

# **Physiological, phytochemical and molecular characterization of pigmented cereals**



## **Dissertation**

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## Content

<b>Content</b> .....	<b>I</b>
<b>Abstract</b> .....	<b>V</b>
<b>Zusammenfassung</b> .....	<b>VII</b>
<b>Abbreviations</b> .....	<b>IX</b>
<b>1. Introduction</b> .....	<b>1</b>
1.1. Pigments in wheat and barley varieties .....	2
1.1.1. Anthocyanins .....	3
Wheat .....	3
Barley .....	4
1.1.2. Oxidized proanthocyanidins .....	6
Wheat .....	7
Barley .....	7
1.2. The role of seed pigments .....	8
1.2.1. Physiological function .....	9
1.2.2. Agronomic importance .....	9
1.3. The genetics underlying seed pigments in cereals .....	10
1.4. Scientific aims of the present research .....	13
<b>2. Materials &amp; Methods</b> .....	<b>14</b>
2.1. Crops .....	14
2.1.1. Wheat .....	14
2.1.2. Barley .....	15
2.2. Growth conditions .....	17
2.2.1. Greenhouse .....	17
2.2.2. Climate chamber .....	17
2.2.3. Field .....	18
2.3. Measurements of phenotypic traits .....	19
2.3.1. Agronomic parameters .....	19
2.3.2. Seed appearance .....	20
2.4. Analysis of primary and secondary metabolites .....	20
2.4.1. Preparation of anthocyanin and proanthocyanidin extracts .....	20
2.4.2. Amino acids extraction .....	20

## II | CONTENT

---

2.4.3. AccQ•Tag derivatization.....	21
2.4.4. Extraction of primary metabolites .....	21
2.4.5. Derivatization of metabolites for GC-MS analysis.....	21
2.4.6. UPLC-MS and UPLC-MS/MS analyses for anthocyanins and proanthocyanidins.....	22
2.4.7. Determination of total monomeric anthocyanin content by the pH differential method .	22
2.4.8. UPLC-Fluorescence detection for free amino acids .....	23
2.4.9. GC-MS analysis for primary metabolites .....	23
2.4.10. Elemental analysis by ICP-OES .....	24
2.5. Physiological experiments of seeds .....	24
2.5.1. Determination of seed moisture content .....	24
2.5.2. Accelerated ageing treatment (AA) .....	24
2.5.3. Germination test.....	24
2.6. Gene expression analysis .....	25
2.6.1. RNA extraction and cDNA synthesis .....	25
2.6.2. Quantitative RT-PCR.....	25
2.7. Statistical analyses .....	27
2.7.1. T-test and One-way ANOVA .....	27
2.7.2. Chi <sup>2</sup> -test.....	27
2.7.3. PCA.....	27
<b>3. Results .....</b>	<b>28</b>
<b>Part A. Anthocyanin accumulation in wheat grains and its potential effects on growth, grain yield, and seed germination.....</b>	<b>28</b>
3.1. Generation of bulk materials differing only on anthocyanins.....	29
3.2. Verifying if the seed compositions except for anthocyanins of non-colored and colored offspring were well equilibrated .....	32
3.2.1. Anthocyanin profiles in wheat bulk+ materials .....	32
3.2.1.1. Tentative identification of anthocyanins using LC-MS/MS .....	34
3.2.1.2. Quantification of anthocyanin concentrations by LC-MS and Spectrophotometry..	39
3.2.2. Comparison of other seed constituents .....	42
3.2.2.1. Primary metabolites .....	42
3.2.2.2. Free amino acids .....	45
3.2.2.3. Elements.....	50

3.3. Evaluation of growth parameters, seed yield components and germination of wheat parental lines and offspring lines .....	54
3.3.1. Comparison of growth and grain yield parameters .....	54
3.3.2. Comparison of seed germination .....	56
3.3.2.1. Determination of accelerated ageing period .....	57
3.3.2.2. Standard germination test after experimental ageing.....	58
3.4. Summary of Part A .....	60
<b>Part B. Understanding the metabolic and molecular mechanisms controlling barley grain pigmentation at different developmental stages.....</b>	<b>61</b>
3.5. Screening for total anthocyanins in different-colored barley varieties .....	61
3.6. Characterization of developing seeds from yellow, purple and black-grained barley genotypes .....	63
3.6.1. Differential pigmentation in the developing seed coat of barley .....	63
3.6.2. Changes in fresh weight during seed development.....	64
3.7. Developmental deposition of anthocyanins and proanthocyanidins in barley seeds .....	66
3.7.1. PCA of phenolic compounds for differential seed pigmentation.....	66
3.7.2. Tentative identification of anthocyanins and proanthocyanidins .....	68
3.7.3. Quantification of anthocyanins and proanthocyanidins in developing barley grains .....	77
3.8. Differential expression of anthocyanin and proanthocyanidin genes during seed development	84
3.8.1. Common genes – HvF3H and HvDFR .....	84
3.8.2. Anthocyanin synthetic genes – HvANS and HvUFGT .....	85
3.8.3. proanthocyanidin synthetic genes – HvLAR and HvANR.....	86
3.9. The detection of substrate candidates producing presumptive melanin in barley grains.....	87
3.10. Summary of Part B.....	90
<b>4. Discussion.....</b>	<b>91</b>
<b>Part A. Anthocyanin accumulation in wheat grains and its potential effects on growth, grain yield, and seed germination.....</b>	<b>92</b>
4.1. Chemical constituents except for anthocyanins of wheat grains were well balanced between wheat bulks- and bulks+ .....	92
4.1.1. The strategy used for the equilibration of seed compositions.....	92
4.1.2. Phytochemical characterization of wheat bulk- and bulk+ materials .....	93
4.1.2.1. Pigments observed in wheat bulk+ were related to the presence of anthocyanins ...	94

## IV | CONTENT

---

4.1.2.2. Most of the other seed components were evenly balanced between wheat bulk- and bulk+ .....	95
4.2. The influence of anthocyanins accumulating in wheat grains was insignificant on the parameters measured.....	97
<b>Part B. Understanding the metabolic and molecular mechanisms controlling barley grain pigmentation at different developmental stages.....</b>	<b>97</b>
4.3. Phenotypic and developmental variations of grain color in barley ( <i>Hordeum vulgare</i> L.) ....	99
4.4. Biochemical mechanisms underlying the differences in kernel color of barley .....	101
4.4.1. Anthocyanins were not the sole factor contributing to grain coloration in pigmented barley .....	102
4.4.2. Accumulation of catechin and proanthocyanidins were not related to the black pigmentation in HOR 4802 .....	103
4.5. The expression of genes involved in flavonoid biosynthesis in relation to anthocyanin and proanthocyanidin levels in yellow-, purple- and black-grained barley during seed development.....	104
4.6. Do melanin-like compounds contribute to the black pigmentation in HOR 4802? .....	106
<b>5. Summary.....</b>	<b>107</b>
<b>6. References.....</b>	<b>108</b>
<b>7. Appendix.....</b>	<b>124</b>
<b>8. Acknowledgements.....</b>	<b>128</b>
<b>9. Curriculum Vitae .....</b>	<b>130</b>
<b>10. Affirmation .....</b>	<b>131</b>

## Abstract

Pigmented cereals are distinguished by the cereal grains with blue, purple to black colors in the covering layers. Flavonoids, such as anthocyanins and proanthocyanidins (PAs), are the predominant compounds found mainly in the pericarp of pigmented cereals. The presence of these compounds has been associated with potential health benefits mainly due to their antioxidant properties. Wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.), which are two crops of great economical importance worldwide, present significant genotypic diversity in seed pigmentation, rendering them as ideal models to examine flavonoid biosynthesis and metabolism in cereals.

In the first part of this research, potential effect of anthocyanins on seed yield was investigated using wheat mapping populations by crossing two pigmented cultivars ‘purple’, ‘purple feed’(‘PF’) with two non-colored cultivars ‘Saratovskaya 29’ (‘S29’) and ‘Novosibirskaya 67’ (‘N67’), i.e. ‘N67’×‘Purple’, ‘N67’×‘PF’, ‘S29’×‘Purple’, ‘S29’×‘PF’. Wheat materials that differed only in the anthocyanin contents were obtained by pooling equal numbers of yellow (**bulk-**) and purple offspring (**bulk+**) randomly. Targeted and untargeted metabolic analyses demonstrated that the substances of wheat bulk- and bulk+ grains were well balanced except for the anthocyanin contents. The measured yield components did not constantly differ between bulk- and bulk+ in consecutive years, suggesting that anthocyanins accumulation in wheat grains do not influence growth parameters, yield parameters, and seed germination.

Besides, the biochemical and genetic factors that characterize seed pigmentation were studied at different stages of barley seed development. Screening 23 barley varieties with different seed pigmentation for the total anthocyanin content showed that pigments in some cultivars were not extractable. The initial speculation regarding the non-extractable pigments was oxidized PA polymers. To explore this possibility, two dark-grained barley varieties (**HOR 2199** & **HOR 4802**) with extractable or non-extractable pigments were selected to explore the pigment accumulation during seed development. The yellow-grained cultivar **HOR 3011** was used as a control. According to LC-MS/MS analysis, anthocyanins were only found in the purple-grained barley cultivar HOR 2199. The levels of PAs and the accumulated precursors did not differ among the three barley lines during grain maturity. At early developmental stages, the concentration of catechin first increased

## VI | ABSTRACT

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and then decreased during seed maturation. This decrease was accompanied by a concomitant increase in the concentration of PA dimers and trimers. Transcript levels of genes involved in the flavonoid pathway (*F3H*, *Flavanone 3-hydroxylase*; *DFR*, *dihydroflanonol 4-reductase*) and proanthocyanidin biosynthesis (*LAR*, *leucoanthocyanidin reductase*) confirmed the findings of phytochemical analysis. These results demonstrated that neither catechin nor PAs was responsible for the black coloration of barley grain. Accordingly, it was further proposed that the black pigments might be melanin-like compounds synthesized from L-tyrosine and L-3, 4-dihydroxyphenylalanine (L-DOPA). The significantly positive correlation between the potential melanin precursors and black pigmentation in barley grains provided promising evidence for this new speculation. However, the isolation and identification of melanin-like pigment are still needed.



## Zusammenfassung

Pigmentierte Getreidesorten zeichnen sich durch ihre blaue, violette bis schwarze Farbgebung des Getreidekornes aus. Die Pigmente, welche hauptsächlich im Pericarp akkumulieren, wurden als Flavonoide, wie zum Beispiel Anthocyane und oxidierte Proanthocyanidine (PA), beschrieben. Aufgrund ihrer antioxidativen und vor degenerativen Krankheiten (z.B., Herzkrankheiten, Krebs) schützenden Wirkungen werden diesen Farbstoffen gesundheitsfördernde Effekte zugeschrieben. Es befinden sich zahlreiche pigmentierte Vertreter unter den weltweit am meisten konsumierten Getreidesorten wie Weizen (*Triticum aestivum* L.) und Gerste (*Hordeum vulgare* L.).

Im ersten Teil dieser Arbeit wurde ein möglicher Einfluss der Anthocyane auf den Ertrag unter Verwendung von zwei Weizen-Populationen untersucht. Dafür wurden zunächst zwei pigmentierte Sorten „Purple“ und „Purple Feed“ (PF) mit zwei nicht pigmentierten Kultivaren „Saratowskaja 29“ (S29) und „Nowosibirskaya 67“ ('N67') nach folgendem Schema gekreuzt: 'N67' × 'Purple', 'N67' × 'PF', 'S29' × 'Purple', 'S29' × 'PF'. Um Weizenmaterial zu erhalten, welches sich lediglich durch den Anthocyanengehalt unterschied, wurde eine gleiche Anzahl von gelben (**bulk-**) und lila gefärbten (**bulk+**) Nachkommen zufällig ausgewählt und vereint. Die anschließend durchgeführten gerichteten und nicht gerichteten Analysen der Inhaltsstoffe zeigten, dass sich **bulk-** und **bulk+** nur im Anthocyanengehalt unterschieden. Weiterhin zeigten die Ertragsparameter in aufeinanderfolgenden Jahren keine Unterschiede zwischen **bulk-** und **bulk+**, was darauf hindeutet, dass Anthocyane weder einen Einfluss auf Ertrags- und Wachstumsparameter noch die Keimung haben.

Zudem wurde untersucht, welche biochemischen und genetischen Faktoren die Pigmentierung während der Gerstenentwicklung beeinflussen. Bei der Bestimmung der Anthocyankonzentration von 23 unterschiedlichen Gerstensorten, welche sich durch eine unterschiedliche Kornfarbe auszeichneten, gab es zudem Vertreter, von denen die Pigmente nicht extrahierbar waren. Zuerst wurde spekuliert, dass es sich bei diesen nicht extrahierbaren Pigmenten um oxidierte PA-Polymere handeln könnte. Zur Überprüfung dieser Hypothese wurden zwei Gerstensorten mit dunklen Körnern (**HOR 2199** & **HOR 4802**), welche über extrahierbare bzw. nicht extrahierbare Pigmente verfügten, ausgewählt und die Akkumulation der Farbstoffe während der Samenentwicklung untersucht. Die gelb-körnige Line **HOR 3011** wurde für Vergleichsanalysen verwendet. Mittels

## VIII | ABSTRACT

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LC-MS/MS konnten Anthocyane lediglich in der Linie HOR 2199 mit violett gefärbten Körnern nachgewiesen werden. Die Gehalte der PA und deren Vorstufen wiesen keinen Unterschied zwischen den drei Linien während der Samenreife auf. In den frühen Entwicklungsstadien erhöhte sich zunächst die Konzentration von Catechin und nahm im Anschluss mit steigendem Gehalt von PA-Dimeren und Trimeren ab. Zusätzlich wurde die Expression wichtiger Gene des Flavonoidstoffwechsels (*F3H*, Flavanon 3-Hydroxylase, *DFR*, Dihydroflanonol 4-Reduktase) und der Proanthocyanidin-Biosynthese (*LAR*, Leukoanthocyanidin-Reduktase) auf transkriptioneller Ebene untersucht. Die Ergebnisse zeigten, dass weder Catechin noch PAs für die schwarze Färbung der Gerstenkörner verantwortlich sind. Demnach wurde vermutet, dass es sich bei den schwarzen Pigmenten um Melanin-ähnliche Verbindungen handeln könnte, die aus L-Tyrosin und L-3, 4-Dihydroxyphenylalanin (L-DOPA) synthetisiert werden. Die signifikant positive Korrelation zwischen den möglichen Melaninvorstufen und der schwarzen Pigmentierung in Gerstenkörnern verstärkte diese Vermutungen. Allerdings müssen die Melanin-ähnlichen Verbindungen noch isoliert und identifiziert werden.

## Abbreviations

AA	Accelerated ageing
Al	Aluminum
Ala	L-alanine
ANOVA	One-way analysis of variance
ANS	Anthocyanin synthase
Arg	L-arginine
Asn	L-asparagine
Asp	L-aspartic acid
B	Boron
BCC	Barley Core Collection
BNF	British Nutrition Foundation
C	Catechin
Ca	Calcium
Cd	Cadmium
Cu	Copper
CF	Calculation factor
C4H	Cinnamate 4-hydroxylase
CHI	Chalcone isomerase
CHS	Chalcone synthase
CoA	4-coumaroyl-coenzyme A
cv.	Cultivar
Cys	L-cysteine
4CL	4-coumarate: CoA
DAF	Days after flowering
DF	Days to flowering
DFR	Dihydroflavonol 4-reductase
DNA	Deoxyribonucleic acid
DRE	Dynamic range enhancement
DW	Dry weight
EC	Epicatechin
<i>eEF1a1</i>	Elongation factor-a

## X | ABBREVIATIONS

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EIC	Extracted ion chromatogram
FAO	The food and agriculture organization of the United Nations
F3H	Flavanone 3-hydroxylase
F3'H	Flavonoid 3'-hydroxylase
F3'5'H	Flavonoid 3', 5'-hydroxylase
FLS	Flavonol synthase
FW	Fresh weight
Fe	Iron
<i>GADPH</i>	Glyceraldehyde 3-phosphate dehydrogenase
GH	Greenhouse
Glu	L-glutamic acid
Gln	L-glutamine
GMD	Golm Metabolome Database
GN	Grain number per spike
GS	Gas chromatography
GW	Grain weight per spike
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
HOR	Hordeum
ICP-OES	Inductively Coupled Plasma Atomic Emission Spectrometry
IG	Initial germination
IPK	The Leibniz Institute of Plant Genetics and Crop Plant Research
ISTA	International Seed Testing Association
K	Potassium
LAR	Leucoanthocyanidin reductase
LC	Liquid chromatography
L-DOPA	L-3, 4-dihydroxyphenylalanine
LDOX	Leucoanthocyanidin dioxygenase
MAH	Methoxyamine hydrochloride
Met	L-methionine
MHOR	Mutant Hordeum
MSTFA	N-Methyl-N-(trimethylsilyl) trifluoroacetamide
Mg	Magnesium
Mn	Manganese

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Mo	Molybdenum
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
m/z	Mass-to-charge
N67	Novosibirskaya 67
Na	Sodium
Ni	Nickel
NILs	Near-isogenic lines
P	Phosphorus
PA	Proanthocyanidins
PAL	Phenylalanine ammonia-lyase
PCA	Principal component analysis
PDA	photodiode array detector
PF	Purple feed
Phe	L-phenylalanine
Pp	Purple pericarp
PPO	Polyphenol oxidase
Pro	L-proline
Q-TOF	Quadrupole time-of-flight
QTL	Quantitative trait loci
RTI	Retention time index
RNA	Ribonucleic acid
RT	Retention time
RT-qPCR	Real time - quantitative polymer chain reaction
S	Sulfur
S29	Saratovskaya 29
Se	Selenium
SL	Spike length
SN	Spikelet number per spike
SPL	Spike length
SS-2D	Seed size-two dimensions
SW	Seed width
TAC	Total anthocyanin content

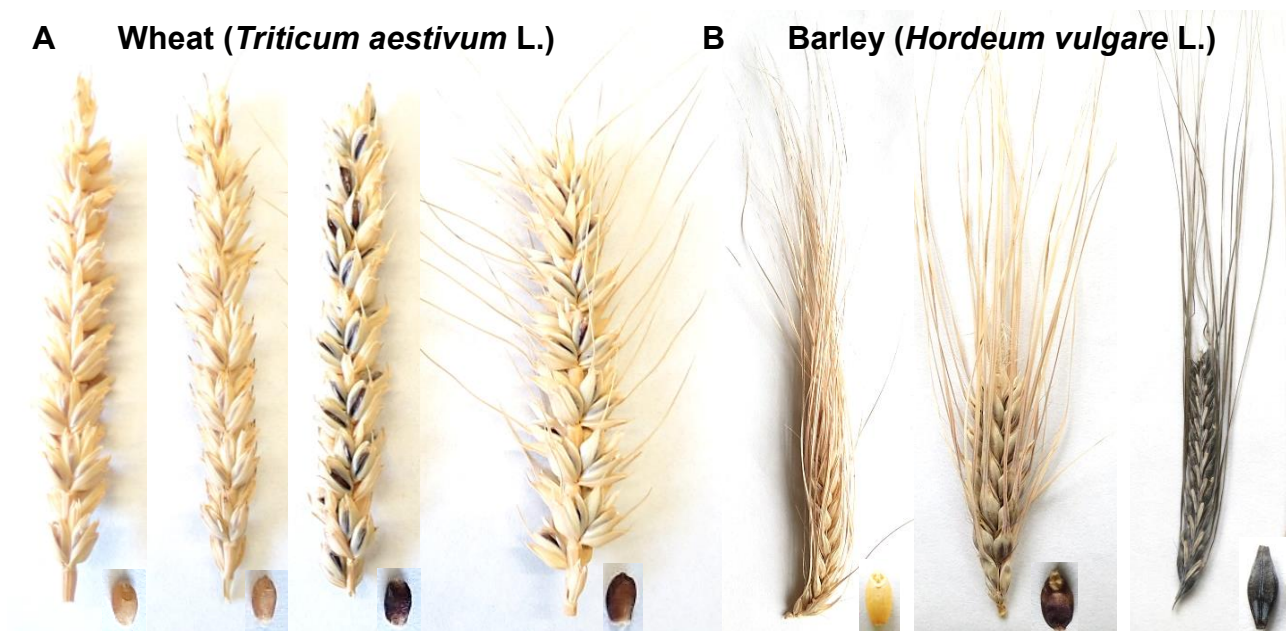
## XII | ABBREVIATIONS

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TGY	Total grain yield
T <sub>max</sub>	Maximum air temperature
T <sub>min</sub>	Minimum air temperature
TPC	Total proanthocyanidin content
TKW	Thousand kernel weight
<i>tt</i>	Transparent Testa
TY	Total yield
TYR	Tyrosinase
UFGT	Uridine diphosphate (UDP)-glucose: flavonoid-O-glycosyltransferase
UPLC	Ultra Performance Liquid Chromatography
vs	Versus
λ <sub>max</sub>	Maximum absorbance

## 1. Introduction

Cereals are the most important sources of food (FAO 2014) and they have been staple crops both directly for human consumption and indirectly via livestock feed since the beginning of civilization (BNF 1994). In addition to high amounts of carbohydrates, protein, and lipids (McKevith, 2004), cereal grains contain many health-promoting components such as vitamins, minerals, and phytochemicals (i.e. carotenoid, flavonoids) which lead to a great diversity in grain coloration (Dykes & Rooney, 2007). In recent times, pigmented cereals with characteristic blue, purple or black colors in seed pericarp have captured continuous interest of scientists from many different disciplines because of their ecological significance, possible alternative to artificial food colorants, and potential sources for functional foods (Escribano-Bailon et al., 2004). Although phytonutrients are present in relatively large amounts in fresh vegetables and fruits, due to the insufficient consumption (Butelli et al., 2008; McGuire, 2013) and short-term storage of vegetables and fruits, pigmented grain-based foods are promising sources for developing dietary products rich in beneficial compounds. Among the most common cereals consumed worldwide, wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) include a wide range of colored varieties as shown in Figure 1.



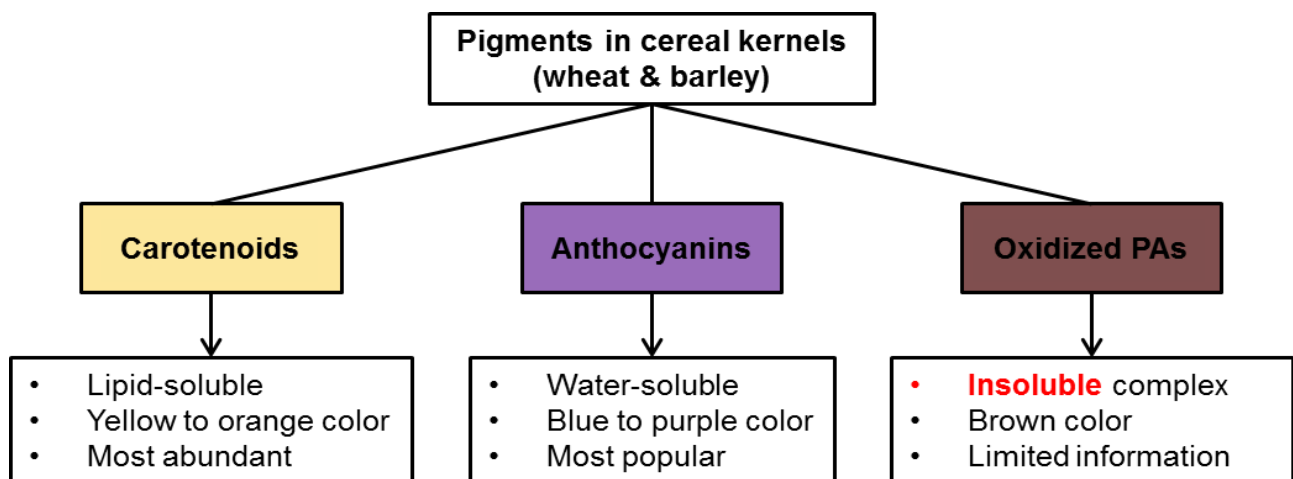
**Figure 1. Spikes and seeds of diverse wheat and barley cultivars.** (A) From left to right are spikes and seeds from yellow-grained wheat cultivars ‘Saratovskaya 29’ (‘S29’) and ‘Novosibirskaya 67’ (‘N67’); purple-grained wheat cultivars ‘purple’, ‘purple feed’ (‘PF’). (B) From left to right are spikes and seeds from yellow-grained barley cultivar HOR 3011; purple-grained barley cultivar HOR 2199; black-grained barley cultivar HOR 4802.

## 2 | INTRODUCTION

The following sections provide an overview of the pigments found in wheat and barley varieties (1.1.) and the genetic studies of pigments-related compounds (1.2.). In section 1.3., the physiological and agronomic roles of seed pigments in plants are introduced, while the remaining questions of current studies as well as the scientific aims of the present research are outlined in 1.4.

### 1.1. Main pigments found in wheat and barley varieties

In general, cereals (wheat and barley) display three major types of pigments and their corresponding characteristics are summarized in Figure 2. The colors of cereal grains vary from bright light-yellow or off-white to purple and black, which are mainly caused by the levels of carotenoids, flavonoids (anthocyanins or/and oxidized proanthocyanidins) in the hull, pericarp, or/and aleurone layer (Dykes & Rooney, 2007; Baik & Ullrich, 2008). The yellow to orange colors in grains are attributed to different levels of carotenoid accumulation in chromoplasts, which are lipid-soluble bioactive components among the abundant families (von Lintig & Sies, 2013; Nisar et al., 2015). Anthocyanins in the aleurone layer of the wheat and barley kernel are responsible for the blue color, whereas purple coloration comes from anthocyanins located in the lemma, palea or pericarp layers (Abdel-Aal et al., 2006). Oxidized proanthocyanidins (PAs) are insoluble complex compounds, associated with the brown to black pigmentation of the seed coat or testa (Pourcel et al., 2007). However, due to the technical limitations in their extraction, little information about oxidized PAs is known. This section is focused on the coloring compounds (anthocyanins, oxidized PAs) in cereals.

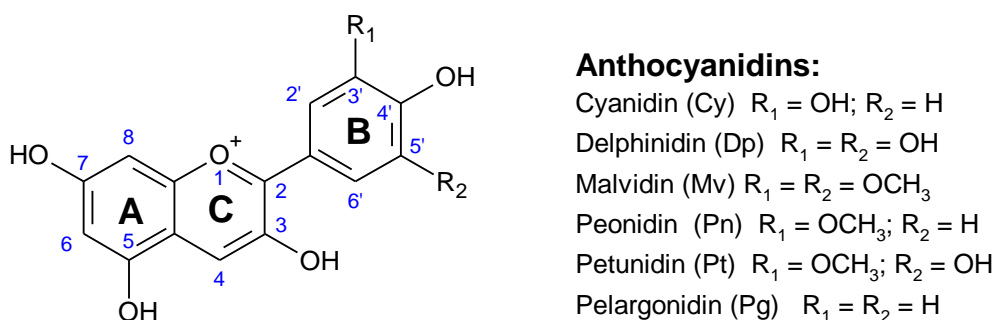


**Figure 2. The coloration of different phytochemical compounds.** Carotenoids are among the abundant families which responsible for yellow to orange color in cereals; Anthocyanins, the most studied pigments, are responsible for red to purple color in cereal grains; Colorless PAs convert into brown after oxidization. Due to the insoluble property, oxidized PAs are the lack of information up to date.



### 1.1.1. Anthocyanins

Anthocyanins are the major water-soluble pigments that generate the characteristic bluish and purple hues in cereals. They constitute the largest subgroup of flavonoids and structurally comprise an anthocyanidin (or aglycone) united to one or more sugar moieties. According to the literature, cyanidin, delphinidin, malvidin, peonidin, petunidin and pelargonidin are the six main anthocyanidins usually found in cereals (Figure 3). At present, more than 700 naturally occurring anthocyanins have been identified in the plant kingdom (Andersen & Jordheim, 2013; Santos-Buelga et al., 2014), yet the constituent anthocyanins of cereals are not well characterized (Escribano-Bailon et al., 2004). As listed in Table 1, more than 40 anthocyanin species from wheat and barley varieties have been reported. The putatively identified anthocyanins in barley are even less than in wheat. Except for cyanidin-3-(3, 6-di-malonyl-glucoside), anthocyanins detected in barley were also found in wheat. However, the anthocyanin profiles vary significantly among different varieties and genotypes and mainly depend on environmental conditions and developmental stages.



**Figure 3. Chemical structures of the most common anthocyanidins in cereals.** Cyanidin glucoside and delphinidin glucoside are the most common anthocyanins found in barley and wheat.

#### *Wheat*

Total anthocyanin contents of blue (103.3-160.0  $\mu\text{g/g}$ ) and purple wheat (81.6-476.9  $\mu\text{g/g}$ ) was significantly higher than in yellow or white wheat grains, which contained only trace amount or no anthocyanins (Escribano-Bailón et al., 2004; Knievel et al., 2009). Apart from that, anthocyanin composition of blue wheat differs from purple wheat lines as well. In blue wheat, Abdel-Aal et al., (2006) reported that delphinidin-3-glucoside was the most abundant pigment followed by delphinidin-3-rutinoside. Besides, the presence of cyanidin-3-glucoside and peonidin-3-glucoside, as well as their acylated derivatives have also been found in blue wheat (Hu et al., 2007). These

## 4 | INTRODUCTION

findings agree with the results by Knievel et al. (2009). For purple pericarp wheat, the main anthocyanins were cyanidin-3-glucoside, cyanidin-3-rutinoside, cyanidin-succinyl-glucoside, and peonidin-3-glucoside (Abdel-Aal et al., 2006; Knievel et al., 2009). With advanced Q-TOF LC-MS/MS, Garg et al. (2016) identified 22, 23, and 26 different anthocyanins from blue, purple and black wheat, respectively.

### *Barley*

As early as 1958, Mullick et al. showed that three common anthocyanidins including delphinidin, cyanidin, pelargonidin were presented in black, blue and purple barley varieties. Later by advances in analytical techniques, cyanidin-3-glucoside, delphinidin-3-glucoside, and petunidin-3-glucoside, are the three most common anthocyanins found in barley cultivars (Abdel-Aal et al., 2006; Kim et al., 2007; Kohyama et al., 2008; Bellido & Beta, 2009; Yao et al., 2010). The most comprehensive study of pigmented barley was carried out by Kim et al. (2007), who found that the highest total anthocyanin content was in the purple and blue lines compared to the black lines. Among 127 colored barley lines, cyanidin-3-glucoside (239.3 µg/g), pelargonidin-3-glucoside (42.2 µg/g), and peonidin-3-glucoside (37.5 µg/g), were the major anthocyanin components in the purple lines, whereas delphinidin-3-glucoside was the most abundant in the blue (167.6 µg/g) and the black lines (42.4 µg/g). Kohyama et al. (2008) was the first to identify cyanidin-3-(3-malonyl-glucoside) from barley grains and determined a decrease in anthocyanins during the late stages of seed maturation (35 to 42 DAF) in purple-grained barley cv. Daishimochi.

**Table 1. Anthocyanins reported in wheat and barley grains.** Cy, Cyanidin; Dp, Delphinidin; Pn, Peonidin; Pt, Petunidin; Pg, Pelargonidin; Mv, Malvidin. Ara, arabinoside; Glc, Glucoside; Gal, Galactoside; Rut, rutinoside; Sam, sambubioside; Caf, caffeoyl; Mal, malonyl; Suc, succinyl; Fer, feruloyl; Xyl, xylosyl; <sup>3,2</sup> indicate three isomers or two isomers. TAC: Total anthocyanin content; DW, dry weight; The anthocyanin content of the unit in nmol/seed was labeled with \*.

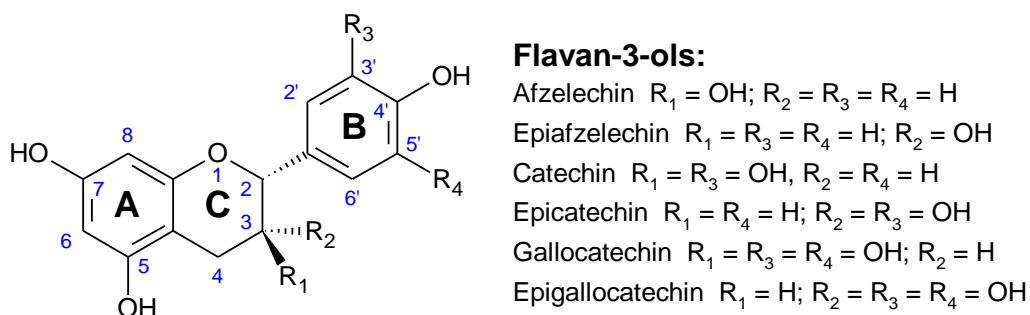
Cereal species	Compounds	[M+H] <sup>+</sup> [m/z]	Fragments [m/z]	Amount [µg/g] DW	References
Wheat	Cy	287	-	5.2	Dinelli et al., 2009; Hosseinian et al., 2008
	Cy-3-ara	419	287	25.1	Hosseinian et al., 2008
	<b>Cy-3-glc</b>	449	287	0.59-103.0	Abdel-Aal et al., 2006, 2008; Dinelli et al., 2009; Hirawan et al., 2011; Hu et al., 2007; Knievel et al., 2009; Ficco et al., 2014; Trojan et al., 2014; Garg et al., 2016
	Cy-3-gal	449	287	72.0	Hosseinian et al., 2008; Hu et al., 2007
	Cy-mal-glc	535	287	1.2	Abdel-Aal et al., 2006; Knievel et al., 2009; Garg et al., 2016

3 Cy-suc-glc	549	287	1.2	Abdel-Aal et al., 2006; Knievel et al., 2009, Garg et al., 2016
Cy-3-rut	595	287	2.3-32.53	Abdel-Aal et al., 2006, 2008; Knievel et al., 2009; Ficco et al., 2014; Garg et al., 2016
Cy-3-di-mal-glc	621	287	-	Garg et al., 2016
Cy-3-(2G-xyl-rut)	727	287	-	Garg et al., 2016
Cy-3-rut-5-glc	757	287	-	Garg et al., 2016
Cy-3-(fer-glc)-glc	787	287	-	Garg et al., 2016
Dp-3-ara	435	303	16.7	Hosseinian et al., 2008
<b>Dp-3-glc</b>	465	303	22.2-140	Abdel-Aal et al., 2006, 2008; Knievel et al., 2009; Ficco et al., 2014; Trojan et al., 2014; Garg et al., 2016
Dp-3-gal	465	303	38.3	Hosseinian et al., 2008
Dp-3-mal-glc	551	303	-	Garg et al., 2016
Dp-3-sam	597	303	-	Garg et al., 2016
Dp-3-rut	611	303	27.8-43.9	Abdel-Aal et al., 2008; Knievel et al., 2009; Ficco et al., 2014; Garg et al., 2016
Dp-caf-glc	627	303	-	Garg et al., 2016
Pn-3-ara	449	301	0.95-28.4	Hosseinian et al., 2008; Ficco et al., 2014
<b>Pn-3-glc</b>	463	301	0.76-2.7	Abdel-Aal et al., 2006; Hu et al., 2007; Hosseinian et al., 2008; Knievel et al., 2009; Hirawan et al., 2011; Ficco et al., 2014; Garg et al., 2016
Pn-3-gal	463	301	0.06-3.87	Ficco et al., 2014
Pn-mal-glc	549	301	0.6	Abdel-Aal et al., 2006; Knievel et al., 2009
2 Pn-suc-glc	563	301	0.6-0.9	Abdel-Aal et al., 2006
Pn-3-rut	609	301	1.2	Abdel-Aal et al., 2006
Pn-3,5-di-glc	625	301	-	Garg et al., 2016
Pn-mal-suc-glc	649	301	0.5	Abdel-Aal et al., 2006
Pn-3-rut-5-glc	771	301	-	Garg et al., 2016
Pt-3-glc	479	317	2.2-40.4	Abdel-Aal et al., 2006; Hosseinian et al., 2008; Garg et al., 2016
Pt-3-rut	625	317	2.2	Abdel-Aal et al., 2006
Pt-3-rut-5-glc	787	317	-	Garg et al., 2016
Pg-3-ara	403	271	9.3	Hosseinian et al., 2008
Pg-3-glc	433	271	28.8	Hu et al., 2007; Hosseinian et al., 2008; Dinelli et al., 2009; Garg et al., 2016
Pg-3-gal	433	271	26.1	Hosseinian et al., 2008
Pg-3-mal-glc	519	271	-	Garg et al., 2016
Mv-3-glc	493	331	0.06-51.6	Hosseinian et al., 2008; Ficco et al., 2014
Mv-rut	639	331	2.0	Abdel-Aal et al., 2006; Garg et al., 2016
Mv-caf-glc	655	331	-	Garg et al., 2016
Mv-3-rut-5-glc	801	331	-	Garg et al., 2016
<b>TAC</b>			1.3-447.6	Abdel-Aal et al., 2006; Liu et al., 2010
<b>Barley</b>				
Cy	287	-	-	Bellido & Beta, 2009
<b>Cy-3-glc</b>	449	287	1.2-99	Abdel-Aal et al., 2006; Kim et al., 2007; Kohyama, et al., 2008; Bellido & Beta, 2009; Yao et al., 2010
Cy-3-(3-mal-glc)	535	287	1.22 *	Kohyama et al., 2008
Cy-3-(6-mal-glc)	535	287	2.07 *	Kohyama et al., 2008
Cy-3-(3,6-di-mal-glc)	621	287	5.43 *	Kohyama et al., 2008
<b>Dp-3-glc</b>	465	303	93-104	Bellido & Beta, 2009; Yao et al., 2010
Dp-3-rut	611	303	-	Bellido & Beta, 2009
Pn-3-glc	463	301	3.3-37.5	Kim et al., 2007

Pt-3-glc	579	317	2.9-37	Abdel-Aal et al., 2006; Bellido & Beta, 2009; Yao et al., 2010
Pg-3-glc	433	271	12.1-42.2	Kim et al., 2007
Mv-3-glc	493	331	0.6-38.6	Kim et al., 2007
TAC			34.6-573	Abdel-Aal et al., 2006; Kim et al., 2007; Bellido & Beta, 2009

### 1.1.2. Oxidized proanthocyanidins

Oxidized proanthocyanidins (PAs) have been associated with the brown pigmentation in seed coat or testa of cereals by the following observations: (1) PAs concentration positively correlated with mature seed coat color in wheat (Mccallum & Walker, 1990); (2) barley products made from PA-containing lines showed darker color than from PAs-free lines (Quinde-Axtell & Baik, 2006). The darkening of cereal grains is correlated with the autoxidation or enzymatic reactions of catechin and proanthocyanidins (PAs), as well as further polymerization of oxidized PAs (Quinde-Axtell & Baik, 2006; Matus-Cadiz et al., 2008). Non-oxidized PAs are the colorless mixture of oligomers and polymers, composing of flavan-3-ols named afzelechin, epiafzelechin, catechin, epicatechin, galocatechin, and epigallocatechin (Figure 4) through the linkages of C4→C8, C4→C6 (both are called B-type) or by an additional ether bond between C2→C7 (A type). During seed desiccation, a high proportion of soluble PAs is oxidized in the presence of polyphenol oxidase (PPO), leading to seed darkening and insoluble complex (Pourcel et al., 2007). Although the quantification of insoluble polymers is difficult, PAs concentration could be considered as an important factor due to its positive correlation with the dark pigmentation of seeds (Quinde-Axtell et al., 2005). Therefore, the existing PAs and the precursors are introduced in this section. So far, more than 50 different PAs have been reported in wheat and barley grains, and the major patterns are listed in Table 2.



**Figure 4. Flavan 3-ol units of PAs.** Proanthocyanidins (PAs) are oligomers and polymers of flavan-3-ol units (e.g., catechin, epicatechin, and epigallocatechin *etc.*).

### Wheat

The types and amounts of extractable PAs in wheat appear to be significantly less than those reported for barley. Only catechin, procyanidin B3, and prodelphinidin B3 were found in wheat. Miyamoto and Everson (1958) observed a close positive correlation between kernel color of mature grain and PA concentrations in the immature grain, suggesting that the main pigment in wheat seed coat was formed by oxidation of catechin and PAs. This hypothesis was later supported by Mccallum and Walker (1990).

### Barley

Similar observations were made by McMurrough (1983) on barley. They reported that methanolic solutions of (+)-catechin, procyanidin B3 and prodelphinidin B3 from barley turned from colorless to brown after 1-4 weeks exposure to air. The dark brown polymeric substances were formed due to oxidation of the oligomeric PAs. There is a heterogeneous diversity of PA polymers in their monomer composition, the extent of polymerization, linkage patterns, and stereochemistry in different species, tissues and developmental stages of a single plant (Tanner et al., 2003). The most abundant compounds are the dimeric PAs: procyanidin B3 and prodelphinidin B3 and the main trimers are T1 (GC-GC-C), T2 (GC-C-C), T3 (C-GC-C), T4 or procyanidin C2 (C-C-C). Total PAs varied substantially from 169 to 3618  $\mu\text{g}\cdot\text{g}^{-1}$  between barley varieties (Klausen et al., 2010). Moreover, the contents of (+)-catechin and PA dimers differed substantially during seed maturation. (+)-catechin accumulated at the early stages and decreased drastically later, while procyanidin B3 and prodelphinidin B3 accumulated slowly in contrary with (+)-catechin and kept stable until seed maturity (Kohyama et al., 2008).

**Table 2. Flavan 3-ols and proanthocyanidins reported in wheat and barley grains.** C, catechin; EC, epicatechin; GC, gallic catechin; glc, glucoside; TPC, total proanthocyanidin contents. There are larger diversities of PA species found in barley than in wheat.

Cereal species	Compounds	[M+H] <sup>+</sup> [m/z]	Fragments [m/z]	Amount [ $\mu\text{g/g}$ ] DW	References
Wheat	(+)-catechin (C)	291	-	-	Mccallum & Walker, 1990
	Procyanidin B3	579	289	-	Mccallum & Walker, 1990
	Prodelphinidin B3	595	305	-	Mccallum & Walker, 1990
	TPC			20-40	Mccallum & Walker, 1990
Barley	Catechin (C)	291	-	4.9-113	McMurrough et al., 1983, 1996; Goupy et al., 1999; Holtekjølén et al., 2006; Quinde-Axtell & Baik, 2006; Kim et al., 2007; Dvorakova et al., 2008; Kohyama et al., 2008;

## 8 | INTRODUCTION

Monomers	Epicatechin (EC)	291	-	-	Hambira, 2009; Klausen et al., 2010 Jende-Strid & Møller, 1981; McMurrough et al., 1983, 1996; Gu et al., 2004;
	Catechin-3-glc	453	289	6-45	Quinde-Axtell & Baik, 2006; Hambira, 2009
	Catechin-7-glc	453	289	-	Friedrich & Galensa, 2002; Klausen et al., 2010
Dimers	Procyanidin B1 (EC-C)	579	289	-	Klausen et al., 2010
	Procyanidin B2 (EC-EC)	579	289	4-19	Jende-Strid & Møller, 1981; Gu et al., 2004; Dvorakova et al., 2008 Mulkay et al., 1981; McMurrough et al., 1983, 1996; Goupy et al., 1999; Holtekjølen et al., 2006;
	<b>Procyanidin B3 (C-C)</b>	579	289	2.4-475	Quinde-Axtell & Baik, 2006; Dvorakova et al., 2008; Kohyama et al., 2008; Hambira, 2009; Klausen et al., 2010
	Procyanidin B4 (C-EC)	579	289	-	Jende-Strid & Møller, 1981
	7 Prodelphinidins	595	303	-	Mulkay et al., 1981 Jende-Strid & Møller, 1981; McMurrough et al., 1983, 1996; Goupy et al., 1999; Holtekjølen et al., 2006; Quinde-Axtell & Baik, 2006; Dvorakova et al., 2008; Kohyama et al., 2008; Hambira, 2009; Klausen et al., 2010
	<b>Prodelphinidin B3 (GC-C)</b>	595	303	2.9-769	Mulkay et al., 1981; McMurrough et al., 1983, 1996
Trimers	Propelargonidin	563	276	-	Jende-Strid & Møller, 1981; Mulkay et al., 1981; McMurrough et al., 1983, 1996; Goupy et al., 1999; Holtekjølen et al., 2006; Quinde-Axtell & Baik, 2006; Dvorakova et al., 2008; Hambira, 2009
	Procyanidin C2 (C-C-C)/T4	867	289	1.7-130	Goupy et al., 1999; Holtekjølen et al., 2006; Quinde-Axtell & Baik, 2006; Dvorakova et al., 2008; Hambira, 2009
	Procyanidin T1 (GC-GC-C)			40-450	Goupy et al., 1999; Holtekjølen et al., 2006; Quinde-Axtell & Baik, 2006
	Procyanidin T2 (GC-C-C)			67-230	
	Procyanidin T3 (C-GC-C)			33-170	
	<b>TPC</b>			12.4-3618	Griffiths & Welch, 1982; McMurrough et al., 1983; Goupy et al., 1999; Holtekjølen et al., 2006; Quinde-Axtell & Baik, 2006

### 1.2. The role of seed pigments

Apart from coloration, a variety of activities has also been ascribed to the pigments in seeds, both with positive and negative attributes to plants and humans. This section presents an overview of investigations into the physiological and agronomic aspects of seed pigments.

### 1.2.1. Physiological function

In common with the other flavonoids, anthocyanins in plants are responsible for antioxidant and UV/photoprotection (Kong, 2003). As reported in earlier studies, anthocyanins and proanthocyanidins seem to play crucial roles in defense against pathogens and predators. Particular anthocyanins with demonstrable antiviral, antibacterial, and fungicidal activities are considered to protect plants from infections by pathogenic microorganisms (Konczak & Zhang, 2004; Wrolstad, 2004). Pelargonidin-3-glucoside in seed coats of *Phaseolus vulgaris* was shown to inhibit pathogenic growth and to induce the growth of symbiotic bacteria (Stanton & Francis, 1966), while proanthocyanidins in sorghum were reported to have resistance to grain mold with increased levels (Menkir et al., 1996). In cowpea, defense functions have also been attributed to proanthocyanidins for the resistance to infestation by insects such as the cowpea weevil (Oigiangbe, 1996).

There is also evidence that both anthocyanins and proanthocyanidins play a role in seed dormancy and germination. Anthocyanins may participate in nonspecific plant defenses such as pre-harvest sprouting (PHS) (Groos et al., 2002). It has been observed that red-kernel wheat appear to be more resistant to PHS than white-kernel wheat. Additionally, the evidence accumulate to support the idea that PAs oxidation has a protection function during plant development and growth (Pourcel et al., 2007). A positive correlation exists between the oxidation of PAs and their cross-linking to the cell wall (Marles et al., 2003). Such modification of testa characteristics might reinforce coat-imposed dormancy by increasing the physical resistance of the tissues, impeding the entrance of germination-stimulating agents such as oxygen or water, or the leakage of inhibiting substances such as abscisic acid or carbon dioxide (Lenoir et al., 1986; Himi & Taketa, 2015). Moreover, the scavenging of oxygen and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) through oxidation might protect seeds from deterioration and therefore prolong the period for which they can be stored (Debeaujon et al., 2000; Bailly, 2004).

### 1.2.2. Agronomic importance

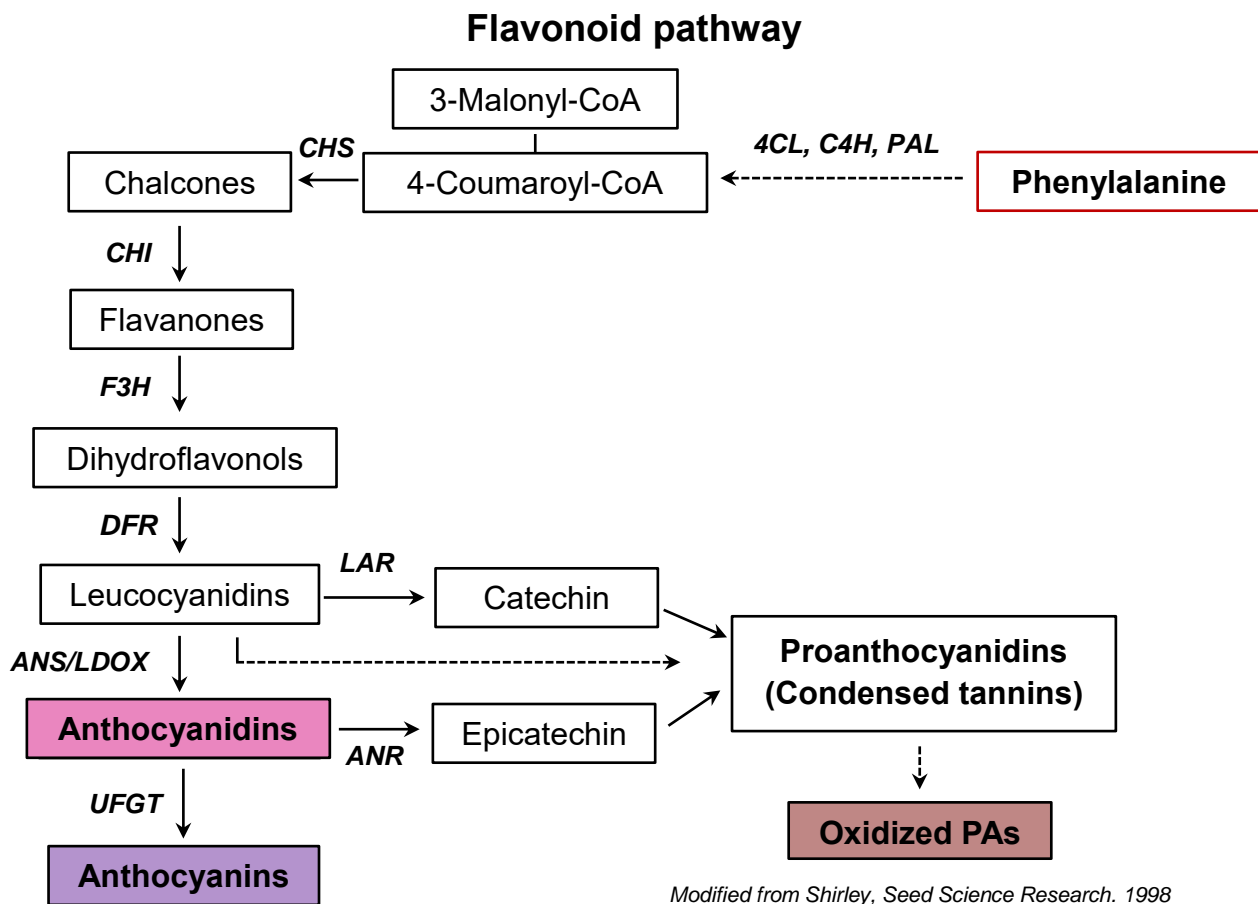
Pigmented cereals have already started to attract high attention of breeders and scientists around 100 years ago, because the grain color was considered as an important indicator for superior varieties (Kornicke, 1885; Aufhammer, 1933; Takahashi et al., 1950). For example, barley cultivars with blue kernel were associated with high malting quality which means better beer products (Kornick & Hansen, 1902). Recently colored cereals gained further interest due to their health-

promoting functions and disease preventing effects originating from phytochemical pigments such as anthocyanins (Escribano-Bailon et al., 2004; Toufektsian et al., 2008). Additionally, barley is known to accumulate proanthocyanidins during grain development. These compounds have undesirable consequences for beer production because they can precipitate with proteins, causing cloudiness in beer at 0 °C (Horsley et al., 1991). Therefore, more than 1000 proanthocyanidin-deficient (*ant*) mutants of barley have been isolated to overcome this problem (von Wettstein et al., 1977; Jende-Strid, 1991).

### 1.3. The genetics underlying pigmentation of cereal grains

Anthocyanins and oxidized PAs are synthesized by the branched flavonoid pathway (Figure 5). The pathway leading to anthocyanin and oxidized PAs biosynthesis is complex and involves several substrates and enzymes, which in turn give rise to high color variability. Flavonoids in plants are synthesized in the cytosol and transported conjugated to glutathione-S-transferase (GST) in particular cell organelles: vacuole or cell walls (Carletti et al., 2014). Anthocyanins and PAs share several common biosynthetic steps of the general flavonoid pathway, which begins with the condensation of one molecule of 4-coumaroyl-coenzyme A (CoA) and three molecules of malonyl-CoA by the enzyme chalcone synthase (*CHS*). The first intermediate chalcone is converted to naringenin with catalysis by chalcone isomerase (*CHI*), and subsequently is hydroxylated by flavanone 3-hydroxylase (*F3H*), flavonoid 3'-hydroxylase (*F3'H*) or flavonoid 3', 5'-hydroxylase (*F3'5'H*) to different dihydroflavonols. Then leucocyanidins are formed by the last mutual enzyme dihydroflanonol 4-reductase (*DFR*). Following this, the pathway diverges into side branches leading to anthocyanidins by leucoanthocyanidin dioxygenase/anthocyanin synthase (*LDOX/ANS*) and catechin by leucoanthocyanidin reductase (*LAR*). Eventually, anthocyanidins are further glycosylated to anthocyanins by uridine diphosphate (UDP)-glucose: flavonoid-O-glycosyltransferase (*UFGT*); on the other hand, catechin is polymerized to colorless PAs which can be further oxidized to brown complex. The anthocyanins and PAs firstly accumulated in the cytosol and then transported to vacuoles or cell walls (Falcone et al., 2012).





**Figure 5. Biosynthetic pathway of flavonoids.** The synthesis of two pigment candidates of cereals starts from the same precursor (phenylalanine). Enzyme abbreviations: *PAL*, phenylalanine ammonia-lyase; *C4H*, cinnamate 4-hydroxylase; *4CL*, 4-coumarate:CoA; *CHS*, chalcone synthase; *CHI*, chalcone isomerase; *F3H*, flavone 3-hydroxylase; *FLS*, flavonol synthase; *DFR*, dihydroflanonol 4-reductase; *LAR*, leucoanthocyanidin reductase; *ANR*, anthocyanidin reductase; *ANS*, anthocyanidin synthase; *LDOX*, leucoanthocyanidin dioxygenase; *UFGT*, uridine diphosphate-glucose:flavonoid 3-O-glucosyltransferase.

