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**Growth-related changes in
subcellular ion and protein patterns
in maize and field bean leaves under
salt stress**

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D - 24118 Kiel
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**Growth-related changes in subcellular ion and protein patterns
in maize and field bean leaves under salt stress**

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der Agrar- und Ernährungswissenschaftlichen Fakultät
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Muhammad Shahzad, M. Sc.
aus Pakistan

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Dekanin: Prof. Dr. Karin Schwarz

1. Berichterstatter: Prof. Dr. Karl H. Mühling

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Chapter 1

General introduction

1. General introduction:

Salinity stress negatively impacts agricultural yield throughout the world affecting plant production, whether it is for subsistence or economic gain. It has been estimated that nearly 80 million hectares of arable lands worldwide is affected by salinity (FAO, 2008). The soil solution of saline soils comprise of a range of dissolved salts, such as NaCl, Na₂SO₄, MgSO₄, CaSO₄, MgCl₂, KCl, and Na₂CO₃, each of which can contribute to salinity stress, but NaCl is the most prevalent salt and has been the focus of much of the work on salinity to date (Rengasamy, 2002; Munns and Tester, 2008; Tavakkoli et al., 2010; Shahzad et al., 2011). Salinity threat to global agriculture is also aggravating by becoming more prevalent with the intensity of land use increase worldwide (Meloni et al., 2003; Rangasamy, 2006). In arid and semi-arid regions, saline soils are especially abundant, because the evaporation is intense and the amount of rainfall is insufficient for substantial leaching. Thus, salinity is one of the main limiting factors for agricultural production in these areas.

Also, global food production will need to increase by 70% by 2050 (Rahnama, 2011) requiring gains in agricultural productivity with less land and water (Fischer et al., 2010). When the accumulated salt in soil layers is above 4 dS m⁻¹ it can adversely affects crop production, choosing salt-resistant crops and managing soil salinity are important strategies to boost agricultural economy (Rangasamy, 2010). Therefore, increasing the yield of crop plants in salinized lands is an urgent need for feeding the world population that is estimated to increase by 1.5 billion over the next 20 years (Yamaguchi and Blumwald, 2005; Rangasamy, 2006). Since soil salinization is the major reason of land degradation, salinity is one of the main problems for agriculture nowadays. This work focuses on NaCl as the cause of salinity. For this reason, the word salt will interchangeably used with NaCl in this thesis.

1.1. Causes of soil salinity

One of the major causes of salinity is the presence of a shallow water-table. If the groundwater is 2 metres deep or less for substantial periods of time, especially in summer, then salt dissolved in the soil water will diffuse to the soil surface. The growth and survival of agriculturally important plants can then become affected. A soil having electrical conductivity of its saturation extract (EC_e) above 4 dS m⁻¹ (Richards, 1954) is considered saline. Most of this salt-affected land has arisen from the accumulation of salts over substantial periods of time in arid and semi-arid zones (Rengasamy, 2002; Munns and Tester, 2008). The problem of salinization is increasing often due to bad agricultural practices (e.g., use of fertilizers on a massive scale), draining of aquifers, and a limited regional rainfall (Tester and Davenport, 2003), leading to inefficient resource management.

Saline soils are widespread on earth, and their genesis may be natural or accelerated by the extension of irrigated agriculture, the extensive use of water resources combined with high evaporation rates and human activity (Lambers, 2003; Arzani, 2008). Most of the cultivated lands has turned saline following land clearing or irrigation (secondary salinity; Munns and Tester, 2008), both of which cause water tables to rise and concentrate the salts within the root zone. In irrigated soils the problem of salt accumulation has exacerbated due to poor quality of irrigation water (Rengasamy, 2006; Rozema and Flowers, 2008). As a consequence, in every minute three hectares of arable land worldwide are lost because of soil salinity (FAO, 2005).

1.2. Plant growth under salinity

Growth inhibition is the primary injury that leads to other symptoms although programmed cell death may also occur under severe salinity shock (Zhu, 2007). As a result, salinity negatively influences the yield of the crop plants (Läuchli and Grattan,

2007). One of the big breakthroughs in the consideration of salt stress was the proposal of the two-phase model of growth response to salt stress by Munns (1993) (Läuchli and Lüttge, 2002). According to this model, salt stress in a first phase decreases plant growth due to osmotic problems. The osmotic effect initially reduces the ability of the plant to absorb water. This effect is similar to water stress and little variation among genotypes has been observed in this phase. After continuous salinization to the root medium, cells dehydrate and shrink, there is a gradual recovery of the growth rate until a new steady rate is reached (Munns, 2002). Plant shows shoot growth reduction after application of 100 mM NaCl for 14 days, while leaves showed no salt injury symptoms (Fig. 2).

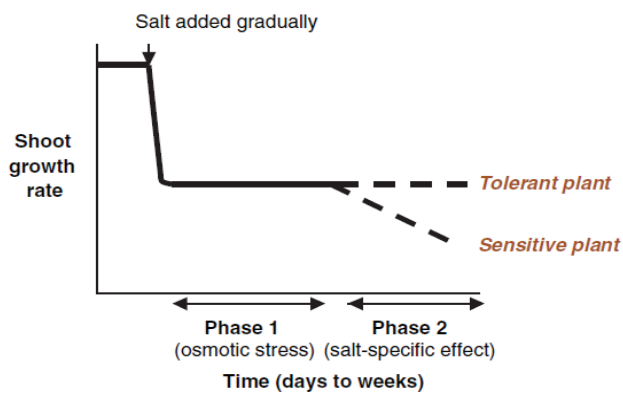


Figure 1. Schematic illustration of the two-phase growth response to salinity for genotypes that differ in the rate at which salt reaches toxic levels in leaves (Munns, 2005).

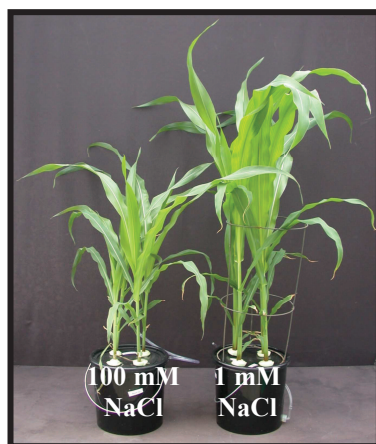


Figure 2. Influence of the NaCl concentration outside the roots, the osmotic stress phase, on the plant growth. Left pot: The presence of 100 mM NaCl at the roots over a period of 14 days decreases the growth of maize (*Zea mays* L.). Right pot: Control-treated maize (1 mM NaCl).

Despite of large body of literature on salt stress, physiological mechanisms behind this shoot growth inhibition are not fully understood (Munns and Tester, 2008). Also, in comparison to Na^+ toxicity and Na^+ exclusion, much less is known about the osmotic stress phase, which are encountered during the first phase of salt stress.

Especially in this phase monocotyledons plants show physiological Ca^{2+} deficiency (Mizrahi and Pasternak, 1985; Fortmeier and Schubert, 1995) as Na^+ induced inhibition of Ca^{2+} uptake and transport. In general salt-stressed maize plants in the first phase show stunted growth with dark-green leaves but without toxicity symptoms. Salinity often reduces shoot growth more than root growth (Läuchli and Epstein, 1990). However, when plants are growing in high salt concentrations, an adequate sequestration of ions in the vacuole can become a limiting factor, especially in the case of glycophytes. In this scenario, undue amounts of Na^+ accumulated by plants in their cytosol, disrupt many aspects of cellular physiology. Antagonistic effects on nutrient uptake may occur, causing deficiencies, particularly of K^+ and Ca^{2+} under conditions of excessive Na^+ content. For example, the physiochemical similarities between Na^+ and K^+ lead to competition at transport and catalytic sites that normally bind the essential cation K^+ and maintaining a high cytosolic K^+/Na^+ ratio is believed to improve salt resistance (Maathuis and Amtmann, 1999; Zhu, 2001).

In the second phase, much slower ionic effect is the result of salt accumulation in leaves, leading to salt toxicity in the plant, primarily in the older leaves. Salt toxicity can lead to the death of leaves and reduce the total photosynthetic leaf area toxicity builds up, limiting growth of sensitive genotypes.

Salinity stress involves different ways such as osmotic effects, specific-ion toxicity and/or nutritional disorders to affect plants (Läuchli and Epstein, 1990). Based on this two-phase concept, the initial growth reduction for both salt-sensitive and salt resistant plants is caused by an osmotic effect of the salts in the medium outside the roots. In contrast, in the second phase, a salt-sensitive species or genotype differs from a more salt resistant one by its inability to prevent salt from accumulating in transpiring leaves to toxic levels (Munns et al., 2006).

1.3. Alleviation by silicon of salinity stress

Silicon is ubiquitous in our environment; it is the second most prevalent element in the earth's crust (Epstein, 1999). Silicon is absorbed by plants in the form of uncharged silicic acid, Si(OH)_4 , and is ultimately irreversibly precipitated throughout the plant as amorphous silica. Therefore, although silicon is plentiful, most sources of silicon are insoluble and not in a plant available form in soils (Richmond and Sussman, 2003). The element has beneficial effects in enhancing the resistance of plants to biotic and abiotic stresses (Epstein, 1999; Ma, 2004), and benefits of silicon in enhancing salt resistance have been found in many plant species e.g. barley, tomato and cucumber (Liang et al., 2003; Al-Aghbary et al., 2004; Zhu et al., 2004). Matoh et al. (1986) reported that silicate at 0.89 mM reduces the translocation of Na^+ to the shoots and increase dry matter production of salt-stressed rice plants compared to control. In addition, silicon mediated resistance was also observed in the leaf apoplast of *Cucumis sativus* L. (Rogalla and Römheld, 2002). Silicon, taken up as Si(OH)_4 by plants is mainly deposited in the apoplast (Handreck and Jones, 1968). However, in recent investigation with dicot pumpkin an influx transporter for silicon has been reported.

1.4. Significance of apoplast in plants growth processes under salinity

The apoplast of plant cells is a dynamic compartment involved in many processes, including maintenance of tissue shape, development, nutrition, signalling, detoxification and defence (Dani et al., 2005). The metabolic and ion concentrations in the apoplastic fluid of leaves are of interest in several fields of plant biology (Lohaus et al., 2001) i.e. response to stress (Mühling and Läuchli, 2002, 2003; Shahzad et al., 2011), leaf growth, enzyme activities, exchange between plant and environment (Husted and Schjoerring, 1995), phloem loading, plant-pathogen

interactions, and perception and transduction of environmental signals (Hoson, 1998).

The apoplast is the portion of the plant cell outside the cell membrane (Fig. 3). This region includes the cell walls and intercellular space of the plant (Dietz, 1997). To date, most identified salt stress resistance-related signaling components have been localized to the cytosol or cell membrane. Although it is an important component of the plant cell, the plant apoplast has been ignored in studies of the salt stress response. The apoplast of plant cells, which carries out multiple functions in plant metabolism, is not only a barrier but also the linker between the environment and the protoplast.

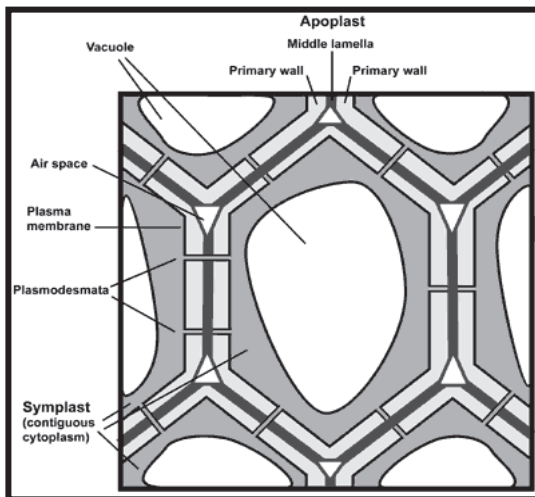


Figure 3. Apoplast and symplast; Primary cell wall structure (Albersheim, 1976).

The plant apoplast comprises many organic and inorganic molecules, as well as some enzymes. These molecules have essential functions in plant cell metabolism (Nielsen and Schjoerring, 1998), with responses to pathogen stress (Misas-Villamil and van der Hoorn, 2008), cell division and proliferation, cell differentiation (Takeda et al., 2003), and, particularly, responses to drought and salt stress (Dietz, 1997; Ramanjulu et al., 1999).

Previously, Oertli (1968) suggested that salt accumulation in the extracellular space of the leaves can lead to plant growth reduction, later wilting of the leaves and finally the cell death. Later, Flowers et al. (1991) supported this idea by finding very high 600 mM NaCl in the leaf apoplast of rice plants by presenting only 50 mM NaCl to the roots for 7 days. However, later was criticised as the data were produced by x-ray microanalysis, with problem in resolution to detect precisely the Na⁺ concentration in the small apoplastic space. In the same year, Speer and Kaiser (1991) found 87 mM Na⁺ and 204 mM Cl⁻ in the leaf apoplast of the salt-sensitive pea compared with 7 mM Na⁺ and 13 mM Cl⁻ in the leaf apoplast of more salt-resistant spinach plants after the application of 100 mM NaCl for 14 days. Unfortunately, 100 mM NaCl was supplied only in one step without allowing plants to adjust to the altered osmotic conditions.

Later, very low salt accumulation was observed in the leaf apoplast under conditions of salinity in investigations with salt-sensitive maize (Lohaus et al., 2000; Mühling and Läuchli, 2002) and wheat (Mühling and Läuchli, 2003; Wimmer et al., 2003). However, considering above mentioned investigations contradictory results have been found in previous decades for Na⁺ accumulation in the leaf apoplast of different plant species.

Proteins present in the plant apoplast reflect this broad functional diversity. Studies on the dynamic change of apoplastic protein composition revealed new insights into plant responses to abiotic stress (Mühling and Läuchli, 2003; Dani et al., 2005; Tasgin et al., 2006; Ramanjulu et al., 1999), nutrient supply (Wimmer et al., 2003; Alves et al., 2006; Fecht-Christoffers et al., 2003), wounding (Soares et al., 2009), water deficiency (Bhushan et al., 2007; Pandey et al., 2010), pathogen response (Floerl et al., 2008; Goulet et al., 2010) and xylem composition (Djordjevic et al., 2007; Alvarez et al., 2006). To date, however, biological functions have been

reported for only a few apoplastic proteins. The selection of a suitable extraction protocol is a crucial step in proteomics surveys as proteins reveal a high degree of biochemical heterogeneity and investigated plant materials can be categorized by the presence of non-protein components interfering with subsequent analytical techniques, e.g. two-dimensional gel electrophoresis (2-DE) or liquid chromatography-mass spectrometry (LC-MS).

Proteomics is now emerging as an influential tool for studying these protein dynamics, especially in plant stress responses (Lee et al., 2004; Zhang et al., 2009). A number of studies have been carried out using proteomic approaches to identify pathogen response proteins in rice and arabidopsis (Kim et al., 2003; Ndimba et al., 2003) and salt stress response proteins in the rice microsome (Lee et al., 2004), root (Zhang et al., 2009), and leaf sheath (Abbasi and Komatsu, 2004). Dani et al. (2005) used a proteomic approach to analyze the proteome changes in the tobacco (*Nicotiana tabacum*) leaf apoplast during long-term (20 d) salt stress. Nevertheless, few studies have addressed the changes in the apoplast proteome in response to salt stress, especially during the initial phase of salt stress. Fecht-Christoffers et al. (2003) also pointed to an enhanced release of pathogen-related-like protein in the leaf apoplast of cowpea as a late response of manganese toxicity. These data suggest that there might be a number of proteins in the plant apoplast that are involved in the plant stress response. The identification of these proteins during the initial phase of salt stress will be an important step toward understanding the role of these apoplastic proteins in the plant salt stress response

1.5. Objectives

The primary reaction of plants to salinity is the reduction in leaf growth due to the osmotic effects caused by the salt outside the roots. The present study also focuses in osmotic phase or before the start of the second phase.

In this study the roles of ion changes in relation to growth reduction under salinity stress was investigated in the leaf apoplast of salt sensitive maize (monocot) and field bean (dicot). An infiltration-centrifugation technique has been used to observe ion and protein changes in the maize leaf apoplast under salinity. In addition in field bean investigation, in terms of ion accumulation silicon role in the subcellular compartments of the leaf such as apoplast and symplast under salinity stress was also examined.

Increase in apoplastic salt accumulation has been explained as possible reason for reduction in leaf growth, leaf wilting, and finally cell death (Oertli 1969; Flowers et al. 1991; Speer and Kaiser 1991). However, there is still need to prove whether or not the hypothesis is still valid.

To investigate the apoplastic protein the composition of the infiltration solution is essential as it has to fulfil certain prerequisites, such as maintenance of osmotic pressure to prevent collapsing of plasma membrane and stringency for extracting cell wall-bound proteins. In this study, we examined protein extracts obtained by six different infiltration solutions already described for apoplast proteins from different plant species.

This thesis also focused to evaluate the changes in the maize leaf apoplast proteins during the initial phase of salt stress by two-dimensional electrophoresis (2-DE). This study was conducted to unravel the possible contribution of apoplastic proteins to the salt induced reduction in leaf growth under salt stress.

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Chapter 2

Growth-Related Changes in Subcellular Ion Patterns in Maize Leaves (*Zea mays* L.) under Salt Stress

M. Shahzad, K. Witzel, C. Zörb & K. H. Mühling

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SALINITY STRESS

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M. Shahzad, K. Witzel, C. Zörb & K. H. Mühling

Institute of Plant Nutrition and Soil Science, Christian Albrechts University, Kiel, Germany

Keywords

apoplast; growth; ion chromatography; maize; salinity

Correspondence

K. H. Mühling
Institute of Plant Nutrition and Soil Science
Christian Albrechts University
Hermann-Rodewald-Str. 2
24118 Kiel, Germany
Tel.: +49 0431 880 3189
Fax: +49 0431 880 1625
Email: khmuehling@plantnutrition.uni-kiel.de

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Abstract

Na⁺ accumulation in the leaf apoplast has been suggested to lead to dehydration, later wilting and finally, the death of the affected leaves. Our aim has been to evaluate whether the reduction in the plant growth of sensitive maize in response to salinity is correlated with higher amounts of Na⁺ and Cl⁻ concentrations in the leaf apoplast. Subcellular ion patterns in intact leaves were investigated by using deionised water infiltration. We found an increase in soluble Na⁺ and Cl⁻ concentrations of about 16- and 4-fold, respectively, compared with the control. These concentrations characterized the apoplasts of expanding leaves that had entirely developed under salinity. Interestingly, the K⁺ concentration was significantly reduced by 64 % compared with its control in the symplast under salinity. Our finding of a significantly decreased Ca²⁺ concentration in shoots suggested a possible association of Ca²⁺ concentration with the reduction in leaf expansion under salinity. As the absolute increase in the apoplastic Na⁺ concentration during salt treatment was much lower compared with the increase in the symplastic Na⁺ concentration, salt treatment in maize appears not to result in osmotic stress imposed by a high apoplastic Na⁺ concentration as has been suggested for other plant species (Oertli hypothesis).

Introduction

Salinity is one of the cardinal stress factors affecting crop plants by shoot growth inhibition, thus limiting agricultural production worldwide (Läuchli and Lüttge 2002). The sensitivity of crop species and genotypes determines the level of growth reduction by salinity (Mühling and Läuchli 2002). Excessive Na⁺ is commonly held to be responsible for the reductions in growth and yield under salinity (Chi Lin and Huei Kao 2001, Tsai et al. 2004, Hong et al. 2009). The mechanisms by which Na⁺ ions enter the cells have therefore been extensively studied, and the Na⁺ exclusion mechanism, which aids salt resistance, is known in molecular detail (Amtmann and Sanders 1999, Tester and Davenport 2003, Apse and Blumwald 2007). During the continuous exposure of salt to the plant, the rate of export from the root can exceed the rate of compartmentalization in the shoot. If this occurs, salt ions will accumulate outside the vacuole, either in the cytoplasm or in the apoplast (Volkmar et al. 1998), and produce toxicity symptoms.

An additional explanation for toxicity symptoms is that an accumulation of salt in the leaf apoplast leads to the death of leaves by dehydration of leaf cells and turgor loss (Oertli 1968). Munns (1993) has suggested that older leaves die as a result of a fast increase in the salt concentration in the apoplast or cytoplasm when vacuoles can no longer sequester incoming salts. The transport of Na⁺ into older leaves and their subsequent death as a result of the build-up of high Na⁺ has previously been shown under conditions of salinity (Fortmeier and Schubert 1995). Therefore, Na⁺ accumulation in leaves, particularly in the leaf apoplast, might be responsible for Na⁺ toxicity (Volkmar et al. 1998). An increased Na⁺ efflux from the cytoplasm into the apoplast might result from the plasma membrane ATPase being stimulated under salt stress (Niu et al. 1995) or be caused by incoming salt from the root into the leaf apoplast (Munns 1993).

The apoplast comprises the entire volume of the tissue external to the plasma membrane and is also an important compartment for the transport and delivery of ions,

assimilates and other metabolites (Grignon and Sentenac 1991, Dietz 1996, Sattelmacher et al. 1999). It is thought to participate in numerous physiological processes in plants, such as development, growth, leaf movement, gas exchange and pathogen defence. However, it is still not as well characterized as the symplast. Although most research has concentrated on the root apoplast, knowledge of the regulation of the ionic status of the apoplast in leaf tissue is also essential for gaining a better understanding for the decline in leaf growth under salinity.

Despite being the predominant anion in saline soils, the importance of high Cl^- concentrations to salt resistance and the mechanism of Cl^- resistance are less well understood compared with Na^+ (Britto et al. 2004, Teakle and Tyerman 2010). Chloride is a vital micronutrient that regulates enzyme activities in the cytoplasm, is a co-factor in photosynthesis, acts as a counter anion to stabilize membrane potential and is involved in turgor and pH regulation (Xu et al. 2000, White and Broadley 2001). We have focused, in the present study, on the chloride distribution under salinity in various parts of a plant, including the subcellular apoplastic and cytoplasmic concentrations of Cl^- .

Only a limited number of studies have dealt with the importance of the leaf apoplast under saline conditions (Flowers et al. 1991, Speer and Kaiser 1991, 1994, Mühling and Läuchli 2002) compared with the large body of literature concerning salt stress. Contradictory results have been found with regard to ion concentrations in the leaf apoplast of various plant species. Initially, Flowers et al. (1991) presented data in support of the Oertli hypothesis by finding accumulations of Na^+ ions up to 600 mM in the apoplast of rice leaves given a 50 mM Na^+ supply for 7 days, as shown by means of X-ray microanalysis. Speer and Kaiser (1991) found 87 mM Na^+ and 204 mM Cl^- in the leaf apoplast of the salt-sensitive pea compared with 7 mM Na^+ and 13 mM Cl^- in the leaf apoplast of more salt-resistant spinach plants after the application of 100 mM NaCl for 14 days. In agreement with the latter, Ramanjulu et al. (1999) detected low apoplastic Na^+ in leaves (up to about 7 mM) of more salt-resistant barley plants under 100 mM NaCl salinity.

Extremely low salt accumulation was observed in the leaf apoplast under conditions of salinity in investigations with salt-sensitive maize (Lohaus et al. 2000, Mühling and Läuchli 2002) and wheat (Mühling and Läuchli 2003). However, a short-term salt treatment of 8 days was applied compared with the present investigation of 16 days when plants were still in the first phase of adaptation. Different from the maize study by Mühling and Läuchli (2002), only expanding leaves with no salt injury were used for apoplastic fluid extraction and these leaves had developed entirely under salinity. Therefore, the aim

of the present study has been to assess the importance of ions in expanding leaves, together with subcellular concentrations in salt-sensitive maize under conditions of salinity.

Materials and Methods

Plant cultivation and NaCl treatment

Seeds of maize (*Zea mays* L. cv Lector, Limagrain GmbH) were soaked overnight in aerated 1 mM CaSO_4 solution and germinated at 28 °C in the dark between two layers of filter paper moistened with 0.5 mM CaSO_4 . Seedlings were transferred after 4 days to light in constantly aerated plastic pots (four plants per pot) with 4.3 l of a one-fourth concentrated nutrient solution. The concentration of the nutrient solution was increased after 2 and 4 days of cultivation to half- and full-strength, respectively. The full-strength nutrient solution had the following concentrations: 2.0 mM $\text{Ca}(\text{NO}_3)_2$, 1.0 mM K_2SO_4 , 0.2 mM KH_2PO_4 , 0.5 mM MgSO_4 , 2.0 mM CaCl_2 , 5.0 μM H_3BO_3 , 2.0 μM MnSO_4 , 0.5 μM ZnSO_4 , 0.3 μM CuSO_4 , 0.01 μM $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$, 200 μM Fe-EDTA. The nutrient solution was changed twice a week to avoid nutrient deficiencies. The NaCl treatment started when the full-nutrient concentration was applied and at the emergence of the 5th leaf stage. Later shoot fractions above the 5th leaf and including 5th leaf are referred to as expanding shoot tissue. The tested levels of NaCl concentrations were 1 mM for controls and 100 mM for salt treatments unless otherwise described. The plants were slowly acclimated to salinity in daily increments of 25 mM until a final concentration of 100 mM NaCl was reached. Each treatment was run with ten replicates. The experiments were carried out in a greenhouse with an average day/night temperature of 28/18 °C and a photoperiod of 14 h ($\approx 400 \mu\text{mol m}^{-2}$ per s PAR) for 5 weeks at a relative humidity of about $70 \pm 5\%$. For apoplastic washing fluid (AWF) collection, the 5th and 6th two youngest expanded leaves were used. These leaves showed no visible salt stress symptoms and were excised 16 days after reaching the maximum salinity level at 100 mM NaCl.

