Ectopic expression of a *Vicia faba* amino acid permease1 (*VfAAP1*) improves grain yield and stimulates seedling root growth in wheat (*Triticum aestivum*)

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Abbreviations

AAP -amino acid permease

AAT -aspartate aminotransferase

ABC- ATP-binding cassette

ANTs-aromatic and neutral amino acid transporters

ARF-auxin response factors

ASN- asparagine synthetase

ATF-amino acid transporter superfamily

Aux–auxin

CDKs-cyclin-dependent kinases

CPP-copalyl pyrophosphate

Ct - cycle threshold

DAF- days after flowering

DAS-days after sowing

DEs-differentially expressed genes

FC- fold change

FEH-fructan 6-exohydrolase

FLAs-fasciclin-like arabinogalactan proteins

GA-gibberellin

GATs- gamma-aminobutyric acid transporters

GS-glutamine synthetase

GDH-glutamate dehydrogenase

GFP-green fluorescent protein

GGPP-geranylgeranyl pyrophosphate

GH3- Gretchen hagen3

HMW-Glu- high molecular weight glutenin

ICP-OES-optical emission spectrometers with inductively coupled plasm

IAA-indole-3-acetic acid

KA-ent-kaurenoic acid

KIP- kinase inhibitors protein

KOA-ent-Kaurenoic acid hydroxylase

KRPs-KIP-related protein

LHTs-lysine and histidine transporters

LMW-Glu- low molecular weight glutenin

NA- nicotianamine

ProTs-proline transporters

SAUR-small auxin-up RNA

SEM- S-adenosylmethionine

SPAD-soil and Plant Analysis Development

ROS-reactive oxygen species

TGW-thousand grain weight

TSP-trehalose-6-phosphate synthase

UDP glucose- uridine diphosphate glucose

Summary

Wheat (*Triticum aestivum*) is one of the primary grains consumed by humans and is growing around the globe in diverse environments. Given the rapid growth of the world's population, there is an urgent need to increase wheat yield. Grain yield of wheat is a complex trait and influenced by the formation, translocation, partitioning and accumulation of assimilates during grain development and maturation. Previous studies frequently suggest that grain sink limitation is one of the major grain yield-limiting factors in wheat. Amino acids represent the transport form of organic nitrogen, which is a crucial assimilate for proper plant growth and development. Improving the amino acid uptake capacity of developing grains might be fundamental to analyze the current yield-limiting factors in wheat. The present study analyzed the potential of improving amino acids uptake capacity of developing grains on yield formation in wheat. Transgenic winter wheat lines overexpressing the well-characterized *Vica faba* amino acid permease *VfAAP1* under the control of the wheat HMW-Glu-1Ax1 promoter (XAP) were analyzed. The aim of the studies in frame of this thesis was (i) to evaluate the potential of improved amino acid uptake capacity of developing grains for yield and quality of wheat grains, (ii) to characterize the seedling root phenotype of the new transgenic XAP winter wheat lines, (iii) to evaluate the influence of *VfAAP1* expression on grain development at the transcript level.

The yield potential of XAP lines was evaluated under field-like conditions over two consecutive cropping seasons. Grain biomass shows significant increment in developing and in mature XAP grains, the number of grains per plot was not reduced. Consequently, grain yield per plot was found significantly increased in XAP lines across all seasons. The increase in grain biomass was evident from 10 days after flowering (DAF), which corresponds to the dramatic increases of *VfAAP1* expression. The data suggest that *VfAAP1* expression increases amino acid uptake into developing grains resulting in formation of higher grain dry mass, improved TGW and stimulated storage protein synthesis. The result suggests that increased transcriptional activity of sink located amino acid transporters positively affects sink strength and that N assimilate uploading into developing grains might be a limiting step for sink development in wheat.

Considering the often reported transgene expression instability and unexpected phenotype variability in transgenic plants, the HMW-Glu-1Ax1 promoter activity in transgenic wheat was evaluated. The result reveals that the 1Ax1 promoter is primarily active in the developing endosperm. In addition, the 1Ax1 promoter shows considerable activity in maternal grain tissues specifically in the nucellar projection during the pre-storage phase. The HMW-Glu-1Ax1 promoter also shows slight activity in vegetative tissue of transgenic wheat which is highest in developing roots.

Introducing new genetic information in the genome may influence the growth and development of roots in many aspects. In this study, the seedling root phenotype of XAP lines was evaluated.

The result reveals that XAP lines have altered seedling root architecture when compared to the non-transformed control Certo. XAP lines displayed more seminal roots per seedling, shorter seminal roots but higher root dry mass, and more root branching points when compared to the non-transformed control. qPCR analysis showed significantly different expression of the *VfAAP1* gene across distinct root parts of XAP seedlings. Highest expression was observed in the cell maturity zone. On the contrary, expression was negligible in the cell elongation zone. Obviously, expression of *VfAAP1* in the root tissue influences root morphology.

Grain development is associated with massive changes in gene expression. Availability of assimilates in developing seeds has the potential to influence gene expression and metabolite profiles, resulting in altered biological processes and grain development traits. Transcriptional changes in developing XAP grains in comparison to the non-transgenic control were evaluated by transcript profiling. According to moderate t-test with corrected P-value (Westfall-Young, $P \leq 0.05$) differentially expressed genes (DEs) were identified at five stages of grain development (4, 6, 8, 10 and 14 DAF). Results are presented in two sets of data, which reflect the transcriptome of pericarp and endosperm tissues according to the stages of grain development. Based on gene ontology terms analysis, transport-related and storage proteins were the largest upregulated groups in XAP pericarp and endosperm, respectively. Cell wall related genes like expansins, COBRA-like and fasciclin-like arabinogalactan and cellulose synthase were preferentially upregulated in XAP pericarp. Cluster of genes related to transporters like ABC transporters show upregulation in the XAP pericarp. These findings suggest that pericarp growth might contribute to the improved TGW and grain size in XAP lines. Genes related to cell division including cyclins and CDKs are upregulated in XAP endosperm. Cell-wall related genes like pectin modifying enzymes and water transporters (aquaporins), which may stimulate cell wall extension, are upregulated in the XAP endosperm. The result suggests that cell division and cell growth might be stimulated in XAP endosperm cells. Cluster of genes related to storage protein synthesis, specifically genes encoding proteins of the prolamin class show upregulation in the XAP endosperm. Subsequently, mature grains analysis indicates that XAP grains have increased percentages of total nitrogen and prolamins. These findings suggest that synthesis of prolamin storage protein might be stimulated in XAP endosperm. The expression of metal transporter genes including Zn and Fe transporters were upregulated in XAP endosperm. Correspondingly, genes related to the biosynthesis of nitrogen chelator compounds like nicotianamine synthase are upregulated in the XAP endosperm. Consistent with this result, significant increment of Zn and Fe was observed in mature XAP grains. The availability of N assimilates in XAP endosperm obviously enhances the biosynthesis of nitrogenous chelator compounds and metal transporters, which might accelerate the uploading of Zn and Fe into the developing grain.

Zusammenfassung

Weizen (*Triticum aestivum*) ist eine der wichtigsten Kulturarten für die menschliche Ernährung und wird überall in der Welt unter den unterschiedlichsten Bedingungen angebaut. Aufgrund des rasanten Wachstums der Weltbevölkerung besteht eine dringende Notwendigkeit, den Weizenertag zu steigern. Der Kornertrag von Weizen ist ein komplexes Merkmal und wird durch die Bildung, den Transport, die Verteilung und die Akkumulation von Assimilaten während der Kornentwicklung und –reifung beeinflusst. In der Vergangenheit führten viele Untersuchungen zu der Vermutung, dass die limitierte Sink-Stärke der sich entwickelnden Körner hauptsächlich verantwortlich sein könnte für die Begrenzung des Kornertrages. Aminosäuren sind die Transportform für organischen Stickstoff und grundlegend wichtige Assimilate für normales Wachstum und normale Entwicklung von Pflanzen. Die Erhöhung der Aufnahmekapazität für Aminosäuren in sich entwickelnden Körnern könnte deshalb eine Möglichkeit sein, die ertragslimitierenden Faktoren bei Weizen zu beeinflussen. In der vorliegenden Arbeit wurde untersucht, welchen Einfluss die Erhöhung der Aufnahmekapazität für Aminosäuren in sich entwickelnden Körnern auf das Ertragspotenzial von Weizen hat. Transgene Winterweizenlinien (XAP) wurden analysiert, die die cDNA des gutcharakterisierten *Vicia faba* Aminosäuretransporter VfAAP1 unter Kontrolle des 1Ax1-Promotors aus Weizen exprimieren. Die Arbeiten wurden durchgeführt, (i) um das Potenzial der erhöhten Aufnahmekapazität für Aminosäuren in das sich entwickelnde Korn in Bezug auf Kornertrag und Kornqualität zu evaluieren, (ii) um den Phänotyp der Keimlingswurzeln der transgenen XAP-Linien zu charakterisieren und (III) um den Einfluss der *VfAAP1*-Expression auf das Transkriptom der sich entwickelnden Körner zu untersuchen.

Das Ertragspotenzial der XAP-Linien wurde in zwei aufeinanderfolgenden Anbauperioden unter feldnahen Bedingungen evaluiert. Die Biomasse der sich entwickelnden und der reifen XAP-Körner war signifikant erhöht, die Kornzahl pro Parzelle war nicht reduziert. Demzufolge war der Kornertrag pro Parzelle für alle XAP-Linien und in beiden Anbauperioden signifikant erhöht. Die Erhöhung der Kornbiomasse war nachweisbar ab dem 10. Tag nach der Blüte (*day after flowering*, DAF) und stimmte überein mit der dramatischen Erhöhung der *VfAAP1*-Expression in den sich entwickelnden Körnern. Aus den Daten kann abgeleitet werden, dass die Expression von *VfAAP1* die Aufnahme von Aminosäuren in die sich entwickelnden Körner verstärkt, was zu einer höheren Korntrockenmasse führt, verbunden mit einer Steigerung der Tausendkornmasse und einer stimulierten Synthese von Speicherproteinen. Aus diesem Ergebnis kann abgeleitet werden, dass die erhöhte transkriptionelle Aktivität von Sink-lokalisierten Aminosäuretransportern die Sinkstärke positiv beeinflusst und dass die Entladung von N-Assimilaten in sich entwickelnde Körner ein limitierender Schritt für die Entwicklung des Sinks während der Kornentwicklung sein könnte.

In einer Vielzahl von Veröffentlichungen wird von Instabilitäten der Transgenexpression und von unerwarteter Variabilität des Phänotyps transgener Pflanzen berichtet. Deshalb wurde die Aktivität des HMW-Glu-1Ax1-Promotors im transgenen Weizen untersucht. Die Ergebnisse zeigten, dass der 1Ax1-Promotor hauptsächlich im sich entwickelnden Endosperm aktiv ist. Darüber hinaus zeigte der 1Ax1-Promotor bemerkenswerte Aktivität in maternalen Korngeweben, besonders in der nucellaren Projektion während der Vorspeicherphase. Der HMW-Glu-1Ax1-Promotor zeigt geringe Aktivität auch in vegetativen Geweben des transgenen Weizens. Hier ist die Aktivität am höchsten in Keimlingswurzeln.

Wird neue genetische Information in das Genom integriert, kann sich die Entwicklung und das Wachstum von Wurzeln auf vielfältige Weise verändern. In dieser Arbeit wurde der Phänotyp der XAP-Keimlingswurzeln untersucht. Die Ergebnisse zeigten, dass die Architektur der XAP-Keimlingswurzeln im Vergleich zur nichttransformierten Kontrolle Certo verändert ist. Die Keimlinge der XAP-Linien entwickeln mehr und kürzere seminale Wurzeln, zeigen aber eine höhere Wurzeltrockenmasse und eine größere Zahl von Wurzel-Verzweigungspunkten als die nichttransformierte Kontrolle. Die qRT-PCR-Analyse zeigte, dass *VfAAP1* in verschiedenen Teilen der XAP-Keimlingswurzeln unterschiedlich stark exprimiert wird. Die höchste Expression wurde im ausdifferenzierten Teil der Wurzel detektiert. Im Gegensatz dazu war die Expression im Bereich der Zellstreckung zu vernachlässigen. Es ist offensichtlich, dass die Wurzel-spezifische *VfAAP1*-Expression die Wurzelmorphologie beeinflusst.

Die Kornentwicklung ist verbunden mit großen Veränderungen in der Genexpression. Die Verfügbarkeit von Assimilaten im sich entwickelnden Korn kann potenziell sowohl Genexpression als auch metabolische Profile verändern. In Folge dessen können sich sowohl biologische Prozesse als Kornmerkmale verändern. Die transkriptionellen Veränderungen in sich entwickelnden XAP-Körnern im Vergleich zur nicht-transgenen Kontrolle wurden mittels *transkript profiling* analysiert. Entsprechend des moderaten t-Tests mit korrigierten P-Werten (Westfall-Young $P \leq 0.05$) wurden differenziell exprimierte Gene (DEs) in fünf Stadien der Kornentwicklung (4, 6, 8, 10 und 15 DAF) identifiziert. Es resultierten zwei Datensätze, die das Transkriptom von Pericarp und Endosperm den Stadien der Kornentwicklung entsprechend widerspiegeln. Auf der Grundlage von *gene ontology terms* wurden zwei Gruppen von Genen identifiziert, die eine ausgeprägte Hochregulation zeigen: Transport-assoziierte Gene im Pericarp und Gene mit Relevanz zur Speicherprotein-Synthese im Endosperm. Gene mit Assoziation zur Zellwand wie z. B. Expansin- und COBRA-like Gene, weiterhin Fasciclin-like Arabinogalactan- und Zellulosesynthasegene sind im XAP-Pericarp hochreguliert. Ein Cluster von Genen mit Hochregulation im Pericarp hat Beziehung zu Transportprozessen und enthält z. B. ABC-Transporter. Diese Ergebnisse weisen darauf hin, dass verstärktes Pericarp-Wachstum einen Betrag zur Erhöhung des Tausendkorngewichtes und zu größeren Körnern leisten könnte. Im XAP-Endosperm sind verstärkt Gene mit Bezug zu Zellteilungsprozessen exprimiert, wie z. B.

Cyclin-Gene und Gene, die für CDKs codieren. Weiterhin sind Gene exprimiert mit Bezug zur Ausdehnung der Zellwand, wie z. B. Gene für Pectin-modifizierende Enzyme und Wassertransporter (Aquaporine). Die Ergebnisse weisen darauf hin, dass Zellteilung und Zellstreckung (Wachstum) im XAP-Endosperm stimuliert sein könnten. Weiterhin werden Gene über-exprimiert, die für die Synthese von Speicherproteinen codieren, vor allem Gene der Prolamin-Klasse. Die Analyse reifer XAP-Körner zeigte einen erhöhten prozentualen Gehalt an Gesamtstickstoff und Prolaminen. Insgesamt weisen diese Ergebnisse darauf hin, dass die Synthese von Speicherproteinen der Prolamin-Klasse im XAP-Endosperm stimuliert sein könnte.
Die Expression von Metalltransporter-Genen, unter ihnen Transporter für Zn und Fe, ist im XAP-Endosperm hochreguliert. Dem entsprechend sind Gene mit Bezug zur Biosynthese von Stickstoffchelator-Verbindungen wie Nicotianamin-Synthase hochreguliert. In Übereinstimmung mit diesem Ergebnis wurde eine signifikante Erhöhung von Zn- und Fe-Gehalten in reifen XAP-Körnern gefunden. Offensichtlich verstärkt die Verfügbarkeit von N-Assimilaten im XAP-Endosperm die Biosynthese von N-Chelatorverbindungen, was die Entladung von Zn und Fe in das sich entwickelnde Korn fördert.

Chapter 1

General introduction

1.1 Wheat

Wheat (*Triticum Spp)* is one of the most important crops grown around the world, and it is one of the primary grains consumed by humans(Curtis, 2002). Wheat evolved from wild grasses, and it is believed that wheat had been first cultivated between 15,000 and 10,000 BC, and thought wheat is one of the Neolithic founder crops, domesticated alongside other cereals in the Near-Eastern Fertile Crescent (Lev-Yadun, 2000). It is an annual plant belonging to the genus *Triticum* which includes common bread wheat (*Triticum aestivum)* and durum wheat (*Triticum turgidum*). Bread wheat is a major global cereal grain essential to human nutrition. Bread wheat (*Triticum aestivum*) is a hexaploid species with 42 chromosomes, and its genomes are designated as ABD, composed of three closely related and independently maintained genomes resulting from a series of naturally occurring hybridizations. Today, wheat occupies more than 200 million hectares of land worldwide, it accounts for 19% of the total production among the major cereal crops and provides one-fifth of the total calories of the world's population (Braun et al., 2010). Wheat is the second largest world producing food crop next to rice and cultivated in diverse environments from cool rain-fed to hot, dry land areas (Curtis, 2002; FAOSTAT, 2013; Nuttonson, 1955; Ray et al., 2012).

Wheat is a cool season crop, and it has a very broad agronomic adaptability. Moreover, widely differing pedigree varieties exist, which allow its cultivation under many environmental conditions. Although successfully grown between the ranges 30-60°N and 27- 40°S of latitude, it can also be cultivated well beyond these limits. For instance, it grows from within the Arctic Circle to the equator and from sea level on the Dutch Polders to 4500m altitude in Tibet (Nuttonson, 1955). Furthermore, the growing conditions of wheat are very diverse regarding variability in soil types and crop management (Curtis, 2002). World leaders of wheat production are China, India, the United States, the Russian Federation, France, Germany, Pakistan, Canada, Australia and Ukraine in respected order (FAOSTAT, 2013). These countries contribute more than two-thirds of the global wheat production. By growing habit, wheat is classified as winter and spring. Winter wheat is planted in autumn considering the fact that it requires a cold period (vernalization) during early growth for normal heading under long day conditions. Spring wheat, as the name implies, is usually planted in spring and matures in late summer but can be sown in autumn in countries that experience mild winters, such as in South Asia, North Africa, the Middle East and the lower latitudes.

Recent estimates indicate that by 2050 world must produce 50-70% more food, due to a combination of increasing population and shifts in consumption patterns (FAO, 2009).

Accordingly, global wheat production should increase to 1090 million tons by 2050 from its current production level of 680 million tons (Raj Paroda, 2012). Unfortunately, except in China where growth is still above 3% yield increment per annum, in the world's 20 major wheat producing countries, accounting for 85% of all wheat production, yields rise annually by only 1.1% during 1995–2006 (Dixon et al., 2009). Based on a 1.0% global yield growth rate, wheat production will be only 15% higher by 2025. To make things worse, climate-change induced temperature increases are estimated to reduce wheat production in developing countries (where around 66% of all wheat produced) by 20-30% (Easterling et al., 2007; Lobell et al., 2008; Reynolds et al., 2011). In summary, improving wheat productivity in the coming decades is crucial for achieving sustainable agriculture and food security. Nevertheless, the current approaches to yield improvement are not sufficient to double production by 2050. The overall information indicates that there is an urgent need for new approaches to address the problem.

1.2 Yield improvement and grain quality

Since the development of modern plant breeding techniques, wheat breeders have been successfully increased grain yield in different environments (Calderini et al., 1995; Khush, 2001; Slafer and Andrade, 1991). The major factor contributing to the improvement in yield over the last century was due to harvest index increment (Austin et al., 1980; Reynolds et al., 1999; Slafer and Andrade, 1991). As a result of the green revolution, wheat yield sharply increased due to the use of improved varieties and intensive agronomical practices. During the green revolution era, the introduction of reduced height alleles has been a key player to improve harvest index and to allow the application of more nitrogen without crop lodging. As a result, yield stability also increased during this period (Evenson and Gollin, 2003; Smale, 1996). However, at the later period, yields at the farm gate have stagnated in many countries or are increasing at less than half the rate required to meet the projected demand (Brisson et al., 2010; Calderini and Slafer, 1999; Fischer and Edmeades, 2010). Combined with reduced rates of yield improvement, increasing global population (Rosegrant and Cline, 2003) and climate change effects (Asseng et al., 2014; Asseng et al., 2013; Challinor et al., 2014) are the challenges for wheat breeders.

In wheat, grain yield is mostly the combination result of the development and growth of yield components during the life of the crop (Reynolds et al., 2011; Slafer and Rawson, 1994). However, a dynamic view of yield components spotted that, at each level of integration, components are not independent and correlated in a complex manner (Gambin and Borras, 2010; Sadras and Denison, 2009; Slafer et al., 2015; Slafer and Rawson, 1994). Accordingly, grain number per unit area and indivisible grain weight are major grain yield components in wheat. Negative correlation between the number of grains and grain weight has been commonly reported in wheat, but only partially understood (Garcia et al., 2013; Griffiths et al., 2015; Sadras, 2007; Slafer et al., 2015). Consequently, compensations among components are one of the main barriers improving grain yield and its quality. Therefore understanding genetics and

physiologic dynamics behind the complex relationship of yield components might be fundamental for improving the current knowledge of yield-limiting factors to design future breeding strategies.

Wheat is the staple food for 35% of the world's population and provides more calories and protein in the world's diet than any other crop (Dixon et al., 2009; Gupta et al., 2008). Grain quality is an important trait along with yield in wheat breeding. Grain quality is defined by a range of physical and compositional properties, which describe the nutritional and end-use class of the grain. The quality of the wheat grain depends on several characteristics, grain hardness, protein content and composition of high molecular weight glutenin subunits (HMW) are the most important quality traits (Groos et al., 2004; Kuchel et al., 2006; Martin et al., 2001; Payne et al., 1987; Salmanowicz et al., 2008; Shewry et al., 1992; Surma et al., 2012). Grain protein content and composition are main determinants of grain nutrition and grain quality (Shewry and Halford, 2002; Weegels et al., 1996). Genetics, growing conditions, and genotype by environment interaction are the major regulators of the quality and quantities of stored protein in wheat endosperm (Martre et al., 2003; Nuttall et al., 2016; Surma et al., 2012). However, the inverse relationship between grain yield and protein content in cereals was repeatedly reported. Improving these two traits simultaneously in conventional approaches could be challenging (Brancourt-Hulmel et al., 2003; Delzer et al., 1995; Stewart and Dwyer, 1990). One challenge for global nutrition in the next decade is to increase food yield per unit ground area in a sustainable manner while maintaining its nutritional value (Cassman, 1999; McLaughlin and Kinzelbach, 2015; Tilman et al., 2011). Therefore, understanding the crucial factors of associated yield and grain quality is critical for further grain yield improvement without affecting the grain quality.

In summary, world wheat yields are not improving fast enough to keep up with projected demands in 2050. Grain yield is a complex trait influenced by the genetic composition of the plant, environmental conditions and interaction between genotype and environment. Negative correlations among the major yield components of wheat are frequently reported. Grain protein content is an important trait in wheat breeding. However, the negative correlation of grain protein content and grain yield is a potential challenge for breeders. Genetic and physiological understanding of these trade-offs among yield traits could be fundamental to increasing grain yield and grain quality in wheat.

1.3 Amino acid supply of the developing seed

Amino acids have highly diverse and essential roles in plants. These compounds are the building blocks of enzymes and proteins that provide essential components for plant metabolism and structure. Moreover, amino acids act as precursors or amino group donors for the synthesis of an array of compounds critical for plant development including nucleotides, chlorophyll,

hormones and secondary metabolites (Epstein, 2005). Plants can take up amino acids directly from the soil or assimilate from inorganic nitrogen (Nasholm et al., 2009; Tegeder and Rentsch, 2010). Most plants take up inorganic N compounds from the soil and following their uptake, nitrate and ammonium are metabolized into amino acids. Therefore, for proper plant growth and development the concerted uptake, assimilation and partitioning of amino acids are vital. In general, amino acids are the currency of nitrogen exchange between sources and sink tissues in plants and constitute a major source of the components used for cellular growth and differentiation. Plant seeds synthesize storage compounds (proteins and starch) representing the primary source of protein and calories for human and animal nutrition. In several crops, seed proteins are predominantly synthesized in the endosperm/cotyledon during seed development. Storage reserve accumulation and seed growth are strongly dependent on sugars, and nitrogen compounds imported into the developing seeds (Weber et al., 1997; Weber et al., 1998; Wobus and Weber, 1999). Amino acids are the major transported organic nitrogen, which are mainly assimilated within plant roots or leaves, and then translocated to developing sink tissues (Rentsch et al., 2007). Plants have fundamental processes for selective partitioning of amino acids among different organelles, cells, tissues, and organs mediated by various transport mechanisms. These mechanisms must be coordinated and regulated to achieve normal physiological cell functions (Lalonde et al., 2003; Stitt et al., 2002).

Currently, around 11% of the world population suffers from malnutrition, and some diseases are caused by poor quality protein and lack of vitamins and other micronutrients (FAO, 2015). Enhancing the nutritional quality of cereals is a means of improving human nutrition and health. Wheat contains different contents of grain protein, which is an important part of the total protein in human food. Grain protein content is a key factor in determining the nutritional quality of the grains. The import of organic nitrogen (amino acids) has a critical relevance for storage protein biosynthesis and grain development (Blumenthal et al., 1990; Rolletschek et al., 2005). Therefore, understanding the amino acid transport activities and flows towards and within developing grains might be an important step to improve grain quality and enhance nutrition value of the grain.

1.4 Amino acid transporters

Amino acid transporters are integral membrane proteins mediating the transport of amino acids across cellular membranes in higher plants. Partitioning of amino acids within and between cells and from source to sink organs requires proteins functioning as importers and/or exporters in cellular or subcellular membranes (Bush, 1993). Physiological and genetic evidence for the activity of multiple-carrier mediated transport systems responsible for the uptake and transfer of amino acids were reported for many plant species (Li and Bush, 1990; Li and Bush, 1992; Weston et al., 1995). After a proton-coupled uptake system for amino acids had been proposed by Bush (1993), numerous amino acid transporters have been isolated from different crops.

Amino acid permease *AtAAP1*/*NAT2* was the first plant amino acid transporter isolated by functional complementation in yeast (Frommer et al., 1993; Hsu et al., 1993). Subsequently, several members of the amino acid transporter superfamily (ATF) have been identified. ATF is classified into subfamilies based on sequence similarities and uptake properties (Hirner et al., 1998; Neelam et al., 1999; Ortiz-Lopez et al., 2000; Rentsch et al., 1996). So far, six subfamilies of AFT, such as amino acid permeases (AAPs), lysine and histidine transporters (LHTs), proline transporters (ProTs), gamma-aminobutyric acid transporters (GATs), auxin transporters (AUXs), and aromatic and neutral amino acid transporters (ANTs) are identified (Rentsch et al., 2007; Schwacke et al., 2003). In Arabidopsis more than 60 distinct amino acid transporter genes have been identified by heterologous expression systems and database screening with known transporters (Rentsch et al., 2007).

The amino acid permease (AAP) subfamily has been characterized and identified in more detail based on heterologous complementation experiments and sequence homology in different plant species. AAPs may classify either as neutral and acidic amino acid transporters or as general amino acid transporters. In Arabidopsis, eight members were found in the AtAAPs subfamily. AtAAP members can transport neutral and charged amino acids with varying specificities and affinities. Among the members, *AtAAP1* to *AtAAP6* and *AtAAP8* have been characterized in more detail using heterologous expression systems. The results indicate that these AAP members have a preference in the transport of neutral amino acids and glutamate (Fischer et al., 1995; Fischer et al., 2002; Rentsch et al., 2007; Svennerstam et al., 2008). Accordingly, the functions of *AAP* family members from other plant species had also been studied, such as *PsAAP1* (Tegeder et al., 2000), *VfAAP1* and *VfAAP3* (Miranda et al., 2001), *StAAP1* (Koch et al., 2003), *PvAAP1* (Tan et al., 2008), *PtAAP11* (Couturier et al., 2010). In monocots, the biochemical transport function of AAPs is not well characterized. However, recently four AAPs (*OsAAP1*, *OsAAP3*, *OsAAP7* and *OsAAP16*) from rice were characterized in more detail (Taylor et al., 2015).

During reproductive growth, seeds represent the major sink for organic nitrogen. Assimilated nitrogen and remobilized organic nitrogen derived from protein breakdown during senescence of vegetative plant organs are transported to the seed sinks. Since the filial parts of the seeds are symplasmically isolated, organic N is exported from the maternal seed coat into the seed apoplast, followed by uptake into filial cells (Wang and Fisher, 1994; Weber et al., 1997). Imported N compounds might be needed for seed development and stored as storage proteins in the endosperm or the embryo/cotyledons. AAPs are expressed in developing seeds of different plant species. *AtAAP1, AtAAP2* and *AtAAP8* (Hirner et al., 1998; Kwart et al., 1993) from Arabidopsis and legume *PsAAP1* (Tegeder et al., 2000) and *VfAAP1* (Miranda et al., 2001) are active in developing seed. In summary, amino acid transport activity is involved in all processes associated with nitrogen allocation during plant growth. Therefore, their detailed

physiological and biological function in different plant species might be critical for understanding the current yield limiting factors.

1.5 Objectives

Current evidence suggests that wheat grain yield is at plateau rates in the major production regions (Brisson et al., 2010; Calderini and Slafer, 1999; Fischer and Edmeades, 2010). Previous studies indicated grain sink strength as a critical yield limiting factor in wheat and thus, sink capacity needs improvement to increase grain yield. Recent studies suggest that facilitating the import of assimilates into the developing seed could be a potential option to improve yield and seed quality in different crop species (Rolletschek et al., 2005; Saalbach et al., 2014; Wang et al., 2015; Weichert et al., 2010; Weigelt et al., 2008). Amino acids represent the major transport form of organic nitrogen, which is a crucial assimilate for appropriate seed development. Therefore, improving the amino acid uptake capacity of developing grains in wheat might be fundamental to analyze the current yield-limiting factors in wheat. So far, little is known about the influence of improved amino acids uptake capacity of developing grains on yield and quality in wheat grains. This study investigates the yield potential and grain quality of novel winter wheat lines (XAP), which ectopically express the *Vicia faba* amino acid permease 1 (*VfAAP1*) under the control of the wheat high molecular weight glutenin (HMW-Glu) *1Ax1* promoter.

The specific objectives of the study are:

I. to evaluate the potential of improved amino acid uptake capacity of developing grains for improving yield and quality of wheat grains,
II. to characterize the seedling root phenotype of the new transgenic XAP winter wheat lines,
III. to analyze the molecular-physiological parameters of developing XAP grains in comparison to the non-transgenic control at the molecular level by transcript profiling.

Chapter 2

Preliminary work: establishment of transgenic winter wheat lines

Wheat grain yield has been steadily increased over the past decade. The concerns about the ability of our agricultural system to meet future demand and the central role of wheat in maintaining food security would be the challenge for wheat breeders. Past experiences in wheat improvement have led crop physiologists to suggest that grain sink limitation is one of the major grain yield-limiting factors in wheat. Increasing the sink capacity of developing grains could be an opportunity to improve the grain yield potential in wheat (Fischer, 2007; Miralles and Slafer, 2007; Reynolds et al., 2012). Based on this hypothesis new transgenic winter wheat lines ectopically expressing the *Vicia faba* amino acid permease 1 (*VfAAP1*) under the control of the HMW-Glu-1Ax1 wheat promoter were established at the IPK Gatersleben, Germany. The aim was to increase the sink strength of developing grains of winter wheat, and evaluate the influence of improved amino acid uptake capacity on grain development and grain yield and composition.

2.1 Establishment of marker gene free XAP lines

The *Vicia faba* amino acid permease 1 (*VfAAP1*) cDNA (1590bp, Acc. No AJ318809) was fused to the high molecular weight glutenin (HMW-Glu) 1Ax1 wheat promoter (2376bp, Acc. No M22208) and the terminator of the octopine synthetase gene (*OCS*) (727bp Acc. No *AF242881.1)*. The construct was cloned into the binary vector p6U and transformed into winter wheat (*Triticum aestivum* L. cv. Certo). A schematic drawing of the used vectors is given in Fig. 1. Co-transformation method was used with two independent binary vectors to separate integration of the target (XAP) gene from that of the selectable marker gene. The transformation procedure was performed based on the Agrobacterium method following the protocol of Hensel et al. (2009). Seeds were collected from each independent PCR positive T0 progeny to produce T1 plants. The inheritance of the transgene in the T1 progeny was tested by PCR amplification and Southern blot analysis. Three independent transgenic lines, two of the lines carrying one copy of the transgene at different genomic loci (BB, CC) and a third line (BBCC) containing two copies of the transgene (combining the two transgenic loci) were selected from the T1 generation. From the three lines, homozygous plants were established by the doubled haploid methods (DH) (collaboration with NORDSAAT Saatzucht GmbH, Böhnshausen, Germany). The inheritance of the transgene in the DH lines and subsequent generations was analyzed by using Southern blotting and PCR amplification. Southern blotting analysis was used to detect the absence of selectable marker genes (*HPT* or *aadA*) and the presence of the target gene *(Vf*AAP1) (Fig. 2).

2.2 Establishment of *1Ax1::GFP* lines

Efficient genetic modification of crops for targeted traits requires the use of well-characterized promoters, to drive the expression of transgenes in specific plant tissues or at certain developmental stages. The use of gene fusion constructs carrying a defined promoter sequence and reporter genes is a common approach to analyzing the activity of promoters (Jefferson et al., 1987; Medberry et al., 1992; Pauk et al., 1995). Accordingly, transgenic winter wheat carrying the 1Ax1::GFP construct was developed to analyze the strength, tissue specificity and the expression patterns of the 1Ax1 wheat promoter in transgenic winter wheat. Green fluorescent protein gene (*GFP*) (Gene ID: 7011691) was fused to the 1Ax1 wheat promoter (2376bp, Acc. No M22208) and terminator of octopine synthetase (*OCS*) (727bp Acc. No *AF242881.1)*. The construct was cloned into the p6U vector and transformed into winter wheat (*Triticum aestivum* L. cv. Certo). The transformation procedure was performed based on the Agrobacterium method following the protocol of Hensel et al. (2009). Independent PCR positive T0 plants were selected for further analysis. According to Southern blot analysis, two independent lines carrying one copy of the transgene were selected from T1 progeny for promoter activity experiments.

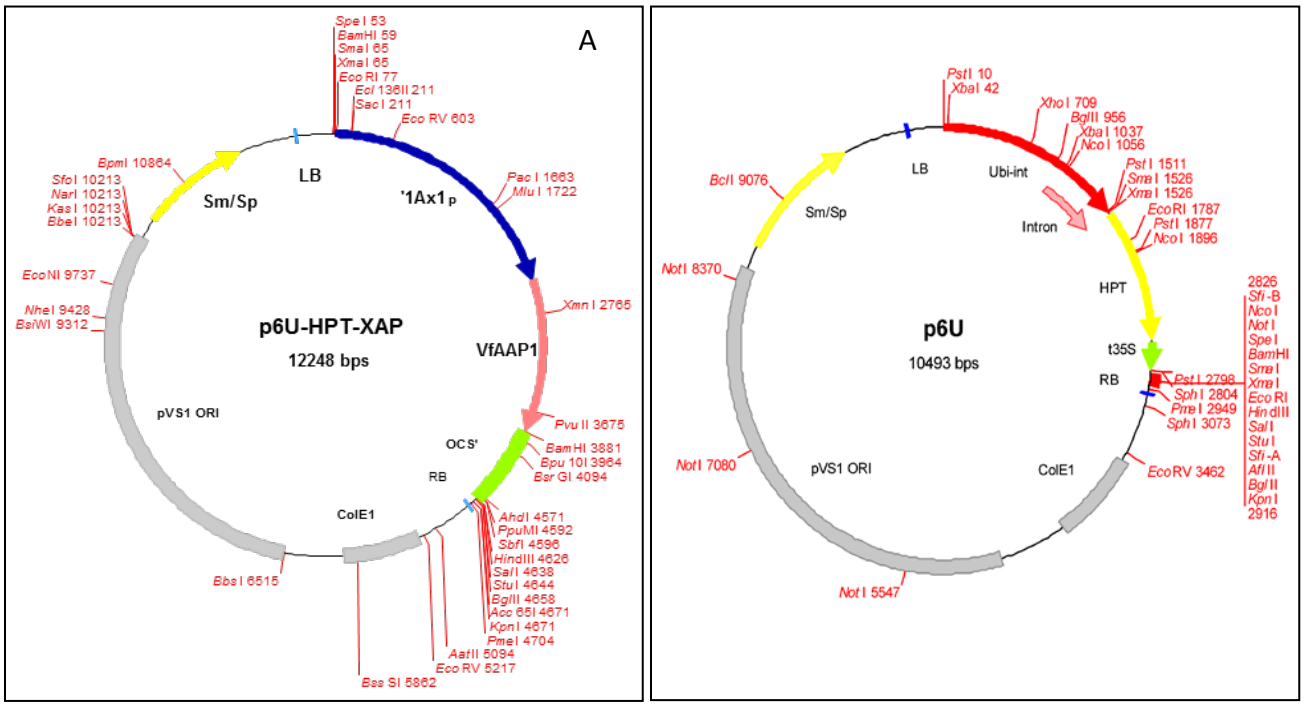


Fig. 1 Binary vector construct (p6U(-)HPT-XAP used for wheat transformation and the original p6U vector carrying the HPT gene.

A. Plasmid construct p6U (-)HPT-XAP. B. p6U plasmid containing the marker gene *HPT*. Co-transformation has been used for XAP lines establishment.

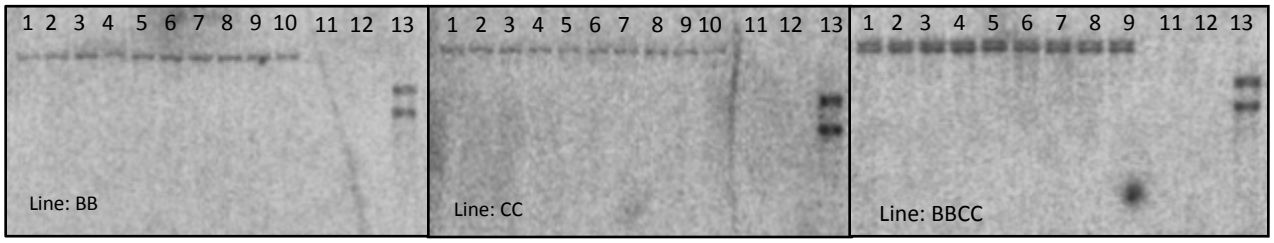


Fig. 2 Detection of *VfAAP1* by Southern blot analysis in XAP lines.

Ten randomly chosen plants of each line at T5 generation were analyzed; genomic DNA (15µg) digested with *Eco*RI was used. In each blot, 1-10, XAP lines, 11-12, negative control (Certo) and 13, positive control (*V. faba).* The blots were hybridized with ^{32}P labeled *V. faba AAP1* cDNA.

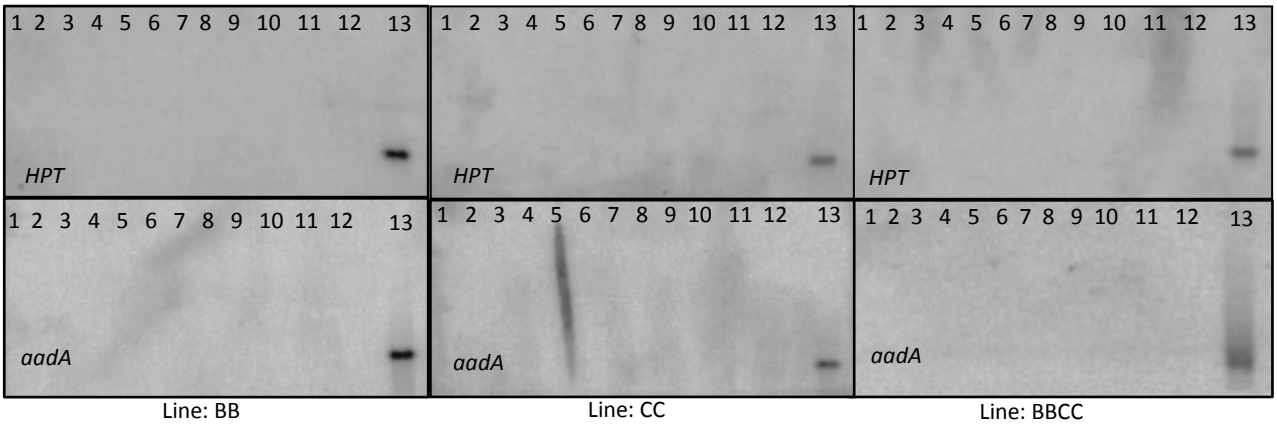


Fig. 3 Southern blot analysis of XAP lines for the absence of selectable marker genes.

Ten randomly chosen plants from each line were analyzed. Genomic DNA (15µg) of the T5 generation was digested with *Eco*RI (*hpt*) and HindIII (aadA), the blots hybridized with ^{32}P labelled *HTP* and *aadA* sequences*.* Each blot, 1-10, XAP lines, 11-12, negative control (Certo), 13, positive control, respective fragments were used as a probe.

Chapter 3

Ectopic expression of the *V. faba* amino acid permease 1 (*VfAAP1*) in winter wheat increases grain size and improves grain yield

3.1 Introduction

Grain yield in monocots is a complex trait that is directly associated with grain size and number of grains per unit area. Many studies comparing major yield components of wheat cultivars were released at different periods. These studies suggested that most of the modern cultivars have a greater number of grains per unit areas but lower average individual grain weight than their ancestors (Perry and D'Antuono, 1989; Sadras and Lawson, 2011; Siddique et al., 1989; Slafer and Andrade, 1989; Waddington et al., 1986). In general, grain yield improvement in wheat has been highly associated with grain number per unit area (Calderini and Slafer, 1999; Fischer, 2007; Sadras and Lawson, 2011; Slafer et al., 1990). Recent studies reported that grain yield and grain number show curvilinear association in different wheat genotypes (Bustos et al., 2013; Garcia et al., 2013; Reynolds et al., 2013). This suggests that the efficiency of grain yield improvement will be limited in the near future if it focuses only on grain number. Accordingly, increasing individual grain weight might be a complementing approach for further yield potential improvement in wheat.

Grain weight is mainly determined by grain filling, i.e. between anthesis and grain maturity (Charmet et al., 2005; González et al., 2014; Wan et al., 2008; Xie et al., 2015). Grain/seed development largely dependents on the supply of assimilates to the developing grains and metabolic regulation (Borisjuk et al., 2004; Weber et al., 2005; Weber et al., 1998). The proper control of assimilate partitioning towards sink tissues is fundamental to improve crop yield (Braun, 2012; Calderini et al., 1995; Reynolds et al., 2005). Increasing sink strength by enhancing assimilate uptake capacity of targeted sink organs results in increased yield and improved quality in cultivated crops (Rosche et al., 2002; Saalbach et al., 2014; Sun et al., 2011; Wang et al., 2015; Weichert et al., 2010; Zhang et al., 2015a). Amino acids are very basic compounds for plant growth and development. In most plant species, they are predominantly assimilated in roots and transported to other plant parts. These compounds are the building blocks of proteins and act as amino group donors for the synthesis of an array of N metabolites essential for cell structure and function and plant defense. Assimilate movement from the sieve tubes into sink tissues is often presumed to occur by simple diffusion along concentration gradients (Patrick and Offler, 2001). Developing seeds can import assimilates immediately after release from the maternal tissue (Tegeder and Rentsch, 2010; Weber et al., 1997; Weber et al., 2005; Zhang et al., 2007). Transport of amino acids to the developing seed plays a significant role for proper seed development and biosynthesis of storage compounds (e.g. oil and protein) (Barneix, 2007; Tegeder and Rentsch, 2010; Thorne, 1985), and finally results in successful seed production

(Gifford et al., 1984; Pate et al., 1980; Patrick and Stoddard, 2010). Therefore, facilitating the import of amino acids into developing seeds might be a vital approach to improve seed yield and quality in field crops.

Recently, physiological and biochemical consequences of increased seed sink strength through transporter genes expression have been reported in cultivated crops. Expressing a *Vicia faba* amino acid permease (*VfAAP1)* in *Vicia narbonensis* and *Pisum sativum* under the control of a storage parenchyma specific promoter resulted in increased seed size and seed storage protein content (Rolletschek et al., 2005; Weigelt et al., 2008). Tan et al. (2010), documented an increased seed number and biomass as a result of yeast S-Methylmethionine Permease1 (*MMP1*) expression in pea plants under the control of the *AtAAP1* promoter, which directs expression throughout the phloem and developing seed. Moreover, ectopic expression of a barley sucrose transporter (*HvSUT1*) in wheat increased sucrose uptake capacity, stimulated storage protein synthesis (Weichert et al., 2010), and increased grain size and yield (Saalbach et al., 2014). Similarly, phloem-specific expression of Arabidopsis sucrose transporter (*AtSUC2)* in rice increased grain size and yield (Wang et al., 2015). However so far, it has not been proved whether overexpression of amino acid transporters increases grain yield and quality in wheat. Therefore, in this study, the yield potential of novel transgenic winter wheat lines which express the *Vicia faba* amino acid permease 1 *(VfAAP1*) under the control of the wheat high molecular weight glutenin (HMW-Glu) 1Ax1 promoter is evaluated. The aim of this study is to assess the potential of improving amino acid uptake capacity of developing grains on yield and quality of wheat grains.

3.2 Material and methods

3.2.1 Cultivation of transgenic lines

In this study, transgenic winter wheat lines developed by overexpressing *VfAAP1* in the winter wheat variety Certo under the control of HMW-Glu 1Ax1 promoter were evaluated (refer chapter 2 section 2.1). Three homozygous marker gene free lines were assessed, two of the lines carry one copy of the transgene at different genomic loci (BB, CC) and the third line (BBCC) contains two copies of the transgene (combining the two transgenic loci). All the three homozygous marker gene free lines of the T5 generation together with its non-transformed control Certo were cultivated over two consecutive growing seasons (2012/13 and 2013/2014 cropping season). The lines were grown in small greenhouses (6.1m x 3.4m) in soil beds without regulating temperature, humidity, and light. Cultivation was done in randomized mini-plots of $0.5m^2$ with a planting density of 200 plants per plot. In each small greenhouse, 16 plots were planted from October (sowing) to August (harvest) at the IPK campus, Gatersleben, Germany. Grains were harvested separately from the 12 middle plots per greenhouse for analysis of yield and yield related parameters. In each growing season, eight plots per line harvested from two small greenhouses were combined for statistical analysis.

To evaluate the 1Ax1 promoter activity two independent lines of the T3 generation of 1Ax1::GFP plants (refer chapter 2, section 2.2) together with the non-transformed control Certo were cultivated under controlled conditions. Seeds were germinated in trays with substrate mix (Klasmann-Deilmann GmbH, Germany), in the greenhouse (20°C) for two weeks, followed by eight weeks of incubation in vernalization chambers (4°C/4°C, 8-h/16-h day/night regime). Plantlets were transferred to 2.5-L pots containing cultivation substrate (Klasmann-Deilmann GmbH) and grown in a greenhouse with 18°C/14°C, 16-h/8-h day/night regime (SON-T-Agro 400 high-pressure sodium bulbs with 25,000 lux). Fertilization of greenhouse plants was conducted as described by Hensel et al. (2009).

3.2.2 Determination of grain biomass accumulation

Biomass accumulation of developing XAP grains was evaluated under field-like conditions. All lines were cultivated in 2013/14 and 2014/2015 cropping seasons under semi-controlled conditions (refer section 3.2.1). Biomass of developing grains was recorded in 4 days interval starting from 4 Days After Flowering (DAF). From each plot, 120 grains were harvested from 15 spikes at every developing stage, and biomass (fresh and dry weight) accumulation was measured accordingly, grain water content was calculated based on wet base calculation, i.e.,

Water content (%) = $\left(\frac{Fresh\ biomass - dry\ biomass}{Fresh\ biomass}\right)$ x 100.

