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Expression analysis of abscisic acid (ABA) and metabolic signalling factors in developing endosperm and embryo of barley

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ABSTRACT

The expression of genes encoding components of ABA and metabolic signalling pathways in developing barley endosperm and embryo was investigated. The genes included *HvRCAR35_47387* and *HvRCAR35_2538* (encoding ABA receptors), *HvABI1d* (protein phosphatase 2C), *HvSnRK2.4*, *HvSnRK2.6* and *HvPKABA1* (SnRK2-type protein kinases) and *HvABI5* (ABA response element binding protein; AREBP), as well as two genes encoding SnRK1-type protein kinases. Both SnRK1 and SnRK2 phosphorylate AREBPs, but SnRK2 is activated by ABA whereas SnRK1 may be broken down. Multiple cereal AREBPs with two conserved SnRK1/2 target sites and another class of BZIP transcription factors with SnRK1/2 binding sites, including HvBLZ1, were identified. Barley grain (cv. Triumph) was sampled at 15, 20, 25 and 30 days post-anthesis (dpa). *HvRCAR35_47387*, *HvABI1d*, *HvSnRK2.4* and *HvABI5* were expressed highly in the endosperm but at much lower levels in the embryo. Conversely, *HvPKABA1* and *HvRCAR35_2538* were expressed at higher levels in the embryo than the endosperm, while *HvSnRK2.6* was expressed at similar levels in both. *HvRCAR35_47387*, *HvABI1d*, *HvSnRK2.4* and *HvABI5* all peaked in expression in the endosperm at 20 dpa. A model is proposed in which ABA brings about a transition from a SnRK1-dominated state in the endosperm during grain filling to a SnRK2-dominated state during maturation.

1. Introduction

The transition between grain filling and maturation in cereal seed development involves the cessation of starch and storage protein synthesis as well as the expression of genes that initiate processes that prepare the seed for dormancy, and dramatic changes in gene expression in the endosperm have been described (Wan et al., 2008). The hormone abscisic acid (ABA) is one of the major players in this process and the recent elucidation of the ABA signal transduction pathway (Cutler et al., 2010) means that there is now the opportunity to advance our understanding of the mechanisms by which ABA influences seed development.

Integral to ABA signalling is **s**ucrose **n**onfermenting-1-**r**elated protein **k**inase-2 (SnRK2). In the absence of ABA, SnRK2 is kept in an inactive state through the action of protein phosphatase 2C (PP2C). In the presence of ABA, ABA receptors PYR/PYL/RCAR bind to and inhibit PP2C, allowing the accumulation of active SnRK2 and subsequent phosphorylation of ABA-response element binding proteins (AREBPs) (Cutler et al., 2010). AREBPs (also known as ABFs) are a family of basic leucine zipper (bZIP) transcription factors that recognize the ABA response elements (ABREs) present in some ABA-regulated genes (Cutler et al., 2010).

SnRK2s phosphorylate AREBPs at serine (preferentially) or threonine residues with a basic residue at −3 with respect to the serine/threonine residue (Fujii et al., 2007; Furihata et al., 2006; Yoshida et al., 2010). Two of the SnRK2 target sites in AREBPs are potential targets for phosphorylation by a related protein kinase, SnRK1, which also phosphorylates serine residues with a basic residue at −3, but has an additional requirement for hydrophobic residues at −5 and +4. These SnRK1 target sites are conserved throughout the

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Arabidopsis AREBP family and in all of the AREBPs that have been identified so far in other species (Zhang et al., 2008). Peptides with amino acid sequences based on these sites have been shown to be phosphorylated by both SnRK1 and 2 (Zhang et al., 2008).

SnRK1 is functionally as well as structurally related to SNF1 of fungi and adenosine monophosphate (AMP)-activated protein kinase (AMPK) of animals, both of which are major regulators of metabolism and a range of cellular processes in their respective systems (reviewed by Hey et al., 2010). SnRK2-type protein kinases, on the other hand, do not have any direct functional counterpart in fungal or animal cells. They are encoded by a medium-sized gene family with, for example, 10 members in Arabidopsis (*Arabidopsis thaliana*) and are involved with or implicated in responses to abiotic stresses such as drought, salinity, cold and osmotic stress (Hey et al., 2010). Kobayashi et al. (2004) divided Arabidopsis SnRK2s into three classes, based on phylogeny and their response to ABA: Class 1, which are not activated in response to ABA, Class 2, which are activated weakly by ABA and Class 3, which are strongly activated by ABA.

In a recent study, we investigated the interactions between ABA, SnRK1 and SnRK2 in wheat and showed contrasting effects of ABA on SnRK1 and SnRK2 protein levels and phosphorylation state (Coello et al., 2012). Application of ABA to wheat roots brought about a dramatic decrease in SnRK1 protein, and phosphorylation/activation of a 42 kDa SnRK2. Unusually for SnRK2, this member of the family was calcium-dependent. These results imply differential roles for SnRK1 and SnRK2 in ABA signalling and antagonistic effects of SnRK1 and SnRK2 on gene expression. Consistent with this, transient repression of *SnRK1* has been shown to repress the activity of an α-amylase gene (*α-Amy2*) promoter in cultured wheat embryos (Laurie et al., 2003), while a SnRK2, PKABA1, has been shown to down-regulate α-amylase genes in response to ABA (Gomez-Cadenas et al., 1999). Recently, ABA-responsive *cis*-elements have been identified in the promoters of key genes of starch biosynthesis in barley:*HvSUS1*, encoding sucrose synthase, and *HvAGP-L1*, encoding the large subunit of ADP-glucose pyrophosphorylase (AGPase) (Seiler et al., 2011). Homologous genes are up-regulated in response to SnRK1 over-expression in potato tubers (McKibbin et al., 2006), with a concomitant increase in starch content.

