



RESEARCH PAPER

# Field studies on the regulation of abscisic acid content and germinability during grain development of barley: molecular and chemical analysis of pre-harvest sprouting

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## Abstract

To investigate whether the regulation of abscisic acid (ABA) content was related to germinability during grain development, two cDNAs for 9-*cis*-epoxycarotenoid dioxygenase (*HvNCED1* and *HvNCED2*) and one cDNA for ABA 8'-hydroxylase (*HvCYP707A1*), which are enzymes thought to catalyse key regulatory steps in ABA biosynthesis and catabolism, respectively, were cloned from barley (*Hordeum vulgare* L.). Expression and ABA-quantification analysis in embryo revealed that *HvNCED2* is responsible for a significant increase in ABA levels during the early to middle stages of grain development, and *HvCYP707A1* is responsible for a rapid decrease in ABA level thereafter. The change in the embryonic ABA content of imbibing grains following dormancy release is likely to reflect changes in the expression patterns of *HvNCEDs* and *HvCYP707A1*. A major change between dormant and after-ripened grains occurred in *HvCYP707A1*; the increased expression of *HvCYP707A1* in response to imbibition, followed by a rapid ABA decrease and a high germination percentage, was observed in the after-ripened grains, but not in the dormant grains. Under field conditions, *HvNCED2* showed the same expression

level and pattern during grain development in 2002, 2003, and 2004, indicating that *HvNCED2* expression is regulated in a growth-dependent manner in the grains. By contrast, *HvNCED1* and *HvCYP707A1* showed a different expression pattern in each year, indicating that the expression of these genes is affected by environmental conditions during grain development. The varied expression levels of these genes during grain development and imbibition, which would have effects on the activity of ABA biosynthesis and catabolism, might be reflected, in part, in the germinability in field-grown barley.

Key words: ABA 8'-hydroxylase, abscisic acid, dormancy, field-grown barley, germination, nine-*cis*-epoxycarotenoid dioxygenase, pre-harvest sprouting.

## Introduction

Seed dormancy is an important trait in cereal crops to prevent premature seed germination while still on the mother plant (i.e. pre-harvest sprouting or vivipary). Pre-harvest sprouting of cereal grain causes not only reduced grain yield but also damages the quality of the end-product,

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Abbreviations: ABA, abscisic acid; DAP, days after pollination; NCED, 9-*cis*-epoxycarotenoid dioxygenase; PA, phaseic acid; PCR, polymerase chain reaction; QRT-PCR, quantitative reverse transcription-PCR.

resulting in economic losses of cultivars. In Japan, there are often cool wet weather conditions during the barley harvest, which occurs at the beginning of the rainy season. It has been shown that temperatures around 15 °C appear to be critical to the induction of pre-harvest sprouting in barley, because a temperature of around 15 °C allows grains to germinate as soon as dormancy is broken (Black *et al.*, 1987). Since the average temperature during the barley harvest in Japan is around 15 °C, a high level of dormancy before harvest is desirable to prevent pre-harvest sprouting. On the other hand, strong dormancy limits grain replanting immediately after the harvest. For malting barley in particular, dormancy at harvest causes a problem in malting newly harvested grain, because the malting process requires grain germination. To obtain barley cultivars with moderate dormancy to prevent pre-harvest sprouting, it is important to clarify the mechanisms determining dormancy in developing and maturing barley grain.

Research into the mechanism of seed dormancy suggests the strong involvement of the plant hormone abscisic acid (ABA). Mutants impaired in ABA biosynthesis and responsiveness produce precociously germinating seeds. These include maize *viviparous* (*vp*), and *Arabidopsis* ABA-deficient (*aba*) and ABA-insensitive (*abi*) mutants (Koorneef and Karssen, 1994; MaCarty, 1995). There are few barley mutants impaired in ABA biosynthesis or responsiveness (Walker-Simmons *et al.*, 1989; Visser *et al.*, 1996; Molina-Cano *et al.*, 1999). In barley, research has indicated that ABA sensitivity and/or ABA content are important in maintaining dormancy (Goldbach and Michael, 1976; Dunwell, 1981; Wang *et al.*, 1995; Romagosa *et al.*, 2001; Jacobsen *et al.*, 2002). In such cases, however, the regulation mechanism of ABA content in the grains themselves in response to these treatments (including imbibition) has been investigated, although, much remains to be examined to clarify the physiological roles of ABA in barley grain dormancy.

Several studies using ABA-deficient mutants have reported that ABA synthesized in the embryo is important for the prevention of germination during seed development. In *Arabidopsis*, reciprocal crosses between *aba* mutants and wild-type plants show that dormancy is initiated when the embryo produces ABA (Karssen *et al.*, 1983). It has also been demonstrated in maize mutants that *de novo* ABA synthesis in the embryo is necessary for the dormant state (Robertson, 1955; McCarty, 1995). In barley, comparisons of dormant versus non-dormant grains have indicated that dormancy is correlated with ABA biosynthesis during imbibition (Wang *et al.*, 1995). Jacobsen *et al.* (2002) have shown that the dormancy release of mature grains is correlated with changes in ABA content during imbibition. To investigate further the correlation between endogenous ABA and dormancy during grain development, it is important to clarify the regulation of ABA content during imbibition.

The first committed step toward ABA biosynthesis in plants is the oxidative cleavage of a 9-*cis*-epoxycarotenoid

to form xanthoxin, a precursor of ABA. Current evidence indicates that the key regulatory step in ABA biosynthesis is the oxidative cleavage reaction (Taylor *et al.*, 2000). The gene encoding the cleavage enzyme was first cloned from the ABA-deficient mutant *viviparous14* (*vp14*) of maize (Schwartz, 1997; Tan *et al.*, 1997). Molecular and biochemical analyses revealed that the *Vp14* gene encodes a 9-*cis*-epoxycarotenoid dioxygenase (NCED), which cleaves 9-*cis*-xanthophylls to form xanthoxin. Since the cloning of *Vp14*, a number of genes with sequence similarity to *Vp14* have been reported in many species, and are found in databases.

The level of ABA in plants is controlled not only by its biosynthesis, but also through its catabolism. ABA is catabolized into inactive forms either by oxidation or conjugation (Cutler and Krochko, 1999; Nambara and Marion-Poll, 2005). One of the primary catabolites of ABA is phaseic acid (PA). The conversion of ABA to PA begins with the hydroxylation of the 8' position by ABA 8'-hydroxylase. The 8' hydroxyl appears to be an unstable intermediate that spontaneously rearranges to form PA. Recently, a family of cytochrome P450s (*CYP707A*) that catalyses the 8'-hydroxylation of ABA to PA has been isolated from *Arabidopsis* (Kushiro *et al.*, 2004; Saito *et al.*, 2004). The gene expression of *CYP707A2*, one of the *CYP707A* genes, is rapidly increased after seed imbibition. T-DNA insertional mutants of *CYP707A2* have higher ABA content and exhibit increased dormancy. These results indicate that ABA catabolism plays an important role in reducing ABA content in *Arabidopsis* seeds. In the embryo of after-ripened barley grain, the ABA content decreases rapidly after hydration and ABA appears to be metabolized to PA (Jacobsen *et al.*, 2002). To examine the regulatory mechanisms of ABA content in barley grains, it is important to clone genes related to ABA biosynthesis and catabolism and analyse the expression patterns of these genes, which combine with the quantification analysis of endogenous ABA content.

The correlation between ABA content and dormancy during seed development has been studied for a long period. In wheat and barley, ABA content is highest in developing grains and declines as the grains undergo maturation drying (Goldbach and Michael, 1976; Walker-Simmons, 1987; Benech-Arnold *et al.*, 1999). Environmental conditions, including temperature, rainfall, and humidity surrounding mother plants, have an effect on the appearance of ABA peaks during grain development (Goldbach and Michael, 1976; Radley, 1976; Wiedenhoft *et al.*, 1988; Walker-Simmons and Sessing, 1990; King, 1993; Romagosa *et al.*, 2001). Although dormancy maintenance is essential to prevent pre-harvest sprouting under field conditions in cereal crops, field studies on the regulation of ABA content and germinability during grain development are few. In the present study, molecular and chemical analyses were performed to elucidate the mechanisms of

dormancy maintenance and release using field-grown barley grains. cDNAs encoding NCED and CYP707A homologues were cloned from barley, and the expression patterns of these genes analysed during grain development and imbibition. ABA content was measured and compared with the expression patterns of ABA-related genes to elucidate the regulation of ABA content in barley.

