# Metabolic Regulation in Developing Barley Seeds – Novel Insights from Transcriptome Analyses

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# Metabolic Regulation in Developing Barley Seeds – Novel Insights from Transcriptome Analyses.


## Abstract

The barley (*Hordeum vulgare*) seed, termed caryopsis, is of high importance for feed and food industry. In addition, it provides an excellent model to study the development and physiology of starch-storing cereal seeds.

Gene expression in caryopses, like all plant organs, is to a large extent regulated by the cellular sugar status. As part of the sugar signalling cascade, we have previously identified the sucrose-inducible barley WRKY transcription factor SUSIBA2 which activates an isoamylase gene involved in starch biosynthesis. Subsequently, the aims of this thesis work were i) to clarify the phylogenetic relationship of *SUSIBA2* with other *WRKY* genes and ii) to investigate the effects of diurnal changes and heat stress on metabolite levels and global gene expression in order to identify new potentially sugar status-dependent processes in developing barley caryopses.

A total number of 45 barley *WRKY* gene family members were identified. Based on comparative phylogenetic analyses with genes from Arabidopsis (*A. thaliana*) and rice (*Oryza sativa*), barley *WRKY* genes putatively involved in development and stress response were assigned. In an attempt to non-invasively study the effect of altered sugar levels, we applied the 22K Barley1 GeneChip microarray to monitor the diurnal changes of global gene expression in barley caryopses. Among 2,091 diurnally regulated genes, almost 900 were highly correlated or anti-correlated to sugar levels, and, thus, potentially sugar status-regulated. Investigations of the short-term (0.5h, 3h and 6h) exposure to heat identified stress response mechanisms and revealed a global repression of carbon metabolism-related genes after as short as 3h of heat stress.

Summarized, this thesis provides the barley research community with a repository of genes involved in metabolic regulation of barley caryopsis development during its adaptation to diurnal changes and heat stress. Candidate genes were identified which merit further investigation of their functions in order to understand and, thereon, modify sugar signalling and carbon metabolism in the barley caryopsis as a model for cereal seeds.



*Keywords:* transcriptome, cereal, seed, storage, diurnal, abiotic stress, heat shock, WRKY, sucrose, starch



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*The important thing is not to stop questioning.*
Albert Einstein

# Contents

# List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Mangelsen, E., Kilian, J., Berendzen, K.W., Kolukisaoglu, H.Ü., Harter, K., Jansson, C., Wanke, D. (2008). Phylogenetic and comparative gene expression analysis of barley (*Hordeum vulgare*) WRKY transcription factor family reveals putatively retained functions between monocots and dicots. *BMC Genomics* 9, 194.

II Mangelsen, E., Wanke, D., Kilian, J., Sundberg, E., Harter, K., Jansson, C. (2010). Significance of Light, Sugar and Amino Acid Supply for Diurnal Gene Regulation in Developing Barley Caryopses. *Plant Physiology* 153(1), 14-33.

III Mangelsen, E., Kilian J., Harter, K., Jansson, C., Wanke, D., Sundberg, E. (2010). Global gene expression analysis of high-temperature stress in developing barley caryopses: Early stress response mechanisms and effects on storage compound biosynthesis. Accepted in *Molecular Plant*.

Papers I and II are reproduced with the permission of the publishers.

# Additional Publications

Sun C.X., Hoglund A.S., Olsson H., Mangelsen E., Jansson C. (2005) Antisense oligo-deoxynucleotide inhibition as a potent strategy in plant biology: identification of SUSIBA2 as a transcriptional activator in plant sugar signalling. *Plant Journal* 44, 128-138.

Wanke, D., Kilian, J., Bloss, U., Mangelsen, E., Supper, J., Harter, K., Berendzen, K.W. (2009). Integrating biological perspectives: A quantum leap for microarray expression analysis. In: Accardi, L., Freudenberg, W.,Ohya, M. (Eds.) QP-PQ: Quantum Probability and White Noise Analysis - Vol. 21 "Quantum Bio-Informatics: From Quantum Information to Bio-Informatics". Quantum Bio-Informatics. Japan, World Scientific.

The contribution of EM to the papers included in this thesis was as follows:

I EM initiated the project, performed molecular genetics and phylogenetics analyses together with the co-authors and wrote the first draft of the manuscript.

II EM initiated and designed the project, performed the majority of the lab work, analyzed the results of the different bioinformatics approaches together with the co-authors, wrote the first draft of the manuscript and acts as corresponding author.

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# Abbreviations

| | |
|---|---|
| AAP | amino acid permease |
| ABA | abscisic acid |
| AGPase | ADP-glucose pyrophosphorylase |
| AMY | alpha-amylase |
| BAM | beta-amylase |
| BE | branching enzyme |
| CWINV | cell wall invertase |
| DE | differentially expressed |
| d.p.a. | days post anthesis |
| DBE | debranching enzyme |
| DPE | disproportionating enzyme |
| EC | endospermal cavity |
| EC: | Enzyme Commission number |
| ESR | embryo surrounding region |
| EST | expressed sequence tag |
| ETC | endosperm transfer cells |
| GBSS | granule-bound starch synthase |
| GWD | glucan, water dikinase |
| HSF | heat shock factor |
| HSP | heat shock protein |
| HSR | heat shock response |
| HXK | hexokinase |
| INVINH | invertase inhibitor |
| ISA | isoamylase |
| LD | limit dextrinase |
| NP | nucellar projection |
| PWD | phosphoglucan, water dikinase |
| RNAi | RNA interference |
| ROS | reactive oxygen species |

| | |
|---|---|
| SBE | starch branching enzyme |
| SnRK | sucrose non-fermenting 1 related protein kinase |
| SS | soluble starch synthase |
| SuSy | sucrose synthase |
| SUT | sucrose transporter |
| STP | sugar transporter |
| TF | transcription factor |
| TILLING | targeting induced local lesions in genomes |
| TPS | trehalose 6-phosphate synthase |
| T6P | trehalose-6-phosphate |
| UGPase | UDP-glucose pyrophosphorylase |

Sugars and amino acids are abbreviated according to international three-letter codes.

# 1 Introduction

Billions of humans are every day dependent on supply of staple food in form of cereal seeds, which constitute more than 50% of the worldwide crop production (www.faostat.fao.org). But even though cereal seeds have attracted great scientific interest, many aspects of the development and physiology of these complex organs are yet far from understood. For example, the exact role and coordination of several enzymes vital for the accumulation of storage starch in net carbon-importing cereal seeds has not been clarified, and little is known about the seed's adaptation to alterations of incoming nutrients provided by net carbon-exporting tissues.

In this thesis the cereal crop barley was used as a model plant to study metabolic regulation in developing starch-storing cereal seeds, termed caryopses. As a follow-up of our previous work on WRKY transcription factor (TF)-mediated sugar signalling in barley (Sun *et al.*, 2005; Sun *et al.*, 2003), members of the barley *WRKY* gene family were identified. The ability of barley caryopses to adapt to diurnal changes and heat stress was investigated by transcriptome analyses and resulted in the identification of about 3,500 differentially expressed (DE) genes, among them many genes related to sugar signalling and starch metabolism. Based on these data, we suggest candidate genes for future studies to enhance our understanding of metabolic regulation in developing barley caryopses.

## 1.1 Barley

### 1.1.1 Origin and use as a cereal crop plant

Barley is a member of the grass family (*Poaceae*) which is estimated to have diverged from a common ancestor 50 to 70 MYA (Kellogg, 2001). As illustrated in Figure 1, barley is phylogenically related to wheat,

*Brachypodium* and oat, all belonging to the clade of *Pooideae*. Based on archeological evidence, human use of the wild ancestor of barley, *Hordeum spontaneum*, started in the Near East about 17,000bc (Zohary & Hopf, 2000). Remains of cultivated barley (*Hordeum vulgare*), distinguishable by its non-brittle spikes, were found in the Fertile Crescent and date back to 8,000bc (Zohary & Hopf, 2000). Since then, barley cultivation has spread to virtually everywhere in the world from sub-Arctic to sub-tropical climate zones (Grando & Macpherson, 2005). In the meanwhile, barley food consumption has decreased markedly. And even though barley is still the major staple food in many regions of the world, the main purpose of barley cultivation is considered to be to produce animal feed as well as beer and whiskey (www.gramene.org; www.grains.org; Grando & Macpherson, 2005). In 2008 the barley world production was estimated to 157MT and, thus, ranked fourth in cereal crop world production after maize, wheat and rice (www.faostat.fao.org).

