The HvDOF19 transcription factor mediates the abscisic acid-dependent repression of hydrolase genes in germinating barley aleurone

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Summary

Upon germination of seed cereals, mobilization of the reserves stored in the endosperm is regulated by the phytohormones gibberellins (GA) and abscisic acid (ABA). In barley, the *cis* regulatory elements and the *trans*-acting factors mediating the ABA response of hydrolase genes remain elusive. Two new barley genes, *HvDof17* and *HvDof19*, encoding transcription factors (TFs) of the DNA binding with one finger (DOF) class have been characterized and their role upon germination investigated. HvDOF19 binds in a specific manner to the pyrimidine box within the GARC of a thiol-protease gene (*Al21*), and mediates the ABA repression of this gene in the barley aleurone. Silencing of *HvDof19* in transient expression assays diminishes the inhibitory effect of ABA upon expression of the *Al21* gene promoter. Transcripts from *HvDof17* and *HvDof19* accumulate early in germinating aleurones with a peak at 16 h after seed imbibition (hai), whereas the mRNAs of the GA-induced activator GAMYB remain little expressed. At 48 hai, mRNA content of both genes is comparatively insignificant compared with that of GAMYB, which reaches a maximum. Both TFs repress, in transient expression assays, the GA- and the GAMYB-mediated activation of this thiol-protease gene (*Al21*). In addition, HvDOF17 and HvDOF19 interact with GAMYB in plant cell nuclei, and HvDOF17, but not HvDOF19, interferes with the DNA binding of GAMYB to its target site in the promoter of the *Al21* gene. A regulatory model of hydrolase gene expression upon germination is proposed.

Keywords: abscisic acid-induced DOF, barley seeds, gibberellin-repressed DOFs, germinating aleurone, HvDOF19, HvDOF17.

Introduction

The hormonal balance between gibberellins (GA) and abscisic acid (ABA) is a crucial factor in seed germination, although other phytohormones such as brassinosteroids and ethylene, as well as light and sugars, also influence this physiological process (Aoki *et al.*, 2006; Brocard-Gifford *et al.*, 2003; Lovegrove and Hooley, 2000; Steber and McCourt, 2001). ABA is involved in the maintenance of dormancy, inhibits germination and is required in the glucose-mediated developmental arrest during post-germination (Arenas-Huertero *et al.*, 2000; Gubler *et al.*, 2005; Lopez-Molina *et al.*, 2001, 2002). ABA perception seems to occur both at the plasma membrane and in the cytoplasm, and it has been suggested that different receptors might perceive this hormone in different response pathways (Allan

et al., 1994; Finkelstein *et al.*, 2002). In Arabidopsis, an ABA receptor (FCA) involved in RNA metabolism and in the control of flowering time has been identified (Razem *et al.*, 2006). In barley, the protein ABAP1 could be an ABA receptor located in the plasma membrane of the aleurone cells (Razem *et al.*, 2004). Genetic approaches have led to the characterization of several intermediates of the ABA signal transduction pathway. However, a complete identification of the network components is still lacking (Finkelstein *et al.*, 2002).

In cereals, imbibition triggers a decrease in ABA seed content and promotes the synthesis of GA by the embryo that diffuses to the aleurone (Jacobsen *et al.*, 2002; Kaneko *et al.*, 2002). In this tissue, GA is perceived by a soluble

receptor promoting the degradation of the DELLA proteins that repress the GA response (Ueguchi-Tanaka *et al.*, 2005). In addition, this hormone could be perceived by an unknown membrane-localized GA receptor. This would trigger a signal transduction pathway involving increases in cytoplasmic calcium and in pH values, as well as increases in heterotrimeric G proteins, protein kinases and Ser/Thr O-linked N-actylglucosamine transferases (Gilroy and Jones, 1994; Hartweck and Olszewski, 2006; Jacobsen *et al.*, 1995; McCubbin *et al.*, 2004; Robertson *et al.*, 1998; Sun and Gubler, 2004). Eventually, transcription of genes encoding hydrolases is activated. These enzymes, mainly α -amylases and proteases, are secreted into the endosperm where they mobilize stored reserves, releasing the nutrients that will support embryo growth until photosynthesis is fully established (Eastmond and Russell, 2005). Most of GA effects can be antagonized by ABA. In dormant seeds and under unfavourable germinating conditions, ABA suppresses GA-induced expression of hydrolase genes (Gomez-Cadenas *et al.*, 2001b; Lovegrove and Hooley, 2000).