In this study, we have investigated the expression of genes encoding components of the ABA sensing and signalling pathway in developing barley endosperm and embryo, compared expression patterns of different SnRK1 and SnRK2 family members, and identified cereal AREBP and other BZIP transcription factors with SnRK target sites. The aim was to provide new knowledge of the interactions between SnRK1, SnRK2, PP2C, ABA and transcription factors in the control of cereal seed development.

2. Materials and methods

2.1. Database screening

BLAST searches of the non-redundant (nr) protein sequence database were performed using the National Center for Biotechnology Information (NCBI) portal (www.ncbi.nlm.nih.gov/). Barley1 22k Microarray data (Dash et al., 2012) was accessed via the PLEXdb portal (www.plexdb.org/modules/tools/plexdb_blast.php).

2.2. Sample preparation

Plants of the two-rowed barley (*Hordeum vulgare*) cultivar Triumph were grown in 15 cm diameter pots in a glasshouse with a 16 h day-length (supplemental lighting was used as necessary) and a minimum temperature of 14 °C by night and 18 °C by day. There was 1 plant per pot and pots were laid out in a randomised design. Ears were tagged at anthesis, grain sampled at 15, 20, 25 and 30 days post-anthesis (dpa), and caryopses dissected under a dissecting microscope. There were three biological replicates for each of the four time points. Dissected samples were immediately frozen in liquid nitrogen and stored at −80 °C. Images of the dissected grain were obtained with a Leica M205 FA Stereomicroscope (Leica Microsystems, Milton Keynes, UK).

2.3. RNA extraction and cDNA synthesis

Total RNA was isolated using TRIzol® Reagent (Ambion, USA) according to the manufacturer's instructions. The quality and the concentration of RNA were measured by spectrophotometry using a NanoDrop ND-100 (Thermo Fisher, Wilmington, DE, USA; supplied by Labtech International Ltd, Uckfield, UK) and confirmed by electrophoresis through an agarose gel. The RNA was treated with RQ1 RNase-Free DNase (Promega, USA); cDNA was then synthesised using SuperScript® III Reverse Transcriptase (Invitrogen, USA). The cDNA was checked for purity by polymerase chain reaction (PCR) amplification using primers CATCAAGCTCAAGGACGACA and GCCTTGTCCTTGTCAGTGAA, which anneal to sites flanking an intron within the *HvGAPDH* gene.

2.4. PCR primer design and quantitative real-time PCR

Primers were designed using either Primer Express® Software Version 3.0 (Applied Biosystems, USA) or Primer 3 (http://primer3.wi.mit.edu/). PCR reactions were performed in 96-well plates on a 7500 Real Time PCR System (Applied Biosystems, USA) using SYBR® Select Master Mix (Applied Biosystems, USA). The reactions for biological replicates were separated across three plates, thus forming statistical blocks for subsequent data analysis. Reactions contained 10 μL 2 × mix, 0.6 μL of each primer (10 μM) and 100 ng cDNA template in a final volume of 20 μL. The same 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; data collection was carried out during the 60 °C step. The dissociation curves/melting curves analyses were conducted after cycle 45.

2.5. Data analysis

The efficiency of the PCR was estimated using the LinReg PCR programme (Ramakers et al., 2003). A Ct value was obtained by 7500 software v2.0.5 (Applied Biosystems, USA) and the *Ct* and efficiency values were then used to calculate the Relative Quantity (*RQ*), and the Normalised Relative Quantity (NRQ) of a target gene's expression with respect to three reference genes, *HvActin*, *HvCyclophilin* and *HvGAPDH*. NRQ was calculated using the following formula:

$$NRQ = \frac{E_{target}^{-Ct,target}}{\sqrt{E_{HvActin}^{-Ct,HvActin} \cdot E_{HvCyclophilin}^{-Ct,HvCyclophilin} \cdot E_{HvGAPDH}^{-Ct,HvGAPDH}}}$$

Where E_{target}, $E_{HvActin}$, $E_{HvCyclophilin}$ and $E_{HvGAPDH}$ are the estimated PCR efficiencies for a particular target gene and the two reference genes and where *Ct*, target, *Ct*, *HvActin*, *Ct*, *HvCyclophilin* and *Ct*, *HvGAPDH* are the corresponding *Ct* values. The *Ct* values of each reference gene were found to be stable across the time points, prior to using the geometric mean of the *RQ* values as shown in the formula.

Statistical analysis of the NRQ data was performed using the GenStat statistical package (2011, 14th edition, © VSN International Ltd, Hemel Hempstead, UK). Taking account of the three plates as statistical blocks, ANOVA was applied to the log (to base 2)-

transformed inverse of the NRQ data (Rieu and Powers, 2009). This transformation ensured homogeneity of variance and effectively provided values back on the Ct-scale. Therefore, as for *Ct* values ($Ct = \log_2(1/NRQ)$), a low $\log_2(1/NRQ)$ indicated a high gene expression, whereas a high $\log_2(1/NRQ)$ indicated low gene expression. Following a significant ($p < 0.05$, *F*-test) result from ANOVA, the analysis assessed the statistical significance of differences in gene expression between time points using the least significant difference (LSD) at the 5% ($p < 0.05$) level of significance.

