          | Score  |
|------------------|---------------------------------|-----|------|---------|--------|------|----------------------|--------|--------|--------|--------------------|--------|--------|--------|---|---|---------------------------------|--|
|                  |                                 |     |      |         |        |      | Lam                  | C      | Lam    | S      | TopC               | TopS   | Lam    | C      |   |   |                                 |  |
| <b>Cluster 1</b> |                                 |     |      |         |        |      |                      |        |        |        |                    |        |        |        |   |   |                                 |  |
| 400039130        | Defensin-like protein           | 4   | 4    | 42.67   | 0.0250 | 1.56 | 531.95               | 832.41 | 828.67 | 709.06 | 42.10              | 280.10 | 86.50  | 192.57 | DSNCASVCETER<br>IAEAR<br>CFCTKPC<br>FSGGNCHGFR  | 21.96<br>44.41<br>25.21<br>25.15                            | 2<br>1<br>1<br>2                | 714.2839.26<br>559.3066.16<br>972.3909.22<br>569.7548.81   |
| <b>Cluster 2</b> |                                 |     |      |         |        |      |                      |        |        |        |                    |        |        |        |   |   |                                 |  |
| 400079443        | Hemoglobin                      | 5   | 4    | 38.4    | 0.0102 | 3.17 | 134.69               | 45.61  | 144.51 | 131.87 | 29.29              | 8.88   | 36.62  | 170.56 | EASPEMWSVDMK<br>YALLETIK<br>SSFSEEQEALVVK<br>YGVADEHFVTK  | 54.89<br>54.52<br>56.59<br>41.14                            | 2<br>2<br>1<br>3                | 705.3368.42<br>475.7807.91<br>1494.726.82<br>465.5577.55   |
| 400083124        | Uncharacterized protein         | 9   | 7    | 68.4    | 0.0074 | 1.93 | 411.61               | 212.96 | 401.79 | 360.50 | 102.06             | 57.28  | 80.27  | 111.07 | LDIDSTSSVEEITK<br>FFVSFNCDK<br>GGNYGWSEYEGPYLYTP<br>LTDLWK<br>DNPYTEDKELQPEIWAL<br>SVICAKCDK<br>SMTDPCMHGR                                    | 51.38<br>57.11<br>62.79<br>47.66<br>73.24<br>21.21<br>21.64 | 2<br>2<br>2<br>1<br>3<br>2<br>2 | 768.8868.2<br>582.2727.95<br>1084.5<br>775.4207.9<br>802.3967.4<br>540.7586.4<br>596.2927.44         |
| <b>Cluster 3</b> |                                 |     |      |         |        |      |                      |        |        |        |                    |        |        |        |   |   |                                 |  |
| 400005571        | Glutathione S-transferase-like  | 2   | 2    | 15.39   | 0.0121 | 5.74 | 34.52                | 30.72  | 176.36 | 51.27  | 5.13               | 6.37   | 40.39  | 37.78  | SPLLLESNPIYK<br>ILDNELKDK   | 60.69<br>33.06  | 2<br>2                          | 687.3818.3<br>544.2597.09  |
| 400040518        | Putative cinnamyl alcohol       | 9   | 5    | 88.93   | 0.0000 | 2.78 | 723.38               | 530.70 | 1474.2 | 655.17 | 119.05             | 84.38  | 418.01 | 75.40  | ETQEMVDFAAK<br>VGVGCLVGSCR<br>LGADSFLVSR<br>DTSGVLSPFNFSR<br>VMYCGICHSDLHQLK  | 43.92<br>42.61<br>52.75<br>66.23<br>39.32                   | 2<br>2<br>2<br>2<br>2           | 634.8018.56<br>582.2898.57<br>532.7908.73<br>713.8548.7<br>930.9236.03                               |
| <b>Cluster 4</b> |                                 |     |      |         |        |      |                      |        |        |        |                    |        |        |        |   |   |                                 |  |
| 400035262        | Sucrose synthase                | 4   | 2    | 28.51   | 0.0492 | 1.85 | 75.20                | 60.82  | 86.82  | 112.40 | 11.78              | 9.88   | 17.55  | 44.95  | FQEIGLER<br>NKPIIFTMAR  | 43.59<br>49.99  | 2<br>2                          | 496.2626.91<br>595.8246.91   |
| 400047710        | Luminal-binding protein 5       | 15  | 3    | 139.15  | 0.0192 | 2.17 | 682.37               | 548.89 | 683.11 | 1193.4 | 53.72              | 42.10  | 199.15 | 691.39 | NSLETYVYNMR<br>ITPSWVAFTDGER<br>TVFDVKR   | 46.65<br>62.47<br>43.07                                     | 2<br>2<br>1                     | 695.3376.57<br>739.8688.43<br>864.4516.81  |
| 400035380        | ADP,ATP carrier protein         | 3   | 2    | 27.79   | 0.0127 | 4.34 | 103.00               | 55.79  | 94.99  | 241.97 | 48.86              | 10.14  | 45.57  | 162.83 | LLIQNQDEMLK<br>SSLDAFSQIVK  | 51.67<br>59.74  | 2<br>2                          | 672.8236.69<br>597.8137.08   |
| 400018820        | Putative ABC transporter family | 23  | 12   | 210.42  | 0.0064 | 3.92 | 253.30               | 298.42 | 369.30 | 992.20 | 17.36              | 131.14 | 218.13 | 721.87 | FISGLQQS YD TTVGER<br>TILQQDIAFYDKETNTGE<br>EGNATEAEVLA A AELAN<br>FYDPQSGQVLIDGINLK<br>QAEKANTVPFYK<br>QQMGLVSQEPVLFNDTIR<br>IDPSDDSGMTLDTVK | 52.23<br>62.33<br>58.82<br>68.44<br>49.63<br>68.34<br>64.93 | 2<br>3<br>2<br>2<br>2<br>2<br>2 | 900.9448.85<br>833.0928.43<br>1005.008.3<br>954.0009.01<br>698.3606.81<br>1038.048.65<br>797.3716.89 |

|           |  |    |    |        |        |          |        |        |        |        |       |        |        |        |  |  |                    |       |   |             |
|-----------|--|----|----|--------|--------|----------|--------|--------|--------|--------|-------|--------|--------|--------|--|--|--------------------|-------|---|-------------|
|           |  |    |    |        |        |          |        |        |        |        |       |        |        |        |  |  | FIDKLPQGLDTMVGEHG  | 58.86 | 3 | 886.1188.34 |
|           |  |    |    |        |        |          |        |        |        |        |       |        |        |        |  |  | MMYEEASQVANDAVGG   | 60.66 | 2 | 970.9737.06 |
|           |  |    |    |        |        |          |        |        |        |        |       |        |        |        |  |  | SAAASVFAILDR       | 73.81 | 2 | 610.8358.03 |
|           |  |    |    |        |        |          |        |        |        |        |       |        |        |        |  |  | ETNTGEVVGRMSGDTVL  | 61.38 | 3 | 879.7557.47 |
|           |  |    |    |        |        |          |        |        |        |        |       |        |        |        |  |  | MASSGQDAYAK        | 18.75 | 2 | 564.7527.93 |
| 400025670 | <b>U- box domain containing protein</b>  | 2  | 2  | 14.36  | 0.0189 | 139.26   | 4.06   | 0.32   | 3.19   | 44.35  | 2.66  | 0.51   | 4.52   | 38.03  |  |  | EAAISLLYELSK       | 76.37 | 2 | 668.8737.16 |
|           |  |    |    |        |        |          |        |        |        |        |       |        |        |        |  |  | ITFVLNDEPNVSLCR    | 64.54 | 2 | 924.4677.2  |
| 400083041 | <b>Plasma membrane ATPase</b>            | 49 | 13 | 564.74 | 0.0018 | 4.26     | 564.00 | 522.11 | 627.74 | 2224.8 | 93.16 | 94.97  | 345.70 | 1662.2 |  |  | GEKPEVLDVAVLK      | 59.57 | 2 | 670.3709.19 |
|           |  |    |    |        |        |          |        |        |        |        |       |        |        |        |  |  | GVDADTVVLMMAAR     | 57.84 | 1 | 1317.699.5  |
|           |  |    |    |        |        |          |        |        |        |        |       |        |        |        |  |  | LFSESTNFNQLAEAA    | 66.79 | 2 | 1170.589.46 |
|           |  |    |    |        |        |          |        |        |        |        |       |        |        |        |  |  | TENQDAIDTAIVGMLSDP | 77.56 | 2 | 1009.499.36 |
|           |  |    |    |        |        |          |        |        |        |        |       |        |        |        |  |  | DESIASLPIDELIEK    | 79.00 | 2 | 836.4389.74 |
|           |  |    |    |        |        |          |        |        |        |        |       |        |        |        |  |  | EGLTGTAQER         | 25.21 | 2 | 566.7849.63 |
|           |  |    |    |        |        |          |        |        |        |        |       |        |        |        |  |  | WNEEDAAVLVPGDIISIK | 78.09 | 2 | 985.0209.18 |
|           |  |    |    |        |        |          |        |        |        |        |       |        |        |        |  |  | LFSESTNFNQLAEAA    | 70.05 | 2 | 1092.538.9  |
|           |  |    |    |        |        |          |        |        |        |        |       |        |        |        |  |  | TLHGLQVPDTK        | 38.70 | 2 | 604.8318.98 |
|           |  |    |    |        |        |          |        |        |        |        |       |        |        |        |  |  | NLVEVFAK           | 53.80 | 1 | 919.5278.95 |
|           |  |    |    |        |        |          |        |        |        |        |       |        |        |        |  |  | LTVDKNLVEVFAK      | 61.45 | 2 | 738.4149.12 |
|           |  |    |    |        |        |          |        |        |        |        |       |        |        |        |  |  | DGKWNEEDAAVLVPGDI  | 73.92 | 2 | 1135.099.19 |
|           |  |    |    |        |        |          |        |        |        |        |       |        |        |        |  |  | LGMGTNMYPSALLGQT   | 85.87 | 3 | 1174.598.6  |
| 400019462 | <b>Uncharacterized protein</b>           | 2  | 2  | 15.66  | 0.0288 | 16.27    | 21.74  | 10.47  | 14.82  | 170.40 | 15.54 | 14.43  | 21.82  | 194.68 |  |  | NYELNDDALGLNPELR   | 61.98 | 2 | 923.4508.12 |
|           |  |    |    |        |        |          |        |        |        |        |       |        |        |        |  |  | SIFYEMTLEQR        | 61.91 | 2 | 708.8527.54 |
| 400036847 | <b>Pyrophosphate- energized vacuolar</b> | 4  | 2  | 34.57  | 0.0099 | Infinity | 0.06   | 0.00   | 0.79   | 49.75  | 0.14  | 0.00   | 1.28   | 66.87  |  |  | QFNTIPGLMEGLAKPDY  | 72.41 | 3 | 818.4168.27 |
|           |  |    |    |        |        |          |        |        |        |        |       |        |        |        |  |  | TDALDAAGNTTAAIGK   | 43.63 | 1 | 1489.738.78 |
| 400031304 | <b>High-affinity nitrate transporter</b> | 4  | 4  | 31.57  | 0.0306 | 9.33     | 62.13  | 30.57  | 34.67  | 285.23 | 12.49 | 12.59  | 23.26  | 272.84 |  |  | MDVGNAGVASVSGSILS  | 58.40 | 2 | 860.4428.28 |
|           |  |    |    |        |        |          |        |        |        |        |       |        |        |        |  |  | EPVLAFSVASPMVPTDTT | 69.95 | 2 | 1031.047.99 |
|           |  |    |    |        |        |          |        |        |        |        |       |        |        |        |  |  | GSEEHYYTSEYTEAER   | 35.00 | 2 | 975.9047.87 |
|           |  |    |    |        |        |          |        |        |        |        |       |        |        |        |  |  | DNLNLTK            | 28.93 | 1 | 817.4347.43 |
| 400005236 | <b>Hypersensitive induced response</b>   | 9  | 3  | 79.81  | 0.0003 | 4.18     | 92.85  | 46.19  | 79.23  | 193.04 | 22.95 | 27.39  | 28.50  | 90.09  |  |  | MGLDAAFEQK         | 49.73 | 1 | 1109.518.06 |
|           |  |    |    |        |        |          |        |        |        |        |       |        |        |        |  |  | GQIQSYVFDVIR       | 71.03 | 2 | 712.8687.84 |
|           |  |    |    |        |        |          |        |        |        |        |       |        |        |        |  |  | DSVLAFSENVPGTSPR   | 57.87 | 2 | 838.4258.28 |
| 400001904 | <b>Ras-related protein RABG3f like</b>   | 5  | 2  | 39.78  | 0.0381 | 2.26     | 204.11 | 132.94 | 154.72 | 299.88 | 28.17 | 41.78  | 75.22  | 131.01 |  |  | EGTNVEEAFQCIK      | 58.20 | 2 | 798.3887.89 |
|           |  |    |    |        |        |          |        |        |        |        |       |        |        |        |  |  | TSLMNQYVVK         | 40.64 | 2 | 599.3077.24 |
| 400057709 | <b>Hexose transporter 1</b>              | 4  | 4  | 37.79  | 0.0454 | 2.00     | 252.21 | 282.94 | 207.42 | 413.92 | 20.51 | 53.25  | 47.21  | 202.19 |  |  | FMTEVDYPGNR        | 45.21 | 2 | 664.8248.01 |
|           |  |    |    |        |        |          |        |        |        |        |       |        |        |        |  |  | EHWFWSK            | 52.89 | 1 | 1019.517.16 |
|           |  |    |    |        |        |          |        |        |        |        |       |        |        |        |  |  | GIENVDEEFNDLVVASEA | 76.48 | 2 | 1097.028.32 |
|           |  |    |    |        |        |          |        |        |        |        |       |        |        |        |  |  | IRGIENVDEEFNDLVVAS | 73.36 | 3 | 821.4117.14 |
| 400029027 | <b>Tetraspanin-8</b>                     | 5  | 5  | 49.32  | 0.0350 | 1.66     | 1008.1 | 1059.8 | 920.28 | 1528.6 | 48.00 | 108.72 | 56.98  | 461.50 |  |  | ICDTLIQGSNTK       | 37.98 | 1 | 1349.679    |
|           |  |    |    |        |        |          |        |        |        |        |       |        |        |        |  |  | VLCYGCQSK          | 28.37 | 2 | 637.7748.47 |
|           |  |    |    |        |        |          |        |        |        |        |       |        |        |        |  |  | FGDYSNLQK          | 56.86 | 2 | 629.3077.7  |
|           |  |    |    |        |        |          |        |        |        |        |       |        |        |        |  |  | AGLLDNVK           | 41.39 | 1 | 829.4478.14 |
|           |  |    |    |        |        |          |        |        |        |        |       |        |        |        |  |  | EDNAWK             | 23.99 | 1 | 762.3876.72 |
| 400027156 | <b>Ubiquitin</b>                         | 7  | 2  | 59.99  | 0.0009 | 1.76     | 193.02 | 246.60 | 219.78 | 339.82 | 23.07 | 48.34  | 59.15  | 49.35  |  |  | TLADYNIQK          | 36.54 | 2 | 533.2817.93 |
|           |  |    |    |        |        |          |        |        |        |        |       |        |        |        |  |  | EGIPDQQR           | 34.96 | 1 | 1039.486.44 |

|           |                                   |    |       |        |        |        |        |        |        |        |        |        |        |                    |                   |       |              |              |
|-----------|-----------------------------------|----|-------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------------------|-------------------|-------|--------------|--------------|
| 400046727 | ABC transporter A family member 7 | 5  | 62.22 | 0.0006 | 2.31   | 145.89 | 191.76 | 176.54 | 336.81 | 11.69  | 26.24  | 16.53  | 145.49 | DPPTDTSSSGFLR      | 42.34             | 2     | 690.3547.54  |              |
|           |                                   |    |       |        |        |        |        |        |        |        |        |        |        | LLTDFFAELQDR       | 82.78             | 2     | 734.3788.33  |              |
|           |                                   |    |       |        |        |        |        |        |        |        |        |        |        | CPGALHFVER         | 40.41             | 2     | 593.3057.18  |              |
|           |                                   |    |       |        |        |        |        |        |        |        |        |        |        | TTTINCLTGITPVTAGDA | 77.83             | 3     | 908.4677.77  |              |
|           |                                   |    |       |        |        |        |        |        |        |        |        |        |        | GVFEDPTFTFQIPLQLAA | 86.99             | 2     | 1140.097.98  |              |
| 400046278 | Calmodulin-2/4                    | 9  | 4     | 80.29  | 0.0001 | 2.31   | 229.01 | 310.51 | 272.45 | 529.55 | 10.57  | 82.27  | 55.76  | 164.63             | VMMAK             | 31.15 | 1            | 579.3036.89  |
|           |                                   |    |       |        |        |        |        |        |        |        |        |        |        | LTDEEVDEMIR        | 45.33             | 2     | 675.3456.84  |              |
|           |                                   |    |       |        |        |        |        |        |        |        |        |        |        | EAFRVFDK           | 41.46             | 2     | 506.2567.86  |              |
|           |                                   |    |       |        |        |        |        |        |        |        |        |        |        | HVMTNLGEK          | 24.72             | 2     | 514.7657.87  |              |
| 400032134 | Aquaporin PIP1;3                  | 8  | 6     | 130.76 | 0.0214 | 4.48   | 624.68 | 824.68 | 1108.6 | 2797.2 | 119.50 | 299.24 | 799.51 | 1478.4             | ETQPLGTSAQTDK     | 24.82 | 2            | 688.3419.92  |
|           |                                   |    |       |        |        |        |        |        |        |        |        |        |        | ETQPLGTSAQTDKDYK   | 28.13             | 3     | 594.6249.96  |              |
|           |                                   |    |       |        |        |        |        |        |        |        |        |        |        | SLGAAIIFNDK        | 58.03             | 2     | 574.8189.51  |              |
|           |                                   |    |       |        |        |        |        |        |        |        |        |        |        | AMPFR              | 31.68             | 1     | 621.3208.39  |              |
|           |                                   |    |       |        |        |        |        |        |        |        |        |        |        | GFMVGPYQR          | 47.11             | 2     | 527.7748.88  |              |
|           |                                   |    |       |        |        |        |        |        |        |        |        |        |        | ETQPLGTSAQTDKDYKE  | 77.83             | 4     | 1017.998.74  |              |
| 400065168 | Plasma membrane ATPase            | 45 | 8     | 503.25 | 0.0002 | 2.34   | 288.52 | 295.94 | 354.71 | 676.04 | 35.17  | 46.55  | 47.84  | 263.96             | IFSETTNFNLNQLAEEA | 66.20 | 2            | 1177.588.83  |
|           |                                   |    |       |        |        |        |        |        |        |        |        |        |        | GIDADTVVLMMAAR     | 61.22             | 2     | 666.3479.08  |              |
|           |                                   |    |       |        |        |        |        |        |        |        |        |        |        | YALSGK             | 26.37             | 1     | 638.3006.78  |              |
|           |                                   |    |       |        |        |        |        |        |        |        |        |        |        | IFSETTNFNLNQLAEEA  | 69.29             | 2     | 1099.548.4   |              |
|           |                                   |    |       |        |        |        |        |        |        |        |        |        |        | VFGVSTLEK          | 50.48             | 1     | 979.5336.52  |              |
|           |                                   |    |       |        |        |        |        |        |        |        |        |        |        | TNFFPR             | 36.20             | 1     | 781.4027.44  |              |
|           |                                   |    |       |        |        |        |        |        |        |        |        |        |        | DGKWDEEDAAVLVPGDI  | 75.24             | 3     | 757.3908.55  |              |
|           |                                   |    |       |        |        |        |        |        |        |        |        |        |        | DESIAALPIDELIEK    | 80.05             | 2     | 828.4408.81  |              |
| 400036643 | Transmembrane protein             | 4  | 4     | 31.54  | 0.0192 | 3.75   | 120.84 | 159.93 | 168.06 | 453.29 | 11.27  | 27.74  | 67.45  | 187.28             | LVDATSGQESLAGK    | 33.91 | 2            | 688.3627.88  |
|           |                                   |    |       |        |        |        |        |        |        |        |        |        |        | ISSAAETLETETEK     | 40.76             | 2     | 754.8648.1   |              |
|           |                                   |    |       |        |        |        |        |        |        |        |        |        |        | ELLDCTFLR          | 61.81             | 2     | 583.8047.71  |              |
|           |                                   |    |       |        |        |        |        |        |        |        |        |        |        | NPLAHTALDDIIPCVDTA | 72.80             | 3     | 1003.837.85  |              |
| 400012780 | Aquaporin PIP1;1                  | 7  | 4     | 89.78  | 0.0238 | 6.22   | 276.21 | 392.67 | 428.37 | 1717.1 | 77.70  | 128.54 | 274.61 | 1004.7             | ETQPLGTAAQTDK     | 27.11 | 2            | 680.3409.71  |
|           |                                   |    |       |        |        |        |        |        |        |        |        |        |        | ETQPLGTAAQTDKDYK   | 31.00             | 2     | 883.4249.96  |              |
|           |                                   |    |       |        |        |        |        |        |        |        |        |        |        | ETQPLGTAAQTDKDYKE  | 78.26             | 4     | 1013.999.31  |              |
|           |                                   |    |       |        |        |        |        |        |        |        |        |        |        | GFMVGPYER          | 42.83             | 2     | 528.2606.7   |              |
| 400010497 | Plasma membrane ATPase            | 62 | 37    | 815.46 | 0.0000 | 5.25   | 3331.8 | 3555.8 | 4266.5 | 17505. | 306.60 | 627.69 | 2575.2 | 9435.4             | NPGDEVFSGSTCK     | 35.35 | 2            | 699.29710.21 |
|           |                                   |    |       |        |        |        |        |        |        |        |        |        |        | GAPEQILDLCNCK      | 53.90             | 2     | 759.3519.62  |              |
|           |                                   |    |       |        |        |        |        |        |        |        |        |        |        | MITGDQLAIK         | 45.33             | 2     | 580.8149.43  |              |
|           |                                   |    |       |        |        |        |        |        |        |        |        |        |        | ADIGIADVADATDAAR   | 52.94             | 2     | 715.37210.04 |              |
|           |                                   |    |       |        |        |        |        |        |        |        |        |        |        | IPIEEVFEQLK        | 76.15             | 2     | 672.8689.94  |              |
|           |                                   |    |       |        |        |        |        |        |        |        |        |        |        | GVDKEYVLLLAAR      | 59.60             | 2     | 723.91110.18 |              |
|           |                                   |    |       |        |        |        |        |        |        |        |        |        |        | TALTYIDNNGNWHR     | 46.65             | 2     | 837.90310.13 |              |
|           |                                   |    |       |        |        |        |        |        |        |        |        |        |        | WSEQEAAILVPGDIISVK | 76.48             | 2     | 978.00310.08 |              |
|           |                                   |    |       |        |        |        |        |        |        |        |        |        |        | VENQDAIDACMVGMLA   | 67.49             | 2     | 1038.9610.22 |              |
|           |                                   |    |       |        |        |        |        |        |        |        |        |        |        | LQIFGPNK           | 45.33             | 1     | 916.5018.98  |              |
|           |                                   |    |       |        |        |        |        |        |        |        |        |        |        | LQIFGPNKLEEK       | 49.24             | 2     | 708.3939.7   |              |
|           |                                   |    |       |        |        |        |        |        |        |        |        |        |        | LGMGTNMYPSASLLGQD  | 61.64             | 2     | 941.9579.77  |              |
|           |                                   |    |       |        |        |        |        |        |        |        |        |        |        | EVHFLPFNPVDR       | 56.13             | 2     | 799.4209.8   |              |
|           |                                   |    |       |        |        |        |        |        |        |        |        |        |        | DSSIASLPVEELIEK    | 75.39             | 2     | 815.4329.97  |              |



|                  |  |    |    |        |        |      |        |        |        |        |        |        |        |        |  |  |                    |       |   |             |
|------------------|--|----|----|--------|--------|------|--------|--------|--------|--------|--------|--------|--------|--------|--|--|--------------------|-------|---|-------------|
|                  |  |    |    |        |        |      |        |        |        |        |        |        |        |        |  |  | AYEAATATASSDLAPTHP | 42.54 | 3 | 681.6747.92 |
|                  |  |    |    |        |        |      |        |        |        |        |        |        |        |        |  |  | NLVSVGKY           | 38.86 | 1 | 879.4727.84 |
| 400075291        | <b>40S ribosomal protein S6</b>            | 3  | 2  | 21.93  | 0.0006 | 2.70 | 43.30  | 37.52  | 54.50  | 20.21  | 5.06   | 5.82   | 8.57   | 9.15   |  |  | LRAFYDK            | 37.39 | 2 | 456.7557.02 |
|                  |  |    |    |        |        |      |        |        |        |        |        |        |        |        |  |  | LVTPLTLQR          | 52.36 | 2 | 520.8256.93 |
| 400014945        | <b>Putative vacuolar proton ATPase</b>     | 18 | 12 | 159.06 | 0.0003 | 2.42 | 2644.2 | 2659.8 | 3251.7 | 1342.3 | 67.51  | 492.07 | 545.93 | 661.64 |  |  | VLQAQDDLVNTMK      | 49.28 | 2 | 737.8819.1  |
|                  |  |    |    |        |        |      |        |        |        |        |        |        |        |        |  |  | IVCENTLDAR         | 32.44 | 2 | 595.7969.11 |
|                  |  |    |    |        |        |      |        |        |        |        |        |        |        |        |  |  | MNDADVSK           | 29.91 | 1 | 921.4068.44 |
|                  |  |    |    |        |        |      |        |        |        |        |        |        |        |        |  |  | NDADVSK            | 22.03 | 1 | 748.3396.66 |
|                  |  |    |    |        |        |      |        |        |        |        |        |        |        |        |  |  | KHDVHLVEHVLEGVK    | 29.00 | 3 | 580.3067.23 |
|                  |  |    |    |        |        |      |        |        |        |        |        |        |        |        |  |  | LEVVFR             | 47.46 | 1 | 762.4548.25 |
|                  |  |    |    |        |        |      |        |        |        |        |        |        |        |        |  |  | EEYAEK             | 36.34 | 1 | 768.3797.33 |
|                  |  |    |    |        |        |      |        |        |        |        |        |        |        |        |  |  | LPEIR              | 30.47 | 1 | 627.3618.06 |
|                  |  |    |    |        |        |      |        |        |        |        |        |        |        |        |  |  | KCLFGQVAA          | 46.48 | 1 | 993.5187.87 |
|                  |  |    |    |        |        |      |        |        |        |        |        |        |        |        |  |  | EPCILLR            | 47.53 | 1 | 900.4836.19 |
|                  |  |    |    |        |        |      |        |        |        |        |        |        |        |        |  |  | IKVLQAQDDLVNTMK    | 53.28 | 2 | 858.4647.18 |
|                  |  |    |    |        |        |      |        |        |        |        |        |        |        |        |  |  | ANEISVSAEEEFNIEK   | 42.54 | 2 | 904.9646.73 |
| 400068704        | <b>Vacuolar ATPase subunit B</b>           | 20 | 4  | 263.26 | 0.0050 | 2.06 | 1115.9 | 1038.1 | 1247.8 | 607.24 | 57.55  | 62.45  | 316.11 | 453.79 |  |  | DISGSSINPSER       | 32.62 | 1 | 1261.619.57 |
|                  |  |    |    |        |        |      |        |        |        |        |        |        |        |        |  |  | QAGLVK             | 37.52 | 1 | 615.3436.5  |
|                  |  |    |    |        |        |      |        |        |        |        |        |        |        |        |  |  | TVSGVAGPLVILEK     | 67.66 | 2 | 691.9199.2  |
|                  |  |    |    |        |        |      |        |        |        |        |        |        |        |        |  |  | GYPGYMYTDLATIYER   | 71.39 | 2 | 956.9448.74 |
| 400004125        | <b>Peptidyl-prolyl cis-trans isomerase</b> | 5  | 4  | 55.04  | 0.0030 | 1.73 | 627.99 | 576.37 | 697.54 | 403.27 | 101.42 | 93.54  | 158.54 | 113.90 |  |  | CSKPVVVADCGQL      | 42.76 | 2 | 716.8549.09 |
|                  |  |    |    |        |        |      |        |        |        |        |        |        |        |        |  |  | VVMELFADTPK        | 62.47 | 2 | 675.8539.26 |
|                  |  |    |    |        |        |      |        |        |        |        |        |        |        |        |  |  | TEWLDGK            | 37.85 | 1 | 848.4188.23 |
|                  |  |    |    |        |        |      |        |        |        |        |        |        |        |        |  |  | VIPGFMCQGGDFTAGNG  | 63.91 | 2 | 1346.117.75 |
| 400033202        | <b>Uncharacterized protein</b>             | 10 | 6  | 120.89 | 0.0350 | 1.60 | 1963.4 | 1959.8 | 2305.8 | 1442.7 | 110.78 | 164.88 | 648.71 | 1148.8 |  |  | ASTEGDQVTLDTGVDSS  | 38.86 | 2 | 919.4289.84 |
|                  |  |    |    |        |        |      |        |        |        |        |        |        |        |        |  |  | GLTIFAPNDEAFK      | 64.69 | 1 | 1422.729.65 |
|                  |  |    |    |        |        |      |        |        |        |        |        |        |        |        |  |  | KDPISTLATNGASK     | 38.02 | 2 | 702.3667.24 |
|                  |  |    |    |        |        |      |        |        |        |        |        |        |        |        |  |  | LADEINSR           | 27.91 | 2 | 459.2547.33 |
|                  |  |    |    |        |        |      |        |        |        |        |        |        |        |        |  |  | TFASLLVK           | 57.15 | 1 | 878.5249.11 |
|                  |  |    |    |        |        |      |        |        |        |        |        |        |        |        |  |  | NAGNVNTPALLK       | 49.07 | 2 | 606.3176.34 |
| <b>Cluster 7</b> |  |    |    |        |        |      |        |        |        |        |        |        |        |        |  |  |                    |       |   |             |
| 400047481        | <b>Actin</b>                               | 15 | 3  | 151.29 | 0.0450 | 2.24 | 442.22 | 197.68 | 222.36 | 200.69 | 48.78  | 100.30 | 103.80 | 161.33 |  |  | NYELPDGQVITIGAER   | 62.20 | 2 | 887.9529.1  |
|                  |  |    |    |        |        |      |        |        |        |        |        |        |        |        |  |  | LAYVALDYEQELETAR   | 65.78 | 2 | 942.4778.41 |
|                  |  |    |    |        |        |      |        |        |        |        |        |        |        |        |  |  | DLTDSLMLK          | 48.22 | 1 | 922.4518.3  |
| 400058342        | <b>CASP-like protein</b>                   | 3  | 3  | 28.56  | 0.0003 | 2.07 | 758.80 | 679.45 | 365.74 | 424.13 | 128.18 | 93.72  | 38.47  | 193.81 |  |  | EICTLYGKFCR        | 45.82 | 1 | 1446.656.59 |
|                  |  |    |    |        |        |      |        |        |        |        |        |        |        |        |  |  | EICTLYGK           | 35.98 | 1 | 983.4948.03 |
|                  |  |    |    |        |        |      |        |        |        |        |        |        |        |        |  |  | GNTYVGWR           | 39.07 | 1 | 952.4717.35 |
| 400024318        | <b>Uncharacterized protein</b>             | 5  | 5  | 60.99  | 0.0003 | 1.87 | 1256.5 | 1121.1 | 822.83 | 672.18 | 100.42 | 254.58 | 154.89 | 198.51 |  |  | VLVPSEFTDPK        | 52.89 | 1 | 1231.668.99 |
|                  |  |    |    |        |        |      |        |        |        |        |        |        |        |        |  |  | FYTFDDLQTVSNPVR    | 64.66 | 2 | 901.4439.17 |
|                  |  |    |    |        |        |      |        |        |        |        |        |        |        |        |  |  | EGQYNTFIK          | 41.36 | 1 | 1099.558.8  |
|                  |  |    |    |        |        |      |        |        |        |        |        |        |        |        |  |  | NPPLAVYQLDK        | 54.16 | 2 | 629.8397.5  |
|                  |  |    |    |        |        |      |        |        |        |        |        |        |        |        |  |  | VQLILNHVIPK        | 56.16 | 2 | 637.3718.74 |
| 400062832        | <b>Uncharacterized protein</b>             | 4  | 3  | 31.47  | 0.0000 | 1.61 | 301.34 | 274.63 | 211.42 | 187.68 | 16.76  | 36.76  | 11.27  | 39.70  |  |  | LDVDSTPSAAEITK     | 43.92 | 2 | 723.8677.93 |
|                  |  |    |    |        |        |      |        |        |        |        |        |        |        |        |  |  | CDQFSADLFSTSSAPR   | 58.79 | 2 | 894.9017.9  |
|                  |  |    |    |        |        |      |        |        |        |        |        |        |        |        |  |  | FFASFNC DK         | 49.07 | 2 | 568.2667.6  |