Functional analyses of the promoters of GA-responsive hydrolase genes in the barley aleurone (Jacobsen *et al.*, 1995) have identified a conserved *cis*-acting response complex, the GARC. This complex comprises three motifs necessary for a full GA response: (1) the 5'-TAACAA/GA-3' box or GA-responsive element (GARE); (2) the pyrimidine box, 5'-C/TCTTTTC/T-3'; and (3) the 5'-TATCCAC-3' element. In addition, in low-pI α -amylase genes, a putative Opaque-2 binding sequence (O2S: 5'-CTTGACCATCATC-3') is also involved in the GA response (Gomez-Cadenas *et al.*, 2001a; Lanahan *et al.*, 1992; Rogers *et al.*, 1994). However, the *cis*-motifs responsible for the ABA repression of these hydrolase genes have not yet been identified.

Over the past few years, several transcription factors (TFs) have been demonstrated to interact with the three motifs of the GARC. In barley, these TFs belong to the R2R3MYB, R1MYB and DOF classes (Gubler *et al.*, 1995; Isabel-LaMoneda *et al.*, 2003; Mena *et al.*, 2002; Rubio-Somoza *et al.*, 2006a,b). In rice, TFs of the WRKY class have been also described as regulators of hydrolase gene expression through binding to the O2S motif (Xie *et al.*, 2006; Zhang *et al.*, 2004).

The GAMYB protein of the R2R3MYB class, which is GA induced, interacts with the GARE and *trans*-activates a number of hydrolase gene promoters downstream of the barley DELLA protein SLN1 (Gubler *et al.*, 1995, 1999, 2002; Sun and Gubler, 2004). SLN1 is an indispensable intermediate of GA signaling, and its silencing leads to derepression of α -amylase gene expression in aleurone cells (Gubler *et al.*, 2002; Zentella *et al.*, 2002). This derepression is suppressed by exogenous ABA, so it has been suggested that molecular interaction between the signaling cascades

mediated by GA and ABA occurs midway in the pathway that leads to the synthesis of hydrolytic enzymes (Zentella *et al.*, 2002). The barley PKABA1 protein kinase, which is ABA induced, participates in the ABA signaling cascade in aleurone cells. Its transient overexpression represses the GA induction of *GAMYB* (Gomez-Cadenas *et al.*, 2001b), and consequently represses hydrolase gene expression (Gomez-Cadenas *et al.*, 1999; Shen *et al.*, 2001). However, silencing of PKABA1 seems not to affect the ABA repression of α -amylase genes (Zentella *et al.*, 2002).

TFs of the DOF class, BPBF and SAD, regulate hydrolase gene expression in germinating barley aleurones through binding to the pyrimidine box of the GARC. They have antagonistic functions. BPBF is a transcriptional repressor whereas SAD is a transcriptional activator, and both interact with GAMYB in cell nuclei (Diaz *et al.*, 2005; Isabel-LaMoneda *et al.*, 2003; Mena *et al.*, 2002).

This work deals with the molecular characterization of two novel genes encoding DOF proteins from barley, *HvDof19* and *HvDof17*. These genes are highly expressed in aleurone cells early after seed imbibition (up to 16 h after seed imbibition; 16 hai), and their mRNAs decrease thereafter. Both TFs are repressors of the GA-induced cathepsin B-like thiol protease gene *A121*, and they interact with GAMYB in plant cell nuclei. Whereas *HvDof19* is induced by ABA, *HvDof17* is not, although both genes are GA repressed. In addition, the *HvDof19* RNAi construct diminishes the inhibitory effect of ABA upon the expression of the *A121* gene promoter in transient expression assays, indicating that this factor is necessary for the ABA repression of the GA signaling in aleurone cells. Possible physiological implications are discussed.