3. Results

3.1. Identification of genes involved in ABA signalling in barley

The aim of the project was to analyse expression of genes encoding proteins potentially involved in the abscisic acid (ABA) signalling pathway during barley grain development, including the ABA receptor protein, RCAR, protein phosphatase 2C (PP2C), sucrose nonfermenting-1-related protein kinase 2 (SnRK2) and related protein kinase SnRK1, and ABA response element binding proteins (AREBPs), as well as additional transcription factors that could be substrates for SnRK1 or SnRK2.

Two genes encoding ABA receptors were studied; these were *HvRCAR35_47387* and *HvRCAR35_2538* (Seiler et al., 2011). The protein phosphatase 2C gene was *HvABI1d* (Seiler et al., 2011). The *ABI1* name derives from the fact that this gene was first identified in Arabidopsis in a screen of mutants that were ABA insensitive (Finkelstein, 1994).

Two *SnRK2* genes from barley have been described previously: *HvSnRK2.6* (Mangelsen et al., 2011; Sreenivasulu et al., 2006; Seiler et al., 2011) and *HvPKABA1* (Yamauchi et al., 2002), and these were both included in the study. A third *SnRK2* gene was identified from Barley1 22k Microarray data (Dash et al., 2012) and also included. It has been annotated as *HvSnRK2.4* but there is no experimental evidence that the SnRK2 that it encodes is a functional equivalent of Arabidopsis SnRK2.4, which is ABA-independent.

Two *SnRK1* genes have been characterised previously in barley:*HvSnRK1a* (also known as *BKIN2*) and *HvSnRK1b* (also known as *BKIN12* and related *BKIN9*) (Halford et al., 1992). *HvSnRK1a* has been shown to be expressed in all tissues that have been analysed to date, whereas *HvSnRK1b* is expressed at high levels in the seed but at relatively low levels elsewhere (Hannappel et al., 1995). Despite its relatively low levels of expression in non-seed tissues, antisense inhibition of *HvSnRK1b* gene expression in anthers has been shown to result in impaired pollen development, a failure of pollen grains to accumulate starch, and pollen inviability (Zhang et al., 2001). Southern analysis of the gene family has suggested that it comprises 10–20 members (Halford et al., 1992).

A BLAST search of the non-redundant (nr) protein sequence database was performed with the *HvSnRK1a* amino acid sequence using the National Center for Biotechnology Information (NCBI) portal. This identified one barley *SnRK1a* entry, accession number CAA57898.1, and seven barley *SnRK1b* entries, accession numbers BAJ95249.1, BAK03923.1, CAA46555.1, CAA46556.1, BAK05223.1, CAA07813.1 and CAA46554.1. Primers with which to amplify *HvSnRK1a* and *HvSnRK1b* products were designed to reveal any of these homologues with the aim being to distinguish between the two types. SnRK1 interacts with two non-catalytic subunits to form a heterotrimeric complex and the expression of a barley gene, *HvSnRK1β*, encoding one of these was