Many genes encoding flavonoid biosynthetic enzymes in wheat and barley have been elucidated using different biotechnologies. The genetic loci corresponding to structural or regulatory genes for flavonoid biosynthesis in these two species are summarized in Table 3. In wheat, the expression of four genes (*CHS*, *CHI*, *F3H*, and *DFR*) in white colored seeds reduced in comparison with red ones, suggested that three *R* genes (*R1*, *R2*, *R3*) encoding the seed color and located at the chromosomes 3A, 3B, and 3D (Mcintosh et al., 1998; Himi et al., 2005; Khlestkina et al., 2011). *R* genes are also transcription activators (*Myb* transcription factors) of genes in flavonoid synthesis (Himi & Noda, 2005). Knievel et al. (2009) reported that purple colored wheat pericarp was controlled by two loci with a segregation ratio of 11 purple: 5 white in F<sub>2</sub> populations whereas blue aleurone appeared to be controlled by a single dominant gene in the backcross populations. This conclusion was

supported by available genetic maps of wheat chromosomes 2A and 7B, mapped genes *Pp3* and *Pp1*, and linked molecular markers (Khlestkina et al., 2010; Tereshchenko et al., 2012). Moreover, the comparison of near-isogenic lines (NILs) differing by the allelic state of the *Pp* (purple pericarp) gene and transcriptional activity of the anthocyanin biosynthesis structural genes (*CHS*, *CHI*, *F3H*, *DFR*, and *ANS*) showed that alleles conferring strong pigmentation induced high transcription levels of the structural genes. This result suggested that the gene *Pp* was the transcriptional regulator in the anthocyanin biosynthesis in wheat (Tereshchenko et al., 2013; Shoeva et al., 2014). A recent study by Ma et al. (2016) showed nine phenolic acid biosynthesis pathway genes (*PAL1*, *PAL2*, *C3H1*, *C3H2*, *C4H*, *4CL1*, *4CL2*, *COMT1*, and *COMT2*) exhibiting three distinct expression patterns in developing grains of white, purple, and red wheat, which might be closely related to phenolic acids accumulation.

In barley, Jende-Strid (1993) reported that more than 700 mutations in genes with affected anthocyanin and/or proanthocyanidin biosynthesis had been isolated since 1977. Besides, most of the mutants localized to 28 *Ant* genes, in which ten genes resulted in a blocked PAs formation in the testa layer of the grains, whereas 18 *Ant* gene loci only affected anthocyanin accumulation in different organs. With the comparison of mutants in various *Ant* genes and wildtypes, the author also analyzed the contents of flavonoids and other phenolic compounds and the activity levels of several of the enzymes in the flavonoid pathway. The results indicated that *Ant17*, *Ant18*, *Ant19*, *Ant22*, and *Ant26* were structural genes, while *Ant13* was a regulatory gene coding for a transcription factor of structural genes in the flavonoid pathway. Besides, the genetic model of *Hordeum vulgare* 'Bowman' NILs was used to investigate the regulation of the flavonoid biosynthesis in white, purple, and black barley grains (Shoeva et al., 2016). In their research, the isolated dominant *Ant2* allele of the purple-grained line showed a high level of sequence similarity with the recessive Bowman's *ant2* in the coding region. However, an insertion of 179 bp in the promoter region of *ant2* underlaid different expression in grain pericarp between *Ant2* and *ant2* alleles. *Ant2* was up-regulated in the purple-grained line with coordinately co-expressed flavonoid biosynthesis structural genes (*CHS*, *CHI*, *F3H*, *F3'H*, *DFR*, and *ANS*), whereas it was not transcribed in Bowman.

**Table 3. Genetic loci for flavonoid structural and regulatory genes in wheat and barley.**

Enzyme	Wheat	Barley
Chalcone synthase ( <i>CHS</i> )	<i>Myb</i>	<i>Chs; Ant 13</i>
Chalcone isomerase ( <i>CHI</i> )	<i>Chi-B1, Chi-D1</i>	
Flavanone 3-hydroxylase ( <i>F3H</i> )	<i>F3h-A1, F3h-B1, F3h-B2, F3h-D1</i>	<i>Ant17; Ant 13</i>
Flavonoid 3'-hydroxylase ( <i>F3'H</i> )		
Dihydroflanonol 4-reductase ( <i>DFR</i> )		<i>Ant18; Ant 13</i>
Leucoanthocyanidin dioxygenase/Anthocyanin synthase ( <i>LDOX/ANS</i> )	<i>Ans-A1, Ans-A2, Ans-B1, Ans-B2, Ans-D1</i>	
Leucoanthocyanidin reductase ( <i>LAR</i> )		<i>Ant19</i>
Uridine diphosphate (UDP)-glucose: flavonoid-O-glycosyltransferase ( <i>UGT</i> )		

#### 1.4. Scientific aims of the present research

Anthocyanins as the most abundant coloring compounds are increasingly attractive for consumers and breeders due to the potential health benefits. Thus, it was important to investigate the possible effects of anthocyanins accumulation on seed germination and other yield traits (**part A**). Moreover, cereal kernel color shows a great diversity of kernel colors ranging from white yellowish to blue, purple and black underlying different pigmentation. Despite the flavonoid biosynthetic mutants identified in barley, it is still largely unclear how various classes of pigments are synthesized. Therefore, to classify and characterize distinct pigmentation cereal genotypes based on grain color would provide useful information for the mechanisms underlying the differential pigmentation in cereal grains (**part B**). The primary objectives of each part were summarized below:

- **Part A.** Initially, the seed compositions except for anthocyanins of wheat materials were needed to be equilibrated to avoid the interference from the other substances. The major compounds in wheat grains were compared to verify whether the seed compositions were well balanced or not. Further, the comparisons of crop yield parameters and seed germination were performed to explore the possible effects of anthocyanins accumulation in wheat grains.
- **Part B.** Total anthocyanin concentrations of different barley varieties with various grain colors were primarily screened for the classification of seed pigmentation. Then the temporal variation tendency of pigment-related phenolic compounds was determined in developing seeds and the expression patterns of corresponding genes were compared among selected barley genotypes to investigate the biochemical and genetic factors that cause differential seed pigmentation during developmental stages in barley.

## 2. Materials & Methods

### 2.1. Crops

#### 2.1.1. Wheat

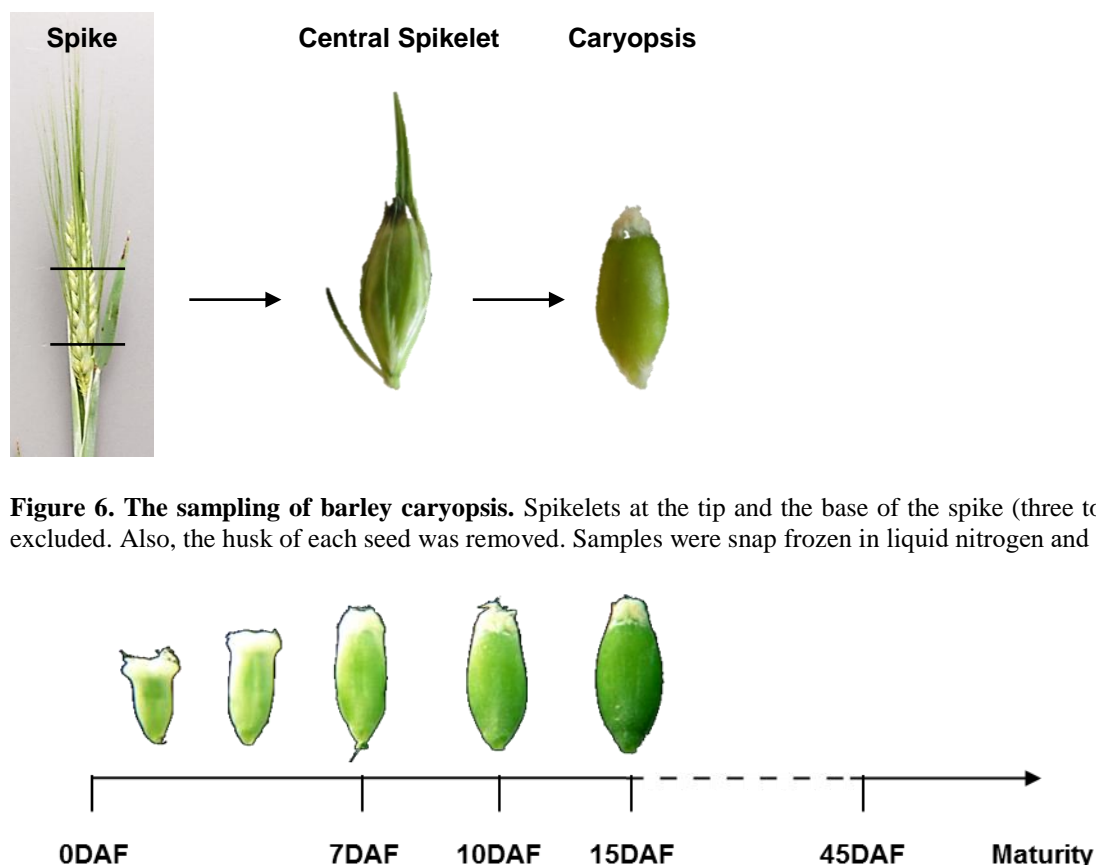
Wheat materials were provided by PD. Dr. Andreas Börner (IPK Gatersleben, Resources Genetics and Reproduction Group). As described in Table 4, F<sub>3</sub> families were generated from F<sub>2</sub> mapping populations by crossing between two pigmented cultivars ‘purple’, ‘purple feed’ (‘PF’) and two non-colored cultivars ‘Saratovskaya 29’ (‘S29’) and ‘Novosibirskaya 67’ (‘N67’) i.e. ‘N67’×‘Purple’, ‘N67’×‘PF’, ‘S29’×‘Purple’, ‘S29’×‘PF’ (Dobrovolskaya et al., 2006). According to the grain color, arbitrary lines of F<sub>3</sub> offspring were divided into ‘**bulk+**’ materials containing only pigmented seeds and ‘**bulk-**’ materials including only non-colored seeds. Whole grains were ground using a Disc Mill RS 200 (Retsch, Germany) with variable speed 1,000 min<sup>-1</sup> for 1min, and this process was repeated if wheat grains were not fine milled. Grain samples were kept at room temperature, while grain flour samples were stored at -80 °C and then freeze-dried at -55 °C before use.

**Table 4. Description of wheat cultivars used as parents of the mapping population and their F<sub>3</sub> offspring.** Firstly, F<sub>2</sub> mapping populations were developed from non-colored cultivars ‘Saratovskaya 29’ and ‘Novosibirskaya 67’ crossing with the purple-colored Canadian cultivar ‘Purple’ and the Australian cultivar ‘Purple Feed’ respectively. Then their F<sub>3</sub> families were distinguished between ‘bulk+’ materials with pigmented seeds and ‘bulk-’ materials with non-colored seeds by the grain color (Dobrovolskaya et al., 2006).

	Cultivars	Label	Description	Grain color
<b>Parental lines</b>	Saratovskaya 29 (‘S29’)	1.1	Russian spring wheat	Non-colored
	Novosibirskaya 67 (‘N67’)	1.2	Russian spring wheat	Non-colored
	Purple	2.1	Australian spring wheat ‘k-46990’	Dark purple
	Purple Feed (‘PF’)	2.2	Canadian spring wheat ‘k-49426’	Dark purple
<b>F<sub>3</sub> families</b>	‘S29’ × ‘Purple’ bulk-	3	Crossed from ‘S29’ × ‘Purple’	Non-colored
	‘S29’ × ‘Purple’ bulk+	4	Crossed from ‘S29’ × ‘Purple’	Purple
	‘S29’ × ‘PF’ bulk-	5	Crossed from ‘S29’ × ‘PF’	Non-colored
	‘S29’ × ‘PF’ bulk+	6	Crossed from ‘S29’ × ‘PF’	Purple
	‘N67’ × ‘Purple’ bulk-	7	Crossed from ‘N67’ × ‘Purple’	Non-colored
	‘N67’ × ‘Purple’ bulk+	8	Crossed from ‘N67’ × ‘Purple’	Purple
	‘N67’ × ‘PF’ bulk-	9	Crossed from ‘N67’ × ‘PF’	Non-colored
	‘N67’ × ‘PF’ bulk+	10	Crossed from ‘N67’ × ‘PF’	Purple

### 2.1.2. Barley

Twenty-three different-colored barley accessions from IPK genome center (Gatersleben, Germany) as following were used: two hull-less and seven hulled yellow-grained cultivars, one hull-less blue-grained cultivar, four hull-less and five hulled purple-grained cultivars, two hulled and two hull-less lines with black seeds (Table 5). As shown in Figure 6, spikelets in the middle of the spikes from three selected barley cultivars HOR 2199, HOR 4802 and HOR 3011 (labeled in gray) were collected for further research. At each developmental stage, approximately 20-50 grains from single barley cultivar were randomly pooled as one biological replicate with 5-day intervals throughout seed development from 7 days after flowering (DAF) until maturity (Figure 7). After the husk removal, samples were snap frozen in liquid nitrogen and stored at  $-80\text{ }^{\circ}\text{C}$ . Whole grains were ground using a Mixer Mill MM 400 (Retsch, Germany) with variable speed  $1,000\text{ min}^{-1}$  for 1 min.



**Figure 6. The sampling of barley caryopsis.** Spikelets at the tip and the base of the spike (three to four nodes) were excluded. Also, the husk of each seed was removed. Samples were snap frozen in liquid nitrogen and stored at  $-80\text{ }^{\circ}\text{C}$ .

**Figure 7. Barley grains at different developmental stages were harvested for experimental use.** Nine developmental stages were examined in this study. Seeds were harvested 7, 10, 15, 20, 25, 30, 35, 40, 45 days after flowering (DAF) and maturity. (Displayed pictures are taken from W. Weschke, IPK Gatersleben)

## 16 | MATERIALS & METHODS

**Table 5. Description of barley genotypes used for screening.** Twenty-three barley genotypes with yellow, blue, purple and black grain colors were provided from IPK Genbank. Among them, Betzes und Class are cultivars/breeding material from breeders. Detailed information of the barley genotypes can be found on the following website. [https://gbis.ipk-gatersleben.de/GBIS\\_I/suche.jsf;jsessionid=Pyfj8ZtIzEapp5lxkw1O27uo\\_tBoc4mt\\_FBZmL0dRZQ0SAdMrLjj!-2140960688!1472803740488?autoScroll=0,122](https://gbis.ipk-gatersleben.de/GBIS_I/suche.jsf;jsessionid=Pyfj8ZtIzEapp5lxkw1O27uo_tBoc4mt_FBZmL0dRZQ0SAdMrLjj!-2140960688!1472803740488?autoScroll=0,122)

Accession Nr.	Accession name	Country of origin	Grain color	Seed type
HOR 13475	Golden Promise	United Kingdom	yellow	hulled
HOR 1648	Heines Haisa	Germany (before 1945)	yellow	hulled
HOR 22192	Germina	Germany	yellow	hulled
HOR 4394	Studina 623	Romania	yellow	hulled
HOR 98	Heines Haisa	Germany (before 1945)	yellow	hulled
BCC 819/HOR 16844	Betzes	Germany	yellow	hulled
-	Class	-	yellow	hulled
HOR 3011	Schweizer Nackte	Switzerland	yellow	hull-less
MHOR 380	-	Germany-IPK	yellow	hull-less
HOR 3710	Blaue Nackte	Unknown	blue	hull-less
HOR 6866	-	Ethiopia	light purple	hulled
HOR 9420	-	Ethiopia	light purple	hulled
HOR 16912	ADDIS ABEBA	Ethiopia	light purple	hull-less
HOR 16915	SOGGIO SOUDAN	India	light purple	hull-less
HOR 17020	RUSSIAN 68	Union of Soviet Socialist Republics	light purple	hull-less
HOR 10458	-	Ethiopia	purple	hulled
HOR 10636	-	Ethiopia	purple	hulled
HOR 18098	ALEQUA-PASS	Ethiopia	purple	hulled
HOR 2199	Schwarze Nackte Kraftborn	Germany (before 1945)	purple	hull-less
MHOR 403	-	Germany-IPK	purple	hull-less
HOR 2444	Tschermaks Vierzeilige Schwarze Wintergerste	Austria	black	hulled
HOR 4802	Schwarze Zweizeilige	Ethiopia	black	hulled
HOR 1606	Weihenstephaner Schwarze Nackte	Germany (before 1945)	black	hull-less

Note: HOR=Hordeum; MHOR= Mutant Hordeum; BBC=Barley Core Collection. IPK: The Leibniz Institute of Plant Genetics and Crop Plant Research.

## 2.2. Growth conditions

As summarized in Table 6, plants of all cereals in this study were grown at IPK (latitude 51° N and longitude 11° E) with different growth conditions from 2014 to 2015. Wheat cultivars in 2014 were grown in the greenhouse (section 2.2.1), while in 2015 the same materials were conducted with three biological replicates at two plots in the field (section 2.2.3). For barley, plants of each genotype were grown in a controlled environment chamber (section 2.2.2) in 2014 and greenhouse (section 2.2.1) in 2015 respectively. More detailed information regarding growing conditions is described below.

**Table 6. Summary of crop growth conditions.** Wheat plants were grown in greenhouse and field in 2014 and 2015 respectively, while barley plants were grown in climate chamber and greenhouse in 2014 and 2015 respectively.

Crop species	Year	Growth conditions				
		Location	Photoperiod [h]	Light intensity [ $\mu\text{moles m}^{-2} \text{s}^{-1}$ ]	Relative humidity	Temperature (Day/Night)
Wheat	2014	Greenhouse	13-15	200	-	22 °C/18 °C
	2015	Field: two plots		See Figure 8		
Barley	2014	Climate chamber	12	70	30%	14 °C/12 °C
	2015	Greenhouse	13-15	200	-	22 °C/18 °C

### 2.2.1. Greenhouse

Germinated seeds were placed in the dark at 5 °C for three weeks. After that, the plants were transferred to 13 × 13 cm pots filled with red substrate 1 (Klasmann-Deilmann GmbH, Geeste, Germany) in the greenhouse at constant 20±2 °C during day supplied with a light intensity of 200  $\mu\text{moles m}^{-2} \text{s}^{-1}$  for 13-15 h and 17±1 °C at night in the dark, respectively. All pots were watered every second day and randomly rearranged every three days to minimize microenvironmental effects.

### 2.2.2. Climate chamber

Plants were grown in 13 cm × 13 cm pots under a controlled environment chamber maintained at 14 °C/12 °C day/night temperatures, 30% relative humidity, and a 12 h photoperiod with a light intensity of 70  $\mu\text{moles m}^{-2} \text{s}^{-1}$ . All pots were watered every second day and randomly rearranged every three days to minimize micro environmental effects.

### 2.2.3. Field

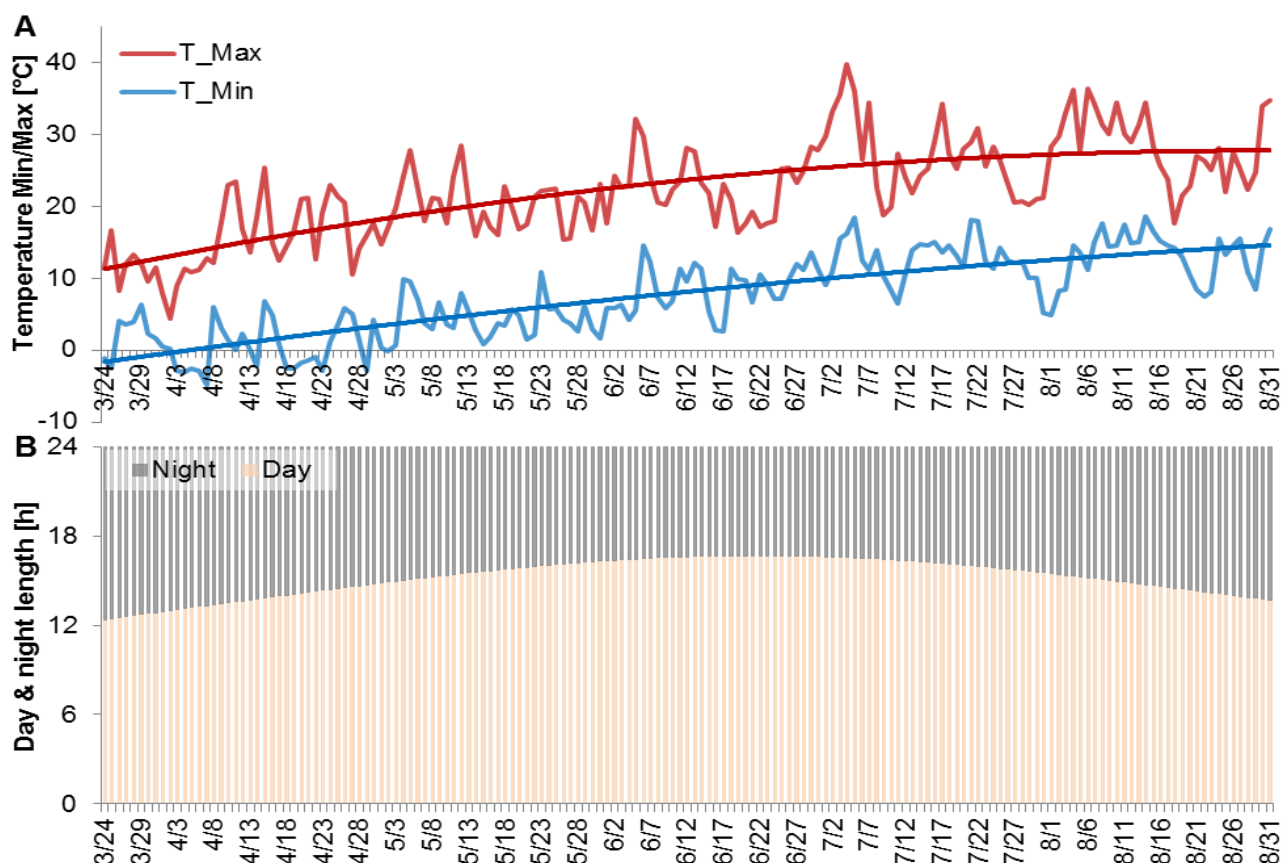
Wheat plants were grown with three biological replicates at two plots of IPK in 2015. According to the soil evaluation report supplied by AGROLAB (Table 7), the composition of soil inside IPK (Plot 1) had a distinct condition with that at plot 2 which locates outside IPK. The soil was detected with higher pH value at plot 1 (7.5) than at plot 2 (7.1). Moreover, soil at plot 1 consists of higher K content, but lower P, Mg, NO<sub>3</sub> and mineral N contents when compared with soil at plot 2.

**Table 7. Soil properties at two experimental sites.** The components of soil at plot 1 differed from that at plot 2. Investigation of soil characteristics was provided by AGROLAB group (AGROLAB Boden- und Pflanzenberatungsdienst GmbH) in March 2015.

Items	Plot 1	Plot 2
pH	7.5	7.1
P (mg·100g <sup>-1</sup> )	8.2	11.8
K (mg·100g <sup>-1</sup> )	17.4	10.7
Mg (mg·100g <sup>-1</sup> )	10.5	19.9
NO <sub>3</sub> (kg N·ha <sup>-1</sup> )	63	84
NH <sub>4</sub> (kg N·ha <sup>-1</sup> )	4	4
Mineral N (kg N·ha <sup>-1</sup> )	50	88

The sowing date was carried out on the 24<sup>th</sup> March for plot 1 and on 26<sup>th</sup> March for plot 2 in 2015. During the period from planting to harvest, daily meteorological data including maximum and minimum temperature and day length was obtained from websites <http://wetter.ipk-gatersleben.de/apex/f> and <http://www.weather.com/>. As shown in Figure 8, the maximum temperature and the minimum temperature increased from 5 °C to 25 °C and -5 °C to 15 °C respectively. The day length firstly rose from 12 h to 17 h and then decreased to 14 h when the plants were harvested at the end of August.





**Figure 8. Daily meteorological parameters during wheat development.** (A) The daily maximum and minimum air temperatures. (B) Day and night length of climate records in Gatersleben, Germany. The original data was obtained from websites <http://wetter.ipk-gatersleben.de/apex/f> and <http://www.weather.com/>.

## 2.3. Measurements of phenotypic traits

### 2.3.1. Agronomic parameters

Flowering dates of wheat plants were recorded when half of them in one plot started flowering. At the stage of the full maturity on 21<sup>st</sup> August, plant heights of three randomly selected plants were measured from the ground to the head, and 20 spikes were harvested from each plot. The final harvest was conducted on 31<sup>st</sup> August in 2015. After one-week storage in a drying chamber, total grain yield (TGY), spike length (SL) and spikelet number per spike (SN) of wheat materials were measured. Then the other yield parameters including thousand kernel weight (TKW), grain number per spike (GN), grain weight per spike (GS), seed size (SS-2D), seed length (SL) and seed width (SW) were determined by the MARVIN device.

### 2.3.2. Seed appearance

The images of cross sections, whole seeds and barley grains at different developmental stages were captured by using DimensionOne™ software from Vision Engineering's stereo microscope. The important setups were White Balance: daylight; Gamma 1.50; Edge enhancement: strong; Flip frame vertical: click; Enable color correction: off.

## 2.4. Analysis of primary and secondary metabolites

### 2.4.1. Preparation of anthocyanin and proanthocyanidin extracts

Anthocyanin and PA extracts were prepared by a modified method from Wrolstad (2001). Grounded wheat or barley (around 100 mg) were homogenized with 1000 µl of acetone, water and formic acid (70:28:2, v/v) for 45 s twice at 4500 rpm. Subsequently, the mixture was centrifuged for 15 min at 13000 g 4 °C. After a second extraction, combined supernatant was participated with 500 µl chloroform to remove chlorophyll and lipids. Only the upper phase was applied for LC-MS analysis followed by a further purification. Extracts were done in triplicates.

For the determination of total monomeric anthocyanin content, freeze dried wheat flour (around 100 mg) were firstly added with 500 µl of 70% acetone acidified with 2% formic acid. After 10 min ultrasonic bath, the extracts were centrifuged for 10 min at 28000 g 4 °C. Then depending on the color of precipitation, the extract procedures were repeated two times with additional 300 µl of acidified acetone. After centrifugation, the combined supernatants were participated with 800 µl chloroform and mixed thoroughly for 2 min at 2000 rpm followed by the centrifugation at 28000 g at 4 °C for 5 min. Finally, the upper phase (350-380 µl) were filled to 400 µl and applied for the pH differential method described in Section 2.4.7 using plate photometer. Extracts were done in triplicates.

### 2.4.2. Amino acids extraction

Amino acids were extracted from 20 mg grounded wheat/barley seeds with 500 µl methanol. The mixture was incubated for 30 min at 70 °C 1000 rpm, then 250 µl chloroform and 500 µl distilled water were added successively and vortexed and centrifuged for 15 min. Cellular debris was

removed by centrifugation at 13500 rpm at 4 °C for 15 min, and 2×50 µl of the upper phase were transferred to clean tube and dried under vacuum. The dried extracts were stored at -80 °C.

#### 2.4.3. AccQ•Tag derivatization

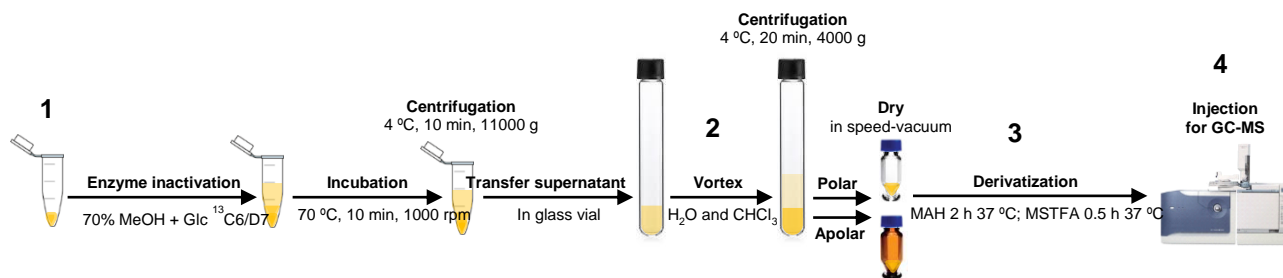
Amino acid derivatization with AccQ•Tag reagents was conducted according to the manufacturer's protocol. Briefly, 10 µl of either a standard amino acid mix solution, or a biological extract were mixed with 70 µl of AccQ•Tag Ultra borate buffer, and 20 µl of AccQ•Tag reagent previously dissolved in 1.0 ml of AccQ•Tag Ultra reagent diluent were added. The reaction was allowed to proceed for 10 min at 55 °C.

#### 2.4.4. Extraction of primary metabolites

The extract method of primary metabolites was adopted from Lisec et al. (2006). Grounded wheat grains (25 mg) were mixed with 1000 µl 70% methanol containing 50 µM glucoside (Glc) <sup>13</sup>C<sub>6</sub>/D<sub>7</sub> as an internal standard. The samples were incubated for 10min at 70 °C with 1000rpm, and the supernatants were transferred to 15 ml glass vials after a centrifugation procedure (11000 g, 10 min, 4 °C). Then 1000 µl distilled water and 500 µl chloroform were added successively and vortexed. A second centrifugation at 4 °C was applied with 4000 g by HERAEUS Multifuge 1S-R for 20 min. Later 60 µl from both polar and apolar phases were transferred into 1.5 ml reaction tubes. The dried extracts were stored at -80 °C or proceed to derivatization. The extraction was performed by Teresa Pérez-Piñar López (IPK Gatersleben, Applied Chemistry Group).

#### 2.4.5. Derivatization of metabolites for GC-MS analysis

According to the method of Lippmann et al. (2009), the dried extracts from wheat grains were mixed with 40 µl of methoxyamine hydrochloride (MAH, 20 mg·ml<sup>-1</sup> in pyridine) for 2 h at 37 °C to convert keto- and aldehyde groups. Followed with an incubation of 70/80 µl N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) for 30 min at 37 °C, the samples were ready for GC-MS injection.



**Figure 9. Experimental procedures for sample preparation of GC-MS analysis.** (1) The sample was mixed with 1000  $\mu\text{l}$  70% methanol containing 50  $\mu\text{M}$  internal standard  $\text{Glc}^{13}\text{C}_6/\text{D}_7$  to inactivate enzymes. The mixture was shaken for 10 min at 70  $^\circ\text{C}$  in a thermomixer at 1000 rpm. Then the sample was centrifuged for 10 min at 4  $^\circ\text{C}$  at 11000 g, and the supernatant was transferred to a glass tube. (2) Add 1000  $\mu\text{l}$   $\text{dH}_2\text{O}$  and 500  $\mu\text{l}$  chloroform to the sample and vortex. After centrifuge, 60  $\mu\text{l}$  extracts from both polar and apolar phases were transferred to GC-MS glass vials and dried under vacuum. (3) Store the samples at -80  $^\circ\text{C}$  or proceed to derivatization with an addition of 40  $\mu\text{l}$  MAH (shake for 2 h at 37  $^\circ\text{C}$ ) and 80  $\mu\text{l}$  MSTFA (shake for 0.5 h at 37  $^\circ\text{C}$ ) continuously. (4) Analyze aliquot by GC-MS.

#### 2.4.6. UPLC-MS and UPLC-MS/MS analyses for anthocyanins and proanthocyanidins

Sample analysis was carried out with an Acquity UPLC system (Waters) coupled to a tandem mass spectrometer. The column used for analysis was a 100 mm  $\times$  2.1 mm i.d. 1.7  $\mu\text{m}$ , Acquity UPLC CSH phenyl-Hexyl column (Waters Corp., Wexford, Ireland). The flow rate of the eluent was 0.5 ml/min. The elution profile used two solvents, water with 0.5% formic acid (A) and acetonitrile with 0.5% aqueous formic acid (B): 0-3 min, 98% A in B; 3-7 min, 90% A in B; 7-10 min, 80% A in B; 10-13.5 min, 2% A in B; 13.5-15 min, 98% A in B. Positive ionization mode was used for MS analyses. ESI conditions: capillary voltage, 4 kV; source temperature, 200  $^\circ\text{C}$ . Cyanidin-3-glucoside (1  $\mu\text{g}/\mu\text{l}$ ) was used to monitor the stability of the ionization efficiency of the mass spectrometer.

#### 2.4.7. Determination of total monomeric anthocyanin content by the pH differential method

According to the method of Giusti and Wrolstad (2001), two times of 180  $\mu\text{l}$  aliquots of the anthocyanin extracts were added with 20  $\mu\text{l}$  25 mM KCl buffer, pH 1.0 and 20  $\mu\text{l}$  0.4 M Na-acetate buffer pH 4.5 respectively. The dilutions were equilibrated for 15 min at 1000 rpm 40  $^\circ\text{C}$  (Thermomixer) and centrifuged for 10 min at 14000 rpm 4  $^\circ\text{C}$ . The absorbance of each dilution was measured at 515 nm and 700 nm. Finally, the monomeric anthocyanin pigment concentration was calculated as cyanidin-3-glucoside using the following formula:

Monomeric anthocyanin pigment (cyanidin-3-glucoside equivalents,  $\mu\text{g}/\mu\text{l}$ ) =  $A/CF$ , where  $A = (A_{515\text{nm}} - A_{700\text{nm}})_{\text{pH}1.0} - (A_{515\text{nm}} - A_{700\text{nm}})_{\text{pH}4.5}$ ; CF is the calculation factor depended on a dilution series of calibration standard cyanidin-3-glucoside.

#### 2.4.8. UPLC-Fluorescence detection for free amino acids

UPLC analysis was performed on a Waters Acquity UPLC system, equipped with a binary solvent manager, an autosampler, a column heater, a PDA detector, and interfaced to a tandem quadrupole detector. The separation column was a Waters AccQ•Tag Ultra column (2.1 mm i.d. × 100 mm, 1.7 µm particles). The column heater was set at 60 °C, and the mobile phase flow rate was maintained at 0.7 ml/min. Eluent A was 10% AccQ•Tag Ultra concentrate solvent A, and eluent B was 100% AccQ•Tag Ultra solvent B. The non-linear separation gradient was as follows: 0-0.5 min, 99.9% A in B; 5.7 min, 98% A in B; 7.5 min, 91% A in B; 8.7 min, 90.3% A in B; 9.2 min, 87% A in B; 12 min, 86% A in B; 12.2-13 min, 40% A in B; 13-14 min, 99.9% A in B. A VanGuard™ Waters column (2.1 mm i.d. × 5 mm, 1.7 µm particles) was used as the guard column. One µl of sample was injected for each chromatographic separation. The detection of photodiode array UV detector was at 260 nm; the fluorescence detector was excitation at 266 nm and emission at 473 nm.

#### 2.4.9. GC-MS analysis for primary metabolites

Measurements were carried out by Teresa Pérez-Piñar López (IPK Gatersleben, Applied Chemistry Group). Inject 1 µl of the sample at 240 °C in splitless mode with helium carrier gas flow set to 1 ml·min<sup>-1</sup>. The GC-MS system consisted of a gas chromatograph A7890 (Agilent Technologies, Böblingen, Germany), a multipurpose sampler (GERSTEL GmbH & Co.KG Mülheim an der Ruhr, Germany) and a TOF mass spectrometer GCT Premier (Waters Corporation). Chromatography was performed on a 30 m DB-35 capillary column (Phenomenex, Aschaffenburg, Germany) with 0.25 mm inner diameter (Restek, Bad Homburg, Germany) and 0.25 µm film thickness including a 5 m guard column. The temperature program for GC separation was isothermal for 3 min at 80 °C, followed by a 5 °C per min ramp to 300 °C, and holding at this temperature for 5 min. The dynamic range enhancement (DRE) modus was used to record mass data with Mass Lynx 4.1 (Waters Corporation) in a range of 50-700 m/z in centroid mode at 10 scans s<sup>-1</sup>. The detector voltage was 2700 V.

Identification and annotation were facilitated by reference to the NIST 05 library (Waters), while quantification was obtained using internal software MassLynx (Waters). Retention time indices of the compounds were calculated for validation purposes and compared to those documented in the Golm Metabolome Database (<http://gmd.mpimp-golm.mpg.de/>). Normalization was achieved using

the deviation-from-the-mean factor, calculated by dividing the mixed sample value by the average value of all the combined samples.

### **2.4.10. Elemental analysis by ICP-OES**

The elemental composition was measured in collaboration with Dr. Yudelsy Antonia Tandron Moya (IPK Gatersleben, Molecular Plant Nutrition Group). Approximately 50 mg of pulverized and freeze-dried wheat grains were weighed into PTFE digestion tubes. Nitric acid (2 ml) was added each tube. Samples were digested under pressure using a high-performance microwave reactor (Ultraclave 4, MLS). Afterward, samples were transferred to Greiner centrifuge tubes and diluted with de-ionized water (Milli-Q) to a final volume of 15ml. Mineral constituents were identified and quantified using inductively coupled plasma optical emission spectroscopy technique (iCAP 6500 dual OES spectrometer, Thermo Fischer Scientific).

## **2.5. Physiological experiments of seeds**

### **2.5.1. Determination of seed moisture content**

High constant temperature oven method was carried out in an oven at 130 °C for 2 h, using two samples of 10.0 g of milled seeds, for each lot. Results were expressed as mean percentages for each lot (ISTA, 2013).

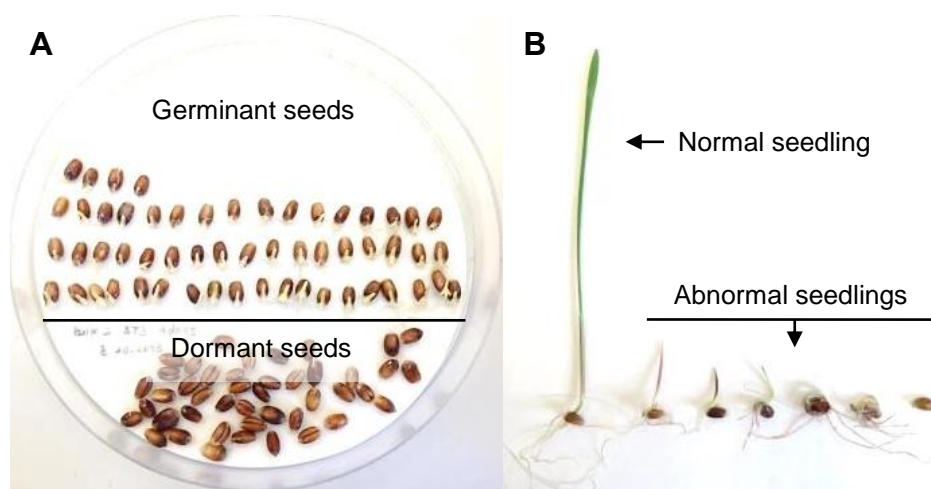
### **2.5.2. Accelerated ageing treatment (AA)**

Controlled deterioration test was performed according to the method described in the Handbook of vigor test method, 1995. Samples with three replicates of 100 seeds from each cultivar were placed in aluminum bags with their moisture content adjusted to 18%. They were kept for 48 h, 72 h, and 96 h in the climate chamber at 45 °C under dark condition.

### **2.5.3. Germination test**

According to ISTA rules (2013), 50 or 100 seeds with three replicates were used for each accession. All the seeds were placed over moistened filter papers on Petri dishes with the climate condition that a temperature regime of 25 °C during the day and 20 °C during the night. Germinated seeds

were put on the other side of petri dishes every day. Normal seedlings were counted when the majority of seedlings were up after 7 days (Figure 10).



**Figure 10. Performance of seeds and seedlings during germination test.** (A) Germinated (up) and dormant (down) seeds. Sprouting grains with at least 2 mm-shoots/roots were considered as germinant ones, and they were separated with dormant seeds. (B) Normal and abnormal wheat seedlings after 7 days. A normal seedling has all of the essential structures present for normal growth, for example, the root, the shoot or the terminal bud. However, an abnormal seedling is categorized as such missing one or more of its essential seedling structures.

## 2.6. Gene expression analysis

### 2.6.1. RNA extraction and cDNA synthesis

Total RNA was isolated from the various developmental grains with a slightly modified method by Furtado (2014). The quality of RNA was verified by the absorbance ratios ( $A_{260}/A_{280}$ ) of 1.8-2.0. Before cDNA synthesis, total RNA was treated with RNase-Free DNase Set (QIAGEN) to remove trace amount of DNA contamination. Then one microgram of total RNA was reverse transcribed using oligo d(T)<sub>18</sub> and Maxima Reverse Transcriptase (Thermo Scientific) following the protocol of the supplier.

### 2.6.2. Quantitative RT-PCR

Six pathway genes, including *F3H*, *DFR*, *ANS*, *UFGT*, *LAR*, and *ANR* were analyzed to evaluate their expression levels during seed development. Nine seed development stages of four barley genotypes were harvested for RT-qPCR analysis. Real-time quantitative PCR (RT-qPCR) was performed using SYBR green method on a CFX96 cycler (Bio-Rad, USA). Each pair of specific RT-qPCR primers (Table 8) were designed by Primer 3 program according to gene sequences. To

test the primer accuracy, DNA was purified according to the protocol of QIAquick Gel Extraction Kit after gel electrophoresis and sent to EUROFINs company for sequencing. Among 12 frequently used candidate reference genes, elongation factor- $\alpha$  (*eEF1 $\alpha$* ) and glyceraldehyde 3-phosphate dehydrogenase (*GADPH*) were stably expressed among varieties and grain developmental stages. Final PCR reaction (6  $\mu$ l) contained 2  $\mu$ M primer (each), 1  $\mu$ l cDNA (diluted 1:50), and 3  $\mu$ l SYBR Green supermix. The thermal cycling conditions were 95 °C for 3 min followed by 95 °C for 10 s, 60 °C-64 °C (depend on the product) for 30 s for 40 cycles, followed by a melt cycle from 60 °C to 95 °C. PCR amplification efficiency for each gene was preliminarily determined by using cDNA mixture in 1-104 folding dilutions. All samples were measured in triplicate, and the expression of genes was normalized to two reference genes *eEF1 $\alpha$*  and *GADPH*. Relative expression values of the genes were calculated by Q-base software (Hellemans et al., 2007). Reported values represented the average of three technical and three biological replicates.

**Table 8. Primers design for qRT-PCR analyses of candidate genes from flavonoid pathway and reference genes based on published cDNA sequences of barley.** Barley sequences were selected from IPK Barley BLAST Server <http://webblast.ipk-gatersleben.de/barley/>, and the primers of target genes and reference genes were designed by online software BatchPrimer 3 with general settings: product size, 180-210 bp; primer size, 18-23 bp; primer T<sub>m</sub>, 59.0 °C-61.0 °C; Max T<sub>m</sub> difference, 2 °C; primer GC%, 45-60%.

	Genes	Accession	Sequences (5'-3')	Amplicon (bp)	Annealing T <sub>m</sub>
Target genes	<i>F3H</i>	MLOC_64248.1	Forward-ACGGCGGCAAGAACTGGATC Reverse-GTGGTCCGCGTCTTGAACCT	110	60 °C
	<i>DFR</i>	MLOC_65788.2	Forward-GAGTACGACATCCCGCAGAAG Reverse-GTGGTGTACCGGAAGCTGAAG	107	60 °C
	<i>ANS</i>	MLOC_37146.1	Forward-CTCGGCTACCATGCGTGTGA Reverse-TGCGTCTCCTTTGCGTACCTG	148	60 °C
	<i>UFGT</i>	AK375231	Forward-TCGGCGACCAGACGATGAAC Reverse-TCCTGCGCCTTTGCCCTCAT	154	64 °C
	<i>LAR</i>	MLOC_10899.1	Forward-CACGCTTAGCAAGGAGGAGCT Reverse-ATGCCGAAGTTGGTCTGGCAG	117	64 °C
	<i>ANRlike-1</i>	AK373696	Forward-CGTCACCCTCTCCCTCTTGT Reverse-CGTGGCATATCGACACGGAGT	96	60 °C
	<i>ANRlike-2</i>	MLOC_70051.1	Forward-GCCATGTCTGACGAGGATT Reverse-AGCACCTTCGAGGCAGGGTA	91	60 °C
Reference genes	<i>eEF1<math>\alpha</math></i>	MLOC_21774.1	Forward-CTTGGCTGGTGTGGGGATTG Reverse-GCAGTGGTGCCAGGGACATT	99	60 °C
	<i>GADPH</i>	MLOC_72170.1	Forward-GTTGGCAAGGTGCTCCCAGA Reverse-GCTCATAGGTGGCTGGCTTG	121	60 °C



## 2.7. Statistical analyses

### 2.7.1. T-test and One-way ANOVA

Statistical analyses of metabolites levels and agronomic parameters were performed with SigmaPlot 13.0 software (Systat Software Inc., UK). Data sets were evaluated by T-test or one-way analysis of variance (ANOVA) to compare different groups corresponding to different cultivars to find statistical significance of differences between means within groups.  $P < 0.05$  was considered to be statistically significant. P values are represented as \*  $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

### 2.7.2. Chi<sup>2</sup>-test

The Chi<sup>2</sup> statistic compares the difference of the counts of germinated seeds between two (or more) independent wheat cultivars. Note: Chi<sup>2</sup> test can only be used on actual numbers rather than percentages, proportions, means, *etc.*

### 2.7.3. PCA

For the PCA of phenolics levels of LC-MS analyses using Bruker Compass PofileAnalysis 2.1 (Bruker Daltonik GmbH) with following settings: 20 masses per retention time were isolated at a threshold at 5% of base peak intensity. The retention time window was set between 1.5 and 8.5 min, and the mass window was set between 100 and 1000 with a mass tolerance at 5 ppm Da Pareto algorithm was used for visualization of Loading and Score Plot.

### 3. Results

The present work firstly aims at the eventual effects of anthocyanins accumulating in grains on wheat growth parameters, grain yield and seed germination (**part A**), and another emphasis lies to the metabolite and molecular mechanisms of pigmentation during barley grain development (**part B**).

The following chapter in **section A** begins with the generation of bulk materials from mapping population crossed by anthocyanin-containing wheat cultivars and anthocyanin-free wheat cultivars. Their metabolite profiles are compared to verify if the composition of bulk- and bulk+ materials are well equilibrated except for anthocyanin contents. Further, the comparisons of crop yield parameters and germination rates are presented. The following **section B** shows primarily with seed pigmentation in developing grains of three different-colored barley genotypes. The temporal variation tendency of pericarp color related phenolic compounds and the expression patterns of corresponding genes during seed development are described.

#### ***Part A. Anthocyanin accumulation in wheat grains and its potential effects on growth, grain yield, and seed germination***

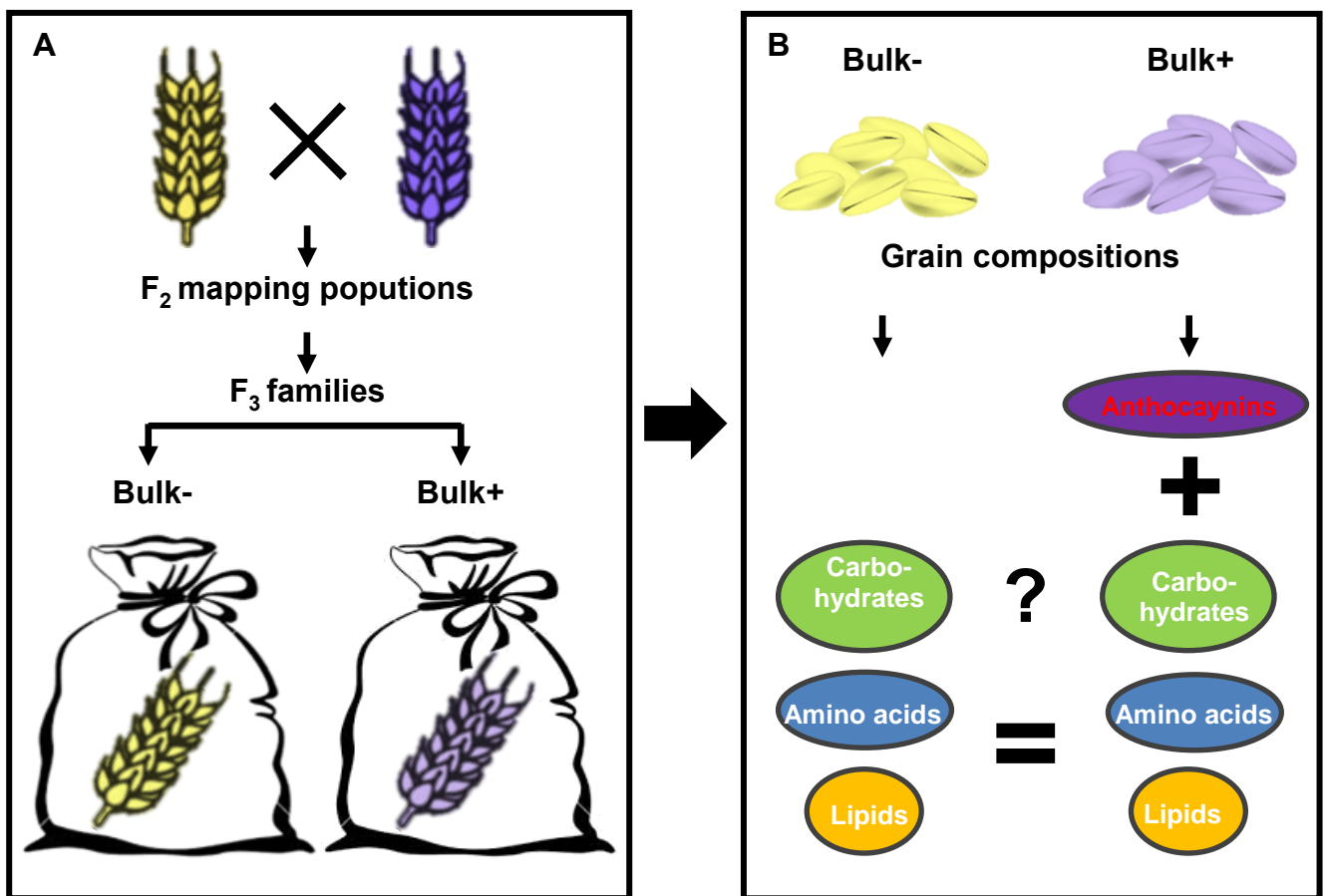
To investigate the possible consequence of anthocyanins accumulation on wheat yields such as seed germination and other key parameters, the first step was to create wheat materials differing only in anthocyanins contents (**section 3.1.**). Then, metabolite analyses were carried out to examine how well the bulk materials were balanced (**section 3.2.**). Finally, comparisons between yellow and purple-grained wheat revealed the impacts of anthocyanins on yield (**section 3.3.**). As summarized in Table 9, parental wheat cultivars and bulk offspring lines were grown either in the greenhouse (2014) or at two plots in the field (2015). The contents of various seed compositions such as anthocyanins, primary metabolite, amino acid and mineral element in wheat samples were determined by a number of techniques. In addition, major yield parameters and seed germination after accelerated ageing treatment were measured and compared among yellow- and purple-grained wheat lines.

**Table 9. Summary of wheat materials used in different experiments.** Wheat materials were grown in the greenhouse in 2014 and at two plots of the field in 2015. The seed compositions from different-colored bulks were compared in section 3.2., while measurements of yield parameters and germination test were showed in section 3.3..

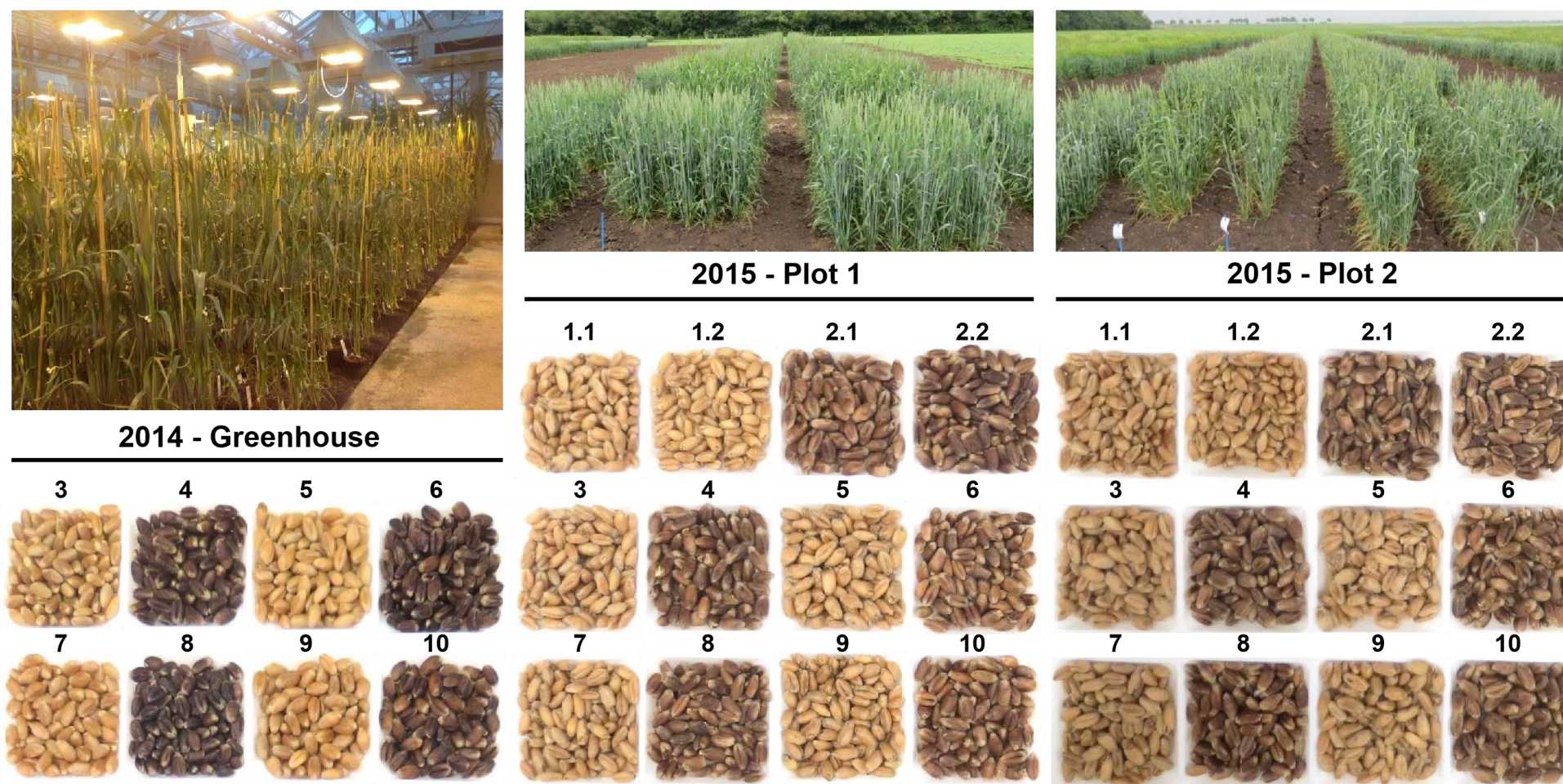
Year	Location	Wheat Materials	Experiments/Analyses	Section
2014	Greenhouse	'N67'×'Purple' bulk±; 'N67'×'PF' bulk±; 'S29'×'Purple' bulk±; 'S29'×'PF' bulk±;	Anthocyanins determination by LC-MS Free amino acids by UPLC-fluorescence Elemental analysis by ICP-OES	3.2.
			Germination test after accelerated ageing	3.3.
2015	Field Plot 1, 2	'N67' 'S29' 'Purple' 'PF' 'N67'×'Purple' bulk±; 'N67'×'PF' bulk±; 'S29'×'Purple' bulk±; 'S29'×'PF' bulk±;	Anthocyanins determination by LC-MS <b>Primary metabolites using GC-MS</b> Free amino acids by UPLC-fluorescence Elemental analysis by ICP-OES	3.2.
			<b>Measurement of yield parameters</b> Germination test after accelerated ageing	3.3.

