Journal of Experimental Botany, Vol. 62, No. 8, pp. 2615–2632, 2011
doi:10.1093/jxb/erq446 Advance Access publication 2 February, 2011
This paper is available online free of all access charges (see http://jxb.oxfordjournals.org/open_access.html for further details)

Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

ABA biosynthesis and degradation contributing to ABA homeostasis during barley seed development under control and terminal drought-stress conditions

Christiane Seiler, Vokkaliga Thammegowda Harshavardhan, Kalladan Rajesh, Palakolanu Sudhakar Reddy, Marc Strickert, Hardy Rolletschek, Uwe Scholz, Ulrich Wobus and Nese Sreenivasulu*

Leibniz-Institute of Plant Genetics and Crop Plant Research (IPK), Correnstrasse 03, D-06466 Gatersleben, Germany

* To whom correspondence should be addressed. E-mail: srinivas@ipk-gatersleben.de

Received 11 October 2010; Revised 2 December 2010; Accepted 3 December 2010

Abstract

Drought is one of the most severe environmental stress factors limiting crop yield especially when occurring during anthesis and seed filling. This terminal drought is characterized by an excess production of the phytohormone abscisic acid (ABA) which plays an important role during seed development and dormancy. All the genes putatively involved in ABA biosynthesis and inactivation in barley were identified and their expression studied during plant ontogeny under standard and drought-stress conditions to learn more about ABA homeostasis and the possible mode of cross-talk between source and sink tissues. Out of 41 genes related to ABA biosynthesis and inactivation 19 were found to be differentially regulated under drought stress in both flag leaves and developing seed during seed filling. Transcripts of plastid-located enzymes are regulated similarly in flag leaf and seed under terminal drought whereas transcripts of cytosolic enzymes are differentially regulated in the two tissues. Detailed information on the expression of defined gene family members is supplemented by measurements of ABA and its degradation and conjugation products, respectively. Under drought stress, flag leaves in particular contain high concentrations of both ABA and the ABA degradation products phaseic acid (PA) and diphaseic acid (DPA); whereas, in seeds, besides ABA, DPA was mainly found. The measurements also revealed a positive correlation between ABA level and starch content in developing seeds for the following reasons: (i) genes of the ABA controlled SnRK2.6 and RCAR/PP2C-mediated signal transduction pathway to the ABF transcription factor HvABI5 are activated in the developing grain under drought, (ii) novel ABA- and dehydration-responsive *cis*-elements have been found in the promoters of key genes of starch biosynthesis (*HvSUS1, HvAGP-L1*) and degradation (*HvBAM1*) and these transcripts/activity are prominently induced in developing seeds during 12 and 16 DAF, (iii) spraying of fluridone (an ABA biosynthesis inhibitor) to drought-stressed plants results in severely impaired starch content and thousand grain weight of mature seeds.

Key words: ABA homeostasis, abscisic acid, barley endosperm, genomics, gene expression, hormone profiling, starch, storage product accumulation, terminal drought.

Introduction

The phytohormone abscisic acid (ABA) is involved in the regulation of various processes of plant development like seed dormancy, germination, senescence, and adaptive responses to environmental stress. ABA biosynthesis and signalling genes have been well studied in non-seed tissues but little is known about the situation in seeds (Nambara and Marion-Poll, 2003). In *Arabidopsis,* genes encoding the enzymes for most steps of the ABA biosynthesis pathway have been cloned or their functions were characterized using ABA-deficient mutants. ABA biosynthesis occurs in plastids with the exception of the last two steps where xanthoxin is converted to ABA in the cytosol (Marin *et al.*, 1996; Tan

et al., 1997; Seo and Koshiba, 2002). The first biosynthetic step in ABA biosynthesis is the conversion of zeaxanthin to all-*trans*-violaxanthin, a two-step epoxidation process catalysed by zeaxanthin epoxidase (ZEP/AtABA1; Marin et al., 1996; Audran et al., 2001) .The enzyme(s) involved in the conversion of all-*trans*-violaxanthin to 9-*cis*-violaxanthin or 9-*cis*-neoxanthin have not yet been identified. 9-*cis*-epoxycarotenoid dioxygenase (NCED) catalyses the next step, the oxidative cleavage of 9-*cis*-violaxanthin and/or 9-*cis*-neoxanthin to produce xanthoxin. This reaction is considered to be rate-limiting in ABA biosynthesis and occurs in plastids (Tan et al., 1997; Burbidge et al., 1999; Chernys and Zeevaart, 2000; Iuchi et al., 2001; Qin and Zeevaart, 2002). Xanthoxin is eventually exported to the cytosol and converted to abscisic aldehyde by a short-chain dehydrogenase/reductase (SDR/AtABA2; Cheng et al., 2002; Gonzalez-Guzman et al., 2002). Abscisic aldehyde is then oxidized to ABA by aldehyde oxidase (AAO/AO; Seo et al., 2004). AO needs the sulphurylated form of a molybdenum cofactor (moco/AtABA3) for its activity (Bittner et al., 2001).

In many species, two peaks of ABA accumulation during seed development have been reported. The first peak is maternally derived and the second one depends on ABA biosynthesis in the seed itself. The latter one is required for the induction of storage protein accumulation and, generally, for seed maturation and dormancy (Karssen et al., 1983; McCarty, 1995). In barley too, two ABA peaks during seed development have been reported (Jacobsen et al., 2002; Millar et al., 2006). Temporal patterns of ABA biosynthetic gene expression patterns related to seed tissues have been described for ZEP (Audran et al., 2001), NCED (Lefebvre et al., 2006) and AO (Seo et al., 2004) in *Arabidopsis*, but little is known for cereal grains with the exception of the aleurone (Ritchie et al., 2000). According to our previous work in barley, endogenous production of ABA is likely to take place in both the endosperm and the embryo during seed maturation as reasoned by the activation of ABA biosynthesis genes (Sreenivasulu et al., 2006, 2008, 2010*a*). However, a comprehensive knowledge of ABA biosynthesis and catabolism events involved in the regulation of ABA levels in different grain tissues is still lacking.

Reduction of elevated ABA levels is possible by two mechanisms: (i) catabolism of ABA and (ii) inactivation of ABA. Higher levels of ABA are known to trigger the major catabolic route, which leads to the formation of 8′-hydroxy ABA catalysed by the cytochrome P450 monooxygenase ABA 8′-hydroxylase. The 8′-hydroxy ABA is subsequently converted to phaseic acid by spontaneous isomerization (Kushiro et al., 2004). Except the gene for this first step of ABA catabolism, other genes involved in ABA breakdown are not known. A different form of ABA inactivation is the conjugation with glucose by ABA glucosyltransferase to form the inactive ABA glucose ester (ABA-GE; Xu et al., 2002). This reaction is reversible, and the deconjugation of ABA-GE is catalysed by β-glucosidase (Lee et al., 2006).

ABA is well known to be produced under drought stress in vegetative tissues. The hormone is thought to be an essential messenger in the plant's response to drought (Wilkinson and Davies, 2002; Sreenivasulu et al., 2007). In cereals such as wheat and barley, drought stress reduces the grain-filling rate mainly due to a decrease in photosynthetic efficiency. Physiological data from rice and wheat provide evidence that a higher ABA content in leaves and stems is positively correlated with efficient seed filling by enhancing remobilization (Yang et al., 2000, 2006; Yang and Zeevaart, 2006). Enhanced ABA levels in water-stressed grains are also closely related to an increased grain-filling rate (Yang et al., 2000). However, little is known about the likely role of ABA in enhancing sink strength and in the accumulation of starch. Furthermore, it is unclear whether ABA is synthesized in the grain itself or imported from vegetative tissues. To address these issues in barley (i) gene family members of ABA biosynthesis, catabolism, and deconjugation pathways were defined using the 50 000 unigene collection (HarvEST database) and (ii) *in silico* genome wide expression patterns of these genes were estimated in a wide variety of tissues covering plant ontogeny. To develop a comprehensive view of pathways contributing to ABA homeostasis in seeds under terminal drought stress, (iii) ABA-metabolites were measured and (iv) a qRT-PCR platform was established to reveal the differential response of these genes in flag leaves and developing seeds. Finally, (v) the effects of exogenous ABA treatments of spikes under field conditions were studied to investigate the role of ABA in seed-filling efficiency under terminal drought.

Materials and methods

Plant material and growth conditions

Barley plants (*Hordeum vulgare,* LP110 breeding line of KWS, Lochow GmbH) were cultivated in a growth chamber with a 16/8 h light/dark cycle at 20/15 °C. Spikes were labelled at anthesis and four different developmental stages of seed material were harvested as described by Weschke et al. (2000). With phytochamber-cultivated plants drought stress was imposed 1 week after anthesis and these plants were maintained at 10% soil moisture levels. Throughout the period, soil moisture levels were monitored using a moisture meter HH2 with soil moisture sensor SM200 probes (Delta T devices). The remaining batch of plants was treated as a control and continuously watered. The developing seeds were harvested at 12, 16, 20, and 25 days after flowering (DAF) from the mid-region of the ear from stress and control plants. In addition, flag leaf material at the same stages was harvested. Two replications were maintained by growing them independently. The stages 12, 16, 20, and 25 DAF were chosen to cover the middle and late stages of seed development, including the start of the storage phase (12 DAF), the peak of storage at 16 and 20 DAF, and the seed maturation phase (25 DAF). According to the Zadoks system for barley, those stages are related to the late milk, soft dough, and hard dough physiological stages (Tottman and Broad, 1987).

For field studies, plants were grown in plots with 50 plants per plot. A random block design was used for assessing reproducibility. In the field study a rain shelter was installed at anthesis to protect plants from natural rainfall. Control plants were continuously watered. One week after anthesis and again 10 d later the spikes of two plots of stress-treated plants were sprayed with 200 μM ABA and 50 μM fluridone, respectively. After ripening mature seeds from control, stress-treated, and ABA/fluridone-treated

plants were collected and grain yield as well as thousand kernel weight per plot was calculated.

Annotation of barley genes

To identify genes encoding enzymes of ABA biosynthesis, catabolism, deconjugation, and signalling in barley, sequence similarity searches were performed using the HarvEST Barley database (http://harvest.ucr.edu/) with 50 000 unigenes by BLASTN, BLASTX, and tBLASTX based on known *Arabidopsis* and rice sequence annotations. The five best EST hits of each BLAST search were selected. The corresponding cDNA sequences were extracted from the database and subjected to multiple alignments by using ClustalW (DNAstar). cDNA sequences were translated and searched for conserved domains known from the corresponding *Arabidopsis* proteins using the NCBI database. Phylogenetic trees comprising barley, rice, and *Arabidopsis* translated cDNA sequences have been generated.

RNA isolation and cDNA synthesis

Total RNA was isolated from developing seeds and flag leaves using the TRIZOL reagent (Invitrogen GmbH, Karlsruhe, Germany) and RNAeasy columns (Qiagen, Hilden, Germany). Tissue was ground in liquid nitrogen and 100 mg of the homogenized powder was added to 1 ml TRIZOL and incubated at 60 °C for 5 min. Samples were centrifuged at 10 000 rpm for 10 min and the supernatant was transferred to a new tube. 200 µl of chloroform were added and incubated at room temperature for 2–3 min. Samples were again centrifuged as described above and the aqueous supernatant was transferred to the Qia shredder column and centrifuged for 30 s at 10 000 rpm. 350 µl of RLT buffer (plus β-mercaptoethanol) and 250 µl of absolute ethanol were added to the flow-through and passed through an RNAeasy spin column. All the following steps were performed as described in the manufacturer's protocol followed by in-column DNAse digestion. The quality of RNA was checked on a 1% agarose gel. RNA concentration was measured using the NanoDrop photometer (Peqlab) according to the manufacturer's instructions. Absence of genomic DNA was confirmed by PCR, using primers derived from an intron sequence of a control gene. RNA quality was checked on a 1% (w/v) agarose gel. 2 µg of total RNA were taken for cDNA synthesis using SuperScript™III (Invitrogen GmbH), according to the manufacturer's instructions. To test cDNA yield, qPCR was performed using primers of a reference gene (serine/threonine protein phosphatase PP2A-4, catalytic subunit, EST clone HZ44D03; forward primer: GATGACTGCAACGCTCACAC, reverse primer: CTCAAAGGAAATAATCAGGCGTTC) that is stably expressed under the experimental conditions tested. cDNA was diluted according to C_T values until all samples ranged between ±1 C_T. The quality of cDNA was assessed by using two primer pairs for a reference gene (elongation factor 1α, EST clone HZ42K12) from the 5′ and 3′ regions (forward primer 5′ region: AAGGAAGCC GCTGAGATGAACA, reverse primer 5′ region: AGCACC-CACGCG TACTTGAATG; forward primer 3′ region: TTACCCTCCTCTTGGTCGTTTTG, reverse primer 3′ region: TCTTCTTGATGGCAGCCTTGG). The C_T value of the 5′-end primer did not exceed that of the 3′-end primer by more than one C_T, indicating a uniform cDNA synthesis.

