Barley Grain Maturation and Germination: Metabolic Pathway and Regulatory Network Commonalities and Differences Highlighted by New MapMan/PageMan Profiling Tools[1][W][OA]

Nese Sreenivasulu[2]*, Björn Usadel[2], Andreas Winter, Volodymyr Radchuk, Uwe Scholz, Nils Stein, Winfriede Weschke, Marc Strickert, Timothy J. Close, Mark Stitt, Andreas Graner, and Ulrich Wobus

Leibniz-Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK), D–06466 Gatersleben, Germany (N.S., V.R., U.S., N.S., W.W., M. Strickert, A.G., U.W.); Max-Planck-Institut für Molekulare Pflanzenphysiologie, D–14476 Potsdam-Golm, Germany (B.U., M. Stitt); and Department of Botany and Plant Sciences, University of California, Riverside, California 92521 (T.J.C.)

Plant seeds prepare for germination already during seed maturation. We performed a detailed transcriptome analysis of barley (*Hordeum vulgare*) grain maturation, desiccation, and germination in two tissue fractions (starchy endosperm/aleurone and embryo/scutellum) using the Affymetrix Barley1 GeneChip. To aid data evaluation, *Arabidopsis thaliana* MapMan and PageMan tools were adapted to barley. The analyses allow a number of conclusions: (1) Cluster analysis revealed a smooth transition in transcription programs between late seed maturation and germination within embryo tissues, but not in the endosperm/aleurone. (2) More than 12,000 transcripts are stored in the embryo of dry barley grains, many of which are presumably activated during germination. (3) Transcriptional activation of storage reserve mobilization events occurs at an early stage of germination, well before radicle protrusion. (4) Key genes of gibberellin (GA) biosynthesis are already active during grain maturation at a time when abscisic acid peaks suggesting the formation of an endogenous store of GA in the aleurone. This GA probably acts later during germination in addition to newly synthesized GA. (5) Beside the well-known role of GA in gene activation during germination spatiotemporal expression data and cis-element searches in homologous rice promoters confirm an equally important gene-activating role of abscisic acid during this developmental period. The respective regulatory webs are linked to auxin and ethylene controlled networks. In summary, new bioinformatics PageMan and MapMan tools developed in barley have been successfully used to investigate in detail the transcriptome relationships between seed maturation and germination in an important crop plant.

In most flowering plants, seed development and germination are separated by a period of quiescence, which in many cases is also a phase of dormancy. Only after breaking dormancy, the quiescent embryo is able to germinate after imbibition. These processes have been studied intensively at the physiological and molecular level (Bewley and Black, 1994; Bewley, 1997). Two hormones, abscisic acid (ABA) and GA, are the major players during seed maturation and germination (Yamaguchi et al., 2007), and other hormones are also involved (Kucera et al., 2005). Whereas ABA levels peak during maturation and dormancy, GA increases during imbibition and remains high during germination and postgerminative growth. However, it is apparently not absolute concentration but the ratio of the two hormones that determines the different events. ABA as well as GA biosynthesis and deactivation have been only studied in sufficient detail in Arabidopsis (*Arabidopsis thaliana*; Yamaguchi and Nambara, 2007). Much less is known for cereal grains (Ritchie et al., 2000) with the exception of the aleurone (Ritchie et al., 2000). ABA can be synthesized during seed maturation in the maternal tissue and the embryo, and its content decreases rapidly during imbibition (Jacobsen et al., 2002; Millar et al., 2006). GA is synthesized and stored at least in the embryo, and is released during imbibition to trigger the synthesis and secretion of hydrolytic enzymes in the aleurone for endosperm storage product mobilization (Bewley, 1997). Cellular localization of key biosynthetic transcripts suggests GA biosynthesis occurs in specific cell types of both embryo axis and radicle (Yamaguchi et al., 2001; Ogawa et al., 2003). Such temporal and spatial patterns of hormone biosynthesis and responsiveness

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[2] These authors contributed equally to the article.

* Corresponding author; e-mail srinivas@ipk-gatersleben.de.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Nese Sreenivasulu (srinivas@ipk-gatersleben.de).

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are important for seed development and germination but have not been investigated in cereal grains in any detail mainly due to technical constraints.

It has now become possible to use omics technologies to achieve a more global insight into the complex events of seed maturation and germination. Due to the advanced set of tools, technologies and available data, Arabidopsis has been chosen for almost all of those studies (Bradford and Nonogaki, 2007). Genome-wide expression analysis in dry and imbibed seeds provided hints that nearly 12,000 mRNA species are stored in mature dry seeds (Nakabayashi et al., 2005). Based on the use of a translational inhibitor, cycloheximide, these authors concluded that the stored mRNAs are used for de novo synthesis of enzymes that are required for reserve mobilization during seed germination. These results confirmed the view that initial protein synthesis initiates from preexisting stored mRNA (Rajjou et al., 2004). A recent comprehensive study of metabolites by gas chromatography-mass spectrometry profiling has provided additional evidence that seed maturation and germination are associated with coordinated changes in primary metabolism during this defined transitory period and that metabolic preparation for germination is already initiated during late stages of seed maturation (Fait et al., 2006).

Rather little comparable data are available from crop plants, especially cereal grains. Recent transcriptome studies in barley (*Hordeum vulgare*) have provided information about seed development (Sreenivasulu et al., 2002, 2004, 2006; Nielsen et al., 2006) and seed germination (Potokina et al., 2002; Druka et al., 2006) including malting (White et al., 2006). However, the two processes were analyzed separately and, therefore these studies do not reflect the detailed links between grain maturation and germination at the transcriptome level. To close this gap we analyzed barley grain batches with the help of the Affymetrix Barley1 GeneChip (Close et al., 2004) at several time points during both grain maturation and germination with the following aims: (1) to catalog gene expression patterns during grain development across the dormant quiescence period; (2) to analyze the expression patterns by new bioinformatics tools adapted for barley to define groups of carefully annotated, coregulated genes that are specific for two hand-prepared tissue fractions, starchy endosperm/aleurone and embryo/scutellum; (3) to infer interaction networks by postulating DNA-protein interactions based on defined cis-elements in orthologous rice (*Oryza sativa*) gene upstream regions; and (4) to extend our knowledge on hormonal regulation and interaction during seed development and germination by analyzing the temporal and spatial changes in expression of hormone biosynthetic and deactivating genes in the two analyzed tissue fractions. Our data provide evidence that large numbers of transcripts are accumulated in aleurone and embryo that are later required during germination, among them many transcription factors (TFs). Overall transcription programs are characterized by a smooth transition between maturation and germination within embryo tissues. The gene expression data provide evidence that reserve mobilization is activated early during germination, i.e. long before radicle protrusion. Germination events are governed by both GAs and ABA and interconnected to auxin and ethylene-controlled processes. Further hints for the deduced regulatory networks come from specific cis-elements found in the 5′-upstream regions of homologous rice genes.

RESULTS

Barley MapMan and PageMan to Index Overrepresented Functional Categories and to Visualize Metabolic Pathways Derived from Transcriptome Data

The PageMan (Usadel et al., 2006) and MapMan (Usadel et al., 2005) software tools have been developed to map Arabidopsis transcriptome data to defined functional categories, display them onto pathway diagrams, and allow time course analyses for functional gene groups. In this study we adapted these tools to barley by generating unique mapping files of the Affymetrix Barley1 GeneChip (see ''Materials and Methods''). The automatic annotations using the Affymetrix Barley1 GeneChip exemplary sequences allowed the assignment of more than 11,000 sequence classifications into 35 BINs. About another 11,000 could only be classified as unknown or not assigned; 3,396 entries of the classified ones belong to known metabolic pathways and large enzyme families. Arabidopsis and barley seeds store different storage components such as triacyl-glycerols (TAGs) and starch, respectively. However, no major differences in the classified gene sets of primary metabolism were found. The number of sequences assigned to the different categories/BINs is provided in Supplemental Table S1. Some abundant storage protein genes (hordeins) and genes of inhibitor families, which are specific to barley, were put into subBINS of the category ''development''.

The described mapping file was used in the context of barley PageMan and MapMan software tools to display the transcriptome data onto pathway diagrams and perform time course analyses for identifying significantly overrepresented functional groups based on Fisher's exact test and Wilcoxon rank summary test statistics of pathways. This allowed us to explore the global activation of endosperm and embryo specific metabolic pathways and gene regulatory networks activated during barley seed development and germination at two levels: (1) identification of enriched functional categories of metabolic pathways, cellular processes, and hormone metabolism during seed development and germination (see Figs. 2 and 7A; Supplemental Fig. S2) and visualization in MapMan of individual gene responses in metabolic pathways (see Figs. 3, 5, 6, and 7B; Supplemental Figs. S3 and S4); (2) definition of regulators based on enriched cluster groups and identification of those showing distinct

time course expression patterns during seed development and germination (see Fig. 9).

Transcriptome Characteristics of Developing and Germinating Barley Grains

We investigated the expression of 21,439 nonredundant genes during seed development and germination (four time points each: 4, 8, 16, and 25 days after flowering [DAF], and 0, 24, 48, and 72 hours after imbibition [HAI]; Fig. 1A) from endosperm (starchy endosperm plus aleurone fraction [E/A-fraction]) and embryo (embryo axis plus scutellum fraction [Em-fraction]) of barley seeds in 'Barke' by using the 22K Affymetrix Barley1 GeneChip (Close et al., 2004). The degree of radicle protrusion at 72 HAI (Fig. 1A) indicates that this developmental time point represents already a postgerminative stage.

Results were reproduced with two independently grown and harvested seed lots. The high degree of reproducibility was substantiated by a hierarchical clustering-based classification procedure. In a dendrogram, replicates clustered as neighbor clades with the exception of the replicates Em48_1 and Em48_2 (Fig. 1B). The latter difference might be partially explained by a difference in stage assignment, which is especially difficult in the embryo during key transitional stages of development. Affymetrix GeneChip pseudoimages produced by the probe-level modeling procedure and RNA degradation plots did not show serious artifacts for any of the 32 experiments (data not shown). The raw expression data derived from Affymetrix Microarray suite 5.0 software (MAS) gave each transcript an absolute expression level, and a present or absent call based on the signal-noise ratio. The number of sequences called present was 60% to 62% (approximately 13,000 transcripts), except in the endosperm and embryo at 0 HAI (dry seed) where the percentage was 46% (approximately 9,860 transcripts) and 56% (approximately 12,100 transcripts), respectively. Most of these transcripts are supposed to be presynthesized and stored during seed maturation (see the introduction). During early germination (24 HAI) the number of transcripts detected in the Em-fraction increased to approximately 13,050 (61%), but remained low (49%, approximately 10,480 transcripts) within the E/A-fraction. After radicle protrusion (72 HAI) the embryo maintains its highly active transcriptional state (Supplemental Fig. S1).

