FUSCA3 from barley unveils a common transcriptional regulation of seed-specific genes between cereals and Arabidopsis

Miguel Ángel Moreno-Risueno , Noemi González , Isabel Díaz , François Parcy , Pilar Carbonero and Jesús Vicente-Carbajosa

[1]*Laboratorio de Bioquímica y Biología Molecular, Departamento de Biotecnología, Centro de Biotecnología y Genómica de Plantas – Universidad Politécnica de Madrid, ETS Ingenieros Agrónomos, 28 040 Madrid, Spain, and*
[2]*Laboratoire de Physiologie Cellulaire Vegétale Centre National de la Recherche Scientifique (CNRS), Commissariat à l'Energie Atomique (CEA), Institut National de la Recherche Agronomique (INRA), Université Joseph Fourier, 17 rue des Martyrs, Batiment C2-38 054, Grenoble Cedex 9, France*

Summary

Accumulation of storage compounds in the embryo and endosperm of developing seeds is a highly regulated process that allows seedling growth upon germination until photosynthetic capacity is acquired. A critical regulatory element in the promoters of seed storage protein (SSP) genes from dicotyledonous species is the RY box, a target of B3-type transcription factors. However, the functionality of this motif in the transcriptional regulation of SSP genes from cereals has not been fully established. We report here the identification and molecular characterization of barley FUSCA3, a B3-type transcription factor as yet uncharacterized in monocotyledonous plants. Our results show that both the barley and Arabidopsis *FUS3* genes maintain a conserved functionality for the regulation of SSP genes and anthocyanin biosynthesis in these two distantly related phylogenetic groups. Complementation of the loss-of-function mutant *fus3* in Arabidopsis by the barley *HvFus3* gene resulted in restored transcription from the *At2S3* gene promoter and normal accumulation of anthocyanins in the seed. In barley, HvFUS3 participates in transcriptional activation of the endosperm-specific genes *Hor2* and *Itr1*. HvFUS3, which specifically binds to RY boxes in EMSA experiments, *trans*-activates *Hor2* and *Itr1* promoters containing intact RY boxes in transient expression assays in developing endosperms. Mutations in the RY boxes abolished the HvFUS3-mediated *trans*-activation. *HvFus3* transcripts accumulate in the endosperm and in the embryo of developing seeds, peaking at mid maturation phase. Remarkably, HvFUS3 interacts with the Opaque2-like bZIP factor BLZ2 in yeast, and this interaction is essential for full *trans*-activation of the seed-specific genes *in planta*.

Keywords: FUSCA3, SSP genes, seed gene regulation, barley seeds, developing endosperm, anthocyanin accumulation.

Introduction

In the evolutionary history of plants, seed formation is a key step that favours dispersal and makes possible interruption of the life cycle, enabling the plant to withstand adverse conditions in a quiescent state. Under a newly favourable environment, seeds can resume growth using storage compounds that supply nutrients to the embryo until photosynthetic capability is acquired. During seed formation, reserves are stored throughout the maturation phase, a period that is characterized by growth arrest, acquisition of desiccation tolerance and subsequent entry into a quiescent phase of variable length (Harada, 1997; Vicente-Carbajosa and Carbonero, 2005). In *Arabidopsis thaliana*, essential transcription factors (TFs) that participate in this process have been characterized, such as FUSCA3 (FUS3), ABSCISIC ACID-INSENSITIVE3 (ABI3), LEAFY COTYLEDON1 (LEC1) and LEC2, which genetically control various aspects of the

maturation phase. FUS3, ABI3 and LEC2 belong to the VP1/ABI3-like B3 family of TFs, whereas LEC1 is a CBF-A protein (Giraudat *et al.*, 1992; Lotan *et al.*, 1998; Luerssen *et al.*, 1998; Stone *et al.*, 2001). Loss-of-function mutations in any of these genes severely affect the establishment of seed traits, leading to reduced expression of seed storage proteins (in *fus3*, *abi3*, *lec1* and *lec2*), absence of chlorophyll degradation (in *abi3*) and of anthocyanin accumulation (in *fus3*, *lec1* and *lec2*), reduced sensitivity to abscisic acid (in *abi3* and *lec1*), intolerance to desiccation (in *fus3*, *abi3* and *lec1*) and altered cotyledon identity (in *fus3*, *lec1* and *lec2*) (Bäumlein *et al.*, 1994; Keith *et al.*, 1994; Kroj *et al.*, 2003; Lotan *et al.*, 1998; Meinke, 1992; Meinke *et al.*,1994; Nambara *et al.*, 1995; Parcy *et al.*, 1994, 1997; Raz *et al.*, 2001; To *et al.*, 2006; West *et al.*, 1994). In addition, these regulators are interlocked in a complex hierarchical network of mutual interactions (To *et al.*, 2006).

In dicotyledonous plants, cotyledons are the main tissue for reserve accumulation. In Arabidopsis, only a single layer of endosperm remains in the dry seed, and storage compounds are deposited in the embryo itself (Berger, 2003; Higgins, 1984; Vicente-Carbajosa and Carbonero, 2005). SSP genes are under tight tissue-specific and temporal transcriptional control during the maturation phase. In *Brassica napus*, precise *cis* regulatory elements (CREs) conferring seed-specific expression have been identified in the *NapA* gene promoter, including the B-box comprising the DistB (5′-GCCACTTGTC-3′) and ProxB (5′-CAAACACC-3′) elements, the RY box (5′-CATGCA-3′) and the G-box (5′-CACGTG-3′; Ezcurra *et al.*, 1999). These CREs are also conserved and functionally relevant in the promoters of genes encoding 2S albumins from *A. thaliana* (Kroj *et al.*, 2003). The importance of RY boxes and B3-type TFs in such regulation has been extensively documented. FUS3 and LEC2 seem to regulate SSP genes by directly binding to the RY box in their promoters. In particular, FUS3 specifically binds *in vitro* to the RY box in the *At2S3* and *NapA* gene promoters (Kroj *et al.*, 2003; Reidt *et al.*, 2000). However, FUS3 has also been reported to regulate the synthesis of SSPs through TRANSPARENT TESTA GLABRA 1 (TTG1) or some other intermediate protein (Gazzarrini *et al.*, 2004; Tsuchiya *et al.*, 2004). Although ABI3 regulation of SSP genes is also dependent on the RY box, it is as yet unclear whether it takes place through a direct interaction or by activation of downstream targets such as FUS3 or LEC2. Moreover, a protein complex of ABI3 and two OPAQUE2-like (O2-like) bZIP factors, AtbZIP10 and AtbZIP25, that specifically bind to the G-box of the *At2S1* gene promoter, has been reported to synergistically activate its expression (Lara *et al.*, 2003).

In contrast to dicotyledonous plants, the endosperm is the prevailing reserve tissue in which starch and SSPs accumulate in the majority of monocot seeds. Regulation of SSP genes has been intensively studied, and numerous important *cis*-elements have been identified. In the promoters of genes encoding cereal SSPs, a bipartite endosperm box encompassing the GCN4 like-motif (GLM, 5′-ATGAGTCAT-3′) and the prolamin box (PB, 5′-TGTAAAG-3′), together with the AACA motif, are important regulatory elements (Forde *et al.*, 1985; Takaiwa *et al.*, 1996; Wu *et al.*, 2000). These CREs are bound by TFs of the bZIP, DOF (DNA binding with one finger) and R2R3MYB families, respectively. In barley, the O2-like bZIPs, BLZ1 and BLZ2, and the DOF proteins, BPBF and SAD, activate expression of the *Hor2* gene encoding the B-hordein storage protein, through these *cis*-elements (Díaz *et al.*, 2005; Mena *et al.*, 1998; Oñate *et al.*, 1999; Vicente-Carbajosa *et al.*, 1998). GAMYB, a member of the R2R3MYB family, has been reported to activate the expression of endosperm-specific genes such as the *Itr1* gene, which encodes the trypsin inhibitor BTI-CMe, through the 5′-(C/T)AACA-3′ motif (Díaz *et al.*, 2002). In addition, HvMCB1 and HvMYBS3, which belong to the R1MYB-SHAQYF class, regulate endosperm-specific genes through the 5′-GATA-3′ element (Rubio-Somoza *et al.*, 2006a,b). Transcriptional regulation of SSPs and other endosperm-specific genes is achieved by the combinatorial effect of various TFs.

Most interestingly, the prolamins (the main group of SSPs from cereals) and a major group of dicot seed albumins can be phylogenetically traced to a common ancestor (Kreis and Shewry, 1989). Their conservation relates not only to the protein structure, but also to regulatory elements that are functionally exchangeable between the groups (Vincentz *et al.*, 1997). In maize, VIVIPAROUS1, which is the ABI3 orthologue, is also involved in abscisic acid signalling, establishment of dormancy, and activation of maturation-specific genes during seed development (McCarty *et al.*, 1989, 1991; Suzuki *et al.*, 2003). Natively expressed in the embryo and aleurone, maize VP1 can complement Arabidopsis *abi3* mutants that are impaired in the expression of SSP genes (Suzuki *et al.*, 2001).

In support of the evolutionary relationship between prolamins and the dicot SSPs, the RY box is also conserved in the promoters of SSP genes from cereals, but has not been reported to play a role in their transcriptional regulation or to directly interact with a B3-type TF. Given that cereal prolamins are expressed in the endosperm and not in the embryo, and that VP1 is practically absent from the endosperm (McCarty *et al.*, 1989), we explored the possibility that they could be under the regulation of a FUS3-like protein that, in Arabidopsis, is expressed both in the embryo and in the endosperm. Here, we report the identification and characterization of barley FUSCA3 (HvFUS3, encoded by the *HvFus3* gene, accession number AM418838), and show that it specifically binds to and activates the *Hor2* and *Itr1* genes through the RY box in their promoters. Moreover, we have determined that HvFUS3 interacts *in vivo* with the O2-like bZIP factor BLZ2, and that both contribute to transcriptional activation of these two seed-specific genes. Complementation of the *A. thaliana* loss-of-function mutant

fus3 by *HvFus3* resulted in SSP gene activation and restoration of anthocyanin accumulation to wild-type levels in the Arabidopsis seed.