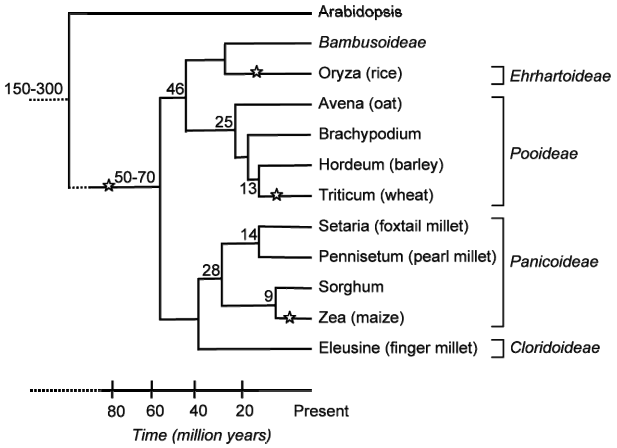


*Figure 1.* Phylogenic relationship between cereal genomes. Divergence times from a common ancestor between the different species are indicated on the branches of the phylogenic tree (in million years). Duplication or polyploidization events are shown with a star. The five main grass subfamilies are indicated in italics (modified from Salse & Feuillet, 2007).

### 1.1.2 Importance as model for cereal genetics and genomics

Arabidopsis, rice and the moss *Physcomitrella* (*P. patens*) are currently the best-established model organisms in plant molecular biology and genetics. This is mainly due to their easy handling, their sequenced genomes and, in case of the second, the high economic value. Compared to the genomes of these models (*A.t.*, 125Mb; *O.s.*, 272Mb, *P.p.,* 511Mb*)* and the recently

sequenced cereal crops sorghum (*Sorghum bicolor*, 730Mb) and *Brachypodium* (*B. distachyon*, 272Mb), the barley genome (estimated 5,000Mb) is huge, as explained by its high content of 80% of repetitive DNA (International Brachypodium Initiative, 2010; Paterson *et al.*, 2009; Schulte *et al.*, 2009; Rensing *et al.*, 2008; International Rice Genome Sequencing Project, 2005; Arabidopsis Genome Initiative, 2000; Bennett & Smith, 1976). The International Barley Sequencing Consortium (IBSC) initiated the sequencing of the barley genome in 2006, but the time for its completion is currently hard to estimate (minutes of the IBSC Business Meeting at Plant Animal Genome XVIII, January 2010; http://barleygenome.org). Nevertheless, several features have led to the establishment of barley as a model for cereal genetics and genomics: Importantly, shedding of the pollen in barley occurs prior to opening of the floret and makes barley a predominantly self-pollinated plant highly suitable for breeding. Also, due to its simpler diploid genome (2n=14) barley has often been used as a genomic model in favour of its close relative, the hexaploid bread wheat (*Triticum aestivum*) (Devos & Gale, 2000). Gene banks, so-called *ex situ* banks, cover at least 370,000 accessions of barley (van Hintum & Menting, 2003), and mutants carrying induced mutations are available to study the genetic variability of barley (http://wheat.pw.usda.gov/ggpages/bgn/; Barley Genetics Newsletter 26;).

In recent years, the concept of functional genomics, defined as use of different "omics"-approaches to study functions of genomes, has further enhanced the value of barley as a model plant (Sreenivasulu *et al.*, 2008a; Lim *et al.*, 2007; Langridge *et al.*, 2006). A broad range of online bioinformatics tools and databases, such as Gramene, GrainGenes, PlantGDB and HarvEST (for websites and refs. see Sreenivasulu *et al.*, 2008a) allow scientists to formulate and test their genetic and genomic hypotheses and to share their results with the barley research community. The expansion of genomic resources for barley becomes also evident by the number of available barley expressed sequence tags (ESTs) on NCBI (www.ncbi.nlm.nih.gov/sites/entrez), which has grown from just 42 ESTs in 1997 to more than half a million (525,777 ESTs on July 9, 2010). These ESTs currently represent a minimum of 23,595 UniGenes, regarded as the minimum number of genes to be found in the barley genome.

Latest technological developments in barley genomics and genetics include the Diversity Arrays Technology (DArT), which allows for the simultaneous analysis of several hundred gene loci without relying on a fully sequenced genome, and has resulted in a high-density map of barley molecular markers linked to agricultural traits (Wenzl *et al.*, 2006; Kilian *et*

*al.*, 2003; Jaccoud *et al.*, 2001). Target Induced Local Lesions IN Genomes (TILLING) facilitates the recovery of individuals carrying allelic variants in candidate genes of mutants and cultivars (Talame *et al.*, 2008). Last but not least, the availability of barley microarrays has enabled global transcriptome analyses. Next to the recently launched 44K Agilent microarray (www.agilent.com), the 22K Affymetrix Barley1 GeneChip (Close *et al.*, 2004) is well-established and allows for interspecies comparisons with e.g. rice, Arabidopsis, *Brachypodium* or wheat, for which corresponding microarrays exist (www.affymetrix.com). In addition to classical expression profiling studies and to overcome the lack of a fully sequenced genome, the Barley1 GeneChip has been utilized to identify single nucleotide polymorphisms, so-called gene expression quantitative trait loci (eQTL) (Wang *et al.*, 2009; Potokina *et al.*, 2008). Summarized, the combination of classical breeding strategies and what Lim *et al.* (2007) termed "genomeless genomics" is considered to provide excellent tools to improve the performance of barley traits like yield, abiotic and biotic stress tolerance (Sreenivasulu *et al.*, 2008a; Varshney *et al.*, 2006).

## 1.2 The barley caryopsis

The caryopsis may be considered the most valuable organ of the cereal plant. First, like seeds of any seed plant, the barley caryopsis as the product of the sexual reproduction has the very central biological function to develop, carry, protect, spread and finally nourish the next generation of the plant, i.e. the plant embryo. Second, seen from the human perspective, the caryopsis as the starch-storing part of the plant is of high nutritional value and thus, third, has a huge economical impact (Grando & Macpherson, 2005).

### 1.2.1 Morphology

The barley caryopsis, alike all cereal seeds, comprises the filial endosperm and embryo as well as the maternal pericarp and seed coat (Figure 2). The endosperm, which comprises the largest part of the caryopsis, constitutes four different cell types: i) starchy endosperm cells which accumulate storage starch, ii) aleurone cells which release catabolic enzymes to promote degradation of storage compounds during germination, iii) endosperm transfer cells (ETC), which facilitate nutrient transport from the maternal to filial tissues and iv) cells of the embryo surrounding region (ESR) which form a physical barrier to the embryo and, presumably, enable its nourishment (Olsen, 2004). As part of the floret, the caryopsis is surrounded

by the floral bracts palea and lemma, which are often summarized as husk or hull. The dorsal lemma partly encloses the ventral palea and elongates into an awn. In addition to their function in seed dispersal and protection against abiotic and biotic stresses, the floral bracts are like the pericarp photosynthetically active and supply the developing seed with carbohydrates (Elbaum *et al.*, 2007; Duffus & Cochrane, 1993). Unloading of assimilates to the filial tissues occurs via the main vascular bundle of the pericarp in the region of the ventral crease. Inside the ventral crease, nutrients from the main vascular bundle are unloaded and transported via the nucellar projection (NP), specialized transfer cells of the nucellar epidermis, into the endospermal cavity (EC), an apoplastic lumen that segregates maternal and filial tissues. From the EC, nutrients are transported into the ETCs of the developing endosperm.

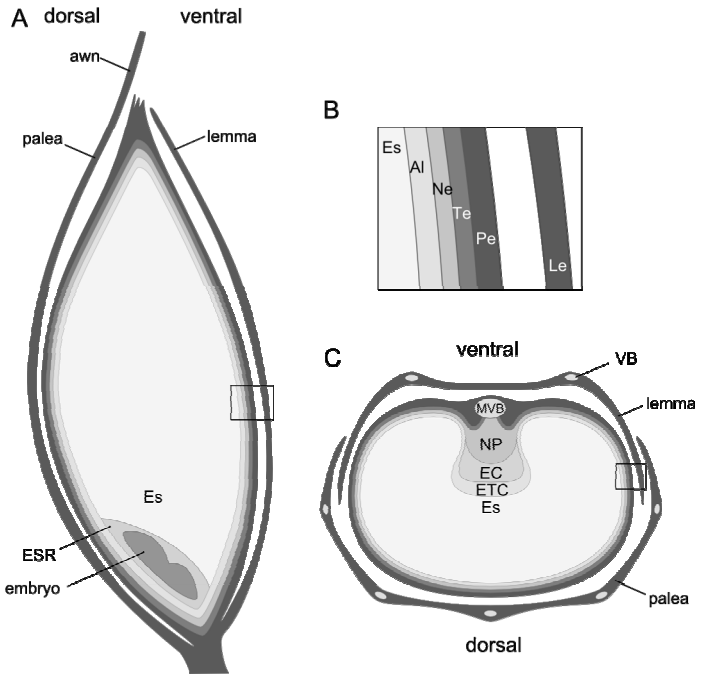


*Figure 2.* Schematic illustration of a developing barley caryopsis and surrounding floral bracts during seed maturation. A, longitudinal cross-section; B, enlargement as indicated by rectangles in A and C; C, median transverse cross-section. Abbreviations are as follows: Al, aleurone, EC, endosperm cavity, Es, endosperm, ESR, endosperm surrounding region, ETC, endosperm transfer cells, Le, lemma, MVB, main vascular bundle, Ne, nucellar epidermis, Pe, pericarp, Te, testa, VB, vascular bundle.