Hierarchical clustering of the data (Fig. 1B) shows tissue relationships (pericarp, endosperm, and embryo) during seed development and germination. The dendrogram reveals three major structures, based on the variation in the transcript populations. The middle part of the tree (colored in blue) contains all embryo tissues including both grain maturation and germination. The clustering of 25 DAF embryo tissues together with germinating embryos from 0 to 24 HAI suggests that there is a smooth transition between late seed maturation to germination in the embryo tissues. The grouping of embryo 16 DAF with 72 HAI data may be due to the occurrence of similar physiological processes such as cell proliferation and elongation at these two stages. At the left side of the tree (pale

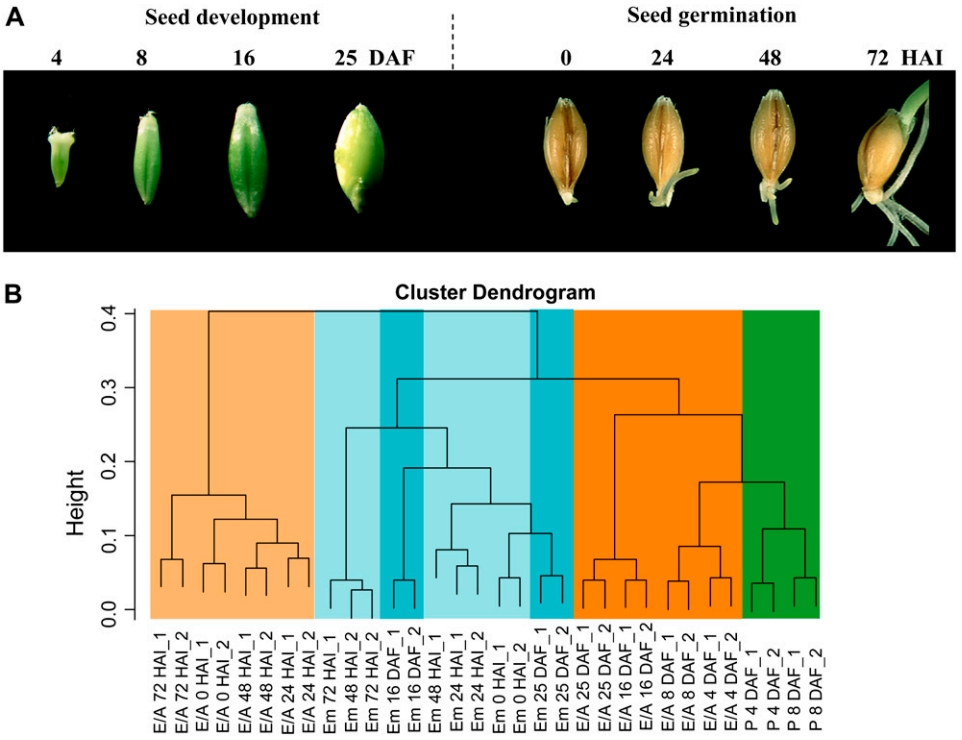


Figure 1. Key stages of grain development and germination used for transcriptome analysis. A, Grain appearance. B, Hierarchical cluster dendrogram of normalized transcript abundances from 32 experiments including biological replicates based on complete distance linkage. Two tissue fractions (E/A, brown; Em, blue) were analyzed during grain development (DAF; dark coloring) and germination (HAI; light coloring). Pericarp (P; green) was analyzed during grain development only.

brown) E/A tissues from germinating caryopses (0–72 HAI) are grouped together, whereas the same tissue from maturing grains (4–25 DAF) clusters toward the right side (brown color) next to the pericarp (green; Fig. 1B). These results suggest a large systematic divergence in transcriptional programs between endosperm (E/A) and embryo (Em) tissues and also between the maturing and the germinating E/A-fraction. This contrasts with the embryo transcriptome, where the differences between the maturation phase of grain development and germination are much less pronounced. One obvious reason for the dramatic changes in the E/A transcriptome could be the programmed cell death in the endosperm during maturation/desiccation.

Inferred Metabolic Pathway Alterations between Seed Development and Germination in Endosperm and Embryo Tissues

Pathways Activated during Reserve Accumulation

During desiccation, metabolic activities within the seed are drastically down-regulated, and are then activated again during imbibition, germination, and postgerminative growth. Reserve accumulation in the endosperm is initiated around 16 DAF by the activation of Suc synthase-mediated Suc cleavage (Supplemental Table S2) and the induction of starch biosynthetic genes. By 25 DAF the expression levels of genes involved in the Suc-to-starch pathway are generally reduced, whereas the activation of hordein storage protein transcription continues along with genes involved in defense-related secondary metabolite biosynthesis pathways (Fig. 2). In embryo tissues, TAG synthesis genes of lipid metabolism are preferentially activated (Fig. 2A; Sreenivasulu et al., 2004, 2006). Interestingly, mRNA species abundantly stored in the dry seed include not only sequences for seed reserve synthesis prone to rapid degradation but also RNAs related to DNA synthesis (histones) and protein synthesis (ribosomal proteins, elongation factors; Supplemental Fig. S2). These transcripts are needed to resume synthetic processes early during germination.

Pathways Activated during Reserve Mobilization

According to our gene expression data, mobilization of deposited starch and storage proteins starts early during seed germination (Supplemental Fig. S3C) as indicated by the transcriptional activation of respective genes in the E/A-fraction. Parallel to the transcriptional activation of starch degrading genes (type A and B amylases), there is also an increase of transcripts of genes encoding cell wall-bound and vacuolar invertases, Suc synthases as well as Suc-P synthases in aleurone and embryo at 24 HAI (Supplemental Figs. S3B and S4B). This is probably to supply hexoses and synthesize Suc from the mobilized starch reserves. Genes of the β-oxidation pathway and of phospholipases are also induced in both the endosperm/aleurone and the embryo from 24 HAI onward (Supplemental Figs. S3B and S4C), implying a more general event of lipid degradation, which eventually results in energy release by peroxisomes and supply of carbon resources for the production of Suc.

Genes involved in cell wall modification (expansins, endoxyloglucan transferase) and cell wall degradation (cellulases and β-1,4-glucanase) represent another set of important components that are active by 48 HAI in both the Em-fraction and the E/A-fraction (Fig. 3, A and B; Supplemental Figs. S3B and S4B). These events are expected to depolymerize cell wall components during the beginning of storage mobilization. During this period, transcripts for Cys proteases (CP1, EP-B1, and EP-B4) and Ser carboxypeptidases (SCPL51) were also significantly increased in aleurone and, less prominent, embryo (cluster group 2, Fig. 4). Both enzymes probably play a pivotal role in endosperm storage protein mobilization. In addition, transcripts of cathepsin B-like Cys proteinase, OTU (ovarian tumor)-like Cys proteinase, Cys protease 1 (γ-vacuolar processing enzyme), and Ser carboxypeptidase I and III were abundant during seed germination in both the E/A- and Em-fraction but were equally prominent already during seed maturation (cluster group 1, Fig. 4). It is likely that protein mobilization in storage tissues occurs in a gradient manner based on both stored and de novo formed proteases. In addition to the described proteases/peptidases, genes related to the ubiquitin/proteasome complex were found to be expressed during early seed germination. This suggests that in addition to the well-known involvement of proteases in mobilizing storage proteins, the ubiquitin/proteasome pathway may also be involved in selective proteolysis during seed germination.

Energy Provision for Storage Mobilization

Along with starch and lipid mobilizing transcripts, the genes related to energy metabolism (glycolytic pathway, TCA cycle, and, in particular, malate dehydrogenases, oxidative phosphorylation, and mitochondrial electron transport) are initiated in the E/A-fraction and the Em-fraction 24 to 48 HAI, indicating the activation of respiratory activities in mitochondria and ATP release (Fig. 3, A and B; Supplemental Figs. S3B and S4B). In accordance, at least 28 transcripts from proton and vacuolar ATPase and mitochondrial ATP synthase complexes, mitochondrial carrier proteins, and mitochondrial-related ATP transporters are preferentially up-regulated in the E/A-fraction during 24 to 72 HAI (Fig. 5A). Also activated early during germination are alcohol dehydrogenase genes of the fermentation pathway (Fig. 3, A and B). These results suggest that the aleurone in germinating seeds plays a pivotal role in energy provision during early seed germination. This burst of energy release probably helps to initiate storage mobilizing events under hypoxic conditions (Perata et al., 1997).

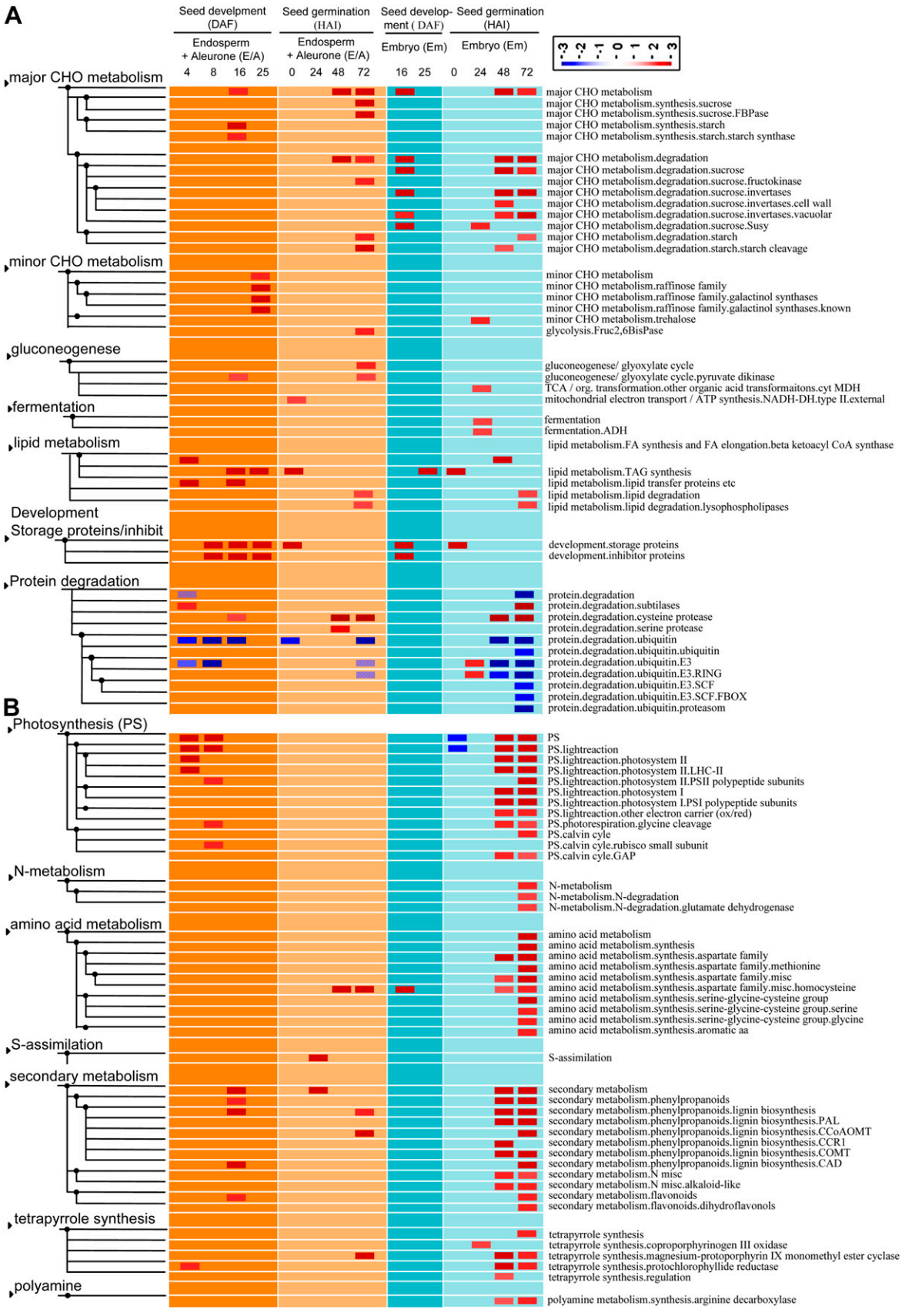


Figure 2. (*Legend appears on following page.*)

Activation of Photosynthesis and Nitrogen Metabolism Genes in the Embryo during Imbibition

During germination the embryo establishes photosynthesis, as is evident in our experiments at 48 and 72 HAI, i.e. slightly later as the initiation of reserve mobilization (Figs. 2B and 3B). During this period the tetrapyrrole biosynthesis pathway is also activated as well as photorespiration genes (Figs. 2B and 3B), which probably help to supply Suc and prevent anoxia (Rolletschek et al., 2004). At around the same time genes for amino acid metabolism, including Asp-derived Met, homo-Sers, and homo-Cys pathways, are activated in the embryo (Fig. 3B). Triggering of amino acid biosynthesis genes during this time of germination provides a major nitrogen source for the developing embryo, where large numbers of amino acid, oligo peptide, and ABC transporters are abundantly expressed in comparison to endosperm/aleurone (Fig. 5). These transport processes in the germinating embryo seem to be vital as reported previously for peptide transporters of the barley scutellum (Waterworth et al., 2000).

Interestingly, genes encoding a set of methylation cycle enzymes, methyl transferases, core histones, and chromatin-modifying genes are coinduced in the embryo during 48 and 72 HAI (Supplemental Table S2). These results point to the importance of methylation events during radicle protrusion. Recently, epigenetic regulation was shown to work during seed imbibition of Arabidopsis, where cytosine methylation was observed in different silent gene clusters (Nakabayashi et al., 2005).

Prevention of Rehydration-Induced Cell Damage in Germinating Aleurone and Embryo

During seed maturation and early seed germination, raffinose metabolism genes are activated specifically in endosperm/aleurone, whereas transcripts for trehalose and aldose metabolism are found both in the E/A- and the Em-fractions at 48 HAI (Fig. 6). While galactinol synthase genes involved in raffinose production are expressed in the E/A-fraction during seed desiccation, the α-galactosidase genes involved in raffinose mobilization are expressed during early seed germination (25 DAF to 48 HAI; Supplemental Table S2). Similarly, raffinose metabolites were shown to increase during late maturation of Arabidopsis seeds (Fait et al., 2006). Raffinose family oligosaccharides are supposed to constitute an important energy source during germination and, accordingly, blocking of raffinose breakdown drastically inhibits germination (Blöchl et al., 2007).