In total, 6 plots per line were combined for statistical analyses.

3.2.3 Amino acid determination

For free amino acid determination developing grains harvested from plants grown under field-like conditions during 2013/14 cropping season was used. The free amino acids level of developing grains at four developing stages (4, 8, 10 and 14 DAF) was evaluated. At two stages (4 and 14 DAF) whole developing grains were used for analysis. Since, pericarp and endosperm have almost equal contribution to the grain volume at the transition phase (Pielot et al., 2015; Weschke et al., 2000), the free amino acids were measured in endosperm and pericarp separately at 8 and 10 DAF. To extract free amino acids 20mg of powdered sample was incubated with 80% ethanol for 60 min at 60^{o}C under constant agitation; internal standard of 150nmol was added to each sample. Probes were centrifuged for 15 min, at 10 000g and 4^{o}C, 10µl of each extract were taken for derivatization. Derivation was performed using the Waters AccQ-Tag derivatization kit (Waters Corporation, USA) according to the manufacturer's instruction. Amino acid derivatives were determined by Acquity UPLC system for amino acid analysis from Waters, using column AccQ-Tag Ultra RP 1.7µm particles. Mobile phase A corresponded to AccQ-Tag Ultra Eluent A and mobile phase B to AccQ-Tag Ultra Eluent B both from Waters. Amino acid derivatives were detected at UV@260nm with 700µl/min flow rate. Standards used for calibration were the amino acid standard solutions from Waters®, to which asparagine (132.12g/mol), glutamine (146.15g/mol), tryptophan (204.23g/mol), GABA

(103.12g/mol) and norvaline (117.15g/mol) were added fresh according to the manufacturer's recommendation. Data acquisition and chromatogram analysis were performed with Waters EMPOWER 2 software.

3.2.4 Analysis of grain morphology and grain composition

Morphology of mature grains harvested from the three XAP lines together with its non-transformed control (Certo) was analyzed. Grains harvested from plants grown in mini plots in two consecutive growing seasons (section 3.2.1) were evaluated. From each plot three randomized samples (20-30g/sample) of grains were taken and scanned by using the digital seed analyzer MARVIN (GAT Sensoric GmbH, Neubrandenburg, Germany). Thousand grain weight (TGW), width, length and area of mature grains were determined.

The composition of mature grains harvested from two growing seasons (the same that were used for the yield parameter analyses) was evaluated. In total, grains harvested from 16 plots per line were assessed. In each line, grains harvested from 4 plots (from the same small greenhouse) were mixed. From each mixed pool ten random samples with 5g/sample were taken and ground. Sucrose and starch content of dried flour were determined using a coupled enzymatic assay as described by Rolletschek et al. (2002). Relative contents of total carbon and nitrogen were measured using an elemental analyzer 2 (Vario EL; Elementar analysensysteme (http://www.elementar.de) and total protein content was calculated from percentages of total nitrogen by multiplying with a conversion factor of 5.7 for wheat grains (Sosulski and Imafidon, 1990). Major protein fractions albumin/globulin and gliadin were sequentially extracted as described by Wieser et al. (1998). Extracted wheat protein fractions were quantified using the colorimetric Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific) according to the product protocol. Bovine serum albumin and gliadin from wheat (G3375; Sigma-Aldrich) were used as standards.

Amino acid composition of total protein lysate of mature grains was determined in grains of the XAP lines along with the non-transformed control Certo. Grain samples used for total protein analysis were used. 10mg grounded sample was weighed in 2ml Sarstedt-Microtube, and 1.5ml of 6M HCL was added to each sample. The Microtube was vacuated and concealed with Argon. To hydrolyze the samples, the sealed tubes were incubated at 100°C for 24h. To filter the hydrolysates, probes were centrifuged for 15min, at 10 000g and 22^{o}C; 50µL hydrolysate sample was dissolved with 200µl of 1.5M NaOH, and 10µl of each extract was taken for derivatization. Derivation was performed using the Waters AccQ-Tag derivatization kit (Waters Corporation, USA) according to the manufacturer's instruction. Amino acid derivatives were determined by Acquity UPLC system for amino acid analysis from Waters, using column AccQ-Tag Ultra RP 1.7µm particles. Mobile phase A corresponded to AccQ-Tag Ultra Eluent A and mobile phase B to AccQ-Tag Ultra Eluent B both from Waters. Amino acid derivatives were detected at

UV@260nm with 700µl/min flow rate. Standards used for calibration were the amino acid standard solutions from Waters®, to which asparagine (132.12g/mol), glutamine (146.15g/mol), tryptophan (204.23g/mol), and norvaline (117.15g/mol) were added fresh according to the manufacturer's recommendation. Data acquisition and chromatogram analysis were performed with Waters EMPOWER 2 software.

Macro and micro elements were determined in mature grains of the XAP lines along with the non-transformed control Certo. Grains of three independent XAP lines (BB, CC, and BBCC) harvested from two consecutive seasons (the same that were used for the yield parameter analyses) were used for evaluation of nutrients accumulated in the grains. For each line, grains harvested from 4 plots (from the same small greenhouse) were mixed. From each mixed pool, eight random samples with 15g/sample were taken and ground by vibratory disc mill RS 200 (Retsch Miller with non-metal grinding chamber) (Retsch GmbH, Haan, Germany). The grounded samples were dried overnight at 60°C. The dried samples were weighed (40-50mg) into polytetrafluoroethylene (PTFE) digestion tubes and digested in HNO_3 under pressure using a microwave digester (Ultraclave 4; MLS GmbH). The concentrations of macro- and microelements were determined by optical emission spectrometers with inductively coupled plasma (ICP-OES) using an iCAP 6500 Dual OES Spectrometer (Thermo Fischer Scientific).

3.2.5 Assessment of vegetative parameters

The vegetative phenotype of XAP wheat lines was evaluated. The lines were grown under semi-controlled conditions in small greenhouses (refer section 3.2.1) over two consecutive cropping seasons along with the non-transformed control Certo. All lines were cultivated in 2013/14 and 2014/2015 cropping seasons. A total of 6 plots per line was evaluated, each plot had a size of $0.5m^2$ and a planting density of 200 plants per plot. Chlorophyll content of leaves was measured at three different growing stages (seedling, tillering and flowering) by using Soil-Plant Analysis Development (SPAD) 502meter (Spectrum Technologies, Inc. Plainfield, USA). In each plot, 40 plants were randomly analyzed, and nine measurements per plant were taken from 3 different leaves at each stage. The flowering date was recorded (yellow anthers of the second spikelet) at the middle part of the spike. In each plot, 60 plants were randomly labeled; the flowering date was recorded. The flowering rate over the entire flowering period was estimated based on sampled plants per plot. It is calculated based on the number of flowered plants per plot on respective days:

$$\text{Flowering rate (\%)} = \left(\frac{Number\ of\ total\ flowered\ plant\ per\ plot\ on\ respective\ date}{number\ of\ sampled\ plants\ (60)}\right)x100.$$

3.2.6 Sample preparation and RNA isolation

Developing grains and vegetative tissues were collected from the XAP lines and Certo. After sampling, all samples were immediately frozen in liquid nitrogen. Total RNA was isolated using

phenol-chloroform extraction as described by Heim et al. (1993) and treated with Turbo DNase I (Invitrogen). For tissue-specific qRT-PCR, the respective tissue was dissected from developing grains by using Laser Microdissection and Pressure Catapulting (LMPC) (Thiel et al., 2008). Total RNA was extracted from microdissected grain tissue using Absolutely RNA Nanoprep Kit (Agilent, catalog No 400753) according to manufacturer's protocol. First-strand cDNA was synthesized with oligo (dT) primer and Superscript™ III (Invitrogen, Life Technologies, Darmstadt, Germany).

3.2.7 Quantitative Real-Time PCR (qRT-PCR)

The ABI 7900HT Fast Real-Time PCR system (Applied Biosystems, Foster City, USA) was utilized for the qRT-PCR analysis. For each reaction 2µl of each primer (200pmol), as well as 1µl cDNA and 5µl SYBR® Green PCR Master Mix (Applied Biosystems, Warrington, UK) was used. The primers were designed with Primer Express® software (Applied Biosystems, Foster City, USA) and are listed in the Appendix I. Five technical replications were performed for each sample. The measured dissociation curves confirmed the presence of one certain amplicon in each reaction. Expression levels were computed with the data set derived from the ABI 7900HT Fast Real-Time PCR system. The cycle threshold (Ct) values were calculated by SDS2.2.1 software, PCR efficiency (E) was determined by using LinReg PCR (Version 7.5). The expression value was computed by E and ΔCt (value of ΔCt was calculated by subtracting the Ct value of the target gene from Ct value of the reference gene). Wheat *actin* (accession no. AB181991) was used as reference gene.

3.2.8 Detection of GFP fluorescence

Transverse median sections of developing grains were prepared from a homozygous 1Ax1::GFP line and Certo grains using Leica VT1000 S vibrating blade microtome (Leica Biosystems, Wetzlar, Germany) (section size 70-100µm). The sections were mounted on glass slides for fluorescence microscopy analysis. Fluorescence microscopy was carried out using a LSM 780 confocal laser scanning microscope (Carl Zeiss, Jena, Germany). To analyzed GFP fluorescence, a 488 nm laser line in combination with 505–530 nm band pass filter was used. The quality of the GFP signal was confirmed by lambda stack analysis. The autofluorescence was captured by 364 nm laser line in combination with 385 nm long pass filter. The detection of promoter-directed expression of GFP in transgenic tissue was evaluated by comparing to the corresponding tissues of the non-transformed control.

3.2.9 Statistics

All collected data were subject to statistical analysis; it was assessed by analysis of variance or student's t-test, using Sigma Stat software (3.5 version) (Jandel Scientific, Erkrath, Germany).

3.3 Results

3.3.1 Grain development

3.3.1.1 Biomass accumulation

Fresh and dry weight accumulation of developing XAP grains were evaluated in comparison to the non-transformed control (Certo); grain water content was calculated based on wet base calculation (refer section 3.2.2). XAP lines show a significant increment in dry grain biomass between 14 and 30 DAF. During this development phase, in average 25-30% dry grain biomass increments were observed for XAP lines (Fig. 4). At these developmental stages, the difference between XAP lines and Certo in dry grain biomass is statistically different. At early stages (4-10 DAF) grain dry biomass was not significantly different between XAP lines and the non-transformed control Certo. Among the XAP lines, CC has shown the tendency of increment in dry grain mass accumulation during the entire period of grain filling (14 to 30 DAF). At later stages (26 and 30 DAF), the difference between the two XAP lines BB and CC was statistically significant in grain dry biomass. However, the difference between line CC and BBCC of the respective parameters was not stastically significant at all stages. Similarly, the difference beteewn BB and BBCC in grain dry biomass was not statistically different at all stage (Fig. 4A). Moreover, the result confirmed that XAP lines have a significant increment in grain water content at 10 and 14 DAF when compared to Certo. In XAP lines, grain moisture content accounted on average, 70% and 66% of grain biomass at 10 and 14 DAF, respectively, whereas the grain moisture content was 65% and 61% of the biomass in Certo at 10 DAF and 14 DAF, respectively (Fig. 4B). At these two developmental stages, the difference between XAP lines and Certo in grain water content is statistically significant. In summary, the data confirmed that XAP grains accumulate higher grain dry mass during grain filling when compared to the non-transformed control Certo. In addition, a significant increase in XAP grain water content is evident during the transition phase and beginning of grain filling (10 and 14 DAF).

3.3.1.2 Amino acid pools in developing XAP grains

Developing grains grown under field-like conditions were harvested at four developmental stages (4, 8, 10 and 14 DAF). At two stages (4 and 14 DAF) whole developing grains were used for analysis. At the transition phase (8 and 10 DAF), the free amino acids of endosperm and pericarp were measured separately. XAP line CC has shown the highest increment in grain dry mass accumulation during the entire period of grain filling when compared to the other XAP lines (BB and BBCC) (refer section 3.3.1.1). qRT-PCR analysis indicates that among the XAP lines, CC has highest *VfAAP1* expression level in developing grains (refer section 3.3.3.1). Based on these facts line CC was selected for free amino acid pools analysis. Results across grain development are separated into two sets of data, which reflect the level of free amino acids in pericarp (4, 8 and 10 DAF) and endosperm (8, 10 and 14 DAF). Among the 20 measured amino acids, the level of 15 amino acids shows a significant difference between XAP and Certo. From

significantly altered amino acids, 10 and 9 correspond to pericarp and endosperm, respectively. Accordingly, endosperm and pericarp share common set of 4 amino acids (Glu, Met, Thr, and Ser), which significantly increased their level in XAP grains. At the pre-storage phase (4-8 DAF) the level of four amino acids (Arg, Asp, Gln, and Glu) showed a significant increment in XAP pericarp. In a similar manner, the level of two amino acids (Ile and Val) shows a significant increment in XAP pericarp during the later stage (8-10 DAF). The level of three amino acids (Met, Thr, and Ser) was significantely increased in XAP pericarp at all stages. However, one amino acid (His) shows a significant decrement in the pre-storage phase. Accordingly, at transition phase (8-10 DAF) free levels of six amino acids (Ala, Gly, Leu, Trp, Glu and Tyr) were significantly increased in XAP endosperm. In addition, the levels of three amino acids (Met, Thr, and Ser) were significantly higher in the XAP endosperm at all stages (Fig. 5). However, there were no significant changes in the level of five amino acids (Asn, Lys, Phe, Pro, and GABA) at all stages in both groups.

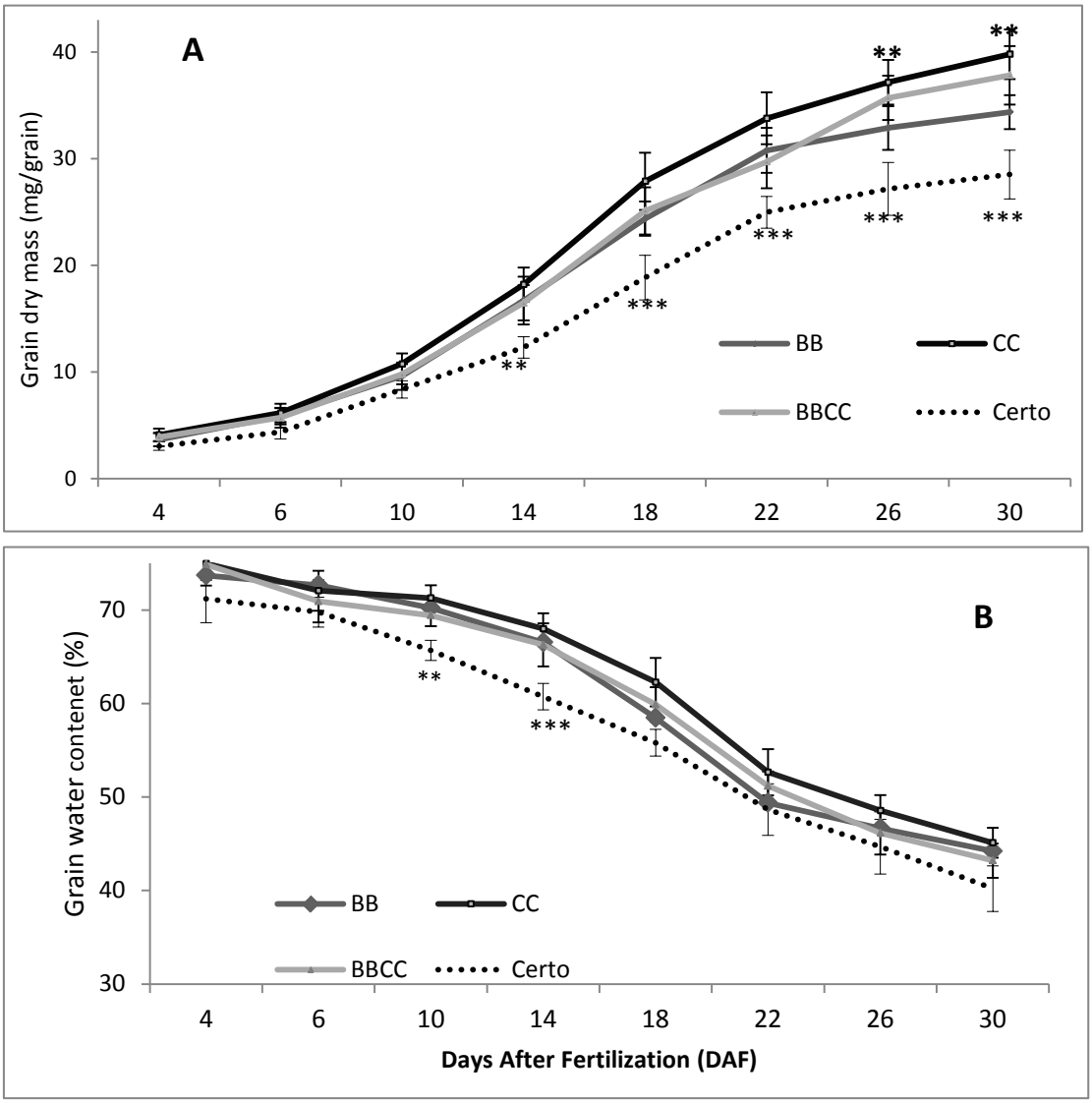


Fig. 4 Biomass accumulation and grain water content in developing XAP grains.

For each developmental stage, 40 grains per sample from 5 spikes were harvested. Data represent the mean of 18 samples grown over two consecutive growing seasons. A. Grain dry weight accumulation. During storage and late maturity phases (14-30 DAF) grain dry weight is significantly increased in all XAP lines (BB, CC, and BBCC) when compared to the non-transformed control Certo according to t-test (*** P≤0.001, ** P≤ 0.01, *P ≤0.05), and during the later stage (26-30 DAF), significant differences between two XAP lines (CC and BB) was registered according to t-test (**P≤ 0.01). B. Water content of developing grains during the entire period of grain growth, during early storage phase (10-14 DAF) grain moisture content was significantly increased in all XAP lines (BB, CC, and BBCC) when compared with the non-transformed control Certo according to t-test (*** P≤0.001, ** P≤ 0.01).

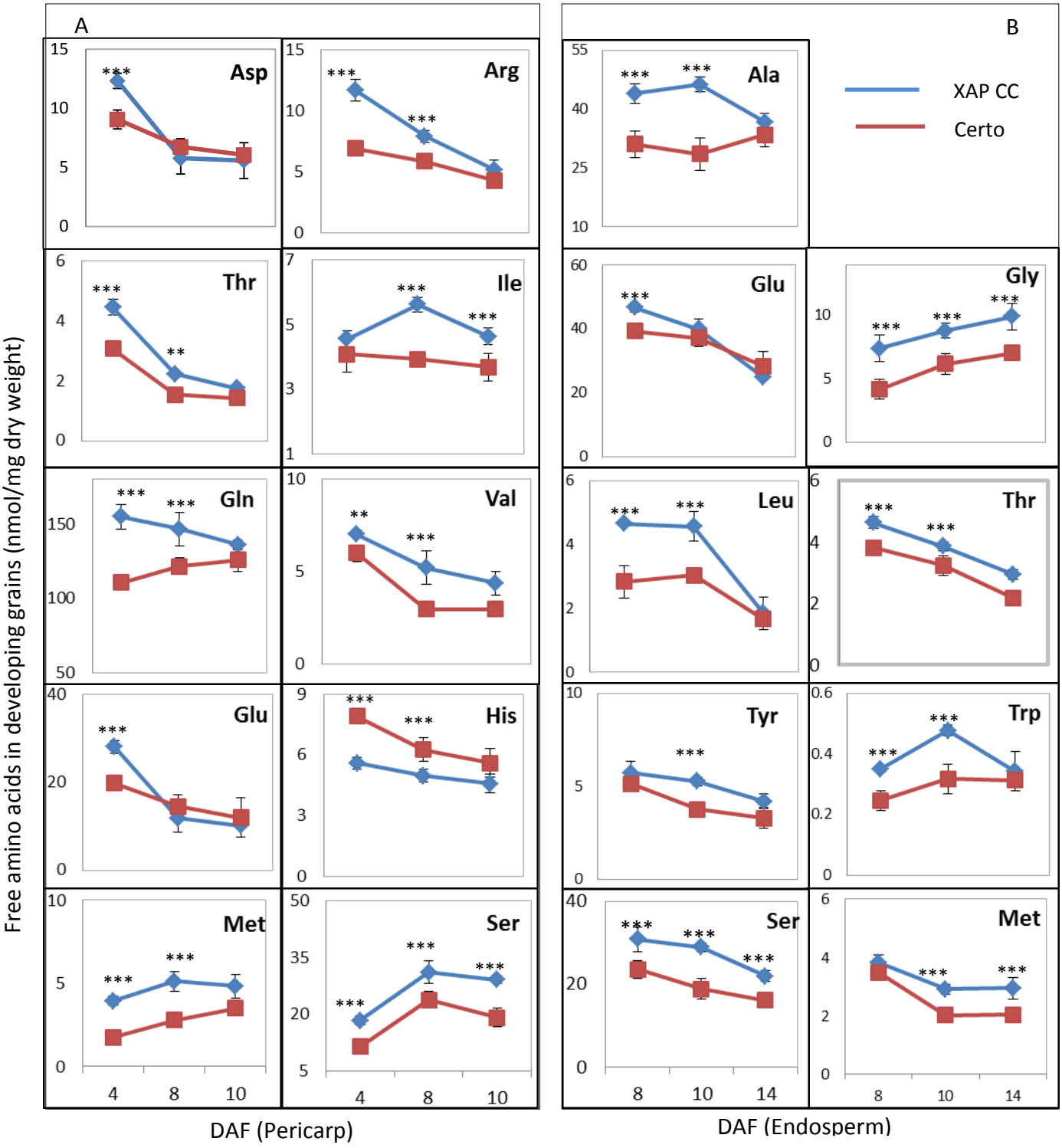


Fig. 5 Level of free amino acids in developing grains of line XAP (CC) and non-transformed control Certo.

A. Free amino acids level in the pericarp. B. Free amino acids level in the endosperm. Data are means from three replicates with the error bar of the standard deviation, significant statistical levels, according to t-test (*** P≤0.001, ** P≤ 0.01, *P ≤0.05).

3.3.2 Grain yield and grain composition

3.3.2.1 Yield-related parameters

The yield potential of the three XAP lines was evaluated under semi-controlled conditions. To analyze yield-related parameters, plants of the T5 generations of XAP lines together with the non-transformed control Certo were cultivated in the season 2012/13 and 2013/2014. A total of 16 plots per line were evaluated with each plot having 0.5m^2 with 200 plants resulting in 2.5cm distance between plants in rows. The yield parameters and the average increase for each transgenic line relative to the control Certo are shown in Table 1. On average, grain yield per transgenic plot was 125.7% of that of Certo (100%) and was significantly higher for all lines in the two seasons. All XAP lines showed a significant increase in thousand grain weight (TGW) when compared to Certo: on average 24.1% increment was registered for XAP lines in comparison to Certo (Table 1). All XAP lines showed a significant increment in nitrogen yield per plot in both seasons; XAP grains contained on average 22.1% more nitrogen per plot than Certo (Table 1). However, only line CC showed a statistically significant increment on the percentage of total grain nitrogen (Table 2). Number of spikes per plot and average grain number per spike did not show any significant difference between XAP lines and non-transformed control in both growing seasons (Table 1). However, there is a tendency to higher number of spikes per plot but lower number of grains per spike in all lines. In summary, expression of *VfAAP1* in transgenic winter wheat increased grain yield by 22-30% under field-like conditions. Additionally, XAP grains contained on average 22% more nitrogen per plot than Certo.

3.3.2.2 Grain morphology

Morphology of XAP grain harvested from two consecutive growing seasons under field like condition was assessed. Grain morphology was analyzed using the digital seed analyzer MARVIN 24. The average grain area was significantly increased by about 20% in all XAP lines in comparison to Certo (Fig. 6A). Furthermore, all XAP lines showed significantly increased grain width relative to Certo, whereas grain length was increased only for one line (CC) (Fig 6). In general, XAP lines have bigger grains and higher TGW than Certo (Table 2). In summary, the results indicate that yield improvement in XAP lines is mainly due to higher thousand grain weight. Moreover increasing of thousand grain weight of XAP lines is primarily due to increased grain width and a minor degree to higher grain length in CC line.

Table 1 Grain yield and yield related parameters of XAP lines in comparison to the non-transformed control Certo in two cropping seasons.

The data represent the data of the three independent XAP lines in comparison to the non-transformed control Certo (100%). Number of plots per cropping season was 8-12 for each line; values are the mean of harvested plots per line ± standard deviations. Significant differences between XAP lines and Certo according to student t-test (*** P≤0.001, ** P≤ 0.01, *P ≤0.05).

Line	BB		CC		BBCC	
Growing season	**2012/13**	**2013/14**	**2012/13**	**2013/14**	**2012/13**	**2013/14**
Grain yield /plot (%)	125.8±5.2***	121.5±3.15***	130.9±7.2***	126.5±5.3***	124.3±4.5***	122.7±4.35***
TGW (%)	120.7±4.5***	122.4±4.3***	129.9 ±5.7***	127.7±4.9***	122.6 ±4.0	121.5±4.3***
Total nitrogen (%)	97.1±9.5	98.2±3.5	103.2±1.3*	109.2±4.1*	98.2±8.6	97.8±5.1
Nitrogen yield/plot (%)	122.0±8.5***	118.2±9.1**	131.8±6.7***	122.7±6.0***	120.5±8.1***	119.7±9.4**
Spikes No /plot (%)	103.4±8.6	104.2±7.1	101.7±9.3	106.7±8.2	107.5±8.7	101.9±6.87
Grain No /spike (%)	99.7±6.2	96±8.1	98.2±5.2	97.4±7.4	96.1±8.5	97.7±5.8

Table 2 Grain area, grain length, width and thousand grain weight of XAP lines and Certo.

Grains harvested from two consecutive growing seasons were evaluated; number of plots per cropping season was 8-12 for each line, the data represent the mean of harvested plots per line ± standard deviations. Significant differences between XAP lines and Certo according to student t-test (*** P≤0.001, **P ≤ 0.01, *P ≤0.05).

Line	BB		CC		BBCC		Certo	
Growing season	**2012/13**	**2013/14**	**2012/13**	**2013/14**	**2012/13**	**2013/14**	**2012/13**	**2013/14**
Grain area (mm^2)	23.2±0.4***	19.2±0.5***	23.8±0.4***	20.7±0.7***	23.6±0.4***	19.2±0.4***	21.6±0.6	17.7±0.3
Grain length (mm)	7.1±0.87*	6.8±0.07	7.2±0.06**	6.9±0.09**	7.0±0.08	6.8±0.06	6.9±0.1	6.7±0.1
Grain width (mm)	4.1±0.08***	3.5±0.09***	4.1±0.06***	3.5±0.11***	4.1±0.7***	3.5±0.9***	3.74±0.1	3.2±0.05
TGW (g)	55.6+1.1***	54.2+1.9***	58.5+1.5***	56.8+2.2***	54.61+1.2	55.1+1.7***	45.78+1.6	44.9+1.6

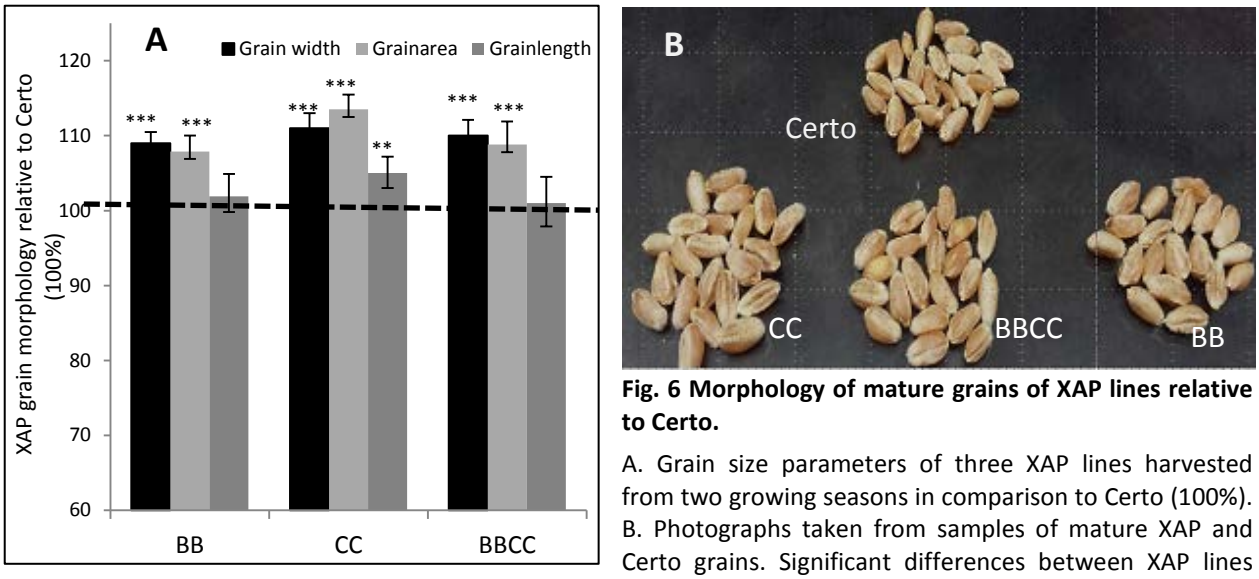


Fig. 6 Morphology of mature grains of XAP lines relative to Certo.

A. Grain size parameters of three XAP lines harvested from two growing seasons in comparison to Certo (100%). B. Photographs taken from samples of mature XAP and Certo grains. Significant differences between XAP lines and Certo according to student t-test (*** P≤0.001, **P ≤ 0.01, *P ≤0.05).

3.3.2.3 Grain composition of XAP lines

To analyze the impact of *VfAAP1* overexpression on grain composition, mature grains harvested from 16 plots per line over two growing seasons (used for analysis of the yield parameters in section 3.3.2.1) were evaluated. Among the three XAP lines, only CC showed a significant increase of total grain protein content when compared with the non-transformed control Certo (Table 3). In average 3-9% increment of total protein content was observed. The composition of main storage protein classes was analyze in mature XAP grains. Storage protein was extracted from mature grains and separated into the fractions albumins/globulins and the prolamins/gliadins. Grains from CC line showed a significant increase of gliadins (13-17%) under all growing seasons, while the increment of gliadin in line BBCC was significant only in the 2012/13 growing season. Gliadin content of line BB grains was unchanged in both seasons (Table 3). On the other hand, the difference among XAP lines in total proteins and gliadin was significant. In both growing seasons, a significant difference between the two XAP lines BB and CC was observed in total grain protein and gliadin content. However, the difference between CC and BBCC lines of the respective parameters was significant only in the second cropping season (2013/14). In contrast, XAP grains showed a tendency to slight decrement in starch content, though this reduction is statistically significant only in line CC when compared with the non-transformed control Certo (Table 3). Further, sucrose content was reduced in mature grains of all XAP lines in the 2013/14 growing season.

3.3.2.4 Micronutrients in grains of XAP lines

The composition of essential nutrients in mature XAP grains from two consecutive cropping seasons was measured in comparison to Certo. The grain content of the main micronutrients was significantly increased in XAP grains from both cropping seasons. In XAP grains dramatic increment in Fe (35-40%), Zn (20-25%) and Ni (23-33%) were registered when compared with the non-transformed control, while Mn contents increase to a relatively lower level (12-18%) (Fig. 7). However, on average 9-13% decrement of Mo was registered in XAP lines. In summary, the data suggest that ectopic expression of *VfAAP1* in winter wheat has a potential to change accumulation of essential nutrients in grains.

3.3.2.5 Amino acid composition of total protein lysate

Amino acids composition of total grain proteins in three independent XAP lines along with the non-transformed control Certo were evaluated in mature grains. Mature grains harvested from two growing seasons (which were used for the yield parameter analyzed in section 3.3.2.1) were used. Levels of the major lysate amino acids in matured grain of XAP and Certo were evaluated. The results indicate a significant increase of Cys (10-12%) and Leu (5-10%) which was observed in all three lines. Besides, a significant increment of the Pro and Glu content was registered in

CC grains (Fig. 8). Overall, the data confirmed that ectopic expression of *VfAAP1* is associated with the trend to increase the content of some primary amino acids in the protein lysate of mature grains. Among the major amino acids, the amount of Cys, Pro, and Glu/Gln showed a significant increment in XAP grains.

Table 3 Composition of mature grains of XAP lines as compared to Certo (100%).

The three independent XAP lines were grown in two cropping seasons, number of plots per cropping season was 8-12 for each line. Data represented as percentages of the non-transformed control variety Certo (100%). Values followed by the same letter in the respective growing season are not significantly different at P≤0.05 according to Student's t-*test*.

Growing season	**2012/13**				**2013/14**			
Line	**BB**	**CC**	**BBCC**	**Certo**	**BB**	**CC**	**BBCC**	**Certo**
Total protein (%)	97.24±7.9^{b}	104.5±2.9^{a}	100.3±3.5^{ab}	100^{b}	103.5±5.5^{b}	109.6+4.7^{a}	97.8±4.52^{b}	100^{b}
Gliadin (%)	104.5±8.2^{b}	117.5±5^{a}	109.3±4.3^{a}	100^{b}	99.3±3.1^{b}	114.8±8.5^{a}	105.82±7.4^{b}	100^{b}
Albumin (%)	104.2+6.4	99.5+6.3	102.6+6.3	100	101.2+5.2	98.7+5.2	99.2+3.69	100
Protein yield /plot (%)	116±6.2^{b}	131±5.5^{a}	119±7.2^{b}	100^{c}	122±5.7^{ab}	127±5.6^{a}	114±6.7^{b}	100^{c}
Starch content (%)	98.7±7.7^{a}	92.5±5.5^{b}	94.1±6.2^{a}	100^{a}	99.6±3.3	97.6±2.9	98.3±3.7	100
Sucrose content (%)	93.8±10.2	95.7±9.6	93.81±8.1	100	88.9±7.9^{b}	83.49+7.4^{b}	87.6+7.6^{b}	100^{a}

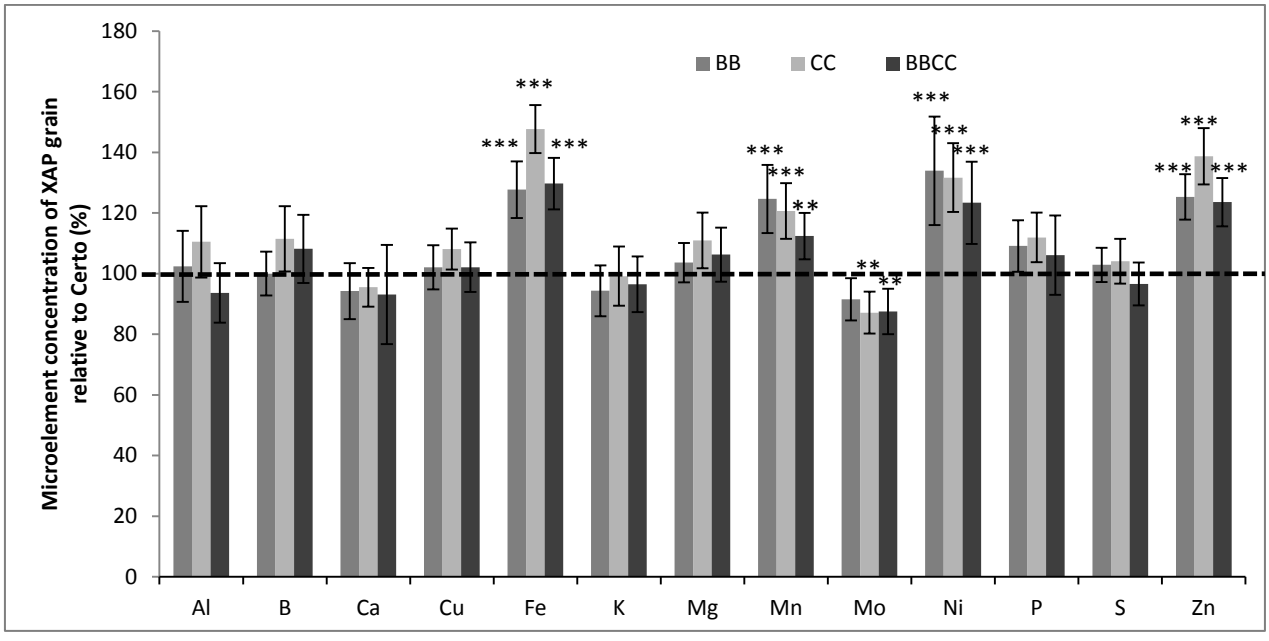


Fig. 7 Micronutrient composition of mature XAP grains in comparison to Certo.

Data are presented as percentages of the non-transformed control Certo (100%). Data from two consecutive cropping seasons (2012/13 and2013/14) was combined for statistical analyses. Significant differences between XAP lines and Certo according to student t-test (*** P≤0.001, ** P≤ 0.01, * P≤0.05).

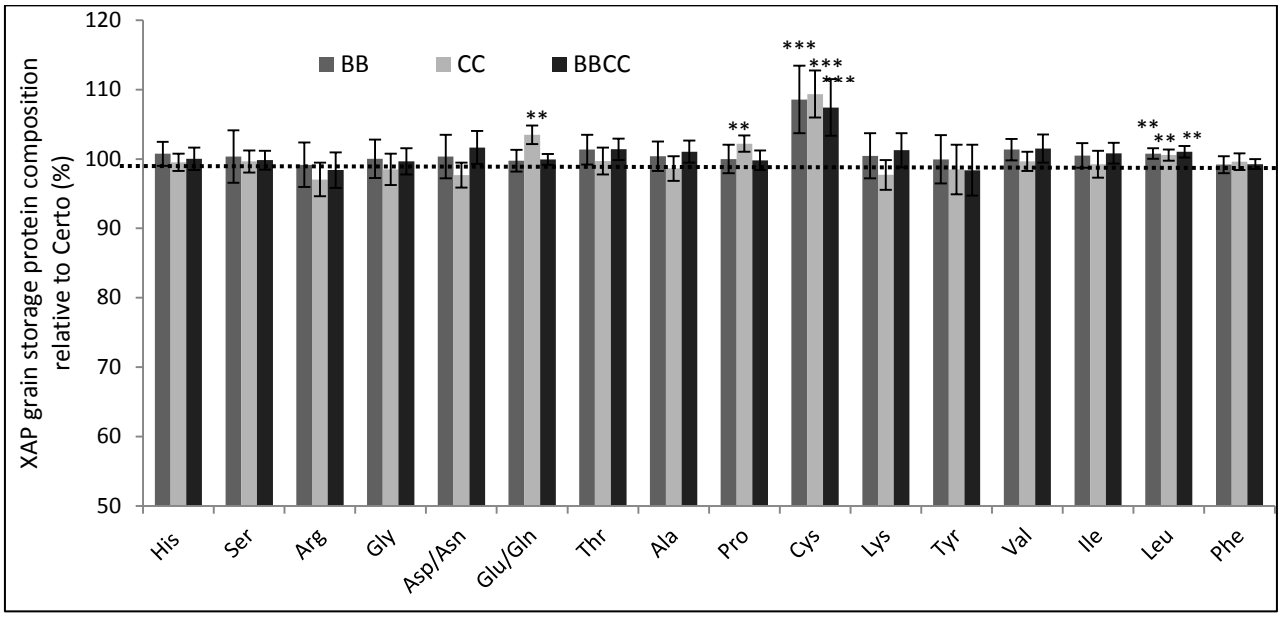


Fig. 8 Amino acids composition of the total protein lysate of mature XAP grains in comparison to Certo.

Data presented as percentages of the non-transgenic control variety Certo (100%). Harvest from two consecutive cropping seasons (2012/13 and2013/14) was combined for statistical analyses. Significant differences between XAP lines and Certo according to student t-test (*** P≤0.001, ** P≤ 0.01, *P≤0.05).

3.3.3 Vegetative parameter

3.3.3.1 Chlorophyll content of the leaves

The average SPAD value was significantly higher in XAP lines over all growing stages when compared to Certo. At the seedling stage, XAP lines showed on average 21% increment in SPAD value. At tillering and flowering, the average increments of SPAD values in XAP lines were 13% and 11%, respectively (Fig. 9). In summary, the transgenic wheat lines which ectopically express *VfAAP1* have shown a significant increment in leaf chlorophyll content under field like condition when compared to the non-transformed control across different stages of plant development.

3.3.3.2 Flowering time

The difference between XAP lines and Certo regarding flowering time plants grown under field-like conditions was evaluated. In each plot, 60 plants were randomly labeled, and the flowering date of the labelled plant was recorded (yellow anthers of the second spikelet) in the middle of the main spike. The flowering rate over the entire flowering period was estimated based on the number of flowering plants at the respective days. The two-year data indicate that on average XAP lines began flowering at 243 days after sowing, while the non-transformed control Certo starts flowering 5-7 days later. Over two cropping seasons under field-like conditions, XAP lines flower on average 5.5 days earlier than control Certo (Fig. 10).

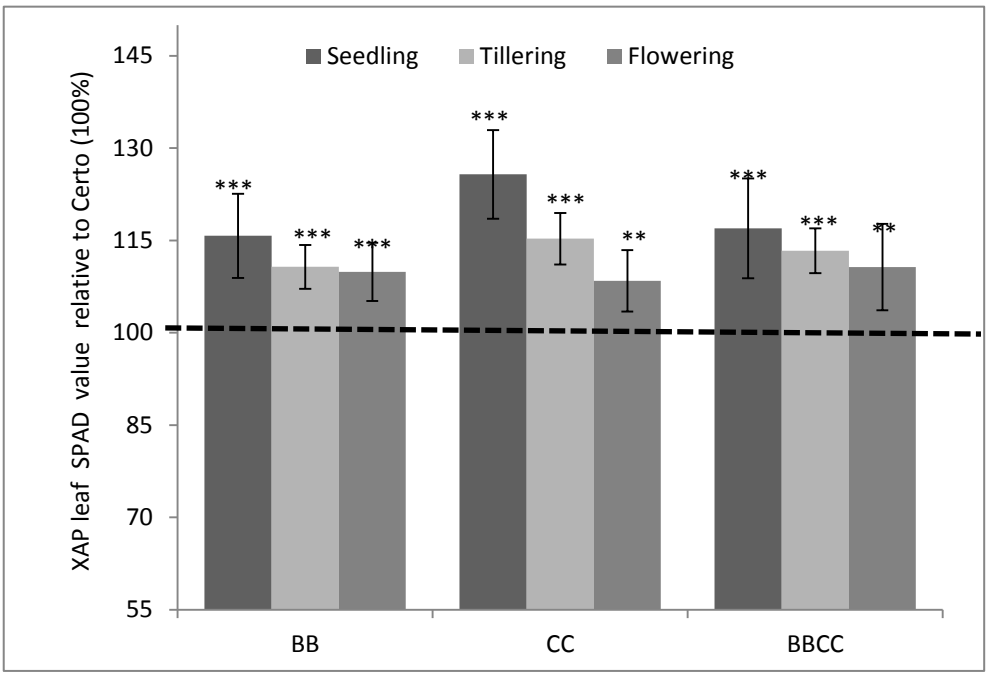


Fig. 9 SPAD measurement of XAP leaves in comparison to the non-transgenic control Certo

From each plot 40 plants were measured, across three developmental stages. At seedling stage and tillering the average SPAD values from the first two leaves per plant were measured, at flowering the SPAD values were taken from the flag leaf. Data represent the mean of 6 plots per line ± standard deviations. Significant differences between XAP lines and Certo according to t-test (*** P≤0.001, ** P≤ 0.01, *P≤0.05).

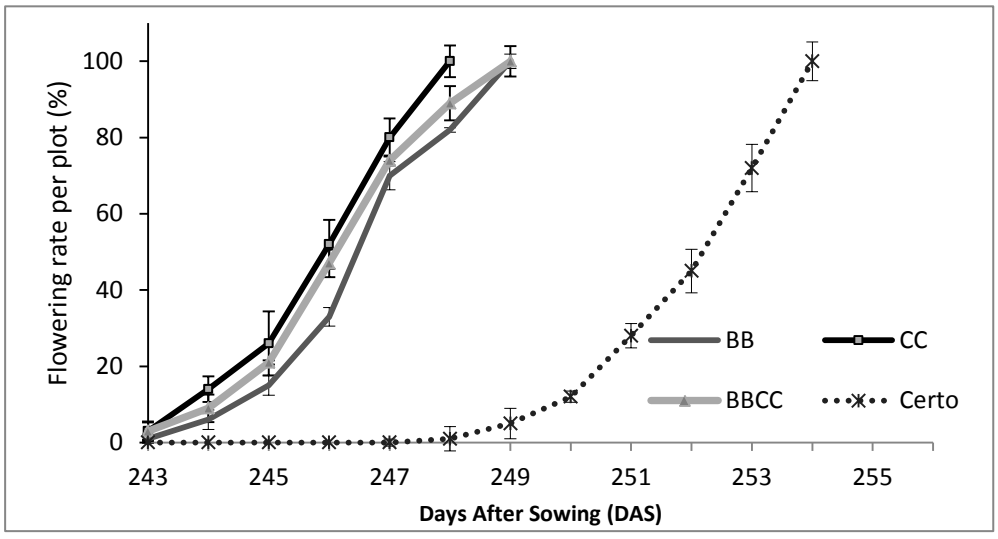


Fig. 10 Flowering time of XAP lines along with non-transgenic control (Certo).

In each plot, 60 plants were randomly sampled, and the first flowering date of the sampled plants was recorded. The flowering rate was estimated based on the number of flowering plants on the respective day relative to all labelled plants (100%=60 flowered plant per plot). The data represent the mean of 6 plots ± standard deviations.

3.3.4 XAP gene expression in developing grains

3.3.4.1 VfAAP1 expression in whole grains

To analyze the level of *VfAAP1* expression in developing grains of XAP lines quantitative real-time PCR (qRT-PCR) was performed. Total RNA was extracted from whole developing grains of the three XAP lines and Certo at five stages (4, 6, 8, 10 and 14 DAF), and the expression of *VfAAP1* was estimated by qRT-PCR. The result indicated that *VfAAP1* expression was detected as early as 4 DAF in developing grains. *VfAAP1* expression increased intensively from 8 DAF to 14 DAF in the three XAP lines, which reflects the activity profile of the HWM-Glu-1Ax1 promoter(Alvarez et al., 2000; Furtado et al., 2008) (Alvarez et al., 2000; Furtado et al., 2008). Among the three XAP lines, CC showed the highest *VfAAP1* expression at all stages. However, statistically, significant differences in *VfAAP1* expression were recorded among the XAP lines starting from 8 DAF (Fig. 11). As expected, the qRT-PCR did not show any signal for the non-transformed control Certo (negative control). In general, *VfAAP1* expression in XAP developing grains started in the pre-storage phase followed by a dramatic increment starting from 8 DAF. Moreover, the result of the qRT-PCR analysis suggests that expression of *VfAAP1* is influenced by position effects and/or correspondence between the transgene copies in XAP lines. Line XAP CC which carries one copy of the transgene has shown highest *VfAAP1* expression among the three lines.