Results

Identification of conserved putative VP1/ABI3-like B3 binding sites in the promoters of cereal seed-specific genes

As a first step in a systematic search for the FUSCA3 transcription factor (TF) from barley, we explored whether the putative *cis*-motif recognized by this TF (the RY box; Reidt *et al.*, 2000) is present in the promoters of cereal endosperm-specific genes. We analysed the promoters of the barley *Hor2* and *Itr1* genes, which encode a B-hordein storage protein and the trypsin inhibitor BTI-CMe, respectively (Díaz *et al.*, 1995, 2005; Rodriguez-Palenzuela *et al.*, 1989). As shown in Figure 1(a), we found putative RY boxes in both promoters within the first 300 bp upstream of the translation start codon. Extending this analysis to wheat, rice, maize and *Coix* SSP gene sequences showed that putative RY boxes appear to be conserved in many of the studied promoters from these species: α-gliadins and high-molecular-weight glutenins from wheat, type A and B rice glutenins, maize 22 and 15 kDa zeins, and *Coix* α-coixin genes. This observation supports a putative role for the RY box in endosperm-specific gene expression, and prompted us to search for the orthologue of the *Fus3* gene in one monocotyledonous species, namely barley.

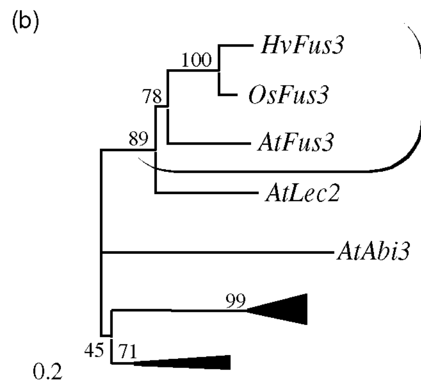
Cloning of the barley HvFus3 *gene*

We initially searched barley EST databases for genes with homology to *AtFUS3* (gene At3g26790), but failed to identify any sequence homologous to the Arabidopsis gene. We extended the search to the rice databases, and found that the rice locus Os01g51610 matched the sequence of the Arabidopsis *Fus3* gene with an e-value of 4.0 e^{-31}. The sequence of this gene, which we designated *OsFus3*, was used in a new search for barley ESTs, resulting in the identification of clones BM376703 and BM376595, with significant homologies. Based on their sequences, we amplified the full-length

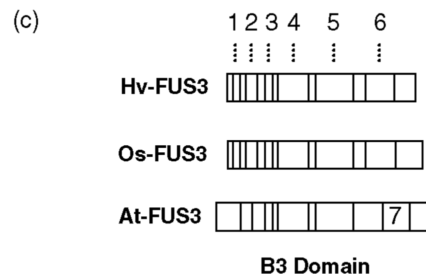
(a)

CEREAL/GENE	RY BOX SEQUENCE	LOCATION
Barley		
B-Hordein	AGGTGAGT**CATGCAT**ACCAAACCT	-280
CMe	AAAATTTC**CATGCA**GCGGCACTCC	-147
Wheat		
-gliadin	AGATGAGT**CATGCATG**TTATCACA	-295
HMW-glutenin	ACCTTATC**CATGCAAG**CTACCTTC	-473
Rice		
GluB-glutelin	TGTCTATA**CATGCA**ACAAACTCTA	-122
GluA-glutelin	AGCAAAAG**CATGCATG**GATATAAT	-271
Maize		
22-kD Zein	GAAGAGAT**CATGCATG**TCATTCCA	-314
15-kD Zein	TTCCACGT**CATGCA**ACGCAACATT	-136
Coix		
-Coixin	GAAGTGAT**CATGCATG**TCATTCCT	-314

(b)



(c)



1 KRRSPSAST

2 VTRKRRSGGRCPR

3 GLRVILQKELK

4 SDVGKLNRLVIPKQHAEAYFPALSCKEGIPLKMQDI

5 WTFKYRYWPNNKSRMYVLENTGDFIQTHGLQAGDFIMIY RDDPCNKYIIGAKKAGDD

6 DISAFIPQADENHEIFDGIFNSLPEIPVANVRYSDFFDP FDDCMDM

7 FNHHINNHNFNFGSNTNKCARFYPVI

Figure 1. Conservation of the RY box in the promoters of cereal endosperm-specific genes and phylogenetic relationships of the Arabidopsis, barley and rice *Fus3* putative orthologues.

(a) Promoter sequence alignment of barley genes encoding seed storage proteins and trypsin inhibitor BTI-CMe in the region of the RY box element. The location of RY box sequences is given as the number of nucleotides upstream of the translation start codon. The genes are *Hor2* (X87232), *Itr1* (X65875), α-gliadin (K03076), high-molecular-weight glutelin (X12929), GluB glutelin (X54193), GluA glutelin (Os01g55690), 22 kDa zein (X55722), 15 kDa zein (M13507) and α-coixin (X63113) (accession numbers in parentheses).

(b) Unrooted phylogenetic tree of the Arabidopsis VP1/ABI3-like B3 genes [At3g26790 (*AtFus3*), At1g28300 (*AtLec2*), At3g24650 (*AtAbi3*), At1g01030, At2g30470, At2g36080, At2g46870, At3g11580, At3g61970, At4g01500, At4g21550, At4g32010, At5g06250] and the barley (HvFus3, AM418838) and rice (OsFus3, Os01g51610) putative orthologues of the *AtFus3* gene. The tree was constructed using the neighbour-joining method based on alignment of the full-length protein sequences. Bootstrapping values are indicated as percentages, and branches that are less related to AtFUS3 were collapsed.

(c) Identification and distribution of conserved motifs among the *HvFus3*, *OsFus3* and *AtFus3* putative orthologues. The motifs, located in their relative positions within the protein sequences, were identified using MEME software based on the deduced amino acid sequences of the proteins encoded by the genes shown in (a).

cDNA of the barley *Fus3* gene (*HvFus3*) from a barley (cv. *Bomi*) developing endosperm sample.

We performed a phylogenetic analysis on *FUS3*- and *ABI3*-related genes from several species. The un-rooted phylogenetic tree obtained (Figure 1b) showed that the genes *HvFus3*, *OsFus3* and *AtFUS3* are phylogenetically related with a bootstrap value of 78 (out of 100), indicating that they are orthologous genes in barley, rice and Arabidopsis. Protein sequence analysis using MEME software identified seven conserved motifs in the sequences of *HvFus3*, *OsFus3* and *AtFUS3*. The rice and barley FUS3 proteins shared motifs 1–6 and motifs 2–5, respectively, with the AtFUS3 protein. Motif 1 was only present in the cereals, and motif 7 was shared in AtFUS3 and the protein encoded by the At4g01500 gene.

In the Arabidopsis fus3 *mutant background,* HvFus3 *activates transcription from the* At2S3 *gene promoter and restores anthocyanin accumulation to wild-type levels*

To test the hypothesis of a conserved function between *HvFus3* and *AtFUS3*, we transformed the Arabidopsis *fus3* mutant with *HvFus3*, and, as a control, with *AtFus3*. Binary plasmids P_{35S}:*HvFus3* and P_{35S}:*AtFUS3* were transformed into the *fus3-3 gl1* mutant containing the GFP gene under the control of the promoter of the *At2S3* gene (accession number At4g27160; Kroj *et al.*, 2003; Luerssen *et al.*, 1998) that encodes the 2S albumin seed storage protein. Albumin 2S and its expression have been shown to be strongly reduced in *fus3* mutant seeds. Seeds of wild-type and *fus3-3 gl1* plants, both transformed with the GFP gene under the control of the *At2S3* gene promoter, were analysed for green fluorescence and under bright-field microscopy. As shown in Figure 2, transcription of the GFP gene, which is strongly diminished in the *fus3* mutant background, was restored to almost wild-type levels in seeds constitutively expressing HvFUS3 or AtFUS3. In contrast to wild-type seeds, *fus3* mutant seeds are purple at the end of their development because anthocyanin biosynthesis is de-repressed in this mutant background (Figure 2) (Luerssen *et al.*, 1998). In *fus3-3 gl1* plants complemented with either the *HvFus3* or *AtFUS3* genes, we observed green-coloured seeds similar to those of the wild-type plants (Figure 2).

Expression patterns of HvFus3 *during barley seed development*

Extensive homology searches using the HvFUS3 sequence in EST databases only produced positive matches with clones derived from seed tissues. The expression pattern of the *HvFus3* gene in barley developing endosperms was investigated using real-time quantitative PCR. Endosperms were separated from the rest of the seed and collected on various days after pollination (10, 14, 18, 22 and 26 d.a.p.). In addition, embryos at 22 d.a.p. were also sampled. Variation in the transcript levels was analysed and normalized to those for the barley *Actin2* gene.

As shown in Figure 3(a), the transcripts of *HvFus3* increased by 1.5-fold between 10 and 22 d.a.p., then decreased at 26 d.a.p. to the same level detected at 10 d.a.p.. This expression pattern is similar to that of *HvBlz2* (accession number Y10834, a known regulator of the *Hor2* gene), which encodes a barley bZIP factor of the Opaque-2 class (Oñate *et al.*, 1999). Therefore, the *HvFus3* transcript expression profile is consistent with the possibility of this factor being a regulator of the *Hor2* and *Itr1* genes in the developing endosperm.