Interestingly, with respect to trehalose metabolism we observe that certain members of the small trehalose-6-P synthase (TPS) and trehalose-6-P phosphatase (TPP) gene families are expressed preferentially during late seed maturation in the E/A-fraction. These results indicate that transcripts involved in trehalose biosynthesis are expressed both during seed desiccation and early seed germination (Fig. 6). The discussed carbohydrates may not only protect aleurone and embryo cells during seed maturation from desiccation, but also help to stabilize proteins that are activated during early seed germination. Arabidopsis mutants impaired in biosynthesis of trehalose due to a disruption of the TPS1 gene have been shown to exhibit embryo lethality during late seed maturation (Eastmond et al., 2002). Whether in seeds trehalose acts as an effective osmoticum, stabilizer of proteins, and/or energy source or whether the effect is due to the signaling role of the TPS1 gene product, trehalose-6-phosphate, is still unknown (see Grennan [2007] and Stitt et al. [2007] for a recent discussion).

The transcriptional activation of genes for ascorbate and glutathione peroxidases, redox metabolism, and super oxide dismutases during seed germination (48 HAI) in endosperm/aleurone and embryo suggests an involvement in scavenging reactive oxygen species (Fig. 6). These sets of genes may be activated due to reoxygenation events, which occur during germination. Also preferentially expressed in the E/A-fraction during germination and in the Em-fraction during maturation and early germination are LEA (late embryogenesis abundant) protein genes (Fig. 6). These results together indicate that activation of genes related to sugar alcohols (osmoprotectants), antioxidants, and desiccation-related LEA proteins seem to play a role in maintaining cell integrity against rehydration-induced damage in aleurone and embryo cells during early grain germination.

Hormone Biosynthetic Pathway Alterations during the Switch from Seed Maturation to Germination in Endosperm and Embryo Tissues

As shown in Figures 7 and 8, ABA and GA biosynthetic genes are expressed during both seed maturation and early germination (see below for further details). Surprisingly, transcripts for genes involved in the biosynthesis of ethylene, brassinosteroids (BA), auxin (IAA), and jasmonate (JA) are also most prominent in germinating seeds in both the E/A- and the

Figure 2. PageMan display of coordinated changes of gene categories activated during barley seed development and germination. A, Storage product accumulation and its mobilization. B, Selected major gene categories of primary and secondary metabolism pathways. Affymetrix Barley1 GeneChip normalized gene expression data collected from E/A- and Em-fractions during seed development (4, 8, 16, and 25 DAF) and seed germination (0, 24, 48, and 72 HAI) were subjected to overrepresentation analysis of functional categories using PageMan. Fisher's exact test was used to test whether significantly more genes in a given category at a given developmental point were up-regulated when normalized to their average expression. (Color scale is: red, significant enrichment of up-regulated genes; blue, significant depletion of up-regulated genes). In the display, the overrepresented MapMan functional categories are given by collapsing nonsignificant categories. The complete analysis and its display are provided in Supplemental Figure S2.

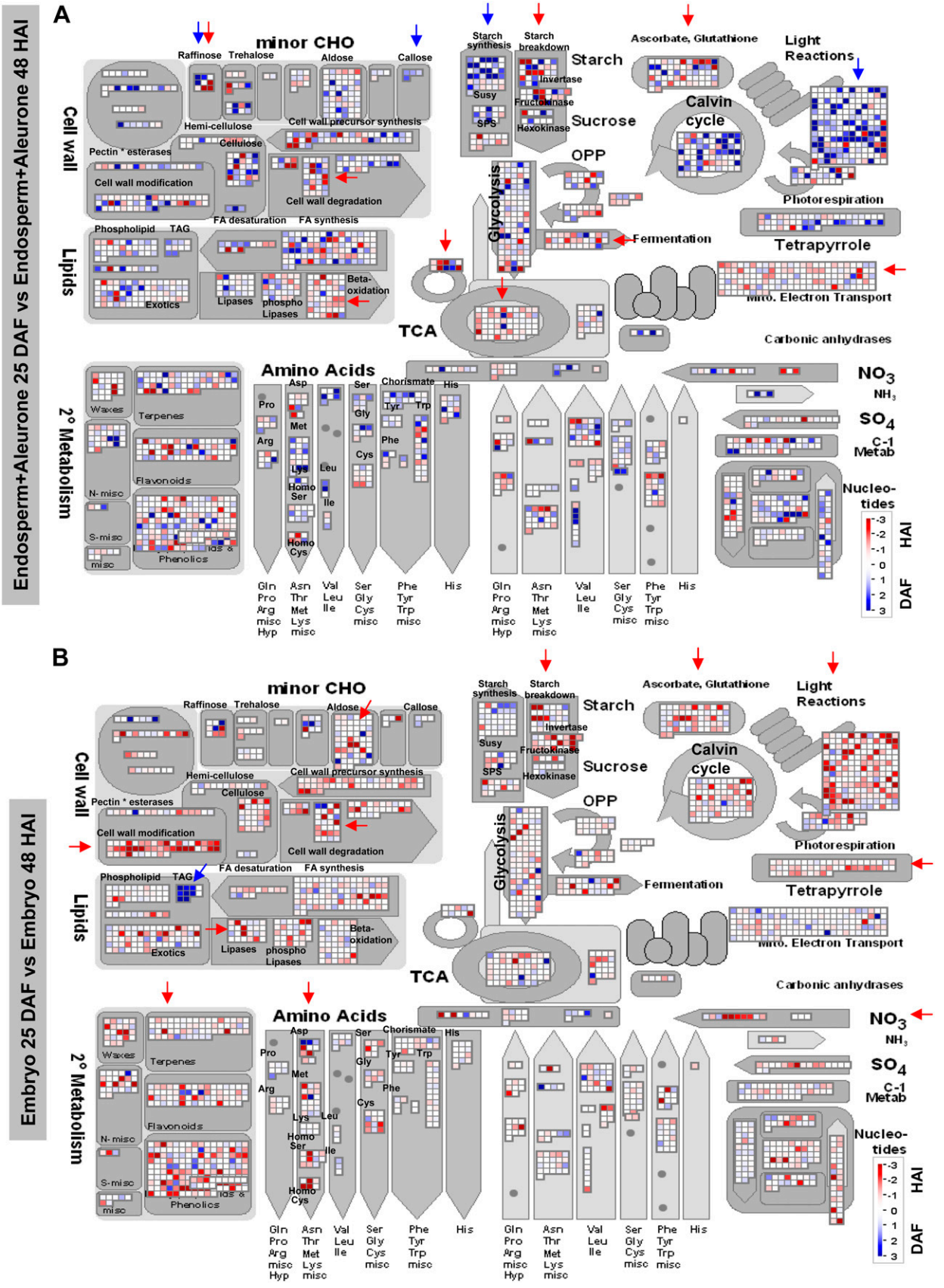


Figure 3. (*Legend appears on following page.*)

Em-fractions (Fig. 7, A and B), underlining the recently developed view (Kucera et al., 2005; Feurtado and Kermode, 2007) that besides participation of ABA and GA in controlling germination events, other hormones are apparently part of an intricate interaction web regulating the onset and early course of grain germination. However, in the following analysis we focus on the protagonists GA and ABA.

The Antagonistic Hormones GA and ABA Are Both Synthesized during Seed Maturation and Germination

Although ABA is known to play an important role in seed dormancy and GA in germination, their coexistence and antagonistic interplay during these two processes has not been explored fully in cereals. Our expression profiling studies show that key GA biosynthetic transcripts such as *ent*-kaurene synthase A (GA2), *ent*-kaurene oxidase (GA3), *ent*-kaurenoic acid oxidase (KAO1), GA 20-oxidases (GA20OX1, GA20OX2, and GA20OX3), GA3 oxidase (GA3OX), and GA2-oxidases (GA2OX1, GA2OX2) are already expressed at high levels during the maturation phase of grain development in endosperm/aleurone and to a lesser extent in the embryo. In addition, we observed a second peak of expression of GA biosynthetic transcripts in the embryo during early seed germination, from 24 HAI onward (Fig. 8; note isoform differences). Whether the different transcripts are confined to specific cell types as shown for Arabidopsis seeds (Ogawa et al., 2003) needs to be investigated. If the expression of the GA biosynthetic genes indicates the synthesis of the respective compounds (Kaneko et al., 2003), we would expect increased levels of *ent*-kaurenoids and bioactive GA compounds including GA1. In agreement, Jacobsen et al. (2002) reported the accumulation of large amounts of *ent*-kaurenoids and different GAs in the embryo of dry barley seeds. Based on transcript data we furthermore assume that bioactive GA compounds and several of its precursors accumulate during seed maturation to a higher extent in the barley endosperm/aleurone than in the embryo. Surprisingly, during germination GA3OX, a marker gene for GA biosynthesis, is present at only very low levels. Of the GA 20-oxidases, which provide the substrate for GA3OX, transcripts of only one isoform (GA20OX2) are found in the embryo fraction at relevant levels (Fig. 8). One explanation for the absence of GA3OX transcripts could be that a second isoform, active in the embryo, is missing on the array since two differentially expressed GA3OX isoforms have been reported in Arabidopsis (Ogawa et al., 2003). Among GA signaling genes, GAMYB transcript levels increase drastically in the aleurone of germinating barley seeds whereas transcripts of SPINDLY and RGA1/SLN1, which act as negative regulators of GA signaling, are rather reduced. These results are in agreement with a previous report that GAMYB transcript is increased within 2 h of GA treatment in barley aleurone cells whereas SLN1 protein is degraded 10 min after GA application (Gubler et al., 2002).

Distinct gene family members of the ABA-biosynthetic pathway in endosperm (NCEDs and aldehyde oxidase 1) and embryo (aldehyde oxidase 2 and molybdenum cofactor sulfurase) are activated during seed maturation as already described (Sreenivasulu et al., 2006). Interestingly, the same genes remain active during seed germination. This is consistent with the observation that ABA levels in nondormant barley grains stayed high even after an initial drop during imbibition at about 40% of the dry-grain value (Millar et al., 2006). Our results also provide hints that ABA signaling network genes such as ABA binding protein, protein phosphatase 2C (ABI2), ABA-responsive element binding protein, and ABA-insensitive protein 3 (ABI3/VP1) are expressed to a higher extent in the endosperm/aleurone and the embryo during germination than during maturation (Fig. 8).

Putative GA and ABA Interaction Networks during Seed Germination as Revealed by cis-Element Analysis

Whereas the action of GA in barley aleurone is well understood (Gubler et al., 2002), possible interactions between the GA and ABA networks are clearly less understood. One way to approach the problem is to analyze gene upstream regions for cis-regulatory elements that are known to mediate responses to either GA or ABA. To this end, we selected a set of genes that was recently identified by Chen and An (2006) as GA- and/or ABA-regulated. The authors used the Affymetrix Barley1 GeneChip and de-embryonated barley aleurone tissue to identify, among others, genes encoding hydrolytic enzymes. More than 80% of the gene sets were GA regulated and the remaining were regulated by ABA. We identified a set of homologous rice

Figure 3. MapMan Metabolism overview maps showing differences in transcript levels between late seed maturation (25 DAF) and seed germination (48 HAI) in two genetically distinct tissues. A, E/A-fraction. B, Em-fraction. Log_2 ratios for average transcript abundance based on two independent replicates of Affymetrix Barley1 GeneChip normalized gene expression data of E/A 25 DAF versus E/A 48 HAI and Em 25 DAF versus Em 48 HAI were calculated. The resulting file was loaded into the MapMan Image Annotator module to generate the metabolism overview map. On the logarithmic color scale ranging from −3 to 3, dark blue represents at least 6-fold higher gene expression during seed maturation in comparison to seed germination, and red represents 6-fold higher gene expression during seed germination in comparison to late seed maturation. Color saturates at an 8-fold or higher change. Significant coregulation is indicated with arrow marks (blue, 25 DAF seed maturation; red, 48 HAI seed germination). The complete set of genes, derived functional categories, normalized expression values, and calculated ratios are given in Supplemental Tables S3 and S4.