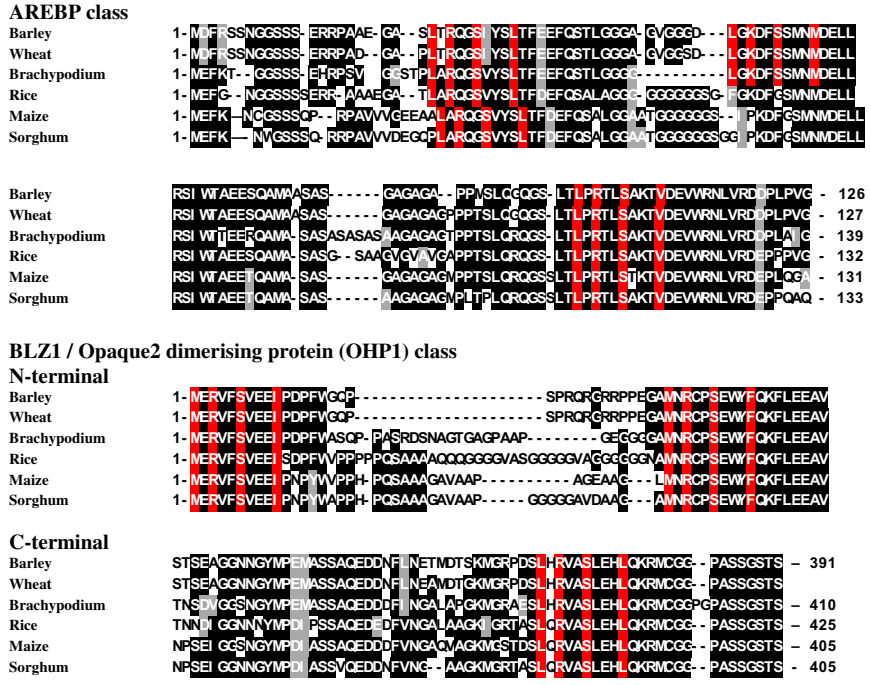

Fig. 1. SnRK1/2 target sites in cereal transcription factors Top: Alignment of the N-terminal region of representative ABA-response element binding proteins (AREBPs) from barley (*Hordeum vulgare*) (GenBank: AAO06115.1); wheat (*Triticum aestivum*) (GenBank: BAD97366.1); Brachypodium (*Brachypodium distachyon*) (NCBI Reference Sequence: XP_003578228.1); rice (*Oryza sativa*) (GenBank: EEC84700.1); maize (*Zea mays*) (NCBI Reference Sequence: NP_001132507.1); and sorghum (*Sorghum bicolor*) (NCBI Reference Sequence: XP_002460329.1). Bottom: Alignment of the N- and C-terminal regions of BLZ1 and related transcription factors from barley (*Hordeumvulgare*) (GenBank: CAA56374.1), wheat (*Triticum aestivum*), from wheat genome data (Brenchley et al., 2012); Brachypodium (*Brachypodium distachyon*) (NCBI Reference Sequence: XP_003557420.1); rice (*Oryza sativa*) (Genbank ABF99347.1); maize (*Zea mays*) Opaque2 heterodimerizing protein1 (OHP1) (NCBI Reference Sequence: NP_001105687.1); and sorghum (*Sorghum bicolor*) (NCBI Reference Sequence: XP_002463740.1). Residues that make up SnRK1/2 target sites (a serine residue with a hydrophobic residue at −5 and +4 with respect to the serine, and a basic residue at −3) are highlighted in red. Other conserved residues are highlighted in black, with like-for-like substitutions highlighted in grey. Numbers refer to the position in the amino acid sequence; note that the length of the wheat BLZ1-like transcription factor is not known.

identified from Barley1 22k Microarray data (Dash et al., 2012) and this gene was also included in the study.

The screen of ABA-insensitive Arabidopsis mutants that identified ABI1 (Finkelstein, 1994) also identified genes encoding transcription factors of the ABA response element binding protein (AREBP) class. Arabidopsis contains 14 genes encoding AREBP-type transcription factors (Zhang et al., 2008); of these, ABI5 has been the most intensively studied because of its role in germination (*abi5* mutant seeds germinate in the presence of ABA at levels that prevent germination of wild-type seeds). However, it is important to note that other AREBPs may have different but equally important roles in controlling plant development. Unfortunately, the prominence of ABI5 in the literature has led to AREBPs from other species being annotated as ABI5 analogues, in some cases without any evidence that they have the same function as ABI5.

SnRK2 phosphorylates AREBPs at multiple sites, requiring only a serine or threonine residue with a basic residue at −3 for substrate recognition (Furihata et al., 2006). Two of the sites in AREBPs that can be phosphorylated by SnRK2 have also been shown to be phosphorylated by SnRK1 (Zhang et al., 2008). SnRK1 phosphorylates target proteins at serine residues with a hydrophobic residue at −5 and +4 with respect to the serine as well as the basic residue (preferably arginine) at −3 or (sub-optimally) at −4.

In order to identify cereal AREBPs, BLAST searches were performed of the non-redundant protein sequence database using a barley AREBP amino acid sequence identified previously (Zhang et al., 2008). This identified members of the AREBP family from barley, wheat, Brachypodium, rice, maize and sorghum. The N-terminal regions of one AREBP from each species are shown in Fig. 1, with SnRK1 target sites highlighted in red. Two such sites are present in all six of the AREBPs shown and also in all but one (ABI5 itself) of the AREBPs in Arabidopsis (Zhang et al., 2008). Synthetic peptides based on both sites have been shown to be phosphorylated by partially-purified SnRK1 and SnRK2 from wheat roots (Coello et al., 2012). For the present study, primers for analysis of AREBP gene expression were based on the barley gene annotated as *HvABI5* (Casaretto and Ho, 2003).

3.2. Identification of additional transcription factors with SnRK target sites

The tolerance of multiple similar amino acids within the SnRK1 target site makes computational identification of putative SnRK1-regulated proteins and protein families difficult. A pipeline has therefore been developed consisting of a basic motif search, PROSITE family searches (Falquet et al., 2002) and multiple alignments within each PROSITE family. AREBPs were identified as potential substrates for SnRK1 using this pipeline (Zhang et al., 2008). For the present study, the database that was compiled using this pipeline was mined for additional bZIP transcription factors that could also be phosphorylated by SnRKs. This process identified another class of cereal bZIPs with N-terminal SnRK1 target sites, comprising Opaque2 dimerising protein (OHP1) from maize (NP_001105687; 542704_ohp1) and sorghum, BLZ1 from barley (HvBLZ1; CAA56374) and multiple uncharacterised BZIPS from rice (NP_001051558; AAP44683; ABF99347; BAF13472; Os03g0796900; BAA11431; EAY92182; BAA36492). These are aligned in Fig. 1. BLZ1 (Vicente-Carbajosa et al., 1997) recognizes the so-called N motif of the prolamin box of wheat, barley and rye storage protein gene promoters and was therefore included in the analysis of gene expression.