|           |  |    |    |        |        |      |        |        |         |        |        |        |       |        |                    |       |   |             |
|-----------|--|----|----|--------|--------|------|--------|--------|---------|--------|--------|--------|-------|--------|--------------------|-------|---|-------------|
| 400022574 | <b>Alpha-tubulin</b>                       | 11 | 11 | 97.37  | 0.0000 | 1.64 | 1177.6 | 1080.1 | 1942.61 | 718.52 | 100.38 | 123.52 | 34.18 | 139.92 | EDLAALEK           | 37.32 | 1 | 888.4677.52 |
|           |  |    |    |        |        |      |        |        |         |        |        |        |       |        | GDVVPK             | 26.69 | 1 | 614.3356.87 |
|           |  |    |    |        |        |      |        |        |         |        |        |        |       |        | TVGGGDDAFNTFFSETG  | 67.42 | 2 | 989.4529.28 |
|           |  |    |    |        |        |      |        |        |         |        |        |        |       |        | CGINYQPPTVVPGGDLA  | 55.31 | 2 | 943.48 8.86 |
|           |  |    |    |        |        |      |        |        |         |        |        |        |       |        | DVNAAVATIK         | 38.93 | 1 | 1001.578.14 |
|           |  |    |    |        |        |      |        |        |         |        |        |        |       |        | EIVDLCLDR          | 54.30 | 2 | 566.7938.52 |
|           |  |    |    |        |        |      |        |        |         |        |        |        |       |        | LSVDYGKK           | 26.30 | 2 | 455.2498.35 |
|           |  |    |    |        |        |      |        |        |         |        |        |        |       |        | AVFVDLEPTVIDEVR    | 71.56 | 2 | 851.4539.04 |
|           |  |    |    |        |        |      |        |        |         |        |        |        |       |        | YMACCLMFR          | 48.26 | 2 | 634.3266.9  |
|           |  |    |    |        |        |      |        |        |         |        |        |        |       |        | AVCMISNSTSVAEVFSR  | 63.39 | 2 | 929.4538.99 |
|           |  |    |    |        |        |      |        |        |         |        |        |        |       |        | AYHEQLSVAEITNSAFEP | 48.16 | 3 | 1066.817.05 |
| 400070407 | <b>ATP synthase D chain</b>                | 3  | 2  | 21.35  | 0.0459 | 2.35 | 119.58 | 108.10 | 95.66   | 50.85  | 33.36  | 26.51  | 10.54 | 24.71  | AFDDVNSQLQTK       | 39.29 | 2 | 683.3447.3  |
|           |  |    |    |        |        |      |        |        |         |        |        |        |       |        | FSQEPEPINWEYYR     | 60.27 | 2 | 929.4437.25 |
| 400035497 | <b>Uncharacterized protein</b>             | 2  | 2  | 14.23  | 0.0107 | 1.70 | 191.01 | 174.87 | 163.75  | 112.44 | 32.86  | 31.17  | 13.13 | 52.85  | ELILR              | 37.71 | 1 | 643.3706.87 |
|           |  |    |    |        |        |      |        |        |         |        |        |        |       |        | IESLEILTAR         | 59.18 | 2 | 572.8327.36 |
| 400078507 | <b>ATP synthase subunit beta</b>           | 13 | 3  | 116.69 | 0.0413 | 1.65 | 454.61 | 395.14 | 362.88  | 275.60 | 103.46 | 55.43  | 65.22 | 129.06 | FTQANSEVSALLGR     | 58.62 | 2 | 746.8918.96 |
|           |  |    |    |        |        |      |        |        |         |        |        |        |       |        | ITDEFTGAGSVGK      | 39.75 | 2 | 641.3228.54 |
|           |  |    |    |        |        |      |        |        |         |        |        |        |       |        | ESINSFQGVLDGK      | 56.40 | 2 | 697.3557.91 |
| 400023732 | <b>Putative leucine-rich repeat</b>        | 5  | 4  | 38.41  | 0.0002 | 2.77 | 518.04 | 410.86 | 404.44  | 187.01 | 53.61  | 127.63 | 26.82 | 87.29  | VLYSSLNSPGQLTK     | 47.80 | 2 | 753.9097.96 |
|           |  |    |    |        |        |      |        |        |         |        |        |        |       |        | MVDPALEGLYPVK      | 62.47 | 2 | 716.3828.26 |
|           |  |    |    |        |        |      |        |        |         |        |        |        |       |        | TNEASSAPIVK        | 27.11 | 2 | 558.7987.81 |
|           |  |    |    |        |        |      |        |        |         |        |        |        |       |        | WNANGGDPCGESWTGIT  | 52.06 | 2 | 1198.497.1  |
| 400010174 | <b>Actin 1</b>                             | 15 | 2  | 148.33 | 0.0000 | 4.91 | 437.35 | 323.33 | 349.51  | 89.11  | 197.52 | 136.43 | 79.48 | 84.06  | DLTDNLMKILTER      | 56.89 | 2 | 781.4117.4  |
|           |  |    |    |        |        |      |        |        |         |        |        |        |       |        | EIVRDMK            | 55.91 | 1 | 890.4696.26 |
| 400058377 | <b>Pollen-specific lysine-rich protein</b> | 5  | 2  | 37.34  | 0.0422 | 7.42 | 109.21 | 58.58  | 22.99   | 14.72  | 49.52  | 64.03  | 21.68 | 13.08  | NETTTPVSVVENDETPV  | 57.91 | 2 | 1229.618.05 |
|           |  |    |    |        |        |      |        |        |         |        |        |        |       |        | KNENEETSPVSVVAVVE  | 51.84 | 2 | 979.5047.36 |
| 400039213 | <b>Probable inactive receptor kinase</b>   | 5  | 3  | 42.66  | 0.0098 | 2.18 | 329.80 | 229.00 | 220.49  | 151.08 | 42.69  | 16.32  | 45.98 | 100.00 | APNQASLGEEGIDLPR   | 51.80 | 2 | 833.9177.96 |
|           |  |    |    |        |        |      |        |        |         |        |        |        |       |        | LGSIPSDFSNLK       | 54.52 | 2 | 682.8557.31 |
|           |  |    |    |        |        |      |        |        |         |        |        |        |       |        | QDNQDACVSDYGLNPLF  | 62.44 | 3 | 969.1046.71 |
| 400073467 | <b>Phospholipase D</b>                     | 3  | 3  | 21.58  | 0.0183 | 2.00 | 191.20 | 137.73 | 140.75  | 95.82  | 22.15  | 38.77  | 29.41 | 48.86  | SIDGGAAFPGPDPEDAA  | 58.89 | 2 | 933.4377.76 |
|           |  |    |    |        |        |      |        |        |         |        |        |        |       |        | IIVVDSALPSGESEK    | 50.19 | 2 | 772.4086.96 |
|           |  |    |    |        |        |      |        |        |         |        |        |        |       |        | SGEYEPSEPEPDSNYMR  | 40.58 | 2 | 1037.436.86 |
| 400045607 | <b>Somatic embryogenesis receptor</b>      | 4  | 2  | 32.04  | 0.0177 | 1.97 | 135.69 | 99.89  | 98.77   | 68.94  | 37.20  | 26.79  | 16.29 | 21.79  | ELQVASDNFSNR       | 39.66 | 2 | 690.3408.1  |
|           |  |    |    |        |        |      |        |        |         |        |        |        |       |        | VLVYPYMENGSVASR    | 55.14 | 2 | 843.4227.13 |
| 400041634 | <b>14-3-3 protein</b>                      | 16 | 5  | 151.47 | 0.0015 | 2.35 | 334.10 | 229.59 | 235.00  | 141.95 | 38.14  | 65.84  | 23.95 | 88.93  | ALPENLTR           | 49.70 | 1 | 955.5166.51 |
|           |  |    |    |        |        |      |        |        |         |        |        |        |       |        | ILDQYLIPASAGESK    | 57.31 | 2 | 846.4438.44 |
|           |  |    |    |        |        |      |        |        |         |        |        |        |       |        | YLAEFKVGNER        | 45.39 | 2 | 663.3446.74 |
|           |  |    |    |        |        |      |        |        |         |        |        |        |       |        | LVIGSGSSELTVEER    | 59.52 | 2 | 788.4097.22 |
|           |  |    |    |        |        |      |        |        |         |        |        |        |       |        | AAQDIAVAELAPTHPIR  | 52.62 | 3 | 591.6588.36 |
| 400007182 | <b>Dihydroipoamide Dehydrogenase</b>       | 9  | 6  | 80.54  | 0.0390 | 2.24 | 263.46 | 171.51 | 157.67  | 117.57 | 40.42  | 28.37  | 40.85 | 51.65  | ALAENEGELAK        | 31.25 | 1 | 1201.617.98 |
|           |  |    |    |        |        |      |        |        |         |        |        |        |       |        | YGDTVITAK          | 31.39 | 1 | 967.5037.4  |
|           |  |    |    |        |        |      |        |        |         |        |        |        |       |        | APFTQGLGLENINVQTR  | 62.37 | 2 | 993.5167.85 |
|           |  |    |    |        |        |      |        |        |         |        |        |        |       |        | SNLTNSMK           | 29.35 | 1 | 894.4386.75 |
|           |  |    |    |        |        |      |        |        |         |        |        |        |       |        | ANTSSPVAEPVAA      | 37.68 | 1 | 1213.617.51 |
|           |  |    |    |        |        |      |        |        |         |        |        |        |       |        | VIDASGELVPHLYCIGDA | 62.62 | 3 | 743.3747.11 |

|           |  |    |    |        |        |      |        |        |        |        |        |        |        |        |                    |       |   |             |
|-----------|--|----|----|--------|--------|------|--------|--------|--------|--------|--------|--------|--------|--------|--------------------|-------|---|-------------|
| 400079310 | <b>60S ribosomal protein L13</b>       | 4  | 3  | 46.53  | 0.0398 | 4.18 | 89.86  | 27.34  | 55.33  | 21.47  | 12.74  | 20.43  | 35.02  | 23.81  | SLEGLQTNVQR        | 38.15 | 1 | 1244.668.38 |
|           |  |    |    |        |        |      |        |        |        |        |        |        |        |        | GFSLEELK           | 55.50 | 1 | 922.4907.52 |
|           |  |    |    |        |        |      |        |        |        |        |        |        |        |        | AGDSSAEELATATQVQG  | 62.33 | 2 | 1233.117.65 |
| 400012216 | <b>Cupredoxin superfamily protein</b>  | 14 | 10 | 126.32 | 0.0001 | 1.79 | 1894.8 | 1323.1 | 1562.1 | 1057.0 | 175.54 | 304.36 | 91.07  | 154.10 | ASGGFGGITINNR      | 46.65 | 2 | 632.3338.51 |
|           |  |    |    |        |        |      |        |        |        |        |        |        |        |        | VTGVGILK           | 41.78 | 2 | 393.7588.47 |
|           |  |    |    |        |        |      |        |        |        |        |        |        |        |        | DLGKPDGVLINGK      | 42.27 | 2 | 663.3568.27 |
|           |  |    |    |        |        |      |        |        |        |        |        |        |        |        | WDGIAR             | 33.03 | 1 | 717.3728.23 |
|           |  |    |    |        |        |      |        |        |        |        |        |        |        |        | TFSMNQARSIR        | 44.58 | 2 | 656.3356.51 |
|           |  |    |    |        |        |      |        |        |        |        |        |        |        |        | NKLDEELLHWSGVQQK   | 60.69 | 3 | 679.6947.74 |
|           |  |    |    |        |        |      |        |        |        |        |        |        |        |        | TELPMPDNTLFCGQLGK  | 68.15 | 2 | 960.9698.84 |
|           |  |    |    |        |        |      |        |        |        |        |        |        |        |        | TENLDSWFLGQETYIR   | 76.34 | 2 | 986.4829.24 |
|           |  |    |    |        |        |      |        |        |        |        |        |        |        |        | VTGVGILKYTNSK      | 56.13 | 2 | 690.3607.02 |
|           |  |    |    |        |        |      |        |        |        |        |        |        |        |        | STWQDGLPGTNCPIPPK  | 52.97 | 2 | 934.4597.61 |
| 400057418 | <b>Glycerophosphodiesterase-like</b>   | 15 | 12 | 166.17 | 0.0000 | 2.14 | 4149.2 | 2695.4 | 3060.8 | 1935.5 | 328.50 | 621.38 | 242.79 | 584.38 | AASDGDILGCPVQMTK   | 53.84 | 1 | 1733.699.44 |
|           |  |    |    |        |        |      |        |        |        |        |        |        |        |        | LQSANLPVYVR        | 50.06 | 1 | 1259.729.29 |
|           |  |    |    |        |        |      |        |        |        |        |        |        |        |        | DGVGICFPDIK        | 63.84 | 2 | 610.8058.78 |
|           |  |    |    |        |        |      |        |        |        |        |        |        |        |        | FLNEDDIEPSTSQTYGSL | 59.42 | 3 | 748.3768.86 |
|           |  |    |    |        |        |      |        |        |        |        |        |        |        |        | DLAPVSLK           | 44.12 | 1 | 842.4888.39 |
|           |  |    |    |        |        |      |        |        |        |        |        |        |        |        | SSNVTSK            | 26.93 | 1 | 723.3566.2  |
|           |  |    |    |        |        |      |        |        |        |        |        |        |        |        | TFAAGILVPK         | 57.70 | 1 | 1016.628.8  |
|           |  |    |    |        |        |      |        |        |        |        |        |        |        |        | SIASR              | 55.45 | 1 | 533.3016.75 |
|           |  |    |    |        |        |      |        |        |        |        |        |        |        |        | NSVFPSEGAFIGQTNVV  | 68.44 | 2 | 1068.068.85 |
|           |  |    |    |        |        |      |        |        |        |        |        |        |        |        | YSDYLSR            | 32.10 | 1 | 990.4748.68 |
|           |  |    |    |        |        |      |        |        |        |        |        |        |        |        | SVIANYISSPEVNFLR   | 73.36 | 2 | 904.9848.55 |
|           |  |    |    |        |        |      |        |        |        |        |        |        |        |        | SFVISLSR           | 55.41 | 1 | 908.4977.86 |
| 400008687 | <b>60S ribosomal protein L12</b>       | 4  | 2  | 29.32  | 0.0155 | 2.84 | 146.74 | 83.89  | 128.69 | 51.60  | 47.08  | 44.19  | 37.83  | 30.76  | VSVVPSAAALVIK      | 62.83 | 2 | 627.3848.82 |
|           |  |    |    |        |        |      |        |        |        |        |        |        |        |        | IGPLGLSPK          | 47.27 | 2 | 441.2707.16 |
| 400061841 | <b>Xyloglucan</b>                      | 3  | 3  | 22.52  | 0.0293 | 1.62 | 244.47 | 182.04 | 213.61 | 151.02 | 47.00  | 46.58  | 23.15  | 56.97  | TGEPYLIQTNVYK      | 52.36 | 2 | 763.3928.28 |
|           |  |    |    |        |        |      |        |        |        |        |        |        |        |        | KPEECWLNPNWE       | 65.84 | 2 | 744.3447.75 |
|           |  |    |    |        |        |      |        |        |        |        |        |        |        |        | NQPFVSSYK          | 35.35 | 1 | 1069.536.49 |
| 400036347 | <b>Uncharacterized protein</b>         | 2  | 2  | 25.25  | 0.0013 | 3.59 | 358.88 | 171.81 | 288.67 | 99.94  | 63.39  | 53.42  | 40.53  | 65.66  | GPCLNGPICK         | 35.22 | 1 | 1115.548.69 |
|           |  |    |    |        |        |      |        |        |        |        |        |        |        |        | GNDIFSGK           | 51.70 | 1 | 950.4957.87 |
| 400006894 | <b>Fructose-bisphosphate aldolase</b>  | 17 | 9  | 138.94 | 0.0001 | 2.53 | 737.55 | 457.71 | 667.29 | 291.34 | 70.50  | 135.01 | 50.13  | 123.33 | IGANEPSQLAINDNANGL | 48.82 | 2 | 1019.528.26 |
|           |  |    |    |        |        |      |        |        |        |        |        |        |        |        | YAGASNLSEGASESLHV  | 42.51 | 2 | 910.4478.04 |
|           |  |    |    |        |        |      |        |        |        |        |        |        |        |        | VDKGTVELPGTDGETTT  | 55.41 | 3 | 887.1048.1  |
|           |  |    |    |        |        |      |        |        |        |        |        |        |        |        | EAGVLPGIK          | 44.12 | 1 | 883.4397.67 |
|           |  |    |    |        |        |      |        |        |        |        |        |        |        |        | LSSINVENVESNRR     | 38.93 | 3 | 539.6177.97 |
|           |  |    |    |        |        |      |        |        |        |        |        |        |        |        | RLSSINVENVESNR     | 38.93 | 3 | 539.6177.22 |
|           |  |    |    |        |        |      |        |        |        |        |        |        |        |        | AQAFLTR            | 36.76 | 1 | 877.4727.55 |
|           |  |    |    |        |        |      |        |        |        |        |        |        |        |        | AWGGKEENVQK        | 22.59 | 2 | 623.3108.31 |
|           |  |    |    |        |        |      |        |        |        |        |        |        |        |        | EAGVLPGIKVDK       | 45.17 | 2 | 613.3137.49 |
|           |  |    |    |        |        |      |        |        |        |        |        |        |        |        | NAAYIGTPGK         | 29.18 | 1 | 991.5258    |
| 400010366 | <b>V-type proton ATPase subunit E2</b> | 5  | 2  | 57.96  | 0.0313 | 3.05 | 217.74 | 147.70 | 198.06 | 71.48  | 30.44  | 40.11  | 22.30  | 33.89  | ISGEKNYK           | 44.41 | 1 | 1053.536.79 |
|           |  |    |    |        |        |      |        |        |        |        |        |        |        |        | QIQQMVR            | 28.72 | 1 | 902.4928.64 |
| 400013297 | <b>Uncharacterized protein</b>         | 14 | 9  | 101.34 | 0.0000 | 1.74 | 1198.9 | 951.86 | 1108.7 | 690.29 | 155.72 | 153.34 | 84.40  | 95.63  | SDVILLK            | 45.53 | 1 | 787.4968.13 |





**Appendix Table 4.** The abundance of proteins responsive to the 24 h and 72 h treatment, Supplementary Table S1, chapter IV

| Average protein abundance |         |            |                  |             |                 |  |           |           |           |           |       |        |        |       | Normalized peptide abundance average of 3 biological replicates and 2 technical |          |             |            |               |              |
|---------------------------|---------|------------|------------------|-------------|-----------------|--|-----------|-----------|-----------|-----------|-------|--------|--------|-------|---|----------|-------------|------------|---------------|--------------|
| Accession                 | Peptide | Uniqueness | Confidence score | Anova (p)   | Max fold change | Description Blast NCBI   | Lam C     | Lam S     | Top C     | Top S     | RT    | Charge | m/z    | Score | Sequence  | Anova    | Lam control | Lam stress | Topas control | Topas stress |
| Lambada 24hours           |         |            |                  |             |                 |  |           |           |           |           |       |        |        |       |   |          |             |            |               |              |
| 400001303                 | 5       | 5          | 429.4            | 0           | 2.42            | <b>Formate dehydrogenase</b>                                       | 31499507  | 47365275  | 48626894  | 55235151  | 43.3  | 2      | 413.25 | 24.0  | IVGVFYK   | 0.00E+00 | 4360846     | 6838810    | 7702899       | 8429403      |
|                           |         |            |                  |             |                 |  |           |           |           |           | 33.9  | 4      | 554.51 | 53.7  | GHQYIVTPDKEGPDCELEK   | 8.39E-08 | 5293875     | 6799891    | 9436196       | 11234246     |
|                           |         |            |                  |             |                 |  |           |           |           |           | 63.5  | 2      | 751.40 | 49.2  | CDIVVINTPLTEK   | 0.00E+00 | 3049768     | 5261440    | 4554751       | 5154913      |
|                           |         |            |                  |             |                 |  |           |           |           |           | 84.5  | 3      | 818.71 | 61.4  | ANEYAEMNPNFLGCAENALGIR  | 3.06E-10 | 3332860     | 4640393    | 6289071       | 8124626      |
|                           |         |            |                  |             |                 |  |           |           |           |           | 80.4  | 3      | 878.44 | 74.8  | AAAAAGLTVAEVTGSNTVSVAEDEL<br>MR   | 1.42E-04 | 7639149     | 9251378    | 9642682       | 10832204     |
| 400002454                 | 12      | 12         | 749.6            | 0           | 2.62            | <b>2,3-bisphosphoglycerate-independent phosphoglycerate mutase</b> | 207424891 | 311242094 | 359263893 | 378428829 | 49.3  | 2      | 493.80 | 33.4  | LVDLALASGK  | 0.00E+00 | 31472302    | 42292079   | 50281049      | 51321323     |
|                           |         |            |                  |             |                 |  |           |           |           |           | 44.1  | 2      | 584.27 | 23.5  | YENDWDVVK   | 3.41E-12 | 22915691    | 30009056   | 32451060      | 32430402     |
|                           |         |            |                  |             |                 |  |           |           |           |           | 51.6  | 2      | 594.82 | 35.0  | YLVSPPEIDR  | 0.00E+00 | 30123151    | 45004023   | 47930663      | 48746770     |
|                           |         |            |                  |             |                 |  |           |           |           |           | 80.3  | 2      | 534.85 | 43.1  | LDQLLLLLK   | 0.00E+00 | 18088419    | 31522293   | 38909690      | 41568337     |
|                           |         |            |                  |             |                 |  |           |           |           |           | 41.7  | 3      | 566.28 | 24.9  | VNIPNSDMVGHTGDIK  | 2.26E-08 | 15065236    | 23872252   | 27189019      | 30334677     |
|                           |         |            |                  |             |                 |  |           |           |           |           | 51.0  | 3      | 516.57 | 30.7  | ALEYEDFDKFDR  | 7.22E-15 | 15625510    | 21161029   | 26583461      | 28518776     |
|                           |         |            |                  |             |                 |  |           |           |           |           | 100.5 | 2      | 1070.5 | 100.2 | DVLDGSSVGFMETLENDLAK  | 3.47E-13 | 18863516    | 29888721   | 32576461      | 36315803     |
|                           |         |            |                  |             |                 |  |           |           |           |           | 47.8  | 4      | 430.24 | 42.4  | GTLHLGLVSDGGVHSR  | 1.90E-13 | 8678736     | 11878070   | 19412156      | 19368948     |
|                           |         |            |                  |             |                 |  |           |           |           |           | 65.8  | 3      | 755.72 | 45.0  | LEEYVEIPSDSGITFNVKPK  | 0.00E+00 | 10977068    | 14406679   | 17166425      | 18989596     |
|                           |         |            |                  |             |                 |  |           |           |           |           | 88.0  | 3      | 978.56 | 74.4  | NGNIQILTSHLEPVPIAIGGPGLLPGV<br>R  | 8.08E-13 | 9842987     | 16617702   | 16740813      | 16157482     |
|                           |         |            |                  |             |                 |  |           |           |           |           | 42.8  | 3      | 916.09 | 56.4  | AHGNAVGLPTEDDMGNSEVGHNALG<br>AGR  | 2.67E-06 | 5455629     | 9250051    | 7556067       | 7296068      |
|                           |         |            |                  |             |                 |  |           |           |           |           | 102.8 | 3      | 967.49 | 38.8  | MILEAIEQVGGIFLVTADHGNAEDMV<br>K   | 6.83E-05 | 5217579     | 8966892    | 9114895       | 10884729     |
| 400006337                 | 2       | 2          | 94.08            | 5.16254E-14 | 4.89            | <b>Alpha-1,4 glucan phosphorylase L-1 isozyme</b>                  | 730309    | 1343326   | 1762904   | 2757333   | 103.3 | 2      | 636.83 | 35.6  | DIWNIEAVEIA   | 1.73E-05 | 418867      | 708534     | 1136794       | 1736264      |
|                           |         |            |                  |             |                 |  |           |           |           |           | 87.3  | 2      | 1263.0 | 58.4  | SGAFGSYNYDDLIGSLEGNEGFR   | 2.00E-15 | 311441      | 634792     | 626111        | 1021070      |
| 400009804                 | 3       | 3          | 149.06           | 5.32917E-11 | 3.09            | <b>Elongation factor Tu</b>  | 5162096   | 9400349   | 11114644  | 12110452  | 37.4  | 3      | 586.30 | 25.8  | SGAFGSYNYDDLIGSLEGNEGFR   | 2.00E-15 | 1187670     | 2116885    | 3373978       | 4088596      |
|                           |         |            |                  |             |                 |  |           |           |           |           | 76.6  | 2      | 730.89 | 23.3  | GSALSALQGTNNEIGKK   | 2.41E-08 | 2574205     | 4014333    | 4468022       | 4412593      |
|                           |         |            |                  |             |                 |  |           |           |           |           | 40.3  | 2      | 802.42 | 59.1  | GITIAAHVEYETAK  | 4.74E-05 | 684176      | 1817083    | 1861544       | 1838314      |
| 400010497                 | 7       | 7          | 311.18           | 0           | 2.21            | <b>Plasma membrane ATPase 4</b>                                    | 15190926  | 25090175  | 29966463  | 33615138  | 26.3  | 4      | 435.22 | ---   | AAHLVDSTNNVGHFQK  | 1.10E-05 | 1254740     | 1860427    | 3050612       | 3166410      |
|                           |         |            |                  |             |                 |  |           |           |           |           | 56.9  | 2      | 467.78 | 23.2  | TLVEVFVK  | 0.00E+00 | 1951660     | 2356118    | 3609408       | 3975891      |
|                           |         |            |                  |             |                 |  |           |           |           |           | 53.3  | 2      | 779.92 | 71.4  | IDQSALTGESLPVTK   | 1.74E-13 | 2624835     | 4385531    | 5049926       | 6196564      |
|                           |         |            |                  |             |                 |  |           |           |           |           | 85.4  | 3      | 668.36 | 36.7  | QGELEAVVIATGVHTFFGK   | 1.72E-08 | 3507123     | 4984287    | 6602766       | 7147050      |







|           |    |    |        |                 |      |   |           |           |           |           |       |   |                     |      |                                  |          |          |          |          |          |
|-----------|----|----|--------|-----------------|------|---|-----------|-----------|-----------|-----------|-------|---|---------------------|------|----------------------------------|----------|----------|----------|----------|----------|
| 400063205 | 3  | 3  | 135.36 | 1.87149<br>E-08 | 2.42 | <b>Betaine aldehyde<br/>dehydrogenase, chloroplastic-<br/>like</b>                              | 4836214   | 9704967   | 7367593   | 10242446  | 76.3  | 2 | 806.97              | 41.9 | LIQETIAPQFLAR                    | 1.04E-08 | 2275735  | 4680398  | 3833265  | 5467767  |
|           |    |    |        |                 |      |   |           |           |           |           | 70.2  | 2 | 986.47              | 39.4 | QVTQYVTPDEPWAFYK                 | 2.97E-04 | 1777435  | 3143883  | 2223638  | 2989226  |
|           |    |    |        |                 |      |   |           |           |           |           | 96.3  | 2 | 1504.2<br>9         | 54.0 | LPIINPANEIIGYIPAATEEDVDIAVK      | 3.01E-13 | 783043   | 1880685  | 1310690  | 1785453  |
| 400063697 | 2  | 2  | 73.17  | 9.16145<br>E-12 | 4.47 | <b>40S ribosomal protein S10-1-<br/>like</b>  | 8244007   | 13903742  | 19223805  | 22939353  | 78.1  | 2 | 829.97              | 53.4 | TYLNLPSIIVPATLK                  | 1.18E-10 | 5316760  | 9934920  | 12997049 | 15483135 |
|           |    |    |        |                 |      |   |           |           |           |           | 64.8  | 3 | 495.96              | 19.8 | HPLIDVPNLQVIK                    | 4.48E-12 | 2927247  | 3968822  | 6226756  | 7456219  |
| 400066574 | 3  | 3  | 187.5  | 0               | 4.48 | <b>Scopoletin glucosyltransferase</b>   | 10552793  | 22782238  | 19331980  | 27002155  | 67.9  | 2 | 810.94              | 47.7 | ATIITPLNESVFSK                   | 0.00E+00 | 5143041  | 11698376 | 9514134  | 13143031 |
|           |    |    |        |                 |      |   |           |           |           |           | 63.5  | 2 | 839.87              | 48.4 | TGNEDWLPEGFEER                   | 0.00E+00 | 3483643  | 7663922  | 6517596  | 9798702  |
|           |    |    |        |                 |      |   |           |           |           |           | 107.9 | 2 | 1494.1<br>9         | 91.5 | EAIEEGGSSYNGWATLLQDITSYTSTG<br>H | 1.03E-02 | 1926109  | 3419940  | 3300249  | 4060422  |
| 400072411 | 6  | 4  | 311.74 | 4.1831E-<br>12  | 2.77 | <b>Pyruvate kinase 1, cytosolic</b>   | 17941343  | 30814355  | 31425556  | 38560787  | 55.3  | 2 | 660.86              | 75.3 | LGDLSQTQIFAK                     | 5.75E-10 | 6160432  | 11067462 | 11403461 | 14197826 |
|           |    |    |        |                 |      |   |           |           |           |           | 52.6  | 2 | 587.30              | 28.9 | VFNQDLYFK                        | 2.49E-11 | 6584028  | 10239529 | 10820149 | 13432180 |
|           |    |    |        |                 |      |   |           |           |           |           | 43.3  | 2 | 630.83              | 35.5 | EVISTWGVQNK                      | 5.58E-05 | 3798366  | 5548289  | 5496063  | 6215739  |
|           |    |    |        |                 |      |   |           |           |           |           | 67.5  | 3 | 543.32              | 40.3 | YRPTMPVLSVVIPR                   | 1.38E-14 | 1398518  | 3959074  | 3705883  | 4715042  |
| 400080580 | 5  | 3  | 188.9  | 0               | 3.68 | <b>Heat shock 70 kDa protein 15-<br/>like</b>   | 6202016   | 10162830  | 14182016  | 16131840  | 55.3  | 2 | 1156.1<br>0         | 46.3 | ALAEAGLTTEINIHAVEVVGSSSR         | 1.73E-02 | 408231   | 994747   | 832483   | 1023369  |
|           |    |    |        |                 |      |   |           |           |           |           | 53.0  | 2 | 714.87              | 29.9 | ISTYITIGPFQSSK                   | 1.01E-08 | 1747729  | 1925595  | 2697258  | 2061329  |
|           |    |    |        |                 |      |   |           |           |           |           | 90.4  | 4 | 635.32              | 21.3 | NLNAAVVDCCIGIPVYFTDLQR           | 1.36E-08 | 1057034  | 1626052  | 2909993  | 3915724  |
| 400080588 | 8  | 6  | 476.09 | 0               | 2.90 | <b>Heat shock 70 kDa protein 15-<br/>like</b>   | 25744908  | 42716769  | 55640809  | 64079608  | 59.1  | 2 | 1163.1<br>0         | 52.5 | ALAEAGLTTEINIHAVEVIGSSSR         | 1.26E-12 | 754071   | 1573731  | 1512868  | 1735109  |
|           |    |    |        |                 |      |   |           |           |           |           | 62.4  | 2 | 738.89              | 71.2 | GAALQCAILSPTFK                   | 1.69E-13 | 8931717  | 12812673 | 15490469 | 17146333 |
|           |    |    |        |                 |      |   |           |           |           |           | 91.7  | 3 | 545.62              | 22.2 | VNESFPPIALSWK                    | 5.77E-04 | 367441   | 477641   | 872382   | 1096735  |
|           |    |    |        |                 |      |   |           |           |           |           | 46.7  | 3 | 764.38              | 30.0 | GPAPDAQNGALENHQSTIVFPK           | 0.00E+00 | 2518788  | 4231128  | 5620472  | 5932792  |
|           |    |    |        |                 |      |   |           |           |           |           | 96.1  | 4 | 645.83              | 64.6 | NLNAVVDCCIGIPIYFTDLQR            | 2.04E-10 | 1725744  | 2493585  | 5525310  | 7210548  |
|           |    |    |        |                 |      |   |           |           |           |           | 33.0  | 3 | 863.07              | 24.8 | GAAAASGAENGVPESGDKPVQMETD<br>AK  | 7.84E-09 | 1505215  | 3252061  | 3889917  | 4556968  |
| 400083137 | 4  | 4  | 134.31 | 0               | 4.01 | <b>Putative delta-1-pyrroline-5-<br/>carboxylate dehydrogenase<br/>12A1, mitochondrial-like</b> | 6672580   | 11145730  | 17427465  | 20962133  | 34.9  | 3 | 660.33              | 26.1 | AEEVSGSQPAEVHNLVQGK              | 2.79E-11 | 1129543  | 2498764  | 3550179  | 4690373  |
|           |    |    |        |                 |      |   |           |           |           |           | 37.8  | 3 | 603.62              | 22.9 | TTGAPQNHWFPGAGDPR                | 4.57E-13 | 1423182  | 2647149  | 3841631  | 4549500  |
|           |    |    |        |                 |      |   |           |           |           |           | 85.7  | 2 | 734.40              | 32.1 | DNQLPLVLDALEK                    | 0.00E+00 | 1089247  | 1472979  | 4391544  | 5465404  |
|           |    |    |        |                 |      |   |           |           |           |           | 78.6  | 2 | 778.90              | 37.0 | EIFGPFQVVTEYK                    | 0.00E+00 | 2576006  | 3561876  | 4715245  | 5327438  |
| 400084201 | 14 | 13 | 689.04 | 5.66214<br>E-15 | 2.89 | <b>NADP-dependent malic enzyme</b>  | 126226802 | 199033786 | 199654601 | 264744542 | 39.5  | 3 | 473.26              | 28.2 | RPQGLYISLNEK                     | 8.48E-07 | 8304166  | 14595664 | 14075537 | 19966975 |
|           |    |    |        |                 |      |   |           |           |           |           | 35.1  | 3 | 486.29              | 22.4 | AVKPTVLIGTSGTGR                  | 1.96E-04 | 8295426  | 14052282 | 10962234 | 14138439 |
|           |    |    |        |                 |      |   |           |           |           |           | 51.7  | 2 | 658.36<br>44.3<br>4 |      | SIQVIVTDGER                      | 2.84E-08 | 15811522 | 24750623 | 20527656 | 27713617 |
|           |    |    |        |                 |      |   |           |           |           |           | 87.5  | 3 | 712.66<br>35.1<br>8 |      | ATGQEYYDFLHEFMSAVK               | 3.46E-13 | 15531952 | 25515104 | 28180668 | 35922330 |
|           |    |    |        |                 |      |   |           |           |           |           | 84.0  | 3 | 740.39<br>21.4<br>4 |      | ILVQFEDFANHNAFELLAK              | 4.77E-15 | 20671904 | 29614317 | 30739209 | 41174849 |
|           |    |    |        |                 |      |   |           |           |           |           | 71.5  | 2 | 662.86<br>66.4<br>4 |      | LLNDEFYIGLK                      | 0.00E+00 | 8051456  | 9856189  | 13457232 | 19070593 |
|           |    |    |        |                 |      |   |           |           |           |           | 27.2  | 2 | 569.83<br>37.7<br>7 |      | ISAHIAASVAAK                     | 1.33E-02 | 935644   | 2734659  | 1245198  | 1600035  |