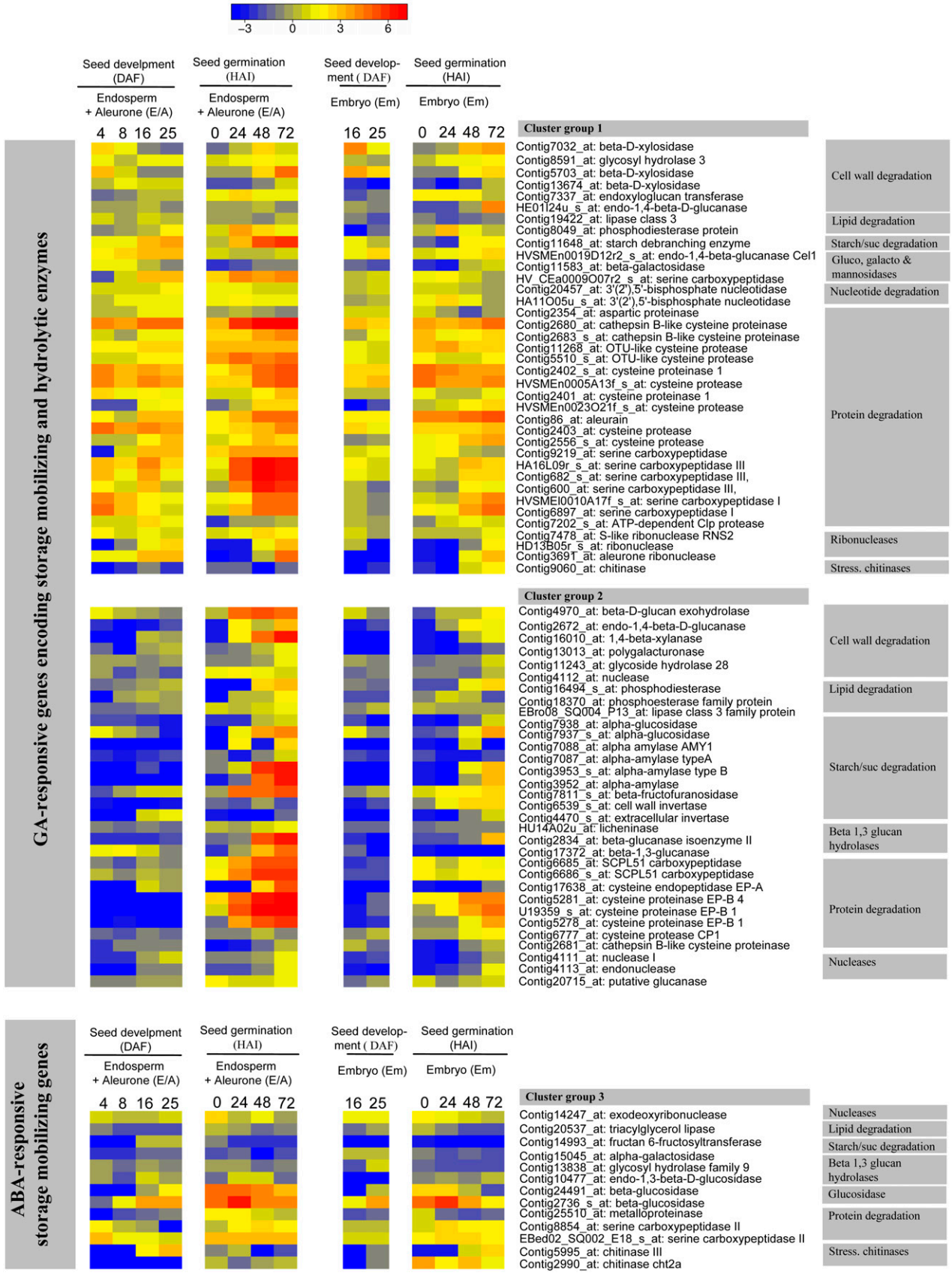


Figure 4. (*Legend appears on following page.*)

sequences and used it to extract 1,000-bp promoter regions because a barley genomic sequence is not yet available, followed by a search for the GA-responsive tripartite GA-response complex (GARC) including the GA-response element (GARE), the pyrimidine box, and the R1MYB motif. All three motifs are known to be responsible for a full GA response. In addition, we searched for the ABRE cis-motif and coupling element known to be responsible for ABA-mediated gene expression. The 69 GA-responsive genes in this set are involved in degradation processes of cell wall proteins, polysaccharides, proteins, nucleic acids, and lipids. The GARE motif was found in 22 (44 times), the R1MYB motif in 10, and the pyrimidine box in 61 (124 times) gene upstream regions, with multiple copies as the most frequently occurring arrangement (Fig. 8; Supplemental Table S5). Based on the calculation of genome-wide probabilities motif-specific enrichments were indexed (Fig. 8; Supplemental Table S5). The results suggest that GARE and R1MYB are the most significant and the pyrimidine box the most abundant motifs in GA-induced gene expression among the defined tripartite cis-elements. Interestingly, among the set of 69 GA-induced genes, we also found ABRE cis-elements (cacgtg) in 26 genes (41 times) with multiple copies and (acgt) in 34 genes (93 times). This suggests that these GA-regulated genes are also responsive to ABA (Fig. 8; Supplemental Table S5).

Another interesting question is whether GA-responsive transcripts encoding storage mobilizing and hydrolytic enzymes found in de-embryonated aleurone tissue (Chen and An, 2006) are newly synthesized during germination, or already formed and stored during maturation and only translationally activated upon germination. Nearly all of the transcripts of cluster group I (Fig. 4), which encode storage mobilizing and hydrolytic enzymes, are apparently synthesized and stored during grain maturation and used during germination. On the other hand, most of cluster group II members (Fig. 4) are newly synthesized during germination. The functional meaning of this differential expression is unknown. Of the GA-responsive genes of cluster group II (Fig. 4) most are induced in the aleurone (E/A-fraction; 0–48 HAI) and a smaller set is also induced in the embryo. The respective enzymes belong to similar functional groups as those being stored and activated.

Regulators Determining the Transition between Grain Maturation and Germination in Endosperm and Embryo

Although the presence of stored mRNA species in the dry seed has been known for several decades (Dure and Waters, 1965), the types of stored mRNAs and their functional role has not been explored in cereals by genome-wide gene expression studies. Especially the expression patterns of all putative regulatory genes during the transition from seed maturation to germination have not been investigated so far. Therefore, 1,508 TF gene transcripts were analyzed at different developmental stages in the E/A- and in the Em-fraction, respectively. Of these, 453 TFs (30%) were found to be significantly expressed during seed development and germination. A combination of the K-mean method and self-organizing map (SOM) clustering was used to identify 11 different clusters, which were grouped into six major cluster groups characterized by coexpressed TFs associated with specific tissues during development, or confined to either developing or germinating grains (Fig. 9A; Supplemental Table S6). We used PageMan to calculate the likelihood of over-representation of TF members (see legend of Fig. 2) defined in each category among TF families across the 11 cluster members. TFs that are preferentially expressed in pericarp, endosperm, and embryo of developing grains are represented in cluster groups 1, 2, and 3, respectively (Fig. 9A). Although MADS and YABBY TF family members are found to be expressed in pericarp (Supplemental Table S6), the bHLH CCAAT TF members were found to be enriched in endosperm during seed maturation (Fig. 9A, Supplemental Table S6). Further analysis was focused on regulators with overlapping functions during both seed maturation and germination in endosperm and embryo.

The set of TFs that is expressed preferentially during maturation and has reduced transcript levels during imbibition was provisionally defined as ''stored'' and the set with preferential expression during seed germination as ''newly synthesized''. Those TFs together are categorized in six subclusters of cluster group 6, which contains a total of 337 TFs. The subclusters 6(1) and 6(2) represent genes that are about equally active during both seed maturation and germination. TF genes in subcluster 6(3) are expressed preferentially in the embryo and subcluster 6(5) transcripts are found especially in the aleurone (i.e. the E/A-fraction) of

Figure 4. Temporal expression profiles of transcripts involved in storage product mobilization. Storage mobilizing transcripts reported by Chen and An (2006) to be induced in barley aleurone during seed germination by GA or ABA were chosen. By using K-mean and SOM clustering methods, three cluster groups based on temporal expression patterns were identified, two related to GA and one related to ABA. Note that cluster group 1 genes are mostly present during both grain maturation and germination in the two tissue fractions Em and E/A, i.e. they are presumably already synthesized during maturation and become reactivated during germination. On the other hand, cluster group 2 transcripts are preferentially newly synthesized during germination. The same tendency is seen in cluster group 3 transcripts regulated by ABA. Expression values are given in logarithmically scaled (base 2) signal intensities: red, high expression; yellow, moderate expression; blue, low expression. Horizontal rows represent gene expression patterns. Vertical lines represent the developmental stages indicated on top for the two tissue fractions. The predicted function of genes and functional classifications are indicated on the right. The presence of cis-elements shown to be involved in GA or ABA response in 1-kb upstream regions of homologous rice genes is given in Supplemental Table S5.

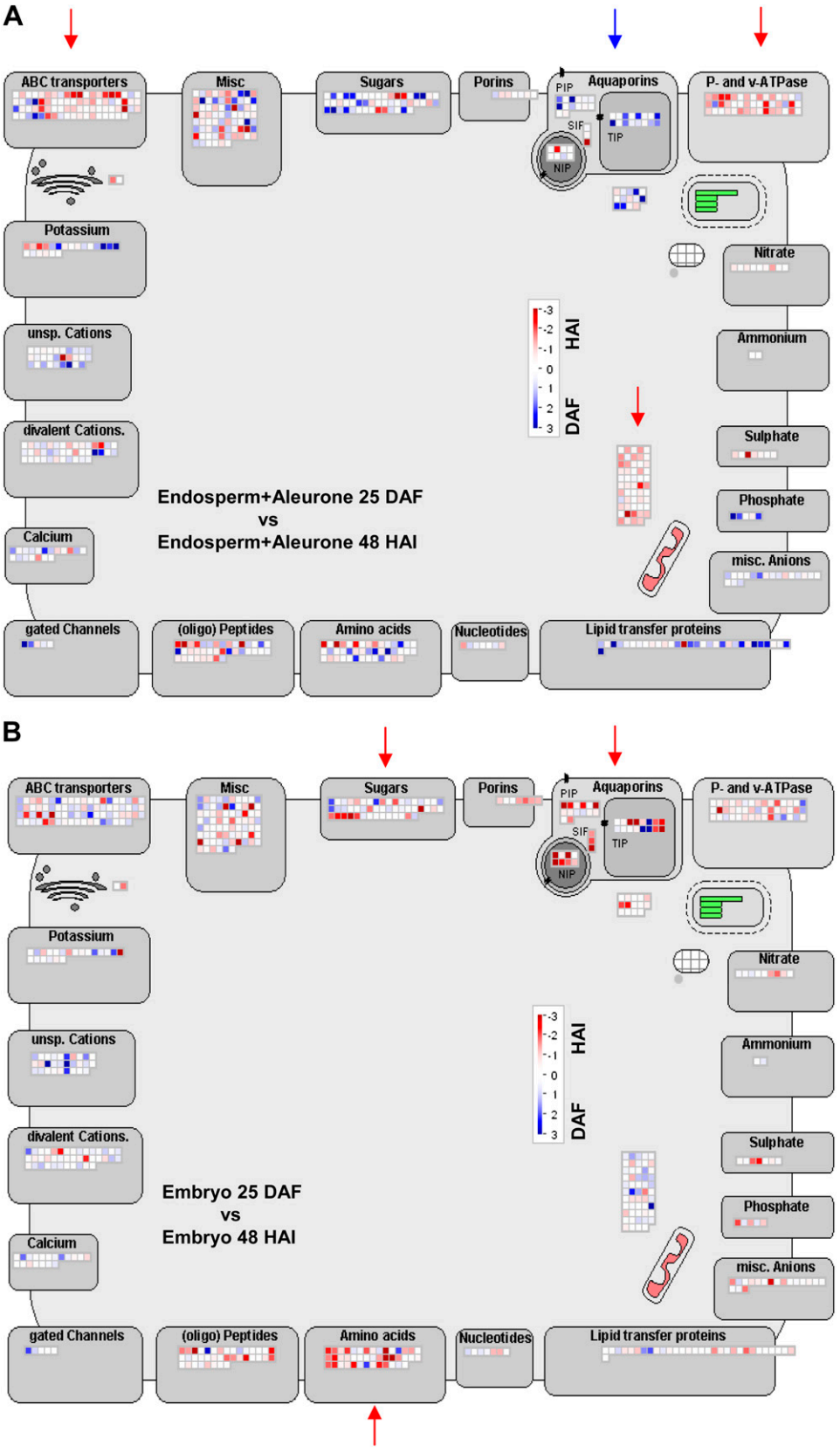


Figure 5. MapMan transport overview maps showing differences in transcript levels between late seed maturation (25 DAF) and germination (48 HAI) in both the E/A- and the Em-fraction. In the color scale, blue represents at least 6-fold higher gene expression during seed maturation in comparison to seed germination and red represents 6-fold higher gene expression during seed germination in comparison to late seed maturation. For further details, see legend of Figure 3. For a detailed gene list, normalized expression values and calculated ratios see Supplemental Tables S3 and S4.