3.3. Expression analyses

Plants of barley (*Hordeum vulgare*) cultivar Triumph were grown in a glasshouse and grain sampled at 15, 20, 25 and 30 days post-anthesis (dpa) (Fig. 2). Caryopses were dissected to separate the embryo (including the scutellum) and endosperm. For the endosperm analysis, only material from the central, starchy endosperm was analysed (Fig. 2). RNA was purified from the samples and used as a template for quantitative, real-time polymerase chain reaction (qRT-PCR) analysis of gene expression for the *HvRCAR35_47387*, *HvRCAR35_2538*, *HvABI1d*, *HvSnRK2.4*, *HvSnRK2.6*, *HvPKABA1*, *HvABI5*, *HvSnRK1a*, *HvSnRK1b*, *HvSnRK1β*, and *HvBLZ1* genes described above. The primers used for the PCR reactions, mean $\log_2(1/\text{NRQ})$ values and results of ANOVA to consider significance of differences in gene expression between time points for the genes of interest are given in Supplementary Information. For comparison, expression of a gene encoding a late embryogenesis abundant protein, *HvLEA3*, was also included. Each analysis included three biological replicates from different pots and the expression level of a gene was calculated as the Normalised Relative Quantity (NRQ) with respect to three reference genes, *HvActin*, *HvCyclophilin* and *HvGAPDH*. The results for expression in the endosperm are shown in Fig. 3 and the embryo in Fig. 4. The differences in scales on the graphs within and between Figs. 3 and 4 should be considered when comparing the expression levels of different genes or the same gene in different tissues.

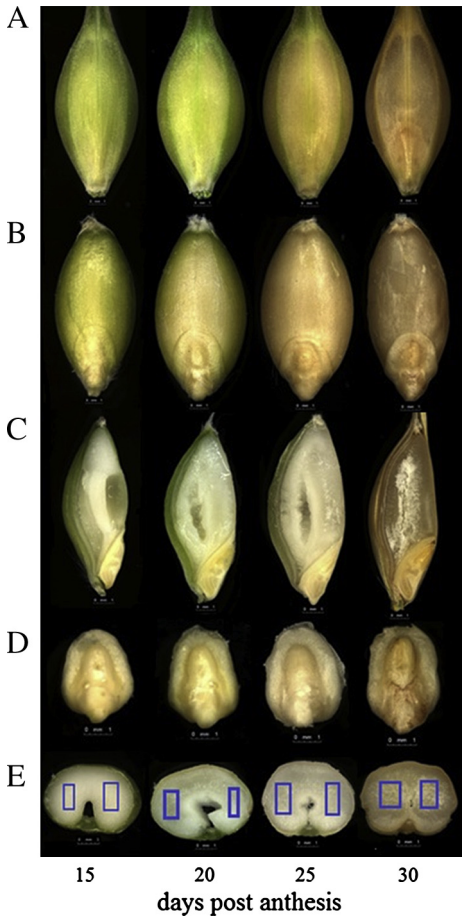

Fig. 2. Seeds and samples at 15, 20, 25 and 30 days post anthesis (dpa). The scale beneath each picture shows 1 mm. A. Whole developing seeds. B. Whole developing seeds without pericarp. C. Longitudinal sections of developing seeds. D. Sections of embryo. E. Seed transection; rectangles indicate sections manually dissected to give endosperm samples.

The expression of the *HvLEA3* gene was as expected, rising through mid-development to its highest level in the endosperm at 30 dpa and the embryo at 25–30 dpa. *HvRCAR35_47387* gene expression, in contrast, was readily detectable in the endosperm,