PCR primer design and quantitative real-time PCR analysis

Gene-specific primers were designed by using the Primer Select Software (DNAstar) with the following criteria: *Tm*=60 ±1 °C, 18–25 bp length, close to the 3'-end if possible, GC content between 40% and 60% which generate unique, short PCR products between 60 bp and 150 bp (Udvardi *et al.*, 2008). The sequences of each primer pair are given in Supplementary Table S1 at *JXB* online.

PCR reactions were performed in optical 384-well plates with an ABI PRISM® 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using SYBR® Green to monitor dsDNA synthesis. Reactions contained 5 µl 2× Power SYBR® Green Mastermix reagent (Applied Biosystems), 1 µl cDNA, and 200 nm of each gene-specific primer in a final volume of 10 µl. To reduce pipetting errors, an electronic Eppendorf multipipette was used to pipette the cDNA and the mastermix (SYBR® Green and primers). The following standard thermal profile was used for all PCR reactions: 50 °C for 2 min, 95 °C for 10 min, 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Amplicon dissociation curves, i.e. melting curves, were recorded after cycle 45 by heating from 60 °C to 95 °C with a ramp speed of 1.9 °C min^{-1}. Data were analysed using the SDS2.2.1 software (Applied Biosystems). To generate a baseline-subtracted plot of the logarithmic increase in fluorescence signal (ΔR_n) versus cycle number, baseline data were collected between cycles 3 and 15. All amplification plots were analysed with an R_n threshold of 0.2 to obtain C_T values. To compare data from different PCR runs or cDNA samples, C_T values for all genes were normalized to the C_T value of the housekeeping gene (serine/threonine protein phosphatase PP2A-4, catalytic subunit, EST clone HZ44D03) included in each PCR run. PCR efficiency (E) was estimated from the data obtained from the exponential phase of each individual amplification plot and the equation $+E=10^{slope}$ (Ramakers *et al.*, 2003). The expression level of each gene of interest (GOI) is presented as $(1+E)^{-\Delta CT}$, where $\Delta C_T=C_{TGOI}-C_{TREF}$.

Expression analysis using the Barley1 GeneChip and data analysis

RNA isolation was performed as described in Sreenivasulu *et al.* (2008). Probe synthesis, labelling, and hybridization were performed according to the manufacturer's protocol (Affymetrix) at the University of California Irvine MicroArray Core Facility. Arrays were scanned on a GeneChip Scanner 3000. The purified labelled cRNA samples prepared from developing and germinating seed samples were hybridized to Barley1 GeneChips as described in Close *et al.* (2004). The raw data obtained were analysed according to Sreenivasulu *et al.* (2008) and the normalized expression values were shown in heat maps for ABA metabolism genes.

12 K barley seed cDNA macroarray analysis for showing differential expression of starch biosynthesis and degradation pathway

RNA isolation of developing seeds from 12, 16, and 20 DAF was performed as described above. Probe synthesis and labelling were performed according to Sreenivasulu *et al.* (2006). The 12 K barley seed array was hybridized and processed, exposed to phosphor image screens for 6 h, and scanned at 100 mm resolution using a Fuji BAS 3000 phosphor scanner. The quantified gene expression data were subjected to quantile normalization, genes with marginal expression values or diverging double spot ratios were eliminated. The normalized expression values of starch biosynthesis and degradation pathway genes were extracted.

In silico promoter analysis

The upstream region of beta-amylase (*HvBAM1*), ADP-glucose pyrophosphorylase small subunit (*HvAGP-S1*), and sucrose synthase (*HvSUS1*) genes were identified by BLAST in the NCBI database. All these identified promoters were amplified and verified by re-sequencing. In order to identify any putative conserved plant *cis*-acting regulatory elements of *HvBAM1*, *HvAGP-S1*, and *HvSUS1* promoter sequences were analysed by using PLACE (Higo *et al.*, 1999) and plantCARE (Lescot *et al.*, 2002) databases as well as motifs extracted from the literature. To find out the regulatory elements, their types and positions, whole promoter sequence were searched in both the forward and reverse

strands of the promoter regions of the genes and distributed evenly. The presence of noteworthy regulatory elements resulting from diverse functions like stress, structural and developmental stages, are reported in Supplementary Table S5 at *JXB* online.

ABA and ABA-catabolite measurements

ABA was extracted from lyophilized plant material using ethyl acetate (100%) (Sigma/Germany). Isotopically labelled D6-ABA (ICON/USA) was used as an internal standard and added to each sample during the extraction procedure. Extraction was carried out twice each with 1 ml of ethyl acetate at 4 °C. The supernatant collected after centrifugation (13 000 *g*, 10 min, 4 °C) was evaporated using a vacuum concentrator until dryness at room temperature. The dried samples were redissolved in acetonitrile:-methanol (1:1 v/v) and filtered using a 0.8 μm filter (Vivaclear mini; 0.8 μm; PES; Sartorius/Germany). The filtrate (10 μl) was used for subsequent quantification using HPLC-ES-MS/MS (Dionex Summit coupled to Varian 1200L). Chromatographic separation was carried out on a GENESIS column (4 μm, 100 mm; Vydac/USA). As solvents, 0.1% acetic acid (A), 100% acetonitrile (B), and distilled water (C) were used with the following gradient: start at 0 min with 70%B and 29.2%C, within 4 min to 89% B and 10.2% C; 4–4.5 min 99.2% B and 4.5–5 min 70%B and 29.2%CSolvent A was held constant at 0.8%. Solvent flow rate was 200 μl min^{-1}. The MS was operated with ESI in the negative mode with the following parameters: collision gas 1.7 mTorr, API drying gas 20 psi, 250 °C, API nebulizing gas 51 psi, needle –5000 V, shield –200 V, detector 1500 V. MRM and quantification was done using the mass traces 263/153 for ABA and 269/159 for D6-ABA. The validity of the extraction and measurement procedure was checked by recovery experiments (approximately 82–95%). Quantification was based on calibration with known ABA standards (Sigma, Deutschland) and individual recovery rates for the samples. The ABA catabolites and ABA-glucose esters analysis was performed at the National Research Council of Canada Plant Biotechnology Institute (Saskatoon, Canada) (http://www.pbi.nrc.gc.ca/ENGLISH/technology-platforms/plant-hormone-profiling.htm) by high performanceliquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC-ES-MS/MS) using deuterated internal standards as described in Chiwocha *et al.* (2003) and Kong *et al.* (2008).

β-glucosidase assay

β-glucosidase activity was measured by the release of *p*-nitrophenol from the synthetic substrate *p*-nitrophenyl-β-D-glucopyranoside (p-NPG), as described in Simos and Georgatsos, (1988). Enzyme was extracted from finely ground seed material using ammonium acetate (pH 6) as extraction buffer with a 1: 3 ratio of sample to buffer (mg μl^{-1}) at 4 °C. The supernatant obtained after centrifuging at 10 000 *g* for 10 min at 4 °C was used as the crude enzyme extract for the assay. The assay was carried out in a volume of 800 μl consisting of 450 μl of assay buffer (ammonium acetate pH 5), 250 μl of 7 mM substrate (p-NPG), and 100 μl of the crude enzyme extract incubated at 37 °C for 15 min. The reaction was stopped using 800 μl of 0.2 M sodium carbonate and the amount of *p*-nitrophenol liberated was determined spectrophotometrically at 400 nm after the development of a yellow colour in alkaline conditions. Enzyme activity is expressed as μmoles of *p*-nitrophenol produced per gram fresh weight tissue per minute (μmol g^{-1} min^{-1}).

Starch measurements and enzyme activities of sucrose synthase and AGPase

Starch measurements were performed as described by Rolletschek *et al.* (2002). Enzyme activities of AGPase and sucrose synthase were measured spectrophotometrically at 340 nm according to the protocols of Weber *et al.* (1995) and Weber *et al.* (1996), respectively. AGPase activity was measured in the reverse reaction (G-1-P synthesis).

Results and discussion

Annotation of ABA biosynthesis, catabolism and deconjugation pathway genes in barley

To derive homologous sequences of ABA biosynthesis and inactivation pathways in barley, the available unigene set of 'barley HarvEST assembly' was used and different sequence comparisons (BLASTN, BLASTX, tBLASTX) were performed against the *Arabidopsis* and rice genome sequences. The hit descriptions retrieved for gene family members of ABA biosynthesis, catabolism, and deconjugation were subjected to motif search, and sequence alignments of putative members to each family were performed using ClustalW to define specific members. In total, 41 sequences were assigned to gene families of ABA metabolism, catabolism and deconjugation pathways. The identified genes were clustered to different gene families and phylogenetic trees comprising barley, rice, and *Arabidopsis* translated cDNA sequences have been generated (Fig. 1).

Zeaxanthin epoxidase (ZEP) catalyses the first step of ABA biosynthesis, the conversion of zeaxanthin to violaxanthin which takes place in the plastid. In *Arabidopsis*, one gene, named *AtZEP*/*ABA deficient 1 (ABA1)* is known. In rice, one zeaxanthin epoxidase gene has been identified, which exists in two alternative splice variants. In barley five members belonging to the ZEP family, were identified. *HvZEP1* is full-length and its amino acid sequence contains the same conserved domains as found in AtZEP. These are the Pyr-redox-domain (pyridine nucleotide-disulphide oxidoreductase) and a FHA domain (forkhead associated domain). In addition, *HvZEP2, 3, 4*, and *5*, all partial clones, contain the Pyr-redox-domain, but the sequence covering the FHA domain is missing. The phylogenetic tree of the ZEP family has two clear branches. One branch comprises HvZEP1 and HvZEP2, the two most closely related members to AtZEP (71% amino acid sequence identity). The second branch contains the remaining members HvZEP3, 4, and 5, which also share the Pyr-Redox-domain but form a subgroup and seem to be more distantly related to AtZEP (Fig. 1A). HvZEP1 shows high similarity to the homologous proteins from rice and *Arabidopsis* based on amino acid sequence comparisons.

As described in the introduction 9-*cis*-violaxanthin and 9-*cis*-neoxanthin are oxidized by 9-*cis*-epoxycarotenoid dioxygenase (NCED) to generate the intermediate product xanthoxin. This step also takes place in plastids and is considered to be the rate-limiting step in ABA biosynthesis. Thus, NCED is the key gene involved in ABA biosynthesis. In *Arabidopsis* nine *NCED*/*CCD* genes have been described but only five (*AtNCED2, 3, 5, 6*, and *9*) are thought to play a role in ABA production (Lefebvre *et al.*, 2006). The remaining four proteins differ from NCEDs and have been classified as carotenoid cleavage dioxigenases (CCDs). In the rice genome, six genes related to NCED have been