## Materials and methods

### Plant materials and growth

Barley (*Hordeum vulgare* L. cv. Misato Golden) seeds were sown in an experimental field at the National Institute of Crop Science on 25 October 2001, 28 October 2002, and 1 November 2003, and grown under normal field conditions. Misato Golden is a two-rowed and hulled type of normal barley. To provide the same developmental stage for all samples, approximately 1600 spikes were tagged at pollination. Misato Golden is a closed type of flower, and 'pollination' indicates when anthers reached the top inside floret. When the centre part of florets on a spike started to pollinate, each spike was tagged with its pollination date. Twelve to fourteen grains per spike were collected from the centre part of the tagged spike at 5 d intervals after pollination and subjected to the following experiment. The dry weight of grains was measured after incubating 50 grains at 135 °C for 4 h. The water content (%) of grains was estimated by (fresh weight-dry weight)/fresh weight. The temperature and precipitation data at the experimental field were obtained from the web site of the National Agricultural Research Center (<http://mserver.narc.affrc.go.jp/>). After the water content of grains fell lower than 25%, the spikes to be used for the preparation of storage grains were harvested to avoid heavy rainfall and pre-harvest sprouting in the field. In 2004, the tagged spikes were harvested from the field at 45 d after pollination (DAP) when the water content of grains was 20%, kept at room temperature at around 22 °C to dry for 1 month, and then the grains collected from the dried spikes were stored at 4 °C for 1 year before analysis. To eliminate the effect of embryo isolation before imbibition on changes of gene expressions and ABA content, whole grains were allowed to imbibe and then the embryos were isolated. The isolated embryos were immediately frozen in liquid nitrogen.

### Germination tests

Grains were subjected to germination tests. Thirty whole grains were placed in a Petri dish (9 cm diameter) containing two Whatman No. 2 filter papers and 6 ml of water. The dishes were placed in plastic bags to prevent evaporation, and kept in a growth cabinet at various temperatures in darkness. Germinated grains (those showing the emergence of the coleorhiza beyond the seed coats) were counted every 24 h and removed from the dishes. Germination tests were performed in triplicate except for those of freshly harvested grains in 2002 and 2003. The average mean and standard error (SE) values of germination percentage were calculated. The germination tests of freshly harvested grains in 2002 and 2003 were not replicated.

### Molecular cloning

cDNAs derived from immature embryos, imbibed embryos, and leaves treated with drought stress were prepared using the same method described in a previous paper (Chono *et al.*, 2003). For the cloning of NCED homologues, a polymerase chain reaction (PCR) was performed with degenerated primers designed from FDGDGM(V/I)H (forward primer) and PD(Q/H)Q(V/M)VF(K/R)L (reverse primer) using cDNA as a template, and nested PCR was performed with a forward primer and a primer designed from

M(M/I)HDF(A/V)IT. The nested PCR fragment was cloned into a pGEM-T easy vector (Promega, Madison, WI, USA). DNA sequencing was performed using a Big Dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 3100Avant Genetic Analyzer (Applied Biosystems). Sequence analysis was performed using DNASIS pro software (Hitachi Software Engineering Co., Tokyo, Japan). RACE reactions were performed using a Marathon cDNA amplification kit and Advantage cDNA PCR kit (Clontech, CA, USA) following the manufacturer's instructions, using gene-specific sense and antisense primers. Amplification products were cloned into a pGEM-T easy vector and sequenced. To clone the CYP707A homologue, gene-specific primers designed from the sequence data of EST clones (accession numbers CA001959 and CA008406) on the database were used to perform a RACE reaction. End-to-end PCR was performed to obtain the full-length cDNA of barley cv. Misato Golden using 5' and 3' end primers (5'-CTCGAATCGATCGGACCAGCTCT-3' and 5'-GTGATGAG-TAACCGCCGCTAACTG-3' for *HvNCED1*, 5'-CTCTCTCGCAACAACAAAACCCACG-3' and 5'-CTGGCAACTTCCTCTTTC-CATGTCC-3' for *HvNCED2*, and 5'-CCTCCGTTGCAGGTTG-CAGGTAACA-3' and 5'-GCCGTTGCCTCTATCGTGTGTTGA-3' for *HvCYP707A1*) and high-fidelity DNA polymerase (*Pyrobest* DNA polymerase, Takara shuzo Co. Ltd, Kyoto, Japan). The amplified PCR fragment was cloned into a pCR 4 Blunt-TOPO vector (Invitrogen, Carlsbad, CA, USA) and sequenced. DNA sequences were determined from at least five independent clones, thus avoiding potential PCR-induced errors.

### Functional expression of barley CYP707A homologue

Function analysis of the barley CYP707A1 homologue was performed using the same method described by Kushiro *et al.* (2004). Full-length cDNA of the candidate gene was cloned into a yeast expression plasmid, pYeDP60 (Pompon *et al.*, 1996), and the resulting plasmid was transferred into *Saccharomyces cerevisiae* strain WAT11 (Pompon *et al.*, 1996). Transformants were grown in SGI medium for 24–36 h, transferred to SLI medium, and induced by galactose for 12 h. The cells were collected, resuspended in 0.1 M potassium phosphate buffer (pH 7.6) and passed through a French press (20 000 psi). Microsomal fractions were suspended in 0.1 M potassium phosphate buffer (pH 7.6). To assay ABA 8'-hydroxylase, (t)-S-ABA was incubated with 2 µg of microsomal protein (in a 100 µl volume) containing 0.5 mM NADPH and 0.5% (w/v) Triton X-100, at 22 °C for 12–15 h. The amount of protein was measured by the Bradford method. The reaction was stopped by adding 1 N HCl, extracted with EtOAc and analysed by HPLC using a PEGASIL-B ODS (4.6 mm i.d.×250 mm; Senshu Scientific Co. Ltd, Tokyo, Japan) column (flow rate 1.0 ml min<sup>-1</sup>, UV detection at 254 nm) with the following gradient condition at ambient temperature: (A) 10% MeOH, 0.1% acetic acid, (B) 60% MeOH, 0.1% acetic acid, 0–3 min, 50% B, 3–33 min, 50–100% B linear gradient. For GC-MS analysis, samples were treated with ethereal diazomethane at room temperature for 5 min to obtain methyl esters of PA. GC-MS analysis was performed using the same method described by Kushiro *et al.* (2004).

### Quantitative reverse transcription-PCR (QRT-PCR)

The plant tissues were powdered in a mortar and pestle with liquid nitrogen, and the powdered tissue was divided in two. One was used for ABA quantification analysis, and the other was used for gene expression analysis. For gene expression analysis, total RNA was prepared from plant tissues using TRIZOL reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. Total RNA (2 µg) was treated with RNase-free DNase I (Roche Molecular Biochemicals, Mannheim, Germany) to eliminate genomic DNA contamination. First-strand cDNA was synthesized using a first-strand cDNA synthesis kit according to the manufacturer's instructions

(Amersham-Pharmacia Biotech Ltd, Amersham, Bucks, UK). Gene-specific primer pairs, capable of amplifying a 250 base 3' end fragment of *HvNCED1*, a 259 base 3'-end fragment of *HvNCED2*, and a 243 base 3'-end fragment of *HvCYP707A1*, respectively, were *HvNCED1.Fw* (5'-CCCCTATGGCTTCCACGGCACATT-3'); *HvNCED1.Rv* (5'-GTGATGAGTAACCGCCGCTAACTG-3'), and *HvNCED2.Fw* (5'-CGGGTACATTCACCTTCGTGCA-3'); and *HvNCED2.Rv* (5'-CTGGCAACTTCCTCTTTC-CATGTCC-3'), *HvCYP707A1.Fw* (5'-CTGGCCAAGCTGGAGAT-GCTCGTC-3'), and *HvCYP707 A1.Rv* (5'-GCACGGACACT-GACGGATGGAGAAC-3'). RT-PCR was performed with a gene-specific primer pair and an 18S primer pair as an internal control (QuantumRNA 18S internal standards, Ambion, Austin, TX, USA) according to the manufacturer's instructions. PCR products were analysed on a 1.5% (w/v) agarose gel containing ethidium bromide and the signal intensities were determined with an EDAS gel documentation system (Invitrogen) and 1D image analysis software (Invitrogen). Each experiment was repeated at least three times using the same cDNA source. For the *HvNCED1*, *HvNCED2*, and *HvCYP707A1* reactions, the average mean and SE values of relative transcript abundance were calculated.

#### ABA quantification

After  $^2\text{H}_6$ -labelled ABA (purchased from S Abrams, Plant Biotechnology Institute, National Research Council of Canada) was added as an internal standard, powdered plant tissues, as mentioned in previous parts of the gene expression analysis, were extracted by methanol. The methanol extract was mixed with 20% volume of water, and then loaded onto a Bond Elut  $\text{C}_{18}$  (Varian, Palo Alto, CA, USA) cartridge. This cartridge was washed with 5 ml of 80% of methanol and the effluent was evaporated to dryness. The residue was dissolved in methanol, added to an equal volume of water, and subjected to the following LC/MS/MS analysis. The LC/MS/MS analysis was carried out by a quadrupole tandem mass spectrometry (TSQ7000, Thermo Electron Corporation, Waltham, MA, USA) coupled with liquid chromatograph (HP1100, Agilent Technology, Palo Alto, CA, USA) with an HPLC column of CAPCELL PAK  $\text{C}_{18}$  MG (2.0 mm i.d.  $\times$  150 mm; Shiseido Fine Chemicals, Yokohama, Japan), and eluted for 10 min by 80% aqueous methanol with 0.1% acetic acid. The elution was run at a flow rate of 0.2 ml  $\text{min}^{-1}$ , and the column was maintained at 40 °C. An identification of ABA was performed by a selected reaction monitoring mode using electrospray ionization which monitors negative daughter ions of  $m/z$  153 and 159 derived from parental negative ions of  $m/z$  263 (ABA) and 269 ( $^2\text{H}_6$ -ABA), respectively, and those retention times on LC were compared with those of authentic samples. The content of the ABA identified was estimated from peak area ratios of both daughter ions using Xcalibur (ver1.1) software (Thermo Electron Corporation).