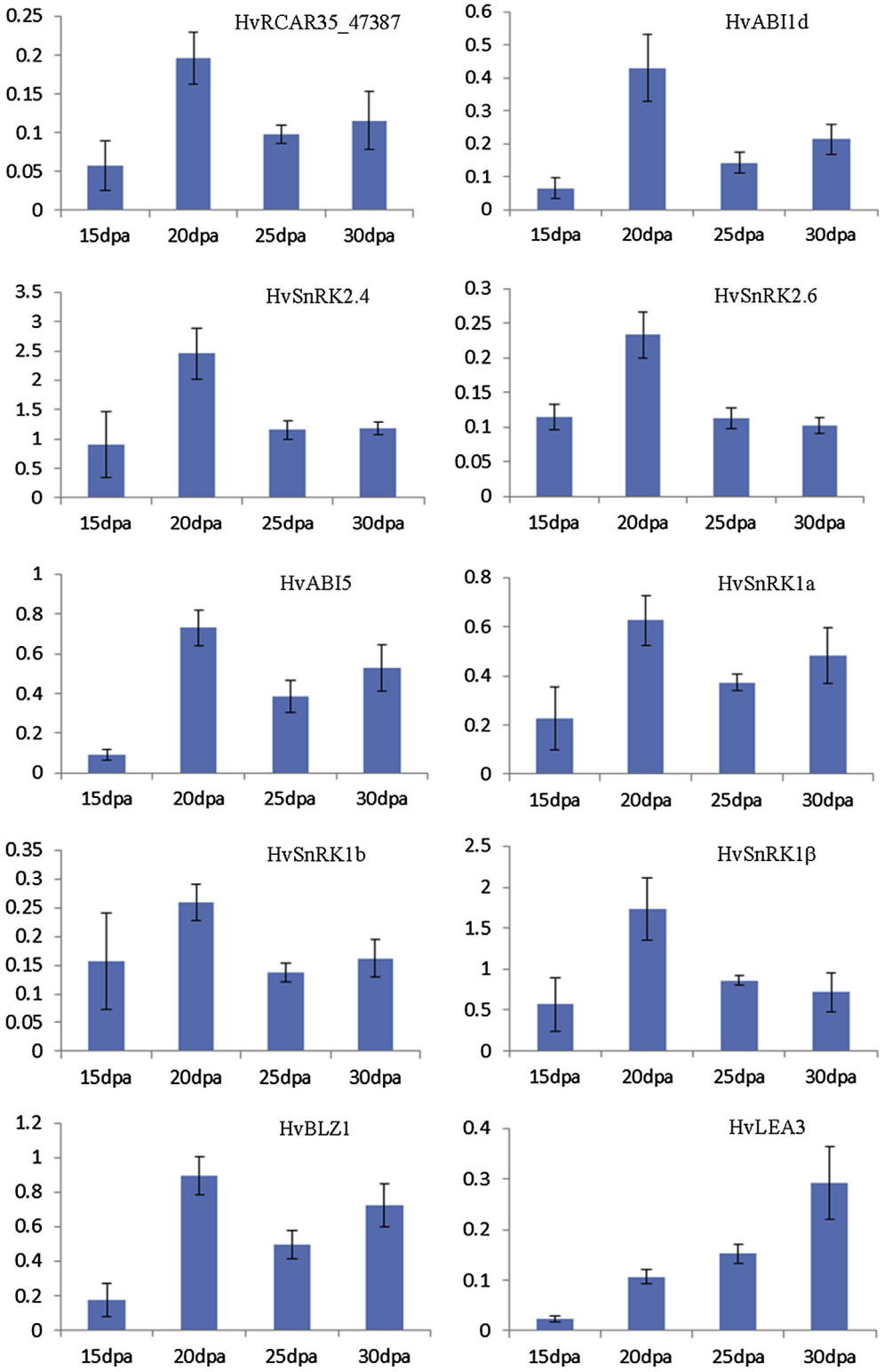

Fig. 3. Differential expression of genes encoding components of ABA and metabolic signalling pathways in endosperm of barley (*Hordeum vulgare*) cv. Triumph analysed by qRT-PCR. Seeds were sampled for analysis at 15, 20, 25 and 30 days post-anthesis (dpa), as indicated. Expression is represented as the Normalised Relative Quantity (NRQ) of a target gene's expression with respect to three reference genes: *HvActin*, *HvCyclophilin* and *HvGAPDH*. Means and standard errors are shown from the analysis of three biological replicates. See Supplementary Information for results of statistical comparisons using the $\log_2(1/\text{NRQ})$ transformed data.

with expression peaking at 20 dpa (Fig. 3), but almost undetectable in the embryo (not shown), while *HvRCAR35_2538* was expressed at low but detectable levels in the embryo, with expression declining through seed development (Fig. 4), but not in the endosperm (not shown). The expression of the PP2C-encoding gene, *HvABI1d*, showed a similar pattern to *HvRCAR35_47387* in the endosperm, with a peak at 20 dpa. However, it was also expressed in the embryo, with expression increasing through