To determine the spatial expression of *HvFus3* within developing seeds, *in situ* hybridization assays were performed in samples collected at 22 d.a.p.. In samples hybridized with the corresponding specific antisense probe, a strong signal was observed in endosperm and aleurone cells, as well as in the embryo, but not in the testa or the pericarp (Figure 3b, upper and middle panels). Signal intensities were in agreement with quantitative PCR values obtained for developing endosperm (approximately 1.0) and

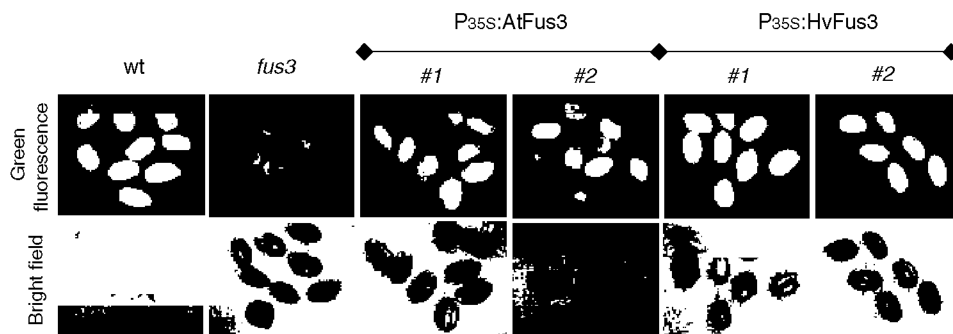


Figure 2. Complementation of the Arabidopsis *fus3* mutant (Luerssen *et al.*, 1998) with *HvFus3* and *AtFus3*.
Wild-type and *fus3* plants containing the GFP gene under the control of the *At2S3* (2S albumin) gene promoter were transformed with the barley and Arabidopsis *Fus3* genes under the control of the CaMV 35S promoter. Seeds of transformed plants were analysed for green fluorescence and under bright-field microscopy, to assess expression from the 2S3 promoter and anthocyanin accumulation in seeds, respectively. #1 and #2 correspond to independent transgenic lines.

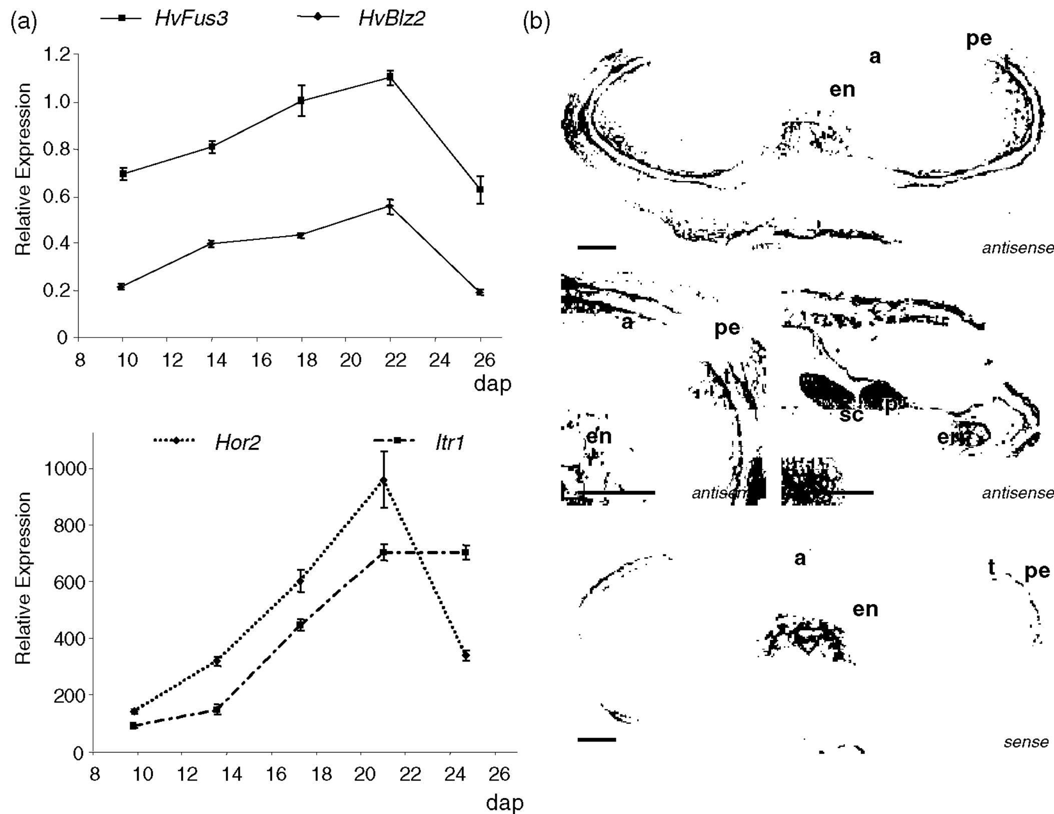


Figure 3. Expression analyses of the barley *HvFus3* gene by real-time quantitative PCR and *in situ* hybridization during barley seed maturation.
(a) Relative mRNA content of the *HvFus3* and *Blz2* TF genes and the *Hor2* and *Itr1* genes encoding the seed storage protein B-hordein and the trypsin inhibitor BTI-CMe, respectively, in developing endosperms from 10 to 26 d.a.p. All values were standardized to the barley *Actin2* mRNA levels.
(b) Spatial expression pattern of the HvFus3 gene in developing barley seeds at 22 d.a.p. by *in situ* hybridization analysis. Hybridization signals were obtained using the antisense HvFus3 probe (upper and middle panels) or the sense probe (lower panel) as a negative control. a, aleurone; en, endosperm; er, embryo root; fp, foliar primordia; pe, pericarp; sc, scutellum; t, testa. Scale bars = 250 μm.

embryo (0.54). When samples were hybridized with the sense probes, used as negative controls, no signal above background was detected (lower panel).

HvFUS3 binds to the RY box in the promoters of the At2S3, Hor2 *and* Itr1 *genes*

As HvFUS3 activates transcription of the *At2S3* promoter in Arabidopsis, and putative *cis*-motifs capable of being recognized by this TF are conserved in the promoters of many seed-specific genes, we tested whether HvFUS3 could specifically bind *in vitro* to the RY boxes present in the promoters of *Hor2*, *Itr1* and *At2S3* genes using electrophoretic mobility shift assays (EMSA).

The HvFUS3 protein and a mutated version in which the aspartic acid residue at position 75 was changed to asparagine (HvFUS3*), with native Arabidopsis FUS3 as a control, were expressed as GST fusions in *Escherichia coli*. The HvFUS3* mutation was designed on the basis of an *abi3* mutant that contained this amino acid change at the equivalent residue of the B3 domain, which presumably impairs its DNA-binding capacity. As shown in Figure 4(a), when the three proteins were incubated with the radioactively labelled oligonucleotide probe *At2S3-RY* derived from the *At2S3* gene promoter, a retarded band was observed for the HvFUS3 and AtFUS3 recombinant proteins but not for the mutated version HvFUS3*. Similarly, when we assayed the probe *Hor2-RY* derived from the *Hor2* gene promoter, we detected a retardation band for the barley HvFUS3 protein but not for its mutated version. In addition, HvFUS3 also bound *in vitro* to the *Itr1-RY* probe derived from the *Itr1* gene promoter (Figure 4b). Binding specificities were confirmed by competition titrations, using up to 50-fold molar excess, using non-labelled *Itr1-RY* and *Itr1-ry* probes in which the CATGCA core sequence was changed to ATCCTG.

HvFUS3 activates transcription of the Hor2 *and* Itr1 *promoters in a RY box-dependent manner*

To evaluate the functional relevance of the interaction observed *in vitro* between HvFUS3 and the RY box in the

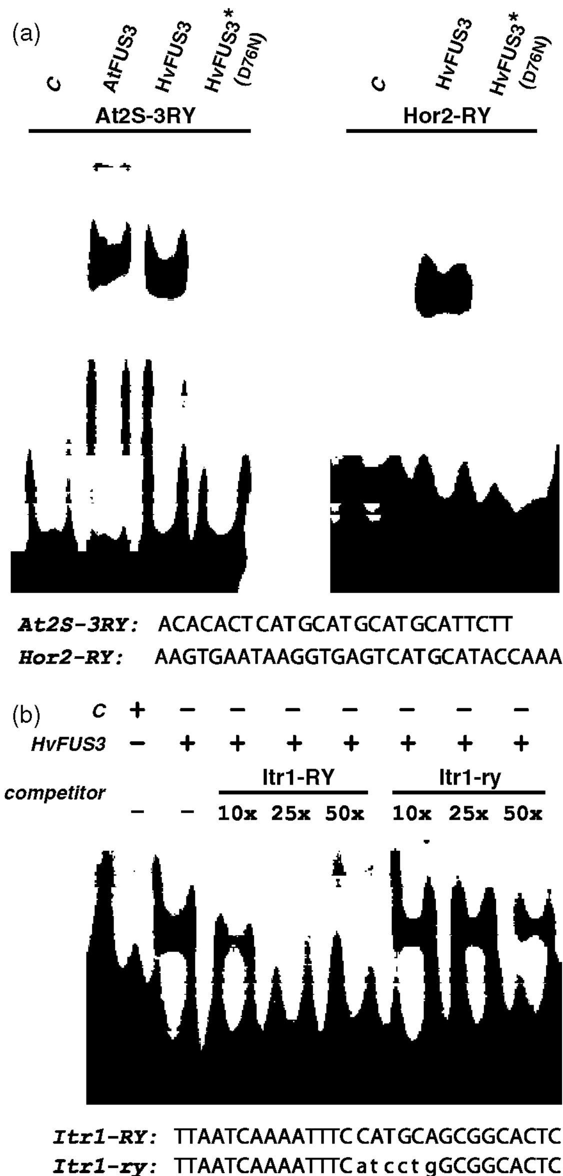


Figure 4. Electrophoretic mobility shift assays (EMSA) of recombinant AtFUS3, HvFUS3 and mutated HvFUS3* proteins using probes derived from the *At2S*, *Hor2* and *Itr1* gene promoters.