#### Drought-stress treatment

Grains were sown on moist soil in pots and grown in a greenhouse at 20 °C day/15 °C night temperatures under a supplemental photoperiod of 16 h. Daily watering was continued for 3 weeks, and stopped 3 d before harvesting. The second and third leaves (young leaf) and the fourth and fifth leaves (mature leaf) from the top were cut and frozen in liquid nitrogen. Total RNA isolated from frozen leaf tissues was used for RNA expression analysis and molecular cloning of ABA-related genes.

## Results

#### Molecular cloning of NCED homologues in barley

Genes closely related to NCED were found in ESTs from barley; however, their sequences were too short to

categorize them as *NCED* genes. A start was made by attempting to amplify fragments of the barley NCED homologue by using PCR with degenerate primers designed from conserved amino acid sequences of plant NCED homologues. Primer sites were chosen to amplify the barley NCED gene, using cDNAs derived from immature barley (cv. Misato Golden) embryos as a PCR template. One DNA fragment, approximately 520 bp long, was amplified, cloned, and sequenced. The results of nucleotide sequencing indicated that the 520 bp fragment contained two cDNAs. The same partial cDNAs were also obtained from a cDNA template derived from a drought-treated leaf. New gene-specific primers were constructed for each fragment and used for RACE experiments, and then end-to-end PCR performed using 5'- and 3'-end primers with high-fidelity DNA polymerase. From the end-to-end PCR, 2091 bp and 1959 bp fragments were cloned, which were then sequenced. The predicted amino acid sequences of these genes showed high sequence identity with each other (76.5%). BLAST search showed that the predicted polypeptides showed high sequence similarity with plant NCEDs including *Zea mays* (Tan *et al.*, 1997), *Arabidopsis thaliana* (Neill *et al.*, 1998), *Lycopersicon esculentum* (Burbidge *et al.*, 1997), *Persea americana* (Chernys and Zeevaart, 2000), and *Phaseolus vulgaris* (Qin and Zeevaart, 1999). Genomic DNA corresponding to these genes was also isolated from the barley genome and sequenced, revealing that they have intronless gene structures. This intronless structure is the same feature of known NCED genes in *Arabidopsis* and rice. The predicted polypeptide of 2091 bp and 1959 bp fragments showed 75.23% and 80.6% amino acid identity to VP14 (U95953), 81.1% and 86.5% to rice NCED3 (AY838899), and 80.9% and 73.9% to rice NCED5 (AY838901). Then the barley genes corresponding to the 2091 bp and 1959 bp fragments were named *HvNCED1* and *HvNCED2*, respectively (GenBank accession numbers AB239297 and AB239298).

#### Molecular cloning of CYP707A1 homologue in barley

Recently, ABA 8'-hydroxylase genes (*CYP707A1*, 2, 3, and 4) have been cloned from *Arabidopsis* (Kushiro *et al.*, 2004; Saito *et al.*, 2004). Expression analysis showed that one of the *CYP707A* genes, *CYP707A2*, is responsible for the rapid decrease in ABA levels during seed imbibition. The *CYP707A2* sequence was used to identify its rice homologues in BLAST search, and two candidate genes encoding ABA 8'-hydroxylase (AP004129 and AP004162) were found when tested. Then these rice sequences were used to search the barley homologues. One gene corresponding to barley ESTs (CA008406 and CA001959) showed high sequence similarity with rice candidates. To clone the full-length cDNA of the gene, RACE and end-to-end PCR were performed. Finally, a 1503 bp fragment was obtained, which was sequenced. BLAST search showed

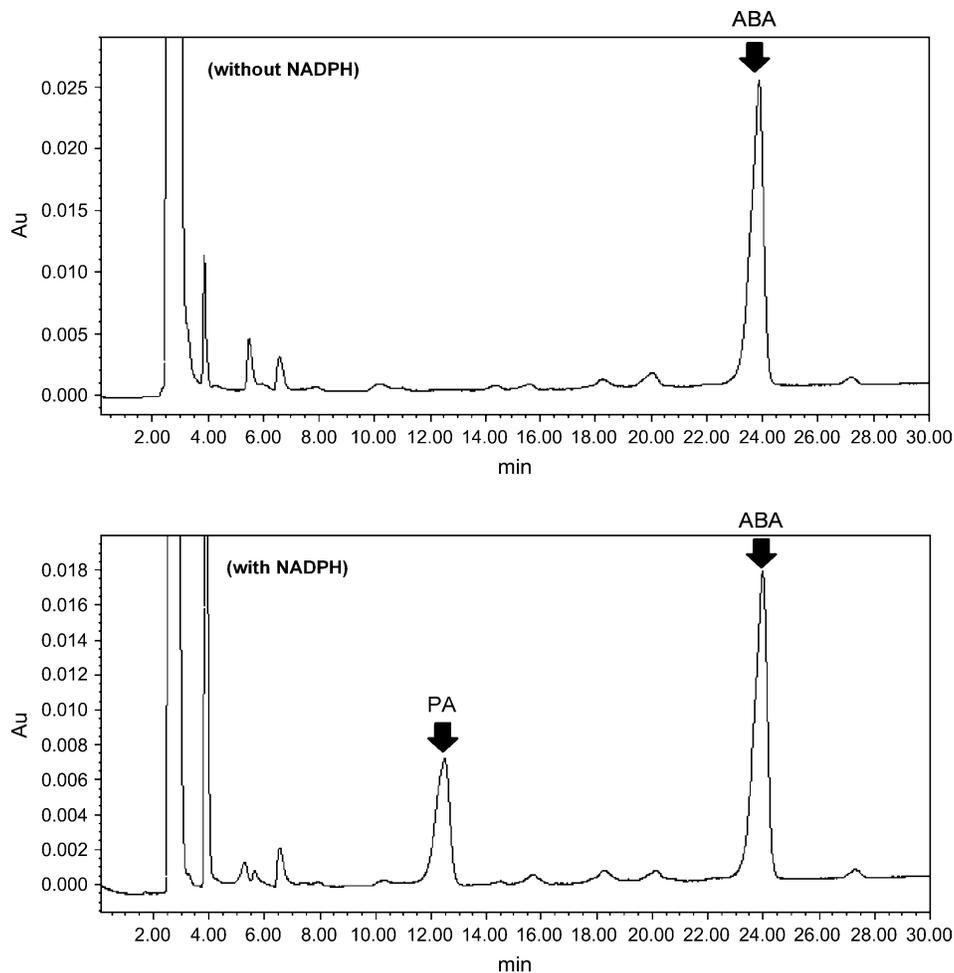
that the predicted polypeptide of the 1503 bp fragment shows the highest sequence similarity with the rice full-length cDNA sequence of AK067007 (1477 bits), corresponding to genomic clone AP004129. Although an attempt was made to amplify the cDNA fragment of the barley CYP707A homologue using PCR with degenerate primers, only the partial cDNA of the cloned fragment was identified.

To test whether a product encoded by the 1503 bp fragment would catalyse the 8'-hydroxylation of ABA to PA, full-length cDNA was introduced into yeast and expressed. The microsomal fraction of the transformant was used for *in vitro* enzyme assay where it was incubated with NADPH and (+)-S-ABA, and analysed by HPLC and GC-MS. The expression of the candidate gene produced a peak equal in retention time to authentic PA (Fig. 1). This peak was not seen in the vector-only control (data not shown). The sample was further analysed by GC-MS after methylation to confirm that the compound corresponding to the peak was indeed PA; molecular ion peaks at  $m/z$  294, 276, 244, 217, and 125 (data not shown). The production