### 1.2.2 Development

From anthesis to late stages of seed maturation, the different cell types of the caryopsis' suborgans change greatly in their number, morphology and functional specification (Olsen, 2004; Duffus & Cochrane, 1993; Kirby & Rymer, 1975). Based on the developmental processes in the endosperm, barley caryopsis development has been divided into three phases (Borisjuk *et al.*, 2004). Briefly, during the prestorage phase, which lasts from fertilization to about four days post anthesis (d.p.a.), the triploid endosperm develops from a syncytial mass to a fully cellularized tissue. During the transition phase around 5 to 10 d.p.a., the caryopsis grows longitudinal and undergoes fundamental metabolic re-programming before it enters the maturation phase at around 11d.p.a.. Early maturation is characterized by high accumulation rates of storage starch inside the endosperm which level off at around 16d.p.a. when the onset of programmed cell death (PCD) in the central region of the endosperm indicates the onset of the late maturation phase (Sabelli & Larkins, 2009). At maturity, the embryo is fully developed, the endosperm cells are tightly packed with starch granules and the floral bracts are adhered to the pericarp. The caryopsis is in a dormant stage, which will be abolished upon seed imbibition when endospermal storage compounds are remobilized to nourish the germinating embryo.

## 1.3 Plant carbon metabolism

### 1.3.1 Sucrose and starch as regulators of carbon balance

Growth and survival of plants is dependent on the balance of carbon assimilation and storage under different conditions and in different organs. As primary products of photosynthesis, sucrose and starch play essential roles in the maintenance of this balance (Rolland *et al.*, 2006). Whilst the disaccharide sucrose is the major transport form of carbon between net carbon-exporting (source) and net carbon-importing (sink) organs (Lim *et al.*, 2006; Patrick & Offler, 2001), starch is a water-insoluble alpha-glucan polymer which acts as carbon storage in two different ways: During daytime, excess carbon produced by photosynthesis is stored as transitory starch in chloroplasts. This intermediate carbon storage pool is utilized at night, when it is degraded to sugars in order to sustain metabolic functions (Zeeman *et al.*, 2010). In contrast, storage starch is accumulated as a long-term energy pool in amyloplasts of sink organs such as roots, tubers and seeds (Comparot-Moss & Denyer, 2009). Next to sucrose and starch, which are arguably the most common carbon balancing compounds in plants,

several other mono-, oligo- and polysaccharides have the potential to affect the carbon balance by acting as cellular carbon storage pools. These include water-soluble sucrosyl-oligosaccharides like fructan and raffinose family oligosaccharides (RFO) as well as water-insoluble cell wall storage polysaccharides like mannans, xyloglucans and galactans (Ritsema & Smeekens, 2003; Peterbauer & Richter, 2001; Blum, 1998).

### 1.3.2 Structure and metabolism of starch

Starch consists of the polyglucans amylose and amylopectin, which form water-insoluble, semicrystalline granules in plastids of plant cells (for a display of starch structures please refer to Zeeman *et al.*, 2010). Amylose is composed of alpha-1,4-linked, mainly linear D-glucose chains. In contrast, amylopectin molecules are highly branched through alpha-1,6-linked glucose bonds which connect the linear alpha-1,4-linked chains. Whilst amylopectin is crucial in the formation of granules and constitutes their major compound, amylose makes up a minor part with about 25% (Zeeman *et al.*, 2010).

Starch biosynthesis and degradation have been extensively studied in source and sink tissues of both model and crop plants (for recent reviews, please refer to Jeon *et al.*, 2010; Zeeman *et al.*, 2010; Comparot-Moss & Denyer, 2009). By now, a minimum of four enzyme classes are considered crucial in the biosynthesis of starch, including ADP-glucose pyrophosphorylase (AGPase; EC: 2.4.1.21), starch synthases (SS; EC: 2.4.1.21), branching enzymes (BE; EC: 2.4.1.18) and debranching enzymes (DBE; ECs: 3.2.1.41 and 3.2.1.68). However, the coordination of their activities by phosphorylation, redox regulation and protein complex formation requires further investigations (Kotting *et al.*, 2010). Several isoforms exist for most of these enzymes and, as demonstrated in several mutant studies, have specific functions in the initiation, elongation, branching and debranching of transient and/or storage starch biosynthesis in source tissues and sink tissues, respectively (Jeon *et al.*, 2010).

### 1.3.3 Enzymes involved in starch biosynthesis and degradation

AGPase catalyzes the initial, rate-limiting step of starch biosynthesis by converting UDP-glucose to ADP-glucose. AGPase is a heterotetrameric enzyme composed of two large (AGP-L) and two small (AGP-S) subunits, encoded by distinct genes (Comparot-Moss & Denyer, 2009). Interestingly, AGPase has a plastidial localization in all plant tissues except the cereal endosperm, where the major portion of AGPase activity is found in the cytosol (Rosti *et al.*, 2006; Johnson *et al.*, 2003). In the cereal endosperm,

the subcellular localization of the enzyme is determined by two alternative splicing products exist for the AGP-S subunit (Thorbjornsen *et al.*, 1996). Upon synthesis of ADP-glucose in the cytosol, ADP-glucose is transported via an ADP-glucose transporter across the amyloplast membrane into the stroma and site of starch synthesis (Patron *et al.*, 2004). Inside the plastid, four different classes of soluble starch synthases (SSI to SSIV) utilize ADP-glucose to synthesize amylopectin. SS mutants showed altered amylopectin structures, suggesting that all SS have particular functions in amylopectin biosynthesis and cannot substitute each other (Jeon *et al.*, 2010). Branching points of the amylopectin macromolecule are inserted by the action of branching enzymes (BE), which cut existing α-1,4-glucan chains and transfer the cut segment to the C6 position of a glucosyl residue of another glucan chain. Isoamylases (ISA) and limit dextrinases (LD, also called pullanases), summarized as DBEs, play and important role in the determination of amylopectin structure by selectively cleaving branch points. In DBE mutants, granular starch is often reduced and replaced by water-soluble phytoglycan, which is characterized by a higher degree of glucan chain branching (for refs. see Zeeman *et al.*, 2010). Amylose is synthesized by granule-bound starch synthases (GBSS), and mutants lacking this enzyme are essentially amylose free (Denyer *et al.*, 2001).

Enzymes involved in starch degradation processes include alpha-amylases (AMY; EC: 3.2.1.1), beta-amylases (BAM; EC: 3.2.1.2), DBEs, the glucan-phosphorylating enzymes glucan, water dikinase (GWD; EC: 2.7.9.4) and phosphoglucan, water dikinase (PWD; EC: 2.7.9.5) as well as disproportionating enzymes (DPE; EC: 2.4.1.25). Today's understanding of starch degradation is mainly based on studies in leaves, where transitory starch is utilized during the night, and investigations of germinating caryopses, where endospermal storage starch is utilized to provide the growing embryo with carbon-based energy. The physiological circumstances and, thus, regulation and coordination of these two processes differ greatly (Zeeman *et al.*, 2010).

Starch In barley, a minimum of 38 genes encoding different classes and isoforms of enzymes involved in starch biosynthesis and/or degradation have been annotated based on publicly available EST sequence information (Radchuk *et al.*, 2009). Some of these genes have been analyzed in mutant-based studies, such as *HvAGP-S* (*Risø16* mutant), *HvSSIIa* (*sex6* mutant) and *HvGBSSI* (*waxy* mutant), but their majority lacks a detailed functional characterization (Jeon *et al.*, 2010; Johnson *et al.*, 2003; Morell *et al.*, 2003; Patron *et al.*, 2002).