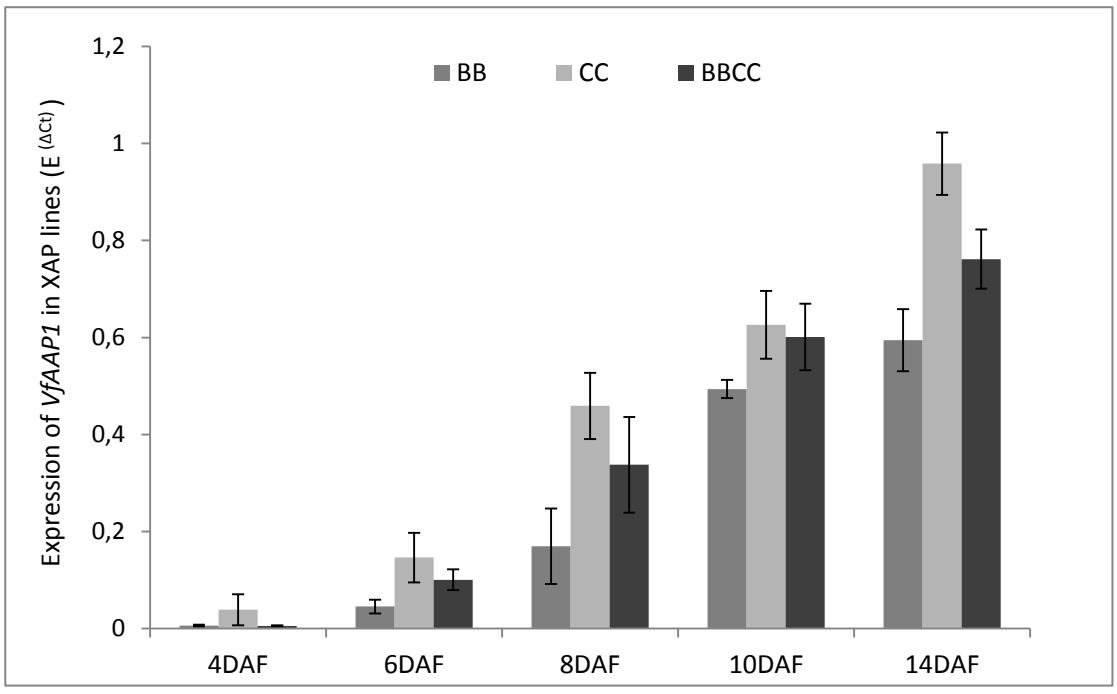


Fig. 11 *VfAAP1* expression in developing grains of XAP lines.

Total RNA was extracted from whole grains, *VfAAP1* expression in three independent XAP lines was evaluated by qRT-PCR, and values are means of three biological replicates ±standard deviation. Actin gene sequence was used as a reference. Significant difference among XAP lines according to student t-test (*** P≤0.001, ** P≤0.01, *P≤0.05).

3.3.3.2 1Ax1 promoter activity in developing grains of 1Ax1::GFP wheat line

To identify the *1Ax1* promoter activity in tissues of developing grains 1Ax1::GFP transgenic winter wheat was used. The 1Ax1::*GFP* transgenic line was established by expressing *GFP* in the winter wheat variety Certo under the control of the wheat HMW-Glu-1Ax1 promoter. Three homozygous lines with one copy of the transgene were established. At T3 generation the 1Ax1 promoter activity was evaluated. Cross sections of developing grains at five stages (4, 6, 8, 10 and 14 DAF) were assessed by using a confocal microscope; and the *GFP* expression has been evaluated by qRT-PCR. The result from confocal microscopy analysis indicates that GFP fluorescence was detected in developing grains, at 4 DAF in the nucellar projection and in the pericarp and as early as 6 DAF in the starchy endosperm. Among the endosperm tissues GFP signal was highest in endosperm wings; whereas in the prismatic endosperm, the GFP signal was relatively low across all stages (Fig. 12). Results from qRT-PCR confirm confocal microcopy. *GFP* was primarily expressed in developing grains starting from the pre-storage phase, the expression level increases at later stages; highest expression was found at 14 DAF (Fig. 13). In summary, qRT-PCR results and confocal microscopy analysis showed that the 1Ax1 wheat promoter activates gene expression in developing grains starting as early as 4 DAF in maternal tissues. A dramatic increment in gene expression starts from 8 DAF in the endosperm. Furthermore, GFP data revealed that the *1Ax1* promoter has highest activity in the endosperm wings.

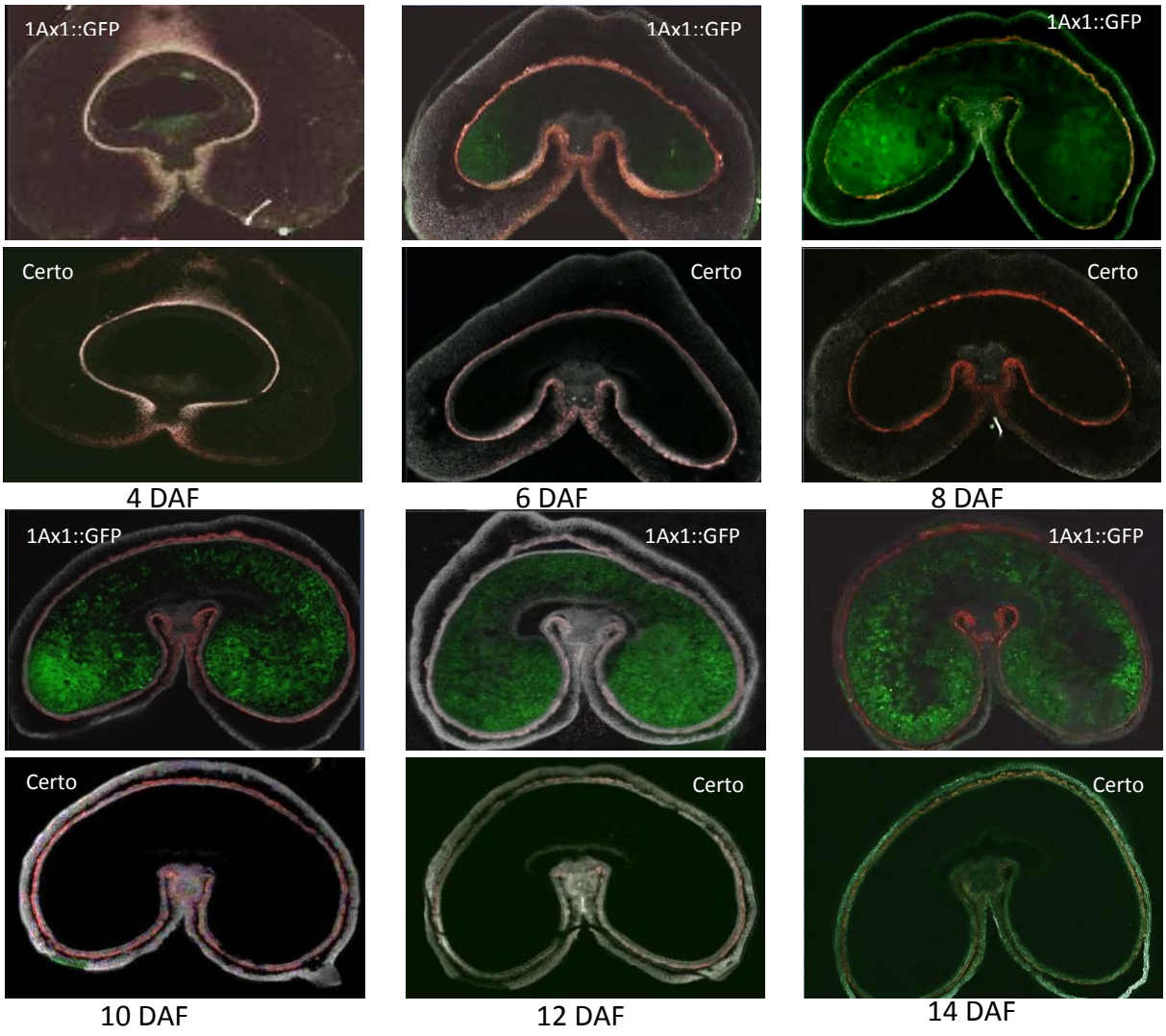


Fig. 12 GFP fluorescence in transverse sections of developing 1Ax1::*GFP* grains.

Homozygous transgenic 1Ax1::GFP T3 grains were analyzed using a confocal microscope to detect reporter gene activity by GFP fluorescence. Cross sections of developing grains at different developmental stages (4-14 DAF) are compared with the respective sections from non-transformed Certo grains (negative control).

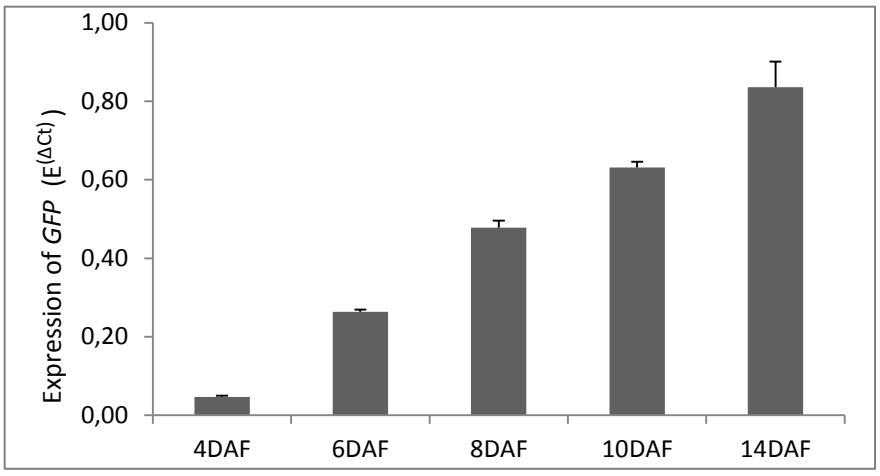


Fig. 13 GFP gene expression as estimated by qRT-PCR in grains of a homozygous 1Ax1::*GFP* line.

Total RNA was extracted from homozygous transgenic 1Ax1::*GFP* T3 grains (line253/8/2). Values are means of three biological replicates and actin gene sequences was used as a reference.

3.3.4.3 Tissue-specificity of VfAAP1 expression in developing XAP grains

Tissue-specific expression patterns of *VfAAP1* in developing grains of line CC were evaluated by qRT-PCR. Developing grain tissues (pericarp, nucellar projection, main vascular bundle, endosperm wings (outer part), endosperm wings (center) and prismatic endosperm were dissected at five developmental stages (4, 6, 8, 10, and 14 DAF) by laser coupled microdissection. From each tissue, the expression level of *VfAAP1* was evaluated by qRT-PCR using the same tissue dissected from Certo for negative control. Results, as presented here, reflect the temporal gradient of tissue formation during grain development. In the pre-storage phase (4 and 6 DAF) remarkable *VfAAP1* expression was detected in the nucellar projection, main vascular bundle, and pericarp. At 4 DAF, the highest expression of *VfAAP1* was registered in nucellar projection. At this stage, *VfAAP1* expression was not detectable in the endosperm. At 6 DAF expression of *VfAAP1* was detected in the endosperm wings (Fig. 14). At transition and beginning storage phases (8-14 DAF) the expression of *VfAAP1* increases in endosperm tissues. Among endosperm tissues, highest expression was observed in the outer part of the endosperm wings. In contrast, at the same phase, the expression level of *VfAAP1* was very limited in the prismatic endosperm and the center of the endosperm wings. In maternal tissues (nucellar projection, vascular bundles, and pericarp) *VfAAP1* expression was deteriorating at the storage phase (Fig. 14). In summary, expression of *VfAAP1* differs between maternal tissues and starchy endosperm in XAP wheat. The per-storage phase is characterized by relatively high *VfAAP1* expression in maternal tissues (nucellar projection, vascular bundles, and pericarp) the expression is specifically high at 4 DAF in the nucellar projection. At this developmental stage expression in the starchy endosperm was negligible. In the storage phase *VfAAP1* expression reached maximum level in endosperm tissues, especially in the endosperm wings whereas *VfAAP1* expression is barely detectable in maternal tissues.

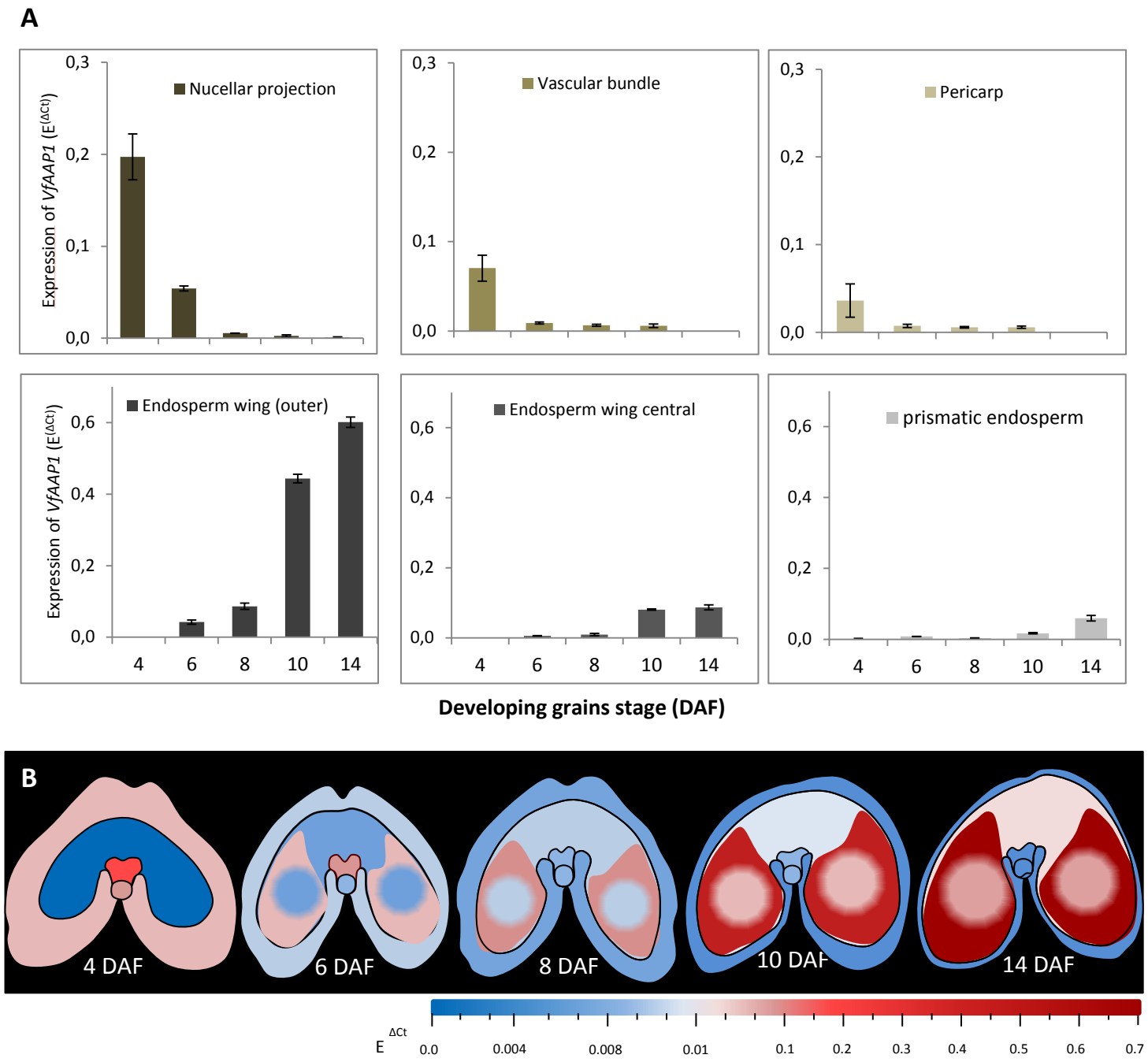


Fig. 14 Pattern of *VfAAP1* expression in developing grains of XAP line CC.

A. The result of quantitative real-time PCR analysis of *VfAAP1* expression in different parts of developing XAP grains selected by microdissection. Values are means of three biological replicates; actin gene sequence was used as a reference. B. Schematic visualization of qRT-PCR results on artificial grain sections. The color code indicates $E^{(\Delta Ct)}$value ≤ 0.009 (blue, low expression) ≥0.05 (red high expression).

3.3.4.4 VfAAP1 expression in XAP vegetative tissue

VfAAP1 expression in vegetative tissues (flag leaf, glumes and stem) of XAP wheat was evaluated during grain development. Total RNA was extracted from the respective vegetative tissues of line CC at five stages (0, 4, 8, 10, 14 DAF) and *VfAAP1* expression was evaluated by qRT-PCR. *VfAAP1* expression was detected in the flag leaf and glumes starting from anthesis (0 DAF). However, the expression levels of *VfAAP1* in vegetative tissues were very low when compared with those in developing grains. Nevertheless, remarkable *VfAAP1* expression increment was observed in flag leaves and glumes during the storage phase of grain development (10-14 DAF) (Fig. 15). In stem tissue, *VfAAP1* signal was undetectable during grain development. On average, the expression of *VfAAP1* in vegetative tissues (leaves and glumes) of line XAP CC was approximately 2-6% as compared to whole developing grains (100%).

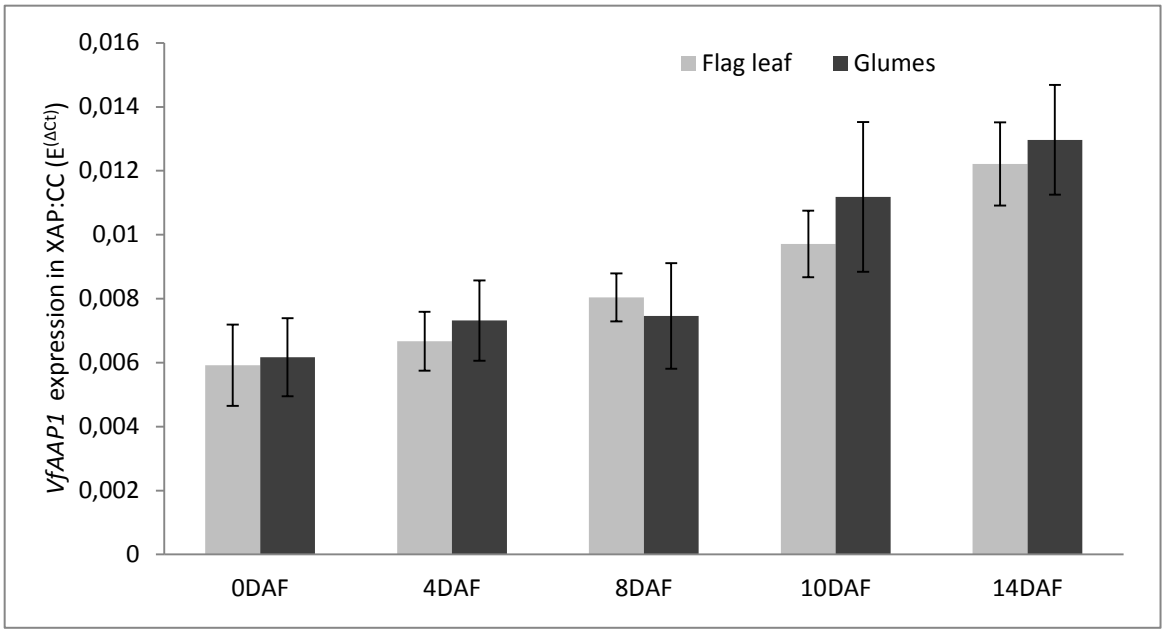


Fig. 15 Expression pattern of *VfAAP1* in flag leaf and glumes of line XAP CC during grain development.

Values are means of three biological replicates n=3±standard deviations. RNA was extracted from flag leaves and glumes, and the expression level of *VfAAP1* was evaluated by qRT-PCR; actin gene sequence was used as a reference.

3.4 Discussion

In wheat, grain number per unit area and individual grain weight are the two major grain yield components. However, so far grain yield improvement largely relates to grain number rather than grain weight (Fischer, 2007; Miralles and Slafer, 2007), as grain weight is less plastic and more heritable than grain number in cereals (Sadras and Slafer, 2012). Tradeoff relation between these two main grain yield components reported frequently. Understanding the determinants of grain weight potential and the mechanisms accounting for the tradeoff

between grain weight and grain number remains elusive in wheat and other grain crops (Gambin and Borras, 2010; Reynolds et al., 2012; Sadras, 2007; Slafer et al., 2015). Nevertheless, numerous studies indicate that the inverse relation between grain number and grain weight is mainly sink-limited (Acreche and Slafer, 2006; Borras et al., 2004; Miralles and Slafer, 1995; Slafer and Savin, 1994). Therefore, enhancing assimilate partitioning to developing grains and improving assimilate uptake capacity of developing grains might be at high priority for improving the individual grain weight and yield potential in wheat (Reynolds et al., 2011; Ruan et al., 2012).

In the present study, the yield potential of new transgenic winter wheat lines (XAP) over-expressing *VfAAP1* in the winter wheat variety Certo under the control of HMW-Glu-1Ax1 wheat promoter was analyzed. The result showed 25-30% grain yield increment in XAP lines under field-like conditions. The data revealed that XAP lines have on average 24.1% higher TGW when compared to the non-transformed control. However, grain number per spikes and spike number per plot did not show any significant difference between XAP lines and non-transformed control although spike number per plot showed a clear tendency to higher levels. All these results proof that the enhancement of grain yield in XAP lines is mainly due to an increase in grain weight. *VfAAP1* functions as an uptake system for amino acids at the level of the parenchyma storage cells of developing seeds in *Vicia faba* (Miranda et al., 2001). Recent studies reported increased seed sink strength through transporter genes expression and its positive impact on yield in cultivated crops. *VfAAP1* over-expression in *Vicia narbonensis* and pea plants showed increased amino acid uptake of developing seed resulting in higher individual dry seed mass (Rolletschek et al., 2005). Similarly, phloem and embryo-specific expression of *PsAAP1* in pea *(Pisum sativum*), improves plant biomass production and seed productivity (Zhang et al., 2015a). Furthermore, overexpressing barley sucrose transporter (*HvSUT1*) under barley hordein (HO) promoter in winter wheat ensures enhanced grain weight and yield (Saalbach et al., 2014; Weichert et al., 2010). In a similar manner, phloem-specific expression of the Arabidopsis sucrose transporter (*AtSUT2)* showed an increase in grain size and grain yield in rice (Wang et al., 2015). In summary, analysis of the XAP lines suggests that improving amino acid uptake capacity of developing grains could be a promising approach for grain yield enhancement through increasing individual grain weight. Analysis of the yield components in XAP lines confirmed that improving amino acid uptake capacity of developing grains in wheat can increase individual grain mass without remarkably affecting grain number.

Availability of nitrogen assimilates is one of the major factors which can change the kinetics of accumulation of dry mass in developing grains (Charmet et al., 2005; Oscarson, 2000; Singh and Jenner, 1984; Triboi et al., 2003). Unloading of assimilates from the vascular bundle into the grains represents a potential bottleneck for limiting assimilates in developing grains (Ruan et al., 2012). Subsequently, improving the capacity of transport activities might be a critical approach

facilitating assimilate availability in developing grains. In conclusion, XAP lines that express *VfAAP1* under the control of HMW-Glu-1Ax1 wheat promoter has shown a significant increment in the biomass of developing grains, which reflects the sink potential of developing grains. Grain biomass has direct relation to the growth rate of developing grains, which indicates the status of number and size of cells in developing grains (Dunstone and Evans; Gleadow et al., 1982; Saini and Westgate, 1999; Singh and Jenner, 1984). The free amino acid pool of XAP developing grains has shown significant increment for most amino acids. These results suggest proper functioning of the *VfAAP1* in XAP lines. In summary, the data suggest that improving amino acid uptake capacity of developing grains might facilitate the availability of N assimilate in developing grains, which might enhance the grain growth rate.

The study of transgene expression is necessary whenever transgenic plants are analysed. Transgene expression levels are influenced by many factors, in particular by the site of integration of the transgene within the plant genome, gene silencing, copy number and the strength of the attached promoter (Dean et al., 1988; Jones et al., 1985; Prols and Meyer, 1992). Based on this fact, the quantification and localization of XAP gene expression were evaluated. The result revealed that *VfAAP1* is mainly expressed in developing XAP grains, and the expression level among the three XAP lines differs significantly. The highest expression was observed in line CC whereas the lowest expression was observed in line BB. Both lines contain one copy of the transgene but at different loci. Therefore, the differences might be explained by the position effect, i.e., influences of the adjacent genomic DNA. The observation that *VfAAP1* expression differs between line CC and BB corresponds to previous reports on position-dependent differences in gene expression of transgenic petunia (Prols and Meyer, 1992) and transgenic tobacco (Dean et al., 1988; Jones et al., 1985). Some studies showed insignificant position effects on transgene expression in tobacco (Hobbs et al., 1990) and *Arabidopsis thaliana* (Schubert et al., 2004). Furthermore, the analysis of XAP lines showed no additive effect of transgene copy number on expression level: line BBCC contains two copy of the transgene by combining the CC and the BB locus. Nevertheless, the analysis showed that the expression level of *VfAAP1* is significantly higher in CC than BBCC. The result clearly indicates that copy number and gene expression are not linked to each other in XAP BBCC. In summary, the expression analysis suggests that the level of transgene expression in XAP lines might be influenced rather by position effect than copy number.

The HMW-Glu promoters have been widely used in wheat genetic manipulation. Many studies support the idea of endosperm-specificity of HMW-Glu promoters (Altpeter et al., 1996; Alvarez et al., 2000; Furtado et al., 2009; Lamacchia et al., 2001; Yao et al., 2006). In the present study the pattern of activity of HMW-Glu subunit 1Ax1 promoter was analyzed in *1Ax1::GFP* transgenic wheat and by tissue-specific qRT-PCR analysis of XAP wheat. The result of *GFP* expression revealed that HMW-Glu-1Ax1 promoter is active in developing grain tissue as early

as 4 DAF, however in the maternal nucellar projection, and in the endosperm from 6 DAF. Besides, the result clearly showed that 1Ax1 promoter activity dramatically increased during storage phase especially in the wings of the endosperm. qRT-PCR data for the expression pattern of *VfAAP1* in specific grain tissues of XAP wheat showed 1Ax1 promoter activity in maternal tissue (nucellar projection, pericarp, and main vascular bundle) during the pre-storage phase of grain development. At the beginning of storage phase, the expression increased dramatically in endosperm tissues specifically in the endosperm wings. In previous studies, the spatial and temporal activities of different promoters from HMW-Glu subunits were reported. Accordingly, in transgenic durum wheat, the HMW-Glu-1Dx5 promoter showed activity primary in the endosperm wings during mid-development, and distribute throughout the starchy endosperm in the later grain development stages (Lamacchia et al., 2001). Similarly, Thilmony et al. (2014) reported that 1Dy10 HMW-Glu promoter is active in the endosperm of transgenic *Brachypodium distachyon* as early as 6 DAF. However, HMW-Glu-1Ax1 promoter activity pattern is largely unknown; the result of the present study pointed out that in addition to the endosperm, HMW-Glu 1Ax1 promoter is active in maternal tissue especially in the nucellar projection at early grain development (4 DAF). qRT-PCR analysis of XAP wheat showed the slight activity of 1Ax1 promoter also in the flag leaf and glumes during grain development, and it is interesting to note that the *VfAAP1* expression level in XAP flag leaf and glumes displayed a tendency of increment during plant aging. Similarly, Furtado and his colleagues reported that HMW-Glu promoters were leaky in transgenic rice, and recorded *GFP* expression in the leaf and root tissue in addition to that in the endosperm (Furtado et al., 2008).

Chapter 4

Ectopic expression of the *V. faba* amino acid permease 1 (*VfAAP1*) alters seedling root architecture

4.1 Introduction

Root system architecture is a critical factor for nutrient and water uptake, anchorage, nutrient storage, and plant-microbe interactions. Roots are vital for crop plants, and it serves as the primary organ, which interfaces between the plant and various biotic and abiotic factors in the soil environment. Root systems have secondary functions, such as synthesis of growth regulators and storage (Fitter, 2002). The synthesis of organic substances is crucial for plant growth; by releasing of these organic substances from roots, the physical and biochemical characteristics of the soil can be modified in the rhizosphere (Grayston et al., 1997). Roots have a major role in sensing and responding to abiotic and biotic stresses through signaling pathways. In root signaling pathways phytohormones are the key players. ABA (abscisic acid), ethylene and auxin are well-known hormones which mediate root and shoot communication signals (Bano et al., 1993; Kazan, 2013; Krouk, 2016; Zhang et al., 2015b). Similarly, previous studies suggest that cytokinins and strigolactones as key signal molecules in root- shoot communication (Koltai, 2011; Kudo et al., 2010; Kumar et al., 2015; Matsumoto-Kitano et al., 2008). The seedling root system is essential for efficient, healthy and vigorous mature plant establishment and linked to mature plant traits such as growth rate and yield (Lynch, 1995; Lynch, 2007; Wasson et al., 2012).

Root architectural traits are important for the selection of wheat cultivars that are efficient in the acquisition of nutrients and water from soil (Ford et al., 2006). It is well known that the functions of absorption and support of the root system are an important guarantee for biological yield and grain yield of wheat (Lynch, 2007; Smith and De Smet, 2012). Plant breeders have largely ignored root architecture regarding potential yield increases in previous yield improvement programs. Root traits were not a major selection criterion as part of the crop development programs during green revolution era (Waines and Ehdaie, 2007), mainly due to the difficulty of observing and quantifying the traits in soil and the complexity and plasticity of root phenotypes (Waines and Ehdaie, 2007). It is believed that the introduction of reduced height *(Rht*) genes in wheat had an influence on root cell proliferation and root dry mass accumulation (Bai et al., 2013; Waines and Ehdaie, 2007; Wojciechowski et al., 2009). Miralles et al. (1997), reported the impact of dwarfing genes on total root length and root dry mass in spring wheat. Similarly, Bai et al. (2013) investigated that yield-related traits and root morphology in wheat seedlings has strong genetic relationships. These experiences suggest the necessity of exploring and understanding development and architecture of roots to improve agricultural land use efficiency and yield.

The spatial configuration of the root systems varies widely among plant species and cultivars, soil composition, and particularly on water and mineral nutrients availability (Malamy, 2005). Root traits are believed to be complex and controlled by many genes, each with a small genetic effect (Sharma et al., 2011) and often with the highest degree of interaction effects with environmental conditions (Cooper et al., 2009; de Dorlodot et al., 2007). The plant root system results from the coordinated control of endogenous genetic programs (regulating growth and organogenesis) and the action of abiotic and biotic environmental stimuli (Malamy, 2005). Consequently, the introduction of new alleles in the genome may influence the growth and development of roots in many aspects. In this study, the seedling root phenotype of new transgenic winter wheat lines was evaluated, which express *VfAAP1* under the control of the 1Ax1 wheat promoter (XAP lines).

4.2 Material and methods

4.2.1 *In-vitro* experiments

Three homozygous selectable marker gene free XAP lines, two of the lines carry one copy of the transgene at different genomic loci (BB, CC) and a third line (BBCC) containing two copies of the transgene which combines the two transgenic loci (refer Chapter 2), were evaluated. From all three lines homozygous grains of the T5 generation together with its non-transformed control Certo were sowed and grown in a controlled growth chamber (IPK campus, Gatersleben, Germany), the growth chamber was adjusted at 15/12^{o}C day/night temperature and 60–70% relative humidity with a photoperiod of 12h. All seedlings were grown in 45mm diameter plots of 20cm height. In each pot, five XAP and five non-transformed pre-germinated seeds (10 seeds per pot) were planted in vermiculite (Kakteen-Schwarz, Germany) and watered with nutrition solution (Appendix IIA) as described by Song et al. (2007). Each line was planted in 10 replicated pots. Seedling roots at the three leave stage were harvested, and root growth parameters were evaluated. Data from three independent experiments were combined for statistical analysis.

4.2.2 Analysis of traits of roots under field-like conditions

Seedling root phenotype of XAP wheat lines was evaluated under semi-controlled environmental conditions over two consecutive cropping seasons along with the non-transformed control Certo. The lines were cultivated in small greenhouses (6.1m x 3.4m) in soil beds without regulation of temperature, humidity, and lighting. Planting was done in randomized mini-plots of 0.5m^2 with 200 plants per plot at the IPK campus, Gatersleben, Germany. Seedling roots at three stages (one, two and three-leaf stage) were harvested, and root growth parameters were evaluated. From each plot, 60 seedlings were harvested, and root traits (seminal root length, the number of seminal roots per plant and root biomass) were assessed. Data from four plots per line were combined for statistical analyses.

4.2.3 qRT-PCR analysis

The expression of *VfAAP1* in XAP seedling roots was analyzed by qRT-PCR. Tissues from five different regions of the seedling root were collected from seedlings grown under *in vitro* conditions (refer section 4.2.1). Line CC was used for the expression analysis, and non-transformed Certo was a negative control. Total RNA was isolated using phenol-chloroform extraction as described by Heim et al. (1993) and treated with Turbo DNase I (Invitrogen). First-strand cDNA was synthesized with oligo (dT) primer and Superscript™ III (Invitrogen, Life Technologies, Darmstadt, Germany). ABI 7900HT Fast Real-Time PCR system (Applied Biosystems, Foster City, USA) was used for the qRT-PCR analysis. For each reaction 2µl of each primer (200pmol), 1µl cDNA and 5µl SYBR® Green PCR master mix (Applied Biosystems, Warrington, UK) were used. Five technical replications were applied for each sample. The measured dissociation curves confirmed the present of one amplicon in each reaction. The primers were designed with Primer Express® software (Applied Biosystems, Foster City, USA) and are listed in the Appendix (I). Expression levels were computed with the data set derived from the ABI 7900HT Fast Real-Time PCR system. The cycle threshold (Ct) values were calculated by SDS2.2.1. PCR efficiency (E) was determined by using LinReg PCR (Version 7.5). The expression value was computed by E and ΔCt (value of ΔCt was calculated by subtracting the Ct value of gene interest from the Ct value of the reference gene). The wheat actin gene (accession no. AB181991) was used as a reference.

4.2.4 Root branching

Root branching of XAP seedlings was characterized through Sholl analysis techniques. Seedlings of line XAP CC together with the non-transformed control were grown in controlled chambers as described in section 4.2.1. At the three-leaves stage, roots were harvested. The roots were carefully washed in tap water, and the top 20cm of each seedling root system was properly fixed on a glass plate. The image was taken from the fixed roots, and each image was analyzed with Sholl technique (collaboration with the Fraunhofer IFF Magdeburg, Germany). Images from 10 seedlings harvested from 10 different pots per line were analyzed (Appendix IIB).

4.2.5 Statistics

All collected data were subject to statistical analysis; it was assessed with student's t-test, using Sigma Stat software (3.5 version) (Jandel Scientific, Erkrath, Germany).

4.3 Results

4.3.1 Phenotype of XAP roots under *in-vitro* condition

Seedlings from three XAP lines were grown in a controlled growth chamber. Homozygous XAP lines at the T5 generation along with the non-transformed control Certo were sown in vermiculite and watered with nutrition solution. In total roots of 150 seedlings per line from three independent experiments were evaluated. Seminal root traits and root dry mass were

determined. All measured root traits showed significant differences between XAP lines and the non-transformed control Certo. Seminal root number per plant was significantly increased in XAP. On average, 6.7 seminal roots per plant were registered in XAP lines at the three-leaves stage, while the mean number of seminal roots per plant was 5.01 in Certo seedlings. In contrast, XAP seedlings had a significant decrease in seminal root length when compared with Certo. On average, the non-transformed control had 10.5cm longer seminal roots than the XAP lines. However, root dry mass of XAP seedlings was significantly higher than that of the non-transformed control, on average 20% higher root dry biomass was registered for XAP seedlings (Fig. 16). In summary, the results of root phenotyping under *in-vitro* condition showed that XAP lines had altered seedling root architecture when compared to the non-transformed control. XAP seedlings have a higher number of seminal roots per plant, reduced root length but higher root dry biomass than Certo.

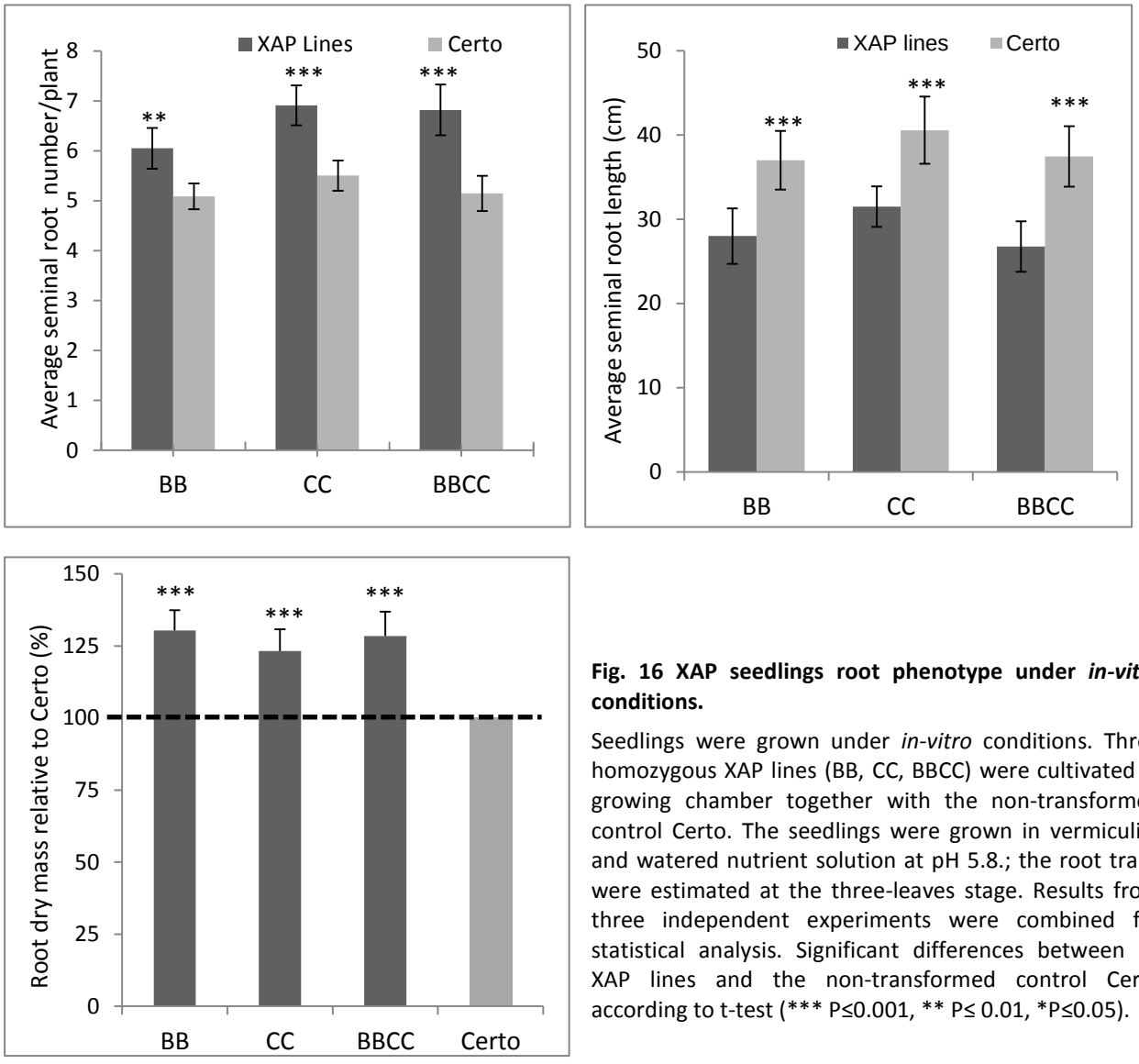


Fig. 16 XAP seedlings root phenotype under *in-vitro* conditions.

Seedlings were grown under *in-vitro* conditions. Three homozygous XAP lines (BB, CC, BBCC) were cultivated in growing chamber together with the non-transformed control Certo. The seedlings were grown in vermiculite and watered nutrient solution at pH 5.8.; the root traits were estimated at the three-leaves stage. Results from three independent experiments were combined for statistical analysis. Significant differences between all XAP lines and the non-transformed control Certo according to t-test (*** P≤0.001, ** P≤ 0.01, *P≤0.05).

4.3.2 XAP root phenotype under field like condition

The *in-vitro* experiments presented in section 4.3.1 clearly showed that XAP seedlings had a higher number of seminal roots per plant, shorter seminal roots and an increase in root dry mass accumulation in comparison to the control Certo. To evaluate root performance under field like conditions XAP seedlings were grown in the seasons of 2013/14 and 2014/2015 along with the non-transformed control Certo. A total of 160 seedlings per line was evaluated at the three-leaves stage. Under field-like conditions, seminal root number and root dry mass were significantly increased in all XAP lines when compared with the non-transformed control. The average number of seminal roots per plant was 4.85 and 3.51 in XAP and non-transformed control seedlings, respectively. In contrast at the same stage, seminal root length was significantly reduced in the XAP seedlings. The non-transformed control had 4.2cm longer roots than the XAP lines; however, this difference was more pronounced (10cm, 2.4 times higher) under *in-vitro* conditions. Root dry mass was significantly increased in XAP seedlings under field-like conditions. On average, 22–30% higher root dry mass was recorded in XAP seedling when compared to Certo (Fig. 17). However, differences in all seedling root traits were not significant at the one-leaf and the two-leaves stages. In summary, the result of the field-like experiment confirmed the differences in the root architecture between XAP seedlings and the non-transformed control at the three-leaves stage.

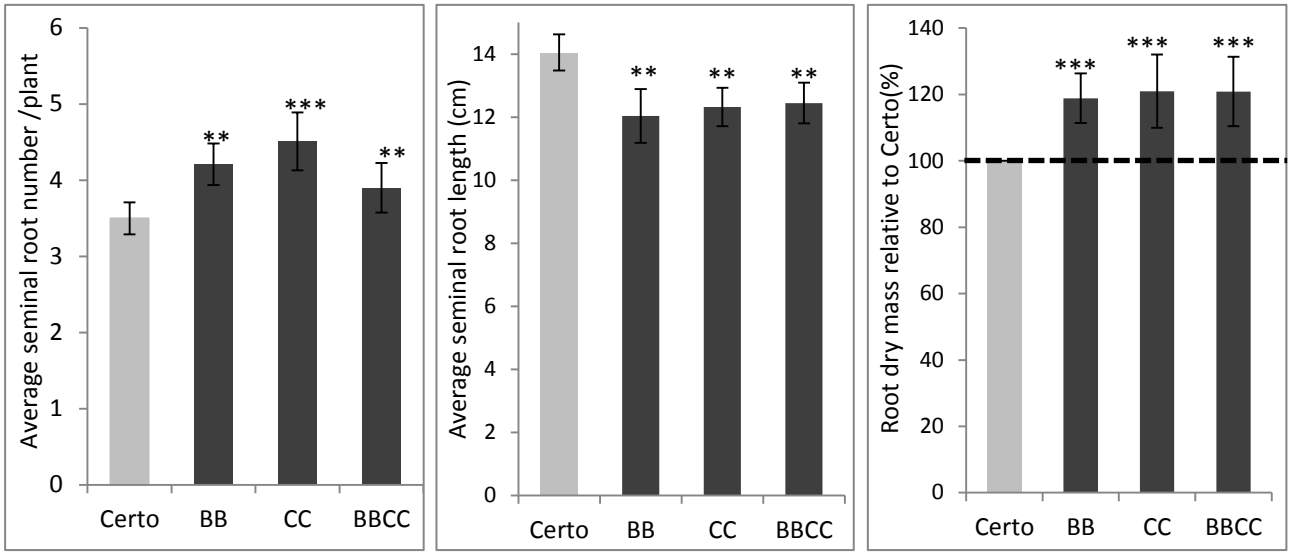


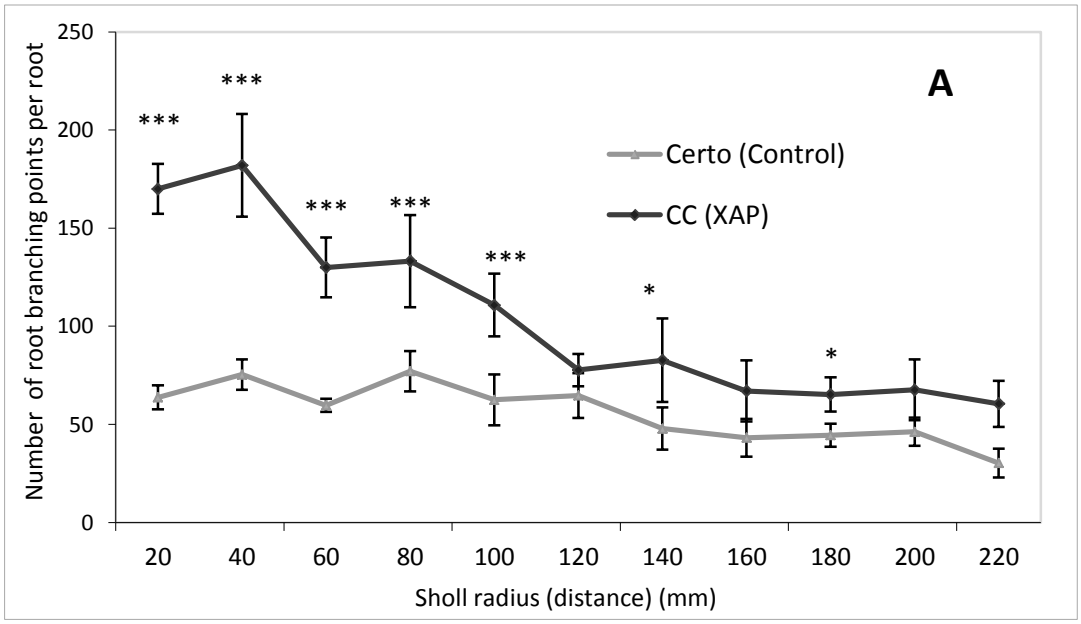
Fig. 17 XAP seedlings root phenotype under field like condition at three-leaves stage.

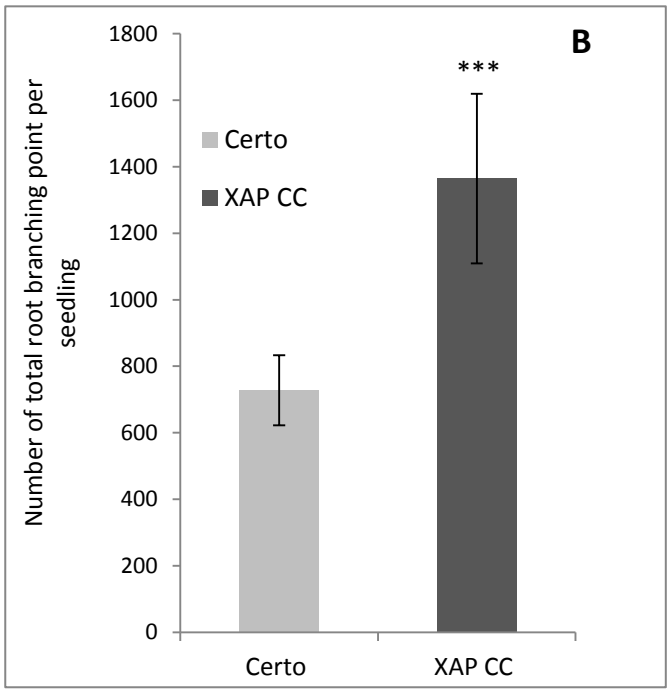
Seedlings are grown under semi-controlled environmental conditions in two consecutive cropping seasons. Three XAP lines (BB, CC, and BBCC) together with the non-transformed control Certo were planted in small greenhouses, in randomized 0.5m^2 soil plots without regulation of temperature, humidity. Data from two cropping seasons, 160

seedlings per line harvested from four plots was combined for statistical analysis. Significant differences between all XAP lines and the non-transformed control Certo according to t-test (*** P≤0.001, ** P ≤ 0.01, *P ≤0.05).