|                         |    |   |        |          |      |  |          |          |          |          |       |   |        |      |                             |          |          |          |          |          |
|-------------------------|----|---|--------|----------|------|--|----------|----------|----------|----------|-------|---|--------|------|-----------------------------|----------|----------|----------|----------|----------|
| 400054431               | 5  | 5 | 239.11 | 7.46E-05 | 1.75 | <b>Carboxylesterase 15</b>                                   | 16767676 | 25824839 | 19853723 | 26402068 | 91.4  | 2 | 880.96 | 56.6 | LPAACDASFAGLLWLR            | 6.22E-04 | 8022277  | 12638803 | 8430339  | 10867468 |
|                         |    |   |        |          |      |  |          |          |          |          | 49.6  | 2 | 767.38 | 63.7 | IAVDMDPVTSATEK              | 1.49E-02 | 3292000  | 4336502  | 4096309  | 5011017  |
|                         |    |   |        |          |      |  |          |          |          |          | 83.9  | 2 | 647.86 | 58.6 | LFEAIAEFINK                 | 6.55E-04 | 2404866  | 3874001  | 2634951  | 4151822  |
|                         |    |   |        |          |      |  |          |          |          |          | 54.5  | 3 | 460.59 | 16.2 | LAGAIPHPGFMR                | 1.11E-02 | 1245874  | 2084399  | 1919524  | 2391473  |
|                         |    |   |        |          |      |  |          |          |          |          | 86.7  | 3 | 615.64 | 44.0 | DLIKDTEMEFYEALK             | 5.47E-03 | 1534029  | 2400076  | 2272080  | 3321667  |
| 400047047               | 4  | 4 | 139.36 | 5.64E-12 | 1.89 | <b>Glutathione S-transferase zeta class-like</b>             | 28712765 | 44222846 | 40844401 | 54127265 | 41.2  | 3 | 598.64 | 38.0 | VGPNETTPWVQSHITK            | 1.41E-11 | 8669806  | 15877322 | 13633873 | 20417475 |
|                         |    |   |        |          |      |  |          |          |          |          | 83.7  | 2 | 805.41 | 49.1 | FEVDMNQFPTLLR               | 6.30E-03 | 5945946  | 9991606  | 7702184  | 9599734  |
|                         |    |   |        |          |      |  |          |          |          |          | 59.1  | 2 | 564.79 | 29.2 | LQLYSYWR                    | 1.94E-05 | 9439249  | 11037331 | 11839190 | 13553047 |
|                         |    |   |        |          |      |  |          |          |          |          | 85.5  | 4 | 635.07 | 23.0 | YATGDEVYMDLFLAPQIHAAIK      | 1.94E-02 | 2244933  | 3135922  | 3937094  | 5636852  |
| 400029318               | 4  | 4 | 193.3  | 0        | 2.48 | <b>Caffeic acid 3-O-methyltransferase-like</b>               | 11807916 | 18300429 | 20053406 | 21488999 | 65.3  | 2 | 671.39 | 40.9 | TLVDVGGGLGVNLIK             | 2.00E-15 | 4199396  | 7241689  | 7865177  | 8620081  |
|                         |    |   |        |          |      |  |          |          |          |          | 57.0  | 3 | 533.61 | 38.8 | LSALEIVSHMPNCK              | 6.77E-14 | 3232088  | 4757134  | 5979223  | 6214526  |
|                         |    |   |        |          |      |  |          |          |          |          | 26.1  | 2 | 642.33 | 36.6 | VHGVHAFEYPK                 | 9.15E-03 | 3244806  | 4585741  | 3740298  | 3768309  |
|                         |    |   |        |          |      |  |          |          |          |          | 98.1  | 3 | 591.63 | 77.1 | DEGASMGPLLALLQDK            | 6.89E-06 | 862614   | 1248613  | 1773543  | 1976096  |
| 400078045               | 3  | 3 | 100.37 | 2.65E-12 | 1.76 | <b>Probable pyridoxal 5'-phosphate synthase subunit PDX1</b> | 36588375 | 57359222 | 43376028 | 55500633 | 32.9  | 3 | 505.94 | 42.6 | TKGEAGTGNIEAVR              | 5.78E-04 | 10049131 | 16282130 | 13147084 | 16740594 |
|                         |    |   |        |          |      |  |          |          |          |          | 42.6  | 2 | 504.74 | 17.5 | IPFVCGCR                    | 1.41E-05 | 12182603 | 18952373 | 12269185 | 17242682 |
|                         |    |   |        |          |      |  |          |          |          |          | 60.4  | 2 | 703.87 | 40.3 | IQAPYDLVMQTK                | 3.08E-09 | 12060962 | 17970872 | 15492297 | 18349263 |
| 400058377               | 2  | 2 | 85.51  | 0        | 3.36 | <b>Nucleolar protein 58-like</b>                             | 4006143  | 2553074  | 1512847  | 1395163  | 32.0  | 2 | 708.38 | 21.7 | TEETIKPIEVK                 | 2.63E-13 | 1382209  | 918838   | 616441   | 544821   |
|                         |    |   |        |          |      |  |          |          |          |          | 63.5  | 2 | 1229.6 | 63.8 | NETTTPVSVVENDETTPVAVVEK     | 0.00E+00 | 2623934  | 1634236  | 896406   | 850342   |
|                         |    |   |        |          |      |  |          |          |          |          |       |   |        |      |                             |          |          |          |          |          |
| 400001180               | 10 | 2 | 886.46 | 0        | 3.03 | <b>Heat shock cognate 70 kda protein</b>                     | 3007834  | 4768293  | 6482631  | 8441543  | 33.5  | 2 | 691.87 | 31.2 | SGVHDVVVLVGSTR              | 1.66E-02 | 542475   | 1015648  | 1072577  | 1320743  |
|                         |    |   |        |          |      |  |          |          |          |          | 50.1  | 2 | 686.86 | 45.2 | ELESVCNPIIAK                | 2.53E-14 | 1103293  | 1435011  | 2146236  | 2713474  |
| 400032252               | 3  | 3 | 163.86 | 8.78E-12 | 2.96 | <b>Protein DJ-1 homolog B-like</b>                           | 6766298  | 11596897 | 11181327 | 14494747 | 75.6  | 3 | 704.72 | 22.0 | ANAQVVVASLEDKLEIVASR        | 3.15E-06 | 2087845  | 3522601  | 3032067  | 3874327  |
|                         |    |   |        |          |      |  |          |          |          |          | 109.4 | 2 | 1005.5 | 78.4 | GPATSIEY AVALVEELYGK        | 1.79E-07 | 2843367  | 4748152  | 4162006  | 5537671  |
|                         |    |   |        |          |      |  |          |          |          |          | 107.8 | 3 | 799.43 | 18.9 | ILVPIANGSEEMEATIHDVLR       | 3.65E-03 | 414675   | 659623   | 923058   | 1083774  |
| 400066954               | 2  | 2 | 83.84  | 6.49E-07 | 1.74 | <b>T-complex protein 1 subunit alpha</b>                     | 6532229  | 3803909  | 4756295  | 3751764  | 106.6 | 2 | 935.54 | 44.6 | EQLAIAQFAESLLVIPK           | 4.55E-10 | 5160166  | 2348993  | 2695586  | 1846537  |
|                         |    |   |        |          |      |  |          |          |          |          | 100.0 | 2 | 908.02 | 39.3 | EVGDGTTSVVIAAELLK           | 4.34E-03 | 909822   | 1018316  | 1171839  | 1068530  |
| 400054791               | 5  | 4 | 211.97 | 3.46E-12 | 2.22 | <b>Phosphoenolpyruvate carboxylase</b>                       | 3661383  | 7164796  | 5616747  | 7468378  | 47.1  | 2 | 656.35 | 44.2 | LDDLEAAPAAVAR               | 3.14E-05 | 1466388  | 2372714  | 1809456  | 2573837  |
|                         |    |   |        |          |      |  |          |          |          |          | 91.0  | 2 | 682.88 | 44.6 | VTIDL VEMVFAK               | 1.35E-09 | 1055667  | 2098035  | 2175329  | 2404883  |
|                         |    |   |        |          |      |  |          |          |          |          | 102.2 | 2 | 888.98 | 33.2 | LLVSEDLWSFGELLR             | 1.02E-06 | 901991   | 2186341  | 1438098  | 2156245  |
|                         |    |   |        |          |      |  |          |          |          |          | 97.2  | 2 | 1439.2 | 44.4 | SAAEVLTLNPTSEYAPGLEDTLILTMK | 9.67E-04 | 237337   | 507706   | 193863   | 333413   |
|                         |    |   |        |          |      |  |          |          |          |          |       |   |        |      |                             |          |          |          |          |          |
| Both cultivars 72 hours |    |   |        |          |      |  |          |          |          |          |       |   |        |      |                             |          |          |          |          |          |
| 400047644               | 2  | 2 | 45.98  | 2.97E-11 | 3.13 | <b>Protein yippe-like At4g27740</b>                          | 3011912  | 6736304  | 4635272  | 9235747  | 39.0  | 2 | 542.80 | 25.2 | ICQNPIALR                   | 7.71E-05 | 1720615  | 3609805  | 2255823  | 5030393  |
|                         |    |   |        |          |      |  |          |          |          |          | 62.5  | 2 | 538.80 | 20.8 | EGGFHIEIAK                  | 7.51E-08 | 1291297  | 3126499  | 2379448  | 4205354  |
| 400066574               | 3  | 3 | 187.5  | 0        | 4.48 | <b>Scopoletin glucosyltransferase</b>                        | 7997499  | 15402487 | 19054821 | 29279415 | 67.9  | 2 | 810.94 | 47.7 | ATIHTPLNESVFSK              | 0.00E+00 | 3814797  | 7884089  | 9498230  | 14678428 |
|                         |    |   |        |          |      |  |          |          |          |          | 63.5  | 2 | 839.87 | 48.4 | TGNEDWLPFGFEER              | 0.00E+00 | 2468173  | 5348641  | 6874154  | 10650962 |
|                         |    |   |        |          |      |  |          |          |          |          | 107.9 | 2 | 1494.1 | 91.5 | EAIIEGGSSYNGWATLLQDITSYTSTG | 1.03E-02 | 1714529  | 2169757  | 2682437  | 3950025  |
|                         |    |   |        |          |      |  |          |          |          |          |       |   |        |      |                             |          |          |          |          |          |



|                |   |   |        |          |      |   |           |           |           |          |       |   |        |       |                           |          |          |          |          |          |
|----------------|---|---|--------|----------|------|---|-----------|-----------|-----------|----------|-------|---|--------|-------|---------------------------|----------|----------|----------|----------|----------|
| 400001674      | 2 | 2 | 103.16 | 1.90E-09 | 2.35 | <b>Signal recognition particle 54 kda protein 2</b>       | 6520794   | 4061416   | 5587683   | 3134629  | 53.0  | 2 | 566.82 | 54.4  | ALLQADVQFK                | 3.68E-07 | 3187610  | 1734309  | 2844682  | 1359633  |
|                |   |   |        |          |      |   |           |           |           |          | 50.4  | 2 | 593.36 | 48.8  | QSVAVGAVIITK              | 1.61E-09 | 3333184  | 2327107  | 2743001  | 1774996  |
| 400007287      | 5 | 5 | 375.42 | 8.03E-11 | 2.84 | <b>Universal stress protein A-like protein isoform X2</b> | 79292154  | 130858870 | 50832028  | 82636565 | 28.7  | 2 | 816.40 | 81.4  | AFLGSVSNHCAQNVK           | 7.52E-05 | 6523589  | 11715474 | 3509977  | 5516241  |
|                |   |   |        |          |      |   |           |           |           |          | 35.6  | 2 | 715.82 | 56.7  | YSSEVAQCVM EK             | 6.50E-05 | 13010737 | 19846126 | 6517006  | 9673550  |
|                |   |   |        |          |      |   |           |           |           |          | 96.7  | 2 | 1255.6 | 122.1 | AVYSTLDGTGYLFSSDILATMER   | 8.79E-05 | 14112323 | 24322521 | 12426829 | 19502100 |
|                |   |   |        |          |      |   |           |           |           |          | 27.7  | 2 | 723.82 | 38.0  | YSSEVAQCVM EK             | 1.09E-14 | 13122791 | 22262530 | 5506702  | 12158131 |
| 400030676      | 6 | 6 | 364.58 | 0        | 2.66 | <b>Hyoscyamine 6-dioxygenase-like</b>                     | 182379357 | 119864572 | 141519537 | 84764201 | 86.3  | 2 | 1263.6 | 38.6  | AVYSTLDGTGYLFSSDILATMER   | 3.21E-02 | 1580261  | 1716167  | 1117053  | 1142940  |
|                |   |   |        |          |      |   |           |           |           |          | 47.2  | 2 | 648.85 | 51.1  | ALVGPENPPQFK              | 2.03E-13 | 40942081 | 24943904 | 32866826 | 16181019 |
|                |   |   |        |          |      |   |           |           |           |          | 58.7  | 2 | 942.97 | 69.6  | TSIGTFICPHDIVEPAK         | 1.09E-04 | 12140988 | 9356411  | 8987137  | 7560068  |
|                |   |   |        |          |      |   |           |           |           |          | 39.1  | 3 | 426.56 | 18.5  | SVYHASLEPFK               | 5.10E-03 | 21909311 | 13969224 | 18345684 | 10375949 |
|                |   |   |        |          |      |   |           |           |           |          | 59.9  | 2 | 569.79 | 39.4  | EFFSLPAAEK                | 0.00E+00 | 25817837 | 18375566 | 14267154 | 10193097 |
|                |   |   |        |          |      |   |           |           |           |          | 98.9  | 3 | 967.46 | 71.8  | AFEEYGFFQIINHGVVPVDMDEAMK | 7.61E-03 | 21813994 | 13650231 | 21721703 | 11936350 |
|                |   |   |        |          |      |   |           |           |           |          | 71.3  | 2 | 908.50 | 86.1  | NAFVVNSGLPITVVSNGK        | 0.00E+00 | 13898213 | 8533578  | 6024171  | 3035894  |
| 400041150      | 2 | 2 | 51.67  | 8.09E-10 | 2.71 | <b>Probable polyamine oxidase 2</b>                       | 986214    | 1500780   | 1201494   | 2253012  | 78.5  | 2 | 940.53 | 22.3  | AEASPSVIVIGGGIAGLAAAR     | 2.81E-05 | 591855   | 1009865  | 721960   | 1265509  |
|                |   |   |        |          |      |   |           |           |           |          | 107.2 | 2 | 1373.2 | 29.4  | YGEVDLFQPVMGEDTPVAVPLLISR | 1.25E-02 | 223424   | 241288   | 285045   | 405650   |
| Topas 72 hours |   |   |        |          |      |   |           |           |           |          |       |   |        |       |                           |          |          |          |          |          |
| 400067456      | 3 | 3 | 213.93 | 8.21E-12 | 2.25 | <b>Expansin-like B1</b>                                   | 18837468  | 26234183  | 16428369  | 25595026 | 85.2  | 2 | 799.91 | 73.6  | WVQLADVLPDEWK             | 5.55E-16 | 8455617  | 11312041 | 6402149  | 10551549 |
|                |   |   |        |          |      |   |           |           |           |          | 75.2  | 3 | 911.76 | 22.7  | AYSDMATSGMANHLLAYGVVDVEYR | 1.21E-03 | 5382016  | 7058747  | 5737143  | 7372881  |
|                |   |   |        |          |      |   |           |           |           |          | 55.8  | 3 | 865.02 | 60.5  | ATYYGSPDCYGTSPGACGFGEYGR  | 3.24E-03 | 2723918  | 4142525  | 2750763  | 4447753  |
| 400075261      | 3 | 3 | 132.29 | 7.18E-12 | 2.17 | <b>Uncharacterized protein</b>                            | 13843105  | 11919220  | 24069407  | 16151759 | 52.9  | 2 | 655.32 | 40.9  | IVYGDYGGFR                | 9.28E-10 | 10257009 | 8350965  | 14309547 | 10209263 |
|                |   |   |        |          |      |   |           |           |           |          | 102.5 | 2 | 817.46 | 65.0  | TYVPLIIDGIMSAIK           | 1.79E-08 | 2589450  | 2651364  | 7369786  | 4187983  |
|                |   |   |        |          |      |   |           |           |           |          | 112.0 | 3 | 785.42 | 26.4  | IITDYIASALNLPFVNAYL DK    | 1.54E-03 | 996646   | 916891   | 2390074  | 1754513  |

**Appendix 5.** The abundance of proteins participating in in the C and N metabolism, protein synthesis and catabolism as well as in lignin synthesis pathway, Supplementary table S2, chapter IV.

| Accession                                     | Peptide | Unique peptides | Confidence | Anova (p)    | Max fold | Description blast ncbi                          | R T | Ch arg | m/z               | Score | Sequence                         | Max fold | Ano        | Max  | Normalized abundance average of three biological and two technical replicates |        |      |       |       |        |        |        |            |       |        |      |       |       |        |        |        |
|---|---------|-----------------|------------|--------------|----------|---|-----|--------|-------------------|-------|----------------------------------|----------|------------|------|---|--------|------|-------|-------|--------|--------|--------|------------|-------|--------|------|-------|-------|--------|--------|--------|
|   |         |                 |            |              |          |   |     |        |                   |       |                                  |          |            |      | 10 T0   | 10 min | 1h   | 1h N+ | 1h N- | 24h N+ | 24h N- | 72h N+ | 72h N-     | 10 T0 | 10 min | 1h   | 1h N+ | 1h N- | 24h N+ | 24h N- | 72h N+ |
|   |         |                 |            |              |          |   |     |        |                   |       |                                  |          |            |      | Lambada   |        |      |       |       |        |        |        | Topas      |       |        |      |       |       |        |        |        |
| Proteins of N assimilation and reassimilation |         |                 |            |              |          |   |     |        |                   |       |                                  |          |            |      |   |        |      |       |       |        |        |        |            |       |        |      |       |       |        |        |        |
| 4000<br>4791                                  | 4       | 4               | 157.73     | 4.77<br>E-15 | 1.58     | <b>Glutamate--glyoxylate aminotransferase 2</b> | 45  | 2      | 394 16.<br>.24 31 | 16.   | GILESLR                          | 1.62     | 1.53 35.01 | 5017 | 5561652920  | 6828   | 5627 | 6116  | 6310  | 5855   | 6674   | 7278   | 6815368554 | 7483  | 7627   | 7093 | 6690  | 6712  | 8114   |        |        |
|   |         |                 |            |              |          |   | 42  | 2      | 711 58.<br>.35 61 | 1.42  | ALDYENLNENVK                     | 1.42     | 7.21 25.45 | 8262 | 8224684135  | 9445   | 9523 | 8346  | 8888  | 8511   | 9667   | 1017   | 9780910403 | 1154  | 1166   | 1058 | 1057  | 1127  | 1165   |        |        |
|   |         |                 |            |              |          |   | 74  | 2      | 593 39.<br>.35 63 | 1.84  | VLLDMGLPLSK                      | 1.84     | 5.11 25.96 | 2870 | 2977027956  | 3520   | 3278 | 3129  | 3116  | 2903   | 3486   | 4257   | 3983840467 | 5155  | 4512   | 4309 | 4545  | 4794  | 4817   |        |        |
|   |         |                 |            |              |          |   | 11  | 3      | 101 43.<br>3.2 18 | 5.63  | QVIALCQAPFLDDP<br>NVGLIFPADAIAR  | 5.63     | 1.70 97.08 | 2661 | 3185229504  | 4382   | 3896 | 2326  | 5365  | 3743   | 4394   | 4805   | 4389446026 | 7155  | 5726   | 8822 | 1111  | 1055  | 1309   |        |        |
| 4000<br>4038                                  | 8       | 8               | 486.03     | 7.13<br>E-07 | 1.86     | <b>Aspartate aminotransferase</b>               | 41  | 2      | 473 42.<br>.77 56 | 1.49  | VGALSIVCK                        | 1.49     | 2.68 39.78 | 1970 | 2028320143  | 2547   | 2308 | 2505  | 2935  | 2217   | 2694   | 2627   | 2558025635 | 2906  | 2678   | 2849 | 2737  | 2466  | 2663   |        |        |
|   |         |                 |            |              |          |   | 43  | 3      | 485 71.<br>.28 28 | 2.32  | TEEGKPLVLNVVR                    | 2.32     | 4.40 62.12 | 1055 | 1306110492  | 1717   | 1562 | 1722  | 1929  | 1381   | 1654   | 2125   | 2011919675 | 2430  | 2134   | 2415 | 2411  | 2296  | 2179   |        |        |
|   |         |                 |            |              |          |   | 66  | 2      | 524 44.<br>.82 18 | 2.00  | IFTLAGLSVK                       | 2.00     | 2.77 41.43 | 1294 | 1530913593  | 2016   | 1992 | 1841  | 2322  | 1555   | 1930   | 2211   | 2252420851 | 2593  | 2121   | 2405 | 2319  | 2332  | 2131   |        |        |
|   |         |                 |            |              |          |   | 39  | 3      | 530 21.<br>.61 51 | 2.29  | TVPHLADAMHAAVT<br>R              | 2.29     | 2.94 46.76 | 7483 | 8458365340  | 1072   | 1002 | 9572  | 1343  | 8859   | 1030   | 1134   | 1132211023 | 1499  | 1227   | 1244 | 1253  | 1220  | 1104   |        |        |
|   |         |                 |            |              |          |   | 74  | 2      | 735 55.<br>.88 07 | 2.23  | EYLSITGLADFNK                    | 2.23     | 2.60 32.65 | 7789 | 8371075446  | 9726   | 9597 | 9872  | 1062  | 8256   | 9914   | 1469   | 1438813962 | 1685  | 1463   | 1552 | 1548  | 1503  | 1438   |        |        |
|   |         |                 |            |              |          |   | 96  | 3      | 916 86.<br>.10 06 | 3.16  | GLLPFFDSAYQGFAS<br>GNLDAQSVR     | 3.16     | 3.10 55.46 | 1047 | 1086894456  | 1388   | 1630 | 1289  | 1713  | 1266   | 1450   | 1640   | 1886515828 | 1836  | 1615   | 2550 | 2984  | 2512  | 2738   |        |        |
|   |         |                 |            |              |          |   | 63  | 3      | 529 44.<br>.62 81 | 1.74  | APEDPILGVTVAYNK                  | 1.74     | 2.13 40.91 | 9769 | 1052893656  | 1270   | 1346 | 1290  | 1404  | 1183   | 1442   | 1227   | 1207912129 | 1392  | 1259   | 1256 | 1571  | 1583  | 1627   |        |        |
|   |         |                 |            |              |          |   | 63  | 2      | 721 74.<br>.34 99 | 1.64  | QMYDEWTVLTK                      | 1.64     | 3.40 29.67 | 4298 | 4419637160  | 4575   | 4951 | 4848  | 5632  | 4854   | 5384   | 4823   | 5019351773 | 6098  | 5507   | 5208 | 5172  | 5660  | 5186   |        |        |
| 4000<br>3469                                  | 3       | 3               | 92.05      | 0            | 1.83     | <b>Glutamate decarboxylase</b>                  | 49  | 2      | 464 16.<br>.24 5  | 1.78  | YFEVELK                          | 1.78     | 1.46 25.03 | 6316 | 6776862357  | 7626   | 6820 | 7383  | 9015  | 7579   | 9003   | 8748   | 8720789898 | 1046  | 9940   | 9722 | 1108  | 9461  | 1067   |        |        |
|   |         |                 |            |              |          |   | 83  | 3      | 804 29.<br>.75 53 | 1.84  | FGWIVPAYTMPDDAQ<br>HITVLR        | 1.84     | 2.62 34.86 | 5543 | 6463358764  | 6761   | 7174 | 6187  | 8338  | 6779   | 8469   | 7557   | 7531777154 | 8662  | 8264   | 8839 | 1018  | 8750  | 9843   |        |        |
|   |         |                 |            |              |          |   | 96  | 3      | 680 46.<br>.99 02 | 2.23  | LNLASFVTTWMEPEC<br>DK            | 2.23     | 0.00 24.63 | 2793 | 2559625274  | 2534   | 2498 | 2537  | 2642  | 2709   | 2735   | 5094   | 5122254748 | 4685  | 5131   | 5357 | 5457  | 5562  | 5213   |        |        |
| 4000<br>6496                                  | 4       | 3               | 261.38     | 1.01<br>E-06 | 1.92     | <b>Gamma aminobutyrate transaminase 2-like</b>  | 99  | 3      | 938 29.<br>.52 74 | 2.18  | ALSGGYVPIGAVLVS<br>PEISNVILSESNK | 2.18     | 4.71 53.82 | 3931 | 3904235607  | 4898   | 4789 | 4597  | 3891  | 4285   | 2513   | 3859   | 3778144999 | 3754  | 3574   | 5473 | 4427  | 4219  | 2579   |        |        |
|   |         |                 |            |              |          |   | 86  | 3      | 850 38.<br>.05 84 | 2.23  | SPNDPPFYDWAAGTY<br>FGAQCK        | 2.23     | 4.03 48.72 | 1382 | 1529315439  | 1721   | 1894 | 1656  | 1467  | 1398   | 9521   | 1342   | 1420615634 | 1564  | 1386   | 2127 | 1836  | 1700  | 1004   |        |        |
|   |         |                 |            |              |          |   | 85  | 2      | 102 85.<br>8.5 04 | 1.59  | YLDALSGLWCTSLGG<br>SEAR          | 1.59     | 2.04 37.51 | 6402 | 6066659715  | 6154   | 6753 | 5371  | 4965  | 5416   | 4989   | 4678   | 4861449791 | 5306  | 4428   | 4801 | 4737  | 4665  | 4255   |        |        |
| 4000<br>2131                                  | 6       | 3               | 272.77     | 0            | 3.52     | <b>Ferredoxin--nitrite reductase</b>            | 67  | 2      | 619 35.<br>.87 95 | 3.60  | IDALLNEPLLK                      | 3.60     | 0.00 34.73 | 3451 | 3544833753  | 4944   | 4847 | 4996  | 7648  | 3583   | 4439   | 8848   | 8898590271 | 1215  | 9391   | 1203 | 1198  | 1151  | 8604   |        |        |
|   |         |                 |            |              |          |   | 57  | 2      | 440 30.<br>.73 15 | 5.20  | DGYWVLK                          | 5.20     | 0.00 58.19 | 9348 | 1232696161  | 1564   | 1400 | 1765  | 2417  | 1023   | 1426   | 3362   | 3319833256 | 4673  | 3666   | 4761 | 4860  | 4589  | 3239   |        |        |
|   |         |                 |            |              |          |   | 58  | 2      | 106 48.<br>2.5 32 | 2.32  | NLVACTGNQFCGQAI<br>IETK          | 2.32     | 3.52 58.64 | 1347 | 1567615576  | 1662   | 1679 | 1540  | 2232  | 1437   | 1433   | 2413   | 2564023560 | 3128  | 2363   | 2856 | 2851  | 2820  | 2154   |        |        |
|   |         |                 |            |              |          |   | 0   | 2      | 514 51.<br>80 840 | 2.32  | IETK                             | 2.32     | E-10       | 514  | 51  | 80     | 840  | 210   | 211   | 973    | 323    | 492    | 403        | 72    | 64     | 039  | 452   | 850   | 229    | 672    | 329    |