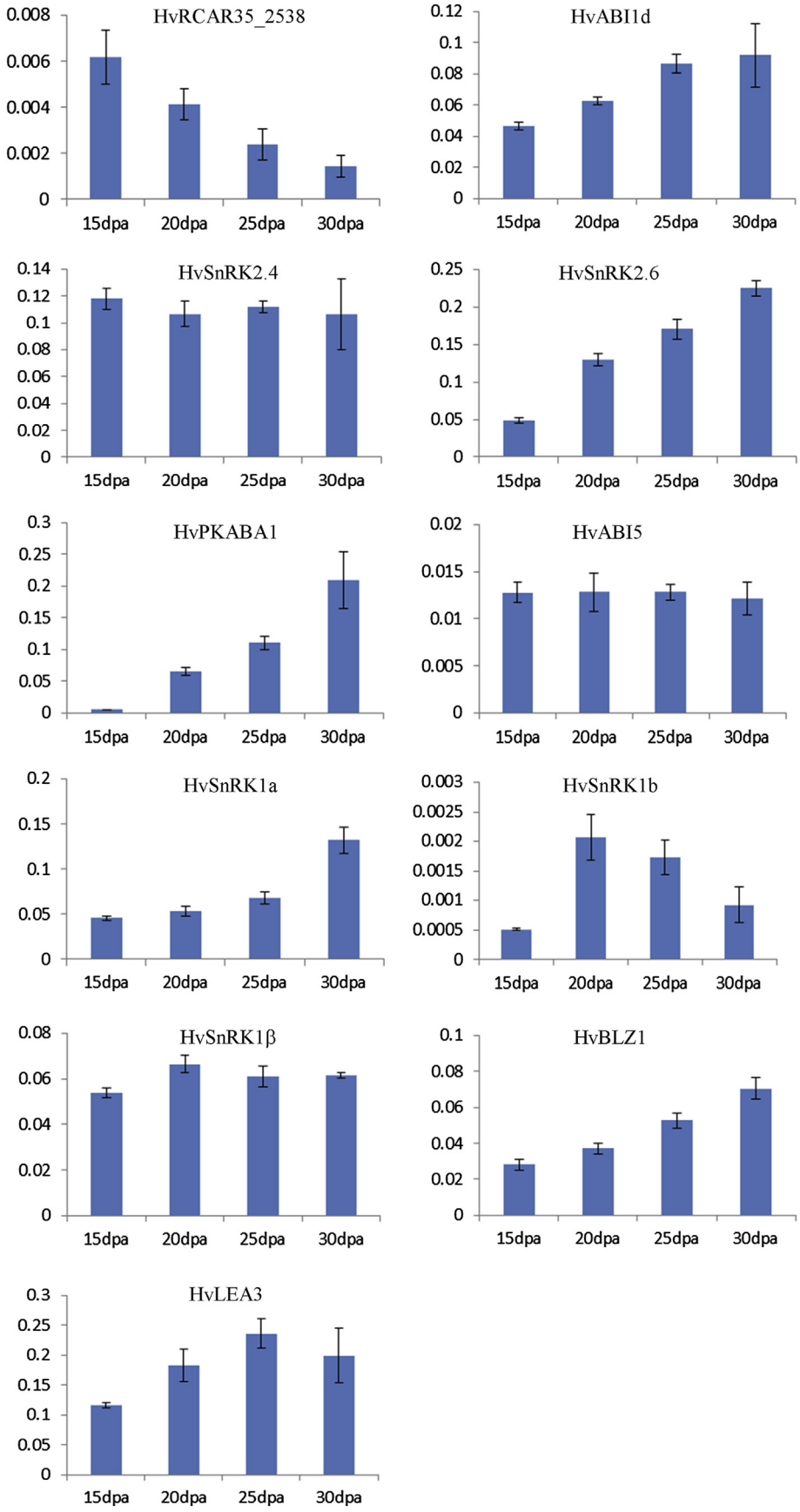

Fig. 4. Differential expression of genes encoding components of the ABA and metabolic signalling pathways in embryo of barley (*Hordeum vulgare*) cv. Triumph analysed by qRT-PCR. Seeds were sampled for analysis at 15, 20, 25 and 30 days post-anthesis (dpa), as indicated. Expression is represented as the Normalised Relative Quantity (NRQ) of a target gene's expression with respect to three reference genes, *HvActin*, *HvCyclophilin* and *HvGAPDH*. Means and standard errors are shown from the analysis of three biological replicates. See Supplementary Information for results of statistical comparisons using the $\log_2(1/\text{NRQ})$ transformed data.

development to its highest point at 30 dpa. At 20 dpa (the peak of its expression in the endosperm), the level of expression in the endosperm was approximately six times that in the embryo, but by 30 dpa, with expression declining in the endosperm but increasing in the embryo, there was only a two-fold difference.

Analysis of expression of the three *SnRK2* genes, *HvSnRK2.6* (Mangelsen et al., 2011; Sreenivasulu et al., 2006; Seiler et al., 2011), *HvPKABA1* (Yamauchi et al., 2002) and *HvSnRK2.4* (Barley1 22k Microarray data; Dash et al., 2012), revealed clear differences. *HvSnRK2.4* was expressed at the highest levels of the three in the endosperm, with expression peaking at 20 dpa. It was also detectable in the embryo but at levels 20 times or more lower than in the endosperm, with expression showing no change through development. *HvSnRK2.6* showed a similar pattern of expression in the endosperm, peaking at 20 dpa, but its expression levels were ten times lower than *HvSnRK2.4*. It showed comparable levels of expression in the embryo, but in contrast to *HvSnRK2.4*, its expression increased from 15 dpa through to 30 dpa, so that by 30 dpa it was expressed more highly than *HvSnRK2.4*. SnRK2.4 is considered to be ABA-independent in Arabidopsis, but the similar expression pattern of *HvSnRK2.4* to other genes encoding components of the ABA signalling pathway suggests that this is not the case in barley.

HvPKABA1 was not expressed at detectable levels in the endosperm, but was expressed in the embryo, with expression increasing from low levels at 15 dpa to its highest at 30 dpa. In other words, expression of this SnRK2 was associated with mid- to late-development in the embryo. Previous studies in wheat and barley have implicated this SnRK2 with a role in controlling the onset of seed dormancy, ABA-induction of gene expression and the suppression of genes that are induced by GA, as well as responses to cold and drought (Gómez-Cadenas et al., 1999; Yamauchi et al., 2002).

Expression of *HvSnRK1a* (also known as *BKIN2*;Halford et al., 1992; Hannapel et al., 1995) increased ($p < 0.05$, LSD) between 15 and 20 days post-anthesis, then did not change significantly (Fig. 3). This gene was also expressed in the embryo (Fig. 4), but levels increased steadily after 15 dpa and were at their highest at 30 dpa. At this point, transcription in the endosperm was still approximately four times higher than in the embryo. *HvSnRK1b* (also known as *BKIN12*) showed a similar pattern of expression in the endosperm, although its level was about half that of *HvSnRK1a*. However, its expression in the embryo, while detectable, was approximately 100 times lower (Fig. 4). This is broadly consistent with the findings of Hannappel et al. (1995), although that study suggested that *HvSnRK1b* was expressed more highly in the endosperm than *HvSnRK1a*.

Currently there is insufficient knowledge of the different roles of SnRK1a and SnRK1b to assess the significance of their different expression patterns. However, specific down-regulation of *HvSnRK1b* in anthers has been shown to have profound effects on pollen development, including a failure to accumulate starch, arrested development and inviability (Zhang et al., 2001). *SnRK1b*homologues have also been implicated in controlling starch accumulation in sorghum and rice grain (Jain et al., 2008; Kanegae et al., 2005).