### 3.1. Generation of bulk materials differing only on anthocyanins

Wheat materials differing only on anthocyanin contents had to be developed to exclude the interference from other seed constituents. The wheat mapping populations were generated by Dobrovolskaya et al. (2006) through crossing between two non-colored cultivars 'Saratovskaya 29' ('S29') and 'Novosibirskaya 67' ('N67') and two pigmented cultivars 'Purple' and 'Purple feed' ('PF') i.e. 'N67' × 'Purple', 'N67' × 'PF', 'S29' × 'Purple', 'S29' × 'PF' (Figure 11A). In the presented work, different-colored offspring lines were randomly selected and propagated under the same conditions. Upon that equal numbers of non-colored seeds (bulk-) and pigmented seeds (bulk+) were collected separately for creating materials with equilibrated metabolite compounds (such as carbohydrates, amino acids, and lipid) except for anthocyanins (Figure 11B). Using the method described above, wheat samples were planted in the greenhouse in 2014 and at two individual plots in the field in 2015. As shown in Figure 12, wheat cultivars 'S29', 'N67' and all bulk- displayed yellow pericarp, while cultivars 'Purple', 'PF' and all bulk+ appeared with purple color. Purple grains harvested in the greenhouse were observed with darker coloration than grown in the field. However, pigmented wheat planted at two plots in 2015 showed a similar shade of purple.



**Figure 11. The strategy used for the generation of bulk materials with balanced grain compositions except for anthocyanins.** Eight wheat bulks $\pm$  crossed from ‘S29’ $\times$ ‘Purple’, ‘S29’ $\times$ ‘PF’, ‘N67’ $\times$ ‘Purple’, ‘N67’ $\times$ ‘PF’ were developed by Dobrovolskaya et al. (2006), in which bulk- contained only yellow seeds and bulk+ contained only purple seeds. However, whether the compositions of bulk- and bulk+ materials differ only on anthocyanins need to be examined.



**Figure 12. Seed appearance of wheat materials with different germplasm color.** (1.1-2.2) From left to right are parental wheat cultivars ‘S29’, ‘N67’, ‘Purple’ and ‘PF’; (3-6) from left to right are bulk- and bulk+ by the crossings of ‘S29’×‘Purple’, ‘S29’×‘PF’; (7-10) from left to right are bulk- and bulk+ by the crossings of ‘N67’×‘Purple’, ‘N67’×‘PF’. In 2014, plants of wheat were grown in the greenhouse; while in 2015, wheat materials were planted with three biological replicates at two plots. Bulk+ materials in 2014 showed darker pigmentation than those in 2015. The numbers 1-10 indicate the labeling of the wheat materials referred in Table 4.



### **3.2. Verifying if the seed compositions except for anthocyanins of non-colored and colored offspring were well equilibrated**

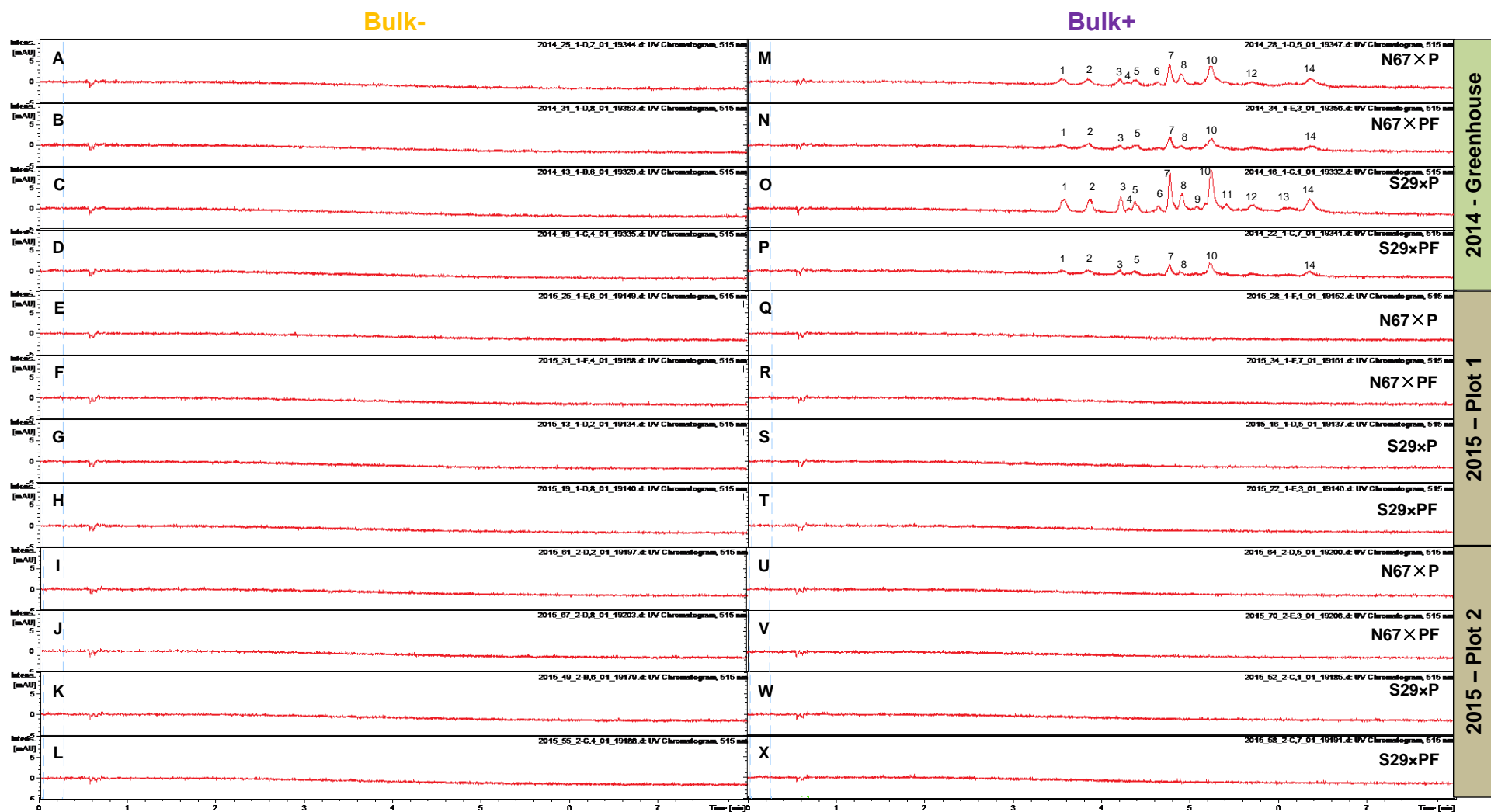
For the purpose of testifying the generation concept of bulk materials, non-targeted and targeted metabolomic analysis were applied to the compositional equivalence of the bulk materials. The determination of:

- anthocyanin occurrence by LC-MS (section 3.2.1),
- primary metabolites contents by GC-MS (section 3.2.2.1),
- free amino acids concentrations by UPLC-fluorescence (section 3.2.2.2),
- elemental contents by ICP-OES (section 3.2.2.3)

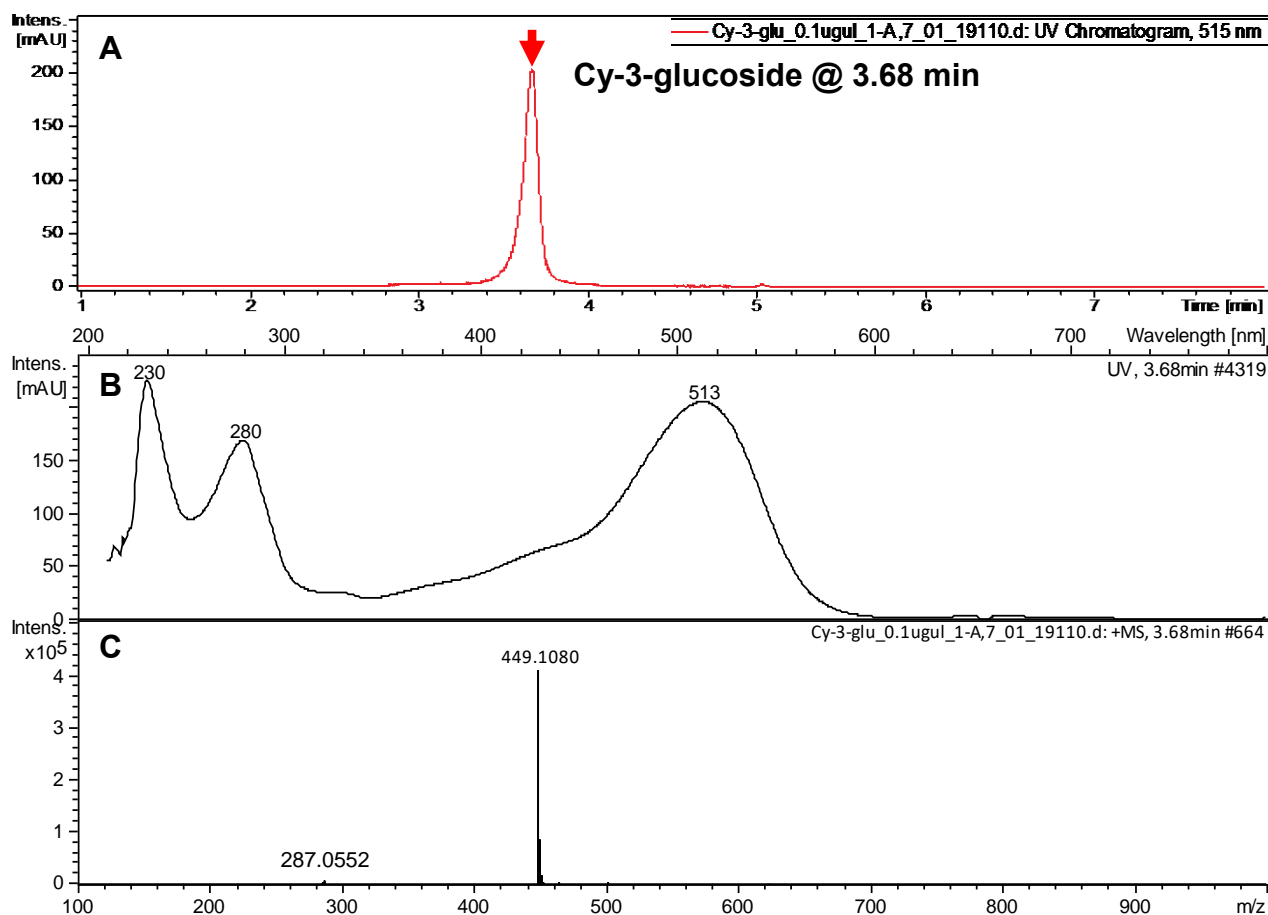
in the grain of wheat parental varieties and offspring lines from F<sub>2</sub> mapping population were performed.

#### **3.2.1. Anthocyanin profiles in wheat bulk+ materials**

When the anthocyanins content is low, anthocyanins locate in the inner layer rather than in pericarp or other pigment patterns accumulate on the wheat seed, it is necessary to determine the occurrence of anthocyanins and assure that they are the only pigments responsible for the color difference between bulk- and bulk+ seeds. Therefore, the anthocyanin profiles of extracts from bulk- and bulk+ materials were compared using LC-MS and LC-MS/MS approaches. According to the LC measurements obtained at 515 nm in Figure 13, a clear separation of eight major anthocyanins (peak 1-3, 5, 7, 8, 10 and 14) and several minor compounds was observed in wheat bulks+ in 2014, in contrast with absent peak in all bulks- for both years. Moreover, no peak was found in bulk+ extracts of 2015 as well, as shown in the examples of anthocyanin profiles. The chromatograms of wheat bulk+ extracts crossed from different parental lines in 2014 were very similar, with the major peaks occurring at retention times of 3.5-6.5 min. A comparison with the anthocyanin standard shown in Figure 14 revealed that one of these peaks was cyanidin-3-glucoside. However, due to the lack of authentic standards, other anthocyanins peaks are inconclusive, and the identification is required using MS and MS/MS data (section 3.2.1.1). The relative quantification of different anthocyanin patterns detected in bulk materials is given in section 3.2.1.2.



**Figure 13.** LC chromatograms of extracts from bulk materials with detection at 515 nm. (A-L) No peak at 515 nm was detected in bulk- materials; (M-P) several anthocyanin candidates were found in bulk+ materials in 2014. (Q-X) No peak was observed in purple wheat offspring in 2015. The absence of peaks was mainly due to the low anthocyanin concentration and the limit of LC detection. Therefore, a more sensitive measurement named pH differential method was applied to quantify the total anthocyanin contents of bulk+ grown in the field in 2015 (Figure 18B, 18C). The numbering of 1-14 indicate the detected anthocyanins, further information of each peak was provided in Table 10.



**Figure 14. Characterization of the standard cyanidin-3-glucoside.** (A) HPLC chromatogram at 515 nm. The peak detected at 3.68 min was corresponding to the standard of cyanidin-3-glucoside. (B) UV-vis spectrum in the wavelength range of 200-800 nm. The cyanidin-3-glucoside standard showed absorption maxima at 280 nm and 513 nm. (C) MS spectrum. A  $[M+H]^+$  at  $m/z$  449.1080 and a fragment ion at  $m/z$  287.0552 were comprised of the spectrum of cyanidin-3-glucoside, and the loss of  $[M-162]^+$  indicate the cleavage of one glucosyl unit.

### 3.2.1.1. Tentative identification of anthocyanins using LC-MS/MS

Anthocyanin compounds are not always commercially available as standards for identification, and UV-visible spectra do not allow a clear-cut identification of individual anthocyanin peak. Therefore, anthocyanin patterns were further confirmed basing on the data from LC-MS/MS measurements agreement with previous reports and database searches. Fourteen anthocyanins (peak 1-14) derived from cyanidin and peonidin were identified in this section.

#### *Extracted ion chromatogram (EIC) analysis*

To avoid interference with unsuspected co-eluting compounds and to highlight potential isomers, EICs were obtained at specific  $m/z$  values corresponding to the measured mass of the anthocyanins



that were tentatively assigned in wheat bulk+ extract (Figure 15). Mass values at  $m/z$  449.1084, 595.1668, 609.1827, 549.1252, 621.1103, 591.1350, and 635.1255 showed only one peak in EICs, while the EICs for  $m/z$  491.1188, 535.1103, and 505.1341 showed two peaks with different retention times, suggesting the existence of isomers of these anthocyanins. Although further literature searches confirmed the identity of ten compounds (peak 1-4, 6-11), masses at  $m/z$  491.1193, 621.1113, 591.1339 and 635.1254 were documented for the first time in wheat grain.

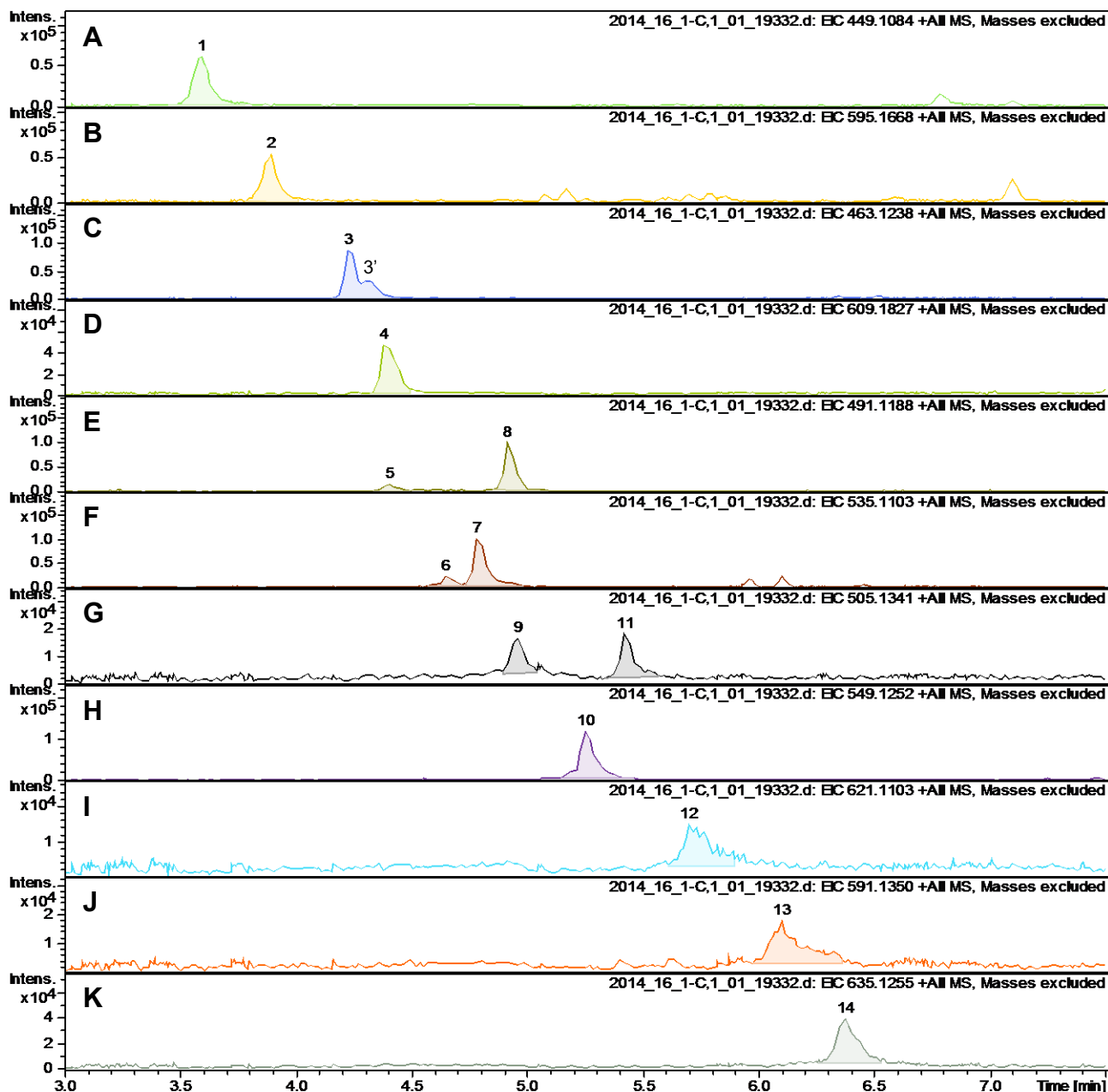
### ***Fragmentation patterns of wheat anthocyanins***

Anthocyanin characterization was obtained by LC-MS/MS technique due to its highly sensitive ionization producing intact molecular ions and the corresponding anthocyanidin fragments. Accordingly, the compounds were tentatively determined depending on their fragmentation behaviors. In this work, fragment ions associated with the elimination of known moieties attached to an aglycone of common anthocyanins were analyzed as shown in Figure 16. For example, the molecular ion furnishing the aglycon with  $m/z$  287 and 301 indicated cyanidin and peonidin derivatives respectively. On the other hand,  $[M-162]^+$  pointed to a loss of one molecule of hexose, while fragment ions of acylated glycoside can be identified by  $m/z$  values at  $[M-162-42]^+$ ,  $[M-162-86]^+$ ,  $[M-162-146]^+$  for acetic acid, malonic acid, and *p*-coumaric acid moieties, respectively. Table 10 summarized the mass spectrometric data including the retention time (RT), molecular ions, fragments and  $\lambda_{\max}$  of UV-visible spectra of all peaks.

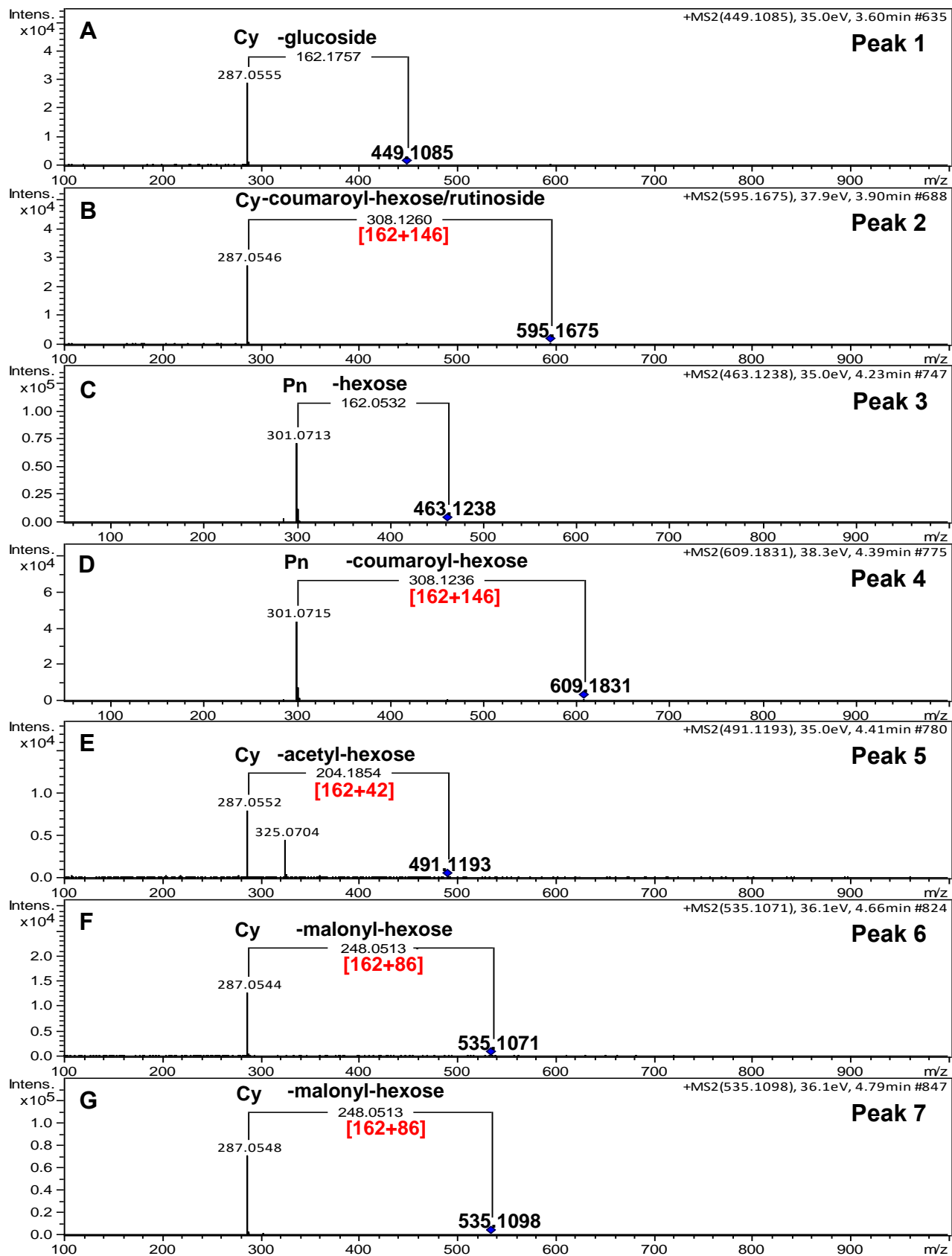
Peak 1 was confirmed as cyanidin-3-glucoside with the help of the authentic standard; it had the molecular ion  $[M+H]^+$  at 449 and the mass fragment ion at  $m/z$  287 was detected by MS/MS analysis due to the elimination of one molecule of glucose  $[M-162]^+$ . For peak 2 with a molecular ion at  $m/z$  595, its fragment ion pattern of  $m/z$  287 [cyanidin] $^+$  indicated that it might be cyanidin-3-coumaroyl-hexose or cyanidin-3-rutinoside. Peak 3 had molecular ion at  $m/z$  463 and aglycon of  $m/z$  301 pointed to a tentative identification as peonidin-3-hexose. Due to the similar fragmentation pattern as peak 2, two possible structures of peak 4, either peonidin-coumaroyl-hexose or peonidin-3-rutinoside, can be assigned. Peak 5 and 8, 6 and 7, 9 and 11 which appeared with the same molecular ions (491, 535 and 505) and the fragment ions (287, 287 and 301) but different retention times, revealed three types of anthocyanins isomers. They were tentatively identified as cyanidin-3-malonyl-hexose, cyanidin-3-acetyl-hexose, and peonidin-3-acetyl-hexose respectively. The molecular ions at  $m/z$  549 and  $m/z$  301 in the fragment ion patterns of peak 10 suggested a

## 36 | RESULTS

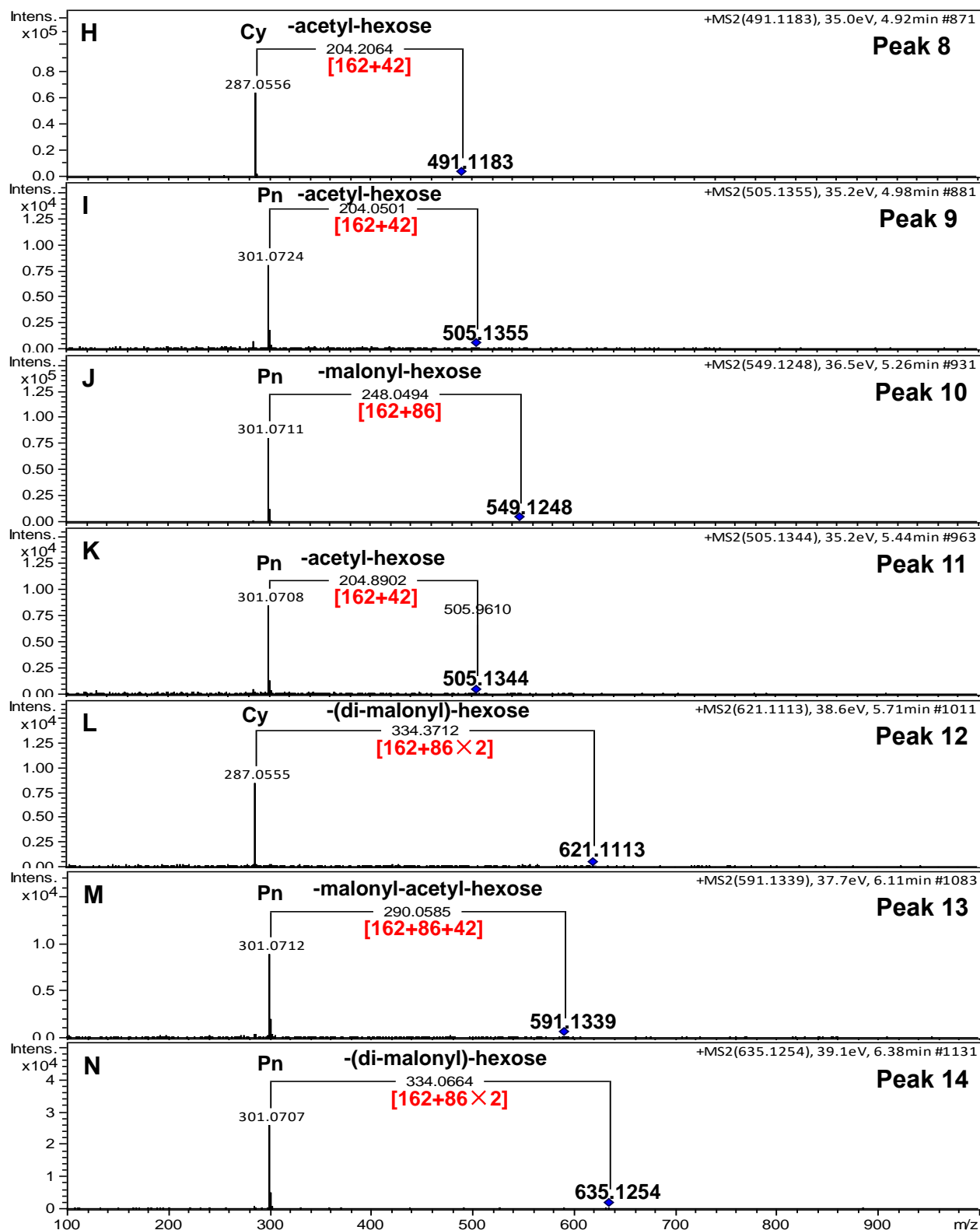
peonidin derivative likely peonidin-3-malonylhexose. Peak 12 was also a cyanidin compound ( $m/z$  287) with moieties of malonyl-hexose,  $m/z$  621  $[M-162-86 \times 2]^+$ . Both peak 13 and 14 were detected with fragment ion at  $m/z$  301 pointed to tentative identification as peonidin-3-malonyl-acetylhexose and peonidin-3-(3, 6-di-malonyl)-hexose respectively. Comparing to published data (see Table 1), peak 5 and 12-14 were compounds that had not been identified in previous reports about anthocyanins in wheat grain.



**Figure 15. Extracted ion chromatograms (EIC) of anthocyanins detected in wheat bulk+.** Wheat plants were grown in the greenhouse in 2014. (A-K) The detailed information obtained by LC-MS were listed in Table 10. The numbers of 1-14 indicate the numbers of the detected anthocyanin peaks according to their retention times.



The figure continues on the following page.



**Figure 16.** MS/MS spectra of the molecular ions corresponding to anthocyanin peaks detected in the wheat bulk extract. Wheat plants were grown in the greenhouse in 2014. (A-N) Positive ion mode MS spectra of peak 1-14 were detected by LC-MS/MS measurements. The fragmentation pattern for each peak was shown with proposed assignment which labeled in red according to the corresponding loss of substitute. Cy stands for cyanidin, Pn stands for peonidin.

**Table 10. Anthocyanin profiles detected in purple wheat materials.** The data were obtained by LC-MS/MS measurements. According to the retention time (RT), molecular ion, fragmentation pattern, maximum absorbance ( $\lambda_{\max}$ ) and information from literature, fourteen different anthocyanin patterns were tentatively identified in bulk+ materials. The molecular ions with same m/z value but different retention times might be anthocyanin isomers. Cy stands for cyanidin, Pn stands for peonidin, <sup>a</sup>, <sup>b</sup> stand for various isomers of anthocyanins, <sup>a</sup> was eluted at earlier retention time when compared to <sup>b</sup>.

Peak	RT [min]	Molecular ion [M] <sup>+</sup> /[M+H] <sup>+</sup> [m/z]	Fragment ion M <sup>+</sup> [m/z]	$\lambda_{\max}$ [nm]	Tentative identification
1	3.58	449.1083	287.0558	280;513	Cy-3-glucoside
2	3.89	595.1675	287.0546	280;513	Cy-3-coumaroyl-hexose / Cy-3-rutinoside
3	4.22	463.1238	301.0713	280;513	Pn-3-hexose <sup>a</sup>
4	4.38	609.1831	301.0715	280;513	Pn-3-coumaroyl-hexose / Pn-3-rutinoside
5	4.41	491.1193	287.0552	280;513	Cy-3-acetyl-hexose <sup>a</sup>
6	4.66	535.1071	287.0544	280;513	Cy-3-malonyl-hexose <sup>a</sup>
7	4.79	535.1071	287.0544	280;516	Cy-3-malonyl-hexose <sup>b</sup>
8	4.92	491.1193	287.0552	280;513	Cy-3-acetyl-hexose <sup>b</sup>
9	4.98	505.1355	301.0708	280;512	Pn-3-acetyl-hexose <sup>a</sup>
10	5.26	549.1248	301.0711	280;516	Pn-3-malonyl-hexose
11	5.44	505.1343	301.0708	280;512	Pn-3-acetyl-hexose <sup>b</sup>
12	5.71	621.1113	287.0555	280;512	Cy-3-(di-malonyl)-hexose
13	6.08	591.1339	301.0717	280;513	Pn-3-malnoyl-acetyl-hexose
14	6.38	635.1254	301.0707	280;512	Pn-3-(di-malonyl)-hexose

### 3.2.1.2. Quantification of anthocyanin concentrations by LC-MS and Spectrophotometry

In this study, the anthocyanin levels in wheat extracts were quantified by two measurements: **MS detection** and **pH differential method**. According to the calibration curve based on a diluted series of cyanidin-3-glucoside, anthocyanin amounts were expressed as cyanidin-3-glucoside equivalents for each measurement.

#### *According to MS detection*

The concentrations obtained for individual anthocyanins as well as the sum of these concentrations for each extract are presented in Table 11. The concentration of each anthocyanin in bulks+ from different parental lines did not differ significantly. Peonidin-3-malonylhexose was the most abundant anthocyanin in wheat bulks+ representing 16%-18% of the total anthocyanins, which was followed by Cy-3-malonylhexose<sup>b</sup> and Pn-3-hexose<sup>a</sup>. The total concentration of anthocyanins determined in purple wheat bulk+ varied from 15.6 to 27.0  $\mu\text{g/g}$  FW with the LC-MS method. The

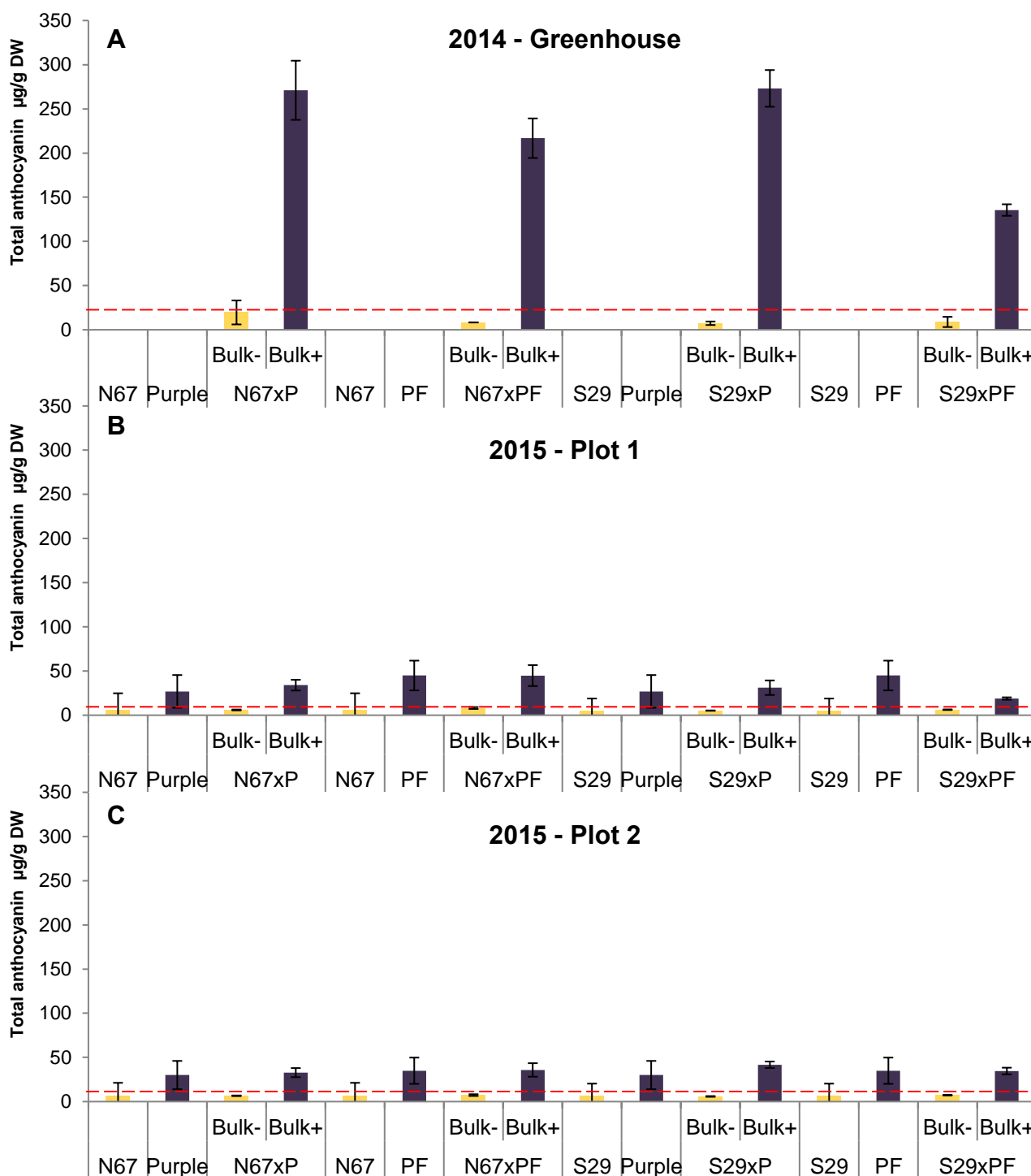
highest concentration of total anthocyanins was obtained in the bulk+ crossing from ‘S29’ with ‘Purple’, while the lowest concentration was found in bulk+ from the parental lines ‘S29’ and ‘PF’.

**Table 11. Anthocyanin contents in wheat materials by LC-MS measurements.** Wheat plants were grown in the greenhouse in 2014. The proposed anthocyanin individuals were relatively quantified in the unit of  $\mu\text{g/g}$  FW according to the authentic standard cyanidin-3-glucoside. Cy stands for cyanidin, Pn stands for peonidin, <sup>a</sup>, <sup>b</sup> stand for different isomers of anthocyanins, <sup>a</sup> was eluted at earlier retention time when compared to <sup>b</sup>.

Peak	Anthocyanin pattern	N67×P		N67×PF		S29×P		S29×PF	
		Bulk-	Bulk+	Bulk-	Bulk+	Bulk-	Bulk+	Bulk-	Bulk+
1	<b>Cy-3-glucoside</b>	-	2.19	-	1.71	-	2.39	-	1.34
2	<b>Pn-3-hexose<sup>a</sup></b>	-	2.76	-	2.07	-	3.20	-	1.66
3	Cy-3-acetyl-hexose <sup>a</sup>	-	0.51	-	0.34	-	0.51	-	0.29
4	Cy-3-acetyl-hexose <sup>b</sup>	-	1.72	-	0.82	-	1.65	-	0.63
5	Pn-3-acetyl-hexose <sup>a</sup>	-	0.41	-	0.27	-	0.50	-	0.35
6	Pn-3-acetyl-hexose <sup>b</sup>	-	0.65	-	0.35	-	0.63	-	0.29
7	Cy-3-malonyl-hexose <sup>a</sup>	-	0.40	-	0.33	-	0.46	-	0.26
8	<b>Cy-3-malonyl-hexose<sup>b</sup></b>	-	3.00	-	2.25	-	2.96	-	1.73
9	<b>Pn-3-malonyl-hexose</b>	-	4.27	-	3.35	-	5.13	-	2.78
10	Pn-3-malonyl-acetyl-hexose	-	1.38	-	1.03	-	1.34	-	0.79
11	Cy-3-coumaroyl-hexose / Cy-3-rutinoside	-	1.79	-	1.83	-	1.50	-	1.17
12	Pn-3-coumaroyl-hexose / Pn-3-rutinoside	-	1.56	-	1.78	-	1.44	-	1.20
13	Cy-3-(3,6-di-malonyl-hexose)	-	1.18	-	1.14	-	1.29	-	0.89
14	<b>Pn-3-(3,6-di-malonyl-hexose)</b>	-	2.38	-	2.15	-	2.82	-	1.64
	<b>Sum</b>	-	25.37	-	20.49	-	27.01	-	15.64

### By UV-Visible spectroscopy

The total monomeric anthocyanin results measured by the pH differential method are provided in Figure 17. The monomeric anthocyanin levels of wheat samples varied highly between yellow and purple grains. The yellow-grained wheat samples displayed the concentrations in the range of 5.22 - 19.64  $\mu\text{g/g}$  DW, while purple-grained wheat materials varied from 18.89 to 273.16  $\mu\text{g/g}$  DW. Besides, the concentration of anthocyanins also varied among different pigmented-wheat genotypes. The parental line ‘PF’ had higher anthocyanin content than ‘Purple’ grown at both plots in the field in 2015. For the F<sub>3</sub> offspring, bulk+ from the cross of ‘S29’ and ‘PF’ were observed with the lowest concentration in two years. The anthocyanin levels obtained from the same pigmented wheat grains differed significantly in growth conditions. Wheat with pigmented grains planted under greenhouse condition in 2014 process significantly higher amount of total anthocyanins when compared with wheat grown in the field in 2015. There was no significant difference of total monomeric anthocyanin from wheat samples grown between two locations in 2015.



**Figure 17. Total monomeric anthocyanin levels of wheat samples.** Anthocyanins of wheat grains were extracted with 99% methanol acidified with 1% HCl solution. The absorbance at pH 1.0 and 4.5 was measured at 515 nm and 700 nm by plate photometer to determine the total anthocyanin content. Relative quantification of total anthocyanin contents was calculated by the pH differential method according to cyanidin-3-glucoside standard. (A) The wheat samples including bulk- without anthocyanins and bulk+ with anthocyanins were grown in the greenhouse in 2014, (B, C) while parental wheat lines were also grown at two plots in the field in 2015. Means and standard errors from three technical replicates in 2014 and three biological replicates in 2015 were shown respectively. The color of the bars was responding to the wheat grain color, for example, yellow-grained samples were labeled in yellow, while purple-grained samples were labeled in purple. Trace amount of anthocyanins was also observed in yellow-grained wheat samples.

### 3.2.2. Comparison of other seed constituents

The main aim of this section is to validate whether the seed constituents except for anthocyanins from bulk materials were well equilibrated or not. Therefore, the principal components such as most primary metabolites, free amino acids, common microelements and macroelements from wheat grains were compared between anthocyanin-free materials (bulk-) and anthocyanin-containing materials (bulk+).

#### 3.2.2.1. Primary metabolites

To evaluate the difference in the composition of primary metabolites between bulk- and bulk+ samples, gas chromatography coupled to mass spectrometry (GC-MS) was applied for conducting comprehensive metabolic profiling of extracts derived from polar fractions. About 50 different metabolites including amino acids, organic acids, sugars, and sugar alcohols *etc.* with the high signal intensities from polar phase were annotated according to the unique mass spectrum and retention time index (RI) (Table 12).

During peak assignment, the retention times of each chromatogram were corrected separately by the calculation of RI for all compounds. As shown in Appendix 1, primary compounds in wheat seed extract were relatively quantified with the unit of peak area/g. The quantitative estimation of sucrose found in grains of wheat was more than 76703.9 area/g. Based on the results sucrose was the highest amount in all tested wheat materials, followed by unknown<sup>14</sup>, two sugars <sup>13</sup>C-glucose and D-Galactose, and malic acid. On contrast, the presence of pentose I was detected with the lowest level in the wheat grains. In general, primary metabolites of wheat materials grown at plot 2 were found to be higher as compared to materials grown at plot 1. The statistical results of parental lines and the corresponding offspring in 2015 are summarized in Table 13. Most significant differences in the content of specific metabolites were observed between the parental lines. Even though the quantitative estimation of several primary metabolites showed significant differences between bulk- and bulk+ wheat offspring, there was no constant result found within bulk materials from different mapping populations.



**Table 12. Putative identity of primary metabolites present in wheat grains.** Metabolites were identified depend on the comparison of observed mass spectra and retention times (RT) with reference libraries such as the NIST library and the Golm Metabolome Database (GMD).

Comp. Nr.	RT [min]	Specific ion [m/z]	Identity	Comp. Nr.	RT [min]	Specific ion [m/z]	Identity
1	7.01	147	Lactic acid	26	22		Unknown <sup>8</sup>
2	8.02	116	L-alanine	27	22.07	116.09	Asparagine
3	10.9	144.12	L-valine	28	22.71		Unknown <sup>9</sup>
4	12.34	314.09	Phosphoric acid	29	22.95		Unknown <sup>10</sup>
5	12.46	205	Glycerol	30	23.26	217.1	Pentose alcohol I
6	12.99	142.1	L-proline	31	24.09		Unknown <sup>11</sup>
7	13.24	174.11	Glycine	32	24.31		Unknown <sup>12</sup>
8	13.48	247.09	Succinic acid	33	25.29	273.1	Citric acid
9	14.45	245.1	Fumaric acid	34	26.4	437.2	D-fructose II
10	14.69	204.12	L-serine	35	26.59	437.2	D-fructose III
11	14.9	141.1	Unknown <sup>1</sup>	36	26.82	204.1	<sup>13</sup> C-glucose <sup>1</sup>
12	15.35	217.99	L-Threonine	37	26.88	191.1	D-Galactose
13	17.04		Unknown <sup>2</sup>	38	27.18	204.1	<sup>13</sup> C-glucose <sup>2</sup>
14	17.91	233.1	Malic acid	39	27.25	319.15	Hexose I (glucose)
15	18.14		Unknown <sup>3</sup>	40	27.53		Glucitol
16	18.57	156.08	Pyroglutamic acid	41	27.64	319.15	Hexose II (D-glucose)
17	18.67	218.1	Aspartic acid	42	30.54	305.13	Myo-inositol
18	18.85		GABA	43	32.63	202.1	L-Tryptophan
19	18.95		Unknown <sup>4</sup>	44	35.46	194.14	Glucuronic acid
20	19.45		Unknown <sup>5</sup>	45	39.52	342.30	Sucrose
21	20.78		Unknown <sup>6</sup>	46	40.9	361	Maltose I
22	20.99	246.14	L-glutamic acid	47	40.94	361	Maltose II
23	21.04	218.1	L-phenylalanine	48	41.27	361	Trehalose
24	21.18		Unknown <sup>7</sup>	49	49.47		Unknown <sup>13</sup>
25	21.84	103.06	Pentose I	50	49.67		Unknown <sup>14</sup>

## 44 | RESULTS

**Table 13. Summary of comparison between yellow and purple wheat samples in 2015.** Wheat samples were grown in the field at two locations (plot 1, 2). The comparison among parental lines and offspring were analyzed by one-way ANOVA using SigmaPlot 13.0. – stand for no significant difference. \*, \*\*, \*\*\* stand for significant at  $p < 0.05$ ,  $p < 0.01$ ,  $p < 0.001$  respectively. Star symbols labeled in red indicated the significantly higher concentrations in purple-grained samples than yellow-grained samples, while green star symbols indicated the significantly lower levels in purple-grained samples. Number 1-50 referred to the putative identification in Table 12. Compound 20 and 28 were detected in trace amounts in wheat samples.

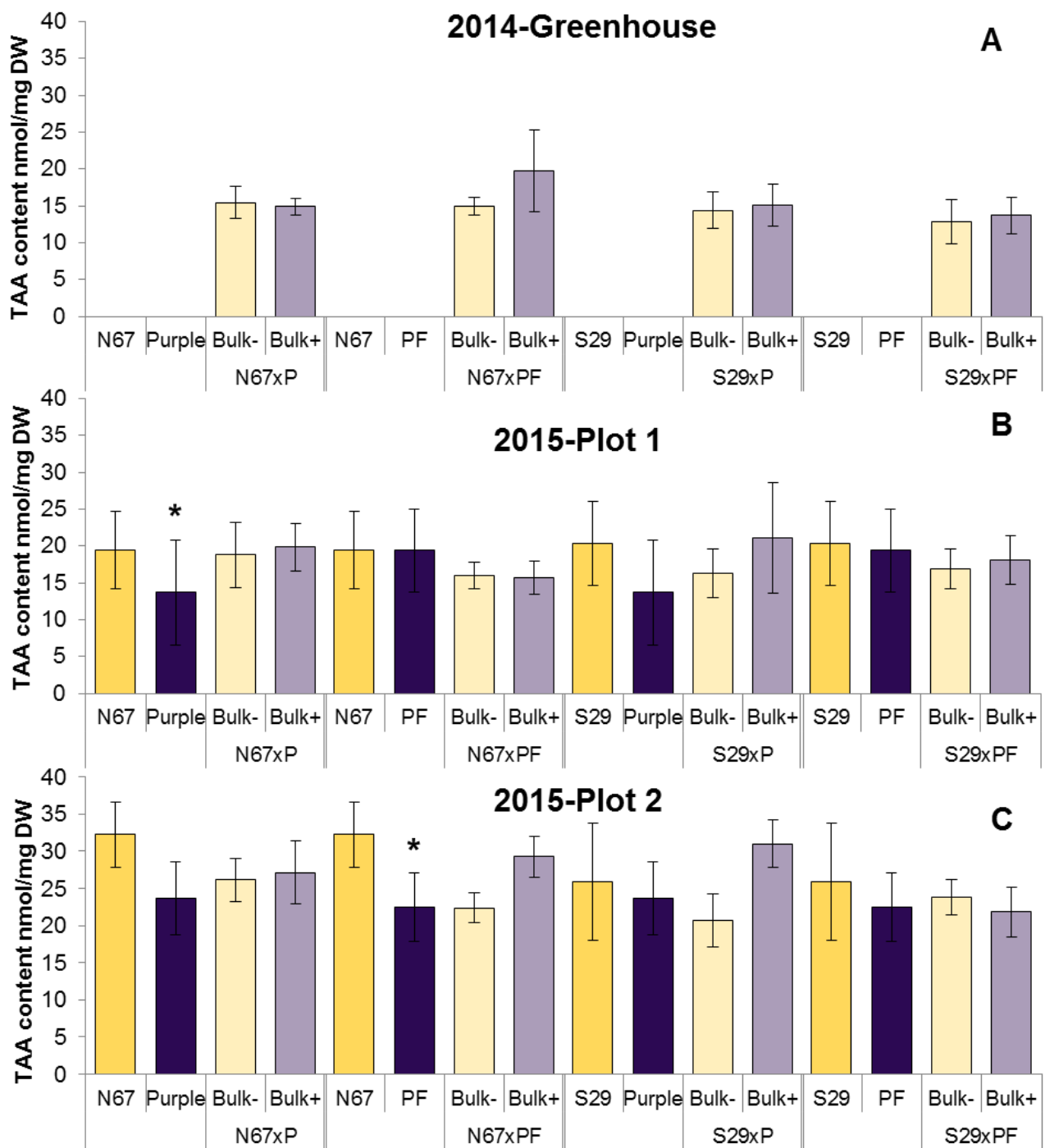
Comp. Nr.	S29 vs Purple		S29×P Bulk- vs Bulk+		S29 vs PF		S29×PF Bulk- vs Bulk+		N67 vs Purple		N67×P Bulk- vs Bulk+		N67 vs PF		N67×PF Bulk- vs Bulk+	
	Plot 1	Plot 2	Plot 1	Plot 2	Plot 1	Plot 2	Plot 1	Plot 2	Plot 1	Plot 2	Plot 1	Plot 2	Plot 1	Plot 2	Plot 1	Plot 2
1	-	-	-	-	-	-	-	-	-	-	-	-	-	*	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	**	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	***	-	-	-	-	-	-	*	-
6	-	-	-	-	-	-	-	-	**	-	-	-	**	-	-	-
7	-	-	-	-	-	-	-	-	*	-	-	-	-	-	-	-
8	*	***	-	*	-	***	-	**	-	-	-	-	-	-	-	-
9	-	***	-	*	**	-	-	-	-	**	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	**	-	-	-	-	-	**	-
11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	*	-	-	-	-	**	-	-
13	*	-	-	-	-	-	-	*	-	-	-	-	-	-	-	-
14	-	*	-	-	-	*	-	*	-	-	-	-	**	-	-	-
15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
16	-	-	-	*	-	-	-	-	**	-	-	-	-	-	-	-
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48	**	-	*	-	*	*	-	-	*	-	-	-	*	-	-	-
49	-	*	-	*	***	-	-	-	*	-	-	-	-	-	-	-
50	-	**	-	-	-	***	-	-	*	-	-	-	-	***	-	-

### 3.2.2.2. Free amino acids

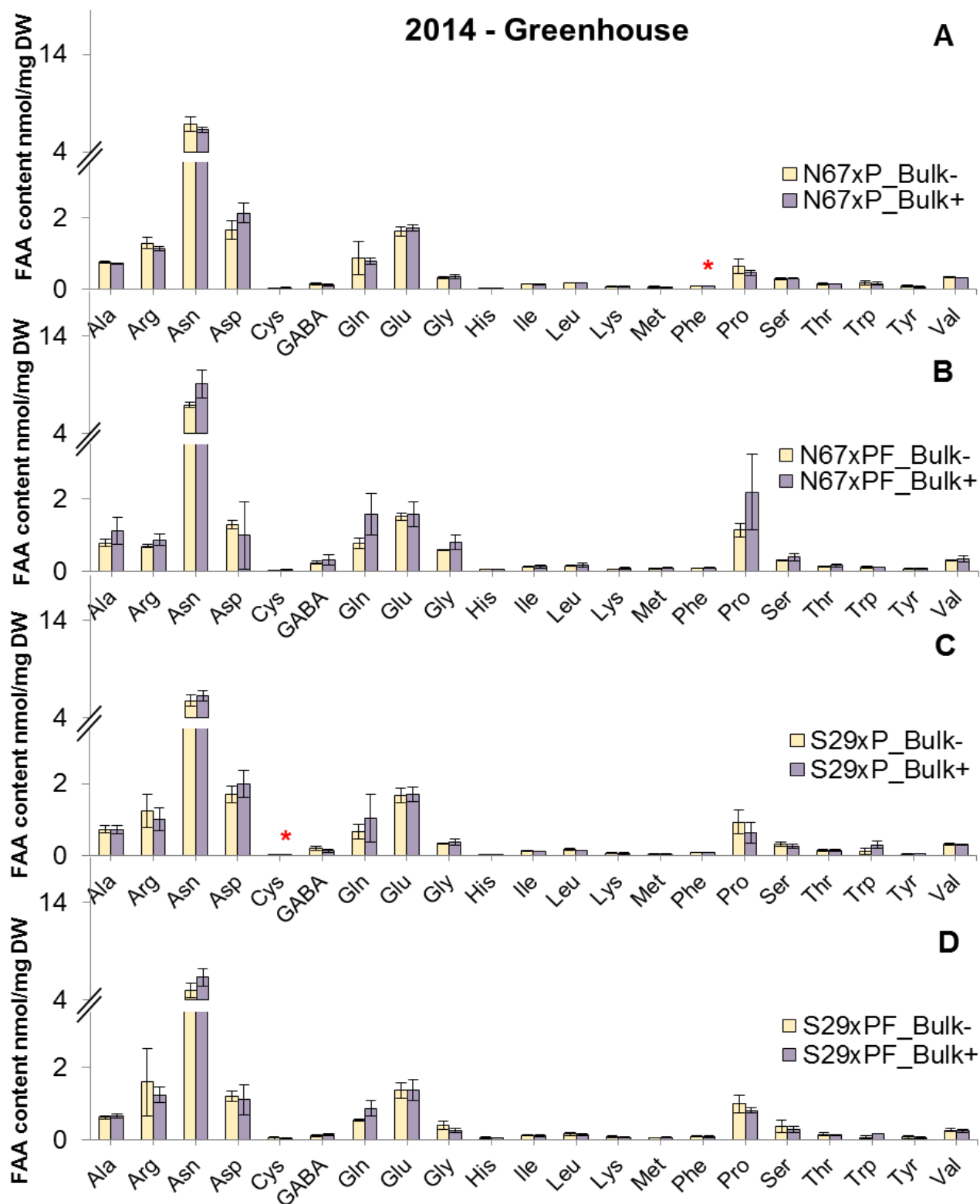
The amino acid content of wheat grains was determined and the compositional data are displayed in Figure 18-21. The wheat parental lines and bulks did not differ in the amino acid pattern but varied in content. Among 22 analytes measured, L-norvaline (Nor) was the only amino acid detected in trace amount of all samples tested.