Relative water content

All mass measurements were made by using an analytical balance with precision of 0.1 mg. Values of fresh weight (FW) and dry weight (DW) were used to calculate the relative water content (RWC) [%] by using the equation below:

$$\text{RWC}(\%) = \frac{\text{FW} - \text{DW}}{\text{FW}} \times 100$$

Parameters of Na⁺ exclusion

Sodium uptake at the root surface was calculated as the ratio of the total plant Na⁺ content to the root DW. High Na⁺ uptake indicated inefficient Na⁺ exclusion. Sodium root-to-shoot translocation was determined as the ratio of the total shoot Na⁺ content to the total root Na⁺ content.

Estimate of apoplastic volumes occupied by water and air

Apoplastic air volume was determined by the silicone oil method (Cosgrove and Cleland 1983, Husted and Schjoerring 1995). A determination of the apoplastic water space was carried out by a modified indigo carmine method (Cosgrove and Cleland 1983, Husted and Schjoerring 1995). The apoplastic water volume was calculated by using the equation $F_{dil} = (V_{water} + V_{air})/V_{air}$ according to Husted and Schjoerring (1995).

Extraction of water-soluble ions from the leaf apoplast

Apoplastic fluid was collected by the infiltration–centrifugation technique (according to Mühling and Sattelmacher 1995, Lohaus *et al.* 2001). Leaves were cut into segments of about 5.5 cm with a razor blade and carefully washed with deionised water. For infiltration, leaf segments were placed in plastic syringes (60 ml) filled with 40 ml deionised water and were infiltrated by pulling the plunger, producing a reduced pressure of an estimated 20 kPa (Lohaus *et al.* 2001). Thereafter, intact leaves were carefully blotted dry, placed in a 10-ml plastic vessel and centrifuged immediately at 400 g, for 5 min at 5 °C. AWF were stored at –80 °C for analysis. The ion concentration in the apoplastic water space of the maize leaves was calculated after multiplication of the ion concentration in the AWF by the dilution factor ($F_{dil} = V_{water} + V_{air}/V_{water}$) of 3.

Preparation of leaf sap

After the separation of the AWF, the residual leaf tissue was shock-frozen in liquid nitrogen at –70 °C, thawed and centrifuged at 715 g, for 5 min, as described above. Thereafter, the leaf sap, which was further described as the symplastic leaf fraction, was stored at –80 °C until analysis.

Determination of ion concentration in the leaf sap and AWF

Samples were mixed with chloroform and centrifuged, and the clear supernatant was collected. The concentration of ions was analysed in the leaf sap and AWF by ion chromatography (DX300; Dionex, Idstein, Germany) on

an IonPac anion exchange column (AS9; Dionex) or on cation exchange column (AS12; Dionex) connected to a conductivity detector module (Dionex). Isocratic elution with H₂SO₄ (15 mM, 1 ml min^{–1}) was used for the separation of the cations, whereas isocratic elution with Na₂CO₃ (1.7 mM) and NaHCO₃ (1.8 mM) was used for anions.

Determination of total ion concentration in leaves and roots

Leaf and root materials were ashed overnight in an oven at 520 °C. Analysis of cations was performed on the ash of 150 mg dried plant material. The ash was dissolved in 2 ml 4 M HNO₃ with gentle heating. This suspension was adjusted to a volume of 10 ml with distilled water and filtered through a paper filter (white 589/3; Schleicher and Schuell, Dassel, Germany). Thereafter, Na⁺, K⁺, Ca²⁺ and Mg²⁺ concentrations were analysed in the filtrate by means of atomic absorption spectrometry (S series AA Spectrometer; Thermo Electron Corporation, MA, USA). Analysis of anions was performed on 30 mg dried plant material that was dissolved in 1.6 ml deionised water by placing it for 5 min in boiling water. After being cooled, each sample was centrifuged and the supernatant was collected. After washing with chloroform, samples were passed through C-18 columns (Bakerbond spe. Octadecyl C18, 7020, 01, Torrance, CA, USA) for cleaning and Cl[–], NO₃[–], SO₄^{2–} and PO₄^{3–} concentrations were analysed by using ion chromatography (DX300; Dionex).

Statistics

Data were normally distributed and significant differences at $P < 0.05$ between treatments were calculated by using the general linear model with a Tukey test (Statistical Product and Service Solutions, SPSS, Chicago, IL, USA) and *t*-test. Error bars in the figures indicate the mean standard error (S.E.).

Results

Physiological effects of salinity stress at various growth stages

The 100 mM NaCl treatment had a considerable adverse effect on the shoot yield, whereas root growth showed no significant effect (Fig. 1a). The yield of expanding shoot tissues that were entirely grown under salt stress showed a 60 % growth reduction compared with its control, whereas the fresh weight of the expanded shoot tissues had no major salt effect compared with its respective control (Fig. 1b).

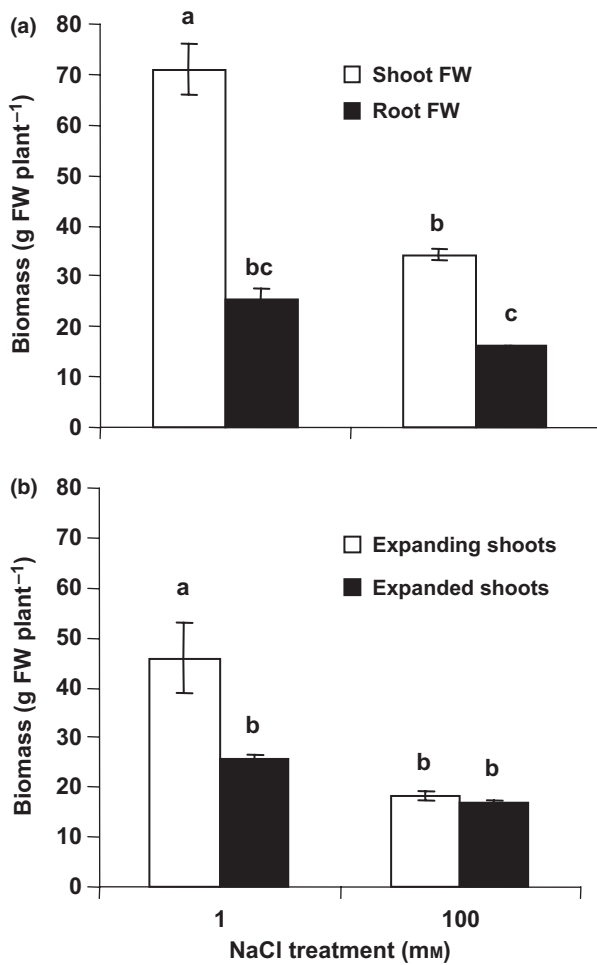


Fig. 1 Effect of NaCl concentration on the fresh weight (FW) of entire shoot and root (a), and the fresh weight of expanding and expanded shoot tissues (b) of maize cv Lector. Different letters indicate significant differences between the treatments at $P < 0.05$ ($n = 3$).

The RWC was significantly reduced in young shoot tissues under salt treatment compared to control with 92 % and 88 % at 1 mM NaCl and 100 mM NaCl, respectively (Fig. 2a). No major difference under salt treatment was observed in older shoot tissue (Fig. 2b) or roots (Fig. 2c).

Yield of AWFs and parameters of Na⁺ exclusion

The yield of AWF was significantly reduced with 62 and 42 $\mu\text{l g}^{-1}$ FW in control and under salt treatment, respectively (Fig. 3a). No considerable reduction was noticed in cell sap under salt treatment. The yield of symplastic fluid extract was 213 $\mu\text{l g}^{-1}$ FW in control and 203 $\mu\text{l g}^{-1}$ FW under salt treatment (Fig. 3b). Na⁺ exclusion parameters values for root to shoot translocation and Na⁺ uptake

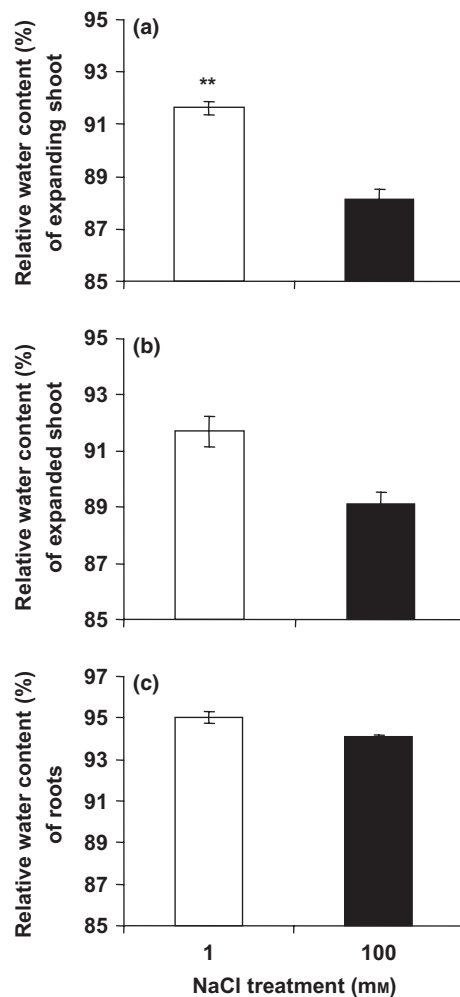


Fig. 2 Effect of NaCl concentration on relative water content in expanding shoot tissue (a), expanded shoot tissue (b) and roots (c) of maize cv Lector (control white vs. salt stress black bars). **Significant differences between the treatments at $P < 0.01$ ($n = 3$).

were 1.86 (Na⁺ shoot/Na⁺ root) and 92.8 (mg Na⁺/g root DW), respectively.

Ratios of Na⁺/K⁺, Na⁺/Ca²⁺ and Na⁺/Mg²⁺ in total plant and subcellular fractions

Higher ratios for Na⁺/K⁺, Na⁺/Ca²⁺ and Na⁺/Mg²⁺ were observed under salinity treatment compared with controls. Significant differences were noticed compared with control in the different plant fractions (Table 1) and in the subcellular compartments of the apoplast (Table 2) and symplast (Table 3). An increasing Na⁺/Ca²⁺ and Na⁺/Mg²⁺ ratio was detected from roots to expanding shoot fractions, whereas Na⁺/K⁺ ratio decreased. However, the maximum Na⁺/Ca²⁺ ratios under salt treatment were 18.4

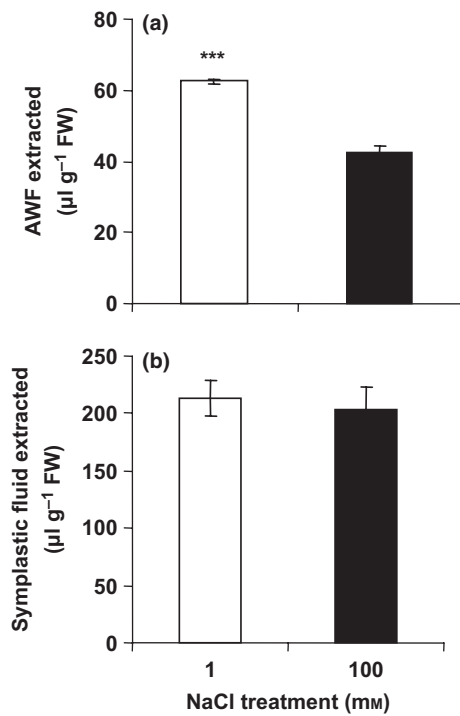


Fig. 3 Effect of NaCl concentration on the yield of extracted apoplastic washing fluid (a) and symplastic fluid extracted (b) of maize cv Lector leaves. ***Significant differences between the treatments at $P < 0.001$ ($n = 7$).

and 22.2 in the apoplast and symplast of expanding shoots, respectively.

Ion concentrations in expanding and expanded leaves and in roots

Each plant was harvested in three parts, i.e. expanding shoots (5th leaf, above 5th leaf), expanded shoots (below 5th leaf) and roots, to differentiate the growing plant parts with respect to their total ion concentration. The addition of 100 mM NaCl reduced the K^+ concentration in all three plant fractions, but no considerable difference was observed compared with their respective controls (Fig. 4a). However, the K^+ concentration was significantly lower in expanding shoot tissues compared with

Table 2 Influence of salinity on apoplastic Na^+/K^+ , Na^+/Ca^{2+} and Na^+/Mg^{2+} ratios. Significant differences are shown at $P < 0.001$ by *t*-test, \pm S.E.

Treatment	Na^+/K^+	Na^+/Ca^{2+}	Na^+/Mg^{2+}
Control	$0.11 \pm (0.02)$	$1.05 \pm (0.25)$	$0.50 \pm (0.14)$
Salt treated	$2.02^{***} \pm (0.12)$	$18.4^{***} \pm (1.11)$	$10.8^{***} \pm (0.36)$

Table 3 Influence of salinity on symplastic Na^+/K^+ , Na^+/Ca^{2+} and Na^+/Mg^{2+} ratios. Significant differences are shown at $P < 0.01$ by *t*-test, \pm S.E.

Treatment	Na^+/K^+	Na^+/Ca^{2+}	Na^+/Mg^{2+}
Control	$0.01 \pm (0.002)$	$0.06 \pm (0.02)$	$0.02 \pm (0.01)$
Salt treated	$3.52^{**} \pm (0.33)$	$22.2^{**} \pm (1.56)$	$9.4^{**} \pm (0.51)$

expanded shoot tissues. The Na^+ concentration was significantly increased during salt treatment from younger to older leaves (Fig. 4b). By comparing all plant fractions, a significant maximum Na^+ concentration was found in expanded shoot tissue ($105 \mu\text{mol g}^{-1}$ FW). An analysis of the total Ca^{2+} concentration revealed a significant reduction in shoots under salt treatment compared with their respective controls (Fig. 4c). This reduction in Ca^{2+} concentration compared with the control was 58 % in expanding shoot tissue and 34 % in expanded shoot tissue. Hereby, a significant reduction in Ca^{2+} concentration was also observed in expanding shoot tissues compared with expanded shoot tissues under salt treatment. However, no significant reduction in Ca^{2+} concentration was observed in roots under salt treatment. The 100 mM NaCl treatment lead to a 31 % significant reduction in Mg^{2+} concentration compared with its control in roots but had no major influence on the two shoot fractions (Fig. 4d).

The same plant parts were used for total anion concentrations as for cations. The concentrations of Cl^- , NO_3^- and SO_4^{2-} increased from younger to older leaves on addition of 100 mM NaCl (Fig. 5). Concentrations in older leaves were $95 \mu\text{mol g}^{-1}$ FW for Cl^- , $15 \mu\text{mol g}^{-1}$ FW for NO_3^- and $0.9 \mu\text{mol g}^{-1}$ FW SO_4^{2-} . The NO_3^- concentration was 54 % reduced in expanding shoot tissue under salt treatment compared with its respective control.

Table 1 Influence of salinity on Na^+/K^+ , Na^+/Ca^{2+} and Na^+/Mg^{2+} ratios. Significant differences are shown at $P < 0.01$ by *t*-test, \pm S.E.

Plant fractions	Na^+/K^+		Na^+/Ca^{2+}		Na^+/Mg^{2+}	
	Control	Salt treated	Control	Salt treated	Control	Salt treated
Expanding shoots	$0.01 \pm (0.001)$	$0.51^{**} \pm (0.06)$	$0.10 \pm (0.01)$	$7.82^{**} \pm (0.96)$	$0.47 \pm (0.02)$	$16.7^{**} \pm (2.31)$
Expanded shoots	$0.01 \pm (0.003)$	$0.52^{**} \pm (0.07)$	$0.04 \pm (0.01)$	$2.26^{**} \pm (0.21)$	$0.46 \pm (0.06)$	$15.5^{**} \pm (2.21)$
Roots	$0.02 \pm (0.001)$	$0.91^{**} \pm (0.10)$	$0.05 \pm (0.01)$	$2.26^{**} \pm (0.28)$	$0.17 \pm (0.05)$	$8.00^{**} \pm (0.51)$

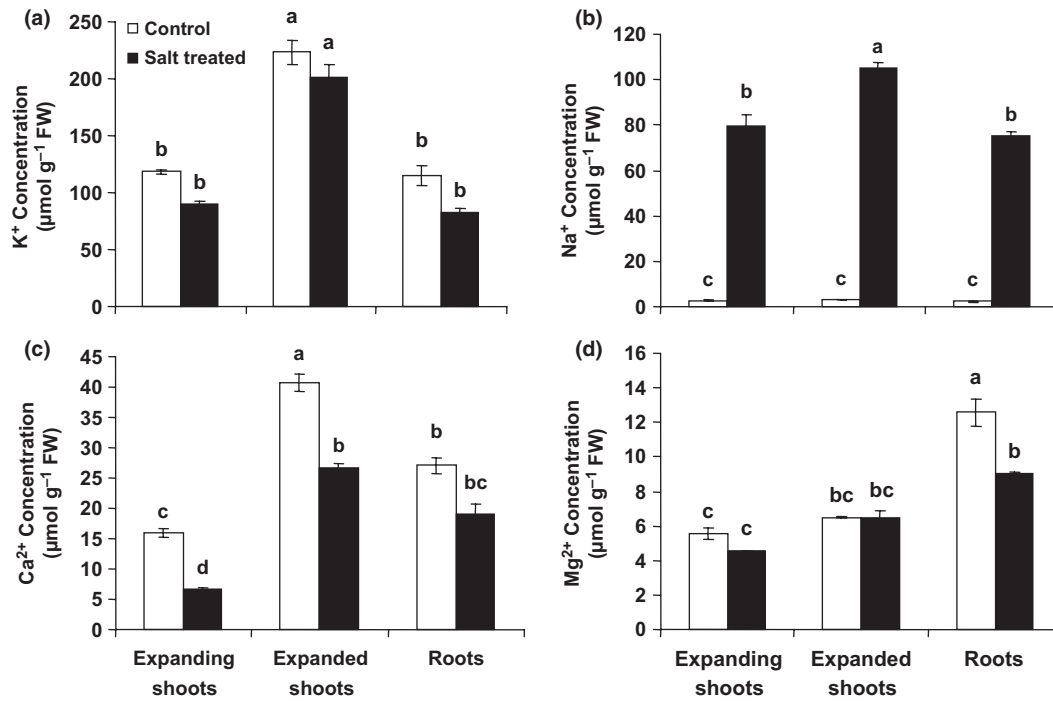


Fig. 4 Effect of 100 mM NaCl supply on cation concentrations of $[\text{K}^+]$, (a), $[\text{Na}^+]$, (b), $[\text{Ca}^{2+}]$ (c) and $[\text{Mg}^{2+}]$ (d) in expanding shoots, expanded shoots and roots of maize cv Lector leaves (control white vs. salt stress black bars). Different letters indicate significant differences between the treatments at $P < 0.05$ ($n = 3$).

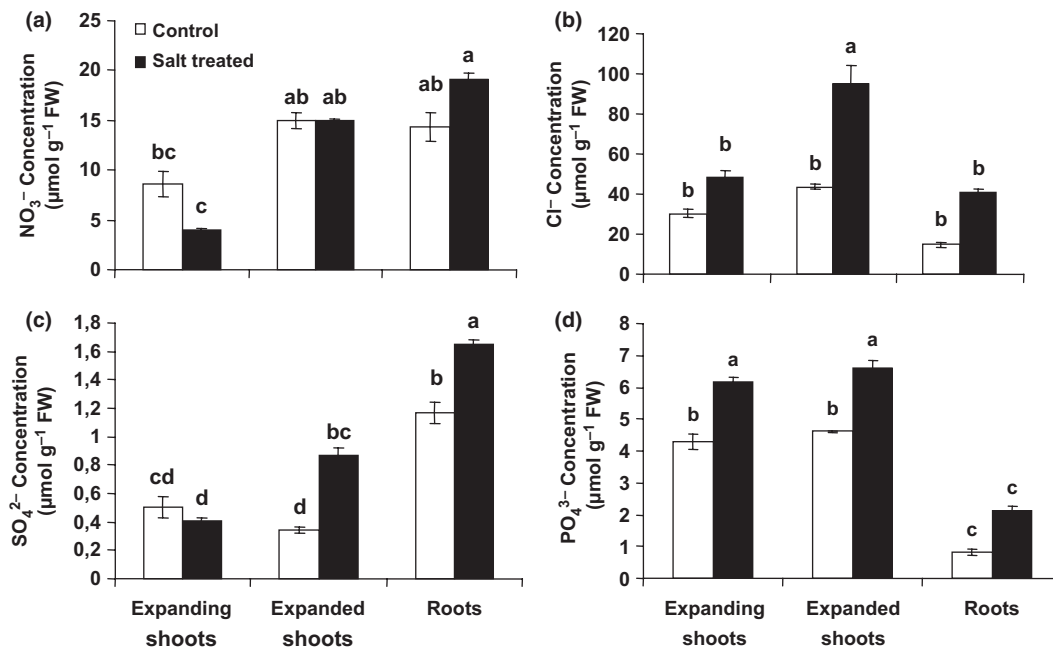


Fig. 5 Effect of 100 mM NaCl supply on anion concentrations of $[\text{NO}_3^-]$ (a), $[\text{Cl}^-]$ (b), $[\text{SO}_4^{2-}]$ (c) and $[\text{PO}_4^{3-}]$ (d) in expanding shoots, expanded shoots and roots of maize cv Lector leaves (control white vs. salt stress black bars). Different letters indicate significant differences between the treatments at $P < 0.05$ ($n = 3$).

A significant increase in SO_4^{2-} concentration was found in expanded shoot tissues and roots under salt treatment. PO_4^{3-} concentration increased significantly under salt treatment compared with control in both expanding and expanded shoot tissues. In roots, NO_3^- and SO_4^{2-} concentrations were higher compared with expanding shoots, whereas PO_4^{3-} significantly decreased in roots under salinity (Fig. 5).

Soluble cations and anions within the leaf apoplast and symplast

The Na^+ concentration in the apoplast of maize leaves was found to be significantly higher under salt treatment (Fig. 6a), and the concentration was 16-fold higher compared with its control. The finding that the K^+ concentration remained relatively constant at below 19 mm also

confirmed that the apoplastic fluid was only slightly contaminated with cytoplasm. The concentrations of other apoplastic cations, i.e. K^+ , Mg^{2+} and Ca^{2+} , were not affected under salt stress (Fig. 6a). In terms of anions, a 4-fold higher Cl^- concentration was found under NaCl treatment compared with its control. The concentrations of other apoplastic anions, i.e. NO_3^- , SO_4^{2-} and PO_4^{3-} , remained lower than 3 mm with no major effect at 100 mM NaCl treatment (Fig. 6b).

When plants were treated with 100 mM NaCl, a 172 mM Na^+ concentration was found in the symplastic space of maize leaves, although soluble ions were extracted from the leaf apoplast by water infiltration (Fig. 7a). Compared with its respective control, the K^+ concentration was 64 % significantly reduced in symplast under the influence of 100 mM NaCl treatment. Mg^{2+}

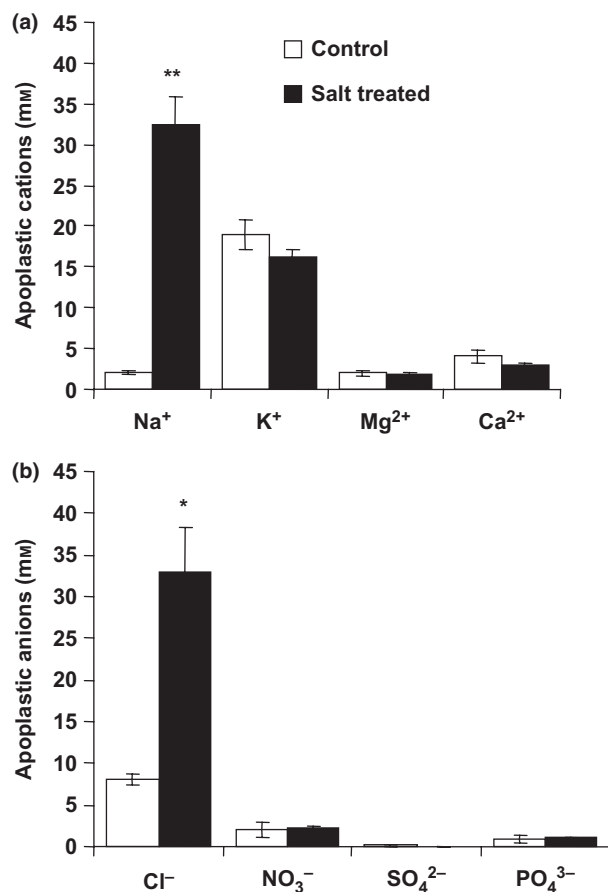


Fig. 6 Influence of salinity on apoplastic concentrations of cation [Na^+], [K^+], [Mg^{2+}] and [Ca^{2+}] (a) and anion [Cl^-], [NO_3^-], [SO_4^{2-}] and [PO_4^{3-}] (b) concentrations in the apoplast of maize cv Lector leaves (control white vs. salt stress black bars). *,**Significant differences between the treatments at $P < 0.05$, 0.01 , respectively ($n = 4$).