## 1.4 Regulation of carbon metabolism

### 1.4.1 Sugar signalling

In addition to their function as prime carbon supply, sugars have important regulatory functions in the control of plant metabolism, development, and stress responses. In general, high sugar levels repress photosynthesis and induce polysaccharide, protein and lipid synthesis whereas sugar depletion stimulates gluconeogenesis and mobilization storage compounds. Different sugars, including mono-, di- and polysaccharides, are synthesized, transported and utilized at different times and locations in the plant body. To enable the specific mediation of different sugar signals, plants have evolved complex signalling networks (Rolland *et al.*, 2006). Figure 3 depicts a selection of single-gene studies which demonstrated the importance of Glc, Suc and Tre as central 'sugar signals' in the regulation of starch metabolism and will be discussed below.

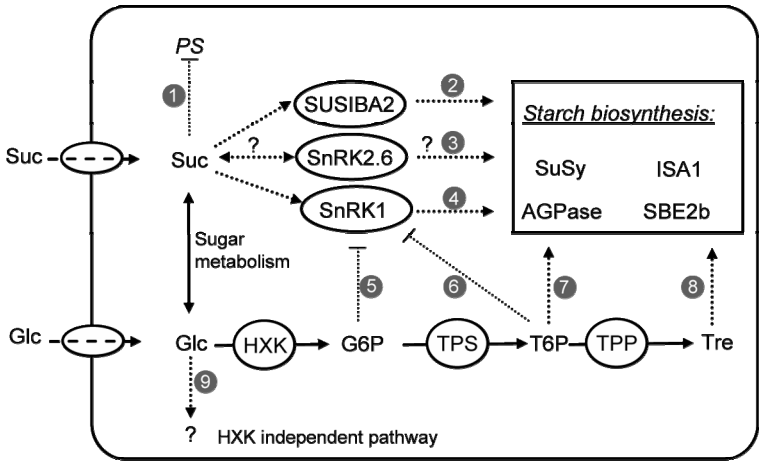


*Figure 3.* Model summarizing sugar signalling cascades related to starch biosynthesis in plant cells. Dashed arrows indicate transport, solid arrows depict metabolic pathways, and dotted arrows represent signalling pathways. Studies that identified the different signalling cascades are indicated by numbers 1 to 9 as follows: 1, Sheen *et al.* (1990); 2, Sun *et al.* (2003); 3, Zheng *et al.* (2010); 4, McKibbin *et al.*(2006) and Tiessen *et al.* (2003); 5, Toroser *et al.*(2000); 6, Zhang *et al.* (2009); 7, Kolbe *et al.* (2005) and Lunn *et al.* (2006); 8, Wingler *et al.* (2000); 9, Xiao *et al.* (2000) and Moore *et al.* (2003). Please observe that the depicted signalling cascades were identified in different species and organs.

As part of the glucose signalling cascade, hexokinase proteins (HXK) act as glucose sensors and mediate the regulation of primary metabolism (Claeyssen & Rivoal, 2007and refs. therein). In addition to the HXK-dependent Glc signalling pathway which act via glucose-6-phosphate, studies of *glucose insensitive* (*gin*) mutants in Arabidopsis have provided evidence for the existence of a HXK-independent Glc signalling pathway (Moore *et al.*, 2003; Xiao *et al.*, 2000).

Sucrose is easily and frequently degraded to glucose and fructose by extra- and intracellular invertases. Nevertheless, several studies have shown a sucrose-specific regulation of gene expression and protein activity. Sucrose-induced expression has for example been shown for genes encoding patatin (Jefferson *et al.*, 1990), UDP-glucose pyrophosphorylase (Ciereszko *et al.*, 2001), a WRKY TF and an isoamylase (Sun *et al.*, 2003) as well as for genes involved in anthocyanin biosynthesis (Solfanelli *et al.*, 2006). Recently, sucrose was also found to repress the translation of AtbZIP11 (Hanson *et al.*, 2008). The sucrose non-fermenting protein kinase 1 (SnRK1) mediates the sucrose-induced activation of sucrose synthase (SuSy) and AGPase transcription and translation in potato tubers (McKibbin *et al.*, 2006; Tiessen *et al.*, 2003). Recently, increased sucrose levels were found in mutants of the paralogous SnRK2.6 gene (Zheng *et al.*, 2010), which further supports the significance of the SnRK gene family in regulating plant carbon metabolism.

The central role of trehalose and its precursor trehalose-6-phosphate (T6P) in plant sugar signalling is nowadays well-established (Paul *et al.*, 2008). For instance, T6P induces the thioredoxin-mediated redox activation of AGPase in Arabidopsis leaves and stimulates starch biosynthesis upon either sucrose or direct T6P feeding (Lunn *et al.*, 2006; Kolbe *et al.*, 2005; Wingler *et al.*, 2000). On the other hand, T6P was shown to inhibit SnRK1 in Arabidopsis seedlings and, consequently, activate processes involved in cell growth which are usually repressed by SnRK1 (Zhang *et al.*, 2009).

In addition to single-gene studies, transcriptome analysis has enabled the investigation of global sugar signalling networks. The combined analysis of sugar feeding and starvation experiments in wild type and starchless *pgm* mutants identified several hundred, potentially sugar-regulated gene candidates in Arabidopsis seedlings (Usadel *et al.*, 2008; Osuna *et al.*, 2007; Blasing *et al.*, 2005; Gibon *et al.*, 2004). And in addition to the well-known connection of sugar and abscisic acid (ABA) signalling networks (Bossi *et al.*, 2009), microarray analyses have revealed a cross-talk between sugar and nitrogen signalling (Palenchar *et al.*, 2004).

### 1.4.2 Diurnal regulation

Light, temperature and the circadian clock, i.e. a free-running internal molecular timekeeper, regulate the adaptation of plants to diurnal light/dark cycles (Yakir *et al.*, 2007; McClung, 2006; Harmer *et al.*, 2000). Carbon metabolism, greatly dependent on incoming light energy, is adapted to these diurnal rhythms, and several genes related to starch biosynthesis and degradation are diurnally regulated in Arabidopsis leaves (Smith *et al.*, 2004). Not surprisingly, Arabidopsis seedlings that are entrained to a particular photoperiod are highly sensitive to extensions of the dark phase, which results in the depletion of cellular carbon pools (Osuna *et al.*, 2007). Based on these findings, Smith and Stitt (2007) conclude that plants have developed an *acclimatory* response to diurnal changes of carbon supply in order to optimize their physiology and growth under these regular and, thus, predictable diurnal changes. In contrast, sudden carbon starvation is met by an *acute* response, which resembles responses to abiotic stresses (Baena-Gonzalez & Sheen, 2008). The complexity of diurnal regulation was further demonstrated by Usadel *et al.* (2008), who combined different microarray datasets to predict carbon-dependent diurnal processes in Arabidopsis seedlings.

## 1.5 Effects of heat stress on seed development

To be able to sustain growth under the impact of abiotic stresses like heat, cold, drought and salinity, plants have evolved complex adaptation strategies. On cellular level, these strategies include sensing of the stress followed by the activation of signalling cascades which amplify the initial signal and, concomitantly, lead to the activation of downstream events involved in stress defence and adaptation. Whilst little is known about the primary sensors of different abiotic stresses, their downstream signalling cascades have been studied extensively (Kotak *et al.*, 2007a; Mittler *et al.*, 2004). Several abiotic stresses occur to share the same upstream signalling components whilst specification of the signal occurs further downstream in a spatially and temporally specific manner (Kilian *et al.*, 2007; Kreps *et al.*, 2002). In addition, genes which do not respond to a single stress show an altered expression during the combined exposure to several stresses (Rizhsky *et al.*, 2004).

Other abiotic stresses alike, heat stress induces global cellular reprogramming. Processes which are induced during heat stress response include the production of the phytohormone ABA, accumulation of heat shock proteins (HSPs) and the production of antioxidants to prevent damage

by reactive oxygen species (ROS) (Kotak *et al.*, 2007a; Mittler *et al.*, 2004). Heat stress further results in the shortening of the plants' developmental phases and reduces the net time for processes related to carbon assimilation, i.e. transpiration, photosynthesis and respiration (Barnabas *et al.*, 2008 and refs. therein). Consequently, the number and size of seeds are negatively affected by heat (Passarella *et al.*, 2008; Nicolas *et al.*, 1985). Among the enzymes involved in the biosynthesis of storage starch, AGPase, GBSS and BEs are repressed by heat (Yamakawa *et al.*, 2007; Linebarger *et al.*, 2005; Singletary *et al.*, 1994). As heat stress is one of the major reasons for worldwide yield losses (Battisti & Naylor, 2009; Peng *et al.*, 2004; Boyer, 1992), one common goal in abiotic stress research is the achievement of an enhanced stress tolerance in cereal crops (Barnabas *et al.*, 2008). But whilst studies on the short-term response of Arabidopsis to heat have led to the identification and validation of global regulators of heat stress response in this model organism (Kotak *et al.*, 2007a), information on the very early regulators of heat stress responses in cereals, and, in particular, cereal seeds, is still limited (Barnabas *et al.*, 2008 and refs. therein).