(a) EMSA of the recombinant proteins AtFUS3, HvFUS3 and mutated HvFUS3* (aspartic acid at position 75 was changed to asparagine) using the ^{32}P-labelled probes *At2S3-RY* and *Hor2-RY*, derived from the *At2S3* and *Hor2* gene promoters.

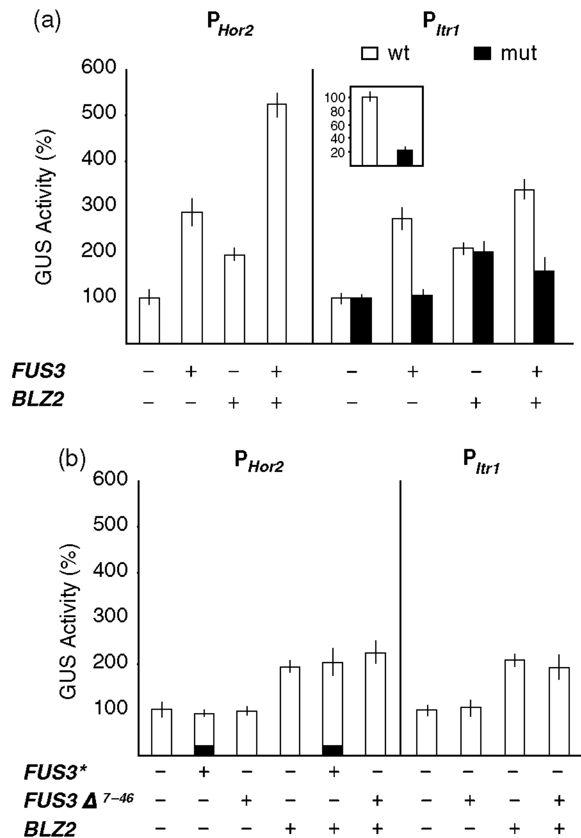
(b) EMSA of the HvFUS3 protein with ^{32}P-labelled probes derived from the *Itr1* gene promoter. *Itr1-RY* corresponds to the native sequence and *Itr1-ry* is a mutated version (lower case). Competition experiments were performed using increasing amounts (10 x, 25 x and 50 x) of the indicated unlabelled wild-type or mutated probes. The B3-binding target sequence is shown in bold.

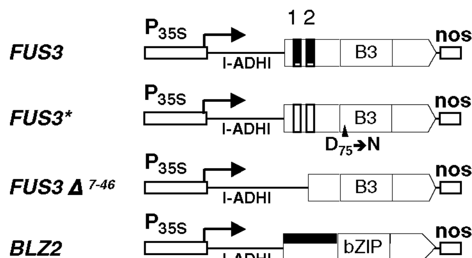
promoters of the *Hor2* and *Itr1* genes, we carried out transient expression assays in co-bombarded barley endosperms (cv. *Bomi*). Promoter:GUS reporters (Figure 5c), corresponding to constructs P_{Hor2} and P_{Itr1} (wt) as described by Díaz *et al.* (2002), and a newly generated construct P_{Itr1} (mut), in which the native RY box (5′-CATGCA-3′) at position -147 was changed to 5′-ATCCTG-3′, unable to interact with HvFUS3 protein *in vitro* were assayed. Effector constructs included *HvFus3* and two mutated versions of it: (i) *HvFus3** (D_{75}N, described above), and ii) *HvFus3*Δ^{7-46}, which has a deletion of the 7th to the 46th amino acid residues. The ORF of *HvBlz2* was also used as an effector. All these effector constructs were driven by the CaMV 35S promoter followed by the first intron of the maize alcohol dehydrogenase I (*AdhI*) gene, and flanked downstream by the 3′ *nos* terminator. Freshly isolated developing barley endosperms were bombarded either with the reporters alone or in combination with the effectors at a 1:1 molar ratio. The GUS activity obtained after bombardment of the reporters without effectors was arbitrarily assigned a value of 100%. As shown in Figure 5(a), when we co-transfected P_{Hor2} and P_{Itr1} (wt) together with *HvFUS3*, the GUS activity increased threefold, but use of P_{Itr1} (mut) did not result in any significant increase (Figure 5a). It is worth noting that this mutation in the RY box of the *Itr1* promoter did not prevent its *trans*-activation by BLZ2, but reduced fivefold (inset to Figure 5a) the intrinsic expression of the native promoter, indicating the importance of this *cis*-motif. Increased activation was observed for combinations of HvFUS3 and BLZ2 on these endosperm-specific gene promoters. Thus, when both *HvFUS3* and *BLZ2* were co-bombarded as effectors with the reporters P_{Hor2} and P_{Itr1} (wt), higher GUS activities were obtained compared to individual effectors (Figure 5a), and this was especially conspicuous in the case of P_{Hor2}. However, when *HvFUS3* and *BLZ2* were co-bombarded using the mutated version P_{Itr1} (mut) as the reporter, we did not observe any increase above the GUS activity detected when *BLZ2* was used as the sole effector. Moreover, a mutation in either the B3 domain (*FUS3**), abolishing DNA binding (Figure 4a), or the mutation *FUS3*Δ^{7-46} (with deletion of a conserved N-terminal region), which lacks activating capacity, did not increase BLZ2-mediated *trans*-activation when both type of effectors were used in the transient expression experiments (Figure 5b).

Taken together, these results indicate that HvFUS3 *trans*-activates the *Hor2* and *Itr1* gene promoters in developing barley endosperm through binding to the RY box in a combinatorial interaction with the bZIP factor BLZ2, among other TFs.

The intrinsic activation capacity of HvFUS3 protein is linked to its C-terminal region

We assayed the activation properties of HvFUS3 in the yeast one-hybrid system. In addition to *HvFUS3* encoding the whole protein, a series of derived fragments containing the B3 domain, the N- and the C-terminal regions, alone or in combination with the B3 DNA-binding domain, were





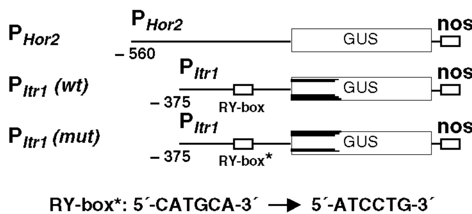


Figure 5. Transient expression assays of GUS activity under the control of the *Hor2* and *Itr1* gene promoters using HvFUS3 and BLZ2 as effectors in barley developing endosperms.

(a) Co-bombardment experiments on barley developing endosperms performed using the indicated combinations of reporter and effector plasmids at a 1:1 molar ratio. β-glucuronidase (GUS) activity in endosperms is expressed as percentage of the GUS activity for the control without effector(s). GUS expression driven by the P_{Itr1} (wt) construct and its mutated version P_{Itr1} (mut) is shown in the insert (white and black, respectively). In each experiment, sets of five endosperms were bombarded, and three replicate experiments were performed. Standard errors are indicated.

(b) Co-bombardment experiments on barley developing endosperms performed as in (a) but using the point mutant *HvFus** ($D_{75} \rightarrow$ N) and the deletion mutant (*HvFUS3*Δ^{7-49}) as effectors.

(c) Schematic representation of the reporter and effector constructs. Reporter constructs P_{Hor2} and P_{Itr1} (wt) have been described previously (Díaz *et al.*, 2002), and P_{Itr1} (mut) is a mutated version differing at the indicated nucleotides in the RY box (see Figure 4). The position of putative *cis* DNA-binding sites for the FUSCA3 TF, or its mutated version, in the *Itr1* promoter is indicated. Effector constructs *HvFus3*, *HvFus3** ($D_{75} \rightarrow$ N), *HvFUS3*Δ^{7-46} and *HvBlz2* contain the indicated ORFs under the control of the CaMV 35S promoter (P35S) followed by the first intron of the maize *AdhI* gene (I-*AdhI*).

fused to the GAL4 binding domain (BD), and β-galactosidase activity was measured in *Saccharomyces cerevisiae*. As shown in Figure 6(a), cells transformed with the BD–*FUS3* construct produced β-galactosidase activity of 200 Miller units, indicating that FUS3 functions as a transcriptional activator in yeast. A deletion construct, containing the C-terminal region alone, increased this activity by approximately 3.5-fold, but none of the other constructs assayed showed significant activity. This indicates the presence of an activation domain within the C-terminal region of HvFUS3, and that the inability of the mutated version *FUS3*Δ^{7-46} to *trans*-activate the *Hor2* and *Itr1* promoters (see Figure 5b) is not due to the lack of a region necessary for direct activation, but probably due to its inability to interact with other transcription factors such as BLZ2 (see below).

HvFUS3 interacts with BLZ2 in the yeast two-hybrid system, and the HvFUS3 N-terminal region is necessary for this interaction

The co-existence of *HvFus3* and *HvBlz2* mRNAs in the barley endosperm, as well as the observation that *trans*-activation of the *Hor2* and *Itr1* gene promoters mediated by HvFUS3 in this tissue is increased by BLZ2, suggested that both TFs might interact *in vivo*. We explored this possibility in the yeast two-hybrid system using the *LacZ* and *His3* genes as reporters. The *HvFus3*, *HvFus3*Δ^{7-46} and *HvBlz2* ORFs were fused in-frame to yeast GAL4-BD and GaL4-AD (activation domain), and used to transform *S. cerevisiae* cells. As shown in Figure 6(c), expression of BD–BLZ2 was sufficient to increase reporter gene activity, consistent with the previous observation that BLZ2 possesses a transcription activation domain (Oñate *et al.*, 1999). Co-expression of BD–BLZ2 with AD–FUS3 led to a further increase in β-galactosidase activity, demonstrating an interaction between BLZ2 and HvFUS3. This increase was not observed with the mutant HvFUS3Δ^{7-46}. No activation of the reporter gene was observed when yeast cells were co-transformed with the two pGBT9 (BD) and pGAD424 (AD) plasmids without inserts, or with the former in combination with AD–*FUS3* or AD–*FUS3*Δ^{7-46}.