The gene encoding the SnRK1 non-catalytic subunit, *HvSnRK1β*, had a similar pattern of expression in the endosperm to *HvSnRK1a* and *HvSnRK1b*, with the peak at 20 dpa. In contrast, its expression in the embryo, while similar to that of *HvSnRK1a* at 15 dpa, did not rise in the way that *HvSnRK1a* gene expression did, resulting in it being substantially lower than *HvSnRK1a* by 30 dpa.

The AREBP-encoding gene, annotated as *HvABI5*, showed a similar expression pattern to *HvRCAR35_47387*, *HvSnRK2.4* and *HvSnRK2.6* in the endosperm, with expression peaking at 20 dpa. Expression was also detectable in the embryo but at much lower levels that remained constant throughout development. The expression of *HvBLZ1* was also more than ten times higher in the endosperm than the embryo, with expression in the endosperm rising significantly ($p < 0.05$, LSD) from 15 to 20 dpa and then remaining relatively high.

4. Discussion

The different events that occur in the endosperm and embryo during the onset of seed dormancy have been reviewed by Sreenivasulu et al. (2006), who considered the cells of the endosperm to undergo a form of programmed cell death, while those of the embryo enter true dormancy, from which they emerge at germination. ABA is implicated in bringing about both of these very different developmental programmes and an important conclusion of the present study is that different ABA receptors and SnRK2s are involved in the different tissues. *HvRCAR35_47387* (encoding an ABA receptor), *HvABI1d* (PP2C), *HvSnRK2.4* and the AREBP-encoding gene annotated as *HvABI5* were expressed highly in the endosperm but at much lower levels in the embryo. Conversely, a second ABA receptor-encoding gene, *HvRCAR35_2538*, and the SnRK2-encoding gene, *HvPKABA1*, were expressed at higher levels in the embryo than the endosperm, while *HvSnRK2.6* was expressed at similar levels in both (although *HvSnRK2.4* was the predominant *SnRK2* transcript in endosperm). This adds a level of complexity to what otherwise might be regarded as a relatively simple ABA signalling pathway. It should be noted here that while differential regulation of gene expression is the most likely explanation of these results, post-transcriptional effects, such as alternative splicing resulting in the loss of regions spanning primer-binding sites, could explain why a transcript was detected in one tissue but not the other. It should also be noted that there is no contradiction in the high expression of *HvABI1* (PP2C) in the endosperm, or the fact that its expression pattern closely matches that of *HvSnRK2.4* and *HvRCAR35_47387*. The presence of PP2C would ensure that SnRK2 would only be activated in the presence of ABA.

HvRCAR35_47387, *HvABI1d*, *HvSnRK2.4* and the AREBP-encoding gene annotated as *HvABI5* all peaked in expression in the endosperm at 20 dpa. ABA is required for both storage compound accumulation and the transition from filling to maturation, and we

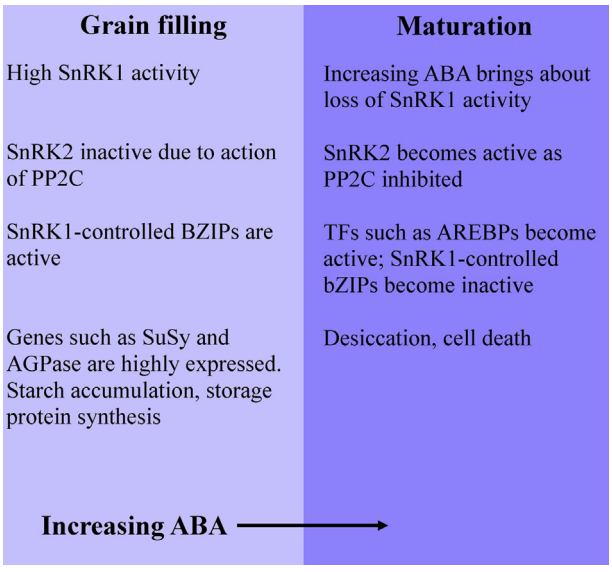

Fig. 5. Diagram summarising a model in which there is a switch in the barley endosperm from a SnRK1-dominated system to one controlled by SnRK2, brought about by the action of ABA, during the transition from grain filling to maturation.

hypothesise that high levels of the signalling components are present at this time to enable these processes to be initiated. SnRK1 on the other hand, has been implicated in the initiation and control of starch biosynthesis in seeds, tubers and pollen (Jain et al., 2008; Kanegae et al., 2005; McKibbin et al., 2006; Zhang et al., 2001). In this study, we found SnRK1 to be expressed in both endosperm and embryo throughout development, with sub-type SnRK1b showing much higher levels of expression in the endosperm than the embryo, while SnRK1a was expressed at more similar levels in both tissues. However, SnRK1 has been shown to be broken down in wheat roots in the presence of ABA, while at least one SnRK2 is activated (Coello et al., 2012). We propose a possible model for the interaction between ABA and the two SnRK families in which the hormone brings about a transition from a SnRK1-dominated state in early grain filling to a SnRK2-dominated state during maturation (Fig. 5).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jcs.2013.06.009.

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