4.3.3 Enhanced root branching in XAP lines

Root branching along the seminal root system was evaluated from digital photographs of seedlings root. Seedlings of XAP line CC together with the non-transformed control Certo were grown in controlled growth chambers. Vermiculite was used as growth media and watered with nutrient solution. At the three-leaves stage, roots were harvested, and digital images were produced from the upper 20cm of the root system (Fig. 18C). The trait root branching was evaluated by Sholl analyses from the photographs of the seedling roots. Cumulative branching points were calculated at each consecutive 20mm Sholl radius (Fig. 18A). XAP seedling roots show a significant increment in cumulative root branching points. The average number of total root branching points was 1364 and 750 per seedling in XAP CC and non-transformed control Certo, respectively (Fig. 18B), the difference is significant (P<0.001). Highest branching point difference was recorded in the upper part (20-100mm Sholl radii) of the root, a maximum difference (three-fold increment) was observed at 40mm Sholl radius. In contrast, at the maximum radial distance (120-200mm) differences in root branching points among XAP and Certo seedlings were not significant (Fig. 18A). In summary, the digital image analysis of seedling roots revealed that XAP seedlings had more root branching points than the non-transformed control; and the highest branching point difference was observed on the upper part of the root system.





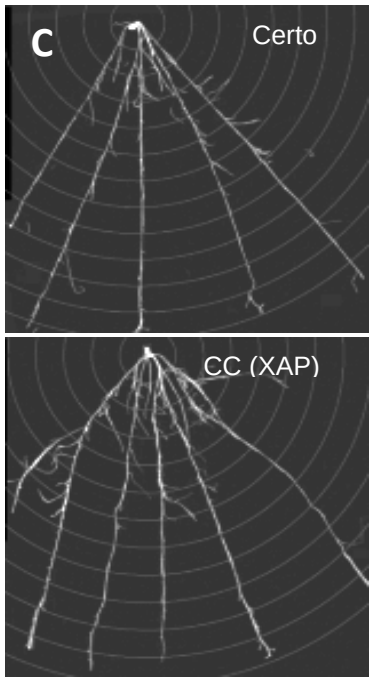


Fig. 18 Branching points in seedling roots of line XAP CC and Certo under *in-vitro* conditions.

A. Number of branching points across subsequent Sholl radii of seedling roots. B. Total root branching point per seedling in XAP CC and Certo. C. Digital image of seedling roots with Sholl radii. The seedlings were grown in growing chambers under *in-vitro* conditions. At the three-leaves stage, digital images were produced from harvested roots. Root branching points were evaluated by Sholl analyses from the digital images. Images from ten seedlings per line harvested from ten pots were analyzed, significant differences of XAP line to the Certo according to t-test (*** P≤0.001, ** P≤ 0.01, *P≤0.05).

4.3.4 *VfAAP1* expression and 1Ax1 promoter activity in seedling roots

The expression pattern of the VfAAP1 gene in seedling roots of XAP line CC was evaluated by qRT-PCR. At the three-leaves stage, total RNA was extracted from five different regions of the seedling root system (root tip (1), cell elongation zone (2), lateral root tip (3), cell maturation zone middle part (4) and the top part(5) (Fig. 19B). The results showed that *VfAAP1* expression differs along the root systems. Highest *VfAAP1* expression was registered in the cell maturation zone and the top part of the root. In the top part of the root, the expression level of *VfAAP1* is 25fold higher than in root tip. As well, in the cell elongation region, the *VfAAP1* expression was insignificant (Fig 19A). However, the expression level of *VfAAP1* in the root is lower compared to that of developing grains (12-17% of that found in whole developing grains at 10 DAF). In conclusion, the *VfAAP1* is expressed in seedling roots of XAP lines. Besides, expression pattern along the root part differs significantly; *VfAAP1* expression is highest in the region of cell maturation and low in young cells of XAP seedling roots.

To investigate the 1Ax1 promoter activity seedlings, 1Ax1::GFP plants were used (refer Chapter 2 section 2.2). Seedlings of the homozygous 1Ax1::GFP line (line253/8/2) at the T3 generation were grown under *in-vitro* conditions as described in section 4.2.1. Total RNA was extracted from seedlings root and leaf at the three-leaves stage. Activity of the 1Ax1 promoter was evaluated by using qRT-PCR. The result confirmed slight activity of 1Ax1 promoter in seedling roots and leaves (Fig. 16). The expression level of *GFP* in seedling roots and leaves was very low when compared with developing grains, on average the expression level of *GFP* in seedling root was 5-7% of that in the developing grains of 1Ax1::GFP line at 8 DAF (100%) (Fig. 16).

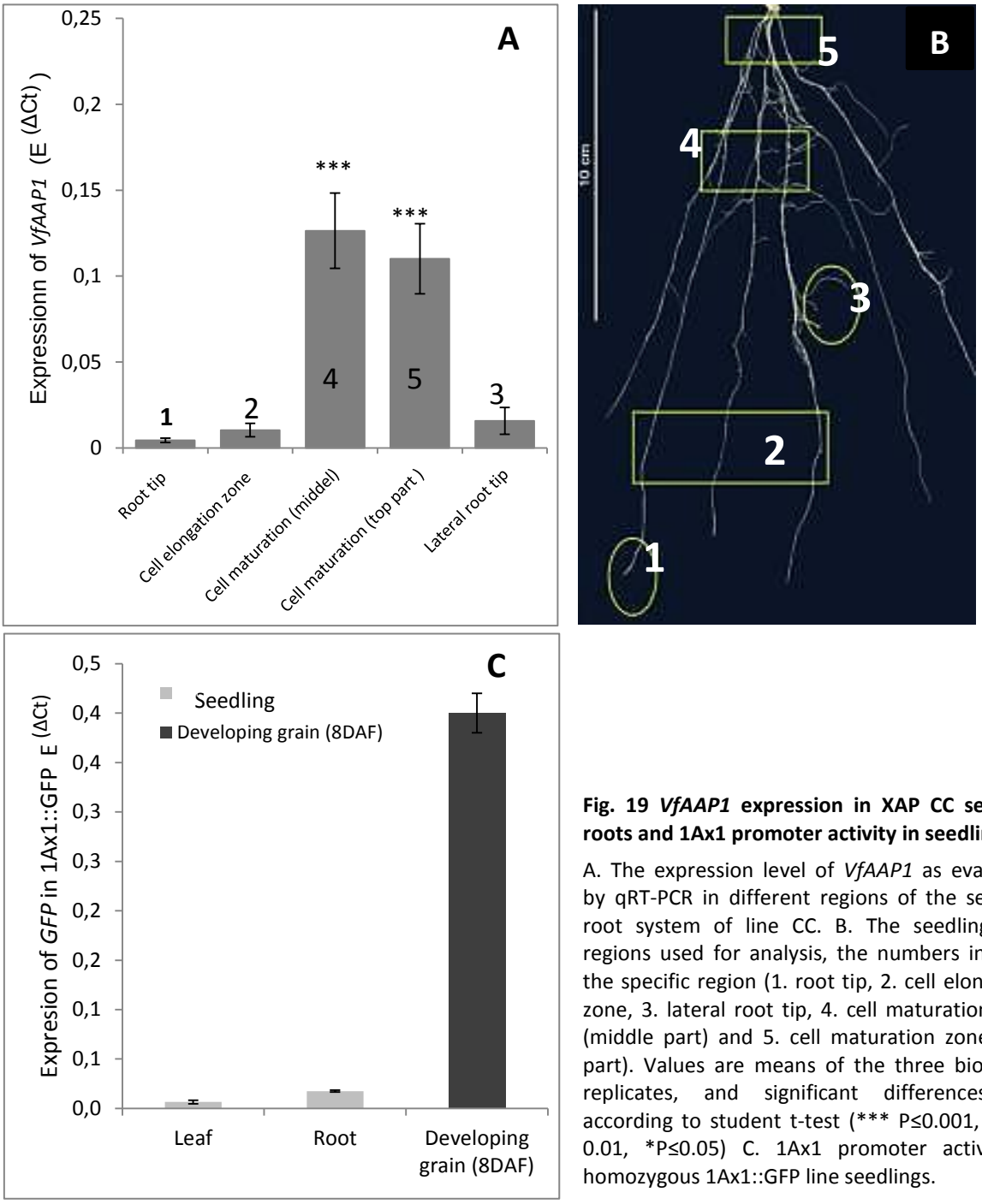


Fig. 19 *VfAAP1* expression in XAP CC seedling roots and 1Ax1 promoter activity in seedlings.

A. The expression level of *VfAAP1* as evaluated by qRT-PCR in different regions of the seedling root system of line CC. B. The seedling root regions used for analysis, the numbers indicate the specific region (1. root tip, 2. cell elongation zone, 3. lateral root tip, 4. cell maturation zone (middle part) and 5. cell maturation zone, (top part). Values are means of the three biological replicates, and significant differences are according to student t-test (*** P≤0.001, ** P≤ 0.01, *P≤0.05) C. 1Ax1 promoter activity in homozygous 1Ax1::GFP line seedlings.

4.4 Discussion

Root system architecture is critical for nutrient and water uptake, anchorage, nutrient storage, and plant-microbe interactions. Root distribution in soil profile has a direct impact on grain yield (Lynch, 1995; Smith and De Smet, 2012). The plant root system strongly influenced its ability to gather water and nutrients are by its root system (Hatzig et al., 2015; Lipiec et al., 2003; Masle and Passioura, 1987). Seedling root architectures are crucial for the efficient establishment of

mature plant and better yield traits in cereal crops (Leskovar, 1995; Lynch, 1995). Changes in root system architecture have occurred as a consequence of domestication and breeding in different cultivated crops (Beebe et al., 2006; Chloupek et al., 2006; de Dorlodot et al., 2007; Kato et al., 2006; Waines and Ehdaie, 2007; Yue et al., 2006). Previous studies reported that the introduction of dwarfing genes (*Rht*) alters the root system architecture in wheat and barley (Chloupek et al., 2006; Miralles et al., 1997; Wojciechowski et al., 2009). In the present study, root phenotypes of seedlings of new transgenic winter wheat lines expressing *VfAAP1* (XAP) under the control of the 1Ax1 promoter were evaluated under *in-vitro* and field-like conditions. The result revealed that XAP lines have a significant increment in seminal root numbers and root dry mass as compared to Certo. On average 20% and 10% increment in seminal roots number were registered under *in-vitro* and field-like conditions, respectively. XAP seedling roots have shown an enhanced root branching capacity as compared to the non-transformed control. However, XAP seedlings roots have shorter seminal roots than Certo.

Root morphology is in most cases regulated by a suite of small-effect loci that interact with the environment. Wojciechowski et al. (2009), suggest direct effects of dwarfing alleles on roots during seedling establishment in modern cultivars of wheat. Similar studies pointed out that the reduction in plant height with the introduction of *Rht* dwarfing genes might change the partitioning of dry matter to the root system, which may affect the root growth, water use and yield (Miralles et al., 1997; Siddique et al., 1990). The altered root architecture of XAP seedlings might be due to the direct effect of the newly introduced gene. Results from qRT-PCR analysis of XAP seedling roots confirmed that *VfAAP1* is expressed especially in the upper part of the seminal roots. It could be expected that the transport of amino acids is facilitated in these parts of the root. The increased sink capacity may enhance the rate of cell division and development of new primordia for root branching, resulting in increased root dry mass accumulation in XAP lines. Moreover, root architecture in plants is mainly regulated by hormonal signals (Liu et al., 2014; Osmont et al., 2007; Wu and Cheng, 2014). Changing the transport rate of amino acids could influence hormone and metabolic activity in the root tissue. Accordingly, the altered root phenotype in XAP lines could be due to hormonal regulation.

In conclusion, the study assessed altered XAP seedling root morphology relative to the non-transformed control Certo. Crucial seedling root architectural traits were evaluated. XAP lines displayed more seminal roots per plant, shorter seminal roots but higher root dry mass, and more branching point when compared to the non-transformed control. Influences from transgene expression in the root system itself on seedling root performance can be expected.

Chapter 5

Ectopic expression of the *V. faba* amino acid permease 1 (*VfAAP1*) causes changes in the transcriptome of developing grains

5.1 Introduction

Grain development in cereals is associated with massive changes in gene expression. Complex anatomical and biochemical changes occur in the developing grains after fertilization (Evers, 1970; Wan et al., 2008). The wheat grain is a composite of different tissues, each with a unique temporal pattern of gene expression during grain development (Laudencia-Chingcuanco et al., 2007; Zheng and Wang, 2011). Grain development proceeds by cell division and histodifferentiation to form the embryonic and endosperm tissues, followed by an influx of water that drives cell expansion, then deposition of storage reserves and finally maturation and drying (Laudencia-Chingcuanco et al., 2007; Opanowicz et al., 2011; Sabelli and Larkins, 2009b). In wheat, grain development is a critical and dynamic process that determines final grain yield. The physiological, biochemical and morphological changes that culminate during grain development determine mature grains size and weight, and its nutrition value at harvest (Howarth et al., 2008; Laudencia-Chingcuanco et al., 2007).

Seed development and storage reserve accumulation are strongly dependent on the availability of assimilates and metabolic regulation in developing seeds of many cultivated crops (Howarth et al., 2008; Rolletschek et al., 2005; Wang et al., 2015; Weber et al., 1997; Weber et al., 2005; Weichert et al., 2010; Weigelt et al., 2008). Amino acids are the major transport form of N, which is a vital nutrient for plant growth and development. It is mainly assimilated within plant roots or leaves and then translocated to developing sink tissues such as fruits and seeds (Lam et al., 1995; Lohaus and Moellers, 2000; Riens et al., 1991). Amino acids play key roles in metabolisms, such as in the production and functioning of enzymes, hormones, and other substances. Consequently, the biosynthesis of several metabolic compounds and plant hormones is mainly affected by the availability of amino acids in developing grains. Therefore, increasing the influx of amino acids into developing grains might have an influence on physiological, biochemical and morphological traits of the developing grain.

In the previous chapters, the grain yield potential, grain nutritional quality and plant phenology of new transgenic winter wheat lines that ectopically express *VfAAP1* under the control of the wheat 1Ax1 promoter (XAP) were discussed. In this chapter, transcriptional changes in developing grains of XAP line (CC) in comparison to the non-transgenic control Certo were assessed. Transcript profiling is a powerful technique, which offers detailed views to monitor the complex traits of developing grains. The aim of this study is to evaluate the effect of increasing amino acid supply to developing grains on grain physiology at the transcript level.

5.2 Material and methods

5.2.1 Sample preparation and RNA isolation

To analyze the differential gene expression during grain development of XAP line (CC), plants were grown in the 2013/14 cropping season under semi-controlled conditions (in small greenhouse on soil beds without regulation of temperature, humidity, and lighting) at the IPK campus, Gatersleben, Germany. Developing grains were sampled at five stages (4, 6, 8, 10 and 14 DAF) with five replications. From three stages (4, 6 and 14 DAF) whole developing grains were used for analysis, since in early development (4 and 6 DAF) the grain volume is mainly determined by the pericarp, and at later stage (14 DAF) grain volume strongly determine by endosperm. During transition phase (8 and 10 DAF) pericarp and endosperm tissues was dissected manually and analyzed separately because at these stages the percentages of pericarp and endosperm contribute to the grain volumes are almost equal (Pielot et al., 2015; Weschke et al., 2000). All samples were frozen in liquid nitrogen, and total RNA was isolated using the phenol-chloroform extraction protocol as described by Heim et al. (1993). RNA integrity and concentration were examined by Agilent 2100 Bioanalyzer using the RNA 6000 Nano LabChip Kit (Agilent Technologies).

5.2.2 Microarray analysis

Total RNA (200ng) was used to synthesize the Cy3 labeled cRNA based on Agilent one-color microarray low input quickAmp labeling kit (cat. no 5190-2305). To remove unincorporated nucleotides cRNA was purified by using the RNeasy mini kit (QIAGEN cat. no. 74104). 600ng labeled cRNA was used for fragmentation and array loading (Agilent Gene Expression Hybridization Kit (Cat. No 5188-5242). Hybridization was performed during 17h at 65°C on an Agilent 8×60K customized wheat array. The hybridized array was washed by using the gene expression wash buffer Kit (Agilent Technologies) and dried. Microarray hybridizations were performed using five independent biological replicates. The array was scanned at 3µm resolution with Agilent microarray scanner G2505C. The data extraction was carried out with Agilent Feature Extraction software 11.5v (Agilent Technologies).

5.2.3 Data processing and analysis

The performance of each microarray scan data was evaluated using statistical results generated in the QC (Quality Control) report from Agilent feature extraction software. The extracted data was further analyzed using GeneSpring 13 package (Agilent Technologies). Cluster analysis among five biological replicates was performed. After removing outliers among the biological replicates and transcripts with low signal intensity value, moderated t-test with a corrected p-value (multiple testing correction Westfall-Young, $P \leq 0.05$) was performed and genes differentially expressed between developing XAP and Certo grains were identified. The identified differentially expressed genes were interpreted based on gene ontology system of classification.

5.3 Result

5.3.1 Transcript profiling of developing XAP grains

According to moderate t-test with corrected P-value (Westfall-Young, $P \leq 0.05$) differentially expressed genes (DEs) at five stages of grain development (4, 6, 8, 10 and 14 DAF) were identified. At three stages (4, 6 and 14 DAF), whole developing grains were used for analysis, whereas at 8 and 10 DAF pericarp and endosperm tissues were analyzed separately, because during early development, the grain volume is predominantly determined by the pericarp, at transition phase (8-10 DAF) the percentage of pericarp and endosperm volumes are almost equal, whereas at 14 DAF the grains volume is strongly dominated by the endosperm (Pielot et al., 2015; Weschke et al., 2000). Transcript profiling was performed based on the Agilent one-color microarray gene expression analysis protocol. Transcriptome data analysis was carried out in the GeneSpring 13 package (Agilent Technologies). The identified DEs from four stages (4, 6, 8, 10 DAF) and three stages (8, 10, 14 DAF) were combined into the two components pericarp and endosperm, respectively, for gene ontology analysis. Results are presented in two sets of data which reflect the transcriptome of pericarp and endosperm tissues according to the grain development stages.

A total of 1,361 DEs were identified from pericarp, 204, 45, 216, and 507 genes (973 genes in total) were upregulated in XAP at 4, 6, 8 and 10 DAF respectively (Fig. 20A). From these genes 68, 16, 98, and 116 (corresponding to 33, 35, 45, and 23% of upregulated genes in the respective stages) show no homology to annotated sequences. 37, 202, 143 and 75 genes (457 genes in total) were downregulated in XAP pericarp at 4, 6, 8 and 10 DAF, respectively (Fig. 20A). From these 13, 41, 26, and 23, (corresponding to 35, 20, 19, and 30% of downregulated genes in the respective stages) have shown no homology to annotated sequences. According to gene ontology term analysis, 325 DEs from pericarp tissue were categorized into 12 functional groups (cell proliferation, cell wall-related, signaling, transport-related, hormone synthesis and function, storage protein, C and N metabolism, stress response and tolerant, lipid metabolism, photosynthesis, cytochrome P450 and ubiquitin). Among these functional groups the largest category was transport-related (which share 17.94% of the functionally annotated genes) and followed by stress response and tolerance (15.04%) (Fig. 20B) (Appendix III).

In endosperm tissue 1,082 DEs were identified at three stages, 62, 587 and 54 genes (703 genes in total) were upregulated in XAP endosperm at 8, 10 and 14 DAF, respectively. From these genes 22, 183, and 19 (corresponding to 36, 31, and 35% of upregulated genes in the respective stage) have shown no homology to annotated sequences. In endosperm 109, 186 and 143 genes (431 genes in total) were downregulated in XAP endosperm at 8, 10, and 14 DAF, respectively (Fig. 20C). From these 29, 71 and 51 (corresponding to 27, 38, and 35% of downregulated genes in the respective stages) have no homology to annotated sequences. A total of 335 DEs were categorized into 14 function groups based on gene ontology terms. The represented functional

groups are cell proliferation, cell wall-related, signaling, hormone biosynthesis and functions, storage protein, transport-related, C and N metabolism, lipid metabolism, cytochrome P450, protease, stress response and tolerance, protein kinase, ribosomal proteins, and ubiquitin. Among represented functional groups storage protein has the highest number of genes (15.01% of the functionally annotated genes) and followed by C and N metabolism (14%) (Fig. 20D).

In summary, at all stages, 2,220 DEs were identified in XAP developing grains as compared to the non-transformed control Certo, 1,361 and 1,082 from pericarp and endosperm, respectively. Accordingly, endosperm and pericarp tissues share a common set of 223 transcripts which significantly alter their expression profile in XAP. From the two data sets in total, 608 DEs were clustered into 15 different functional groups based on gene ontology terms. According to the number of genes in the cluster transport-related and storage proteins were the largest groups in pericarp and endosperm, respectively. Surprisingly, in both tissue fractions, the highest number of differentially expressed/upregulated genes was found at 10 DAF.

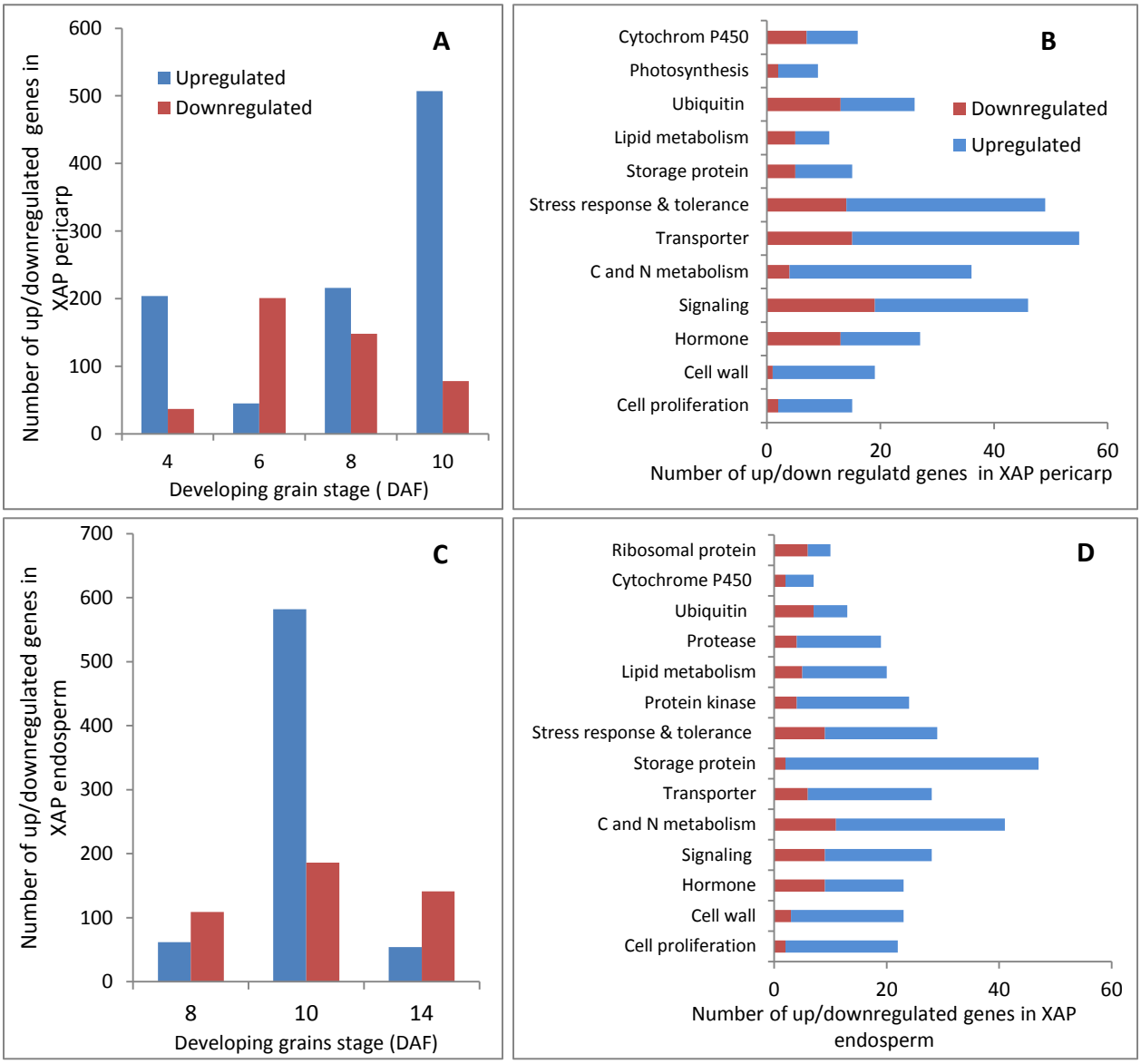


Fig. 20 Differentially expressed genes in developing grains of XAP line CC in comparison to Certo.

A. Number of differentially expressed genes, across stages of grain development in pericarp tissue, B. Differentially expressed genes from pericarp as related to distinct functional groups. C. Number of differentially expressed genes across stages of grain development in endosperm tissue. D. Differentially expressed genes from endosperm as related to distinct functional groups.

5.3.2 Cell wall and cell elongation-related genes

Cell walls have dynamic nature and function; the plant cell wall is a key determinant of overall plant formation like plant growth and development, environmental sensing and signaling. A total of 38 transcripts associated with cell wall were upregulated in developing XAP grains (Appendix III). From these genes, 20 and 18 correspond to endosperm and pericarp, respectively. In the XAP endosperm (8 and 10 DAF) genes encoding enzyme families involved in

pectin catabolism including pectin esterase (responsible for the demethylation of galacturonyl residues in high-molecular weight pectin), pectin lyase and pectin acetylesterase (PAE) (catalyzes the deacetylation of pectin) were upregulated (Table 4). All these enzymes are importantly involved in pectin metabolism and structural regulation of plant development. Pectin metabolism is the key process in cell extension through regulation, remodeling and changing of physiochemical properties of cell wall polysaccharides (Gou et al., 2012; Phan et al., 2007). In the XAP pericarp, a group of genes mainly involved in cell wall organization and modification was upregulated (Table 4). Known genes like cellulose synthase D1 (involved in beta-glycan synthase that polymerizes the backbones of non-cellulosic polysaccharides of plant cell wall), COBRA-like protein 7 (involved in cellulose microfibril organization) and expansins (proteins that mediate cell wall extension in the plant) are among the upregulated genes.

5.3.3 Cell division-related genes

In total 33 genes associated with cell proliferation activity were upregulated in developing XAP grains. Most of these upregulated genes are from the endosperm, in total 20 upregulated genes were identified in XAP endosperm. A cluster known genes, which are involved in cell division process including cyclins, cyclin-dependent kinases, spermidine synthase, cell division control protein and protein kinases, showed upregulation in the XAP endosperm (Table 5). In the XAP pericarp, 13 genes associated with cell division, including cell cycle control, Leu-rich receptor-like protein kinase, actin-related proteins were upregulated, especially during early grain development (4-6 DAF)(Table 5). Besides, in pericarp cyclin-dependent kinase inhibitor-5 which functions as cell cycle arrest shows downregulation in XAP at 8 DAF. In conclusion, the result recommends that improving amino acid uptake capacity of developing wheat grains can stimulate the expression of genes mainly involved in cell division and elongation processes.

Table 4 Upregulated genes in developing grains of XAP line CC related to cell wall and cell expansion.

Data was extracted from Appendix III, significant difference according to moderate t-test with multiple testing correction (Westfall-Young (P<0.05)), *P < 0.05, **, P <0.01, ***, P <0.001, (Endo= endosperm, Peri=pericarp, FC=Fold Change).

Seq ID	Best Blast Hit wheat	Annotation	Stage (DAF)	FC (XAP/Certo)	Function
Wht_37539	Traes_2AL_D3715CC49.1	COBRA-like protein 7	4	4.9***	cell growth/cellulose microfibril organization
Wht_14924	Traes_4DL_BB4D6F40A.1	aquaporin-like superfamily	4	2.4***	Water transport activity
Wht_32050	Traes_2BL_63C8E35E4.1	fasciclin-like arabinogalactan 1	8(Peri)	2.81***	cell adhesion protein
Wht_15402	Traes_3B_258E29040.1	expansin B2	8(Peri)	2.06**	cell wall organization
Wht_12617	Traes_3B_319A2B700.1	expansin/Lol pI	10(Peri	2.93***	cell wall organization
Wht_41194	Traes_XX_37E78F29C.1	cellulosesynthase-like D1	10(Peri	2.81***	cellulose synthase
Wht_14022	Traes_7DL_2FB89480C.1	aquaporin-like superfamily	10(Endo)	4.02***	water transport activity
Wht_00733	Traes_3AL_0ADBF00B9.1	pectinacetylesterase family	10(Endo)	3.37***	cell wall organization
Wht_01390	Traes_3AL_0ADBF00B9.1	pectinacetylesterase family	10(Endo)	3.06***	cell wall organization
Wht_04895	Traes_3DL_2C93D2B5E.1	pectinacetylesterase family	10(Endo)	2.41***	cell wall organization
Wht_36027	Traes_XX_1E007BB6E.1	pectinesterase family	4 10(Endo)	3.76*** 2.0**	cell wall modification
Wht_05701	Traes_2DL_B9663237F.1	pectin lyase-like superfamily	8 (Endo) 10(Endo)	2.46** 3.29***	cell wall organization
Wht_51317	Traes_3B_798C724AC.1	callose synthase 1	10(Endo)	3.34***	cell wall biogenesis
Wht_25903	Traes_5BS_CD20C307C.1	glucan-1,3-β-glucosidase	10(Endo)	3.33***	cell wall biogenesis
Wht_12199	Traes_5DL_A42BBCFD7.1	beta-galactoside alpha-2,6-sialyltransferase 2	10(Endo)	3.46***	cell wall biogenesis /galactosyltransferase
Wht_43708	Traes_6AS_B03B32977.1	endoglucanase 11	10(Endo	2.34**	cellulose degradation/ cell wall organization
Wht_14855	Traes_6AS_EE8C37602.1	endoglucanase 11	10(Endo	2.16***	cellulose organization
Wht_36106	Traes_1AS_B16A9D49C.1	peroxidaseN	10(Endo	2.30**	extensin peroxidase /Cell wall biosynthesis
Wht_26713	Traes_5AL_C1E3FCB4F.1	diacylglycerol glucosyltransferase	10(Endo	2.05*	glycolipid biosynthesis
Wht_25780	Traes_2DS_7146B6135.1	fasciclin-like	10(Endo)	1.78*	cell surface adhesion protein

Table 5 Up/downregulated genes in developing grains of XAP line CC involved in cell proliferation.

Data was extracted from Appendix III, significant difference according to moderate t-test with multiple testing correction: (Westfall-Young (P< 0.05)) *P < 0.05, **, P < 0.01, ***, P < 0.001, (Endo= endosperm, Peri=pericarp, FC=Fold Change).

Seq ID	Best Blast Hit wheat	Annotation	Stage (DAF)	FC (XAP/Certo)	Function
Wht_24047	Traes_5DL_FFF0AA252.1	actin-related protein 4	4	2.04***	chromatin organization /DNA repairing
Wht_03702	Traes_2BL_C085E7F9A.1	Leu-rich receptor-like protein kinase/ RPK2	4	3.40***	regulators of cell specification
Wht_41570	Traes_1AL_789B53D8F.1	cell cycle control protein 50A	4	3.61***	cell cycle control
Wht_55361	Traes_XX_A23164C28.1	cyclin-dependent kinase inhibitor 5	8(Peri)	-3.38***	cyclin /cell cycle arrest
Wht_23688	Traes_4DS_A800BBB3B.1	cytosine-specific methyltransferase	4 6	2.08** 2.87***	DNA methylation
Wht_56221	Traes_2AS_55BD046C1.1	elongation factor, GTP-binding domain	8(Peri) 10(Peri)	4.41*** 4.52***	protein biosynthesis /GTP binding
Wht_03664		Cell differentiation protein Rcd1	4 6 8(Endo) 10(Endo)	10.72*** 4.35*** 4.12*** 3.94***	transcriptional factor
Wht_10658	Traes_6DL_B99906102.1	s-adenosylmethionine decarboxylase proenzyme	4 6 10(Endo)	-2.11** -2.01*** 2.95***	spermidine /polyamine biosynthetic
Wht_26792	Traes_7BL_0E93FBCFF	spermidine synthase 3	8(Endo) 10(Endo	2.01** 2.12***	spermidine synthase
Wht_36029	Traes_4AL_4B411B3A7.1	spermidine synthase 1	8(Endo 10(Endo	3.26*** 3.06***	spermidine /polyamine biosynthetic
Wht_23508	Traes_6BL_724171B65.1	s-adenosylmethionine decarboxylase proenzyme	10(Endo)	3.01***	spermidine/polyamine biosynthetic
Wht_17690	Traes_2AS_09E707C65.1	Cys-rich receptor-like PK	4 10(Endo)	2.16*** 3.74***	Protein serine/threonine kinase /ATP binding
Wht_01667	Traes_5AL_B08AEFEF9.1	Ser/Thr-protein kinase NAK	10(Endo)	4.81***	protein serine/threonine kinase /ATP binding
Wht_04419	Traes_XX_CD5BE62A7.1	phytosulfokine receptor 1/2 (PSK)	10(Endo)	2.01**	receptor of cell growth promoting peptide
Wht_20894	Traes_5BL_32C82DE83	cell division control protein 45	8(Endo)	2.07**	DNA replication initiation
Wht_00172	Traes_1BL_F3035AD07.1	cyclin-dependent kinase D1	10(Endo)	3.42***	cyclin/cell cycle regulation
Wht_12685	Traes_3B_6589A8C2E.1	cyclin D2	10(Endo)	2.72***	cyclin
Wht_44636	Traes_4DL_F63F53885.1	cyclin F3-2	10(Endo)	3.18***	cyclin/cell cycle regulation
Wht_12008	Traes_5DL_B33355172.1	shaggy-related protein kinase alpha	8(Endo) 10(Endo)	2.76*** 3.74***	ATP binding/ GTP binding

5.3.4 C and N metabolism-related genes

A total of 48 and 13 entities, which are potentially involved in C and N metabolism, respectively, altered their expression in XAP grains (Appendix III). From sugar metabolism and glycolytic pathway, 16 upregulated genes were identified in XAP grains (Table 6). Four sequences encoding the enzyme trehalose-6-phosphate synthase involved in trehalose biosynthesis were upregulated in XAP pericarp and endosperm. Enzyme trehalose-phosphate synthase (TPS1) catalyzes the transfer of glucose from UDP-glucose to glucose-6-phosphate to produce trehalose-6-phosphate plus UDP; then trehalose-6-phosphate is hydrolyzed to trehalose by trehalose-6-phosphate phosphatase (TPS2) (Wolf et al., 2003). Trehalose is a known sugar signaling molecule in plants with strong effects on metabolism, growth, and development (Eastmond et al., 2002; Paul et al., 2008). At early grain development (4 and 6 DAF), five genes which encode members of glycoside hydrolase families (fructan 6-exohydrolase, soluble acid invertase, alpha-amylase and 1, 4-alpha-glucan branching enzyme GlgB) were upregulated in XAP. However, two enzymes (cell wall invertase and beta-amylase), from the glycoside hydrolase families were downregulated in XAP grains at 4 and 6 DAF. Cell wall invertase is one of the key enzymes involved in sucrose cleavage into hexose sugars, and beta-amylase is a known polysaccharide catabolic enzyme (hydrolyse 1, 4-alpha-glucosidic linkages in starch-type polysaccharide) (Edner et al., 2007; Hirose et al., 2002; Sherson et al., 2003).

Nine genes encoding enzymes of the amino acid metabolism were upregulated in XAP (Table 7). In the XAP pericarp, four genes which are related to primary amino acid synthesis (asparagine synthetase (ASN1), aspartate aminotransferase (AST), chorismate synthase and NADH glutamate synthase) were upregulated. In the endosperm, three genes involved in amino acid catabolism (glutamate decarboxylase (glutamate catabolism), alpha-aminoadipic semialdehyde synthase (lysine degradation) and serine-glyoxylate aminotransferase (Asn catabolism) were upregulated. Accordingly, in the XAP endosperm two genes potentially involved in amino acid biosynthesis (shikimate kinase1, (biosynthesis of aromatic amino acids) and aspartate kinase, an enzyme catalyzes the first reaction in the aspartate pathway) show downregulation. Similarly, four genes encoding key enzymes, which are involved in polyamines synthesis (spermidine synthase1 (SPDSYN1), s-adenosylmethionine decarboxylase (SAMDC), s-adenosyl-L-methionine-dependent methyltransferases (SAM-dependent (MTases)), nicotianamine synthase (NAS) have shown upregulation in XAP endosperm. SAMDC is one of the most important common enzymes which catalyzes the removal of the carboxylate group of S-adenosylmethionine (SAM) to form S-adenosyl-5'-3-methylpropylamine, which then acts as the propylamine group donor in the synthesis of the polyamines (Mad Arif et al., 1994). Nicotianamine synthase is the key enzyme in the biosynthetic pathway for the mugineic acid family of phytosiderophores (MAs), catalyzes the trimerization of S-adenosylmethionine to form one molecule of nicotianamine (Zhou et al., 2013).

In summary, based on gene ontology term analysis the cluster of genes encoding key enzymes involved in N metabolism shows altered expression in XAP grains. Amino acid biosynthesis enzymes have been upregulated mainly in XAP pericarp, while genes involved in amino acid catabolism were upregulated in endosperm tissue. Enzymes involved in the synthesis of polyamines like spermidine, SAM and nicotianamine have shown upregulation In XAP endosperm.

Table 6 Up/downregulated genes in developing grains of XAP line CC involved in sugar metabolism.

Data was extracted from Appendix III, significant difference according to moderate t-test with multiple testing corrections (Westfall-Young (P< 0.05)), *** P≤0.001, ** P≤ 0.01, *P ≤0.05, (Endo= endosperm, Peri=pericarp, FC=Fold change).

Seq ID	Best Blast Hit wheat	Annotation	Stage (DAF)	FC (XAP/Certo)	Function
Wht_29049	Traes_XX_B58E30BDA.1	pyruvate, phosphate dikinase 2	4	2.68***	pyruvate metabolism
Wht_27710	Traes_4AL_C6DEABC36.1	soluble acid invertase	4	2.54**	sucrose-6-phosphate hydrolase (invertase).
Wht_10055	Traes_7DL_D39A0193F.1	1,4-alpha-glucan branching enzyme GlgB	4	3.0***	glycogenase
Wht_00569	Traes_2AL_FCCDDB9081.1	plantinvertase/Pectin methylesterase inhibitor	8(Peri)	3.01***	pectinesterase inhibitor
Wht_00295	Traes_2BL_2C62185EE.1	1,4-alpha-glucan branching enzyme GlgB	4 8(Peri)	3.34*** 4.20***	glycogenase activity
Wht_20671	Traes_3B_89F6ACF89.1	Cell wall invertase	4 6	-2.78*** -2.4**	sucrose hydrolase
Wht_14865	Traes_XX_A33491620.1	beta-amylase 1	4 6 8(Peri)	-3.01*** -2.62*** -2.48***	glucan maltohydrolase /hydrolysis of α-1,4-glucan
Wht_02573	Traes_5BL_AC845F1C1.1	alpha-amylase-like	4 14	2.04* 4.32***	glycogenase /endoamaylase
Wht_00620	Traes_XX_422555914.1	trehalose phosphate synthase 1/TPS1	10(Peri)	2.52**	trehalose biosynthesis
Wht_09398	Traes_6DL_33F8A5EF4.1	trehalose phosphate synthase /TPS	10(Peri) 8(Endo) 10(Endo)	3.07*** 1.92* 2.68***	trehalose biosynthesis
Wht_08223	Traes_5BL_94F42F9A7.1	trehalose phosphate synthase /TPS	10(Peri) 10(Endo)	2.5** 2.03*	trehalose biosynthesis
Wht_05566	Traes_XX_0A31DDFFA.1	trehalose phosphate synthase /TPS	10(Endo)	2.02**	trehalose biosynthesis
Wht_54683	Traes_4BL_C3267759F.1	fumarate hydratase 1	10(Endo)	3.66***	fumarate hydratase
Wht_14880	Traes_2BL_7F9F8D481.1	fructan 6-exohydrolase	8(Endo) 10(Endo)	2.66*** 3.46***	fructans hydrolase
Wht_01478	Traes_4AS_254B6D654.1	phosphoenolpyruvate carboxy kinase	10(Endo)	-2.81**	gluconeogenesis/ATP binding

Table 7 Up/downregulated genes in developing grains of XAP line CC involved in N metabolism.

Data was extracted from Appendix III, Significant difference according to moderate t-test with multiple testing corrections (Westfall-Young (P< 0.05)), *** P≤0.001, ** P≤ 0.01, *P ≤0.05, (Endo= endosperm, Peri=pericarp FC=Fold change).

Seq ID	Best Blast Hit wheat	Annotation	Stage (DAF)	FC (XAP/Certo)	Function
Wht_08692	Traes_4AS_C19AB3A1A.1	asparagine synthetase (ASN1)	4	3.32**	Asn synthesis
Wht_01089	Traes_6AS_06B794C38.1	aspartate aminotransferase	4	3.56***	Asp metabolism
Wht_36153	Traes_7AS_069A1FA77.1	shikimate kinase 1	6	-3.84***	biosynthesis of aromatic amino acids
Wht_54337	Traes_4DL_A43826645.1	chorismate synthase	10(Peri)	2.36**	biosynthesis/aromatic amino acids
Wht_27143	Traes_3B_D003ACECF.1	NADH glutamate synthase	10(Peri)	2.06**	Glu biosynthesis
Wht_30492	Traes_7AS_089028EAD.1	serine-glyoxylate aminotransferase	8(Peri)	2.53*	aminotransferase/ Asn metabolism
Wht_05816	Traes_6AL_6D310D2B0.1	alpha-aminoadipic semialdehyde synthase	10(Endo)	2.01**	Lys degradation
Wht_03456	Traes_2AL_104945FD6.1	glutamate decarboxylase	10(Endo)	3.08***	Glu catabolism
Wht_14730	Traes_5BL_7657F73D5.1	aspartate kinase	10(Endo)	-2.03*	phosphorylation of Asp
Wht_36200	Traes_1DS_EFDF9CB72.1	glutamate-cysteine ligase B	14	-2.01*	glutathione biosynthesis
Wht_36029	Traes_4AL_4B411B3A7.1	spermidine synthase 1	8(Endo) 10(Endo)	3.48*** 3.14***	polyamine biosynthesis
Wht_15347	Traes_4DL_AF0869DDB.1	nicotianamine synthase 6	8(Endo)	4.06***	nicotianamine biosynthesis
Wht_10658	Traes_6DL_B99906102.1	s-adenosylmethionine decarboxylase	10(Endo)	2.81***	S-adenosylmethioninamine biosynthesis
Wht_23222	Traes_6DS_F3BC1C2DF.1	s-adenosyl-L-methionine methyltransferases	10(Endo)	2.12***	polyamine biosynthesis

5.3.5 Storage protein-related genes

A stimulatory effect on storage protein gene expression might be an indication for improving storage protein content. In total 42 transcripts encoding storage protein-related genes were upregulated in the XAP endosperm (Appendix III). These upregulated genes were primarily identified as encoding the gliadin, glutenin, globulin, and avenin class of proteins. Preferential upregulation of the prolamin class storage protein genes was observed in XAP endosperm. Prolamin storage proteins are the main repository of nitrogen in the endosperm of cereal grains. These stable proteins accumulate at massive levels because of the high-level expression of extensively duplicated genes in starchy endosperm cells. In total, 26 members of the gene family encoding the prolamin class of storage proteins were upregulated in XAP. Among the prolamin class, 14 upregulated genes are members of gliadin, and 12 genes classified as glutenin groups. Accordingly, the upregulated gliadin group genes represent the alpha/beta and gamma gliadin types. From the upregulated glutenin group, 11 genes encode the low molecular weight subunit. Additionally, four upregulated genes encoding the low molecular weight glutenin subunit (Glu-LMW) were identified at 4 DAF. A cluster of genes classified as late embryogenesis

abundant protein (LEA) was downregulated in XAP at early stages of grain development (4 and 6 DAF) (Table 8). LEA proteins are mainly expressed at different stages of late embryogenesis in seeds of higher plants and under conditions of dehydration stress. It is believed that LEA proteins are involved in dehydration tolerance and storage of seeds (Battaglia et al., 2008; Hong-Bo et al., 2005). Also in the pericarp, a cluster of genes related to storage proteins was upregulated. Alpha-amylase inhibitor, hordoindoline-B1, and avenin are among the upregulated genes (Appendix III). In summary, the data indicate that overexpression of *VfAAP1* in developing wheat grains has the potential to stimulate the expression of storage protein genes, especially in the endosperm with a preference of the prolamin class of proteins.

5.3.6 Transport-related genes

Transporters are crucial for the uptake of minerals and water from the soil, and for the transport of assimilates, hormones and nutrient throughout the plant system. Plants have evolved many large transporter families, which differ in their expression pattern, substrate specificity and localization at the levels of cell, tissue, and organ. In total, 90 transport related genes were identified with altered expression pattern in XAP grains, 61 and 29 were from pericarp and endosperm tissues, respectively (Appendix III). The majority of differentially expressed genes related to transport activity have shown upregulation. In pericarp, 17 upregulated genes were classified as ATP-binding cassette transporters (ABC transporters). ABC transporters have a broad participation in the export or import of a range of substrates from small ions to macromolecules in plant cells (Kang et al., 2011; Saurin et al., 1999). The involvement of ABC transporters in diverse biological processes is well documented, as for instance in pathogen response, surface lipid deposition, phytate accumulation in seeds, and transport of the phytohormones auxin and abscisic acid (Theodoulou, 2000; Yazaki, 2006). In a similar manner, a group of genes related to peptide and amino acid transport was upregulated in XAP pericarp. Ten genes related to peptide and amino acid transporters, including peptide transporter genes *PTR1, PTR2*, amino acid permease genes *AAP3/4, AAP2* and the gene encoding a cationic amino acid transporter were upregulated in XAP pericarp (Table 9). Similarly, a cluster of genes related to sugar transport showed altered expression in XAP pericarp in comparison to the non-transformed control. Three genes (*TaSUT1A,* bidirectional sugar transporter genes *TaSWEET4* and *TaSWEET2a*) were upregulated in XAP pericarp. Other sugar transporter genes (*SUT3*-like, sugar transport protein-5 and UDP-glucose transporter-3) were downregulated in XAP pericarp. Besides, nine genes related to metal transport including zinc, iron, and boron transporter genes were upregulated in XAP pericarp and endosperm (Table 10). Transporters of essential metals are vital in the uptake of metal ions from the soil and in the transport of metal ions to different parts of the plant. Metal ion transporters have key roles in maintaining ion homeostasis throughout the plant system. In summary, the result suggests that increasing amino acid uptake capacity of developing grains in wheat stimulates expression of numerous transport-related genes.

Table 8 Up/downregulated genes in developing grains of XAP line CC related to storage protein biosynthesis.

Data was extracted from Appendix III, significant difference according to moderate t-test with multiple testing corrections (Westfall-Young (P< 0.05)), *** P≤0.001, ** P≤ 0.01, *P ≤0.05, (Endo= endosperm, Peri=pericarp FC=Fold Change).