The total amino acid content shown in Figure 18 indicated that wheat harvested in the greenhouse (2014) with the range of 12.9-19.7 nmol/mg dry weight (DW) were lower than at both plots in the field (2015). The total amino acid of wheat grains grown at plot 1 ranged from 13.8-20.3 nmol/mg DW, while the sum of amino acid in samples grown at plot 2 was found between 20.7 to 32.2 nmol/mg DW. No significant difference occurred between the bulk- and bulk+ materials in the total amino acid content, even though the sum of amino acids from parental line 'Purple' at plot 1 and 'PF' at plot 2 showed significantly lower concentrations than those from yellow parent 'N67'.

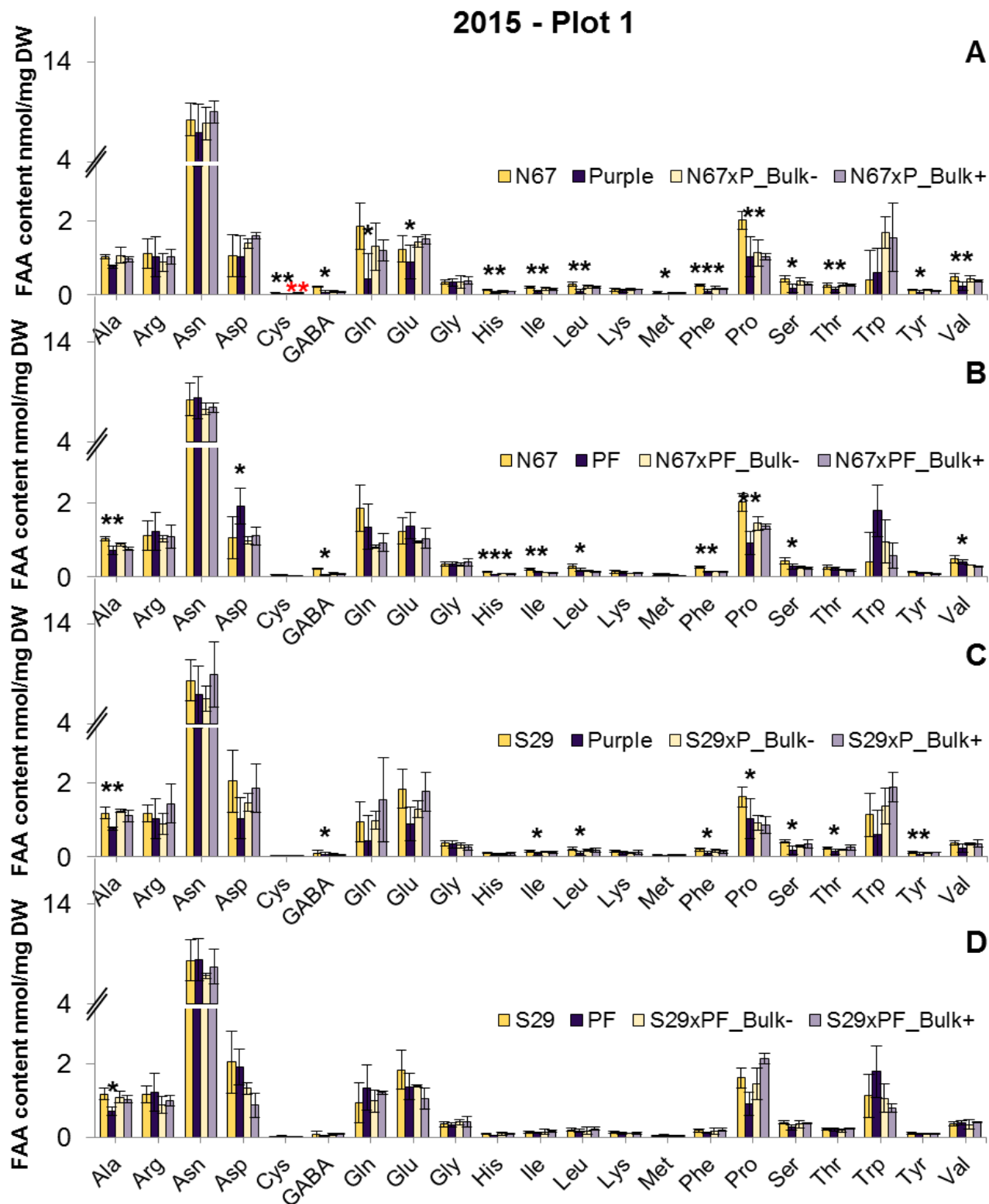
The results of individual amino acid are presented in Figure 19-21. L-asparagine (Asn) was the most abundant free amino acid determined in all the wheat samples for both years. Under greenhouse condition, the main amino acids were Asn, L-aspartic acid (Asp), L-glutamic acid (Glu), L-arginine (Arg) and L-alanine (Ala). Additionally, statistically significant difference in the concentration of L-phenylalanine (Phe) and L-cysteine (Cys) was observed between bulk- and bulk+ crossing from 'N67', 'S29' with 'Purple' respectively. On the other hand, L-glutamine (Gln) and L-proline (Pro) were also found with relatively high amounts in the field conditions. The quantitative difference of most amino acids was observed between different-colored parental wheat lines at both plots. For plot 1, Cys were detected with the significantly higher concentration in bulk+ than in bulk- which is crossed from 'N67' and 'Purple'. However, more amino acids including Arg, Asn, Cys, Gln and L-methionine (Met) behaved significant difference between bulk- and bulk+ materials which grown at plot 2.



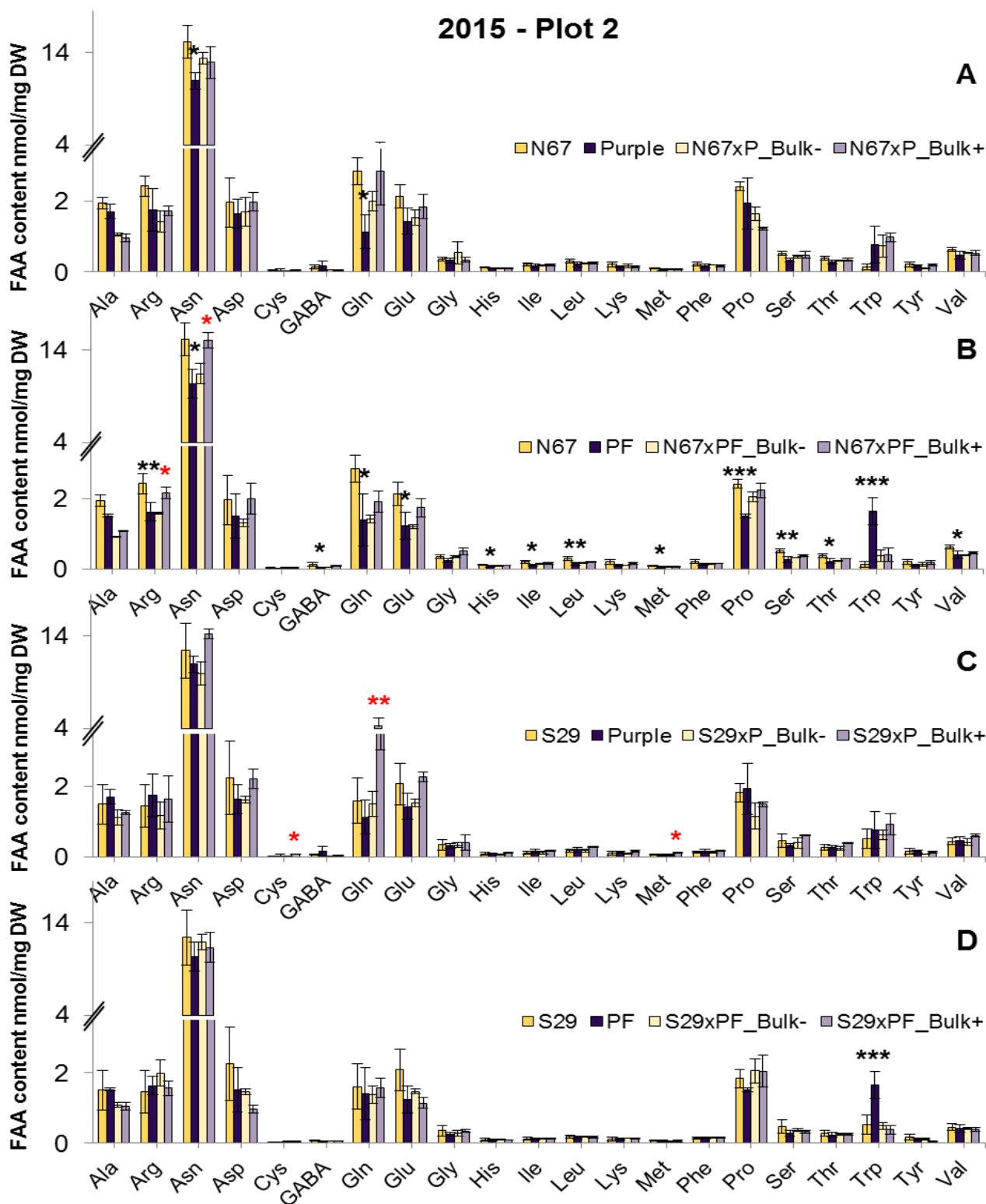
**Figure 18. Total concentrations of free amino acid (nmol/mg DW) in wheat materials.** After derivatization, amino acids were separated and identified by UPLC coupled to fluorescence. The wheat samples including bulk- without anthocyanins and bulk+ with anthocyanins were grown in the greenhouse in 2014, while parental wheat lines were also cultivated in the field in 2015. Means and standard errors from three technical replicates in 2014 and three biological replicates in 2015 are shown respectively. Statistical significances between parental lines and the corresponding bulk- and bulk+ offspring were analyzed by one-way ANOVA using SigmaPlot 13.0. \* significant at  $p < 0.05$ .



**Figure 19. Concentrations of individual amino acid (nmol/mg DW) in wheat bulk materials in 2014.** After derivatization, amino acids were separated and identified by UPLC coupled to fluorescence. (A-D) The bulk samples including bulk- without anthocyanins and bulk+ with anthocyanins crossing from N67×P, N67×PF, S29×P and S29×PF were grown in the greenhouse. Means and standard errors from three technical replicates are shown in each case. Statistical significances between bulk- and bulk+ were analyzed by T-test using SigmaPlot 13.0. \* significant at  $p < 0.05$ . \* labeled in red indicate the significant difference found between bulk- and bulk+.



**Figure 20. Concentrations of individual amino acid (nmol/mg DW) in wheat materials at plot 1 in 2015.** After derivatization, amino acids were separated and identified by UPLC coupled to fluorescence. The parental lines and bulk samples were grown in the field. Means and standard errors from three biological replicates are shown in each case. Statistical significances were analyzed by one-way ANOVA using SigmaPlot 13.0. \* significant at  $p < 0.05$ , \*\* significant at  $p < 0.01$ , \*\*\* significant at  $p < 0.001$ . \* labeled in red indicate the significant difference found between bulk- and bulk+.



**Figure 21. Concentrations of individual amino acid (nmol/mg DW) in wheat materials at plot 2 in 2015.** After derivatization, amino acids were separated and identified by UPLC coupled to fluorescence. The parental lines and bulk samples were grown in the field. Means and standard errors from three biological replicates are shown in each case. Statistical significances were analyzed by one-way ANOVA using SigmaPlot 13.0. \* significant at  $p < 0.05$ , \*\* significant at  $p < 0.01$ , \*\*\* significant at  $p < 0.001$ . \* labeled in red indicate the significant difference found between bulk- and bulk+.

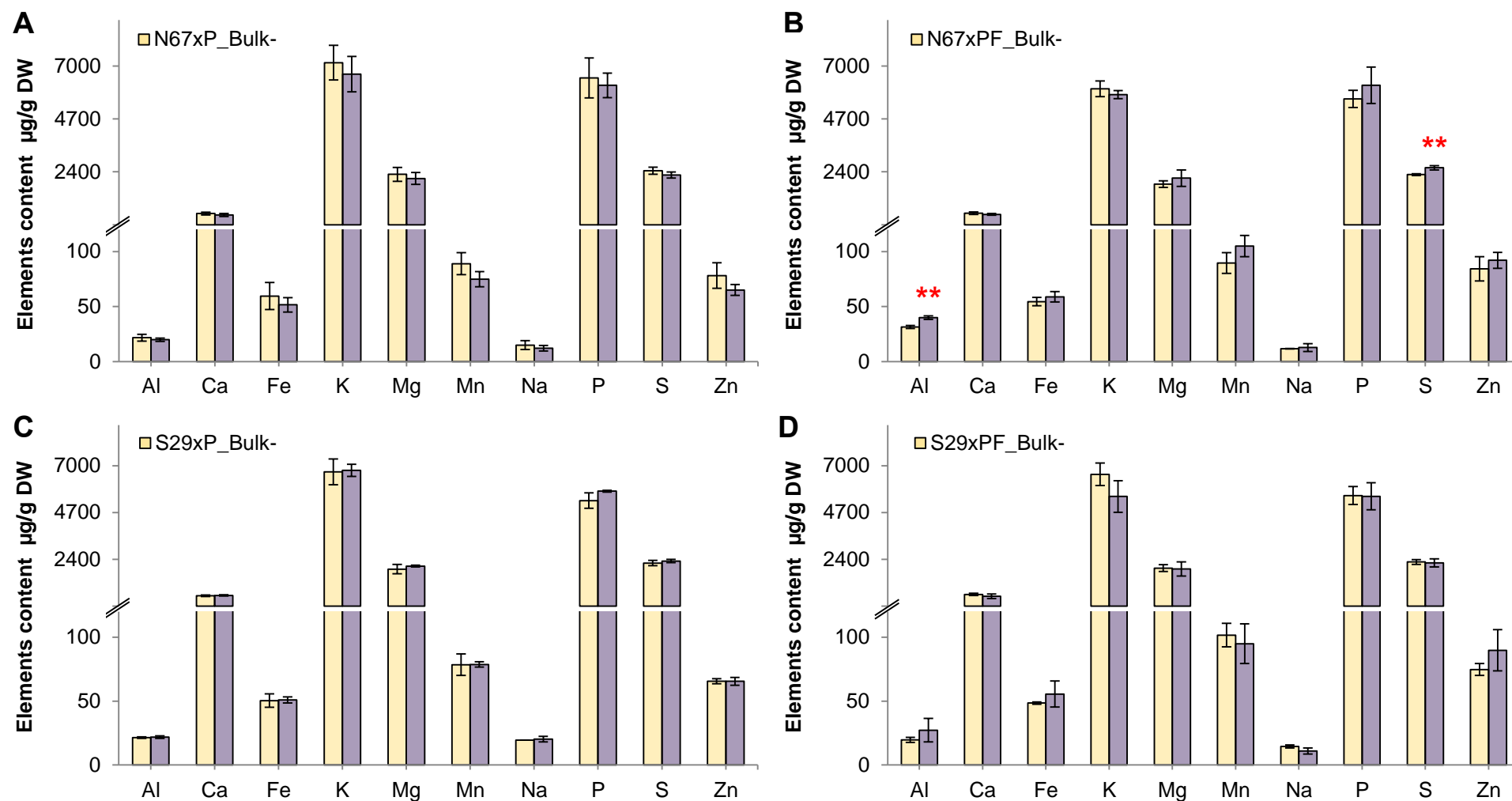
**3.2.2.3. Elements**

The concentrations of elements including aluminum (Al), boron (B), calcium (Ca), cadmium (Cd), copper (Cu), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn), molybdenum (Mo), sodium (Na), nickel (Ni), phosphorus (P), sulfur (S), selenium (Se) in the freeze-dried wheat grain powder were determined. However, 8 out of 15 elements (B, Cd, Co, Rb, Mo, Ni, Se, and Cu) were found under the limit of detection. The quantitative results for three sample preparations of the bulk- and bulk+ materials are shown in Figure 22-24. When compared with elements Al and Na ( $< 5 \mu\text{g.g}^{-1}$ ), the amounts of K and P in wheat varieties were recorded at much higher levels, following by Mg, S, Ca, Mn, Fe, and Zn.

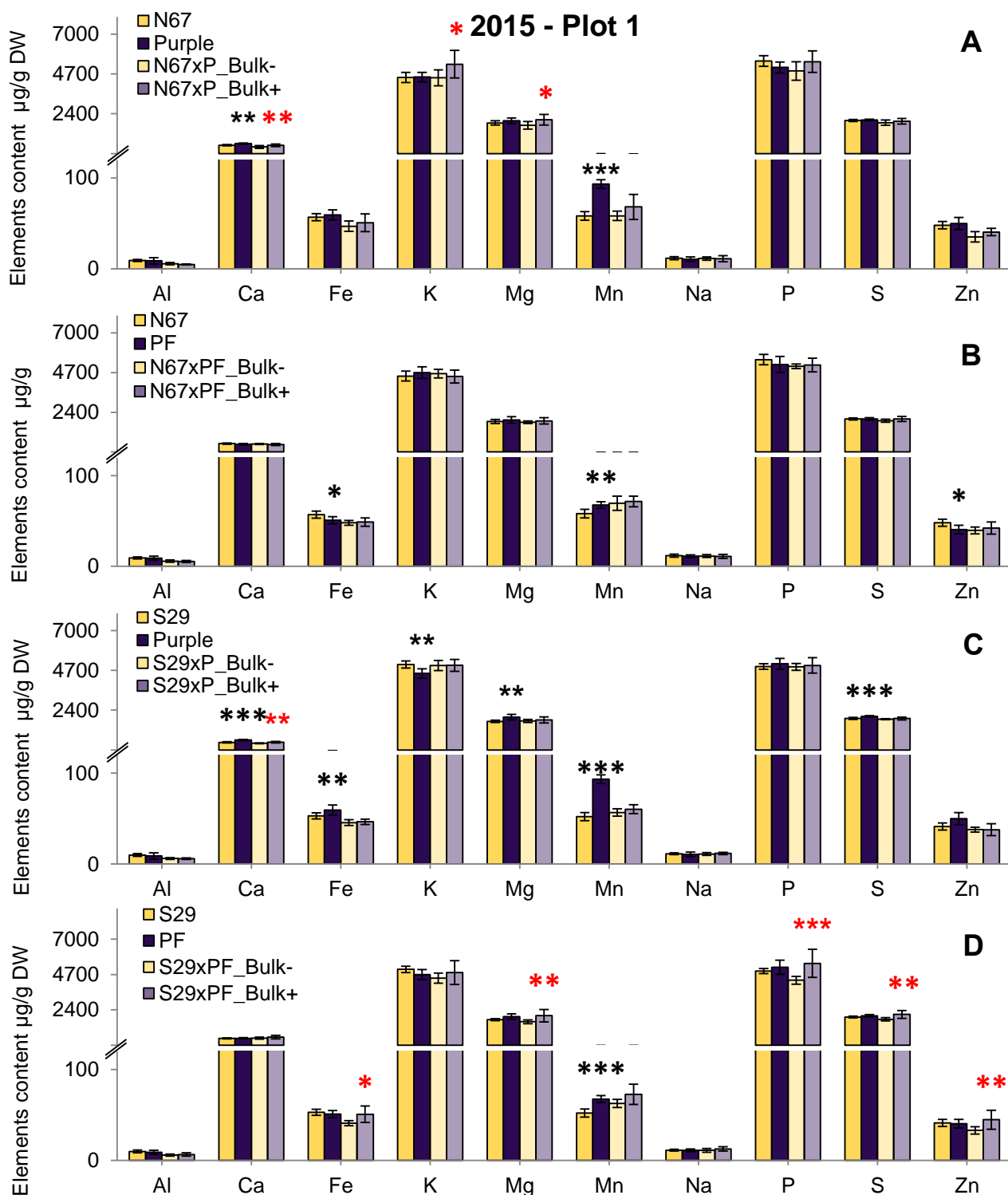
The average concentrations of each element for wheat plants grown in the greenhouse (2014) were much higher than those grown in the field (2015). In the case of K, the amount ranged from 5.5 to 7.1  $\text{mg.g}^{-1}$  in 2014, while in 2015 the measured values were between 4.2 and 5.7  $\text{mg.g}^{-1}$ . Moreover, by conducting the comparison of parental lines and bulked offspring, there were significantly higher amounts of Al and S in bulk+ than in bulk- crossing from 'N67' and 'Purple' under greenhouse condition. Several elements Ca, K, Mg, P, S and Zn from bulk+ offspring grown at plot 1 indicated higher concentrations than from bulk-, while only three elements Al, Ca and Zn were found with significant differences between bulked offspring.



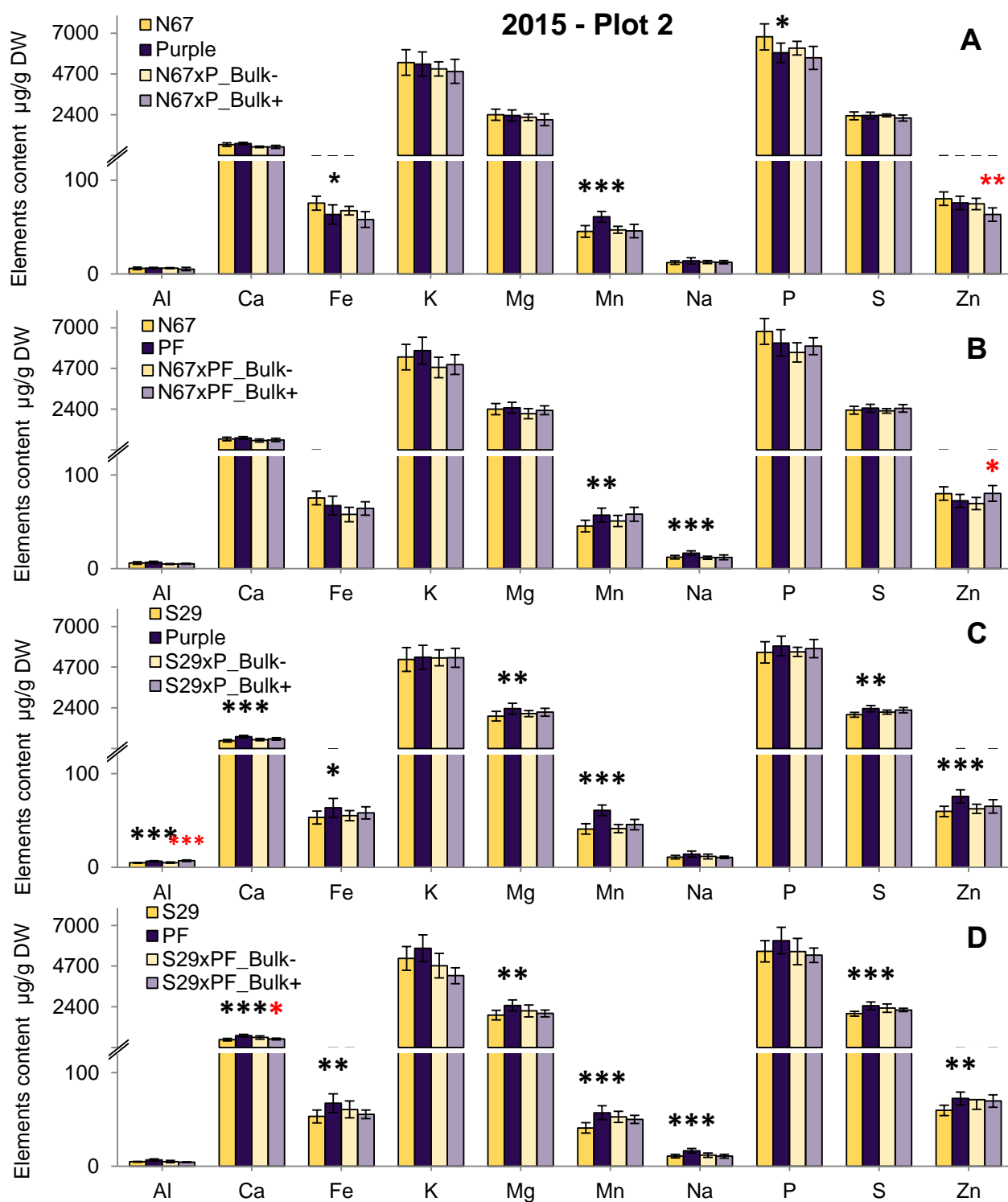
## 2014 - Greenhouse



**Figure 22. Determination of elemental contents for wheat bulk materials in 2014.** (A-D) The bulk samples including bulk- without anthocyanins and bulk+ with anthocyanins crossing from N67×P, N67×PF, S29×P and S29×PF were grown in the greenhouse. Means and standard errors from three technical replicates are shown in each case. Statistical significances between bulk- and bulk+ were analyzed by T-test using SigmaPlot 13.0. \*\* significant at  $p < 0.01$ .



**Figure 23. Determination of elemental contents for wheat materials at plot 1 in 2015.** The parental lines and corresponding bulk samples were grown in the field. Means and standard errors from three biological replicates are shown in each case. Statistical significances were analyzed by one-way ANOVA using SigmaPlot 13.0. \* significant at  $p < 0.05$ , \*\* significant at  $p < 0.01$ , \*\*\* significant at  $p < 0.001$ . \* labeled in red indicate the significant difference found between bulk- and bulk+.



**Figure 24. Determination of elemental contents for wheat materials at plot 2 in 2015.** The parental lines and corresponding bulk samples were grown in the field. Means and standard errors from three biological replicates are shown in each case. Statistical significances were analyzed by one-way ANOVA using SigmaPlot 13.0. \* significant at  $p < 0.05$ , \*\* significant at  $p < 0.01$ , \*\*\* significant at  $p < 0.001$ . \* labeled in red indicate the significant difference found between bulk- and bulk+.

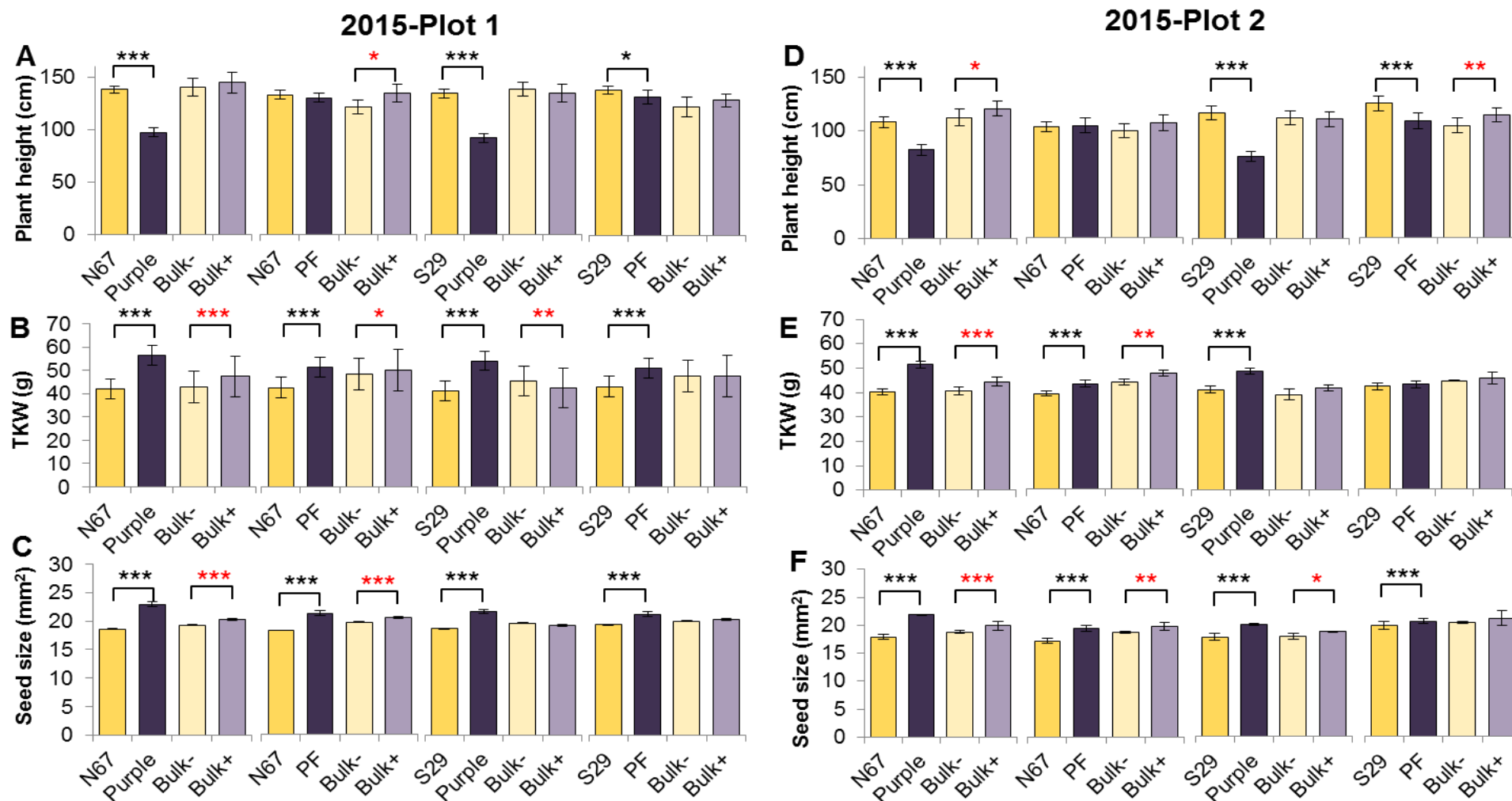
### **3.3. Evaluation of growth parameters, seed yield components and germination of wheat parental lines and offspring lines**

#### **3.3.1. Comparison of growth and grain yield parameters**

The major yield component characters such as days to flowering, plant height (cm), spike length (cm), spikelet number/grain number/grain weight per spike, thousand kernels weight (g), seed size (mm<sup>2</sup>) were measured for both plots in 2015. Mean values of different traits were compared between two plots and within wheat samples. In general, when compared with plot 2, more contrasting yield parameters were measured between non-pigmented and pigmented wheat samples at plot 1.

Plant growth was measured in terms of days to flowering and plant height. As indicated in Table 14, the days to flowering of wheat cultivars varied from 82-89. The flowering date of pigmented wheat cultivar 'Purple' grown at plot 2 were observed 6 days' earlier than yellow parental lines 'N67' and 'S29'. By contrast, the other purple-grained wheat cultivar 'PF' was found with the longest days-to flowering. The data presented in Figure 25A, D revealed that plant height was mainly affected among various wheat cultivars and slightly influenced by soil conditions. For example, yellow wheat cultivars 'N67' and 'S29' were significantly taller than purple-grained varieties 'Purple' and 'PF' for both locations. Besides, all wheat plants grown at plot 1 were 10 - 30 cm higher than grown at plot 2. However, the plant heights of wheat F<sub>2</sub> population did not differ between non-pigmented and pigmented lines.

Additionally, total grain weight and the thousand kernels weight appeared higher values at plot 1 than at plot 2. Purple parental lines 'Purple' and 'PF' produced higher thousand kernels weight and bigger seed size as compared to yellow-grained lines, while bulks- and bulks+ obtained from F<sub>2</sub> families at both plots were observed with much less difference. To sum up, the comparison among wheat samples revealed that the differences in most agronomic traits were statistically significant between anthocyanin-free and anthocyanin-containing parental lines rather than offspring bulks. Furthermore, except for total grain weight and the thousand kernels weight, the other yield components did not differ between two growing locations.



**Figure 25. Comparison of plant height, TKW and seed size for wheat varieties as affected by two planting plots.** Plants were grown in the field at plot 1 (A, B, C) and plot 2 (D, E, F). Means and standard errors were calculated from three biological replicates. (A, D) Measurement of plant height (cm), (B, C) thousand kernel weight (g) and (C, F) seed size (mm<sup>2</sup>) were compared between non-pigmented and pigmented wheat materials. Statistical significances were analyzed by one-way ANOVA using SigmaPlot 13.0. \*\*\* stands for significant difference ( $P < 0.001$ ); \*\* stands for significant difference ( $P < 0.01$ ); \* stands for significant difference ( $P < 0.05$ ). \* labeled in red indicate the significant difference found between bulk- and bulk+.

**Table 14. Yield component characters of wheat genotypes in the field in 2015.** Values are presented as the mean of three independent determinations. Except for days to flowering (DF) and total yield (TY) were measured with three technical replicates, the other parameters consisted of 20 samples for each technical replicate. DF, Days to flowering; TY, Total yield; SPL, Spike length; SN, Spikelet number per spike; GN, Grain number per spike; GW, Grain weight per spike; SL, Seed length; SW, Seed width. Statistical significances were analyzed by one-way ANOVA using SigmaPlot 13.0. \*\*\* stands for significant difference (P<0.001); \*\* stands for significant difference (P<0.01); \* stands for significant difference (P<0.05). \* labeled in red indicate the significant difference found between bulk- and bulk+.

Location	Wheat materials	DF (d)	TY (g)	SPL(cm)	SN	GN	GW(g)	SL (mm)	SW (mm)
Plot 1	S29	84	194.37	9.8	17***	43	1.86***	7.32***	3.60***
	Purple	84	281.56	9.8	15	42	2.38	7.64	3.96
	'S29' × 'Purple' bulk-	86**	475.33	11.1	18	42	2.00	7.38**	3.72*
	'S29' × 'Purple' bulk+	84	522.00	10.7	18	44	1.97	7.59	3.65
	S29	84	194.37	9.8***	17	43	1.86	7.32***	3.60***
	PF	89	209.97	11.0	18	41	2.10	7.55	3.79
	'S29' × 'PF' bulk-	87	527.76	10.2	17	43	2.04	7.37	3.70
	'S29' × 'PF' bulk+	84	559.14	10.0	16	41	1.98	7.24	3.77
	N67	86	199.02*	10.8***	20***	41	1.72***	6.79***	3.59***
	Purple	84	281.56	9.8	15	42	2.38	7.64	3.96
	'N67' × 'Purple' bulk-	84	422.16	11.0	18**	39**	1.69***	7.27	3.51***
	'N67' × 'Purple' bulk+	85	552.90	11.3	19	43	2.06	7.29	3.68
	N67	86	199.02	10.8	20***	41	1.72***	6.79***	3.59***
	PF	89	209.97	11.0	18	41	2.10	7.55	3.79
	'N67' × 'PF' bulk-	83	666.16	10.5	18	48*	2.30	7.23	3.69***
	'N67' × 'PF' bulk+	83	595.25	10.4	18	44	2.16	7.20	3.81
Plot 2	S29	88*	372.67*	9.7	17***	48*	2.11	7.15***	3.59***
	Purple	82	172.32	9.5	14	44	2.30	7.47	3.86
	'S29' × 'Purple' bulk-	86	428.84	10.8	18	46	1.94	7.25*	3.57
	'S29' × 'Purple' bulk+	86	375.74	10.6	18	47	2.09	7.42	3.66
	S29	86	428.84*	10.8	18	46**	1.94*	7.25***	3.57
	PF	89	176.19	10.8	18	43	1.91	7.34	3.63
	'S29' × 'PF' bulk-	85	473.75	9.6	16	42	1.93	7.15	3.70
	'S29' × 'PF' bulk+	83	483.63	9.4	16	41	1.92	7.26	3.75
	N67	88***	150.58	9.9**	19***	42	1.71***	6.69***	3.52***
	Purple	82	172.32	9.5	14	44	2.30	7.47	3.86
	'N67' × 'Purple' bulk-	86	417.85*	10.4	18	45	1.87	7.06*	3.51***
	'N67' × 'Purple' bulk+	85	504.43	10.7	18	43	1.94	7.21	3.64
	N67	88	150.58	9.9***	19	42	1.71	6.69***	3.52***
	PF	89	176.19	10.8	18	43	1.91	7.34	3.63
	'N67' × 'PF' bulk-	85	553.72*	10.2	18	51	2.32	7.07	3.64***
	'N67' × 'PF' bulk+	85	423.41	10.1	17	47	2.31	7.18	3.78

### 3.3.2. Comparison of seed germination

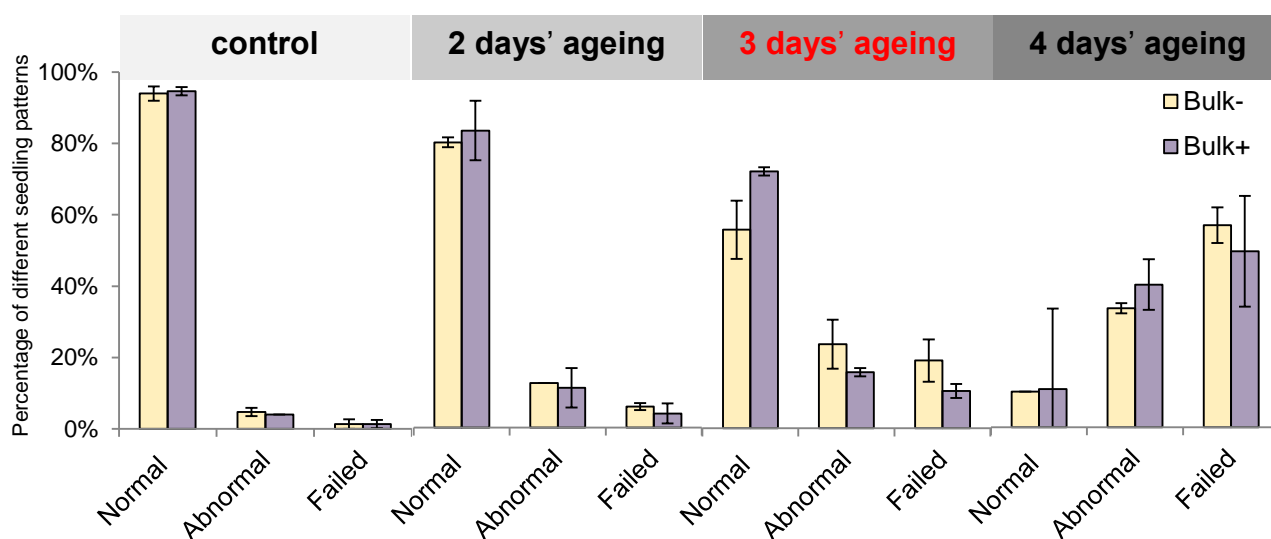
The standard germination test is commonly used to evaluate seed quality and predict seed lot performance before growing in the field. However, because freshly harvested seeds often showed high germination without variation under ambient condition, the accelerated ageing test was utilized to distinguish high vigor seed lots from low vigor lots in short periods. The first step is to determine

the duration of ageing treatment with high temperature and high relative humidity for shortening the seed deterioration process and avoid seed death at the same time.

### 3.3.2.1. Determination of accelerated ageing period

In order to find the optimal treating time, bulk- and bulk+ materials crossed from yellow-grained wheat variety ‘N67’ and purple-grained wheat variety ‘PF’ in both two plots were used for the artificial ageing test. Three accelerated ageing treatments with different processing time (2 days, 3 days and 4 days) were compared after adjusting seed moisture content to 18%.

All the seed lots showed almost perfect germination percentages (around 100%) under control condition, but the normal seedlings of wheat seeds decreased gradually with the increasing ageing period. The other characteristics of seed lots were presented in Figure 26. There was an approximate 5% reduction after 2 days’ ageing treatment when compared to the control. But, in contrast, the number of normal seedlings substantially declined after 4 days’ ageing treatment. For this reason, 3 days’ ageing was the optimal processing time to distinguish the possible effects from different colored offspring. When comparing the germination results of bulk- and bulk+, there was only slight difference found after 3-day ageing treatment between bulk materials grown at plot 1. Nevertheless, bulk- material grown at plot 2 had a significantly lower germinated percentage than bulk+ material. Therefore, the accelerated ageing period of 3 days was applied in further experiments.



**Figure 26. Expression for wheat offspring with different periods of ageing treatments.** Percentages of normal seedlings, abnormal seedlings and dead seeds under control condition and 2 days’, 3 days’ and 4 days’ artificial ageing treatments. Means and standard errors were calculated from three biological replicates, each replicate included 100 seeds. Normal, Abnormal and Failed stand for normal seedlings, abnormal seedlings, and dead seeds respectively.

### **3.3.2.2. Standard germination test after experimental ageing**

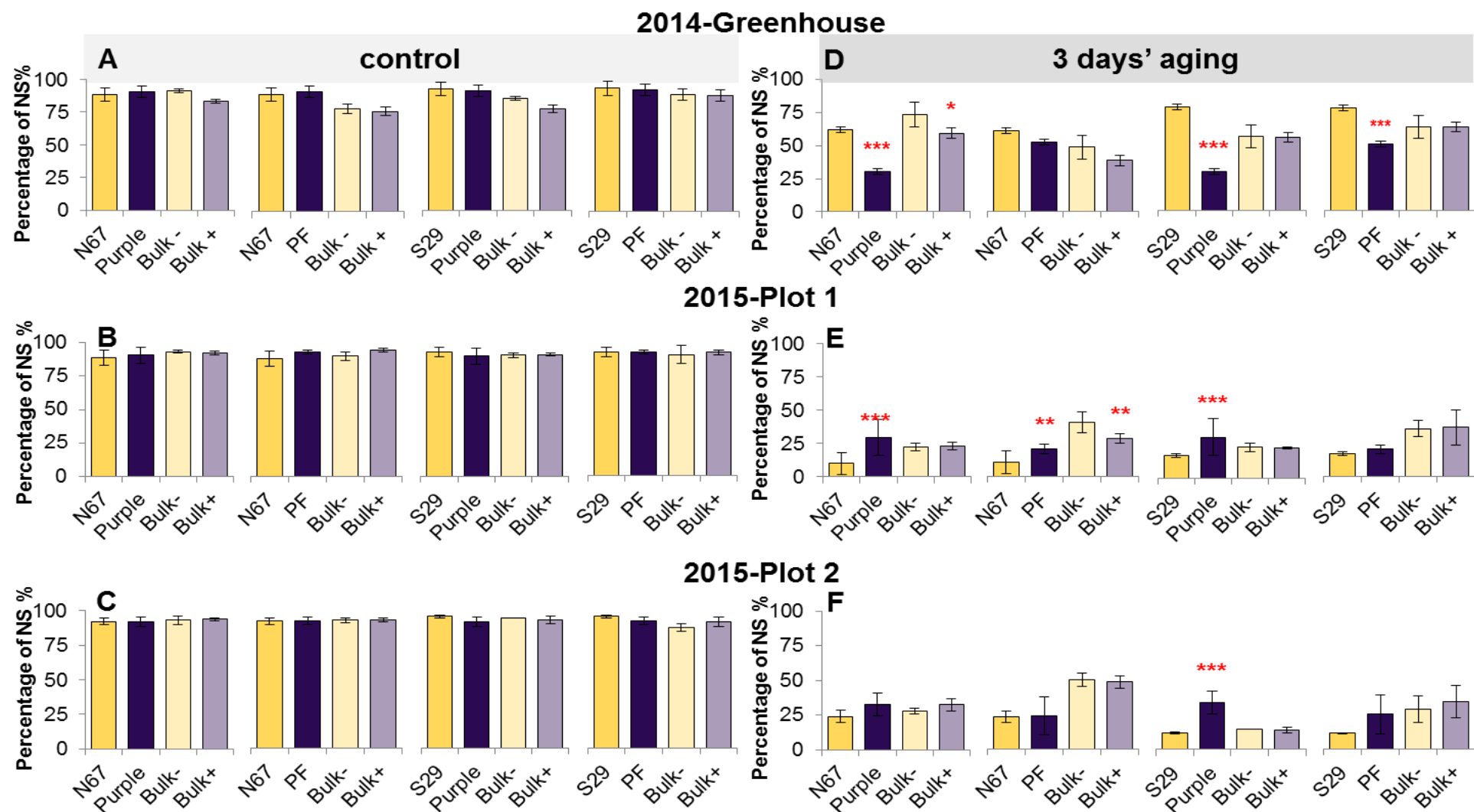
The tested population consisted of 4 groups, in which each included one purple-grained wheat cultivar containing anthocyanins, one yellow-grained cultivar without anthocyanins, their offspring bulk- (anthocyanin-free) and bulk+ (anthocyanin-containing). In total, 4 parental wheat cultivars and 8 offspring lines were investigated. Germination tests of wheat seeds harvested in 2014 (greenhouse) and 2015 (two plots in the field) were conducted before and after 3 days' ageing treatment. Only normal grown seedlings were taken into account, while germinated seeds grown abnormally were excluded before calculation. The results are shown in Figure 27.

In general, the seed lots of all wheat varieties showed high percentages of initial germination (IG) with the mean around or above 80%. The initial germination of wheat grown in the field (2015) was 4% - 16% higher than wheat grown in the greenhouse (2014). On the other hand, the differences of IG of wheat seeds were slight between two plots in the field. When compared IG between different colored parental lines and their offspring respectively, there were also not significant differences between corresponding seed lots.

Accelerated ageing treatment had a dramatic effect on the performance of all seed lots and reduced the mean germination within the range of 10% - 52% for the seeds grown in the field (2015). However, the effects from ageing process on AAG in seeds planted in the greenhouse (2014), ranging from 31% to 82%, were not as evident as the results for the seeds harvested in the field in 2015. In 2014, the minimum value of germination was observed in wheat cultivar 'Purple' (0%) and the maximum was 82% found in cultivar 'S29'. By contrast, results in 2015 showed that parental line 'N67' grown in plot 1 with the lowest germination and bulk- crossed by 'N67' and 'PF' grown in plot 2 with the highest number of germinated seeds.

To find out the ultimate impact of anthocyanin accumulation on seed germination, the comparison between non-pigmented wheat cultivars and pigmented cultivars were carried out. Significant differences in germination percentage were found between parental lines but not for the bulked offspring lines, as shown in Figure 27.





**Figure 27. Comparison of germination capacity of wheat varieties.** (A-C) Percentages of germinated wheat seeds under control condition. (B-E) Percentages of sprouted wheat seeds after 3 days' artificial ageing treatment. Plants were grown in the greenhouse in 2014 (A, D), at plot 1 (B, E) and plot 2 (C, F) in the field in 2015. Means and standard errors were calculated from three biological replicates, each replicate included 100 seeds. Differences of germinated percentages between colored- and non-colored grains were analyzed by CHI-square Test using SigmaPlot 13.0, \*\*\* stands for a significant difference at  $P < 0.001$ ; \*\* stands for a significant difference at  $P < 0.01$ ; \* stands for a significant difference at  $P < 0.05$ . \* labeled in red indicate the significant difference found between bulk- and bulk+.

### **3.4. Summary of Part A**

The major goal of first part is to investigate the possible influence of anthocyanins accumulation on wheat yields such as seed germination and other key parameters. Firstly, seed materials were created by using wheat mapping populations crossed from ‘N67’×‘Purple’, ‘N67’×‘PF’, ‘S29’×‘Purple’ and ‘S29’×‘PF’. Equal numbers of yellow and purple offspring seeds were randomly pooled for bulk- materials with only yellow seeds, while bulk+ materials include only purple wheat seeds. Afterward, the comprehensive comparison between bulk- and bulk+ materials were performed to demonstrate how well the seed compositions in wheat bulks were equilibrated. There were no constantly significant differences of major seed constituents between anthocyanins-free (bulk-) and anthocyanins-containing (bulk+) offspring, in contract to significant difference of many compounds among parental lines.

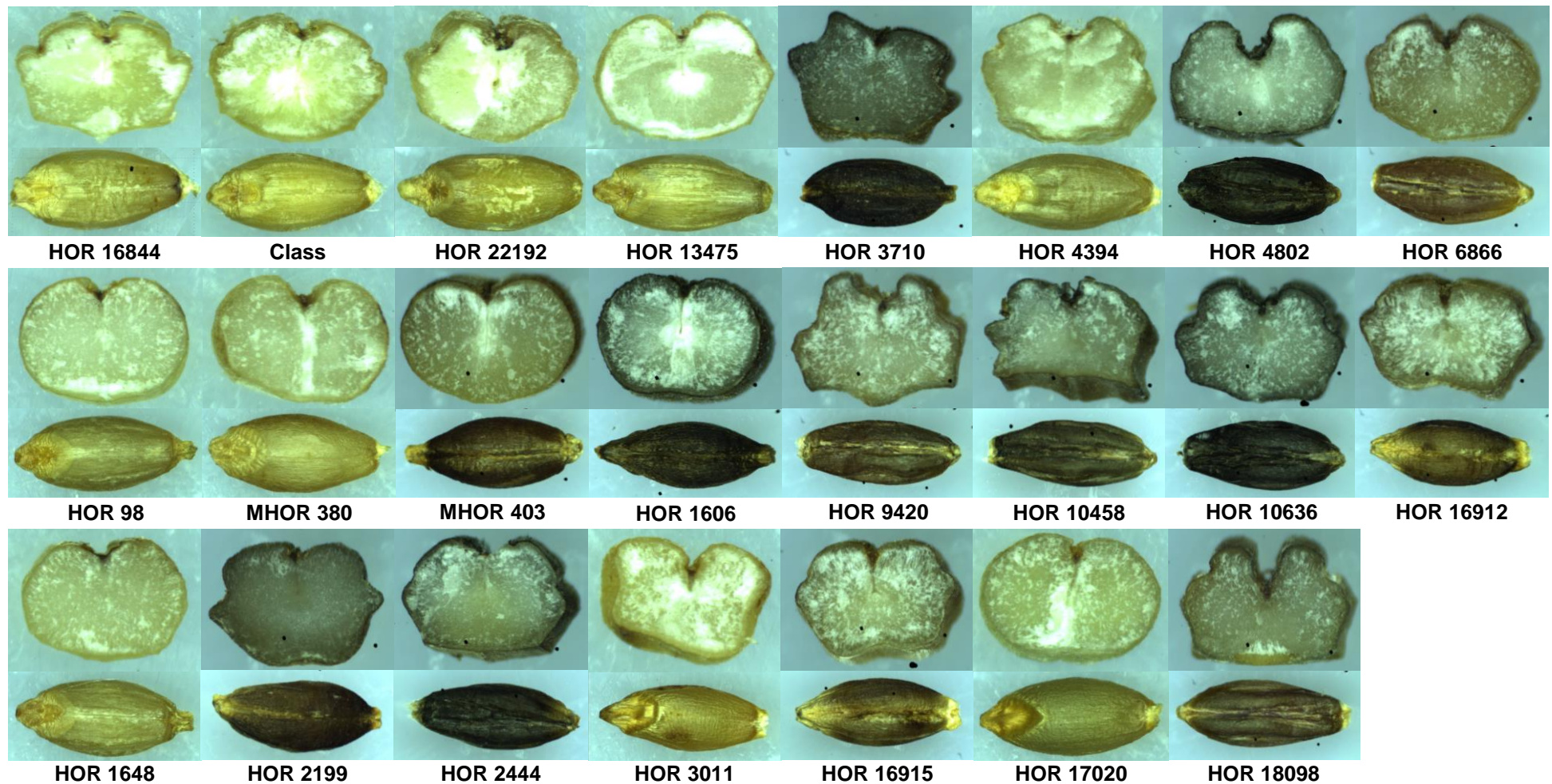
According to the comparison of yield parameters among parental lines and offspring, significant differences were observed in wheat parental genotypes, while yield related characters of bulk- were not significantly different from bulk+. After 3 days’ artificial ageing treatment, the germination capacity of wheat seeds behaved differently in 2014 and 2015. For 2014, purple-grained parental wheat varieties had less germinated seeds than yellow-grained wheat cultivar ‘S29’. However, an opposite result was found in wheat grown at plot 2 in 2015. When compared the germinated bulk materials, anthocyanin-containing bulks did not differ with anthocyanin-free bulks in both years.

## ***Part B. Understanding the metabolic and molecular mechanisms controlling barley grain pigmentation at different developmental stages***

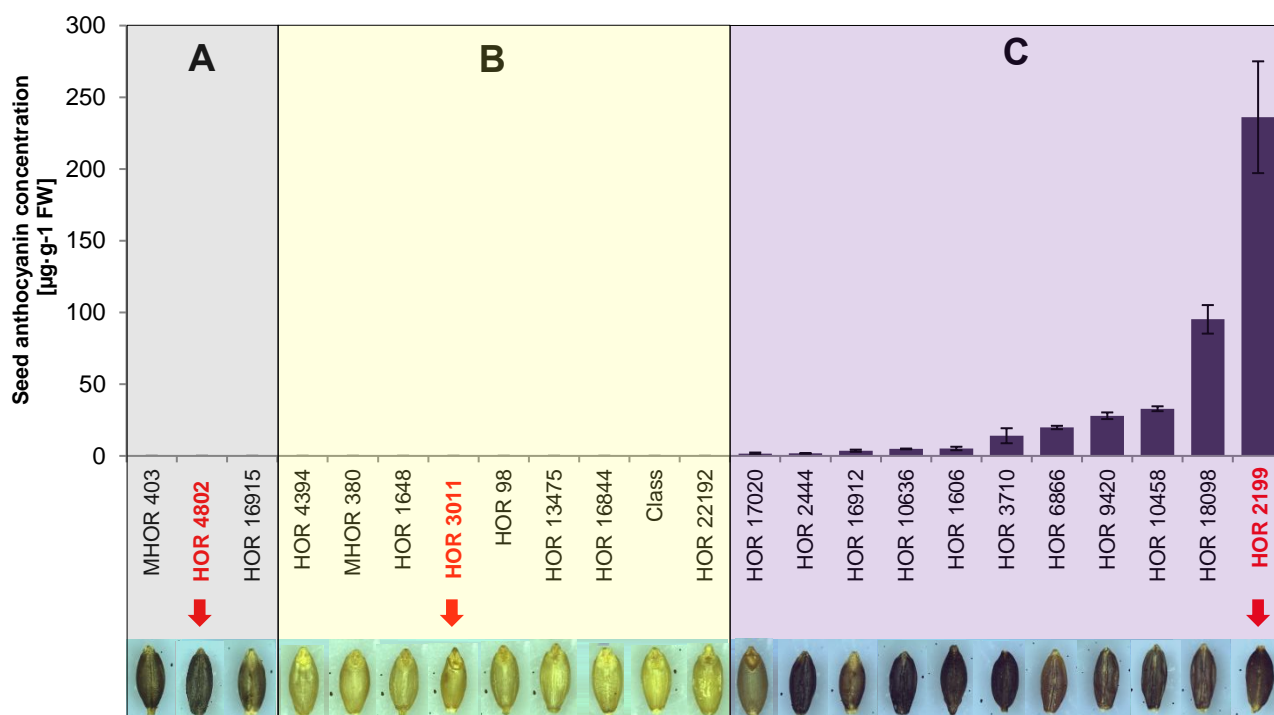
The main emphasis of this section is to investigate the biochemical and molecular factors that characterize grain pigmentation during developmental stages. Therefore, firstly three barley cultivars with different pigmentation patterns were selected according to the screening of grain color and total anthocyanin concentration (section 3.5). After harvesting, the phenotypic properties of barley grain such as seed coloration and fresh weight were recorded at each developmental stage (section 3.6). Then pigments related phenolic compounds in barley grains were identified and relatively quantified by LC-MS/MS and LC-MS respectively (section 3.7), and transcript levels for biosynthetic genes in the flavonoid pathway were analyzed using RT-qPCR (section 3.8). In the end, targeted analysis of potential precursors (L-tyrosine and L-DOPA) of melanin-like polymer were performed using UPLC/Fluorescence analysis (section 3.9).