## 1.6 WRKY TFs

### 1.6.1 Regulation of seed development and stress responses

As recently reviewed by Rushton *et al.* (2010), *WRKY* (pronounced *"worky"*) genes regulate diverse plant stress responses and developmental processes, including heat stress responses and seed development. For example, *AtWRKY7* expression was strongly reduced in heat shock factor (HSF) double knockout mutants *hsfA1a/hsfA1b* (Busch *et al.*, 2005) and suggest a downstream role of this *WRKY* gene in the HSFA1a/b-mediated heat shock response (HSR). And whereas Arabidopsis *wrky25* mutant seedlings showed an increased thermosensitivity (Li *et al.*, 2009), the heat shock promoter-driven expression of *OsWRKY11* led to and activation of raffinose biosynthesis and an enhanced desiccation tolerance in rice seedlings (Wu *et al.*, 2009). The WRKY protein SUGAR SIGNALLING in BARLEY 2 (SUSIBA2) is involved in sucrose-mediated regulation of starch biosynthesis in the barley endosperm (Sun *et al.*, 2005; Sun *et al.*, 2003). TRANSPARENT TESTA GLABRA 2 (TTG2, AtWRKY44) and MINISEED3 (MINI3, AtWRKY10) are other examples of WRKY proteins involved in seed development. Whilst *ttg2* mutants are impaired in seed coat pigmentation, *miniseed3* mutants are characterized by reduced seed

size as a consequence of an impaired embryo development and endosperm cellularization (Zhou *et al.*, 2009; Ishida *et al.*, 2007).

### 1.6.2 Protein structure and phylogeny

The common feature of all WRKY proteins is the existence of either one or two, approximately 60 amino acid-spanning WRKY-domains consisting of an N-terminal WRKYGQK sequence stretch and a C-terminal zinc finger (Figure 4). The three-dimensional structure of the WRKY domain together with nine adjacent N-terminal amino acids consists of a five-stranded beta-sheet forming a zinc-binding pocket. The WRKYGQK motif is located on the second beta-sheet and, judged from the highly conserved sequence and the three-dimensional orientation, a candidate motif to enable specific DNA recognition (Duan *et al.*, 2007; Yamasaki *et al.*, 2005). The conserved consensus sequence of the WRKY binding site, the W-box, is TTGACC/T, even though some WRKY proteins have been found to bind to other cis-elements called SURE and WK-box (van Verk *et al.*, 2008; Sun *et al.*, 2003).

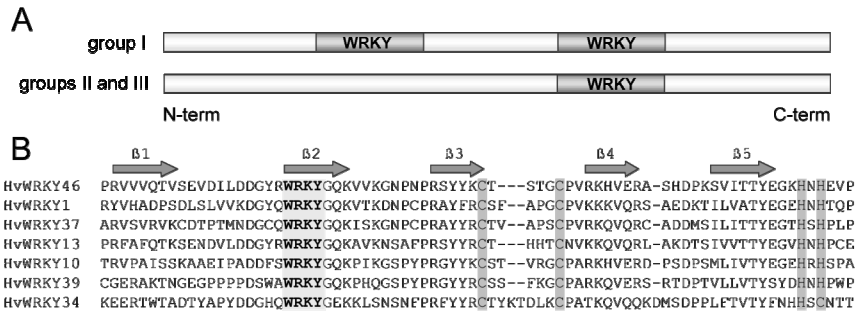


*Figure 4.* The WRKY domain. A, Location of WRKY domains in WRKY protein groups I, II and III. B, HvWRKY protein domain sequence alignment of proteins belonging to WRKY group I (HvWRKY46, C-terminal domain), IIa (HvWRKY1), IIb (HvWRKY37), IIc (HvWRKY13), IId (HvWRKY10), IIe (HvWRKY39) and III (HvWRKY34). The conserved WRKY amino acid signature is highlighted in bold letters and light grey shading, amino acids forming the zinc finger motif are displayed in grey shading, gaps are marked with dashes. Five ß-strands of the three-dimensional protein structure are shown as arrows above the alignment.

Recent studies have shown that the *WRKY* gene family is part of the *WRKY-GCM1* superfamily of zinc finger TF genes (Babu *et al.*, 2006). Whilst the zinc finger motif is conserved in all organism kingdoms, the WRKYGQK motif is limited to the plant lineage. With 74 and 102 members in Arabidopsis and rice, the *WRKY* gene family is one of the

largest TF families in higher plants (Ross *et al.*, 2007; Eulgem *et al.*, 2000). *WRKY* genes have been classified into groups I, IIa to IIe and III based on the number of WRKY domains and the structure of the zinc finger. However, the grouping according to structural features does not reflect the true phylogenetic relationship of the genes, as indicated by the polyphyletic nature of WRKY group II (Zhang & Wang, 2005).

# 2 Aims

The main objective of the studies included in this thesis was to investigate mechanisms underlying the adaptation of developing barley caryopses to diurnal changes and heat stress. To be able to provide a repository of genes involved in the metabolic regulation of barley caryopsis development, global transcriptome analyses were performed with a particular focus on genes involved in sugar signalling and starch biosynthesis. The specific aims were:

- To identify members of the barley *WRKY* gene family, to analyze their phylogenetic relationship and to assign *WRKY* gene candidates potentially involved stress responses and caryopsis development.

- To monitor global mechanisms underlying the caryopsis' adaptation to diurnal changes and high temperature stress.

- To identify genes involved in carbon metabolism and signalling which are responsive to diurnal and/or temperature changes in barley caryopses.

- To suggest candidate genes for future studies and genetic engineering approaches which aim at improving the caryopsis' sink strength and its ability to adapt to heat stress.

# 3 Results and Discussion

## 3.1 The barley *WRKY* gene family consists of a minimum of 45 members (I)

At the time we initiated our project, four barley WRKY proteins (HvWRKY) had been described to play regulatory roles in different processes of barley development: HvWRKY38, involved in drought and cold response as well as the regulation of an alpha-amylase upon seed germination (Xie *et al.*, 2007; Mare *et al.*, 2004), HvWRKY1 and HvWRKY2, regulating the response to powdery mildew (*Blumeria graminis*) infections (Shen *et al.*, 2007), and SUSIBA2, involved in sugar signalling and regulation of starch biosynthesis (Sun *et al.*, 2003). As evident from the processes the HvWRKY proteins were involved in, i.e. seed germination (malting), resistance to abiotic and biotic stresses and accumulation of storage starch, all of them carried a potential economic relevance as targets for barley breeding. However, the lack of knowledge about their phylogenetic relationships prevented both intra- and interspecies comparisons, which would have aided a deeper analysis of this obviously important gene family in barley. To overcome these limitations, we screened the available EST data in GenBank and identified a minimum number of 45 unique *HvWRKY* genes which we annotated in NCBI. Based on a phylogenetic analysis that included the WRKY domains of all proteins from Arabidopsis, rice and barley, HvWRKY proteins were grouped into the pre-defined subgroups I to III (Eulgem *et al.*, 2000). Subclusters consisting solely of rice and barley WRKY domains indicate a monocot-specific diversification of *WRKY* genes.

In comparison with the total number of *WRKY* genes in Arabidopsis and rice, we assume that our study identified about 50% of all *HvWRKY* genes

based on EST sequence data. Since we performed our analysis in 2007, the number of EST sequences deposited at NCBI has increased from roughly 440,000 to about 525,000. However, a repeated screen of EST sequences performed in June 2010 only returned two previously unknown *HvWRKY* sequences (personal observation). Whilst the exact number of *HvWRKY* genes will remain unknown until the barley genome has been fully sequenced, the identified *HvWRKY* genes have often already been assigned orthologs in Arabidopsis and rice and allow for knowledge transfer from these model species to predict putative biological functions of *HvWRKY* genes. Our finding that the previously annotated genes *HvWRKY38* and *HvWRKY1* are actually allelic versions of the same gene further supported the relevance of an unambiguous nomenclature for members of TF gene families in grasses (Gray *et al.*, 2009).