Seq. ID	Best Blast Hit wheat	Annotation	Stage (DAF)	FC (XAP/Certo)	Function
Wht_25252	Traes_4AL_4FF5B8837.1	alpha/beta gliadin	8(Endo) 10(Endo)	3.08*** 2.78***	storage protein
Wht_01191	Traes_4AL_4FF5B8837.1	alpha/beta gliadin	8(Endo) 10(Endo)	2.30*** 2.54***	storage protein
Wht_01438	Traes_4AL_4FF5B8837.1	alpha/beta gliadin	8(Endo) 10(Endo)	2.28** 2.21**	storage protein
Wht_01874	Traes_4AL_4FF5B8837.1	alpha/beta gliadin	4 8(Endo)	2.64*** 2.41**	storage protein
Wht_02736		alpha/beta gliadin	4 8(Endo)	3.67*** 2.34***	storage protein
Wht_27161		gamma gliadin	8(Endo) 10(Endo)	2.68*** 2.57***	storage protein
Wht_01195		gamma gliadin	8(Endo) 10(Endo	3.01*** 2,59***	storage protein
Wht_32308		gamma gliadin	8(Endo) 10(Endo	3.11*** 2.58**	storage protein
Wht_16064		gamma gliadin	8(Endo) 10(Endo	3.14*** 2.88***	storage protein
Wht_02363	Traes_1DS_66B67E9B41.1	glutenin, (LMW)	4 8(Endo)	4.23*** 2.22***	storage protein
Wht_01441	Traes_1DS_66B67E9B41.1	glutenin, (LMW)	8(Endo)	2.42***	storage protein
Wht_01486	Traes_1DS_66B67E9B41.1	glutenin, (LMW)	4 8(Endo)	2.61*** 2.40**	storage protein
Wht_27302	Traes_1DS_66B67E9B41.1	glutenin, (LMW)	8(Endo) 10(Endo	2.54***	storage protein
Wht_03113	Traes_1DS_66B67E9B41.1	glutenin, (LMW)	4 6 8(Endo) 10(Endo)	4.76*** 2.48*** 2.38*** 2.46***	storage protein
Wht_01057	Traes_1DS_66B67E9B41.1	glutenin, (LMW)	8(Endo) 10(Endo	2.56** 2.08**	storage protein
Wht_01903	Traes_1DS_66B67E9B41.1	glutenin, (LMW)	4 8(Endo)	3.52*** 2.24**	storage protein
Wht_02378	Traes_1DL_D861501F5.1	glutenin (HMW)	8(Endo) 10(Endo	3.10*** 2.57***	storage protein
Wht_10505	Traes_7AS_DF9A9B7BF1.1	avenin-like b5	8(Endo) 10(Endo	2.94*** 2.50***	storage protein
Wht_03551		avenin	8(Endo) 10(Endo	2.28***	storage protein
Wht_03519	Traes_4AL_3D862C090.1	vicilin	8(Endo)	2.44***	storage protein
Wht_00914	Traes_XX_994BDAE15.1	LEA-14	6	-4.21***	desiccation tolerance
Wht_02465	Traes_XX_146FE45EA.1	LEA-14	6	-3.84**	desiccation tolerance
Wht_25730	Traes_3B_8B604D9E0.1	LEA-5	4 6	-2.84** -3.02***	response to stress
Wht_31428	Traes_5DS_0BFE4FD87.1	hordoindoline-B1	4 8(Peri)	2.74*** 6.62***	storage protein
Wht_30449	Traes_6DS_1854119F9.1	alpha-amylase inhibitor 0.19	4 8(Peri)	2.78*** 2.25***	grain softness
Wht_30449	Traes_6DS_1854119F9.1	alpha-amylase inhibitor 0.28	4 8(Peri)	3.36*** 4.56***	grain softness

Table 9 Up/downregulated genes in developing grains of XAP line CC related to amino acid and sugar transport.

Data was extracted from Appendix III, significant difference according to moderate t-test with multiple testing corrections (Westfall-Young (P< 0.05)), *** P≤0.001, ** P≤ 0.01, *P ≤0.05, (Endo= endosperm, Peri=pericarp, FC=Fold Change).

Seq ID	Best Blast Hit wheat	Annotation	Stage (DAF)	FC (XAP/Certo)	Function
Wht_14359	Traes_XX_C9723A6CC.1	Na-coupled neutral amino acid transporter 6	10(Peri)	3.20***	amino acid transporter
Wht_35441	Traes_2DS_4EA42CAD7.1	peptide transporter/ *PTR2*	10(Peri)	2.87***	oligopeptide transport
Wht_10484	Traes_2BL_C41ED2148.1	peptide transporter/ *PTR1*	10(Peri)	2.25**	oligopeptide transport
Wht_27616	Traes_XX_7885435D2.1	peptide transporter/PTR-like	10(Peri)	2.64***	oligopeptide transport
Wht_00856	Traes_5DL_BB10A3B4B.1	amino acid permease *AAP 3/4*	10(Peri)	2.84**	amino acid transporter
Wht_39052	Traes_1BL_118B6763E.1	amino acid permease *AAP 5*	10(Peri)	2.25***	amino acid transporter
Wht_14482	Traes_XX_F296C5A02.1	amino-acid permease *AAP2*	10(Peri)	2.18**	amino acid transporter
Wht_36458	Traes_5BS_9384D46B3.1	cationic amino acid transporter 2	10(Peri)	2.12**	amino acid transporter
Wht_22477	Traes_5DL_E85FB920C.1	vesicular glutamate transporter 3	10(Peri)	2.68***	amino acid transporter
Wht_22262	Traes_6DL_EC95AC355.1	bidirectional sugar transporter *SWEET4*	10(Peri)	3.06***	sugar transporter
Wht_03767	Traes_3B_38344FE7E.1	bidirectional sugar transporter *SWEET2*b	10(Peri)	2.54**	sugar transporter
Wht_25431	Traes_4AS_A322DBCB0.1	*TαSUT1*A	10(Peri)	2.48***	sucrose transport
Wht_42583	Traes_4AS_A322DBCB0.1	*SUT3*-like	4 8(Peri) 10(Peri)	-3.64*** -4.21*** -3.01***	sucrose transport
Wht_22090	Traes_2AL_7A16D363B.1	sugar transport protein 5	4	-3.47***	sugar transporter

Table 10 Up/downregulated genes in developing grains of XAP line CC related to metal transport.

Data was extracted from Appendix III, significant difference according to moderate t-test with multiple testing corrections (Westfall-Young (P< 0.05)), *** P≤0.001, ** P≤ 0.01, *P ≤0.05, (Endo= endosperm, Peri=pericarp, FC=Fold Change).

Seq ID	Best Blast Hit wheat	Annotation	Stage (DAF)	FC (XAP/Certo)	Function
Wht_24350	Traes_XX_1B056461B.1	magnesium transporter *NIPA2/4*	10(Endo)	3.04***	Mg^{2+} transmembrane transporter
Wht_42475	Traes_6DL_AA95B2246.1	metal-nicotianamine transporter *YSL2*	4 10(Peri)	2.6** 2.0**	metal ion transporter/iron ion homeostasis/response to zinc ion
Wht_51154	Traes_4AL_7541D0C33.1	vacuolar iron transporter (*VIT*)	10(Endo)	2.16**	transport iron to the vacuole
Wht_42619	Traes_XX_3586E6BE7.1	zinc transporter 1	8(Endo)	3.18***	Zn transport to vacuoles/ maintain Zn homeostasis.
Wht_51893	Traes_XX_4C6DD3A91.1	zinc transporter 1/3	8(Endo)	2.22**	Zn transporter
Wht_39253	Traes_5DL_A98953CFA.1	Ca-activated outward-rectifying K^+ channel 1	6	-4.42***	K^+ transporter/ intracellular K+ redistribution/ K^+ homeostasis maintain
Wht_36094	Traes_3B_75C958395.1	K^+ transporter 5	10(Peri)	3.34**	K^+ ion transmembrane transporter activity
Wht_18196		boron transporter B5	8(Peri)	3.46***	boron transporter
Wht_42319	Traes_5DL_628B86979.1	sodium/sulphate symporter	8(Endo)	2.12***	Na^+ transmembrane transport

5.3.7 Hormone-related genes

The involvement of phytohormones in plant growth and seed development in many plant species is well known (Depuydt and Hardtke, 2011; Gibson, 2004; Ongaro and Leyser, 2008; Vriet et al., 2012). In total 27 and 19 DEs related to hormones were identified in pericarp and endosperm, respectively (Appendix III). Twelve genes, related to the plant hormone auxin were identified with altered expression in XAP grains. These genes were annotated as members of the primary auxin response gene families, Aux/IAA proteins (auxin/indole-3-acetic acid), GH3 (Gretchen hagen3) and SAUR (small auxin-up RNA). Three genes of the Aux/IAA protein family were downregulated in the XAP endosperm (8-14 DAF). While, at the same developing stage two genes from the Aux/IAA protein family were upregulated in the XAP pericarp. Aux/IAA proteins act as repressors of auxin-induced gene expression at low auxin concentrations. However, two genes from the SAUR family which are induced by auxin were upregulated in XAP endosperm (8-10 DAF). As well in pericarp one gene from SAUR family was upregulated. The expression profile of three genes encoding GH3 family members has shown altered pattern across grain development. At the very early stage (4 DAF), GH3 genes were downregulated

followed by upregulation at 8 and 10 DAF in the XAP endosperm. Auxin mainly induces the expression of GH3 genes. The respective gene products maintain hormonal homeostasis by conjugating excess indole-3-acetic acid (IAA) to free amino acids (Staswick et al., 2005). Besides, a gene encoding IAA-amino acid hydrolase was upregulated in the XAP pericarp at 10 DAF (Table 11). This enzyme has a function in hydrolyzing amino acid conjugates of IAA; it is believed that hydrolases of IAA-conjugates release free IAA from the conjugate form and thus are likely to play a major role in regulating free IAA levels (Davies et al., 1999; LeClere et al., 2002).

Five genes involved in gibberellin (GA) biosynthesis and signaling were upregulated in XAP grains. Two transcripts which encode ent-copay diphosphate synthase1 and ent-kaurenoic acid oxidase (KAO) were upregulated in the XAP developing grains. At very early stage (4 DAF), ent-kaurenoic acid oxidase (KAO) was upregulated, and ent-copalyl diphosphate synthase1 shows upregulation in XAP endosperm at 10 DAF. In a similar manner, the receptor of GA (GID1L2) was upregulated in XAP endosperm (8-10 DAF). Ent-kaurene synthetase 1 and KAO are among the key enzymes in GA biosynthesis (Regnault et al., 2014; Sun and Kamiya, 1994; Yamauchi et al., 2004). However, during pre-storage phase (6 DAF) four genes from the GRAS family were downregulated in the XAP; GRAS proteins act as transcriptional factors and play a major role in GA signaling.

Remarkably a cluster of genes related to ethylene synthesis and signaling was downregulated in XAP grains. Four genes (1-aminocyclopropane-1-carboxylate oxidase (ACO), ethylene-responsive transcription factor 1A, and two ethylene-responsive transcription factor4) were downregulated in XAP during early grain development stages (4-6 DAF). ACO is involved in the last steps of the ethylene biosynthesis pathway that catalyze 1-aminocyclopropane-1-carboxylic acid to form ethylene (Qin et al., 2007; Yang and Hoffman, 1984). Ethylene-responsive transcription factors (ERFs) are classes of transcription factors which are involved in the regulation of gene expression by stress factors and components of stress signal transduction pathways mediated by ethylene. ERF1A is a transcription activator, while ERF4 acts as a transcriptional repressor (Fujimoto et al., 2000; Hao et al., 1998; Ohta et al., 2001; Onate-Sanchez and Singh, 2002; Suzuki et al., 1998).

Table 11 Up/downregulated genes in developing grains of XAP line CC involved in hormone biosynthesis and function.

Data was extracted from Appendix III, significant difference according to moderate t-test with multiple testing corrections (Westfall-Young (P< 0.05)), *** P≤0.001, ** P≤ 0.01, *P ≤0.05 (Endo= endosperm, Peri=pericarp, FC=Fold Change).

Seq ID	Best Blast Hit wheat	Annotation	Stage (DAF)	FC (XAP/Certo)	Function
	Auxin-related genes				
Wht_14301	Traes_2AL_A598DE96B.1	auxin-responsive protein IAA14	14	-2.14**	repressors /early auxin
Wht_14871	Traes_1BL_4B1CFEF1C.1	auxin-responsive protein IAA19	6	-2.61***	repressors /early auxin
Wht_14259	Traes_1BS_E5A12CB09.1	auxin-responsive protein IAA16	10(Endo)	-2.68***	repressors/early auxin
Wht_27856	Traes_1DS_CA4185D11.1	auxin-responsive protein IAA4	10(Peri)	2.26***	repressors/early auxin
Wht_52494	Traes_3B_F880EDE81.1	auxin-responsive protein IAA6	10(Peri)	3.56***	repressors/early auxin
Wht_51746	Traes_XX_E252AF3BF.1	indole-3-acetic acid-amido synthetase/ GH3.4	8(Endo)	2.06***	IAA-amino acid conjugates syntheses
Wht_52494	Traes_3B_F880EDE81.1	indole-3-acetic acid-amido synthetase /GH3.5	6 10(Endo)	-3.01*** 2.91***	IAA-amino acid conjugates syntheses
Wht_42622	Traes_7BS_D6FDDD12F1.1	SAUR-like auxin-responsive	8(Endo) 10(Endo)	4.73*** 2.1**	regulation of cell elongation /auxin response
Wht_11606	Traes_7DL_4ABF3A54D.1	SAUR-like auxin-responsive	10(Endo)	1.95**	regulation of cell elongation /auxin response
Wht_35151	Traes_7DL_42A948988.1	SAUR-like auxin-responsive protein family	8(Peri)	3.50***	regulation of cell elongation /auxin response
Wht_02372	Traes_5AS_5A7E40F71.1	3-beta-hydroxysteroid-dehydrogenase/decarboxylase	10(Endo)	2.68*	sterol biosynthetic process
Wht_14716	Traes_XX_A27776CCF.1	IAA-amino acid hydrolase	10(Peri)	2.88***	hydrolyzes amino acid conjugates of IAA
	GA related				
Wht_14711	Traes_2BS_3EF779D1A.1	GA receptor GID1L2, carboxylesterase 3	8(Endo) 10(Endo)	2.08***	GA receptor
Wht_28842	Traes_4DS_26600563E.1	GA receptor GID1L2, carboxylesterase 3	10(Endo)	2.09*	GA receptor
Wht_35975	Traes_XX_522F99811.1	ent-Kaurenoic Acid hydroxylase2/ KAO2,	4	3.51***	GA biosynthesis /GA12
Wht_11452	Traes_7DL_02E7F7D6A.1	ent-copalyl diphosphate synthase 1	10(Endo)	2.44***	GA biosynthesis,
Wht_43009	Traes_2DS_2D035BAFD.1	GRAS family transcription factor	6	-3.05***	transcription factor/ GA Signaling
Wht_26428	Traes_3B_A25F058E5.1	GRAS family transcription factor	6	-2.60***	transcription factor/ GA signaling
	Ethylene related				
Wht_36268	Traes_2DS_9C0C983B2.1	ethylene receptor 2, *EIN4*	6 10(Endo)	2.56*** 2.76***	ethylene detection/ signal transduction
Wht_03207	Traes_1BS_FA0EFB1B8.1	1-aminocyclopropane-1-carboxylate oxidase	6	-2.99***	ethylene biosynthesis
Wht_29975	Traes_XX_907E287B6.1	ethylene-responsive transcription factor 1A	6	-4.9***	transcriptional repressor
Wht_16070	Traes_XX_28D711C9D.1	ethylene-responsive transcription factor 4	6	-5.9***	transcription factor
Wht_00682	Traes_XX_CBC33C43B1.1	ethylene-responsive transcription factor 4	6	-5.4***	transcription factor /stress signal

5.4 Discussion

Improving the assimilate uptake capacity of developing seeds has a potential influence on gene expression and metabolite profiles leading to changes in biological processes and compositional traits (Rolletschek et al., 2005; Wang et al., 2015; Weichert et al., 2010; Weigelt et al., 2008). In this study the transcript profiles of XAP wheat grains, which overexpresses the amino acid permease *VfAAP1*. The transcriptome of developing XAP grains was compared to that of the non-transformed control Certo. The differences in gene expression were presented in the result part of this chapter. For gene ontology analysis differentially expressed genes at four stages (4, 6, 8, 10 DAF) and three stages (8, 10, 14 DAF) were combined into the two components pericarp and endosperm, respectively. Since the proportion of pericarp and endosperm almost equal at transition phase (8 and 10 DAF) the tissues were dissected manually and analyzed separately.

The development of plant organs such as seeds relies on patterned control of cell proliferation, differentiation and growth (Dante et al., 2014; Harashima and Schnittger, 2010). Cell division, cell elongation and cell differentiation play critical roles in the process of cereals grain developmental (Dante et al., 2014; Sugimoto-Shirasu and Roberts, 2003; Thiel et al., 2008). Transcriptome analysis indicates clusters of genes related to cell division and elongation which altered their expression in XAP pericarp and endosperm. At an early stage (4 DAF), genes related to cell division and elongation including protein kinases, cyclin, COBRA-like protein 7, aquaporin were upregulated in XAP pericarp. Interestingly in the pericarp, genes related to cell elongation like expansins, cellulose synthase, and the Fasciclin-like arabinogalactan are upregulated at the transition phase (8-10 DAF). Final grain mass and volume positively correlate with pericarp expansion (Lizana et al., 2010). Cell division in the pericarp is terminated at an early stage of grain development and the later growth depends mainly on cell expansion (Pielot et al., 2015; Radchuk et al., 2011). During the pre-storage phase, an increase in biomass primarily occurs by elongation of the pericarp, and grain length is largely determined by the growth rate of the pericarp during the pre-storage phase (Pielot et al., 2015; Radchuk et al., 2011; Weschke et al., 2000). In cereals, the rate of grain growth and final grain weight are closely related to the final endosperm cell number and cell size (Brocklehurst, 1977; Morita et al., 2005; Nicolas et al., 1984; Singh and Jenner, 1984). Genes related to cell division including cyclins, cyclin dependent kinases (CDKs), protein kinases and spermidine synthase were upregulated in XAP endosperm. In addition, genes related to cell expansion like pectin acetylesterase, pectin lyase, glucan glucosidase, and aquaporins were upregulated in XAP endosperm. The finding suggests that the endosperm cell division and expansion might be stimulated in XAP endosperm cells, which are ultimately responsible for establishing the number and size of cells in the starchy endosperm. Accordingly, increased grain size of the XAP lines may be mediated by intensified endosperm cell division and/or cell expansion. Amino acids are the predominant transportable form of nitrogen in many plant species; these compounds are the building blocks of proteins and act as precursors or amino group donors for the synthesis of N metabolites essential for cell structure and function (Rentsch et al., 2007; Tegeder, 2014). Therefore increased availability of amino acids might stimulate cell division and growth in XAP pericarp and endosperm. In summary, the result suggests that

cell division and expansion process might be activated in the developing XAP endosperm. These phenomena could enhance the endosperm cell number and size and finally, lead to larger grains. Subsequently, the enhanced pericarp growth might contribute to increase TGW by regulating the capacity of grain expansion and volume.

Plant growth and development are highly dependent on the interaction between carbon and nitrogen metabolism. The control of C and N interaction constitutes an extremely complex network. During plant growth, nitrogen assimilation is integrated with photosynthesis, photorespiration, and respiration. Accordingly, signals are emanating among inorganic N compounds (nitrate and ammonium), nitrogen-containing metabolites (like amino acids) and C metabolites in different tissues and organs (Coruzzi and Zhou, 2001; Miller et al., 2008; Nunes-Nesi et al., 2010; Stitt and Krapp, 1999). The availability of substrates, metabolites, and products represent a valuable input to control gene expressions and the activity of key enzymes, and allow coordination of carbon and nitrogen assimilation. Remarkably, clusters of genes potentially involved in N metabolism were upregulated in XAP pericarp and endosperm. Genes involved in primary amino acid synthesis like ASN1, AAT, and NADH glutamate synthase, which is responsible for the synthesis of asparagine, aspartate, and glutamate, respectively, show increased expression in the XAP pericarp. Inorganic nitrogen is assimilated into the amino acids glutamine (Gln), glutamate (Glu), asparagine (Asn), and aspartate (Asp), which serve as major nitrogen carriers in plants. The enzymes glutamine synthetase (GS), glutamate synthase (GOGAT), glutamate dehydrogenase (GDH), aspartate aminotransferase (AAT), and asparagine synthetase (ASN1) are responsible for the biosynthesis of these nitrogen carrying amino acids (Lam et al., 1996). These findings suggest that synthesis of Glu and Asp branching amino acids might be stimulated in XAP pericarp. In addition, a difference in the timely pattern of regulation of genes involved in aromatic amino acids synthesis was observed. At early development (6 DAF) downregulation of shikimate kinase 1, which catalyzes the conversion of shikimate into shikimate-3-phosphate a precursor of chorismate was observed followed by upregulation of chorismate synthase gene expression in the XAP pericarp (10 DAF). The respective enzyme is responsible for the synthesis of chorismate, a precursor of aromatic amino acids. This data suggest that in XAP pericarp, the biosynthesis of aromatic amino acids through the shikimate pathway might be stimulated. In the XAP endosperm, genes involved in amino acid catabolism (glutamate decarboxylase (glutamate catabolism), alpha-aminoadipic semialdehyde synthase (lysine degradation) and serine-glyoxylate aminotransferase (asparagine catabolism) were upregulated. Similarly, cluster of genes involved in polyamines synthesis like spermidine synthase1, s-adenosylmethionine decarboxylase, s-adenosyl-L-methionine-dependent methyltransferases and nicotianamine synthase were upregulated in XAP endosperm. This data suggest that amino acid catabolism might be stimulated in XAP endosperm. In addition the biosynthesis of polyamines like spermidine and nicotianamine might be enhanced in XAP endosperm.

Metabolic utilization of sucrose for either growth and maintenance of cell activity depends on the breakdown of its glycosidic bond. Remarkably, a cluster of genes responsible for sugar metabolism and glycolytic pathway have altered their expression in developing XAP pericarp and endosperm when compared to non-transformed control. At early grain development (4 and 6 DAF), genes encoding glycoside hydrolases like soluble acid invertase and alpha-amylase were upregulated in XAP pericarp. However, at the same stage two genes (cell wall invertase and beta-amylase 1), which participate in sugar metabolism show downregulation in XAP pericarp. Invertases exhibit complex regulation at the transcriptional and posttranscriptional level in response to developmental, environmental, and carbohydrate signals (Bate et al., 2004; Sturm, 1999). Invertases are key players in carbohydrate transport and partitioning, sugar signaling, seed development and plant development (Koch, 1996; Sturm, 1999; Weschke et al., 2003). Cell wall invertases are usually correlated with developmental processes during seed development with highest activities measured in pre-storage phase (Wang and Ruan, 2012; Weschke et al., 2003). Amylases are glucan hydrolases that cleave α-1,4-glycosidic bonds of starch and release maltose units (Smith et al., 2005). Recent studies showed that beta-amylase1 (BAM1) mainly involved in the breakdown of starch in response to drought or salt stress (Monroe et al., 2014; Valerio et al., 2011; Zanella et al., 2016). The tight regulation of several enzymes involved in starch degradation seems consistent with the need to speed up the use of starch under particular conditions, i.e. under stress (Santelia et al., 2015). During transition phase (8-10 DAF) genes encoding key enzymes involved in sugar metabolism like trehalose -6-phosphate synthase and alpha-glucan branching enzyme were upregulated in XAP pericarp. Trehalose-6-phosphate synthase (TSP) is an enzyme, which catalyzes the first step in trehalose synthesis, and it has an essential role in embryo maturation (Eastmond et al., 2002). There is growing evidence that T6P plays a central role in regulating carbohydrate metabolism and act as signaling molecule that modulates critical metabolic and developmental processes in plants (Eastmond et al., 2002; Martinez-Barajas et al., 2011; Paul et al., 2008). In XAP endosperm similar to pericarp genes encoding as trehalose -6-phosphate were upregulated during transition phase (8-10 DAF). From the member of glycoside hydrolases enzayem fructan 6-exohydrolase (6-FEH) shows upregulation in XAP endosperm during transition phase (8-10DAF). Fructan 6-exohydrolase is one of the known enzymes for the breakdown of fructan (Le Roy et al., 2008; Van den Ende et al., 2003). In some plant species, fructan serves as a reserve carbohydrate (Hendry, 1993). Rapid fructan breakdown by FEHs is widely recognized to be an essential process for sugar mobilization in fructan plants whenever carbon supply is needed towards sink organs (Asega and de Carvalho, 2004; Morvan-Bertrand et al., 2001). In summary, transcript profiling of XAP pericarp and endosperm suggests that increasing influxes rate of amino acids into the developing grains of wheat seems to have the potential to change the transcriptional activity of the C and N metabolism in developing grains.

Plants store amino acids in the form of specific storage proteins in different tissues/organs. Developing seeds synthesize storage compounds from imported assimilates during their maturation phase. Protein accumulation in seeds of many flowering plants is mostly related to the sink strength for nitrogen assimilates during seed development. Consequently, it is associated with transport activity for nitrogen assimilates in developing seeds (Golombek et al., 2001; Miranda et al., 2001; Rolletschek et al., 2005). Notably, 42 genes related to storage protein show upregulation in the XAP endosperm. Subsequently, genes encoding the prolamin class of storage proteins were preferentially upregulated in XAP grains; it is in accordance with higher levels of prolamin class proteins in mature XAP grains (refer chapter 3). The availability of sulfur-containing amino acids is the major factor for the synthesis of prolamin storage proteins in developing endosperms (Shewry and Halford, 2002). *VfAAP1* mediates the transport of a wide range of amino acids with a preference of cysteine (Miranda et al., 2001). Cysteine and methionine are the two essential sulfur-containing amino acids that are incorporated into proteins (Brosnan and Brosnan, 2006; Galili and Hofgen, 2002; Leustek, 1996). The transport activity of sulfur-rich amino acids in XAP grains could be facilitated due to the type of amino acids preferentially transported by *VfAAP1*. Consequently, the stimulated synthesis of prolamin class proteins might be the result of the facilitated import of sulfur-rich amino acids into the starchy endosperm cells of developing XAP grains.

Plant transporters participate in various functions including mineral nutrition uptake, carbon and nitrogen metabolism, cell signaling, osmoregulation, cell homeostasis, storage and stress responses (Barbier-Brygoo et al., 2001; Delauney and Verma, 1993; Martinoia et al., 2007; Schroeder and Hedrich, 1989). This study revealed that a big cluster of transport-related genes showed altered expression in developing XAP pericarp and endosperm when compared to Certo. In pericarp tissue, 61 transport related genes are differently expressed; most of them were upregulated in XAP. A high number of upregulated genes were related to the ATP-binding cassette (ABC) superfamily. ABC transporters are a diverse group of transporters and mediate transport across membranes and conveyance of various substrates including lipids, phytohormones, carboxylates, heavy metals, chlorophyll catabolites and xenobiotic conjugates (Kretzschmar et al., 2011; Saurin et al., 1999; Theodoulou, 2000; Yazaki, 2006). Most ABC transporters are primary pumps, which use the energy of ATP hydrolysis to drive transport, but some also modulate the activity of heterologous channels or have intrinsic channel activity. Furthermore, the expression of metal transporter genes including Zn, Fe, and B transporters were upregulated in XAP. These transporters were upregulated in both grain parts (pericarp and endosperm), except B transporter, which is only upregulated in pericarp at 10 DAF. Consistent with this result, in mature XAP grains, significant increment of micronutrients (Fe, Zn, Mn, and Ni) was observed (refer chapter 3). It is frequently reported that the nitrogen status of plants has a positive effect on grain accumulation of Zn and Fe in wheat (Aciksoz et al., 2011; Erenoglu et al., 2011; Kutman et al., 2010). Grain sink strength is the major factor that affects the Zn

accumulation in the wheat endosperm (Stomph et al., 2011). Consequently, improving sink strength in wheat endosperm by overexpression of SUT1 shows higher Fe and Zn concentrations in mature grain (Saalbach et al., 2014). Thus, it seems reasonable to hypothesize that metal transporters might be induced by high levels of amino acids in developing XAP grains, playing a role in the import and accumulation of micronutrient. In summary, the study results confirm that increasing amino acid uptake capacity of developing grains in wheat is related to increased expression of several transporter-associated genes. Accordingly, the availability of N assimilates might regulate the absorption and accumulation of essential nutrients in the developing endosperm by inducing the uptake capacity of the cells. Further, availability of amino acids in developing grains might facilitate the homeostasis of nutrient ions during grain development.

Phytohormones are considered as important regulators of seed development (Davies, 2012; Yang et al., 2001). Auxins, cytokinins, gibberellins (GAs), and abscisic acid (ABA) are among the key hormones known to have a direct involvement in regulating grain development (Davies, 2012; Groot et al., 1987; Hansen and Grossmann, 2000; Kende and Zeevaart, 1997; Swain et al., 1997; Yang et al., 2001). Auxin is involved in many developmental processes including embryo and fruit development, root patterning and elongation, vascular tissue differentiation, organogenesis and apical dominance (Paciorek and Friml, 2006). Remarkably, a cluster of genes related to auxin functions and metabolism altered their expression in developing XAPpericarp and endosperm. Most of these respective genes are members of auxin response gene families. Auxin-responsive genes have been broadly grouped into three classes: auxin/indoleacetic acid (Aux/IAA), GH3, and SAUR gene families (Guilfoyle and Hagen, 2007; Jain et al., 2006). Among the primary auxin response gene families, three genes from the Aux/IAA class were downregulated and two upregulated in XAP endosperm and pericarp, respectively. Aux/IAA proteins are active repressors that inhibit auxin-induced gene expressions at low auxin concentrations (Teale et al., 2006). Besides, two genes of the SAUR class were upregulated in XAP endosperm. At 8 DAF one member of the SAUR gene family shows upregulation in XAP pericarp. SAUR genes encode short transcripts that are induced by auxin, and prior studies have correlated the expression of some SAUR genes with auxin-mediated cell expansion (Esmon et al., 2006; Knauss et al., 2003; McClure and Guilfoyle, 1987; Spartz et al., 2012). Upregulation of genes encoding in GH3 enzymes that conjugate free IAA with amino acids (Staswick et al., 2005) was observed in the XAP endosperm. In conclusion, the altered transcript profiles of XAP endosperm suggest that the level of auxin might be higher in XAP endosperm than in the control Certo.

GAs serves a vital role in coordinating growth and development throughout the life cycle of a plant, including seed development (Nadeau et al., 2011). Cell elongation is one of the major effects of GA in various plant organs. Previous work suggests that GAs play a major role in early seed development. GA-mediated cell elongation is suggested as important stimuli of pericarp

expansion and cell elongation in the nucellar projection of developing barley grains (Pielot et al., 2015; Weier et al., 2014). Similarly, Nadeau et al. (2011) report the role of GA in pea seed coat development during the pre-storage phase. In the present study, a cluster of DEs related to GA function and synthesis were identified in XAP pericarp and endosperm. Genes encoding major GA biosynthesis enzyme ent-Kaurenoic acid hydroxylase (KOA) show upregulation in XAP pericarp at early stage (4 DAF). However, cluster of genes related to GRAS family were down regulated in XAP pericarp. GRAS proteins act as transcriptional factors and play a major role in GA signaling. In XAP endosperm GA biosynthesis enzyme ent-copalyl diphosphate was upregulated at early storage phase (10 DAF). In a similar manner, group of genes encoding the GA receptor GID1L2 were upregulated in the XAP endosperm. GID1 is a soluble GA receptor that plays a key role in GA signaling cascade (Griffiths et al., 2006; Ueguchi-Tanaka et al., 2005). Grain development changes conferred by the expression of the *VfAAP1* in developing wheat grains seems to have a considerable connection with plant hormone (auxin and GA) activities, which resulted in a significant increase in TGW. The availability of N assimilates may induce the levels of auxin and GAs in the developing grains. Increased levels of these hormones could positively influence pericarp growth, endosperm cell division and cell expansion, which may reflect the stimulated grain growth in XAP lines.

Chapter 6

General discussion

Crop seed development is a complex process characterized by progressive differentiation of organs and tissues resulting in developmental gradients (Locascio et al., 2014; Milligan et al., 2005; Weber et al., 2005). The entire process covers multiple tissues and cells with specific patterns of proliferation and differentiation. Growing seeds in monocots, composed of the maternal pericarp and the filial endosperm and embryo, are genetically and physiologically heterogeneous (Milligan et al., 2005; Nowack et al., 2010; Opanowicz et al., 2011; Weschke et al., 2000). Seed development is initiated by the process of double fertilization that gives rise to the embryo and the endosperm (Weterings and Russell, 2004). The diploid embryo and the triploid endosperm develop concertedly inside maternal tissues and are protected by a seed coat (Haughn and Chaudhury, 2005; Milligan et al., 2005). The entire process is intricately and genetically programmed, correlated with changes in metabolite levels and regulated by a complex signaling network (Holdsworth et al., 2008; Lohe and Chaudhury, 2002; Sun et al., 2010; Weber et al., 2005; Wobus and Weber, 1999).

In most plants, amino acids represent the principal nitrogen assimilate transported over long distances in the vascular system to meet the metabolic N demands of highly diverse sinks. For seed development, amino acids distribution and transport is crucial for the biomass and storage product formation. Amino acid translocation depends on several factors, like sink strength and availability of N assimilates (Evans, 1989; Masclaux-Daubresse et al., 2008; White et al., 2016). In seeds, several tissues are symplasmically isolated (Stadler et al., 2005; Werner et al., 2011), i.e. not connected by plasmodesmata, channels in the cell walls (Lucas et al., 1993; Muller et al., 2015). Consequently, amino acids must be imported into the developing seed tissues to support seed development. This import step requires the activity of transporter proteins located in the plasma membrane. The molecular mechanisms of amino acid transport have been well characterized in different plant species (Fischer et al., 1995; Hirner et al., 1998; Miranda et al., 2001; Ortiz-Lopez et al., 2000; Taylor et al., 2015; Tegeder, 2014; Tegeder et al., 2000). Recently, the physiological function of transporters involved in the import of amino acids into developing seeds has been demonstrated in some plant species (Rolletschek et al., 2005; Tegeder et al., 2000; Weigelt et al., 2008; Zhang et al., 2015a). However, information on molecular mechanisms of amino acid transport and its physiological role in yield formation of monocots are limited.

The present study analyzed the positive impact of overexpression of the well-characterized amino acid transporter *VfAAP1* (Miranda et al., 2001) in winter wheat. Transgenic winter wheat lines overexpressing the *Vicia faba* amino acid permease *VfAAP1* under the control of the wheat

HMW-Glu-1Ax1 promoter (XAP) were analyzed. The potential of improved amino acids uptake capacity of developing wheat grains on yield formation was evaluated. The prospective influence of XAP expression on the phenology of seedling roots was analyzed. XAP wheat grain development was investigated at the transcript level based on differential gene expression.

6.1 Uploading of amino acids into developing grains is a limiting step for sink strength

The present study showed, the *VfAAP1* expression in developing wheat grains resulted in increased relative levels of the major free amino acids, and accumulation of higher grain biomass during grain development, which is in accordance with previous observations in legumes (Rolletschek et al., 2005; Weigelt et al., 2008; Zhang et al., 2015a). On the other hand, the hypothesis of the existence of sink limitation during grain development in wheat was supported by dramatic increases in grain dry mass and TGW in XAP lines. Assimilate distribution in plants is hypothesized to be regulated by transport processes located in importing sinks like developing seeds and fruits. The transport of organic nitrogen is a major factor influencing plant productivity, crop yield and reproductive success (Tegeder, 2014). Amino acids are the principal constituent of numerous organic compounds like proteins, nucleic acids, enzymes, phytohormones and secondary metabolites (Mengel et al., 2001). Allocation of nitrogen assimilates such as amino acids in different plant organs is mediated by its complex vascular system and driven by a series of transport steps. In developing seeds, the delivery process could be controlled by the capacity of the seed itself to import amino acids via specific transporters (Thorne, 1985). On the other hand, studies suggest that, sink strength for nitrogen acquired during seed development is associated with amino acid transport in the mother plant (Golombek et al., 2001; Rolletschek et al., 2005; Tan et al., 2010; Zhang et al., 2015a). Mutant analyses and localization studies support that amino acid transporters function in developing seed tissues by regulating amino acid seed loading, which can influence metabolic processes and storage product accumulation in many plant species (Hirner et al., 1998; Miranda et al., 2001; Sanders et al., 2009; Schmidt et al., 2007; Tan et al., 2010; Tegeder et al., 2000). Therefore, the expression or activity of respective transporters might be the key regulatory factor for amino acid import rate of developing seeds.

Notably, an increase in percentages of total nitrogen which reflects grain protein content was observed in mature XAP CC grains. Likewise, XAP lines grown under field-like conditions have shown an increase in gliadin content in mature grains. The data was supported by transcriptome analysis, discovering 42 upregulated genes related to storage protein in the developing XAP endosperm. Among the upregulated genes, 58% were members of the prolamin storage protein gene family. These findings suggest that the uptake system of amino acids could be facilitated in the starchy endosperm due to the expression of *VfAAP1* in XAP lines. Stimulation of storage protein genes expression in the XAP endosperm may be due to nutritional effects of the facilitated amino acid uptake activity. During seed development, N accumulation and protein

synthesis rely on the availability of assimilates like sugars and amino acids (Borisjuk et al., 2004; Rolletschek et al., 2005; Weber et al., 1997; Weichert et al., 2010). Increased storage protein accumulation in wheat might be associated with enhanced grain sink strength by expressing sucrose transporter (Weichert et al., 2010). The concentration and composition of grain storage proteins in mature wheat grain are strongly influenced by N and S supply (Dai et al., 2015; Triboi et al., 2003). Subsequently, sulfur containing amino acids are an important determinant of prolamin synthesis in cereal grains (Dai et al., 2015; Shewry et al., 1995). The developing XAP grains are characterized by a shift towards higher relative abundance of methionine. Functional characterization of *VfAAP*1 in a yeast mutant shows that it has a preference for cysteine uptake (Miranda et al., 2001). Methionine and cysteine are the two common sulfur-containing amino acids which can incorporate into protein synthesis (Brosnan and Brosnan, 2006). Consequently, higher influx rates of sulfur containing amino acids due to *VfAAP1* activity in the XAP starchy endosperm cells might contribute triggering the expression of prolamin genes.

In summary, the data demonstrate that *VfAAP1* expression increases amino acid uptake into developing grains resulting in higher grain dry mass formation, improves TGW and grain yield and stimulates storage protein synthesis. The present study suggests that increased activity of a sink located amino acid transporter positively affects sink strength and that N assimilate uploading into developing grains might be a limiting step for sink strength in wheat.

6.2 Expression of *VfAAP1* leads to enhanced grain growth

Plant growth and morphogenesis are products of localized cell division and anisotropic cell expansion. Seed development results from a combination of three cellular processes: cell division, cell expansion, and cell differentiation. These processes are controlled by the molecular machinery, which regulates cell cycle progression in coordination with nutritional, hormonal, developmental and environmental signals (Lau et al., 2012; Nowack et al., 2010; Sabelli, 2012; Sun et al., 2010; Wobus and Weber, 1999). The mitotic cell cycle consists of DNA synthesis (S-phase) and chromosome condensation and sister chromatid segregation phase (M-phase), separated by the two gap phases G1 and G2. The M-phase is coupled to separation of daughter chromosomes (karyokinesis) and ends with the division of cytoplasm (cytokinesis). However, in several plant tissues, cell types, and developmental stages, alternative cell cycle types can occur. In the context of seed development, cell cycles are characterized by acytokinetic mitoses of the primary endosperm nucleus, resulting in a syncytium, cellularization of syncytial nuclear domains, and cell proliferation through mitotic activity. In monocots, the endosperm undergoes a rapid growth phase with a specialized type of cell cycle known as endoreduplication (Becraft and Yi, 2011; Olsen, 2004; Sabelli and Larkins, 2009a; Sabelli and Larkins, 2009b). It is characterized by one or more rounds of DNA synthesis in the absence of mitosis, resulting in polyploid cells, and frequently associated with cell enlargement and accumulation of storage compounds (Edgar et al., 2014). All these different cell cycle types influence growth and

development of seed structures by influencing cell division and expansion, which determines cell number and cell size in mature seeds.

In cell cycle progression, different molecular processes are involved orderly through the various phases of the cell cycle and their appropriate responses to extracellular signals. These are governed by multiple regulatory mechanisms such as reversible protein phosphorylation, the interaction of proteins and specific protein degradation (Inze and De Veylder, 2006). Protein phosphorylation is one of the widely known major mechanisms that control the progression of cell cycle, and it is centrally governed by members of cyclin-dependent kinases (CDKs), cyclins and CDK inhibitors (ICKs or KIP-related protein (KRPs)) (Inze and De Veylder, 2006; Verkest et al., 2005). Plants possess relatively large sets of genes encoding different CDKs and cyclins (Van Leene et al., 2011). The decision to enter or leave the cell division cycle is taken in the G1 phase of the cell cycle. Concerted action of G1 specific CDK-cyclin complexes is crucial to initiate genome duplication, and drive cells into the cell cycle (Gutierrez et al., 2002; Menges et al., 2006). Interestingly, the transcriptomic analysis of developing XAP grains shows that a group of genes related to protein serine kinases and G1 specific CDKs/cyclin including cyclin-dependent kinase D1, cyclin D2, and cyclin F3-1 shows upregulation in XAP developing endosperm. The D-type cyclins are often mentioned as sensors of external conditions, and associated with cyclin dependent kinase (e.g., CDKA) to regulate cell cycles (Nieuwland et al., 2009). A set of *in-vitro* cell culture studies reported the close correlation between the supply of sucrose and the expressions of cyclins (Hartig and Beck, 2006; Riou-Khamlichi et al., 2000). The involvement of plant growth factors, such as auxins, cytokinins, brassinosteroids, gibberellins and ethylene responsive elements influencing the activity of promoters of cyclins, CDKs, and KRPs is well documented (Burssens et al., 2000; Ferreira et al., 1994; Hemerly et al., 1993; Hu et al., 2000; Richard et al., 2002; Sauter et al., 1995). The expression of D-type cyclin genes is mainly modulated by plant growth factors and extracellular signals (Fuerst et al., 1996; Gaudin et al., 2000; Hu et al., 2000; Meijer and Murray, 2000; Oakenfull et al., 2002; Soni et al., 1995). Increasing availability of assimilates in growing cells can stimulate gene expression and enhance metabolic activity associated with cell division. Availability of N assimilates in the XAP endosperm might stimulate the expression of G1 specific CDKs/cyclin and accelerate cell division in the starchy endosperm. Proliferative cell cycles are ultimately responsible for establishing the number of cells in the starchy endosperm, which has a positive correlation with the final grain size and grain weight. Accordingly, increased TGW and grain size in XAP wheat might result from stimulated endosperm cell proliferation.

Grain size and grain weight are highly influenced by endosperm cell expansion, which is defined as an irreversible increase in cell volume brought up by massive accumulation of storage compounds (proteins, lipids, and/or carbohydrates) and water intake during grain development. The increase in cell volume which is responsible to the seed size during growth is driven by

water movement into the cell. Grain water content is significantly increased in XAP grains at the early storage phase. Remarkably, gene expression analysis revealed that a cluster of cell wall-related genes including pectin modifying enzymes (PME, PG, PLL, and PAE) and water transporters (aquaporins) are upregulated in the XAP endosperm. Turgor pressure drives the growth of plant cells and is controlled by the extensibility of the cell wall (Cosgrove, 2005). Extensive integration of a wide variety of endogenous and exogenous inputs can control the cell expansion process (Passardi et al., 2004; Wolf et al., 2012). In growing cells, the expansion processes are primarily regulated by the cell wall extensibility (Derbyshire et al., 2007; Nicol et al., 1998; Refregier et al., 2004; Taiz, 1984). Improving the influx rate of nutrient ions into the growing plant cells may influence the cell extension process in many aspects. Increasing the flow of nutrient ion in growing cell can develop osmotic potential differences and create turgor pressure. In XAP grains the increased transport capacity for amino acids might facilitate the homeostasis of nutrient ions, which may increase the availability of osmotically active solutes in the growing cell. Thus osmotically active solute may generate a lower osmotic potential to attract water flux into the cell, thereby generating a turgor pressure to drive cell expansion. Subsequently, the availability of N assimilates in XAP endosperm could facilitate the biosynthesis of pectinase modifying enzymes, which may stimulate cell wall extension. The result suggests that the cell expansion process might be enhanced in XAP endosperm leading to accelerated growth of endosperm cells, and ultimately improving the size of endosperm cells. Accordingely, the enhanced endosperm cell size might contribute to the improved XAP grains weight and size.

In monocots, growth of maternal pericarp has a direct influence on mature grain size and volume. Pericarp growth largely depends on cell expansion since cell division in pericarp tissue stops very early (Pielot et al., 2015; Radchuk et al., 2011). In XAP pericarp genes for cell wall-related proteins (expansins, COBRA-like and fasciclin-like arabinogalactan) and cellulose synthase gene shows upregulation. Cell wall-related proteins are believed to play a central role in modulating cell wall extensibility, which mediates cell enlargement and expansion (Cosgrove, 2005; Li et al., 2003; MacMillan et al., 2010; Sampedro and Cosgrove, 2005). It is believed that cell wall proteins like expansins mainly participate in pH-dependent induction of wall extension and stress relaxation. Plant cell walls enlarge more rapidly at low pH, a process mediated by nonlytic proteins called expansins (Cosgrove 2014). Promoting the influx rate of nutrients into growing cells may activate the plasma membrane (PM)-H^+-ATPase, pumping protons into the extracellular space. This phenomena can lowers the pH in cell wall matrix, activating cell wall loosening proteins such as expansins and xyloglucan. Notably, gene expression analysis revealed that a big cluster of ABC transporter was upregulated in the XAP pericarp. These transporters use the binding and hydrolysis of ATP to power the translocation of a diverse assortment of substrates across membranes. These findings suggest that proton pump ATPase transporter activity seems to be enhanced in the XAP pericarp. Consequently, proton pumping out of the

cell might be stimulated, which may facilitate cell expansion by activating cell wall loosening proteins such as expansins and xyloglucan. Accordingly, enhanced pericarp cell expansion could contribute to increased TGW and grain size in XAP by promoting the capacity of grain expansion and volume.

The phytohormone auxin is a well-known regulator of plant growth and development from embryogenesis to senescence. Many aspects of auxin influences on plant growth and development are usually associated with its crosslink effect on cell division and expansion. Primary evidence suggests that auxin has a direct influence on the expression and assembly of the G1 specific CDKA-cyclin complex (del Pozo et al., 2006). More precisely, at the transcriptional level, auxin regulates the expression of the cyclins like *CYCA, CYCB, CYCD* (Ferreira et al., 1994; Richard et al., 2002)*,* and *CDKA* (Hemerly et al., 1993). The transcriptome analysis reveals that groups of auxin responsible genes altered their expression in the XAP endosperm. Remarkably, cluster of genes encoding AUX/IAA protein has shown downregulation in the XAP endosperm. AUX/IAA proteins are active repressors that inhibit auxin-induced gene expressions at low auxin concentrations (Teale et al., 2006). These proteins function as active repressors by dimerizing with auxin response factors (ARF) which negatively regulates the expression of auxin response genes (Gray et al., 2001; Guilfoyle and Hagen, 2007; Tiwari et al., 2001). Degradation of AUX/IAA transcription inhibitor proteins facilitates binding of ARF transcription factors and the subsequent expression of target genes, like CDKs, cyclins (Berleth et al., 2004; Richard et al., 2002). Downregulation of AUX/IAA proteins in XAP endosperm cells might facilitate the upregulation of auxin-dependent cyclins and CDKs. Moreover, gene expression analysis revealed upregulation of SAUR genes in the XAP endosperm. SAUR genes encode short transcripts that are induced by auxin, and prior studies have correlated the expression of some SAUR genes with auxin-mediated cell expansion (Esmon et al., 2006; Knauss et al., 2003; McClure and Guilfoyle, 1987; Spartz et al., 2012). Similarly, the third group of auxin responsible gene families, GH3, shows upregulation in the XAP endosperm. GH3 genes encode IAA-amido synthetases, which help to maintain auxin homeostasis by conjugating excess IAA to amino acids. These findings indicate that the level of auxin might be increased in the XAP endosperm, and ultimately auxin-mediated transcriptional activation might contribute to the enhanced grain growth by facilitating cell division and/or cell growth processes in the XAP endosperm.