|      |   |   |        |      |      |    |         |     |     |                   |      |       |       |       |       |       |      |      |      |      |      |      |       |       |       |      |      |      |      |      |      |
|------|---|---|--------|------|------|----|---------|-----|-----|-------------------|------|-------|-------|-------|-------|-------|------|------|------|------|------|------|-------|-------|-------|------|------|------|------|------|------|
|      |   |   |        |      |      | 49 | 528.34. |     |     |                   | 1.31 | 75.40 | 2884  | 32515 | 31890 | 3611  | 3005 | 3529 | 3934 | 3597 | 3955 | 7960 | 83340 | 76196 | 9168  | 9157 | 7533 | 9024 | 7934 | 8463 |      |
|      |   |   |        |      |      | .5 | 3       | .60 | 73  | EGQEITVSTDYTIK    | 3.18 | E-08  | 712   | 22    | 42    | 601   | 196  | 048  | 653  | 523  | 820  | 712  | 56    | 20    | 331   | 101  | 489  | 188  | 036  | 251  |      |
|      |   |   |        |      |      | 70 | 838.89. |     |     |                   |      | 8.35  | 30.40 | 1850  | 21842 | 20983 | 2560 | 2683 | 2574 | 3115 | 2231 | 2617 | 3078  | 30607 | 29337 | 3163 | 2917 | 3037 | 3182 | 3076 | 3328 |
|      |   |   |        |      |      | .0 | 2       | .93 | 66  | ATDSESTEVEILEAALK | 1.80 | E-08  | 2769  | 851   | 718   | 8976  | 6116 | 4489 | 4344 | 6333 | 1404 | 8358 | 445   | 708   | 5995  | 4823 | 9863 | 7244 | 2051 | 9992 |      |
|      |   |   |        |      |      | 78 | 761.66. |     |     |                   |      | 3.29  | 61.00 | 5901  | 78804 | 63662 | 1173 | 1140 | 1033 | 1522 | 8636 | 1099 | 1313  | 13401 | 12752 | 1588 | 1267 | 1621 | 1607 | 1552 | 1404 |
|      |   |   |        |      |      | .1 | 2       | .40 | 12  | AVVTATQMLESMIK    | 2.75 | E-06  | 326   | 96    | 53    | 3368  | 8518 | 4909 | 3717 | 252  | 4511 | 0084 | 029   | 861   | 7924  | 0949 | 1564 | 1631 | 1199 | 0750 |      |
|      |   |   |        |      |      | 83 | 904.69. |     |     |                   |      | 1.94  | 39.60 | 1374  | 16950 | 14818 | 2341 | 2284 | 2380 | 3090 | 1704 | 2191 | 2705  | 27541 | 25014 | 2752 | 2507 | 2854 | 2847 | 2693 | 2778 |
|      |   |   |        |      |      | .7 | 2       | .51 | 53  | EK                | 2.25 | E-08  | 3038  | 235   | 997   | 6469  | 2326 | 7023 | 5124 | 3518 | 9679 | 4670 | 816   | 022   | 4376  | 1294 | 1309 | 0805 | 1852 | 0676 |      |
|      |   |   |        |      |      | 35 | 580.27. |     |     |                   |      | 4.81  | 56.60 | 6460  | 82927 | 63229 | 1155 | 1027 | 1079 | 1822 | 9980 | 1262 | 1372  | 13336 | 11622 | 1283 | 1433 | 1678 | 1580 | 1457 | 1437 |
|      |   |   |        |      |      | .5 | 4       | .02 | 79  | DNLK              | 2.88 | E-07  | 624   | 07    | 00    | 8884  | 1888 | 6364 | 5441 | 850  | 3155 | 9272 | 178   | 928   | 0728  | 4167 | 6669 | 5775 | 9816 | 4424 |      |
|      |   |   |        |      |      | 78 | 975.10. |     |     |                   |      | 6.93  | 62.50 | 6194  | 70881 | 69671 | 1027 | 1031 | 8495 | 1371 | 7619 | 9827 | 1169  | 11732 | 11437 | 1490 | 1166 | 1359 | 1372 | 1292 | 1280 |
|      |   |   |        |      |      | .0 | 2       | .49 | 5.0 | TK                | 2.41 | E-05  | 798   | 14    | 48    | 4511  | 1798 | 824  | 9016 | 828  | 332  | 0302 | 842   | 442   | 4932  | 2629 | 7348 | 0983 | 4781 | 5127 |      |
|      |   |   |        |      |      | 10 | 892.54. |     |     |                   |      | 3.41  | 66.30 | 3576  | 43229 | 29806 | 8294 | 6228 | 5997 | 1115 | 5371 | 6426 | 1201  | 94068 | 10703 | 1264 | 1297 | 1770 | 1967 | 1503 | 1346 |
|      |   |   |        |      |      | 8. | 3       | .12 | 53  | MIALSFVR          | 6.60 | E-10  | 301   | 47    | 88    | 285   | 888  | 8628 | 066  | 293  | 1774 | 81   | 796   | 2250  | 5177  | 4429 | 9649 | 8960 | 6006 |      |      |
|      |   |   |        |      |      | 69 | 646.18. |     |     |                   |      | 2.22  | 70.50 | 2445  | 32028 | 28744 | 4573 | 5252 | 4223 | 6055 | 3548 | 4703 | 5679  | 56255 | 53326 | 6844 | 5647 | 6260 | 6616 | 6601 | 6657 |
|      |   |   |        |      |      | .8 | 3       | .04 | 7   | TEK               | 2.80 | E-03  | 182   | 14    | 00    | 189   | 099  | 216  | 022  | 791  | 337  | 534  | 67    | 52    | 115   | 097  | 858  | 309  | 252  | 171  |      |
| 4000 |   |   | 2.16   |      |      | 32 | 691.32. |     |     |                   |      | 2.03  | 75.10 | 1568  | 16766 | 17387 | 2473 | 2555 | 2414 | 4656 | 1558 | 2142 | 3292  | 30904 | 31079 | 4151 | 3252 | 3709 | 3193 | 2883 | 2724 |
| 3286 | 3 | 3 | 148.06 | E-13 | 2.19 | .0 | 2       | .36 | 26  | WTPDNPNGKPELK     | 2.99 | E-03  | 761   | 49    | 22    | 232   | 020  | 039  | 473  | 863  | 008  | 854  | 53    | 38    | 227   | 282  | 964  | 768  | 371  | 419  |      |
|      |   |   |        |      |      | 54 | 603.20. |     |     |                   |      | 2.21  | 40.00 | 2694  | 27557 | 28735 | 3293 | 3320 | 3772 | 3874 | 2811 | 2833 | 4091  | 33933 | 38341 | 3836 | 3361 | 4060 | 3784 | 3798 | 3577 |
|      |   |   |        |      |      | .8 | 2       | .31 | 94  | NEIDPTLTFR        | 1.52 | E-03  | 697   | 17    | 93    | 832   | 616  | 307  | 687  | 891  | 514  | 577  | 06    | 93    | 487   | 802  | 268  | 846  | 062  | 666  |      |
|      |   |   |        |      |      | 82 | 875.56. |     |     |                   |      | 1.69  | 35.80 | 4298  | 46034 | 44019 | 5662 | 5742 | 5627 | 6992 | 4807 | 5475 | 6917  | 65258 | 62618 | 8017 | 6743 | 8056 | 9087 | 8331 | 7906 |
|      |   |   |        |      |      | .6 | 2       | .41 | 22  | DLVDMTNYFYQYK     | 2.11 | E-10  | 516   | 67    | 65    | 114   | 245  | 001  | 382  | 566  | 386  | 086  | 50    | 74    | 320   | 266  | 385  | 748  | 534  | 708  |      |
| 4000 |   |   | 3.46   |      |      | 47 | 656.44. |     |     |                   |      | 3.14  | 41.10 | 1469  | 14677 | 13998 | 1742 | 1734 | 1497 | 2854 | 1466 | 2372 | 1632  | 15355 | 15078 | 2036 | 1896 | 1923 | 2397 | 1809 | 2573 |
| 5479 | 5 | 4 | 211.97 | E-12 | 2.22 | .1 | 2       | .35 | 16  | LDDLEAAPAAVAR     | 2.04 | E-05  | 117   | 95    | 79    | 356   | 483  | 310  | 264  | 388  | 714  | 017  | 05    | 05    | 847   | 080  | 693  | 953  | 456  | 837  |      |
|      |   |   |        |      |      | 91 | 682.44. |     |     |                   |      | 1.35  | 54.50 | 1214  | 11087 | 10516 | 1449 | 1307 | 1459 | 2267 | 1055 | 2098 | 1717  | 18372 | 18575 | 2052 | 1818 | 2227 | 2721 | 2175 | 2404 |
|      |   |   |        |      |      | .0 | 2       | .88 | 64  | VTIDLVMEMVFAK     | 2.59 | E-09  | 890   | 08    | 43    | 855   | 610  | 300  | 495  | 667  | 035  | 893  | 98    | 29    | 751   | 685  | 029  | 256  | 329  | 883  |      |
|      |   |   |        |      |      | 10 | 888.33. |     |     |                   |      | 1.02  | 51.80 | 9712  | 77959 | 89589 | 1232 | 1117 | 1137 | 2190 | 9019 | 2186 | 1284  | 12664 | 10824 | 1228 | 1389 | 1399 | 2040 | 1438 | 2156 |
|      |   |   |        |      |      | 2. | 2       | .98 | 16  | LLVSEDLWSFGELLR   | 2.81 | E-06  | 49    | 9     | 7     | 040   | 005  | 588  | 082  | 91   | 341  | 184  | 44    | 86    | 719   | 278  | 847  | 098  | 245  |      |      |
|      |   |   |        |      |      | 97 | 143.44. |     |     |                   |      | 9.67  | 53.00 | 3151  | 18775 | 26191 | 2554 | 2407 | 2728 | 5462 | 2373 | 5077 | 2264  | 23019 | 20947 | 2738 | 2468 | 2473 | 2502 | 1938 | 3334 |
|      |   |   |        |      |      | .2 | 2       | 9.2 | 42  | PGLEDTLILTMK      | 2.91 | E-04  | 06    | 3     | 0     | 72    | 22   | 23   | 97   | 37   | 06   | 31   | 8     | 0     | 60    | 96   | 74   | 19   | 63   | 13   |      |
| 4000 |   |   | 1.13   |      |      | 39 | 652.62. |     |     |                   |      | 2.52  | 43.40 | 1012  | 11125 | 10759 | 1261 | 1084 | 1191 | 1505 | 1125 | 1018 | 1494  | 14212 | 14369 | 1731 | 1556 | 1746 | 1803 | 1368 | 1395 |
| 8179 | 3 | 3 | 133.77 | E-12 | 2.22 | .3 | 2       | .83 | 83  | LEAACIGAVESGK     | 1.78 | E-05  | 8443  | 520   | 391   | 8198  | 5592 | 5387 | 8455 | 2291 | 4709 | 7565 | 870   | 414   | 2531  | 1734 | 8061 | 6789 | 7905 | 1868 |      |
|      |   |   |        |      |      | 10 | 657.46. |     |     |                   |      | 2.95  | 46.60 | 4752  | 50280 | 44747 | 6299 | 5547 | 6384 | 9592 | 5243 | 4740 | 1013  | 85490 | 74515 | 1374 | 9824 | 1199 | 1408 | 9876 | 8111 |
|      |   |   |        |      |      | 0. | 2       | .41 | 92  | LILPFLELDIK       | 3.15 | E-11  | 641   | 12    | 45    | 741   | 270  | 192  | 142  | 079  | 473  | 1306 | 84    | 66    | 2573  | 778  | 5217 | 7069 | 996  | 390  |      |
|      |   |   |        |      |      | 79 | 514.24. |     |     |                   |      | 3.34  | 66.90 | 9747  | 11253 | 81773 | 1107 | 1145 | 1180 | 1482 | 1044 | 9595 | 1623  | 15776 | 15441 | 1960 | 1680 | 2339 | 2952 | 2780 | 2186 |
|      |   |   |        |      |      | .7 | 3       | .58 | 02  | DIFQEVYEANWK      | 3.61 | E-05  | 22    | 87    | 5     | 433   | 464  | 953  | 323  | 611  | 67   | 717  | 17    | 47    | 664   | 364  | 753  | 580  | 464  | 318  |      |
| 4000 |   |   | 3.46   |      |      | 74 | 572.    |     |     |                   |      | 3.54  | 44.60 | 4272  | 55668 | 46108 | 5815 | 5659 | 5910 | 8219 | 5223 | 5027 | 8482  | 75871 | 78323 | 1031 | 8886 | 9521 | 1177 | 8870 | 8523 |
| 4311 | 2 | 2 | 75.75  | E-05 | 2.23 | .5 | 3       | .99 | 49  | LDHPLPLPSVLGSPFK  | 2.76 | E-07  | 215   | 71    | 30    | 168   | 087  | 218  | 610  | 549  | 484  | 083  | 59    | 42    | 0160  | 447  | 557  | 2847 | 852  | 886  |      |
|      |   |   |        |      |      | 56 | 596.26. |     |     |                   |      | 4.53  | 47.90 | 3769  | 40763 | 40443 | 3617 | 4243 | 3930 | 4946 | 3455 | 4284 | 5010  | 46514 | 52443 | 5884 | 5217 | 5844 | 6206 | 4890 | 4294 |
|      |   |   |        |      |      | .8 | 2       | .31 | 75  | AMVELEGAPFK       | 1.80 | E-02  | 100   | 37    | 07    | 536   | 513  | 370  | 930  | 314  | 393  | 113  | 48    | 57    | 976   | 818  | 286  | 301  | 122  | 830  |      |
| 4000 |   |   | 1.19   |      |      | 71 | 549.23. |     |     |                   |      | 1.30  | 43.20 | 1174  | 11592 | 11834 | 1491 | 1496 | 1600 | 2339 | 1419 | 1440 | 2461  | 22175 | 18379 | 2792 | 2286 | 2535 | 2962 | 2740 | 2654 |
| 7026 | 4 | 2 | 180.85 | E-05 | 2.30 | .8 | 2       | .82 | 24  | GIFPILADPR        | 2.56 | E-11  | 805   | 94    | 64    | 440   | 296  | 895  | 802  | 840  | 515  | 337  | 01    | 88    | 741   | 725  | 361  | 470  | 075  | 487  |      |
|      |   |   |        |      |      | 53 | 674.25. |     |     |                   |      | 3.81  | 77.10 | 1610  | 15446 | 15533 | 1553 | 1592 | 1695 | 2223 | 1704 | 1921 | 2613  | 23701 | 20813 | 2560 | 2271 | 2221 | 3244 | 3013 | 2627 |
|      |   |   |        |      |      | .2 | 2       | .36 | 91  | LGDLNQTQIFAK      | 2.10 | E-02  | 899   | 71    | 23    | 728   | 037  | 747  | 227  | 624  | 244  | 064  | 27    | 89    | 415   | 067  | 340  | 296  | 941  | 650  |      |
| 4000 |   |   |        |      |      | 46 | 563.51. |     |     |                   |      | 0.00  | 23.00 | 7412  | 83727 | 72420 | 9341 | 8512 | 9116 | 1276 | 8072 | 9588 | 1299  | 12865 | 13111 | 1651 | 1425 | 1635 | 1655 | 1475 | 1432 |



|      |   |   |        |      |      |    |         |  |    |                  |      |      |         |                 |       |             |      |      |      |      |      |      |      |       |       |      |       |       |      |      |      |      |      |      |      |       |       |      |      |      |      |      |      |
|------|---|---|--------|------|------|----|---------|--|----|------------------|------|------|---------|-----------------|-------|-------------|------|------|------|------|------|------|------|-------|-------|------|-------|-------|------|------|------|------|------|------|------|-------|-------|------|------|------|------|------|------|
|      |   |   |        |      |      | 51 | 65844.  |  |    |                  |      | 2.84 | 35.00   | 1122            | 12676 | 12390       | 1665 | 1566 | 1581 | 2475 | 1539 | 2105 | 1962 | 19921 | 17292 | 2195 | 1921  | 2052  | 2771 | 2237 | 2999 |      |      |      |      |       |       |      |      |      |      |      |      |
|      |   |   |        |      |      | .7 | 2       | .36  | 34 | SIQVIVVTDGER     | 2.67 | E-08 | 0022    | 959             | 256   | 0960        | 1892 | 1522 | 0623 | 2472 | 7658 | 2689 | 208  | 611   | 9996  | 6517 | 7656  | 3617  | 2414 | 2489 |      |      |      |      |      |       |       |      |      |      |      |      |      |
|      |   |   |        |      |      | 87 | 712.35  | ATGQEYYDFLHEFMS  |    |                  |      | 3.46 | 45.80   | 1064            | 12666 | 95421       | 1586 | 1628 | 1553 | 2551 | 1424 | 2056 | 2085 | 22779 | 18631 | 2310 | 2025  | 2818  | 3592 | 2623 | 3567 |      |      |      |      |       |       |      |      |      |      |      |      |
|      |   |   |        |      |      | .5 | 3       | .66  | 18 | AVK              | 3.76 | E-13 | 1381    | 329             | 57    | 3799        | 3668 | 1952 | 5104 | 1777 | 3747 | 7268 | 862  | 185   | 3606  | 4296 | 0668  | 2330  | 8786 | 5501 |      |      |      |      |      |       |       |      |      |      |      |      |      |
|      |   |   |        |      |      | 84 | 740.21  | ILVQFEDFANHNAFE  |    |                  |      | 4.77 | 30.60   | 1663            | 16943 | 14709       | 2010 | 2021 | 2067 | 2961 | 1922 | 2447 | 2655 | 26820 | 22489 | 2578 | 2625  | 3073  | 4117 | 3259 | 4049 |      |      |      |      |       |       |      |      |      |      |      |      |
|      |   |   |        |      |      | .0 | 3       | .39  | 44 | LLAK             | 2.80 | E-15 | 4531    | 785             | 722   | 5749        | 2915 | 1904 | 4317 | 3072 | 1453 | 9059 | 114  | 258   | 1744  | 7448 | 9209  | 4849  | 9801 | 0081 |      |      |      |      |      |       |       |      |      |      |      |      |      |
|      |   |   |        |      |      | 71 | 662.66. |  |    |                  |      | 0.00 | 32.50   | 8090            | 75202 | 69388       | 7611 | 8079 | 8051 | 9856 | 7889 | 9777 | 1336 | 11988 | 12114 | 1621 | 1318  | 1345  | 1907 | 1539 | 2047 |      |      |      |      |       |       |      |      |      |      |      |      |
|      |   |   |        |      |      | .5 | 2       | .86  | 44 | LLNDEFYIGLK      | 2.95 | E+0  | 864     | 22              | 37    | 897         | 494  | 456  | 189  | 797  | 040  | 5322 | 204  | 396   | 8145  | 7141 | 7232  | 0593  | 5097 | 6375 |      |      |      |      |      |       |       |      |      |      |      |      |      |
|      |   |   |        |      |      | 27 | 569.37. |  |    |                  |      | 1.33 | 93.50   | 8511            | 80673 | 98057       | 1105 | 1067 | 9356 | 2734 | 1277 | 2198 | 1707 | 19919 | 14023 | 1935 | 1235  | 1245  | 1600 | 1303 | 2017 |      |      |      |      |       |       |      |      |      |      |      |      |
|      |   |   |        |      |      | .2 | 2       | .83  | 77 | ISAHIAASVAAK     | 3.39 | E-02 | 25      | 5               | 7     | 839         | 459  | 44   | 659  | 110  | 555  | 004  | 17   | 28    | 128   | 797  | 198   | 035   | 738  | 771  |      |      |      |      |      |       |       |      |      |      |      |      |      |
|      |   |   |        |      |      | 47 | 490.26. |  |    |                  |      | 0.00 | 38.50   | 2390            | 24479 | 25386       | 2893 | 2773 | 2890 | 4706 | 2771 | 4075 | 5660 | 57053 | 52752 | 7127 | 5723  | 6722  | 8175 | 6800 | 8827 |      |      |      |      |       |       |      |      |      |      |      |      |
|      |   |   |        |      |      | .9 | 2       | .79  | 34 | LPQPADLVK        | 3.69 | E+0  | 638     | 35              | 64    | 936         | 146  | 683  | 741  | 460  | 852  | 096  | 86   | 29    | 903   | 084  | 362   | 444   | 678  | 347  |      |      |      |      |      |       |       |      |      |      |      |      |      |
|      |   |   |        |      |      | 10 | 135.66. | LLIDNVEELLPIVYTP   |    |                  |      | 3.62 | 55.50   | 4441            | 42921 | 41500       | 6407 | 5597 | 6203 | 1049 | 5034 | 7385 | 9395 | 97887 | 78946 | 1178 | 9573  | 9689  | 1204 | 8552 | 1335 |      |      |      |      |       |       |      |      |      |      |      |      |
|      |   |   |        |      |      | 5. | 2       | 7.7  | 31 | TVGEACQK         | 3.22 | E-07 | 083     | 85              | 69    | 359         | 494  | 272  | 7872 | 004  | 501  | 286  | 71   | 20    | 8649  | 169  | 498   | 2169  | 759  | 7273 |      |      |      |      |      |       |       |      |      |      |      |      |      |
|      |   |   |        |      |      | 76 | 653.59. | VHDDMLLAASEALA   |    |                  |      | 2.09 | 56.10   | 9259            | 10295 | 77193       | 1241 | 1102 | 1199 | 1612 | 1061 | 1388 | 1234 | 12651 | 11154 | 1425 | 1284  | 1344  | 1727 | 1571 | 1996 |      |      |      |      |       |       |      |      |      |      |      |      |
|      |   |   |        |      |      | .8 | 4       | .82  | 02 | AQVTEEHYAK       | 2.59 | E-05 | 484     | 507             | 38    | 7273        | 2865 | 1129 | 9784 | 4184 | 3627 | 9251 | 268  | 844   | 5000  | 5401 | 0820  | 4254  | 3556 | 4431 |      |      |      |      |      |       |       |      |      |      |      |      |      |
|      |   |   |        |      |      | 38 | 777.63. |  |    |                  |      | 1.62 | 97.40   | 1152            | 13016 | 12480       | 1865 | 1887 | 1381 | 4311 | 1588 | 2582 | 2367 | 27074 | 21249 | 3843 | 2656  | 3209  | 4521 | 3417 | 4499 |      |      |      |      |       |       |      |      |      |      |      |      |
|      |   |   |        |      |      | .2 | 2       | .82  | 73 | YAESCMYTPNYR     | 3.92 | E-02 | 251     | 92              | 56    | 757         | 844  | 886  | 913  | 456  | 664  | 907  | 54   | 79    | 848   | 116  | 997   | 592   | 308  | 4020 |      |      |      |      |      |       |       |      |      |      |      |      |      |
|      |   |   |        |      |      | 31 | 785.35. |  |    |                  |      | 8.24 | 28.80   | 1221            | 12267 | 13364       | 1335 | 1283 | 1242 | 1672 | 1257 | 1419 | 1442 | 14474 | 13812 | 1706 | 1451  | 1654  | 1858 | 1613 | 1850 |      |      |      |      |       |       |      |      |      |      |      |      |
|      |   |   |        |      |      | .6 | 2       | .82  | 68 | YAESCMYTPNYR     | 1.52 | E-04 | 6041    | 096             | 484   | 0968        | 3239 | 4889 | 3533 | 7705 | 3808 | 9765 | 763  | 065   | 3931  | 7053 | 6180  | 1435  | 3531 | 1879 |      |      |      |      |      |       |       |      |      |      |      |      |      |
|      |   |   |        |      |      | 81 | 981.22. | LALYALGGVRPSAC   |    |                  |      | 1.42 | 94.80   | 4585            | 48823 | 59254       | 6693 | 7385 | 4912 | 1498 | 5984 | 1094 | 1102 | 11603 | 97716 | 1883 | 1053  | 1454  | 1769 | 1145 | 2105 |      |      |      |      |       |       |      |      |      |      |      |      |
|      |   |   |        |      |      | .8 | 3       | .85  | 92 | LPITIDVGTNNEK    | 4.59 | E-07 | 67      | 2               | 4     | 50          | 23   | 67   | 624  | 32   | 348  | 027  | 66   | 6     | 819   | 125  | 577   | 026   | 082  | 301  |      |      |      |      |      |       |       |      |      |      |      |      |      |
| 4000 |   |   |        | 3.81 |      |    |         | <b>Pyruvate kinase</b>                                   |    |                  |      | 88   | 603.28. | NVNLPGVIVDLPTLT |       |             |      |      |      |      |      |      |      | 4.14  | 83.60 | 1443 | 11747 | 11387 | 2198 | 2157 | 2544 | 3756 | 1354 | 2091 | 2903 | 30700 | 24309 | 3214 | 2433 | 5251 | 7198 | 4408 | 5299 |
| 2830 | 6 | 6 | 329.25 | E-12 | 2.66 |    |         |  |    |                  |      | .0   | 3       | .34             | 06    | DK          | 6.32 | E-06 | 07   | 2    | 0    | 20   | 36   | 88    | 64    | 83   | 29    | 56    | 7    | 6    | 70   | 27   | 76   | 63   | 29   | 408   | 5299  |      |      |      |      |      |      |
|      |   |   |        |      |      | 46 | 784.41. |  |    |                  |      | 3.14 | 42.70   | 3539            | 39604 | 37463       | 5091 | 4894 | 5208 | 9046 | 3752 | 5115 | 5873 | 54707 | 47323 | 6891 | 5511  | 6674  | 7460 | 6761 | 6566 |      |      |      |      |       |       |      |      |      |      |      |      |
|      |   |   |        |      |      | .0 | 2       | .89  | 25 | QGQEITVSTDYSIK   | 2.56 | E-05 | 553     | 49              | 30    | 749         | 280  | 223  | 607  | 838  | 134  | 496  | 02   | 68    | 308   | 285  | 962   | 555   | 755  | 241  |      |      |      |      |      |       |       |      |      |      |      |      |      |
|      |   |   |        |      |      | 66 | 485.66. |  |    |                  |      | 8.39 | 63.90   | 4804            | 60145 | 50249       | 1027 | 1245 | 1173 | 2255 | 6390 | 1167 | 1781 | 21030 | 11353 | 1483 | 1580  | 1770  | 2105 | 1587 | 1731 |      |      |      |      |       |       |      |      |      |      |      |      |
|      |   |   |        |      |      | .9 | 2       | .33  | 58 | AALILVLR         | 4.69 | E-06 | 68      | 7               | 2     | 227         | 595  | 830  | 669  | 27   | 612  | 376  | 08   | 61    | 023   | 751  | 191   | 598   | 568  | 350  |      |      |      |      |      |       |       |      |      |      |      |      |      |
|      |   |   |        |      |      | 74 | 950.73. |  |    |                  |      | 5.06 | 30.10   | 4172            | 41580 | 41669       | 5213 | 5739 | 5532 | 7801 | 4066 | 5179 | 6926 | 68994 | 56754 | 7590 | 6545  | 7300  | 8388 | 6845 | 7721 |      |      |      |      |       |       |      |      |      |      |      |      |
|      |   |   |        |      |      | .3 | 2       | .46  | 9  | ICIEAESTIDYDPVFK | 2.06 | E-09 | 853     | 94              | 10    | 597         | 547  | 558  | 082  | 780  | 226  | 229  | 01   | 28    | 729   | 787  | 292   | 871   | 267  | 251  |      |      |      |      |      |       |       |      |      |      |      |      |      |
|      |   |   |        |      |      | 75 | 600.19. |  |    |                  |      | 2.05 | 71.60   | 1688            | 17299 | 16056       | 2937 | 3061 | 2764 | 5400 | 1940 | 2768 | 3901 | 41309 | 33143 | 4473 | 4001  | 4445  | 5729 | 4804 | 4713 |      |      |      |      |       |       |      |      |      |      |      |      |
|      |   |   |        |      |      | .9 | 3       | .01  | 38 | YRPGMPILSVVPEIK  | 3.57 | E-05 | 334     | 72              | 73    | 509         | 824  | 170  | 309  | 451  | 919  | 927  | 42   | 88    | 019   | 999  | 859   | 820   | 389  | 347  |      |      |      |      |      |       |       |      |      |      |      |      |      |
|      |   |   |        |      |      | 49 | 887.62. |  |    |                  |      | 1.20 | 64.50   | 1909            | 19930 | 18017       | 2821 | 2615 | 3017 | 5872 | 2234 | 3073 | 4710 | 41774 | 36393 | 4953 | 4654  | 4332  | 6225 | 4966 | 5660 |      |      |      |      |       |       |      |      |      |      |      |      |
|      |   |   |        |      |      | .6 | 2       | .36  | 09 | TDSFDWTCSDSPAR   | 3.46 | E-07 | 860     | 73              | 27    | 783         | 513  | 069  | 356  | 769  | 667  | 896  | 48   | 98    | 957   | 899  | 435   | 241   | 262  | 321  |      |      |      |      |      |       |       |      |      |      |      |      |      |
| 4000 |   |   |        | 4.18 |      |    |         | <b>Pyruvate kinase 1</b>                                 |    |                  |      | 55   | 660.75. |                 |       |             |      |      |      |      |      |      |      | 5.75  | 44.30 | 4834 | 48635 | 53295 | 6884 | 6754 | 6160 | 1106 | 5729 | 7047 | 1051 | 99535 | 78304 | 1207 | 9867 | 1140 | 1419 | 1101 | 1215 |
| 7241 | 6 | 4 | 311.74 | E-12 | 2.77 |    |         |  |    |                  |      | .3   | 2       | .86             | 34    | LGDLSTQIFAK | 2.94 | E-10 | 720  | 27   | 96   | 288  | 456  | 432   | 7462  | 394  | 801   | 5661  | 23   | 04   | 4271 | 323  | 3461 | 7826 | 1892 | 4380  |       |      |      |      |      |      |      |
|      |   |   |        |      |      | 52 | 587.28. |  |    |                  |      | 2.49 | 40.50   | 4900            | 52442 | 51062       | 6209 | 6001 | 6584 | 1023 | 5645 | 6624 | 1000 | 95803 | 77694 | 1098 | 9252  | 1082  | 1343 | 1036 | 1135 |      |      |      |      |       |       |      |      |      |      |      |      |
|      |   |   |        |      |      | .6 | 2       | .30  | 86 | VFNQDLYFK        | 2.74 | E-11 | 119     | 60              | 66    | 251         | 616  | 028  | 9529 | 597  | 999  | 0869 | 43   | 93    | 9981  | 958  | 0149  | 2180  | 3524 | 0048 |      |      |      |      |      |       |       |      |      |      |      |      |      |
|      |   |   |        |      |      | 43 | 630.35. |  |    |                  |      | 5.58 | 42.30   | 3391            | 31646 | 33602       | 4122 | 4077 | 3798 | 5548 | 3329 | 3539 | 5455 | 46546 | 45653 | 5762 | 5373  | 5496  | 6215 | 5065 | 4968 |      |      |      |      |       |       |      |      |      |      |      |      |
|      |   |   |        |      |      | .3 | 2       | .83  | 51 | EVISTWGVQNK      | 1.96 | E-05 | 065     | 38              | 72    | 025         | 904  | 366  | 289  | 439  | 763  | 489  | 33   | 89    | 269   | 313  | 063   | 739   | 095  | 365  |      |      |      |      |      |       |       |      |      |      |      |      |      |
|      |   |   |        |      |      | 67 | 543.40. |  |    |                  |      | 1.38 | 64.10   | 8109            | 86748 | 74501       | 1418 | 1334 | 1398 | 3959 | 1097 | 1604 | 3526 | 31350 | 20120 | 3759 | 2846  | 3705  | 4715 | 3385 | 4807 |      |      |      |      |       |       |      |      |      |      |      |      |
|      |   |   |        |      |      | .5 | 3       | .32  | 33 | YRPTMPVLSVVIPR   | 6.45 | E-14 | 97      | 2               | 5     | 185         | 114  | 518  | 074  | 386  | 516  | 141  | 33   | 55    | 333   | 086  | 883   | 042   | 718  | 925  |      |      |      |      |      |       |       |      |      |      |      |      |      |
| 4000 |   |   |        |      |      |    |         | <b>Pyruvate dehydrogenase E1 component subunit alpha</b> |    |                  |      | 47   | 570.30. |                 |       |             |      |      |      |      |      |      |      | 0.00  | 69.60 | 2918 | 27746 | 28041 | 3175 | 2921 | 3734 | 6068 | 2799 | 3348 | 4498 | 47292 | 51055 | 6694 | 5695 | 6621 | 7246 | 6260 | 5036 |
| 5822 | 6 | 6 | 248.41 | 0    | 2.96 |    |         |  |    |                  |      | .8   | 2       | .78             | 35    | MEIAADSLYK  | 2.61 | E+0  | 248  | 29   | 34   | 934  | 199  | 176   | 285   | 765  | 468   | 381   | 99   | 05   | 310  | 257  |      |      |      |       |       |      |      |      |      |      |      |