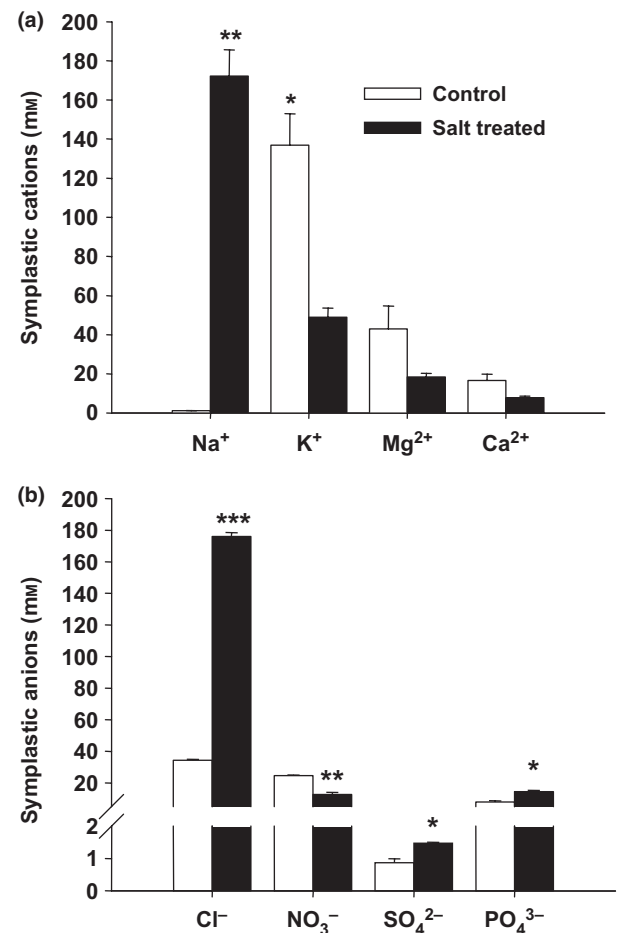


Fig. 7 Influence of salinity on symplastic concentrations of cation [Na^+], [K^+], [Mg^{2+}] and [Ca^{2+}] (a) and anion [Cl^-], [NO_3^-], [SO_4^{2-}] and [PO_4^{3-}] (b) concentrations in the symplast of maize cv Lector leaves (control white vs. salt stress black bars). *,**,*** Significant differences between the treatments at $P < 0.05$, 0.01 , 0.001 , respectively ($n = 4$).

and Ca^{2+} concentrations were also reduced in symplast under salt stress but with no significant difference (Fig. 7a). A significantly higher 176 mM Cl^- concentration was detected in the leaf symplast under the 100 mM NaCl treatment compared with its control (Fig. 7b), whereas NaCl treatment led to a significant decrease in nitrate concentration. However, sulphate and phosphate significantly increased in symplast under salt treatment by 41 % and 44 %, respectively, compared with the control (Fig. 7b).

Discussion

Plant growth under salt stress

Plant growth is rapidly reduced in the osmotic phase of salt stress (Munns and Tester 2008). The reduced growth of expanding maize shoot tissue compared with that of expanded shoot tissue favours the idea, that, when salt is applied to the root medium of maize plants, leaf elongation is inhibited immediately (Cramer 1992, Szalai and Janda 2009) but recovers to a new steady-state rate below that of the control. The main site of Na^+ toxicity for most plants is the expanded leaf tissue in which Na^+ accumulates after being deposited in the transpiration stream (Munns 2002), whereas the decline in leaf elongation is caused by an increase in apoplastic pH (Pitann et al. 2009a,b), a direct effect of salt on the rate of cell division, a slower rate of expansion or a decrease in the duration of expansion (Volkmar et al. 1998). Nonetheless, continuous exposure to elevated root-zone salinity progressively decreases leaf size over time (Munns et al. 1988). The significant reduction in AWF indicates that changes in cell wall structure are caused by salt stress (Zhong and Läuchli 1993).

Many plant enzymes are activated by K^+ . Under Na^+ toxicity, Na^+ competes with K^+ for binding sites resulting in an inactivation of enzymes and cellular functions (Maathuis and Amtmann 1999, Tester and Davenport 2003). The higher ratios of $\text{Na}^+:\text{K}^+$, $\text{Na}^+:\text{Ca}^{2+}$ and $\text{Na}^+:\text{Mg}^{2+}$ under salt treatment observed in the total plant and in the apoplast and symplast indicate that Ca^{2+} , K^+ and Mg^{2+} transport is impaired by Na^+ under saline conditions and might disturb plant metabolism and reduce plant growth. Ca^{2+} is an essential element in all plants (Marschner 1995). Our results support earlier findings that Ca^{2+} transport to the youngest leaves is impaired during the first phase of salt stress (Fortmeier and Schubert 1995). In addition, this 58 % reduction of Ca^{2+} concentration in expanding shoot tissues under salinity possibly affects leaf expansion, as some Ca^{2+} is required to maintain cell membrane integrity and function (Wei et al. 2003, Hu et al. 2007). The increased shoot Cl^- con-

centration is accompanied by a reduced NO_3^- concentration with important implications for the growth rate of plants under saline conditions (Hu and Schmidhalter 2005). The antagonism between Cl^- and NO_3^- under saline conditions might have led to the 54 % and 48 % reduction in NO_3^- concentration in expanding leaves and symplast, respectively. The increased shoot P concentration on the application of NaCl may be attributable to the synergistic effect of Na^+ , which is involved in P uptake and/or transport to the shoot (Grattan and Maas 1988).

Relative water content in leaves is well known as an alternative measure of plant water status, reflecting the metabolic activity in tissues (Flower and Ludlow 1986, Zeid 2009). A decrease in RWC indicates a loss of turgor and results in a limitation in water availability for the cell extension process (Katerji et al. 1997). Recently, salinity stress has been found to reduce the RWC in maize shoot tissues rather than in the roots (Tuna et al. 2008, Zeid 2009). Our findings indicate that, in particular, the expanding shoot tissue is affected by a decrease in RWC.

Ion relationships in the apoplastic and symplastic space in leaves of maize under salt stress

A limited number of studies have dealt with the determination of cations and anions within the leaf apoplast of monocots, such as maize, under salinity. Therefore, we have subjected the roots of salt-sensitive maize plants to a 16-day salt-stress period of 100 mM NaCl and separated the leaf tissues into apoplastic and symplastic fractions prior to analysis. Extremely high symplastic Na^+ and Cl^- concentrations have been observed, as found in earlier literature (Speer and Kaiser 1991). The increase in apoplastic Na^+ and Cl^- concentrations is smaller than the increase in symplastic concentrations. Compared with earlier investigation with maize, the observed higher accumulation of Na^+ and Cl^- in the apoplast of expanding leaves during salt treatment is probably attributable to the higher xylem import of ions. Once saline solutes reach the leaf, two mechanisms can exclude them from the cytoplasm. Salt ions can be concentrated in the apoplast or isolated within the vacuole. An accumulation of 33 mM Na^+ in the leaf apoplast is not high enough to increase the osmotic gradient between the inside and outside of the cell. Our results with maize leaf apoplast do not support Oertli's hypothesis that high extracellular salt accumulation in leaves can lead to cellular dehydration and, eventually, cell death.

In maize, Na^+ is the primary toxic ion (Fortmeier and Schubert 1995, Sümer et al. 2004) that interferes with K^+ and disturbs efficient stomatal regulation resulting in water loss and necrosis. Accordingly, a significant reduction in the concentration of soluble K^+ in the leaf symp-

last has been found under salinity. The finding that the K^+ concentration remains relatively constant with a lower than 19 mM concentration in the leaf apoplast suggests no or even negligible contamination from the cell sap, because damage to the membranes would lead to a higher apoplastic K^+ concentration because of the high K^+ concentration (approximately 100 mM) in the cytoplasm.

High Na^+ causes disturbances in Ca^{2+} nutrition (Läuchli and Schubert 1989, Cramer 2002, Essa 2002, Hu *et al.* 2007) as observed in expanding and expanded shoots. We have found no considerable salinity effects with respect to soluble Ca^{2+} in the apoplast and symplast.

Earlier studies (Lohaus *et al.* 2000, Mühling and Läuchli 2002) have demonstrated extremely low Na^+ concentrations in the leaf apoplast of the salt-sensitive monocot maize. In earlier study of maize leaf apoplasts (Lohaus *et al.* 2000), only 8-day salt treatment was applied together with the selection of older leaves to determine the potential Na^+ concentration. On the basis of the two-phase model of salt injury (Munns *et al.* 1995), specific ionic toxicity is more likely to build up over a longer period. Thus, a relationship that might arise under short-term studies might not necessarily reflect that observed in the longer term. According to Munns (1993), differences in salt tolerance among genotypes are related to the difference in the time that it takes for the salt to reach its maximum concentration in the leaf vacuoles. This mechanism of a high salt accumulation might contribute to the avoidance of the toxic ion concentration in the growing leaf apoplast under stress conditions.

The Na^+ and Cl^- build-up in the symplastic and apoplastic fluids suggests that the salt sensitivity of maize is attributable to the inability of plants to control the total salt flux into the leaf apoplast and, from there, into the cells. During a prolonged salt treatment, the moderately loaded maize apoplast favours salt flux into the symplast, thereby causing high concentrations first in the vacuole and then in the symplast. This is based on the secondary active Na^+ transport into the vacuole which favours Na^+ accumulation more than 100-fold in the vacuole compared with the cytosol. Further experiments with genotypes differing in Na^+ and Cl^- accumulation are needed to resolve fully the contribution of these intercellular ions to the salt-induced decline in leaf growth.

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Chapter 3

Apoplastic Na⁺ in *Vicia faba* leaves rises after short-term salt stress and is remediated by silicon

Muhammad Shahzad, Christian Zörb, Christoph-Martin Geilfus and Karl H. Mühlring

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Apoplastic Na⁺ in *Vicia faba* leaves rises after short-term salt stress and is remedied by silicon

Muhammad Shahzad, Christian Zörb, Christoph-Martin Geilfus and Karl H. Mühling

Institute of Plant Nutrition and Soil Science, Christian Albrechts University,
Hermann-Rodewald-Str. 2, 24118 Kiel, Germany

Corresponding author

Prof. Dr. Karl H. Mühling

Institute of Plant Nutrition and Soil Science, Christian Albrechts University,
Hermann-Rodewald-Str. 2, 24118 Kiel, Germany

Tel: +49 (0431) 880-3189

Fax: +49 (0431) 880-1625

Email: khmuehling@plantnutrition.uni-kiel.de

Running title

Subcellular ion patterns in leaves under salinity

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Apoplast, Growth, Field bean, Na⁺ toxicity, Salinity, Silicon, Oertli hypothesis

Abbreviations

AWF : apoplastic washing fluid

FW : fresh weight

DW : dry weight

Si : silicon

Abstract

Salinity primarily affects plants by inhibiting shoot growth. Salt-sensitive plants have been suggested to accumulate Na^+ within their leaf apoplast under salinity, leading to a reduced water status. Evidence related to apoplastic Na^+ accumulation is still enigmatic. We have focused on the effect of a short-term salt treatment by using the salt-sensitive *Vicia faba*. Moreover, we have examined the role of silicon in alleviating sodium accumulation in the apoplast. Salt-sensitive field beans have been subjected to increasing levels of salinity, with and without the addition of silicon under hydroponic conditions. We have demonstrated that the dicot *Vicia faba* exhibits a rise in Na^+ concentration in the leaf apoplast at higher salinity levels; this is significantly ameliorated by the addition of silicon. Further, enhanced shoot growth under high salt treatment in the presence of added silicon is correlated with a significant decrease in Na^+ concentration in the leaves. The novelty of the current study is the detection of a high Na^+ concentration in the leaf apoplast of the salt-sensitive dicot field bean. Our results support Oertli's hypothesis that extracellular salt accumulation can lead to wilting leaves, plant growth reduction and cell death.

1 Introduction

The over-accumulation of Na^+ is commonly held to be responsible for the reductions in growth and yield of crop plants under salinity (Fortmeier and Schubert 1995, Mühling and Läuchli 2002b, Hong et al. 2009, Shahzad et al. 2012). The toxicity level of Na^+ depends upon its subcellular location. During continuous exposure of the plant to salt, the accumulation of Na^+ in a tissue can exceed the capability of the individual cells to compartmentalize the ions into the vacuole. If this occurs, salt ions will accumulate outside the vacuole, either in the cytoplasm or in the apoplast (Volkmar et al. 1998), resulting in metabolic toxicity or osmotic imbalances (Munns 2002). Osmotic inequality caused by high concentrations of Na^+ in the apoplast leads to cell dehydration and reduced turgor (Flowers et al. 1991).

Previously, Oertli (1968) suggested that salt build-up in the leaf apoplast can lead to death of the leaves by the dehydration of leaf cells and turgor loss. The accumulation of Na^+ ions up to 600 mM in the apoplast of rice leaves (Flowers et al. 1991) with a 50 mM Na^+ supply in plants for 7 days supported the Oertli hypothesis. However, these data were later criticised (Mühling and Läuchli 2002a), as they were produced by X-ray microanalysis in which problems are experienced in the resolution of small apoplastic spaces for the precise detection of the Na^+ and the differentiation between Na^+ and K^+ . Speer and Kaiser (1991) found high Na^+ and Cl^- concentrations in the leaf apoplast of salt-sensitive pea compared with more salt resistant spinach plants, a result that also supported the Oertli's hypothesis. However, in that investigation (Speer and Kaiser 1991), the 100 mM NaCl treatment was given in one step without permitting the plants to adapt to the altered osmotic conditions. In studies with salt-sensitive monocots, such as maize and wheat, extremely low salt accumulation has been observed in the leaf apoplast under salinity (Lohaus et al. 2000, Mühling and Läuchli 2002a; 2003, Wimmer et al. 2003, Masood et al. 2012). Therefore, in the

present investigation, a salt-sensitive dicot, namely the field bean, has been subjected to increments of NaCl in order to address the potential Na⁺ accumulation in the leaf apoplast.

To test the Oertli hypothesis, silicon has been used to alleviate the Na⁺ accumulation in the leaf apoplast of the dicot *Vicia faba*. However, the mechanism(s) for these effects of silicon in stress resistance is(are) still unclear. Ahmad et al. (1992) has suggested that silicon complexes sodium in the root, thereby decreasing sodium transport to the shoot; however, no direct evidence for this has been presented. Earlier, Zuccarini (2008) showed significant reduction in total shoot Na⁺ concentration in bean plants on the addition of silicon under salinity. The present work includes measurements of subcellular salt accumulation, because silicon is deposited mainly in the apoplast (Rogalla and Römheld 2002). In this context, we have examined the subcellular ion accumulation in the leaf apoplast of field bean plants with increasing salinity levels. Silicon has been used as a tool to regulate Na⁺ accumulation in the leaf apoplast under salinity.

2 Materials and Methods

2.1 Plant cultivation and NaCl treatment

Seeds of field bean (*Vicia faba* cv. Scirocco, Hans-Georg Lembke KG, Germany) were soaked overnight in aerated 0.5 mM CaSO₄ solution and germinated in quartz sand. After 1 week, seedlings were transferred into constantly aerated plastic pots (four plants per pot) with 4.3 L of a one-fourth concentrated nutrient solution (see below). The concentration of the nutrient solution was increased after 2 and 4 days of cultivation to half and full strength, respectively. The full-strength nutrient solution had the following concentration: 2.0 mM Ca(NO₃)₂, 0.2 mM KCl, 0.1 mM KH₂PO₄, 1 mM NaCl, 0.5 mM MgSO₄, 1.0 mM K₂SO₄, 10 μM H₃BO₃, 0.2 μM CuSO₄, 0.5 μM ZnSO₄, 2.0 μM MnSO₄, 0.05 μM (NH₄)₆ MO₇O₂₄ and 60 μM Fe-EDTA. Silicon was supplied at a concentration of 1 mM after neutralization of Na₂SiO₃ solution (water glass) with HCl. Nutrient solution was changed twice a week to avoid nutrient deficiencies. The tested levels of NaCl concentration were 50, 75 and 100 mM for salt treatments. The plants were slowly acclimated to salinity in daily increments of 25 mM until a final concentration of 100 mM NaCl was reached. Each treatment was run in five replicates. The whole experiment was carried out twice in a greenhouse with an average day/night temperature of 28/18°C, a photoperiod of 14 h ($\geq 350 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR) for 4 weeks, and a relative humidity of about 70% \pm 5%. For apoplastic washing fluid (AWF) collection, 3rd, 4th, 5th and 6th paired leaves with no salt injury from the bottom of the plants were excised 10 days after reaching the maximum salinity level at 100 mM NaCl.

2.2 Estimate of apoplastic volumes occupied by water and air

Apoplastic air volume was determined by the silicone oil method (Cosgrove and Cleland 1983, Husted and Schjoerring 1995). A determination of the apoplastic water

space was carried out by a modified indigo carmine method (Cosgrove and Cleland 1983, Husted and Schjoerring 1995). The apoplastic water volume was calculated by using the equation $F_{dil} = (V_{water} + V_{air})/V_{air}$ according to Husted and Schjoerring (1995).

2.3 Extraction of water-soluble ions from the leaf apoplast

Apoplastic fluid was collected with the infiltration-centrifugation technique (Mühling and Sattelmacher 1995, Lohaus et al. 2001). Leaves were cut with a razor blade and carefully washed with deionised water. For infiltration, intact leaves were placed in plastic syringes (60 ml) filled with 40 ml deionised water and were infiltrated by pulling the plunger, producing a reduced pressure of an estimated 20 kPa (Lohaus et al. 2001). Thereafter, intact leaves were carefully blotted dry. Intact leaves were positioned with the xylem wound facing upwards between two plastic funnels fitted into a 10-ml vessel located in a centrifugation tube and centrifuged at $71 \times g$ at $5\text{ }^{\circ}\text{C}$ for 5 min. Samples of AWF were stored at $-80\text{ }^{\circ}\text{C}$ for analysis. The calculation of the ion concentration in the apoplastic water space of field bean leaves was performed after multiplication by the dilution factor of 9, as given by Lohaus et al. (2001).

2.4 Preparation of leaf sap

After the separation of AWF, the residual leaf tissue was shock-frozen in liquid nitrogen at $-70\text{ }^{\circ}\text{C}$, thawed and centrifuged at $327 \times g$, for 5 min, as described above. Thereafter, the leaf sap was stored at $-80\text{ }^{\circ}\text{C}$ until analysis; this sample was further designated as the symplastic leaf fraction.

2.5 Determination of ion concentration in the leaf sap and apoplastic washing fluid

Samples were mixed with chloroform and centrifuged and the clear supernatant was collected. The concentration of ions was analysed in the leaf sap and AWF by ion chromatography (Dionex, DX2500, Idstein, Germany) on an IonPac anion exchange column (Dionex, AS9) or cation exchange column (Dionex, AS12) connected with a conductivity detector module (Dionex). Isocratic elution with H_2SO_4 (15 mM, 1 ml min^{-1}) was used for the separation of the cations and isocratic elution with Na_2CO_3 (1.7 mM) and NaHCO_3 (1.8 mM) was used for anions.

2.6 Determination of total ion concentration in leaves

Leaf and root materials were ashed overnight in an oven at 520 °C. Analysis of the cations was performed on the ash of 150 mg dried plant material. The ash was dissolved in 2 mL 4 M HNO_3 with gentle heating. This suspension was adjusted to a volume of 10 mL with distilled water and filtered through a paper filter (Schleicher and Schuell, white 589/3, Dassel, Germany). Thereafter, Na^+ , K^+ , Ca^{2+} and Mg^{2+} concentrations were analysed in the filtrate by means of atomic absorption spectrometry (Thermo Electron Corporation, S series AA Spectrometer, Massachusetts, USA). Analysis of anions was performed on 30 mg dried plant material that was dissolved in 1.6 ml deionised water by placing it for 5 min in boiling water. After cooling, each sample was centrifuged and the supernatant was collected. After washes with chloroform, samples were cleaned by passing through strata C-18 columns obtained from phenomenex (Torrance, CA, USA). Thereafter, anion (Cl^- , NO_3^- , SO_4^{2-} and PO_4^{3-}) concentrations were analysed by ion chromatography (Dionex, DX5000, Idstein, Germany).

2.7 Statistics

Data were distributed normally with homogeneous variances. Significant differences at $P < 0.05$ between treatments were calculated by using a general linear model with related multiple contrast tests by the statistical software 'R' Development Core Team (2011, Vienna, Austria) URL <http://www.R-project.org/>. Error bars in the figures indicate the mean standard error (SE).

3 Results

3.1 Effect of silicon on growth of field bean plants under NaCl stress conditions

Salt treatment alone (50, 75 and 100 mM NaCl) significantly decreased fresh weight by 24%, 35% and 47% compared with the control (white bars in Fig. 1). A NaCl treatment with addition of 1 mM silicon (black bars) led to a significantly increased growth at NaCl concentrations above 50 mM compared with the corresponding NaCl treatment alone. The treatment of 75 and 100 mM NaCl plus 1 mM silicon increased biomass to 14% and 18% compared with the salt treatment without silicon.

3.2 Ratios of Na^+/K^+ , $\text{Na}^+/\text{Ca}^{2+}$ and $\text{Na}^+/\text{Mg}^{2+}$ in whole leaf and subcellular fractions

An increasing Na^+/K^+ , $\text{Na}^+/\text{Ca}^{2+}$ and $\text{Na}^+/\text{Mg}^{2+}$ ratio was detected with increasing salinity treatment, reaching a maximum at 100 mM NaCl in all shoot fractions (Tab. 1). In whole leaves without silicon treatment, the Na^+/K^+ , $\text{Na}^+/\text{Ca}^{2+}$ and $\text{Na}^+/\text{Mg}^{2+}$ ratio increased significantly with increasing salinity levels. The Na^+/K^+ ratio was significantly reduced on addition of silicon at maximum salinity treatment (Tab. 1). Except for $\text{Na}^+/\text{Ca}^{2+}$ at 75 mM NaCl in the symplast, the $\text{Na}^+/\text{Ca}^{2+}$ and $\text{Na}^+/\text{Mg}^{2+}$ ratio was significantly reduced at 75 and 100 mM NaCl in whole leaf, apoplast and symplast (Tab. 2, 3).

3.3 Accumulation of cations in whole leaf, extracted AWF and symplastic fluid of field bean leaves

In whole leaf: The whole leaf Na^+ concentration increased significantly with increasing salinity levels (Fig. 2A). A 100 mM NaCl treatment led to a significant maximum Na^+ accumulation when compared with other salinity treatments in which the Na^+ concentration was significantly reduced by 22% on the addition of silicon (Fig. 2A). When compared with their respective controls in whole leaf tissue, salinity treatments led to more than a 60%, 58% and 34% significant reduction in K^+ , Ca^{2+} and Mg^{2+} concentrations, correspondingly (Fig. 2D, 3A, 3D).

Extracted AWF: In the apoplast of field bean leaves, soluble Na^+ significantly increased at 100 mM NaCl compared with other salt treatments (Fig. 2B). At higher salinity levels, the addition of silicon significantly reduced the Na^+ concentration in the apoplast (Fig. 2B). Moreover, the addition of silicon plus 75 and 100 mM NaCl led to a significant reduction of 53 % and 31 % in the Na^+ concentration in the AWF. Maximum concentrations of 83 and 121 mM Na^+ were found at 100 mM NaCl treatment with and without silicon. The increase in Na^+ concentration compared with control was 13-, 140- and 344-fold at 50, 75 and 100 mM NaCl treatment, accordingly; however, on the addition of silicon, the increase in Na^+ concentration was less prominent at 5-, 25- and 91-fold. Soluble K^+ , Ca^{2+} and Mg^{2+} concentrations also increased with increasing salinity levels (Fig. 2E, 3B, 3E). Addition of silicon significantly increased the soluble Ca^{2+} concentration at maximum salinity level when compared with the respective salinity treatment without silicon (Fig. 3B).