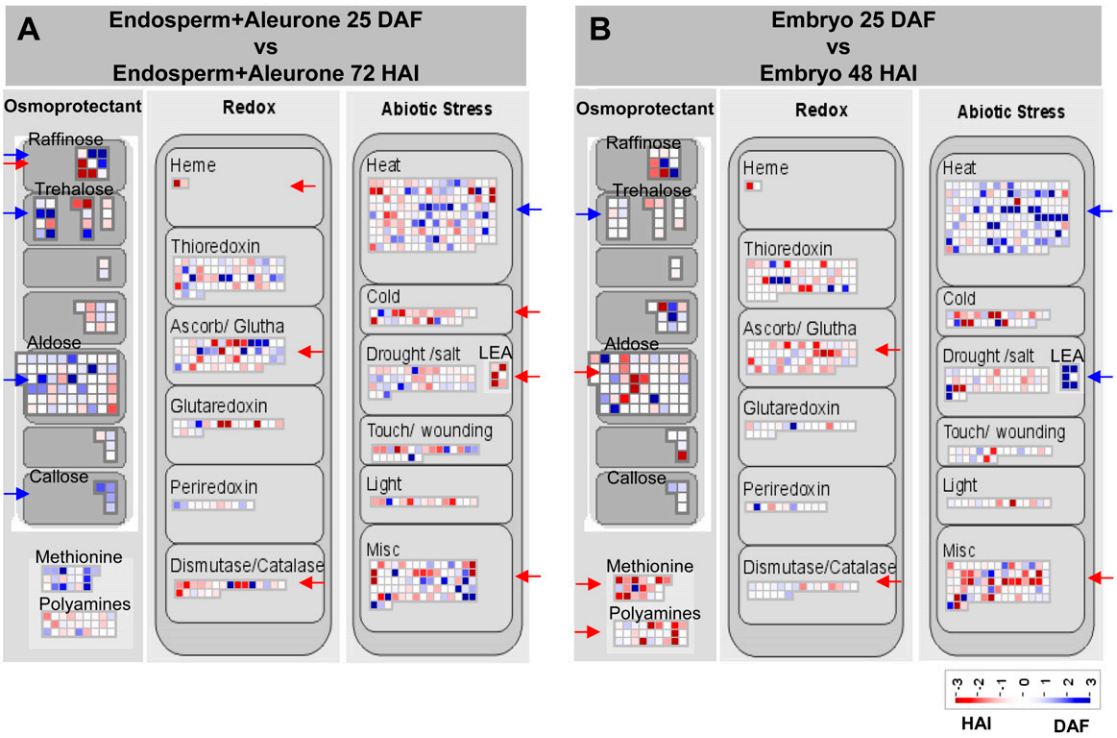


Figure 6. MapMan metabolic overview maps showing differences in transcript levels of osmoprotectant, antioxidant, and stress response genes between late seed maturation (25 DAF) and germination (48 HAI) in both the E/A- and the Em-fraction. For further details, see legend to Figure 5.

germinating seeds (Fig. 9A). Furthermore, we associated the TF genes that are responsive to exogenous GA and ABA, as defined in de-embryonated barley grains by Chen and An (2006), with the temporal expression profiles in this study (Fig. 9C). Surprisingly, among the 337 TFs that are expressed during the transition from maturation to germination (Fig. 9A), only 32 TFs are responsive to GA and ABA by the above described criterion. The time course changes group these TFs mostly in two major clusters (Fig. 9C), 6(2) and 6(5). TF members found in cluster 6(2) are generally presynthesized during seed maturation and triggered during germination whereas cluster 6(5) TF members are preferentially expressed in the aleurone during germination. These results suggest that GA may trigger some TF genes that are presynthesized during seed maturation in addition to its well-documented role during germination.

bZIP and MYB TFs Are Members of TF Families Abundantly Represented during the Transition from Grain Maturation to Germination

The two subclusters 6(1) and 6(2), which are characteristic for the transition between seed maturation and germination in endosperm and embryo, have different kinetic expression patterns. Subcluster 6(1) is represented by 74 TFs that show a high expression during seed maturation in the endosperm, and a decline during germination. In the embryo the overall expression level is slightly lower during maturation and stays at that level or slightly increases during germination. Because of the described expression profile, we assume that the RNAs preferentially in the E/A-fraction (Fig. 9A) represent mainly stored mRNA species. This mRNA population includes members of the S1FA TF family (Fig. 9B; Supplemental Table S6), whose expression behavior is similar in both endosperm/aleurone and embryo. Subcluster 6(2) contains 73 TFs that show preferential expression during seed germination in both endosperm and embryo tissues (Fig. 9A). Representatives (numbers in parentheses) belong to the following TF families: AP2/EREBP (5), bZIP (6), NAC (5), and MYB (10); see Supplemental Table S6. The latter ones are the most abundant (Fig. 9B). Of the AP2 family, high levels of dehydration-responsive TFs homologous to Arabidopsis DREB2A and RAP2.12 were found in both the E/A- and Em-fractions upon imbibition. This indicates an essential role of these factors during germination events, in addition to their known role in stress tolerance. We also observed expression of an ABA-responsive element-binding protein (HY04C04u, Contig9071) of the bZIP family, which is known to participate in activating ABA-dependent gene expression (Finkelstein et al., 2002). Furthermore, dehydrin and LEA genes are preferentially transcribed in the germinating aleurone (Fig. 6) in addition to their already described synthesis

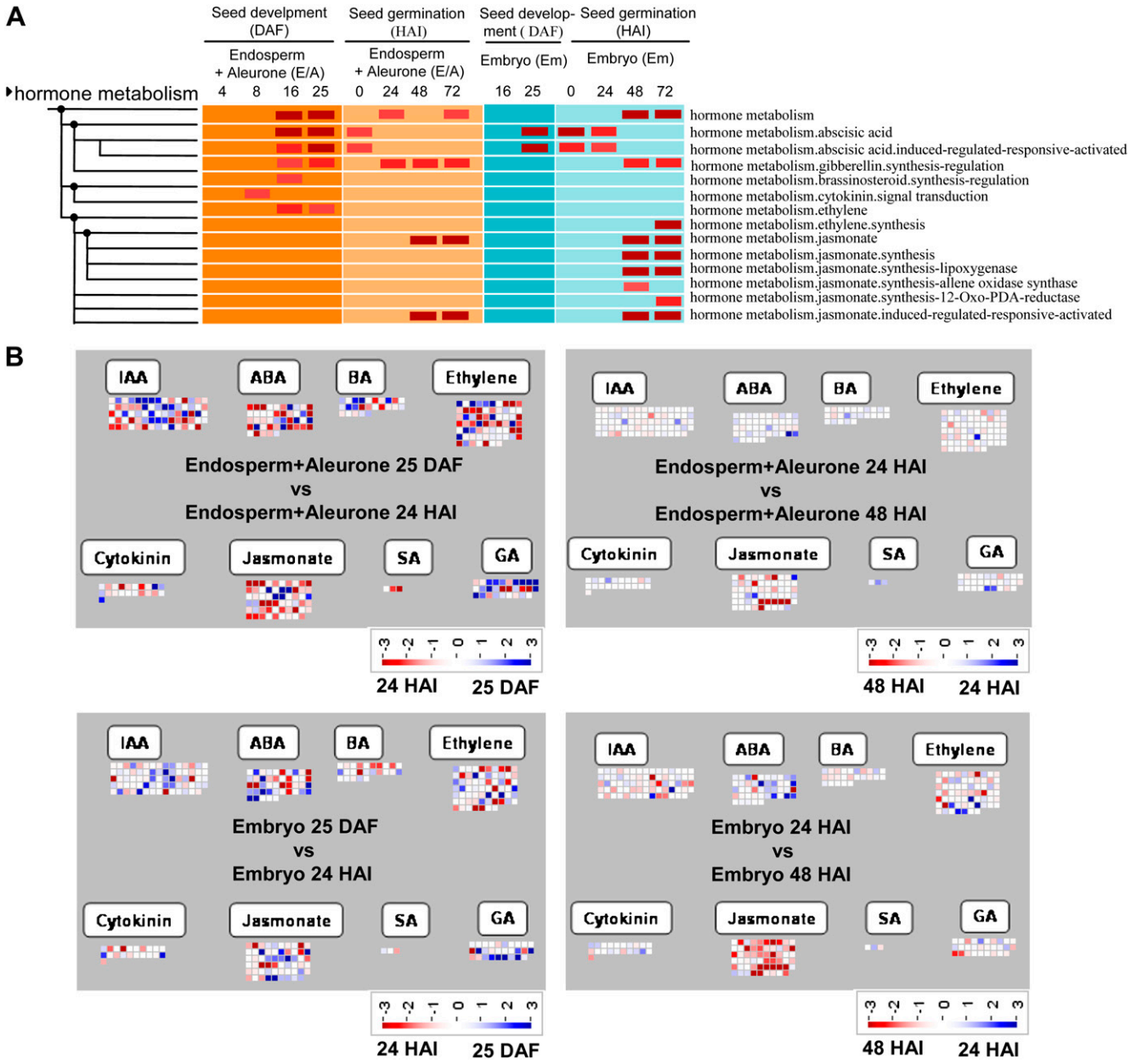
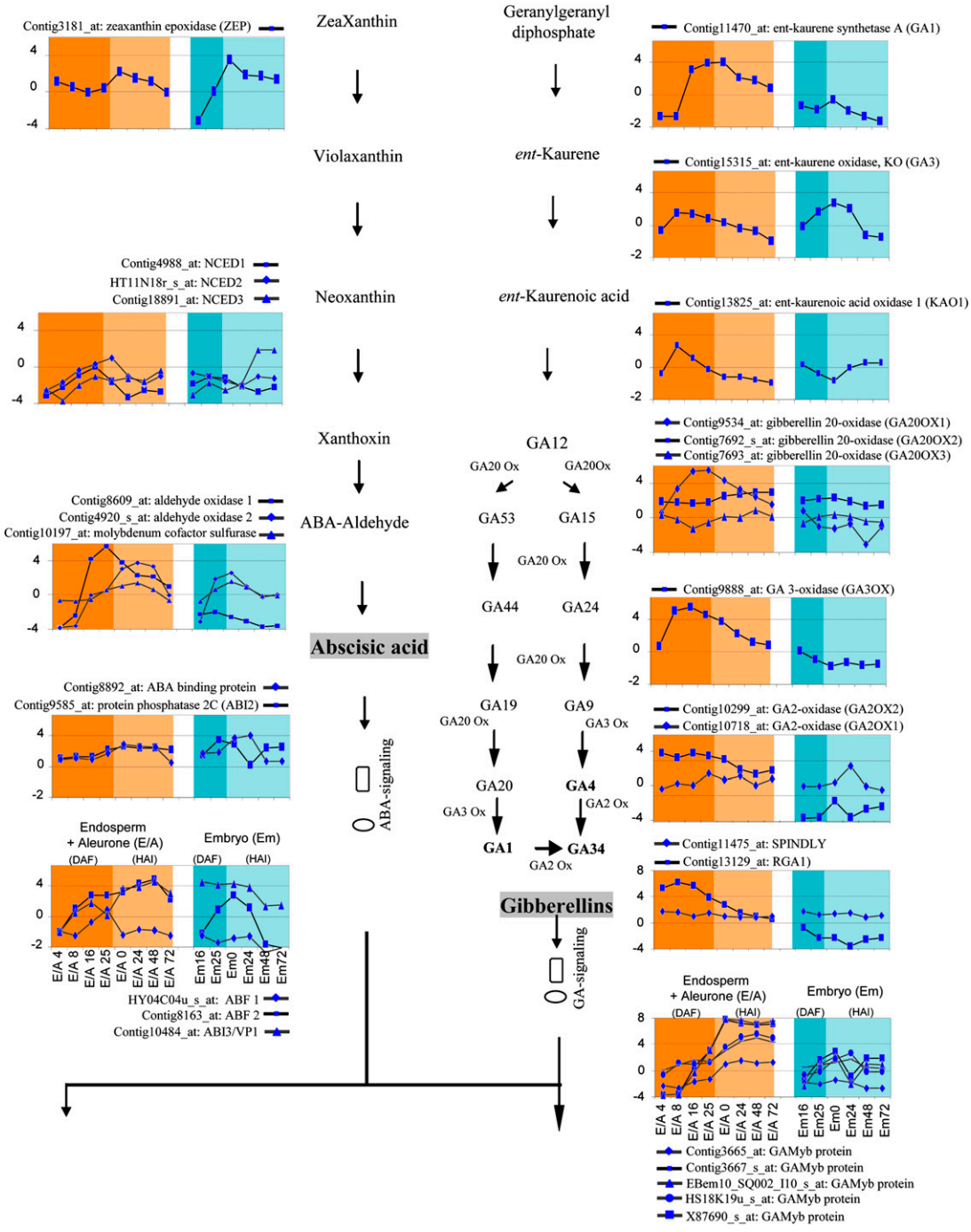


Figure 7. A, PageMan display of coordinated changes of hormone biosynthesis gene categories (for further details, see legend to Fig. 2). B, MapMan hormone overview maps showing differences in transcript levels of hormone-related genes between late seed maturation (25 DAF) and germination (24 HAI; left side) in both the E/A- and the Em-fraction and during germination between 24 and 48 HAI (right side) in the same tissue fractions. For further details, see legend to Figure 5.

in embryo during seed development (Sreenivasulu et al., 2006). These genes have been proposed to be involved in acquisition of desiccation tolerance of the embryo. Our data indicate that they might play an analogous role during rehydration in seed germination, since we found enrichment of ABRE and DREB cis-elements in the promoter regions of homologous rice LEA and dehydrin genes (Sreenivasulu et al., 2006).