|                                   |    |    |        |      |      |    |         |                       |      |       |      |       |       |       |       |      |      |      |      |      |       |       |       |       |      |      |      |      |      |      |
|-----------------------------------|----|----|--------|------|------|----|---------|-----------------------|------|-------|------|-------|-------|-------|-------|------|------|------|------|------|-------|-------|-------|-------|------|------|------|------|------|------|
|                                   |    |    |        |      |      | 81 | 644.27  | ESGFYGGHGIVGAQV       | 0.00 | 44.40 | 1337 | 14868 | 12197 | 1737  | 1844  | 1785 | 2518 | 1349 | 1882 | 3216 | 34097 | 31936 | 4240  | 3501  | 4825 | 5840 | 5048 | 4944 |      |      |
|                                   |    |    |        |      |      | .8 | 4       | .09 37 PLGIGLAFAQK    | 4.79 | E+0   | 440  | 96    | 67    | 334   | 602   | 377  | 058  | 643  | 926  | 914  | 67    | 24    | 268   | 279   | 848  | 323  | 908  | 352  |      |      |
| 4000                              |    |    |        |      |      | 49 | 493.33. |                       |      |       | 0.00 | 22.60 | 2326  | 26348 | 23786 | 3190 | 2885 | 3147 | 4229 | 2526 | 2987  | 4057  | 39809 | 39386 | 5275 | 4407 | 5028 | 5132 | 4670 | 4337 |
| 0245                              | 12 | 12 | 749.6  | 0    | 2.62 | .3 | 2       | .80 38 LVDLALASGK     | 2.27 | E+0   | 1544 | 010   | 697   | 7361  | 9758  | 2302 | 2079 | 3466 | 9186 | 4229 | 793   | 489   | 7135  | 3423  | 1049 | 1323 | 5660 | 0295 |      |      |
|                                   |    |    |        |      |      | 44 | 584.23. |                       |      |       | 3.41 | 26.50 | 1620  | 19239 | 16835 | 2186 | 2067 | 2291 | 3000 | 1819 | 1953  | 2634  | 24784 | 25623 | 2963 | 2922 | 3245 | 3243 | 2574 | 2705 |
|                                   |    |    |        |      |      | .1 | 2       | .27 54 YENDWDVVK      | 2.00 | E-12  | 8836 | 995   | 966   | 6508  | 6134  | 5691 | 9056 | 3424 | 4585 | 1663 | 391   | 984   | 6852  | 5808  | 1060 | 0402 | 0962 | 6088 |      |      |
|                                   |    |    |        |      |      | 51 | 594.35. |                       |      |       | 0.00 | 26.30 | 2056  | 23634 | 21126 | 3062 | 2909 | 3012 | 4500 | 2268 | 2806  | 3966  | 39307 | 39087 | 5035 | 4316 | 4793 | 4874 | 4762 | 4268 |
|                                   |    |    |        |      |      | .6 | 2       | .82 04 YLVSPPEIDR     | 2.45 | E+0   | 3151 | 260   | 775   | 4125  | 4459  | 3151 | 4023 | 1826 | 7207 | 8840 | 750   | 897   | 5851  | 2549  | 0663 | 6770 | 6157 | 1965 |      |      |
| Protein synthesis and degradation |    |    |        |      |      |    |         |                       |      |       |      |       |       |       |       |      |      |      |      |      |       |       |       |       |      |      |      |      |      |      |
| 4000                              |    |    | 0.000  |      |      | 44 | 635.53. |                       |      |       | 1.13 | 34.80 | 4857  | 42746 | 36813 | 3898 | 3620 | 3885 | 4404 | 4254 | 4330  | 4609  | 48070 | 50169 | 5827 | 5155 | 5684 | 5070 | 5548 | 4893 |
| 6380                              | 2  | 2  | 101.66 | 5779 | 1.52 | .2 | 2       | .82 19 QYAGYVEVDVK    | 1.61 | E-03  | 367  | 57    | 69    | 290   | 379   | 899  | 399  | 580  | 213  | 734  | 43    | 14    | 958   | 379   | 794  | 787  | 588  | 951  |      |      |
|                                   |    |    |        |      |      | 93 | 931.48. | IIENHIPVWFSGDQD       |      |       | 8.28 | 37.70 | 3089  | 34549 | 27766 | 3819 | 4167 | 3495 | 4101 | 3086 | 4277  | 3350  | 35002 | 34013 | 4202 | 3824 | 4435 | 4622 | 4232 | 5085 |
|                                   |    |    |        |      |      | .4 | 3       | .16 47 SVVPLGSR       | 1.83 | E-03  | 177  | 26    | 47    | 794   | 221   | 887  | 767  | 360  | 536  | 621  | 88    | 54    | 142   | 219   | 794  | 127  | 260  | 539  |      |      |
| 4000                              |    |    | 6.81   |      |      | 46 | 625.21. | LVGSQGEIGSWGHEY       |      |       | 1.66 | 20.50 | 8707  | 88909 | 76864 | 8599 | 7505 | 8587 | 8787 | 8575 | 1007  | 1029  | 10301 | 10108 | 1165 | 1088 | 1145 | 1171 | 1060 | 1151 |
| 2430                              | 3  | 3  | 76.2   | E-10 | 1.57 | .4 | 3       | .31 82 VR             | 1.56 | E-08  | 811  | 54    | 09    | 322   | 055   | 287  | 816  | 577  | 6522 | 8978 | 188   | 175   | 9652  | 1174  | 8891 | 5108 | 7908 | 1201 |      |      |
|                                   |    |    |        |      |      | 10 | 851.37. |                       |      |       | 2.56 | 53.70 | 2165  | 25143 | 20596 | 2178 | 2255 | 1766 | 2311 | 1768 | 2006  | 2675  | 25487 | 24412 | 3286 | 2968 | 3001 | 3016 | 2623 | 2663 |
|                                   |    |    |        |      |      | 7. | 2       | .51 4 YIPLSPILEGFVILK | 1.86 | E-03  | 221  | 55    | 34    | 112   | 976   | 956  | 891  | 817  | 308  | 477  | 46    | 49    | 438   | 716   | 014  | 553  | 624  | 292  |      |      |
|                                   |    |    |        |      |      | 94 | 928.16. | LSHSDSDSEVAMAAIIS     |      |       | 7.97 | 41.30 | 2617  | 25684 | 24439 | 2561 | 2793 | 2256 | 2905 | 2319 | 2855  | 2643  | 26142 | 26797 | 3315 | 3007 | 3916 | 4377 | 3149 | 3935 |
|                                   |    |    |        |      |      | .9 | 3       | .46 98 LGLIGAGTNNAR   | 1.94 | E-03  | 137  | 31    | 42    | 961   | 651   | 288  | 967  | 922  | 669  | 140  | 40    | 37    | 199   | 606   | 273  | 889  | 600  | 705  |      |      |
| 4000                              |    |    | 4.01   |      |      | 44 | 410.24. |                       |      |       | 1.73 | 23.90 | 6784  | 69243 | 67533 | 6535 | 5647 | 6353 | 6047 | 6826 | 7196  | 8468  | 84911 | 88410 | 9556 | 8831 | 8878 | 8350 | 8433 | 8800 |
| 2076                              | 2  | 2  | 64.36  | E-11 | 1.57 | .1 | 2       | .25 09 VLSLPYK        | 1.69 | E-10  | 511  | 19    | 00    | 174   | 158   | 531  | 286  | 883  | 084  | 328  | 84    | 46    | 905   | 364   | 836  | 858  | 889  | 044  |      |      |
|                                   |    |    |        |      |      | 70 | 593.40. |                       |      |       | 1.29 | 31.60 | 2216  | 24066 | 21987 | 2351 | 2391 | 2043 | 2489 | 2546 | 2770  | 2669  | 23591 | 25519 | 3101 | 2755 | 2550 | 3010 | 2589 | 3044 |
|                                   |    |    |        |      |      | .7 | 2       | .34 27 LVFANALYFK     | 1.52 | E-02  | 441  | 45    | 24    | 241   | 068   | 119  | 451  | 735  | 767  | 315  | 46    | 00    | 597   | 313   | 879  | 871  | 967  | 276  |      |      |
| 4000                              |    |    | 2.37   |      |      | 60 | 552.43. |                       |      |       | 1.14 | 32.30 | 1094  | 10792 | 11134 | 1061 | 1029 | 9262 | 8815 | 1149 | 1124  | 1291  | 12404 | 13191 | 1412 | 1412 | 1309 | 1249 | 1275 | 1277 |
| 4708                              | 2  | 2  | 83.46  | E-07 | 1.61 | .2 | 2       | .82 68 EPSITFIVAK     | 1.60 | E-05  | 5192 | 708   | 576   | 7008  | 1322  | 634  | 953  | 2818 | 5321 | 8740 | 588   | 110   | 7714  | 8988  | 9615 | 8580 | 7055 | 3791 |      |      |
|                                   |    |    |        |      |      | 58 | 644.39. |                       |      |       | 2.54 | 25.10 | 1087  | 10191 | 12533 | 1152 | 1122 | 9712 | 7772 | 1091 | 1103  | 1165  | 11650 | 12417 | 1236 | 1262 | 1112 | 1172 | 1316 | 1209 |
|                                   |    |    |        |      |      | .1 | 2       | .84 78 SSDSDIDIVPLK   | 1.69 | E-04  | 7838 | 547   | 038   | 0942  | 6367  | 593  | 474  | 4013 | 2676 | 2404 | 716   | 422   | 4163  | 2381  | 5229 | 5733 | 4403 | 2350 |      |      |
| 4000                              |    |    | 1.81   |      |      | 52 | 516.35. |                       |      |       | 5.91 | 19.00 | 4601  | 46060 | 43398 | 4379 | 4364 | 4876 | 5242 | 4862 | 4632  | 5853  | 56788 | 53443 | 5922 | 5376 | 5875 | 6030 | 5928 | 5351 |
| 7621                              | 2  | 2  | 94.13  | E-14 | 1.62 | .9 | 2       | .76 78 ENLDWLSR       | 1.39 | E-08  | 030  | 21    | 55    | 467   | 043   | 365  | 685  | 590  | 622  | 967  | 50    | 31    | 593   | 386   | 673  | 171  | 978  | 009  |      |      |
|                                   |    |    |        |      |      | 42 | 558.58. |                       |      |       | 3.38 | 50.60 | 1048  | 96916 | 11141 | 1259 | 8264 | 1199 | 1727 | 1125 | 1292  | 1801  | 17302 | 16572 | 2470 | 1773 | 2286 | 2260 | 1878 | 1608 |
|                                   |    |    |        |      |      | .2 | 2       | .32 35 LPTAVLSTSAR    | 2.99 | E-07  | 727  | 6     | 70    | 285   | 70    | 939  | 724  | 793  | 043  | 599  | 85    | 64    | 164   | 177   | 847  | 568  | 951  | 426  |      |      |
| 4000                              |    |    | 4.54   |      |      | 73 | 642.26. |                       |      |       | 1.33 | 23.90 | 3164  | 32291 | 31683 | 3283 | 3062 | 3109 | 3407 | 3100 | 3344  | 3588  | 36439 | 37638 | 4111 | 3960 | 3586 | 3832 | 4037 | 3853 |
| 6639                              | 3  | 3  | 127.3  | E-06 | 1.67 | .4 | 2       | .33 94 TEDFLYLINR     | 1.34 | E-02  | 938  | 40    | 61    | 717   | 870   | 893  | 234  | 597  | 591  | 067  | 76    | 07    | 956   | 771   | 292  | 583  | 525  | 406  |      |      |
|                                   |    |    |        |      |      | 91 | 765.65. |                       |      |       | 2.40 | 37.80 | 2658  | 28358 | 23650 | 2768 | 3036 | 2536 | 3093 | 2648 | 2887  | 3399  | 31597 | 33006 | 3982 | 3475 | 3487 | 3408 | 3704 | 3445 |
|                                   |    |    |        |      |      | .6 | 2       | .92 96 DLDFVQLNQLIGR  | 1.68 | E-04  | 250  | 68    | 31    | 849   | 820   | 291  | 745  | 024  | 465  | 829  | 94    | 21    | 581   | 378   | 924  | 770  | 036  | 198  |      |      |
|                                   |    |    |        |      |      | 11 | 772.34. | QELLPLVPLFCQSLLE      |      |       | 1.69 | 69.50 | 1274  | 14808 | 10413 | 1505 | 1402 | 1249 | 1551 | 1363 | 1395  | 1818  | 15920 | 18049 | 1786 | 1650 | 2438 | 2517 | 2784 | 2384 |
|                                   |    |    |        |      |      | 0. | 3       | .74 4 MGTK            | 2.67 | E-02  | 147  | 26    | 10    | 744   | 829   | 977  | 763  | 928  | 567  | 575  | 30    | 88    | 011   | 570   | 278  | 207  | 360  | 799  |      |      |
| 4000                              |    |    | 6.82   |      |      | 57 | 662.37. |                       |      |       | 7.09 | 46.00 | 8356  | 93317 | 90306 | 1198 | 1179 | 1152 | 1218 | 9053 | 9996  | 1179  | 12009 | 12768 | 1540 | 1246 | 1395 | 1315 | 1256 | 1008 |
| 2070                              | 3  | 3  | 113.61 | E-06 | 1.71 | .9 | 2       | .89 7 AVAVVVDPIQSVK   | 1.84 | E-04  | 801  | 64    | 47    | 5984  | 0199  | 5816 | 8866 | 151  | 402  | 2738 | 230   | 802   | 4004  | 1718  | 6822 | 9735 | 3475 | 9582 |      |      |
|                                   |    |    |        |      |      | 45 | 530.49. |                       |      |       | 2.33 | 27.70 | 1463  | 15612 | 14802 | 1625 | 1586 | 1769 | 1864 | 1700 | 1943  | 2048  | 19390 | 19226 | 2389 | 2223 | 1991 | 1997 | 2107 | 1948 |
|                                   |    |    |        |      |      | .7 | 2       | .28 6 WTDGLALQR       | 1.63 | E-04  | 1377 | 965   | 836   | 3859  | 4301  | 6341 | 4396 | 0766 | 2073 | 2079 | 686   | 796   | 3328  | 4623  | 2711 | 2112 | 7589 | 9237 |      |      |
|                                   |    |    |        |      |      | 11 | 121.26. | AGVPMMEVMGLMLGD       |      |       | 1.90 | 83.20 | 1176  | 12597 | 11341 | 1781 | 1590 | 1395 | 2757 | 1498 | 1827  | 1845  | 15191 | 13922 | 2432 | 2168 | 2329 | 2708 | 1875 | 2158 |
|                                   |    |    |        |      |      | 3. | 2       | 5.0 31 FVDEYTVR       | 2.43 | E-03  | 168  | 88    | 39    | 725   | 014   | 060  | 324  | 431  | 282  | 002  | 58    | 82    | 143   | 116   | 350  | 924  | 898  | 571  |      |      |
| 4000                              |    |    | 4.66   |      |      | 32 | 532.23. |                       |      |       | 9.97 | 53.10 | 6849  | 57606 | 73318 | 6932 | 7307 | 6754 | 1106 | 7554 | 9844  | 5673  | 55780 | 50582 | 5591 | 5253 | 4241 | 4601 | 4024 | 5447 |
| 3697                              | 2  | 2  | 61.6   | E-15 | 1.91 | .8 | 2       | .81 51 ALHAELVGVR     | 2.75 | E-03  | 47   | 0     | 7     | 02    | 90    | 46   | 740  | 63   | 81   | 92   | 0     | 3     | 81    | 81    | 15   | 73   | 23   | 93   |      |      |
|                                   |    |    |        |      |      | 88 | 106.38. | IPVLIYSGDQDSVVPL      |      |       | 2.54 | 42.10 | 4894  | 51464 | 51897 | 5126 | 5935 | 5458 | 7892 | 5109 | 9824  | 4835  | 47380 | 47419 | 5103 | 4585 | 4733 | 6101 | 5254 | 7805 |
|                                   |    |    |        |      |      | .3 | 2       | 4.5 09 IGSR           | 2.14 | E-05  | 28   | 3     |       |       |       |      |      |      |      |      |       |       |       |       |      |      |      |      |      |      |

|      |   |   |        |      |      |    |        |                           |       |       |      |       |       |       |       |      |      |      |      |      |       |       |       |       |      |      |      |      |      |      |
|------|---|---|--------|------|------|----|--------|---------------------------|-------|-------|------|-------|-------|-------|-------|------|------|------|------|------|-------|-------|-------|-------|------|------|------|------|------|------|
|      |   |   |        |      |      | 81 | 580.50 | ACIVFFDEVDAIGGA           | 4.00  | 50.40 | 2405 | 26031 | 19943 | 2458  | 2874  | 2684 | 2860 | 2199 | 2696 | 3262 | 32802 | 31544 | 3460  | 3179  | 4151 | 5290 | 5036 | 4481 |      |      |
|      |   |   |        |      |      | .3 | 3      | .62 5 R                   | 2.65  | E-03  | 017  | 81    | 39    | 337   | 192   | 835  | 897  | 389  | 016  | 307  | 32    | 77    | 509   | 671   | 303  | 589  | 851  | 758  |      |      |
|      |   |   |        |      |      | 37 |        | 802 16.                   |       |       | 1520 | 15165 | 16677 | 1572  | 1479  | 1591 | 1919 | 1452 | 1479 | 1821 | 19563 | 21032 | 2342  | 2068  | 2272 | 1972 | 1955 | 1765 |      |      |
|      |   |   |        |      |      | .0 | 2      | .38 12 QMMQEEQLQVAR       | 1.61  | E-05  | 588  | 54    | 05    | 217   | 980   | 458  | 819  | 466  | 938  | 230  | 16    | 83    | 325   | 085   | 392  | 797  | 774  | 195  |      |      |
| 4000 |   |   |        |      |      | 47 |        | 550 39.                   |       |       | 4.44 | 33.60 | 1396  | 15415 | 15014 | 1843 | 1856 | 1748 | 2056 | 1629 | 2148  | 2713  | 26247 | 25366 | 3091 | 2869 | 2967 | 2865 | 3149 | 3205 |
| 4460 | 4 | 4 | 325.62 | 0    | 2.07 | .9 | 2      | .30 96 QVSVDPDIK          | 2.30  | E-16  | 071  | 44    | 70    | 998   | 580   | 992  | 952  | 684  | 244  | 261  | 88    | 22    | 153   | 471   | 884  | 018  | 865  | 298  |      |      |
|      |   |   |        |      |      | 10 |        | 108 10 TPGFSGADLANLLNE    |       |       | 1.84 | 47.70 | 5195  | 52875 | 54470 | 6531 | 6106 | 5845 | 6861 | 5472 | 6437  | 8162  | 77915 | 74191 | 8793 | 8640 | 8066 | 8316 | 8498 | 8428 |
|      |   |   |        |      |      | 7. | 2      | 6.05.7 AAILAGR            | 1.69  | E-06  | 472  | 49    | 20    | 778   | 128   | 656  | 628  | 142  | 415  | 932  | 12    | 34    | 633   | 601   | 440  | 783  | 711  | 882  |      |      |
|      |   |   |        |      |      | 86 |        | 140 33. AAEEVIFGEPEVTTG   |       |       | 5.69 | 42.50 | 4349  | 46298 | 53740 | 5787 | 5340 | 5123 | 5032 | 5007 | 6289  | 7743  | 80841 | 74056 | 7607 | 8139 | 6563 | 5661 | 6988 | 7734 |
|      |   |   |        |      |      | .1 | 2      | 8.2 13 AAGDLQQITGLAK      | 1.87  | E-07  | 62   | 7     | 9     | 26    | 11    | 54   | 19   | 54   | 98   | 63   | 8     | 9     | 02    | 42    | 28   | 62   | 39   | 29   |      |      |
|      |   |   |        |      |      | 99 |        | 781 64. VDLFENGTTAIVEAVS  |       |       | 2.51 | 53.10 | 2705  | 29510 | 22496 | 3021 | 3782 | 2742 | 3079 | 3341 | 3221  | 4244  | 46509 | 44375 | 4468 | 4421 | 5578 | 6171 | 6110 | 6469 |
|      |   |   |        |      |      | .8 | 3      | .74 35 PELGNR             | 2.88  | E-05  | 936  | 37    | 15    | 752   | 188   | 320  | 333  | 767  | 356  | 468  | 41    | 50    | 267   | 358   | 238  | 568  | 869  | 837  |      |      |
| 4000 |   |   |        |      |      | 51 |        | 512 25.                   |       |       | 0.00 | 41.90 | 9247  | 10002 | 95952 | 1360 | 1395 | 1347 | 2731 | 1053 | 1622  | 2399  | 25475 | 21232 | 3285 | 2634 | 2846 | 3682 | 2912 | 3567 |
| 3179 | 3 | 3 | 139.99 | 0    | 2.46 | .3 | 2      | .23 89 WDEVFGR            | 3.98  | E+0   | 90   | 68    | 1     | 357   | 689   | 152  | 179  | 965  | 668  | 376  | 82    | 12    | 278   | 075   | 477  | 488  | 305  | 941  |      |      |
|      |   |   |        |      |      | 35 |        | 875 71.                   |       |       | 4.84 | 46.60 | 2839  | 26320 | 30292 | 3389 | 3055 | 3276 | 4712 | 2620 | 3474  | 4331  | 47302 | 37083 | 4356 | 4078 | 4626 | 5153 | 3587 | 5151 |
|      |   |   |        |      |      | .8 | 2      | .35 11 SSGNFCTQCEAEGFR    | 1.97  | E-07  | 534  | 35    | 95    | 233   | 147   | 292  | 571  | 805  | 089  | 793  | 24    | 62    | 314   | 648   | 507  | 977  | 105  | 057  |      |      |
|      |   |   |        |      |      | 11 |        | 132 42. EYDLDFNIVA VPDF   |       |       | 4.55 | 230.8 |       |       |       |      |      |      |      |      |       |       |       |       |      |      |      |      |      |      |
|      |   |   |        |      |      | 1. | 2      | 3.1 99 NMGAMENK           | 39.29 | E-03  | 0    | 7297  | 6622  | 6682  | 8     | 9881 | 3582 | 66   | 7214 | 3590 | 6     | 38110 | 19198 | 9863  | 6    | 1    | 51   | 1    | 2    |      |
| 4000 |   |   | 2.22   |      |      | 37 |        | 625 34.                   |       |       | 5.27 | 83.70 | 2744  | 29355 | 33396 | 2638 | 2170 | 2012 | 1649 | 2570 | 2256  | 1701  | 14257 | 16128 | 1845 | 1416 | 1733 | 9541 | 1235 | 1159 |
| 6427 | 3 | 3 | 105.36 | E-16 | 2.53 | .9 | 2      | .33 55 YTPSQINSLAR        | 3.50  | E-04  | 454  | 25    | 09    | 427   | 834   | 098  | 947  | 622  | 987  | 555  | 25    | 62    | 138   | 987   | 764  | 36   | 423  | 019  |      |      |
|      |   |   |        |      |      | 65 |        | 923 34.                   |       |       | 5.41 | 43.40 | 2312  | 23161 | 26845 | 1966 | 1887 | 1822 | 1894 | 2106 | 1895  | 1572  | 14957 | 16325 | 1554 | 1534 | 1545 | 1442 | 1245 | 1190 |
|      |   |   |        |      |      | .0 | 2      | .99 52 IIVPETVSFTQINEK    | 2.25  | E-08  | 460  | 26    | 23    | 186   | 792   | 896  | 701  | 336  | 732  | 879  | 85    | 79    | 416   | 411   | 895  | 257  | 439  | 619  |      |      |
|      |   |   |        |      |      | 80 |        | 968 36. YLFSDPNPTAELIGG   |       |       | 0.00 | 24.20 | 2256  | 23283 | 24714 | 2045 | 1915 | 1806 | 1988 | 2092 | 1936  | 1044  | 10847 | 10633 | 1007 | 1047 | 1042 | 1064 | 9838 | 1003 |
|      |   |   |        |      |      | .5 | 2      | .50 29 TK                 | 2.51  | E+0   | 380  | 26    | 14    | 865   | 829   | 803  | 701  | 542  | 239  | 872  | 88    | 13    | 798   | 209   | 591  | 201  | 02   | 712  |      |      |
| 4000 |   |   | 1.28   |      |      | 29 |        | 606 13. LGNKEEFEGESAFHP   |       |       | 1.22 | 107.3 | 7580  | 43214 | 45980 | 5038 | 7983 | 3787 | 1454 | 7137 | 5376  | 2360  | 23094 | 40359 | 2759 | 3267 | 3743 | 2554 | 1785 | 2190 |
| 3252 | 2 | 2 | 30.19  | E-08 | 2.56 | .9 | 3      | .96 22 K                  | 8.15  | E-03  | 0    | 32    | 2     | 3     | 56    | 00   | 25   | 617  | 18   | 87   | 48    | 2     | 8     | 28    | 59   | 33   | 03   | 75   | 69   |      |
|      |   |   |        |      |      | 29 |        | 641 16.                   |       |       | 1.45 | 86.50 | 9600  | 10033 | 11310 | 1102 | 1080 | 8779 | 1937 | 9395 | 1229  | 4027  | 33470 | 43350 | 4135 | 2664 | 4289 | 3379 | 2694 | 2569 |
|      |   |   |        |      |      | .9 | 2      | .33 97 SAHPTWSPSAIK       | 7.54  | E-12  | 53   | 77    | 58    | 556   | 545   | 26   | 881  | 60   | 889  | 21   | 7     | 4     | 72    | 36    | 89   | 04   | 49   | 36   |      |      |
| 4000 |   |   |        |      |      | 32 |        | 368 21.                   |       |       | 4.57 | 48.60 | 1737  | 18642 | 15297 | 1554 | 1165 | 1477 | 1423 | 2219 | 2179  | 8259  | 78771 | 81736 | 7888 | 8328 | 7915 | 8264 | 9170 | 9310 |
| 3949 | 7 | 7 | 477.94 | 0    | 2.68 | .6 | 3      | .20 31 ASYHIVSigr         | 2.82  | E-09  | 6647 | 533   | 540   | 8264  | 3365  | 6822 | 1550 | 0117 | 0545 | 202  | 89    | 13    | 881   | 501   | 867  | 900  | 483  | 894  |      |      |
|      |   |   |        |      |      | 40 |        | 751 72.                   |       |       | 0.00 | 29.70 | 3941  | 35057 | 38870 | 3181 | 3091 | 3016 | 3997 | 4619 | 4556  | 1707  | 15452 | 15837 | 1621 | 1591 | 1468 | 1503 | 1724 | 1693 |
|      |   |   |        |      |      | .0 | 2      | .88 63 YNSDVGQPIGTPVR     | 3.15  | E+0   | 6749 | 925   | 075   | 2287  | 2603  | 8833 | 8023 | 7917 | 6471 | 0251 | 641   | 052   | 1771  | 4271  | 3699 | 6678 | 0235 | 2013 |      |      |
|      |   |   |        |      |      | 53 |        | 526 26.                   |       |       | 0.00 | 22.80 | 2144  | 22390 | 22560 | 2036 | 1852 | 1993 | 2224 | 2884 | 2949  | 1150  | 11226 | 11731 | 1083 | 1094 | 1090 | 1171 | 1390 | 1310 |
|      |   |   |        |      |      | .8 | 2      | .26 73 DQPFVNFK           | 2.72  | E+0   | 5676 | 869   | 090   | 5803  | 8609  | 9126 | 8461 | 3283 | 3686 | 3838 | 329   | 674   | 5340  | 6673  | 0020 | 7446 | 7095 | 7698 |      |      |
|      |   |   |        |      |      | 41 |        | 576 42.                   |       |       | 6.22 | 43.10 | 1994  | 20084 | 22424 | 2022 | 1865 | 2238 | 2288 | 2974 | 2921  | 1405  | 12484 | 15000 | 1207 | 1112 | 1094 | 1107 | 1198 | 1471 |
|      |   |   |        |      |      | .3 | 2      | .29 73 VGDYDVS LGAR       | 2.72  | E-09  | 1333 | 648   | 939   | 6349  | 8490  | 0447 | 5010 | 7275 | 7026 | 7470 | 355   | 019   | 3416  | 8039  | 0600 | 2697 | 0623 | 0238 |      |      |
|      |   |   |        |      |      | 66 |        | 898 96. LLETGGTLGQGDSSW   |       |       | 0.00 | 25.70 | 3043  | 29544 | 32180 | 2693 | 2605 | 2458 | 2896 | 3362 | 3371  | 1628  | 15360 | 16417 | 1555 | 1513 | 1514 | 1578 | 1608 | 1617 |
|      |   |   |        |      |      | .5 | 2      | .45 34 FK                 | 2.23  | E+0   | 1827 | 243   | 398   | 7718  | 0261  | 7793 | 3644 | 2560 | 0480 | 4420 | 319   | 320   | 5656  | 5529  | 8266 | 1545 | 8913 | 3442 |      |      |
|      |   |   |        |      |      | 38 |        | 719 67.                   |       |       | 5.23 | 82.60 | 5808  | 46521 | 64748 | 4755 | 4628 | 3588 | 5607 | 5315 | 5246  | 1832  | 15712 | 15488 | 1965 | 1730 | 1607 | 1214 | 1713 | 1809 |
|      |   |   |        |      |      | .0 | 2      | .37 98 LVLDTNGNLDHR       | 5.33  | E-11  | 497  | 68    | 45    | 376   | 730   | 798  | 222  | 301  | 017  | 924  | 24    | 07    | 483   | 401   | 828  | 096  | 770  | 789  |      |      |
|      |   |   |        |      |      | 76 |        | 137 10 SDYSTGSGIFENQDV    |       |       | 0.00 | 34.90 | 8206  | 77035 | 81221 | 6881 | 6338 | 6528 | 7238 | 9907 | 9387  | 3086  | 28393 | 29383 | 2497 | 2585 | 2412 | 2463 | 2627 | 2666 |
|      |   |   |        |      |      | .7 | 2      | 5.65.3 NVQFDIATSR         | 4.11  | E+0   | 567  | 88    | 99    | 881   | 503   | 745  | 874  | 019  | 222  | 973  | 77    | 80    | 612   | 417   | 211  | 942  | 667  | 233  |      |      |
| 4000 |   |   |        |      |      | 33 |        | 474 33.                   |       |       | 8.96 | 94.00 | 4955  | 69287 | 55886 | 8694 | 9025 | 8923 | 2412 | 6273 | 1044  | 1479  | 13950 | 11411 | 2371 | 1515 | 1966 | 2081 | 1453 | 1583 |
| 5301 | 4 | 3 | 214.99 | 0    | 2.81 | .8 | 3      | .28 38 ELLKPSASVALHR      | 4.87  | E-13  | 55   | 8     | 3     | 91    | 49    | 16   | 908  | 81   | 001  | 118  | 49    | 93    | 598   | 556   | 294  | 498  | 765  | 381  |      |      |
|      |   |   |        |      |      | 10 |        | 101 68. ENAPAIIFIDEVDAIAT |       |       | 5.37 | 40.30 | 4942  | 49648 | 46981 | 6329 | 6457 | 6014 | 9955 | 5137 | 6567  | 9050  | 97900 | 75390 | 1149 | 9143 | 9830 | 1154 | 9298 | 9860 |
|      |   |   |        |      |      | 3. | 2      | 5.0 33 AR                 | 2.46  | E-14  | 277  | 00    | 64    | 680   | 592   | 298  | 200  | 813  | 555  | 566  | 64    | 85    | 6051  | 319   | 782  | 8907 | 191  | 139  |      |      |
|      |   |   |        |      |      | 54 |        | 104 35. TETSITDVPDGEDDL   |       |       | 2.01 | 43.80 | 1318  | 15291 | 14765 | 1115 | 1267 | 1161 | 1406 |      |       |       |       |       |      |      |      |      |      |      |