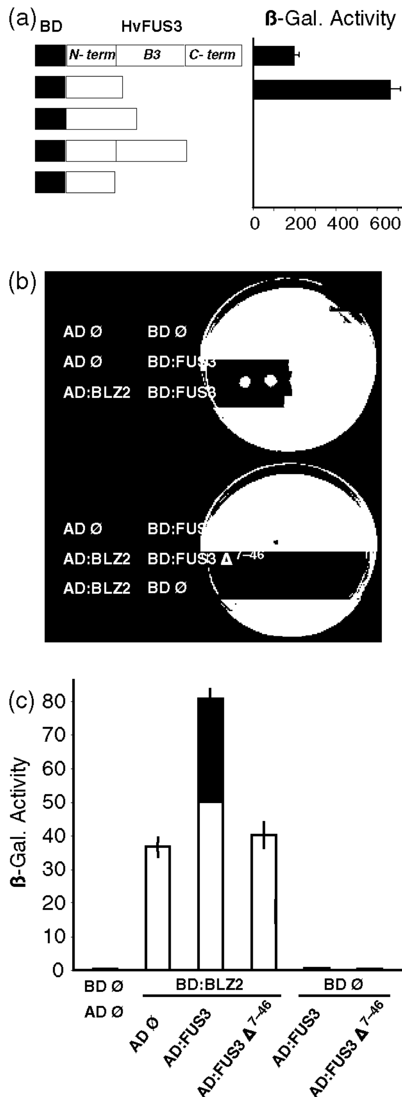


Figure 6. Yeast *S. cerevisiae* one- and two-hybrid system assays of HvFUS3.
(a) The indicated constructs containing the *HvFus3* ORF and a series of derived fragments were used to transform *S. cerevisae* strain SFY526. *LacZ* induction was measured to detect activation domains within this protein.
(b) Effector constructs were used to transform *S. cerevisae* strain HF7C containing the reporter gene *His3* under the control of the *Gal1* promoter. Growth of yeast cells on minimum His$^-$ agar medium containing 60 mM 3-aminotriazole was assayed to test the interaction between HvFUS3, or HvFUS3Δ^{7-46}, with BLZ2. Each colony cluster comes from a different transformation event.
(c) Protein–protein interaction of HvFUS3 and BLZ2 in the yeast two-hybrid system. The indicated effector constructs were used to transform *S. cerevisae* strain SFY526 containing the reporter gene *LacZ* under the control of the *Gal1* promoter. *LacZ* induction was measured to test the interaction between HvFUS3 and BLZ2, and β-galactosidase activity in liquid cultures was calculated from six independent replicates in two assays. Standard errors are indicated.
HvFus3, full-length ORF; N-term, 1st to 60th amino acids of the *HvFus3* ORF; B3, DNA-binding domain (61th to 171th amino acids) of the *HvFus3* ORF; C-term, 172th to 285th amino acids of the *HvFus3* ORF; BD, Gal4 DNA-binding domain; AD, Gal4 DNA activation domain; FUS3Δ^{7-46}, deletion from the 7th to the 46th amino acids of the *HvFus3* ORF; BLZ2, full-length HvBlz2 ORF.

The interaction between FUS3 and BLZ2 was confirmed using activation of the *HIS3* reporter gene and growth in His$^-$ medium (Figure 6b).

Discussion

FUS3 from *A. thaliana* is a central regulator of seed development that is involved in the establishment of dormancy, desiccation tolerance and cotyledon identity, as well as in the synthesis of SSPs and anthocyanin accumulation (Gazzarrini *et al.*, 2004; Keith *et al.*, 1994). Participation of FUS3 protein in these processes is coordinated within a hierarchical network involving other key regulators, namely ABI3, LEC1 and LEC2 (Kroj *et al.*, 2003; To *et al.*, 2006). In this study, we have isolated and functionally characterized the first FUS3-like factor from a monocot species. Our data show that barley *HvFUS3* is the putative orthologue of AtFUS3, as both factors are not only phylogenetically related but can perform similar functions in distantly related systems. Like AtFUS3 in Arabidopsis seeds, HvFUS3 promotes the activation of seed-specific genes in barley. Furthermore, when introduced into a *fus3* loss-of-function background in the model plant *A. thaliana*, it is able to restore the synthesis of SSPs and anthocyanin accumulation.

FUSCA3-mediated control of SSP genes and anthocyanin accumulation is conserved among various angiosperm species

A major goal of this investigation was to establish the possible existence of a *FUS3* gene in monocot species, and to determine the degree of conserved functionality with the gene described in Arabidopsis. Following an extensive search of database sequences, we could identify the rice *OsFus3*, and thus isolated a barley cDNA corresponding to a gene that was the putative ortholog of *AtFUS3*. When the barley *FUS3* cDNA was introduced into Arabidopsis *fus3* plants, we observed restoration of features that are typically impaired in the mutant, such as activation of SSP gene transcription and accumulation of anthocyanins (Figure 2). Complementation of the *fus3* phenotype in these respects indicates that HvFUS3 might be participating in the same regulatory network as the AtFUS3 protein, probably by interacting with other transcription factors and controlling the subsequent downstream target genes.

The capacity of HvFUS3 to activate SSP gene expression in Arabidopsis prompted us to investigate whether the *FUS3* orthologous gene from barley, a monocot species, might also be involved in the transcriptional regulation of SSP and other seed-specific genes in this cereal. We addressed this question by analysis of seed gene promoters that contain conserved putative binding sites for this TF (the RY box in Figure 1) (Reidt *et al.*, 2000) in transient expression experiments involving particle bombardment of barley developing

endosperms. These experiments demonstrated that (i) HvFUS3 *trans*-activates the promoters of the genes *Hor2* and *Itr1* encoding the B-hordein storage protein and the BTI-CMe trypsin inhibitor, respectively, and (ii) the RY box is an essential element in *trans*-activation of these seed-specific genes. This was particularly significant in the *Itr1* gene promoter, where mutation of this *cis*-element resulted in an 80% decrease of the GUS activity controlled by the *Itr1* promoter (Figure 5).

Activation by HvFUS3 of barley SSP genes and its interchangeability with the Arabidopsis FUS3 protein is consistent with the current hypothesis of a shared origin for dicotyledonous seed globulins/albumins and monocotyledonous prolamins. Identification of HvFUS3 provides further evidence in support of the idea that evolutionary conservation affects not only protein structural domains, but it also extends to gene regulatory *cis*-regions and *trans*-acting factors (Lara *et al.*, 2003; Vincentz *et al.*, 1997).

HvFUS3-mediated transcriptional activation through interaction with RY boxes in target gene promoters

Arabidopsis FUS3 has been reported to activate transcription of SSP genes by binding to the RY box in their promoters (Kroj *et al.*, 2003; Reidt *et al.*, 2000). However, other studies show that FUS3 regulates the synthesis of SSPs through the epidermal morphogenesis regulator TTG1 or another protein functioning in a cell non-autonomous manner (Gazzarrini *et al.*, 2004; Tsuchiya *et al.*, 2004). Our results show that HvFUS3 binds specifically to the RY box in the promoters of the *Hor2* and *Itr1* genes, and activates transcription from their promoters in transient expression assays (Figures 4 and 5). Furthermore, when these assays were performed with HvFUS3*, a mutated version that is unable to bind to the RY box in EMSA experiments, the *trans*-activation capacity upon the *Hor2* and *Itr1* gene promoters was lost. We also showed in reciprocal experiments that mutations of the RY box, abolishing HvFUS3 binding to the promoter of the *Itr1* gene, impaired HvFUS3-mediated *trans*-activation. Taken together, our data indicate that activation of these target genes *in planta* most likely occurs through direct interaction of FUS3 with the RY boxes present in their promoters. If a parallel scenario to Arabidopsis is assumed, the possibility cannot be ruled out that HvFUS3 might also act through an intermediate factor (TTG1 or another protein), which would direct the final interaction with RY boxes in target promoters, or even operate in a redundant manner with other B3-type TFs, such as ABI3 or LEC2. In this respect, it is worth mentioning that cereal seeds accumulate prolamins and hydrolase inhibitors in the starchy endosperm, in contrast to the SSPs of Arabidopsis, which are deposited in the cotyledons. This situation may account for potential differences in the precise mode of action of FUS3 in the two systems. In the case of barley, our data showing co-expression of HvFUS3 and prolamin genes in the starchy endosperm are in favour of a direct interaction, whereas the non-overlapping expression of FUS3 and SSPs in the embryo of Arabidopsis might require additional connections (Tsuchiya *et al.*, 2004).

Involvement of HvFUS3 and BLZ2 in the combinatorial regulation of seed-specific gene expression

Transcriptional regulation is normally exerted by the concerted action of multiple transcription factors responding to distinct signals. Consequently, native levels of gene expression arise from the effects of various TFs acting on a particular promoter and their combinatorial interactions. The importance of such interactions (e.g. ABI3–bZIP10/25, ABI3–ABI5, VP1–TRAB, O2–PBF, GAMYB–BPBF, etc.) has already been reported in the regulation of seed-specific genes of both monocot and dicot species (Hobo *et al.*, 1999; Lara *et al.*, 2003; Nakamura *et al.*, 2001).