### **3.5. Screening for total anthocyanins in different-colored barley varieties**

As shown in Figure 28, twenty-three barley accessions supplied by the IPK Genebank have the following characteristics: nine yellow-grained cultivars, one hull-less line with blue grains, nine lines with purple seeds, four lines with black seeds. Their total anthocyanin contents were determined according to the authentic standard cyanidin-3-glucoside (Figure 29). The result showed that all yellow barley grains had no anthocyanins detected, on the other hand, pigmented barley varieties contained various anthocyanin concentrations in the range of 0-236  $\mu\text{g/g}$ . The highest amount of anthocyanin was found in cultivar HOR 2199 followed by HOR 18098 with 95  $\mu\text{g/g}$ , in comparison to relatively low levels of anthocyanin content (below 30  $\mu\text{g/g}$ ) in the other pigmented barley varieties. However, three cultivars namely HOR 4802, MHOR 403 and HOR 16915 were discovered with unextractable anthocyanins or pigments, even though their pericarp presented brown to black color. As the first hypothesis, the insoluble pigments were supposed to associate with the polymerization of soluble anthocyanins or proanthocyanidins during the developmentally determined desiccation phase. To verify this hypothesis, two barley cultivars HOR 2199, HOR 4802 with different levels of anthocyanin extractability were selected to explore the characterization of pigment accumulation during seed development, and yellow-grained cultivar HOR 3011 was used as the control.



**Figure 28. Cross sections and the whole grains of 23 barley varieties with or without pigmentation.** For all kernels, the cross sections of the seed were shown on the upper part and the whole seed were shown on the lower part. Based on the seed color, these barley cultivars can be classified into two groups: yellow-grained varieties (HOR 16844, Class, HOR 22192, HOR 13475, HOR 4394, HOR 98, MHOR 380, HOR 1648, HOR 3011 and HOR 17020) and purple/black-grained varieties (HOR 3710, HOR 4802, HOR 6866, MHOR 403, HOR 1606, HOR 9420, HOR 10458, HOR 10636, HOR 16912, HOR 2199, HOR 2444, HOR 16915 and HOR 18098). Detailed information about examined barley cultivars can be found in Table 5. Cross sectioning was performed in the middle of seeds and images were captured using a stereo microscope as described in Materials and Methods. HOR stands for *Hordeum*, MHOR stands for mutant *Hordeum*. Note: *Class* is the accession name.



**Figure 29. Seed anthocyanin concentrations from 23 barley genotypes.** Anthocyanins extracted from barley grains in 70% acetone acidified with 2% formic acid solution. (A) Seeds with brown to black pericarp; (B) seeds with yellow pericarp; (C) seeds with purple to black pericarp. Barley cultivars labeled in red were selected for further investigation. Detailed information about examined barley varieties can be found in Table 5. The images were captured using a stereo microscope as described in materials and methods. HOR stands for *Hordeum*, MHOR stands for Mutant *Hordeum*. Note: *Class* is the accession name.

### 3.6. Characterization of developing seeds from yellow, purple and black-grained barley genotypes

Changes in seed coat pigmentation and fresh mass of seeds occur at each developmental stage. Accordingly, barley seeds from selected cultivars were collected at 5-d intervals between 7 DAF and maturity. In parallel, the appearance and fresh weight of developing seeds were measured to record the major phases of seed formation and maturation. Two independent experiments under different climate conditions were performed in 2014 (12 h, photoperiod; 14 °C/12 °C, day/night temperature) and 2015 (13-15 h, photoperiod; 22 °C/18 °C, day/night temperature).

#### 3.6.1. Differential pigmentation in the developing seed coat of barley

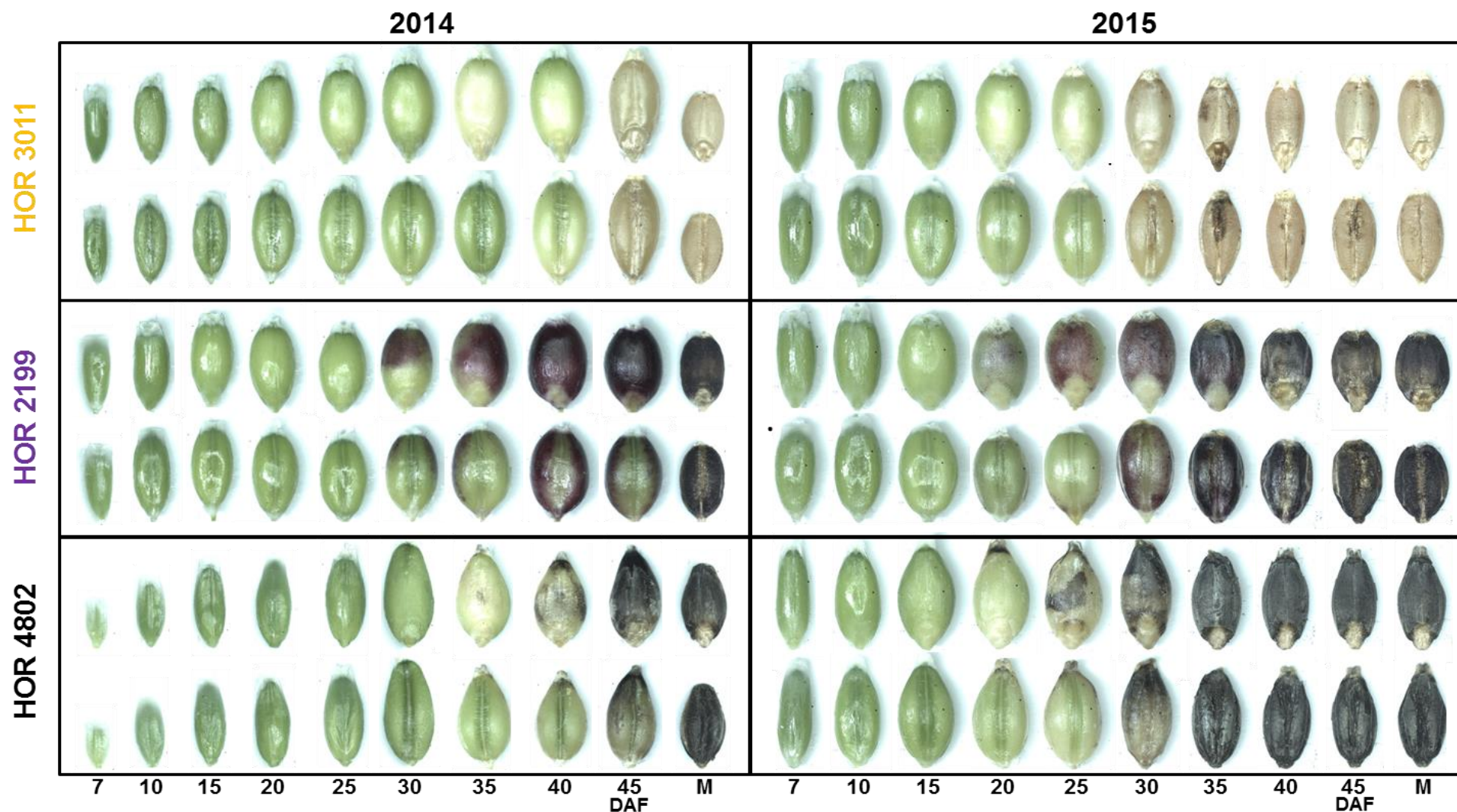
Developing grains of barley varieties HOR 3011, HOR 2199 and HOR 4802 shown in Figure 30 were freshly harvested from 7 DAF to maturity. The variations in seed coat pigmentation through

different developmental stages appeared not only among different-colored barley lines but also in various planting years. According to seed coat color, the developmental stages of barley grain exhibited non-pigmented and pigmented phases during seed maturity. During non-pigmented phase (0-25/30 DAF in 2014 and 0-15/20 DAF in 2015) the seed color changed from pale green to dark green. In pigmented phase (approximately from 30/35 DAF till maturation in 2014 and from 20/25 DAF to maturation in 2015) the differential coloration of grain started and continued to intensify until seed maturation. Different appearance of pigments genotype-dependently accumulated in the seed coat of barley lines in both growth experiments. In 2014, the grains from barley cultivars HOR 2199 and HOR 4802 started to accumulate red or black pigments partly in seed-pericarp from 30 DAF or 35 DAF, while grains of HOR 3011 turned to yellow during seed maturation. Similar changes in seed pigmentation were found in the grains harvested in 2015, but the developmental processes occurred more rapidly.

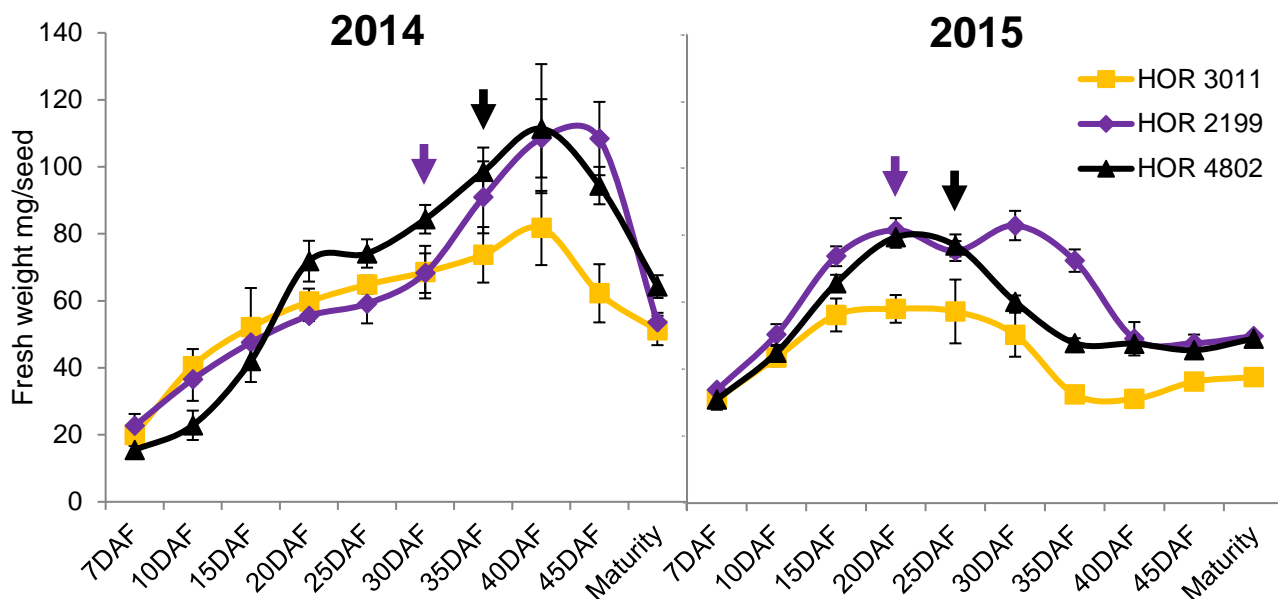
### **3.6.2. Changes in fresh weight during seed development**

Throughout the course of development, barley seeds underwent differential changes of fresh weight in both years (Figure 31). Seeds that matured under consistently warm temperature developed faster when compared with seeds grown in cold condition. During development, the fresh weights of three barley cultivars in 2014 increased rapidly between 7 and 40 DAF and declined dramatically afterward during seed desiccation. Mature seeds were the only half weight of developing seeds at 40 DAF. With higher temperature and longer illumination duration in 2015, the highest fresh weights occurred 20 days earlier in comparison with the result of 2014, and furthermore, the final grain weights were 10 mg less than those from 2014. The caryopsis weights remained constant after 35-40 DAF, when seeds entered the desiccation phase and dried out. In comparison with dark grained cultivars, yellow-grained cultivar HOR 3011 showed a considerable lower weight during seed development. In general, seeds of black-grained cultivar HOR 4802 was approximately 10 mg heavier than yellow-grained cultivar HOR 3011 and purple-grained cultivar HOR 2199.





**Figure 30. Images of barley seeds of three genotypes at different developmental stages.** Barley plants were grown in 2014 (Conditions: climate chamber; light length, 12 h; day/night temperature, 14 °C/12 °C) and 2015 (Conditions: greenhouse; light length, 13-15 h; day/night temperature, 22 °C/ 18 °C). Grains located in the middle of spikes were harvested every 5 days after flowering until maturity. The figures represent days after flowering, the upper panels are the front sides of seeds and the lower panels are the back sides. Grains from barley cultivars **HOR 2199** and **HOR 4802** turned to fully pigmented after maturation, while grains from barley cultivar **HOR 3011** changed from green to yellowish. The images were captured using a stereo microscope as described in methods and materials. DAF: days after flowering; M: maturity.



**Figure 31. Fresh weight of barley grains grown in the course of development.** Barley plants were grown in the climate chamber in 2014 and in the greenhouse in 2015. Grains located in the middle of spikes were harvested every 5 days after flowering until maturation. Due to the limited number of barley plants, data points in 2014 showed the average of 10 measurements, while in 2015 total weight of 50 seeds were calculated with three replicates. Error bars represent s.e. (not visible if smaller than symbols). The arrows indicate the starting points of seed pigmentation.

### 3.7. Developmental deposition of anthocyanins and proanthocyanidins in barley seeds

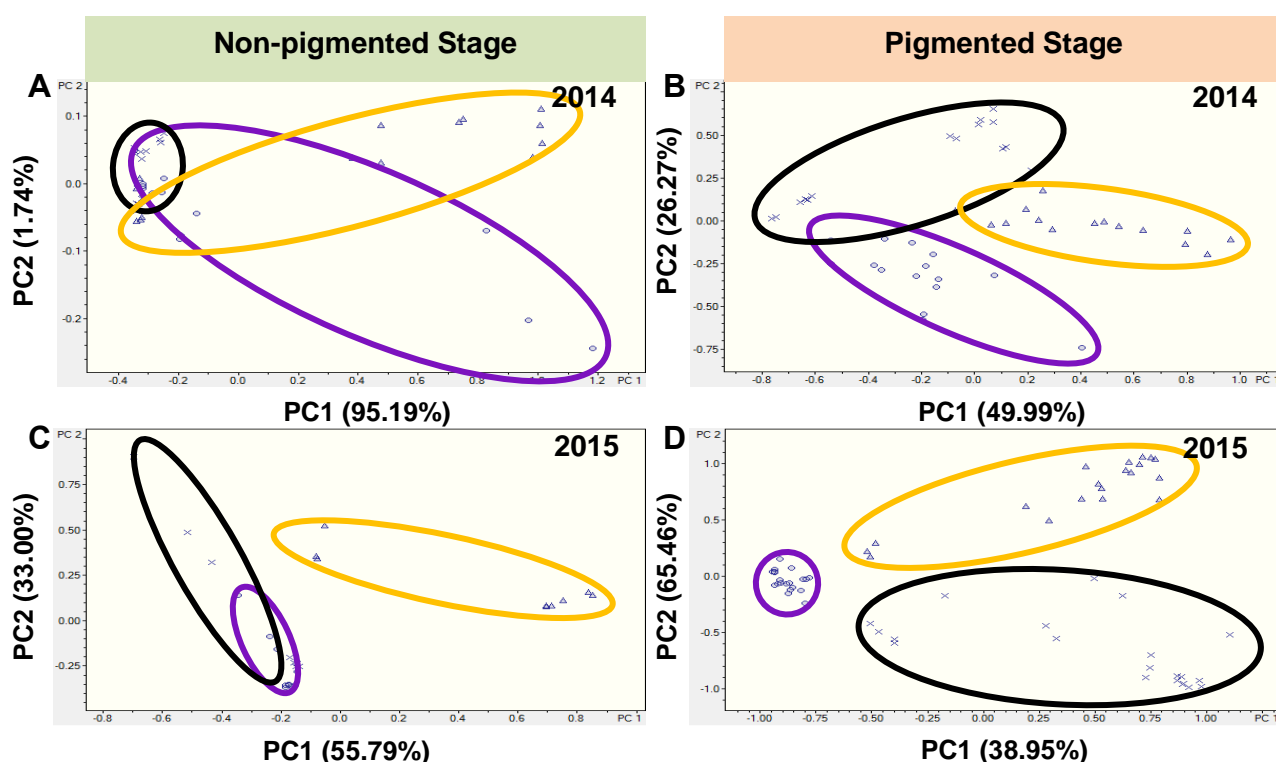
The phenolic compounds which might be responsible for the different pigmentation during seed development were determined in this section. Candidate compounds such as anthocyanins and PAs of seeds at different developmental stages were analyzed by LC-MS and LC-MS/MS. Principal component analysis (PCA) was primarily conducted to obtain a general overview of the measured dataset of barley seed extracts (section 3.7.1). Furthermore, tentative identification (section 3.7.2) and relatively quantification (section 3.7.3) of candidate compounds regarding pigments were applied to demonstrate differential grain coloration among pigmented barley genotypes.

#### 3.7.1. PCA of phenolic compounds for differential seed pigmentation

LC/MS profiles of phenolic compounds were explored using PCA to visualize the extent of sample dispersion in relation to barley phenotype during the characteristic stages of seed development, namely: non-pigmented stages and pigmented stages. With the deposition of pigments, which starts at 30 DAF in 2014 and 20 DAF in 2015, considerable changes also occur in different developmental stages. Score plots of the first principal component (PC1) versus PC2 from PCA of



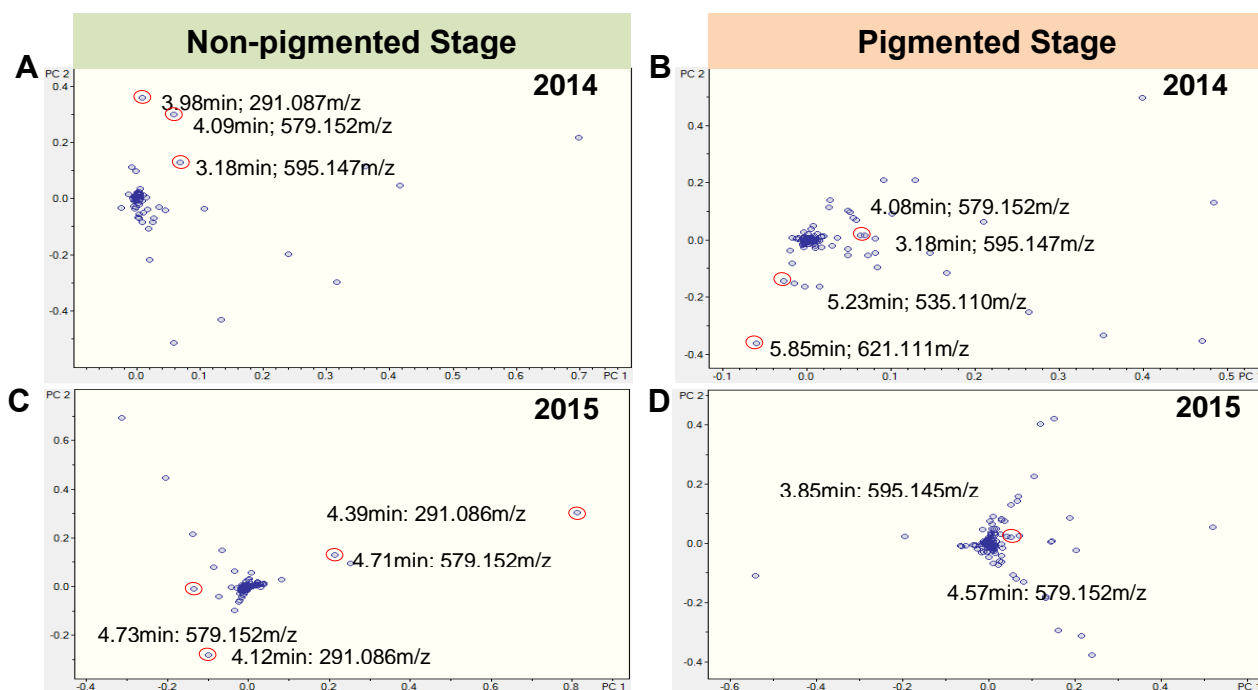
mass spectra were shown in Figure 32. During the non-pigmented stages, the first two components describe more than 85% of the total variation in mass spectra, and there was some degree of overlaps in the clustering of three barley cultivars in the PCA scores plots for both years. However, after seed pigmentation, the PCA of the LC-MS data set exhibited clear discrimination among the barley varieties by PC1 and PC2 with 76.3% in 2014, 65.5% in 2015 of the total variation in the spectra respectively, pointing out three distinct metabolic profiles. In addition, the comparison of PCA results from two planting years revealed considered patterns under different growth conditions/in different harvested years. As shown in Figure 32A, PCA result of 2014 overlapped at a higher level than that of 2015.



**Figure 32. PCA score plots for LC-MS data from barley seed extracts.** PC1 is plotted versus PC2. Score plots in PCA of samples from 2014(A) and 2015 (C) at non-pigmented stages; from 2014 (B) and 2015 (D) at pigmented stages. Each point represents one metabolite profile of one biological replicate. Barley cultivars **HOR 3011**, **HOR 2199** and **HOR 4802** were marked in yellow, purple and black respectively. Separation among cultivars is visible after the pigments formation. PCA was conducted using ProfileAnalysis 2.1 software from Bruker Compass.

The relationship among identified ions for phenolic compounds was interpreted in loading plots shown in Figure 33. Each dot stands for one particular ion at certain retention time, and dots that appear closer together on the plot are more highly correlated. By this, the loading plots could provide the first hint that ions separated with others are candidate ions responsible for differential LC-MS profiles of barley. When pigmentation was not visible, the candidate ions for

proanthocyanidins (listed in Introduction)  $m/z$  291.087, 579.152 and 595.147 were found away from the other main ions, indicating that they made the most effect on clustering among different-pigmented barley genotypes. After grain coloration, ion candidates of anthocyanins (listed in Introduction)  $m/z$  535.110 and 621.111 were also discerned from correlated ions.



**Figure 33. PCA loading plots for LC/MS data of barley seed extracts.** Loading plots in PCA of samples from (A) 2014 and (C) 2015 at non-pigmented stages; from (B) 2014 and (D) 2015 at pigmented stages. Spots labeled in red indicate the ion candidates might be related to pigments. PCA was conducted using ProfileAnalysis 2.1 software from Bruker Compass.

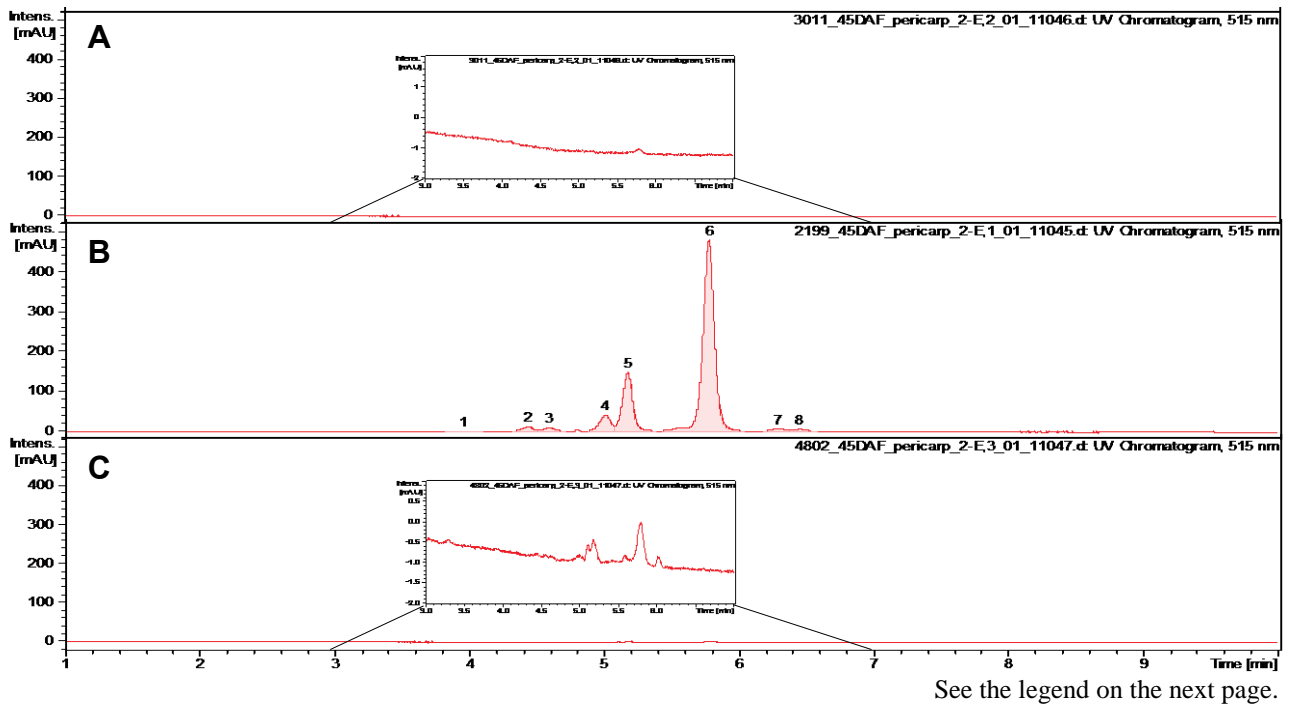
### 3.7.2. Tentative identification of anthocyanins and proanthocyanidins

In the previous section, subjecting the evaluated mass-retention time matrix to PCA separated most samples regarding LC-MS profiles at different levels, independently from barley genotypes and growing years. The main objective of this study focuses on the identification of candidate compounds which might be responsible for differential seed pigmentation. A combination of MS/MS measurements, comparison to literature data and database searches were used for the tentative identification of anthocyanins and PAs from barley grain extracts.

#### *Anthocyanins*

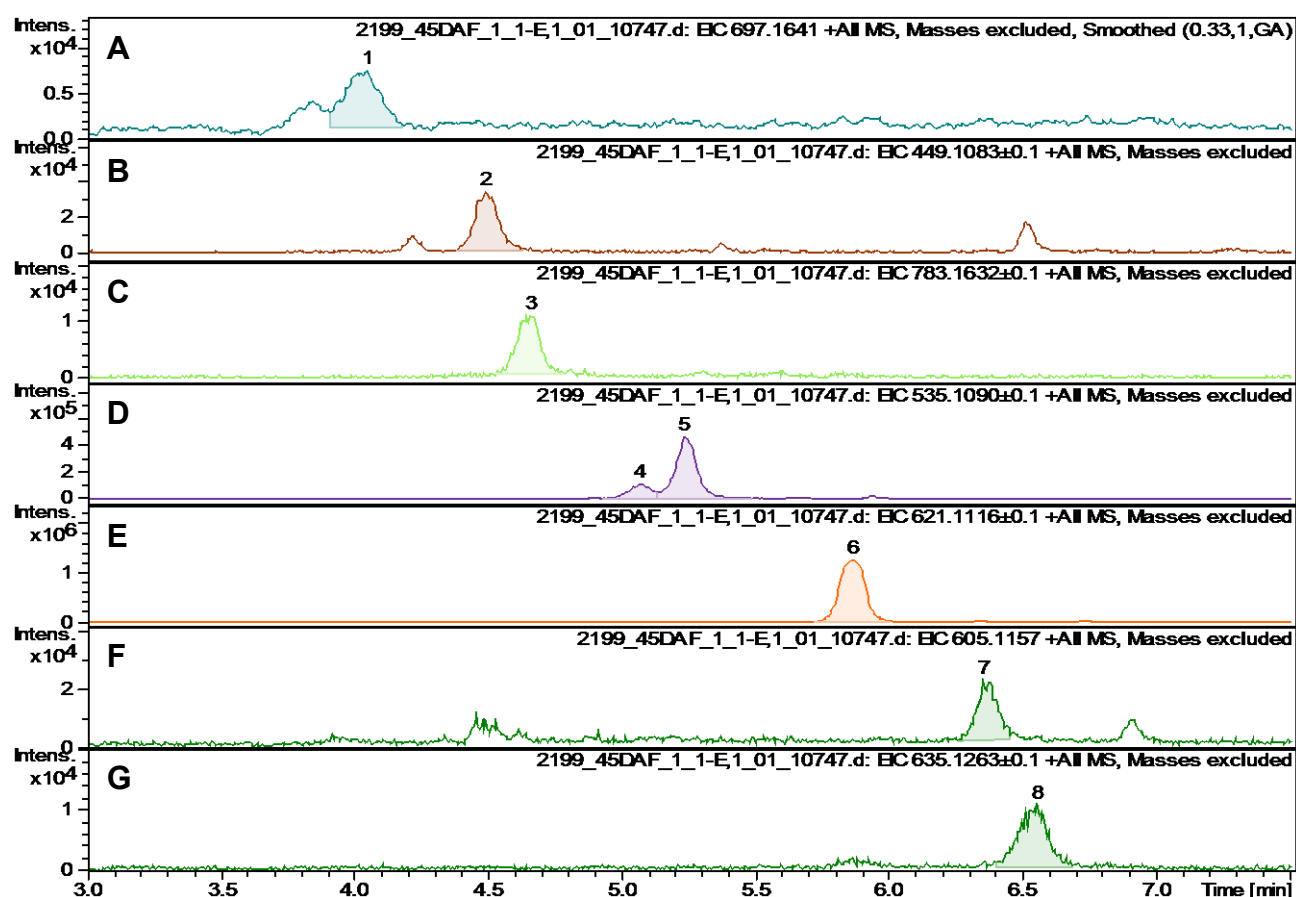
Representative LC chromatograms corresponding to anthocyanins in barley grains of different genotypes at 45 DAF in 2014 were detected at 515 nm (Figure 34). Eight major peaks were

observed from the waxy hull-less barley cultivar HOR 2199 with purple grains, contrasting to the observation that only minor peaks were found in the extracts from the yellow-grained barley cultivar HOR 3011 and the black-grained cultivar HOR 4802. Further identification of the compounds was carried out based on the relative elution order and tandem mass spectrometry measurements (Figure 35, 36). All tentatively identified molecular ions are listed in Table 15, among which compound 2, 4, 5 and 6 showed intense ion peak at  $m/z$  449.1, 535.1, 535.1 and 621.1 with the same product ion of cyanidin at  $m/z$  287.0. Compound 2 was assigned to cyanidin-3-glucoside, as it has the same molecular ion and eluted at the same time as the commercially available. Compound 4 and 5 shared the same ion value were eluted at different retention times, indicating that they were anthocyanin isomers. According to the peak intensities of identified compounds, the most abundant anthocyanin was Cy-3-(di-malonyl)-hexose, followed by Cy-3-malonyl-hexose<sup>1,2</sup> and Cy-3-glucoside. The other peak 1, 3, 7 and 8 assigned positive molecular ions  $[M]^+$  at  $m/z$  697.16, 783.16, 605.11 and 635.13, which were considered as Cy-malonyl-di-hexose, Cy-di-(malonyl-hexose), Pg-3-(di-malonyl)-hexose and Pn-3-(di-malonyl)-hexose respectively. Except for Cy-malonyl-di-hexose, same anthocyanin patterns were identified in both years. By comparing the fragmentation patterns of detected anthocyanins with published anthocyanins, possible structures were given in Figure 37. Peak 1 and peak 3 might be Cy-3-(6-malonyl-glucoside)-6-glucoside and cy-3,6-di-malonyl-glucoside. Peak 4/5 can be assigned to two anthocyanin isomers Cy-3-(3-malonyl-glucoside) or Cy-3-(6-malonyl-glucoside). Peak 6-8 might be cyanidin, pelargonidin, peonidin with the same possible substitution 3,6-di-malonyl-glucoside.



See the legend on the next page.

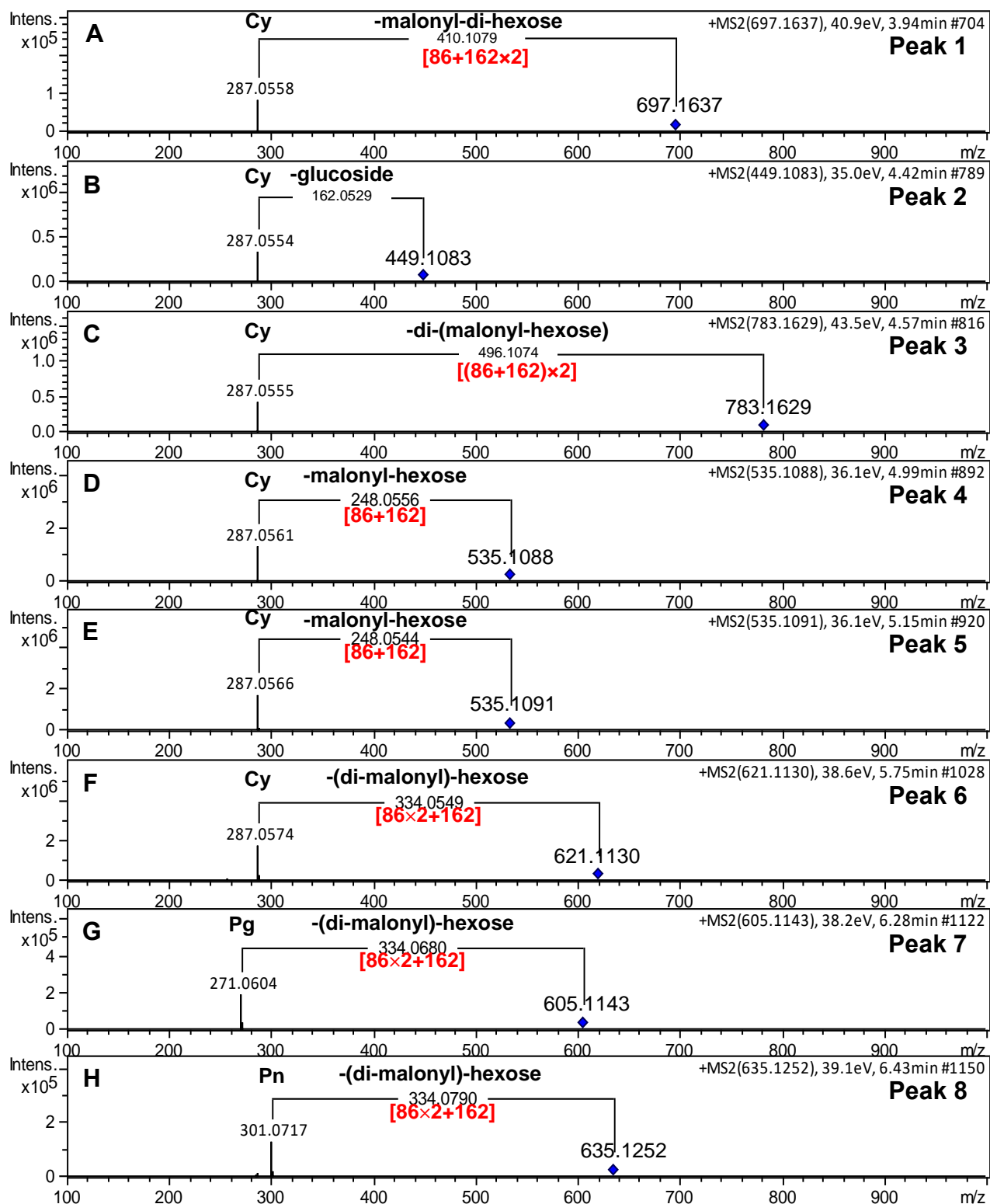
**Figure 34. Representative LC chromatograms of anthocyanins from the barley grains captured at 515 nm.** Barley plants were grown in climate chamber in 2014. (A) No peak was detected in the seed extract from the yellow-grained barley cultivar **HOR 3011**. (B) Eight anthocyanins were separated and defined in the purple-grained barley cultivar **HOR 2199**. (C) Several small peaks were observed in black-grained barley cultivar **HOR 4802**. Peak numbering is corresponding to the identification information of anthocyanins in Table 15.



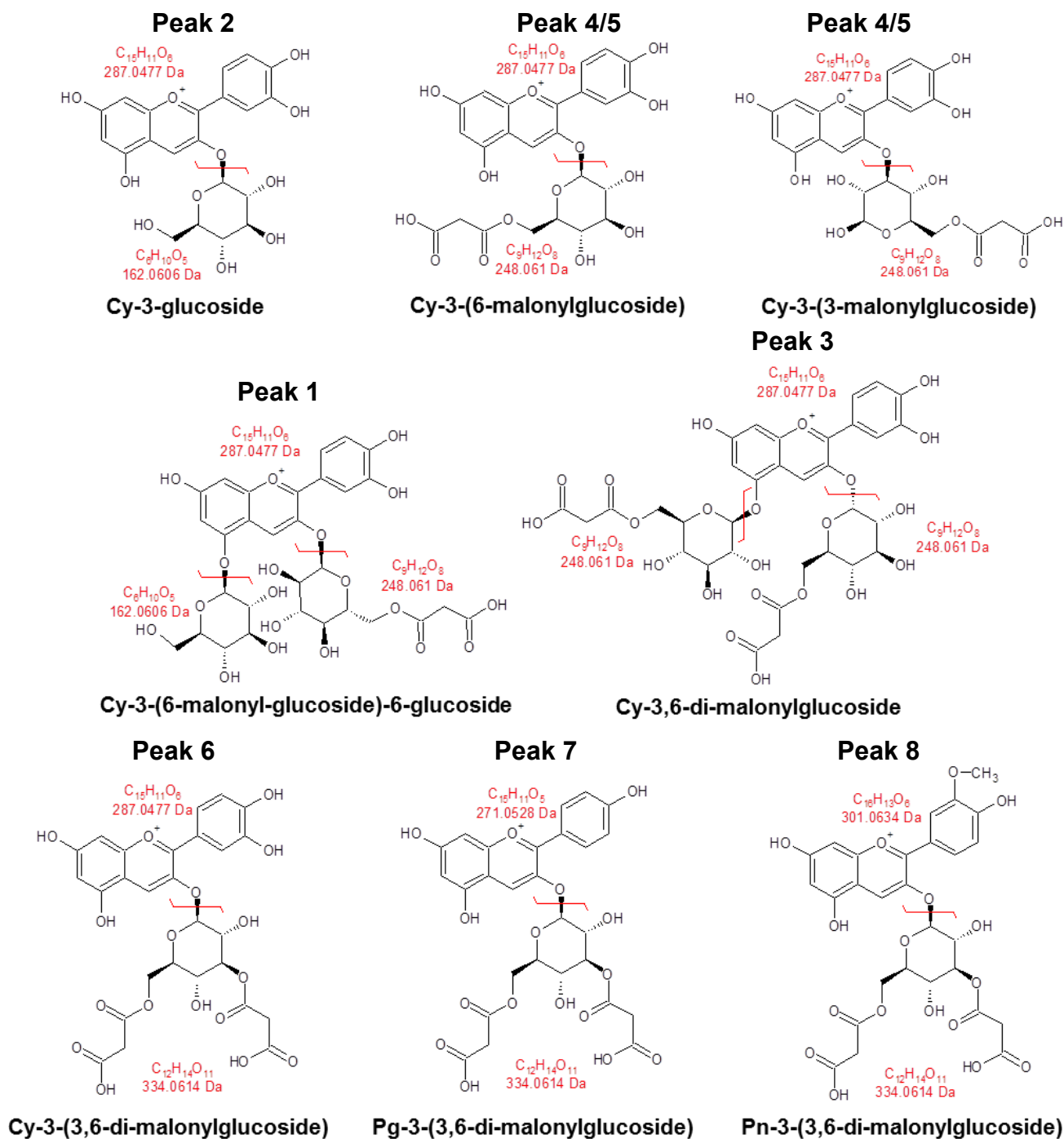
**Figure 35.** Extracted ion chromatograms (EIC) for specific detection of individual anthocyanin from seed extract of purple barley HOR 2199. Barley plants were grown in climate chamber in 2014. (A-G) The chromatographic peaks 1-8 are represented with specific  $m/z$  values: 697.1641, 449.1083, 783.1650, 535.1078, 535.1081, 621.1116, 605.1145 and 635.1258. Further information of assigned anthocyanins is given in Table 15.

**Table 15.** Tentative identification of anthocyanins from purple barley grains of HOR 2199 using LC-MS/MS. According to the retention time (RT), positive charged molecular ion, fragmentation pattern, maximum absorbance ( $\lambda_{\max}$ ) and information from literature, eight different anthocyanin patterns were tentatively identified in barley cultivar HOR 2199. <sup>a,b</sup> indicate two isomers of cy-3-malonyl-hexose; *Cy* stands for cyanidin, *Pg* stands for pelargonidin, *Pn* stands for peonidin, *n.d.* means not observed.

Peak	RT [min]		Molecular ion [M] <sup>+</sup> /[M+H] <sup>+</sup> [m/z]	Fragment ion M <sup>+</sup> [m/z]	$\lambda_{\max}$ [nm]	Tentative identification
	2014	2015				
1	4.02	n.d.	697.1641	287	515	Cy-malonyl-di-hexose
2	4.49	3.45	449.1083	287	510	Cy-3-glucoside
3	4.63	4.25	783.1650	287	518	Cy-di-(malonyl-hexose)
4	5.05	4.52	535.1078	287	515	Cy-3-malonyl-hexose <sup>a</sup>
5	5.22	4.68	535.1081	287	515	Cy-3-malonyl-hexose <sup>b</sup>
6	5.88	5.56	621.1116	287	515	Cy-3-(di-malonyl)-hexose
7	6.34	6.01	605.1145	271	515	Pg-3-(di-malonyl)-hexose
8	6.51	6.24	635.1258	301	515	Pn-3-(di-malonyl)-hexose



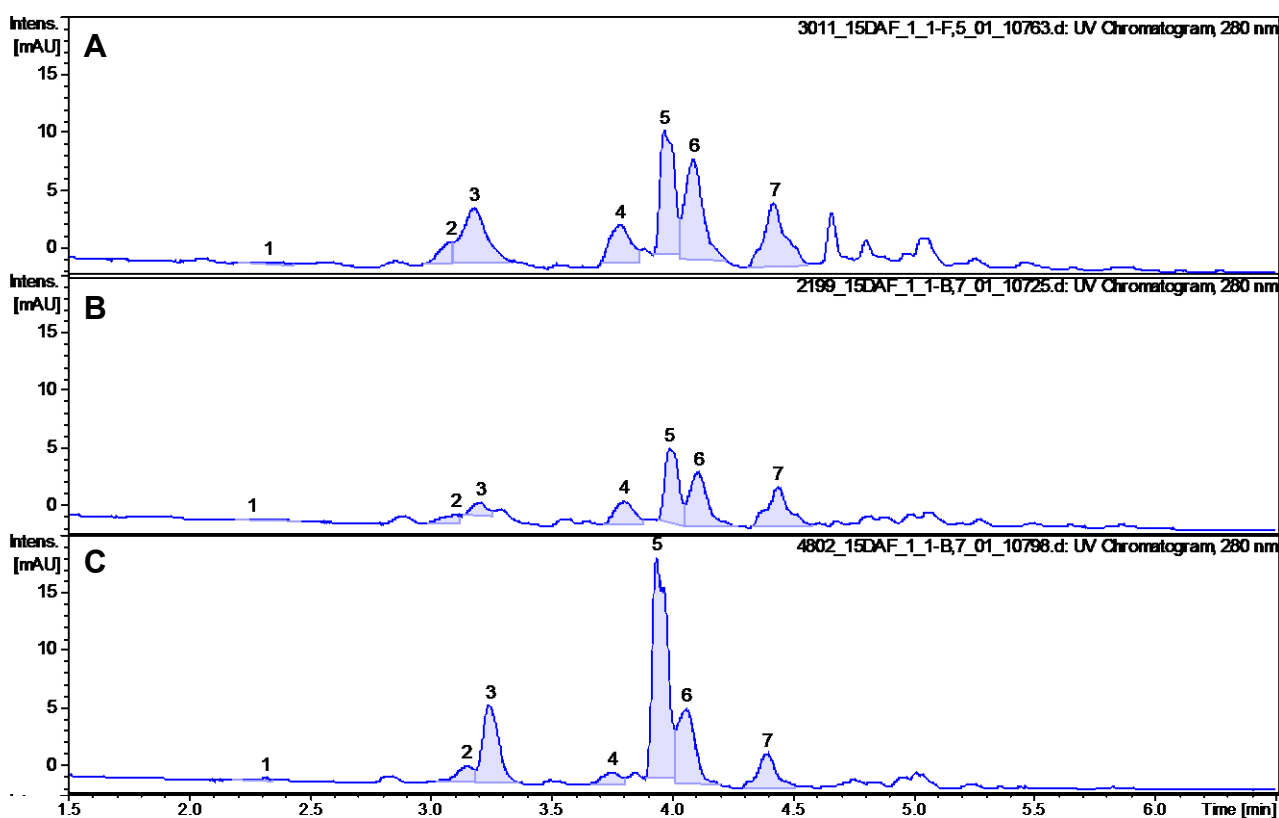
**Figure 36.** MS/MS spectra and proposed assignments of identified anthocyanins in purple barley HOR 2199. Barley plants were grown in climate chamber in 2014. (A-F) The fragment ion at  $m/z$  287 was associated with the aglycone cyanidin, while (G, H) were observed with fragment ions of pelargonidin ( $m/z$  271) and peonidin ( $m/z$  301) respectively. The fragmentation patterns labeled in red to some extent indicate the loss of specific moieties: e.g. one hexose  $[M-162]^+$ , malonyl-di-hexose  $[M-86-162-162]^+$ , malonyl-hexose  $[M-86-162]^+$  etc.



**Figure 37. Proposed structures of anthocyanins in the grain of purple barley cultivar HOR 2199.** Barley plants were grown in climate chamber in 2014. The possible structures of peak 1-8 were given according to the LC-MS/MS results and chemical databases. The red lines indicated the cleavage positions in the chemical structures, while the fragmentation patterns and corresponding formulas were labeled in red as well. Cy stands for cyanidin, Pg stands for pelargonidin, Pn stands for peonidin. Compounds were drawn using ACD/ChemSketch Freeware.

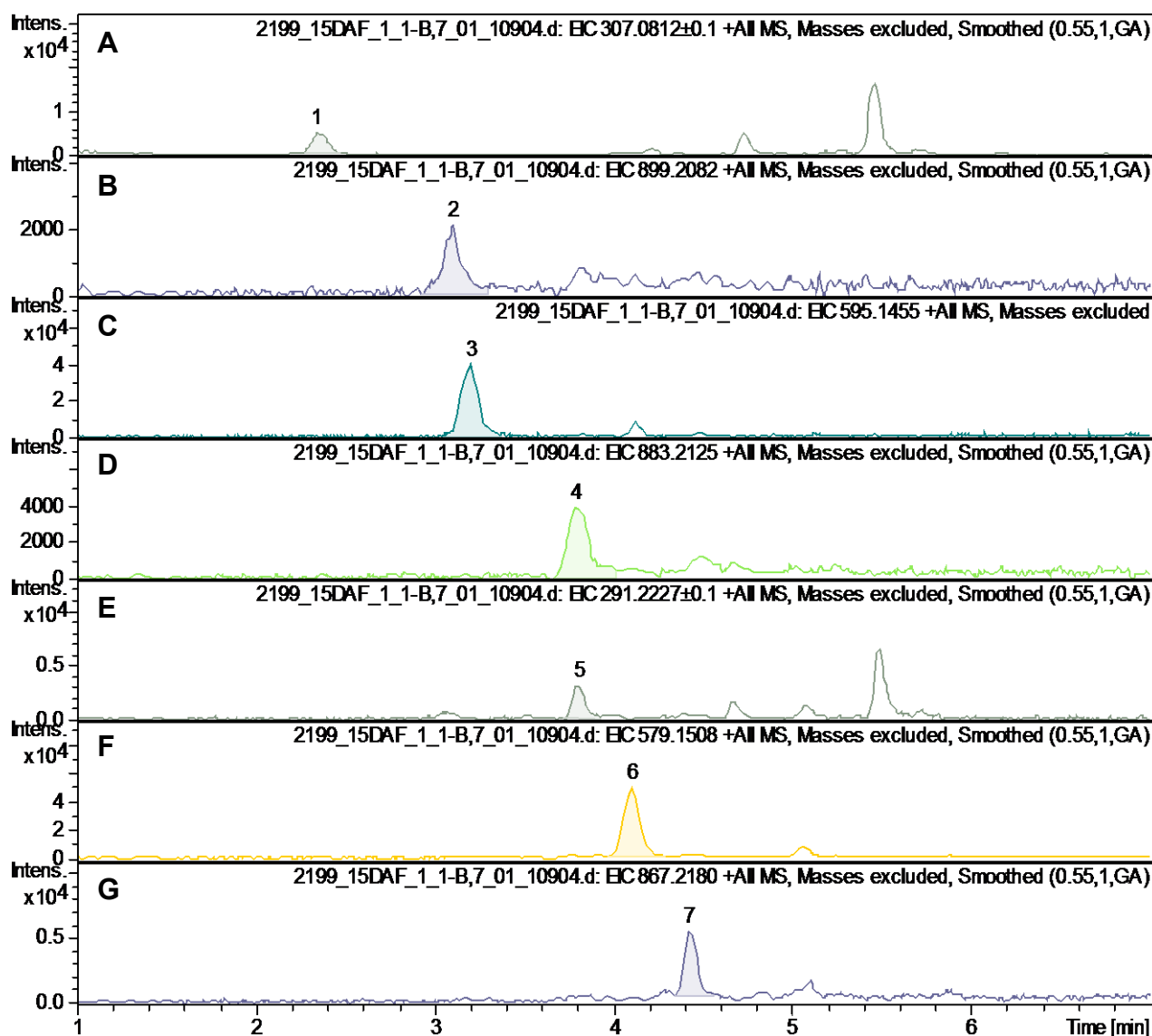
### Flavan-3-ols & Proanthocyanidins

Representative LC chromatograms indicating seven flavan-3-ols and PA species were detected at 280 nm from barley grain extracts (Figure 38). The same flavan-3-ols and PA patterns were detected from three barley cultivars for both years. Two monomers catechin (C) and gallic catechin (GC), two PA dimers namely procyanidin B3 (C-C) and prodelphinidin B3 (GC-C), and three trimeric PAs tentatively identified as procyanidin C2 (C-C-C), prodelphinidin T2 (GC-C-C) and Trimer 1 (GC-GC-C) in barley grains (Figure 39, 40). Peak 4 was assigned to catechin with the help of the authentic standard. Together with peak 1, 6 and 3 identified as gallic catechin, procyanidin B3 (C-C), and prodelphinidin B3 (GC-C) were the major flavan-3-ols found in barley grains. For unambiguous identification of all of the present PAs in both years are shown in Table 16. These findings suggested that the composition of the PAs did not differ among the tested barley genotypes and between harvested years. Besides, the proposed structures of detected PAs were given in Figure 41.



**Figure 38.** Representative LC chromatograms of PAs from the barley grains captured at 280 nm. Barley plants were grown in climate chamber in 2014. (A-C) Seven flavan-3-ols and PAs were separated and defined in yellow-grained barley cultivar **HOR 3011**, purple-grained barley cultivar **HOR 2199**, and black-grained barley cultivar **HOR 4802**. Peak numbering is corresponding to the identical information of PAs in Table 16.