|                                 |    |   |             |  |    |                          |                 |   |
|---------------------------------|----|---|-------------|--|----|--------------------------|-----------------|---|
| 4000                            |    |   | 4.45        | <b>60S ribosomal protein L12</b>               | 45 | 441 44.                  | 8.1337.70       | 4803 57773 54129 6892 6500 6640 7279 5167 4825 7459 7281078551 9448 7900 9144 7943 8481 5903  |
| 0868                            | 5  | 5 | 274.61 E-08 | 1.69   | .7 | 2 .28 65IGPLGLSPK        | 1.97 E-06       | 4253 345 057 2969 5216 6918 9077 2408 3631 0070 349 714 1567 1025 7716 9479 8006 0909         |
|                                 |    |   |             |  | 36 | 672 57.                  | 4.9148.20       | 7980 94311 87408 1093 9585 1077 1046 9254 8437 1122 10572 12247 1235 1183 1278 1115 9719 9572 |
|                                 |    |   |             |  | .7 | 2 .36 42VTGGEVGAASSLAPK  | 1.60 E-02       | 8540 918 333 6249 2657 8322 2066 5580 7898 0242 8294 6972 3672 8389 6387 4853 7573 3293       |
|                                 |    |   |             |  | 66 | 627 38.                  | 1.6446.50       | 5246 61423 63876 7595 7743 7427 8057 5973 5623 8439 8381690046 9512 7563 9729 7923 8854 5906  |
|                                 |    |   |             |  | .0 | 2 .39 76VSVVPSAAALVIK    | 1.85 E-02       | 3255 297 658 3433 9718 5567 5138 8550 1767 9447 474 724 9372 9685 8637 3321 0948 7406         |
|                                 |    |   |             |  | 53 | 847 83.EILGTCVSVGCTVDG   | 4.9018.60       | 4063 45457 45226 5295 5257 5482 5649 4490 4362 5803 5843261326 6530 6116 6265 6075 6398 5068  |
|                                 |    |   |             |  | .6 | 2 .91 6K                 | 1.61 E-13       | 1685 798 615 0690 8658 1806 5192 3411 2358 2420 029 167 4315 2094 8445 4381 1335 5898         |
|                                 |    |   |             |  | 63 | 546 50.                  | 2.0836.70       | 1938 23592 20170 2699 2632 2776 2541 2256 2155 2953 29347 31592 3462 3213 3491 3503 3626 2849 |
|                                 |    |   |             |  | .6 | 3 .62 18HNGNISLDDVIEIAK  | 1.87 E-07       | 2815 851 480 1941 0019 3436 3632 2136 9357 9749 830 886 5933 8024 4513 1799 9966 5241         |
| 4000                            |    |   | 5.38        | <b>Ribosomal protein L17-like protein</b>      | 53 | 475 21.                  | 4.2960.40       | 5125 70649 65387 7807 7112 8063 9947 5787 5714 1315 11965 12094 1551 1224 1598 1523 1505 1005 |
| 0977                            | 2  | 2 | 68.27 E-05  | 2.14   | .7 | 2 .29 12NLYHSVK          | 3.12 E-05       | 960 51 72 690 849 966 799 196 184 7633 170 733 7044 1974 8603 7359 9649 1568                  |
|                                 |    |   |             |  | 65 | 789 47.LPSACVGDMMATV     | 4.0534.60       | 7181 66755 69245 7128 7235 7083 8441 6168 6688 8602 81465 78594 9993 8219 9582 9337 8305 7087 |
|                                 |    |   |             |  | .3 | 2 .90 15K                | 1.62 E-03       | 548 37 85 249 061 529 317 125 751 311 67 02 057 551 273 271 899 423                           |
| 4000                            |    |   | 0.000       | <b>60S acidic ribosomal protein P0</b>         | 89 | 990 82.NVLAIAIETDYSFPLA  | 3.9833.60       | 5419 54603 52507 6056 5996 5686 5673 5010 4907 7293 7286072113 7548 6999 7778 7695 7546 6400  |
| 6021                            | 3  | 3 | 409.07 3934 | 2.30   | .6 | 2 .52 92DK               | 1.59 E-04       | 1446 319 922 2025 3370 3275 9574 9094 3694 6427 489 104 5194 9760 8855 2559 5612 5120         |
|                                 |    |   |             |  | 11 | 103 12MCQLLDDFTQVLVA     | 2.7364.70       | 1762 18155 12767 2994 2244 2090 2794 2002 2036 2704 22424 26015 3362 2663 3948 4560 4115 3540 |
|                                 |    |   |             |  | 4. | 3 6.1 1.2AADNVGSNQLQSIR  | 3.57 E-03       | 1552 087 586 8368 5663 4858 2932 0995 6561 8844 777 681 8326 8945 7588 6285 9158 7124         |
|                                 |    |   |             |  | 11 | 104 87.MCQLLDDFTQVLVA    | 4.9270.40       | 1458 17478 12065 2326 2050 1729 1964 1958 1751 2373 20085 22062 2089 2022 2798 3112 3465 3003 |
|                                 |    |   |             |  | 2. | 3 1.5 58AADNVGSNQLQSIR   | 2.87 E-02       | 7465 373 358 1503 6327 5851 4090 5881 3040 9640 943 580 0877 7602 9932 2308 1980 1783         |
| 4000                            |    |   |             | <b>60s acidic ribosomal protein p3-like</b>    | 51 | 479 31.                  | 0.0021.20       | 8338 87310 88155 8367 7728 7892 6786 8477 6496 5835 56235 64767 6414 6124 6211 5258 6103 4355 |
| 2195                            | 2  | 2 | 204.3 0     | 2.38   | .9 | 2 .25 29GVFTFVCK         | 2.02 E+0        | 4555 587 212 5162 3836 7988 4774 2851 3762 5644 930 799 5098 7179 4987 6588 0282 6270         |
|                                 |    |   |             |  | 42 | 562 72.                  | 2.7571.80       | 6325 69345 61553 6295 5499 6822 4968 7322 5360 3469 33250 36703 3472 3564 3546 3570 3550 2592 |
|                                 |    |   |             |  | .8 | 3 .59 63GDLEASASCTYDLQR  | 2.82 E-04       | 3047 691 108 2416 9125 3667 7259 1904 7446 7259 623 672 0229 7546 3579 2517 9615 7070         |
|                                 |    |   |             |  | 42 | 843 10                   | 0.0042.80       | 7922 74739 81699 7171 7204 6720 7246 7419 5711 3621 35623 39196 3912 3833 3836 3335 3658 2794 |
|                                 |    |   |             |  | .9 | 2 .38 0.3GDLEASASCTYDLQR | 2.92 E+0        | 1520 048 739 8352 1259 6431 4583 6749 1024 7297 849 606 6105 3723 4928 1070 1958 6391         |
| 4000                            |    |   | 9.16        | <b>40S ribosomal protein S10-like</b>          | 78 | 829 53.                  | 1.1859.70       | 3258 40486 39569 5385 5273 5316 9934 3944 4520 1180 10993 88987 1324 1027 1299 1548 1180 9791 |
| 6369                            | 2  | 2 | 73.17 E-12  | 4.47   | .1 | 2 .97 4TYLNLPEIVPATLK    | 4.75 E-10       | 559 12 50 243 231 760 920 723 226 4075 571 58 8969 9850 7049 3135 2742 1160                   |
|                                 |    |   |             |  | 64 | 495 19.                  | 4.4852.70       | 1875 22762 20940 2729 2595 2927 3968 2169 2398 5409 47151 38755 6533 4659 6226 7456 5795 5067 |
|                                 |    |   |             |  | .8 | 3 .96 77HPLIDVPLQVIK     | 3.98 E-12       | 663 14 12 218 363 247 822 138 875 549 24 04 566 496 756 219 597 280                           |
| Proteins of lignin biosynthesis |    |   |             |  |    |                          |                 |   |
| 4000                            |    |   |             | <b>Putative cinnamyl alcohol dehydrogenase</b> | 53 | 532 49.                  | 0.00 32.50 1038 | 11447 11642 1200 1205 1292 1461 1121 1435 3363 32037 35051 3807 3621 3597 3649 3859 3674      |
| 4051                            | 12 | 5 | 726.45 0    | 2.76   | .7 | 2 .79 81 LGADSFLVSR      | 3.72 E+0        | 3456 1108 1732 6138 1742 2555 2106 8863 7500 2184 9688 0353 2262 1057 4853 4355 3870 0494     |
|                                 |    |   |             |  | 99 | 597 19.LVMVGAPEKPLELPV   | 2.50 109.2      | 4762 43251 31415 3057 6428 3920 4348 3814 4335 1307 15086 13413 7987 9387 2382 2474 1576 2333 |
|                                 |    |   |             |  | .0 | 4 .86 31 FPLLLGR         | E-06            | 0 61 0 0 03 64 12 74 95 48 037 37 98 62 92 685 844 078 475                                    |
|                                 |    |   |             |  | 95 | 802 47.LVMVGAPEKPLELPV   | 0.00 40.50 1143 | 11905 12685 1114 1315 1272 1381 1216 1452 2449 26587 23956 1922 2389 2465 2440 2417 2750      |
|                                 |    |   |             |  | .7 | 3 .14 81 FPLLLGR         | 2.47 E+0        | 2467 7283 2558 1363 5980 1088 7786 5889 4793 7812 2423 8559 3290 8943 5532 6218 7120 9910     |
|                                 |    |   |             |  | 60 | 648 34.HSITPDVEVVPMDYV   | 0.00 33.40      | 7727 10007 91245 1014 1006 1149 1146 1027 1267 3386 30592 30472 2921 2906 2787 3134 3053 2672 |
|                                 |    |   |             |  | .8 | 3 .32 18 NK              | E+0             | 983 636 11 8322 6051 4199 9768 2908 6022 902 15 28 871 329 377 739 250 126                    |
|                                 |    |   |             |  | 77 | 141 17.NEWSNSIYPMVPGHE   | 2.16 109.8      | 1601 14070 22296 1676 1604 1606 2055 1633 1757 4103 39568 49722 4412 3656 3163 2958 2727 2745 |
|                                 |    |   |             |  | .9 | 2 4.6 43 VVGVVTEIGSK     | 8.17 E-10       | 0 032 16 67 724 480 937 415 243 055 85 1 6 19 76 32 93 87 09                                  |
| 4000                            |    |   |             | <b>Putative cinnamyl alcohol dehydrogenase</b> | 74 | 120 59.HNITPDVEVVPMDYV   | 6.66 52.60      | 1196 10535 13091 1051 1163 1013 1282 9701 1102 2970 29537 29890 3301 2981 2866 2741 2596 2405 |
| 8082                            | 4  | 2 | 270.9 0     | 3.60   | .9 | 2 6.5 09 NTALER          | E-16            | 6718 518 383 1502 0892 0550 9234 801 3810 8584 388 038 9920 3817 2266 7762 2961 1910          |
|                                 |    |   |             |  | 66 | 810 33.HNITPDVEVVPMDYV   | 0.00 31.80      | 2540 23027 27792 2518 2710 2567 2674 2614 2780 6150 64362 61511 5877 6168 5647 5919 6571 7774 |
|                                 |    |   |             |  | .0 | 3 .07 02 NTALER          | 3.38 E+0        | 8697 795 197 7391 4888 8610 3793 0809 1277 3554 643 715 3179 7075 2879 4618 1998 2263         |
| 4000                            |    |   | 9.88        | <b>Lignin-forming anionic peroxidase-like</b>  | 67 | 927 62.                  | 1.18 24.60      | 5943 46755 59483 4483 5412 4635 4571 5068 6184 6422 71491 74467 6612 6863 5906 6397 6913 9040 |
| 8188                            | 4  | 4 | 205.19 E-15 | 2.16   | .4 | 2 .42 04 STPDEFDNYFTNLK  | 2.02 E-13       | 565 84 20 736 185 482 751 425 435 958 75 22 411 721 877 090 743 000                           |
|                                 |    |   |             |  | 40 | 824 46.LCPQGGNGGTFANLD   | 1.83 63.60      | 2331 23637 22950 2077 2264 1848 1795 1820 2451 2839 28478 33043 3177 3182 3184 2693 3161 3783 |
|                                 |    |   |             |  | .6 | 2 .89 21 K               | E-07            | 627 56 36 278 202 680 137 597 196 812 89 30 708 254 641 071 567 133                           |
|                                 |    |   |             |  | 93 | 121 70.SGANSDIPSPETLAV   | 4.83 51.10      | 6129 52724 68491 5399 5976 5573 5630 5431 6878 6841 71368 74828 7597 7264 7270 6876 7694 9099 |
|                                 |    |   |             |  | .0 | 2 6.6 62 MIPQFTR         | 1.73 E-06       | 710 74 90 124 020 783 949 104 246 471 91 87 148 016 076 662 097 086                           |

|      |   |   |       |      |      |    |   |  |           |      |       |       |      |      |      |      |      |      |      |       |       |      |      |      |      |      |      |      |
|------|---|---|-------|------|------|----|---|--|-----------|------|-------|-------|------|------|------|------|------|------|------|-------|-------|------|------|------|------|------|------|------|
|      |   |   |       |      |      | 77 | 10826.LSPTFYDCTCPNVIDI                        |  | 3.5154.60 | 1515 | 12436 | 16155 | 1344 | 1643 | 1263 | 1407 | 1188 | 1659 | 2151 | 23859 | 23130 | 2517 | 2139 | 2171 | 2352 | 2569 | 3176 |      |
|      |   |   |       |      |      | .7 | 2 5.532 VR                                    |  | 2.67      | E-05 | 178   | 13    | 97   | 524  | 619  | 837  | 235  | 038  | 706  | 121   | 90    | 65   | 383  | 033  | 653  | 217  | 998  | 700  |
| 4000 |   |   | 2.68  |      |      |    | <b>Lignin-forming anionic peroxidase-like</b> |  |           |      |       |       |      |      |      |      |      |      |      |       |       |      |      |      |      |      |      |      |
| 8200 | 2 | 2 | 78.32 | E-13 | 2.20 | 80 | 62055.GMDITDLVALSGAHT                         |  | 1.8855.60 | 6145 | 55118 | 52797 | 5427 | 5834 | 5789 | 5135 | 5440 | 6902 | 8156 | 81123 | 95269 | 9060 | 9085 | 9950 | 9986 | 1131 | 1318 |      |
|      |   |   |       |      |      | .4 | 3 .9809 FGR                                   |  | 2.57      | E-12 | 121   | 85    | 02   | 325  | 236  | 961  | 962  | 924  | 146  | 324   | 51    | 05   | 653  | 531  | 672  | 488  | 9445 | 8363 |
|      |   |   |       |      |      | 51 | 74323.  |  | 3.4370.60 | 1531 | 16427 | 18595 | 1280 | 1721 | 1508 | 1783 | 1544 | 1542 | 3617 | 29969 | 44486 | 3938 | 4129 | 3834 | 3691 | 3431 | 4962 |      |
|      |   |   |       |      |      | .3 | 2 .4023 LGNIGALTGTNGEIR                       |  | 3.88      | E-07 | 399   | 13    | 30   | 709  | 592  | 307  | 684  | 765  | 826  | 076   | 66    | 76   | 260  | 910  | 418  | 824  | 081  | 830  |
| 4000 |   |   |       |      |      | 65 | 67140.  |  | 2.0031.70 | 4063 | 40286 | 39822 | 5291 | 5577 | 5748 | 9650 | 4199 | 7241 | 7706 | 72660 | 70371 | 9312 | 7453 | 9244 | 1024 | 7865 | 8620 |      |
| 2931 | 4 | 4 | 193.3 | 0    | 2.48 | .3 | 2 .3992TLVDVGGGLGVNLK                         |  | 2.57E-15  | 936  | 20    | 68    | 536  | 736  | 119  | 739  | 396  | 689  | 782  | 20    | 86    | 862  | 566  | 408  | 3643 | 177  | 081  |      |
|      |   |   |       |      |      | 57 | 53338.  |  | 6.7742.80 | 2773 | 32104 | 27581 | 3936 | 4299 | 3948 | 5688 | 3232 | 4757 | 5506 | 52249 | 49433 | 6711 | 5592 | 6532 | 7715 | 5979 | 6214 |      |
|      |   |   |       |      |      | .0 | 3 .6176LSALEIVSHMPNCK                         |  | 2.80E-14  | 995  | 25    | 70    | 339  | 697  | 576  | 974  | 088  | 134  | 090  | 48    | 74    | 932  | 276  | 075  | 931  | 223  | 526  |      |
|      |   |   |       |      |      | 26 | 64236.  |  | 9.1561.60 | 2679 | 29361 | 34283 | 4272 | 4387 | 3825 | 8004 | 3244 | 4585 | 4511 | 44601 | 39786 | 4958 | 4164 | 5120 | 4929 | 3740 | 3768 |      |
|      |   |   |       |      |      | .1 | 2 .3355VHGVHAFEYPK                            |  | 2.99E-03  | 810  | 67    | 14    | 770  | 717  | 324  | 483  | 806  | 741  | 539  | 91    | 67    | 692  | 378  | 519  | 564  | 298  | 309  |      |
|      |   |   |       |      |      | 98 | 59177.DEDGASMGPLLALLQ                         |  | 6.8975.70 | 9361 | 88845 | 63964 | 1072 | 1131 | 1088 | 1762 | 8626 | 1248 | 1202 | 12020 | 12319 | 1312 | 1069 | 2136 | 2782 | 1773 | 1976 |      |
|      |   |   |       |      |      | .1 | 3 .6307DK                                     |  | 4.35E-06  | 85   | 1     | 0     | 496  | 907  | 475  | 431  | 14   | 613  | 080  | 83    | 05    | 637  | 933  | 159  | 446  | 543  | 096  |      |

**Appendix 6.** The specific masses of the quantified fragment ion (m/z) along with retention time index of the 65 metabolites, Supplementary Table S3, chapter IV.

|    | <b>Compound name</b>          | <b>TMS</b> | <b>m/z</b>    | <b>RI</b> |
|----|-------------------------------|------------|---------------|-----------|
| 1  | <b>Lactic acid</b>            | 2          | 117.02        | 1071      |
| 2  | <b>L-Alanine (1)</b>          | 2          | 116.081       | 1100      |
| 3  | <b>Oxalic acid</b>            | 2          | 100.02        | 1137      |
| 4  | <b>Urea</b>                   | 2          | 171.07        | 1144      |
| 5  | <b>Phosphoric acid</b>        | 2          | 241.047       | 1173      |
| 6  | <b>L-Valine</b>               | 2          | 144.11        | 1212      |
| 7  | <b>Ethanolamine</b>           | 3          | 174.1         | 1262      |
| 8  | <b>Nicotinic acid</b>         | 1          | 180.047       | 1288      |
| 9  | <b>L-Isoleucine</b>           | 2          | 158.136       | 1290      |
| 10 | <b>L-Proline</b>              | 2          | 142.104       | 1293      |
| 11 | <b>Glycine</b>                | 3          | 174.106       | 1302      |
| 12 | <b>Succinic acid</b>          | 2          | 247.08        | 1310      |
| 13 | <b>Glyceric acid</b>          | 3          | 292.13        | 1328      |
| 14 | <b>Uracil</b>                 | 2          | 241.08        | 1333      |
| 15 | <b>Fumaric acid</b>           | 2          | 245.06        | 1348      |
| 16 | <b>L-Serine</b>               | 3          | 204.01        | 1358      |
| 17 | <b>L- Threonine</b>           | 3          | 117           | 1384      |
| 18 | <b>Butyric acid</b>           | 3          | 103.06        | 1410      |
| 19 | <b>beta Alanine</b>           | 3          | 174.11        | 1427      |
| 20 | <b>Malic acid</b>             | 3          | 147.06        | 1486      |
| 21 | <b>L- Asparagine (1)</b>      | 2          | 115.08        | 1495      |
| 22 | <b>Threitol</b>               | 4          | 205.1         | 1508      |
| 23 | <b>Oxoproline</b>             | 2          | 156.06        | 1514      |
| 24 | <b>Aspartic acid</b>          | 3          | 231.98        | 1518      |
| 25 | <b>4- Aminobutyric acid</b>   | 3          | 174.09        | 1526      |
| 26 | <b>Threonic acid</b>          | 4          | 292.13        | 1541      |
| 27 | <b>Trihydroxybutyric acid</b> | 4          | 220.09        | 1559      |
| 28 | <b>Oxoglutarate</b>           | 2          | 198.058       | 1571      |
| 29 | <b>Hydrocinnamic acid</b>     | 2          | 193.1         | 1577      |
| 30 | <b>L- Glutamine (1)</b>       | 3          | 227.13        | 1585      |
| 31 | <b>Glutaric acid</b>          | 3          | 247.1         | 1600      |
| 32 | <b>Glutamic acid</b>          | 3          | 246.12        | 1617      |
| 33 | <b>L- Asparagine (2)</b>      | 4          | 188.09        | 1626      |
| 34 | <b>L- Lysine (1)</b>          | 3          | 218.98        | 1630      |
| 35 | <b>Gluconic acid</b>          | 5          | 204.09        | 1647      |
| 36 | <b>Arabinose</b>              | 4          | 217.1         | 1655      |
| 37 | <b>L-Asparagine (3)</b>       | 3          | 116.08        | 1665      |
| 38 | <b>Putrescine</b>             | 4          | 174.1         | 1733      |
| 39 | <b>Glycerol-3-phosphate</b>   | 4          | 299.07        | 1760      |
| 40 | <b>L- Glutamine (3)</b>       | 3          | 155.98        | 1770      |
| 41 | <b>Glucaric acid</b>          | 1          | 333.13        | 1803      |
| 42 | <b>Shikimic acid</b>          | 4          | 204.09        | 1809      |
| 43 | <b>Citric acid</b>            | 4          | 273.09        | 1816      |
| 44 | <b>Quinic acid</b>            | 5          | 345.17        | 1856      |
| 45 | <b>D- Fructose (1)</b>        | 5          | 306.97        | 1870      |
| 46 | <b>D- Fructose (2)</b>        | 6          | 216.97        | 1880      |
| 47 | <b>Glucose</b>                | 5          | 319.15        | 1885      |
| 48 | <b>Internal standard</b>      |            | 327.17/210.11 | 1891      |
| 49 | <b>Mannose\glucose</b>        | 5          | 318.96        | 1894      |
| 50 | <b>L- Lysine (2)</b>          | 4          | 174.11        | 1919      |
| 51 | <b>Sorbitol</b>               | 1          | 319.15        | 1927      |
| 52 | <b>Octopamine</b>             | 4          | 174.11        | 2020      |
| 53 | <b>Palmitic acid</b>          | 1          | 313.25        | 2043      |
| 54 | <b>Inositol</b>               | 6          | 304.96        | 2089      |
| 55 | <b>Galactonic acid</b>        | 6          | 245.1         | 2107      |
| 56 | <b>Sedoheptulose</b>          | 3          | 319.15        | 2132      |
| 57 | <b>L- Tryptophan</b>          | 3          | 202.1         | 2205      |

|    |                              |   |        |      |
|----|------------------------------|---|--------|------|
| 58 | <b>Stearic acid</b>          | 1 | 341.28 | 2239 |
| 59 | <b>Galactose-6-phosphate</b> | 4 | 315.1  | 2315 |
| 60 | <b>Glucose-6-phosphat</b>    | 6 | 387.13 | 2334 |
| 61 | <b>Inositol-2-phosphate</b>  | 7 | 318.14 | 2408 |
| 62 | <b>Trehalose</b>             | 8 | 331.15 | 2551 |
| 63 | <b>Sucrose</b>               | 1 | 216.96 | 2641 |
| 64 | <b>Melibiose</b>             | 8 | 204.09 | 2944 |
| 65 | <b>Ononitol</b>              | 5 | 259.11 | 2971 |

**Appendix 7. Peptides changed in the phosphorylation status in the response to N starvation, Supplementary Table S1, chapter V.**

| RT (min)    | Neutral mass | Peptide Sequence              | Variable Modifications | Localization Probability | Ascore          | Pep. Score | Protein Accession | Description NCBI   | Function               | Anova    | Normalized abundance |           |          |          |  |  |
|-------------|--------------|-------------------------------|------------------------|--------------------------|-----------------|------------|-------------------|--|------------------------|----------|----------------------|-----------|----------|----------|--|--|
|             |              |                               |                        |                          |                 |            |                   |  |                        |          | Lam N+               | Lam N-    | Topas N+ | Topas N- |  |  |
| 10 min      |              |                               |                        |                          |                 |            |                   |  |                        |          |                      |           |          |          |  |  |
| Cluster 1 a |              |                               |                        |                          |                 |            |                   |  |                        |          |                      |           |          |          |  |  |
| 91.05       | 2833.31      | TLSEEGLEDLHLISSmEIGGQNLPR     | S3 Phos, M16 Oxid      | 96%, 100%                | 13.85, 1,000.00 | 141.18     | 400079398         | <b>CCR4-NOT transcription complex subunit 10</b>   | transcription          | 2.38E-02 | 193967               | 1329310   | 169431   | 265328   |  |  |
| 102.76      | 3193.519     | NNPFDVHNDNYDLGLPPIGsAPSVLVR   | S21 Phos               | 100%                     | 29.09           | 109.04     | 400072493         | <b>Uncharacterized protein</b>   | unclassified           | 5.00E-03 | 276071               | 2759973   | 301669   | 388614   |  |  |
| Cluster 1 b |              |                               |                        |                          |                 |            |                   |  |                        |          |                      |           |          |          |  |  |
| 58.55       | 1512.806     | SVtGVSPVIRPPK                 | T3 Phos                | 97%                      | 14.49           | 79.43      | 400050062         | <b>Uncharacterized protein</b>   | unclassified           | 1.70E-02 | 1026540              | 437345    | 1817471  | 4051448  |  |  |
| 80.51       | 1742.861     | IALVGLGsPTSSTAAYR             | S8 Phos                | 100%                     | 46.21           | 213.04     | 400020254         | <b>Leucine aminopeptidase 2</b>  | Prot. Destin. storage  | 3.15E-02 | 108432               | 39759     | 229776   | 1825671  |  |  |
| 67.41       | 2666.296     | ATTSVPQsPFKPLASNVTTESAPTR     | S8 Phos                | 100%                     | 50.51           | 217.49     | 400019030         | <b>Putative vegetative cell wall protein gp1-like</b>                                    | cell structure         | 4.95E-02 | 1630044              | 1104151   | 2366366  | 13925722 |  |  |
| Cluster 1 c |              |                               |                        |                          |                 |            |                   |  |                        |          |                      |           |          |          |  |  |
| 67.71       | 1969.996     | SSPIRPsPILNGVDSVPR            | S7 Phos                | 100%                     | 19.08           | 93.42      | 400063603         | <b>Putative topless-related protein 3-like</b>   | transcription          | 4.89E-03 | 34334747             | 57933070  | 3756647  | 20938937 |  |  |
| 84.28       | 1915.834     | NSPFTFEDSVPGsPLSR             | S13 Phos               | 100%                     | 29.88           | 75.31      | 400025666         | <b>Putative actin cytoskeleton-regulatory complex protein PAN1-like</b>                  | cell structure         | 2.62E-02 | 47616939             | 79211438  | 5423223  | 31747869 |  |  |
| 67.91       | 1838.88      | APDELLsPQHLPEVSK              | S7 Phos                | 100%                     | 77.82           | 218.63     | 400015182         | <b>Putative R3H and coiled-coil domain-containing protein 1-like (Solanum chacoense)</b> | unclear classification | 1.35E-02 | 17749756             | 29996516  | 2798811  | 14542889 |  |  |
| 81.50       | 2210.055     | mSLGLsPLSSTPTGSITPGPK         | M1 Oxid, S6 Phos       | 100%, 100%               | 1,000.00, 39.20 | 146.88     | 400014926         | <b>AT-hook motif nuclear-localized protein 5-like isoform X1</b>                         | transcription          | 3.70E-02 | 2466964              | 4207679   | 553377   | 2835013  |  |  |
| 90.01       | 2105.928     | EGsPLGFGSESAGSGSLFGVK         | S3 Phos                | 100%                     | 106.01          | 218.75     | 400042565         | <b>Nuclear pore complex protein NUP50B-like</b>  | transporter            | 1.75E-02 | 1724120              | 3062284   | 486916   | 2672246  |  |  |
| 84.28       | 2109.122     | VVLVsPPQSPVLQANGISK           | S5 Phos                | 100%                     | 54.03           | 195.87     | 400027620         | <b>Vesicle-associated protein 2-2-like isoform X2</b>                                    | intracellular traffic  | 2.64E-02 | 6379609              | 8053738   | 1064587  | 6140928  |  |  |
| 71.31       | 1973.923     | LHLGLQPPEASNLFNFK             | T1 Phos                | 100%                     | 174.34          | 192.41     | 400010497         | <b>Plasma membrane ATPase</b>  | transporter            | 1.13E-02 | 6825906              | 9041938   | 1028677  | 5051633  |  |  |
| 92.55       | 2159.906     | DDDGILMQPPsPNELHSL            | M8 Oxid, S12 Phos      | 100%, 100%               | 1,000.00, 34.90 | 171.67     | 400012001         | <b>Probable histone-arginine methyltransferase 1.3</b>                                   | transcription          | 1.24E-02 | 60112220             | 106829415 | 7819990  | 61200856 |  |  |
| 75.01       | 2630.268     | SLSmPTSPPTPGTPVTPTNIsPTVR     | M4 Oxid, S21 Phos      | 100%, 98%                | 1,000.00, 20.98 | 313.73     | 400076456         | <b>Auxin repressed/dormancy associated protein</b>                                       | disease-defence        | 1.48E-02 | 15709455             | 22152028  | 1448774  | 11873348 |  |  |
| 60.46       | 3116.345     | INHQNEIDEVQGTDCtIENLGSPPSR    | S23 Phos               | 100%                     | 34.55           | 280.11     | 400007972         | <b>Uncharacterized protein</b>   | unclassified           | 2.33E-02 | 21015106             | 31922559  | 899833   | 7290223  |  |  |
| 80.85       | 1806.874     | LPDSIQTLINQsPSK               | S13 Phos               | 96%                      | 17.01           | 270.61     | 400019030         | <b>Putative vegetative cell wall protein gp1-like</b>                                    | cell structure         | 1.30E-02 | 28204701             | 41940439  | 2457450  | 22656266 |  |  |
| 72.06       | 2036.017     | TKLPDSIQTLINQsPSK             | S15 Phos               | 96%                      | 17.01           | 141.31     | 400019030         | <b>Putative vegetative cell wall protein gp1-like</b>                                    | cell structure         | 6.04E-03 | 7533205              | 15058641  | 1132627  | 5775725  |  |  |
| 90.40       | 2214.053     | NLSPTSPIFTPTSLGPASGYK         | S3 Phos                | 100%                     | 36.69           | 203.16     | 400011739         | <b>Uncharacterized protein</b>   | unclassified           | 4.45E-02 | 804050               | 1610451   | 219648   | 1239812  |  |  |
| 82.55       | 2568.174     | LVLPSDLPsPNSVSESSVMSGQR       | S9 Phos, M20 Oxid      | 100%, 100%               | 41.02, 1,000.00 | 173.33     | 400015310         | <b>Putative activating signal cointegrator 1 complex subunit 2-like</b>                  | transcription          | 1.73E-02 | 9180867              | 23134048  | 2172298  | 8506125  |  |  |
| 86.03       | 2529.324     | DSTILLKPPPPAPLsPVATDAR        | S17 Phos               | 100%                     | 18.11           | 243.53     | 400052626         | <b>Putative adaptin ear-binding coat-associated protein 1-like</b>                       | intracellular traffic  | 1.53E-02 | 2538601              | 6923243   | 763973   | 2938680  |  |  |
| 80.85       | 2582.124     | MYQQA VSGISQISFGDEDTV sPK     | M1 Oxid, S21 Phos      | 100%, 98%                | 1,000.00, 17.98 | 317.85     | 400045704         | <b>Uncharacterized protein</b>   | unclassified           | 2.19E-02 | 9696281              | 30849187  | 2229557  | 12011273 |  |  |
| 74.61       | 3157.492     | NLEEGGSTVDRPQLPVQPADsVEGTVIDR | S21 Phos               | 100%                     | 48.04           | 165.28     | 400079854         | <b>Uncharacterized protein</b>   | unclassified           | 4.70E-03 | 6984669              | 18242944  | 552138   | 5677928  |  |  |
| Cluster 1 d |              |                               |                        |                          |                 |            |                   |  |                        |          |                      |           |          |          |  |  |
| 92.80       | 1616.72      | sPNAFVLDPSFTR                 | S1 Phos                | 100%                     | 175.33          | 212.32     | 400007881         | <b>Plant UBX domain-containing protein 8 isoform X1</b>                                  | unclear classification | 4.92E-02 | 9283733              | 18432854  | 2661182  | 9928714  |  |  |
| 96.28       | 2261.114     | LLATPtiPIGTPLYSIPEENR         | T6 Phos                | 98%                      | 16.24           | 106.89     | 400072223         | <b>Splicing factor 3B subunit 1</b>  | transcription          | 1.62E-02 | 101074               | 209787    | 42688    | 159432   |  |  |
| 82.45       | 2207.065     | IASPtVPGTVPAQVGYWQEK          | T5 Phos                | 100%                     | 27.61           | 74.48      | 400008036         | <b>Uncharacterized protein</b>   | unclassified           | 4.90E-02 | 4676583              | 9193615   | 973360   | 4006843  |  |  |
| 93.04       | 1886.74      | GYsFDDADPFGSTGPFK             | S3 Phos                | 98%                      | 17.98           | 177.89     | 400025666         | <b>Uncharacterized protein</b>   | unclassified           | 2.07E-02 | 11710858             | 19797385  | 2308740  | 9299644  |  |  |