Our transient expression assays show that co-transfection with HvFUS3 and BLZ2 increased the GUS activity above values obtained when each effector was used independently, indicating that a combination of HvFUS3 and BLZ2 is necessary for full *trans*-activation of the *Hor2* and *Itr1* genes. In addition, the TFs are able to interact *in vivo* in the yeast two-hybrid system. When the mutated versions HvFUS3*, lacking DNA-binding capacity (Figure 4), or HvFUS3Δ^{7-46}, which is unable to interact with BLZ2 (Figure 6), were used in transient experiments, *trans*-activation of the *Hor2* and *Itr1* promoters did not occur (Figure 5). Moreover, the increased activation capacity expected for combination with BLZ2 was also lost in both cases, pointing to the importance of interaction of these two TFs as a requisite for their functionality.

The spatial distribution of transcripts within the barley seed is also in agreement with the regulation of target genes *Hor2* and *Itr1* and the importance of a combined action of HvFUS3 and BLZ2. During seed development, *HvFus3* transcripts are present at significant levels in both the endosperm and the embryo (Figure 3, and data not shown). However, transcripts of *Blz2* are almost undetectable in the embryo at the same developmental stage (2500-fold less than *HvFus3* in the endosperm and 2000-fold less than *HvFus3* in the embryo). Transcripts of the *Hor2* and *Itr1* genes are abundant in the endosperm (Figure 3) and nearly absent from the embryo (data not shown). The absence of BLZ2 in the embryo could explain the lack of expression of the *Hor2* and *Itr1* genes in embryos even at high levels of *HvFUS3*. However, when the promoter of one of these genes (*Itr1*) driving expression of the GUS reporter was introduced into tobacco plants, reporter activity also appeared in the embryo (Díaz *et al.*, 1995). As described here for the transgenic effect of *HvFUS3* in Arabidopsis, a plausible explanation is that the dicotyledonous genes corresponding to

HvFUS3 and *Blz2* (*AtFUS3* and *AtbZIP10/25* in Arabidopsis, Lara *et al.*, 2003; Parcy *et al.*, 1997), natively co-expressed in the embryo, are responsible for the observed pattern.

The results of our studies and those of others indicate that expression of a conserved set of SSP genes occurs in different tissues (endosperm and embryo) depending on the prevailing site of accumulation of reserves, and relies on the combined action of various regulators. Some TFs, such as FUS3, are common to both tissues and seem to have maintained this functionality within them. In this respect, the HvFUS3 regulatory activity in the cereal endosperm described here is in line with the regulation of SSP genes by FUS3 in the Arabidopsis embryo, which has also been reported for At2S3:GFP in the endosperm layer (Kroj *et al.*, 2003). Similarly, ABI3 in dicotyledonous species and VP1 in monocots are expressed in both the embryo and the endosperm aleurone. By contrast, other TFs central to this regulation show overlapping patterns that differ from those above. For instance, the bZIP Opaque2, which is exclusively expressed in the cereal endosperm, seems to be functionally replaced by Opaque2-like bZIPs that are present in dicot embryos (Lara *et al.*, 2003).

Interactions between B3-type and bZIP transcription factors that have important implications in seed gene regulation have been reported, both in Arabidopsis (ABI3 and AtbZIP10/25, Lara *et al.*, 2003; ABI3 and ABI5, Nakamura *et al.*, 2001) and in cereals (TRAB1–VP1 in rice, Hobo *et al.*, 1999). The novel capacity of HvFUS3 to interact with bZIP factors presented in this work prompts the search for additional interactions. Comparison of such investigations between Arabidopsis and cereals is particularly attractive, as the existence of specific TFs with distinct patterns of expression opens up various possibilities for combinatorial interactions that could eventually explain the allocation of storage compounds into embryo or endosperm.

Experimental procedures

Cloning of the barley Fus3 *gene, and generation of mutated constructs* Fus3* *and* Fus3Δ^{7-46} *and derived fragments*

The ORF of the *HvFus3* gene was amplified from first-strand cDNA of developing endosperm (22 d.a.p.) of barley cv. Bomi by PCR using the primers 5′-ATGGCCGCCATCAGCAGCA-3′ (primer 1) and 5′-TCACATCAGAGGCCCAGACT-3′ (primer 2) derived from the publicly available sequences BM376703 and AJ484391, respectively. The resultant amplified fragment was cloned in pGEM T-easy (Promega, http://www.promega.com/) and used for molecular characterization of the *Fus3* gene and generation of its derived fragments and mutated versions. *Fus3*Δ^{7-46}, corresponding to a deletion of the 7th to 46th amino acids of the ORF, was generated by PCR using primer 5′-ATGGCCGCCATCAGCAGCGGCGGTG-TCCGGTGGATG-3′ and primer 2. In the mutated form of *Fus3*, the nucleotides encoding aspartic acid at position 75 were altered to encode asparagine, using site-directed mutagenesis with a PCR approach. The base changes were introduced in two overlapping fragments. One of them, spanning 232 bp, was amplified by PCR using primer 1 and the primer 5′-GGCTTATGTTACTGTTACGG-3′, and the other, of 646 bp, was obtained using primer 5′-CCGTAA-CAGTAACATAAGCC-3′ and primer 2. These fragments were annealed and extended by a PCR reaction using the two external primers 1 and 2. Other fragments derived from *HvFus3* ORF were obtained by PCR using the combinations (i) primer 1 and 5′-CCCAGGCACTTGATGTGGT-3′ to amplify the N-terminal region, (ii) 5′-TTACGAGTTATTCTGCAGAAGG-3′ and 5′-ATCATCTCCCG-CCTTCTTTG-3′ (primer 3) to amplify the B3 domain, (iii) primers 1 and 3 to amplify the N-terminal part plus the B3 domain, and (iv) 5′-CTAGTTGCTGCTATGCCACA-3′ and primer 2 to amplify the C-terminal domain.

Phylogenetic tree and identification of conserved motifs

The amino acid sequences of the Arabidopsis VP1/ABI3-like B3 domain genes At1g01030, At1g28300 (*AtLec2*), At2g30470, At2g36080, At2g46870, At3g11580, At3g24650 (*AtAbi3*), At3g26790 (*AtFus3*), At3g61970, At4g01500, At4g21550, At4g32010 and At5g06250 were obtained from http://www.Arabidopsis.org/ .The rice putative *AtFus3* orthologue (Os01g51610) was obtained from http://www.tigr.org/tdb/e2k1/osa1. The amino acid sequence of the barley HvFUS3 protein was deduced using the 'Translate' tool at http://www.expasy.ch/. Alignment of full-length amino acid sequences through CLUSTAL W, phylogeny construction, and bootstrap tests of phylogeny by means of the neighbour-joining method and the Poisson correction model were performed using MEGA version 3.1 (Kumar *et al.*, 2004). Conserved motifs within the amino acid sequences specified above were identified using MEME software http://meme.sdsc.edu/meme/intro.html) as described by Bailey and Elkan (1994). Default parameters were used with the following exceptions: the occurrence of a single motif was set to any number of repetitions, the maximum number of motifs to find was set to 50, and the minimum width of each motif was set to eight amino acid residues.

Expression of HvFUS3 in Arabidopsis fus3 *loss-of-function and wild-type genetic backgrounds*

Col-0 and *fus3-3 gl1* (Luerssen *et al.*, 1998) transgenic plants carrying a fusion of the *At2S3* promoter and the coding sequence of the GFP gene have been previously described by Kroj *et al.* (2003). The P35S:*HvFus3* and P35S:*AtFus3* constructs were created by combination of the respective barley and Arabidopsis *Fus3* ORFs as described by To *et al.* (2006). The resulting plasmids were used to transform *fus3* p*At2S3*:GFP plants by the *Agrobacterium*-mediated floral dip method (Clough and Bent, 1998). Seeds from the transformed plants were harvested and plated on medium containing Basta to select transgenic plants harbouring p35S:*HvFus3* and p35S:*AtFus3*. Specific primers for *HvFus3* and *AtFus3* were used to verify the presence of the respective constructs by PCR.

Real-time quantitative RT-PCR analyses

Real-time quantitative RT-PCR studies were performed to analyse variation in mRNA accumulation for the genes *HvFus3*, *HvBlz2*, *Hor2* and *Itr1*. Developing endosperms of barley cv. *Bomi* at 10, 14, 18, 22 and 26 days after pollination (d.a.p.) and immature embryos at 22 and 26 d.a.p. were isolated and collected. Total RNA was purified from frozen tissues by phenol/chloroform extraction, followed by precipitation with 3 M LiCl (Lagrimini *et al.*, 1987) and digestion with

DNase (DNase I, RNase-free; Roche Diagnostics; http://www.roche-applied-science.com). Aliquots (50 μg) of these samples were used to isolate mRNA using a QuickPrep™ Micro mRNA purification kit (GE Healthcare; http://www.gehealthcare.com), and duplicate first-strand cDNA samples were synthesized in the presence of an oligo(dT)$_{17}$ primer according to the manufacturer's instructions (first-strand cDNA synthesis kit, GE Healthcare). Real-time quantitative PCR analyses were performed for duplicate samples using an Applied Biosystems 7300 real-time PCR system (http://www.appliedbiosystems.com/) and SYBR Green detection system. Quantification was standardized to barley *Actin2* mRNA levels (accession number BQ768853). The primers used for PCR amplification were as follows: barley *Actin2* forward, 5′-TCCAGCTATCGTCCACAGGAA-3′; reverse, 5′-TGTCCAACAAAACCACCACT-3′; *HvFus3* forward, 5′-TCTTTGTTTCCCAACCCGAA-3′; reverse, 5′-AAGGCTTGGTGACTCCGAACT-3′; *HvBlz2* forward, 5′-TACATTGGTGCCTACCCCACA-3′; reverse, 5′-AATTCCGGCTCAGGTCTCTCA-3′; *Hor2* forward, 5′-AGGCAATCCGTGCAATCGT-3′; reverse, 5′-TTGTCCAACTTGCTCCTGCTG-3′; *Itr1* forward, 5′-TCCTCACCTCGGACATGAAGA-3′; reverse, 5′-AACGCACCCTGCCAAGTTACT-3′).