Members of the MYB TF family show preferential expression during transition from seed maturation to germination in the E/A- and Em-fractions (clusters 6[2] and 6[5]; Fig. 9B; Supplemental Table S6). AtMYBR1 and AtMYBR2, two MYB TFs found during very late stages of seed maturation and in dry seeds (in barley grains represented by the Contig10662 and Contig5841) are down-regulated in the Arabidopsis *fus3* mutant, where late embryogenesis programs are affected (Kirik et al., 1998). These results hint that the two TFs act downstream of FUS3 and might play a role in embryo longevity and early seed germination. In summary, the data suggest that TFs functional during seed maturation can be stored to later initiate germination programs.



ABA-responsive storage mobilizing and LEA genes

***cis*-element prediction from 13 orthologous rice promoters**

GARE:	**0.081**	**(3/2)**
R1-MYB:	**0.053**	**(1/1)**
Pyrimidine box:	**0.319**	**(19/12)**
ABRE [acgt]:	**0.251**	**(29/12)**
ABRE [cacgt]:	**0.143**	**(14/7)**

GA-responsive genes encoding storage mobilizing and hydrolytic enzymes

***cis*-element prediction from 69 orthologous rice promoters**

GARE:	**0.161**	**(44/22)**
R1-MYB:	**0.042**	**(10/10)**
Pyrimidine box:	**0.320**	**(124/61)**
ABRE [acgt]:	**0.272**	**(93/34)**
ABRE [cacgt]:	**0.257**	**(41/26)**

Figure 8. (*Legend appears on following page.*)

Regulatory Genes Preferentially Expressed in the Aleurone of Germinating Grains Act under the Concerted Action of GA

The 39 TFs from subcluster 6(5) represent germination-specific genes of the E/A-fraction (Fig. 9A; Supplemental Table S6). Most probably this set of TFs is expressed only in aleurone cells since endosperm cells die as a result of programmed cell death during late seed maturation. Among the coexpressed TFs is the GAMYB factor transcript (Contig3665_at, Contig3667_s_at, EBem10_SQ002_I10_s_at, HS18K19u_s, X87690_s). In addition to GAMYB we found three other MYB transcripts with an encoded DNA binding domain homologous to rice sequences that have been shown to bind a GA-response peptide motif. Also 4 WRKY TFs (Contig10471_at, Contig15657_at, Contig13375_at, Contig10167_at) expressed in the aleurone during imbibition are most likely connected to the GA signaling pathway (Zhang et al., 2004). Based on this correlative evidence, it appears that many of the TFs characterized by a peak of expression in the aleurone of imbibed seeds are regulated in concert by GA.

To provide more supportive data, we compared 39 TFs from subcluster 6(5) characterized by expression in aleurone cells (see above) with the GA-responsive TF list of Chen and An (2006). As a result, eight TFs were identified as preferentially expressed in aleurone during germination and responsive to GA (Fig. 9C; Supplemental Table S8). These candidate genes include an NAC TF (Contig9031_at), a DOF TF (Contig13717_at), an ARR-B family member (Contig8572_s_at), an AP2 EREBP member (Contig7722_at), a homeobox member (Contig12869_at), two EIL (ethylene insensitive 3-like) members (Contig4395_at; HVSMEa0017I09r2_s_at), and the abovementioned GAMYB factor represented by two sequences (X87690_s_at, HS18K19u_s_at; Supplemental Table S8). In addition, we found in subcluster 6(2) TFs such as BPBF DOF (Contig9071_at), a bHLH TF member (Contig15975_at), an ABA signal transduction TF member from the ABI3/VP1 family (Contig10484_at), an ABA response element binding factor from the bZIP family (Contig8163_at), two members of the NAC family (Contig6233_at, Contig6235_s_at), and two homeobox family members (Contig4741_s_at, Contig6168_at), which are all GA responsive in the aleurone during seed germination (Chen and An, 2006) and are presumably synthesized already during grain maturation (Fig. 9C; Supplemental Table S8). In summary, these TFs are synthesized during seed development in endosperm/aleurone, probably play an active role in storage product accumulation, and are also responsive to endogenous GA in the aleurone during germination.

ARF and AUX/IAA TF Family Members Are Preferentially Expressed in the Embryo of Germinating Grains

Subcluster 6(3) consists of 66 TFs that are activated preferentially in the embryo of germinating seeds. Prominent TF families represented in this cluster are ARF, AUX/IAA, C2C2-GATA, and C3H (Fig. 9A; Supplemental Table S6). ARF (auxin-responsive factor) genes expressed in this cluster share a high degree of homology with Arabidopsis and rice sequences. The preferential expression of these sequences (ARF6, ARF7, and ARF8) in the embryo of imbibed barley seeds suggests an important role in cell expansion during seed germination. The AUX/IAA family members are coexpressed in subcluster 6(3) but also in cluster 4 (Fig. 9A) and are known to regulate auxin-mediated gene expression by interacting with ARF TFs (Overvoorde et al., 2005). Two such ARF TF transcripts (Contig14766_s_at, HO13O09S_s_at), which are known to be induced by exogenous GA treatment, were found (Supplemental Table S8). The described genes were scrutinized for overrepresented cis-elements in the promoter regions of homologous rice genes. These regions of ARF and AUX/IAA genes are enriched with GA-responsive pyrimidine box and GARE cis-elements. However, only two AUX/IAA family members (Contig322_at, Contig9991_at) were found to possess ARF cis-elements (Supplemental Table S7). Based on the correlative data provided here it appears that ARF and AUX/IAA TF family members are most probably involved in the concerted action of GA and auxin. Another important class of TF genes, the C2C2-GATA family members expressed preferentially in the embryo during germination shows homology to GATA zinc finger proteins. A loss of function of the GATA-gene BME3-ZF in Arabidopsis seeds leads to reduced expression of GA20-oxidase and GA3-oxidase (Liu et al., 2005). These data suggest a connection to the GA signal transduction pathway. Altogether, our results indicate an interaction between GA and auxin in regulating ARF and AUX/IAA gene expression and, eventually, in the establishment of cell division events in the embryo.

DISCUSSION

In this study we extend our investigation on barley grain development (Sreenivasulu et al., 2006) by a combined analysis of grain maturation, desiccation, and germination. We used the Affymetrix Barley1 GeneChip for transcriptome analysis in contrast to the 12K macro array of earlier work with genes only expressed during grain development (Sreenivasulu et al., 2004,

Figure 8. Transcript levels of ABA (left) and GA (right) biosynthetic and signaling pathways integrated into a hypothetical scheme related to the induction of storage mobilizing transcripts. Expression values are given in logarithmically scaled (base 2) signal intensities. The Affymetrix Contig identification and predicted function of each gene is indicated. In the lower part, the presence of GA-responsive motifs (GARE, R1-MYB, and Pyrimidine box) and ABA-responsive motifs (ABRE) in the 1-kb upstream region of rice homologous storage mobilizing genes is indicated (for predicted cis-elements see Supplemental Table S5); for detailed expression of storage mobilizing genes see Figure 4. For details about motif specific enrichment see "Materials and Methods".

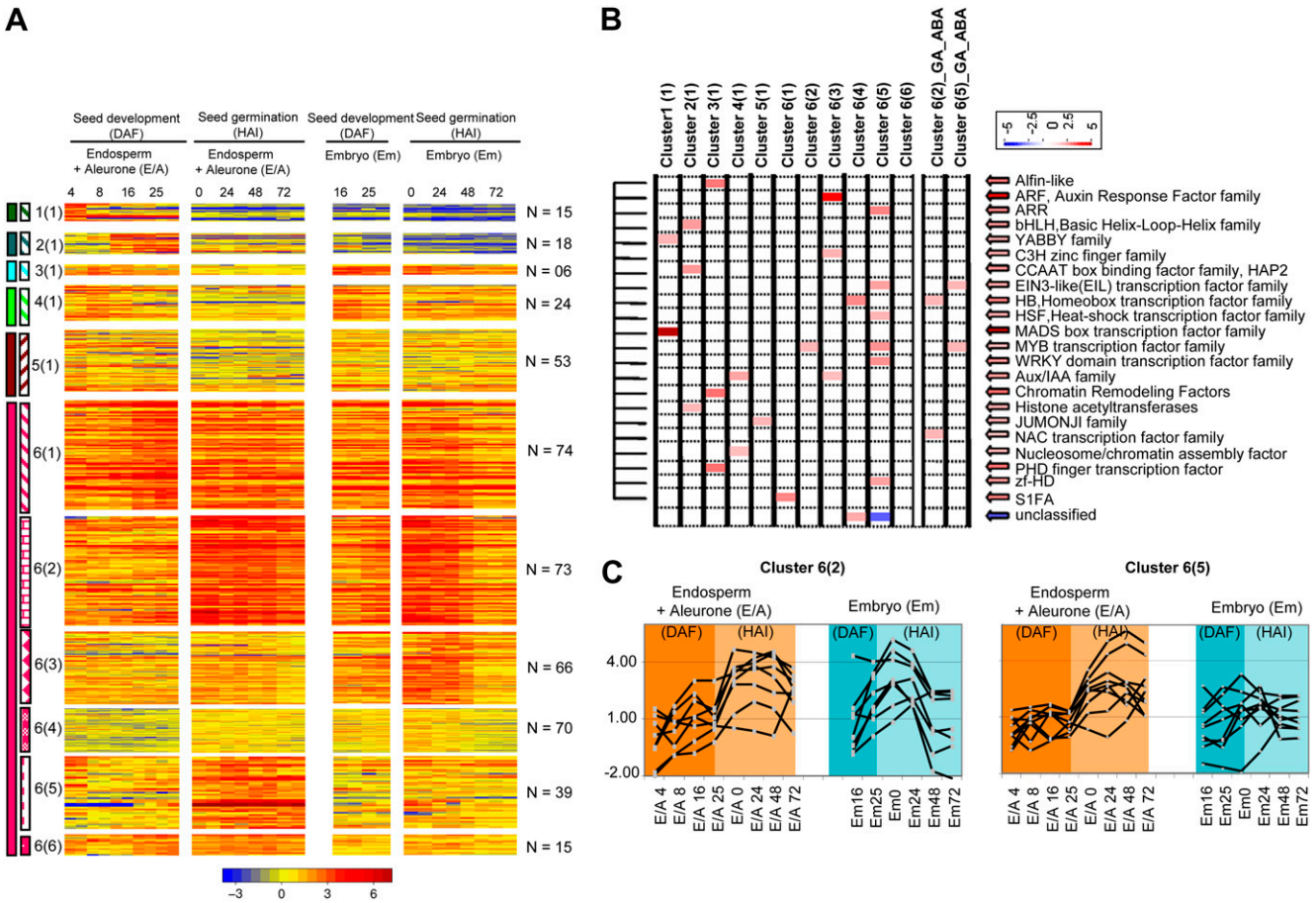


Figure 9. A, Expression profiles of TFs showing tissue- and development-specific patterns during the period of seed development and germination. Major clusters (1–6) obtained by K-mean clustering and subclusters 6(1) and 6(6) based on the SOM method (subclusters 6[1] to 6[6]) are indicated on the left by vertical bars; the number of genes (*N*) represented in each individual cluster/subcluster is on the right. Log_2 expression values are plotted visualized on the color scale: red, high expression; yellow, moderate expression; blue, low expression. Horizontal rows represent gene expression patterns; the developmental stage is assigned to vertical columns on the top. B, Overrepresented functional groups predicted based on Fisher's exact test in PageMan for every individual subcluster. C, TF genes responsive to exogenous GA and ABA (Chen and An, 2006) were used to associate temporal expression profiles obtained from seed development (DAF) and germination (HAI), which fall into two major cluster groups, 6(2) and 6(5). For a detailed gene list and normalized expression values, see Supplemental Table S8.