|       |          |                              |                             |                  |                           |        |           |  |                       |          |           |           |         |          |
|-------|----------|------------------------------|-----------------------------|------------------|---------------------------|--------|-----------|--|-----------------------|----------|-----------|-----------|---------|----------|
| 75.86 | 1827.844 | VLDYPISSGITmPsPR             | M12 Oxid, S14 Phos          | 100%, 100%       | 1,000.00, 30.27           | 155.54 | 400035478 | <b>Putative RNA-binding protein 38-like</b>                              | transcription         | 4.15E-02 | 7072687   | 12129475  | 2104305 | 8034169  |
| 51.08 | 2841.212 | AHGNAVGLPTEDDmGNsEVGHNALGAGR | M14 Oxid, S17 Phos          | 100%, 100%       | 1,000.00, 69.01           | 207.54 | 400002454 | <b>Phosphoglycerate mutase</b>   | energy                | 2.46E-02 | 13598835  | 24692755  | 1893974 | 7655487  |
| 70.86 | 1726.853 | VQEIVSEIFGKsPSK              | S12 Phos                    | 97%              | 15.01                     | 186.72 | 400027703 | <b>Heat shock 70 kDa protein,</b>  | disease-defence       | 3.41E-03 | 1248828   | 2280847   | 319211  | 1154229  |
| 90.01 | 1317.611 | SLFSAGFLGsPR                 | S10 Phos                    | 100%             | 76.06                     | 155.33 | 400076377 | <b>VQ motif-containing protein 4-like</b>                                | transcription         | 4.18E-03 | 2761991   | 5672245   | 1137928 | 3579340  |
| 93.84 | 1931.01  | TASVAQTPSILSLPitPR           | T16 Phos                    | 100%             | 53.57                     | 106.82 | 400048002 | <b>Lysine histidine transporter-like 8</b>                               | transporter           | 9.26E-03 | 499452    | 1051588   | 225836  | 712198   |
| 50.55 | 1898.839 | YAGASNLsEGASESLHVK           | S8 Phos                     | 100%             | 43.35                     | 175.63 | 400006894 | <b>Fructose-bisphosphate aldolase</b>                                    | energy                | 1.40E-02 | 5807538   | 10704426  | 1464138 | 4467764  |
| 74.36 | 2016.925 | SmsFVGTHEYLAPEIik            | M2 Oxid, S3 Phos            | 100%, 100%       | 1,000.00, 26.58           | 138.33 | 400050818 | <b>Serine/threonine-protein kinase D6PK-like</b>                         | signal transduction   | 1.32E-02 | 1521629   | 2894700   | 629905  | 1931891  |
| 80.16 | 1444.717 | LsLPPDEVALSK                 | S2 Phos                     | 100%             | 121.14                    | 98.33  | 400000744 | <b>MAPKK</b>   | signal transduction   | 1.47E-02 | 1900103   | 2414327   | 364677  | 1388327  |
| 66.09 | 1939.964 | NLPGKPPDAEVIAlsPK            | S16 Phos                    | 100%             | 1,000.00                  | 194.85 | 400008694 | <b>Protein indeterminate-domain 9-like</b>                               | transcription         | 1.62E-02 | 3082015   | 4124781   | 739125  | 2675130  |
| 60.20 | 1793.929 | QGLNLTQSPsLSLKK              | S11 Phos                    | 100%             | 30.07                     | 143.43 | 400022015 | <b>Putative VHS domain-containing protein-like</b>                       | intracellular traffic | 4.61E-03 | 3205774   | 4385040   | 472393  | 1694225  |
| 73.46 | 2046.952 | DLDKEFAPIAEPAitPK            | T16 Phos                    | 100%             | 1,000.00                  | 243.53 | 400001563 | <b>Probable serine/threonine-protein kinase kinX</b>                     | signal transduction   | 3.79E-02 | 71819927  | 87530427  | 8110379 | 26435438 |
| 77.10 | 1849.868 | TAsGVSLPPLPAETSSEK           | S3 Phos                     | 98%              | 17.98                     | 161.1  | 400022815 | <b>Uncharacterized protein</b>   | unclassified          | 3.47E-02 | 74174010  | 83702386  | 7026189 | 22866294 |
| 80.06 | 1392.609 | FGsFGSPPSVGFK                | S3 Phos                     | 100%             | 72.17                     | 144.22 | 400049833 | <b>Uncharacterized protein</b>   | unclassified          | 2.90E-02 | 3310176   | 4600384   | 1255204 | 3859471  |
| 80.01 | 1606.709 | LVQsPNSFFmDVK                | S4 Phos, M10 Oxid           | 100%, 100%       | 84.18, 1,000.00           | 157.87 | 400042183 | <b>40S ribosomal protein S27</b>   | protein synthesis     | 3.89E-02 | 6828528   | 9859677   | 2096023 | 6990532  |
| 35.60 | 2181.947 | EVDEPPsPPATTHDEEKPK          | S7 Phos                     | 100%             | 37.18                     | 96.52  | 400046956 | <b>Uncharacterized protein</b>   | unclassified          | 2.22E-02 | 600072610 | 120668840 | 4571343 | 8773995  |
| 74.50 | 2361.065 | LATASQLmASSNFPLsPmAANK       | M8 Oxid, S16 Phos, M18 Oxid | 100%, 100%, 100% | 1,000.00, 66.73, 1,000.00 | 108.98 | 400017605 | <b>Putative zinc finger CCCH domain-containing protein 53-like</b>       | transcription         | 5.77E-03 | 1834561   | 3835071   | 972130  | 2299915  |
| 78.16 | 1976.806 | TASSGSFESFDSNSLsFK           | S16 Phos                    | 98%              | 17.99                     | 160.01 | 400018572 | <b>Probable ADP-ribosylation factor GTPase-activating protein AGD14</b>  | intracellular traffic | 3.98E-02 | 1643364   | 3289609   | 701831  | 1505795  |
| 65.91 | 1663.667 | HFFDSTDFGAsPTR               | S11 Phos                    | 100%             | 24.15                     | 176.76 | 400025666 | <b>Uncharacterized protein</b>   | unclassified          | 3.64E-02 | 17762362  | 28662079  | 3622881 | 8073401  |
| 56.85 | 1685.813 | PINQITIGsHEELR               | S9 Phos                     | 100%             | 30.92                     | 104.31 | 400018375 | <b>Tonoplast intrinsic protein</b>                                       | transporter           | 6.93E-04 | 3329078   | 5163386   | 930566  | 2060700  |
| 94.94 | 1406.588 | AFFDsADWALGK                 | S5 Phos                     | 100%             | 1,000.00                  | 159.18 | 400047433 | <b>Uncharacterized protein</b>   | unclassified          | 2.49E-02 | 4219853   | 7070900   | 2694183 | 5528160  |
| 82.84 | 1319.59  | GLEQSFsIGFR                  | S7 Phos                     | 100%             | 20.98                     | 90.45  | 400002592 | <b>Chloroplast calcium-dependent protein kinase 7</b>                    | signal transduction   | 8.63E-04 | 657389    | 1114623   | 514724  | 1009527  |
| 65.76 | 1711.735 | HDTsPQFGGSPLSFR              | S4 Phos                     | 97%              | 15                        | 107.24 | 400073848 | <b>AT-hook motif nuclear-localized protein 14-like</b>                   | transcription         | 2.82E-02 | 1361804   | 2156761   | 252269  | 666151   |
| 94.91 | 1822.881 | ADISGPPsQIFILETR             | S8 Phos                     | 100%             | 35.76                     | 89.36  | 400058304 | <b>Formin-like protein</b>   | cell structure        | 4.48E-02 | 417754    | 703145    | 158430  | 454576   |
| 65.41 | 1841.834 | FSDQALSQAVFsPthk             | S12 Phos                    | 100%             | 17.99                     | 212.36 | 400021057 | <b>Putative zinc finger CCCH domain-containing protein 30-like</b>       | transcription         | 2.31E-02 | 9344386   | 17792206  | 3175991 | 8057514  |
| 63.97 | 1711.731 | HDTsPQFGGSPLSFR              | S10 Phos                    | 100%             | 55.93                     | 112.15 | 400073848 | <b>AT-hook motif nuclear-localized protein 14-like (Capsicum annuum)</b> | transcription         | 4.03E-02 | 1043995   | 1886516   | 171248  | 433696   |
| 77.78 | 2089.004 | ILPESEImLETPAQPtPK           | M8 Oxid, T16 Phos           | 100%, 100%       | 1,000.00, 52.35           | 100.71 | 400039343 | <b>Putative CDT1-like protein a, chloroplastic-like</b>                  | cell growth-division  | 5.42E-03 | 1273245   | 2288770   | 592415  | 1534001  |
| 85.00 | 1485.728 | LStvPLNFVAmAK                | T3 Phos, M11 Oxid           | 98%, 100%        | 16.22, 1,000.00           | 114.32 | 400075869 | <b>65-kDa microtubule-associated protein 6</b>                           | cell structure        | 1.49E-02 | 1708551   | 2995076   | 749714  | 1630710  |
| 84.13 | 1275.566 | GIEGFGsFNIR                  | S7 Phos                     | 100%             | 1,000.00                  | 44.08  | 400001041 | <b>Epsin-3-like isoform X1</b>   | Prot. Destin. storage | 1.88E-02 | 624785    | 1172549   | 465535  | 1029361  |
| 83.29 | 1320.665 | ISDLsPLLVER                  | S5 Phos                     | 100%             | 88.3                      | 156.46 | 400072493 | <b>Uncharacterized protein</b>   | unclassified          | 7.52E-04 | 780234    | 1059115   | 371929  | 916568   |
| 67.86 | 1735.76  | LsPLPHEPADFYDR               | S2 Phos                     | 100%             | 189.74                    | 201.2  | 400028073 | <b>Secretory carrier-associated membrane protein</b>                     | transporter           | 3.61E-02 | 7380073   | 10574347  | 1908641 | 4530480  |
| 78.51 | 1244.579 | TTFsVLEGVGR                  | S4 Phos                     | 100%             | 60.65                     | 149.51 | 400023089 | <b>Uncharacterized protein</b>   | unclassified          | 3.93E-02 | 4587620   | 6507832   | 1736362 | 4033402  |



|             |          |                        |                             |                 |                           |        |           |   |                        |          |          |          |         |         |
|-------------|----------|------------------------|-----------------------------|-----------------|---------------------------|--------|-----------|---|------------------------|----------|----------|----------|---------|---------|
| 71.31       | 1712.816 | EEHQIPILSPDLR          | S10 Phos                    | 100%            | 26.31                     | 154.81 | 400069247 | Putative zinc finger CCCH domain-containing protein 56-like       | transcription          | 2.41E-02 | 2361030  | 3294617  | 1088319 | 2540683 |
| 90.01       | 1225.542 | sYGVFLEGFK             | S1 Phos                     | 100%            | 23.98                     | 122.49 | 400061873 | Uncharacterized protein   | unclassified           | 5.50E-03 | 937479   | 1179504  | 428448  | 932313  |
| 83.13       | 1402.624 | sLSELEFEELK            | S1 Phos                     | 100%            | 40.2                      | 129.24 | 400052590 | Uncharacterized protein   | unclassified           | 1.24E-02 | 1282979  | 1506439  | 883781  | 1853383 |
| 81.21       | 1724.753 | LNNFLFSGGGGGGSFK       | S15 Phos                    | 100%            | 20.98                     | 131.33 | 400065029 | Putative transcription factor bHLH-like                           | transcription          | 4.48E-02 | 3754775  | 4458454  | 1686031 | 3599559 |
| 35.60       | 1330.628 | HAVATENAtIPK           | T9 Phos                     | 100%            | 81.34                     | 103.06 | 400058377 | Pollen-specific lysine-rich protein SBgLR                         | unclear classification | 2.24E-02 | 5176717  | 6408900  | 1320108 | 2673346 |
| 76.81       | 1661.758 | DTSGVLSPFNFsRR         | S12 Phos                    | 99%             | 25.91                     | 28.59  | 400040518 | Putative cinnamyl alcohol dehydrogenase                           | secondary metabolism   | 3.05E-02 | 510728   | 639968   | 320905  | 672915  |
| 70.31       | 1223.578 | TSsLELEPLR             | S3 Phos                     | 100%            | 40.2                      | 105.67 | 400050818 | Serine/threonine-protein kinase D6PK-like                         | signal transduction    | 4.88E-02 | 796269   | 840892   | 365009  | 935505  |
| 55.55       | 971.3939 | mSLsPWR                | M1 Oxid, S4 Phos            | 100%, 100%      | 1,000.00, 29.38           | 52.94  | 400037294 | Protein PLASTID MOVEMENT IMPAIRED 1                               | cell structure         | 3.29E-02 | 457803   | 494798   | 233538  | 597493  |
| cluster 1 e |          |                        |                             |                 |                           |        |           |   |                        |          |          |          |         |         |
| 49.19       | 2069.899 | VNLEVsEDEKPSVSSDEK     | S6 Phos                     | 100%            | 67.91                     | 125.4  | 400009545 | Uncharacterized protein   | unclassified           | 3.95E-02 | 4265240  | 5023043  | 1385376 | 1320012 |
| 32.24       | 1367.552 | GLDHYESAsPGR           | S9 Phos                     | 100%            | 48.19                     | 66.87  | 400067153 | Probable alkaline/neutral invertase D                             | metabolism             | 2.35E-03 | 500607   | 833901   | 192396  | 160261  |
| 64.96       | 1383.687 | SSHtLLPPLPSR           | T4 Phos                     | 100%            | 18.56                     | 69.21  | 400019190 | Vacuolar amino acid transporter 1                                 | transporter            | 6.31E-04 | 1251445  | 1988255  | 533482  | 632624  |
| 55.85       | 875.4261 | LNLsPPR                | S4 Phos                     | 100%            | 1,000.00                  | 59.54  | 400015190 | Uncharacterized protein   | unclassified           | 1.57E-02 | 15170118 | 19689060 | 4043937 | 6100505 |
| 57.81       | 1351.661 | tPPVLELHPGGR           | T1 Phos                     | 100%            | 1,000.00                  | 102.81 | 400073788 | Putative mitogen-activated protein kinase kinase kinase YODA-like | signal transduction    | 6.73E-03 | 782812   | 986816   | 377025  | 552414  |
| 44.75       | 1294.57  | LHLDYSgPGR             | T8 Phos                     | 98%             | 19.86                     | 40.66  | 400039491 | Cbs domain-containing protein cbscbpb5                            | disease-defence        | 2.38E-02 | 1451253  | 2071767  | 669517  | 929124  |
| 44.95       | 1145.559 | HLLSsPIGSR             | S5 Phos                     | 95%             | 13.27                     | 48.94  | 400002750 | Putative deSI-like protein-like                                   | Prot. Destin. storage  | 1.80E-03 | 3652822  | 5538525  | 1292182 | 2001828 |
| 34.57       | 1192.512 | HYLPPmsPR              | M6 Oxid, S7 Phos            | 100%, 100%      | 1,000.00, 47.47           | 58.36  | 400069225 | Autophagy-related protein 13a-like                                | disease-defence        | 8.56E-03 | 3173413  | 4905979  | 662969  | 1046317 |
| 60.15       | 2276.076 | NTTPTLAsPISITSHGGTQSAR | S8 Phos                     | 100%            | 30.92                     | 99.2   | 400036790 | Putative uncharacterized protein PPS3                             | unclassified           | 4.05E-02 | 2231246  | 3226217  | 679667  | 1078359 |
| 42.96       | 883.3943 | FsPVAGAR               | S2 Phos                     | 100%            | 1,000.00                  | 84.22  | 400037271 | Putative heterogeneous nuclear ribonucleoprotein 1-like           | transcription          | 3.08E-02 | 2878301  | 4100010  | 638737  | 997102  |
| 100.12      | 1572.69  | GVsAWNFLEDLKL          | S3 Phos                     | 100%            | 1,000.00                  | 134.88 | 400031593 | Putative serine/threonine-protein kinase fray2-like               | signal transduction    | 4.89E-02 | 1003714  | 1416354  | 769415  | 1220894 |
| 99.87       | 1359.644 | TIFsFVVPDQK            | S4 Phos                     | 100%            | 65.16                     | 137.1  | 400002261 | Mitochondrial outer membrane protein porin of 36 kDa              | transporter            | 1.22E-02 | 1976095  | 3415931  | 1615727 | 2425836 |
| 44.40       | 1894.795 | GsPSPVGSIDmmQHVGK      | S2 Phos, M11 Oxid, M12 Oxid | 99%, 100%, 100% | 24.15, 1,000.00, 1,000.00 | 79.07  | 400078305 | Putative KH domain-containing protein-like                        | unclear classification | 4.35E-02 | 1935894  | 3540340  | 522445  | 916130  |
| 56.00       | 1735.832 | SESETIKPEILtPR         | T13 Phos                    | 100%            | 44.2                      | 112.19 | 400011621 | Putative RPM1-interacting protein 4-like                          | disease-defence        | 1.07E-02 | 1564465  | 2672337  | 795615  | 1421213 |
| 56.93       | 1923.938 | LRPAQsVEEVTYPTVR       | S6 Phos                     | 100%            | 127.18                    | 166.14 | 400063603 | Putative topless-related protein 3-like                           | transcription          | 2.49E-03 | 7606405  | 9788575  | 2690628 | 4774599 |
| 66.62       | 1068.499 | AsNPFVNLK              | S2 Phos                     | 100%            | 1,000.00                  | 47.95  | 400004878 | Alpha-1,4-glucan-protein synthase [UDP-forming] 2                 | metabolism             | 2.67E-02 | 1342737  | 1656571  | 961553  | 1601505 |
| 47.10       | 1567.756 | GILAADESTGtHGKR        | T11 Phos                    | 97%             | 17.98                     | 91.06  | 400006894 | Fructose-bisphosphate aldolase                                    | energy                 | 1.67E-02 | 5373853  | 6583263  | 1853432 | 3176207 |
| 90.01       | 1450.633 | sADFLETSNFLK           | S1 Phos                     | 100%            | 176.61                    | 158.14 | 400024491 | Uncharacterized protein   | unclassified           | 1.96E-02 | 3055665  | 4450405  | 1940717 | 3578282 |
| 47.40       | 1454.699 | QPAAsPGAIATLHGR        | S4 Phos                     | 100%            | 119.42                    | 158.97 | 400032509 | AT-hook motif nuclear-localized protein 26-like                   | transcription          | 1.23E-02 | 913961   | 1264039  | 292618  | 517222  |
| 69.96       | 1542.685 | AmLSPINTNFsPR          | M2 Oxid, S11 Phos           | 100%, 100%      | 1,000.00, 41.02           | 139.58 | 400021057 | Putative zinc finger CCCH domain-containing protein 30-like       | transcription          | 4.72E-03 | 2101428  | 3165292  | 1458253 | 2864720 |
| 58.75       | 1841.864 | tPNSINLHEVVEAPR        | T1 Phos                     | 100%            | 58.89                     | 119.84 | 400072493 | Uncharacterized protein   | unclassified           | 4.95E-02 | 2783356  | 3989759  | 1035103 | 2069708 |
| 85.53       | 1317.629 | LGsSFLASSLTR           | S3 Phos                     | 100%            | 40                        | 163.74 | 400019190 | Vacuolar amino acid transporter 1                                 | transporter            | 3.26E-02 | 3980003  | 5522337  | 1644177 | 3299608 |
| 70.46       | 941.4732 | IVsFIQR                | S3 Phos                     | 100%            | 1,000.00                  | 87.33  | 400018674 | Acylsugar acylhydrolase 1   | secondary metabolism   | 3.32E-02 | 1981088  | 2707054  | 877637  | 1787810 |

|              |          |                                 |                    |            |                 |        |           |   |                        |          |         |          |         |         |
|--------------|----------|---------------------------------|--------------------|------------|-----------------|--------|-----------|---|------------------------|----------|---------|----------|---------|---------|
| 58.00        | 1304.578 | SQsFTSLSNVR                     | S3 Phos            | 100%       | 43.2            | 118.5  | 400061873 | Uncharacterized protein   | unclassified           | 2.98E-02 | 6491335 | 5846962  | 2428221 | 3076696 |
| 52.77        | 1333.662 | KLDESIPLPR                      | S7 Phos            | 96%        | 13.28           | 31.06  | 400017000 | Uncharacterized protein   | unclassified           | 1.70E-02 | 1032158 | 1218123  | 657695  | 744755  |
| 35.16        | 1580.756 | RAGSTSGSGIPVsPTK                | S13 Phos           | 100%       | 45.56           | 117.19 | 400038105 | Putative histone-lysine N-methyltransferase SUVR2-like                | transcription          | 2.62E-03 | 686080  | 828281   | 283797  | 347206  |
| 48.19        | 1337.659 | KVsGPLDSIGSAK                   | S3 Phos            | 100%       | 103.22          | 131.62 | 400023758 | Uncharacterized membrane protein At1g16860-like                       | unclassified           | 1.22E-02 | 3131625 | 3455396  | 1531581 | 1954846 |
| 60.61        | 1585.757 | HTNLLENLsPNVR                   | S9 Phos            | 100%       | 72.55           | 78.34  | 400000751 | Nucleosome assembly protein 12-like                                   | cell structure         | 9.04E-04 | 1843658 | 2101210  | 588617  | 875337  |
| 59.50        | 1423.705 | sFDKPLLATLQK                    | S1 Phos            | 100%       | 62.98           | 61.03  | 400075283 | Putative dentin sialophosphoprotein-like                              | unclear classification | 1.21E-02 | 2540643 | 2598171  | 969959  | 1424593 |
| 69.12        | 919.4198 | QAsFFLK                         | S3 Phos            | 100%       | 1,000.00        | 62.66  | 400001041 | Epsin-3-like isoform X1   | Prot. Destin. storage  | 3.06E-02 | 1953996 | 2046636  | 1709465 | 2859949 |
| Cluster I f  |          |                                 |                    |            |                 |        |           |   |                        |          |         |          |         |         |
| 69.71        | 1987.951 | NIEHPLLQASFGVsSPGR              | S15 Phos           | 95%        | 13.27           | 251.42 | 400017546 | Zinc finger CCCH domain-containing protein 30-like                    | transcription          | 1.16E-03 | 1251861 | 4627462  | 532668  | 2072969 |
| 78.46        | 2540.213 | IVQQAANGISQISFSTEENIsPK         | S21 Phos           | 100%       | 43.7            | 149.34 | 400056112 | Uncharacterized protein   | unclassified           | 1.33E-02 | 523291  | 2138176  | 409311  | 1253573 |
| 82.79        | 2189.11  | TVEAPPFEALRsPIEAAAIK            | S12 Phos           | 100%       | 108.48          | 127.5  | 400063603 | Putative topless-related protein 3-like                               | transcription          | 2.89E-04 | 1372683 | 5332524  | 617996  | 1905156 |
| 98.97        | 1379.694 | SIPsIIDLDSLK                    | S4 Phos            | 100%       | 47.7            | 104.11 | 400034699 | UTP--glucose-1-phosphate uridylyltransferase                          | metabolism             | 2.47E-02 | 775148  | 1841419  | 470690  | 1184757 |
| 87.57        | 2154.988 | FGAADiFKIPEEVDISHIS             | T6 Phos            | 100%       | 210.94          | 236.38 | 400055773 | Diacylglycerol kinase   | signal transduction    | 2.27E-03 | 1496327 | 3840605  | 824559  | 2616792 |
| 73.61        | 2464.211 | QGGVsPAPVTNVVGYPLRPGEK          | S5 Phos            | 100%       | 62.71           | 157.73 | 400058323 | Zinc finger CCCH domain-containing protein 34                         | transcription          | 1.94E-02 | 854435  | 1939121  | 465503  | 1451443 |
| 73.51        | 2082.968 | GRsPAFNALAAATFENPNAR            | S3 Phos            | 100%       | 140.68          | 149.84 | 400056449 | Putative villin-4-like  | cell structure         | 1.63E-04 | 529081  | 1277783  | 169924  | 492533  |
| 91.50        | 2034.993 | ALsLDALTNLNDQAQQIK              | S3 Phos            | 100%       | 46.94           | 88.37  | 400073591 | Uncharacterized protein   | unclassified           | 8.68E-03 | 2416154 | 5751672  | 1023658 | 3026216 |
| 98.16        | 3260.357 | ESEGDEVFEEAIEPEsPGFGVEDGVVSE GR | S16 Phos           | 100%       | 69.44           | 98.14  | 400004034 | Translocase of chloroplast 120, chloroplastic                         | Prot. Destin. storage  | 1.99E-02 | 772375  | 2392136  | 489288  | 1410658 |
| 118.88       | 2058.849 | GNLlFNALDALGASDSDNGQ            | T4 Phos            | 100%       | 119.04          | 105.38 | 400002331 | Calcium-dependent protein kinase 11-like                              | signal transduction    | 2.38E-03 | 1378242 | 4179686  | 1150362 | 2823313 |
| 81.55        | 1813.836 | SFLETIPsFSIHNSR                 | S8 Phos            | 100%       | 33.56           | 171.71 | 400022129 | Uncharacterized protein   | unclassified           | 2.88E-03 | 1810209 | 5365609  | 934693  | 2398622 |
| 107.65       | 2805.442 | QNsgPIPPILPTTLITSGPLNSAGAPR     | S3 Phos            | 100%       | 38.5            | 90.91  | 400025018 | Putative membrane protein   | unclassified           | 1.64E-02 | 391290  | 1146774  | 261135  | 505839  |
| 96.23        | 2312.062 | ALELLDGSAAFTAEEEDIsPR           | S20 Phos           | 100%       | 45.98           | 131.38 | 400007895 | Heat stress transcription factor B-2b-like isoform X1                 | disease-defence        | 1.28E-02 | 511879  | 1476919  | 468263  | 990529  |
| 90.65        | 1808.852 | EGGVINNLISNLmsPR                | M13 Oxid, S14 Phos | 100%, 100% | 1,000.00, 64.00 | 147.49 | 400075055 | Uncharacterized protein   | unclassified           | 2.07E-04 | 1124847 | 2840285  | 626529  | 1216663 |
| 46.25        | 838.3752 | VPFsPGR                         | S4 Phos            | 100%       | 1,000.00        | 107.54 | 400048013 | Uncharacterized protein   | unclassified           | 2.96E-02 | 3103981 | 8175709  | 845162  | 1887985 |
| 102.71       | 2126.039 | VQsLNYLASLIGEDQQIR              | S3 Phos            | 95%        | 13.28           | 208.4  | 400073591 | Uncharacterized protein   | unclassified           | 6.75E-03 | 663570  | 1713050  | 641447  | 1394211 |
| 95.84        | 2301.197 | ILSQLQGLSLDAVSPQLQsPK           | S19 Phos           | 100%       | 36.31           | 160.91 | 400078050 | Zinc finger CCCH domain-containing protein 66-like                    | transcription          | 1.79E-02 | 510201  | 1351697  | 517073  | 645556  |
| 43.71        | 854.4257 | ILSSIsR                         | S6 Phos            | 95%        | 16.24           | 53.55  | 400012843 | Soluble inorganic pyrophosphatase PPA1                                | metabolism             | 2.37E-03 | 520405  | 1265090  | 242694  | 322045  |
| 44.05        | 966.4518 | sQPISISR                        | S1 Phos            | 100%       | 38.94           | 45.31  | 400045033 | Putative dentin sialophosphoprotein-like                              | unclear classification | 1.57E-02 | 955265  | 2038952  | 373395  | 495710  |
| 114.50       | 1914.877 | LPEsPLNPDFLFGFDK                | S4 Phos            | 100%       | 1,000.00        | 183.96 | 400078796 | Uncharacterized protein   | unclassified           | 1.57E-02 | 357284  | 794136   | 339597  | 418501  |
| 102.51       | 2186.883 | AAAsDNNNDVVTDFPHYR              | S3 Phos            | 100%       | 106.42          | 265.25 | 400018674 | Acylsugar acylhydrolase 1   | secondary metabolism   | 5.93E-03 | 1874708 | 5447325  | 1502485 | 2256686 |
| 108.74       | 2265.073 | sEFQPPVTTVDLFLPQPR              | S1 Phos            | 100%       | 79.07           | 134.76 | 400066508 | Uncharacterized protein   | unclassified           | 2.28E-02 | 273344  | 858774   | 216855  | 311011  |
| 50.15        | 835.3951 | GGLLsPR                         | S6 Phos            | 100%       | 1,000.00        | 102.24 | 400035480 | Trafficking protein particle complex subunit 11-like isoform X1       | intracellular traffic  | 3.40E-02 | 3367034 | 10289091 | 1829042 | 2371848 |
| 32.24        | 1667.693 | AGNFLHSSSPsNENK                 | S11 Phos           | 97%        | 16.24           | 84.92  | 400030663 | VQ motif-containing protein 22-like                                   | disease-defence        | 3.35E-02 | 4324707 | 14234787 | 1166990 | 1471416 |
| 100.87       | 2677.261 | EmLPsPVADKNDPPIVTLDDLFR         | M2 Oxid, S5 Phos   | 100%, 100% | 1,000.00, 71.94 | 130.81 | 400070812 | Putative apoptotic chromatin condensation inducer in the nucleus-like | transcription          | 2.26E-02 | 214363  | 637454   | 280381  | 287044  |
| 110.53       | 2485.19  | SLSQPAFFSLDSLPLsPSPYR           | S17 Phos           | 100%       | 20.98           | 134.66 | 400004178 | Putative transcription factor RF2a-like                               | transcription          | 3.54E-02 | 438195  | 1642554  | 542790  | 639633  |
| 1 hour       |          |                                 |                    |            |                 |        |           |   |                        |          |         |          |         |         |
| Cluster II a |          |                                 |                    |            |                 |        |           |   |                        |          |         |          |         |         |

|               |          |                              |                    |            |                    |        |           |   |                        |          |          |         |          |          |
|---------------|----------|------------------------------|--------------------|------------|--------------------|--------|-----------|---|------------------------|----------|----------|---------|----------|----------|
| 93.55         | 1690.734 | GFVFPVPGsPTEPSGH             | S9 Phos            | 100%       | 20.98              | 167.42 | 400027658 | <b>Sodium/hydrogen exchanger</b>  | transporter            | 1.59E-02 | 699865   | 739974  | 4334592  | 1594060  |
| 93.69         | 1739.797 | VAtVAGVLSLDDDES              | T3 Phos            | 100%       | 107.58             | 134.56 | 400045229 | <b>Alpha, alpha-trehalose-phosphate synthase [UDP-forming] 5-like</b>         | metabolism             | 3.14E-02 | 374881   | 402410  | 1346376  | 557568   |
| 74.91         | 2597.946 | EGDGNdSDEDISYNEYEDDLPK       | S7 Phos            | 100%       | 200                | 334.69 | 400002103 | <b>Protein decapping 5-like isoform X1</b>                                    | transcription          | 1.64E-03 | 6951037  | 6365497 | 70121555 | 27276444 |
| 93.84         | 1931.01  | TASVAQTPSILSLPlPR            | T16 Phos           | 100%       | 53.57              | 106.82 | 400048002 | <b>Lysine histidine transporter-like 8</b>                                    | transporter            | 3.64E-02 | 382477   | 650843  | 1262402  | 572951   |
| 107.65        | 2805.442 | QNsGPIPPILPTTGLITSGPLNSAGAPR | S3 Phos            | 100%       | 38.5               | 90.91  | 400025018 | <b>Putative membrane protein</b>  | unclassified           | 1.82E-02 | 333990   | 433594  | 1008430  | 404064   |
| Cluster II b  |          |                              |                    |            |                    |        |           |   |                        |          |          |         |          |          |
| 35.60         | 1330.628 | HAVATENAIPK                  | T9 Phos            | 100%       | 81.34              | 103.06 | 400058377 | <b>Pollen-specific lysine-rich protein SBgLR</b>                              | unclear classification | 2.45E-02 | 375955   | 274431  | 183628   | 156872   |
| 95.34         | 1320.571 | sLSELsIGLSR                  | S1 Phos, S6 Phos   | 100%, 100% | 32.28, 38.16       | 71.88  | 400067153 | <b>Probable alkaline/neutral invertase D</b>                                  | metabolism             | 2.66E-02 | 3095891  | 3104854 | 5456504  | 3764407  |
| 46.65         | 1112.421 | ETEPWsER                     | S6 Phos            | 100%       | 33.56              | 45.65  | 400046406 | <b>RNA-binding KH domain-containing protein PEPPEP-like</b>                   | transcription          | 6.63E-03 | 4785247  | 4800423 | 2076194  | 1510485  |
| 33.75         | 1599.718 | KPTsPAPETSSFGSK              | S4 Phos            | 97%        | 14.98              | 74.07  | 400032086 | <b>Reticulon-like protein</b>   | unclassified           | 1.99E-02 | 459809   | 438008  | 2583320  | 2076575  |
| 40.21         | 1893.776 | IEASAEDTHGLAAsGDDR           | S14 Phos           | 100%       | 24.92              | 109.73 | 400070812 | <b>Putative apoptotic chromatin condensation inducer in the nucleus-like</b>  | transcription          | 1.12E-04 | 703796   | 706823  | 325650   | 254811   |
| 52.93         | 1150.457 | FmEAAsPFR                    | M2 Oxid, S6 Phos   | 100%, 100% | 1,000.00, 1,000.00 | 114.3  | 400010406 | <b>Uncharacterized protein</b>  | unclassified           | 1.57E-02 | 711777   | 620730  | 3744381  | 1992758  |
| 48.89         | 1218.525 | ISQSVsFSER                   | S6 Phos            | 100%       | 34.2               | 108.82 | 400045422 | <b>Uncharacterized protein</b>  | unclassified           | 2.64E-02 | 3642050  | 3049175 | 14119940 | 9026125  |
| 49.19         | 2069.899 | VNLEVsEDEKPSVSSDEK           | S6 Phos            | 100%       | 67.91              | 125.4  | 400009545 | <b>Uncharacterized protein</b>  | unclassified           | 9.52E-03 | 4323614  | 3634968 | 2240648  | 1330004  |
| 46.50         | 1741.783 | SEsTKGENGVAEIVSR             | S3 Phos            | 100%       | 26.31              | 222.58 | 400058377 | <b>Pollen-specific lysine-rich protein SBgLR</b>                              | unclear classification | 2.80E-02 | 3673647  | 2822845 | 2016556  | 1395945  |
| 59.35         | 842.404  | sIAFVAR                      | S1 Phos            | 100%       | 1,000.00           | 70.96  | 400041657 | <b>Uncharacterized protein</b>  | unclassified           | 1.99E-03 | 529091   | 624894  | 391646   | 266668   |
| 76.06         | 1049.552 | SLsLPVINK                    | S3 Phos            | 98%        | 16.23              | 62.84  | 400074985 | <b>Uncharacterized protein</b>  | unclassified           | 7.80E-03 | 299889   | 350357  | 687537   | 394410   |
| 69.12         | 919.4198 | QAsFFLK                      | S3 Phos            | 100%       | 1,000.00           | 62.66  | 400001041 | <b>Epsin-3-like isoform X1</b>  | Prot. Destin. storage  | 8.13E-03 | 1739969  | 2027933 | 3521897  | 2175112  |
| 43.06         | 1487.703 | VsDAQIITEHPAK                | S2 Phos            | 100%       | 57.53              | 80.37  | 400075360 | <b>Pyrophosphate--fructose 6-phosphate 1-phosphotransferase subunit alpha</b> | energy                 | 4.14E-02 | 874294   | 934180  | 1291409  | 714582   |
| Cluster II c  |          |                              |                    |            |                    |        |           |   |                        |          |          |         |          |          |
| 73.81         | 1505.672 | DLNIGsPPESELR                | S6 Phos            | 100%       | 94.5               | 62.59  | 400032855 | <b>Putative membrane protein</b>  | unclassified           | 3.66E-02 | 387107   | 459286  | 677949   | 564193   |
| 35.16         | 1580.756 | RAGSTSGSGIPVsPTK             | S13 Phos           | 100%       | 45.56              | 117.19 | 400038105 | <b>Putative histone-lysine N-methyltransferase SUV2-like</b>                  | transcription          | 2.27E-02 | 300887   | 349053  | 208743   | 189267   |
| 43.71         | 854.4257 | ILSSIsR                      | S6 Phos            | 95%        | 16.24              | 53.55  | 400012843 | <b>Soluble inorganic pyrophosphatase PPA1</b>                                 | signal transduction    | 2.82E-02 | 722173   | 1003177 | 231096   | 227364   |
| Cluster II d  |          |                              |                    |            |                    |        |           |   |                        |          |          |         |          |          |
| 80.51         | 1742.861 | IALVGLGsPTSSAAAYR            | S8 Phos            | 100%       | 46.21              | 213.04 | 400020254 | <b>Leucine aminopeptidase 2, chloroplastic</b>                                | Prot. Destin. storage  | 3.46E-02 | 140777   | 90060   | 3079633  | 900327   |
| 70.56         | 2889.139 | VESEGVVDVDEEEdADEEETEITR     | S14 Phos           | 100%       | 146.43             | 460.22 | 400045835 | <b>Acidic leucine-rich nuclear phosphoprotein 32-related protein</b>          | transcription          | 1.78E-02 | 2479017  | 1331549 | 5286720  | 1709644  |
| 67.41         | 2666.296 | ATTsVPQsPFKPLASNVTTESAPTR    | S8 Phos            | 100%       | 50.51              | 217.49 | 400019030 | <b>Putative vegetative cell wall protein gp1-like</b>                         | cell structure         | 1.99E-03 | 1593406  | 881617  | 29511234 | 1254444  |
| 69.71         | 2001.795 | EGSSLEGAsDDEIEDLEKR          | S9 Phos            | 100%       | 57.1               | 123.14 | 400028137 | <b>Protein phosphatase inhibitor 2-like</b>                                   | signal transduction    | 3.24E-02 | 10141633 | 5069538 | 7210377  | 3436387  |
| 58.55         | 1512.806 | SVtGVSPVIPRPPK               | T3 Phos            | 97%        | 14.49              | 79.43  | 400050062 | <b>Uncharacterized protein</b>  | unclassified           | 9.50E-06 | 686193   | 463509  | 9072508  | 3913800  |
| 61.97         | 2270.894 | HQDYFADsPSAGLDPASREE         | S8 Phos            | 100%       | 41.53              | 219.47 | 400075275 | <b>Transcription initiation factor TFIID subunit 10</b>                       | transcription          | 1.97E-02 | 11826226 | 7572674 | 7513989  | 3736234  |
| Cluster II e  |          |                              |                    |            |                    |        |           |   |                        |          |          |         |          |          |
| 47.60         | 1885.797 | ETPQPEPENVPESPEK             | S13 Phos           | 100%       | 297.92             | 234.8  | 400013297 | <b>Putative patellin-2-like</b>   | transporter            | 4.02E-02 | 22511054 | 6868064 | 51113669 | 15485507 |
| 24 hours      |          |                              |                    |            |                    |        |           |   |                        |          |          |         |          |          |
| Cluster III a |          |                              |                    |            |                    |        |           |   |                        |          |          |         |          |          |
| 104.35        | 2820.351 | SGGIASLVSPCPVTPAVLPTPMFsPSR  | M22 Oxid, S24 Phos | 100%, 100% | 1,000.00, 19.23    | 100.26 | 400061835 | <b>Uncharacterized protein</b>  | unclassified           | 6.57E-03 | 649808   | 532224  | 1905486  | 360250   |
| 110.71        | 2485.185 | SLSQPAFFSLDLSLPLsPSPYR       | S17 Phos           | 100%       | 27.61              | 115.32 | 400004178 | <b>Putative transcription factor RF2a-like</b>                                | transcription          | 3.90E-02 | 1545447  | 1265642 | 2502269  | 881059   |
| 59.10         | 2816.359 | SATDSAKPIPDGSASVSQGHIAQsPOK  | S25 Phos           | 100%       | 75.83              | 108.63 | 400025768 | <b>La-related protein 1C-like</b>   | transcription          | 1.07E-05 | 76127    | 60927   | 536822   | 205936   |