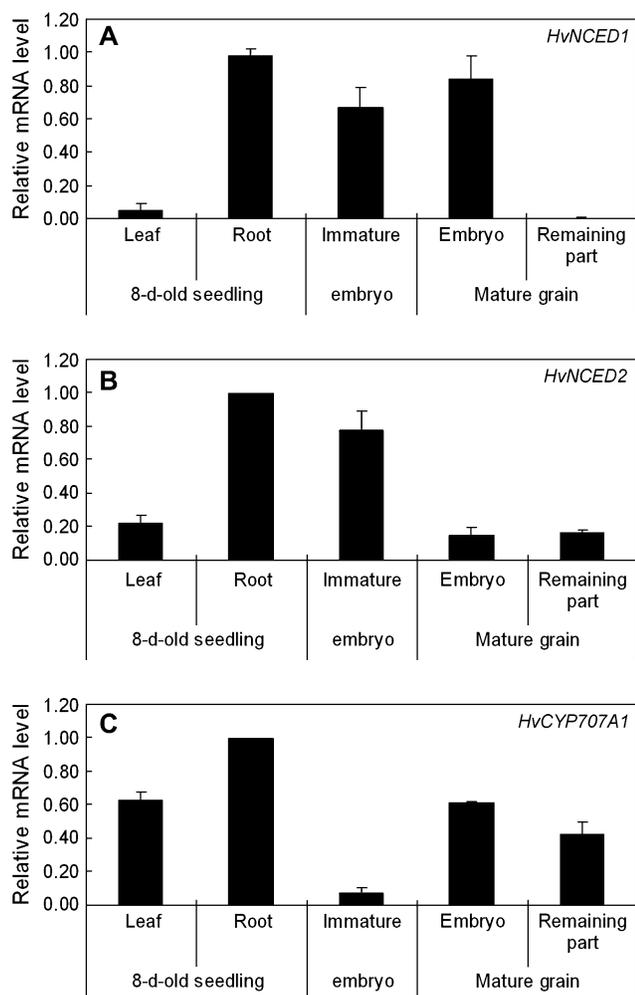
of PA was not seen when NADPH was omitted from the incubation mixture (Fig. 1). Therefore, the gene corresponding to the 1503 bp fragment was named *HvCYP707A1* (GenBank accession number AB239299).

#### Characterization of *HvNCEDs* and *HvCYP707A1* in gene expression

The expression of *HvNCEDs* and *HvCYP707A1* was measured in different tissues by QRT-PCR. Quantification analysis of ABA was performed with LC/MS/MS using the same plant materials for QRT-PCR. *HvNCEDs* and *HvCYP707A1* were expressed in the tissues tested, although the relative abundance differed between these genes. At the seedling stage (8-d-old seedlings), the expression levels of *HvNCEDs* in roots were higher than those in leaves (Fig. 2A, B). *HvCYP707A1* was highly expressed in both roots and leaves, and the level in roots was higher than that in leaves (Fig. 2C). Endogenous ABA content was low in these tissues at the seedling stage (Table 1). The induction of *HvNCED* mRNAs by drought-stress treatment in



**Fig. 1.** Functional expression of *HvCYP707A1* in yeast. HPLC profiles of reaction products on incubation of (+)-S-ABA with 2  $\mu$ g of microsomal protein are shown. Retention time is given in minutes, while the vertical axis indicates UV absorbance at 254 nm. The positions of authentic ABA and PA (with an arrow) under the same conditions are labelled. The enzyme assays were performed with (lower) or without (upper, control) NADPH.



**Fig. 2.** Gene expression of *HvNCEDs* and *HvCYP707A1* among various organs. (A) The expression level of *HvNCED1*. (B) The expression level of *HvNCED2*. (C) The expression level of *HvCYP707A1*. The expression levels of these genes were analysed by QRT-PCR, which was performed using leaves and roots of 8-d-old seedlings, immature embryos, and mature grains. Mature grains were divided into two parts: the embryo and the remaining part of the grain, including the endosperm, pericarp, testa, and husk. The results were normalized to the expression of 18S ribosomal RNA (internal control), and then the highest value was set to '1'. Results from three independent PCR are shown with error bars (SE).

**Table 1.** Endogenous levels of ABA in leaves and roots of 8-d-old seedlings, immature embryos and mature grains

Mature grains were divided into two parts: the embryo and the remaining part of the grain including the endosperm, pericarp, testa, and husk.

	8-d-old seedling		Immature embryo	Mature grain	
	Leaf	Root		Embryo	Remaining part
ABA (ng g <sup>-1</sup> FW)	1.6	1.4	230.6	251.6	10.4
ABA (ng grain <sup>-1</sup> )			0.92	0.65	0.56

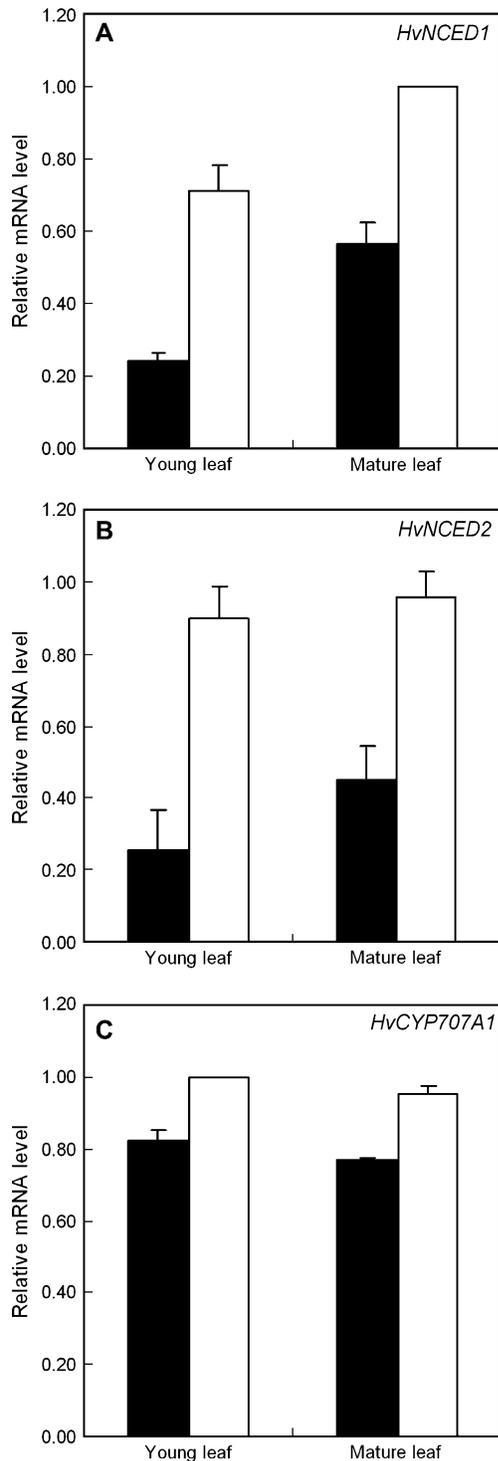
young and mature leaves of 3-week-old plants was clear (Fig. 3A, B), but the induction of *HvCYP707A1* mRNA was relatively small (Fig. 3C). The ABA content in these leaves was greatly induced by drought stress treatment (Table 2).

The fresh weight of immature embryos gradually increased after pollination (inset of Fig. 4A). Immature embryos collected from the grains at around 25–30 DAP and mature grains harvested at 60 DAP were used for QRT-PCR. *HvNCED1* was expressed in immature and mature embryos, but not in the part of mature grains remaining after embryo removal, including the endosperm, the pericarp/testa underling the husk, and the husk (Fig. 2A). The level of *HvNCED2* in immature embryos was higher than in the embryo and the remaining part of mature grains (Fig. 2B). The level of *HvCYP707A1* mRNA in mature embryos was higher than in immature embryos (Fig. 2C). *HvCYP707A1* was also expressed in the part of mature grains remaining after embryo removal. The level of ABA was higher in immature and mature embryos than in the remaining part of mature grains (Table 1).

#### Change in ABA content and the expressions of *HvNCEDs* and *HvCYP707A1* during grain development

Patterns of grain germination, ABA accumulation, and gene expressions were examined three times using developing grains collected from field-grown barley in 2002, 2003, and 2004. The grains were harvested at 5 d intervals from 20 to 55 (in 2002 and 2003) or 60 (in 2004) DAP, and subjected to germination assay, ABA quantification analysis, and gene expression analysis. The grains were allowed to imbibe at 15 °C, which is the most problematic temperature for pre-harvesting sprouting of barley in Japan. The 2004 results are shown in Fig. 4. The differences between the results in each year are shown below.

The germination percentage at 7 d after imbibition is shown in Fig. 4A. The fresh weight of embryos is shown in the inset of Fig. 4A. The grains germinated precociously between 20 and 30 DAP. When the grain water content rapidly decreased, maturing grains started to germinate at 40 DAP, and the germination percentage increased thereafter. To compare the change of grain germination with the change of ABA content during grain development, the level of embryonic ABA was quantified by LC/MS/MS. The ABA content increased gradually and peaked (2.45 ng embryo<sup>-1</sup>) at 35 DAP, and then rapidly decreased (0.80 ng embryo<sup>-1</sup>) at 40 DAP and maintained a low level (Fig. 4B). Precocious germination occurred around 25 DAP, just before ABA accumulation. When the embryo contained the maximum level of ABA, the grains did not germinate (35 DAP) during imbibition for 7 d. The grains started to germinate again after the decrease in ABA.