2006). A comparison of annotated genes on both arrays revealed that nearly 8,600 sequences derived from developing seed libraries are found both on the Barley1 GeneChip and the 12K array (N. Sreenivasulu, unpublished data). The remaining 14,000 sequences of the Affymetrix Barley1 GeneChip originate from various cDNA libraries that represent various developmental stages including germinating seeds as well as abiotic and biotic stress treatments of vegetative tissues. For analysis we used two hand-prepared fractions of maturing and germinating grains, (1) the EM-fraction consisting of embryo axis and scutellum and (2) the E/A-fraction, i.e. the de-embryonated grain consisting of the storage endosperm and the covering endospermal aleurone layer. Because the storage endosperm dies during late maturation, any increase in transcript abundance in this fraction during germination must be due to RNA that is newly synthesized in the aleurone. Despite increased resolution by tissue separation, both isolated fractions are still heterogeneous, but they also exhibit some functional overlap. Thus, both aleurone and scutellum are involved in regulating endosperm reserve mobilization.

MapMan and PageMan Tools for Barley

PageMan (Usadel et al., 2006) is an efficient tool to get a quick overview of the overrepresented functional gene groups from multiple Arabidopsis transcriptome experiments such as developmental time course analyses. Once the index of overrepresentation of functional categories in a time series data set is identified by PageMan, the response can be inspected in more detail using the MapMan tool (Usadel et al., 2005) based on MapMan ontology. The mapping of Affymetrix Barley1 GeneChip probe identifiers into the MapMan

functional categorization (http://mapman.mpimp-golm.mpg.de/) allows these tools to be applied to barley, an important crop plant. Regulatory or metabolic processes are easily identified, avoiding tedious manual sorting through gene lists. For instance, TAG synthesis or GA/ABA metabolism/regulation can be studied in great detail by simply selecting the appropriate pathway in MapMan (see Figs. 3, 5, 6, and 7) or by pinpointing changed pathways during plant development in PageMan (see Figs. 2 and 7A). Furthermore, as most genes are now categorized into processes, a more unbiased approach can be taken by using the PageMan software tool to have a look at many pathways at once across the developmental time course. On the other hand, individual genes may still be inspected by using MapMan as shown in e.g. Figure 3 and thus enables one to make connections based on the biological context.

mRNAs Stored in Mature Barley Grains

Most transcripts in both the E/A-fraction and the Em-fraction of dry seeds (0 HAI) can be regarded as stored or conserved mRNA. Nakabayashi et al. (2005) described nearly 12,500 stored mRNA species of all ontological categories from dry Arabidopsis seeds. A similar number was detected in 24-HAI imbibed seeds. In addition, Ogawa et al. (2003) reported no dramatic changes in gross total mRNA levels during germination. Our analysis revealed nearly 12,100 mRNAs in the dry barley grain embryo. Most abundant stored mRNA species are related to seed reserve synthesis, DNA synthesis as well as general protein synthesis and degradation (Supplemental Fig. S2) as previously reported for Arabidopsis (Rajjou et al., 2004; Nakabayashi et al., 2005). During germination, transcription is slightly activated with an increase of less than 1,000 in the number of transcripts registered as present at 24 HAI in both the Em- and the E/A-fraction. Altogether, our study supports the notion that like in Arabidopsis (Rajjou et al., 2004) the potential for germination is largely programmed during seed maturation. The entire set of differentially expressed genes identified during seed maturation and germination, along with functional annotation and corresponding sequences, is searchable at http://pgrc.ipk-gatersleben.de/seeds/.

Transcriptional Activation of Storage Reserve Mobilization Occurs Early during Germination

Seed storage reserves such as starch, storage proteins, and lipids are predominantly mobilized during and after radical protrusion (Bewley, 1997). Our results demonstrate that genes involved in sugar, starch, and lipid catabolism are expressed much earlier during seed germination, i.e. already 24 HAI (Supplemental Fig. S3). Thus, activation of reserve mobilization is one of the early distinct events taking place before radical protrusion. Studies of imbibed Arabidopsis seeds suggest that the majority of found Suc is derived from storage lipid degradation and not from other soluble sugars within the seed (Pritchard et al., 2002). Similarly, we observed lipid but also starch mobilization transcripts together with coexpressed Suc synthase and SPS mRNAs, which are key transcripts of Suc cleavage and synthesis. The early activation of transcripts of starch and lipid reserve mobilization pathways is expected to supply Suc and hexoses, which provide energy until the cotyledon becomes photoautotrophic (see below for further details). Collectively, we assume based on the transcript data that reserve mobilization already starts during germination, i.e. before radicle protrusion.

Characteristic for germination is the large group of transcripts of storage mobilizing and hydrolytic enzymes, which are either synthesized and stored during seed maturation for usage during germination (cluster group 1 of Fig. 4) or preferentially synthesized during early germination (cluster groups 2 of Fig. 4). Although it has been previously reported that GA positively regulates a large number of proteases in aleurone cells (Chen and An [2006] for barley and Bethke et al. [2006] for rice), no general overview is available at a genome-wide level about stored proteinases and newly synthesized proteinases involved in storage protein mobilization of germinating barley grains. Based on the transcript data we assume that Ser carboxypeptidases (types I and III) and cathepsin-like Cys protease transcripts are presynthesized during seed development and stored as transcripts and, according to Degan et al. (1994), as enzyme in both the E/A- and Em-fractions (see Fig. 4). During germination new transcripts were reported to be GA induced in aleurone during germination resulting in additional enzyme activity (Degan et al., 1994; Chen and An, 2006). Their secretion into endosperm adds to the already stored pool of proteases, which trigger the breakdown of storage proteins in endosperm (Degan et al., 1994). Stored carboxypeptidases might be the major source of peptidases that release free amino acids for very early protein biosynthesis during imbibition. Interestingly, the Cys proteinase gene family members EP-B and EP-A, which are also responsive to GA (Chen and An, 2006), are not expressed during maturation but early during germination at 24 HAI in both fractions (see Fig. 4). This result is in accordance with the reported localization of EP-B transcripts in the scutellar epithelium within the first 24 h of germination and the later prominent presence in the aleurone (Mikkonen et al., 1996). Taken together, our data and results from Chen and An (2006) suggest that the phytohormone GA not only plays an important role in triggering major proteinases during early seed germination but probably also triggers these set of proteases during seed maturation leading to their storage in the dry seed. The data presented in Figure 4 also suggest that GA plays an additional role in triggering cell wall modifying transcripts during germination. As a result the physical constraint of the embryo-surrounding seed coat and endosperm will be overcome by inducing their debilitation during radicle protrusion.

Deduced Roles of ABA and GA in Grain Maturation and Germination

Whereas the interwoven roles of GA and ABA in Arabidopsis seed germination are roughly known mainly due to mutant studies (Feurtado and Kermode, 2007; Yamaguchi et al., 2007), the situation is much less clear in cereals. As reported here, both ABA and GA biosynthetic genes are expressed during barley grain maturation and germination. As a consequence, ABA-GA ratios are established that control seed maturation, dormancy, and germination (White et al., 2000). The cereal aleurone transcriptome is known to be especially responsive to GA but also to ABA. Studies in rice (Bethke et al., 2006) and barley (Chen and An, 2006) reported surprisingly that in the aleurone during germination ABA up-regulates many more genes than it down-regulates. After imbibition, ABA content decreases rapidly (Jacobsen et al., 2002), and the embryo is supposed to release GA to aleurone cells (Ritchie et al., 2000; Olszewski et al., 2002). Although the hypothesis of active GA transport from embryo to aleurone has not yet been proven in cereals, import of bioactive GA or its synthesis is required for the production of hydrolases in the aleurone tissue of imbibed barley grains. Key GA biosynthesis transcripts such as AtGA3OX1 and AtGA3OX2 have been localized in the cortex and endodermis of the embryonic axis of germinating Arabidopsis seeds, suggesting active GA synthesis in the embryo (Ogawa et al., 2003). In contrast to Arabidopsis, cereals need to mobilize distinct storage products in distinct tissues, i.e. starch and storage proteins in the endosperm as well as lipids and another set of storage proteins in the embryo and aleurone. Therefore, it is interesting to deduce the regulation of GA and ABA biosynthesis and the role of the two hormones during the switch between seed maturation and germination in the two genetically distinct fractions of the barley grain (E/A and Em). Our microarray data support the assumptions that (1) expression of GA biosynthesis transcripts in the E/A-fraction during seed maturation leads to preferential accumulation of GA or GA biosynthetic transcripts in the aleurone of the mature dry seed and that (2) the prominent peak of most of the GA biosynthesis genes (KAO1, GA20Ox2, and GA2OX1) in the embryo during early seed germination (see Fig. 8) leads to the production of active GAs necessary for germination as measured by Jacobsen et al. (2002). However, the key GA biosynthetic gene on the Barley1 GeneChip encoding GA 3-oxidase is not expressed in the embryo during germination (Fig. 8). In Arabidopsis two genes encode GA 3-oxidases. Although AtGA3OX1 exhibits early up-regulation within 8 HAI, the AtGA3OX2 gene displays a peak of expression during 40 HAI, which corresponds to the accumulation of GA_4 (Mitchum et al., 2006). Based on these data we assume that the Barley1 GeneChip is missing a second isoform of GA3OX. Our study confirms on the one hand the accepted knowledge that increased synthesis of active GA in the embryo is required for triggering germination events. On the other hand, the presence of key biosynthetic transcripts in the aleurone hints to the contribution of stored GA precursors in triggering expression of hydrolases and storage mobilizing genes in that tissue. A statistical search of homologous rice promoter regions of genes of GA-regulated proteases and cell wall loosening enzymes revealed the frequent occurrence of multiple copies of the ABRE motif in addition to GA-responsive GARE and pyrimidine box motifs (Supplemental Table S5). Accordingly, we observe higher expression of key regulators of the GA response as GAMYB and ABA signaling genes (ABF2, ABI3/VP1) in aleurone cells during germination (see Fig. 8). Taken together our results indicate that GA and ABA signals seem to influence the expression of a subset of stored as well as newly synthesized proteases and cell wall loosening gene sets in an antagonistic manner by interacting independently with distinct cis-regulatory sequences of the abovementioned gene sets.

A Suite of TFs Acts under the Concerted Control of GA and/or ABA Interconnected with Ethylene and Auxin Signaling

Of 453 TFs identified as temporally regulated during late grain maturation and germination only 34 (7.5%) are listed by Chen and An (2006) as to be regulated by GA and/or ABA. These TFs are supposed to be part of transcription networks operating in aleurone (cluster 6[5], Fig. 9C; embryo, cluster 6[3]). They are presynthesized during seed maturation and/or induced during seed germination in both tissue fractions (cluster 6[2], Fig. 9C). By developing barley PageMan and MapMan tools we demonstrate that the preferential activation of transcripts of GA-biosynthesis takes place already during seed maturation in endosperm/aleurone at a time when ABA peaks too (Sreenivasulu et al., 2006). These results reinforce the importance of endogenous stored GA and/or GA biosynthetic transcripts in endosperm/aleurone in addition to the de novo GA biosynthesis occurring during early seed germination in the embryo. A combination of temporal expression profiling data and cis-element prediction additionally provided data regarding the importance of ABA during seed germination. The hormone triggers TFs during the onset of seed germination in connection with ethylene and auxin signaling networks.