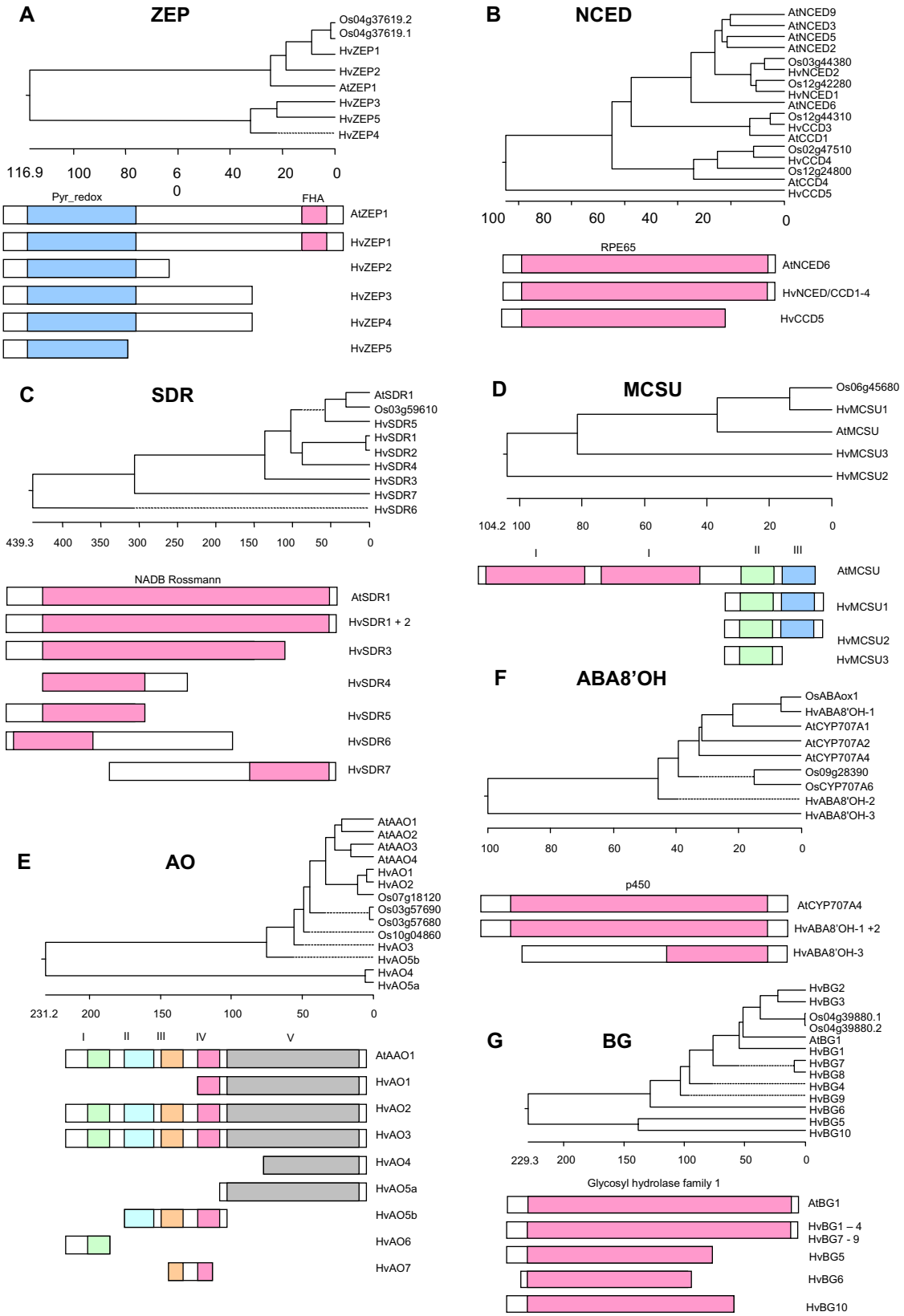


Fig. 1. Phylogenetic trees of different families of ABA biosynthesis and catabolism as well as domain structure comparisons of these families. (A) ZEP; (B) NCED; (C) SDR; (D) MCSU, I: AAT_I (aspartate aminotransferase superfamily), II: MOSC_N domain, III: MOSC domain; (E) AO, I: Fer2_2, 2Fe–2S binding domain, II: FAD binding domain, III: CO dehydrogenase flavoprotein C-terminal domain, IV: aldehyde oxidase and xanthine dehydrogenase hammerhead a/b domain, V: aldehyde oxidase and xanthine dehydrogenase molybdopterin binding domain; (F) ABA8'OH; (G) BG.

found and one related to the CCD family (TIGR database; http://rice.plantbiology.msu.edu). In barley two NCED genes, *HvNCED1* and *HvNCED2* have already been identified (Leymarie *et al.*, 2008). Three more putative members of that NCED/CCD family comprising two full-length sequences named *HvCCD3* and *HvCCD4* are described here. The third sequence, *HvCCD5*, is only partial. *Arabidopsis* NCED proteins contain a so-called RPE65-motif (retinal pigment epithelial membrane protein). The same motif could also be identified in all *HvCCD* sequences (Fig. 1B). Sequence comparisons revealed that HvNCED1 and HvNCED2 seem to be more closely related to AtNCED2, 3, 5, and 9, whereas HvCCD3 shows similarity to AtCCD1 (Fig. 1B, upper part). HvCCD4 could be the barley homologue to AtCCD4 as both sequences share 64% sequence identity on the amino acid level. HvCCDs were named that way because they seem to be more closely related to *Arabidopsis* CCDs than NCEDs (Fig. 1B). Less sequence homology is seen for HvCCD5 with known *Arabidopsis* and rice NCED/CCD members, possibly due to the reduced sequence length available.

The intermediate product xanthoxin is transported to the cytosol and further converted to abscisic aldehyde by a short-chain dehydrogenase/reductase (SDR). In *Arabidopsis* the *aba2* gene encodes AtSDR1. In the barley genome, seven putative SDR genes showing homology to AtSDR1 were identified. Two cDNA clones are full-length and were named *HvSDR1* and *HvSDR2*. A characteristic motif found in SDR protein sequences is the so-called NADB Rossmann motif which was identified in all putative members from barley as well. In the phylogenetic tree, HvSDR5 is located within the same group as AtSDR1 and LOC_Os03g59610 indicating that it could be the orthologous protein to AtSDR1. The full-length sequences HvSDR1 and HvSDR2 form a separate subgroup within the clade where AtSDR1 belongs to HvSDR5. Furthermore, HvSDR6 and HvSDR7 seem to be more distantly related to AtSDR1 (Fig. 1C, upper part). Besides LOC_Os03g59610, a search within the rice genome revealed seven more genes related to the SDR family.

Aldehyde oxidases catalyse the last step in ABA biosynthesis by converting ABA aldehyde to ABA. In *Arabidopsis* four aldehyde oxidases (AO) are known, named AtAAO1–4. In barley eight putative genes belonging to the AO family have been identified. Searches in the Barley database containing 5000 full-length cDNAs (http://www.shigen.nig.ac.jp/barley) revealed that two full-length AO cDNA clones exist (*HvAO2* and *HvAO3* based on our nomenclature). HvAO2 and HvAO3 contain the same conserved domains common to the *Arabidopsis* aldehyde oxidases. The remaining partial sequences (HvAO1, HvAO4, HvAO5a and b, HvAO6, and HvAO7) contain, depending on their length, at least one conserved domain common to other aldehyde oxidases (Fig. 1E). The aldehyde oxidase protein sequence of *Arabidopsis* contains five different domains: 2Fe-2S binding domain, FAD binding domain, CO dehydrogenase flavoprotein C-terminal domain, aldehyde oxidase and xanthine dehydrogenase hammerhead a/b domain, and molybdopterin binding domain (Fig. 1E). The putative HvAO1 and HvAO2 sequences show similar identities on amino acid level to the *Arabidopsis* AAOs AtAAO1–AtAAO4 (56–62%), and it cannot be resolved which barley isoform is the homologue of a specific *Arabidopsis* AO. The HvAO3 and HvAO5b proteins show highest similarity to several rice aldehyde oxidases as shown in the phylogenetic tree (Fig 1E). HvAO2 and HvAO3 share 62% identity. It seems that the barley genome contains slightly more members of the AO family than the *Arabidopsis* genome. This is also true for rice as a search within the rice genome provides evidence for the existence of six genes related to aldehyde oxidases. The corresponding amino acid sequences contain all five characteristic domains found in the *Arabidopsis* AOs. One of these genes, LOC_Os03g31550, produces two alternative splice forms. For some of the partial AO sequences it cannot be excluded that they might form a single gene contig as not all of the short sequences overlap.

Aldehyde oxidases need a molybdenum cofactor sulphurase (MCSU) for their activity. The AtMCSU protein contains two AATI-motives (aspartate aminotransferase superfamily) as well as a MOSC-N-terminal and MOSC-domain in its amino acid sequence. Only one MCSU isoform seems to be present in the rice genome (LOC_Os06g45680). In barley, three genes showing homology to AtMSCU were identified, but no full-length sequence is among them (Fig. 1D). HvMCSU1 and HvMCSU2 contain the two MOSC-domains; HvMCSU3 only contains the MOSC-N domain. The phylogenetic tree shows that HvMCSU1 seems to be more closely related to AtMCSU and the homologous rice clone compared to the other barley genes (Fig. 1D, upper part).

ABA catabolism is the major route of ABA degradation. ABA8' hydroxylase (ABA8'OH) degrades active ABA into 8'hydroxy-ABA. This enzyme belongs to the cytochrome p450 family (CYP707). In barley two genes, *HvABA8'OH-1* and *HvABA8'OH-2*, have been identified recently (Millar *et al.*, 2006). With our approach one additional member was found, named *HvABA8'OH-3* which is related more distantly to the other two members (Fig. 1F). Another way of inactivating ABA is the conjugation with glucose to form ABA glucose ester which is catalysed by an ABA glucosyltransferase (Xu *et al.*, 2002). ABA-GE can be hydrolysed back by a beta-glucosidase (BG). Beta-glucosidases comprise a big gene family with about 47 genes in *Arabidopsis* and also in rice. However, to date only AtBG1 is proved to be functional in *Arabidopsis* to release active ABA through deconjugation (Lee *et al.*, 2006). Hence this sequence was used to identify the orthologous sequences from barley and 10 putative genes showing homology to AtBG1 were identified; seven of them are full-length (Fig. 1G). All putative members of the BG family contain the conserved domain of glycosyl hydrolase family 1 within their protein sequence, as the largest domain covering more than 450 amino acids. In addition, beta galactosidase and beta-glucosidase domains were also found in all predicted barley beta-glucosidase genes (see Supplementary Fig. S1 at *JXB*

online). Within the functionally important active site of the beta-glucosidase domain, the highly conserved unique amino acid triplet 'ENG' is found in all barley sequences as well as in the functionally characterized AtBG1 sequence (see Supplementary Fig. S1 at *JXB* online). Among the defined barley beta-glucosidase genes the closest homologue to *Arabidopsis* BG1 is *HvBG1* (Fig. 1G) which has an endoplasmic reticulum-targeting signal peptide like AtBG1 (Table 1). In addition, *HvBG2* and *HvBG3* belong to the same clade as *AtBG1* but possess signal peptides targeting proteins to chloroplasts and vacuoles, respectively (Table 1).

In summary, putative gene family members of ABA biosynthesis genes in barley have been annotated and several full-length sequences based on sequence comparisons with known *Arabidopsis* and rice sequences were identified. In almost all gene families of ABA biosynthesis (ZEP, SDR, AO, and MCSU), more members seem to be present in the barley genome as compared to the *Arabidopsis* genome. These results suggest that proteins of the gene families described have expanded in a species-specific manner in the large barley genome compared with the small *Arabidopsis* genome. In the medium-sized rice genome these gene families also comprise more members compared with *Arabidopsis*. An exception is the NCED/CCD family where nine members are described in *Arabidopsis* and, so far, only five putative genes have been identified in barley but it may be that not all of the genes related to the NCED family have yet been discovered. In *Arabidopsis* only five of the nine NCEDs are thought to be involved in ABA production (Tan *et al.*, 2003) and *HvNCED1* and *HvNCED2* have been found to be closely related. It remains to be investigated if all members of a gene family play a role in ABA biosynthesis because the phylogenetic trees have only been created based on sequence similarities and the existence of common domains in the respective proteins.

Expression patterns of ABA biosynthesis and inactivation genes during the reproductive phase of barley development

For the defined set of ABA biosynthesis and inactivation pathway gene family members (Fig. 1), homologous sequences from the Affymetrix barley1 GeneChip were identified to characterize expression patterns of these genes using publicly available experimental data (Druka *et al.*, 2006; Sreenivasulu *et al.*, 2008). The data from plant ontogeny cover diverse vegetative tissues (root, radicle, crown, coleoptile, and leaf), later stages of plant development including reproductive tissues (floral bracts, immature inflorescence, pistil, and anthers) and developing and germinating seed tissues (pericarp, endosperm, and embryo). To assess the expression patterns of these genes, Affymetrix Cel files were downloaded from the experiments mentioned (Druka *et al.*, 2006; Sreenivasulu *et al.*, 2008), subjected to MAS5 normalization, and the normalized expression values plotted as heat maps. The analysis revealed, on the one hand, a set of more ubiquitously

Table 1. List of β-glucosidase genes identified in barley and their sequence characteristics