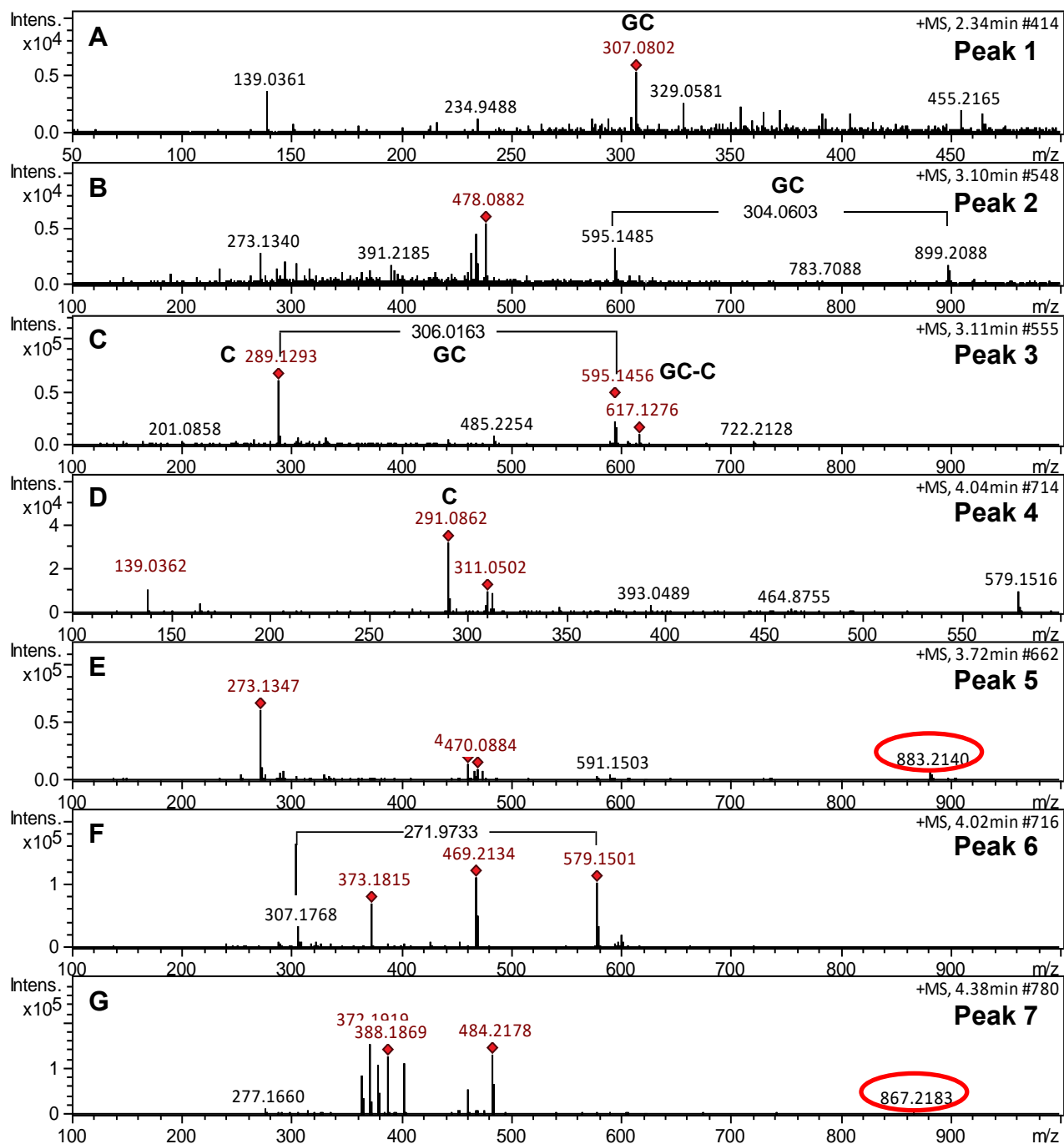




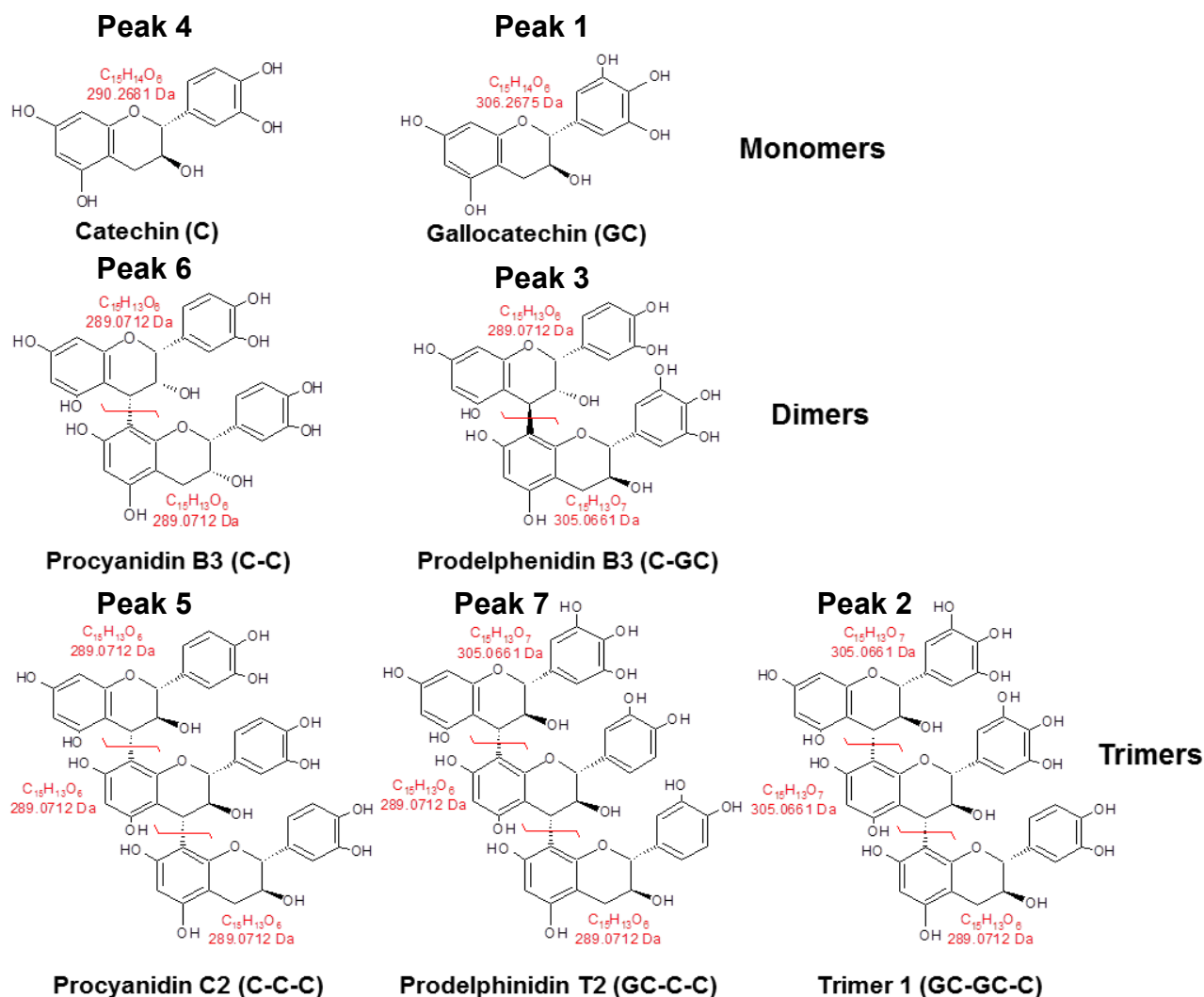
**Figure 39. Extracted ion chromatograms (EIC) for specific detection of individual PA from seed extract of purple barley HOR 2199.** Barley plants were grown in climate chamber in 2014. (A-G) The chromatographic peaks 1-7 are represented with specific  $m/z$  values: 307.0812, 899.2079, 595.1447, 291.0868, 883.2107, 579.1508 and 867.2192. Further information of identified PAs is described in Table 16.

**Table 16. Tentative identification of PAs from barley grains using LC-MS/MS.** According to the retention time (RT), molecular ion, fragmentation pattern, maximum absorbance ( $\lambda_{\max}$ ) and information from literature listed in Table 2. Seven different proanthocyanidin patterns were tentatively identified in three tested barley cultivars.

Peak	RT [min]		Molecular ion [M] <sup>+</sup> /[M+H] <sup>+</sup> [m/z]	Fragment ion M <sup>+</sup> [m/z]	$\lambda_{\max}$ nm	Tentative identification
	2014	2015				
1	2.33	2.53	307.0812	307	280	Gallocatechin (GC)
2	3.11	3.11	899.2079	291	280	Trimer 1 (GC-GC-C)
3	3.21	3.81	595.1447	306	280	Prodelphinidin B3 (GC-C)
4	4.01	4.16	291.0868	291	280	Catechin (C)
5	3.82	4.41	883.2107	306	280	Procyanidin C2 (C-C-C)
6	4.11	4.51	579.1508	289	280	Procyanidin B3 (C-C)
7	4.44	4.92	867.2192	289	280	Prodelphinidin T2 (GC-C-C)



**Figure 40. MS/MS spectra and proposed assignments of identified PAs.** (A, D) Peak 1 and 4 with the molecular ions at  $m/z$  306 and 291 were assigned to two flavan-3-ols: gallocatechin (GC) and catechin (C). (C, F) Peak 3 and 6 were tentatively assigned to PA dimers, while (B, E, G) peak 2, 5, and 7 were PA trimers.



**Figure 41. Structures of PAs in the grain of barley cultivars.** The possible structures of peak 1-7 were given according to the LC-MS/MS results and chemical databases. Peak 1 and 4 with the molecular ions at  $m/z$  306 and 291 were assigned to two flavan-3-ols: gallocatechin (GC) and catechin (C). Peak 3 and 6 were tentatively assigned to PA dimers, while peak 2, 5, and 7 were PA trimers. The red lines indicated the cleavage positions in the chemical structures, while the fragmentation patterns and corresponding formulas were labeled in red as well. Compounds were drawn using ACD/ChemSketch Freeware.

### 3.7.3. Quantification of anthocyanins and proanthocyanidins in developing barley grains

With standards cyanidin-3-glucoside and catechin, the relative quantification of individual anthocyanin and PA were calculated in  $\mu\text{g}/\text{seed}$  to avoid the dilution from dry matter accumulation. The total anthocyanin and PA concentrations were quantified by the sum of individual compounds.

### *Anthocyanins*

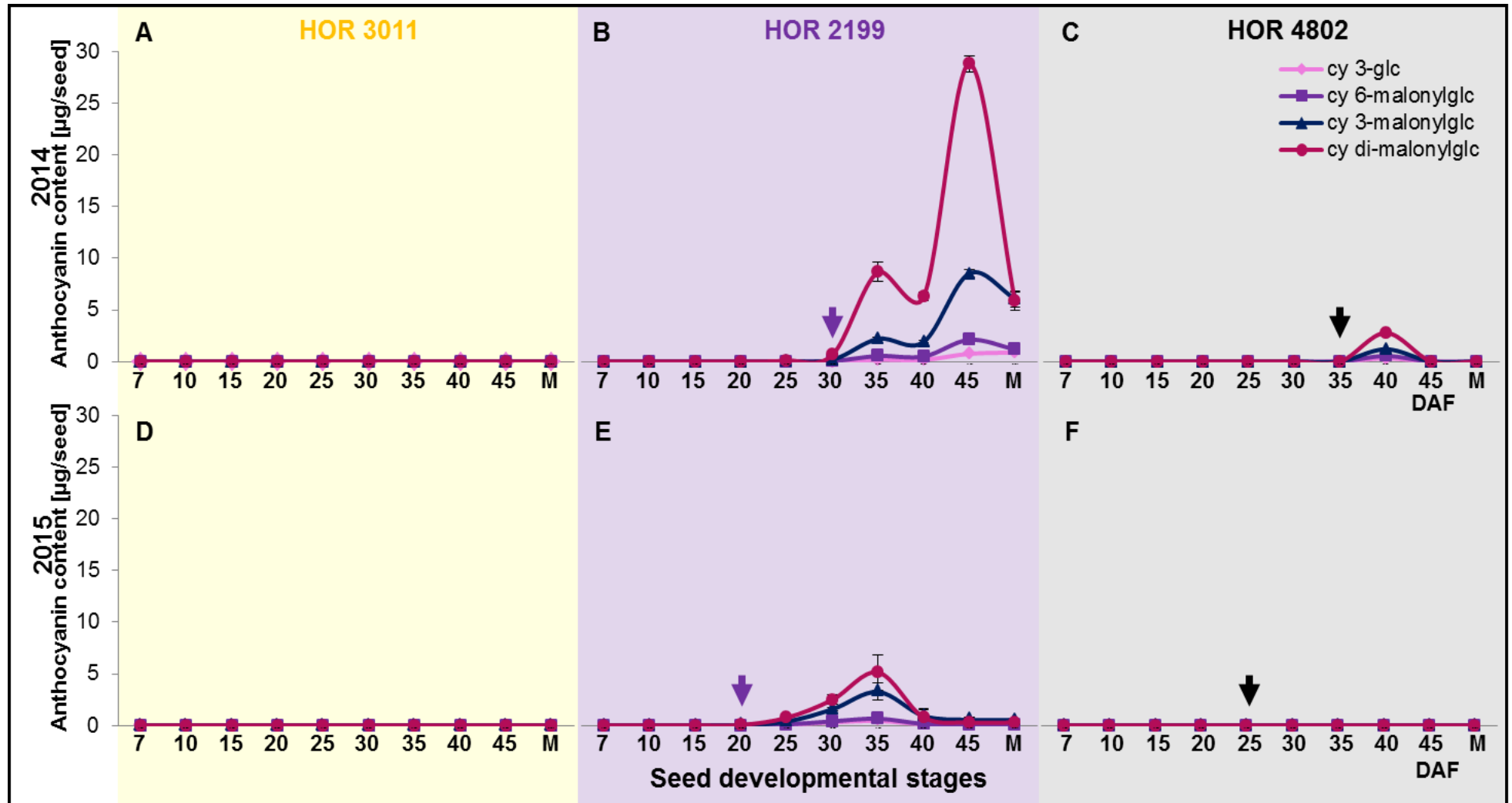
The content of four major anthocyanins in grains during maturation are shown in Figure 42. No anthocyanin was detected in yellow-grained cv. HOR 3011 in two growing years. On the contrary, anthocyanins from purple-colored cv. HOR 2199 were found in grains from 25 DAF in 2014, and 20 DAF in 2015, but not every anthocyanin compound started accumulation at the same growth stage. The most abundant anthocyanins, Cy-3-malonyl-hexose<sup>b</sup> and Cy-3-(di-malonyl)-hexose, appeared around 5-10 days earlier than the other anthocyanin types. Additionally, grain anthocyanins detected in two growing years were found with different change tendencies. A double-peak trend was observed in 2014 which differed with one single peak in 2015. From 8 anthocyanins identified in purple-grained barley, only Cy-malonyl-di-hexose was absent in black-grained cultivar HOR 4802 at 40 DAF in 2014, even though with relatively low concentrations. However, no anthocyanins could be detected in cultivar HOR 4802 through the whole developmental stages in 2015.

According to Figure 46, the total anthocyanin concentrations were observed differently in two years. In 2014 the highest anthocyanin content with the value of 41.2 µg/seed occurred at 45 DAF from cultivar HOR 2199, while only 10.3 µg/seed of total anthocyanin content was detected in 2015 at 35 DAF. On the other hand, changes in extractable anthocyanins content per seed were consistent with the appearance of grains from dark-grained cultivars at early stages of seed pigmentation. However, the total anthocyanins declined dramatically in cv. HOR 2199. In more details, the decrease of total anthocyanin content in 2014 started from 45 DAF, but the anthocyanin amount kept stable after a sharp reduction at 35 DAF in 2015. The most abundant anthocyanin, Cy-3-(di-malonyl)-hexose, took up around 70% of total anthocyanin content amount in cultivar HOR 2199.

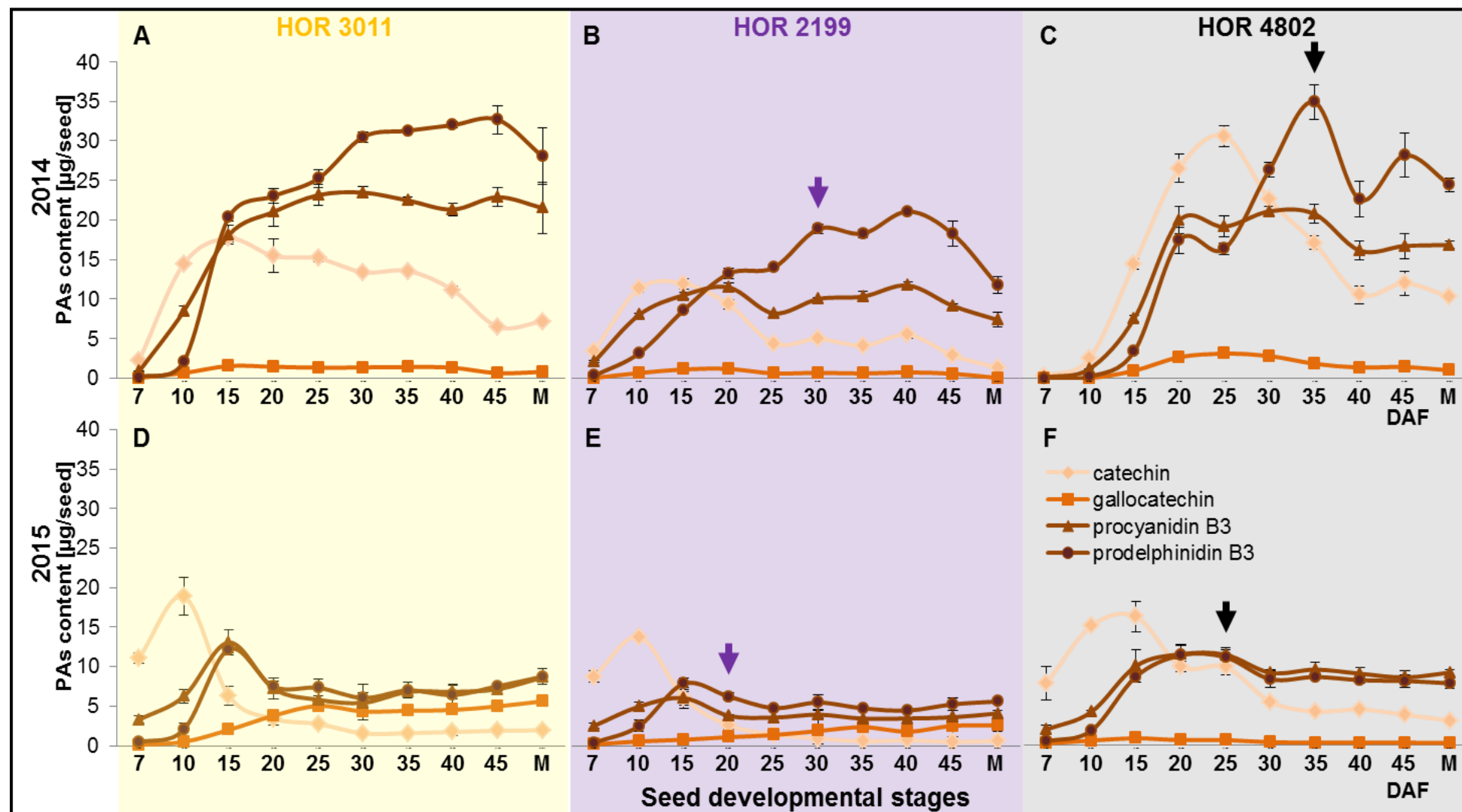
### *Flavan-3-ols & Proanthocyanidins*

The contents of catechin, gallic catechin and PA dimers during seed maturation are shown in Figure 43. PAs contents differed not only in barley varieties at different growth stages but also under separate climate conditions. Catechin always accumulated largely at early growth stages (10-25 DAF), then the concentrations decreased to relatively low level afterward. Another monomer gallic catechin stayed in low concentrations ( $\leq 3.1$  µg/seed) through growth in 2014, while there appeared to be a slightly ascending trend in 2015. Two PA dimers procyanidin B3 and

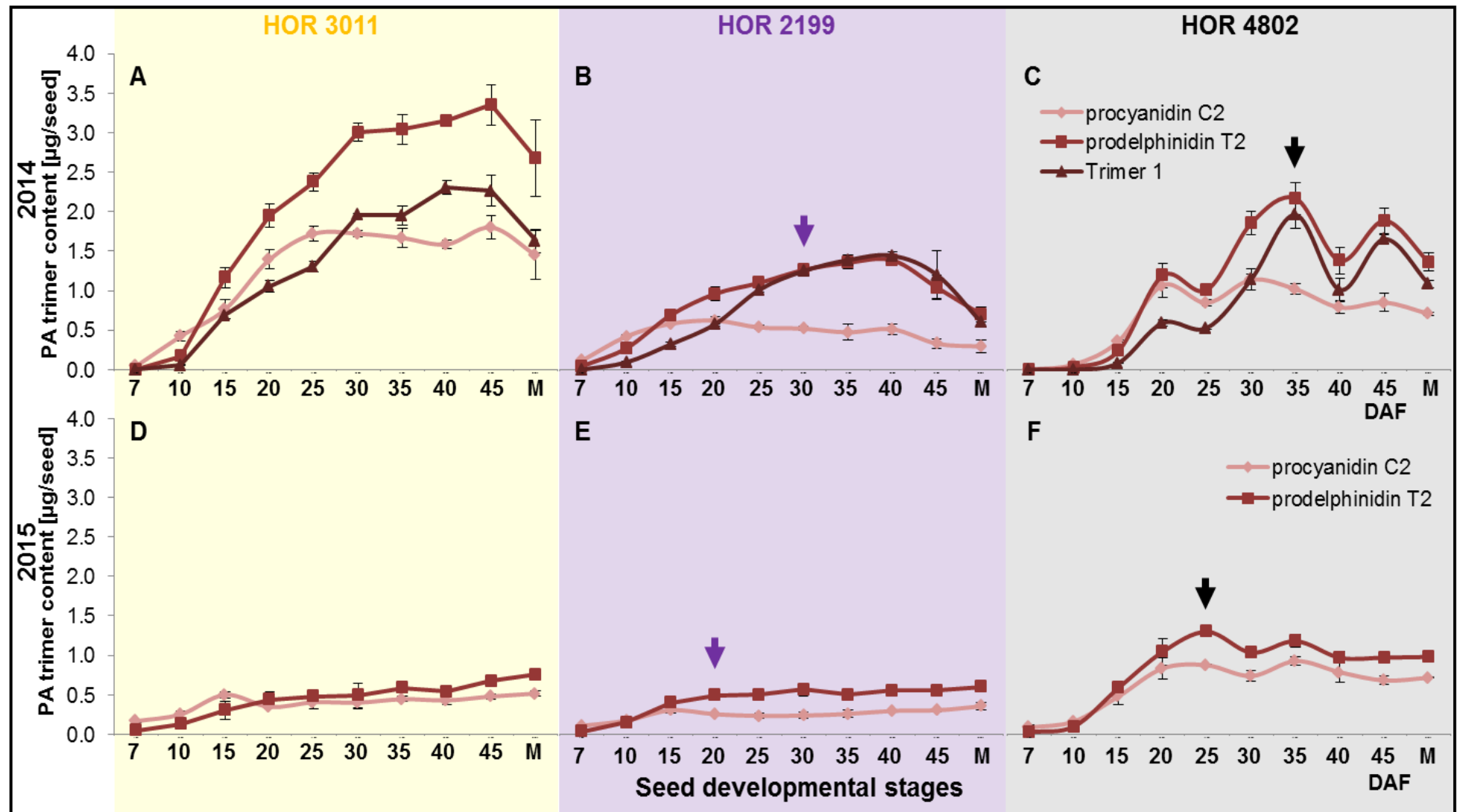
prodelphinidin B3 formed 5-10 days later than catechin, then synthesis declined slightly and kept stable after their contents arrived at the peak. All three PA trimers procyanidin C2, prodelphinidin T2 and Trimer 1 presented relatively low amounts ( $\leq 3.4 \mu\text{g}/\text{seed}$ ) during barley grain development (Figure 44). Additionally, the same flavan-3-ol compound showed similar variation tendency among different barley varieties in the same year. Mature seeds always ended up with higher values of PA dimers than those of PA precursors for the tested barley accessions. Yellow-grained cv. HOR 3011 containing no anthocyanins and black-grained cv. HOR 4802 turned to have higher PA contents than anthocyanins containing cultivar HOR 2199. Barley varieties grown in 2014 gained much higher PA contents, especially the PA dimers, than in 2015. PAs started to accumulate 5-10 days' earlier in 2015 than in 2014. Concerning PA derivatives, cultivar HOR 3011 and HOR 4802 contained high levels of PA precursor catechin during seed maturation. In cultivar HOR 2199, only reduced levels of monomers were detected. The metabolic profile of cv. HOR 3011 was similar to that of the black-grained cv. HOR 4802.



**Figure 42. Anthocyanin profiles during seed development of three barley genotypes.** Yellow-grained cv. **HOR 3011**, purple-grained cv. **HOR 2199** and black-grained cv. **HOR 4802** were grown in greenhouse in 2014 (A, B, C) and 2015 (D, E, F). Relative quantification of main anthocyanins was calculated according to cyanidin-3-glucoside standard. Error bars represent SD of three independent measurements. The arrows indicate the starting point of pigmentation. Cy stands for cyanidin, glc stands for glucoside.

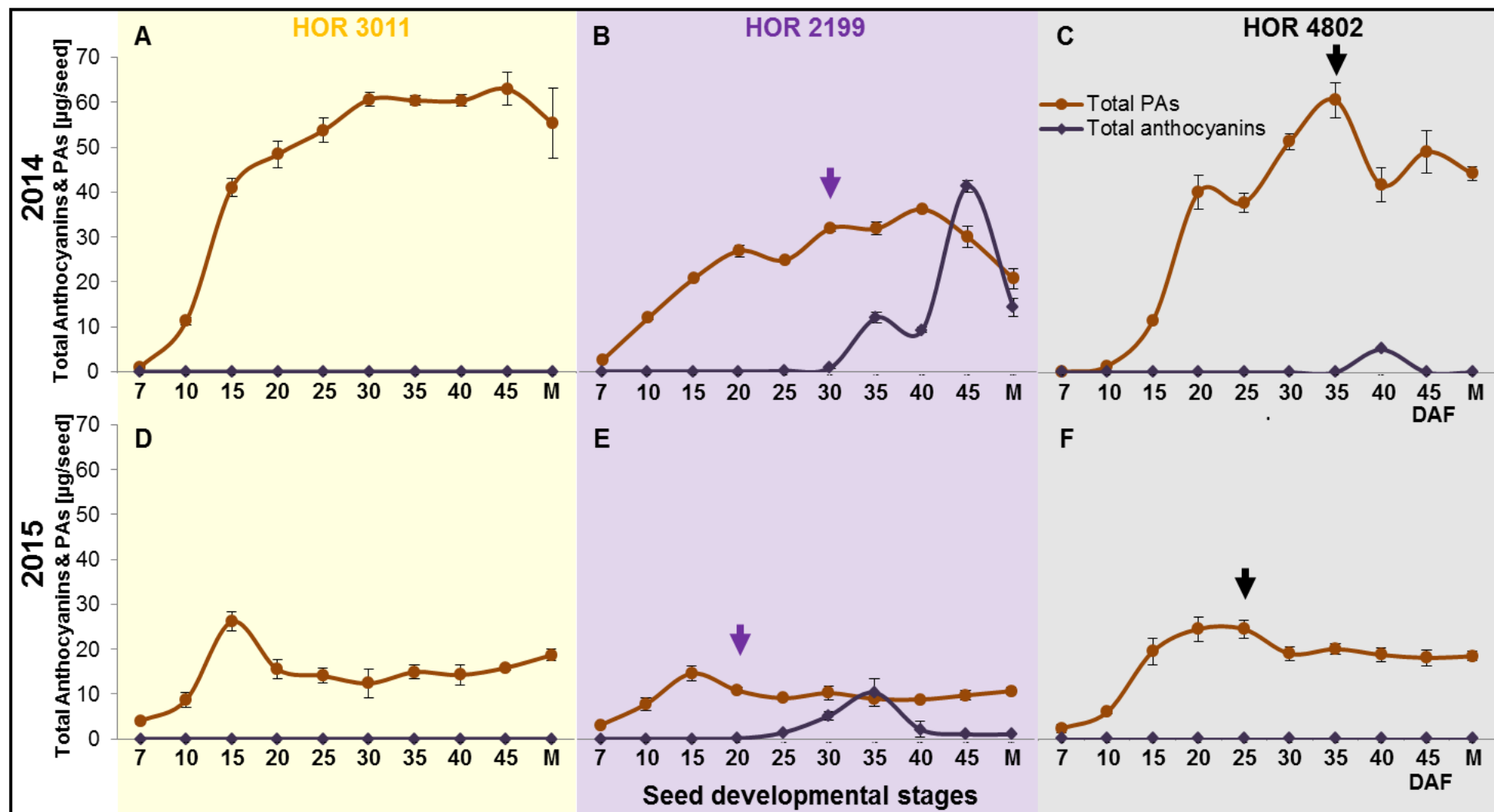


**Figure 43. Proanthocyanidin profiles during seed development of three barley genotypes.** Yellow-grained cv. **HOR 3011**, purple-grained cv. **HOR 2199** and black-grained cv. **HOR 4802** were grown in greenhouse in 2014 (A, B, C) and 2015 (D, E, F). Relative quantification of PA precursors and main PAs were calculated according to catechin standard. Error bars represent SD of three independent measurements. The arrows indicate the starting point of pigmentation.



**Figure 44.** Contents of proanthocyanidin trimers during seed development of three barley genotypes. Yellow-grained cv. **HOR 3011**, purple-grained cv. **HOR 2199** and black-grained cv. **HOR 4802** were grown in greenhouse in 2014 (A, B, C) and 2015 (D, E, F). Relative quantification of PAs was calculated according to catechin standards. Trimer 1 was not detected in 2015. Error bars represent SD of three independent measurements. The arrows indicate the starting point of pigmentation.

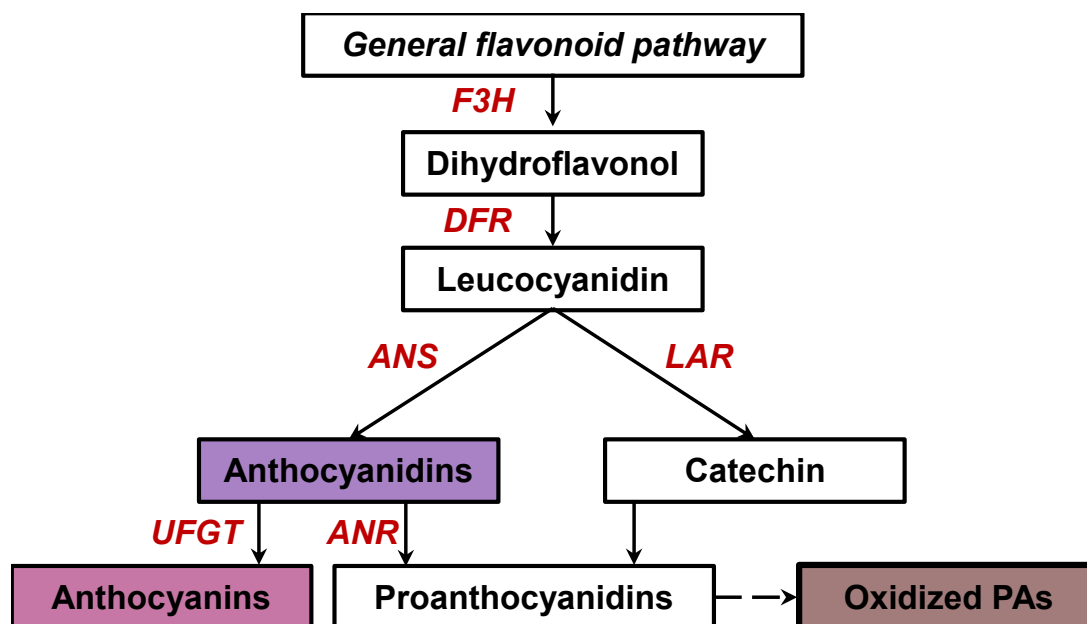




**Figure 45. Total Anthocyanin and proanthocyanidin contents during seed development of three barley genotypes.** Yellow-grained cv. **HOR 3011**, purple-grained cv. **HOR 2199** and black-grained cv. **HOR 4802** were grown in green house in 2014 (A, B, C) and 2015 (D, E, F). Relative quantification of total anthocyanins and PAs were calculated by the summary of individual anthocyanin and PA according to cyanidin-3-glucoside and catechin standards respectively. Error bars represent SD of three independent measurements. The arrows indicate the starting point of pigmentation.

### 3.8. Differential expression of anthocyanin and proanthocyanidin genes during seed development

In this section, the expression of genes involved in flavonoid pathway was investigated to elucidate the molecular mechanisms for purple to black coloration in different barley genotypes. Therefore, the transcripts of anthocyanin and proanthocyanidin structural genes were examined in non-colored and colored cultivars at different developmental phases by RT-qPCR. Specific primers for the candidate genes (Figure 46) were generated based on published gene sequences for *HvF3H*, *HvDFR*, *HvANS*, *HvLAR*, *HvUFGT*, and *HvANR* from barley. The exact pattern of change of expression of genes associated with seed pigmentation was clearly different (Figure 47, 48 and 49).

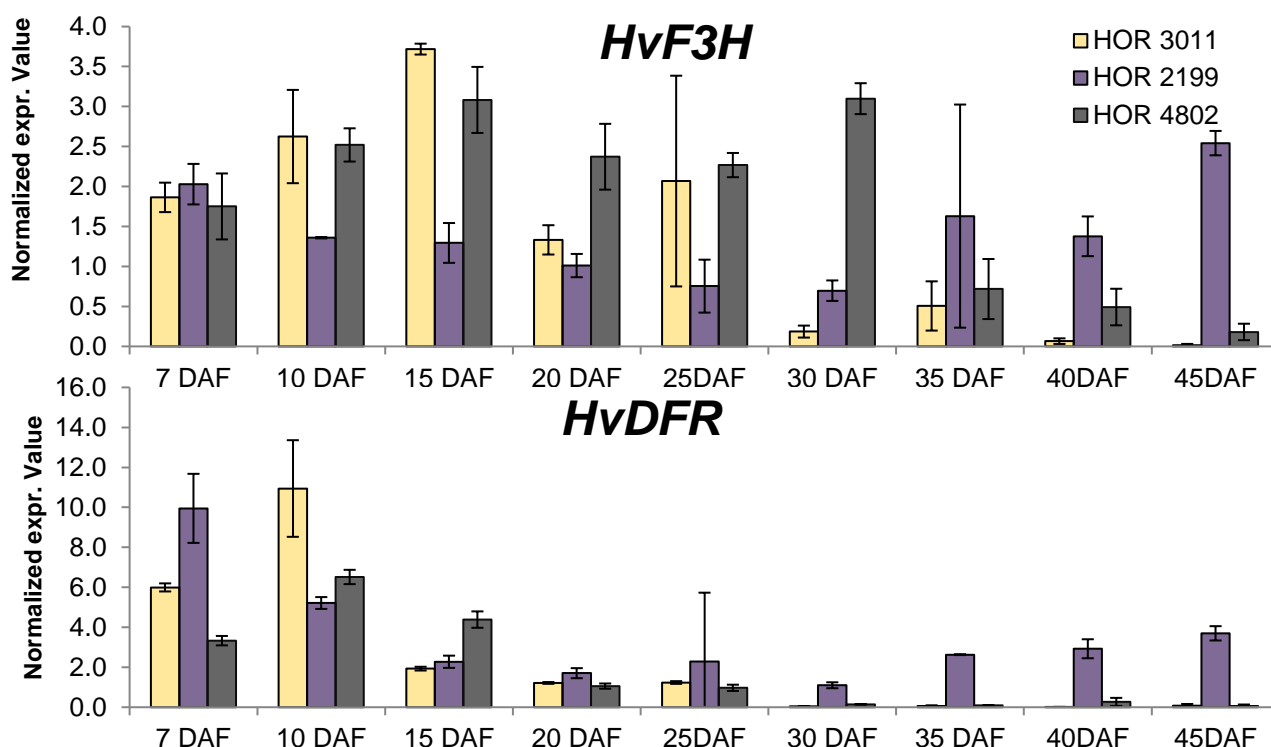


**Figure 46. Schematic view of flavonoid pathway (modified from Bogs et al. 2007).** Abbreviations for enzymes: *F3H*, flavonol 3-hydroxylase; *DFR*, dihydroflavonol 4-reductase; *ANS*, anthocyanidin synthase; *LAR*, leucoanthocyanidin reductase; *ANR*, anthocyanidin reductase; *UFGT*, UDP-glucose: sterol glucosyltransferase.

#### 3.8.1. Common genes – *HvF3H* and *HvDFR*

Acting in the early steps of the flavonoid pathway, the general genes (*HvF3H* and *HvDFR*) behaved similar expression trends in non-colored variety HOR 3011 and black variety HOR 4802 during seed maturation (Figure 47). The relative expression values of *HvF3H* and *HvDFR* increased at early developmental stages, then significantly and continuously reduced until the seeds became mature. While in HOR 2199, these two genes first presented increasing expression values at the beginning of seed development, their relative expression value subsequently decreased to the low

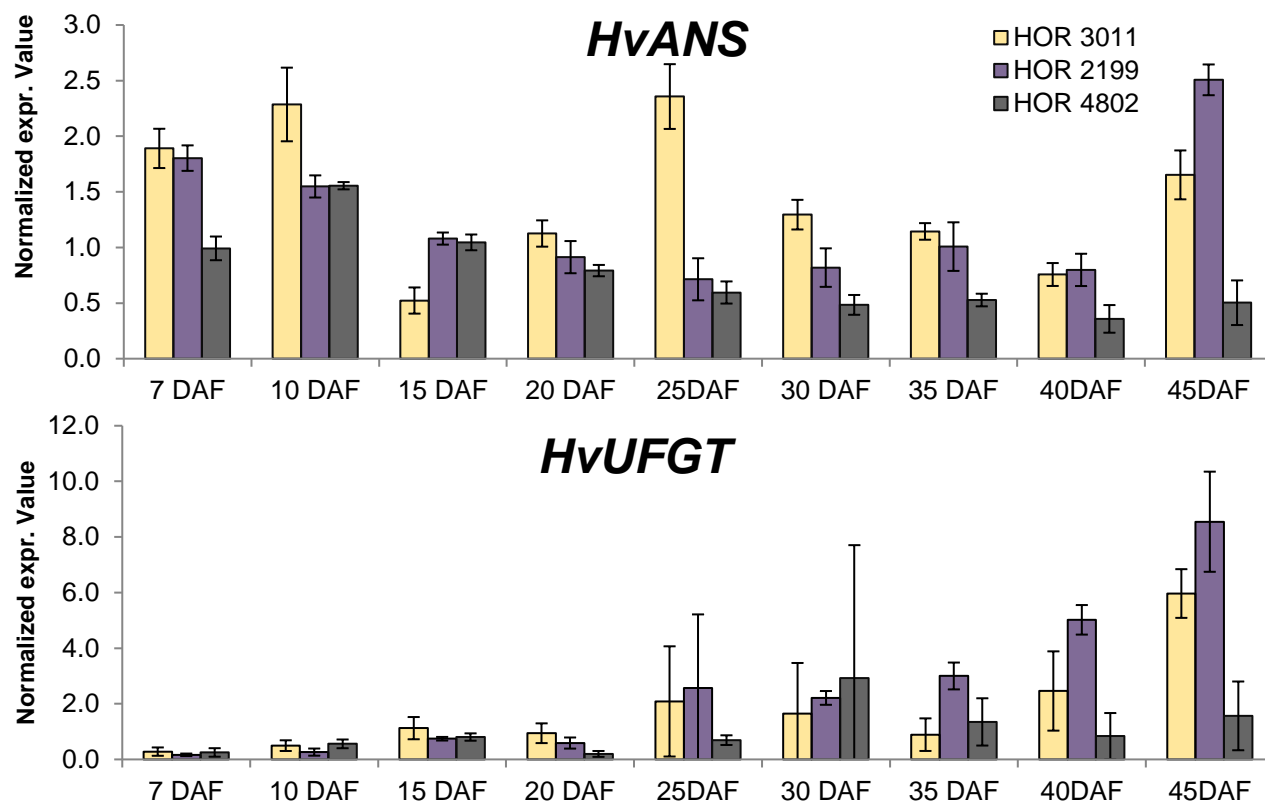
level. However, *HvF3H* and *HvDFR* started increasing again as the seed color turned from green to purple. In general, *HvDFR* expressed at higher levels compared to *HvF3H*.



**Figure 47. Transcript levels of the general genes in flavonoid pathway during barley grain development.** Barley plants were grown in climate chamber in 2014. Expression of *HvF3H* and *HvDFR* transcripts was analyzed in the grains of different-colored barley varieties (yellow-grained cultivar **HOR 3011**, purple-grained cultivar **HOR 2199** and black-grained cultivar **HOR 4802**) at different developmental stages. Expression values of targeted genes were normalized by two reference genes *eEF121* and *GADPH*. Error bars represent SD of three technical replicates in three independent measurements.

### 3.8.2. Anthocyanin synthetic genes – *HvANS* and *HvUFGT*

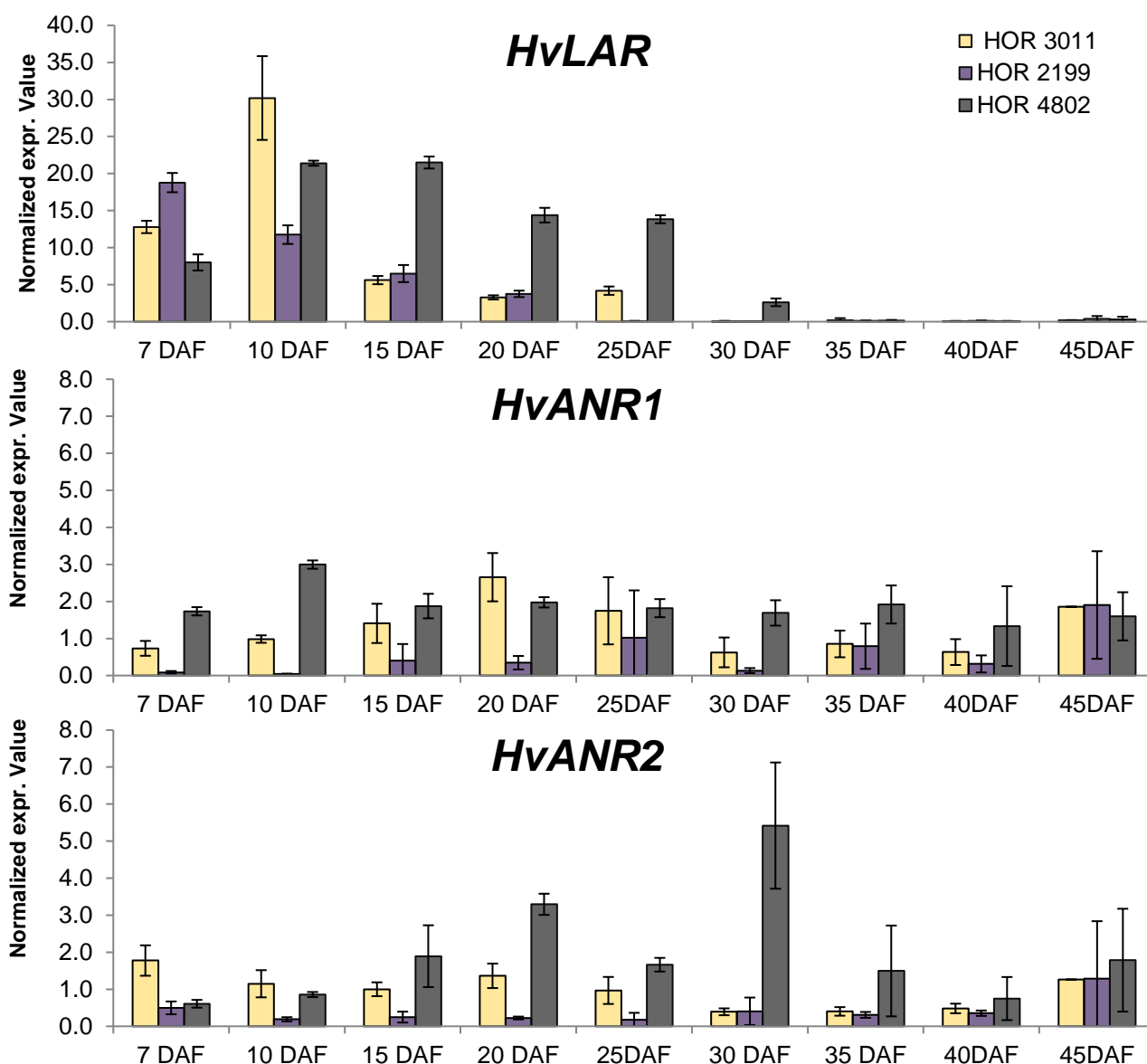
To examine whether the color difference between barley cultivars is due to expression of the anthocyanin biosynthesis genes, the expression of anthocyanin-specific genes (*HvANS* and *HvUFGT*) were also analyzed in different colored barley cultivars (Figure 48). *HvANS* in anthocyanin-free cv. HOR 3011 behaved multiple changes of expression through grain development, the transcriptional value increased at the mature stage after two-time decreases. While the expression of *HvANS* in anthocyanin-containing cv. HOR 2199 firstly presented a declining trend and rose continuously as anthocyanin accumulated on seed pericarp. On the contrary, the expression of *HvUFGT* did not differ in anthocyanin-free and anthocyanin-containing cultivars, their transcriptional levels enhanced during seed development. *HvANS* and *HvUFGT* in black-grained cv. HOR 4802 expressed at low levels through grain development.



**Figure 48. Transcript levels of anthocyanin-specific genes during barley grain development.** Barley plants were grown in climate chamber in 2014. Expression of *HvANS* and *HvUFGT* transcripts was analyzed in the seeds of different-colored barley varieties (yellow-grained cultivar **HOR 3011**, purple-grained cultivar **HOR 2199** and black-grained cultivar **HOR 4802**) at different developmental stages. Expression values of targeted genes were normalized by two reference genes *eEF121* and *GADPH*. Error bars represent SD of three technical replicates in three independent measurements.

### 3.8.3. Proanthocyanidin synthetic genes – *HvLAR* and *HvANR*

The expression of proanthocyanidin-specific genes (*HvLAR*, *HvANR1*, and *HvANR2*) was investigated in developing grains (Figure 49). Data indicated that *HvLAR* is highly expressed at the beginning of ripening stages in all three barley varieties, but they were observed with dramatical decreases until seed maturation. The time course of transcriptional levels of proanthocyanidin synthesis related gene *HvLAR* paralleled the catechin accumulation. By contract, both anthocyanidin transferred genes *HvANR1* and *HvANR2* performed relatively low expression values during the progress of seed maturation.

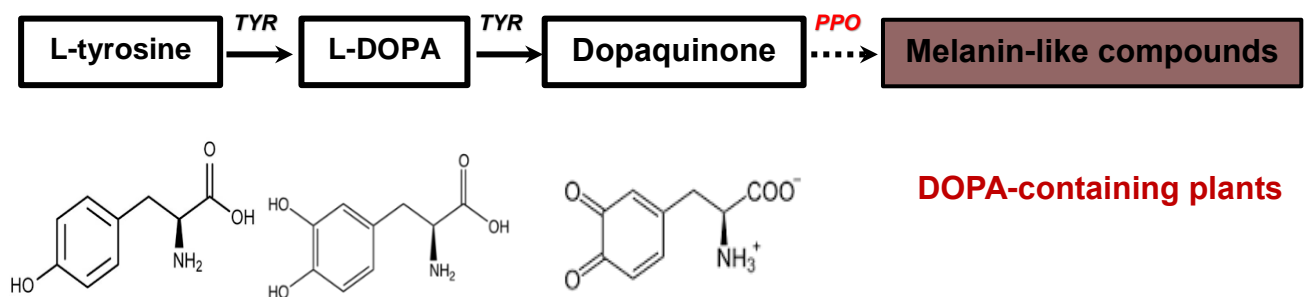


**Figure 49. Transcript levels of proanthocyanidin-specific genes during barley grain development.** Barley plants were grown in climate chamber in 2014. Expression of *HvLAR* and *HvANR1*, *HvANR2* transcripts was analyzed in the grains of different-colored barley varieties (yellow-grained cultivar **HOR 3011**, purple-grained cultivar **HOR 2199** and black-grained cultivar **HOR 4802**) at different developmental stages. Expression values of targeted genes were normalized by two reference genes *eEF121* and *GADPH*. Error bars represent SD of three technical replicates in three independent measurements.

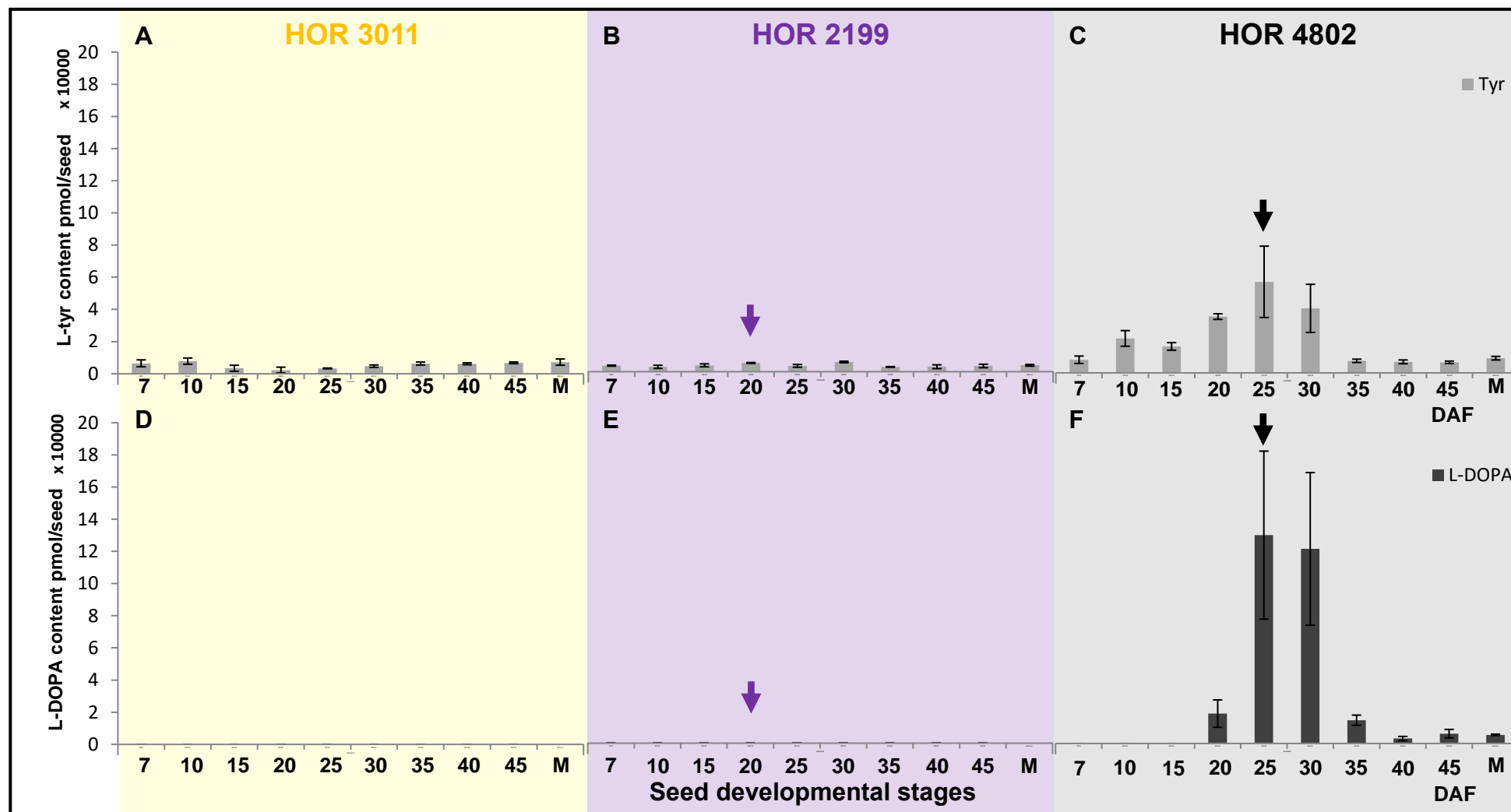
### 3.9. The detection of substrate candidates producing presumptive melanin in barley grains

In DOPA-containing plants, melanin-like compounds are proposed to be synthesized by polyphenol oxidase/tyrosinase from L-tyrosine and L-DOPA (Figure 50). To determine whether melanin accumulation was involved in the black pigmentation of barley grains, targeted analyses of two

potential melanin precursors (L-tyrosine and L-DOPA) from the developmental grains of three barley cultivars were performed by UPLC-Fluorescence. As shown in Figure 51, significant differences in L-tyrosine and L-DOPA contents were observed between cultivars and also at different developmental stages. When compared with black-grained cultivar HOR 4802 L-tyrosine accumulated at relatively low levels in yellow- and purple-grained barley. L-DOPA was only detected in black-grained barley rather than the other two cultivars. Moreover, the highest contents of L-tyrosine and L-DOPA perfectly matched with the starting point of seed pigmentation in barley cultivar HOR 4802, their contents declined substantially during seed maturation. L-tyrosine accumulated around 15 days earlier than L-DOPA.



**Figure 50. Proposed melanin synthesis pathway (Andrews & Pridham, 1966).** Melanin biosynthesis in DOPA-containing plants occurs by an oxidation process starting with the amino acid L-tyrosine. The major step is the oxidation of tyrosine to dopaquinone by the enzyme tyrosinase. Subsequent steps involve the L-DOPA. *PPO*, polyphenol oxidase; *TYR*, tyrosinase; L-DOPA, L-3, 4-dihydroxyphenylalanine.



**Figure 51. Changes in the concentrations of L-Tyr and L-DOPA during seed development of three barley genotypes.** Barley plants were grown in the greenhouse in 2015. L-Tyr (A, B, C) and L-DOPA (D, E, F) of yellow-grained cv. **HOR 3011**, purple-grained cv. **HOR 2199** and black-grained cv. **HOR 4802**. Relative quantification of potential melanin precursors was calculated according to commercial amino acid standards mixed with L-DOPA. Error bars represent SD of three independent measurements. The arrows indicate the starting point of seed pigmentation.

### **3.10. Summary of Part B**

The purpose of this part is to explore pigment accumulation during seed development using barley as the model. The quantification of the total anthocyanin concentrations from 23 different barley accessions showed that the pigments in some colored cultivars were not extractable. The initial speculation was that this non-extractable pigments might be related to oxidized proanthocyanidins (PAs) polymers derived from flavan-3-ol. For further exploration, two dark-grained barley cultivars (HOR 2199 & HOR 4802) with extractable or non-extractable pigments using the solvent for anthocyanin extraction and without extractable pigments were selected to explore pigment accumulation during seed development, and the yellow-grained cultivar HOR 3011 was used for comparative analysis.

Based on LC-MS/MS analysis, anthocyanins were only found in the purple-grained barley cultivar HOR 2199. The levels of PAs and the accumulated precursor did not differ among the three lines during grain maturity. At early developmental stages, the concentration of catechin increased but decreased with ascending concentrations of PA dimers and trimers during seed maturation. A common gene of the flavonoid pathway (*DFR*) and the proanthocyanidin synthesis related gene (*LAR*) at transcriptional levels paralleled the phytochemical variations. These results demonstrated that neither catechin nor PAs was linked to the dark coloration of barley cultivar HOR 4802. Therefore, a new hypothesis was proposed that melanin-like substances might be responsible for the black pigmentation in barley grains. Two potential precursors of melanin-like substances (L-tyrosine and L-DOPA) in black-grained cultivar HOR 4802 were found with a significant increase at the onset of pigmentation, whereas relatively low levels of L-tyrosine and no L-DOPA were detected in both anthocyanin-containing cultivar HOR 2199 and yellow-grained cultivar HOR 3011.