Symplastic fluid: Increasing salinity levels led to an increase in Na^+ concentration in the symplast of the field bean leaves (Fig. 2C). When compared with the Na^+ treatment alone, salinity treatment of 100 mM NaCl with the addition of silicon resulted in a significant reduction of 28% in the Na^+ concentration in the symplast.

Moreover, maximum Na^+ concentrations of 136 and 174 mM were found in the leaf symplast at 100 mM NaCl with and without silicon, respectively. A significant increase in the Na^+ concentration was observed in a comparison of 50 mM NaCl with 75 and 100 mM NaCl, with and without silicon treatment (Fig. 2C). When compared with their respective controls, symplastic K^+ , Ca^{2+} and Mg^{2+} concentrations were significantly reduced and showed more than a 50 % reduction at all salinity levels (Fig. 2F, 3C, 3F). However, the addition of silicon had no considerable influence on this reduction of K^+ , Ca^{2+} and Mg^{2+} concentrations in the symplast under salinity.

3.4 Accumulation of anions in whole leaf, extracted AWF and symplastic fluid of field bean leaves

In whole leaf: After a 10-day exposure of plants to salt, an analysis of the Cl^- concentration in whole leaf tissue revealed a significantly higher concentration compared with that of the control (Fig. 4A). However, maximum salt treatment plus the addition of silicon resulted in a decrease by 14% in Cl^- concentration but was not significant (Fig. 4A). Surprisingly, the NO_3^- concentration was significantly higher on the addition of silicon in the control (Fig. 4D). Under salinity, the addition of silicon did not however change the NO_3^- concentration when compared with its respective sole salinity treatment (Fig. 4D).

Extracted AWF: A significant maximum soluble Cl^- concentration was detected at 100 mM NaCl (Fig. 4B). When compared with non silicon-treated plants, silicon-treated plants showed a significantly lower Cl^- concentration in their leaf apoplast at maximum salinity level (Fig. 4B). Furthermore, when compared with the control, the NO_3^- concentration was significantly reduced at 74, 75 and 90 % at 50, 75 and 100 mM NaCl (Fig. 4E).

Symplastic fluid: An increase in the Cl^- concentration in the symplast of field bean leaves was noticed with increasing salinity levels (Fig. 4C). Moreover, silicon addition significantly reduced the Cl^- concentration (by 31%) in the symplast at maximum salinity treatment when compared with its respective sole salinity treatment. Further, maximum 127 and 166 mM Cl^- concentrations were found at 100 mM NaCl with and without silicon, respectively. Additionally, the NO_3^- concentration was significantly reduced, with a more than 90% reduction at all salinity levels (Fig. 4F).

4. Discussion

4.1 Reduction of leaf growth under NaCl stress

A significant decline in plant biomass under salinity reflects the rapid decrease in shoot growth and wall extensibility in the osmotic phase of salt stress (Munns and Tester 2008, Szalai and Janda, 2009, Geilfus et al. 2011). Earlier studies have reported the predominant importance of Na^+ over, for example, Cl^- for many crop plant species as a reason for ion-specific damage during salt stress (Fortmeier and Schubert 1995, Tester and Davenport 2003, Slabu 2009). In addition, Sümer et al. (2004) have demonstrated a contribution of Na^+ toxicity in the first phase of salt stress. In the current investigation, added NaCl resulted in a significant increase in leaf apoplastic Na^+ concentration (Fig. 2B). This has previously been suggested as a possible reason for the decline in leaf growth (Oertli 1968, Flowers et al. 1991). Later reports have also indicated that a possible reason for the differential salt resistance of plants is their ability to control total salt fluxes into the leaf apoplast and into the cells. With increasing salinity, a highly loaded apoplast might favour salt flux into the symplast of salt-sensitive plants. This accumulation in the cytoplasm will then cause enzyme inhibition (Kaiser et al. 1986) and the breakdown of

metabolism (Speer and Kaiser 1991). High Na^+ and Cl^- concentrations in the leaf apoplast of dicots such as pea (Speer and Kaiser 1991) and field bean (in current study) might contribute to the salt sensitivity of these plant species.

4.2 Subcellular ion distribution in leaves under increasing NaCl

A limited number of studies have investigated cations and anions at the subcellular level in field bean leaves under salinity. Stress by salinity has often been reported to cause a reduction in plant growth via ion toxicity, in particular Na^+ toxicity (Kaiser et al. 1986, Fortmeier and Schubert 1995, Tester and Davenport 2003).

The accumulation of Na^+ and Cl^- that we have observed in the apoplast of field bean leaves during salt treatment without silicon addition might be attributable to higher xylem import. Upon the arrival of saline solutes in the leaf apoplast, two mechanisms are available to exclude them from the cytoplasm. Salt ions can be concentrated in the apoplast or isolated within the vacuole. Accumulation of Na^+ and Cl^- in the apoplast (Fig. 2B, 4B) gradually increases the osmotic gradient between the inside and outside of the cell. As a result, intracellular water moves outwards into the apoplast to achieve a thermodynamic equilibrium, leading to progressive cellular dehydration and, eventually, cell death (Volkmar et al. 1998).

The high Na^+ concentration in the apoplast of field bean leaves under salt stress supports the results of an earlier study of salt-sensitive rice plants grown under mild saline conditions (50 mM NaCl), which produced a high apoplastic Na^+ concentration of up to 600 mM (Flowers et al. 1991). In addition, the dicot salt-sensitive pea (Speer and Kaiser 1991) shows a higher apoplastic Na^+ concentration. In contrast, other studies have demonstrated extremely low Na^+ concentrations in the leaf apoplast of the salt-sensitive monocots maize and wheat (Lohaus et al. 2000, Mühling and Läuchli 2002a; 2003, Wimmer et al. 2003, Shahzad et al. 2012).

Therefore, the high Na^+ concentration in the leaf apoplast of the salt-sensitive field bean (Fig. 2B) and pea (Speer and Kaiser 1991) suggest that Oertli's hypothesis is only valid for dicot plants.

In the cytosol, Na^+ competes with K^+ for binding sites resulting in the inactivation of enzymes and cellular functions on binding of Na^+ (Tester and Davenport 2003). We have shown that K^+ concentrations are significantly reduced in whole leaf tissue and the symplast of field bean under salinity (Fig. 2D, 2F), this indicates that the $\text{Na}^+:\text{K}^+$ ratio should be considered when determining Na^+ toxicity. Moreover, high Na^+ causes disturbances in Ca^{2+} nutrition (Cramer 2002, Essa 2002, Hu et al. 2007). We have found a significant reduction in Ca^{2+} and Mg^{2+} concentrations under salinity in the symplast and whole leaf tissue (Fig. 3A, 3D, 3C, 3F), even under low salt-stress conditions (50 mM NaCl). Indeed, higher ratios of $\text{Na}^+:\text{K}^+$, $\text{Na}^+:\text{Ca}^{2+}$ and $\text{Na}^+:\text{Mg}^{2+}$ under salt treatment have been observed in whole leaf tissue, apoplast and symplast (Tab. 1, 2, 3). These higher ratios indicate that Ca^{2+} , K^+ and Mg^{2+} transport is impaired by high Na^+ concentrations under saline conditions and might be responsible for the disturbance of plant metabolism and reduced plant growth.

In addition, salinity exposure leads to a significant increase in Cl^- concentration in all leaf compartments (Fig. 4A, 4B, 4C). However, Tavakkoli et al. (2011) have demonstrated that Cl^- is more important than Na^+ when plants are grown in salt-affected soils. In contrast to Na^+ , little is known about the molecular mechanisms behind the substantial Cl^- influx that results from salinization (Flower and Colmer 2008). After NaCl exposure, high Cl^- concentrations in the chloroplasts, amplified by a simultaneously high Na^+ concentration, can lead to a reduction of chlorophyll in the leaves of the faba bean (Slabu et al. 2009). The increased shoot Cl^- concentration under salinity is often found to reduce the NO_3^- concentration, nitrogen playing a critical role in determining the growth of salinized plants (Hu and Schmidhalter

2005). For nitrate, we have observed a strong depletion in all shoot compartments under salt stress (Fig. 4D, 4E, 4F). Most likely, under high salinity, the antagonism between Cl^- and NO_3^- results in a significant reduction in the NO_3^- concentration, both with and without the addition of silicon.

4.3 Effect of silicon on shoot biomass and subcellular ion distribution in leaves under salinity

Silicon is well known as being able to increase the stress resistance of many plant species (Zuccarini 2008, Saqib et al. 2008, Pei et al. 2010). The addition of silicon to the hydroponic solution significantly increases the shoot fresh weight at higher salinity levels (Fig. 1). No significant effect of added silicon has been observed on the fresh weight of shoots of control (1 mM NaCl) plants; this suggests that silicon available from the water and chemicals used to prepare the standard culture solution satisfies any specific requirement in the faba bean (Match et al. 1986, Yeo et al. 1999). Furthermore, we have found a significant reduction in the volume of extracted AWF at higher salinity levels, a volume that is significantly greater following the addition of silicon (data not shown). Changes in cell wall structure (Zhong and Läuchli 1993) together with the water stress imposed by salinity might lead to the differences in extracted AWF (Mühling and Läuchli 2002a). However, the beneficial effects of added silicon on plant growth have been linked to reduced Na^+ translocation and decreased transpiration under salinity (Gong et al. 2006, Pei et al. 2010). The effect of added silicon at 75 and 100 mM NaCl on the apoplastic Na^+ concentration is significantly reduced when compared with the respective salinity treatments alone (Fig. 2B). An ameliorative effect of silicon with a significant decrease in Na^+ concentration has been observed in whole leaf tissue and the symplast of the field bean at higher salinity levels (Fig. 2A, 2C). Silicon application

in previous studies also been reported to influence manganese detoxification via the binding of manganese to the cell wall and thus decreasing its presence in the cell sap (cytoplasmic and apoplastic fluid) (Iwasaki et al. 2002, Rogalla and Römheld 2002). Moreover, on the application of silicon, the significantly higher Ca^{2+} concentration in the leaf apoplast (Fig. 3B) might have a positive role in enhanced leaf expansion, as a reasonable amount of Ca^{2+} is required to maintain plasma membrane integrity and function (Wei et al. 2003, Cramer 2002), together with cell-wall extensibility. In plants, calcium is transported via the xylem and its transport depends on transpiration (Arndt et al. 2000). One possible explanation for the improved Ca^{2+} transport in silicon-treated plants under salt stress is increased transpiration as observed in previous studies of stressed plants treated with silicon (Gong et al. 2005, Hattori et al. 2005). The addition of silicon at higher salinity significantly reduces the salt concentration in the leaf apoplast of field bean plants (Fig. 2B, 4B). The build-up of Na^+ and Cl^- in the apoplast suggests that the sensitivity of the field bean is attributable to their inability to control the total salt flux into the leaf apoplast.

Conclusion

In contrast to earlier publications on monocots (wheat, maize) (Mühling and Läuchli 2002a; 2003, Shahzad et al. 2012) we now demonstrate that the dicot *Vicia faba* exhibits a rise in the Na^+ concentration in the leaf apoplast at higher salinity levels and that this rise is significantly ameliorated by the addition of silicon. Similar findings have been detected for the Cl^- concentration in the leaf apoplast. Furthermore, the addition of silicon significantly decreases the Na^+ concentration in the symplast and whole leaf tissue at maximum salinity levels. A high Na^+ concentration in the leaf apoplast of the salt-sensitive dicot field bean support Oertli's hypothesis that extracellular salt accumulation can lead to wilting leaves, growth reduction and cell death.

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Tables:

Table 1 Influence of salinity on $\text{Na}^+:\text{K}^+$, $\text{Na}^+:\text{Ca}^{2+}$ and $\text{Na}^+:\text{Mg}^{2+}$ ratios in whole leaf. Significant differences between salinity and silicon treatments ($P < 0.05$) are indicated by small and capital letters, respectively ($n \geq 3$).

NaCl Treatment (mM)	$\text{Na}^+:\text{K}^+$		$\text{Na}^+:\text{Ca}^{2+}$		$\text{Na}^+:\text{Mg}^{2+}$	
	- Si	+ Si	- Si	+ Si	- Si	+ Si
Control	0.04 Ad	0.01 Ac	0.24 Ad	0.09 Ac	0.44 Ad	0.15 Ac
50	1.32 Ac	1.28 Ab	8.61 Ac	7.76 Ab	7.99 Ac	9.27 Ab
75	2.93 Ab	2.30 Aa	15.3 Ab	10.9 Bb	17.5 Ab	13.8 Bab
100	4.94 Aa	3.32 Ba	23.9 Aa	16.6 Ba	27.8 Aa	17.6 Ba

Table 2 Influence of salinity on apoplastic $\text{Na}^+:\text{K}^+$, $\text{Na}^+:\text{Ca}^{2+}$ and $\text{Na}^+:\text{Mg}^{2+}$ ratios. Significant differences between salinity and silicon treatments ($P < 0.05$) are indicated by small and capital letters, respectively ($n \geq 3$).

NaCl Treatment (mM)	$\text{Na}^+:\text{K}^+$		$\text{Na}^+:\text{Ca}^{2+}$		$\text{Na}^+:\text{Mg}^{2+}$	
	- Si	+ Si	- Si	+ Si	- Si	+ Si
Control	0.05 Ac	0.09 Ac	2.24 Ab	18.5 Ab	0.37 Ab	0.98 Ba
50	0.23 Ac	0.25 Ab	3.29 Ab	5.32 Ab	2.45 Ab	3.09 Aa
75	1.26 Ab	0.53 Aab	30.8 Aa	7.82 Bab	27.3 Aa	12.8 Ba
100	1.67 Aa	1.44 Ba	25.7 Aa	10.8 Ba	33.2 Aa	19.3 Ba

Table 3 Influence of salinity on symplastic $\text{Na}^+:\text{K}^+$, $\text{Na}^+:\text{Ca}^{2+}$ and $\text{Na}^+:\text{Mg}^{2+}$ ratios. Significant differences between salinity and silicon treatments ($P < 0.05$) are indicated by small and capital letters, respectively ($n \geq 3$).

NaCl Treatment (mM)	$\text{Na}^+:\text{K}^+$		$\text{Na}^+:\text{Ca}^{2+}$		$\text{Na}^+:\text{Mg}^{2+}$	
	- Si	+ Si	- Si	+ Si	- Si	+ Si
Control	0.02 Ac	0.02 Ab	0.04 Ac	0.03 Ac	0.16 Ad	0.11 Ab
50	2.36 Ac	2.19 Ab	3.65 Ab	3.18 Abc	17.8 Ac	15.0 Aab
75	8.06 Ab	7.89 Aa	7.46 Ab	6.61 Aab	49.2 Ab	29.6 Ba
100	16.8 Aa	8.71 Ba	12.2 Aa	7.37 Ba	65.7 Aa	35.4 Ba

Figure legends:

Figure 1. Influence of increasing NaCl concentration on the fresh weight of the entire shoot of *Vicia faba* cv Scirocco. The plants were treated for 12 days in a greenhouse with (black bars) and without (white bars) 1 mM silicon treatment. Significant differences between salinity and silicon treatments ($P < 0.05$) are indicated by small and capital letters, respectively ($n \geq 3$).

Figure 2. Effect of increasing NaCl supply on $[Na^+]$ and $[K^+]$ concentrations in whole shoot (A) & (D), in extracted AWF (B) & (E) and in symplastic fluid (C) & (F) of *Vicia faba* cv Scirocco with (black bars) and without (white bars) 1 mM silicon treatment. Significant differences between salinity and silicon treatments ($P < 0.05$) are indicated by small and capital letters, respectively ($n \geq 3$).

Figure 3. Effect of increasing NaCl supply on $[Ca^{2+}]$ and $[Mg^{2+}]$ concentrations in whole shoot (A) & (D), in extracted AWF (B) & (E) and in symplastic fluid (C) & (F) of *Vicia faba* cv Scirocco with (black bars) and without (white bars) 1 mM silicon treatment. Significant differences between salinity and silicon treatments ($P < 0.05$) are indicated by small and capital letters, respectively ($n \geq 3$).

Figure 4. Influence of increasing salinity on $[Cl^-]$ and $[NO_3^-]$ concentrations in whole shoot (A) & (D), in extracted AWF (B) & (E) and in symplastic fluid (C) & (F) of *Vicia faba* cv Scirocco with (black bars) and without (white bars) 1 mM silicon treatment. Significant differences between salinity and silicon treatments ($P < 0.05$) are indicated by small and capital letters, respectively ($n \geq 3$).