## 3.2 *HvWRKY* genes are involved in stress responses and development (I, II, III)

As an initial step towards their functional characterization, we analyzed the expression patterns of *HvWRKY* genes applying the Barley1 GeneChip (Close *et al.*, 2004). Among the 45 *HvWRKY* genes, 20 were represented by at least one probe set on the microarray and used for further investigations. A screen of the dataset by Caldo *et al.* (2004) revealed that several of these *WRKY* genes were induced upon an infection by powdery mildew (*Blumeria graminis*) and suggested their involvement in pathogen response. In contrast, *HvWRKY* genes which were not DE during powdery mildew infection often depicted different expression levels in different plant organs. A comparative expression analysis using developmental datasets from Arabidopsis and barley (Druka *et al.*, 2006; Schmid *et al.*, 2005) demonstrated that the expression of orthologous *WRKY* genes is often correlated in analogous organs of these two species. And thus, their function in the development of Arabidopsis and barley may be conserved. A recently published developmental dataset for rice (Wang *et al.*, 2010) will enable similar comparative expression analyses among the three species to allow for further testing of this hypothesis.

Based on the results of our own microarray experiments, transcripts of a minimum of 13 *HvWRKY* genes are present in developing caryopses at the age of 12 d.p.a., including *HvWRKY1*, *4* to *10*, *13*, *34*, *41*, *42* and *SUSIBA2*, classified as *HvWRKY46*. Only two of them, *HvWRKY4* and *HvWRKY5*, are also responsive to infections with powdery mildew in barley leaves, which allows speculating that the majority of the caryopsis-

expressed genes have other functions than the regulation of responses to pathogen attacks. To our surprise, even though sucrose levels fluctuated greatly during the day and were at least slightly increased during heat stress exposure, transcript levels of *SUSIBA2* were unaffected in both experiments. An investigation of the expression profile of its ortholog *AtWRKY20* during development and under the exposure to various stresses (Kilian *et al.*, 2007; Schmid *et al.*, 2005) did not reveal a transcriptional response under any of the circumstances tested (data not shown). Hence, the exact molecular circumstances that trigger *SUSIBA2* expression during sucrose feeding experiments (Sun *et al.*, 2003) remains elusive. *HvWRKY41*, like *SUSIBA2* a group I *WRKY* gene, is the only caryopsis-expressed *HvWRKY* which is diurnally regulated and repressed upon exposure to high temperature. Nevertheless, the distinct temporal and spatial expression patterns of several caryopsis-expressed *HvWRKY* genes suggest their significance for caryopsis development and physiology, such as the predominantly endosperm-expressed genes *HvWRKY7* and *SUSIBA2* or the pericarp-expressed *HvWRKY13*. A pilot study on the orthologs of *HvWRKY13* in Arabidopsis, *AtWRKY24*, *AtWRKY56* and *AtWRKY43*, which depict developmental expression patterns similar to *HvWRKY13*, has indicated an impaired development of seeds and siliques in T-DNA insertion mutants of these genes (E. Mangelsen, unpublished data).

## 3.3 Diurnal changes affect gene expression in barley caryopses (II)

Global expression profiling has been applied to study the developmental processes in the barley caryopsis from early time points after flowering until late maturation stages (Sreenivasulu *et al.*, 2008b; Druka *et al.*, 2006; Sreenivasulu *et al.*, 2006; Sreenivasulu *et al.*, 2004). A particular focus has also been set on the developmental expression patterns of genes involved in starch biosynthesis and degradation, which were analyzed using Northern blots (Radchuk *et al.*, 2009). But whilst a strong impact of diurnal changes on genes involved in carbon metabolism was shown for net carbon-exporting Arabidopsis leaves (Blasing *et al.*, 2005; Smith *et al.*, 2004), the diurnal impact on net carbon-importing developing caryopses remained unknown.

In an attempt to identify diurnally regulated processes in barley caryopses, we monitored their gene expression at six time points during a 24h cycle and identified 2,091 DE genes, which were assigned to six major expression clusters. 45% of all DE genes grouped into the diurnal clusters Set 1 and Set

3, which are characterized by a pronounced up- or downregulation *in anticipation* rather than *in response* to light/dark shifts. Several hundred transcript profiles were highly correlated or anti-correlated to sucrose, glutamine, glutamate, asparagine and aspartate, known as major carbon and nitrogen transport forms in the phloem (Lam *et al.*, 1995), thereby suggesting a tight adaptation of metabolic and regulatory processes to imported metabolites. This was further supported by the differential expression of genes with a presumable function in phloem unloading, such as amino acid permeases (AAP) and aquaporins (Thiel *et al.*, 2008; Patrick & Offler, 2001). A comparison with the previously published dataset by Sreenivasulu *et al.* (2008b) revealed that pericarp, endosperm and embryo tissues contribute equally to diurnal regulation in barley caryopses. At the same time, many tissue-specific transcripts allow speculating that tissue-specific pathways for diurnal regulation exist in barley caryopses.

In addition to the predicted effects on photosynthesis and starch metabolism (see below), our analysis revealed the diurnal cycling of embryo development, cell growth and chaperone activity – all being unexpected targets of diurnal regulation. The distinct peak of late embryogenesis abundant (LEA; Battaglia *et al.*, 2008) gene expression at the early light phase and maximum expression of many polysaccharide biosynthesis genes at the later light phase indicates that fluxes into carbon and nitrogen storage pools are not only developmentally regulated (Borisjuk *et al.*, 2004) but also temporally separated on day-to-day basis. Further, maximum transcript levels of starch biosynthesis genes often preceded expressional peaks of genes related to cell wall expansion, and it remains elusive whether cell wall expansion is somehow mechanically triggered by the enlargement of starch granules in a diurnal manner. In general, the pronounced cycling of cell wall polysaccharide biosynthesis and degradation genes hints at the existence of a short-term storage pool for excess carbon in cell walls of the caryopsis. Thus, next to the developmentally triggered remobilization of cell wall polysaccharides from stems towards the end of caryopsis maturation (Blum, 1998), diurnal remobilization of cell wall polysaccharides inside the caryopsis may occur as a strategy to avoid carbon starvation. Summarized, our data demonstrate that a fine-tuned acclimatory response to diurnal changes of metabolite supply and light exists in barley caryopses, as previously described for Arabidopsis leaves (Smith & Stitt, 2007). The fact that diurnal regulation exists for processes central to caryopsis development should be well-considered in future studies.

## 3.4 Early HSR events in barley caryopses (III)

The very early events during heat stress exposure in cereal seeds had, despite a recent study in wheat (Qin *et al.*, 2008), remained unknown. To elucidate these events and to identify expressional regulators which have the potential to improve heat stress tolerance in barley caryopses via genetic engineering, we initiated a study in which we monitored the transcriptome of barley caryopses after 0.5h, 3h and 6h of heat exposure.

Heat stress had a very early and pronounced effect on gene expression in barley caryopses, as indicated by a total number of 956 heat-induced and 1,122 heat-repressed genes. The induction of *HSF* and *HSP* genes demonstrated that conserved HSR mechanisms exist in barley caryopses. The immediate effect (3h and 6h) on genes involved in very central, sink-strength determining functions of the barley caryopsis, was, however, unexpected. For example, the repression of genes encoding transporters for sugars, amino acids and peptides implies an early negative effect on global metabolite transport in the caryopsis, most likely in the area of phloem unloading, including NP and ETC (Thiel *et al.*, 2008). Repression of starch biosynthesis genes and induction of starch degradation genes suggest an impairment of the central function of the endosperm in this very short timeframe. The induction of several maternal embryo effect (*MEE*), embryo deficient (*EMB*) and *LEA* genes (Battaglia *et al.*, 2008; Tzafrir *et al.*, 2004) further indicates an immediate speed-up of processes which favour embryo development and desiccation tolerance, most likely to ensure survival of the next generation. The dramatic repression of 49 histone genes may indicate a severe impact on endosperm growth, which involves endoreduplication processes in nuclei of endosperm cells (Ngyuen, 2007). Alternatively, global histone repression could be an indicator for the epigenetic reprogramming induced by heat, as recently described for Arabidopsis seedlings (Kumar & Wigge, 2010). Indeed, seeds as carrier of the next generation are the major target for an epigenetically inherited trans-generational adaptation to stress (Boyko & Kovalchuk, 2008). The predicted suborgan-localization of DE genes based on the comparison with data of Sreenivasulu *et al.* (2008b) indicates that all suborgans are responsive to heat stress. Compared to the embryo-specific genes, a larger proportion of the predominantly endosperm- and pericarp-expressed genes were affected by the heat stress, suggesting that these metabolically active tissues require more pronounced adaptations during heat exposure. Summarized, our data provide novel insights into the regulatory processes that trigger heat responses and adaptation in barley caryopses and allow for the selection of candidate genes for future functional studies (see below).