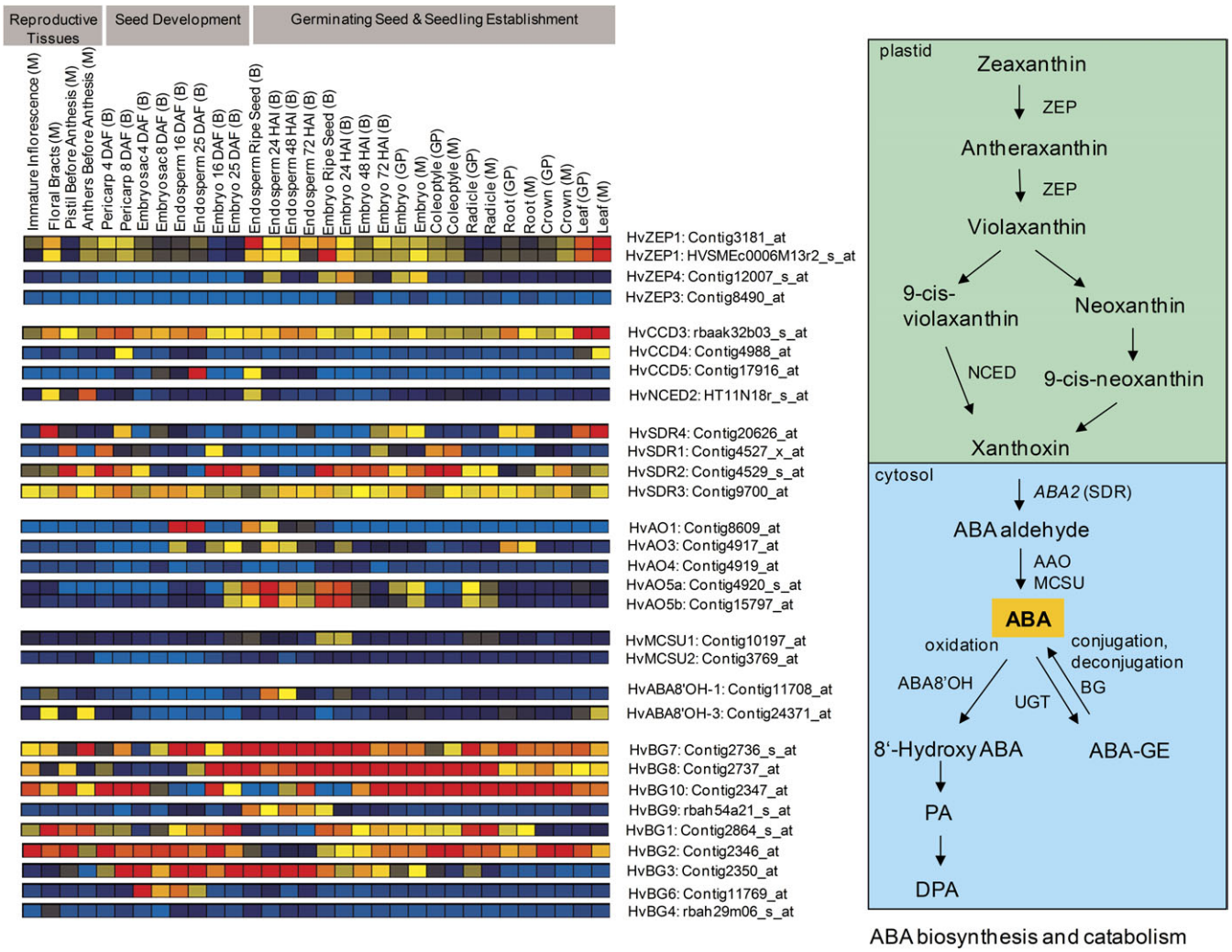
Further details of sequences, full length/partial, open reading frame (ORF) size, predicted protein molecular weight (Mwt) and localization of the genes are provided. Numbers in brackets indicate the position of the important functional domains of the β-glucosidase family.
Glyco_hydro_1, glycosyl hydrolase family 1; BGL, beta-galactosidase; BglB, beta-glucosidase/6-phospho-beta-glucosidase/beta-galactosidase (carbohydrate transport); PLN02849, glycosyl hydrolase family 1 protein; PLN0299, hydrolase, hydrolysing *O*-glycosyl compounds/glucosidase; PLN02814, glycosyl hydrolase family 1 protein; PRK13511, 6-phospho-beta-galactosidase, Provisional; celA, 6-phospho-beta-glucosidase; Reviewed; lacG, This enzyme is part of the tagatose-6-phosphate pathway of galactose-6-phosphate; arb, 6-phospho-beta-glucosidase; PRK09852, cryptic 6-phospho-beta-glucosidase.

Name	Full or Partial	ORF (bp)	Mwt (kDa)	Localization	Glyco_ hydro_1	BGL	BglB	PLN02849	PLN02998	PLN02814	PRK13511	celA	lacG	arb	PRK09852
AtBg1	Full	1587	60,5	ER	476(40–516)	467(44–511)	469(41–524)	479(37–516)	478(38–516)	478(37–515)	470(43–513)	472(44–516)	467(44–511)	469(44–513)	467(44–511)
HvBg1	Full	1530	57,3	ER	466(29–495)	460(29–489)	462(29–491)	470(25–495)	471(26–497)	480(26–506)	461(29–490)	462(29–491)	462(28–490)	461(29–490)	461(29–490)
HvBg2	Full	1659	61,0	Chloroplast	468(29–497)	455(33–488)	466(32–498)	464(29–493)	466(29–495)	470(29–495)	457(32–489)	463(33–496)	458(33–491)	464(32–496)	465(31–496)
HvBg3	Full	1530	57,4	Vacuolar	463(44–507)	455(44–499)	461(44–505)	466(40–506)	463(40–503)	465(39–504)	463(44–507)	461(43–504)	458(44–502)	463(41–504)	460(44–504)
HvBg4	Full	1515	56,5	Vacuolar	460(27–487)	453(27–480)	460(27–487)	482(22–504)	465(20–485)	462(22–484)	453(27–480)	462(25–487)	453(27–480)	460(27–487)	462(25–487)
HvBg5	Partial–3'	1100		ER	310(34–342)	309(35–344)	309(32–341)	330(30–340)	314(30–344)	314(30–344)	310(34–344)	307(35–342)	309(35–344)	307(35–342)	309(33–342)
HvBg6	Partial–5'			–	117(9–126)	110(9–119)	110(9–119)	122(3–125)	114(11–125)	113(11–124)	110(9–119)	108(11–119)	110(9–119)	95(24–119)	101(18–119)
HvBg7	Full	1542	57,7	Vacuolar	470(28–498)	464(28–492)	464(28–492)	474(24–498)	474(24–498)	474(24–498)	464(28–492)	465(28–493)	468(26–494)	469(23–492)	430(28–458)
HvBg8	Full	1536	57,4	Vacuolar	463(33–496)	453(37–490)	454(37–491)	463(33–496)	463(33–496)	463(33–496)	453(37–490)	453(37–490)	453(37–490)	453(37–490)	453(37–490)
HvBg9	Full	1524	56,5	Mitochondrial	465(23–488)	454(27–481)	461(27–488)	465(23–488)	463(23–486)	474(23–497)	454(27–481)	461(27–488)	456(27–483)	465(27–492)	461(27–488)
HvBg10	Partial–3'			Chloroplast	354(34–388)	306(38–344)	320(37–357)	323(34–357)	347(34–381)	311(28–339)	308(37–345)	300(38–338)	307(38–345)	266(38–304)	304(36–340)

expressed ABA biosynthetic genes with higher expression levels in specific tissues and, on the other hand, a minor set of genes showing a clear tissue-specific expression (Fig. 2). After revealing the developmental specific patterns, an altered response of the ABA biosynthesis transcriptome to terminal drought was also identified using a qRT-PCR platform.

ABA pathway gene expression in reproductive organs and early seed development

Not much is known about the regulation of ABA biosynthesis and ABA inactivation during early seed establishment. As suggested earlier, ABA accumulation in reproductive tissues and during the early stages of seed development might be due to translocation from leaves to seeds (Setter *et al.*, 1980; Ober and Setter, 1990). However, based on transcriptome data there is some indirect evidence pointing to ABA biosynthesis in the maternal coat of *Arabidopsis* seeds (Nambara and Marion-Poll, 2003). Our expression analysis data indicate that specific members of ABA biosynthesis gene families seem to be involved in ABA production in reproductive organs (Fig. 2). *HvZEP1* shows a moderate expression in reproductive tissues, mainly in floral bracts, anthers before anthesis, in 4 DAF pericarp and embryosac fractions (nucellar projection and nucellus maternal tissue parts are also represented in this fraction; Sreenivasulu *et al.*, 2002). *HvZEP1* transcripts were also detected at high levels in leaves during seedling establishment besides its appearance during seed development. *HvNCED2* is expressed highest in anthers as well as in floral bracts. Besides, it was observed that *HvCCD4* transcripts have only been detected in leaves and pericarp (8 DAF) and *HvCCD3* shows a more ubiquitous expression



ABA biosynthesis and catabolism

Fig. 2. Expression profiles of ABA biosynthetic, catabolic, and deconjugation genes during seed development and germination analysed by the Affymetrix 22K barley gene chip. A scheme of the main steps of ABA biosynthesis and inactivation is given on the right. Horizontal rows represent gene expression patterns. Vertical lines represent the developmental stages and investigated tissues. Signal intensities: red, high expression; yellow, moderate expression; blue, low expression. Trivial names of genes as well as the corresponding Affymetrix IDs are given. M, cultivar 'Morex'; B, cultivar 'Barke'; GP, cultivar 'Golden Promise'. Expression values are given as log2.

in seeds (Fig. 2). With respect to the SDR family, *HvSDR1* shows high expression in pistils and pericarp 4 DAF. *HvSDR4* mRNA was detected in floral bracts besides its expression in leaves during seedling establishment (Fig. 2). These results indicate that nearly the complete gene machinery required for ABA biosynthetic reactions in both plastids and cytosol is active in reproductive tissues. Interestingly, expression of AO or MCSU genes, which are responsible for the last step of ABA biosynthesis, was not found in any of the reproductive tissues and in maternal pericarp and embryosac fractions during early onset of seed development. Based on these results, it is assumed that the down-stream reactions required to convert ABA-aldehyde to ABA might be completely missing in reproductive tissues leading to the synthesis of only precursors during the early stages of seed development, which could be utilized for *de novo* synthesis of ABA in seeds from intermediate stage onwards. However, the possibility cannot be ruled out that additional AO and MCSU gene family members were missed.

Several β-glucosidase genes (*HvBG1, HvBG2*, and *HvBG10*) are expressed at high levels in reproductive tissues as well as in developing seed pericarp and embryo sac fractions (Fig. 2). Since the respective enzyme(s) is expected to hydrolyse glucose-conjugated inactive ABA it may increase the pool of active ABA during these developmental stages. It is known that glucose-conjugated inactivated ABA as a long-distance export form of ABA is transported from vegetative tissues to reproductive tissues and early developing seeds through vascular bundles (Jiang and Hartung, 2008). Transcripts of ABA hydrolysing enzymes were also found to be expressed mainly in floral bracts (*HvABA8'OH-1*, *HvABA8'OH-3*) and in anthers (*HvABA8'OH-3*; Fig. 2), indicating the activation of key genes of ABA catabolism. It is known that higher levels of ABA regulate ABA8'OH gene expression to bring ABA to its basal levels (Cutler and Krochko, 1999). By contrast with reproductive tissues, the ABA catabolic gene is not activated during the early stages of seed development, indicating the differential regulation of ABA biosynthesis and catabolism to maintain the maternal derived active ABA levels during early seed development. The maternally effected shrunken endosperm mutant *seg8* was found to have lower ABA levels compared with the wild type during the pre-storage phase but higher levels during the transition from cell division/differentiation to storage product accumulation. These ABA level changes were accompanied by changes in cell cycle regulation, ploidy levels and, finally, the starch content (Sreenivasulu *et al.*, 2010*b*). Taken together our results suggest that fine-tuned ABA levels play a very important role in early seed development.

ABA pathway gene expression in maturing and imbibed seeds

The second peak of ABA accumulation in developing seeds in *Arabidopsis* is a result of *de novo* biosynthesis typically connected to embryo maturation and desiccation tolerance (Koornneef *et al.*, 2002). The situation seems to be similar in barley according to our recent results on the expression of a partial set of ABA biosynthesis genes in endosperm and embryo tissues during seed maturation (Sreenivasulu *et al.*, 2008). An update on the expression patterns of defined gene family members of ABA biosynthesis, deconjugation, and catabolism has been provided here based on the approach described above.

In contrast to reproductive organs, the complete gene machinery of ABA biosynthesis covering both plastid and cytosolic reactions is active during seed maturation and germination in the endosperm and embryo tissues, suggesting *de novo* synthesis of ABA in both tissues during storage and desiccation (Fig. 2). Among the ABA biosynthetic genes, *HvZEP1*, *HvSDR2*, and *HvSDR3* are ubiquitously expressed in seeds, reproductive organs and other vegetative tissues during plant development, indicating their broader role in ABA biosynthesis in diverse tissue types. *HvZEP3* and *HvZEP4* mRNA levels are specifically up-regulated in imbibed embryos (Fig. 2). In *Arabidopsis,* Lefebvre *et al.* (2006) showed that AtNCED6 and AtNCED9 are required for ABA biosynthesis during seed development. Both genes are expressed specifically in the seed, whereas *AtNCED6* was expressed exclusively in the endosperm and *AtNCED9* in both the embryo and endosperm. Expression of *HvNCED1* and *HvNCED2* has already been described in embryos of imbibing barley grains (Millar *et al.*, 2006). In our analysis based on the barley1 GeneChip, *HvNCED1* is not included in the chip and for *HvNCED2* expression could not be observed in imbibed seeds but rather in the endosperm/aleurone fraction of ripe seeds. Millar *et al.* (2006) reported that *HvNCED2* expression was higher in non-dormant embryos compared with dormant embryos during imbibition where ABA content was much lower in non-dormant embryos compared with dormant ones. Among SDR members, *HvSDR2* is likely to play a role in ABA biosynthesis during early seed development. At the later stages of seed maturation and during imbibition, transcripts of *HvSDR1*, *HvSDR2*, and *HvSDR4* were detected at high levels in the embryo. Nearly no expression was seen in the endosperm except for ripe seeds (Fig. 2).