Figures

Figure 1

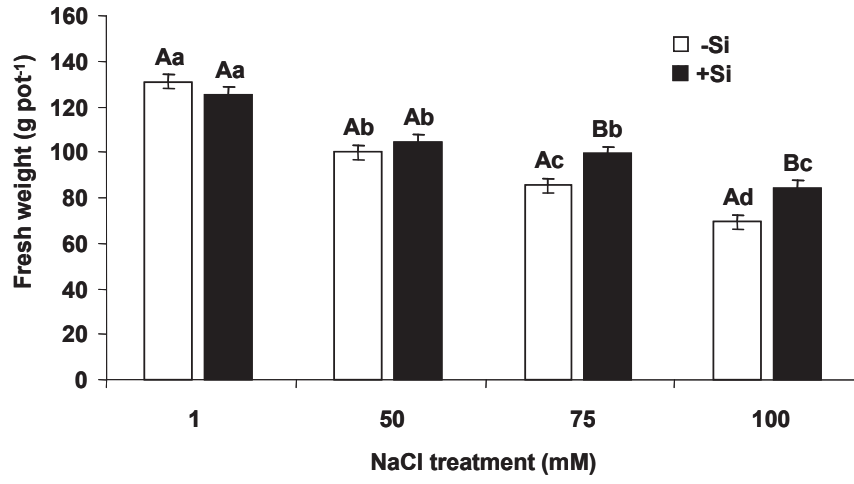


Figure 2

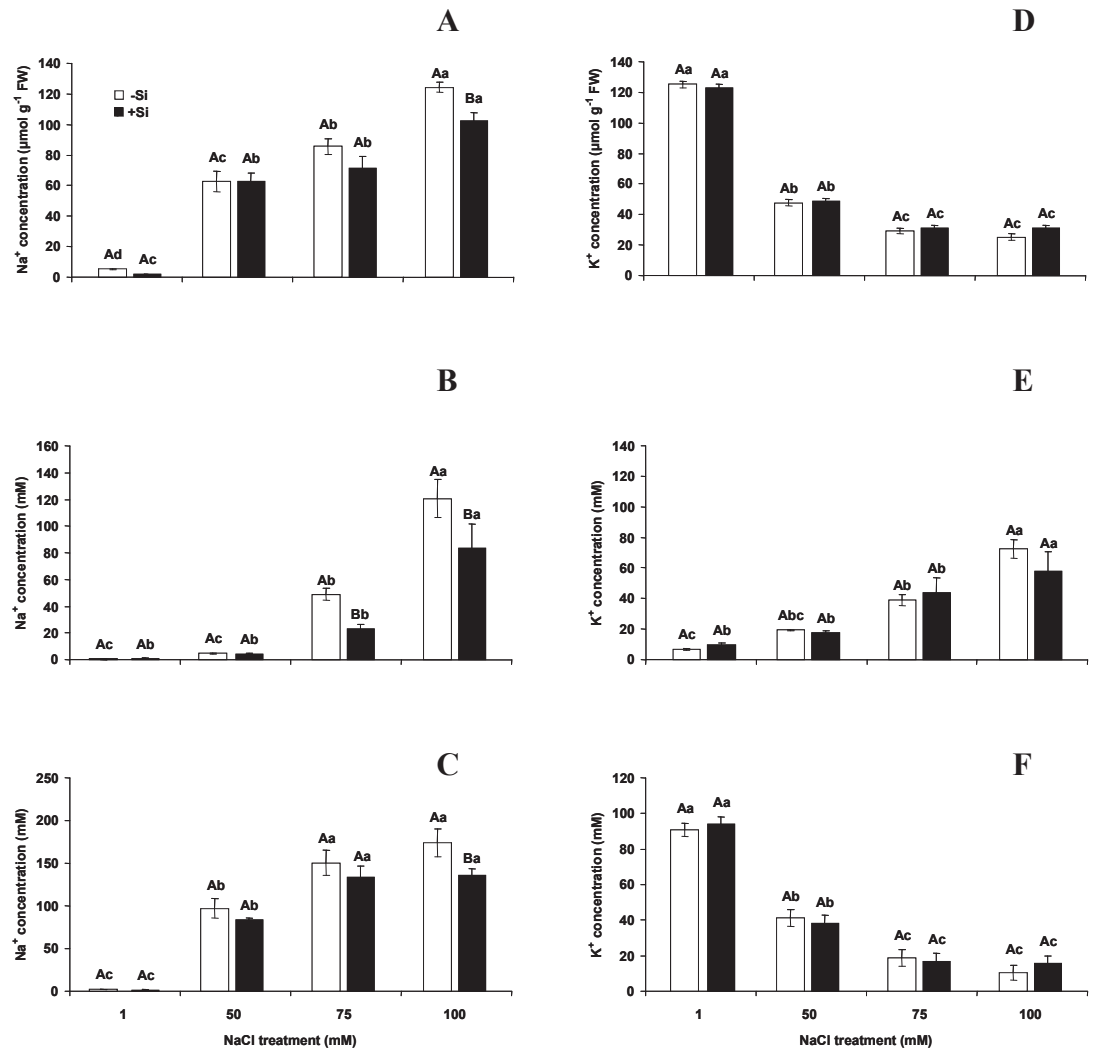


Figure 3

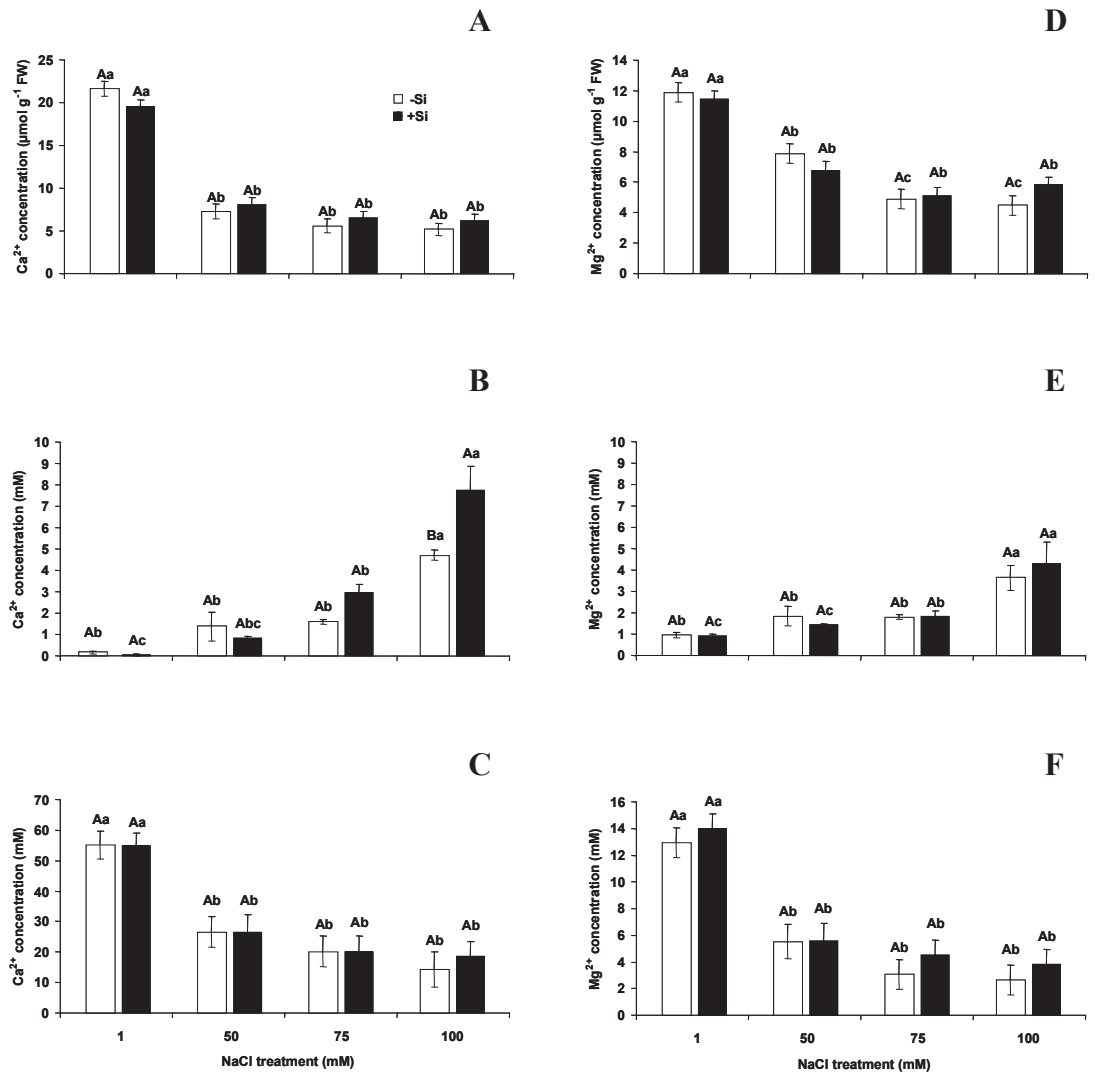
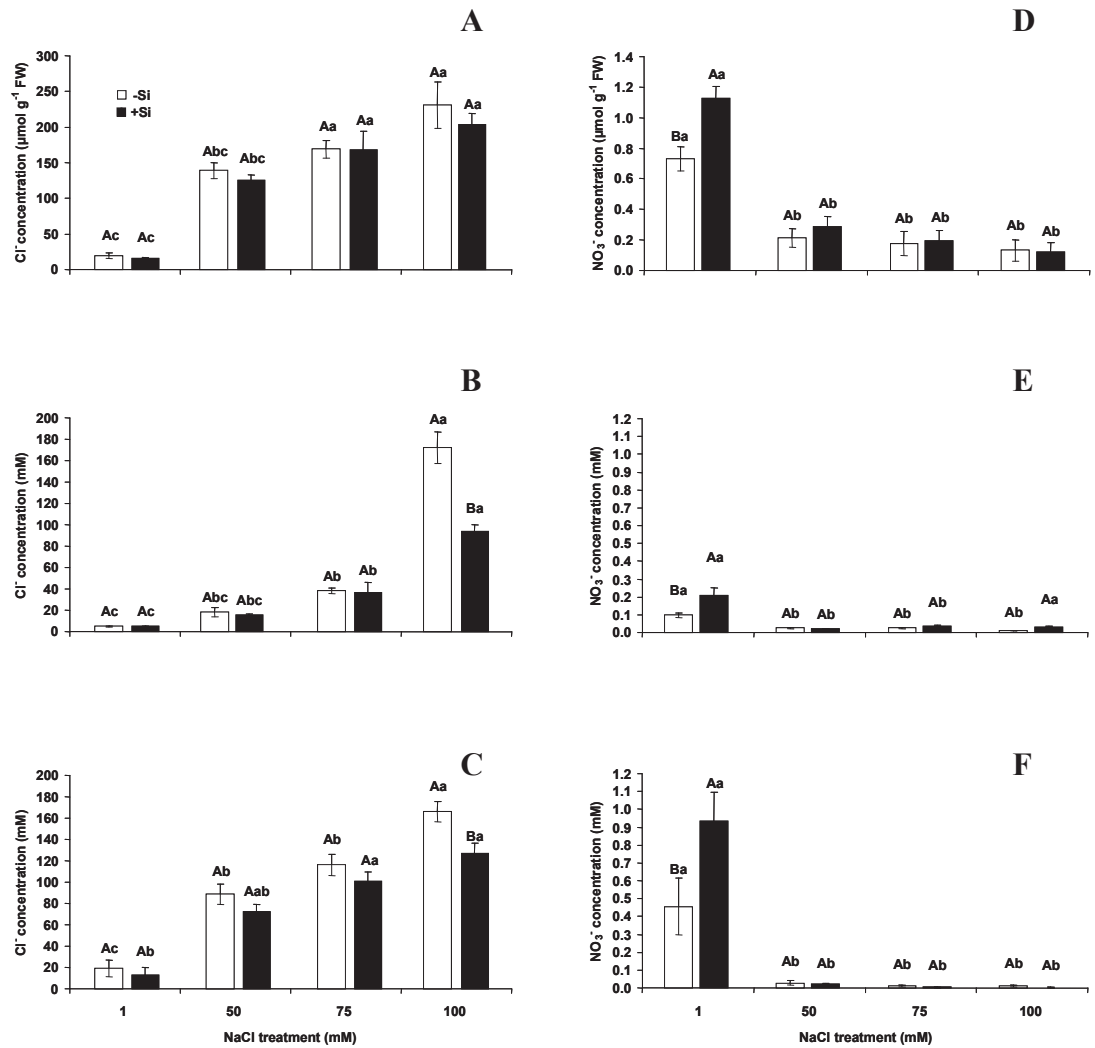


Figure 4



Chapter 4

Comparative evaluation of extraction methods for apoplastic proteins from maize leaves

Katja Witzel, Muhammad Shahzad, Andrea Matros, Hans-Peter Mock and
Karl H. Mühling

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METHODOLOGY

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Comparative evaluation of extraction methods for apoplastic proteins from maize leaves

Katja Witzel^{1,3}, Muhammad Shahzad¹, Andrea Matros², Hans-Peter Mock² and Karl H Mühling^{1*}

Abstract

Proteins in the plant apoplast are essential for many physiological processes. We have analysed and compared six different infiltration solutions for proteins contained in the apoplast to recognize the most suitable method for leaves and to establish proteome maps for each extraction. The efficiency of protocols was evaluated by comparing the protein patterns resolved by 1-DE and 2-DE, and revealed distinct characteristics for each infiltration solution. Nano-LC-ESI-Q-TOF MS analysis of all fractions was applied to cover all proteins differentially extracted by infiltration solutions and led to the identification of 328 proteins in total in apoplast preparations. The predicted subcellular protein localisation distinguished the examined infiltration solutions in those with high or low amounts of intracellular protein contaminations, and with high or low quantities of secreted proteins. All tested infiltration solution extracted different subsets of proteins, and those implications on apoplast-specific studies are discussed.

Keywords: Apoplast, liquid chromatography mass spectrometry, maize, proteome analysis, two-dimensional gel electrophoresis

Background

The plant apoplast comprises the cell wall matrix and the intercellular spaces, and plays a major role in a wide range of physiological processes, including water and nutrient transport [1], plant-pathogen interactions, and perception and transduction of environmental signals [2,3]. Proteins present in the plant apoplast reflect this broad functional diversity. Studies on the dynamic change of apoplast protein composition revealed new insights into plant responses to abiotic stress [4-7], nutrient supply [8-10], wounding [11], water deficiency [12,13], pathogen response [14-16] and xylem composition [17,18]. The selection of a suitable extraction protocol is a crucial step in proteomics surveys as proteins reveal a high degree of biochemical heterogeneity and investigated plant materials can be characterized by the presence of non-protein components interfering with subsequent analytical techniques, e.g. two-dimensional gel electrophoresis (2-DE) or liquid chromatography-mass spectrometry (LC-MS). These biological realities led to the establishment of sample preparation methods

for numerous plant species and tissues, such as Arabidopsis leaves [19], papaya leaves [20], sunflower leaves [21], cotton seedlings [22], apple and strawberry fruit [23], potato tuber [24], grapevine leaves and roots [25], grape berry cell wall [26], rubber latex [27], cotton fibers [28], banana meristem [29] and chloroplast [30], among others. Despite their biological significance, investigations on apoplastic proteins are hampered due to their low abundance compared to intracellular protein concentrations. The extraction of proteins from the leaf and root apoplast is mainly based on the principle of vacuum infiltration with an extraction solution, followed by a mild centrifugation step to collect the apoplastic washing fluid. The composition of the infiltration solution is essential as it has to fulfil certain prerequisites, such as maintenance of osmotic pressure to prevent collapsing of plasma membrane and stringency for extracting cell wall-bound proteins. Borderies et al. [31] compared different solutions to extract loosely bound cell wall proteins of Arabidopsis cell suspension cultures and showed that the composition of extraction solution determines the efficiency of preparation. Similarly, Boudart et al. [32] investigated weakly cell wall-bound proteins in rosettes of Arabidopsis. Here, we compared protein extracts obtained by six different infiltration

* Correspondence: khmuehling@plantnutrition.uni-kiel.de

¹Institute of Plant Nutrition and Soil Science, Christian Albrechts University, Hermann-Rodewald-Strasse 2, 24118 Kiel, Germany

Full list of author information is available at the end of the article

solutions already described for apoplastic proteins from different plant species. We aimed at identifying a protocol most suitable for the extraction of leaf apoplast proteins of maize, a crop of high economic importance. We evaluated the protein patterns as resolved by 1-DE or 2-DE, identified the proteins using LC-MS and located them to cellular compartments.

Results and discussion

In this study, six different solutions were tested for the ability to extract proteins from the maize leaf apoplast: water [8], 20 mM ascorbic acid/20 mM CaCl_2 [6], 100 mM sorbitol [4], 25 mM Tris-HCl [9], 100 mM sodium phosphate buffer [16] and 50 mM NaCl [33] (Figure 1). In most cases, the infiltration solutions were applied for wheat leaves and no comparison of the efficiency of protein extraction for each method was performed. Thus, this study focussed on identifying the optimal method for extracting apoplastic proteins from maize leaves.

Proteins from the leaf apoplast and symplast extracted with the six infiltration solutions were compared on 1-DE (Figure 2A, Additional file 1). A sharp band pattern was obtained from all apoplast extracts with a high number of protein bands in each extract. While the yield of protein extraction was similar, the protein profiles showed distinct differences. A prominent band of

about 20 kDa was present in extracts of 100 mM sodium phosphate buffer, 25 mM Tris-HCl, 20 mM ascorbic acid/20 mM CaCl_2 and 50 mM NaCl, but not in water or 100 mM sorbitol. One protein band of high molecular weight (approximately 100-130 kDa) was apparent in extracts of water, 100 mM sodium phosphate buffer and 100 mM sorbitol, but not in 25 mM Tris-HCl, 20 mM ascorbic acid/20 mM CaCl_2 or 50 mM NaCl. While there were similarities, each extract revealed specific protein bands indicating that different subsets of proteins were isolated by the six infiltration solutions. Proteins with a molecular weight < 15 kDa were underrepresented in all extracts and this corresponds to previous proteomic reports on some of the infiltration solutions [4,16]. The observed selective protein patterns generated by the individual infiltration solutions emphasize the necessity of careful selection of isolation method [34]. Band patterns from symplast preparations did not reveal significant differences among the infiltrates and the overall band patterns were more complex as from apoplastic preparations. This demonstrates an apparent subfractionation of the cellular compartments.

Equal amounts of apoplast proteins were separated by 2-DE to assess the protein patterns in more detail (Figure 2B). We found areas of good and poor resolved proteins

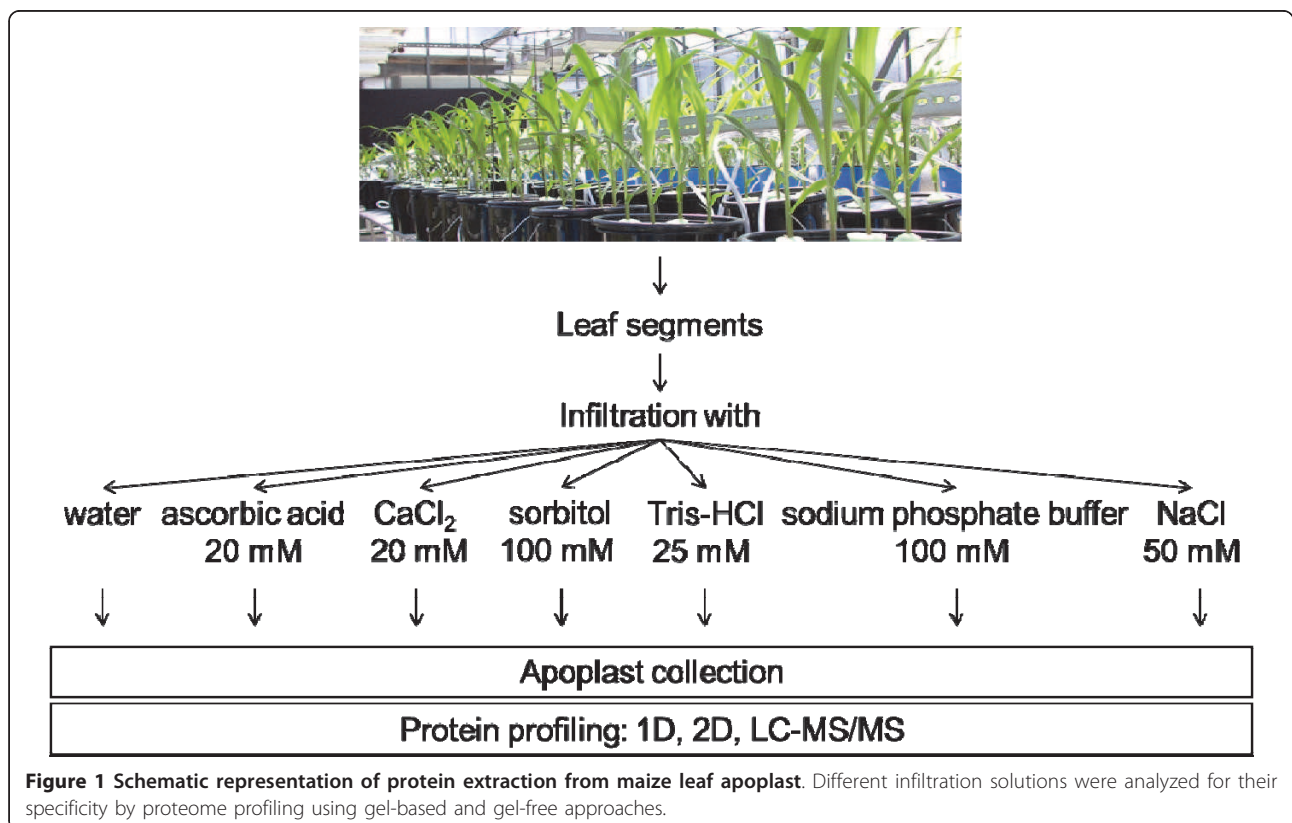
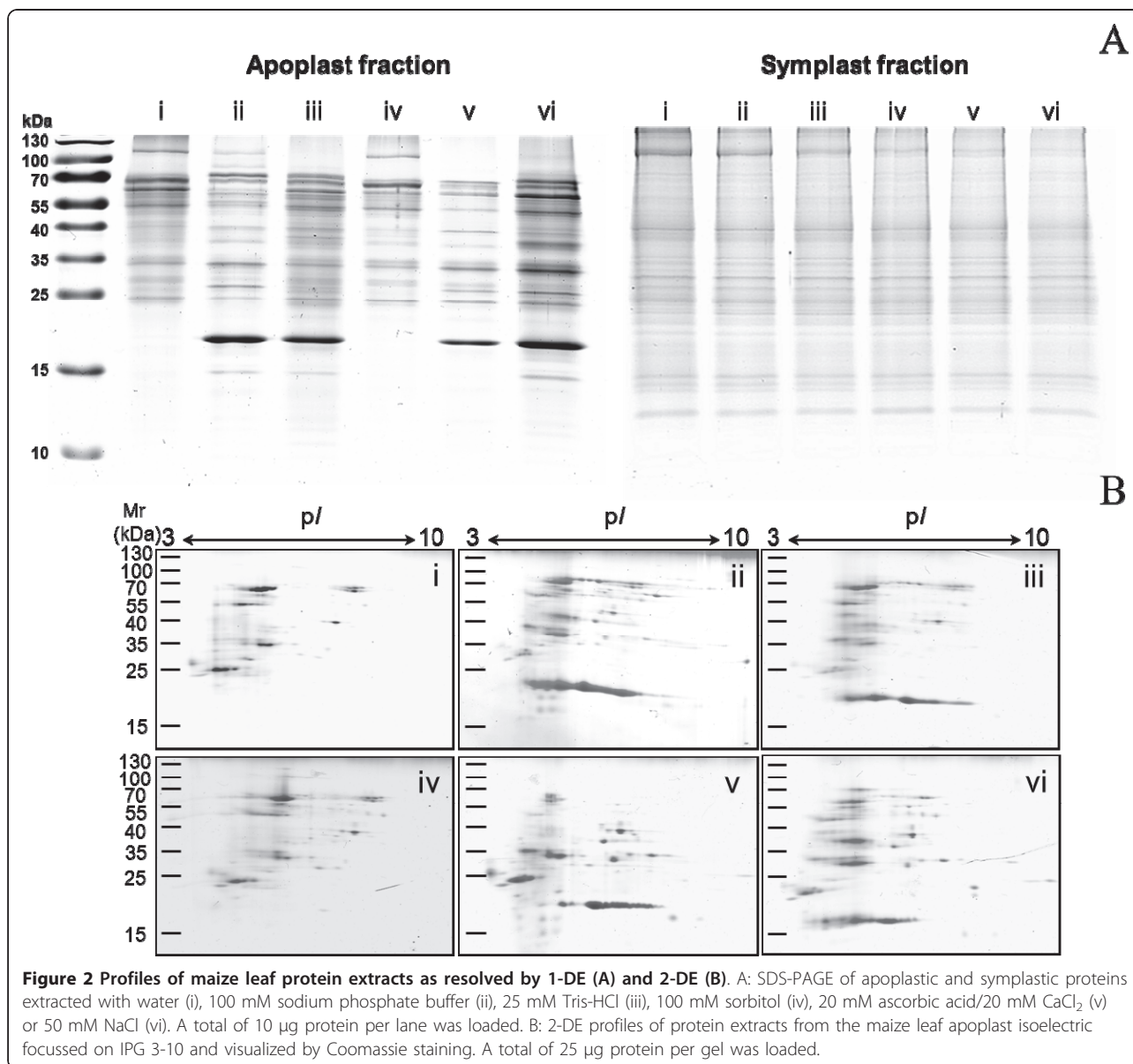


Figure 1 Schematic representation of protein extraction from maize leaf apoplast. Different infiltration solutions were analyzed for their specificity by proteome profiling using gel-based and gel-free approaches.



spots on all 2-D gels. Proteins in the acidic gel region of pH 4-6 showed horizontal streaking. Although all samples were precipitated, dissolved in urea-containing buffer system and dialyzed prior to 2-DE to avoid the contamination with nucleic acids or other interfering substances, these poorly separated spots were observed. Contrary to this, proteins in the basic region of 2-D gels near the pH 6-10 interval showed a superior resolution with minimal streaking. The spot patterns resembled the band patterns to a certain extent, e.g. as observed for the 20 kDa band that was prominent also on 2-D gels of the respective apoplastic extracts. The best resolution of proteins in 25-45 kDa intervals was achieved on extracts of 20 mM ascorbic acid/20 mM CaCl₂ infiltration solution, while high

molecular weight proteins separated best in extracts of 100 mM sodium phosphate buffer infiltration solution. The latter was applied with success to extract proteins from the leaf apoplast of lupin and resulted in the generation of well resolved protein maps containing about 50 spots to evaluate the effect of water and boron deficiency [9]. Our results showed that this separation was not reached, probably due to substances present in the maize apoplast interfering with isoelectric focusing. As 2-DE did not result in a comprehensible evaluation of the employed infiltration solutions, we used nano-LC-ESI-Q-TOF MS for proteomic analysis of all extracts.

In order to obtain an overview of all proteins present in the six different extracts, we aimed at establishing

qualitative protein profiles by LC-MS analysis. An automatic data directed analysis mode was applied as described in materials and methods section. Results exceeding the PLGS score of 12 for protein identification and probability score of 50% for *de novo* sequencing of peptides were accepted.

A total of 328 proteins were identified from all extracts. Additional file 2 shows the identities of those proteins, along with the predicted subcellular localization and detection in the six apoplastic extracts. Additional file 3 provides the respective identifier, PLGS score, number of peptides, protein coverage, peptide sequences and peptide sequence probability score for all identified proteins. In order to visualize and identify infiltration solutions with similar protein abundance patterns, a hierarchical clustering method was applied. Two main clusters were found, with the first represented by the 100 mM sodium phosphate buffer and the second containing all other infiltration solutions indicating the isolation of a rather different set of proteins by the first one than compared to all other solutions under examination (Figure 3). The most similar abundance patterns derived from leaf infiltration with 25 mM Tris-HCl and 50 mM NaCl reflecting a comparable degree of protein extraction efficiency.

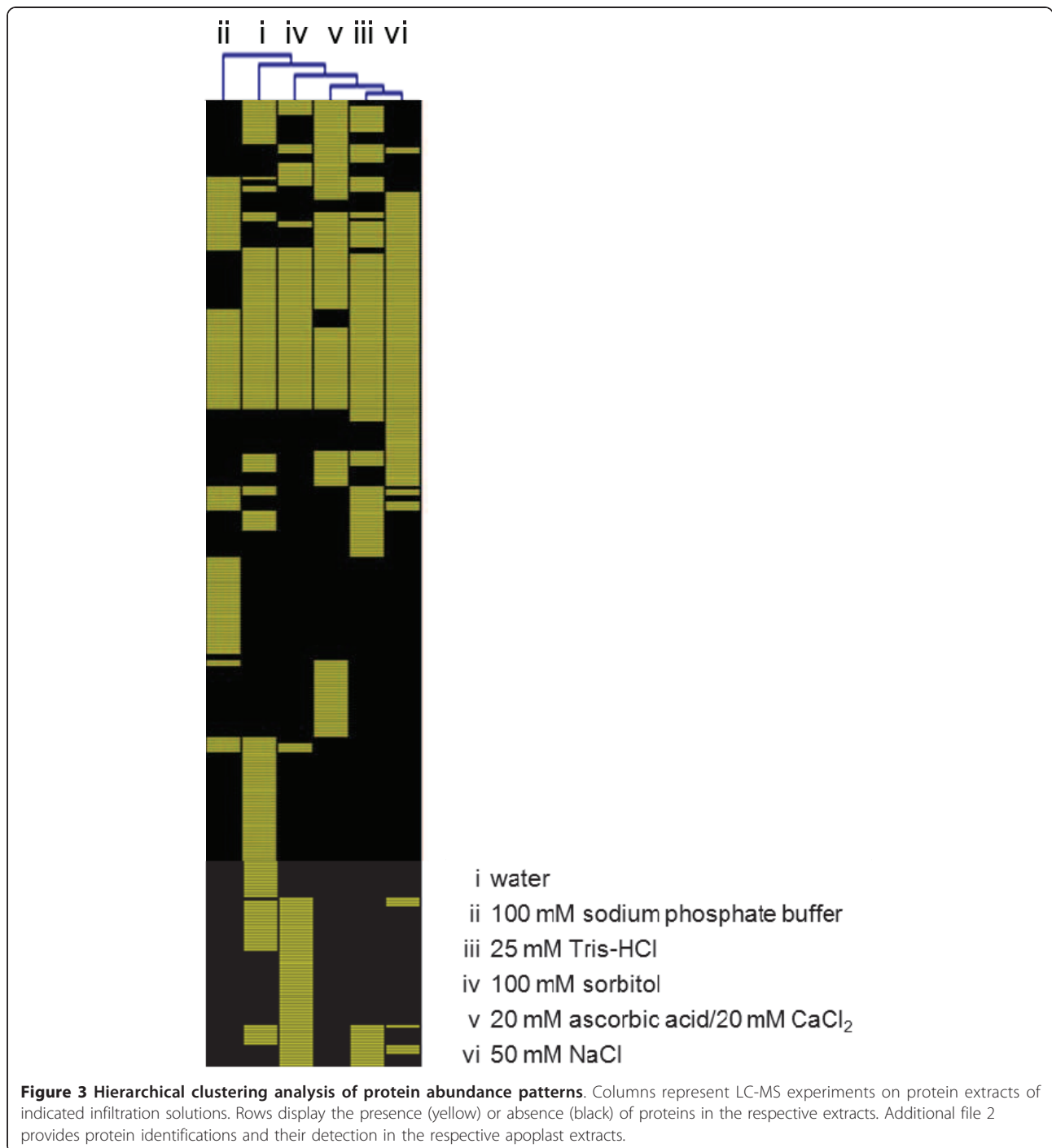
The highest number of proteins was found in apoplastic extracts using water as infiltration solution. Here, 171 proteins were detected. Extracts of 25 mM Tris-HCl, 100 mM sorbitol and 20 mM ascorbic acid/20 mM CaCl₂ yielded in the identification of a similar number of 131, 133 and 133 proteins, respectively. We found 114 proteins in extracts of 50 mM NaCl solution and 107 proteins in those of 100 mM sodium phosphate buffer. Out of all 328 proteins, only 28 proteins were common across all six extracts (Additional file 4). A similar observation was made for Arabidopsis cell wall proteins when extracted by different solutions; here, only 11 out of 96 proteins were found to be common in all extracts [31]. Exhydrolase II [UniProt: Q9XE93] was found in all extracts and its identification is illustrated in Additional file 5 as an example. Here, the amino acid sequence is shown and the 12 detected peptides are marked within, resulting in protein sequence coverage of 28.7%.