## 3.5 Identification of temperature-responsive, diurnally regulated genes (II, III)

A comparison of the DE genes in our two approaches revealed that 306 and 284 genes in developing barley caryopses were diurnally regulated *and* heat-induced and –repressed, respectively (Figure 5 and Table 1). This suggests that about one fourth (590 out of 2,091genes) of all diurnally expressed genes are, at least partly, regulated by daily temperature changes. Coherently, the majority of heat-induced genes cluster into the diurnal Sets 3 and 5, which depict expressional maxima at midday and in the afternoon when the temperature is high, whereas heat-repressed genes predominantly group in the diurnal Set 2 with maximum expression levels at night. Interestingly, several *HSP* and *HSF* genes which are induced during heat stress response are also diurnally regulated, which demonstrates that chaperone-mediated protein stabilization occurs on a daily basis and that their function goes beyond protein protection during acute stress responses.

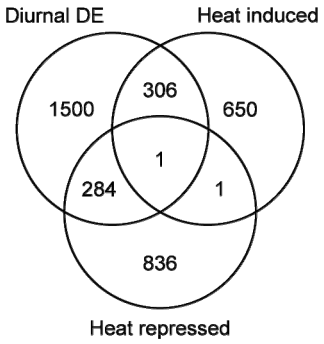


*Figure 5.* Venn Diagram depicting the number of overlapping diurnally and heat stress-regulated genes in developing barley caryopses.

Assuming that diurnally cycling genes are generally important for normal metabolic regulation and caryopsis development, their overlap with heat-responsive genes identifies processes which are particularly important targets for genetic engineering towards a proceeding normal caryopsis development during (transient) heat stress. The overlapping 284 diurnally regulated and heat-*repressed* genes (Figure 5) are particularly interesting candidates as they are potentially involved in processes that lead to a reduced performance under heat exposure. Not surprisingly, several of these genes encode heat-labile proteins of the photosystem, such as the rubisco activase and light harvesting complexes (Allakhverdiev *et al.*, 2008). In addition, several transporters and expansin genes should be considered as targets for genetic

engineering to maintain normal phloem unloading and cell expansion under (transient) heat stress exposure.

*Table 1. Number of overlapping diurnally regulated and heat-responsive genes in developing caryopses of barley.*

| Diurnal Set | heat induced genes ([%]) | heat repressed genes ([%]) |
|---|---|---|
| 1 | 28 (9) | 45 (16) |
| 2 | 16 (5) | **86 (30)** |
| 3 | **86 (28)** | 50 (17) |
| 4 | 17 (6) | 51 (18) |
| 5 | **104 (34)** | 28 (10) |
| 6 | 55 (18) | 24 (9) |
| Total | 306 (100) | 284 (100) |

## 3.6 Starch biosynthesis genes as targets for abiotic regulation in barley caryopses (II, III)

Summarizing our data from papers II and III we identified several genes involved in the conversion from sucrose to starch which are responsive to diurnal changes and/or heat stress (Figure 6). A general notice is that diurnal regulation occurs at all levels in the conversion from sucrose to starch and that genes involved in sugar transport and starch biosynthesis are repressed during heat stress whereas starch degradation, as indicated by *BAM* gene induction, is promoted. The previously described functional differences of the sucrose transporter genes *HvSUT1* and *HvSUT2* (Weschke *et al.*, 2000) is supported by their distinct responsiveness to heat and diurnal cycling, respectively. Also, the specific regulation of several barley *HXK* and *TPS* genes under these circumstances suggest their involvement in metabolic regulation in developing barley caryopses, as previously described for their orthologs in other plant species and tissues (Paul *et al.*, 2008; Claeyssen & Rivoal, 2007). Genes encoding different starch biosynthesis protein isoforms (Jeon *et al.*, 2010) are often either diurnally regulated or heat-responsive. For instance, genes of the different SS isoforms respond to heat (*SSI* and *SSIIa*) and diurnal changes (*SSIIIb*) or remain unaffected by both (*SSIV*). Judged from their functions in amylopectin chain elongation in rice and barley (Fujita *et al.*, 2006; Morell *et al.*, 2003), the repression of genes encoding SSI and SSIIa during heat stress allows assuming that starch of heat-stressed barley caryopses comprises glucans with a reduced chain length. The recently annotated barley *SSIIIb* gene shows a distinct

expressional peak in developing barley endosperm tissues at 12d.p.a. (Radchuk *et al.*, 2009). Its pronounced diurnal regulation suggests an important function during this developmental stage, which might even be related to the initiation of starch granule formation, as recently demonstrated for *SSIII* and *SSIV* genes in Arabidopsis (Szydlowski *et al.*, 2009).

Even though obvious for the critical user of microarray data, it needs to be stated at this point that conclusions on single gene functions involved in carbon metabolism only allow to *formulate* but not to *prove* any hypotheses – clearly, additional functional analyses on protein level are required to draw further conclusions. Nevertheless, based on transcriptome analyses – by now the most sensitive way of monitoring plant responses on a systems-level – we are able to identify (and reduce!) the number of interesting gene candidates for functional studies, a topic that will be discussed in the next section.

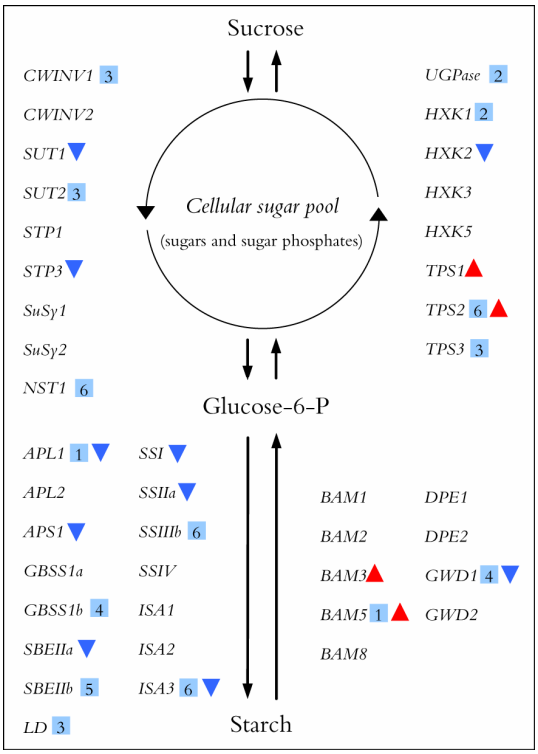


*Figure 6.* Summary of diurnally regulated and heat-responsive genes involved in the conversion from sucrose to starch. Diurnally regulated genes are labelled by light-blue squares with numbers indicating the diurnal clusters 1-6. Heat induced and repressed genes are indicated by red and blue arrows, respectively. For complete gene names please refer to the text.

## 3.7 Suggestions for candidate genes for future studies (I, II, III)

The roughly 3,500 DE genes expressed in developing barley caryopsis under diurnal and heat stress conditions provide a large repository of gene candidates to choose from. And even though it is premature at this point to suggest precise molecular breeding approaches, we would like to assign genes related to carbon metabolism and transcriptional regulation that should be tested for their potential to increase the sink strength of the barley caryopsis and/or to improve its tolerance to high temperature exposure. The suggested gene candidates may very well serve both purposes, as carbon metabolism and stress response are generally two tightly interweaved cellular processes (Baena-Gonzalez, 2010).

Among regulatory genes – usually manipulated in order to obtain a global effect on several downstream targets (Umezawa *et al.*, 2006) – the diurnally regulated *HvWRKY41* constitutes an interesting candidate. *HvWRKY41* is diurnally expressed (Set3) and highly correlated to sucrose levels (correlation coefficient 0.862), downregulated during heat stress exposure and mainly expressed in pericarp and endosperm tissues. Speculating that this gene is somehow involved in the regulating of photoassimilate flux from pericarp to endosperm tissues via the EC, its tissue-specific induction might increase sink strength under both normal and heat stress conditions.

The three *HSF* genes *HvHSFA2c*, *HvHSFA2d* and *HvHSFB2b* are rapidly and consistently induced during heat stress in barley caryopses. In addition, they are all grouped into the diurnal cluster Set 5 with maximum expression at the middle of the light phase. A phylogenetic analysis demonstrates that neither of them is orthologous to the previously described, seed-expressed *HSFA9* genes in Arabidopsis and sunflower (E. Mangelsen, unpublished; Almoguera *et al.*, 2009; Kotak *et al.*, 2007b). Rather, *HvHSFA2c* and *HvHSFA2d* belong to a monocot-specific subcluster of HSF genes. Also, the *HSF* genes' predominant expression in the endosperm of heat exposed caryopses supports their relevance for caryopsis development and adaptation to stress. Functional studies should investigate whether these *HSF* genes play a monocot-specific role in seed development and HSR.