**Fig. 3.** Effect of drought stress on the gene expressions of *HvNCEDs* and *HvCYP707A1*. (A) The expression level of *HvNCED1*. (B) The expression level of *HvNCED2*. (C) The expression level of *HvCYP707A1*. The expression levels of these genes were analysed by QRT-PCR. Daily watering of 3-week-old plants was continued or stopped 3 d before harvest. The second and third leaves (young leaves) and the fourth and fifth leaves (mature leaves) from the top after the control (black columns) or dehydration treatment (white columns) were harvested and used for QRT-PCR. Results were normalized to the expression of 18S ribosomal RNA (internal control), and then the highest value was set to '1'. Results from three independent PCR are shown with error bars (SE).

**Table 2.** Effect of drought stress on the level of ABA

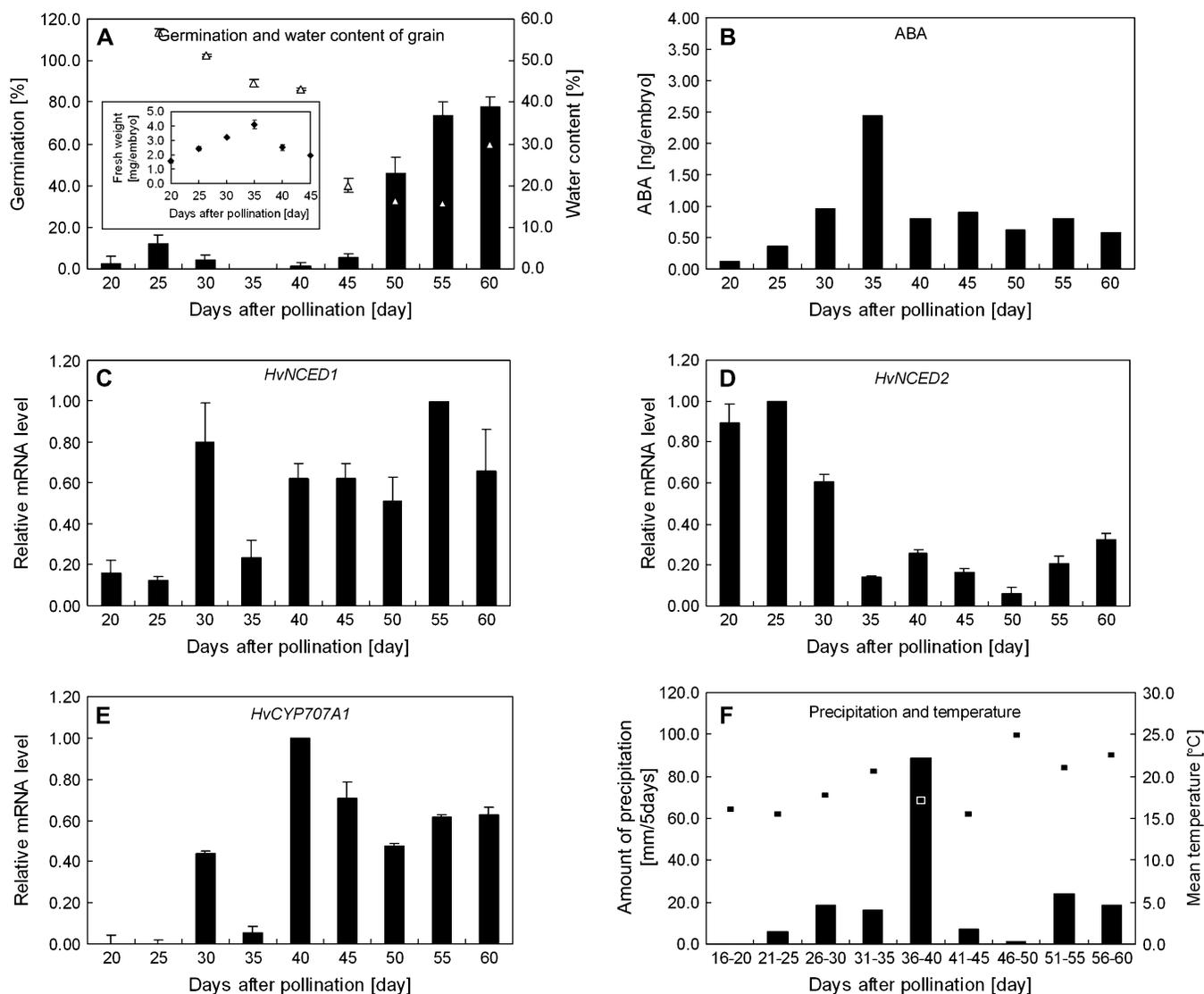
Three-week-old plants were used. Daily watering was continued (control) or stopped (dehydration) 3 d before harvesting. The second and third leaves (young leaf) and the fourth and fifth leaves (mature leaf) from the top were used.

	Young leaf		Mature leaf	
	Control	Dehydration	Control	Dehydration
ABA (ng g <sup>-1</sup> FW)	17.5	1014.5	15.3	557.0

The expression patterns of *HvNCEDs* and *HvCYP707A1* in embryos during grain development were examined. In this experiment, *HvNCED1* needed two more PCR cycles (33 cycles) to detect the gene expression than *HvNCED2* (31 cycles), meaning that the level of *HvNCED2* mRNA is higher than that of *HvNCED1* mRNA. *HvNCED2* was mainly expressed in the early stage of grain development (Fig. 4D). The trend in the expression pattern of *HvNCED1* was that the level of *HvNCED1* mRNA was relatively low, while the level of *HvNCED2* mRNA was high (Fig. 4C, D). After *HvNCED2* expression peaked, ABA content peaked at 35 DAP (Fig. 4B). When the highest expression of *HvCYP707A1* was observed (40 DAP), a rapid decrease in ABA content occurred (Fig. 4E). While the level of *HvCYP707A1* mRNA was high, the level of embryonic ABA was low (Fig. 4B, E). The expression patterns of these genes in embryos were consistent with the embryonic ABA content during grain development.

#### Changes in ABA content and the expressions of *HvNCEDs* and *HvCYP707A1* during imbibition of premature and mature grains

The germination pattern of imbibing grains at different maturation states was examined using the freshly harvested grains at 40 and 60 DAP in 2004 (Fig. 5A). These grains were allowed to imbibe at 15 °C without drying and storage. Grains at 40 DAP (premature grains) did not germinate for 2 d after imbibition; however, grains at 60 DAP (mature grains) showed 20% germination at 1 d after imbibition, and 41% germination at 2 d after imbibition. At 7 d after imbibition, the germination percentage of grains at 40 DAP was poor (1.1%); however, the germination percentage of grains at 60 DAP reached 78%. The change in the embryonic ABA content during the first 48 h of imbibition is shown in Fig. 5B. The amounts of embryonic ABA in the dry grains at 40 DAP and 60 DAP were 0.80 ng embryo<sup>-1</sup> and 0.59 ng embryo<sup>-1</sup>, respectively. In the grains at 40 DAP, the embryonic ABA content was sustained for 8 h after the start of imbibition (0.80 ng embryo<sup>-1</sup>), and then decreased at 16 h (0.61 ng embryo<sup>-1</sup>). After the first decrease, the ABA content increased at 24 h (0.71 ng embryo<sup>-1</sup>), and then decreased again at 48 h (0.59 ng embryo<sup>-1</sup>). In the grains at 60 DAP, the embryonic ABA