Of the eight identified putative AO family members, five are represented on the chip and therefore amenable to expression analysis. Interestingly, the genes of cytosol-located aldehyde oxidase gene family members are preferentially expressed in the seed (Fig. 2), indicating its potential importance in seed ABA biosynthesis. *HvAO1* is specifically expressed in endosperm at the late stages of seed development (16 and 25 DAF) and during early imbibition (24 HAI). Therefore, this aldehyde oxidase isoform should play a role in ABA biosynthesis during the later stages of seed development and early germination. *HvAO3* is expressed in both endosperm and embryo. Moreover, transcripts have been detected in roots. Similarly, *HvAO5a* and *HvAO5b* mRNA levels are highest in the endosperm and embryo of ripe and imbibed seeds. Lower expression levels have already been detected in the developing embryo at 25 DAF. In addition, *HvAO5a* and *HvAO5b* transcripts have

been seen in the radicle. Thus their expression is not restricted to seeds. *HvAO5a* and *HvAO5b* transcripts show a rather similar expression pattern indicating that they may originate from the same gene (Fig. 2). However, since their sequences do not overlap both clones were treated independently in our analysis. Expression analysis using the qRT-PCR platform revealed that *HvAO1* transcripts were highly abundant in developing seeds around 16 DAF. The approximately 5000-fold higher expression in seeds compared with flag leaves (see Supplementary Table S at *JXB* online) argues for its seed specificity. In *Arabidopsis*, *AtAAO1* and *AtAAO4* are abundantly expressed in seeds compared with *AtAAO3*. *In vitro* analysis of the protein based on abscisic aldehyde oxidase activity in different *Arabidopsis aao* mutants showed that only AtAAO3 has high specific activity for the oxidation of abscisic aldehyde (Seo *et al.*, 2004). Thus it would be important to clarify which of the barley AO isoforms plays an important role in ABA biosynthesis in barley grains. But to answer this question full-length gene sequences are needed. Nevertheless, the different transcript levels of the AO isoforms in the endosperm and embryo may be interpreted as hints for tissue-specific roles in seed ABA biosynthesis. Aldehyde oxidases need a molybdenum cofactor sulphurase (MCSU) for their activity. From the two putative barley MCSU sequences only *HvMCSU1* shows expression in the embryo of ripe seeds and during early germination as well as, to some extent, in the endosperm fraction whereas *HvMCSU2* transcripts are generally of low abundance (Fig. 2).

In summary, activation of individual gene family members of ABA biosynthesis showing preferential expression either in endosperm or embryo tissues during seed maturation and imbibition was noted. Other gene family members are ubiquitously expressed with higher basal levels in developing and imbibed seeds thus certainly contributing to the *de novo* synthesis of ABA.

Besides the likely activation of the ABA biosynthesis pathway during seed development and germination, expression was noted of all eight members of the beta-glucosidase genes (with the exception of *HvBG4*; Fig. 2). The respective enzymes are expected to hydrolyse glucose-conjugated ABA to free ABA. In particular, *HvBG6* transcripts were detected exclusively in the embryo sac and endosperm during seed development, and *HvBG9* is highly expressed in the endosperm and embryo of ripe seeds. Other BG members show a rather ubiquitous expression pattern. Taken togethe, higher levels of ABA in endosperm/aleurone and embryo tissues could be expected during seed maturation due to both *de novo* synthesis and deconjugation events. Interestingly, higher gene expression of the ABA catabolic enzyme HvABA8'OH-1, with higher preference for the endosperm/aleurone tissue fraction, was noted compared with the embryo of ripe and imbibed seeds (Fig. 2). The results underline the importance of ABA catabolism during the transition from maturation to imbibition to bring down the active levels of ABA during early seed imbibition and to promote seed germination.

ABA accumulation and catabolism in source (flag leaf) and sink (seed) tissues under terminal drought stress

Quantitative real-time PCR was used to investigate the expression of putative ABA biosynthetic and inactivation pathway genes in an elite line, LP 110, which shows a senescing phenotype under terminal drought stress. A list of genes putatively involved in ABA biosynthesis and the primer sequences used for qRT-PCR are provided in Supplementary Table S1 at *JXB* online. First, expression patterns of ABA metabolism genes in flag leaves and seeds were investigated to understand the role of source/sink relationships. Plants at 8 DAF were subjected to drought stress by withholding water and maintaining 10% soil moisture throughout seed filling. Developing seeds were collected at 12, 16, 20, and 25 DAF and flag leaf material at 16 and 20 DAF. Out of 41 putative genes related to ABA biosynthesis and inactivation pathways, 19 members were found to be differentially regulated upon terminal drought-stress treatments. Most of the genes were found to be up-regulated rather than down-regulated. For example, in flag leaves, at least one member of each gene family of the ABA biosynthesis pathway is up-regulated under stress conditions. A similar pattern was observed in developing seeds but to a lesser extent (Fig. 3).

ABA biosynthesis

Plastid-located transcripts of the ABA biosynthetic pathway genes *HvZEP2* and *HvNCED2* are significantly up-regulated in flag leaves as well as in developing seeds under terminal drought stress. Interestingly, *HvCCD5* is also significantly up-regulated in 20 DAF flag leaves under drought, but at 16 DAF both *HvCCD5* and *HvCCD3* are down-regulated (Fig. 3A). On the other hand, *HvNCED2* is up-regulated upon drought stress treatment in flag leaves at 16 DAF but down-regulated at 20 DAF, indicating differential fine tuning of NCED and CCD expression in flag leaves for short- and long-term drought exposure. HvNCED2 shows 66% sequence identity at the amino acid level with AtNCED3 which is thought to play a major role in ABA biosynthesis in response to stress (Iuchi *et al.*, 2001; Tan *et al.*, 2003). The expression level of *HvZEP1* is about 60-fold higher in flag leaves than in seeds, but the gene is not influenced by drought (see Supplementary Table S2 at *JXB* online).

Among the cytosolic enzymes of the ABA biosynthesis pathway, *HvSDR3, 6*, and *7* mRNA levels are increased under drought stress only in flag leaves (Fig. 3A). *HvSDR3* and *HvSDR7* are up-regulated at both stages (16 and 20 DAF) whereas *HvSDR6* transcripts are high in flag leaves at 20 DAF. In seeds, different members of the SDR family are regulated by drought. *HvSDR2* transcripts increase at 16 DAF and *HvSDR4* transcripts at 20 DAF (Fig. 3B). Among aldehyde oxidases which catalyse the last step of ABA biosynthesis, only *HvAO1, HvAO4, HvAO5a*, and *HvAO5b* seem to be drought-regulated. As already shown by Affymetrix expression analysis, aldehyde oxidases are

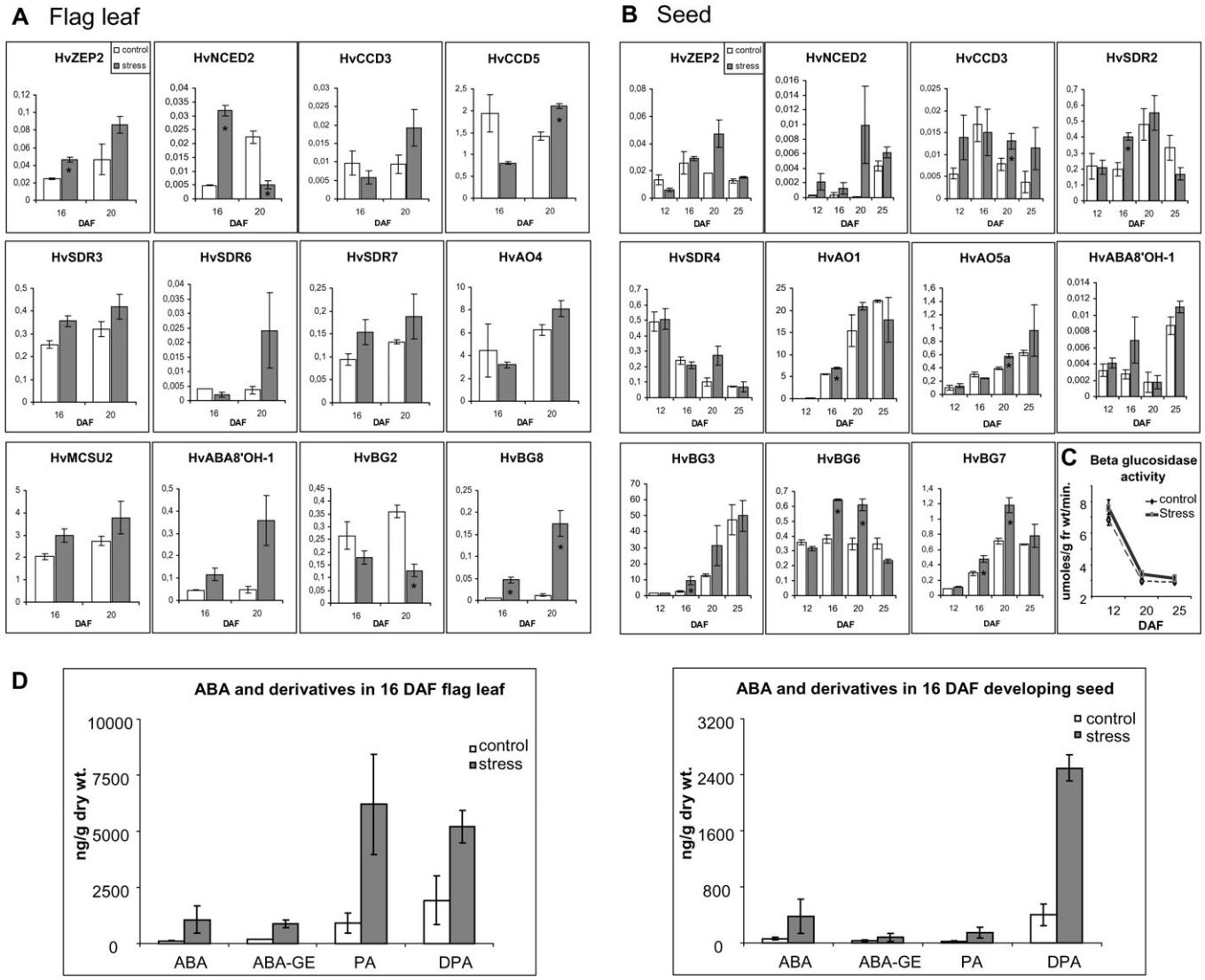


Fig. 3. Differential expression of putative ABA biosynthetic, catabolic, and deconjugation genes in a barley senescing genotype under drought-stress conditions analysed by qRT-PCR. (A) Differentially expressed genes in flag leaves at 16 and 20 DAF. (B) Differentially expressed genes in developing seeds at 12, 16, 20, and 25 DAF. The graphs show mean values from two replicates of qRT-PCR experiments from biological independent material (n=2) with an additional two technical replications. Relative mRNA levels are shown by white and grey bars (means ±SD). The significance of differences between control and stress was determined using Student's t test (* $P \leqslant 0.05$). (C) Beta glucosidase activity. (D) Measurements of ABA and ABA metabolites; ABA, abscisic acid; PA, phaseic acid; DPA, diphaseic acid; ABA-GE, ABA glucose ester. Developmental stages are shown on the x-axis in days after flowering (DAF). Stress treatment was given for one set of plants starting 1 week after anthesis, keeping a soil moisture content of 10%. Whole caryopses were collected at 12, 16, 20, and 25 DAF, flag leaves at 16 and 20 DAF.

preferentially expressed in seeds but transcript levels of *HvAO4* are also increased in flag leaves at 20 DAF under drought. In seeds, expression of *HvAO1* and *HvAO5a* is induced under drought during seed development. This correlates with the Affymetrix expression data where *HvAO1* is preferentially expressed during the late stages of seed development under stress. In general, *HvAO4*, *HvAO5a*, and *HvAO5b* show a rather similar expression pattern indicating that these three contigs may belong to the same gene (see Supplementary Table S2 at *JXB* online). The expression of the putative molybdenum cofactor sulphurase gene, *HvMCSU2*, is slightly increased under drought stress in both flag leaf stages whereas, in seeds, its expression is not significantly changed by drought stress.

Taken together, the expression data indicate that transcripts of genes encoding plastid-located enzymes (ZEP2, NCED2, and CCD3) are up-regulated under drought in both sink (seeds) and source (flag leaves) tissues. By contrast, transcripts of genes encoding cytosolic enzymes involved in the later stages of ABA biosynthesis are differentially regulated between leaf and seed under drought with the participation of different members of the SDR, MCSU and AO families in flag leaves and seeds, respectively (Fig. 3A, B).

To relate our expression data obtained by qRT-PCR to the level of ABA and its metabolites, those in seeds were measured as well as in flag leaves from control and stress-treated plants at 16 DAF (Fig. 3D). The ABA content under stress in flag leaves was increased 10-fold. In seeds, ABA levels are comparatively lower but also increase under drought stress up to 6-fold.