The quality of apoplastic protein preparations is estimated in many cases by enzymatic measurements of specific proteins such as malate dehydrogenase [5,9] and glucose-6-phosphate dehydrogenase [6]. However, it is known that the activity of those enzymes is detectable in respective cellular compartment as well [35]. To assess the amount of symplast contaminations in our samples, we used topology prediction tools. The identified proteins were classified for their subcellular localization as deduced by Expasy tools Target P and WoLF

PSORT (Figure 4). A number of proteins in this study were allocated to other cellular compartments than the apoplast, suggesting considerable amounts of intracellular protein contaminations. However, previous reports using different plant species and extraction methods described the detection of cytosolic, mitochondrial or vacuolar proteins in cell wall or apoplast preparations [31,36-38]. These consistent findings point to the occurrence of non-classical secretory pathways for proteins lacking signal sequences [39,40] and therefore, differentiation between yet unknown apoplastic proteins and ones resident in other organelles remains difficult. Water-infiltrated leaves revealed 23 protein identifications localized to the apoplast, while a high number of intracellular proteins were detected from the vacuole (19), cytosol (46) and chloroplast (23). This observation is indicative for the disruption of plasma membrane during the infiltration process. Also, apoplastic extracts with 100 mM sorbitol as infiltration solution contained a superior proportion of chloroplast (26) and cytosolic (41) proteins with only 15 predicted apoplastic proteins. This result was unexpected as the sugar alcohol sorbitol was applied to maintain the osmotic cell pressure. Similar numbers of proteins in infiltrates with 25 mM Tris-HCl, 20 mM ascorbic acid/20 mM CaCl₂ and 50 mM NaCl were assigned to the chloroplast (14, 17, 14), the cytosol (36, 34, 27), and the apoplast (25, 31, 25). Of all tested infiltration solutions, 100 mM sodium phosphate buffer contained the lowest number of proteins assigned to intracellular compartments (chloroplast: 10, cytosol: 16) and the highest number of proteins targeted to the extracellular apoplast with 34 identified proteins.

Table 1 presents the 67 proteins allocated to the apoplast of maize leaves and grouped according to their function into 7 classes. The largest class consisted of 39 proteins related to cell structural processes, including carbohydrate metabolism (e.g.: lichenase 2, alpha N-arabinofuranosidase, beta galactosidase, exoglucanase, exhydrolase II) and cell wall modification (e.g.; pectinesterase, xyloglucan endotransglycosylase hydrolase, peroxidases). Synthesis and integration of polysaccharides into the cell wall and extension of this network during plant growth are the major biological functions of proteins present in the apoplast [41] and our findings reflect this reality. Fifteen proteins were involved in disease and defense reactions, the second prime function of the apoplast [42]. The third class was related to proteins with transporting function and here, 7 proteins were identified. Further classes were related to cell growth/division, protein destination/storage, secondary metabolism and signal transduction.

The number of proteins identified exclusively in any of the extracts was compared and revealed that 16 out of 34 apoplastic proteins were found only in extracts of



100 mM sodium phosphate buffer, representing the highest number of unique proteins in all tested infiltration solutions (Table 1). Usage of this infiltration solution appears to prevent damaging the plasma membrane and enables extraction of proteins adhesive to the cell wall. Most polypeptides found in the analysis were annotated as hypothetical based on an *in silico* match to a genome sequence, or putative due to a homology to a

protein with known function (Figure 4). The identification of these proteins in apoplastic preparations reveals the potential inherited in proteomic surveys for establishing comprehensive maps of all translated polypeptides present in a subcellular compartment. A number of 12 proteins with unknown function were exclusively identified using the 100 mM sodium phosphate buffer infiltration solution (see Additional file 2). As this

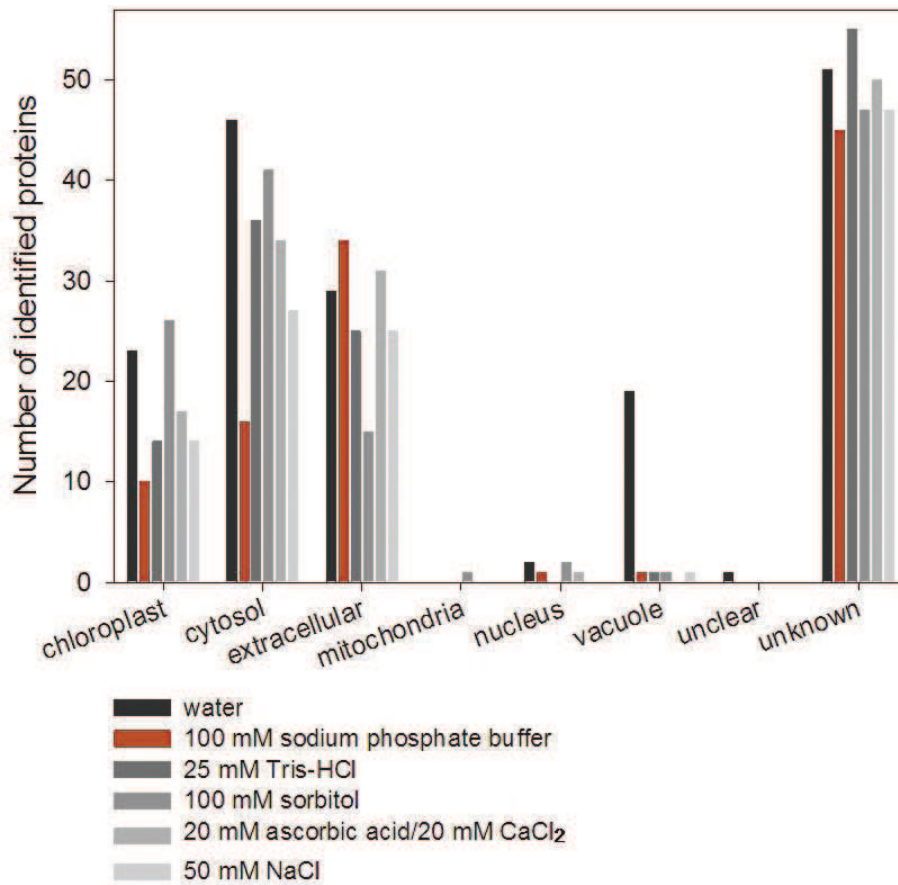


Figure 4 Predicted subcellular distribution of identified proteins from the maize leaf apoplast extracted by different infiltration solutions. Topology prediction was performed with ExPASy tools Target P (<http://www.cbs.dtu.dk/services/TargetP/>) and WoLF PSORT (<http://wolfpsort.org/>).

protein fraction performed best regarding contaminations from other cellular compartments and contained most of the apoplastic proteins, we assume that these yet unknown proteins are involved in physiological processes of the apoplast.

Conclusions

The plant apoplast is a dynamic compartment with a broad range of physiological functions. To study proteins involved in nutrition, growth, signaling or transport processes, it is crucial to apply extraction methods selective for apoplastic proteins. In this study, we compared six different infiltration solutions already reported for the isolation of this protein subset. The protein patterns resolved by 1-DE revealed clear differences between apoplast and symplast preparations. We found the lowest number of intracellular protein contaminants with the highest number of extracted proteins present in the apoplastic fluid obtained with 100 mM sodium phosphate buffer. Also, the number of secreted proteins

exclusively found in a single fraction was highest for that buffer. Those findings are now employed in comparative proteomic studies aiming at identifying proteins involved in abiotic stress responses.

Materials and methods

Plant cultivation

Maize grains cv. Lector (LG Seeds, <http://www.lgseeds.com>) were imbibed overnight in aerated 1 mM CaSO₄ solution and germinated at 28°C in the dark between two layers of filter paper moistened with 0.5 mM CaSO₄. After 4 days, seedlings were transferred to light in constantly aerated plastic pots containing one-fourth concentrated nutrient solution. The concentration of nutrient solution was increased to half and full strength after 2 and 4 days of cultivation, respectively. The full strength nutrient solution had the following concentrations: 2.0 mM Ca(NO₃)₂, 1.0 mM K₂SO₄, 0.2 mM KH₂PO₄, 0.5 mM MgSO₄, 2.0 mM CaCl₂, 5.0 μM H₃BO₃, 2.0 μM MnSO₄, 0.5 μM ZnSO₄, 0.3 μM CuSO₄,

Table 1 Identification of proteins assigned to the apoplast.

Entry	Description	mW (Da)	pI (pH)	Infiltration solutions									
				i	ii	iii	iv	v	vi				
<u>Cell growth and division</u>													
UniRef90_Q6ZDE3	Abscisic acid 8'-hydroxylase 2, <i>Oryza sativa</i>	56519	9.83	x									
<u>Cell structure</u>													
UniRef90_A5H454	Peroxidase 66, <i>Zea mays</i>	33398	8.02						x				
UniRef90_A5H8G4	Peroxidase 1, <i>Zea mays</i>	38330	6.89							x			
UniRef90_A5JTQ2	Alpha N arabinofuranosidase, <i>Medicago varia</i>	83673	6.22		x								
UniRef90_B4FKV6	Peroxidase 54, <i>Zea mays</i>	36178	4.95									x	
UniRef90_B5AK47	Dhurrinase-like B glucosidase, <i>Zea mays</i>	64233	7.95			x							
UniRef90_B6SMR2	Peroxidase 52, <i>Zea mays</i>	33504	8.14		x	x				x	x		
UniRef90_B6SUH6	Non-cyanogenic beta glucosidase, <i>Zea mays</i>	56680	5.39	x		x				x			
UniRef90_B6SXU7	Heparanase-like protein 3, <i>Zea mays</i>	58262	9.35	x		x	x			x	x		
UniRef90_B6SXY3	Beta galactosidase, <i>Zea mays</i>	48728	8.38		x								
UniRef90_B6T391	Lichenase 2, <i>Zea mays</i>	34951	5.64	x	x	x	x			x	x		
UniRef90_B6T9B9	Alpha N arabinofuranosidase, <i>Zea mays</i>	74827	5.04			x				x	x		
UniRef90_B6TU39	Peroxidase 2, <i>Zea mays</i>	34941	4.67		x								
UniRef90_B6TU78	Glucan endo-1-3 beta glucosidase 7, <i>Zea mays</i>	45316	5.54	x									
UniRef90_B6TXJ8	Glycoside hydrolase family 28, <i>Zea mays</i>	47066	5.58	x									
UniRef90_B6U063	Carboxylic ester hydrolase, <i>Zea mays</i>	50032	7.85	x		x				x			
UniRef90_B6U0W2	Beta galactosidase, <i>Zea mays</i>	93935	6.48		x								
UniRef90_B9SD68	Hydrolase, <i>Ricinus communis</i>	67825	6.80	x	x	x							
UniRef90_C4N559	Xyloglucan endotransglycosylase hydrolase, <i>Musa acuminata</i>	20309	9.54									x	
UniRef90_C5WQU7	Beta galactosidase, <i>Sorghum bicolor</i>	92893	5.31	x		x	x			x	x		
UniRef90_C5Z534	Beta galactosidase, <i>Sorghum bicolor</i>	79098	7.74		x								
UniRef90_O04943	Alpha galactosidase, <i>Hordeum vulgare</i>	17730	6.33		x	x				x	x		
UniRef90_P93518	PRm 3, <i>Zea mays</i>	30099	3.90	x	x	x	x			x	x		
UniRef90_Q10CU3	Glycosyl hydrolase family 3, <i>Oryza sativa</i>	43916	8.20	x		x							
UniRef90_Q10M79	Alpha L arabinofuranosidase, <i>Oryza sativa</i>	73965	4.73							x			
UniRef90_Q10NX8	Beta galactosidase 6, <i>Oryza sativa</i>	92780	5.52	x			x						
UniRef90_Q2R3E0	Glycosyl hydrolases family 38, <i>Oryza sativa</i>	114085	5.85		x							x	
UniRef90_Q2RAZ2	Alpha L arabinofuranosidase, <i>Oryza sativa</i>	73421	4.57	x		x	x			x	x		
UniRef90_Q43417	Peroxidase, <i>Cenchrus ciliaris</i>	32473	7.50							x			
UniRef90_Q53MP2	Beta D-xylosidase, <i>Oryza sativa</i>	82557	6.62		x								
UniRef90_Q5CCP6	Beta galactosidase, <i>Pyrus pyrifolia</i>	94782	8.12		x								
UniRef90_Q5J3F3	Peroxidase 5, <i>Triticum monococcum</i>	27533	5.72		x								
UniRef90_Q6L619	Beta galactosidase, <i>Raphanus sativus</i>	92580	8.36	x						x			
UniRef90_Q7G3T8	Beta galactosidase 13, <i>Oryza sativa</i>	91940	6.06		x								
UniRef90_Q8GUY1	Pectinesterase, <i>Lolium perenne</i>	24837	7.81		x								
UniRef90_Q8RUV9	Beta galactosidase 1, <i>Oryza sativa</i>	91652	5.71		x								
UniRef90_Q9FXT4	Alpha galactosidase, <i>Oryza sativa</i>	45792	7.91							x	x		
UniRef90_Q9LLB8	Exoglucanase, <i>Zea mays</i>	66900	6.99	x	x	x	x					x	
UniRef90_Q9XE93	Exhydrolase II, <i>Zea mays</i>	68330	6.16	x	x	x	x			x	x		
UniRef90_Q9XEI3	Beta D-glucan exohydrolase isoenzyme, <i>Hordeum vulgare</i>	67862	6.24	x	x		x						
<u>Disease and defence</u>													
UniRef100_P25272	Kunitz-type trypsin inhibitor 1, <i>Glycine max</i>	22531	4.77	x									
UniRef90_A7I2L3	Invertase inhibitor, <i>Coffea canephora</i>	20205	6.68		x								
UniRef90_B6TA80	Thaumatin-like protein, <i>Zea mays</i>	17632	6.75							x			
UniRef90_B6TDW7	Secretory protein, <i>Zea mays</i>	24467	4.64							x			
UniRef90_B6TT00	Endochitinase PR4, <i>Zea mays</i>	28545	4.96	x						x			
UniRef90_B6TTY1	Germin-like protein, <i>Zea mays</i>	26764	7.25	x		x				x	x		
UniRef90_B6TWH6	Lysosomal Pro x carboxypeptidase, <i>Zea mays</i>	59936	5.62	x	x	x	x					x	

Table 1 Identification of proteins assigned to the apoplast. (Continued)

UniRef90_B6UB57	Lysosomal protective protein, Zea maize	53540	5.87	x	x	x		x	x
UniRef90_O24007	Chitinase, Oryza sativa	18956	4.83	x		x	x	x	x
UniRef90_P01063	Bowman-Birk-type proteinase inhibitor C II, Glycine max	9194	4.38	x					
UniRef90_P29022	Endochitinase A, Zea maize	29105	7.85					x	
UniRef90_Q5U1S9	Class III peroxidase 14, Oryza sativa	37174	5.77					x	
UniRef90_Q6EUS1	Class III peroxidase 27, Oryza sativa	33300	8.09		x				x
UniRef90_Q6TM44	Germin-like protein, Zea mays	21873	6.04	x	x	x	x	x	x
UniRef90_Q7M1R1	Chitinase, Gladiolus × gandavensis	30695	5.77	x		x	x	x	x
<u>Protein destination and storage</u>									
UniRef90_B6TG95	Vignain, Zea mays	38823	4.68	x	x	x	x	x	x
UniRef90_B6TYX7	Polygalacturonase inhibitor 1, Zea mays	30011	8.08		x			x	x
<u>Secondary metabolism</u>									
UniRef90_O64411	Polyamine oxidase, Zea mays	56308	5.63		x				
<u>Signal transduction</u>									
UniRef90_B6TWC3	Rhcadhesin receptor, Zea mays	23726	9.20		x	x			
UniRef90_B9MZ47	Fasciclin-like AGP 14 4 protein, Populus trichocarpa	24786	8.63					x	
<u>Transporters</u>									
UniRef90_B4FB54	Non-specific lipid transfer protein, Zea mays	12084	9.60		x				
UniRef90_B6SP11	Non-specific lipid transfer protein, Zea mays	9802	8.73		x				
UniRef90_B6SY96	Non-specific lipid transfer protein, Zea mays	12011	9.29		x				
UniRef90_B6TRB2	Copper ion binding protein, Zea maize	17057	9.78				x		
UniRef90_P05046	Lectin, Glycine max	30908	5.60	x			x		
UniRef90_P19656	Non-specific lipid transfer protein, Zea mays	11697	8.74		x				x
UniRef90_Q04672	Sucrose-binding protein, Glycine max	60484	6.42	x					

UniProt database identifiers, along with molecular weight (mW) and isoelectric point (pI) are shown. The identification of the respective proteins using different extraction solutions is indicated (i: water, ii: 100 mM sodium phosphate buffer, iii: 25 mM Tris-HCl, iv: 100 mM sorbitol, v: 20 mM ascorbic acid/20 mM CaCl₂, vi: 50 mM NaCl).

0.01 μM (NH₄)₆Mo₇O₂₄, 200 μM Fe-EDTA. Nutrient solution was changed twice a week to avoid nutrient deficiencies. The experiments were carried out under greenhouse conditions with an average day/night temperature of 28/18°C and a photoperiod of 14 h for 5 weeks with relative humidity about 70% ± 5%. The fifth and sixth leaf from medium part of the stem was harvested 16 d after reaching the full nutrient solution for collection of apoplast proteins.

Extraction of apoplastic and symplastic proteins

Apoplastic proteins were collected using the infiltration-centrifugation technique [43] with minor modifications. Leaves were cut into segments of about 5.5 cm and washed with deionised water. For infiltration, leaf segments were placed in plastic syringes (60 ml) filled with 40 ml of the respective infiltrating medium and were infiltrated by pulling the plunger, producing a reduced pressure of estimated about 20 kPa. Leaves were infiltrated either with water, 20 mM ascorbic acid/20 mM CaCl₂, 100 mM sorbitol, 0.1 M sodium phosphate buffer (pH 6.5), 25 mM Tris-HCl (pH 8.0) or 50 mM NaCl (Figure 1). Thereafter, intact leaf segments were carefully blotted dry, and then placed in a 10 ml plastic vessel and centrifuged immediately at 400 g for 5 min at 5°

C. The clear infiltrate, now referred to as apoplast fraction, was collected at the bottom of the tube.

After the extraction of the apoplastic fraction, the residual leaf tissue was shock frozen in liquid nitrogen, thawed, and centrifuged at 715 g for 5 min for cell sap extraction, now referred to as symplast fraction. Four pools of extracts from five plants each were combined for subsequent analyses. Extracts were stored at -80°C until analysis.

Gel electrophoretic protein separation

Proteins contained in the different extracts were precipitated by chloroform/methanol method [44]: 200 μl of sample was mixed with 800 μl MeOH, 400 μl chloroform and 600 μl deionized water. The incubation at 4°C for 5 min was followed by a centrifugation step (9,000 g, 2 min, 4°C). The upper phase was removed and 600 μl MeOH was added to the lower and interphase. A further centrifugation sedimented the proteins, the supernatant was removed and the pellet was dried in a vacuum centrifuge.

For one-dimensional separation of proteins, the pellets were dissolved in 10% glycerol, 2.3% SDS, 5% β-mercaptoethanol, 0.25% bromphenol blue, 63 mM Tris-HCl (pH 6.8). The 2-D Quant Kit (GE Healthcare, <http://>

www.gehealthcare.com) was used for determining the protein concentration. A sample of 10 µg was separated by SDS-PAGE according to Laemmli [45]. The two-dimensional separation of proteins was accomplished as described in Zörb *et al.* [46] with the following modifications. Protein pellets were first dissolved in 8 M urea, 2 M thiourea, 0.5% IPG (immobilized pH gradient) buffer, 4% w/v CHAPS, 30 mM DTT, 20 mM Tris and then dialyzed using 3.5 kDa cut-off membrane (Zellu-Trans, Carl Roth, <http://www.carlroth.com>) against the same buffer. The protein concentration was determined with the 2-D Quant Kit (GE Healthcare) and 25 µg of protein were separated on IPG strips of 7 cm in length with pH gradient of 3-10. Protein gels were stained according to the hot-staining protocol with Coomassie R350 tablets (PlusOne Coomassie tablets PhastGel Blue R-350, GE Healthcare) [47] and digitized with an Epson Perfection V700 Photo scanner (Epson, <http://www.epson.com>).

LC-MS-based protein identification

Dialyzed protein extracts were precipitated by chloroform/methanol method and about 30 µg of protein were resolubilized in 50 µl 0.1% Rapigest (Waters Corporation, <http://www.waters.com>) in 50 mM ammonium bicarbonate. Protein concentrations were determined using the Bradford method [48] and bovine serum albumin as standard protein. Five µg of protein were reduced, alkylated and digested with trypsin over night at 37°C as described earlier [49]. The enzymatic reaction was stopped with 1N HCl and peptide solutions were adjusted to 0.1 µg/µl final concentration.

Three µl of protein digest were used for LC-separation on a nanoAcquity UPLC system (Waters) followed by mass spectrometry analysis on a Q-TOF Premier MS instrument (Waters) in a data directed analysis (DDA) mode, as described in Agrawal *et al.* [50].

Peptide separation was performed on a 180 µm × 20 mm Symmetry (5 µm) C18 precolumn (Waters) coupled to a 150 mm × 75 µm BEH130 (1.7 µm) C18 column (Waters), with a gradient of 3-40% acetonitrile over 90 min. The MS operated in a positive ion mode with a source temperature of 80°C, a cone gas flow of 50 l/h, and a capillary voltage of approximately 3 kV. Mass spectra were acquired in a continuum V-mode and spectra integrated over 1 s intervals using MassLynx 4.1 software (Waters). The instrument was calibrated using selected fragment ions of the CID (collision-induced dissociation) of Glu-Fibrinopeptide B (SIGMA-ALDRICH, <http://www.sigmaaldrich.com>). Automatic data directed analysis (DDA) was employed for MS/MS analysis on doubly and triply charged precursor ions. The MS

spectra were collected from m/z 400 to m/z 1600, and product ion MS/MS spectra were collected from m/z 50 to m/z 1600. Lock mass correction of the precursor and product ions was conducted with 500 fmol/µl Glu-Fibrinopeptide B in 0.1% formic acid in AcN/water (50:50, v/v) respectively, and introduced via the reference sprayer of the NanoLockSpray interface. ProteinLynx GlobalSERVER v2.3 software was used as a software platform for data processing, deconvolution, de novo sequence annotation of the spectra, and database search. A 10 ppm peptide, 0.1 Da fragment tolerance, one missed cleavage, and variable oxidation (Met) and carbamidomethylation (Cys) were used as the search parameters.

The resulting mass spectra were searched against the protein index of the UniProt *viridiplantae* database (release: July 2010 with 722,718 protein sequences) for protein identification applying the algorithm implemented in the ProteinLynxGlobalServer software (PLGS, Waters Cooperation). All samples were run as technical triplicates. Protein identifications consistent in two out of three LC-MS runs were considered as present in that sample. The false discovery rate was set to 4% of proteins included in the database.

Hierarchical clustering of protein abundances was performed using Gene Expression Similarity Investigation Suite Genesis v1.7.6 [51]. Average linkage clustering was applied for LC-MS experiments and protein abundances.

Additional material

Additional file 1: Biological reproducibility of protein profiles from the maize leaf apoplast as resolved by 1-DE. Apoplastic proteins were extracted with water (i) or 100 mM sodium phosphate buffer (ii). Two independent experiments were performed to assure consistent protein patterns.

Additional file 2: Identification of proteins from the apoplast of maize leaves. UniProt database identifiers, along with molecular weight (mW) and isoelectric point (pI) are shown. The cellular localisation was assigned using ExPASy tools Target P (<http://www.cbs.dtu.dk/services/TargetP/>) and WoLF PSORT (<http://wolfpsort.org/>). The identification of the respective proteins using different extraction solutions is indicated (i: water, ii: 100 mM sodium phosphate buffer, iii: 25 mM Tris-HCl, iv: 100 mM sorbitol, v: 20 mM ascorbic acid/20 mM CaCl₂, vi: 50 mM NaCl).

Additional file 3: Identification of proteins from the apoplast of maize leaves. Provided are the UniProt database identifiers, the PLGS score, probability score for identification, number of identified peptides, protein coverage and the peptide sequence.

Additional file 4: Proteins identified in apoplast extracts of all six infiltration solutions.

Additional file 5: Example of protein identification from apoplastic extracts using nanoLC-ESI-Q-TOF MS. The database search against the protein index of UniProt led to the identification of exhydrolase II [Q9XE93]. The amino acid sequence of the corresponding protein is shown on top with the detected peptides underlined. The *de novo* sequence of a selected peptide with precursor mass m/z 859.4698 (charge 3) is shown. This peptide is marked in bold within the protein sequence.

Abbreviations

LC-MS/MS: liquid chromatography tandem mass spectrometry; 1-DE: one-dimensional gel electrophoresis; 2-DE: two-dimensional gel electrophoresis

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Author details

¹Institute of Plant Nutrition and Soil Science, Christian Albrechts University, Hermann-Rodewald-Strasse 2, 24118 Kiel, Germany. ²Leibniz Institute of Plant Genetics and Crop Plant Research, Corrensstrasse 3, 06466 Gatersleben, Germany. ³Leibniz Institute of Vegetable and Ornamental Crops, Theodor-Echtermeyer-Weg 1, 14979 Großbeeren, Germany.