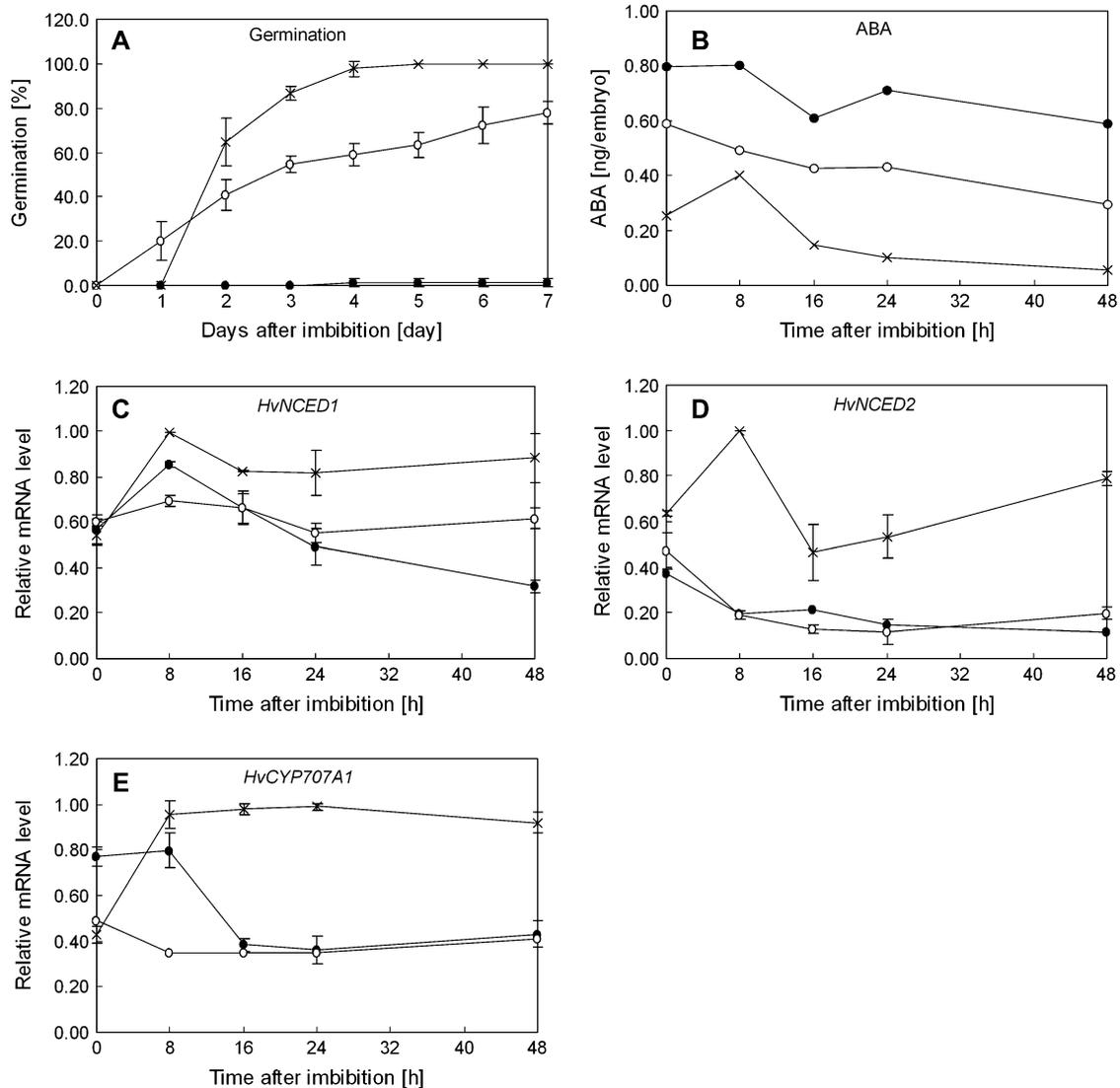


**Fig. 4.** Changes in germination, embryonic ABA content, and the expression levels of *HvNCEDs* and *HvCYP707A1* during grain development in 2004. (A) Changes in the germination percentage and the water content of grains during grain development. The germination percentage (black columns) and the water content of grains (open triangles) are shown with error bars (SE). The fresh weight of embryo is shown in the inset of the figure. (B) Change in the embryonic ABA contents during grain development. Endogenous ABA content in embryos is shown. (C–E) Changes in the expression levels of *HvNCED1*, *HvNCED2*, and *HvCYP707A1* in embryos during grain development. The expression levels of *HvNCED1* (C), *HvNCED2* (D), and *HvCYP707A1* (E) were analysed by QRT-PCR. Results were normalized to the expression of 18S ribosomal RNA (internal control), and then the highest value was set to '1'. Results from three independent PCR are shown with error bars (SE). (F) Environmental conditions during grain development. Amount of precipitation (black columns) and mean temperature (filled squares) are shown.

content decreased continuously during 48 h of imbibition. Forty-eight hours after the start of imbibition, the embryonic ABA content at 40 DAP and 60 DAP decreased to  $0.59 \text{ ng embryo}^{-1}$  (40 DAP) and  $0.30 \text{ ng embryo}^{-1}$  (60 DAP), respectively.

Comparing grains at 40 DAP and 60 DAP, the trends in the expression patterns of *HvNCEDs* in embryos were similar for 24 h after the start of imbibition (Fig. 5C, D). The only difference between the imbibing grains at 40 DAP and 60 DAP for 24 h was that the level of *HvNCED* mRNAs at 40 DAP was slightly higher than at 60 DAP at

8 h (*HvNCED1*) and 16 h (*HvNCED2*) after the start of imbibition. On the other hand, the expression pattern of *HvCYP707A1* was different between grains at 40 DAP and 60 DAP (Fig. 5E). In grains at 40 DAP, the level of *HvCYP707A1* mRNA was high before imbibition and was sustained for 8 h after the start of imbibition, and then greatly decreased at 16 h. After the great decrease of *HvCYP707A1* mRNA, the level of ABA increased (Fig. 5B). In grains at 60 DAP, the level of *HvCYP707A1* mRNA was lower than in grains at 40 DAP before imbibition, and slightly decreased at 8 h after the start of imbibition



**Fig. 5.** Effects of grain maturation on the germination pattern, embryonic ABA content, and the expression levels of *HvNCEDs* and *HvCYP707A1* in embryos during grain imbibition. Results of freshly harvested grains at 40 DAP (filled circles) and 60 DAP (open circles), and the stored grains (crosses) are shown. (A) Change in the germination percentage during grain imbibition. Results are shown with error bars (SE). (B) Change in the embryonic ABA content during grain imbibition. (C–E) Changes in the expression levels of *HvNCED1* (C), *HvNCED2* (D), and *HvCYP707A1* (E) in embryos during grain imbibition. The expression levels of these genes were analysed by QRT-PCR. Results were normalized to the expression of 18S ribosomal RNA (internal control), and then the highest value was set to '1'. Results from triplicate samples in three independent experiments are shown with error bars (SE).

(Fig. 5E). After 16 h, the level of *HvCYP707A1* mRNA was almost the same in grains at 40 DAP and 60 DAP, and the level of *HvCYP707A1* mRNA remained low in both grains for the next 32 h (Fig. 5E).

#### Effects of storage on ABA content and the expressions of *HvNCEDs* and *HvCYP707A1* during imbibition

The grains were harvested at 45 DAP in 2004, and dried for 1 month at room temperature of around at 22 °C, and stored for 1 year at 4 °C (stored grains). The stored grains were allowed to imbibe at 15 °C. The stored grains did not germinate at 1 d after imbibition, and rapidly increased their

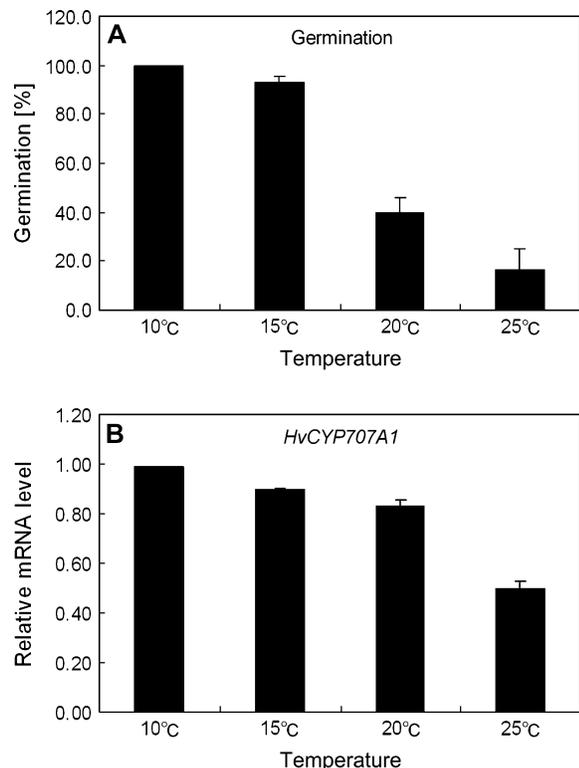
germination percentage to 64% after 2 d (Fig. 5A), reaching 100% after 5 d. In the dry grain, the amount of embryonic ABA was 0.25 ng embryo<sup>-1</sup> (Fig. 5B), which was lower than in freshly harvested grain during grain maturation (Fig. 4B). After the start of imbibition, the embryonic ABA content transiently increased at 8 h (0.40 ng embryo<sup>-1</sup>) and then rapidly decreased at 16 h (0.15 ng embryo<sup>-1</sup>) (Fig. 5B). The level of embryonic ABA content in the stored grain was lower than in grains at 40 DAP and 60 DAP during 48 h of imbibition.

The level of *HvNCED1* mRNA transiently increased at 8 h after the start of imbibition (Fig. 5C, D). The levels of *HvNCED* mRNAs were higher than in freshly harvested

grains at 40 DAP and 60 DAP during imbibition. The level of *HvCYP707A1* mRNA, which was low in the embryos of dry grain, greatly increased at 8 h after the start of imbibition, and was sustained for the next 40 h (Fig. 5E). When transient increases in *HvNCED* expressions were detected, a transient increase of ABA was detected (Fig. 5B). During imbibition, changes in the expression patterns of *HvNCEDs* and *HvCYP707A1* in the embryos of stored grains were consistent with the change in the embryonic ABA content.