ABA deconjugation

ABA can also be present as a glucose ester (ABA-GE), which is a functionally inactive long-distance transport form synthesized via ABA conjugation. This reaction is reversible and free ABA can be released from the conjugates through deconjugation. In *Arabidopsis* ABA is released from the glycosylated form by AtBG1 which functions as β-glucosidase (Lee *et al.*, 2006). In barley ABA-GE might be the long-distance transport form of ABA from roots to shoot (Dietz *et al.*, 2000). When soil drying occurs in the initial growing phase, ABA biosynthesis is known to occur in the roots. Root-borne ABA is afterwards translocated to the shoots in the form of ABA-GE as a long-distance signal to regulate stomatal conductance (Jiang and Hartung, 2008). However, in *Arabidopsis*, the role of ABA in root-to-shoot signalling may be different since Christmann *et al.* (2007) provided evidence for an active role of a hydraulic signal in long-distance signalling. In maize, Ernst *et al.* (2010) discussed that *de novo* ABA biosynthesis in leaves is responsible for increases in xylem sap under short-term drought stress. Not known is the flow of ABA from leaf to developing seeds and the role of conjugates and deconjugation events in source (flag leaf) and sink tissues (developing seeds) during grain-filling under prolonged drought. Hence the expression patterns of ABA deconjugating enzyme genes encoding beta-glucosidases (BG) were studied. The expression of members of that family in barley from stress-treated and control plants is diverse. While *HvBG2* is down-regulated under drought stress in flag leaves, *HvBG8* is found to be up-regulated in the same tissue (Fig. 3A). Interestingly, within developing seeds, *HvBG3*, *HvBG6*, and *HvBG7* are up-regulated under drought stress treatments around 16, 20, and 25 DAF (Fig. 3B). Among them HvBG3 and HvBG7 possess vacuole-targeting signal peptides (Table 1) and ABA-GE is known to be stored in the vacuoles (Dietz *et al.*, 2000). Since HvBG6 is a partial sequence its signal peptide is not known. Within seeds, *HvBG6* expression is confined to endosperm whereas *HvBG3* and *HvBG7* mRNA is found in both endosperm and embryo according to the Affymetrix expression data (Fig. 2). Our metabolite measurements have shown that the content of ABA-GE in flag leaves increase 4.5-fold under drought stress compared with the control (Fig. 3D). ABA levels also increase under stress in flag leaves as mentioned earlier but the ratio between free ABA and the conjugated ABA-GE remains nearly 1:1. This would indicate that conjugation events take place instead of deconjugation under stress in flag leaves. This nicely corresponds to increases in expression of several members of glucosyltransferase genes in the flag leaf as reflected by Affymetrix transcriptome data (not shown), thus supporting the likely possibility that conjugation catalysed by an ABA-specific glucosyltransferase is increased.

In seeds from stress-treated plants, ABA-GE levels are 5-fold lower compared with absolute values of free ABA (Fig. 3D). Correspondingly, up-regulation of *HvBG3*, *HvBG6*, and *HvBG7* transcripts under stress (Fig. 3B) was noticed and also an increase in β-glucosidase activity within developing seeds under terminal drought (Fig. 3C). Taken together, these data indicate that the ABA-GE:free ABA ratio is not dramatically changed in flag leaves but gets reduced in seeds under drought-stress conditions. Our results also point to the possibility that elevated free ABA in the flag leaf might be channelled for the production of ABA-GE, which could, potentially, be uploaded into the phloem and transported along with remobilized nutrients from leaf to seed.

ABA degradation

qRT-PCR experiments of ABA catabolic pathway genes showed that out of three members only *HvABA8'OH-1* is up-regulated during drought stress in both flag leaf and seeds (Fig. 3A, B). Interestingly, *HvABA8'OH-1* is most prominently induced late in the flag leaf (at 20 DAF), i.e. after a long-term drought stress of 2 weeks, and even later in seeds at 25 DAF (Fig. 3A, B). When ABA levels increase during drought stress it is also important to degrade excess ABA rapidly in order to adjust ABA levels, thus, transcripts of ABA catabolic genes like *HvABA8'OH-1* are most prominently increased under these conditions. In *Arabidopsis*, the transcript levels of CYP707As (an ABA 8′ hydroxylase), the major enzyme for ABA catabolism, was induced by dehydration and exogenous ABA treatment (Kushiro *et al.*, 2004; Saito *et al.*, 2004). To reveal the dynamics of ABA biosynthesis and ABA degradation in the flag leaf and seeds, 16 DAF (one week after stress) was chosen where *HvABA8'OH-1* just starts to increase expression. At this time point, phaseic acid (PA) levels had already increased up to 9-fold upon drought treatment, especially in flag leaves (Fig. 3D). PA is further converted to dihydrophaseic acid (DPA) which forms the end-product of ABA degradation. It is noticeable that absolute levels of ABA degradation products in flag leaves are much higher than ABA levels itself. The absolute levels of PA and DPA in flag leaves under drought stress corresponded to 6212 ng g^{-1} and 5198 ng g^{-1}, respectively, compared with the absolute ABA level of 1054 ng g^{-1}, suggesting a nearly 12 times elevation of catabolic rate compared with ABA biosynthesis (Fig. 3D). Since *HvABA8'OH-1* induction is most prominent at 20 DAF the increased rate of ABA catabolism is expected to be a dominant factor during prolonged drought exposure.

ABA, PA, and DPA levels are much lower in seeds compared with flag leaf material, but an increase under drought up to 6-fold could also be observed. In seeds, PA levels under drought stress are lower by 2.5-fold but DPA

levels are again 6.7-fold higher compared with ABA levels (Fig. 3D). This indicates that the ABA catabolic pathway also becomes active in developing seeds under drought stress to down-regulate ABA levels, but the end-product of ABA degradation in seeds is favourably DPA instead of PA.

Presently the role of ABA degradation products is poorly understood. PA is found to be transported in the xylem sap of plants (Hansen and Dörffling, 1999). Under terminal drought stress PA and DPA are the major ABA metabolites in flag leaves whereas, in seeds, DPA is the dominating product of ABA degradation (Fig. 3D). It is possible that these metabolites have an additional function like for instance signalling under drought stress. For instance, the intermediate products of ABA catabolism such as 8'-hydroxy-ABA and PA resulting from hydroxylation of ABA are known to possess ABA-like activity (Zhou *et al.*, 2004). Like ABA, PA treatments also exhibited stomatal closure in barley, maize, and amaranthus (Sharkey and Raschke, 1980). In addition, PA and 8'-hydroxyABA were shown to induce a group of three LEA mRNAs, stimulate long-chain fatty-acid biosynthesis in *Brassica* embryos, and inhibit wheat embryo germination (Jadhav *et al.*, 2008). Also, in *Brassica napus* seed development, DPA was found to accumulate to four times higher values than ABA during seed development from mid-maturation to seed maturity, whereas other ABA catabolites such as 7'-OH ABA and neoPA were only found at extremely low levels (Jadhav *et al.*, 2008). In our present study, significant levels of 7'-OH ABA and neoPA were detected under drought compared with controls in 16 DAF seeds, but DPA levels were overwhelmingly high with a 10-fold increase (Fig. 3D), indicating 8'-hydroxylation as the major route of catabolism in developing seeds under both control and drought-stress conditions.

Impact of ABA on seed filling under drought

Besides the importance of ABA in regulating seed desiccation events and promoting fatty acid biosynthesis in the

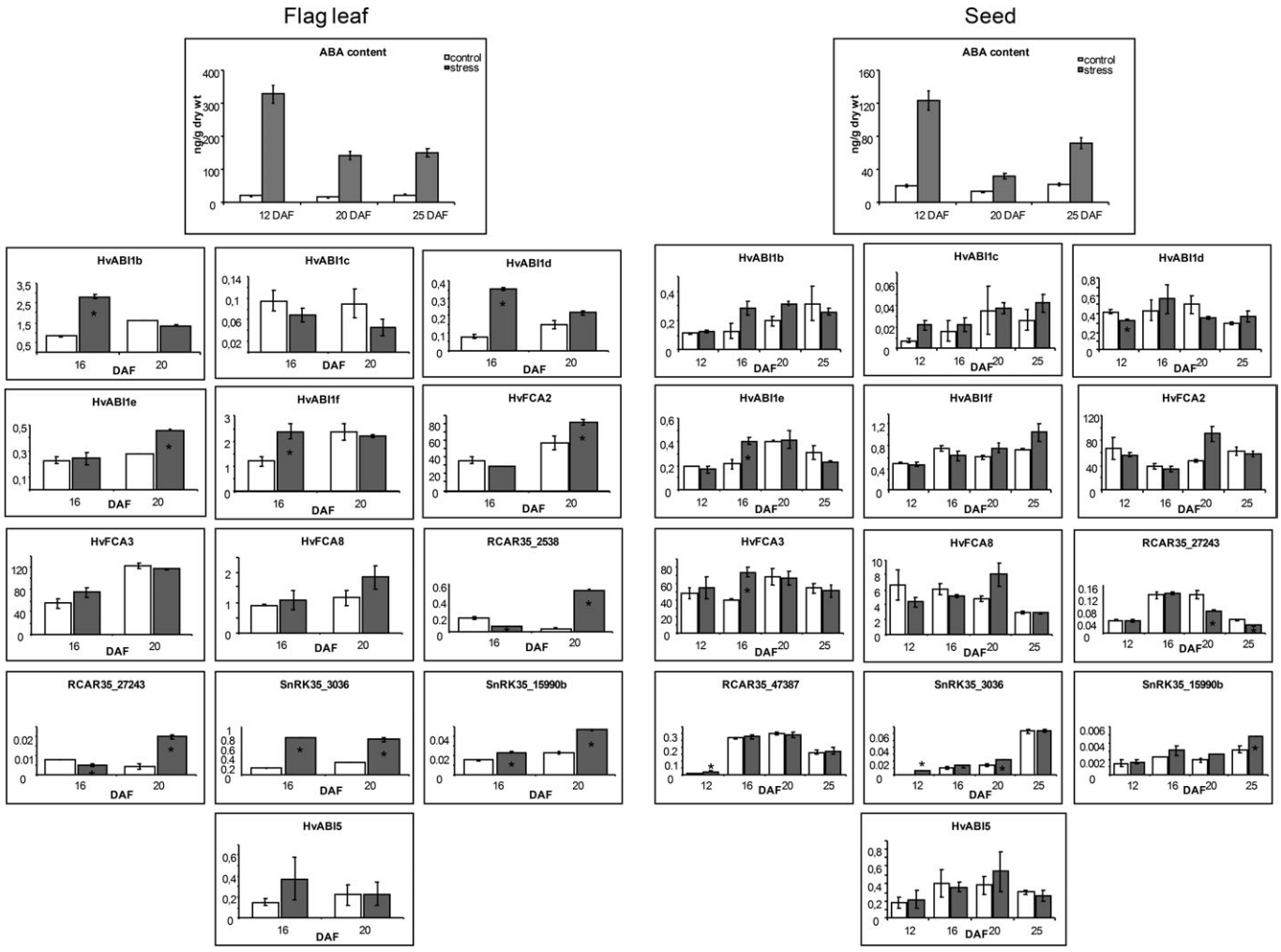


Fig. 4. (Upper graphs) ABA content in flag leaves and seeds in a barley senescing genotype under drought-stress conditions shown in ng g^{-1} dry weight. (Lower graphs) Differential expression of putative ABA signalling genes in a barley senescing genotype under drought-stress conditions analysed by qRT-PCR. Relative mRNA levels are shown. Left: mRNA expression of putative barley ABI1/2, FCA, RCAR, SnRK, and ABI5 gene family members in flag leaves at 16 and 20 DAF. Right: Expression in developing seeds. Developmental stages are shown on the *x*-axis. For further explanation see legend to Fig. 3.

embryo (Sreenivasulu *et al.*, 2006; Jadhav *et al.*, 2008), not much is known about the participatory role of ABA in the endosperm during seed filling. Considering the gene expression data related to ABA metabolism (Figs 2, 3) and the measured ABA levels (Fig. 3) it is concluded that ABA biosynthesis probably takes place in both endosperm and embryo tissues during seed maturation. The levels of induction of ABA within developing seeds under drought exposure are substantial (Fig. 4). The elevated ABA amounts (Fig. 4) correlate with an increased starch content especially at 12 and 16 DAF under stress (see Supplementary Fig. S2 at *JXB* online) but slightly decreases later, indicating the possibility that ABA accelerates the rate of starch accumulation and reduces the duration of seed filling. Correspondingly, the transcript and enzyme activity of sucrose synthase (SUS) and ADP-glucose pyrophosphorylase (AGL) was not affected at 12 and 16 DAF under drought, but decreased at 20 DAF onwards under drought (see Supplementary Fig. S2 at *JXB* online). Also, the genes involved in amylopectin biosynthesis such as starch-branching enzyme (*SBE1*) and starch synthase (*SSIIIa*) were induced at 12 and 16 DAF under drought (see Supplementary Fig. S2 at *JXB* online). Besides starch biosynthesis genes, transcripts of β-amylase (*BAM1, BAM2*) known to be involved in starch breakdown in transfer and aleurone cells (Radchuk *et al.*, 2009) were also induced in developing seeds during 16 DAF under drought (see Supplementary Fig. S2 at *JXB* online). Whether, the induction of β-amylase in developing grains during storage under drought influences starch mobilization is unclear.