The most abundant TF family that is induced in the aleurone during seed germination by GA is the MYB family (Fig. 9, B and C; Supplemental Table S8) including the well-characterized GAMYB TF transcript (HS18K19u_s, X87690_s). GAMYB has been characterized as a positive transcriptional regulator triggering a large set of hydrolytic genes such as α-amylase by binding to the GA-responsive pyrimidine box element (Gubler et al., 1999; Tsuji et al., 2006). Also overrepresented is a TF family with EIL (Contig4395_at, HVSMEa0017I09r2_s_at) and ERF (ethylene response factor; Contig7722_at) members that are suppressed by

GA treatment and induced by exogenous ABA treatment (Chen and An, 2006; Fig. 9, B and C; Supplemental Table S8). This finding suggests that GA down-regulates negative regulators of ethylene signaling. Since ethylene positively influences seed germination (Kucera et al., 2005) both GA and ethylene act synergistically in the process. Accordingly, along with GA biosynthetic genes ethylene biosynthetic genes are coexpressed in aleurone during 24 HAI (Fig. 7B). Likewise, GA treatment leads to an increase in expression of key transcripts of ethylene formation during Arabidopsis seed germination (Ogawa et al., 2003).

GA and ABA also differentially regulate large groups of genes related to auxin influx during seed germination as, for instance, PIN genes (Chen and An, 2006; Ogawa et al., 2003), eventually leading to polar auxin transport. Two possible mechanisms can be discussed by which GA may regulate expression of PIN genes. First, in Arabidopsis, exogenous GA treatment modulates the expression of cytochrom P450 (CYP) mRNAs (Ogawa et al., 2003), which are necessary for auxin biosynthesis. Thus, GA treatment may lead to changes in auxin level, which in turn modulates the expression of auxin influx carrier proteins. Second, according to our transcriptome data GA may trigger the expression of ARFs and by this way influence auxin influx carrier protein gene expression. One more hint supporting the latter suggestion is the finding that in the 5′-upstream sequences of ARF homologous rice genes GA-responsive GARE and pyrimidine box cis-elements were found, specifically in ARF7a (Supplemental Table S7). Barley ARF7a transcripts are induced by GA treatment (Chen and An, 2006) and are expressed specifically in the embryo during imbibition (Supplemental Table S6). In addition, IAA/Aux genes do possess auxin-responsive elements within the upstream 1,000 bp of homologous rice promoter regions (Supplemental Table S7), suggesting the specific involvement of ARF- and Aux/IAA-dependent signaling in the transcriptional regulation of PIN genes. These observations collectively indicate that GA and auxin signaling pathways are interconnected in the germinating embryo tissue but details have to be worked out.

In conclusion, we provide evidence that the above-described phytohormone biosynthetic gene sets and sets of TF transcripts active under the influence of these hormones are synthesized already during late maturation at a tissue-specific level and later on needed during germination. Thus, during late seed maturation the barley grain not only prepares by metabolic switching for enhanced biosynthetic activity during seed germination but also stores the required regulators in the mature grain.

MATERIALS AND METHODS

Plant Material

Barley plants (*Hordeum vulgare* 'Barke', a European two-rowed malting variety), were cultivated in growth chambers at 20°C/18°C under a 16-h light/8-h dark cycle until seed set. The developmental stage of a caryopsis was determined from the midregion of the ear and assigned as 4, 8, 16, and 25 DAF. Two fractions were prepared as described by Sreenivasulu et al. (2002), i.e. at stages 16 and 25 DAF the starchy E/A-fraction and the Em-fraction. To analyze germination, seeds were incubated on a moist filter paper in Microclima 1000 growth chambers (Snijders Scientific B.V.) with 16-h light (17°C; 337–377 mmol m^{-2} s^{-1} light intensity measured by a Sky Quantium light sensor) and 8-h darkness (12°C), 80% humidity, and the fractions dissected 0, 24, 48, and 72 h after imbibition (referred to in the figures as Em0, Em24, Em48, and Em72, and E/A0, E/A24, E/A48, and E/A72, respectively). Plant growth conditions and tissue collection descriptions have been deposited in the Protocols submission section in Gene Expression Omnibus (GSE9365). Samples were collected from two independently grown biological plant batches.

RNA Isolation and Probe Preparation

RNA from Em- and E/A-fractions collected during seed development was isolated by the Trizol method (Gibco BRL Life Technologies), treated with DNase (QIAGEN), and cleaned with RNeasy spin columns (QIAGEN). RNA isolation from germinating samples has been performed according to Potokina et al. (2002). RNA quality was assessed on an Agilent Bioanalyzer 2100 (Agilent Technologies). Probe synthesis, labeling, and hybridization were performed according to the manufacturer's protocols (Affymetrix) at the University of California Irvine MicroArray Core Facility. Arrays were scanned on a GeneChip Scanner 3000. The purified labeled cRNA samples prepared from developing and germinating seeds were hybridized to Barley1 GeneChips according to Close et al. (2004).

Barley1 GeneChip Data Analysis

The results were quantified and analyzed with GCOS 1.1.1 software (Affymetrix) using default values. For quality assessment, probe-level modeling and RNA degradation and relative log expression procedures from Bioconductor (Gentleman et al., 2004) were used. Further analysis and quality controls were done using the R programming environment (R Development Core Team, 2006). Genes detected with *P* values higher than 0.05 were excluded. Because GCOS can lead to very low values, which lead to extremely high differences, a small offset was added to each signal value as described previously (Scheible et al., 2004). After transfer of the data to a logarithmic scale, the average expression for all experimental samples for this probe set was subtracted from each individual expression value, thus leading to a positive value in case of above-average expression levels and a negative value in case of below-average expression levels.

To test for consistency of the data, experiments were clustered using complete linkage hierarchical clustering to best visualize the relationship between the samples obtained from different tissues during seed development and germination. Further samples were colored according to the tissue and the experimental series they came from (see legend of Fig. 1).

To identify potentially differentially expressed genes, the MAS5 expression values were log2 transformed and read into R. For each tissue a linear model was fitted using linear models for microarray data (limma), according to Smyth (2004). This was done separately for the maturation and germination series, leading to five linear models in total. For the pericarp (4 and 8 DAF) and embryo (16 and 25 DAF) samples, a simple moderated *t* test was performed and *P* values were corrected using the Benjamini and Hochberg (1995) false-discovery rate control, applying standard limma procedures. For all other whole time series, a linear model was fitted as described in the limma user manual, and F-test *P* values were extracted from the model and *P* values were adjusted using the Benjamini-Hochberg procedure (see Supplementary Table S2). Differentially expressed genes, between germination samples (0, 24, 48, and 72 HAI) and the 25-DAF sample were identified for both endosperm and embryo tissues using the limma nestedF procedure, applying a significance threshold of 0.05 in combination with Benjamini-Hochberg false-discovery rate control and a minimal log2-fold change value of 1 (Supplemental Tables S3 and S4).

For classification of expression patterns a combination of K-means and SOM clustering was used as described in Sreenivasulu et al. (2006) for obtaining major centroids shown in Figures 4 and 9. The quality and reliability of predicted centroids representing coexpressed gene sets were further validated by several repetitions of the clustering that showed low quantization errors and a good reproducibility of these prominent cluster patterns.

Development of a Barley MapMan Classification

MapMan uses a hierarchical ontology system, into which the barley exemplary sequences were classified. This was done using a simple unidirectional BLAST search against already classified proteins from Arabidopsis (*Arabidopsis thaliana*) and barley Contigs, to maximize sensitivity. Based on the best BLAST search results and using a cutoff *e* value of 10^{-10}, the barley genes were assigned to BINs/subBINS according to the most similar Arabidopsis genes, to obtain a high sensitivity, sacrificing some specificity. In the case where a barley sequence had a match of an arbitrary *e* value of 10^{-50} or better against already classified IPK clusters, the IPK cluster classification was transferred to the Affymetrix probe set, represented by this sequence. For these IPK clusters a MapMan BIN was already inferred based on both automatic and manual annotations (N. Sreenivasulu and B. Usadel, unpublished data). In the case where such a hit could not be found, but an *e* value of 10^{-10} or better was obtained for the blastx search against the Arabidopsis proteome, the classification of the respective Arabidopsis protein was imported. Otherwise the sequence was tagged as unknown in this first classification round. Also a reciprocal BLAST method has been implemented (see Supplemental Table S9).

To identify potential regulators including TFs we implemented a similarity search using blastn and blastx programs to predicted sets of Arabidopsis and rice (*Oryza sativa*) TF families. All hits below an *e* value of 10^{-20} were extracted. Further, we scanned for the presence of motifs using locally installed interpro scan (version 12.1 without TMHMM and signalP) to identify potential TF domains or families. Signaling genes and hormone biosynthesis genes were manually checked to achieve a higher specificity as with the simple BLAST approach, which was tuned for sensitivity (for further details see Supplemental Note S1).

Overrepresentation Analysis of TFs

One of PageMan's main modules is overrepresentation analysis using Fisher's exact test. Cluster analysis was used as a module input as follows. If a given probe set representing a TF was within a cluster it was given a one, otherwise a zero. The data were then loaded into PageMan, which calculated overrepresentation for each cluster, if there were more TFs of a given family than expected by chance. By this way an unbiased statistical analysis of involved TFs was achieved.

Computational Approaches of Identifying cis-Regulatory Elements from Rice Promoters

To search for candidate cis-regulatory sequences 1,000 base regions upstream of all rice gene translational start sites (promoters) were used. First, all the known motifs reported for ABA and GA responses have been scanned in the predicted promoter set of the whole rice genome. Eventually the promoters of candidate genes (homologs of barley candidate genes of storage mobilizing transcripts [Supplemental Table S5] and TFs expressed preferentially in embryo during germination [Supplemental Table S7]) were separated from the rest of the rice genome, which has been treated as noncandidate promoter set. For each motif and each candidate gene promoter the genome-wide probability of occurrence was calculated by determining the motif-specific enrichment, i.e. motif counts for candidate promoter regions that exceed the counts of noncandidate promoters. For a specific motif the average probability is calculated that its frequency f_{cj} of occurrence in all g_i genes j from the list of candidate genes is less or equal to the frequency f_n in the set of noncandidate genes:

$$p = \frac{1}{g_i}\sum_j P(f_{cj} \leq f_n)$$

Small P values indicate specific motif enrichment in candidate genes. Because the relative frequency P involves a comparison of occurrences of cis-elements in candidate gene promoters against a large number of more than 61,000 noncandidate promoters in rice, enrichment is already indicated at relatively large significance thresholds. The calculation is carried out separately for ABA and GA-responsive candidate genes for known core motifs of interest. Out of these, GARE, R1-MYB, Pyrimidine box, ABRE [acgt], and ABRE [cacgt] show interesting enrichment characteristics (Fig. 8; Supplemental Tables S5 and S7).

Microarray data from this article have been deposited with the National Center for Biotechnology Information Gene Expression Omnibus data repository (http://www.ncbi.nlm.nih.gov/geo/) under accession number GS9365.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Histograms of transcript abundance.

Supplemental Figure S2. PageMan display of the overview list of overrepresented gene categories of metabolism and regulators.

Supplemental Figure S3. MapMan metabolism overview maps of E/A-fractions. A, 25 DAF versus 72 HAI; B, 0 HAI versus 24 HAI; C, 24 HAI versus 48 HAI; D, 48 HAI versus 72 HAI.

Supplemental Figure S4. MapMan metabolism overview maps of Em-fractions. A, 25 DAF versus 72 HAI; B, 0 HAI versus 24 HAI; C, 24 HAI versus 48 HAI; D, 48 HAI versus 72 HAI.

Supplemental Table S1. Overview of MapMan functional classes of the Affymetrix Barley1 GeneChip.

Supplemental Table S2. Complete list of normalized expression values of the Affymetrix Barley1 GeneChip.

Supplemental Table S3. Differentially expressed gene list in the E/A-fraction between 25 DAF and 48 HAI.

Supplemental Table S4. Differentially expressed gene list in the Em-fraction between 25 DAF and 48 HAI.

Supplemental Table S5. Expression patterns of GA- and/or ABA-regulated proteases and predicted cis-elements list.

Supplemental Table S6. TF genes (453) preferentially expressed during seed development and germination.

Supplemental Table S7. TF genes preferentially expressed in the embryo during seed germination and predicted cis-elements list.

Supplemental Table S8. GA- and/or ABA-regulated TF genes expressed during seed maturation and germination along with predicted cis-elements.

Supplemental Table S9. Reciprocal BLAST results from Arabidopsis versus barley.

Supplemental Note S1. MapMan ontology classification procedure.

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