Authors' contributions

KW carried out protein extractions, protein separations, data evaluation and manuscript preparation. MS performed plant cultivation, apoplastic preparations and contributed to protein separations. AM conceived mass spectrometry analyses and contributed to manuscript writing. HPM participated in discussions during experimental work and manuscript preparation. KHM conceived the project and worked on manuscript preparation. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Chapter 5

Significance of apoplastic proteins involved in growth in the initial phase of salt stress response in maize (*Zea mays* L.) leaves

Muhammad Shahzad, Christian Zörb and Karl H. Mühling

(In preparation)

Significance of apoplastic proteins involved in growth in the initial phase of salt stress response in maize (*Zea mays* L.) leaves

Muhammad Shahzad, Christian Zörb and Karl H. Mühling

Institute of Plant Nutrition and Soil Science, Christian Albrechts University, Hermann-Rodewald-Strasse 2, 24118 Kiel, Germany

Corresponding author:

Prof. Dr. Karl H. Mühling

Institute of Plant Nutrition and Soil Science, Christian Albrechts University, Hermann-Rodewald-Strasse 2, 24118 Kiel, Germany

Tel: +49 431 880 3189

Fax: +49431 880 1625

Email: khmuehling@plantnutrition.uni-kiel.de

Email addressed:

mshahzad@plantnutrition.uni-kiel.de

czoerb@plantnutrition.uni-kiel.de

khmuehling@plantnutrition.uni-kiel.de

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Evaluation of apoplastic proteins by two-dimensional electrophoresis

Keywords:

Apoplast, maize, proteome analysis, two-dimensional gel electrophoresis

Abbreviations:

2-DE: two-dimensional gel electrophoresis

AWF: apoplastic washing fluid

Abstract

Proteins in the plant apoplast are essential for many physiological processes. We aimed to evaluate the apoplastic proteins to examine their exclusive roles under salinity stress. We also focused to investigate whether changes in maize (*Zea mays*) leaves expansion rate in response to salinity stress are mediated by regular or specific expansins, a class of cell wall proteins known to enhance cell wall extensibility. Plant growth was significantly reduced after 100 mM NaCl treatment for 14-d. Apoplastic washing fluid was extracted by 0.1 M Na-phosphate buffer (pH 6.5). A significant reduction in apoplastic fluid extraction was also noticed under salinity. Although, we extracted small amount of proteins in the extraction solution yet we were able to concentrate and present two-dimensional gels with apoplastic proteins. We could also examine three spots by delta 2D which showed down-regulation under salinity treatment. However, we experienced some streaking problem in acidic gel region because of some other apoplastic constituents. Therefore, comparison of the protein spots on delta 2D and later identification by mass spectrometry are still need to be accomplished.

Introduction

The apoplast is the out of cell membrane fraction of the plant cell. It comprises of cell wall and intercellular space of the plant (Dietz, 1997). It is involved in a variety of functions during plant growth and development as well as in plant defence responses to stress conditions (Pennell, 1998). Because cells transport ions, assimilates and other metabolites from the apoplast and furthermore during the signal-transduction processes, a signal must cross the apoplast and plasma membrane for the cell to produce a response (Chikov, 2004).

The apoplast, which plays important roles in regulating plant physiological and developmental processes, is not only a barrier but also a linker between the environment and the protoplast. There is strong evidence that apoplastic proteins has been greatly changed in abundance in response to salt, chilling (Brune et al., 1994; Song et al., 2011), wounding (Li and McClure, 1990) insect infestation (Westhuizen and Pretorius, 1996) and pathogen invasion (Floerl et al., 2008; Goulet et al., 2010). It has been reported that stress conditions have greatly affected the composition of apoplastic proteins both qualitatively and quantitatively (Dani et al., 2005).

The precise molecular basis of wall properties is still enigmatic, but increasing evidence indicates that wall properties are under enzymatic, protein-biochemical, or chemical control. Expansins are believed to regulate cell wall enlargement in growing cells and have been directly implicated in cell wall-loosening processes (McQueen and Cosgrove, 1995; Rochange et al., 2001; Cosgrove, 2005). Vegetatively expressed growth-mediating expansins are one of significant component of the protein-biochemical activity. In salt-sensitive maize the reduced b-expansin protein abundance was found to correlate positively with the reduced shoot growth under salinity stress conditions (Geilfus, 2010).

Expansins are the prime mediators of acid-induced growth (Cosgrove, 2005). The acid growth theory elaborates growth by auxin-mediated cell wall elongation (Rayle and Cleland 1970; Hager et al., 1971). According to this theory, the acidification of the leaf apoplast is the major

requirement for increasing cell wall extensibility, which then controls extension growth (Hager, 2003). The theory is supported by the finding that auxin mediates the acidification of the apoplast below a pH of 5.0 (Peters and Felle, 1991), thus stimulating cell elongation in maize, *Avena coleoptiles*, and pea (Hager et al., 1971; Jacobs and Ray, 1976). However, only limited reports have evaluated the effects of salinity on the abundance of these growth-mediating proteins. Buchanan et al. (2005) reported a salinity-induced increase in b-expansin transcript abundance for *Sorghum bicolor*. Recently, Pitann et al. (2009) and Geilfus et al. (2010) have shown results derived from two-dimensional gel analyses indicating a decrease in b-expansin protein in a salt-sensitive maize cultivar, whereas b-expansin protein level in a resistant cultivar is less affected by salinity. However, knowledge on the apoplastic protein changes under salinity stress is still lacking.

Over the past few years, great progress has been made in the characterization of plant sub-cellular proteomes due to its advantage of dramatically reducing the complexity of crude cell or tissue extracts and providing important information regarding the subcellular localization of proteins in the cell (Brunet et al., 2003). Based on their potential physiological functions, the salt-stress-regulated root apoplastic proteins identified in Zhang et al. (2009) study were all involved in the plant stress response. Proteins changed in the tobacco leaf apoplast proteome in response to salt have also been identified (Dani et al., 2005). These results showed the advantage of proteomics approach on identifying subcellular proteins involved in specific physiological process. But as an important component of the plant cell, the functional research of plant apoplastic proteins has been obviously ignored in regards to signal pathway. Therefore, we aimed to evaluate the maize apoplastic proteins to identify their exclusive roles under salt stress.

Materials and methods

Plant development

Maize seedlings cv. Logo (LG Seeds, www.lgseeds.com) were imbibed in aerated 1 mM CaSO₄ solution for 1 d and germinated at 28 °C in the dark between two layers of filter paper moistened with 0.5 mM CaSO₄. After 4 d, seedlings were transferred to light in continually aerated plastic pots containing one-fourth concentrated nutrient solution. On the following day, the nutrient solution was increased to 1/2 , and after additional 2 d to full concentration. The full strength nutrient solution had the following concentrations: 2.0 mM Ca(NO₃)₂, 1.0 mM K₂SO₄, 0.2 mM KH₂PO₄, 0.5 mM MgSO₄, 2.0 mM CaCl₂, 5.0 μM H₃BO₃, 2.0 μM MnSO₄, 0.5 μM ZnSO₄, 0.3 μM CuSO₄, 0.01 μM (NH₄)₆Mo₇O₂₄, 200 μM Fe-EDTA. Nutrient solution was changed twice a week to avoid nutrient deficiencies. The experiments were carried out under greenhouse conditions with an average day/night temperature of 28/18°C and a photoperiod of 14 h for 5 weeks with relative humidity about 70% ± 5%. The fifth and sixth leaf with no salt injury from medium part of the stem was harvested 16 d after reaching the full nutrient solution for collection of apoplast proteins.

Extraction of apoplastic proteins

Apoplastic proteins were collected using the infiltration-centrifugation technique (Lohaus, et al., 2001) with minor modifications. Leaves were cut into segments of about 5.5 cm and washed with deionised water. For infiltration, leaf segments were placed in plastic syringes (60 ml) filled with 40 ml of the 0.1 M sodium phosphate buffer (pH 6.5; infiltrating medium) and were infiltrated by pulling the plunger, producing a reduced pressure of estimated about 20 kPa. Thereafter, intact leaves segments were carefully blotted dry, and then placed in a 10 ml plastic vessel and centrifuged immediately at 400 g for 5 min at 5 °C. The clear infiltrate, now referred to as apoplast fraction, was collected at the bottom of the tube.

Cell sap

After the extraction of the apoplastic fraction, the residual leaf tissue was shock frozen in liquid nitrogen, thawed, and centrifuged at 715 g for 5 min for cell sap extraction, now referred to as symplast fraction. Four pools of extracts from five plants each were combined for subsequent analyses. Extracts were stored at -80 °C until analysis.

Gel electrophoretic protein separation

Proteins contained in the extracts were precipitated by chloroform/methanol method (Wessel, 1984): 200 µl of sample was mixed with 800 µl MeOH, 400 µl chloroform and 600 µl deionized water. The incubation at 4°C for 5 min was followed by a centrifugation step (9,000 g, 2 min, 4°C). The upper phase was removed and 600 µl MeOH was added to the lower and interphase. A further centrifugation sedimented the proteins, the supernatant was removed and the pellet was dried in a vacuum centrifuge.

The 2-D Quant Kit (GE Healthcare, www.gehealthcare.com) was used for determining the protein concentration. A sample of 10 µg was separated by SDS-PAGE according to Laemmli (1970). The two-dimensional separation of proteins was accomplished as described in Zörb et al. (2004) with the following modifications. Protein pellets were dissolved in 8 M urea, 2 M, thiourea, 0.5 % IPG (immobilized pH gradient) buffer, 4 % CHAPS, 30 mM DTT, 20 mM Tris and dialyzed using 3.5 kDa cut-off membrane (ZelluTrans, Carl Roth, www.carlroth.com). The protein concentration was determined with the 2-D Quant Kit (GE Healthcare) and 25 µg of protein were separated on IPG strips of 7 cm in length with pH gradient of 4-7. Protein gels were stained according to the hot-staining protocol with Coomassie R350 tablets (PlusOne Coomassie tablets PhastGel Blue R-350, GE Healthcare) (Westermeier and Naven, 2002) and digitized with an Epson Perfection V700 Photo scanner (Epson, www.epson.com).

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Results and discussion

Plant growth under salinity

The reduction in growth rate is a well-documented phenomenon of salt sensitive crops such as maize under salinity (Fortmeier and Schubert, 1995; Munns, 2002). A 100 mM NaCl treatment leads to a considerable reduction in plant growth (Fig. 1). The shoot biomass of the maize cultivar Logo 32.2 was 58 % significantly reduced in comparison to control (Fig. 2A). This finding is in good agreement with previously shown results of 61 % biomass reduction with maize plants (Pitann et al., 2009). In our study for apoplastic protein changes only expanding leaves were used for the collection of apoplastic washing fluid which had entirely developed under salinity. Fresh weight of expanding shoots was also significantly reduced under salt treatment (Fig. 2B). Expanding leaves were used for expansin investigation because it is believed to regulate cell wall enlargement in growing cells and are the primary mediators of acid-induced growth (Cosgrove, 2005; McQueen, 1995). In an earlier investigation Geilfus et al. (2010) also focused on expanding leaves to examine protein changes in total shoot as only those leaves were entirely developed under salinity.

The extraction solution for subcellular proteins in maize leaves

The extraction of proteins from the leaf and root apoplast is mainly based on the principle of vacuum infiltration with an extraction solution, followed by a mild centrifugation step to

collect the apoplastic washing fluid. The composition of the infiltration solution is essential as it has to fulfil certain prerequisites, such as maintenance of osmotic pressure to prevent collapsing of plasma membrane and stringency for extracting cell wall-bound proteins. A 0.1 M Na-phosphate buffer (pH 6.5) was used to extract the apoplastic proteins. This extracting solution was considered best as it is found to produce the lowest number of intracellular protein contaminants with the highest number of extracted proteins present in the apoplastic fluid (Chapter 4, Fig. 4). We also observed significant reduction in fluid extraction from the leaf apoplast of salt treated plants (Fig. 3) which already suggest some changes in cell wall structure by salt stress (Zhong and Läuchli, 1993).

Subcellular protein pattern in maize leaves under salinity

Earlier studies by Zörb et al. (2004) and Pitann et al. (2009) has shown 64 and 61 % changes in shoot proteins of salt sensitive maize plants under comparable treatment. In another investigation Riccardi et al. (1998), the proteome changed in response to water deficit in maize in the same range as described under salinity. These results indicate the involvement of proteins under salt and osmotic stress. However, in previous investigation with tobacco (Dani et al., 2005) many apoplastic proteins have been identified in response to salinity stress. Based on their potential physiological functions, the salt-stress regulated apoplastic proteins identified in Zhang et al. (2009) study are all involved in the plant stress response. These results show the advantage of the proteomics approach on identifying subcellular proteins involved in specific stress process. For that reason, equal amounts of apoplast proteins from control and salt treated extracting solutions were separated by 2-DE to assess the protein patterns in detail (Fig. 4). We were able to present areas of good resolved proteins spots on all apoplastic 2-D gels. Proteins in the acidic gel region showed horizontal streaking. Although all samples were precipitated, dissolved in urea-containing buffer system and dialyzed prior to 2-DE to avoid the contamination with nucleic acids or other interfering substances, these

poorly separated spots were observed. Contrary to this, proteins in the basic region of 2-D gels showed a superior resolution with minimal streaking. Although quality of gels was not good enough for identification yet we were able to find few spots which were down-regulated by salt treatment using delta 2D software (Fig. 5). These changes were also observed in the molecular range of 20-30 kDa. These protein spots on 2-DE gels are of interest as expansins are cell wall proteins which had a molecular size around 22-30 kDa. These specific proteins have been focused in many proteomic studies dealing with total shoot under salinity (Pitann et al., 2009; Geilfus et al., 2010). In addition, 100 mM sodium phosphate buffer infiltration solution also resulted in best resolution of proteins in high molecular weight proteins separation. We believe that the detected down-regulated protein spots have some role in physiological process under salinity stress. Further advancement on this part of research is still in progress to fully identify and examine the physiological role of these subcellular proteins in the initial phase of salinity stress.

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Figure legends:

Figure 1. The effect of the salt outside the roots, viz., the osmotic stress phase, on the plant growth. Right plants: The presence of 100 mM NaCl at the roots over a period of 14 days decreases the growth of maize (*Zea mays* L.). Left plants: Control-treated maize (1 mM NaCl).

Figure 2. Influence of 100 mM NaCl on the whole shoot biomass (A) and expanding leaves fresh weight (B) of *Zea mays*. (Control white vs salt stress black bars). *,**,*** Significant differences between salinity treatments were shown at $P < 0.05$, 0.01, 0.001, respectively (n = 10).

Figure 3. Influence of 100 mM NaCl on the apoplastic washing fluid of maize leaves. (Control white vs salt stress black bars). *,**,*** Significant differences between salinity treatments were shown at $P < 0.05$, 0.01, 0.001, respectively (n = 10).

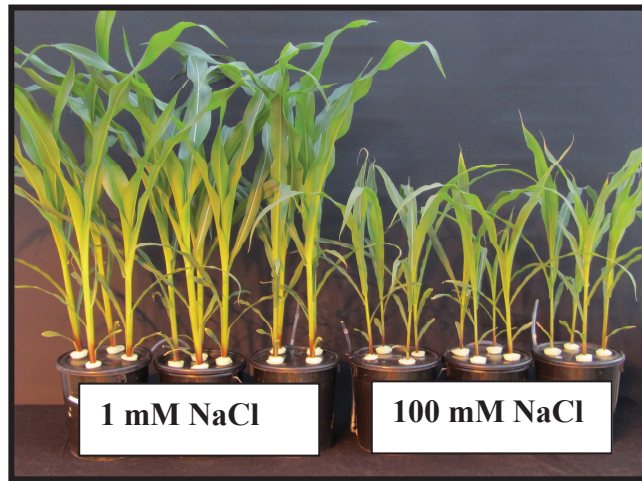
Figure 4.

Representative 2-DE gel images of (A) control and (B) salt-treated, showing protein expression profiles of the maize leaf apoplast after treatment with 100 mM NaCl for 14 d. Highlighted regions indicates good areas of the 2D gels. Gels were stained with Coomassie. Proteins were separated using 7 cm strips using a non-linear gradient pH 3–10 for the first dimension.

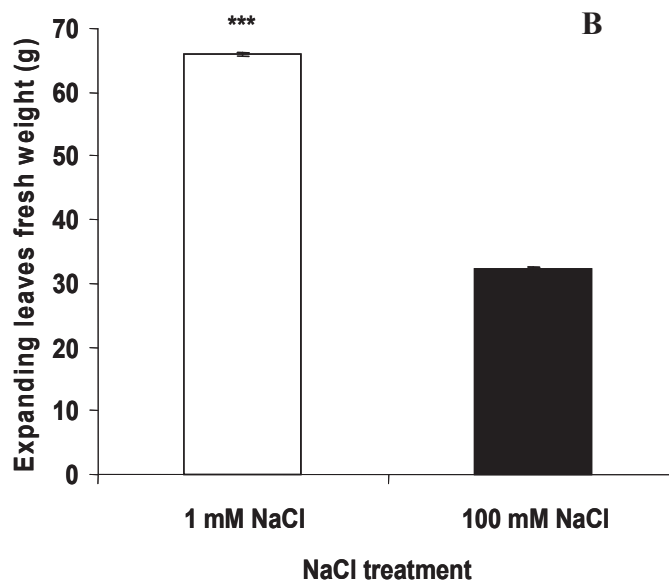
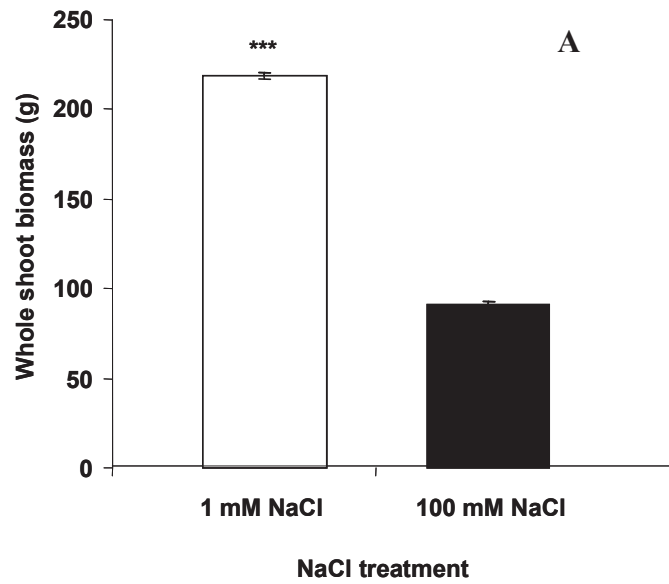
Figure 5.

Zoom in regions of the 2-DE gels of the maize leaf apoplast with detected protein spots whose abundance decreased consistently following NaCl treatments as indicated by arrows of control (A; 30-40 kDa, C; 20-30 kDa) and salt-treatment (B; 30-40 kDa, D; 20-30 kDa).

Figures
Figure 1



Figures 2



Figures 3

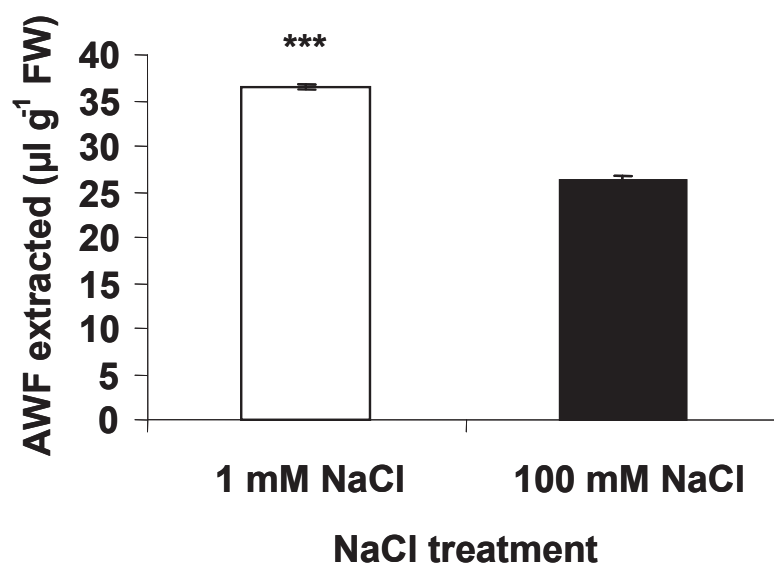


Figure 4

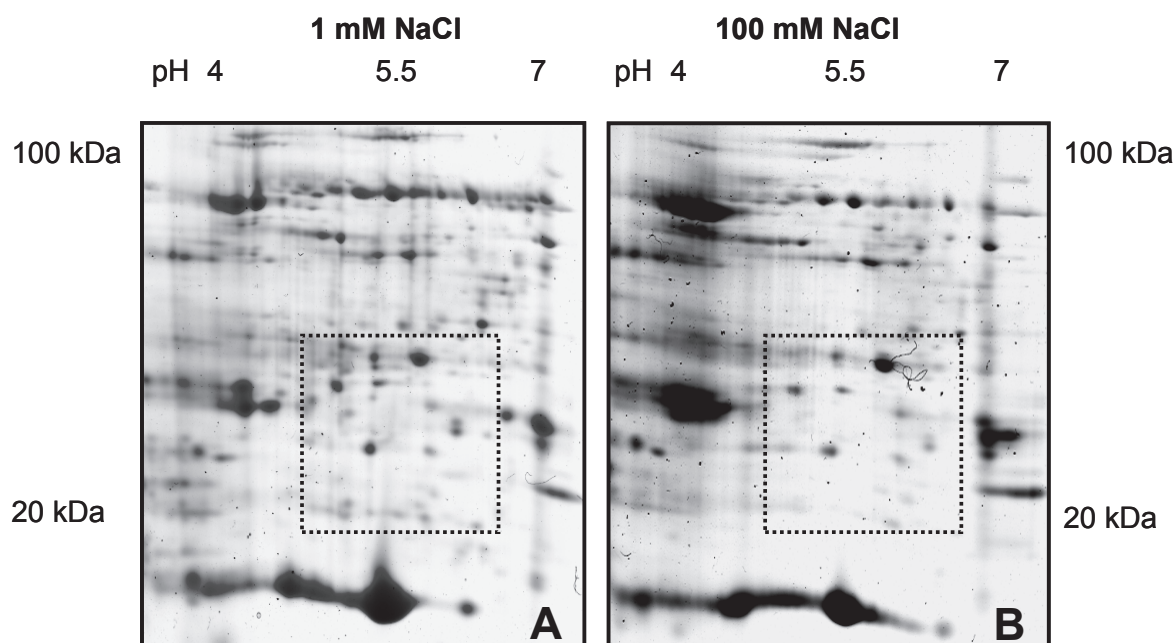
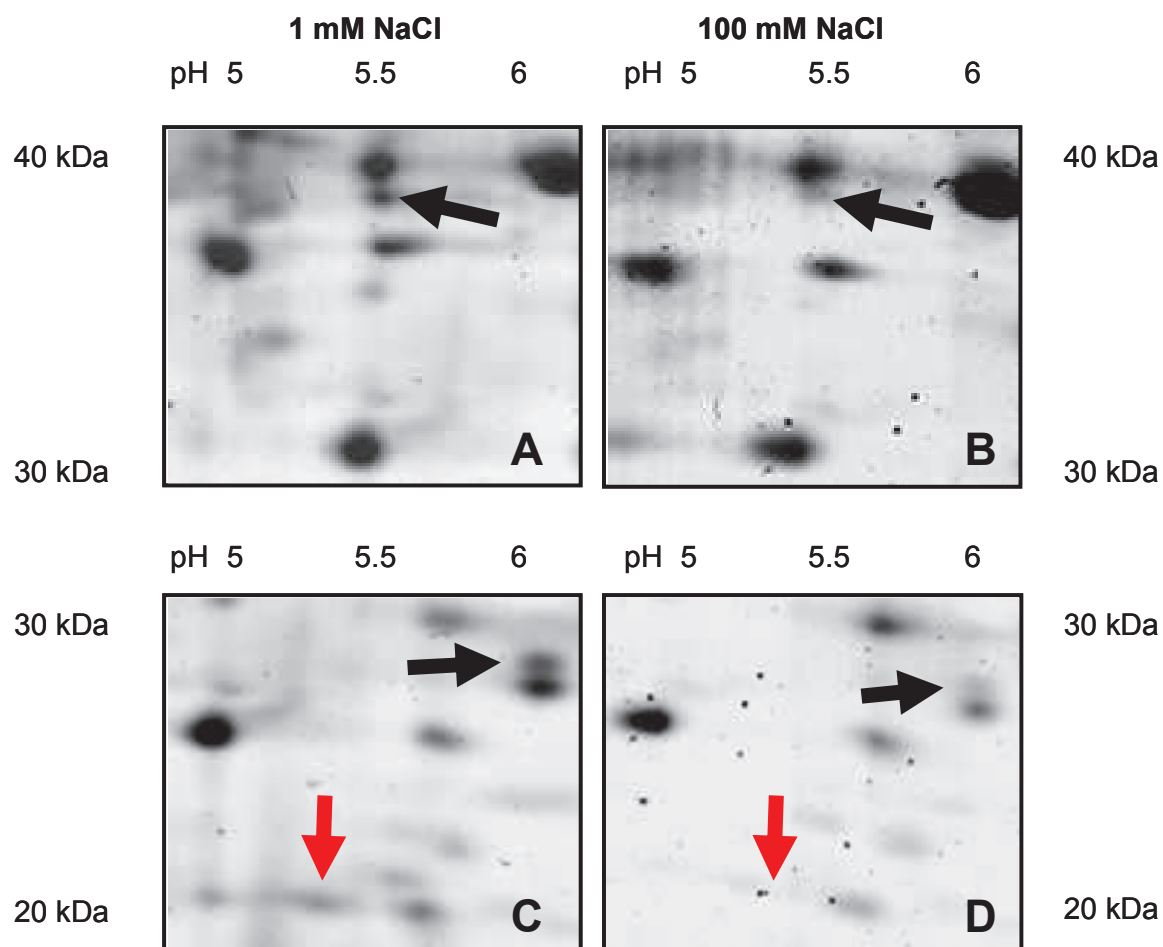


Figure 5



Chapter 6

General discussion

6. General discussion

Endurance and productivity of crop plants exposed to salinity stress is dependent on their capability to develop adaptive mechanisms to avoid or tolerate stress. Accumulating confirmation suggests that the mineral nutritional status of plants greatly affects their ability to adapt to unfavourable saline conditions. In the present thesis the role of the mineral nutritional status of plants in their growth reduction to saline conditions have been discussed, with emphasis on salinity stress. In comparison to earlier studies under salinity stress special consideration has been made to the subcellular compartments of the plant leaves. Of the elements affecting plant adaptation to salt stress conditions, sodium (Na), potassium (K), calcium (Ca), chloride (Cl) and nitrogen (N), are the most extensively studied, therefore, special attention has been paid to them and in addition to the other cations and anions by ion chromatography. However, this study also focussed on identifying the optimal method for extracting apoplast proteins. Furthermore, we also extracted and separated apoplastic proteins on two dimensional electrophoresis gels to facilitate investigations related identification of apoplastic proteins to study their possible role in adaptive processes under salt stress conditions.