The observations described provide some hints for a cross-talk between ABA and sugar signalling mediated through SnRK2.6/SNF1-related kinase in endosperm (Sreenivasulu *et al.*, 2006). SnRK is known to regulate key starch biosynthesis genes such as sucrose synthase and ADP-glucose pyrophosphorylase (Halford and Paul, 2003; Tiessen *et al.*, 2003). The recently established link of SnRK2.6 and RCAR/PP2C-mediated signal transduction to ABF transcription factors also highlights the importance of this signalling gene for integrating ABA responses (Fujii *et al.*, 2009). To find the potential link, ABA signalling homologues in barley were annotated, members specifically expressed in the endosperm were identified, and qRT-PCR expression analysis from developing seed samples after control and drought treatments was performed (seeSupplementary Tables S3 and S4 at *JXB* online). Interestingly, different members of protein phosphatases (HvABI1b, HvABI1c, HvABI1d, HvABI1e), two members of the ABA receptor complex RCAR/snRK (RCAR35_2538, RCAR35_27243, snRK35_3036, snRK35_15990b), and an ABA-binding factor (HvABI5) were up-regulated in the developing seeds under drought at 12 and 16 DAF and also prominently in flag leaves at 16 and 20 DAF (Fig. 4; see Supplementary Table S4 at *JX*B online). Thus, the same ABA signalling components seem to be operative in both source and sink tissues. Furthermore, promoters of sucrose synthase1 (*HvSUS1*; D63574), ADP-glucose pyrophosphorylase small subunit (*HvAGP-S1*; AJ239130), and β-amylase 1 (*HvBAM1*; X73221) were isolated and several novel putative ABA-responsive and dehydration-responsive *cis* elements were found (see Supplementary Table S5 at *JXB* online). In addition, endosperm-specific and light responsive *cis* elements have also been found in the respective promoters. Based on this correlative evidence, it is concluded that elevated ABA under terminal drought within developing seeds could be beneficial to promote faster seed filling by influencing starch biosynthesis events and, at the same time, counteracted by shortening the filling phase, perhaps through the activation of the starch degradation pathway.

To test this concept further ABA and fluridone (an inhibitor of carotenoid/ABA biosynthesis) treatment were used under field conditions in a randomized plot design (for details see the Material and methods). After ripening mature seeds were harvested and thousand grain weight (TGW) per plot was calculated from control, drought stress, and drought stress+ABA treated as well as drought stress+-fluridone treated plants. Under stress conditions, a slight yield reduction (6%) was observed, which is significant. Exogenous treatment of spikes of stress-treated plants with 200 μM ABA compensated the reduction of TGW under stress, whereas fluridone-treated drought-exposed plants showed the opposite effect with a substantial decrease in TGW and starch content of up to 20% in mature seed (Fig. 5).

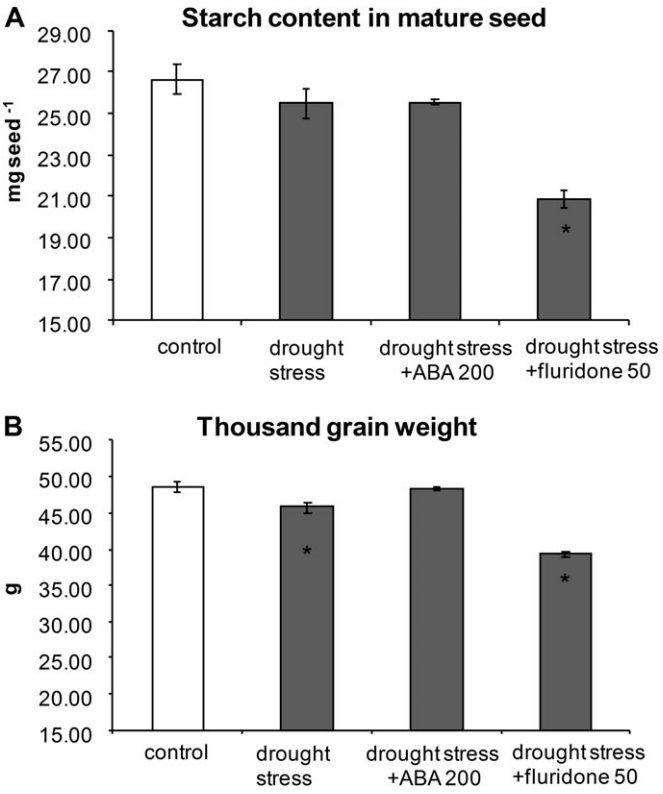


Fig. 5. Starch content (A) and thousand grain weight (B) of field-grown plants under control and stress conditions as well as after exogenous ABA and fluridone treatment. Student's *t* test was used as statistical test and asterisk (*) indicates significance at $P < 0.05$.

These results clearly point to the importance of ABA in promoting starch accumulation in developing seeds.

Controlled soil drying during grain filling enhances carbon remobilization from vegetative tissues to grains, increasing the grain-filling rate in rice and wheat (Yang *et al.*, 2000, 2006). In wheat, it has been shown that exogenous applied ABA to leaves increases carbohydrate accumulation and redistribution to grains resulting in an increase in yield (Travaglia *et al.*, 2007). However, in the present study, spraying ABA only to the spike region resulted already in a minimized yield loss. Our results confirm, on the one hand, the benefits of ABA in promoting drought-induced senescence and remobilization events and shed, on the other hand, new light on the additional regulatory role of ABA within developing seeds in regulating starch metabolism under terminal drought.

Conclusion

Four major conclusions are derived from our study of developing barley grains under terminal drought stress.

(i) Our results demonstrate that developing grains possess the transcript machinery of ABA biosynthesis as well as ABA-deconjugation during seed filling in both endosperm and embryo. The data back the probable scenario that seed ABA content is due to both *de novo* synthesized hormone and imported conjugated ABA-GE deconjugated within the seed.

(ii) Particularly under terminal drought, ABA-GE import and deconjugation in seeds seem to be predominantly responsible for the enhanced ABA levels. This is deduced from the strong activation of the ABA deconjugation pathway transcripts *HvBG3*, *HvBG6*, and *HvBG7* and the 5-fold decreased levels of ABA-GE compared with ABA, wherea, in drought-stressed flag leaves, the ABA-GE:ABA ratio remains at around 1:1.

(iii) Enhanced ABA levels in drought-stressed seeds are positively correlated with elevated starch levels at 12 and 16 DAF, but this rise in starch content is counteracted by a shortened seed-filling period due to a decrease in SUS and AGL activity from 20 DAF onwards. In parallel, the key starch degradation pathway genes and the β-amylase genes *HvBAM1* and *HvBAM2*, were also substantially induced at 16 DAF. It also remains to be seen whether starch biosynthesis and degradation occurs within the same tissue or rather is a divergent response of spatially separated tissues within the caryopsis.

(iv) Overall, the data reveal that ABA is involved in regulating the starch biosynthesis and degradation pathways of developing grains and are suggestive of the involved signalling pathways: (i) spraying of fluridone (an ABA biosynthesis inhibitor) to drought-stressed plants results in severely impaired starch content and thousand grain weight of mature seeds. (ii) genes of the ABA controlled, SnRK2.6 and RCAR/PP2C-mediated signal transduction pathway to the ABF transcription factor HvABI5 are activated in the developing grain under drought, and (iii) novel ABA- and dehydration-responsive *cis*-elements have been found in the promoters of key genes of starch biosynthesis (*HvSUS1, HvAGP-L1*) and degradation (*HvBAM1*). Thus accumulation of ABA in developing seeds is not only an important pre-requisite for grain development under control conditions but also seems to be beneficial under terminal drought.

Supplementary data

Supplementary data can be found at *JXB* online.

Supplementary Fig. S1..Amino acid sequence alignment of putative β-glucosidases in barley including the characterized BG1 from *Arabidopsis*.

Supplementary Fig. S2. Expression profiles of starch biosynthesis and degradation genes during seed development under control and drought stress analysed by the macroarray experiments.

Supplementary Table S1. List of genes putatively involved in ABA biosynthesis and primer sequences used for qRT-PCR.

Supplementary Table S2. Relative mRNA expression levels of ABA biosynthesis, catabolism and deconjugation related genes in LP110 under control and stress conditions analysed by qRT-PCR.

Supplementary Table S3. List of genes putatively involved in ABA signalling and primer sequences used for qRT-PCR.

Supplementary Table S4. Relative mRNA expression levels of ABA signalling related genes in LP110 under control and stress conditions analysed by qRT-PCR.

Supplementary Table S5. Putative *cis*-acting regulatory elements identified in the promoters by *in silico* analysis search against PlantCARE and PLACE databases.

Acknowledgements

This research was funded by BMBF-German Ministry of Education and Research (Project GABI-GRAIN: FKZ; 0315041A) to NS. We thank Dr Irina Zaharia and Dr Selvaraj Gopalan, National Research Council of Canada, Plant Biotechnology Institute, Saskatoon, Canada for providing the service of hormone profiling of the ABA catabolites. We also thank Jana Lorenz, Gabriele Einert, and Katrin Blaschek from IPK for their excellent technical assistance. We also acknowledge Dr Viktor Korzun and Dr Burkhard Schinkel from KWS LOCHOW GMBH for providing elite barley line LP 110.

References

Audran C, Liotenberg S, Gonneau M, North H, Frey A, Tap-Waksman K, Vartanian N, Marion-Poll A. 2001. Localisation and expression of zeaxanthin epoxidase mRNA in *Arabidopsis* in response to drought stress and during seed development. *Australian Journal of Plant Physiology* **28,** 1161–1173.

Bittner F, Oreb M, Mendel RR. 2001. ABA3 is a molybdenum cofactor sulfurase required for activation of aldehyde oxidase and xanthine dehydrogenase in *Arabidopsis thaliana*. *Journal of Biological Chemistry* **276,** 40381–40384.

Burbidge A, Grieve TM, Jackson A, Thompson A, McCarty DR, Taylor IB. 1999. Characterization of the ABA-deficient tomato mutant *notabilis* and its relationship with maize *Vp14*. *The Plant Journal* **17,** 427–431.

Cheng W-H, Endo A, Zhou L, *et al*. 2002. A unique short-chain dehydrogenase/reductase in *Arabidopsis* glucose signalling and abscisic acid biosynthesis and functions. *The Plant Cell* **14,** 2723–2743.

Chernys JT, Zeevaart JA. 2000. Characterization of the 9- *cis*-epoxycarotenoid dioxygenase gene family and the regulation of abscisic acid biosynthesis in avocado. *Plant Physiology* **124,** 343–353.

Chiwocha SD, Abrams SR, Ambrose SJ, Cutler AJ, Loewen M, Ross AR, Kermode AR. 2003. A method for profiling classes of plant hormones and their metabolites using liquid chromatography-electrospray ionization tandem mass spectrometry: an analysis of hormone regulation of thermodormancy of lettuce (*Lactuca sativa* L.) seeds. *The Plant Journal* **35,** 405–417.

Christmann A, Weiler EW, Steudle E, Grill E. 2007. A hydraulic signal in root-to-shoot signalling of water shortage. *The Plant Journal* **52,** 167–174.

Close TJ, Wanamaker SI, Caldo RA, Turner SM, Ashlock DA, Dickerson JA, Wing RA, Muehlbauer GJ, Kleinhofs A, Wise RP. 2004. A new resource for cereal genomics: 22K barley GeneChip comes of age. *Plant Physiology* **134,** 960–968.

Cutler AJ, Krochko JE. 1999. Formation and breakdown of ABA. *Trends in Plant Science* **4,** 472–478.

Dietz KJ, Sauter A, Wichert K, Messdaghi D, Hartung W. 2000. Extracellular beta-glucosidase activity in barley involved in the hydrolysis of ABA glucose conjugate in leaves. *Journal of Experimental Botany* **51,** 937–944.

Druka A, Muehlbauer G, Druka I, *et al*. 2006. An atlas of gene expression from seed to seed through barley development. *Functional and Integrative Genomics* **6,** 202–211.

Ernst L, Goodger JQ, Alvarez S, Marsh EL, Berla B, Lockhart E, Jung J, Li P, Bohnert HJ, Schachtman DP. 2010. Sulphate as a xylem-borne chemical signal precedes the expression of ABA biosynthetic genes in maize roots. *Journal of Experimental Botany* **61,** 3395–3405.

Fujii H, Chinnusamy V, Rodrigues A, Rubio S, Antoni R, Park S-Y, Cutler SR, Sheen J, Rodriguez PL, Zhu J- K. 2009. *In vitro* reconstitution of an abscisic acid signalling pathway. *Nature* **462,** 660–664.