Gibberellin plays important roles in many aspects of seed development. The physiological role of GAs in seed development has been investigated in a range of species. The accumulation of seed storage products and grain fresh weight is associated in a linear fashion with the amount of GA present in the developing seed/grain (Yang et al., 2013). Mostly, the positive effect of GAs in seed development is related to its direct involvement to cell expansion. Recent studies suggest the involvement of GA in nucellar and pericarp cell elongation in developing barley

grains (Pielot et al., 2015; Weier et al., 2014). Transcriptome analysis of developing XAP grains reveals that the GA biosynthesis enzyme ent-Kaurenoic acid hydroxylase (KOA) was transcriptionally upregulated in the XAP pericarp at early grain development. As well, genes of the GA biosynthesis enzyme ent-copalyl diphosphate synthase and the GA receptor GID1L2 were upregulated in the XAP endosperm at the beginning of the storage phase. qPCR analysis indicates remarkable expression of *VfAAP1* in the XAP pericarp during early development (4 DAF), and during the storage phase, the expression peaked in the starchy endosperm. During early development, GA biosynthesis might be stimulated in the XAP pericarp, and GA-mediated cell elongation could enhance pericarp expansion. At beginning storage product accumulation, GA biosynthesis might be stimulated in the endosperm. Altogether GA effects might be responsible for the higher mass of XAP grains.

In summary, pericarp growth might contribute to the improved TGW and grain size in XAP lines. Cell-wall modifying proteins like expansins were preferentially upregulated at the transcript level. Cluster of genes encoding ATP-binding cassette transporters show upregulation in the XAP pericarp, which may facilitate pumping of protons out of growing cells. Eventually apoplastic pH might be decreasing which activates cell wall-loosening enzymes. Thus, pericarp cell expansion seems to be stimulated in developing XAP grains. In the XAP endosperm, a cluster of genes related to cell division including cyclins and CDKs are upregulated. Also genes related to cell-wall modified enzymes, which has a primary role in facilitating the cell expansion process were upregulated in the XAP endosperm. The altered expression profile of auxin responsible genes indicate that also the level of auxin might possibly be enhanced. As well GA biosynthesis enzyme and GA receptor genes show upregulation. Thereby, auxin and GA might facilitate cell division and expansion in pericarp as well as endosperm.

6.3 Activity of the HMW-Glu-1Ax1 wheat promoter in transgenic wheat

Genetic transformation of plants has become a widely used technology that serves multiple purposes in plant biology research. However, it often results in plants characterized by unpredictable transgene expression, silencing and unexpected phenotype variability (Bhat and Srinivasan, 2002; Butaye et al., 2005). These inconsistencies in transgene expression are a major drawback in plant biotechnology, and can also confuse the interpretation of resulting phenotypes. Consequently, screening of individual lines for transgene expression pattern and level is a critical approach.

In the present study, the influence of *VfAAP1* expression on grain development in wheat was evaluated. Transgenic winter wheat lines expressing the amino acid permease *VfAAP1* under the control of the wheat HMW-Glu-1Ax1 promoter (XAP) were used. The pattern of activity of the HMW-Glu subunit 1Ax1 promoter was analyzed in *1Ax1::GFP* transgenic wheat and by tissue-specific qRT-PCR analysis of *VfAAP1* expression in XAP wheat. The result revealed that the

1Ax1 promoter is mainly active in developing grains. The spatial and temporal pattern of 1Ax1 promoter activity is changing in relation to wheat grain development. Very early during grain development, the 1Ax1 promoter is active in maternal tissue (nucellar tissues, pericarp and main vascular bundle) with highest expression in the nucellar projection. At the storage phase, the expression dramatically increases in the starchy endosperm, specifically in the endosperm wings. The high molecular weight (HMW) glutenin subunits represent the main seed storage proteins deposited in the endosperm of developing grains (Payne et al., 1987; Robert et al., 1989). HMW glutenin genes are expressed in the endosperm; they are encoded by 4-5 transcriptionally active genes in the hexaploid wheat genome (Halford et al., 1992). *Glu-1Ax1* is one of the active HMW glutenin genes, and it is considered as an endosperm-specific expressed gene (Altpeter et al., 1996). Previous studies demonstrate the strict endosperm specificity of HMW-Glu gene promoters in diverse species of transgenic plants, i.e., rice, tobacco, maize, *Brachypodium* and wheat (Lamacchia et al., 2001; Norre et al., 2002; Robert et al., 1989; Thilmony et al., 2014). On the contrary, activity of HMW-Glu in pericarp and vascular parenchyma of vegetative organs in addition to endosperm was reported in transgenic rice (Furtado et al., 2008). However, it is not known whether the promoter of the HMW glutenin gene is active in maternal grain tissues during early stages of grain development. The present study reveals evidence for expression of the HMW-Glu-1Ax1 promoter across developing maternal as well as filial grain tissues of transgenic wheat.

Promoter leakage is an often reported problem in transgenic plants. qRT-PCR analysis confirms slight activity of the 1Ax1 promoter in flag leaves and glumes during grain development. In XAP seedlings remarkable activity of the 1Ax1 promoter was observed in the cell maturity zone of seminal roots. Previous studies demonstrated leakiness of seed storage protein promoters in transgenic crops. It has been reported that the rice prolamin promoter is active in vegetative tissue of transgenic rice (Qu le and Takaiwa, 2004). The activity of barley D-hordein (*D-Hor*) storage protein promoter in root tips besides endosperm was detected in transgenic barely (Furtado et al., 2009). Similarly, activity of the globulin seed storage protein promoter, phaseolin (*phas*) from *Phaseolus vulgaris* and *Vicia faba* legumin B4 (*leB4*) were detected in pollen and seed coat of transgenic pea and narbon bean in addition to developing seeds (Zakharov et al., 2004). The expression of eukaryotic genes from their cognate promoters is often regulated by the action of transcriptional enhancer elements. Enhancer-mediated activation of adjacent promoters is a common property of many enhancers (Hily et al., 2009; Singer et al., 2010; Zheng et al., 2007). Previous studies demonstrated enhancer-mediated activation of adjacent tissue-specific promoters, which initiates the activity of promoter in non-targeted tissues (Gudynaite-Savitch et al., 2009; Hily et al., 2009; Liu et al., 2008; Zheng et al., 2007). Due to the random nature of transgene insertion in the majority of higher eukaryotes, transgenic DNA may integrate near to endogenous enhancers, which facilitates the interaction of enhancer and promoter. This interactive nature often provokes unexpected

interference in transgene expression. Most likely, the 1Ax1 promoter leakiness in XAP lines could be due to the influence of enhancer-promoter interactions.

In summary, the study confirms that the HMW-Glu-1Ax1 promoter is primarily active in developing transgenic endosperm. Besides, the 1Ax1 promoter has considerable activity in maternal grain tissues specifically in the nucellar projection during the pre-storage phase. Activity of the HMW-Glu-1Ax1 promoter was also registered in non-seed tissues of transgenic wheat, especially seedling roots. Position effects, which prompt inappropriate enhancer-promoter interactions, might be the possible reason for the leakiness of 1Ax1 promoter in XAP wheat.

6.4 Expression of *VfAAP1* might influence seedling root architecture in wheat

Root architecture is the net product of differential growth dynamics in different parts of the root (Lopez-Bucio et al., 2003). The fibrous root system of cereals is broadly divided into two categories; seminal roots (also called primary roots) that originate from primordia in the embryo, and the crown roots (also called secondary, adventitious, nodal, or brace roots) that arise from foliar nodes of the growing plant (Mattsson et al., 1993). The overall architecture of the creal root system depends on three main processes. The first one is the growth of pioneer roots, such as seminal and nodal roots, second, lateral root formation increases the exploratory capacity of the root system, and third, root-hair formation increases the total surface of seminal and lateral roots (Nibau et al., 2008; Robinson et al., 2016; Wu and Cheng, 2014). Root phenotyping in XAP lines reveal that XAP seedlings have altered root architecture when compared to the non-transformed control Certo. Significant increments in the number of seminal roots per plant, root dry mass and branching were observed in XAP seedling; however seminal roots in XAP lines are shorter than that of the control Certo. Root system architecture is known to be highly plastic, and under tight genetic control and modulated by developmental signals including plant hormones (Jung and McCouch, 2013; Malamy, 2005).

Seminal root traits are critical in cereal seedling because of their early development and association with root-system architecture of mature plants (Richard et al., 2015). Growth and development of seminal roots is largely controlled by inherent genetic factors (Manschadi et al., 2008; Robinson et al., 2016). XAP seedlings have a higher number of seminal roots, but the roots are shorter in length. Seminal root formation is mostly related to enhanced cell differentiation within the primary root meristem. The reduced seminal roots length usually correlates with the reduction of cell proliferation in the root elongation zone (Ticconi et al., 2004; Zhang et al., 2012). The altered seminal root traits of XAP seedlings might be due to the influence of the newly introduced gene. qRT-PCR analysis showed that *VfAAP1* expression was significantly different along the XAP seminal root parts. Highest expression was observed in cell maturation zone, and in cell elongation zone the expression was negligible. It is possible that

amino acid influx rate of cells in the maturity zone of XAP seminal roots might be enhanced, which might be related to stimulated induction of lateral roots along the seedling root system.

Lateral and adventitious root formation represents an important element of the adaptability of the root system to its environment. Analysis of root branching in XAP seedling reveal the dramatic increase of branching point in the cell maturity zone /upper part of XAP seedling roots, which indicates the stimulated lateral root formation. Among the root parts highest expression was observed in cell maturity zone. These findings suggest that *VfAAP1* expression might be coupled to enhanced root branching in XAP seedlings. Lateral root formation is a complex developmental process, which is tightly regulated by a complex interplay of different plant hormones (Nibau et al., 2008). The involvement of auxin in lateral root development is a well-studied phenomenon. Numerous observations suggest a close correlation between the level of auxin and lateral root formation. Elevated levels of auxin (exogenous application or enhanced synthesis) increase lateral root formation (Boerjan et al., 1995; Celenza et al., 1995). Root branching requires that primed pericycle founder cells undergo several rounds of cell division to form the lateral root primodium that gives rise to the new lateral root. The accumulation of high auxin levels in quiescent xylem pole pericycle or endodermal cells induced cell cycle reactivation and lateral root initiation (Benkova and Bielach, 2010; Casimiro et al., 2003; Malamy, 2005). The increased availability of N assimilates in XAP seedling roots might stimulate the auxin level in the respective region. Transcript profiling of XAP grains (highest expression of *VfAAP1*) indicates a significant shift in the expression of auxin-related genes. Accordingly, the enhanced root branching of XAP seedlings could be auxin-mediated.

In summary, the analysis of root phenotype revealed that XAP seedlings had altered root architecture when compared to the non-transformed control. Seminal root formation seems to be enhanced in XAP seedlings; however the seminal root elongation is obviously inhibited. Improved root branching and dry mass accumulation was observed in XAP seedlings. The altered root phenotype of XAP seedlings might be due to the expression of *VfAAP1* across the root system. In the cell maturity zone significant expression of *VfAAP1* was registered. Thereby, the sink capacity of cells in the respective region could be enhanced, which may facilitate the rate of cell division and development of new primordia for root branching.

6.5 Expression of *VfAAP1* stimulates grain Zn and Fe accumulation

Zinc and iron are essential minerals required for various metabolic functions. The cumulative amount of nutrients reserved in plant seeds depends mainly on the availability of each particular element in the soil and the ability of the plant to acquire and translocate the target nutrients to the respective sink organs. In cereals, it is well documented that the uptake of Zn and Fe from soil and their transport from stem or leaf to grain is facilitated by transporter proteins and N status of the plant (Aciksoz et al., 2011; Hall and Williams, 2003; Kobayashi and

Nishizawa, 2012; Menguer et al., 2013; Persson et al., 2016; Ramesh et al., 2004). In the present study, a remarkable improvement in Zn and Fe content of mature XAP grains was registered. Analysis of mature grains confirmed that the Fe and Zn pool are dramatically increased in XAP lines. In average 30-40% Fe and 20-25% Zn increments were registered in XAP lines when compared to the non-transformed control.

The positive effect of improved N nutrition on grain Zn and Fe content in wheat was frequently reported (Barunawati et al., 2013; Cakmak et al., 2010; Erenoglu et al., 2011; Kutman et al., 2010). Mostly, this beneficial effect is related to availability of N assimilates for the biosynthesis of nitrogenous chelator compounds and ion transporter proteins (Aciksoz et al., 2011; Barunawati et al., 2013; Kruger et al., 2002; Shi et al., 2012). Surprisingly, transcriptome analysis of developing XAP grains showed that a cluster of metal-transport related genes including metal-nicotianamine transporter (*YSL2*), vacuolar iron transporter (*VIT*) and Zinc1/3 transporters was upregulated. Most probably, the abundance of metal uptake transporter transcripts in developing grains might be enhanced by increasing the tissue concentration of amino acids. Perhaps, facilitated N assimilate uptake capacity of XAP lines might enhance the production of nitrogenous chelator compounds like nicotianamine and deoxymugineic acid that facilitate Zn and Fe transport activity in plant system (Curie et al., 2009; Palmer and Guerinot, 2009; Suzuki et al., 2008). Nicotianamine (NA) is ubiquitously present in higher plants and appears to be the major metal chelator especially for Fe translocation (Lee et al., 2011; Takahashi et al., 2003; von Wiren et al., 1999). The amino acid methionine is a precursor in NA biosynthesis (Shojima et al., 1989). Developing XAP grains are characterized by a shift towards a higher relative abundance of methionine. Correspondingly, genes related to the biosynthesis of S-adenosylmethionine (SEM) and NA: like s-adenosylmethionine decarboxylase proenzyme (*SAMD*), s-adenosyl-L-methionine-dependent methyltransferase (SAM-dependent MTase), nicotianamine synthase 6 (*NAS6*) are upregulated in the XAP endosperm. The synthesis of SAM is the first committed step in NA biosynthesis (Takahashi et al., 2003). Nicotianamine synthase is the key enzyme in the synthesis of NA; it catalyzes the trimerization of SAM to form one molecule of nicotianamine (Zhou et al., 2013). Overexpression of NAS genes in rice resulted in significant improvement in grain concentration of Fe, Zn, Cu, and NA (Johnson et al., 2011; Lee et al., 2011; Zheng et al., 2010). These findings suggest that synthesis of NA might be stimulated in XAP grains.

In previous studies, the positive effect of N nutrition on Fe and Zn translocation into cereal grains is correlated with improved grain protein content. It has been suggested that grain protein is a sink for Zn and Fe (Cakmak et al., 2010; Kutman et al., 2010; Persson et al., 2009). N assimilate availability can stimulate grain protein synthesis, and thereby the sink strength of the grain for Zn and Fe. The positive correlations between seed protein, Zn, and Fe concentrations have been documented in various studies (Ficco et al., 2009; Morgounov et al.,

2006; Peleg et al., 2007; Zhao et al., 2009). Accordingly, the enhanced grain protein content in XAP lines might have a contribution to the stimulated accumulation of Zn and Fe. Moreover, the availability of each particular element in the soil and /or the potential of the plant that uptake the target nutrients from the soil are directly related to the accumulated nutrients in the plant seeds. Root traits like lateral root production, root hair characteristics and seminal root numbers can influence the acquisition of mineral elements. These root traits are crucial for increasing the volume of soil explored by the root system and the surface area for the uptake of mineral elements. XAP lines show altered seedling root architecture characterized by increased root branching, higher number of seminal roots, and increased root biomass. The changed root architecture of XAP seedlings might improve the efficiency of Zn and Fe uptake.

In summary, the present study clearly shows that improving the availability of N assimilate in developing grains enhances the expression of metal transporters and increases the accumulation of Fe and Zn in mature grains. In a similar manner, the biosynthesis of NA seems to be enhanced in the XAP endosperm. These results suggest that increasing N assimilate uptake capacity of developing grains might facilitate the biosynthesis of metal ion transporters and metal chelator proteins. Accordingly, the translocation of Fe and Zn from the source tissues to developing grains could be facilitated which finally leads to improved Zn and Fe grain content. Thus, enhanced grain protein content and altered root traits of XAP wheat might contribute to stimulating grain Zn and Fe accumulation.

6.6 Concluding remarks

In many aspects, amino acids are the foundation of nitrogen metabolism in the plant system, and understanding their transport within cells and between organs is fundamental in plant biology. The development of sink organs such as seeds strongly depends on the import of carbohydrates and amino acids supplied by the maternal tissues via the phloem. To supply the essential assimilates for symplastically isolated filial tissues in developing seeds, active transport steps involving amino acid transporters like amino acid permeases (AAPs) are necessary. Much work has been concentrated on the molecular characterization of AAPs in different plant species. However, there is a lack of information as to how far amino acid transport activity in the seeds can be rate limiting for seed growth, and what are the physiological and biochemical consequences of increased seed sink strength for N in wheat. The present study analyzed the influence of improved sink strength by expressing the well-characterized *VfAAP1* under the control of the wheat HMW-Glu-*1Ax1* promoter in the winter wheat variety Certo. The possible influence of *VfAAP1* expression on the grain development process was discussed. The activity of the HMW-Glu-1Ax1 wheat promoter in generative and vegetative tissues of transgenic wheat lines was proven. The sink capacity of developing grains seems to be limited for organic nitrogen, and improving the amino acid uptake capacity of developing grains by overexpression of an alien amino acid permease improves grain growth rate. Amino acids imported by VfAAP1

into developing grains probably enhance cell division and expansion processes by modulating the nutrient homeostasis and phytohormones/hormone signaling in developing grains. Intensified cell division and elongation might lead to increased cell number and cell size of the developing endosperm, which is the key determinant of grain size and TGW in mature grain.

The genetic transformation of plants has become an important tool for plant biology research. However, unpredicted transgene expression is a challenge. The desired transgene expression patterns/levels are primarily determined by promoter activities. As a result, evaluating activity of the transgenic promoter in the transgenic plant is an essential approach. The HMW-Glu-1Ax1 gene promoter is active primarily in the endosperm with a preference for the endosperm wings during the storage phase. At early grain development, the 1Ax1 promoter is active in maternal tissues specifically in the nucellar projection. However, the 1Ax1 promoter is leaky in transgenic wheat. Slight activity was observed in flag leaf and glumes during grain development. In addition, remarkable activity of 1Ax1 promoter was registered in XAP seedling roots, specifically in the cell maturation zone of seminal roots. The activity of the 1Ax1 promoter in vegetative parts of XAP wheat might be due to the influence of position effects, which might facilitate inappropriate enhancer-promoter interactions.

The new transgenic winter wheat has shown altered seedling root architecture. Substantial variation in crucial seedling root traits has been observed in XAP lines. XAP lines displayed more seminal roots per plant, shorter seminal roots but higher root dry mass, and more branching points when compared to the non-transformed control. It seems that expression of *VfAAP1* in the root tissues has an influence on root morphology. qRT-PCR reveled that *VfAAP1* was expressed in the cell maturity zone of seminal roots. Thus, amino acid influx rate into cells of the respective region might be stimulated. This may facilitate the rate of cell division and development of new primordia for root branching, resulting in higher number of lateral roots and increased root dry mass accumulation in XAP lines. In cell of the elongation zone expression of *VfAAP1* was negligible, cells in this region might be less competent than the cells from the maturity zone for lateral root formation.

Increasing micronutrient concentration of cereal grains is a global challenge to combat human malnutrition of Fe and Zn. Another importance of enhanced micronutrient content of grains could be in crop production to establish vigorous seedlings. The close relation between N status of the plant and grain Fe and Zn content in wheat is a well-known phenomenon. The present study showed that improving the amino acid influx rate of developing grains by expressing *VfAAP1* can increase the grain Zn and Fe content. Expression of metal-transport related genes is stimulated in XAP lines. Higher relative abundance of methionine which is the precursor of NA is observed in developing XAP grains. Accordingly, genes related to the synthesis of S-adenosylmethionine (SAM), the first step in NA biosynthesis, show upregulation in XAP

developing grains. Furthermore, the gene encoding nicotianamine synthase (*NAS6*), which catalyzes the trimerization of SAM to form NA, is upregulated in the XAP endosperm. Altogether, this study indicates that improving amino acid availability in developing grains represent a promising approach to improve micronutrient contents in wheat grains.

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Appendices

Appendix I-A Yield and yield related parameters in XAP lines under field-like condition.

Growing season	2012/13				2013/14			
Lines	**XAP:BB**	**XAP:CC**	**XAP:BBCC**	**Certo**	**XAP:BB**	**XAP:CC**	**XAP:BBCC**	**Certo**
Grain yield (kg/plot)	0.533±0.05***	0.555±0.08***	0.545±0.05***	0.424±0.06	0.833±0.06***	0.860±0.08***	0.922±0.06***	0.701±0.1
TGW (g)	56.78±1.7***	58.5± 1.36***	54.96±1.12***	45.83±1.5	54.2±2.4***	56.8± 3.8***	52.11± 1.7***	44.88±0.8
Spikes No /plot	365.2 ±18.5	363.8 ±14.2	381.9 ±17.4	359.2±12.1	488.8± 16.1	507.12± 11.5	529.6 ± 8.5	481.8±14
Grain No /spike	25.2 ±1.9	24.8 ±1.6	24.5 ±1.24	26.3 ±3.0	31.74 ±2.01	29.8 ± 1.55	31.53 ± 1.37	33.07±2.5

Appendix I-B Micronutrient concentration in the XAP grains

Line	Cropping season	Grain micronutrient concentration [mg/kg DW]				
		Fe	Zn	Mn	Mo	Ni
XAP:CC	2012/13	84.80 ±5.94***	65.89± 6.43***	80.35±8.14**	1.13±0.03**	0.91±0.15***
	2013/14	80.25±2.8***	61.12±3.1***	65.36±3.01***	1.19±0.06**	0.65± 0.15***
XAP:BB	2012/13	81.90±2.71***	61.17±3.61***	81.99± 9.39***	1.26±0.04	0.88±0.28***
	2013/14	69.22±4.8***	54.35±3.7***	65.31±4.01**	1.31±0.07	0.61±0.09***
XAP:BBCC	2012/13	83.21±7.21***	58.15±1.33***	68.52±4.14***	1.11±0.01**	0.75±0.02***
	2013/14	71.74±3.7***	53.87±3.4***	61.80±2.6***	1.12±0.05**	0.66±0.12***
Certo (WT)	2012/13	56.25±4.26	47.71±4.64	62.23±6.01	1.37±0.06	0.51±0.09
	2013/14	53.72±2.6	45.38±2.25	58.75±2.8	1.41±0.1	0.48±0.08

The data represent the mean ± SD, n=10, plot size =0.5m^2

Significant differences between XAP lines and Certo according to student t-test (*** P≤0.001, ** P≤ 0.01, * P≤0.05).

Appendix II primer list used for qRT-PCR analysis

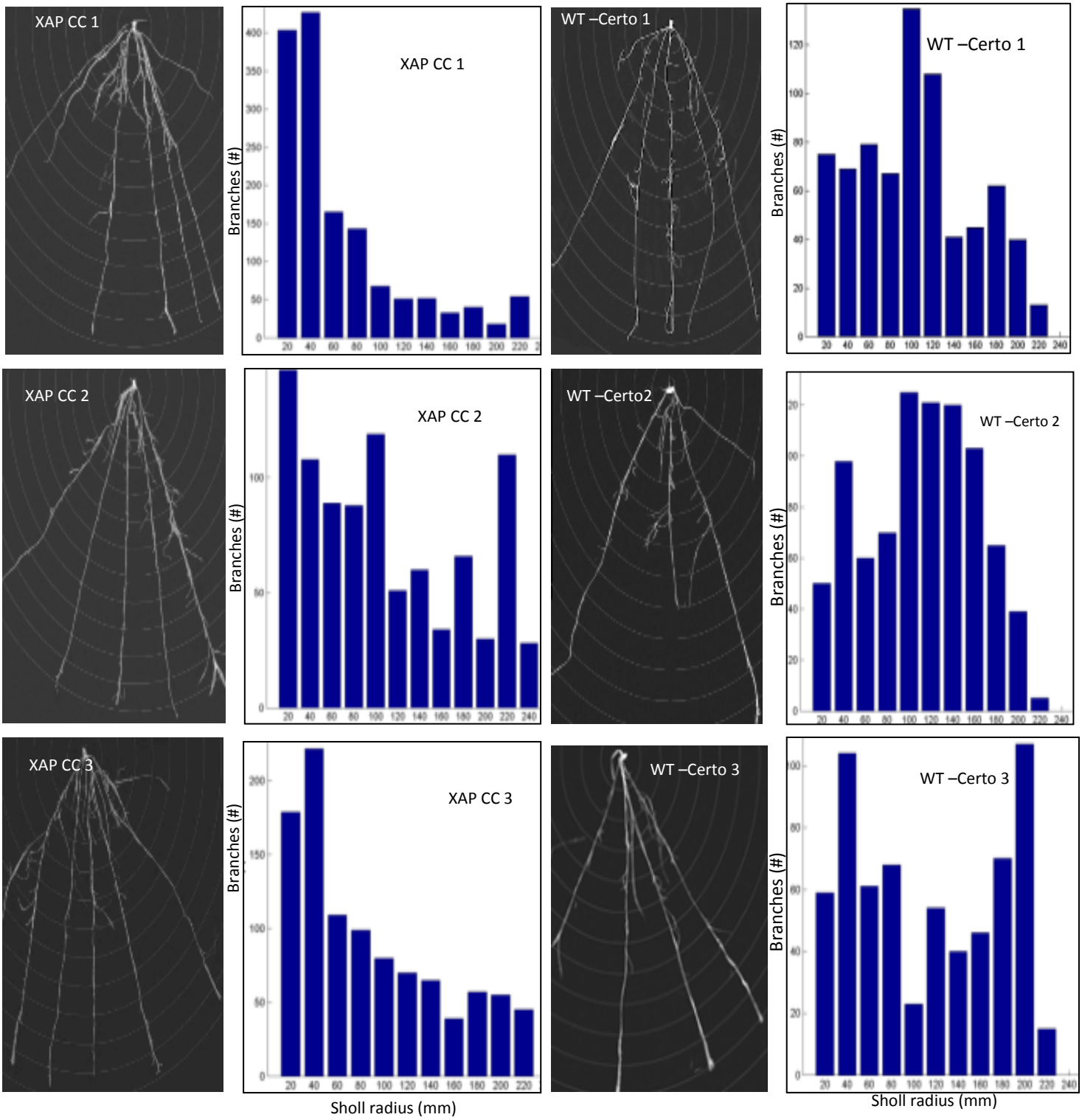
Gene	Accession	Primer Set	Sequence	Start Position	Length	Primer Tm°C
TaActin	AB181991	Forward	GCAACATTGTGCTCAGTGGTG	673	21	59
		Reverse	GCTAAGAGAGGCCAAAATAGAGCC	824	24	59
eGFP	JQ064510	Forward	ATCATGGCCGACAAGCAGA	421	19	57
		Reverse	ATGTTGTGGCGGATCTTGAAG	481	20	60
VfAAP1	AJ318809	Forward	AACGCCGCTCTCATAATTCATC	1011	22	59
		Reverse	CAAATAAGGGTTGCGCGTAAA	1071	21	59

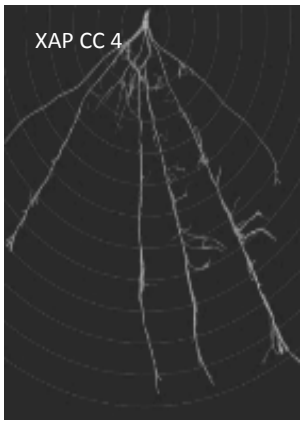
Appendix III-A component and concentration of nutrition solution used for *in-vitro* seedling root experiment. The nutrition solution was prepared as described by Song et al. (2007).

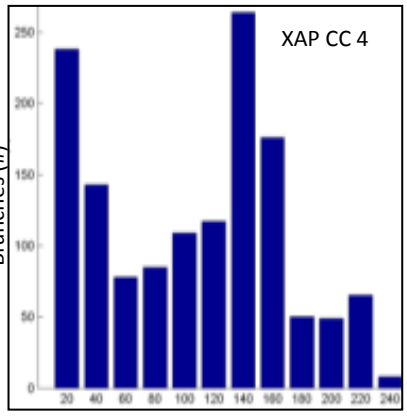
component	MW	concentration (molarity)	Mass per liter of solution (mg/l)
$CaCl_2.2H_2O$	147	1 mM	147.0
KH_2PO_4	136	0.5 mM	68.00
Fe-citrat $C_6H_5FeO_7$	250	10 µM	2.50
$MgSO_4.7H_2O$	246	0.25 mM	61.50
$K2SO_4$	174	0.25 mM	43.50
$MnSO_4.H_2O$	169	1 µM	0.169
H_3BO_3	62	2 µM	0.124
$ZnSO_4.7H_2O$	287	0.5 µM	0.1435
$CuSO_4.5H_2O$	249	0.1µM	0.0498
$CoC_{l2}.6H_2O$	237	0.1µM	0.0237
$Na_2MoO_4.2H_2O$	242	0.1µM	0.0242

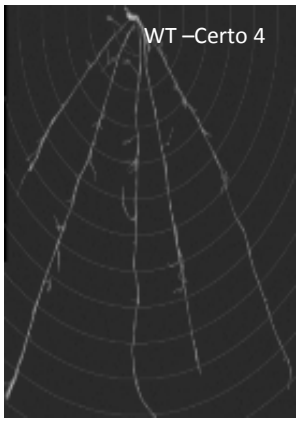
Note:pH adjusted at = 5.8

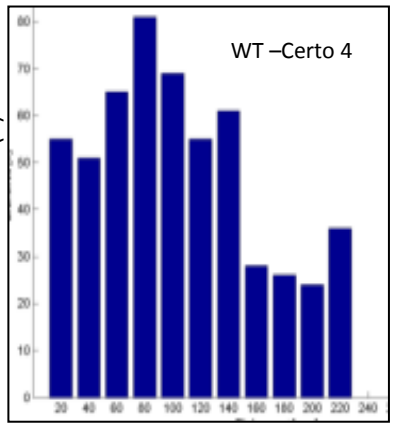
Appendix III-B Sholl analyses of branching points in seedling roots of lines XAP CC and Certo under *in-vitro* conditions. At the three-leaves stage, digital images were produced from harvested roots. Root branching points were evaluated by Sholl analyses from the digital images. Images from ten seedlings per line harvested from ten pots were analyzed; branching points across subsequent Sholl radii were calculated.

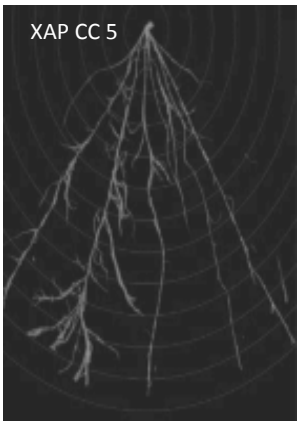


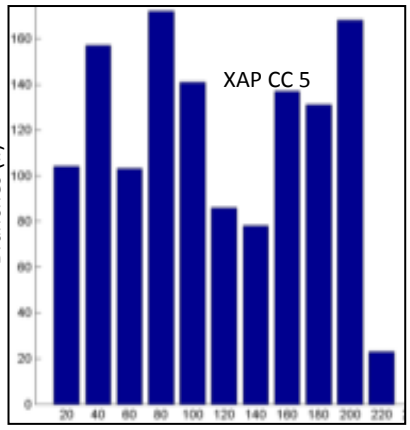


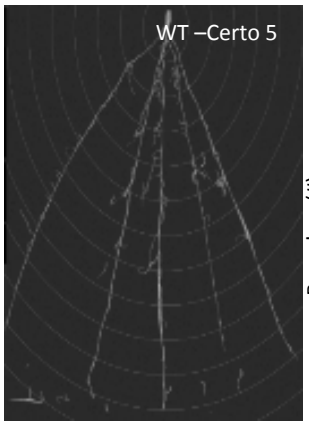


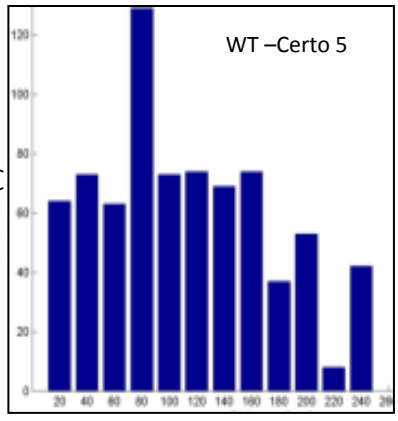


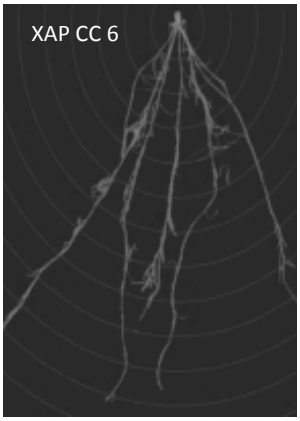


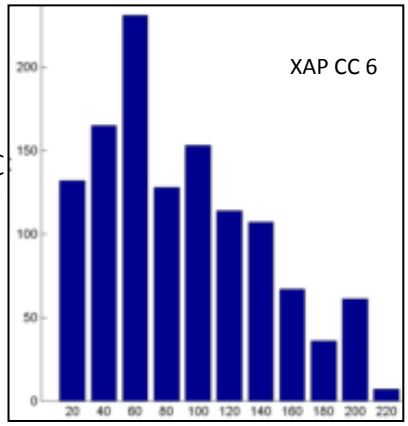


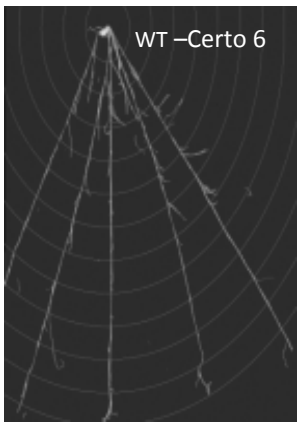


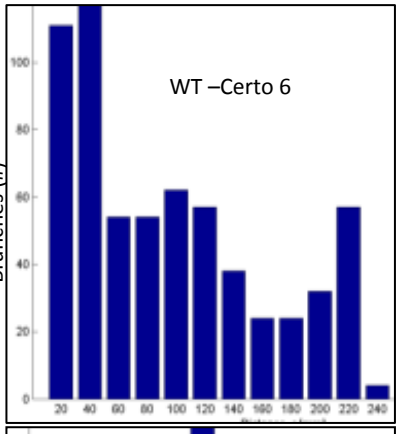


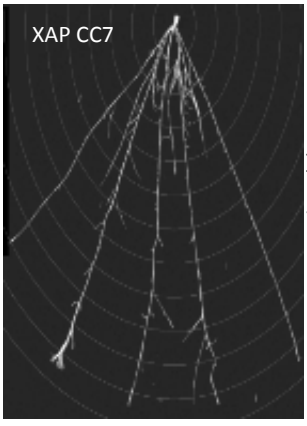


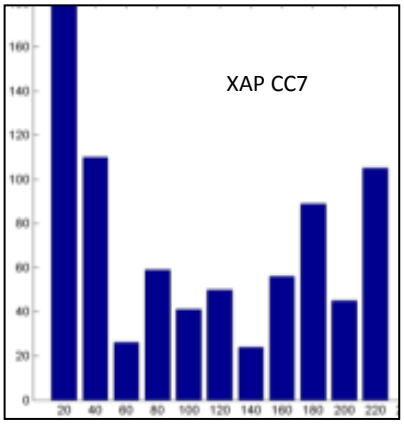


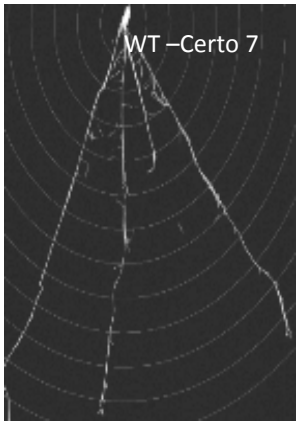


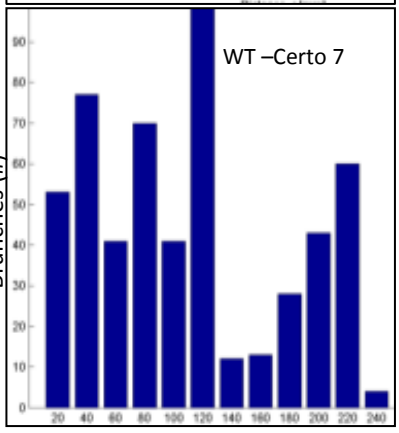






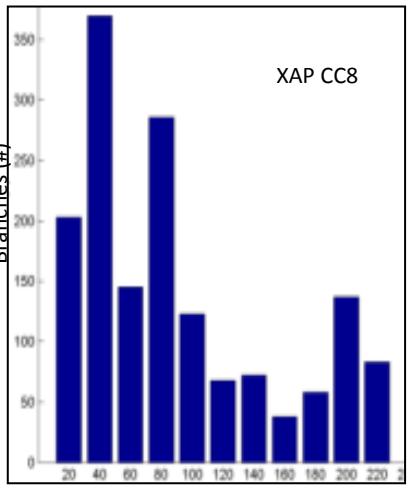


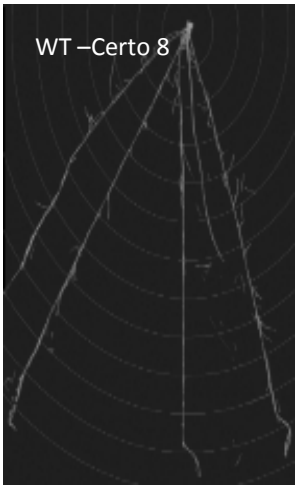


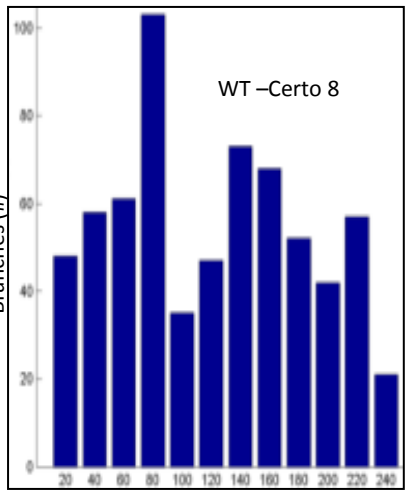


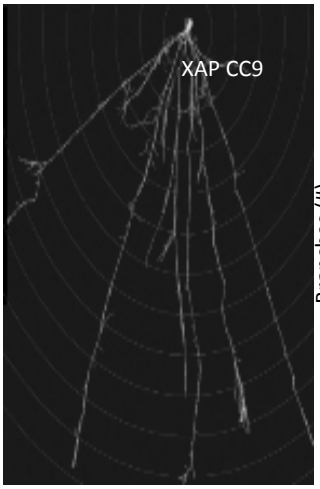
Sholl radius (mm)

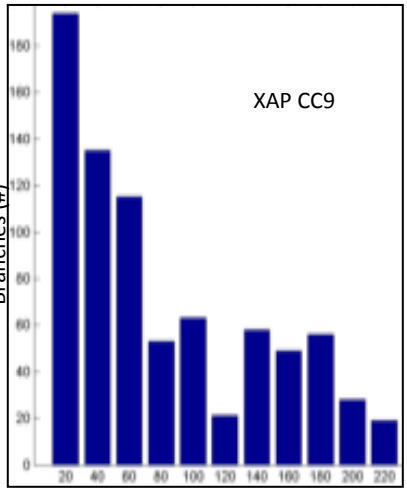
Sholl radius (mm)

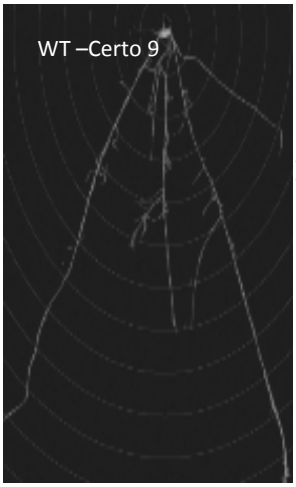


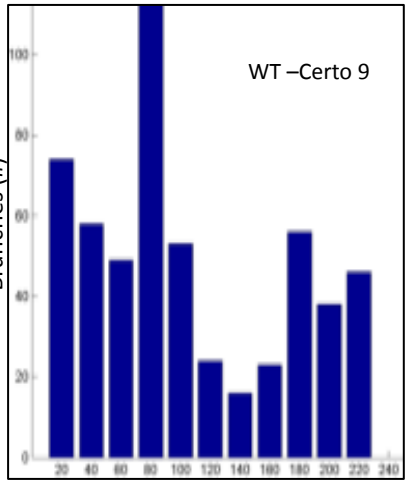


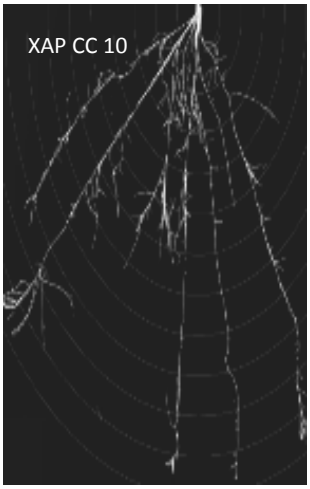


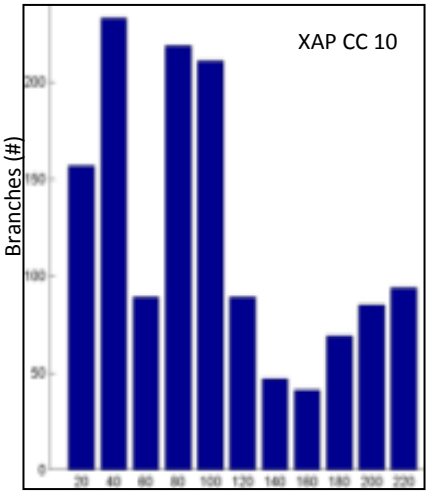


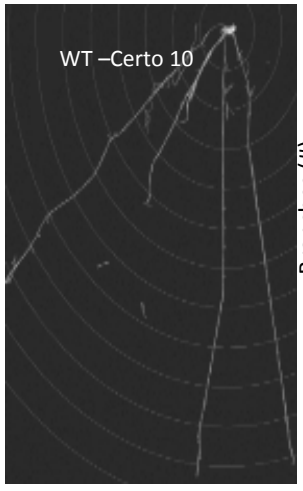


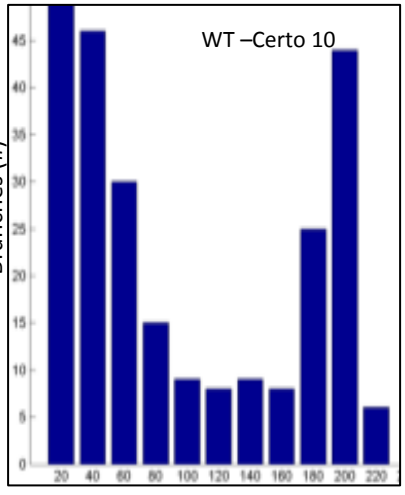












Sholl radius (mm)

Sholl radius (mm)

Appendix IV A List of differentially expressed genes in XAP line CC pericarp group as related to distinct functional clusters. Differentially Expressed genes at five stages of grain development (4, 6, 8 and 10, and 14 DAF) were identified. At three stages (4, 6 and 14 DAF) whole developing grains were used for analysis, whereas at 8 and 10 DAF pericarp and endosperm tissues were analyzed separately. Four stages (4, 6, 8 and 10DAF) grouped as pericarp, moderately t-test with corrected P-value (Westfall-Young. $P \leq 0.05$) was used.