## 4. Discussion

Variation in grain pigmentation not only leads to colorful seed appearances and differential chemical composition but also might involve altered physiological and genetic characteristics. The focus of this chapter was to demonstrate the physiological, biochemical and biosynthetic characterization regarding the pigments in the seed coat of cereals. The first part illustrated whether anthocyanins, the most common pigment, had an effect on the yield and germination of wheat seeds. Additionally, the phytochemical and molecular mechanisms of different seed pigmentation in barley were elucidated in the second part. The key results were discussed as following:

- **Section 4.1.** By classifying the 2<sup>nd</sup> generation of mapping population crossed from non-purple wheat cultivars with purple cultivars into non-pigmented offspring (bulk-) and pigmented offspring (bulk+), most phenolic compounds rather than anthocyanins in the bulked materials were mostly equilibrated.
- **Section 4.2.** No significant variation for major yield components and seed germination between wheat bulks- and bulks+ indicated that anthocyanins deposition in wheat grains had no impact on measured parameters.
- **Section 4.3.** Differential pigmentation in developing caryopses was observed among selected barley varieties also differing in fresh mass.
- **Section 4.4.** LC-MS profiles of barley grain at different developmental stages revealed distinctive patterns of compounds associated with grain pigmentation. Anthocyanins were the main factor contributing to the purple coloration of barley seeds, while PAs accumulation was barely related to the black pigmentation.
- **Section 4.5.** The expression levels of transcripts that encode anthocyanin biosynthetic genes (*HvF3H*, *HvDFR*, *HvANS*, and *HvUFGT*) during the developmental stages were accompanied by the increase in the anthocyanin concentration. However, the catechin and PAs accumulation during seed maturation paralleled with the expression patterns of related genes (*HvLAR*, *HvANR1*, and *HvANR2*) among different-pigmented barley varieties.
- **Section 4.6.** Melanin-like compounds might be the non-extractable pigments for the black coloration due to potential melanin precursors (tyrosine and L-DOPA) accumulated at relatively high levels in black-grained barley than yellow-grained and purple-grained cultivars.

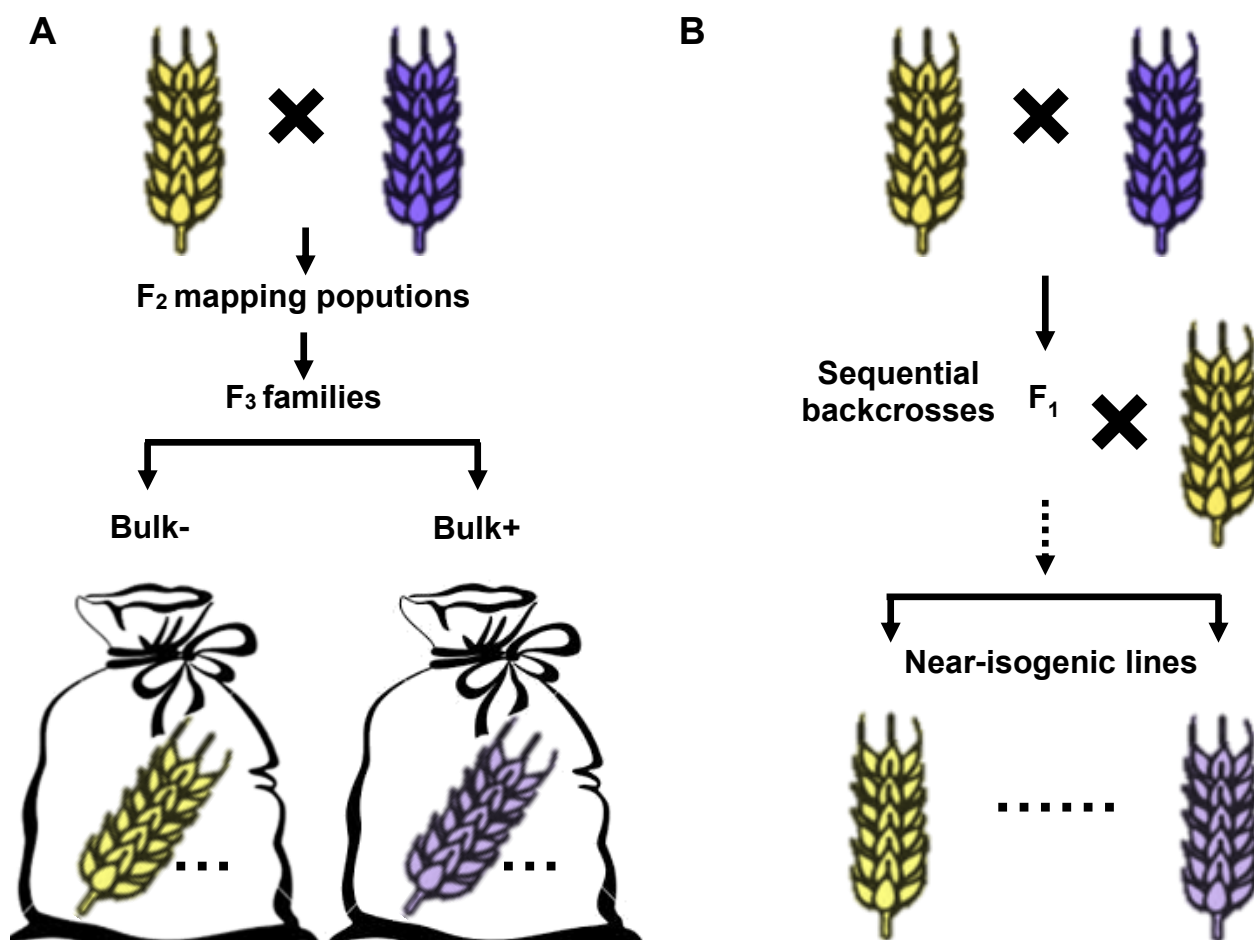
## ***Part A. Anthocyanin accumulation in wheat grains and its potential effects on growth, grain yield, and seed germination***

### **4.1. Chemical constituents except for anthocyanins of wheat grains were well balanced between wheat bulks- and bulks+**

#### **4.1.1. The strategy used for the equilibration of seed compositions**

The materials in this work are the mapping population generated by crossing anthocyanin-free wheat lines ‘N67’ and ‘S29’ with anthocyanin-containing wheat cultivars ‘Purple’ and ‘PF’ followed by the subsequent selfing process (Figure 52A). As described by Dobrovolskaya et al. (2006), further information about mapping populations showed that F<sub>2</sub> generation segregated for wheat genotypes with different shades of grain colors after self-pollination of F<sub>1</sub> plants. Since non-purple and purple parental lines differed not only in anthocyanin occurrences but also in many other substrates such as carbohydrates, free amino acids or lipids. A practical strategy was carried out using bulked F<sub>2</sub> progeny, in which bulk- materials included only yellow seeds and bulk+ materials contained only purple wheat grains. This method was conducted to equilibrate the bulk- and bulk+ materials with most seed composition, so as to avoid possible effects of the primary compounds.

As the traditional method used for comparative studies of the physiological function of a single gene, same property of well-balanced materials can also be obtained via developing near-isogenic lines (NILs). As shown in Figure 52B, the conventional type of experimental materials consisting of a set of closely related genotypes which differ from each other largely only in and around a known target gene, which is achieved through repeated backcrossing and extensive genotyping (Arbuzova et al., 1998). Although NILs represent a powerful means of establishing gene function that the development of NILs can bring aid in elucidating the physiological role of anthocyanins in the wheat pericarp (Gordeeva et al., 2014), the process is often more laborious and time-consuming. For the establishment of wheat NILs, more than eight backcrosses should be done for one genotype to recover a generation by the conventional method, and even advanced generations of NILs can be difficult to phenotype because one gene might be involved in not only one functions (Arbuzova et al., 1998).



**Figure 52. Simplified schemes for generating wheat materials differ only in anthocyanins.** (A) Anthocyanin-free wheat cultivars were crossed with anthocyanin-containing wheat cultivars, after one-generation selfing process, all offspring with and without anthocyanins which named as bulk+ and bulk- were collected individually; (B) A near-isogenic line (NIL) panel is often used for identification of high-likelihood candidates for quantitative trait locus analysis (QTLs). Take pigmented wheat as an example, a donor (anthocyanin-containing) and reference or recurrent (anthocyanin-free) parent are crossed initially. Subsequently, repeated backcrosses to the reference parent lead to a reduction of the donor genome contribution. Therefore, a panel of NILs can be constructed.

#### 4.1.2. Phytochemical characterization of wheat bulk- and bulk+ materials

Phytochemical qualitative analysis exhibited a good range of primary metabolites (amino acids, phenols, and sugars) and targeted secondary metabolites present in wheat grains. The data presented here revealed that the strategy applied to wheat offspring could effectively generate well-balanced materials with an almost equal metabolic background of seed compositions except for anthocyanins.

#### 4.1.2.1. Pigments observed in wheat bulk+ were related to the presence of anthocyanins

As summarized in Table 17, pigmented wheat were characterized by the presence of anthocyanins, which compared to yellow-grained wheat samples with absent anthocyanins by LC-MS approach and trace amounts of total monomeric anthocyanins by pH differential method. This finding was consistent with the result by Liu et al. (2010) that colored wheat varieties (Charcoal, Indigo and Konini) were found to possess significantly higher total anthocyanidin contents than yellow and white (Luteus and AC Vista) wheat. The two methods used in this research presented different quantification results, revealing that anthocyanin measurements were dependent on the extract method and quantitative approach. Similar experiments were carried out on the purple corn cob, total anthocyanins quantified by the pH differential method may overestimate anthocyanins in the material without considering possible presence of other reddish pigments (phlobaphenes or deoxyanthocyanins), contrasted to the LC-MS measurement with acid-hydrolyzed pigments led to underestimating the anthocyanin content (Lao & Giusti, 2016). Abdel-Aal et al. (2006) reported the LC analysis of red and white wheat extracts showed an absence of anthocyanin compounds in wheat grains, the author posed the idea that the small amount of total anthocyanin content may be contributed to additional pigment groups. For mature wheat grain, genotypic variation for anthocyanin concentration was found with statistically significant differences between two growing years, indicating that anthocyanin accumulation was influenced under distinct growth conditions. As demonstrated by several authors, environmental factors such as supplementary UV-B radiation and low temperature tended to induce anthocyanin synthesis (Guo et al., 2008; Sharma et al., 1998).

**Table 17. The detection of anthocyanins in wheat grain extracts using two approaches.** Wheat plants were grown in the greenhouse in 2014 and in the field at two plots in 2015. Anthocyanins in wheat grain extracts were measured by LC-MS and the pH differential method. The presence and absence of anthocyanins in wheat grains were indicated by + and – respectively; \ denotes missing of measurements; TA denotes trace amount. Wheat materials labeled in yellow and purple indicate the yellow and purple grain colors. GH, greenhouse.

Year	Location	Quantitative approach	Parental lines				Offspring							
			N67	S29	Purple	PF	'N67'×'Purple'		'N67'×'PF'		'S29'×'Purple'		'S29'×'PF'	
							Bulk-	Bulk+	Bulk-	Bulk+	Bulk-	Bulk+	Bulk-	Bulk+
2014	GH	LC-MS	\	\	\	\	-	+	-	+	-	+	-	+
		Spectrophotometer	\	\	\	\	TA	+	TA	+	TA	+	TA	+
2015	Plot 1	LC-MS	-	-	-	-	-	-	-	-	-	-	-	-
		Spectrophotometer	TA	TA	+	+	TA	+	TA	+	TA	+	TA	+
	Plot 2	LC-MS	-	-	-	-	-	-	-	-	-	-	-	-
		Spectrophotometer	TA	TA	+	+	TA	+	TA	+	TA	+	TA	+

#### **4.1.2.2. Most of the other seed components were evenly balanced between wheat bulk- and bulk+**

The chemical composition of wheat grains is complex, but it essentially contains carbohydrates, proteins, lipids and the minor components (minerals and vitamins) (Šramková et al., 2009). With respect to quantities of chemical constituents, notable differences exist among wheat varieties which strongly affect the quality of grain products made from wheat (Gutierrez-Alamo et al., 2008; Koehler & Wieser, 2013; Shewry et al., 2013). In this study, the comparison of major grain component contents between yellow and purple wheat cultivars showed that large differences were found in yellow-grained and purple-grained parental wheat lines. By using the equilibrating method, the grain composition of non-pigmented wheat samples which differed significantly with pigmented wheat samples became much less, as summarized in Table 18. Additionally, there were no sustainable differences found among bulked offspring grown under different conditions, implying that this unstable variation in analyzed phytochemical components between wheat bulk- and bulk+ grains mainly contributed to environmental factors. This finding coincided with a number of studies outlined by Gutierrez-Alamo et al. (2008), the growing conditions including geographical locations, growing seasons, soil types, farming management and rainfall could influence certain groups of wheat seed composition to various extents. These results suggested that the differences in grain components detected between bulk- and bulk+ were largely balanced using bulked wheat offspring.

## 96 | DISCUSSION

**Table 18. Summary of wheat grain components showed significant differences between bulk- and bulk+.** Wheat plants were grown in the greenhouse in 2014 and in the field at two plots in 2015. Compounds listed in the following table were found with significantly higher contents in bulk- than in bulk+ or in bulk+ than in bulk-. Even though several grain constituents of wheat grains from bulk- differed significantly with wheat bulk+ samples, there were no sustainable differences found among bulked offspring grown under different conditions. GH, Greenhouse.

Compounds	Year	Location	N67×P		N67×PF		S29×P		S29×PF	
			Bulk-	Bulk+	Bulk-	Bulk+	Bulk-	Bulk+	Bulk-	Bulk+
<b>Primary Metabolites</b>	2014	GH								
		Plot 1			Glycerol, Pentose alcohol I, D-fructose III, Hexose I	Pentose I, D-fructose II, <sup>13</sup> C-glucose <sup>1,2</sup> , D-Galactose, Hexose I, Trehalose			Glycerol, Malic acid, Citric acid, Glucuronic acid	Sucrose
	2015	Plot 2	Unknown <sup>11</sup>			Succinic acid, Fumaric acid, Unknown <sup>13</sup>	Pyroglutamic acid, Unknown <sup>4</sup>	Succinic acid, Unknown <sup>8</sup>		
<b>Amino Acids</b>	2014	GH		Phe			Cys			
		Plot 1		Cys						
	2015	Plot 2			Arg, Asn		Cys, Gln, Met			
<b>Elements</b>	2014	GH				Al, S				
		Plot 1		Ca, K, Mg				Ca		Fe, Mg, P, S, Zn
	2015	Plot 2	Zn		Zn		Al		Ca	

## 4.2. The influence of anthocyanins accumulating in wheat grains was insignificant on the parameters measured

Nilsson-Ehle (1914) first mentioned the physiological function of the pigments in wheat kernel correlated with seed viability, due to the fact that red-kernel wheat have well known to be more resistant to pre-harvest sprouting than white-kernel wheat. From then on, several studies have been performed to investigate the relationship between seed coat color and seed quality in a number of crops. It was commonly agreed that seeds with pigmented seed coats had a longer storage life as the result of better seed impermeability (Mohamed-Yasseen et al., 1994). Gvozdeva and Zhukova (1971) reported that dark-colored chickpeas were longer lived than varieties with light seed coats. Several bean types such as soybean, snap bean, and French bean with colored seeds was more resistant to deterioration under unfavorable storage conditions than pale-seeded lines (Starzinger et al., 1982; Roos, 1984; Powell et al., 1986). Khoroshailov and Zhukova (1973) similarly noted that red wheat cultivar showed longer life than white wheat. Besides, phenolic compounds in seed coats might also play a role in seed germination, and various hypothesis regarding chemical properties and the possible mechanisms were proposed in earlier studies. Tolman and Stout (1940) conclusively demonstrated the germination of sugar beet was inhibited by the presence of water-soluble substances in the seed coat. Later in 1941, they reclaimed that a hydrolytic enzyme was involved in the inhibitory action of the water-extract of beet seed, by which the seed liberated free ammonia in toxic quantities from nitrogenous substances present in the water extract. Nevertheless, Duym et al. (1947) drew a different conclusion that the inhibition of germination was mainly due to the osmotic pressure exerted by inorganic substances rather than the specific inhibiting substances present in the seed coat of sugarbeet. However, these results differed from the germination trials on wheat. It was suggested that phlobaphene was the main pigment responsible for brown color of wheat grain, and a negative relationship between germinated embryo number and concentration of catechin tannin (a suggested precursor to phlobaphene) from green tea leaves indicated the seed extractives were inhibitory action of seed germination before harvest (Miyamoto & Everson, 1958). This finding was agreed by Freed et al. (1976) that red pigmentation of wheat suppressed seed germination. But Beglinger (1995) in her dissertation mentioned an entirely new opinion that coloration of the pericarp and the culm by anthocyanin was found no linkage to the examined morphological and physiological traits of spelt wheat cultivars (*Triticum spelta* L.). A comprehensive study of germination responses in tomato was performed by Atanassova (1997), and

nine tomato genotypes and their anthocyanin-less isogenic line/near-isogenic lines were investigated in their experiment. Although the majority of IL/NILs germinated much faster than wild-type lines, the authors held the opinion that seed coat was not the main reason for more rapid germination because the result was the same after seed testa removal. They further speculated that the loci not only encoded the anthocyanin synthesis genes but also associated with other metabolic processes connected with germination capacities of tomato seeds. In addition to germinate speed, a negative effect was also found in anthocyanin-containing seeds on germination rate. Arabidopsis mutants with affected flavonoid pigmentation exhibited a reduced seed dormancy compared with corresponding wild type, which also indicated a higher germination rate. Moreover, pigmentation mutants of Arabidopsis presented a better capacity to germinate in dark condition (Debeaujon et al., 2000). The comparison of germination test between colorless popcorn line and anthocyanin-containing near isogenic line demonstrated that the yellow genotype had a significantly higher germination percentage, in contrast to less germinated seeds of the pigmented near isogenic line, however, the author did not give any further explanation (Lago et al., 2012). Previous studies about the correlation between seed coat pigmentation and seed germination can be summarized for the following opinions: (1) the genes or quantitative trait loci (QTLs) for seed color closely linked to those for loss of seed dormancy (early seed germination), and there was a significant correlation between these three traits in a number of species (Torada & Amano, 2002); (2) testa pigmentation was thought to improve seed vigor and germination in legumes due to better resistance to solute leakage, imbibition damage, and attack by soil-born fungi (Powell, 1989; Kantar et al., 1996); (3) dark colored testa in legumes and some dark soybean cultivars accompanied with slow water uptake was attributed to the presence of phenolic compounds, and tight adherence of the seed coat to the embryo, seed coats which were loosely attached to the embryo showed a greater rate of imbibition and faster germination (Chachalis & Smith, 2000).

In brief, the positive correlation found between seed coat pigmentation and the inhibitory effect on seed germination has led several authors to conclude that seeds with pigmented seed coat have slower germination behavior in comparison of non-pigmented seed coated cultivars in diverse species, even though the component of seed pigmentation was not always attributed to anthocyanins. By the advances in analytical techniques, the pigmentation of wheat grain color was mainly determined by anthocyanins located in the seed coat (Abdel-Aal & Hucl, 2003; Escribano-Bailon et al., 2004; Abdel-Aal et al. 2006; 2008). However, a search of the literature discloses little attention



has been devoted to the study of the physiological function regarding anthocyanins to wheat seed pigmentation. By the above observations, this study was carried out to investigate the influence of anthocyanin accumulation on grain yield and germinability of wheat varieties. It was found that anthocyanin-containing wheat seeds (**bulk+**) did not differ in most growth and yield parameters as well as seed germination when compared with anthocyanin-free wheat seeds (**bulk-**), but seed morphology (such as size, width, and thousand kernel weight) showed a significant difference between **bulk-** and **bulk+**. These results revealed that the anthocyanin-based characters of wheat grain did not prove to play roles on the examined physiological traits, this conclusion was in conflict with the previous reports that germination rates varied with seed coat colors (Powell, 1989; Debeaujon et al., 2000; Atanassova et al., 2004).

Seed size was considered to positively influence the seed yield especially seed germination in wheat (Mian & Nafziger, 1994; Al-Qasem et al., 2011; Keerio, 2013; Shahwani et al., 2014). In this study, although seed size and thousand kernel weight of **bulk+** achieved from 'N67' crossed with 'Purple/PF' were found with statistically higher values than **bulk-** in 2015, the germination percentage of **bulk+** was not significantly higher than that for **bulk-**. Furthermore, pigmented wheat seeds obtained from 'S29' crossed with 'Purple/PF' did not differ with non-pigmented **bulk-** either in seed size or seed germination. The results again indicated that the accumulation of anthocyanins in wheat grains had no effects on seed size and grain yield traits. The minor differences might be associated with the variation in genotypic behavior or response to different growing conditions.

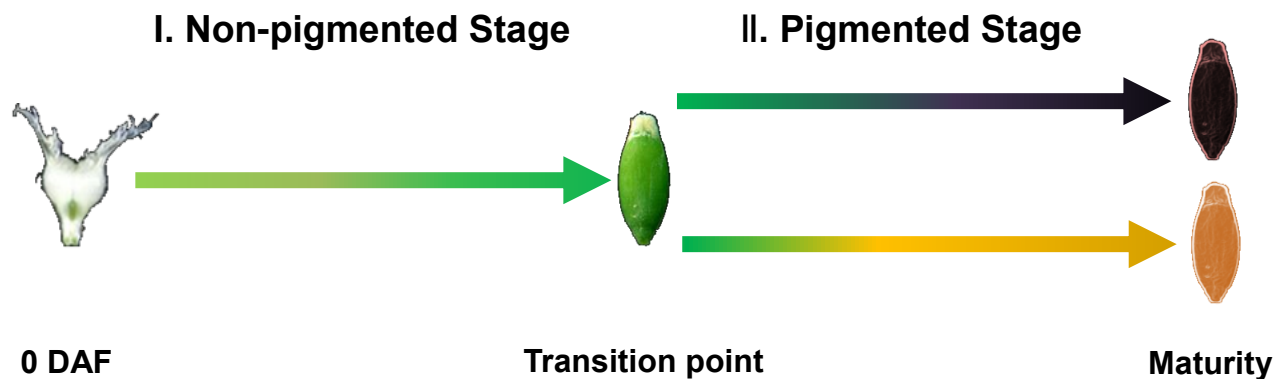
### ***Part B. Understanding the metabolic and molecular mechanisms controlling barley grain pigmentation at different developmental stages***

#### **4.3. Phenotypic and developmental variations of grain color in barley (*Hordeum vulgare* L.)**

The phenotypic diversity regarding kernel or pericarp color in barley has been classified into white/yellow, blue, purple and black due to different concentrations of various chemical compositions (Faris, 1955; Mullick et al., 1958; Mullick, 1966; Demissie & Bjornstad, 1996; von Bothmer et al., 2003; Derbew et al., 2003; Choo et al., 2005; Baik & Ullrich, 2008). Since anthocyanins constitute one of the major groups of natural pigments for colored cereal grains (Escribano-Bailon et al., 2004; Abdel-Aal et al., 2006; Kim et al., 2007), twenty-three barley

accessions with different-pigmented pericarps were screened for their anthocyanin contents in this study. It was noticed that pigments in three dark-seeded barley varieties could not be extracted by common organic solvents. According to the kernel color and extractability of anthocyanins, 23 barley genotypes were divided into three groups: anthocyanin-free varieties with non-colored grains; anthocyanin-containing varieties with colored grains and anthocyanin-free varieties with colored grains. Therefore, yellow-grained cultivar **HOR 3011** as a control, purple-grained barley cultivar **HOR 2199** with the highest anthocyanin concentration, and black-grained barley cultivar **HOR 4802** with non-extractable pigment were selected (1) to determine the phytochemical compositions of purple, black, and anthocyanin-free barleys in developing grains; (2) to explore the molecular mechanism of differential grain pigmentation in barley during development.

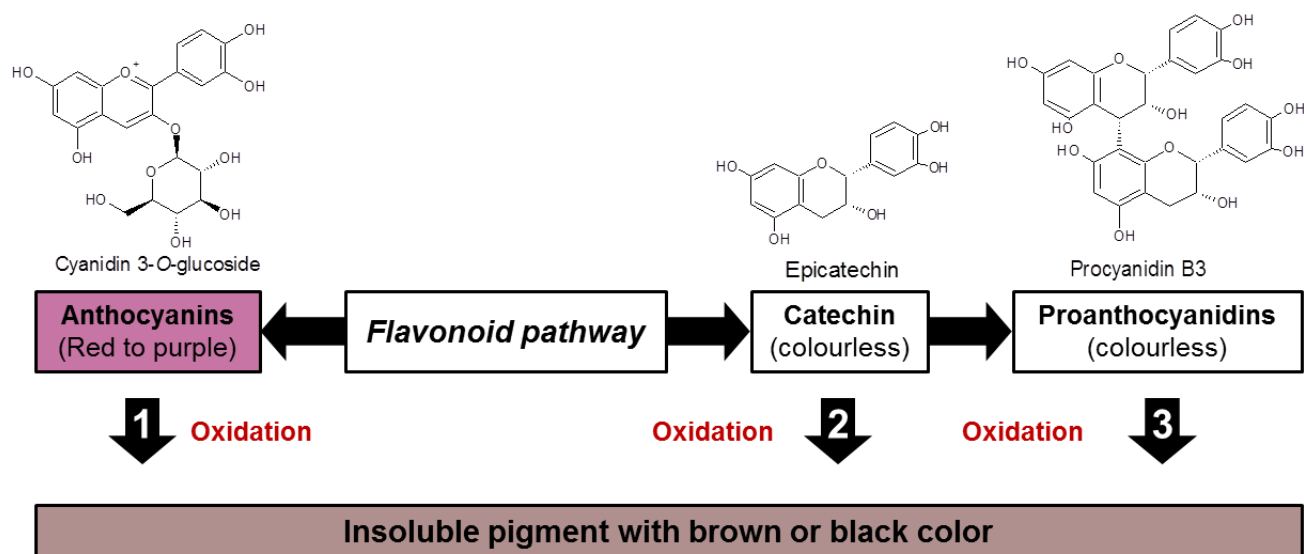
Considerable variations were observed in grain color of barley at different developmental stages among three selected genotypes. Depending on pigment deposition during seed maturation, the developmental grain pigmentation revealed two characteristic phases: non-pigmented and pigmented stages (Figure 53). Green pigments accumulation started after flowering till the transition stage for all three selected barley varieties. Then purple or black pigments continued to intensify until the seed maturity, contrasting to yellow barley with no pigment accumulation. These findings were similar to the coloration of the other grains, particularly wheat and rice, during seed development (Knieval et al., 2009; Žofajová et al., 2012; Trojan et al., 2014; Rahman et al., 2015). In addition, the appearance of pigments in colored barley cultivars varied between two growing years, revealing that environmental conditions play an important part in color development in the barley kernel. Furthermore, although the constituents of pigments in purple barley during seed maturation were already investigated (Kohyama et al., 2008; Bellido & Beta, 2009), this was the first study to provide photo documents recording color development and to demonstrate the black pigmentation observed in barley.



**Figure 53. Development of barley grain pigmentation.** Based on seed coat color, the development of barley grain was divided into two stages from flowering to maturity. Phase I. Color keeps essentially stable from pale to dark green. Phase II. Color changes from dark green to differential colors in different-pigmented barley cultivars. The photos were obtained from website <http://pgrc.ipk-gatersleben.de/seeds/greenlayers.html>. DAF, days after flowering.

#### 4.4. Biochemical mechanisms underlying the differences in kernel color of barley

The seed coat color of barley is determined by the differential accumulation of phenolic compounds (Choo et al. 2005). Previously, researchers showed that the absence and presence of anthocyanins were largely responsible for the pigments deposited in yellow/white, blue and purple barley varieties (Faris, 1955; Mullick et al., 1958; Kim et al., 2007). However, since most of the experimental work emphasized on the inheritance of black pigments in barley caryopses (Buckley, 1930; Woodward, 1941), the biochemistry mechanism involved in black pigmentation of barley had never been clearly elucidated. By contrast, the different characterization of brown/black pigments during seed desiccation in some plant species, especially *Brassica napus*, *Arabidopsis* and *Medicago truncatula*, are well understood (Pourcel et al., 2005; Qu et al., 2013; Liu et al., 2016). As summarized in Figure 54, anthocyanins, epicatechin and PA derivatives are three important phenolic compounds in the synthesis of brown/black pigmentation through complex oxidations. Jiang et al. (2004) correlated seed coat darkening with an increase of anthocyanins dimers, which suggested that brown pigments were produced by the oxidation of anthocyanins by oxidases in Litchi fruits. In *Arabidopsis*, wild-type seeds turned to dark brown at harvest, while seeds of the *TRANSPARENT TESTA10* null (laccase/PPO defected) were yellow with an increase in soluble (non- or poorly oxidized) PAs (Debeaujon et al., 2003; Routaboul et al., 2006). Similar reactions were also observed in pinto bean lines (*Phaseolus vulgaris* var. *Pinto*), pigmented soybean (*Glycine max*), and brown-seeded *Brassica carinata* (Beninger et al., 2005; Marles et al., 2003; Todd & Vodkin, 1993).



**Figure 54. Simplified schematic of the flavonoid pathway (Pourcel et al., 2007).** Three main classes of end-products from flavonoid pathway are presented and their chemical structure illustrated by one example for each class. (1) Anthocyanins which are responsible for red to purple color, colorless (2) catechin and (3) PAs might be oxidized to brown or black color during seed maturation.

Based on the knowledge described above, to determine which phenolic compounds accumulated in grains of **HOR 3011**, **HOR 2199**, and **HOR 4802**, anthocyanins and proanthocyanidins were isolated from developing grains at different stages and analyzed using LC-MS and LC-MS/MS. The results suggested that different pigment patterns of distinct pigmentation pathways were involved in yellow-, purple- and black-grained barley varieties.

#### 4.4.1. Anthocyanins were not the sole factor contributing to grain coloration in pigmented barley

Anthocyanins are one of the most widespread pigments in plant, which display a great diversity of colors from red through purple and blue hues (de Pascual-Teresa & Sanchez-Ballesta, 2007; Santos-Buelga et al., 2014). By 2013, Andersen and Jordheim reported that 702 different anthocyanins with about 200 additional tentative species were identified in nature. A great diversity of anthocyanin patterns was also found in barley. Kim et al. (2007) detected five aglycone forms of anthocyanins including cyanidin, delphinidin, pelargonidin, peonidin, and malvidin from 127 barley lines, their total anthocyanin concentrations ranged from 13.0 to 1037.8  $\mu\text{g/g}$  DW. In this study, eight anthocyanin types were tentatively identified in HOR 2199, among which cy-3-glucoside, two cy-3-malonyl-hexose isomers, and cy-3-(di-malonyl)-hexose were in agreement with earlier identification using NMR by Kohyama et al. (2008). According to previous literature, the other four

isolated anthocyanins, cy-malonyl-di-hexose, cy-3-(malonyl-hexose), pg-3-(di-malonyl)-hexose and pn-3-(di-malonyl)-hexose, were first mentioned in barley grains. In addition that, a dramatic reduction of anthocyanin content observed in HOR 2199 during seed maturation was consistent with previous findings (Mullick, 1959; Kohyama et al., 2008). This observation can be explained by the hypothesis that anthocyanins were bound to the cell wall or some flavonoids and result in products with low or no extractability. Some authors proposed a 'dry matter dilution' theory that increasing starch in endosperm may lead to a dilution impact on total anthocyanin content (Kniewel et al., 2009, Bustos et al., 2012). Besides, the loss of anthocyanins might be caused by the anthocyanin degradation during seed development (Oren-Shamir, 2009).

By contrast, no or trace amount of anthocyanins were detected in the whole developmental stages in HOR 4802, provided promising evidence that other pigment rather than anthocyanins were responsible for the black pigmentation of barley grain. Oxidized PAs was considered as a reasonable candidate pigment in HOR 4802 because proanthocyanidins were the major phenolic compounds in barley grains (McMurrough et al., 1983; Klausen et al., 2010; Verardo et al., 2015). Quinde-Axtell et al. (2005, 2006) observed that flour from PAs-free barley mutants presented whiter color than PAs-containing barley cultivars.

#### **4.4.2. Accumulation of catechin and proanthocyanidins were not related to the black pigmentation in HOR 4802**

The brown to black colors in seed coat are usually attributable to high contents of catechin and PAs, which are colorless molecules at the early developmental stages but undergo enzymatic oxidation to form insoluble complexes with the cell wall or other phenolics in the seed coat during seed maturation, causing brown to black pigmentation (Beninger & Hosfield, 1999; Pourcel et al., 2007; Zhao et al., 2010). In *Arabidopsis*, these genetic mechanisms underlying brown/black color formation has been well characterized by using transparent testa (*tt*) mutants and tannin-deficient seeds (Abrahams et al. 2003; Debeaujon et al. 2003; Baxter et al. 2005; Pourcel et al., 2005). The authors proposed that PAs were prominent flavonoid compounds in seed coat and conferred a brownish color to the testa undergoing oxidation during seed desiccation. In black-seeded *Brassica napus* line was reported to contain a high accumulation of PAs, but only a low amount of PAs was detected in the yellow-seeded line (Marles & Gruber, 2004; Akhov et al., 2009). Moreover, Qu et al. (2013) reported that high content of epicatechin, insoluble PAs, and its derivatives were also related

to the pigments observed in black-seeded *Brassica napus*. In agreement with these findings, Feng et al. (2014) found the total PAs in brown cotton fibers was higher than that in white ones, but it was insoluble PAs rather than soluble PAs contributed to the differences between brown and white cotton fibers. However, Liu et al. (2016) reported different results in the model legume *Medicago truncatula* that the seed color was not associated with soluble PAs or insoluble forms. The author pointed out that although loss function of *ANR* gave large reductions in both soluble and insoluble PAs in seeds, the seed color of *anr* mutants were dark-red comparing to yellow seed from the wild type.

In this research, catechin, gallic acid and five PAs including two dimers and three trimers were detected in both pigmented and non-pigmented grained barley lines. The levels of PAs and the accumulated precursor did not differ among the three lines through the developmental stages. In agreement with Lepiniec et al. (2006), the catechin content increased significantly at early developmental stages, but reduced with ascending concentrations of PA dimers and trimers during seed development, suggested that catechin might be the major precursor of PAs in barley. The most abundant PAs (procyanidin B3 and prodelfinidin B3) appeared constantly stable after the rises in seed extracts of all three barley cultivars. These findings demonstrated that the differences in seed coat color observed in barley were hardly attributable to the catechin and PAs. Therefore, it can be concluded that the black pigmentation in HOR 4802 was not related to the oxidation of catechin and PAs.

#### **4.5. The expression of genes involved in flavonoid biosynthesis in relation to anthocyanin and proanthocyanidin levels in yellow-, purple- and black-grained barley during seed development**

Barley grains contain significant contents of phenolic compounds such as anthocyanins and PAs may responsible for different colors (Mullick et al., 1958; Lundqvist & Franckowiak, 2003; Baik & Ullrich, 2008), these were partially confirmed in the previous section. cDNA fragments of seven structural genes of the flavonoid biosynthesis which lead to anthocyanins and PAs were isolated in this study to clarify their expression patterns in developing barley. Among them, *HvF3H* and *HvDFR* are the last two mutual enzymes before the flavonoid pathway splits into two branches, anthocyanins and PAs, by which their common precursor leucocyanidin is formed from dihydroflavonol (Jende-Strid, 1991; 1993). *HvANS* and *HvUFGT* are genes encoding anthocyanins

as well as catechin (PAs precursor) is controlled by *HvLAR* (Kristiansen, 1984; Jende-Strid, 1991; 1993; Meldgaard, 1992; Himi et al., 2015). Moreover, the biosynthesis of anthocyanins and PAs is related via the enzyme anthocyanidin reductase (*ANR*) by converting anthocyanidin to PAs precursor epicatechin in some plant species (Devic et al., 1999; Xie et al., 2003). Therefore, two presumptive genes (*HvANR1* and *HvANR2*) in barley were characterized in the current experiment as well. The differences in the expression patterns of genes involved in flavonoid biosynthesis throughout seed development revealed distinct genetic characterization of different pigments formation in yellow-, purple- and black-grained barley varieties.

In the present study, quantitative real-time PCR analysis indicated that the expression levels of *HvF3H*, *HvDFR*, *HvANS*, and *HvUFGT* in developing barley increased in concurrence with the accumulation of anthocyanins resulting in purple coloration of HOR 2199 at later stages of grain development. By contrast, *HvF3H* and *HvDFR* were not expressed or expressed at very low levels in anthocyanin-free barley genotypes HOR 3011 and HOR 4802, while transcript levels for *HvANS* and *HvUFGT* were both higher in yellow and purple barley cultivars. These findings suggested that *F3H* and *DFR* played the critical role in anthocyanin production, *ANS* and *UFGT* expressions were not decisive for the pigmentation without or with low transcription of *F3H* and *DFR*. Similar results have been obtained in the comparative transcriptional analysis of the anthocyanin biosynthesis structural genes in different wheat organs such as grains, coleoptiles, and auricles (Himi & Noda, 2005; Ahmed et al., 2009; Tereshchenko et al., 2013; Khlestkina et al., 2014). Moreover, *HvF3H*, *HvDFR*, *HvANS*, and *HvUFGT* all exhibited low expression levels in HOR 4802 during late stages of grain development, which revealed the absence of anthocyanins in black barley cultivar. Thus, a distinct genetic network rather than the flavonoid pathway was underlain with the black pigmentation of barley grain. This result agreed with the differential expression of the flavonoid biosynthesis structural genes in the yellow-grained barley cultivar Bowman and its near-isogenic lines (NILs) with purple or black pericarp (Shoeva et al., 2016).

On the other hand, the temporal pattern of transcript levels for PA-specific genes *HvLAR* was consistent with the timing of catechin (PAs precursor) accumulation in all three barley varieties. The *HvLAR* appeared at high expression level during early developmental stages was followed by a significant decrease during seed maturation. Furthermore, there were no significant differences in the expression level of *HvANR1* and *HvANR2* among the different developmental stages supported

the previous study that the amount of anthocyanin was not related to PA contents in barley (Jendestrid, 1978; Kohyama et al., 2008). These results further demonstrated that neither anthocyanins nor PAs contributed to the black color formation of barley grain at the transcriptional level.

#### **4.6. Do melanin-like compounds contribute to the black pigmentation in HOR 4802?**

As already stated above, both phytochemical and transcriptional results revealed that the two most common pigments in cereals, anthocyanins and oxidized PAs, were not attributed to the black coloration in barley cultivar HOR 4802. In addition to flavonoids, few researchers assumed that melanin-like compounds might also be responsible for the seed color of barley (Buckley, 1930; Woodward, 1941; Faris, 1955; Mullick, 1959; Shoeva et al., 2016), although melanins were not well characterized in plants (Lerner & Fitzpatrick, 1950; Andrews & Pridham, 1967; Riley, 1997). Due to the insolubility and structure complexity of plant melanins, so far no decisive proof has been made to identify these dark pigments. However, the detection of the enzyme (polyphenol oxidase) and substrates (L-tyrosine, L-DOPA, catechol, and dopamine *etc.*) concerned with melanin formation would provide strong presumptive evidence for the occurrence of melanin-like compounds (Bertrand, 1896; Thomas, 1955). For example, both L-tyrosine and L-DOPA had been isolated from *Vicia faba* producing dark pigments (Guggenheim, 1913; Miller, 1920). Moreover, it was notable that aqueous solutions of L-DOPA were oxidized spontaneously to form melanins (Miller, 1920).

In the present study, the black pigments in barley grain of HOR 4802 were not dissolved in most organic solvents, which was in accordance with the insoluble characteristic of melanins. Besides, L-tyrosine and L-DOPA expressed considerably high levels when black pigmentation appeared in HOR 4802, but their contents decrease dramatically as the pigment intensified, suggesting that L-tyrosine and L-DOPA were gradually oxidized to melanin-like compounds in the late stages of grain development. By contrast, only minor L-tyrosine and no L-DOPA were detected in HOR 2199 and HOR 3011 during seed maturation. It seemed that the black pigments were positively correlated with the potential precursors of melanin-like compounds (L-tyrosine and L-DOPA), indicating that the color differences among black-, purple- and yellow-grained barley were related to the melanin-like compound or anthocyanin contents. However, further studies are still needed to determine the presence of the enzyme which oxidizes L-tyrosine and L-DOPA in HOR 4802.



## 5. Summary

In this thesis, the characterization of pigmented cereals was addressed from physiological, phytochemical and genetic aspects. The major findings of this study comprise two parts including (A) possible effects of anthocyanin accumulation in wheat grain on growth parameters, grain yield and seed germination; and (B) metabolic and molecular mechanisms of distinct pigmentation during barley grain development.

- **Part A.** Initially, wheat materials (**bulk-** and **bulk+**) were generated to equilibrate most seed compositions except for anthocyanins. The main compounds such as primary metabolites, amino acids, and mineral elements were well balanced except for anthocyanins. Further, the insignificant differences of crop yield parameters and seed germination between **bulk-** and **bulk+** demonstrated that there was no constant impact of anthocyanins accumulation in wheat grains.
- **Part B.** Total anthocyanin concentrations of 23 barley varieties with different grain colors were primarily screened for the classification of seed pigmentation. Several colored barley cultivars were found containing non-extractable pigments. Therefore, one anthocyanin-containing barley cultivar with purple grain color (**HOR 2199**), and two anthocyanin-free cultivars with black grains (**HOR 4802**) or yellow seeds (**HOR 3011**) were selected to explore pigment accumulation during seed development. To sum up, three phenolic compounds anthocyanins, catechin and PAs were considered to be related to the seed pigmentation in previous studies. It was found that anthocyanins could only be discovered in purple-grained barley cultivar **HOR 2199**, while catechin and PAs in the different-colored barley cultivars exhibited similar changes during grain development. These results suggested that anthocyanins were mainly responsible for the purple-grained barley cultivar **HOR 2199**, however, neither catechin nor PAs contributed to the black pigmentation in barley cultivar **HOR 4802**. The transcriptional expression of flavonoid structural gene expression paralleled with flavonoid accumulation during grain development. Furthermore, tyrosine and L-DOPA in black-grained cultivar **HOR 4802** were found with a significant increase right after pigmentation started, whereas relatively low levels of L-tyrosine and no L-DOPA were detected in both anthocyanin-containing cultivar **HOR 2199** and yellow-grained cultivar **HOR 3011**. But no catechol was found in all the tested materials. Further investigation of melanin-like pigments as the product of tyrosine and L-DOPA is still needed.

## 6. References

- Abdel-Aal el, S. M., & Hucl, P.** (2003). Composition and stability of anthocyanins in blue-grained wheat. *Journal of Agricultural and Food Chemistry*, 51(8), 2174-2180.
- Abdel-Aal el, S. M., Young, J. C., & Rabalski, I.** (2006). Anthocyanin composition in black, blue, pink, purple, and red cereal grains. *Journal of Agricultural and Food Chemistry*, 54(13), 4696-4704.
- Abdel-Aal, E. S. M., Abou-Arab, A. A., Gamel, T. H., Hucl, P., Young, J. C., & Rabalski, I.** (2008). Fractionation of blue wheat anthocyanin compounds and their contribution to antioxidant properties. *Journal of Agricultural and Food Chemistry*, 56(23), 11171-11177.
- Abrahams, S., Lee, E., Walker, A. R., Tanner, G. J., Larkin, P. J., & Ashton, A. R.** (2003). The Arabidopsis *TDS4* gene encodes leucoanthocyanidin dioxygenase (*LDOX*) and is essential for proanthocyanidin synthesis and vacuole development. *The Plant Journal*, 35(5), 624-636.
- Ahmed, N., Maekawa, M., & Noda, K.** (2009). Anthocyanin accumulation and expression pattern of anthocyanin biosynthesis genes in developing wheat coleoptiles. *Biologia Plantarum*, 53(2), 223-228.
- Al-Qasem, A., Kafawin, O., & Duwayri, M.** (1999). Effect of seed size and temperature on germination of two wheat cultivars. *Agricultural Sciences*.
- Andrews, R. S., & Pridham, J. B.** (1967). Melanins from DOPA-containing plants. *Phytochemistry*, 6(1), 13-18.
- Andersen, Ø. M., & Jordheim, M.** (2013). Basic anthocyanin chemistry and dietary sources. *Anthocyanins in Health and Disease*, CRC Press. 13-89.
- Arbuzova, V. S., Maystrenko, O. I., & Popova, O. M.** (1998). Development of near-isogenic lines of the common wheat cultivar 'Saratovskaya 29'. *Cereal Research Communications*, 26(1), 39-46.
- Atanassova, B., Shtereva, L., & Molle, E.** (1997). Effect of three anthocyaninless genes on germination in tomato (*Lycopersicon esculentum* Mill.) I. Seed germination under optimal conditions. *Euphytica*, 95(1), 89-98.
- Atanassova, B., Shtereva, L., Georgieva, Y., & Balatcheva, E.** (2004). Study on seed coat morphology and histochemistry in three anthocyaninless mutants in tomato (*Lycopersicon esculentum* Mill.) in relation to their enhanced germination. *Seed Science and Technology*, 32(1), 79-90.

- Akhov, L., Ashe, P., Tan, Y., Datla, R., & Selvaraj, G.** (2009). Proanthocyanidin biosynthesis in the seed coat of yellow-seeded, canola quality *Brassica napus* YN01-429 is constrained at the committed step catalyzed by *dihydroflavonol 4-reductase*. *Botany*, 87(6), 616-625.
- Bailly, C.** (2004). Active oxygen species and antioxidants in seed biology. *Seed Science Research*, 14(2), 93-107.
- Baik, B.-K., & Ullrich, S. E.** (2008). Barley for food: characteristics, improvement, and renewed interest. *Journal of Cereal Science*, 48(2), 233-242.
- Baxter, I. R., Young, J. C., Armstrong, G., Foster, N., Bogenschutz, N., Cordova, T., Peer, W. A., Hazen, S. P., Murphy, A. S., & Harper, J. F.** (2005). A plasma membrane H<sup>+</sup>-ATPase is required for the formation of proanthocyanidins in the seed coat endothelium of *Arabidopsis thaliana*. *PNAS*, 102(7), 2649-2654.
- Beninger, C. W., & Hosfield, G. L.** (1999). Flavonol glycosides from Montcalm dark red kidney bean: implications for the genetics of seed coat color in *Phaseolus vulgaris* L. *Journal of Agricultural and Food Chemistry*, 47(10), 4079-4082.
- Beninger, C. W., Gu, L., Prior, R. L., Junk, D. C., Vandenberg, A., & Bett, K. E.** (2005). Changes in polyphenols of the seed coat during the after-darkening process in pinto beans (*Phaseolus vulgaris* L.). *Journal of Agricultural and Food Chemistry*, 53(20), 7777-7782.
- Beglinger, C.** (1995). Physiological and genetic studies on early vigour of *Triticum aestivum* L. and *Triticum spelta* L. *Doctoral dissertation*, Swiss Federal Institute of Technology, Zürich.
- Bellido, G. G., & Beta, T.** (2009). Anthocyanin composition and oxygen radical scavenging capacity (ORAC) of milled and pearled purple, black, and common barley. *Journal of Agricultural and Food Chemistry*, 57(3), 1022-1028.
- Bertrand, G.** (1896). Sur la presence simultanee de la laccase et de la tyrosinase dans le suc de quelques champignons. *C. R. Hebd. Seances Academic Science*, 123, 463-465.
- Buckley, G. F. H.** (1930). Inheritance in barley with special reference to the color of caryopsis and lemma. *Scientific Agriculture*, 10(7), 460-492.
- Butelli, E., Titta, L., Giorgio, M., Mock, H. P., Matros, A., Peterek, S., ... & Martin, C.** (2008). Enrichment of tomato fruit with health-promoting anthocyanins by expression of select transcription factors. *Nature Biotechnology*, 26(11), 1301-1308.
- Bustos, D. V., Riegel, R., & Calderini, D. F.** (2012). Anthocyanin content of grains in purple wheat is affected by grain position, assimilate availability and agronomic management. *Journal of Cereal Science*, 55(3), 257-264.

- Cos, P., Bruyne, T. D., Hermans, N., Apers, S., Berghe, D. V., & Vlietinck, A. J.** (2004). Proanthocyanidins in health care: current and new trends. *Current Medicinal Chemistry*, 11(10), 1345-1359.
- Carletti, G., Nervo, G., & Cattivelli, L.** (2014). Flavonoids and melanins: a common strategy across two kingdoms. *International Journal of Biological Sciences*, 10(10), 1159-1170.
- Chachalis, D., & Smith, M. L.** (2000). Imbibition behavior of soybean (*Glycine max* (L.) Merrill) accessions with different testa characteristics. *Seed Science and Technology*, 28(2), 321-331.
- Choo, T. M., Vigier, B., Ho, K. M., Ceccarelli, S., Grando, S., & Franckowiak, J. D.** (2005). Comparison of black, purple, and yellow barleys. *Genetic Resources and Crop Evolution*, 52(2), 121-126.
- Debeaujon, I., Leon-Kloosterziel, K. M., & Koornneef, M.** (2000). Influence of the testa on seed dormancy, germination, and longevity in Arabidopsis. *Plant Physiology*, 122(2), 403-414.
- Debeaujon, I., Nesi, N., Perez, P., Devic, M., Grandjean, O., Caboche, M., & Lepiniec, L.** (2003). Proanthocyanidin-accumulating cells in Arabidopsis testa: regulation of differentiation and role in seed development. *Plant Cell*, 15(11), 2514-2531.
- Derbew, S., Mohammed, H., & Urage, E.** (2013). Phenotypic diversity for qualitative characters of barley (*Hordeum vulgare* (L.)) landrace collections from Southern Ethiopia. *International Journal of Science and Research*, 2(9), 34-40.
- de Pascual-Teresa, S., & Sanchez-Ballesta, M. T.** (2007). Anthocyanins: from plant to health. *Phytochemistry Reviews*, 7(2), 281-299.
- Demissie, A., & Bjornstad, A.** (1996). Phenotypic diversity of Ethiopian barleys in relation to geographical regions, altitudinal range, and agro-ecological zones: As an aid to germplasm collection and conservation strategy. *Hereditas*, 124(1), 17-29.
- Devic, M., Guillemot, J., Debeaujon, I., Bechtold, N., Bensaude, E., Koornneef, M., Pelletier, G., & Delseny, M.** (1999). The *BANYULS* gene encodes a *DFR*-like protein and is a marker of early seed coat development. *The Plant Journal*, 19(4), 387-398.
- Dinelli, G., Carretero, A. S., Di Silvestro, R., Marotti, I., Fu, S., Benedettelli, S., Ghisellid, L., & Gutiérrez, A. F.** (2009). Determination of phenolic compounds in modern and old varieties of durum wheat using liquid chromatography coupled with time-of-flight mass spectrometry. *Journal of Chromatography A*, 1216(43), 7229-7240.

- Dobrovolskaya, O., Arbuzova, V. S., Lohwasser, U., Roder, M. S., & Borner, A.** (2006). Microsatellite mapping of complementary genes for purple grain colour in bread wheat (*Triticum aestivum* L.). *Euphytica*, 150(3), 355-364.
- Duym, C. P. A., Komen, J. G., Ultee, A. J., & Van der Weide, B. M.** (1947). The inhibition of germination, caused by extracts of seed balls of the sugar beet (*Beta vulgaris*). In *Proc. Kon. Ned. Akad. Wet.*, (50), 527-535.
- Dvorakova, M., Moreira, M. M., Dostalek, P., Skulilova, Z., Guido, L. F., & Barros, A. A.** (2008). Characterization of monomeric and oligomeric flavan-3-ols from barley and malt by liquid chromatography-ultraviolet detection-electrospray ionization mass spectrometry. *Journal of Chromatography A*, 1189(1), 398-405.
- Dykes, L., & Rooney, L. W.** (2007). Phenolic compounds in cereal grains and their health benefits. *Cereal Foods World*, 52(3), 105-111.
- Escribano-Bailón, M. T., Santos-Buelga, C., & Rivas-Gonzalo, J. C.** (2004). Anthocyanins in cereals. *Journal of Chromatography A*, 1054(1), 129-141.
- Falcone Ferreyra, M. L., Rius, S. P., & Casati, P.** (2012). Flavonoids: biosynthesis, biological functions, and biotechnological applications. *Frontiers in Plant Science*, 3, 222.
- Faris, D. G.** (1955). Physiology and genetics of the kernel color of barley. *Doctoral dissertation, University of British Columbia*.
- Feng, H., Li, Y., Wang, S., Zhang, L., Liu, Y., Xue, F., Sun, Y., Wang, Y., & Sun, J.** (2014). Molecular analysis of proanthocyanidins related to pigmentation in brown cotton fibre (*Gossypium hirsutum* L.). *Journal of Experimental Botany*, 65(20), 5759-5769.
- Ficco, D. B., De Simone, V., Colecchia, S. A., Pecorella, I., Platani, C., Nigro, F., Finocchiaro, F., Papa, R., & De Vita, P.** (2014). Genetic variability in anthocyanin composition and nutritional properties of blue, purple, and red bread (*Triticum aestivum* L.) and durum (*Triticum turgidum* L. ssp. *turgidum* convar. *durum*) wheats. *Journal of Agricultural and Food Chemistry*, 62(34), 8686-8695.
- Freed, R., Everson, E., Ringlund, K., & Gullord, M.** (1976). Seedcoat color in wheat and the relationship to seed dormancy at maturity. *Cereal Research Communications*, 4(2), 147-149.
- Friedrich, W., & Galensa, R.** (2002). Identification of a new flavanol glucoside from barley (*Hordeum vulgare* L.) and malt. *European Food Research and Technology*, 214(5), 388-393.
- Furtado, A.** (2014). RNA extraction from developing or mature wheat seeds. *Cereal Genomics: Methods and Protocols*, 1099, 23-28.

- Garg, M., Chawla, M., Chunduri, V., Kumar, R., Sharma, S., Sharma, N. K., Kaur, N., Kumar, A., Mundey, J. K., Saini, M. K., & Singh, S. P.** (2016). Transfer of grain colors to elite wheat cultivars and their characterization. *Journal of Cereal Science*, 71, 138-144.
- Giusti, M. M., & Wrolstad, R. E.** (2001). Characterization and measurement of anthocyanins by UV-visible spectroscopy. *Current Protocols in Food Analytical Chemistry*, F1.2.1-F1.2.13.
- Glover, B. J., & Martin, C.** (2012). Anthocyanins. *Current Biology*, 22(5), R147-R150.
- Griffiths, D. W., & Welch, R. W.** (1982). Genotypic and environmental variation in the total phenol and flavanol contents of barley grain. *Journal of the Science of Food and Agriculture*, 33(6), 521-527.
- Groos, C., Gay, G., Perretant, M. R., Gervais, L., Bernard, M., Dedryver, F., & Charmet, G.** (2002). Study of the relationship between pre-harvest sprouting and grain color by quantitative trait loci analysis in a whitexred grain bread-wheat cross. *Theoretical and Applied Genetics*, 104(1), 39-47.
- Gordeeva, E. I., Shoeva, O. Y., & Khlestkina, E. K.** (2014). Marker-assisted development of bread wheat near-isogenic lines carrying various combinations of purple pericarp (*Pp*) alleles. *Euphytica*, 203(2), 469-476.
- Goupy, P., Hugues, M., Boivin, P., & Amiot, M. J.** (1999). Antioxidant composition and activity of barley (*Hordeum vulgare*) and malt extracts and of isolated phenolic compounds. *Journal of the Science of Food and Agriculture*, 79(12), 1625-1634.
- Gu, L., Kelm, M. A., Hammerstone, J. F., Beecher, G., Holden, J., Haytowitz, D., Gebhardt, S., & Prior, R. L.** (2004). Concentrations of proanthocyanidins in common foods and estimations of normal consumption. *The Journal of nutrition*, 134(3), 613-617.
- Guo, J., Han, W., & Wang, M.** (2008). Ultraviolet and environmental stresses involved in the induction and regulation of anthocyanin biosynthesis: a review. *African Journal of Biotechnology*, 7(25), 4966-4972.
- Gupta, R., Min, C. W., Kim, S. W., Wang, Y., Agrawal, G. K., Rakwal, R., Kim, S. G., Lee, B. W., Ko, J. M., Baek, I. Y., & Kim, S. T.** (2015). Comparative investigation of seed coats of brown- versus yellow-colored soybean seeds using an integrated proteomics and metabolomics approach. *Proteomics*, 15(10), 1706-1716.
- Guggenheim, M.**, (1913). Dioxyphenylalanin, eine neue Aminosäure aus *Vicia faba*. *Hoppe-seyler's zeitschrift für physiologische chemie*, 88(7), 276-284.