To accomplish our first objective we used maize (monocot) and field bean (dicot) two different species for accumulating Na⁺ in their leaf apoplast under high salinity, a possible mechanism for growth reduction (Oertli, 1968). Silicon alleviates growth-related effects of salinity; its role in the leaf apoplast of field bean under salinity is also examined in this study. Moreover, to devise a protocol most suitable for the extraction and later implication of that method to study leaf apoplast proteins was used for maize, a crop of high economic importance.

6.1. Whole plant growth response to salinity stress:

All measurements involve only first phase of salinity stress as no salt injury symptoms were noticed on the leaves. After the exposure of 100 mM NaCl to plant roots, tissues adequately revealed the effect on growth-related processes. Plant growth is rapidly reduced in the osmotic phase of salt stress (Munns and Tester, 2008). In our study with maize, shoot growth was significantly reduced under salinity stress (Chapter 2, Fig 1a). Modernity of the experiment was the significant growth reduction of only expanding shoots compared to expanded shoot (Chapter 2, Fig. 1b). The reduced growth of expanding maize shoot tissue compared to that of expanded shoot tissue favours the fact, that, when salinity is applied to the root medium of maize plants, leaf elongation is inhibited immediately (Cramer, 1992), but recovers to a new steady-state rate which is below that of the control. Earlier, possible mechanism for this shoot reduction by accumulation of high Na⁺ concentration in their leaf apoplast was suggested by Oertli (1968) and Flower et al. (1991). Salinity often reduces shoot growth more than root growth (Läuchli and Epstein, 1990). Similar was true in our findings where 100 mM NaCl significantly reduced shoot fresh weight compared to root fresh weight (Chapter 2, Fig. 1a).

In other study with field bean (dicot) again shoot growth was significantly reduced with increasing salinity levels (Chapter 3, Fig. 1A) as, continuous exposure to elevated root-zone salinity progressively decreases leaf size over time (Munns et al., 1988). Silicon has been observed to alleviate the salinity effects on plants in dicots; kidney bean (Zuccarini, 2008), tomato (Alaghbary et al., 2004), cucumber (Zhu et al., 2004) and monocots; wheat (Ahmad et al., 1992; Saqib, 2008), barley (Linag et al., 2003, 2005), rice (Matoh et al., 1986; Yeo et al., 1999), and maize (Shu and Liu, 2001). However, the mechanism(s) for these effects of silicon is still unclear. In this present investigation silicon was added as a tool to modify the apoplastic Na⁺

concentration. Moreover, addition of 1 mM silicon significantly increased the fresh weight of field bean plants at higher salinity levels (Chapter 3, Fig. 1A).

6.2. Mineral uptake in whole leaf tissue under salt stress

Unnecessary Na^+ is generally held responsible for the reductions in growth and yield under salinity (Tsai et al., 2004; Hong et al., 2009) and therefore the mechanisms by which Na^+ ions enter the cells have been studied intensely (Tester and Davenport, 2003; Apse and Blumwald, 2007). In our study with both maize and field bean, we found significant higher Na^+ accumulation in total shoot of both maize and field bean (Chapter 2,3, Fig. 4b,2A). Munns (1993) was suggesting that older leaves may die as a result of a fast increase of the salt concentration into the apoplast or cytoplasm when vacuoles can no longer sequester incoming salts. Accordingly, we found significantly higher Na^+ accumulation in the expanded shoot of maize plants compared to expanding shoots (Chapter 2, Fig. 4b). Moreover, addition of silicon significantly reduced Na^+ concentration in whole leaf tissue of field bean plants at high salinity levels (Chapter 3, Fig. 2A).

As large numbers of plant enzymes are activated by K^+ , Na^+ is the primary toxic ion in maize (Sümer et al., 2004; Fortmeier and Schubert, 1995) that compete with K^+ for binding sites following inactivation of enzymes and cellular functions (Tester and Davenport, 2003). Also, K^+ is an important factor in protein synthesis, glycolytic enzymes, and photosynthesis; an osmoticum mediating cell expansion and turgor-driven movements; and a competitor of Na^+ under saline conditions (Marschner, 1995; Hu and Schmidhalter, 2005). K^+ concentration was significantly reduced in whole leaf tissue of field bean plants under salinity (Chapter 3, Fig. 2D, F).

Calcium availability could be severely affected under salinity, in particular at low $\text{Ca}^{2+} : \text{Na}^+$ ratios because Na^+ readily displaces Ca^{2+} from the plasma membrane

(Cramer et al., 1988). In addition, 58 % reduction of Ca^{2+} concentration in expanding maize shoot tissue under salinity (Chapter 2, Fig. 4C) has possibly affected leaf expansion because, reasonable amount of Ca^{2+} is required to maintain cell-membrane integrity and functions (Wei et al., 2003). Calcium transport is impaired to the younger leaves during the osmotic phase of salt stress (Fortmeier and Schubert, 1995). Also, Ca^{2+} concentration was more than 50 % significantly reduced in whole leaf tissue of field bean plants at all salinity levels (Chapter 3, Fig. 3A).

Na^+ dominates the cations and Cl^- the anions in the majority of saline soils to the extent that NaCl comprises from 50-80 % of the total soluble salts (Rangasamy, 2010). However, Dang et al. (2008) concluded after analysis of a number of field trails that the Cl^- concentration in the soil was more important to growth and yield reduction than Na^+ . Our results with maize and field bean showed very high Cl^- concentration in whole leaf tissue (Chapter 2,3, Fig. 5b,4A) which may have possible role in the reduction of chlorophyll in leaves after NaCl exposure, which gets amplified by a simultaneously high Na^+ concentration in faba bean (Slabu et al., 2009). Also, due to antagonism, this high Cl^- concentration was adversely affecting the NO_3^- concentration in whole leaf tissue for both maize and field bean plants (Chapter 2,3, Fig. 5a,4D).

6.3. Effect of salinity on subcellular ion relations in maize and field bean leaves

As very few studies have dealt with the determination of cations and anions within the leaf apoplast of monocots, such as maize, under salinity. Therefore, we subjected the roots of salt-sensitive maize plants to a 16-day salt-stress period of 100 mM NaCl and separated the leaf tissues into apoplastic and symplastic fractions prior to analysis. Predominantly high symplastic Na^+ and Cl^- concentrations have been observed (Chapter 2, Fig. 7). However, the observed increase in apoplastic Na^+ and

Cl⁻ concentrations is smaller than the increase in symplastic concentrations (Chapter 2, Fig. 6).

In addition investigation with field bean plants was subjected to increasing levels of salinity 1, 50, 75 and 100 mM NaCl for 10 d and separated the leaf tissues into apoplastic and symplastic fractions prior to analysis. In contrast to previous results from maize (monocot), we found huge increase in apoplastic Na⁺ concentration in the leaf apoplast of field bean (dicots). This favours the idea that during continuous exposure of salt to the plant, the rate of export from the root will exceed the rate of compartmentalization. If this occurs, salt ions will accumulate outside the vacuole, either in the cytoplasm or in the apoplast (Volkmar et al., 1998) and lead to toxicity symptoms.

In outline, the observed 33 mM Na⁺ in the maize leaf apoplast is not high enough to increase the osmotic gradient between the inside and outside of the cell. Our results with maize leaf apoplast do not support Oertli's hypothesis that high extracellular salt accumulation in leaves can lead to cellular dehydration and, eventually, cell death. However, in comparison to maize the 4-fold high 120 mM Na⁺ concentration in the leaf apoplast of field bean plants at maximum salinity may have possible role in growth reduction under salinity. The present higher accumulation of Na⁺ and Cl⁻ in the leaf apoplast of field bean plants at high salt treatment is probably attributable to their higher xylem import of ions. After saline solutes enter the leaf, two mechanisms at the plasma membrane or xylem parenchyma cells can exclude them from the cytoplasm of leaf cells. Salt ions can be concentrated in the apoplast or isolated within the vacuole.

A very high Na⁺ and Cl⁻ concentrations were noticed in the leaf symplast of maize and field bean plants. During a prolonged salt treatment, the moderately loaded maize apoplast favours salt flux into the symplast thereby causing high

concentrations first in the vacuole, then in the symplast. This is based on the active Na^+ transport into the vacuole which favours Na^+ accumulation more than 100-fold in the vacuole compared to the cytosol (Nobel, 1991; Niu et al., 1995).

As, Na^+ emulate with K^+ for binding sites under high salinity, resulting in inactivation of enzymes and cellular functions (Tester and Davenport, 2003). Soluble K^+ concentration remains relatively constant with a lower than 19 mM concentration in the maize leaf apoplast. However, a significant 64 % reduction in soluble K^+ concentration in leaf symplast was found under salinity (Chapter 2, Fig. 7a). In experiment with field bean again more than 50 % significant reduction in K^+ concentration was noticed in leaf symplast of field bean plants (Chapter 3, Fig. 2F). Similar to that, the Ca^{2+} concentration was also significantly reduced in the leaf symplast of field bean plants under salinity (Chapter 3, Fig. 3C). As, reasonable amount of Ca^{2+} are required to maintain cell membrane integrity and function, these concentration differences may potentially affect the growth process since Ca^{2+} availability can change with salinity responses (Genc et al., 2010).

Although a few studies in the past have shown the importance of partitioning of Cl^- in the leaf sheath (Boursier et al., 1987; Boursier and Läuchli, 1989) and whole plant level (Tavakkoli et al., 2010) to our knowledge this is the first report on a significant high accumulation of Cl^- at subcellular level of the monocot and dicot plant leaves under salinity. We found significant higher Cl^- concentration in the leaf apoplast and symplast of field bean plants (Chapter 3, Fig. 4B,C). However similar was true in the leaf symplast of maize plants (Chapter 2, Fig. 7b). The Na^+ and Cl^- build-up in the symplastic and apoplastic space suggests that the salt sensitivity of plants is attributable to their inability to control the total salt flux into the leaf apoplast and, from there, into the cells.

6.4. Comparative evaluation of different extraction solutions for the leaf proteome analysis in maize plants

The plant apoplast is a dynamic compartment with a broad range of physiological functions. To study proteins involved in nutrition, growth, signaling or transport processes, it is crucial to apply extraction methods selective for apoplastic proteins. This part of study deals with six different solutions which were tested for their ability to extract proteins from the maize leaf apoplast: water (Wimmer et al., 2003), 20 mM ascorbic acid/20 mM CaCl₂ (Tasgin et al., 2006), 100 mM sorbitol (Mühling and Läubli, 2003), 25 mM Tris-HCl (Alves et al., 2006), 100 mM sodium phosphate buffer (Anand et al., 2004) and 50 mM NaCl (Paetzold, 2006) (Chapter 4, Fig. 1). Proteins from the leaf apoplast and symplast extracted with the six infiltration solutions were compared on 1-DE (Chapter 4, Fig. 2A, Supplementary Figure S1). A sharp band pattern was obtained from all apoplast extracts with a high number of protein bands in each extract. Although, the yield of protein extraction was similar, the protein profiles showed distinct differences. A prominent band of about 20 kDa was present in extracts of 100 mM sodium phosphate buffer, 25 mM Tris-HCl, 20 mM ascorbic acid/20 mM CaCl₂ and 50 mM NaCl, but not in water or 100 mM sorbitol. One protein band of high molecular weight (approximately 100-130 kDa) was apparent in extracts of water, 100 mM sodium phosphate buffer and 100 mM sorbitol, but not in 25 mM Tris-HCl, 20 mM ascorbic acid/20 mM CaCl₂ or 50 mM NaCl. The experienced selective protein patterns generated by the individual infiltration solutions accentuate the necessity of careful selection of isolation method (Robertson et al., 1997).

After 1-D analysis, equal amounts of apoplast proteins were separated by 2-DE to assess the protein patterns in more detail (Chapter 4, Fig. 2B). The spot patterns resembled the band patterns to a certain extent, e.g. as observed for the 20 kDa band

that was prominent also on 2-D gels of the respective apoplastic extracts. The best resolution of proteins with high molecular weight was achieved on the extracts of 100 mM sodium phosphate buffer infiltration solution. As, we found areas of good and poor resolved proteins spots on all 2-D gels overall, 2-DE did not result in a comprehensible evaluation of the employed infiltration solutions.

To obtain an overview of all proteins present in the six different extracts, we aimed at establishing qualitative protein profiles by LC-MS analysis. A total of 328 proteins were identified from all extracts. To assess the amount of symplast contaminations in our samples, we used topology prediction tools. The identified proteins were classified for their subcellular localization as deduced by ExPasy tools Target P and WoLF PSORT (Chapter 4, Fig. 4). Of all tested infiltration solutions, 100 mM sodium phosphate buffer contained the lowest number of proteins assigned to intracellular compartments (Chloroplast: 10, cytosol: 16) and the highest number of proteins targeted to the extracellular apoplast with 34 identified proteins. A number of 12 proteins with unknown function were exclusively identified using the 100 mM sodium phosphate buffer infiltration solution (Chapter 4, see Supplementary Table S1). As this protein fraction performed best regarding less contaminations from other cellular compartments and contained most of the apoplastic proteins, we assume that these yet unknown proteins are involved in physiological processes of the apoplast. These findings are now employed in comparative proteomic studies (Chapter 5) aiming at identifying proteins involved in abiotic stress responses.

6.5. Subcellular protein patterns in maize leaves under salinity stress

The apoplast comprises the entire volume of the tissue external to the plasma membrane, is also an important compartment for the transport and delivery of ions, assimilates and other metabolites (Grignon and Sentenac, 1991; Dietz, 1997;

Sattelmacher et al., 1998). It is thought to be the site of numerous physiological processes in plants, such as development, growth, leaf movement, gas exchange, and pathogen defence. Limited number of studies has dealt with the apoplastic proteins under salinity stress (Dani et al., 2005; Zhang et al., 2009). Based on their potential physiological functions, the salt-stress regulated apoplastic proteins identified in rice (Zhang et al., 2009) are all involved in the plant stress response. These results show the advantage of proteomics approach on identifying subcellular proteins involved in specific physiological process.

In this section to look for apoplastic protein changes only expanding leaves were used for the collection of apoplastic washing fluid which had entirely developed under salinity. Fresh weigh of expanding shoots was significantly affected by salt treatment (Chapter 5, Fig. 2B). Expanding leaves were used as expansins are believed to regulate cell wall enlargement in growing cells and are the primary mediators of acid-induced growth (Cosgrove, 2005; McQueen, 1995).

After the selection of best performing extraction solution from the later study we applied this method for the extraction of subcellular proteins in maize leaves under salinity stress. After some improvements in protein extraction method the 2-DE gels were processed in order to evaluate the protein changes in the maize leaf apoplast under high saline conditions. Although, we were having streaking problems in the acidic part of the gel regions, yet we were able to examine some down-regulated protein spots (Chapter 5, Fig. 5) using delta 2D software. We believe that these protein spots have some physiological roles in maize plants under salinity stress. However, identification will be investigated in further studies.

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7. Conclusion

Salinity stress negatively impacts agricultural yield throughout the world by effecting crop production. The primary reaction of plants to salinity is the reduction in leaf growth due to the osmotic effects caused by the salt outside the roots. However, physiological mechanisms behind this growth reduction are still not fully understood. Therefore, metabolic and ion concentrations in the apoplastic space of leaves are of interest in several fields of plant biology i.e response to stress, leaf growth and enzyme activities. Proteins present in the plant apoplast also reflect its broad functional diversity. Studies on the dynamic change of apoplastic protein composition can reveal new insights into plant responses e.g abiotic stress.

A limited number of studies have dealt with the subcellular determination of cations/anions and proteins within the leaves of plants, under salinity. Therefore, we subjected the roots of salt sensitive maize (monocot) with 100 mM NaCl for 16 d and separated the leaf tissues into apoplastic and symplastic fractions prior to analysis. Similar apoplastic fluid investigation in terms of cations/anions was done in the salt sensitive field bean (dicot) along with silicon application.

The aims of the current study were to test the hypotheses that I) growth reduction is related to subcellular ion distribution within the leaves of maize II) apoplastic Na⁺ concentration is increased only in salt-sensitive dicot (field bean) and is significantly reduced by silicon application under salinity stress III) which solution performed best for the extraction of apoplastic proteins? IV) changes in apoplastic proteins in maize leaves occur to account for possible roles in the growth reduction during the initial phase of salinity stress.

The results obtained in this study favored following conclusions

- I. The absolute increase in the apoplastic Na^+ concentration during salt treatment was much lower compared with the increase in the symplastic Na^+ concentration, salt treatment in maize appears not to result in osmotic stress imposed by a high apoplastic Na^+ concentration as has been suggested for other plant species (Oertli hypothesis).
- II. In contrast to maize leaves high Na^+ concentration found only in the leaf apoplast of the salt-sensitive dicot field bean support the Oertli's hypothesis that extra-cellular salt accumulation can lead to wilted leaves, plant growth reduction, and later cell death. Hereby, silicon can ameliorate adverse salinity effects by lessening Na^+ accumulation in the leaf apoplast under high saline conditions.
- III. 0.1 M Na-phosphate buffer is considered best for apoplastic proteins extraction as it resulted in lowest number of intracellular protein contaminants with the highest number of extracted proteins present in the apoplastic fluid. Also, the number of secreted proteins exclusively found in a single fraction was highest for that buffer.
- IV. Leaf apoplastic proteins extracted by 0.1 M Na-phosphate buffer that were found to be down-regulated may have possible role in growth reduction under salinity stress.

8. Zusammenfassung

Salzstress vermindert weltweit die Produktion pflanzlicher Erzeugnisse. Hohe Salzkonzentrationen außerhalb der Wurzeln verursachen einen osmotischen Effekt, der das Blattwachstum reduziert. Allerdings sind die physiologischen Grundlagen dieser Wachstumsreduktion noch nicht gänzlich verstanden. Aus diesem Grund sind metabolische und ionische Konzentrationen im Blattapoplasten für verschiedene Felder der Pflanzenbiologie, wie z.B. Reaktionen auf Stress, Blattwachstum und Aktivität von Enzymen, von Interesse. Des Weiteren reflektieren die Proteine im Blattapoplast die große funktionelle Vielfalt des Apoplasten. Studien über dynamische Veränderungen der apoplastischen Proteinzusammensetzung können neue Einblicke in die pflanzliche Reaktionen auf z.B. abiotischen Stress bringen.

Nur wenige Studien haben sich mit der subzellulären Bestimmung von Kationen/Anionen und Proteinen in den Blättern von NaCl-behandelten Pflanzen beschäftigt. Aus diesem Grunde wurden Wurzeln einer salzsensitiven Maispflanze (monokotyl) mit 100 mM NaCl über einen Zeitraum von 16 Tagen behandelt. Vor der Analyse wurde das Blattgewebe in apoplastische und symplastische Fraktionen aufgeteilt. Ähnliche Untersuchungen der Kationen und Anionen in der apoplastischen Waschflüssigkeit wurden in salzsensitiven Ackerbohnen (dikotyl) nach einer Siliziumapplikation durchgeführt.

Ziel dieser Studie war es, die Hypothesen zu überprüfen, ob I) die Wachstumsreduktion in einem Zusammenhang mit der subzellulären Ionenverteilung in Maisblättern steht, ob II) die apoplastische Na^+ -Konzentration unter Salinität nur in salzsensitiven Dikotylen (Ackerbohnen) erhöht ist und durch eine Siliziumapplikation signifikant reduziert ist, III) herauszufinden, welche Lösung für die Extraktion von apoplastischen Proteinen am besten geeignet ist, IV) Änderungen der apoplastischen Proteine in den Maisblättern stehen möglicherweise in einem

Zusammenhang mit der Wachstumsreduktion während der initialen Phase der Salinität.

Die Ergebnisse dieser Studie erlauben folgenden Schlussfolgerungen:

- I. Die absolute Zunahme der apoplastische Na^+ -Konzentration während der Salzbehandlung war verglichen mit dem Anstieg der symplastischen Na^+ -Konzentration geringer. Eine Salzbehandlung und die damit in Zusammenhang stehenden hohen apoplastischen Na^+ -Konzentration scheinen bei Mais keine osmotischen Stresssymptome auszulösen, was allerdings für andere Pflanzenarten angenommen wird (Oertli-Hypothese).
- II. Anders als bei Maisblättern unterstützen hohe Na^+ -Konzentrationen im Blattapoplast von salzsensitiven dikotylen Ackerbohnen die Oertli Hypothese, die besagt, dass eine extrazelluläre Salzanreicherung zu welkenden Blättern, Wachstumsreduktionen und zum Zelltod führen kann. Hierbei kann Silizium nachteilige Effekt der Salzbehandlung verbessern, indem Silizium die Na^+ -Anreicherung im Blattapoplasten unter starker Salinität vermindert.
- III. Es ist anzunehmen, dass ein 0.1 M Na-Phosphatpuffer am besten für die Extraktion apoplastischer Proteine geeignet ist, da hiermit die geringsten intrazelluläre Proteinmenge (Kontamination) bei gleichzeitig höchster Proteinmenge aus der apoplastischen Waschflüssigkeit extrahiert werden konnte.
- IV. Blattapoplastischen Proteinen, die mit einem 0.1 M Na-Phosphatpuffer extrahiert wurden und sich als runterreguliert erwiesen, kommt möglicherweise eine Rolle im Zusammenhang mit der Wachstumsreduktion unter Salzstress zu.

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Curriculum vitae

Name: Muhammad Shahzad

Date of birth: 18. June 1983

Place of birth: Muzaffargarh

Nationality: Pakistani

Academic record:

Qualification	Board/University	Year
Matriculation (Science)	Punjab Public School, Muzaffar Garh, Distt. D. G. Khan, Pakistan	1999
F.Sc. (Pre-Medical)	Govt. Higher Sec. School, Rohillanwali (M. Garh), Pakistan	2001
B.Sc. (Hons.) Agriculture (Soil Science)	University of Agriculture, Faisalabad, Pakistan	2005
M.Sc. (Hons.) Agriculture (Soil Science-Salinity)	University of Agriculture, Faisalabad, Pakistan	2007
PhD (Dr. Agrar) Plant Nutrition	Christian Albrechts University of Kiel, Germany	2012

Doctoral thesis: Christian Albrechts University of Kiel (04/2008 to 02/2012)
Institute of Plant nutrition and Soil science, Prof. Dr. K. H. Mühling
Title: Growth- related changes in subcellular ion and protein patterns
in maize and field bean leaves under salt stress

Disputation: Christian Albrechts University of Kiel (02/2012)

Kiel, February 2012