Seq ID	Best Blast Hit wheat	Log 2 FC (XAP: Certo) Pericarp				InterPRO-IDs	Description/Annotation
		4DAF	6DAF	8DAF	10DAF		**Cell proliferation**
Wht_03664		3.97	2.12	2.69	1.34		Protein cell differentiation protein rcd1
Wht_35452	Traes_5BL_CDA23F02D.1	2.41	0.92	1.95	0.56	IPR004314	Villin-4 actin binding
Wht_21163		2.36	1.55	1.59	0.05		BIGYIN, FIS1A tetratricopeptide repeat (TPR) like super family protein
Wht_41570	Traes_1AL_789B53D8F.1	1.87	0.24	0.14	-0.22	IPR005045	Cell cycle control protein 50A
Wht_24047	Traes_5DL_FFF0AA252.1	1.03	0.28	0.19	0.74	IPR004000	Actin-related protein 4
Wht_56221	Traes_2AS_55BD046C1.1	0.15	1.16	2.12	2.18	IPR000795	Elongation factor. GTP-binding domain
Wht_24630	Traes_1DL_F0F3E1096.1	0.01	0.52	1.49	0.24	IPR005225	Gtpase Era, GTP binding protein
Wht_38489	Traes_4AS_4521FE57B.1	0.20	0.29	1.23	2.04	IPR009438	Phytosulfokines 2
Wht_32735	Traes_6AL_7DF57A1F6.1	-0.17	0.52	1.39	0.34	IPR005225	Actin-related protein 3
Wht_26792	Traes_7BL_0E93FBCFF.1	0.19	-0.07	0.14	1.48	IPR001045	Spermidine synthase 3
Wht_27270	Traes_3B_319690489.1	0.02	-0.54	0.40	1.10	IPR004000	Actin 7
Wht_02401	Traes_6AL_50F1E1AF4.1	0.21	-0.48	1.02	-0.04	IPR011009	Cyclin G associated kinase
Wht_15286	Traes_1DL_F6968A58B.1	0.56	-0.16	-0.05	1.03	IPR008921	Replication factor C subunit 5
Wht_12685	Traes_3B_6589A8C2E.1	-0.05	-0.02	-0.50	1.36		Cyclin D2
Wht_51697	Traes_XX_ADA644630.1	-0.22	-0.69	-0.57	1.20	IPR005045	Cell cycle control protein 50A
Wht_55361	Traes_XX_A23164C28.1	-0.12	0.37	-1.64	-0.31	IPR003175	Cyclin-dependent kinase inhibitor 5
							Cell wall
Wht_46745		3.25	3.17	2.02	1.75		Cellulose synthase
Wht_12809	Traes_2BS_FB140D3A3.1	3.11	0.93	1.36	1.60	IPR007118	Expansin 11
Wht_37539	Traes_2AL_D3715CC49.1	2.32	0.51	1.59	0.17	IPR006918	COBRA-like protein 7
Wht_00569	Traes_2AL_FCCDDB9081.1	1.42	0.68	1.50	0.59	IPR006501	Plant invertase/pectinmethylesterase inhibitor
Wht_51406	Traes_XX_F880AD2BF1.1	1.04	-0.07	1.09	1.17	IPR023271	Aquaporin-like
Wht_03705	Traes_2AL_5DC1B4A0F.1	1.54	1.08	0.51	-0.66	IPR011050	Pectinlyase-like s
Wht_35731	Traes_3B_1CB6BA55F.1	1.33	-0.01	0.72	-0.58	IPR011050	Pectinesterase
Wht_15490	Traes_4DL_A950283A1.1	1.10	0.34	0.89	0.92	IPR008985	Xyloglucan endotransglucosylase hydrolase 32
Wht_14924	Traes_4DL_BB4D6F40A.1	1.29	0.27	0.20	0.62	IPR023271	Aquaporin-like
Wht_53088	Traes_2BS_58E7D5315.1	0.06	-0.37	1.63	-0.63	IPR001509	UDP-glucose 4-epimerase
Wht_32050	Traes_2BL_63C8E35E4.1	-0.49	0.32	1.50	-0.72	IPR000782	FASCICLIN-like arabinogalactan 1
Wht_12617	Traes_3B_319A2B700.1	0.11	-0.34	0.54	1.55	IPR007118	Expansin
Wht_41194	Traes_XX_37E78F29C.1	-0.59	0.07	0.35	1.44	IPR005150	Cellulose synthase-like D1
Wht_12917	Traes_4DL_74A03B3BE.1	-0.35	-0.07	0.11	1.17	IPR007118	Expansin-like A1
Wht_03747	Traes_XX_A912CAA50.1	-0.18	0.47	-0.49	1.07	IPR005150	Cellulose synthase-like B4
Wht_41616	Traes_5AL_1EDC5886C.1	-0.64	-0.36	1.19	0.01	IPR026953	Callose synthase 1
Wht_32261	Traes_4AS_A65DDCD05.1	0.29	0.23	1.08	-0.23	IPR007118	Expansin 11
Wht_15402	Traes_3B_258E29040.1	0.57	0.75	1.05	-0.14	IPR007118	Expansin B2
Wht_07268	Traes_3B_66B6D3BD9.1	-0.13	-0.07	1.04	0.09		Expansin
Wht_25168	Traes_2DS_98D6694AB.1	0.26	0.31	-1.07	-0.27		Cellulose synthase-like A3

Seq ID	Best Blast Hit wheat	Log 2 FC (XAP: Certo) Pericarp				InterPRO-IDs	Description/Annotation
		4 DAF	6 DAF	8DAF	10DAF		C&N metabolism
Wht_15662		2.89	1.03	-0.11	0.45		Asparagine synthase
Wht_00295	Traes_2BL_2C62185EE.1	1.72	0.16	2.10	0.41	IPR015902	1.4-alpha-glucan branching enzyme GlgB
Wht_27710	Traes_4AL_C6DEABC36.1	1.27	0.49	1.79	-0.10	IPR001362	Acid beta-fructofuranosidase
Wht_01089	Traes_6AS_06B794C38.1	1.78	0.43	0.03	1.68	IPR015424	Aspartate aminotransferase
Wht_22010	Traes_5DL_A4A06524C.1	1.48	-0.34	0.78	2.14	IPR002213	UDP-glycosyltransferase 73B4
Wht_35541	Traes_XX_C6BDB5B11.1	1.03	0.95	0.38	2.42	IPR015947	Sulfate adenylyltransferase
Wht_45825	Traes_3B_D7F84C82B.1	1.24	0.43	0.88	1.14	IPR012946	Glucan endo-1.3-beta-glucosidase-like 3
Wht_08692	Traes_4AS_C19AB3A1A.1	1.66	0.31	0.53	0.56	IPR006426	Asparagine synthetase 2
Wht_10055	Traes_7DL_D39A0193F.1	1.50	0.31	0.79	0.02	IPR015902	1.4-alpha-glucan branching enzyme GlgB
Wht_29049	Traes_XX_B58E30BDA.1	1.34	0.72	0.32	-0.15	IPR010121	Phosphate dikinase 2.
Wht_46996	Traes_XX_FADEBE202.1	-0.33	0.65	2.18	0.00	IPR009014	Pyruvate dehydrogenasee1.subunit beta
Wht_56117	Traes_XX_EF8FE0BF7.1	0.65	0.51	1.32	0.70	IPR011183	Phosphofructokinase family protein
Wht_30492	Traes_7AS_089028EAD.1	0.15	0.35	1.24	-0.68	IPR015424	Serine-glyoxylate aminotransferase
Wht_53088	Traes_2BS_58E7D5315.1	0.06	0.78	1.63	-0.63	IPR001509	UDP-glucose 4-epimerase
Wht_23335	Traes_2BS_D76508CF5.1	-0.09	0.23	1.36	0.45	IPR000490	Glucan endo-1.3-beta-glucosidase 1
Wht_28217	Traes_XX_7E8AC13B0.1	0.35	0.65	0.45	2.13	IPR002213	UDP-Glycosyltransferase superfamily
Wht_25863	Traes_XX_F3113E1E1.1	0.37	0.11	0.59	1.32	IPR002213	UDP-Glycosyltransferase superfamily
Wht_25475		0.54	-0.27	-0.08	1.44		FAD-binding protein
Wht_08223	Traes_5BL_94F42F9A7.1	0.42	0.98	-0.49	1.22	IPR001830	Trehalose phosphate synthase
Wht_01394	Traes_2BL_7E665182C.1	0.73	0.30	0.55	1.19	IPR014006	Succinate dehydrogenase.
Wht_27143	Traes_3B_D003ACECF.1	0.11	-0.12	-0.16	1.03	IPR023753	NADH glutamate synthase
Wht_54337	Traes_4DL_A43826645.1	0.11	-0.85	0.07	1.18	IPR000453	Chorismate synthase
Wht_09398	Traes_6DL_33F8A5EF4.1	0.08	-0.80	-0.64	1.52	IPR001830	Trehalose phosphate synthase
Wht_24452	Traes_2DL_06E6A2543.1	0.31	-0.54	-0.41	1.10	IPR001282	Glucose-6-phosphate dehydrogenase 6
Wht_00620	Traes_XX_422555914.1	0.15	-0.06	-0.82	1.09	IPR001830	Trehalose phosphate synthase
Wht_14880	Traes_2BL_7F9F8D481.1	-0.15	-0.76	0.10	2.01	IPR001362	Beta-fructofuranosidase. Insoluble
Wht_54798	Traes_1BL_7905E5D6E.1	-0.23	-0.01	-0.33	1.30	IPR006329	AMP deaminase
Wht_36370	Traes_XX_0D4942798.1	-0.52	-0.15	0.22	1.88	IPR002912	Protein-PII. Uridylyltransferase
Wht_25121	Traes_XX_CFE3B7CF1.1	-0.67	-0.31	0.98	0.97	IPR000490	Glucan endo-1.3-beta-glucosidase
Wht_25929	Traes_3B_F88542D34.1	-0.68	-0.39	0.05	1.07	IPR003406	Core-2/i-branching beta-1.6-N-acetylglucosaminyltransferase
Wht_36153	Traes_7AS_069A1FA77.1	-0.07	-1.92	-0.04	0.25	IPR000623	Shikimate kinase 1
Wht_28200	Traes_2BS_891EC5836.1	-0.71	-0.89	0.10	1.66	IPR002213	UDP-glycosyltransferase superfamily
Wht_20671	Traes_3B_89F6ACF89.1	-1.39	-1.20	-0.28	-0.30	IPR001362	Beta-fructofuranosidase. Insoluble
Wht_14865	Traes_XX_A33491620.1	-1.49	-1.31	-0.86	0.09	IPR001554	Beta-amylase 1
							Lipid metabolism
Wht_27764	Traes_XX_D06B51739.1	-1.17	-0.73	2.61	-0.33	IPR012400	Long-chain fatty alcohol dehydrogenase family protein
Wht_27181	Traes_4BL_A71648154.1	0.23	-0.05	2.07	-0.20	IPR012392	3-ketoacyl-CoA synthase 10
Wht_04018	Traes_3DL_2E7AEF61D.1	-0.54	-0.73	1.63	1.05	IPR013537	acetyl-CoA carboxylase 1
Wht_31889	Traes_3B_592CE6F7F.1	0.21	-0.05	1.14	-0.21	IPR002155	3-ketoacyl-CoA thiolase
Wht_35207	Traes_6AL_01081953F.1	0.19	0.19	0.59	0.99	IPR005352	Ergosterol biosynthetic protein 28
Wht_00309	Traes_XX_C827252DA.1	-0.24	-0.09	-0.59	1.61	IPR006694	fatty acid hydroxylase superfamily
Wht_26305	Traes_3B_6577A9FBF.1	0.67	-0.21	-0.32	1.64	IPR000863	sulfotransferase 17
Wht_24291	Traes_XX_5D7D85859.1	0.32	-0.32	-0.24	0.86	IPR005804	fatty acid desaturase 2
Wht_14925	Traes_7DL_CB741047A.1	-0.78	-2.10	-0.75	2.33	IPR006694	fatty acid hydroxylase superfamily
Wht_23660	Traes_6AL_64A7E528C.1	-0.71	-0.44	-1.66	-0.41	IPR006694	fatty acid hydroxylase superfamily
Wht_00480	Traes_4DS_849C911C9.1	-0.61	-0.76	-2.02	-0.27	IPR016040	fatty acyl-CoA reductase 3
Wht_26181	Traes_1BL_6419FCF64.1	-0.43	-1.52	-0.63	0.45	IPR004277	phosphatidylserine synthase 2
Wht_43385	Traes_3AS_9B5BD6076.1	-2.16	-1.77	-0.85	-0.55	IPR026055	fatty acyl-CoA reductase 1
Wht_43492	Traes_XX_FD64FEA65.1	-0.30	-2.50	-1.39	0.47	IPR000873	long-chain-fatty-acid--CoA ligase 1
Wht_14663	Traes_3B_FBE2447AA2.1	-1.91	-1.50	-1.43	1.95	IPR000490	glucan endo-1.3-beta-glucosidase GI

Seq ID	Best Blast Hit wheat	Log 2 FC (XAP: Certo) Pericarp				InterPRO-IDs	Description/Annotation
		4 DAF	6 DAF	8 DAF	10 DAF		**Storage protein**
Wht_02297		2.98	0.70	3.09	-0.53		Avenin-like
Wht_37258	Traes_3B_14528E636.1	2.63	0.99	2.14	1.09	IPR013128	Cathepsin B-like cysteine proteinase 6
Wht_35063	Traes_3DS_D718FF51C1.1	1.97	-0.05	2.21	0.64	IPR006106	Alpha-amylase inhibitor 0.19
Wht_35063	Traes_3DS_D718FF51C1.1	1.97	-0.05	2.21	0.64	IPR006106	Alpha-amylase inhibitor 0.19
Wht_30449	Traes_6DS_1854119F9.1	1.89	0.95	2.31	-0.41	IPR016140	Alpha-amylase inhibitor 0.28
Wht_31428	Traes_5DS_0BFE4FD87.1	1.87	0.25	3.36	-1.13	IPR016140	Hordoindoline-B1
Wht_31428	Traes_5DS_0BFE4FD87.1	1.87	0.25	3.36	-1.13	IPR016140	Hordoindoline-B1
Wht_31410	Traes_2BL_DE4C02E941.1	1.77	0.97	2.33	1.08	IPR027214	Cysteine proteinase inhibitor 8
Wht_35430	Traes_XX_C151A8139.1	0.88	0.04	0.32	0.90	IPR016140	Seed storage 2S albumin s
Wht_11437	Traes_7AS_DF9A9B7BF1.1	-0.16	1.29	2.24	1.77	IPR001954	Glutenin, low molecular weight subunit
Wht_03113	Traes_1DS_66B67E9B41.1	2,76	1,14	0,87	0,61	IPR001954	Glutenin, low molecular weight subunit
Wht_02363	Traes_1DS_66B67E9B41.1	3,82	0,68	0,11	0,41	IPR001954	Glutenin, low molecular weight subunit
Wht_01486	Traes_1DS_66B67E9B41.1	2,51	0,61	0,20	0,18	IPR001954	Glutenin, low molecular weight subunit
Wht_01903	Traes_1DS_66B67E9B41.1	2,61	0,80	0,18	0,86	IPR001954	Glutenin, low molecular weight subunit
Wht_21908	Traes_1DS_D29705572.1	-0.02	-1.81	-0.03	-0.03		Protein early responsive to dehydration 15
Wht_12967	Traes_1AL_DDEE6AC00.1	-0.06	-1.44	0.03	0.75	IPR004864	Late embryogenesis abundant protein
Wht_02465	Traes_XX_146FE45EA.1	-0.31	-1.97	-0.09	-0.12	IPR004864	Late embryogenesis abundant
Wht_00914	Traes_XX_994BDAE15.1	-1.01	-2.10	0.64	-0.34	IPR004864	Late embryogenesis abundant (
Wht_25730	Traes_3B_8B604D9E0.1	-1.20	-1.67	-2.01	-1.73	IPR004926	Late embryogenesis abundant protein Lea5
							Hormone-related
Wht_35400	Traes_4BL_8FD2DD629.1	0.10	0.99	2.13	0.71	IPR004776	Auxin efflux carrier family protein
Wht_35151	Traes_7DL_42A948988.1	-0.13	0.41	1.75	0.38	IPR003676	SAUR-like auxin-responsive protein family
Wht_03477	Traes_3B_A796206A0.1	1.58	0.68	1.29	-0.10	IPR004827	ABSCISIC ACID-INSENSITIVE 5-like protein 2
Wht_36268	Traes_2DS_9C0C983B2.1	0.79	1.06	0.26	2.09	IPR011006	Ethylene receptor 2
Wht_09245	Traes_2DS_CD1FD0261.1	0.90	1.00	-0.02	1.41	IPR001128	Cyp 85a1. Brassinosteroid 6-oxidase (
Wht_14716	Traes_XX_A27776CCF.1	0.29	0.21	0.01	1.45	IPR002933	IAA-amino acid hydrolase ILR1-like 6
Wht_44578	Traes_7DS_E1BAD4250.1	0.58	0.00	-0.33	1.20	IPR013094	Gibberellin receptor GID1L2
Wht_44853	Traes_1AL_DBFD7C798.1	0.66	-0.29	-0.04	2.20	IPR005269	Cytokinin riboside 5'-monophosphate phosphoribohydrolase LOG
Wht_52494	Traes_3B_F880EDE81.1	-0.23	-1.75	-0.27	1.78	IPR003311	Auxin-responsive protein IAA6
Wht_22309	Traes_2AS_A8CCC32D3.1	-0.27	-0.79	0.15	1.32	IPR010399	Jasmonate-zim-domain protein 1
Wht_13212	Traes_3B_82E1F5484.1	-0.97	-0.15	0.05	1.21	IPR016177	Ethylene-responsive transcription factor 11
Wht_27856	Traes_1DS_CA4185D11.1	-0.30	-0.25	-1.08	1.14	IPR003311	Auxin-responsive protein IAA4
Wht_14617	Traes_XX_1BFCC407E.1	-0.05	-0.45	-0.06	1.03	IPR005269	CK riboside 5'-monophosphate phosphoribohydrolase LOG1
Wht_14675	Traes_3B_CE9057560.1	-0.49	-0.02	-1.04	-1.08	IPR016170	Cytokinin dehydrogenase 1
Wht_22240	Traes_5DS_76A4D5D4E.1	-0.50	-0.65	0.38	-1.05	IPR003311	Auxin-responsive protein IAA30
Wht_20386	Traes_4BS_28759156D.1	-1.13	-1.37	-1.86	0.00	IPR003854	Gibberellin-regulated family protein
Wht_26428	Traes_3B_A25F058E5.1	-0.39	-1.38	-0.14	0.16	IPR005202	GRAS family transcription factor
Wht_14871	Traes_1BL_4B1CFEF1C.1	-0.04	-1.41	-0.10	0.52	IPR003311	Auxin-responsive protein IAA19
Wht_25117	Traes_4DS_8075EBF55.1	-1.12	-1.45	-1.66	-0.75	IPR003854	Gibberellin-regulated family protein
Wht_03207	Traes_1BS_FA0EFB1B8.1	0.31	-1.58	-0.20	-0.44	IPR027443	1-aminocyclopropane-1-carboxylate oxidase
Wht_43009	Traes_2DS_2D035BAFD.1	-0.01	-1.81	0.28	0.38	IPR005202	GRAS family transcription factor
Wht_29975	Traes_XX_907E287B6.1	-0.10	-2.06	-0.42	0.60	IPR016177	Ethylene-responsive transcription factor 1A
Wht_22152	Traes_4DL_7564D43A9.1	-0.32	-2.14	0.24	1.03	IPR018467	Jasmonate-zim-domain protein 1
Wht_00682	Traes_XX_CBC33C43B1.1	-0.38	-2.45	0.04	0.04	IPR016177	Ethylene-responsive transcription factor 4
Wht_16070	Traes_XX_28D711C9D.1	-0.54	-2.56	-0.05	0.14	IPR016177	Ethylene-responsive transcription factor 4
Wht_07949	Traes_7DS_0B70FFE9A.1	-0.28	-3.08	0.32	-0.04	IPR018467	Jasmonate-zim-domain protein 6

Seq ID	Best Blast Hit wheat	Log 2 FC (XAP: Certo) Pericarp				InterPRO-IDs	Description/Annotation
		4 DAF	6 DAF	8DAF	10 DAF		Signaling
Wht_03500	Traes_7AS_6A126F5F5.1	3.30	0.99	1.69	0.08	IPR009057	MYB-like transcription factor family protein
Wht_36693	Traes_1AL_FF8F7A939.1	2.91	1.06	0.05	0.51	IPR009057	MYB-like transcription factor family protein
Wht_35504	Traes_XX_E535992C1.1	2.38	0.41	0.62	0.20	IPR012336	Glutaredoxin family protein
Wht_03477	Traes_3B_A796206A0.1	1.58	0.68	1.29	-0.10	IPR004827	BZip type transcription factor TaABI5
Wht_36619	Traes_2AS_5810DD9381.1	1.53	1.00	0.60	0.60	IPR013210	Receptor-like kinase 1
Wht_05735	Traes_2AL_BB7692067.1	0.81	0.58	0.01	0.97	IPR011992	Calcium uptake protein 1. mitochondrial
Wht_11242	Traes_XX_DCD24767C.1	0.69	0.75	-0.28	2.93	IPR009057	MYB-like transcription factor family protein
Wht_14106	Traes_3AL_7A685D3E8.1	0.67	0.71	0.76	1.30	IPR011992	Calcium-dependent protein kinase 34
Wht_54383	Traes_7BL_9032EFE86.1	0.64	0.04	0.31	1.25	IPR011009	Mitogen-activated protein kinase 12
Wht_41561	Traes_7AL_B58112C48.1	0.48	1.30	1.89	2.19	IPR005735	Protein LSD1
Wht_03397	Traes_2BL_6B75B32E3.1	0.35	-0.28	0.16	1.30	IPR003657	WRKY DNA-binding protein 11
Wht_38699	Traes_1DL_EDF770437.1	0.33	-0.12	-0.10	1.04	IPR015404	Sorting nexin 2A
Wht_14693	Traes_4AL_98EA87DFA1.1	0.33	0.31	-0.08	1.24	IPR009057	MYB-like transcription factor family protein
Wht_13021	Traes_1AL_343F2D219.1	0.20	-0.16	1.98	-0.81	IPR009057	MYB domain protein 86
Wht_14167	Traes_1DL_797D879AB.1	0.06	0.40	1.71	0.13	IPR010402	2-comp resp reg.APRR9. CCT-motif
Wht_50241	Traes_5BS_4BF0F2F1B.1	0.14	0.12	1.66	0.44	IPR013210	Receptor-like protein kinase HAIKU2
Wht_08975	Traes_6DL_5A74D7311.1	-0.50	0.15	1.59	-0.69	IPR005512	ROP guanine nucleotide exchange factor 5
Wht_20596	Traes_7DL_F2EF2631B.1	-0.29	0.53	1.50	0.26		Tyrosine-sulfated glycopeptide receptor 1
Wht_45240	Traes_2DL_0B1444B68.1	0.25	-0.45	1.40	1.08	IPR024097	Transcription factor PIF3
Wht_29963		-0.47	0.03	1.35	-1.08		AM7 | CAM7 (calmodulin 7) calcium ion binding
Wht_01847	Traes_6BL_790C095EE.1	-0.41	0.46	1.25	-0.15	IPR017053	2-comp response regulator ARR12
Wht_36126	Traes_2BL_26D525223.1	0.14	-0.45	0.18	1.14	IPR002455	Glutamate receptor 3.4-like
Wht_31811	Traes_2AS_F5D9E6F7B.1	-0.18	-0.10	-0.20	1.09	IPR027417	Ras-related protein Rab-26
Wht_21087	Traes_XX_DBD9507D21.1	-0.11	-0.06	-0.60	-1.07	IPR005512	ROP guanine nucleotide exchange factor 5
Wht_02593	Traes_5AL_F2D4ACCA3.1	0.26	-0.11	-1.25	-0.47	IPR001125	Calcineurin B-like protein 1
Wht_27104	Traes_3B_44B729718.1	0.21	-0.78	0.20	1.00	IPR002041	Ras-related protein Rab-25
Wht_17417		-0.08	-1.32	-0.31	0.38		Calcium ion binding / calmodulin-dependent protein kinase
Wht_39073	Traes_4AS_5015DF7A2.1	-0.02	-1.47	0.12	0.56	IPR011009	Mitogen-activated protein kinase 3
Wht_14864	Traes_2AS_6DA49285E.1	-0.02	-1.80	0.02	0.37	IPR011009	Calcium-dependent protein kinase 19
Wht_03306	Traes_2DL_D39684C41.1	0.23	-1.81	0.19	-0.23	IPR009057	MYB domain protein 63
Wht_57104	Traes_2AL_1368BE0AD.1	-0.41	-1.00	-0.89	-1.44	IPR008801	Ralf-like 33
Wht_21858	Traes_2BS_186EA570A.1	-0.38	-0.31	-1.29	-0.98	IPR017238	Squamosa promoter binding protein 8/13
Wht_04793	Traes_7AL_665B92F4E.1	-1.22	-0.45	-2.07	-0.81	IPR009057	Myb-like transcription factor family protein
Wht_00987	Traes_5DL_9CC4EC839.1	-0.26	-0.52	-0.97	0.43	IPR002100	MADS-box transcription factor 14
Wht_16259	Traes_6BL_3C04C3D2F.1	-1.63	-0.62	-1.22	-0.06	IPR009057	MYB-like transcription factor family protein
Wht_53996	Traes_5AL_57171171A.1	-0.50	-1.51	-0.15	0.31	IPR011992	Calmodulin 1
Wht_34910	Traes_3B_990298FF5.1	-0.25	-2.31	-0.24	-0.38	IPR003657	WRKY family transcription factor family protein
Wht_00691	Traes_XX_BE36CE8421.1	-0.78	-2.38	-0.31	-0.25	IPR003657	WRKY family transcription factor
Wht_03108	Traes_1DS_A6733B734.1	-0.65	-2.72	-0.18	0.42	IPR003657	WRKY DNA-binding protein 33
Wht_20196		-1.06	-3.06	0.27	0.15		Calmodulin-like protein 41
Wht_31708	Traes_XX_41DC51E73.1	-0.20	-3.86	0.03	0.25	IPR009057	MYB-like transcription factor family protein

Seq ID	Best Blast Hit wheat	Log 2 FC (XAP: Certo) Pericarp				InterPRO-IDs	Description/Annotation
		4 DAF	6 DAF	8 DAF	10DAF		Transport related
Wht_00833	Traes_6DS_CA5464FC5.1	2.59	0.66	1.25	0.42	IPR002067	Mitochondrial substrate carrier
Wht_42475	Traes_6DL_AA95B2246.1	1.30	0.35	0.42	0.98	IPR004813	Metal-nicotianamine transporter YSL2
Wht_36094	Traes_3B_75C958395.1	1.12	-0.65	-0.14	1.74	IPR003855	Potassium transporter family protein
Wht_10484	Traes_2BL_C41ED2148.1	1.10	-0.22	0.10	1.11	IPR000109	Peptide transporter PTR1
Wht_25431	Traes_4AS_A322DBCB0.1	1.00	0.43	0.69	1.24	IPR005989	Sucrose transporter 1*TaSut1a*
Wht_00856	Traes_5DL_BB10A3B4B.1	0.82	0.11	0.19	1.37	IPR013057	Amino acid permease 3/4(AAP3/4)
Wht_03894	Traes_2DL_81DE66333.1	0.62	-0.50	0.39	2.26	IPR006121	Heavy metal transport/detoxification superfamily protein
Wht_14482	Traes_XX_F296C5A02.1	0.52	0.40	0.21	1.09	IPR002293	Amino-acid permease 2 (AAP2)
Wht_14314	Traes_XX_BD8E18DC1.1	0.47	0.10	0.26	1.86	IPR000620	Purine permease 3
Wht_35441	Traes_2DS_4EA42CAD7.1	0.47	-0.89	0.16	1.48	IPR000109	Peptide transporter PTR2
Wht_51852	Traes_6DL_94DAE0F8E.1	0.45	-0.35	0.05	2.27	IPR004853	Purine permease 11
Wht_18196		0.41	0.53	1.73	0.32		Boron transporter B5
Wht_51154	Traes_4AL_7541D0C33.1	0.35	0.33	-0.18	2.23		Vacuolar iron transporter (VIT)
Wht_50923	Traes_6AL_22D6C7054.1	0.34	1.18	1.49	0.79	IPR003855	Potassium transporter
Wht_00868	Traes_6BS_9A12C2A1D.1	0.33	0.12	-0.50	1.14	IPR001757	Potassium-transporting at pase B chain 2
Wht_23081	Traes_XX_2C810695E.1	0.27	-0.22	-0.42	0.99	IPR002524	Metal tolerance protein 5
Wht_33615	Traes_6DS_BEE6220D9.1	0.16	0.05	-0.06	1.23	IPR002067	Mitochondrial substrate carrier
Wht_25005	Traes_3B_AB1927EF3.1	0.13	0.22	0.14	1.31	IPR002528	MATE efflux family protein
Wht_36130	Traes_5DL_B41E3B6FF.1	0.06	-0.18	-0.22	1.07	IPR002067	Mitochondrial substrate carrier
Wht_39052	Traes_1BL_118B6763E.1	0.03	-0.76	0.23	1.08	IPR013057	Amino acid permease 5
Wht_03767	Traes_3B_38344FE7E.1	0.00	-0.81	-0.58	1.27	IPR004316	Bidirectional sugar transporter SWEET2b
Wht_50571	Traes_5BL_13AE34978.1	-0.02	-1.29	-0.54	1.03	IPR000620	Purine permease 5
Wht_08144	Traes_7DS_598A2EC31.1	-0.04	-0.83	-0.96	-0.05	IPR002781	Sulfite exporter taue/safe
Wht_54244	Traes_7DS_58DD91508.1	-0.05	-0.21	0.07	2.18	IPR009262	Solute carrier family 35 member F1
Wht_22262	Traes_6DL_EC95AC355.1	-0.07	0.05	0.74	1.53	IPR004316	Bidirectional sugar transporter SWEET4
Wht_36458	Traes_5BS_9384D46B3.1	-0.07	-0.14	0.17	1.06	IPR002293	Cationic amino acid transporter 2
Wht_36003	Traes_2AL_F64B48E36.1	-0.18	-1.11	0.00	1.77	IPR005828	Solute carrier family 22 member 1
Wht_18092	Traes_3AS_AA4DE445B.1	-0.21	0.11	0.71	0.94	IPR001807	Chloride channel C
Wht_27616	Traes_XX_7885435D2.1	-0.26	-0.70	-0.18	1.32	IPR000109	Peptide transporter PTR-like
Wht_01955	Traes_5AL_A7326C4FD.1	-0.35	-1.39	-0.96	-0.30	IPR006043	Nucleobase-ascorbate transporter 7
Wht_03996	Traes_2AL_E80950527.1	-0.54	-0.43	-0.55	1.08	IPR001807	Chloride channel C
Wht_39253	Traes_5DL_A98953CFA.1	0.03	-2.24	-0.19	0.62	IPR003280	Two pore potassium channel a
Wht_15554	Traes_2BL_761F797B8.1	-0.56	-0.83	-1.13	1.08	IPR005828	Solute carrier family 22 member 15-like
Wht_14359	Traes_XX_C9723A6CC.1	-0.71	-0.50	-0.54	1.53	IPR013057	Sodium-coupled neutral amino acid transporter 6
Wht_22477	Traes_5DL_E85FB920C.1	-0.82	-0.48	-0.42	1.33	IPR011701	Vesicular glutamate transporter 3
Wht_22090	Traes_2AL_7A16D363B.1	-1.02	-0.45	-1.32	1.33		Sugar transport protein 5
Wht_10340	Traes_1DL_D58198BB4.1	0.37	0.16	-0.97	-0.55		Purine permease 4
Wht_26178	Traes_2BL_43F81300F.1	-1.13	-0.56	-0.99	1.74		Sugar transport protein 5
Wht_11549	Traes_2AL_7A16D363B.1	-1.46	0.64	0.37	0.09		Sugar transport protein 5
Wht_42583	Traes_1AS_19293C0A9.1	-1.87	-1.54	-2.13	-1.52	IPR016196	SUT3-like

Seq ID	Best Blast Hit wheat	Log 2 FC (XAP: Certo) Pericarp				InterPRO-IDs	Description/Annotation
		4 DAF	6 DAF	8DAF	10 DAF		**ABC transporter**
Wht_53843	Traes_1DL_1F003CA3A.1	-0.10	-0.23	0.02	1.97		ABC transporter F family /Phosphate import ATP-binding protein pstb 1
Wht_43354	Traes_7BL_E4BC8AB8B.1	0.27	0.10	-0.26	1.60	IPR011527	ABC transporter. Transmembrane domain type 1
Wht_31199	Traes_5DL_C334D859A.1	0.20	-0.21	0.17	1.61	IPR013525	ABC-2 type transporter
Wht_35850	Traes_1DS_0CB6BB053.1	0.32	-0.06	-0.09	1.45	IPR013525	ABC transporter G family member 16
Wht_15015	Traes_2DL_BEFA5F419.1	0.17	-0.66	0.00	1.67	IPR013525	ABC transporter G family member 11
Wht_13852	Traes_3B_8DE496E1D.1	0.06	-0.83	-0.39	1.21	IPR002528	Multidrug resistance protein mdtk
Wht_04287	Traes_3DL_BE884863B.1	-0.02	0.46	-0.38	1.01	IPR011701	Major facilitator superfamily
Wht_54982	Traes_1DL_29F70492C.1	-0.10	-0.01	-0.21	1.22	IPR027417	ABC transporter G family member 28
Wht_05554	Traes_7BS_BB0FECDBE.1	-0.18	-0.15	-0.26	1.31	IPR011701	Major facilitator superfamily
Wht_42669	Traes_3DL_0253F1D11.1	-0.20	-0.60	-0.29	1.04	IPR011527	Lipid A export ATP-binding/permease protein msba
Wht_03116	Traes_5BL_D71543428.1	-0.21	0.14	-0.42	1.22	IPR000109	Proton-dependent oligopeptide transporter family
Wht_14112	Traes_1DS_0176B897D.1	-0.22	-0.06	-0.53	1.17	IPR013525	ABC transporter G family member 15
Wht_17799	Traes_2BS_C6941CC6A1.1	-0.24	-0.03	-0.62	1.14	IPR000109	ABC transporter C family member 10
Wht_09083	Traes_7DS_EB0ADD31F.1	-0.25	-0.31	-0.26	1.31	IPR016196	General substrate transporter
Wht_17169	Traes_7BS_4B7568082.1	-0.26	0.28	-0.26	0.97	IPR016196	Zinc induced facilitator-like 2
Wht_01828		-0.52	0.70	2.00	0.14		ABC transporter family protein
Wht_31163	Traes_7AS_FB4D5B81E.1	-0.49	0.22	1.19	-0.51	IPR013525	ABC transporter G family member 14
Wht_20955	Traes_6DL_11D8C3F4C.1	-0.25	-0.09	1.22	0.36	IPR011701	Major facilitator superfamily
Wht_00571	Traes_4DL_84BDB65D9.1	-0.35	-0.20	-1.11	-0.37	IPR002524	Cation-efflux pump fief
Wht_14558	Traes_3DS_2519C6653.1	0.19	-0.37	-1.53	-0.08	IPR011701	Zinc induced facilitator-like 2
Wht_20687	Traes_4AL_AFC780F09.1	-0.52	-0.31	-1.28	0.19	IPR011701	Zinc induced facilitator-like 2
Wht_17539	Traes_3DL_889383227.1	-0.56	-0.94	-1.09	0.12	IPR011701	Zinc induced facilitator-like 2
							Ubiquitin-related
Wht_10432	Traes_4AL_D58CA2281.1	0.86	0.56	0.92	-0.39	IPR013083	RING/U-box superfamily protein
Wht_36968	Traes_1AL_03E5EB067.1	1.42	0.21	-0.21	0.19	IPR001394	Ubiquitin carboxyl-terminal hydrolase 21
Wht_10239	Traes_4AL_D58CA2281.1	0.81	0.39	1.00	0.02	IPR013083	RING/U-box superfamily protein
Wht_23288	Traes_1AL_97A25D0F6.1	-0.54	0.90	1.77	-0.90	IPR008974	Ubiquitin carboxyl-terminal hydrolase 12
Wht_50708		-0.13	0.06	1.43	-0.64		Ubiquitin-conjugating enzyme 9)
Wht_35177	Traes_6AL_34E9B21F7.1	-0.20	0.35	1.42	0.40	IPR013083	RING/U-box superfamily protein
Wht_02147	Traes_XX_8F793D07E.1	-0.43	0.37	1.36	-0.60		UBQ3 (polyubiquitin 3) protein binding
Wht_11125	Traes_XX_DC2A168B4.1	-0.41	0.08	1.14	-0.86	IPR023313	Ubiquitin-conjugating enzyme 13 L
Wht_42373	Traes_1BL_AB728099C.1	-0.07	0.30	1.11	-0.26	IPR020683	Ankyrin repeat family protein
Wht_22674	Traes_1AL_A66077D07.1	-0.43	0.60	1.00	-0.06	IPR022059	UPI000234FA1F related cluster
Wht_04903		0.12	-0.07	0.99	-0.22		Ubiquitin-protein ligase/ zinc ion binding protein
Wht_36603	Traes_1AL_0CF11612B.1	-0.54	-0.58	-0.33	1.15	IPR008942	Vacuolar protein sorting-associated protein 27
Wht_22463	Traes_6AL_2D9CCDCF7.1	0.52	0.14	-0.87	1.18	IPR001394	Biquitin carboxyl-terminal hydrolases family 2)
Wht_07462	Traes_3DL_24BB36F77.1	-0.15	-0.56	-0.99	0.01	IPR019955	Polyubiquitin 10

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		4 DAF	6 DAF	8DAF	10DAF		**Cytochrome P450**
Wht_32639	Traes_4BS_B5F821BD0.1	5.47	4.19	0.00	-0.66		Cytochrome P450
Wht_14441	Traes_6AS_4E2AEFF8F.1	3.36	0.59	2.08	-0.15	IPR001128	Cytochrome P450
Wht_35975	Traes_XX_522F99811.1	1.81	0.38	0.97	0.48	IPR001128	Cytochrome P450 family 88 subfamily A. polypeptide 3
Wht_39079	Traes_6BS_11D740D89.1	1.69	0.63	-0.02	1.70	IPR001128	Cytochrome P450 superfamily protein
Wht_22279	Traes_7BL_A84D60579.1	0.23	0.17	0.36	2.45	IPR001128	Cytochrome P450 superfamily protein
Wht_35700	Traes_XX_438833F7D.1	0.79	0.10	0.68	1.67	IPR001128	Cytochrome P450 superfamily protein
Wht_09245	Traes_2DS_CD1FD0261.1	0.90	1.00	-0.02	1.41	IPR001128	Cytochrome P450 superfamily protein
Wht_37936	Traes_3AL_EFE75B810.1	0.27	-0.50	0.42	1.16	IPR002326	Cytochrome C1 family
Wht_14907	Traes_3B_620A2C0FC.1	0.15	-0.60	0.03	1.15	IPR001128	Cytochrome P450 superfamily protein
Wht_00333	Traes_6AL_933E4F08D.1	0.19	-0.03	-0.33	1.13	IPR001199	Cytochrome b5
Wht_45223	Traes_6AS_142BAAA76.1	0.63	-0.01	-0.01	0.98	IPR001128	Cytochrome P450 superfamily protein
Wht_01974	Traes_2BS_E6A403F63.1	-0.38	-1.50	0.32	-0.21	IPR001128	Cytochrome P450 superfamily protein
Wht_03251	Traes_2BS_E6A403F63.1	-0.19	-1.66	0.19	-0.01	IPR001128	Cytochrome P450 superfamily protein
Wht_28639	Traes_2BL_0783B8D8C.1	0.45	-1.98	-0.02	1.76	IPR001128	Cytochrome P450 superfamily protein
Wht_11710	Traes_2AS_82EDB384F.1	0.04	-2.14	-0.24	0.71	IPR001128	Cytochrome P450 superfamily protein
Wht_36244	Traes_XX_E3D7846FC.1	-0.04	-1.53	-0.78	-0.05	IPR001128	Cytochrome P450. family 707, subfamily A. polypeptide 3
Wht_31279	Traes_2AS_91AF72EAE.1	0.17	-2.07	-1.02	1.18	IPR001128	Cytochrome P450 superfamily protein
							Photosynthesis-related
Wht_25571	Traes_5AL_AD0034878.1	-0.71	-0.07	2.48	-1.10	IPR024680	Ribulose bisphosphate carboxylase small chain chloroplastic
Wht_39279	Traes_4BS_A666283DF.1	-0.62	0.27	2.14	-0.50	IPR022796	Chlorophyll a-b binding protein chloroplastic
Wht_51735	Traes_XX_CAC91FF4B.1	-0.50	0.71	1.71	-0.76	IPR022796	Chlorophyll a-b binding protein 6 chloroplastic
Wht_12245	Traes_XX_B739647B8.1	0.21	0.55	1.62	-0.82	IPR009388	Photosystem II core complex proteins psbY chloroplastic
Wht_00598	Traes_6BL_B51240ACF.1	0.57	0.42	0.53	1.57	IPR006814	Photosystem II 10 kDa polypeptide chloroplastic
Wht_43713	Traes_XX_208A6F4D1.1	-0.02	0.11	-0.09	0.93	IPR009014	Transketolase chloroplastic
Wht_35773	Traes_1BL_436B2D6FC.1	-0.04	-0.52	-0.43	1.05	IPR008545	Protein weak chloroplast movement under blue light 1
Wht_12239		-0.78	-0.19	1.63	-1.01		ELIP1 (early light-inducible protein) chlorophyll binding
Wht_13044	Traes_6AS_D921D884D.1	0.05	0.00	-0.93	-0.21	IPR023329	Chlorophyll A-B binding family protein
Wht_06594	Traes_7AL_112C7294C.1	0.09	-0.38	-1.10	-0.03	IPR002683	PsbP domain-containing protein 5. chloroplastic
							Protein phosphatase2C (PP2Cs)
Wht_34075	Traes_XX_0D1026AFE.1	0.93	1.42	-0.61	1.81	IPR001932	Protein phosphatase 2C 35
Wht_27165	Traes_2AL_4B06CBDD6.1	0.93	0.74	-0.24	1.78	IPR001932	Protein phosphatase 2C 41
Wht_54219	Traes_2AL_937904C9A.1	0.42	-0.36	0.12	1.70	IPR001932	Protein phosphatase 2C 44
Wht_54070	Traes_2BS_6174F3DEB.1	1.42	2.03	-0.42	0.98	IPR001932	Protein phosphatase 2C 64
Wht_31442	Traes_2BS_E2EF5F2CF.1	0.50	-0.41	1.28	0.31	IPR001932	Protein phosphatase 2C 62

Seq ID	Best Blast Hit wheat	Log 2 FC (XAP: Certo) Pericarp				InterPRO-IDs	Description/Annotation
		4 DAF	6DAF	8DAF	10DAF		Stress response and tolerance
Wht_09959	Traes_2DS_F1F7FE1D2.1	6.64	6.12	-0.24	0.32	IPR011008	Stress responsive alpha-beta barrel protein
Wht_42746	Traes_2AS_1002418B1.1	5.74	5.45	0.16	-0.38	IPR016161	Aldehyde dehydrogenase
Wht_37996	Traes_2AS_6BC67DD45.1	4.73	5.62	0.34	-0.53		Disease resistance protein (CC-NBS-LRR)
Wht_37472		4.55	4.86	0.12	-0.46		Heat shock factor protein.HSF30
Wht_35307	Traes_7DL_97626F39E.1	0.97	-0.06	0.65	3.11	IPR001509	Dihydroflavonol 4-reductase-like1
Wht_11360	Traes_2BS_3A775F5F1.1	3.82	2.58	2.47	2.31	IPR006015	Adenine nucleotide alpha hydrolases
Wht_15443		3.81	0.45	1.98	0.80		Peroxidase 2
Wht_07954	Traes_2BS_56373D360.1	2.55	0.16	1.18	1.41	IPR002347	Short-chain dehydrogenase reductase 2a
Wht_02288	Traes_2BL_2B48F410A.1	1.71	1.06	0.74	0.30	IPR008922	Polyphenol oxidase chloroplastic
Wht_15292	Traes_2AS_3947F19A9.1	1.58	1.10	1.02	2.27	IPR002182	Disease resistance protein RPP13
Wht_02014	Traes_XX_4E78886B2.1	-0.54	0.60	1.53	2.13	IPR017853	Chitinase A
Wht_41968	Traes_7DS_4A688D488.1	0.19	-0.54	-0.14	2.08		Lysine-specific demethylase 8
Wht_01932	Traes_1AL_82AAF3E95.1	0.46	-0.34	-0.10	2.08	IPR007541	Plant basic secretory protein (BSP)
Wht_02237	Traes_XX_DAE056F64.1	0.45	-0.41	-0.08	1.82	IPR007541	Plant basic secretory protein (BSP)
Wht_35512	Traes_2DS_F43C1EB35.1	0.16	0.09	-0.12	1.64	IPR015761	Acetyltransferase component of pyruvate dehydrogenase complex
Wht_32643	Traes_3DL_359340FCF.1	0.26	0.19	0.16	1.30	IPR008598	Protein dehydration-induced 19 homolog 5
Wht_03884	Traes_5BL_E956B149A.1	1.18	-0.15	0.44	1.34	IPR010987	Glutathione S-transferase family protein
Wht_00016	Traes_1BS_DDE53AF02.1	0.18	-0.44	-0.04	1.34	IPR010987	Glutathione S-transferase family protein
Wht_00507	Traes_5AL_72929F651.1	0.11	0.16	-0.49	1.17	IPR010987	Glutathione S-transferase
Wht_25509	Traes_3B_23B7A9A57.1	0.13	-0.77	0.16	1.02	IPR010987	Glutathione S-transferase family protein
Wht_06222	Traes_3B_6DA721FB8.1	-0.72	0.39	1.58	-0.70	IPR010987	Glutathione S-transferase 1
Wht_57169	Traes_5DS_4D101E56F.1	-0.09	0.99	1.35	0.33	IPR000767	Disease resistance protein (TIR-NBS-LRR)
Wht_55640	Traes_6BS_47597FFF7.1	0.11	0.88	1.11	-0.51	IPR002182	Disease resistance protein
Wht_31696	Traes_4DL_702BA9725.1	-0.77	0.40	1.43	-1.06	IPR016283	Chitinase family protein
Wht_00735	Traes_3AL_9ADFB3DEA.1	0.41	0.05	1.42	0.14	IPR010255	Peroxidase superfamily protein
Wht_29184	Traes_6DL_C729C41AB.1	-0.30	0.32	1.09	-0.39		Deoxyuridine 5'-triphosphate nucleotidohydrolase
Wht_03341	Traes_XX_59D7D7747.1	0.12	0.79	1.16	-0.26	IPR010987	Glutathione S-transferase family protein
Wht_51725	Traes_5BL_B013EB81C.1	-0.09	-0.06	0.97	-0.03	IPR010987	Glutathione S-transferase T1
Wht_13333	Traes_6DL_134445958.1	0.27	-0.58	0.75	1.25	IPR000167	Cold-shock protein CS120
Wht_10651	Traes_XX_4E78886B2.1	0.27	0.10	0.67	0.90	IPR017853	Chitinase A
Wht_27220	Traes_XX_9CEE2CE36.1	-0.82	-0.61	-0.84	2.52	IPR027725	Heat stress transcription factor C-2b
Wht_36628	Traes_XX_C41FD49F9.1	-0.72	-0.51	-1.19	2.07		Heat stress transcription factor B-2b
Wht_36262	Traes_XX_1CC17A585.1	-0.08	-0.54	0.42	1.16	IPR005200	Endo-1.3(4)-beta-glucanase 1
Wht_08731	Traes_4AS_65C7FDFE9.1	0.59	-0.04	0.20	1.05		Cold-regulated gene 27
Wht_22957	Traes_5DL_E6E00843B.1	0.71	-0.16	0.28	1.20	IPR002085	Oxidoreductase. zinc-binding dehydrogenase family protein
Wht_24662	Traes_3B_5D8EA5CD1.1	-0.20	-0.19	-0.27	1.19	IPR013130	Respiratory burst oxidase protein F
Wht_01233	Traes_6DL_134445958.1	-0.67	-0.39	0.27	1.02	IPR000167	Cold-shock protein CS120
Wht_14818	Traes_XX_400D2F772.1	-0.30	-0.47	-0.01	1.05	IPR002347	Dehydrogenase/reductase SDR family member 11
Wht_00645	Traes_XX_5339C975D.1	-0.24	-0.55	-0.41	1.21	IPR001395	NAD(P)-linked oxidoreductase super
Wht_51260	Traes_1BL_DAACABE3E.1	-0.19	-0.60	0.06	1.17	IPR001080	Chaperone protein dnaj
Wht_00317	Traes_XX_153D7E563.1		-0.11	0.25	-1.17	IPR008978	Heat shock protein 21
Wht_24280	Traes_2BL_137976704.1	-0.20	0.45	-1.40	-1.05	IPR006636	Tetratricopeptide repeat protein 28
Wht_00675	Traes_4DL_1233D47E2.1	-0.25	0.19	-1.01	-0.20	IPR001305	Protein disulfide-isomerase LQY1
Wht_14920	Traes_XX_86A3D56D4.1	-0.18	0.46	-1.23	-0.02	IPR027725	Heat stress transcription factor B-2c
Wht_48285	Traes_3AL_E15419B88.1	-0.67	0.16	-1.46	0.45	IPR027725	Heat stress transcription factor A-2b
Wht_36509	Traes_XX_CD1A48D98.1	-0.77	-1.90	-1.68	0.75	IPR010987	Glutathione S-transferase family protein
Wht_19153	Traes_3B_5DC991EC8.1	0.50	-1.45	0.09	0.50	IPR010255	Peroxidase superfamily protein
Wht_25204	Traes_2BS_19F05C27A.1	0.19	-1.42	-0.10	0.36	IPR010255	Peroxidase superfamily protein
Wht_13766	Traes_2BL_6FDBA749E.1	0.57	-2.09	0.36	0.20		Dehydration-responsive element-binding protein 1E
Wht_11386	Traes_7DL_357BEB7B0.1	-0.54	-2.85	-0.26	-1.02	IPR001623	Chaperone protein dnaj
Wht_00319	Traes_XX_643D0E333.1	-1.64	-0.73	-2.19	-0.31	IPR008978	17.6 kda class II heat shock protein

Appendix IV-B List of differentially expressed genes in XAP line CC pericarp group as related to distinct functional clusters. Differentially Expressed Genes (DEs) at five stages of grain development (4, 6, 8 and 10, and 14 DAF) were identified. At three stages (4, 6 and 14 DAF) whole developing grains were used for analysis, whereas at 8 and 10 DAF pericarp and endosperm tissues were analyzed separately. Three stages (8 and 10 and 14 DAF) group as endosperm, moderately t-test with corrected P-value (Westfall-Young. P ≤ 0.05) was used.