In situ *hybridization*

Developing barley seeds (22 d.a.p.) were collected, fixed in 4% formaldehyde in PBS, dehydrated, embedded in paraffin and sectioned to 8 μm. After de-waxing in Histoclear and rehydration, sections were treated with 0.2 M HCl, neutralized, incubated with 1 μg ml^{-1} proteinase K for 1 h, and dehydrated. Hybridization was performed overnight at 50°C in hybridization solution (100 μg ml^{-1} tRNA, 6x SSC, 3% SDS and 50% formamide), plus approximately 100 ng μl^{-1} of antisense or sense DIG-labelled RNA probes corresponding to a 354 bp fragment (positions 433–787 from the ATG initiation codon) of the *HvFus3* gene. Two washes using 2x SSC and 50% formamide for 90 min at 50°C were then carried out. Antibody incubation and colour detection were performed according to the manufacturer's instructions (Roche Diagnostics).

Transient expression assays in aleurone layers

The reporter constructs P_{Hor2} and P_{Itr1} (wt) were as described by Díaz *et al.* (2002). P_{Itr1} (mut) was created using a PCR-based site-directed mutagenesis approach. The base changes in the RY box (CATGCA to ATCCTG) were introduced using two overlapping fragments. The first, spanning 263 bp, was amplified by PCR using the universal sense primer and the primer 5′-AGTGCCGCCAGGATGAAATTTTG-3′, and the second, spanning 157 bp, was obtained using primer 5′-CAAAATTTCATCCTGGCGGCACT-3′ and the GUS antisense primer (Díaz *et al.*, 2002). These fragments were annealed and extended by a PCR reaction using the two external primers (universal sense and GUS antisense). The resulting fragment replaced the internal *Not*I–*Bam*HI portion of the *Itr1* promoter in the P_{Itr1} (wt) plasmid. The effector constructs were created by cloning the whole ORFs of *HvFus3* and its mutated versions *Fus3*Δ^{7-46} and *Fus3** into the *Bam*HI–*Eco*RI restriction sites of the pBlueScript vector (Stratagene, http://www.stratagene.com/) under the control of the CaMV 35S promoter followed by the first intron of the maize *AdhI* gene and the 3′ *nos* terminator. The effector construct carrying the ORF of *Blz2* is the same as that described by Oñate *et al.* (1999). Preparation of aleurone layers, gold particle coating and bombardment with a biolistic Helium gun device (DuPont PDS-1000, Bio-Rad, http://www.bio-rad.com/) were performed as previously described (Díaz *et al.*, 2002). GUS expression was determined histochemically according to the method described by Jefferson (1987). GUS activity was calculated as the mean number of blue spots per aleurone in each assay and expressed as a percentage, where 100% was the value obtained using the reporter constructs without effectors. The histochemical data were directly correlated with the GUS expression quantified by chemiluminescence per milligram protein, with a correlation coefficient of 0.97 (data not shown). Chemiluminescence was assayed using a GUS Light Kit (Tropix; http://www.appliedbiosystems.com).

Yeast transformation and LacZ *assays*

The whole ORFs of *HvFus3*, its mutated versions *Fus3*Δ^{7-46} and *Fus3**, and the derived fragments described above were cloned into the *Eco*RI–*Sal*I restriction sites of the pGBT9 and/or pGAD424 plasmids (Clontech, http://www.clontech.com/) to generate translational fusions. The complete ORF of *Blz2* fused to the Gal4 DNA-binding domain and the Gal4 activation domain in the pGBT9 and pGAD424 vectors, respectively, were as described by Oñate *et al.* (1999). Two haploid strains of *Saccharomyces cerevisiae* (Clontech) were used: SFY526, carrying a *LacZ* reporter gene under the control of a truncated *Gal1* promoter, which contains the *Gal4*-responsive element, and HF7C, carrying a *His3* auxotrophy gene controlled by the same promoter. Yeast transformation was performed by the polyethylene glycol method, and transformants were screened for β-galactosidase production (*LacZ*) and for growth in histidine-depleted agar medium (*His*$^-$) with 60 mM 3-aminotriazol. Quantification of β-galactosidase activity was calculated using Miller's formula as described by Ausubel *et al.* (1990).

Electrophoretic mobility shift assays

The HvFUS3, HvFUS3* and AtFUS3 proteins were expressed in the *E. coli* BL21 CodonPlus (DE3)-RP strain (Stratagene) by cloning their ORFs into the *Bam*HI restriction site of the pGEX-2T vector (GE Healthcare) as translational fusions to GST. Recombinant proteins were induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 h, and bacterial extracts were obtained by sonication in 8 mM dithiothreitol (DTT) in PBS. Cells carrying the pGEX-2T vector with no insert were identically processed as negative controls. The probes containing the RY DNA-binding site derived from the *Hor2*, *Itr1* and *At2S3* promoters (*Hor2-RY*, *Itr1-RY* and *At2S-3RY*) and the mutated version (*Itr1-ry*) were produced by annealing complementary single-stranded oligonucleotides that generated 5′-protruding ends. End-labelling of the probes using [α-^{32}P]dATP, and DNA–protein binding reactions were performed as described by Mena *et al.* (1998). The primers used were *Hor2-RY*, 5′-AAGTGAATAAGGTGAGTCATGCATACC-3′, and 3′-TTATTCCACTCAGTACGTATGGTTT-5′; *Itr1-RY*, 5′-TTAATCAAAATTTCCATGCAGCGGC-3′ and 3′-GTTTTAAAGGTACGTCGCCGTGAG-5′; *Itr1-ry*, 5′-TTAATCAAAATTTCATCCTGGCGGC-3′ and 3′-GTTTTAAAGTAGGACCGCCGTGAG -5′; *At2S-3RY*, 5′-ACACACTCATGCATGCATGC-3′ and 3′-GTGTACGTACGTACGTAAGTT-5′ (restriction sites underlined).

Acknowledgements

We thank Mar Gonzalez for her valuable technical assistance. Financial support from the Ministerio de Educación y Ciencia, Spain (projects BFU2006-07258 and BIO2004-00168) is gratefully acknowledged. M.A.M.-R. is the recipient of a pre-doctoral fellowship from the Comunidad de Madrid of Spain.

Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Phylogenetic tree obtained for the whole set of B3-type annotated proteins encoded by the rice genome, and protein sequence alignment of the Arabidopsis FUS3, barley HvFUS3 and rice OsFUS3 putative orthologous proteins.

Figure S2. Selection of an internal standard for quantitative PCR analyses in barley seed development.

This material is available as part of the online article from http://www.blackwell-synergy.com

Please note: Blackwell Publishing are not responsible for the content or functionality of any supplementary materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

References

Ausubel, F.M., Brent, R., Dingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1990) *Current Protocols in Molecular Biology*. New York: John Wiley & Sons.

Bailey, T.L. and Elkan, C. (1994) Fitting a mixture model by expectation maximization to discover motifs in biopolymers. *Proc. Int. Conf. Intell. Syst. Mol. Biol.* **2**, 28–36.

Bäumlein, H., Miséra, S., Luerssen, H., Kölle, K., Horstmann, C., Wobus, U. and Müller, A.J. (1994) The FUS3 gene of *Arabidopsis thaliana* is a regulator of gene expression during late embryogenesis. *Plant J.* **6**, 379–387.

Berger, F. (2003) Endosperm: the crossroad of seed development. *Curr. Opin. Plant Biol.* **6**, 42–50.

Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* **16**, 735–743.

Díaz, I., Royo, J., O'Connor, A. and Carbonero, P. (1995) The promoter of the gene Itr1 from barley confers a different tissue specificity in transgenic tobacco. *Mol. Gen. Genet.* **248**, 592–598.

Díaz, I., Vicente-Carbajosa, J., Abraham, Z., Martinez, M., Isabel-La Moneda, I. and Carbonero, P. (2002) The GAMYB protein from barley interacts with the DOF transcription factor BPBF and activates endosperm-specific genes during seed development. *Plant J.* **29**, 453–464.

Díaz, I., Martinez, M., Isabel-LaMoneda, I., Rubio-Somoza, I. and Carbonero, P. (2005) The DOF protein, SAD, interacts with GAMYB in plant nuclei and activates transcription of endosperm-specific genes during barley seed development. *Plant J.* **42**, 652–662.

Ezcurra, I., Ellerstrom, M., Wycliffe, P., Stalberg, K. and Rask, L. (1999) Interaction between composite elements in the napA promoter: both the B-box ABA-responsive complex and the RY/G complex are necessary for seed-specific expression. *Plant Mol. Biol.* **40**, 699–709.

Forde, B.G., Heyworth, A., Pywell, J. and Kreis, M. (1985) Nucleotide sequence of a B1-hordein gene and the identification of possible upstream regulatory elements in endosperm storage protein genes from barley, wheat and maize. *Nucleic Acids Res.* **13**, 7327–7339.

Gazzarrini, S., Tsuchiya, Y., Lumba, S., Okamoto, M. and McCourt, P. (2004) The transcription factor FUSCA3 controls developmental timing in *Arabidopsis* through the hormones gibberellin and abscisic acid. *Dev. Cell*, **7**, 373–385.

Giraudat, J., Hauge, B.M., Valon, C., Smalle, J., Parcy, F. and Goodman, H.M. (1992) Isolation of the *Arabidopsis* ABI3 gene by positional cloning. *Plant Cell*, **4**, 1251–1261.