Gonzalez-Guzman M, Apostolova N, Belles JM, Barrero JM, Piqueras P, Ponce MR, Micol JL, Serrano R, Rodriguez PL. 2002. The short-chain alcohol dehydrogenase ABA2 catalyzes the conversion of xanthoxin to abscisic aldehyde. *The Plant Cell* **14,** 1833–1846.

Halford NG, Paul MJ. 2003. Carbon metabolite sensing and signalling. *Plant Biotechnology Journal* **1,** 381–398.

Hansen H, Dörffling K. 1999. Changes of free and conjugated abscisic acid and phaseic acid in xylem sap of drought-stressed sunflower plants. *Journal of Experimental Botany* **50,** 1599–1605.

Higo K, Ugawa Y, Iwamoto M, Korenaga T. 1999. Plant *cis*-acting regulatory DNA elements (PLACE) database: 1999. *Nucleic Acids Research* **27,** 297–300.

Iuchi S, Kobayashi M, Taji T, Naramoto M, Seki M, Kato T, Tabata S, Kakubari Y, Yamaguchi-Shinozaki K, Shinozaki K. 2001. Regulation of drought tolerance by gene manipulation of 9- *cis*-epoxycarotenoid dioxygenase, a key enzyme in abscisic acid biosynthesis in *Arabidopsis*. *The Plant Journal* **27,** 325–333.

Jacobsen JV, Pearce DW, Poole AT, Pharis RP, Mander LN. 2002. Abscisic acid, phaseic acid and gibberellin contents associated with dormancy and germination in barley. *Physiologia Plantarum* **115,** 428–441.

Jadhav AS, Taylor DC, Giblin M, *et al*. 2008. Hormonal regulation of oil accumulation in *Brassica* seeds: metabolism and biological activity of ABA, 7'-, 8'-, and 9'-hydroxy ABA in microspore derived embryos of *B. napus*. *Phytochemistry* **69,** 2678–2688.

Jiang F, Hartung W. 2008. Long-distance signalling of abscisic acid (ABA): the factors regulating the intensity of the ABA signal. *Journal of Experimental Botany* **59,** 37–43.

Karssen CM, Brinkhorstvanderswan DLC, Breekland AE, Koornneef M. 1983. Induction of dormancy during seed development by endogenous abscisic-acid - studies on abscisic-acid deficient genotypes of *Arabidopsis thaliana* (L.) Heynh. *Planta* **157,** 158–165.

Kong L, Abrams SR, Owen SJ, Graham H, von Aderkas P. 2008. Phytohormones and their metabolites during long shoot development in Douglas-fir following cone induction by gibberellin injection. *Tree Physiology* **28,** 1357–1364.

Koornneef M, Bentsink L, Hilhorst H. 2002. Seed dormancy and germination. *Current Opinion in Plant Biology* **5,** 33–36.

Kushiro T, Okamoto M, Nakabayashi K, Yamagishi K, Kitamura S, Asami T, Hirai N, Koshiba T, Kamiya Y, Nambara E. 2004. The *Arabidopsis* cytochrome P450 CYP707A encodes ABA 8'-hydroxylases: key enzymes in ABA catabolism. *EMBO Journal* **23,** 1647–1656.

Lee KH, Piao HL, Kim HY, Choi SM, Jiang F, Hartung W, Hwang I, Kwak JM, Lee IJ. 2006. Activation of glucosidase via stress-induced polymerization rapidly increases active pools of abscisic acid. *Cell* **126,** 1109–1120.

Lefebvre V, North H, Frey A, Sotta B, Seo M, Okamoto M, Nambara E, Marion-Poll A. 2006. Functional analysis of Arabidopsis NCED6 and NCED9 genes indicates that ABA synthesized in the endosperm is involved in the induction of seed dormancy. *The Plant Journal* **45,** 309–319.

Lescot M, Dehais P, Thijs G, Marchal K, Moreau Y, Van de Peer Y, Rouze P, Rombauts S. 2002. PlantCARE, a database of plant *cis*-acting regulatory elements and a portal to tools for *in silico* analysis of promoter sequences. *Nucleic Acids Research* **30,** 325–327.

Leymarie J, Robayo-Romero ME, Gendreau E, Benech-Arnold RL, Corbineau F. 2008. Involvement of ABA in induction of secondary dormancy in barley (*Hordeum vulgare* L.) seeds. *Plant and Cell Physiology* **49,** 1830–1838.

Marin E, Nussaume L, Quesada A, Gonneau M, Sotta B, Hugueney P, Frey A, Marion-Poll A. 1996. Molecular identification

of zeaxanthin epoxidase of *Nicotiana plumbaginifolia*, a gene involved in abscisic acid biosynthesis and corresponding to the ABA locus of *Arabidopsis thaliana*. *EMBO Journal* **15,** 2331–2342.

McCarty DR. 1995. Genetic-control and integration of maturation and germination pathways in seed development. *Annual Review of Plant Physiology and Plant Molecular Biology* **46,** 71–93.

Millar AA, Jacobsen JV, Ross JJ, Helliwell CA, Poole AT, Scofield G, Reid JB, Gubler F. 2006. Seed dormancy and ABA metabolism in *Arabidopsis* and barley: the role of ABA 8'-hydroxylase. *The Plant Journal* **45,** 942–954.

Nambara E, Marion-Poll A. 2003. ABA action and interactions in seeds. *Trends in Plant Science* **8,** 213–217.

Ober ES, Setter TL. 1990. Timing of kernel development in water-stressed maize: water potentials and abscisic acid concentrations. *Annals of Botany* **66,** 665–672.

Qin X, Zeevaart JA. 2002. Overexpression of a 9- *cis*-epoxycarotenoid dioxygenase gene in *Nicotiana plumbaginifolia* increases abscisic acid and phaseic acid levels and enhances drought tolerance. *Plant Physiology* **128,** 544–551.

Radchuk VV, Borisjuk L, Sreenivasulu N, Merx K, Mock HP, Rolletschek H, Wobus U, Weschke W. 2009. Spatiotemporal profiling of starch biosynthesis and degradation in the developing barley grain. *Plant Physiology* **150,** 190–204.

Ramakers C, Ruijter JM, Deprez RH, Moorman AF. 2003. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neuroscience Letters* **339,** 62–66.

Ritchie S, Swanson SJ, Gilroy S. 2000. Physiology of the aleurone layer and starchy endosperm during grain development and early seedling growth: new insights from cell and molecular biology. *Seed Science Research* **10,** 193–212.

Rolletschek H, Hajirezaei MR, Wobus U, Weber H. 2002. Antisense-inhibition of ADP-glucose pyrophosphorylase in *Vicia narbonensis* seeds increases soluble sugars and leads to higher water and nitrogen uptake. *Planta* **214,** 954–964.

Saito S, Hirai N, Matsumoto C, Ohigashi H, Ohta D, Sakata K, Mizutani M. 2004. *Arabidopsis* CYP707As encode (+)-abscisic acid 8'-hydroxylase, a key enzyme in the oxidative catabolism of abscisic acid. *Plant Physiology* **134,** 1439–1449.

Seo M, Aoki H, Koiwai H, Kamiya Y, Nambara E, Koshiba T. 2004. Comparative studies on the *Arabidopsis* aldehyde oxidase (AAO) gene family revealed a major role of AAO3 in ABA biosynthesis in seeds. *Plant and Cell Physiology* **45,** 1694–1703.

Seo M, Koshiba T. 2002. Complex regulation of ABA biosynthesis in plants. *Trends in Plant Science* **7,** 41–48.

Setter TL, Brun WA, Brenner ML. 1980. Effect of obstructed translocation on leaf abscisic acid, and associated stomatal closure and photosynthesis decline. *Plant Physiology* **65,** 1111–1115.

Sharkey TD, Raschke K. 1980. Effects of phaseic acid and dihydrophaseic acid on stomata and the photosynthetic apparatus. *Plant Physiology* **65,** 291–297.

Simos G, Georgatsos JG. 1988. Lactose-hydrolyzing beta-glycosidases of barley meal. *Biochimica et Biophysica Acta* **967,** 17–24.

Sreenivasulu N, Borisjuk L, Junker BH, Mock HP, Rolletschek H, Seiffert U, Weschke W, Wobus U. 2010*a*. Barley grain development toward an integrative view. *International Review of Cell and Molecular Biology* **281,** 49–89.

Sreenivasulu N, Radchuk V, Alawady A, *et al*. 2010*b*. Deregulation of abscisic acid contents causes abnormal endosperm development in the barley mutant *seg8*. *The Plant Journal* doi: 10.1111/j.1365-313X.2010.04350.x.

Sreenivasulu N, Usadel B, Winter A, *et al*. 2008. Barley grain maturation and germination: metabolic pathway and regulatory network commonalities and differences highlighted by new MapMan/PageMan profiling tools. *Plant Physiology* **146,** 1738–1758.

Sreenivasulu N, Sopory SK, Kavi Kishor PB. 2007. Deciphering the regulatory mechanisms of abiotic stress tolerance in plants by genomic approaches. *Gene* **388,** 1–13.

Sreenivasulu N, Radchuk V, Strickert M, Miersch O, Weschke W, Wobus U. 2006. Gene expression patterns reveal tissue-specific signalling networks controlling programmed cell death and ABA- regulated maturation in developing barley seeds. *The Plant Journal* **47,** 310–327.

Sreenivasulu N, Altschmied L, Panitz R, Hahnel U, Michalek W, Weschke W, Wobus U. 2002. Identification of genes specifically expressed in maternal and filial tissues of barley caryopses: a cDNA array analysis. *Molecular Genetics and Genomics* **266,** 758–767.

Tan BC, Joseph LM, Deng WT, Liu L, Li QB, Cline K, McCarty DR. 2003. Molecular characterization of the *Arabidopsis* 9-*cis* epoxycarotenoid dioxygenase gene family. *The Plant Journal* **35,** 44–56.

Tan BC, Schwartz SH, Zeevaart JA, McCarty DR. 1997. Genetic control of abscisic acid biosynthesis in maize. *Proceedings of the National Academy of Sciences, USA* **94,** 12235–12240.

Tiessen A, Prescha K, Branscheid A, Palacios N, McKibbin R, Halford NG, Geigenberger P. 2003. Evidence that SNF1-related kinase and hexokinase are involved in separate sugar-signalling pathways modulating post-translational redox activation of ADP-glucose pyrophosphorylase in potato tubers. *The Plant Journal* **35,** 490–500.

Tottman DR, Broad H. 1987. The decimal code for the growth stages of cereals, with illustrations. *Annals of Applied Biology* **110,** 441–454.

Travaglia C, Cohen AC, Reinoso H, Castillo C, Bottini R. 2007. Exogenous abscisic acid increases carbohydrate accumulation and redistribution to the grains in wheat grown under field conditions of soil water restriction. *Journal of Plant Growth Regulation* **26,** 285–289.

Udvardi MK, Czechowski T, Scheible WR. 2008. Eleven golden rules of quantitative RT-PCR. *The Plant Cell* **20,** 1736–1737.

Weber H, Buchner P, Borisjuk L, Wobus U. 1996. Sucrose metabolism during cotyledon development of *Vicia faba* L. is controlled by the concerted action of both sucrose-phosphate synthase and sucrose synthase: expression patterns, metabolic regulation and implications for seed development. *The Plant Journal* **9,** 841–850.

Weber H, Heim U, Borisjuk L, Wobus U. 1995. Cell-type specific, coordinate expression of two ADP-glucose pyrophosphorylase genes in relation to starch biosynthesis during seed development of *Vicia faba* L. *Planta* **195,** 352–361.

Weschke W, Panitz R, Sauer N, Wang Q, Neubohn B, Weber H, Wobus U. 2000. Sucrose transport into barley seeds: molecular characterization of two transporters and implications for seed development and starch accumulation. *The Plant Journal* **21,** 455–467.

Wilkinson S, Davies WJ. 2002. ABA-based chemical signalling: the co-ordination of responses to stress in plants. *Plant, Cell and Environment* **25,** 195–210.

Xu ZJ, Nakajima M, Suzuki Y, Yamaguchi I. 2002. Cloning and ;characterization of the abscisic acid-specific glucosyltransferase gene from adzuki bean seedlings. *Plant Physiology* **129,** 1285–1295.

Yang JC, Zhang JH, Liu K, Wang ZQ, Liu LJ. 2006. Abscisic acid and ethylene interact in wheat grains in response to soil drying during grain filling. *New Phytologist* **171,** 293–303.

Yang SH, Zeevaart JA. 2006. Expression of ABA 8'-hydroxylases in relation to leaf water relations and seed development in bean. *The Plant Journal* **47,** 675–686.

Yang JC, Zhang JH, Huang ZL, Zhu QS, Wang L. 2000. Remobilization of carbon reserves is improved by controlled soil-drying during grain filling of wheat. *Crop Science* **40,** 1645–1655.

Zhou R, Cutler AJ, Ambrose SJ, *et al*. 2004. A new abscisic acid catabolic pathway. *Plant Physiology* **134,** 361–369.