#### Effect of temperature on grain germination and the expression level of *HvCYP707A1*

To investigate the effect of temperature on grain germination, grains harvested at 45 DAP in 2004 and then dried at room temperature for 1 month were allowed to imbibe at 10, 15, 20, and 25 °C. At 25 °C, the grains germinated poorly at 7 d after imbibition (Fig. 6A). On the other hand, at 10 °C and 15 °C, the grains germinated efficiently and



**Fig. 6.** Effect of temperature on grain germination and the expression level of *HvCYP707A1*. (A) Germination of grains at various temperatures. The grains, which were harvested at 45 DAP in 2004 and then dried at room temperature for 1 month, were allowed to imbibe at 10 °C, 15, 20, and 25. The germination percentages at 7 d after imbibition are shown. (B) The expression level of *HvCYP707A1* in the embryos of grains imbibed at various temperatures. At 24 h after the start of imbibition, the grain embryos were harvested. The expression level of *HvCYP707A1* in the embryo was analysed by QRT-PCR. Results were normalized to the expression of 18S ribosomal RNA (internal control), and then the highest value was set to '1'. Results from three independent PCR are shown with error bars (SE).

showed 100% and 93% germination, respectively, at 7 d after imbibition. The expression levels of *HvCYP707A1* in the embryos of imbibing grains at various temperatures were examined. At 24 h after imbibition, the level of *HvCYP707A1* mRNA was higher in grains which imbibed at lower temperatures (Fig. 6B).

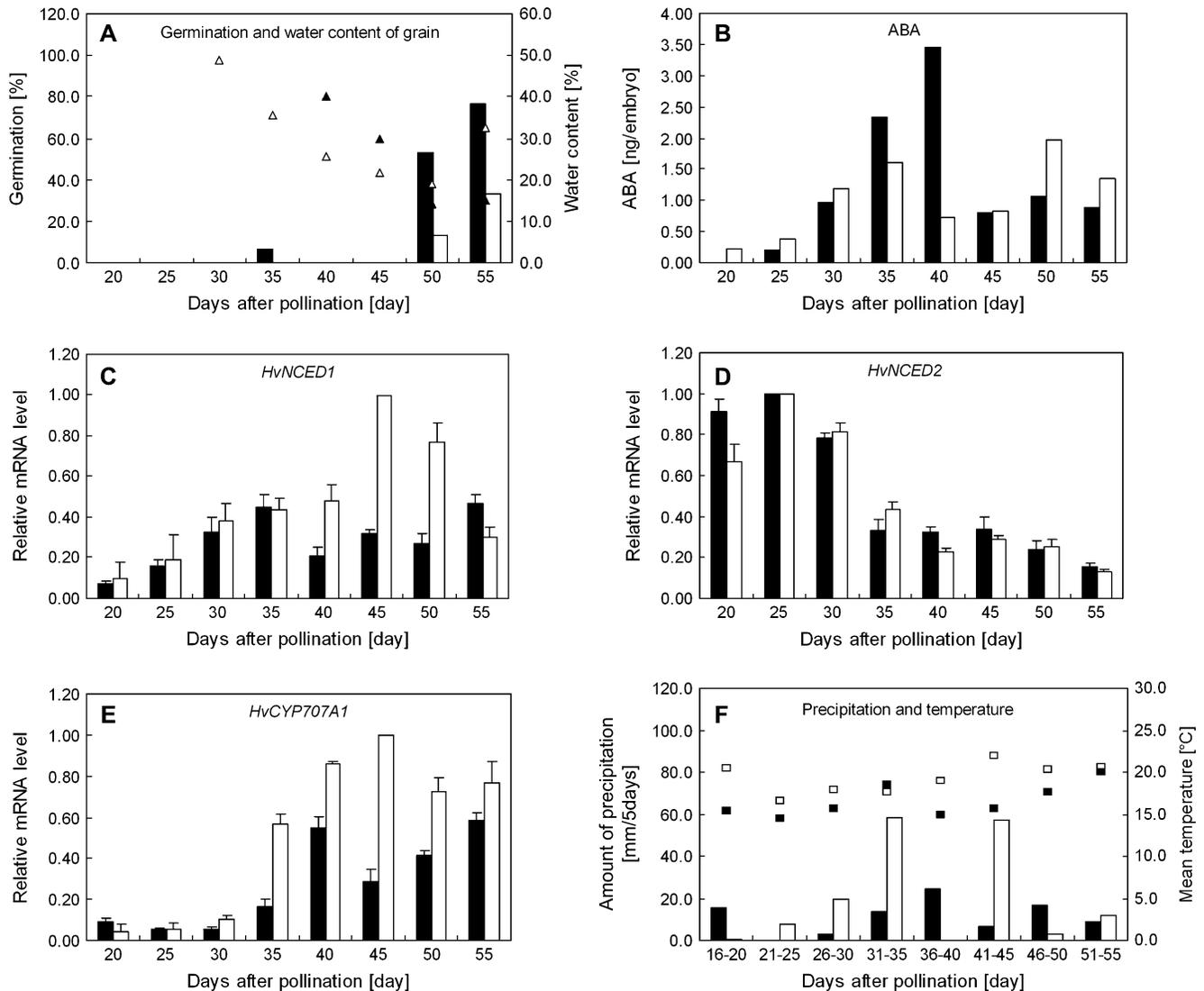
#### Comparisons of germination, ABA accumulation, and the expression patterns of *HvNCEDs* and *HvCYP707A1* during grain development in each year

The regulation of ABA content during grain development was examined three times using freshly harvested grains from field-grown barley plants in 2002, 2003 (Fig. 7), and 2004 (Fig. 4). The trend in the germination patterns of these years was identical with one small exception (Figs 4A, 7A). The germination percentage in the later stages of grain development in 2003 was lower than in 2002 and 2004 (Figs 4A, 7A). In each year, ABA accumulation in immature embryos was detected around 35–40 DAP; however, the maximum amount of ABA varied (Figs 4B, 7B). The second peak of ABA accumulation was only detected in 2003 (Fig. 7B). In the later stages of grain development, the grains in 2003, which contained a higher embryonic ABA content than in 2002 and 2004, showed a lower germination percentage than in 2002 and 2004. In 2002, the average temperature and the amount of precipitation during grain development were relatively lower than in 2003 and 2004 (Figs 4F, 7F). The increase in the water content of grains, which might be affected by rainfall and/or high humidity, was observed at 55 DAP in 2003 and at 60 DAP in 2004 (Figs 4A, 7A).

*HvNCED1* showed different expression patterns and levels in each year (Figs 4C, 7C). In 2003 in particular, the level of *HvNCED1* mRNA clearly increased at 45 and 50 DAP. The peak of *HvNCED1* expression from 45 to 50 DAP in 2003 (Fig. 7C) was followed by a second peak of ABA accumulation (Fig. 7B). By contrast to *HvNCED1*, *HvNCED2* showed the same expression pattern and level during grain development every year (Figs 4D, 7D). After the peak of *HvNCED2* expression, the first peak of ABA accumulation was detected every year (Figs 4B, 7B). *HvCYP707A1* was mainly expressed in the middle to later stages of grain development every year (Figs 4E, 7E). When the level of *HvCYP707A1* mRNA was high, the level of ABA was (or became) low except for 50 and 55 DAP in 2003 (Fig. 7B). Overall, the expression patterns of *HvNCEDs* and *HvCYP707A1* in embryos during grain development correlated well with the accumulation pattern of embryonic ABA in each year.

#### Discussion

A number of NCED genes have been reported in many plant species including *Zea mays* (Tan *et al.*, 1997),



**Fig. 7.** Comparisons of germination, embryonic ABA content, and the expression levels of *HvNCEDs* and *HvCYP707A1* during grain development in 2002 and 2003. Results from grains harvested in 2002 (black columns, filled triangles, filled squares) and 2003 (white columns, open triangles, open squares) are shown. (A) Changes in the germination percentage and the water content of grains during grain development. The germination percentage (columns) and the water content of grains (triangles) are shown. (B) Change in the embryonic ABA content during grain development. Endogenous ABA content in embryos is shown. (C–E) Changes in the expression levels of *HvNCED1*, *HvNCED2*, and *HvCYP707A1* in embryos during grain development. The expression levels of *HvNCED1* (C), *HvNCED2* (D), and *HvCYP707A1* (E) were analysed by QRT-PCR. Results were normalized to the expression of 18S ribosomal RNA (internal control), and then the highest value was set to '1'. Results from three independent PCR are shown with error bars (SE). (F) Environmental conditions during grain development. Amount of precipitation (columns) and mean temperature (squares) are shown.

*Arabidopsis thaliana* (Neill *et al.*, 1998), *Lycopersicon esculentum* (Burbidge *et al.*, 1997), *Persea americana* (Chernys and Zeevaart, 2000), and *Phaseolus vulgaris* (Qin and Zeevaart, 1999), and are found in databases. Two cDNAs of barley *NCED* showed high sequence similarity with *NCED* homologues in monocots, such as rice *NCED* homologues and maize *Vp14*. Expression analyses of maize *Vp14* have shown that *Vp14* mRNA is expressed in embryos and roots and is strongly induced in leaves by water stress (Tan *et al.*, 1997). In barley, both *HvNCEDs* were expressed in embryos and roots, and were induced by

drought stress in leaves (Figs 2, 3). These features of *HvNCEDs* resemble those of maize *Vp14*. Another similarity of *HvNCED2* expression, which appears to be dominant, the fact that *NCED* is expressed in developing embryos (Figs 4D, 7D), is consistent with the hypothesis that *HvNCED2* and *VP14* are orthologues.