As described above, *TPS*, *SnRK* and *HXK* genes have been shown to be involved in the carbon partitioning and/or signalling in different plant species and organs. We have found several members of these gene families to be diurnally expressed and/or heat-responsive in barley caryopses. They can be considered good candidates for phylogenetic and functional characterization. Barley *TPS* genes might serve as targets to modify the T6P

regulation of AGPase activity (Kolbe *et al.*, 2005). At the same time, they could be used to enhance osmotic stress tolerance by increased trehalose levels, as previously described in several crop species (Umezawa *et al.*, 2006).

Finally, judged from the central role of INVINH proteins in suppressing invertases to, among others, promote sucrose supply of developing maize embryos via the ESR (Bate *et al.*, 2004), the role of *HvINVINH1* merits further investigation. *HvINVINH1*, with a predominant localization in the barley endosperm, is diurnally regulated (diurnal cluster Set 3) and strongly induced during heat stress exposure. Again, phylogenetic and subsequent functional analyses will reveal whether genetic engineering of *HvINVINH1* can enhance the sink strength of barley caryopses, as recently demonstrated for *INVINH*-silenced tomato (*Solanum lycopersicum*) seeds (Jin *et al.*, 2009).

## 3.8 Microarray data: Much more than carbon metabolism (II, III)

Transcriptome profiling is performed to gain a *global* overview of the transcriptional state in a particular organ, tissue or cell. As evident from the long gene lists provided as supplements to our publications, diverse processes undergo diurnal regulation and/or respond to heat stress in addition to genes related to (the regulation of) carbon metabolism, a fact which we tried to highlight in form of global summaries (Figures 10 and 11 in papers II and III, respectively). However, the identification of outstanding patterns and functional groups among DE genes requires sound knowledge of the particular biological background. Also, even though holding relevant information on the transcriptional state of the probed sample, genes that are *not* present or *not* DE under the investigated circumstances are usually neglected. In other words: The interpretation of a microarray dataset is often limited by the scope and interest of the analyzing researchers. It is therefore important to stress that the datasets presented in this thesis work, all of them publicly available through the Gene Expression Omnibus (GEO) (Edgar *et al.*, 2002), provide a basis for much more than the analysis of carbon metabolism-related genes. To enhance the accessibility of our own and other publicly available barley microarrays and to increase their global output, we are currently calculating interaction networks, which are highly valuable in the identification of yet unknown gene interactions (Mutwil *et al.*, 2010).

# 4 Conclusions

A minimum of 45 *WRKY* genes exist in barley. *SUSIBA2* clusters into WRKY group I where *HvWRKY41-43* are the most closely related genes. Several *HvWRKY* genes are potentially involved in stress response and barley development.

An acclimatory response of gene expression to day-to-day changes in light, temperature, sugar and amino acid supply occurs in developing barley caryopses.

Several hundred transcript profiles are positively or negatively correlated to diurnal metabolite levels and, hence, demonstrate the significance of carbon and nitrogen for the regulation of diurnal gene expression in developing barley caryopses.

Heat stress results in a pronounced expressional reprogramming in developing barley caryopses. Central processes of barley caryopsis development, including metabolite import and polysaccharide biosynthesis, are repressed in favour of a rapidly induced HSR.

Nearly all steps in the conversion from sucrose to starch have the potential to be diurnally regulated and/or heat-responsive.

Candidate genes with a potential to improve sink strength and/or heat stress adaptation in barley caryopses and therefore merit further investigations include *HvWRKY41*, *HvHSFA2c*, *HvHSFA2d*, *HvHSFB2b* and *HvINVINH1* as well as members of the yet uncharacterized barley *TPS*, *HXK* and *SnRK* gene families.

# 5 Future Perspectives

The transcriptome studies included in this thesis provide a big repository of data which we linked on both intra- and interspecies level to enable the formulation and *in silico* testing of various (almost endless!) biological hypotheses. Next steps in the analysis of the barley genome and its function could be done in several ways, including both desk-top and bench-top strategies as well as approaches in basic and applied research. Some of them include the following:

*Analysis of circadian regulation, carbon starvation and inherited stress adaptation in barley caryopses*

Additional transcriptome and metabolome studies should be performed to improve our understanding of metabolic regulation in the barley caryopsis. For example, the purely circadian regulation in barley caryopses could be studied by analyzing developing caryopses under continuous light. Addressed questions could include the following: Is the expression of any starch metabolic enzymes regulated by the circadian clock? Is the efficiency of storage starch biosynthesis in the endosperm be increased under constant light and presumably consistent sucrose supply? Does continuous light affect the size, structure and composition of the starch granules? Is the ripening of the caryopsis accelerated as a consequence of this "light stress" and high sucrose levels, which are generally known to induce senescence (Wingler & Roitsch, 2008)? The opposite approach would allow raising additional questions: How is the transcriptome and metabolome affected by depletion of carbon pools during an extended night? Are the effects similar to those previously described for Arabidopsis leaves (Usadel et al., 2008)? It could further be investigated whether the short-term exposure to high temperature, after recovery from the stress, has long-term effects on gene expression in barley caryopsis even in the next generation, which would

indicate an epigenetic inheritance of the stress adaptation (Boyko & Kovalchuk, 2008).

*Genome-based analysis of gene families and TF targets*

Once the barley genome is fully sequenced (Schulte et al., 2009) virtually all studies that have been possible on the basis of genomes of the model plants Arabidopsis and rice could also be performed in barley. Based on the work presented in this thesis, these studies should include the refined analysis of the *HvWRKY* gene family. The distribution of WRKY-binding domains in the barley genome could be analyzed and potential targets could be assigned and validated in functional studies. A promoter analysis of, for example, all sucrose-correlated and -anticorrelated genes in barley caryopses could help to identify common *cis*-elements which, subsequently, could help to assign and validate upstream transcriptional regulators in sugar signalling. The barley genome sequence would further enable the identification of full length sequences and promoters of all starch metabolic genes, which are at current mainly assigned based on RNA sequence information.

*Functional analysis of candidate genes based on barley transformation*

A facile protocol for barley transformation would allow for the rapid and high-throughput confirmation and validation of gene function in T-DNA tagged mutant populations or RNA interference (RNAi) lines. Today, the most common way of transforming barley is the *Agrobacterium*-mediated gene transfer into totipotent cell calli derived from immature embryos. However, the protocols for barley transformation are, at least compared to the simple "floral dip" approaches in Arabidopsis (Zhang et al., 2006), tedious. The success rate largely depends on the barley cultivar and is generally low unless in the hands of a few researchers worldwide (Hensel et al., 2009). Transient silencing of genes has been performed using antisense ODN technology in barley leaves and detached spikes (Sun et al., 2007), but the experimental setup does currently not allow long-term developmental studies of caryopses. Virus-induced gene silencing (VIGS) would be another strategy to silence genes under caryopses development, but despite some unpublished results by Scofield and Nelson (2009), in which VIGS was successfully established in flag leaves and floral organs of wheat, this method has not yet been used to study developmental organs in grasses. The development of stable and/or transient barley transformation protocols for the production of (inducible) loss-of-function and/or overexpression lines would provide us with powerful tools to explore the function of several of the above-named gene candidates. Functional studies

in barley caryopses could then be performed over the complete time span of caryopsis development and without detaching the barley spike from the mother plant.

*Improved suborgan localization of transcripts and metabolites*

An important strategy to refine the studies presented in this work would be to clarify the suborgan localization of transcripts and metabolites in the developing caryopsis. Isotope labelling has previously been used to track the path of phloem-unloaded carbon and nitrogen (Thorne, 1985), and RNA in situ hybridization and laser dissection have been used to detect the spatial expression patterns of genes in developing cereal seeds (Thiel et al., 2008; Gubatz et al., 2007; Drea et al., 2005). However, these approaches are labour-intensive and, thus, usually not applied to monitor metabolites and transcripts in the time span of minutes to hours. As our studies have demonstrated that the levels of metabolites and transcripts vary greatly in these time frames, we assume that the same is true for the suborgan localization. Hence, methods for real time monitoring of metabolites and transcripts on suborgan level, resembling approaches to track ion transport in vascular tissues (Metzner et al., 2008), should be developed to enhance our understanding of metabolite fluxes and gene expression activities in developing caryopses.

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