- Gutierrez-Alamo, A., De Ayala, P. P., Verstegen, M. W. A., Den Hartog, L. A., & Villamide, M. J.** (2008). Variability in wheat: factors affecting its nutritional value. *World's Poultry Science Journal*, 64(1), 20-39.
- Gvozdeva, Z. V. & N. V. Zhukova.** (1971). Influence of storage conditions on longevity of seeds of bean, chickpea and soybean. *Trudy po Prikladnoj Botanike*, 45, 161-168.
- Hambira, C.** (2009). Proanthocyanidins, anthocyanins and phenolic acids in food barleys of diverse origin. *Doctoral dissertation*, University of Manitoba.
- Hampton, J. G. & TeKrony, D. M.** (1995). Handbook of vigour test methods. Zürich, *International Seed Testing Association*
- Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., & Vandesompele, J.** (2007). qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome biology*, 8(2), R19.
- Himi, E., Nisar, A., & Noda, K.** (2005). Colour genes (*R* and *Rc*) for grain and coleoptile upregulate flavonoid biosynthesis genes in wheat. *Genome*, 48(4), 747-754.
- Himi, E., & Noda, K.** (2005). Red grain colour gene (*R*) of wheat is a *Myb*-type transcription factor. *Euphytica*, 143(3), 239-242.
- Himi, E., & Taketa, S.** (2015). Barley *Ant17*, encoding *flavanone 3-hydroxylase (F3H)*, is a promising target locus for attaining anthocyanin/proanthocyanidin-free plants without pleiotropic reduction of grain dormancy. *Genome*, 58(1), 43-53.
- Hirawan, R., Diehl-Jones, W., & Beta, T.** (2011). Comparative evaluation of the antioxidant potential of infant cereals produced from purple wheat and red rice grains and LC-MS analysis of their anthocyanins. *Journal of Agricultural and Food Chemistry*, 59(23), 12330-12341.
- Holton, T. A., & Cornish, E. C.** (1995). Genetics and biochemistry of anthocyanin biosynthesis. *Plant Cell*, 7(7), 1071-1083.
- Holtekjølen, A. K., Kinitz, C., & Knutsen, S. H.** (2006). Flavanol and bound phenolic acid contents in different barley varieties. *Journal of Agricultural and Food Chemistry*, 54(6), 2253-2260.
- Honda, C., Kotoda, N., Wada, M., Kondo, S., Kobayashi, S., Soejima, J., Zhang, Z., Tsuda, T., & Moriguchi, T.** (2002). Anthocyanin biosynthetic genes are coordinately expressed during red coloration in apple skin. *Plant Physiology and Biochemistry*, 40(11), 955-962.

- Horsley, R. D., Schwarz, P. B., & Foster, A. E.** (1991). Effects of gene *ant13* on agronomic and malt quality traits of barley. *Crop Science*, 31(3), 593-598.
- Hosseinian, F. S., Li, W., & Beta, T.** (2008). Measurement of anthocyanins and other phytochemicals in purple wheat. *Food Chemistry*, 109(4), 916-924.
- Hu, C., Cai, Y. Z., Li, W. D., Corke, H., & Kitts, D. D.** (2007). Anthocyanin characterization and bioactivity assessment of a dark blue grained wheat (*Triticum aestivum* L. cv. Hedong Wumai) extract. *Food Chemistry*, 104(3), 955-961.
- ISTA** (2005). International rules for seed testing. Bassersdorf, *International Seed Testing Association*.
- Jende-Strid, B.** (1978). Mutations affecting flavonoid synthesis in barley. *Carlsberg Research Communications*, 43(4), 265-273.
- Jende-Strid, B., & Møller, B. L.** (1981). Analysis of proanthocyanidins in wild-type and mutant barley (*Hordeum vulgare* L.). *Carlsberg Research Communications*, 46(1), 53-64.
- Jende-Strid, B.** (1991). Gene-enzyme relations in the pathway of flavonoid biosynthesis in barley. *Theoretical and Applied Genetics*, 81(5), 668-674.
- Jende-Strid, B.** (1993). Genetic control of flavonoid biosynthesis in barley. *Hereditas*, 119(2), 187-204.
- Jiang, Y., Duan, X., Joyce, D., Zhang, Z., & Li, J.** (2004). Advances in understanding of enzymatic browning in harvested litchi fruit. *Food Chemistry*, 88(3), 443-446.
- Kantar, F., Pilbeam, C. J., & Hebblethwaite, P. D.** (1996). Effect of tannin content of faba bean (*Vicia faba*) seed on seed vigour, germination and field emergence. *Annals of Applied Biology*, 128(1), 85-93.
- Keerio, M. U.** (2013). Influence of seed size and seedling rate on growth and yield of wheat. *M.Sc. Thesis*, Sindh Agriculture University Tandojam.
- Khlestkina, E. K., Röder, M. S., & Börner, A.** (2010). Mapping genes controlling anthocyanin pigmentation on the glume and pericarp in tetraploid wheat (*Triticum durum* L.). *Euphytica*, 171(1), 65-69.
- Khlestkina, E. K., Salina, E. A., Matthies, I. E., Leonova, I. N., Börner, A., & Röder, M. S.** (2011). Comparative molecular marker-based genetic mapping of flavanone 3-hydroxylase genes in wheat, rye and barley. *Euphytica*, 179(2), 333-341.



- Khlestkina, E. K.** (2013). Genes determining the coloration of different organs in wheat. *Russian Journal of Genetics: Applied Research*, 3(1), 54-65.
- Khlestkina, E. K., Gordeeva, E. I., Arbuzova, V. S., & Pillen, K.** (2014). Molecular and functional characterization of wheat near-isogenic line 'i:S29Ra' having intensive anthocyanin pigmentation of the coleoptile, culm, leaves and auricles. *Plant Breeding*, 133(4), 454-458.
- Khoroshailov, N. G. & Zhukova, N. V.** (1973). Prolonged storage of collected seed samples. *Trudy po Prikladnoj Botanike*, 49, 269-279.
- Kim, M. J., Hyun, J. N., Kim, J. A., Park, J. C., Kim, M. Y., Kim, J. G., Lee, S. J., Chun, S. C. & Chung, I. M.** (2007). Relationship between phenolic compounds, anthocyanins content and antioxidant activity in colored barley germplasm. *Journal of Agricultural and Food Chemistry*, 55(12), 4802-4809.
- Klausen, K., Mortensen, A. G., Laursen, B., Haselmann, K. F., Jespersen, B. M., & Fomsgaard, I. S.** (2010). Phenolic compounds in different barley varieties: identification by tandem mass spectrometry (QSstar) and NMR; quantification by liquid chromatography triple quadrupole-linear ion trap mass spectrometry (Q-Trap). *Natural Product Communications*, 5(3), 407-414.
- Kniewel, D. C., Abdel-Aal, E.-S. M., Rabalski, I., Nakamura, T., & Hucl, P.** (2009). Grain color development and the inheritance of high anthocyanin blue aleurone and purple pericarp in spring wheat (*Triticum aestivum* L.). *Journal of Cereal Science*, 50(1), 113-120.
- Koehler, P., & Wieser, H.** (2013). Chemistry of cereal grains. *Handbook on sourdough biotechnology*, 11-45. Springer US.
- Kohyama, N., Ono, H., & Yanagisawa, T.** (2008). Changes in anthocyanins in the grains of purple waxy hull-less barley during seed maturation and after harvest. *Journal of Agricultural and Food Chemistry*, 56(14), 5770-5774.
- Konczak, I., & Zhang, W.** (2004). Anthocyanins—more than nature's colours. *Journal of Biomedicine and Biotechnology*, 2004(5), 239-240.
- Kong, J.** (2003). Analysis and biological activities of anthocyanins. *Phytochemistry*, 64(5), 923-933.
- Körnicker, F.** (1885). Die Arten und Varietäten des Getreides, Verlag Emil Strauss, Bonn, Germany, p. 27.
- Kovinich, N., Saleem, A., Arnason, J. T., & Miki, B.** (2011). Combined analysis of transcriptome and metabolite data reveals extensive differences between black and brown nearly-isogenic soybean

(*Glycine max*) seed coats enabling the identification of pigment isogenes. *BMC Genomics*, 12(1), 1-18.

**Kristiansen, K. N.** (1984). Biosynthesis of proanthocyanidins in barley: Genetic control of the conversion of dihydroquercetin to catechin and procyanidins. *Carlsberg Research Communications*, 49(5), 503-524.

**Lago, C., Landoni, M., Cassani, E., Doria, E., Nielsen, E., & Pilu, R.** (2012). Study and characterization of a novel functional food: purple popcorn. *Molecular Breeding*, 31(3), 575-585.

**Lao, F., & Giusti, M. M.** (2016). Quantification of Purple Corn (*Zea mays* L.) Anthocyanins using spectrophotometric and HPLC approaches: method comparison and correlation. *Food Analytical Methods*, 9(5), 1367-1380.

**Lisec, J., Schauer, N., Kopka, J., Willmitzer, L., & Fernie, A. R.** (2006). Gas chromatography mass spectrometry-based metabolite profiling in plants. *Nature Protocols*, 1(1), 387-396.

**Lenoir, C., Corbineau, F., & Côme, D.** (1986). Barley (*Hordeum vulgare*) seed dormancy as related to glumella characteristics. *Physiologia Plantarum*, 68(2), 301-307.

**Lepiniec, L., Debeaujon, I., Routaboul, J. M., Baudry, A., Pourcel, L., Nesi, N., & Caboche, M.** (2006). Genetics and biochemistry of seed flavonoids. *Annual Review of Plant Biology*, 57, 405-430.

**Lerner, A. B., & Fitzpatrick, T. B.** (1950). Biochemistry of melanin formation. *Physiological Reviews*, 30(1), 91-126.

**Lundqvist, U., & Franckowiak, J. D.** (2003). Diversity of barley mutants. *Developments in Plant Genetics and Breeding*, 7, 77-96.

**Lippmann, R., Kaspar, S., Rutten, T., Melzer, M., Kumlehn, J., Matros, A., & Mock, H.-P.** (2009). Protein and metabolite analysis reveals permanent induction of stress defense and cell regeneration processes in a tobacco cell suspension culture. *International Journal of Molecular Sciences*, 10(7), 3012-3032.

**Liu, Q., Qiu, Y., & Beta, T.** (2010). Comparison of antioxidant activities of different colored wheat grains and analysis of phenolic compounds. *Journal of Agricultural and Food Chemistry*, 58(16), 9235-9241.

**Liu, C., Wang, X., Shulaev, V., & Dixon, R. A.** (2016). A role for leucoanthocyanidin reductase in the extension of proanthocyanidins. *Nature Plants*, 2, 16182.

- Ma, D., Li, Y., Zhang, J., Wang, C., Qin, H., Ding, H., Xie, Y., & Guo, T.** (2016). Accumulation of phenolic compounds and expression profiles of phenolic acid biosynthesis-related genes in developing grains of white, purple, and red wheat. *Frontiers in plant science*, 7, 528.
- Marles, M. A. S., Gruber, M. Y., Scoles, G. J., & Muir, A. D.** (2003). Pigmentation in the developing seed coat and seedling leaves of *Brassica carinata* is controlled at the *dihydroflavonol reductase* locus. *Phytochemistry*, 62(5), 663-672.
- Marles, M. A. S., Ray, H., & Gruber, M. Y.** (2003). New perspectives on proanthocyanidin biochemistry and molecular regulation. *Phytochemistry*, 64(2), 367-383.
- Marles, M. A. S., & Gruber, M. Y.** (2004). Histochemical characterisation of unextractable seed coat pigments and quantification of extractable lignin in the *Brassicaceae*. *Journal of the Science of Food and Agriculture*, 84(3), 251-262.
- Mayer, A. M.** (1986). Polyphenol oxidases in plants-recent progress. *Phytochemistry*, 26(1), 11-20.
- Matus-Cadiz, M. A., Daskalchuk, T. E., Verma, B., Puttick, D., Chibbar, R. N., Gray, G. R., Perron, C. E., Tyler, R. T., & Hucl, P.** (2008). Phenolic compounds contribute to dark bran pigmentation in hard white wheat. *Journal of Agricultural and Food Chemistry*, 56(5), 1644-1653.
- Mccallum, J. A., & Walker, J. R. L.** (1990). Proanthocyanidins in wheat bran. *Cereal Chemistry*, 67(3), 282-285.
- McGuire, S.** (2013). State indicator report on fruits and vegetables. Centers for disease control and prevention, Atlanta, GA. *Advances in Nutrition*, 4(6), 665-666.
- McIntosh, R. A., Hart, G. E., Devos, K. M., Gale, M. D., & Rogers, W. J.** (1998). Catalogue of gene symbols for wheat. Proceedings of the 9th international wheat genetics symposium. Saskatoon, 5, 1-235. University Extension Press, Saskatoon, Canada.
- McKevith, B.** (2004). Nutritional aspects of cereals. *Nutrition Bulletin*, 29(2), 111-142.
- McMurrough, I., Loughrey, M. J., & Hennigan, G. P.** (1983). Content of (+)-catechin and proanthocyanidins in barley and malt grain. *Journal of the Science of Food and Agriculture*, 34(1), 62-72.
- McMurrough, I., Madigan, D., & Smyth, M. R.** (1996). Semipreparative chromatographic procedure for the isolation of dimeric and trimeric proanthocyanidins from barley. *Journal of Agricultural and Food Chemistry*, 44(7), 1731-1735.

- Meldgaard, M.** (1992). Expression of *chalcone synthase*, *dihydroflavonol reductase*, and *flavanone-3-hydroxylase* in mutants of barley deficient in anthocyanin and proanthocyanidin biosynthesis. *Theoretical and Applied Genetics*, 83(6-7), 695-706.
- Menkir, A., Ejeta, G., Butler, L., & Melakeberhan, A.** (1996). Physical and chemical kernel properties associated with resistance to grain mold in sorghum. *Cereal Chemistry*, 73(5), 613-617.
- Mian, M. A. R., & Nafziger, E. D.** (1994). Seed size and water potential effects on germination and seedling growth of winter wheat. *Crop Science*, 34(1), 169-171.
- Miller, E. R.** (1920). Dihydroxyphenylalanine, a constituent of the velvet bean. *Journal of Biological Chemistry*, 44(2), 481-486.
- Miyamoto, T., & Everson, E. H.** (1958). Biochemical and physiological studies of wheat seed pigmentation. *Agronomy Journal*, 50(12), 733-734.
- Mohamed-Yasseen, Y., Barringer, S. A., Splittstoesser, W. E., & Costanza, S.** (1994). The role of seed coats in seed viability. *The Botanical Review*, 60(4), 426-439.
- Mullick, D. B., Faris, D. G., Brink, V. C., & Acheson, R. M.** (1958). Anthocyanins and anthocyanidins of the barley pericarp and aleurone tissues. *Canadian Journal of Plant Science*, 38(4), 445-456.
- Mullick, D. B.** (1959). The physiology and genetics of the pigments of barley (*Hordeum vulgare* L.). *M.Sc. thesis*, University of British Columbia.
- Mullick, D. B.** (1966). Biochemical genetics of the anthocyanins of barley (*Hordeum vulgare* L.). *Doctoral dissertation*, University of British Columbia.
- Mulkay, P., Touillaux, R., & Jerumanis, J.** (1981). Proanthocyanidins of barley: separation and identification. *Journal of Chromatography A*, 208(2), 419-423.
- Nagel, M., & Börner, A.** (2010). The longevity of crop seeds stored under ambient conditions. *Seed Science Research*, 20(1), 1-12.
- Nicolaus, R. A.** (1968). Melanins. *Hermann*, Paris.
- Nielsen, S.** (2005). Handbook of food analysis, physical characterization and nutrient analysis. *Journal of Food Quality*, 28(5-6), 507-508.
- Niu, S. S., Xu, C. J., Zhang, W. S., Zhang, B., Li, X., Wang, K. L., Ferguson, I. B., & Chen, K. S.** (2010). Coordinated regulation of anthocyanin biosynthesis in Chinese bayberry (*Myrica rubra*) fruit by a *R2R3 MYB* transcription factor. *Planta*, 231(4), 887-899.

- Nilsson-Ehle, H.** (1914). Zur Kenntnis der mit der Keimungsphysiologie des Weizens in Zusammenhang stehenden inneren Faktoren.
- Nisar, N., Li, L., Lu, S., Khin, N. C., & Pogson, B. J.** (2015). Carotenoid metabolism in plants. *Molecular Plant*, 8(1), 68-82.
- Oigiangbe, N., & Onigbinde, A.** (1996). The association between some physico-chemical characteristics and susceptibility of cowpea (*Vigna unguiculata* (L.) Walp) to *Callosobruchus maculatus* (F.). *Journal of Stored Products Research*, 32(1), 7-11.
- Oren-Shamir, M.** (2009). Does anthocyanin degradation play a significant role in determining pigment concentration in plants? *Plant Science*, 177(4), 310-316.
- Pourcel, L., Routaboul, J. M., Kerhoas, L., Caboche, M., Lepiniec, L., & Debeaujon, I.** (2005). *TRANSPARENT TESTA10* encodes a laccase-like enzyme involved in oxidative polymerization of flavonoids in Arabidopsis seed coat. *Plant Cell*, 17(11), 2966-2980.
- Pourcel, L., Routaboul, J. M., Cheynier, V., Lepiniec, L., & Debeaujon, I.** (2007). Flavonoid oxidation in plants: from biochemical properties to physiological functions. *Trends in Plant Science*, 12(1), 29-36.
- Powell, A. A., Oliveira, M. D. A., & Matthews, S.** (1986). The role of imbibition damage in determining the vigour of white and coloured seed lots of dwarf French beans (*Phaseolus vulgaris*). *Journal of Experimental Botany*, 37(5), 716-722.
- Powell, A. A.** (1989). The importance of genetically-determined seed coat characteristics to seed quality in grain legumes. *Annals of Botany*, 63(1), 169-175.
- Prota, G.** (1988). Progress in the chemistry of melanins and related metabolites. *Medicinal Research Reviews*, 8(4), 525-556.
- Qu, C., Fu, F., Lu, K., Zhang, K., Wang, R., Xu, X., Wang, M., Lu, J., Wan, H., Tang, Z., & Li, J.** (2013). Differential accumulation of phenolic compounds and expression of related genes in black- and yellow-seeded *Brassica napus*. *Journal of Experimental Botany*, 64(10), 2885-2898.
- Quinde-Axtell, Z., Ullrich, S. E., & Baik, B. K.** (2005). Genotypic and environmental effects on color and discoloration potential of barley in food products. *Cereal Chemistry Journal*, 82(6), 711-716.
- Quinde-Axtell, Z., & Baik, B. K.** (2006). Phenolic compounds of barley grain and their implication in food product discoloration. *Journal of Agricultural and Food Chemistry*, 54(26), 9978-9984.

- Rahman, M. M., Lee, K. E., & Kang, S. G.** (2015). Studies on the effects of pericarp pigmentation on grain development and yield of black rice. *Indian Journal of Genetics and Plant Breeding*, 75(4), 426-433.
- Rodriguez-Saona, L. E., & Wrolstad, R. E.** (2001). Extraction, isolation, and purification of anthocyanins. *Current Protocols in Food Analytical Chemistry*, F1.1.1-F1.1.11.
- Roos, E. E.** (1984). Genetic shifts in mixed bean populations. I. Storage effects. *Crop Science*, 24(2), 240-244.
- Routaboul, J.-M., Kerhoas, L., Debeaujon, I., Pourcel, L., Caboche, M., Einhorn, J., & Lepiniec, L.** (2006). Flavonoid diversity and biosynthesis in seed of *Arabidopsis thaliana*. *Planta*, 224(1), 96-107.
- Riley, P. A.** (1997). Melanin. *The International Journal of Biochemistry & Cell Biology*, 29(11), 1235-1239.
- Santos-Buelga, C., Mateus, N., & De Freitas, V.** (2014). Anthocyanins. Plant pigments and beyond. *Journal of Agricultural and Food Chemistry*, 62(29), 6879-6884.
- Stout, M., and Tolman, B.** (1941). Factors affecting the germination of sugar beet and other seeds, with special reference to toxic effects of ammonia. *Journal of agricultural research*, 63(12), 687-713.
- Shahwani, A. R., Baloch, S. U., Baloch, S. K., Mengal, B., Bashir, W., Baloch, H. N., Baloch, R. A., Sial, A. H., Sabiel, S. A. I., Razzaq, K., Shahwani, A. A., & Mengal, A.** (2014). Influence of seed size on germinability and grain yield of wheat (*Triticum aestivum* L.) varieties. *Journal of Natural Sciences Research*, 4(23), 147-155.
- Sharma, P. K., Anand, P., Sankhalkar, S., & Shetye, R.** (1998). Photochemical and biochemical changes in wheat seedlings exposed to supplementary ultraviolet-B radiation. *Plant Science*, 132(1), 21-30.
- Shewry, P. R., Hawkesford, M. J., Piironen, V., Lampi, A.-M., Gebruers, K., Boros, D., Andersson, A. A. M., Åman, P., Rakszegi, M., Bedo, Z., & Bedo, Z.** (2013). Natural variation in grain composition of wheat and related cereals. *Journal of Agricultural and Food Chemistry*, 61(35), 8295-8303.
- Shirley, B. W.** (1998). Flavonoids in seeds and grains: physiological function, agronomic importance and the genetics of biosynthesis. *Seed Science Research*, 8(4), 415-422.
- Shoeva, O. Y., Gordeeva, E. I., & Khlestkina, E. K.** (2014). The regulation of anthocyanin synthesis in the wheat pericarp. *Molecules*, 19(12), 20266-20279.

- Shoeva, O. Y., Mock, H. P., Kukoeva, T. V., Borner, A., & Khlestkina, E. K.** (2016). Regulation of the flavonoid biosynthesis pathway genes in purple and black grains of *Hordeum vulgare*. *PLoS ONE*, 11(10), e0163782.
- Šramková, Z., Gregová, E., & Šturdík, E.** (2009). Chemical composition and nutritional quality of wheat grain. *Acta Chimica Slovaca*, 2(1), 115-138.
- Stanton, W. R., & Francis, B. J.** (1966). Ecological significance of anthocyanins in the seed coats of the Phaseoleae. *Nature*, 211(5052), 970-971.
- Starzinger, E. K., West, S. H., & Hinson, K.** (1982). An observation on the relationship of soybean seed coat color to viability maintenance. *Seed Science and Technology*, 10, 301-305.
- Stout, M., & Tolman, B.** (1941). Factors affecting the germination of sugar beet and other seeds with special reference to the toxic effects of ammonia. *Journal of Agricultural Research*, 63(12), 687-713.
- Tanner, G. J., Francki, K. T., Abrahams, S., Watson, J. M., Larkin, P. J., & Ashton, A. R.** (2003). Proanthocyanidin biosynthesis in plants. Purification of legume *leucoanthocyanidin reductase* and molecular cloning of its cDNA. *The Journal of Biological Chemistry*, 278(34), 31647-31656.
- Tereshchenko, O., Pshenichnikova, T., Salina, E., & Khlestkina, E.** (2012). Development and molecular characterization of a novel wheat genotype having purple grain colour. *Cereal Research Communications*, 40(2), 210-214.
- Tereshchenko, O. Y., Arbuzova, V. S., & Khlestkina, E. K.** (2013). Allelic state of the genes conferring purple pigmentation in different wheat organs predetermines transcriptional activity of the anthocyanin biosynthesis structural genes. *Journal of Cereal Science*, 57(1), 10-13.
- Thomas, M.** (1955). Melanins. Modern methods of plant analysis / Moderne Methoden der Pflanzenanalyse. Berlin, Heidelberg: Springer Berlin Heidelberg, pp. 661-675.
- Todd, J. J., & Vodkin, L. O.** (1993). Pigmented soybean (*Glycine max*) seed coats accumulate proanthocyanidins during development. *Plant Physiology*, 102(2), 663-670.
- Torada, A., & Amano, Y.** (2002). Effect of seed coat color on seed dormancy in different environments. *Euphytica*, 126(1), 99-105.
- Tolman, B., & Stout, M.** (1940). Toxic effect on germinating sugar-beet seed of water-soluble substances in the seed ball. *Journal of Agricultural Research*, 61(11), 817-830.

- Toufektsian, M.-C., De Lorgeril, M., Nagy, N., Salen, P., Donati, M. B., Giordano, L., Mock, H.-P., Peterek, S., Matros, A., Petroni, K., Pilu, R., Rotilio, D., Tonelli, C., de Leiris, J., Boucher, F., & Martin, C.** (2008). Chronic dietary intake of plant-derived anthocyanins protects the rat heart against ischemia-reperfusion injury. *The Journal of nutrition*, 138(4), 747-752.
- Trojan, V., Musilová, M., Vyhnánek, T., Klejdus, B., Hanáček, P., & Havel, L.** (2014). *Chalcone synthase* expression and pigments deposition in wheat with purple and blue colored caryopsis. *Journal of Cereal Science*, 59(1), 48-55.
- van Deynze, A., & Pauls, K. P.** (1993). The inheritance of seed colour and vernalization requirement in *Brassica napus* using doubled haploid populations. *Euphytica*, 74(1), 77-83.
- Verardo, V., Cevoli, C., Pasini, F., Gomez-Caravaca, A. M., Marconi, E., Fabbri, A., & Caboni, M. F.** (2015). Analysis of oligomer proanthocyanidins in different barley genotypes using high-performance liquid chromatography-fluorescence detection-mass spectrometry and near-infrared methodologies. *Journal of Agricultural and Food Chemistry*, 63(16), 4130-4137.
- von Bothmer, R., van Hintum, T., Knüpffer, H., & Sato, K. (Eds.).** (2003). Diversity in barley (*Hordeum vulgare*). *Elsevier*, 7.
- von Lintig, J., & Sies, H.** (2013). Carotenoids. *Archives of Biochemistry and Biophysics*, 539(2), 99-101.
- von Wettstein, D., Jende-Strid, B., Ahrenst-Larsen, B., & Sørensen, J. A.** (1977). Biochemical mutant in barley renders chemical stabilization of beer superfluous. *Carlsberg Research Communications*, 42(5), 341.
- Wang, X., Olsen, O., & Knudsen, S.** (1993). Expression of the *dihydroflavonol reductase gene* in an anthocyanin-free barley mutant. *Hereditas*, 119(1), 67-75.
- Wei, Y. Z., Hu, F. C., Hu, G. B., Li, X. J., Huang, X. M., & Wang, H. C.** (2011). Differential expression of anthocyanin biosynthetic genes in relation to anthocyanin accumulation in the pericarp of Litchi *Chinensis* Sonn. *PLoS ONE*, 6(4), 19455.
- Winkel-Shirley, B.** (2001). Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. *Plant Physiology*, 126(2), 485-493.
- Wrolstad, R. E.** (2004). Symposium 12: Interaction of natural colors with other ingredients - Anthocyanin pigments - Bioactivity and coloring properties. *Journal of Food Science*, 69(5), C419-C421.
- Woodward, R.** (1941). Inheritance of melanin-like pigment in the glumes and caryopses of barley. *Journal of Agricultural Research*, 63(1), 21-28.



**Xie, D. Y., Sharma, S. B., Paiva, N. L., Ferreira, D., & Dixon, R. A.** (2003). Role of *anthocyanidin reductase*, encoded by *BANYULS* in plant flavonoid biosynthesis. *Science*, 299(5605), 396-399.

**Yao, Y., Sang, W., Zhou, M., & Ren, G.** (2010). Antioxidant and  $\alpha$ -glucosidase inhibitory activity of colored grains in China. *Journal of Agricultural and Food Chemistry*, 58(2), 770-774.

**Yu, C. Y.** (2013). Molecular mechanism of manipulating seed coat coloration in oilseed *Brassica* species. *Journal of Applied Genetics*, 54(2), 135-145.

**Zhao, J., Pang, Y., & Dixon, R. A.** (2010). The mysteries of proanthocyanidin transport and polymerization. *Plant Physiology*, 153(2), 437-443.

**Žofajová, A., Pšenáková, I., Havrlentová, M., & Piliarová, M.** (2012). Accumulation of total anthocyanins in wheat grain. *Agriculture*, 58(2), 50-56.

## 7. Appendix

**Appendix 1: Relative quantification of primary metabolites in wheat grains.** Based on targeted analysis of GC-MS measurements, the relative quantification of detected primary metabolites in parental lines and the corresponding bulk- and bulk+ offspring grown in the field at two locations (plot 1, 2) in 2015 was analyzed. Retention time indices of the compounds were calculated for validation purposes and compared to those documented in the Golm Metabolome Database (<http://gmd.mpimp-golm.mpg.de/>). Normalization was achieved using the deviation-from-the-mean factor, calculated by dividing the mixed sample value by the average value of all the combined samples. Sample names labeled in **yellow** and **purple** indicated yellow-grained and purple-grained wheat cultivars respectively.

2015-Plot 1	S29	Purple	S29×P		S29	PF	S29×PF		N67	Purple	N67×P		N67	PF	N67×PF	
			Bulk-	Bulk+			Bulk-	Bulk+			Bulk-	Bulk+			Bulk-	Bulk+
Lactic acid	476.3	417.7	441.3	333.0	476.3	448.9	303.5	636.4	548.6	417.7	828.3	769.8	548.6	448.9	1398.0	668.3
L-alanine	1518.6	1402.1	1154.4	967.4	1518.6	1134.7	1628.6	1817.8	2074.5	1402.1	1623.4	1268.2	2074.5	1134.7	2074.6	2397.2
L-valine	691.0	513.9	617.9	508.9	691.0	811.8	746.2	865.0	1285.6	513.9	864.2	715.2	1285.6	811.8	916.0	1138.2
Phosphoric acid	1440.4	25573.9	1320.3	1187.5	1440.4	2682.5	1329.7	876.6	13977.2	25573.9	9345.7	1505.4	13977.2	2682.5	993.5	2104.9
Glycerol	5404.1	7116.1	4945.1	2809.3	5404.1	5089.3	3559.8	7057.1	6213.6	7116.1	7499.1	7950.5	6213.6	5089.3	7653.1	10135.7
L-proline	5062.5	3382.1	2326.7	1689.0	5062.5	2994.2	4346.4	6940.7	9914.5	3382.1	3074.0	2523.5	9914.5	2994.2	5459.6	8348.9
Glycine	313.1	178.6	214.9	176.4	313.1	265.8	200.8	298.9	381.7	178.6	265.3	297.0	381.7	265.8	334.1	363.7
Succinic acid	681.4	360.0	507.1	367.4	681.4	458.0	400.5	287.4	442.0	360.0	479.2	491.8	442.0	458.0	602.3	671.0
Fumaric acid	892.3	717.3	520.6	336.8	892.3	497.6	358.6	303.3	360.9	717.3	510.8	624.5	360.9	497.6	524.4	712.6
L-serine	468.4	275.5	397.5	385.1	468.4	403.0	504.3	471.0	655.5	275.5	380.2	355.2	655.5	403.0	431.1	437.9
Unknown <sup>1</sup>	376.4	413.9	286.5	279.9	376.4	452.1	265.0	477.4	540.4	413.9	398.1	360.3	540.4	452.1	441.2	670.5
L-Threonine	252.8	257.4	314.8	296.7	252.8	405.1	339.8	325.8	465.4	257.4	350.8	282.1	465.4	405.1	315.4	380.5
Unknown <sup>2</sup>	139.3	245.0	182.9	138.9	139.3	186.5	160.5	178.7	200.5	245.0	173.5	177.2	200.5	186.5	268.5	311.7
Malic acid	10235.8	9249.7	10534.6	5961.0	10235.8	10356.2	5559.0	2761.4	4397.2	9249.7	6812.8	8774.6	4397.2	10356.2	5067.3	6820.6
Unknown <sup>3</sup>	926.9	904.9	638.2	646.1	926.9	1148.6	589.3	1126.3	1334.1	904.9	925.6	802.8	1334.1	1148.6	931.5	1604.7
Pyroglutamic acid	2347.3	1570.7	2261.7	2030.0	2347.3	3005.4	1927.0	2039.3	3878.9	1570.7	2262.2	2093.4	3878.9	3005.4	2131.5	3154.8
Aspartic acid	2156.2	1949.2	2478.5	2202.2	2156.2	3670.1	1768.5	1243.0	2137.8	1949.2	1966.9	2156.0	2137.8	3670.1	2146.9	2560.3
GABA	223.2	282.1	190.2	128.0	223.2	166.2	264.1	291.4	730.0	282.1	244.5	218.5	730.0	166.2	369.5	436.8
Unknown <sup>4</sup>	1375.1	1010.6	655.8	652.0	1375.1	1018.7	424.9	1489.0	1088.7	1010.6	1347.1	897.5	1088.7	1018.7	778.6	1293.3
Unknown <sup>6</sup>	103.2	136.4	71.5	72.6	103.2	84.9	45.5	105.8	84.1	136.4	99.8	74.5	84.1	84.9	83.6	138.1
L-glutamic acid	1136.3	698.0	1193.2	1104.7	1136.3	1278.6	1238.8	1109.1	1228.8	698.0	1195.6	1397.4	1228.8	1278.6	1337.2	1529.0
L-phenylalanine	207.8	166.0	223.1	156.8	207.8	216.6	312.6	262.5	561.1	166.0	206.3	168.8	561.1	216.6	217.1	234.9
Unknown <sup>7</sup>	390.2	460.8	430.8	403.3	390.2	729.8	431.3	350.7	679.7	460.8	331.7	369.1	679.7	729.8	528.0	827.5

Pentose I	56.7	136.0	102.4	64.1	56.7	84.9	123.8	128.0	103.9	136.0	76.1	77.0	103.9	84.9	77.6	102.6
Unknown <sup>8</sup>	161.6	368.8	296.0	194.9	161.6	410.4	470.3	420.8	271.5	368.8	230.4	249.8	271.5	410.4	191.2	276.1
Asparagine	4809.5	4282.9	4271.6	4713.1	4809.5	5757.0	4702.5	6841.0	6942.8	4282.9	5807.4	6261.8	6942.8	5757.0	8970.4	11501.4
Unknown <sup>10</sup>	205.2	295.3	295.5	270.5	205.2	293.6	300.8	248.7	251.2	295.3	243.0	293.5	251.2	293.6	258.3	408.5
Pentose alcohol I	1239.5	2209.8	1472.9	880.7	1239.5	1218.6	2172.5	1612.6	1819.1	2209.8	2151.2	1533.0	1819.1	1218.6	2086.4	3239.9
Unknown <sup>11</sup>	1586.4	1616.7	1540.7	1424.1	1586.4	1641.3	1481.1	1534.4	1624.0	1616.7	1490.7	1169.4	1624.0	1641.3	1397.3	1865.5
Unknown <sup>12</sup>	339.7	148.0	333.0	636.9	339.7	648.1	817.4	767.4	1106.6	148.0	498.3	541.5	1106.6	648.1	577.1	797.4
Citric acid	1104.2	1786.2	1149.1	968.3	1104.2	742.7	2303.0	1462.8	1183.3	1786.2	1022.6	1126.7	1183.3	742.7	1490.9	1478.4
D-fructose II	2759.0	4561.2	4601.2	2894.4	2759.0	5209.7	3732.8	3328.8	4260.2	4561.2	3216.6	3399.5	4260.2	5209.7	3029.5	4707.8
D-fructose III	5231.4	7221.8	7581.6	5830.4	5231.4	8638.0	5988.1	5215.2	7152.1	7221.8	5367.5	5180.6	7152.1	8638.0	6301.9	11022.8
<sup>13</sup> C-glucose	12195.8	13537.0	11448.7	6044.0	12195.8	11292.1	8287.5	8944.9	12375.3	13537.0	8971.0	8755.1	12375.3	11292.1	8563.0	12748.1
D-Galactose	8782.5	10592.3	13013.7	6382.2	8782.5	10469.4	12174.8	14006.1	19812.3	10592.3	9965.1	10783.9	19812.3	10469.4	8614.9	12123.2
<sup>13</sup> C-glucose <sup>2</sup>	1698.8	2123.4	1638.8	827.1	1698.8	1656.0	1192.6	1168.1	1783.9	2123.4	1234.3	1183.0	1783.9	1656.0	1155.9	1939.5
Hexose I (glucose)	1450.8	1887.5	2300.7	1273.2	1450.8	1896.4	2180.0	1987.5	2538.9	1887.5	1815.9	1504.0	2538.9	1896.4	1323.5	1789.4
Glucitol	3650.2	5535.7	5929.3	3464.4	3650.2	5611.1	3917.2	5656.0	4082.1	5535.7	5974.5	6140.4	4082.1	5611.1	4932.1	7862.8
Hexose II (D-glucose)	936.2	825.8	909.2	641.7	936.2	816.3	1016.7	864.0	2131.2	825.8	995.9	1124.8	2131.2	816.3	1020.0	1299.4
Myo-inositol	1306.1	2604.5	1020.1	1042.3	1306.1	769.6	2001.6	1659.7	1342.1	2604.5	1256.6	1061.0	1342.1	769.6	2088.3	1993.8
L-Tryptophan	795.8	834.9	1463.9	1717.9	795.8	2478.4	973.9	524.2	422.6	834.9	2206.8	1687.4	422.6	2478.4	515.3	375.7
Glucuronic acid	327.4	363.6	368.9	382.1	327.4	380.9	411.1	292.6	328.6	363.6	430.5	497.6	328.6	380.9	450.6	442.8
Sucrose	109107.1	104677.9	84357.3	76703.9	109107.1	81595.7	85641.8	112225.3	102928.9	104677.9	103057.6	108319.8	102928.9	81595.7	105284.5	108826.6
Maltose I	4203.6	1027.2	3916.9	2345.9	4203.6	1780.8	3472.5	4189.0	5385.3	1027.2	2855.7	2892.1	5385.3	1780.8	2593.2	2358.7
Maltose II	1580.1	1248.0	1757.3	1567.4	1580.1	1666.6	1600.6	2529.0	1551.3	1248.0	2442.5	2209.0	1551.3	1666.6	1603.4	1886.2
Trehalose	379.0	57.0	363.9	137.6	379.0	134.2	209.7	293.1	561.6	57.0	184.5	200.4	561.6	134.2	175.9	184.1
Unknown <sup>15</sup>	3638.7	8536.7	8355.2	8173.2	3638.7	12325.2	6877.4	5139.6	2920.9	8536.7	6174.9	9235.9	2920.9	12325.2	7095.6	9186.5
Unknown <sup>16</sup>	24396.3	42581.5	35067.6	34368.4	24396.3	29706.9	32610.3	22287.8	13725.7	42581.5	19171.9	21897.6	13725.7	29706.9	16582.0	13466.1

2015-Plot 2	S29	Purple	S29×P		S29	PF	S29×PF		N67	Purple	N67×P		N67	PF	N67×PF	
			Bulk-	Bulk+			Bulk-	Bulk+			Bulk-	Bulk+			Bulk-	Bulk+
Lactic acid	1047.7	1096.4	1060.9	588.6	768.3	1108.5	2143.5	795.5	861.8	1096.4	402.7	301.3	861.8	1108.5	150.2	100.1
L-alanine	2160.5	1962.1	1214.6	1584.9	1444.1	1672.7	1132.1	891.7	2247.7	1962.1	1026.8	620.8	2247.7	1672.7	438.8	206.0
L-valine	1110.1	1182.3	976.0	1394.3	740.2	1299.8	702.8	605.9	1337.1	1182.3	732.5	500.5	1337.1	1299.8	325.4	292.0
Phosphoric acid	2805.0	14146.7	2264.8	14777.2	2121.7	3987.7	1035.8	1899.0	38379.5	14146.7	1379.5	1733.4	38379.5	3987.7	759.6	435.5
Glycerol	9082.3	7819.0	4913.4	5323.5	6196.8	7125.6	3381.5	2642.9	10437.6	7819.0	4503.2	4172.8	10437.6	7125.6	2916.3	2255.3
L-proline	6576.1	8440.8	4210.6	6980.0	4388.1	8615.9	8426.1	7691.0	9773.0	8440.8	2716.8	1323.8	9773.0	8615.9	2402.8	2439.9

## 126 | APPENDIX

<b>Glycine</b>	403.1	324.4	354.6	424.4	272.9	351.3	273.2	230.4	390.2	324.4	310.6	260.7	390.2	351.3	159.8	192.7
<b>Succinic acid</b>	936.4	297.6	502.6	366.4	640.5	332.8	306.0	214.8	383.5	297.6	233.8	287.5	383.5	332.8	147.6	112.6
<b>Fumaric acid</b>	1247.3	409.8	1064.8	732.9	831.8	463.4	457.8	304.6	428.0	409.8	278.6	286.4	428.0	463.4	162.6	175.1
<b>L-serine</b>	591.1	405.2	338.3	489.8	394.1	360.9	249.4	233.0	593.6	405.2	341.1	286.0	593.6	360.9	189.1	173.0
<b>Unknown<sup>1</sup></b>	728.6	768.9	547.3	679.0	488.8	826.9	489.1	394.7	999.7	768.9	637.0	433.1	999.7	826.9	390.9	376.4
<b>L-Threonine</b>	414.0	348.6	257.7	367.7	279.6	306.6	181.0	161.3	459.0	348.6	285.7	212.4	459.0	306.6	151.1	140.9
<b>Unknown<sup>2</sup></b>	164.8	265.2	182.9	161.6	117.3	272.1	138.6	149.0	268.3	265.2	144.1	114.8	268.3	272.1	113.1	101.4
<b>Malic acid</b>	12720.1	4257.5	13047.9	7494.4	8486.9	5480.0	3219.1	1893.0	5000.0	4257.5	4079.8	3672.6	5000.0	5480.0	2092.8	2311.0
<b>Unknown<sup>3</sup></b>	2500.6	2465.8	2266.1	3017.1	1668.1	2801.6	2286.8	1881.5	2880.6	2465.8	1791.9	1162.8	2880.6	2801.6	969.6	1049.0
<b>Pyroglutamic acid</b>	4377.4	2872.2	4455.9	8052.0	2927.8	3700.2	3345.4	2511.2	3676.3	2872.2	2940.8	2918.2	3676.3	3700.2	1489.8	1478.7
<b>Aspartic acid</b>	4090.7	3332.1	4046.3	4022.6	2730.6	3731.9	2085.5	1236.7	3146.4	3332.1	2651.4	2411.6	3146.4	3731.9	1509.0	1342.7
<b>GABA</b>	272.4	699.9	314.0	393.8	188.1	417.7	339.9	305.2	488.3	699.9	116.3	83.7	488.3	417.7	87.1	82.5
<b>Unknown<sup>4</sup></b>	1673.3	2472.7	2903.6	3813.7	1116.7	2935.5	3243.9	2937.6	2564.5	2472.7	2073.0	1245.3	2564.5	2935.5	1079.1	986.5
<b>Unknown<sup>6</sup></b>	263.9	128.8	141.7	153.8	176.1	159.8	158.2	119.7	173.4	128.8	131.1	75.7	173.4	159.8	66.0	68.6
<b>L-glutamic acid</b>	2181.3	1535.4	1467.6	1407.6	1454.2	1395.2	807.3	591.3	1794.7	1535.4	995.0	881.7	1794.7	1395.2	558.5	534.9
<b>L-phenylalanine</b>	239.1	317.3	216.0	250.8	159.4	274.9	172.2	132.6	322.8	317.3	151.9	120.8	322.8	274.9	79.1	68.5
<b>Unknown<sup>7</sup></b>	593.7	916.4	567.1	868.5	452.2	772.0	423.5	442.9	917.8	916.4	435.1	315.6	917.8	772.0	280.2	248.7
<b>Pentose I</b>	45.7	131.4	62.1	77.8	32.6	89.7	57.2	47.6	60.5	131.4	51.1	32.2	60.5	89.7	29.2	25.0
<b>Unknown<sup>8</sup></b>	287.6	421.8	138.3	214.9	196.3	293.4	150.0	135.8	168.4	421.8	172.1	118.7	168.4	293.4	107.3	77.0
<b>Asparagine</b>	13904.5	10289.4	6938.5	8111.1	9286.0	8757.4	6152.6	4342.6	12649.9	10289.4	6584.9	4905.9	12649.9	8757.4	4134.7	4361.5
<b>Unknown<sup>10</sup></b>	310.7	250.2	184.8	211.5	268.1	272.2	177.4	147.9	255.1	250.2	225.3	179.8	255.1	272.2	135.8	136.4
<b>Pentose alcohol I</b>	2025.2	2264.4	1432.5	1309.9	1362.3	1917.6	1588.6	1188.5	2672.6	2264.4	1546.9	734.7	2672.6	1917.6	1014.8	990.0
<b>Unknown<sup>11</sup></b>	1998.0	1944.5	1534.6	1511.4	1383.1	1862.7	1342.2	1152.2	2143.1	1944.5	1512.0	966.3	2143.1	1862.7	948.0	779.5
<b>Unknown<sup>12</sup></b>	978.7	658.5	547.8	1017.1	652.5	793.8	179.8	315.9	1305.7	658.5	571.3	556.2	1305.7	793.8	320.4	297.4
<b>Citric acid</b>	1199.7	2045.5	1294.7	1045.5	800.8	1309.9	1603.1	971.9	1202.5	2045.5	813.2	713.3	1202.5	1309.9	793.5	653.7
<b>D-fructose II</b>	2931.9	4466.2	2928.3	2770.2	1955.6	4240.2	2465.7	1886.5	2808.7	4466.2	2576.0	2560.4	2808.7	4240.2	2303.0	1795.3
<b>D-fructose III</b>	7128.9	9406.1	6836.1	8485.3	4752.6	9224.7	6030.4	5471.7	8429.7	9406.1	5426.9	4928.4	8429.7	9224.7	4469.0	3868.3
<b><sup>13</sup>C-glucose</b>	13472.3	13112.4	18947.9	21458.3	8981.6	18063.8	25474.2	21292.6	9143.7	13112.4	9238.9	8366.3	9143.7	18063.8	8672.9	6781.3
<b>D-Galactose</b>	7408.4	17077.6	7924.0	9266.2	4938.9	9720.4	6943.5	5957.5	8850.4	17077.6	7363.1	5747.8	8850.4	9720.4	5239.3	4541.5
<b><sup>13</sup>C-glucose<sup>2</sup></b>	1928.3	1830.8	2151.0	2415.4	1285.7	2141.7	2728.4	2175.9	1294.4	1830.8	1275.5	1167.8	1294.4	2141.7	1178.3	937.2
<b>Hexose I (glucose)</b>	1185.7	2388.0	1126.6	1330.0	790.5	1449.4	931.3	842.7	1424.4	2388.0	1216.6	937.3	1424.4	1449.4	891.1	717.0
<b>Glucitol</b>	6309.1	12545.1	9463.4	7736.7	4206.0	11855.0	6936.1	4732.3	8536.9	12545.1	5041.3	4533.5	8536.9	11855.0	3628.3	3286.8
<b>Hexose II (D-glucose)</b>	619.1	644.6	550.9	420.7	1126.7	547.6	456.5	358.7	780.5	644.6	1484.0	438.6	780.5	547.6	441.1	437.2

<b>Myo-inositol</b>	1251.7	2371.3	808.0	783.1	1428.2	1468.8	1475.6	1040.3	1587.5	2371.3	774.5	512.1	1587.5	1468.8	938.4	628.1
<b>L-Tryptophan</b>	708.9	1172.9	805.0	1102.1	473.1	2945.9	412.4	389.5	308.5	1172.9	427.1	868.8	308.5	2945.9	138.0	85.4
<b>Glucuronic acid</b>	351.4	408.1	297.9	232.2	234.3	377.1	206.3	140.8	335.2	408.1	238.0	204.0	335.2	377.1	132.8	100.6
<b>Sucrose</b>	109626.6	115255.2	116920.2	111600.5	73084.7	119478.5	132675.0	122728.6	104043.0	115255.2	94808.8	91044.9	104043.0	119478.5	98296.4	86530.8
<b>Maltose I</b>	3745.6	1638.9	1858.9	2022.0	2512.2	1239.0	1067.8	843.4	2382.1	1638.9	1626.7	1230.1	2382.1	1239.0	1218.7	715.9
<b>Maltose II</b>	2294.0	2401.0	2016.7	1428.2	1544.5	2078.1	1230.2	722.2	1855.3	2401.0	1660.8	1252.1	1855.3	2078.1	826.5	594.7
<b>Trehalose</b>	303.4	172.3	308.3	417.1	202.2	162.1	268.5	223.6	189.4	172.3	136.4	106.8	189.4	162.1	107.9	70.1
<b>Unknown<sup>13</sup></b>	8978.8	7296.5	6363.3	4328.9	5985.9	8316.7	3682.0	2948.1	6179.6	7296.5	3201.6	3569.7	6179.6	8316.7	2194.9	2087.3
<b>Unknown<sup>14</sup></b>	18014.1	62713.5	84163.5	74469.6	12009.7	76756.4	55468.1	47694.4	22846.0	62713.5	11462.2	9841.3	22846.0	76756.4	11904.9	8212.8

## 8. Acknowledgements

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## 9. Curriculum Vitae

**Name:** Rongfan Wang  
**Date of Birth:** 14.12.1987  
**Place of Birth:** Hubei, China  
**Nationality:** P.R. China  
**Marital Status:** Unmarried

### Education Background

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<b>10/2012-present</b> Gatersleben, Germany	<b>PhD student</b> Applied Biochemistry Group, Leibniz Institute of Plant Genetics and Crop Plant Research
<b>09/2009-06/2012</b> Yangling, P.R. China	<b>Master of Silviculture</b> Faculty of Forestry, Northwest A & F University
<b>09/2005-06/2009</b> Wuhan, P.R. China	<b>Bachelor of Biology Science (Normal)</b> College of Life Science, Hubei University

### Scientific Experience

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<b>05/2016</b>	<b>Scientific course:</b> Academic writing
<b>10/2015</b>	<b>Scientific course:</b> Communication & presentation in the academic context: How to be confident and persuasive
<b>03/2015</b>	<b>Scientific course:</b> Career strategies accompanying the doctorate: Developing potentials – opening up professional pathways
<b>11/2013</b>	<b>Technical course:</b> Histological and ultrastructural analysis of plant tissue using light, scanning and transmission electron microscopy
<b>09/2013</b>	<b>Summer School "Barlomics":</b> Yield formation in cereals - overcoming yield-limiting factors
<b>03/2013</b>	<b>Technical course:</b> Microdissection and expression analysis
<b>Since 10/2012</b> Gatersleben, Germany	<b>PhD thesis:</b> Physiological, phytochemical and molecular characterization of pigmented cereals, Applied Biochemistry Group, Dept. Physiology and Cell Biology, Leibniz Institute of Plant Genetics and Crop Plant Research



- 09/2009-06/2012**  
Yangling, P.R. China      **Master thesis:** Compared two statistic methods on dividing suitable climatic and ecological region for *Pinus ponderosa* introduction, Faculty of Forestry, Northwest A & F University
- 12/2010-06/2011**  
Yunnan, P.R. China      **Guest Graduate Student:** Study on the extraction of ferulic acid from *Fructus Toosendan* by ultrasonic technique, Pharmacy Department of Dali Medical College
- 09/2008-06/2009**  
Wuhan, P.R. China      **Bachelor thesis:** Study on the extraction and stability of the anthocyanin from *Loropetalum Chinese var. Rubrums*, College of Life Science, Hubei University

### Oral presentations

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Phytochemical characterization of cereals accumulating anthocyanins with potential health beneficial effects. First Eduard Strasburger-Workshop of the German Botanical Society, Nürnberg/Germany, 21.-23.09.2014 & Plant Science Student Conference 2015, Halle/Germany, 02.-05.06.2015, **3rd Best talk awarded by the jury**

Phytochemical and molecular characterization of grain pigmentation of barley varieties. First Eduard Strasburger- Meeting of “Natural Products”, Deutsche Botanische Gesellschaft, Meisdorf/Germany, 11.-13.09.2016

### Poster presentations

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**Wang, R.**, K.J. Dehmer & H.-P. Mock: Relationship between Anthocyanins and Other Phenolic Compounds in Colored Crops. – Plant Science Student Conference 2013, Halle/Germany, 28.-31.05.2013

**Wang, R.**, A. Matros, M. Nagel, A. Börner & H.-P. Mock: Phytochemical characterization of cereals accumulating anthocyanins with potential health beneficial effects. – Institutes’ Day IPK, 25.-26.09.2013 & – Plant Science Student Conference 2014, Gatersleben/Germany, 02.-05.06.2014 & – Joint EUCARPIA Cereal Section & I•T•M•I Conference, Wernigerode/Germany, 29.06.-04.07.2014

**Wang, R.**, M. Nagel, A. Börner & H.-P. Mock: Physiological differences between purple and yellow-grain wheat with different anthocyanins characteristics during artificial ageing. – 1<sup>st</sup> Seed Longevity Workshop, Wernigerode/Germany, 05.-08.07.2015

**Wang, R.**, A. Matros, M. Nagel, A. Börner & H.-P. Mock: Phytochemical characterization of cereals accumulating anthocyanins with potential health beneficial effects. – 8<sup>th</sup> International Workshop on Anthocyanins, Montpellier/France, 16.-18.09.2015

**Wang, R.**, A. Matros, M. Nagel, A. Börner & H.-P. Mock: Phytochemical and molecular characterization of grain pigmentation for barley varieties. – Plant Science Student Conference 2016, Gatersleben/Germany, 07.-04.07.2016 & – The XXVIIIth International Conference on Polyphenols, Vienna/Austria, 11.-15.07.2016



## 10. Affirmation

Hereby, I declare that I have composed my PhD thesis independently (apart from the supervisor's guidance) using only those resources mentioned and that I have as such identified all passages which I have taken from publications verbatim or in substance. Neither this thesis nor any extract of it has been previously submitted to an examining authority, in this or a similar form.

I have ensured that this thesis has been prepared subject to the Rules of Good Scientific Practice of the German Research Foundation.

Gatersleben, July 2017

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