Recent studies have identified a family of cytochrome P450s (*CYP707A*) that catalyse the 8'-hydroxylation of ABA to PA in *Arabidopsis* (Kushiro *et al.*, 2004; Saito *et al.*, 2004). In the present study, one cDNA of the barley gene, *HvCYP707A1*, encoding ABA 8'-hydroxylase was cloned.

Since the *Arabidopsis* CYP707A family consists of four genes, other barley CYP707A genes might also exist in the barley genome. As no other barley EST clone of the gene encoding ABA 8'-hydroxylase has been identified in databases, *HvCYP707A1* might be a major gene expressed in barley.

Under field conditions, the regulation of ABA content during grain development was examined three times using freshly harvested grains in 2002, 2003 (Fig. 7), and 2004 (Fig. 4). *HvNCED2* showed the same expression level and pattern during grain development every year. The growth of embryos might affect the expression of *HvNCED2*. These results indicate that the expression of *HvNCED2* is regulated in a growth-dependent manner in the grains. The peak *HvNCED2* expression was followed by the peak ABA accumulation in the early to middle stages of grain development. Since a maize NCED mutant *vp14* shows a viviparous phenotype (McCarty, 1995), its barley orthologue *HvNCED2* plays an important role in the prevention of premature germination in barley grains.

By contrast to *HvNCED2*, *HvNCED1* and *HvCYP707A1* showed a different expression pattern in each year (Figs 4, 7). In wheat and barley, it has been found that the appearance of ABA peaks during grain development varies in different growth conditions (Radley, 1976; Wiedenhoft *et al.*, 1988; Walker-Simmons and Sessing, 1990; Romagosa *et al.*, 2001). Goldbach and Michael (1976) have shown that ABA peaks earlier in high-temperature-grown barley than in low-temperature-grown barley. King (1993) has shown that during wheat grain maturation, ABA content in embryos is higher in grains produced under wet conditions (90–100% relative humidity) than in grain produced under dry conditions (35–40% relative humidity). As the average temperature in 2002 was lower than in 2003 and 2004 (Figs 4F, 7F), the embryonic ABA in 2002 peaked later than in 2003 and 2004 (Figs 4B, 7B). In the later stages of grain development, as a relatively higher amount of precipitation was detected in 2003 (Figs 4F, 7F), the embryonic ABA content in 2003 was higher than in 2002 and 2004 (Figs 4B, 7B). The amount of precipitation from 42 to 43 DAP in 2003 was 57.5 mm, and the mean humidity was almost 80% (data not shown). The spikes were fully wet on these days, and then dried in the field. The wetting of grains during heavy rainfall and/or the following drying in the field might affect the embryonic ABA content thereafter. After the heavy rainfall from 42 to 43 DAP, the grains did not show pre-harvest sprouting in the field. In the present experiment, grains harvested from field-grown barley plants were used. Environmental conditions, including temperature, rainfall, and humidity during grain development, might affect the expressions of *HvNCED1* and *HvCYP707A1*. Overall, the expression patterns of these genes in embryos during grain development were consistent with the accumulation pattern of embryonic ABA in each year. Differences in the expression patterns of

ABA-biosynthetic and -catabolic genes during grain development, which would affect ABA biosynthesis and catabolism, might result in the different ABA contents of barley grains in each year. In this experiment, the focus was on the regulation of ABA content in the embryo. It was not possible to show any information about the import of ABA from the mother plant, which might affect the embryonic ABA content during grain development.

In general, embryonic ABA content is highest in the early to middle stages of grain development and is relatively low in mature grains. In 2004, the maximum amount of ABA (2.45 ng embryo<sup>-1</sup>) at 35 DAP rapidly decreased to 0.80 ng embryo<sup>-1</sup> over 5 d. After a significant decrease of ABA, the embryonic ABA content varied from 0.89 ng embryo<sup>-1</sup> to 0.59 ng embryo<sup>-1</sup> (Fig. 4B). In comparison with the ABA decrease in all stages of grain development, the ABA decrease in the later stages of grain development was small. Although the difference in the embryonic ABA content during grain maturation was small, the regulation of ABA content in response to imbibition might change during grain maturation. As shown in Fig. 5, the decrease in *HvCYP707A1* expression in response to imbibition by premature grains (40 DAP), which would reduce the rate of ABA catabolism during imbibition, was followed by a lesser extent of ABA decrease and a lower germination percentage than of mature grain (60 DAP). Wang *et al.* (1995) has shown that *de novo* ABA synthesis in embryos plays a role in the control of germination. In the grains at 40 DAP, a small increase in *HvNCED1* expression was observed at 8 h after the start of imbibition (Fig. 5C), when the amount of ABA content was sustained (Fig. 5B). The increase in ABA biosynthesis after the start of imbibition might be effective in reducing the rate of ABA decrease during imbibition. Not only ABA content, but also ABA sensitivity might influence grain germination. In addition to the difference in ABA content before imbibition, the change in the activity of ABA biosynthesis and catabolism in imbibing grain during maturation could, in part, be reflected in the germinability (pre-harvest sprouting) of barley. In this experiment, the embryos from the imbibing grains which will or will not germinate after several hours or days of imbibition were collected and subjected to analysis. The regulation of ABA content in germinating embryos might be different from that in non-germinating embryos.

The expression of seed dormancy is very dependent on temperature and light during imbibition (Dunwell, 1981; Black *et al.*, 1987; Noda *et al.*, 1994). In the Cape Verde Islands (Cvi) ecotype of *Arabidopsis*, dormant seed does not germinate at 27 °C, but germinates at 13 °C (Ali-Rachedi *et al.*, 2004). ABA content in dormant Cvi seeds decreases faster at 13 °C than at 27 °C. In barley, grains before the 1-year-storage period germinated poorly at 25 °C, but germinated much better at 10 and 15 °C (Fig. 6A). When the grains showed a high germination percentage at

these temperatures, the expression of *HvCYP707A1* was high in the embryos of imbibing grains. ABA decay in embryos imbibed at low temperature might be interpreted by the induction of *HvCYP707A1* expression during the early imbibition of barley grains. In barley, Jacobsen *et al.* (2002) has shown that after-ripening involves the increased ability of embryos to reduce ABA content to a non-inhibitory level when grains are hydrated in the light. Freshly harvested grains at 60 DAP germinated when they were allowed to imbibe in the dark at 15 °C, but they did not germinate in light at 15 °C (data not shown). An investigation into the light effect on the expression of *HvCYP707A1* in imbibing dormant and after-ripened grains is important to understand the mechanism of dormancy release. The wetting of grains during heavy rainfall in the field indicates the imbibition of grains in day/night (light/dark) cycles. Concerning the field experiment of 2003, the increased ABA content and lower germination percentage in the later stages of grain development might, in part, be explained by the light effect on dormancy maintenance during grain imbibition.

In this study, it was indicated that the expression patterns of *HvNCEDs* and *HvCYP707A1* during the first 48 h of imbibition were not identical in the embryos in premature (40 DAP), mature (60 DAP), and after-ripened (stored) grains. A major change between grains before and after the storage period occurred in the expression pattern of *HvCYP707A1* in embryos during grain imbibition. In response to imbibition, the level of *HvCYP707A1* mRNA in the embryos in 60 DAP grains did not increase for 48 h, but that in stored grains greatly increased at 8 h and remained high for the next 40 h (Fig. 5E). The increased expression of *HvCYP707A1* in response to imbibition, followed by a rapid ABA decrease in embryos, and a high germination percentage, would increase the rate of ABA catabolism in embryos during imbibition. The regulation of ABA catabolism in embryos might play an important role in dormancy release following after-ripening. These results are consistent with previous studies in barley, which have found that the ABA content in embryos from after-ripened grain decreases during imbibition (Yamada, 1985), and that ABA is rapidly converted to PA in the embryos of imbibing after-ripened grain (Jacobsen *et al.*, 2002).

## Conclusion

Many studies have shown that ABA-biosynthetic and -catabolic genes play important roles in dormancy onset and release but, in many cases, it is unclear how these genes relate to pre-harvest sprouting under field conditions in cereal crops. In this study, the regulation of embryonic ABA content during grain development in field-grown barley was examined for 3 years by using *HvNCEDs* and *HvCYP707A1* as molecular markers. Expression analysis revealed that *HvNCED2* is associated with a significant

increase in ABA levels during early to middle stages of grain development, and its expression is regulated in a growth-dependent manner in the grains. By contrast, *HvNCED1* and *HvCYP707A1* showed a different expression pattern in each year, indicating that the expression of these genes is affected by environmental conditions during grain development. The varying patterns of *HvNCEDs* and *HvCYP707A1* expression in response to imbibition, which might be affected by conditions such as temperature and light (or dark) during grain imbibition, were not the same at different grain maturation stages. All variations in the expression of ABA-biosynthetic and -catabolic genes, which could change the rate of ABA biosynthesis or catabolism, might be reflected, in part, in the germinability of barley grains under field conditions.

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