Seq ID	Best Blast Hit wheat	Log2 FC(XAP:Certo) Endosperm			InterPRO-IDs	Description/Annotation
		8 DAF	10 DAF	14DAF		**Cell Wall**
Wht_14022	Traes_7DL_2FB89480C.1	1.20	2.01	-0.43	IPR000425	Aquaporin-like superfamily protein
Wht_00733	Traes_3AL_0ADBF00B9.1	1.05	1.74	-0.74	IPR004963	Pectinacetylesterase family protein
Wht_51317	Traes_3B_798C724AC.1	1.10	1.74	-0.45	IPR026953	Callose synthase 1
Wht_25903	Traes_5BS_CD20C307C.1	1.87	1.74	-0.75	IPR008999	Glucan 1.3-beta-glucosidase
Wht_05701	Traes_2DL_B9663237F.1	1.21	1.72	-0.49	IPR000743	Pectin lyase-like superfamily protein
Wht_01390	Traes_3AL_0ADBF00B9.1	1.09	1.62	-0.75	IPR004963	Pectinacetylesterase family protein
Wht_14855	Traes_6AS_EE8C37602.1	1.54	1.11	-0.34	IPR001701.	Endoglucanase 11
Wht_37539	Traes_2AL_D3715CC49.1	1.02	0. 7	-0.59	IPR006918	COBRA-like protein 7
Wht_35472	Traes_2BS_58E7D5315.1	0.97	1.04	-0.08	IPR001509	UDP-glucose 4-epimerase
Wht_36027	Traes_XX_1E007BB6E.1	0.75	1.00	-0.99	IPR006501	Pectinesterase family protein
Wht_25780	Traes_2DS_7146B6135.1	-0.05	0.85	0.04		Fasciclin-like protein FLA29
Wht_12199	Traes_5DL_A42BBCFD7.1	-0.67	1.56	-0.60	IPR001675	Beta-galactoside alpha-2.6-sialyltransferase
Wht_14663	Traes_3B_FBE2447AA2.1	-0.84	1.47	-0.67	IPR017853	Glucan endo-1.3-beta-glucosidase GI
Wht_04895	Traes_3DL_2C93D2B5E.1	0.81	1.27	-0.72	IPR004963	Pectinacetylesterase family protein
Wht_36106	Traes_1AS_B16A9D49C.1	-0.27	1.25	0.05	IPR010255	Peroxidasen
Wht_43708	Traes_6AS_B03B32977.1	0.17	1.23	0.03		Endoglucanase 11
Wht_00959	Traes_1BL_20258AC56.1	-0.52	1.19	0.52	IPR000778	Respiratory burst oxidase protein F
Wht_26713	Traes_5AL_C1E3FCB4F.1	0.03	1.04	0.00	IPR007235	Diacylglycerol glucosyltransferase
Wht_25780	Traes_2DS_7146B6135.1	-0.05	0.85	0.04		Fasciclin-like protein FLA29
						Cell proliferation
Wht_03664		2.17	1.968	0.874		Cell differentiation protein Rcd1
Wht_36029	Traes_4AL_4B411B3A7.1	1.686	1.581	-0.359	IPR001045	Spermidine synthase 1
Wht_06381	Traes_2BS_65836FEE4.1	1.661	1.570	-0.713	IPR000269	Amine oxidase 1
Wht_03702	Traes_2BL_C085E7F9A.1	1.70	0.82	0.40	IPR001611.	Leu-rich receptor-like protein kinase RPK2
Wht_12008	Traes_5DL_B33355172.1	1.357	1.837	-0.371	IPR011009	Shaggy-related protein kinase alpha
Wht_23688	Traes_4DS_A800BBB3B.1	1.01	1.30	0.80		Cytosine-specific methyltransferase
Wht_51317	Traes_3B_798C724AC.1	0.915	1.737	-0.016	IPR023175	Callose synthase 1
Wht_20894	Traes_5BL_32C82DE83.1	1.036	0.952	0.113	IPR003874	Cell division control protein 45 homolog
Wht_17690	Traes_2AS_09E707C65.1	0.868	1.872	0.368	IPR011009	Cys-rich recept.-like PK (Phytosulfok. Receptor)
Wht_00172	Traes_1BL_F3035AD07.1	0.608	1.712	-0.094	IPR011009	Cyclin-dependent kinase D1
Wht_12685	Traes_3B_6589A8C2E.1	0.80	1.36	-0.028		Cyclin D2
Wht_14421	Traes_5DL_9824E97A8.1	0.449	1.204	-0.029	IPR011009	MPK/WNK8.with no lysine kinase
Wht_23222	Traes_6DS_F3BC1C2DF.1	0.142	1.035	-0.062		S-adenosyl-L-methionine-dependent methyltransferases superfamily protein
Wht_43304	Traes_3B_82AF16D45.1	0.059	1.094	0.044		Heavy meromyosin-like. Adhesin-related
Wht_47429	Traes_2BL_5CF7B7FB3.1	0.001	1.255	-0.733	IPR000795	Elongation factor 1-alpha
Wht_23508	Traes_6BL_724171B65.1	-0.580	1.406	-0.098	IPR001985	S-adenosylmethionine decarboxylase
Wht_44636	Traes_4DL_F63F53885.1	-0.594	1.590	-0.243	IPR014400	Cyclin F3-2
Wht_04419	Traes_XX_CD5BE62A7.1	-0.261	0.788	-0.039	IPR003591	Phytosulfokine receptor 1/2
Wht_26792	Traes_7BL_0E93FBCFF.1	0.776	0.732	-0.129	IPR001045	Spermidine synthase 3
Wht_33945	Traes_7BS_DB305006A.1	0.730	0.957	-0.232	IPR006461	Cell number regulator 11
Wht_09497	Traes_5AL_9557D27B3.1	-0.923	-0.396	-0.056	IPR022043	Chromatin assembly factor 1 subunit A
Wht_10658	Traes_6DL_B99906102.1	-1.038	1.119	0.336	IPR001985	S-adenosylmethionine decarboxylase

Seq ID	Best Blast Hit wheat	Log2 FC (XAP:Certo) Endosperm			InterPRO-IDs	Description/Annotation
		8 DAF	10 DAF	14 DAF		**Metabolism**
Wht_12650	Traes_7DL_2EB3442BE.1	1.46	0.71	-0.50	IPR002213	Anthocyanin 5-O-glucosyltransferase
Wht_48821	Traes_3AS_44927A043.1	0.48	1.66	0.08	IPR001128	Trans-cinnamate 4-monooxygenase
Wht_35541	Traes_XX_C6BDB5B11.1	0.46	1.20	-0.04	IPR002650	ATP sulfurylase sulfate adenylyltransferase
Wht_50471	Traes_5BL_545CD9F8B1.1	-0.50	-0.65	-0.29	IPR002030	Mitochondrial uncoupling protein 5
Wht_00376	Traes_XX_F0B5CF41A.1	-1.19	-0.35	-0.48	IPR004508	APS-reductase (APR). Phosphoadenosine phosphosulfate reductase
						C metabolism
Wht_25903	Traes_5BS_CD20C307C.1	1.77	1.74	0.03	IPR017853	Glucan 1,3-beta-glucosidase
Wht_14880	Traes_2BL_7F9F8D481.1	1.33	1.73	-0.87	IPR001362	Fructan 6-exohydrolase
Wht_56117	Traes_XX_EF8FE0BF7.1	1.31	0.84	-0.73	IPR011183	Phosphofructokinase family protein
Wht_27710	Traes_4AL_C6DEABC36.1	1.27	0.49	-0.12	IPR001362	Soluble acid invertase
Wht_10055	Traes_7DL_D39A0193F.1	1.50	0.31	0.24	IPR015902	4-alpha-glucan branching enzyme GlgB
Wht_00295	Traes_2BL_2C62185EE.1	1.72	0.16	0.40	IPR015902	4-alpha-glucan branching enzyme GlgB
Wht_29049	Traes_XX_B58E30BDA.1	1.34	0.72	0.31	IPR010121	Pyruvate. Phosphate dikinase 2
Wht_09398	Traes_6DL_33F8A5EF4.1	0.90	1.34	-0.37	IPR001830	Trehalose 6-phosphate synthase
Wht_13574	Traes_XX_5D93FCB5C.1	0.96	1.19	-0.20	IPR002213	UDP-Glycosyltransferase superfamily
Wht_54683	Traes_4BL_C3267759F.1	0.44	1.83	0.37	IPR000362	Fumarate hydratase 1
Wht_44521	Traes_2DS_4C162DAFF.1	0.41	1.36	-0.94	IPR003385	4-alpha-glucanotransferase
Wht_14663	Traes_3B_FBE2447AA2.1	-0.88	1.47	0.70	IPR000490	Glucan endo-1.3-beta-glucosidase GI
Wht_09558	Traes_XX_122113E79.1	0.43	0.80	-0.23	IPR001772	SNF1-likeprotien kinase
Wht_05566	Traes_XX_0A31DDFFA.1	0.61	0.95	-0.03	IPR001830	Trehalose 6-phosphate synthase
Wht_08223	Traes_5BL_94F42F9A7.1	0.36	0.72	-0.14	IPR001830	Trehalose 6-phosphate synthase
Wht_03706	Traes_3DL_84CDD48B8.1	0.28	0.80	-0.23	IPR001772	Trehalose 6-phosphate synthase
Wht_02573	Traes_5BL_AC845F1C1.1	-0.20	-0.18	2.16	IPR015902	Alpha-amylase-like protein
Wht_17086	Traes_5DL_207BCA00D.1	-0.49	-0.14	1.19	IPR002213	UDP-Glycosyltransferase
Wht_20671	Traes_3B_89F6ACF89.1	-1.39	-1.20	0.53	IPR001362	Cell wall invertase
Wht_14865	Traes_XX_A33491620.1	-1.24	-0.44	-0.27	IPR001554	Beta-amylase 1
Wht_01478	Traes_4AS_254B6D654.1	-1.41	0.09	-0.15	IPR001272	Phosphoenolpyruvate carboxykinase
Wht_57222	Traes_2AS_649FE91DA.1	0.09	-0.97	0.00	IPR001557	L-lactate dehydrogenase A
Wht_28864	Traes_2AS_29EA43021.1	-0.24	-0.76	0.20		UDP-Glycosyltransferase superfamily p
Wht_31804	Traes_7DL_70FDF85F0.1	-0.18	-0.04	-1.05	IPR002213	UDP-Glycosyltransferase superfamily
						N metabolism
Wht_15347	Traes_4DL_AF0869DDB.1	2.03	0.42	0.55	IPR004298	Nicotianamine synthase 6
Wht_01089	Traes_6AS_06B794C38.1	1.78	0.43	0.73	IPR015424	Aspartate aminotransferase
Wht_08692	Traes_4AS_C19AB3A1A.1	1.66	0.31	0.05	IPR006426	Asparagine synthetase (ASN1)
Wht_36029	Traes_4AL_4B411B3A7.1	1.686	1.581	-0.359	IPR001045	Spermidine synthase 1
Wht_23764	Traes_1AL_F6A19E672.1	0.72	1.77	0.24	IPR024462	Non-lysosomal glucosylceramidase
Wht_03456	Traes_2AL_104945FD6.1	0.11	1.54	0.57	IPR002129	Glutamate decarboxylase
Wht_23508	Traes_6BL_724171B65.1	-0.180	1.406	-0.098	IPR001985	S-adenosylmethionine decarboxylase proenzyme
Wht_23222	Traes_6DS_F3BC1C2DF.1	0.142	1.035	-0.062		S-adenosyl-L-methionine-dependent methyltransferases
Wht_26792	Traes_7BL_0E93FBCFF.1	0.776	0.732	-0.129	IPR001045	Spermidine synthase 3
Wht_36370	Traes_XX_0D4942798.1	0.82	0.79	-0.14	IPR002912	Protein-PII, uridylyltransferase
Wht_05816	Traes_6AL_6D310D2B0.1	-0.10	0.75	0.20	IPR005097	Alpha-aminoadipic semialdehyde synthase
Wht_50560	Traes_XX_0E3ED1CD61.1	0.25	0.19	-1.49	IPR001544	Branched-chain-amino-acid aminotransferase-like protein 1
Wht_45345	Traes_4DS_A903AA984.1	-0.63	0.20	-1.61		Glutamine dumper 3
Wht_36153	Traes_7AS_069A1FA77.1	-0.07	-1.92	0.06	IPR000623	Shikimate kinase 1
Wht_14730	Traes_5BL_7657F73D5.1	-0.53	-0.95	-0.35	IPR001341	Apartate kinase
Wht_36200	Traes_1DS_EFDF9CB72.1	-0.88	-0.78	-0.69	IPR006336	Glutamate-cysteine ligase B

Seq ID	Best Blast Hit wheat	Log2 FC(XAP:Certo) Endosperm			InterPRO-IDs	Description/Annotation
		8 DAF	10DAF	14 DAF		Storage protein
Wht_03721	Traes_5BL_22ED571AE.1	0.73	1.78	-0.71	IPR000215	Serine protease inhibitor
Wht_14245	Traes_7DS_255D13EDA.1	1.26	1.66	-0.50	IPR000215	Serine protease inhibitor
Wht_00308	Traes_5AL_1C01224F9.1	1.19	1.63	-0.51	IPR000215	Serine protease inhibitor
Wht_18168	Traes_7DS_0FFF2FB02.1	1.25	1.64	-0.40	IPR016453	Coatomer. Beta' subunit
Wht_01242	Traes_1DS_4426B6725.1	1.42	1.41	-0.51	IPR006044	11S globulin seed storage protein G3
Wht_26003	Traes_7DS_9920F7B04.1	1.43	1.38	-0.48	IPR001810	F-box/RNI-like superfamily protein
Wht_27797	Traes_6AL_D896C3DBD.1	1.60	1.30	-0.27	IPR006106	Alpha-amylase/trypsin inhibitor
Wht_25252	Traes_4AL_4FF5B8837.1	1.54	1.39	-0.39	IPR001376	Alpha/beta gliadin
Wht_01191	Traes_4AL_4FF5B8837.1	1.15	1.27	-0.27	IPR001376	Alpha/beta gliadin
Wht_01438	Traes_4AL_4FF5B8837.1	1.14	1.08	-0.15	IPR001376	Alpha/beta gliadin
Wht_01874	Traes_4AL_4FF5B8837.1	1.20	1.06	-0.04	IPR001376	Alpha/beta gliadin
Wht_02736		1.17	0.88	-0.09		Alpha/beta gliadin
Wht_27161		1.34	1.30	0.37		Gamma-gliadin
Wht_01195		1.51	1.29	-0.19		Gamma-gliadin
Wht_32308		1.55	1.29	0.06		Gamma-gliadin
Wht_16064		1.57	1.24	-0.19		Gamma-gliadin
Wht_02300		1.35	1.19	0.01		Gamma-gliadin
Wht_10565		1.19	0.77	0.14		Gamma-gliadin
Wht_01212		1.13	0.83	0.20		Gamma-gliadin
Wht_11437	Traes_7AS_DF9A9B7BF1.1	1.46	1.29	-0.39	IPR001954	Avenin-like b5
Wht_10505	Traes_7AS_DF9A9B7BF1	1.47	1.25	-0.14	IPR001954	Avenin-like b5
Wht_03551		1.14	0.96	-0.25		Avenin
Wht_25274		1.28	1.27	-0.04		Albumin No
Wht_17454	Traes_5DL_5CF73B088.1	1.20	1.26	-0.40	IPR000877	Bowman-Birk type trypsin inhibitor
Wht_02364	Traes_1DS_66B67E9B41.1	1.36	1.05	-0.02	IPR001954	Glutenin. Low molecular weight subunit
Wht_01057	Traes_1DS_66B67E9B41.1	1.28	1.04	-0.12	IPR001954	Glutenin. Low molecular weight subunit
Wht_01721		1.31	1.00	-0.18		Globlin
Wht_27302	Traes_1DS_66B67E9B41.1	1.27	0.98	-0.26	IPR001954	Glutenin. Low molecular weight subunit
Wht_25543		1.33	0.97	-0.14		Avenin-like protein
Wht_03519	Traes_4AL_3D862C090.1	1.22	0.94	-0.84	IPR014710	Vicilin
Wht_03113	Traes_1DS_66B67E9B41.1	1.19	1.28	-0.28	IPR001954	Glutenin. Low molecular weight subunit
Wht_02363	Traes_1DS_66B67E9B41.1	1.11	0.91	-0.04	IPR001954	Glutenin. Low molecular weight subunit
Wht_01441	Traes_1DS_66B67E9B41.1	1.21	0.90	-0.02	IPR001954	Glutenin. Low molecular weight subunit
Wht_01486	Traes_1DS_66B67E9B41.1	1.20	0.88	-0.05	IPR001954	Glutenin. Low molecular weight subunit
Wht_01903	Traes_1DS_66B67E9B41.1	1.12	0.86	-0.05	IPR001954	Glutenin. Low molecular weight subunit
Wht_13615	Traes_7DL_8880576D9.1	1.37	1.31	-0.37	IPR007011	Seed maturation protein
Wht_08122	Traes_3B_E7D10A09E.1	0.55	1.45	-0.26	IPR016460	Coatomer. Beta subunit
Wht_44786	Traes_4AS_5335AE1B0.1	0.21	1.42	-0.32	IPR026285	Seed maturation protein PM36 homolog
Wht_00523	Traes_1BL_472C9200C.1	0.07	1.31	-0.51		Alpha-2-purothionin
Wht_27790	Traes_3B_D5384DBB3.1	0.80	1.10	-0.41	IPR004238	Late embryogenesis abundant protein
Wht_09833		-0.30	0.89	-0.13		Late embryogenesis abundant protein
Wht_02378	Traes_1DL_D861501F5.1	1.06	1.13	-0.02	IPR001419	Glutenin. High molecular weight subunit
Wht_27308	Traes_6DL_5A9314D3D.1	-1.52	-2.06	-0.20	IPR016140	Seed storage 2S albumin superfamily

Seq ID	Best Blast Hit wheat	Log2 (FC XAP:Certo) Endosperm			InterPRO-IDs	Description/Annotation
		8DAF	10 DAF	14DAF		**Fatty Acid/Lipids**
Wht_13512	Traes_3AL_82D3F1A30.1	2.39	0.78	0.53	IPR001087	GDSL esterase/lipase
Wht_42829	Traes_1DS_EBA34AFC4.1	1.445	1.842	-0.689	IPR016040	Fatty acyl-coa reductase 1
Wht_17651	Traes_7BL_D1AFF31A0.1	-0.256	1.573	-0.194	IPR009075	Acyl-coenzyme A oxidase 3
Wht_13587		1.121	1.055	-0.001		Kcs6 (3-ketoacyl-coa synthase 6)
Wht_45701	Traes_7DL_3111E3A31.1	0.822	1.070	-0.009	IPR001087	GDSL esterase/lipase
Wht_22264	Traes_1DL_47D4F60E6.1	0.536	0.998	-0.612	IPR001087	GDSL esterase/lipase
Wht_01457	Traes_XX_4EEEFFFB0.1	0.304	0.905	0.290	IPR008973	Phospholipase D P2
Wht_51829	Traes_XX_232C90776.1	-0.009	1.433	-0.163	IPR002123	1-acyl-sn-glycerol-3-phosphate acyltransferase
Wht_44031	Traes_4DL_291AD27BE.1	0.034	0.830	0.395	IPR006694	Methylsterol monooxygenase 1-1
Wht_14711	Traes_2BS_3EF779D1A.1	0.564	1.041	0.116	IPR013094	Acetyl esterase
Wht_27089	Traes_XX_9F318D119.1	0.009	0.95	0.036	IPR000873	2-succinylbenzoate--coa ligase
Wht_37754	Traes_6DS_C59479AB5.1	0.244	0.060	1.764	IPR001087	GDSL esterase/lipase
Wht_16616	Traes_2AS_EE37ACF7F.1	0.240	0.152	-0.859	IPR006694	Fatty acid hydroxylase superfamily
Wht_10639	Traes_XX_EA98E1D78.1	0.249	-0.471	-1.325	IPR001104	3-oxo-5-alpha-steroid 4-dehydrogenase family protein
Wht_00480	Traes_4DS_849C911C9.1	-1.641	-0.767	-0.304	IPR016040	Fatty acyl-coa reductase 3
						Transport related
Wht_07871		1.18	2.31	-0.15		VDAC1
Wht_24350	Traes_XX_1B056461B.1	1.67	1.87	-0.17	IPR008521	Magnesium transporter NIPA2/4
Wht_55042	Traes_7BL_C6E6BE7FC.1	1.10	1.88	-0.22	IPR011527	MDR-like ABC transporter
Wht_46338	Traes_XX_86355B87B.1	0.96	1.78	-0.04	IPR008972	Early nodulin-like protein 9
Wht_20389		0.85	1.63	0.74		Carbohydrate transport
Wht_24582	Traes_1BS_95AD57294.1	1.14	0.47	-0.40	IPR018108	Dicarboxylate/tricarboxylate carrier
Wht_42619	Traes_XX_3586E6BE7.1	1.58	0.68	0.11	IPR003689	Zinc transporter 1
Wht_51893	Traes_XX_4C6DD3A91.1	1.11	0.44	-0.04	IPR003689	Zinc transporter 1/3
Wht_42319	Traes_5DL_628B86979.1	1.06	0.16	-0.76	IPR001898	2-OG/malate translocator. Plastidic
Wht_22090	Traes_2AL_7A16D363B.1	0.67	0.87	-0.21		Sugar transport protein 5
Wht_08251	Traes_1DL_F77EAE8EA.1	0.55	1.01	-0.28	IPR018108	Mitochondrial carnitine/acylcarnitine carrier
Wht_32259	Traes_4BL_985CBED5D.1	0.54	1.50	0.09	IPR000109	NRT1.2 low affinity
Wht_55178	Traes_1AL_9873095B9.1	0.53	1.37	0.30	IPR002067	ADP.ATP carrier protein 1
Wht_51154	Traes_4AL_7541D0C33.1	0.49	1.08	0.39		Vacuolar iron transporter (VIT)
Wht_26082	Traes_7AS_1713E44CD.1	0.41	1.11	0.30	IPR003855	Potassium transporter 1
Wht_49861	Traes_6AL_6239EF6E0.1	0.38	1.21	-0.40	IPR008972	Early nodulin-like protein 19
Wht_14370	Traes_2BS_7093A1D1A.1	0.26	1.01	0.46	IPR002528	Alf5 (aberrant lateral root formation 5
Wht_22927	Traes_6BS_A679AA5FF.1	0.25	1.48	0.07	IPR006840	Cation transport regulator-like protein
Wht_31199	Traes_5DL_C334D859A.1	-0.04	1.09	0.04	IPR010658).	Major facilitator superfamily protein
Wht_14359	Traes_XX_C9723A6CC.1	-0.04	1.08	-0.56	IPR013057	Sodium-coupled neutral amino acid transporter 6
Wht_22262	Traes_6DL_EC95AC355.1	0.30	0.65	0.16	IPR004316	Bidirectional sugar transporter SWEET4
Wht_34306	Traes_2BS_C52A9FD78.1	0.07	0.00	-1.67	IPR011527	ABC transporter C family member 14
Wht_05530	Traes_3DS_45058B0D3.1	0.06	0.25	-2.33	IPR013525	ABC transporter G family member 32
Wht_04239	Traes_XX_2AA10F27D.1	0.05	-0.49	-1.46	IPR005828.	Solute carrier family 2, facilitated glucose transporter member 3
Wht_05775	Traes_2DS_8E56CA0E3.1	0.81	-0.16	-2.29	IPR008962	Vesicle associated protein
Wht_35827	Traes_7DL_B0EA9E3F4.1	-0.17	-0.77	-0.42	IPR013657	UDP-galactose/UDP-glucose transporter
Wht_13469	Traes_5BL_F05005DE1.1	-1.26	-0.80	-0.95	IPR002528	MATE efflux family protein

Seq ID	Best Blast Hit wheat	Log2 (FC XAP:Certo) Endosperm			InterPRO-IDs	Description/Annotation
		8 DAF	10DAF	14DAF		**Protease**
Wht_37258	Traes_3B_14528E636.1	2.63	0.99	0.28	IPR013128	Cathepsin B-like cysteine proteinase 6
Wht_17829	Traes_3DS_02841F4FB.1	1.67	1.89	-0.02	IPR001375	prolyl oligopeptidase family protein
Wht_22096	Traes_1DS_A477459F0.1	1.61	1.83	-0.42	IPR001461	aspartic proteinase A1
Wht_35354	Traes_4DL_6104363D7.1	1.61	0.98	-0.69	IPR004595	RING/U-box superfamily protein
Wht_56032	Traes_7DL_DA620801B.1	1.22	1.81	-0.29	IPR003137	Signal peptide peptidase like 2
Wht_30327	Traes_2BS_45AA651ED.1	-0.17	1.89	-0.26	IPR000223	Mitochondrial inner membrane protease
Wht_54110	Traes_4BS_9B7EED6CC.1	-0.15	1.64	-0.21	IPR013128	Cathepsin B
Wht_37731	Traes_4DS_FBA13E320.1	0.95	0.79	-0.29	IPR001461	Eukaryotic aspartyl protease family
Wht_05992	Traes_XX_35281064A.1	1.01	0.94	0.47	IPR005937	26S protease regulatory subunit 4 homolog
Wht_25685	Traes_3AL_9C9F6D261.1	0.92	1.04	-0.28	IPR001461	aspartic proteinase A1
Wht_14835	Traes_XX_5A42D11FC.1	1.00	0.74	0.35	IPR001461	Eukaryotic aspartyl protease family
Wht_22588	Traes_2DS_7E6DBE401.1	0.32	1.07	-0.31	IPR002885	Pentatricopeptide repeat-containing protein
Wht_28738	Traes_XX_F5FEEFED0.1	0.77	0.95	-0.34	IPR013083	RING/U-box superfamily protein
Wht_51508	Traes_XX_2DA2BA453.1	0.99	0.85	0.58	IPR000426	Proteasome subunit alpha type-2
Wht_14944	Traes_1DL_9B3CB7027.1	0.93	0.22	-0.13	IPR015500	Subtilisin-like protease
Wht_15204	Traes_5BL_0421DC1F4.1	-0.65	-0.24	1.95	IPR001563	serine carboxypeptidase-like 19
Wht_07073	Traes_XX_982B391EE.1	-0.89	-0.64	0.09	IPR001375	Prolyl oligopeptidase family protein
Wht_18814	Traes_2DL_D13018A01.1	-0.34	-0.70	-0.11	IPR000223 I	plastidic type i signal peptidase 1
Wht_00689	Traes_5BS_333253B8B.1	-1.48	-0.57	-0.02	IPR001461	Eukaryotic aspartyl protease family
Wht_16893	Traes_4DL_B1B379CB5.1	-2.51	-1.52	-0.23	IPR001461	Eukaryotic aspartyl protease family
						Hormones
Wht_42622	Traes_7BS_D6FDDD12F1.	2.23	1.06	-0.71	IPR003676	SAUR-like auxin-responsive protein family
Wht_11606	Traes_7DL_4ABF3A54D.1	0.91	0.97	-0.57	IPR003676	SAUR-like auxin-responsive protein family
Wht_51746	Traes_XX_E252AF3BF.1	1.00	0.94	-0.24	IPR004993	indole-3-acetic acid-amido synthetase GH3.4
Wht_52494	Traes_3B_F880EDE81.1	0.62	1.54	0.10	IPR003311	indole-3-acetic acid-amido synthetase GH3.5
Wht_02372	Traes_5AS_5A7E40F71.1	1.28	1.40	-0.38	IPR016040	3beta-hydroxysteroid-dehydrogenase/decarboxylase
Wht_36268	Traes_2DS_9C0C983B2.1	0.74	1.47	0.26	IPR011006	Ethylene receptor 2. EIN4
Wht_11452	Traes_7DL_02E7F7D6A.1	0.09	1.29	0.07	IPR008930	Ent-copalyl diphosphate synthase 1
Wht_14711	Traes_2BS_3EF779D1A.1	0.60	1.04	-0.27	IPR013094	GA receptor GID1L2. Carboxylesterase 3
Wht_28842	Traes_4DS_26600563E.1	0.85	0.84	0.00	IPR013094	GA receptor GID1L2. Carboxylesterase 3
Wht_00260	Traes_2AL_538DC10D0.1	1.28	1.38	-0.33	IPR003496	Abscisic stress-ripening protein 3
Wht_02292	Traes_2BS_647C82888.1	1.44	1.37	-0.54	IPR023278	Ethylene insensitive 3 family protein
Wht_14259	Traes_1BS_E5A12CB09.1	-0.26	-1.41	-0.33	IPR003311	Auxin-responsive protein IAA16
Wht_31259	Traes_XX_4DDE022D9.1	0.17	0.55	-1.49	IPR005018	Auxin-responsive family protein
Wht_14301	Traes_2AL_A598DE96B.1	-0.56	0.10	-1.14	IPR003311	Auxin-responsive protein IAA14
Wht_31681	Traes_3B_4E90802BA1.1	-0.06	-0.36	-1.35	IPR016177	Ethylene-responsive transcription factor
Wht_48536	Traes_3B_0BA89B2AC.1	-0.18	-0.29	-1.90	IPR016170	Cytokinin dehydrogenase 5
Wht_02997	Traes_7DL_F51807989.1	-1.02	-0.15	-0.37	IPR003854	Gibberellin-regulated family protein
Wht_20386	Traes_4BS_28759156D.1	-2.28	-0.40	-0.04	IPR003854	Gibberellin-regulated family protein
Wht_25117	Traes_4DS_8075EBF55.1	-1.54	-1.33	0.09	IPR003854	Gibberellin-regulated family protein

Seq ID	Best Blast Hit wheat	Log2 (FC XAP:Certo) Endosperm			InterPRO-IDs	Description/Annotation
		8 DAF	10 DAF	14 DAF		Signaling
Wht_35230	Traes_2DL_4416C3D7B.1	2.04	1.42	0.72		Two-component response regulator ARR12
Wht_11281	Traes_7DS_5527ABEE9.1	2.00	0.31	1.31	IPR008914	Phosphatidylethanolamine-binding protein PEBP
Wht_42406	Traes_XX_FC39CD06C.1	1.88	0.70	0.19	IPR015300	ABI3 / VP1
Wht_47712		1.36	1.99	-0.47		MYB-type TF APL
Wht_27107	Traes_3DL_30CF35BB3.1	1.39	0.85	0.20	IPR009057	MYB domain protein 0
Wht_00965	Traes_4AL_72C3E1EAD.1	1.15	0.00	0.41	IPR010402	response regulator 2
Wht_08023	Traes_2AL_409AB7647.1	1.01	1.55	-0.49	IPR003657	WRKY family transcription factor family
Wht_04793	Traes_7AL_665B92F4E.1	-1.35	1.73	0.04	IPR009057	MYB -like transcription factor family p
Wht_02893		-0.08	1.63	-0.10		MIKC-type MADS-box transcription factor, WM31B
Wht_23309	Traes_XX_4857F7A23.1	0.60	1.60	-0.28	IPR025422	TaDOG1L4. Delay of germination
Wht_20329	Traes_6BS_45CC6CFDC.1	0.53	1.37	-0.40		bHLH-TF ABORTED MICROSPORES
Wht_37691	Traes_2AS_B421BB2AE.1	-0.16	1.34	-0.20	IPR024752	protein n
Wht_13755	Traes_1DL_011784D10.1	0.25	1.31	0.03	IPR015300	AP2/ERF and B3 domain-containing protein
Wht_02930	Traes_2AL_5C7E76139.1	0.15	1.31	-0.31	IPR011992	Calcium-binding EF-hand family protein
Wht_13021	Traes_1AL_343F2D219.1	0.15	1.28	0.78	IPR009057	MYB domain protein 86
Wht_01006		0.31	1.21	-0.65		WRKY79 transcription factor
Wht_14106	Traes_3AL_7A685D3E8.1	0.99	1.00	-0.06	IPR011009	calcium-dependent protein kinase 34 L
Wht_14102	Traes_XX_5B27B392D.1	0.97	0.95	-0.15	IPR002100.	MADS-box transcription factor 47
Wht_39073	Traes_4AS_5015DF7A2.1	0.61	0.90	0.24	IPR011009	mitogen-activated protein kinase 3
Wht_49364	Traes_2BL_8101DFF11.1	-0.10	0.86	0.09	IPR002913	Homeobox-leucine zipper ROC4. GLABRA 2
Wht_35300	Traes_3DL_2C29058E6.1	0.26	0.82	0.25	IPR015300	FUSCA3. barley
Wht_11242	Traes_XX_DCD24767C.1	-0.19	0.86	0.89	IPR009057	MYB -like transcription factor family protein
Wht_56301	Traes_3B_25320ED72.1	0.62	0.78	-0.50	IPR009057	MYB domain protein 33
Wht_14472	Traes_6BL_7E9C131F4.1	-0.16	0.76	0.22	IPR009057	MYB -like transcription factor family protein
Wht_37020	Traes_4DS_27CCFA657.1	-0.29	0.67	0.31	IPR011333	Phototropic-responsive NPH3 family protein
Wht_00460	Traes_5BS_439DB52FE.1	-0.22	0.64	-0.15	IPR009057	WUSCHEL related homeobox 2
Wht_03306	Traes_2DL_D39684C41.1	0.74	0.28	-0.08	IPR009057	MYB domain protein 63
Wht_49995	Traes_4BS_5D04C29FF.1	-0.99	0.68	-0.33	IPR015300	B3 domain-containing protein
Wht_00260	Traes_2AL_538DC10D0.1	0.41	-1.10	1.28	IPR003496	Abscisic stress-ripening protein 3
Wht_14920	Traes_XX_86A3D56D4.1	-1.35	-0.13	-0.86	IPR011991.	Heat stress transcription factor B-2c
Wht_05860	Traes_1BS_2BD5792851.1	-1.68	-0.46	0.10	IPR007275	evolutionarily conserved C-terminal region 5
Wht_35135	Traes_2AL_8DF89AA72.1	-1.47	-0.56	0.17	IPR002100	MADS-box transcription factor 31
Wht_39333	Traes_4AL_7A841CAD2.1	-1.19	-0.61	-0.81	IPR009057	MYB domain protein 52
Wht_17912	Traes_6BL_5D8EE6802.1	0.80	0.68	-1.24	IPR009057	MYB -like transcription factor f
Wht_14640	Traes_2BS_9DDBD8C10.1	-0.45	-0.55	-2.48	IPR001192	1-phosphatidylinositol 4.5-bisphosphate phosphodiesterase 1
Wht_37509	Traes_5AL_50D72F0A4.1	-0.18	-0.68	0.24	IPR007527	FAR1-related sequence 5
Wht_46815	Traes_7AL_4CA176EEB.1	-0.24	-0.81	-0.17	IPR001932	ABI1. PP2C
Wht_35646	Traes_3B_5FB67D762.1	-0.79	-1.08	0.18	IPR000406	Rho GDP-dissociation inhibitor 1
Wht_14485	Traes_6BL_5AA3D02B3.1	-0.38	-1.32	0.24	IPR011598.	Transcription factor SPATULA
Wht_03298	Traes_1BL_73811B853.1	-0.81	-1.69	0.24	IPR003657	WRKY DNA-binding protein 70
Wht_22346	Traes_XX_67815B6FD.1	-0.38	-2.13	0.04	IPR009057	Two-component response regulator ARR1
Wht_00691	Traes_XX_BE36CE8421.1	-0.78	-2.38	0.53	IPR003657	WRKY family transcription factor
Wht_03108	Traes_1DS_A6733B734.1	-0.65	-2.72	0.56	IPR003657	WRKY DNA-binding protein 33
Wht_14250	Traes_XX_139418459.1	0.33	-3.33	0.64	IPR011009	calcium-dependent protein kinase 32

Seq ID	Best Blast Hit wheat	Log2 (FC XAP:Certo) Endosperm			InterPRO-IDs	Description/Annotation
		8 DAF	10 DAF	14 DAF		Stress tolerance
Wht_11360	Traes_2BS_3A775F5F1.1	2.07	1.94	-1.66	IPR006015	Adenine nucleotide alpha hydrolases-like superfamily protein
Wht_37801	Traes_1BS_55BE23975.1	1.74	0.33	-0.68	IPR027725	chitinase A
Wht_00923	Traes_XX_B5EA6B4BC.1	1.63	1.46	-1.10	IPR005746 I	Chalcone synthase
Wht_29597		1.02	1.978	-0.32		FDH (Formate dehydrogenase
Wht_15784		1.58	2.20	-0.42		Alcohol dehydrogenase-like protein
Wht_19574	Traes_5DL_F86DF1028.1	1.22	1.53	-0.16	IPR011004	15-hydroxyprostaglandin dehydrogenase
Wht_41162	Traes_3B_BA3224D44.1	1.34	1.64	-0.26	IPR011141 I	gamma carbonic anhydrase 1
Wht_00454	Traes_2BS_76FEB367C.1	1.39	1.07	-0.35	IPR006015	Adenine nucleotide alpha hydrolases-like superfamily protein
Wht_50670	Traes_1AS_F64BAC19D.1	1.06	1.22	-0.01	IPR002283	Fe(II) and 2-oxoglutarate (2OG dependent oxygenase
Wht_00904	Traes_4DL_34762DC69.1	1.07	0.63	-0.94	IPR000167	Adenine nucleotide alpha hydrolases-like superfamily protein
Wht_33730	Traes_7DS_B1A1906F8.1	0.08	1.79	-0.21	IPR010987	Glutathione S-transferase family protein
Wht_06989	Traes_4AL_DEF089EE0.1	-0.14	1.36	0.93	IPR010987	Glutathione S-transferase family protein
Wht_22944	Traes_2DS_5A8D0F48E.1	0.13	1.32	0.062	IPR014710	thioredoxin F2 LENGTH=185
Wht_03173	Traes_5AL_61A545DCD.1	0.71	1.06	0.31	IPR002226	germin-like protein 10
Wht_55980	Traes_6DS_3522B8EF6.1	0.49	0.65	0.039	IPR006015	catalase 2
Wht_00523	Traes_1BL_472C9200C.1	0.03	1.31	0.23		Alpha-2-purothionin
Wht_36106	Traes_1AS_B16A9D49C.1	-0.27	1.25	-0.05	IPR010255	Peroxidase superfamily protein
Wht_04146	Traes_7DL_AA90E8AF5.1	0.35	0.77	-0.39	IPR001623	Chaperone protein DnaJ 1
Wht_01218	Traes_6DL_134445958.1	0.64	1.46	0.53	IPR000167	Cold-shock protein CS120
Wht_13333	Traes_6DL_134445958.1	0.71	1.42	0.67	IPR017853	Cold-shock protein CS120
Wht_00319	Traes_XX_643D0E333.1	-1.80	1.55	-1.28	IPR008978	17.6 kDa class II heat shock protein
Wht_48285	Traes_3AL_E15419B88.1	-0.85	-1.376	-0.36	IPR013126	Heat stress transcription factor A-2b
Wht_25139	Traes_3AL_D23DC53CB.1	-1.12	-1.17	-0.90	IPR001270	Chaperone protein ClpB
Wht_04353	Traes_5BL_D8D745F37.1	-0.96	-0.84	0.048	Wht_04353	Chaperone protein DnaK
Wht_09766	Traes_5AL_C2CE5D766.1	-0.63	-0.73	-0.14	IPR001623	Chaperone protein DnaJ
Wht_09959	Traes_2DS_F1F7FE1D2.1	-0.11	0.01	-2.0	IPR011008	Stress responsive alpha-beta barrel domain protein
Wht_29416	Traes_5BL_E86097AA2.1	0.29	0.11	-1.66	IPR016087	Chalcone--flavonone isomerase
Wht_35368	Traes_XX_74917BCE5.1	0.23	-0.81	0.63	IPR002347	Dehydrogenase/reductase SDR family member 7
Wht_36509	Traes_XX_CD1A48D98.1	-2.35	-0.18	0.04	IPR010987	Glutathione S-transferase family protein
Wht_25111	Traes_1AL_CFF403F3E.1	-1.25	-1.3	0.06		Glutathione S-transferase family protein

Seq ID	Best Blast Hit wheat	Log2 (FC XAP:Certo) Endosperm			InterPRO-IDs	Description/Annotation
		8 DAF	10 DAF	14 DAF		**Ubiquitin-related**
Wht_34900	Traes_4BS_EE7B661C1.1	2.13	0.79	-0.20	IPR000014	Kelch domain-containing protein 10
Wht_36968	Traes_1AL_03E5EB067.1	1.42	0.21	-0.30	IPR001394	Ubiquitin carboxyl-terminal hydrolase 21
Wht_14748		1.10	1.33	-0.40		ubiquitin-protein ligase/ zinc ion binding
Wht_36526	Traes_1BS_940F2E757.1	0.08	0.79	-0.18	IPR004162	E3 ubiquitin-protein ligase SIAH2
Wht_06704	Traes_3DL_4ECE85DE1.1	0.39	0.74	-0.25	IPR000555	STAM-binding protein-like A
Wht_35945	Traes_2BL_B347A3B82.1	0.94	-0.50	-0.21	IPR013083	U-box domain-containing protein
Wht_50117	Traes_2BS_821123BD4.1	0.43	0.95	-0.38	IPR004331	E3 ubiquitin-protein ligase BAH1
Wht_27902	Traes_3B_B5548BCF3.1	0.29	-0.67	-1.08	IPR001810	F-box/RNI-like/FBD-like domains-containing protein
Wht_35361	Traes_6DS_DB325C573.1	-0.99	-0.23	-0.25	IPR019193	UPI000234FE13 related cluster
Wht_07462	Traes_3DL_24BB36F77.1	-0.89	-1.01	-0.52	IPR019955.	polyubiquitin 10
Wht_44222	Traes_3AL_F1C79F0A5.1	-1.00	-1.03	-0.41	IPR019955	polyubiquitin 3
Wht_00884	Traes_5AL_F2EE841DC.1	-1.28	-0.13	0.35	IPR025704	E3 ubiquitin-protein ligase
Wht_05711	Traes_3DL_24BB36F77.1	-0.73	-1.09	-0.48	IPR019955	polyubiquitin
Wht_00935	Traes_2BS_864AB1284.1	0.65	0.89	-0.46	IPR022170	E3 Ubiquitin ligase family protein
Wht_17633	Traes_3AL_997D025FD.1	0.49	0.18	-0.99	IPR019955	polyubiquitin 3
Wht_52187	Traes_XX_F5E62A31C.1	-0.11	0.06	-1.03	IPR016135	Tumor susceptibility gene 101 protein
						Cytochrome P450
Wht_14441	Traes_6AS_4E2AEFF8F.1	3.36	0.59	0.64	IPR001128	Cyp 71d7
Wht_39079	Traes_6BS_11D740D89.1	1.69	0.63	0.10	IPR001128	Cyp 71d7
Wht_48821	Traes_3AS_44927A043.1	-0.05	1.17	0.48	IPR001128	Epoxide hydrolase (low score)
Wht_35700	Traes_XX_438833F7D.1	0.79	0.10	0.73	IPR001128	Cyp 89a2
Wht_38189	Traes_5DS_737779260.1	-0.58	0.48	1.28	IPR001128	Cyp 81d1
Wht_11710	Traes_2AS_82EDB384F.1	0.04	-2.14	0.54	IPR001128	Cyp 709c1
Wht_03251	Traes_2BS_E6A403F63.1	-0.19	-1.66	0.35	IPR001128	Cyp 709c1
Wht_01974	Traes_2BS_E6A403F63.1	-0.38	-1.50	0.28	IPR001128	Cyp 709c1
Wht_31279	Traes_2AS_91AF72EAE.1	0.17	-2.07	-0.83	IPR001128	Cyp 71d10. premnaspirodiene oxygenase
Wht_28639	Traes_2BL_0783B8D8C.1	0.45	-1.98	0.46	IPR001128	Cyp 81d1
						Ribosomal proteins
Wht_42583	Traes_7DL_2F52DA66F.1	3.1922	3.192	1.67	IPR012606	40S ribosomal protein S13-1
Wht_22215	Traes_XX_A2ECE91D6.1	2.2102	2.21	1.14	IPR000529	30S ribosomal protein S6
Wht_10015	Traes_1DS_F0D75DD34.1	2.1647	-2.165	-1.11	IPR011990	30S ribosomal protein S1
Wht_15713	Traes_7DS_304EAFD6B.1	2.5273	-2.527	-1.34	IPR001848	30S ribosomal protein S10
Wht_26880	Traes_1BS_A097EE9C8.1	2.1034	-2.103	-1.07	IPR012340	30S ribosomal protein S1
Wht_27081	Traes_1AS_B0405DDE8.1	2.2702	-2.27	-1.18	IPR008847	30S ribosomal protein S1
Wht_27336	Traes_3B_842771C4C.1	2.8741	-2.874	-1.52	IPR000892	40S ribosomal protein S26-B
Wht_41838	Traes_6DS_BC25BBB52.1	1.9269	-1.927	-0.94	IPR000915	60S ribosomal protein L6
Wht_43449	Traes_3B_EE1F29F0A.1	1.74	-1.74	-0.80	IPR000456	50S ribosomal protein L17

Curriculum Vitae

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Education

10/2012 – present: PhD student in frame of the Leibniz-Graduate school
'Yield formation in cereals –overcoming yield-limiting factor'
Seed development group, Molecular Genetics, Department, Leibniz Institute of Plant Genetics and Crop Plant Research (IPK)

09/2008 - 08/20011: MSc in Plant Biotechnology
Specialization plants for human and animal health
Wageningen University Netherlands
Title of MSc.thesis

Immunological Mechanism of Mushroom Derived β-glucans on Macrophage Endocytosis (Major thesis)

Biological Activity of plant produced human Interleukin-10 on Mast cell proliferation (Minor thesis)

09/2000 - 07/2005: BSc in Horticulture, Jimma University, Jimma, Ethiopia

09/1996- 06/2000: Nefas Mewcha Senior Secondary School, Amhara region Gondar, Ethiopia

Work Experience

02/2011 – 09/2012: Researcher as Plant tissue culture assistant researcher,
Amhara Regional Agricultural Research institute, Bahirdar, Ethiopia

09/2010 -01/2011: Researcher as Horticultural crop assistant researcher
Gondar Agricultural Research Center, Gondar, Ethiopia

10/2005-08/2008 Horticultural crop junior researcher
Gondar Agricultural Research Center, Gondar, Ethiopia

Awards

08/2008 -08/2010-Netherlands Fellowship Programs (NFP), Master's degree program Scholarship, the Netherland

Declaration

I hereby declare that the above particulars are true and correct to the best of my knowledge and belief.

Date: Septemeber 5th, 2016

Place: Gatersleben Yemisrach Melkie Abebaw

Declaration

I, the undersigned, declare that all the work presented in this dissertation was my own, carried out solely at the Seed Development research group of the Leibniz-Institute of Plant Genetics and Crop Plant Research (IPK) Gatersleben. The content of this work has not been previously submitted for a degree in any educational institution. I have not used any other than the permitted reference sources or materials. All references and sources used in the presented work have been appropriately acknowledged.

Gatersleben, September 5th, 2016

Yemisrach Melkie Abebaw

Erklärung über bestehende Vorstrafen und anhängige Ermittlungsverfahren / *Declaration concerning Criminal Record and Pending Investigations*

Hiermit erkläre ich, dass ich weder vorbestraft bin noch dass gegen mich Ermittlungsverfahren anhängig sind. / *I hereby declare that I have no criminal record and that no preliminary investigations are pending against me.*

_____ _____

Datum / Date Unterschrift des Antragstellers / *Signature of the applicant*