|               |          |   |                   |            |                    |        |           |  |                        |          |         |         |          |          |
|---------------|----------|---|-------------------|------------|--------------------|--------|-----------|--|------------------------|----------|---------|---------|----------|----------|
| 42.23         | 1202.603 | RPPSLLEsPK                                  | S8 Phos           | 100%       | 33.56              | 81.53  | 400073451 | <b>Putative ovule protein</b>  | unclear classification | 7.45E-05 | 851340  | 753369  | 6109703  | 2311319  |
| 107.88        | 2805.434 | QNsGPIIPILPTTGLITSGPLNSAGAPR                | S3 Phos           | 100%       | 58.27              | 105.66 | 400025018 | <b>Putative membrane protein</b>   | unclassified           | 3.14E-02 | 683740  | 654206  | 1494036  | 397979   |
| 74.68         | 2597.94  | EGDGNdSDEDISYNEYEDDLPK                      | S7 Phos           | 100%       | 209.69             | 328.98 | 400002103 | <b>Protein decapping 5-like isoform X1</b>                               | transcription          | 6.23E-04 | 2961932 | 3275703 | 36112226 | 10700829 |
| 58.29         | 1512.803 | SvtGVSPVIPRPPK                              | T3 Phos           | 97%        | 14.49              | 79.43  | 400050062 | <b>Uncharacterized protein</b>   | unclassified           | 2.13E-06 | 631104  | 702639  | 11797687 | 3919557  |
| 75.12         | 1676.787 | SPLSFLNsPITEHR                              | S8 Phos           | 100%       | 31.51              | 91.81  | 400013664 | <b>Uncharacterized protein</b>   | unclassified           | 4.87E-02 | 313529  | 330665  | 607095   | 211797   |
| Cluster III b |          |   |                   |            |                    |        |           |  |                        |          |         |         |          |          |
| 35.52         | 1330.627 | HAVATENAIPK                                 | T9 Phos           | 100%       | 30.07              | 63.26  | 400058377 | <b>Pollen-specific lysine-rich protein SBgLR</b>                         | unclear classification | 9.36E-03 | 5640122 | 4589672 | 1754440  | 1322514  |
| 48.87         | 2069.896 | VNLEVsEDEKPSVSSDEK                          | S6 Phos           | 100%       | 67.91              | 125.4  | 400009545 | <b>Uncharacterized protein</b>   | unclassified           | 2.73E-02 | 3475087 | 2829353 | 1590763  | 1042342  |
| 76.46         | 1111.565 | SfPVALAVK                                   | T3 Phos           | 100%       | 35.26              | 83.02  | 400061684 | <b>Uncharacterized protein</b>   | unclassified           | 1.73E-02 | 491248  | 439552  | 884043   | 601675   |
| 45.02         | 1424.651 | AGSTSGSGIPVsPTK                             | S12 Phos          | 99%        | 20.98              | 206.55 | 400038105 | <b>Putative histone-lysine N-methyltransferase SUVR2-like</b>            | transcription          | 4.90E-02 | 4633604 | 3039229 | 1863541  | 1301794  |
| 47.98         | 1498.727 | QPTsPGAVVTLHGR                              | S4 Phos           | 100%       | 28.23              | 89.54  | 400033248 | <b>AT-hook motif nuclear-localized protein 22</b>                        | transcription          | 6.76E-03 | 415166  | 298701  | 268468   | 168304   |
| 35.42         | 2181.946 | EVDEPPsPPATTHDEEKPK                         | S7 Phos           | 100%       | 112.55             | 120.52 | 400046956 | <b>Uncharacterized protein</b>   | unclassified           | 2.96E-02 | 155198  | 90294   | 69377    | 54533    |
| 79.29         | 2537.168 | NETTtPVSvVVENDETTpVAVVEK                    | T5 Phos           | 97%        | 17.98              | 138.1  | 400058377 | <b>Pollen-specific lysine-rich protein SBgLR</b>                         | unclassified           | 7.21E-04 | 985203  | 643188  | 233839   | 187719   |
| 87.53         | 1460.716 | NLsFNvPIQPPR                                | S3 Phos           | 100%       | 1,000.00           | 55.44  | 400073575 | <b>Putative E3 ubiquitin-protein ligase ATL6-like</b>                    | Prot. Destin. storage  | 1.06E-02 | 568390  | 785979  | 1248167  | 940908   |
| 108.08        | 4360.866 | AD2VAGEVEAVAETENQGQTGEFAAD PGLMGDYVPPEVDVDR | S3 Phos, M31 Oxid | 100%, 100% | 61.11, 1,000.00    | 144.51 | 400079854 | <b>Uncharacterized protein</b>   | unclassified           | 3.90E-02 | 230989  | 269572  | 370975   | 256786   |
| 73.54         | 1505.672 | DLNIGsPPESELR                               | S6 Phos           | 100%       | 56.52              | 56.55  | 400032855 | <b>Putative membrane protein</b>   | unclassified           | 2.64E-02 | 442961  | 507246  | 674610   | 467516   |
| 54.38         | 1710.8   | LLsEKNELEDTAAAK                             | S3 Phos           | 100%       | 108.52             | 101.33 | 400079571 | <b>Carbonic anhydrase</b>  | metabolism             | 4.05E-02 | 2820904 | 3123813 | 2193230  | 1696529  |
| 68.82         | 960.5024 | IGPLGLsPK                                   | S7 Phos           | 100%       | 1,000.00           | 103.52 | 400020871 | <b>60S ribosomal protein L12</b>   | protein synthesis      | 2.10E-02 | 1442031 | 1505109 | 3137707  | 1963038  |
| 61.64         | 2270.896 | HQDYFADsPSAGLDPASREE                        | S8 Phos           | 100%       | 30.61              | 153.19 | 400075275 | <b>Transcription initiation factor TFIIID subunit 10</b>                 | transcription          | 4.21E-02 | 6173919 | 6790737 | 4317219  | 2525988  |
| 114.22        | 2856.221 | DNEsLPDEQVADILVETTSACDLDK                   | S4 Phos           | 100%       | 75.29              | 87.71  | 400007986 | <b>Uncharacterized protein</b>   | unclassified           | 2.37E-02 | 1206510 | 1192111 | 2079037  | 1373935  |
| 113.13        | 2546.158 | NYFELNPLPsPSNNNFISPR                        | S11 Phos          | 100%       | 20.98              | 159.81 | 400002024 | <b>Protein MKS1-like</b>   | signal transduction    | 3.44E-02 | 664696  | 689054  | 1112677  | 779704   |
| 47.17         | 1885.796 | ETPOPEPENVPESPEK                            | S13 Phos          | 100%       | 264.45             | 237.07 | 400013297 | <b>Putative patellin-2-like</b>  | transporter            | 2.15E-04 | 6507855 | 8748563 | 25940787 | 15425936 |
| 57.50         | 1351.66  | tPPVLELHPGGR                                | T1 Phos           | 100%       | 1,000.00           | 102.81 | 400073788 | <b>Putative mitogen-activated protein kinase kinase kinase YODA-like</b> | signal transduction    | 9.86E-03 | 868181  | 1134055 | 730714   | 387820   |
| 58.14         | 1595.749 | APMGEHLsPLVPR                               | M3 Oxid, S8 Phos  | 100%, 100% | 1,000.00, 1,000.00 | 59.29  | 400017173 | <b>Uncharacterized protein</b>   | unclassified           | 2.60E-02 | 1107146 | 1621086 | 1197988  | 588410   |
| 89.71         | 1317.607 | SLFSAGFLGsPR                                | S10 Phos          | 100%       | 90.57              | 169.51 | 400076377 | <b>VQ motif-containing protein 4-like</b>                                | transcription          | 3.18E-02 | 3225167 | 4936236 | 5360328  | 2807803  |
| 46.57         | 2373.059 | SQPPPPTASHINyANSVDSsPK                      | S20 Phos          | 100%       | 27.22              | 121.25 | 400015310 | <b>Putative activating signal cointegrator 1 complex subunit 2-like</b>  | transcription          | 4.17E-02 | 1115872 | 1052980 | 770947   | 353808   |
| 102.97        | 3193.517 | NNPFVDVHNDNYDLGLPPIPGsAPSVLVP R             | S21 Phos          | 100%       | 29.09              | 109.04 | 400072493 | <b>Uncharacterized protein</b>   | unclassified           | 4.51E-02 | 557282  | 497334  | 1056206  | 617145   |
| 33.58         | 1599.714 | KPtSPAPETSSFGSK                             | T3 Phos           | 98%        | 17.01              | 77.44  | 400032086 | <b>Reticulon-like protein</b>  | cell structure         | 3.91E-02 | 344126  | 353068  | 2315776  | 1247142  |
| 107.73        | 2485.145 | QVsFELFPEVENLGNAAGVTER                      | S3 Phos           | 100%       | 166.73             | 149.62 | 400051755 | <b>DNA-directed RNA polymerase III subunit rpc31 isoform X2</b>          | transcription          | 1.21E-02 | 527796  | 507672  | 978730   | 528385   |
| 60.94         | 1585.748 | HTNLELNsPNVR                                | S9 Phos           | 100%       | 72.55              | 78.34  | 400000751 | <b>Nucleosome assembly protein 12-like</b>                               | cell structure         | 2.10E-02 | 2104533 | 2070140 | 1634629  | 898372   |
| Cluster III c |          |   |                   |            |                    |        |           |  |                        |          |         |         |          |          |
| 106.07        | 1708.812 | DSSIAsLPVEELIEK                             | S6 Phos           | 99%        | 24.15              | 39.6   | 400010497 | <b>Plasma membrane ATPase</b>  | transporter            | 1.66E-02 | 206759  | 547474  | 539565   | 312574   |
| 82.45         | 1179.603 | FITPLGsPLR                                  | S7 Phos           | 100%       | 77.52              | 122.85 | 400048002 | <b>Lysine histidine transporter-like 8</b>                               | transporter            | 4.79E-02 | 925479  | 2488308 | 2812403  | 1994852  |
| 28.23         | 1348.549 | VKDMsQADFR                                  | M4 Oxid, S5 Phos  | 100%, 100% | 1,000.00, 1,000.00 | 23.76  | 400011624 | <b>Adenosylhomocysteinase</b>  | metabolism             | 4.34E-02 | 307334  | 701667  | 295956   | 234613   |
| Cluster III d |          |   |                   |            |                    |        |           |  |                        |          |         |         |          |          |
| 80.17         | 1145.534 | ADsSVSLLFK                                  | S3 Phos           | 100%       | 34.89              | 119.05 | 400047243 | <b>Callose synthase 12-like</b>  | disease-defence        | 2.63E-02 | 1782307 | 2820119 | 3273645  | 3028236  |
| 92.93         | 1769.755 | DAILDLIsDDESEQK                             | S8 Phos           | 100%       | 53.57              | 71.69  | 400001389 | <b>Polyribonucleotide nucleotidyltransferase 2, mitochondrial-like</b>   | transcription          | 1.57E-02 | 1183768 | 2089224 | 2673557  | 2521552  |

|                 |          |  |                   |            |                    |        |           |   |                        |          |         |         |          |          |
|-----------------|----------|--|-------------------|------------|--------------------|--------|-----------|---|------------------------|----------|---------|---------|----------|----------|
| 71.11           | 1068.533 | VIALSPsFR                                  | S7 Phos           | 100%       | 29.38              | 52.05  | 400061642 | <b>Probable alpha, alpha-trehalose-phosphate synthase [UDP-forming] 9</b> | metabolism             | 5.00E-02 | 554315  | 968617  | 984393   | 1236133  |
| 50.21           | 750.3635 | LPGLGsK                                    | S6 Phos           | 100%       | 1,000.00           | 19.28  | 400020254 | <b>Leucine aminopeptidase 2, chloroplastic</b>                            | Prot. Destin. storage  | 2.87E-02 | 317775  | 279123  | 166247   | 213032   |
| 68.92           | 919.4193 | QAsFFLK                                    | S3 Phos           | 100%       | 1,000.00           | 54.72  | 400001041 | <b>Epsin-3-like isoform X1</b>  | Prot. Destin. storage  | 1.82E-02 | 1450649 | 1923222 | 2738795  | 2476895  |
| 48.52           | 1090.514 | sLESGIHLR                                  | S1 Phos           | 100%       | 67.98              | 93.92  | 400045033 | <b>Putative dentin sialoPhosprotein-like</b>                              | unclear classification | 7.86E-03 | 448277  | 518423  | 304514   | 284528   |
| 57.30           | 886.3948 | SLsFPTR                                    | S3 Phos           | 100%       | 22.19              | 96.99  | 400072553 | <b>Protein WVD2-like 4 isoform X1</b>                                     | cell structure         | 1.83E-02 | 4609128 | 5371360 | 3103515  | 2690028  |
| 52.72           | 1150.454 | FMEAA_sPFR                                 | M2 Oxid, S6 Phos  | 100%, 100% | 1,000.00, 1,000.00 | 121.19 | 400010406 | <b>Uncharacterized protein</b>  | unclassified           | 2.80E-02 | 523664  | 562508  | 1989359  | 1929273  |
| 53.11           | 1083.518 | LSEQLsLSK                                  | S6 Phos           | 99%        | 20.74              | 15.98  | 400080800 | <b>Uncharacterized protein</b>  | unclassified           | 3.24E-02 | 1055197 | 1092439 | 673636   | 713205   |
| 48.62           | 1218.527 | ISQSVsFSER                                 | S6 Phos           | 100%       | 46.21              | 94.36  | 400045422 | <b>Uncharacterized protein</b>  | unclassified           | 1.22E-02 | 2792980 | 2735538 | 9310974  | 8572657  |
| 70.46           | 2162.88  | TETSITDVPsDGEDDLYAR                        | S10 Phos          | 100%       | 29.88              | 51.37  | 400053010 | <b>26S proteasome regulatory subunit 6B homolog</b>                       | Prot. Destin. storage  | 3.39E-02 | 681430  | 700173  | 1645405  | 1380635  |
| <b>72 hours</b> |          |  |                   |            |                    |        |           |   |                        |          |         |         |          |          |
| Cluster IV a    |          |  |                   |            |                    |        |           |   |                        |          |         |         |          |          |
| 37.22           | 1994.791 | TEDEVVcHDDsATPVK                           | S12 Phos          | 97%        | 15.01              | 106.99 | 400016354 | <b>Phosenolpyruvate carboxykinase</b>                                     | energy                 | 8.21E-04 | 500666  | 1382461 | 268030   | 1518974  |
| Cluster IV b    |          |  |                   |            |                    |        |           |   |                        |          |         |         |          |          |
| 108.08          | 4360.866 | ADsVAGEVEAVAEITENQGQTGEFAAD PGLmGDYVPEEVDR | S3 Phos, M31 Oxid | 100%, 100% | 61.11, 1,000.00    | 144.51 | 400079854 | <b>Uncharacterized protein</b>  | unclassified           | 3.39E-02 | 229842  | 188804  | 323506   | 734013   |
| 47.17           | 1885.796 | ETPQPEPENVPesPEK                           | S13 Phos          | 100%       | 264.45             | 237.07 | 400013297 | <b>Putative patellin-2-like</b>   | transporter            | 2.75E-03 | 6813373 | 7166262 | 16709951 | 28217112 |
| 113.13          | 2546.158 | NYFELPNFLPsPSNNNFISPR                      | S11 Phos          | 100%       | 20.98              | 159.81 | 400002024 | <b>Protein MKS1-like</b>  | signal transduction    | 6.11E-03 | 698702  | 685167  | 1045951  | 2299190  |
| 83.04           | 1319.588 | GLEQSFsIGFR                                | S7 Phos           | 100%       | 46.21              | 123.99 | 400002592 | <b>Chloroplast calcium-dependent protein kinase 7</b>                     | signal transduction    | 1.27E-02 | 963908  | 600385  | 1514891  | 2280819  |
| 76.71           | 1661.755 | DTSGVLSPFNFsRR                             | S12 Phos          | 100%       | 54.58              | 54.77  | 400040518 | <b>Putative cinnamyl alcohol dehydrogenase</b>                            | secondary metabolism   | 2.77E-02 | 884299  | 718211  | 1714740  | 2408669  |
| 114.22          | 2856.221 | DNEsLPDEQVADILVETTSAcDLDK                  | S4 Phos           | 100%       | 75.29              | 87.71  | 400007986 | <b>Uncharacterized protein</b>  | unclassified           | 1.29E-02 | 1387845 | 1089478 | 1981795  | 2582324  |
| 91.68           | 1795.92  | LDHPLPLPSVLGsPFK                           | S13 Phos          | 100%       | 56.52              | 139.15 | 400043111 | <b>Pyrophosphate--fructose 6-phosphate 1-Phostransferase subunit beta</b> | energy                 | 3.82E-02 | 255327  | 238530  | 312723   | 470481   |
| 42.23           | 1202.603 | RPPSLLESsPK                                | S8 Phos           | 100%       | 33.56              | 81.53  | 400073451 | <b>Putative ovule protein</b>   | unclear classification | 5.13E-04 | 762464  | 799575  | 7104915  | 7223717  |
| 48.62           | 1218.527 | ISQSVsFSER                                 | S6 Phos           | 100%       | 46.21              | 94.36  | 400045422 | <b>Uncharacterized protein</b>  | unclassified           | 6.80E-03 | 2639148 | 3322400 | 10026695 | 9506083  |
| 28.23           | 1727.812 | KKPiSPAPETSSFGSK                           | T4 Phos           | 100%       | 20.98              | 92.41  | 400032086 | <b>Reticulon-like protein</b>   | cell structure         | 1.96E-03 | 102548  | 122469  | 424791   | 389325   |
| 92.93           | 1769.755 | DAILDLI_sDDESEQK                           | S8 Phos           | 100%       | 53.57              | 71.69  | 400001389 | <b>Polyribonucleotide nucleotidyltransferase 2, mitochondrial-like</b>    | transcription          | 2.55E-03 | 1387006 | 1286851 | 2276024  | 2295626  |
| 38.81           | 1346.622 | KISQSVsFSER                                | S7 Phos           | 99%        | 21.72              | 56.41  | 400045422 | <b>Uncharacterized protein</b>  | unclassified           | 1.36E-02 | 401413  | 413569  | 1056895  | 1138269  |
| 35.52           | 1330.627 | HAVATENaIPK                                | T9 Phos           | 100%       | 30.07              | 63.26  | 400058377 | <b>Pollen-specific lysine-rich protein SBgLR</b>                          | unclear classification | 1.62E-02 | 4313940 | 4288153 | 1502672  | 1649216  |
| 48.52           | 1090.514 | sLESGIHLR                                  | S1 Phos           | 100%       | 67.98              | 93.92  | 400045033 | <b>Putative dentin sialoPhosprotein-like</b>                              | unclear classification | 7.64E-03 | 383922  | 405747  | 229048   | 271852   |
| Cluster IV c    |          |  |                   |            |                    |        |           |   |                        |          |         |         |          |          |
| 94.86           | 1822.874 | ADISGPPsQIFILETR                           | S8 Phos           | 100%       | 53.61              | 147.54 | 400058304 | <b>Formin-like protein</b>  | cell structure         | 8.93E-03 | 168277  | 66140   | 194391   | 55385    |
| 91.58           | 2034.993 | ALsLDALTNLNDQAQQIK                         | S3 Phos           | 100%       | 46.94              | 88.37  | 400073591 | <b>Uncharacterized protein</b>  | unclassified           | 4.92E-02 | 3600466 | 858460  | 4204402  | 3135280  |
| 76.21           | 2277.138 | KVSNuIVALPDDEITSGPTLK                      | T5 Phos           | 99%        | 22.93              | 215.22 | 400006608 | <b>Protein phosphatase 2C ABI2 homolog</b>                                | signal transduction    | 4.57E-02 | 1688727 | 534602  | 2193231  | 1748984  |
| 79.09           | 1174.634 | LLsPSQIPLK                                 | S3 Phos           | 100%       | 51.64              | 129.9  | 400075536 | <b>Gamma-glutamylcyclotransferase</b>                                     | disease-defence        | 1.53E-03 | 3344203 | 1149030 | 4356575  | 2999856  |
| 84.27           | 2109.119 | VVLVsPPQSPVLQPANGISK                       | S5 Phos           | 100%       | 54.03              | 211.36 | 400027620 | <b>Vesicle-associated protein 2-2-like isoform X2</b>                     | intracellular traffic  | 1.93E-02 | 6372371 | 1885985 | 7923875  | 5595186  |
| 123.26          | 2885.405 | QN_sGPIIPILPTTGLITsGPLNSAGAPR              | S3 Phos, S18 Phos | 100%, 94%  | 53.70, 12.61       | 105.37 | 400025018 | <b>Putative membrane protein</b>  | unclassified           | 8.02E-03 | 1500165 | 442685  | 2549293  | 2092472  |

| cluster IV d |          |                              |                            |                  |                           |        |           |   |                        |          |         |         |          |          |
|--------------|----------|------------------------------|----------------------------|------------------|---------------------------|--------|-----------|---|------------------------|----------|---------|---------|----------|----------|
| 121.46       | 1978.922 | NiLNLGEALGLVQSEENV           | T2 Phos                    | 100%             | 41.95                     | 26.43  | 400060262 | Calcium dependent protein kinase                      | signal transduction    | 5.17E-03 | 1105597 | 528933  | 1844881  | 2040457  |
| 83.53        | 1518.778 | KDsVGDLLLNLPR                | S3 Phos                    | 100%             | 1,000.00                  | 145.1  | 400074432 | Uncharacterized protein                               | unclassified           | 1.42E-02 | 1379869 | 696098  | 1912928  | 2225945  |
| 84.02        | 1275.561 | GIEGFGsFNIR                  | S7 Phos                    | 100%             | 1,000.00                  | 60.09  | 400001041 | Epsin-3-like isoform X1                               | Prot. Destin. storage  | 7.84E-03 | 1208390 | 602012  | 2546028  | 3308879  |
| 107.88       | 2805.434 | QNsgPIPPILPTTGLITSGPLNSAGAPR | S3 Phos                    | 100%             | 58.27                     | 105.66 | 400025018 | Putative membrane protein                             | unclassified           | 2.83E-02 | 827509  | 300622  | 1100476  | 1343381  |
| 102.87       | 2126.038 | VQsLNYLASLIGEDQQIR           | S3 Phos                    | 95%              | 13.28                     | 208.4  | 400073591 | Uncharacterized protein                               | unclassified           | 3.97E-02 | 704365  | 279159  | 981036   | 1298560  |
| cluster IV e |          |                              |                            |                  |                           |        |           |   |                        |          |         |         |          |          |
| 74.68        | 2597.94  | EGDGNdsDEDISYNEYEDDLPK       | S7 Phos                    | 100%             | 209.69                    | 328.98 | 400002103 | Protein decapping 5-like isoform X1                   | transcription          | 4.90E-03 | 3074844 | 2334874 | 30801226 | 15624171 |
| 58.29        | 1512.803 | SVtGVSPVIPRPPK               | T3 Phos                    | 97%              | 14.49                     | 79.43  | 400050062 | Uncharacterized protein                               | unclassified           | 3.22E-05 | 577148  | 681072  | 10847787 | 8787117  |
| 59.10        | 2816.359 | SATDSAKPIPDGSASVSQGPIAqSPQK  | S25 Phos                   | 100%             | 75.83                     | 108.63 | 400025768 | La-related protein 1C-like                            | transcription          | 6.33E-03 | 73368   | 78538   | 370786   | 246082   |
| 76.46        | 1111.565 | SfPVALAVK                    | T3 Phos                    | 100%             | 35.26                     | 83.02  | 400061684 | Uncharacterized protein                               | unclassified           | 8.53E-03 | 404242  | 302474  | 619407   | 616506   |
| 52.72        | 1150.454 | FmEAAsPFR                    | M2 Oxid, S6 Phos           | 100%, 100%       | 1,000.00, 1,000.00        | 121.19 | 400010406 | Uncharacterized protein                               | unclassified           | 3.31E-02 | 636533  | 548112  | 2313477  | 2317035  |
| 73.54        | 1505.672 | DLNIGsPPESELR                | S6 Phos                    | 100%             | 56.52                     | 56.55  | 400032855 | Putative membrane protein                             | unclassified           | 1.91E-03 | 445506  | 382411  | 679877   | 563017   |
| 68.92        | 919.4193 | QAsFFLK                      | S3 Phos                    | 100%             | 1,000.00                  | 54.72  | 400001041 | Epsin-3-like isoform X1                               | Prot. Destin. storage  | 5.24E-05 | 1598619 | 1253374 | 2863938  | 2391356  |
| 73.00        | 1184.553 | TFsGVLGPATR                  | S3 Phos                    | 100%             | 22.23                     | 54.16  | 400049440 | WD repeat-containing protein 44                       | signal transduction    | 1.43E-02 | 497044  | 404055  | 688650   | 578447   |
| 67.38        | 1569.692 | ATsNENLLGLESR                | S4 Phos                    | 98%              | 20.98                     | 131.53 | 400061157 | Serine hydroxymethyltransferase                       | metabolism             | 3.07E-03 | 1485096 | 1187919 | 2521091  | 2192981  |
| 82.45        | 1179.603 | FITPLGsPLR                   | S7 Phos                    | 100%             | 77.52                     | 122.85 | 400048002 | Lysine histidine transporter-like 8                   | transporter            | 4.44E-02 | 1666112 | 1006084 | 2658201  | 2338299  |
| 83.23        | 1320.665 | ISDLsPLLVER                  | S5 Phos                    | 100%             | 88.3                      | 156.46 | 400072493 | Uncharacterized protein                               | unclassified           | 3.92E-02 | 554584  | 325954  | 598279   | 569091   |
| 95.84        | 2312.06  | ALELLDGSAAFTAEEEDIsPR        | S20 Phos                   | 100%             | 70.71                     | 117.19 | 400007895 | Heat stress transcription factor B-2b-like isoform X1 | disease-defence        | 4.22E-02 | 649922  | 356990  | 1368932  | 1397647  |
| 67.48        | 1493.724 | NVAPGLSSSIslNR               | S11 Phos                   | 100%             | 33.56                     | 65.61  | 400038619 | Uncharacterized protein                               | unclassified           | 1.39E-03 | 662176  | 451824  | 853442   | 750321   |
| 82.89        | 1402.626 | sLSELEFEELK                  | S1 Phos                    | 100%             | 40.2                      | 129.24 | 400052590 | Uncharacterized protein                               | unclassified           | 6.88E-03 | 2126753 | 1373698 | 2727846  | 2509469  |
| 87.53        | 1460.716 | NLsFNVIQPPR                  | S3 Phos                    | 100%             | 1,000.00                  | 55.44  | 400073575 | Putative E3 ubiquitin-protein ligase ATL6-like        | Prot. Destin. storage  | 2.03E-03 | 822165  | 518186  | 1274592  | 1199761  |
| 35.42        | 2181.946 | EVDEPPsPPATTHDEEKPK          | S7 Phos                    | 100%             | 112.55                    | 120.52 | 400046956 | Uncharacterized protein                               | unclassified           | 2.08E-03 | 170362  | 112988  | 54950    | 65896    |
| 68.82        | 960.5024 | IGPLGLsPK                    | S7 Phos                    | 100%             | 1,000.00                  | 103.52 | 400020871 | 60S ribosomal protein L12 (Solanum tuberosum)         | protein synthesis      | 1.23E-04 | 1313498 | 863101  | 2718218  | 1824147  |
| 78.01        | 1034.54  | SIsLPTPLK                    | S3 Phos                    | 100%             | 43.2                      | 114.58 | 400049451 | Uncharacterized protein                               | unclassified           | 1.10E-02 | 4101387 | 2630482 | 5466448  | 3949054  |
| 98.56        | 1733.832 | DLsLNVIPLDQqGR               | S13 Phos                   | 100%             | 140.13                    | 130.76 | 400076613 | Putative RNA-binding protein NOB1-like                | transcription          | 2.34E-02 | 780790  | 521960  | 1860724  | 1455512  |
| 69.77        | 929.4359 | LAsGIFSR                     | S3 Phos                    | 100%             | 129.43                    | 145.05 | 400015781 | Probable ubiquitin-conjugating enzyme E2 23           | Prot. Destin. storage  | 6.39E-03 | 2411244 | 1532438 | 3838389  | 3065553  |
| 77.71        | 1546.704 | ISAASFFTGGsPPK               | T12 Phos                   | 98%              | 17.01                     | 168.42 | 400044237 | Uncharacterized protein                               | unclassified           | 1.30E-02 | 145471  | 77079   | 491522   | 317540   |
| 51.32        | 808.3833 | SIsLPGR                      | S3 Phos                    | 99%              | 19.23                     | 58.94  | 400096962 | Uncharacterized protein                               | unclassified           | 2.20E-02 | 989980  | 630845  | 986684   | 518124   |
| 107.59       | 2551.175 | NVQqATsPADQLASLsLNDNLVS      | S8 Phos                    | 97%              | 15                        | 90.61  | 400039842 | Polyadenylate-binding protein                         | transcription          | 1.63E-02 | 4420442 | 2090563 | 8748780  | 6937800  |
| 79.29        | 2537.168 | NETTtPVSsVVENDETPVAVVEK      | T5 Phos                    | 97%              | 17.98                     | 138.1  | 400058377 | Pollen-specific lysine-rich protein SBgLR             | unclear classification | 3.73E-02 | 467179  | 217133  | 211893   | 177045   |
| 82.74        | 1079.517 | NIGPLPsFR                    | S7 Phos                    | 100%             | 1,000.00                  | 97.34  | 400040626 | Serine/threonine-protein kinase At5g01020             | signal transduction    | 9.89E-03 | 4940920 | 2598145 | 6416343  | 4955116  |
| 33.98        | 2116.914 | LmNsPSKPVEQSTEATmR           | M2 Oxid, S4 Phos, M17 Oxid | 100%, 100%, 100% | 1,000.00, 17.99, 1,000.00 | 153.26 | 400042400 | WRKY protein  | transcription          | 4.10E-04 | 1272435 | 720145  | 152616   | 122555   |