Harada, J.J. (1997) Seed maturation and control of germination. In *Cellular and Molecular Biology of Plant Seed Development* (Larkins, B.A. and Vasil, I.K., eds). Dordrecht, The Netherlands: Kluwer Academic Publishers, pp. 545–592.

Higgins, T.J.V. (1984) Synthesis and regulation of major proteins in seeds. *Annu. Rev. Plant Physiol.* **35**, 191–221.

Hobo, T., Kowyama, Y. and Hattori, T. (1999) A bZIP factor, TRAB1, interacts with VP1 and mediates abscisic acid-induced transcription. *Proc. Natl Acad. Sci. USA*, **96**, 15348–15355.

Jefferson, R.A. (1987) Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Mol. Biol. Rep.* **5**, 387–405.

Keith, K., Kraml, M., Dengler, N.G. and McCourt, P. (1994) Fusca3: a heterochromic mutation affecting late embryo development in *Arabidopsis*. *Plant Cell*, **6**, 589–600.

Kreis, M. and Shewry, P.R. (1989) Unusual features of cereal seed protein structure and evolution. *Bioessays*, **10**, 201–207.

Kroj, T., Savino, G., Valon, C., Giraudat, J. and Parcy, F. (2003) Regulation of storage protein gene expression in *Arabidopsis*. *Development*, **130**, 6065–6073.

Kumar, S., Tamura, K. and Nei, M. (2004) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief. Bioinform.* **5**, 150–163.

Lagrimini, L.M., Burkhart, W., Moyer, M. and Rothstein, S. (1987) Molecular cloning of DNA encoding the lignin forming peroxidases from tobacco: molecular analysis and tissue specific expression. *Proc. Natl Acad. Sci. USA*, **84**, 7542–7546.

Lara, P., Onate-Sanchez, L., Abraham, Z., Ferrandiz, C., Díaz, I., Carbonero, P. and Vicente-Carbajosa, J. (2003) Synergistic activation of seed storage protein gene expression in *Arabidopsis* by ABI3 and two bZIPs related to OPAQUE2. *J. Biol. Chem.* **278**, 21003–21011.

Lotan, T., Ohto, M., Yee, K.M., West, M.A., Lo, R., Kwong, R.W., Yamagishi, K., Fischer, R.L., Goldberg, R.B. and Harada, J.J. (1998) *Arabidopsis* LEAFY COTYLEDON1 is sufficient to induce embryo development in vegetative cells. *Cell*, **93**, 1195–1205.

Luerssen, H., Kirik, V., Herrmann, P. and Misera, S. (1998) FUSCA3 encodes a protein with a conserved VP1/AB13-like B3 domain which is of functional importance for the regulation of seed maturation in *Arabidopsis thaliana*. *Plant J.* **15**, 755–764.

McCarty, D.R., Carson, C.B., Stinard, P.S. and Robertson, D.S. (1989) Molecular analysis of *viviparous-1*: an abscisic acid-insensitive mutant of maize. *Plant Cell*, **1**, 523–532.

McCarty, D.R., Hattori, T., Carson, C.B., Vasil, V., Lazar, M. and Vasil, I.K. (1991) The *Viviparous-1* developmental gene of maize encodes a novel transcriptional activator. *Cell*, **66**, 895–905.

Meinke, D.W. (1992) A homeotic mutant of *Arabidopsis thaliana* with leafy cotyledons. *Science*, **258**, 1647–1650.

Meinke, D.W., Franzmann, L.H., Nickle, T.C. and Yeung, E.C. (1994) Leafy cotyledon mutants of *Arabidopsis*. *Plant Cell*, **6**, 1049–1064.

Mena, M., Vicente-Carbajosa, J., Schmidt, R.J. and Carbonero, P. (1998) An endosperm-specific DOF protein from barley, highly conserved in wheat, binds to and activates transcription from the prolamin-box of a native B-hordein promoter in barley endosperm. *Plant J.* **16**, 53–62.

Nakamura, S., Lynch, T.J. and Finkelstein, R.R. (2001) Physical interactions between ABA response loci of *Arabidopsis*. *Plant J.* **26**, 627–635.

Nambara, E., Keith, K., McCourt, P. and Naito, S. (1995) A regulatory role for the ABI3 gene in the establishment of embryo maturation in *Arabidopsis thaliana*. *Development*, **121**, 629–636.

Oñate, L., Vicente-Carbajosa, J., Lara, P., Díaz, I. and Carbonero, P. (1999) Barley BLZ2, a seed-specific bZIP protein that interacts with BLZ1 in vivo and activates transcription from the GCN4-like motif

of B-hordein promoters in barley endosperm. *J. Biol. Chem.* **274**, 9175–9182.
Parcy, F., Valon, C., Raynal, M., Gaubier-Comella, P., Delseny, M. and Giraudat, J. (1994) Regulation of gene expression programs during *Arabidopsis* seed development: roles of the ABI3 locus and of endogenous abscisic acid. *Plant Cell*, **6**, 1567–1582.
Parcy, F., Valon, C., Kohara, A., Misera, S. and Giraudat, J. (1997) The ABSCISIC ACID-INSENSITIVE3, FUSCA3, and LEAFY COTYLEDON1 loci act in concert to control multiple aspects of *Arabidopsis* seed development. *Plant Cell*, **9**, 1265–1277.
Raz, V., Bergervoet, J.H. and Koornneef, M. (2001) Sequential steps for developmental arrest in *Arabidopsis* seeds. *Development*, **128**, 243–252.
Reidt, W., Wohlfarth, T., Ellerstrom, M., Czihal, A., Tewes, A., Ezcurra, I., Rask, L. and Baumlein, H. (2000) Gene regulation during late embryogenesis: the RY motif of maturation-specific gene promoters is a direct target of the FUS3 gene product. *Plant J.* **21**, 401–408.
Rodriguez-Palenzuela, P., Royo, J., Gomez, L., Sanchez-Monge, R., Salcedo, G., Molina-Cano, J.L., Garcia-Olmedo, F. and Carbonero, P. (1989) The gene for trypsin inhibitor CMe is regulated in trans by the lys 3a locus in the endosperm of barley (*Hordeum vulgare* L.). *Mol. Gen. Genet.* **219**, 474–479.
Rubio-Somoza, I., Martínez, M., Abraham, Z., Díaz, I. and Carbonero, P. (2006a) Ternary complex formation between HvMYBS3 and other factors involved in transcriptional control in barley seeds. *Plant J.* **47**, 269–281.
Rubio-Somoza, I., Martinez, M., Díaz, I. and Carbonero, P. (2006b) HvMCB1, a R1MYB transcription factor from barley with antagonistic regulatory functions during seed development and germination. *Plant J.* **45**, 17–30.
Stone, S.L., Kwong, L.W., Yee, K.M., Pelletier, J., Lepiniec, L., Fischer, R.L., Goldberg, R.B. and Harada, J.J. (2001) LEAFY COTYLEDON2 encodes a B3 domain transcription factor that induces embryo development. *Proc. Natl Acad. Sci. USA*, **98**, 11806–11811.
Suzuki, M., Cao, C.Y., Cocciolone, S. and McCarty, D.T. (2001) Maize VP1 complements *Arabidopsis* abi3 and confers a novel ABA/auxin interaction in roots. *Plant J.* **28**, 409–418.
Suzuki, M., Ketterling, M.G., Li, Q.B. and McCarty, D.R. (2003) Viviparous1 alters global gene expression patterns through regulation of abscisic acid signaling. *Plant Physiol.* **132**, 1664–1677.
Takaiwa, F., Yamanouchi, U., Yoshihara, T., Washida, H., Tanabe, F., Kato, A. and Yamada, K. (1996) Characterization of common cis-regulatory elements responsible for the endosperm-specific expression of members of the rice glutelin multigene family. *Plant Mol. Biol.* **30**, 1207–1221.
To, A., Valon, C., Savino, G., Guilleminot, J., Devic, M., Giraudat, J. and Parcy, F. (2006) A network of local and redundant gene regulation governs *Arabidopsis* seed maturation. *Plant Cell*, **18**, 1642–1651.
Tsuchiya, Y., Nambara, E., Naito, S. and McCourt, P. (2004) The FUS3 transcription factor functions through the epidermal regulator TTG1 during embryogenesis in *Arabidopsis*. *Plant J.* **37**, 73–81.
Vicente-Carbajosa, J. and Carbonero, P. (2005) Seed maturation: developing an intrusive phase to accomplish a quiescent state. *Int. J. Dev. Biol.* **49**, 645–651.
Vicente-Carbajosa, J., Onate, L., Lara, P., Díaz, I. and Carbonero, P. (1998) Barley BLZ1: a bZIP transcriptional activator that interacts with endosperm-specific gene promoters. *Plant J.* **13**, 629–640.
Vincentz, M., Leite, A., Neshich, G. ***et al.*** (1997) ACGT and vicilin core sequences in a promoter domain required for seed-specific expression of a 2S storage protein gene are recognized by the opaque-2 regulatory protein. *Plant Mol. Biol.* **34**, 879–889.
West, M., Yee, K.M., Danao, J., Zimmerman, J.L., Fischer, R.L., Goldberg, R.B. and Harada, J.J. (1994) LEAFY COTYLEDON1 is an essential regulator of late embryogenesis and cotyledon identity in *Arabidopsis*. *Plant Cell*, **6**, 1731–1745.
Wu, C., Washida, H., Onodera, Y., Harada, K and Takaiwa, F. (2000) Quantitative nature of the prolamin-box, ACGT and AACA motifs in a rice glutelin gene promoter: minimal cis-element requirements for endosperm-specific gene expression. *Plant J.* **23**, 415–421.