Results

Molecular characterization of new DOF genes from barley

We had previously reported the role of BPBF and SAD (*HvDof24-Pbf* and *HvDof23-Sad* genes, respectively) as regulators of hydrolase gene expression in aleurone cells upon barley seed germination (Isabel-LaMoneda *et al.*, 2003; Mena *et al.*, 2002). Therefore, we decided to explore whether other DOF proteins could also participate in this transcriptional network. As five DOF proteins from rice had been identified as regulators of the expression of a peptidase gene following germination (Washio, 2001), and in our annotation of 26 different DOF genes from barley we had inferred their phylogenetic relationships with genes from rice (Moreno-Risueno *et al.*, 2007), we focused on the barley putative orthologs of these five DOF genes from rice. Four clear couples of barley-rice orthologs were: *HvDof8*-Os02g49440, *HvDof2*-Os02g45200, *HvDof23-Sad*-Os04g47990 and *HvDof24-Pbf*-Os02g15350. Os01g55340 had two putative orthologous genes in barley, the *HvDof19* and *HvDof17* genes.

The expression of these six DOF genes in barley aleurones during an early phase of seed germination and post-germination was explored by real time quantitative PCR (RT-qPCR). In our growing conditions, radicle emerges at 18 hai ($n = 200$, $84.2 \pm 5.1\%$) indicating the beginning of the post-germination stage. At different times after water imbibition (8, 12, 18, 24, 36 and 48 hai), aleurones were separated from the rest of the seed and collected. Samples at 8, 12 and 18 hai (germination) and at 24, 36 and 48 hai (post-germination) were mixed in a 1:1:1 ratio. The results, expressed as relative content of the *HvActin2* mRNA levels, indicated that the six barley DOF genes were expressed during the germination stage (Figure 1a). During post-germination, the most abundant transcripts were those of *HvDof19*, followed by *HvDof24-Pbf* and *HvDof17*, and the lowest levels corresponded to *HvDof2*, followed by *HvDof23-Sad* and *HvDof8* (Figure 1a). When the transcript content of these genes was analogously analyzed by RT-qPCR in germinating embryos collected at the same times as the aleurones, we observed that they were comparatively less expressed (data not shown). Based on this expression pattern and considering that we had already studied the *HvDof24-Pbf* gene, the *HvDof19* and *HvDof17* genes were selected for further characterization.

The nucleotide and deduced amino acid sequences of the cDNAs of *HvDof19* and *HvDof17* showed that both genes encoded for putative proteins of 201 and 215 residues, with a predicted molecular mass of 21.9 and 23.0 kDa, respectively. Their DOF domains shared 89/98% of identical/similar residues, whereas along the protein, the identity/similarity percentage dropped to 41/49%. Neither of the two genes had introns within their open reading frames (ORFs) (Moreno-Risueno *et al.*, 2007).

The expression of the HvDof19 and HvDof17 genes in barley aleurone layers is hormonally regulated

A more detailed expression pattern of the *HvDof19* and *HvDof17* genes in germinating barley aleurones at 8, 16, 24, 36, 48 and 72 hai was investigated by RT-qPCR. As shown in Figure 1(b), a rapid increase in the mRNA accumulation of the *HvDof17* and *HvDof19* genes that peaked at 16 hai was detected. However, at 24 hai the *HvDof19* transcripts had fallen to the same levels detected at 8 hai, whereas the *HvDof17* mRNAs decreased only by 25%. In contrast, the mRNAs levels of the gene encoding a GA-responsive cathepsin B-like protease (Martinez *et al.*, 2003) exponentially increased between 16 and 24 hai. They continued to rise until 48 hai and declined thereafter. The expression pattern of the *HvDof24-Pbf* gene, previously described as a repressor of hydrolase genes in aleurone (Mena *et al.*, 2002), resembled that obtained for *HvDof17* and *HvDof19*, whereas the mRNAs of the GA-induced *GAMYb* gene encoding a potent activator of the same hydrolase genes (Gubler *et al.*, 1999) increased to a maximum at 48 hai.

Figure 1. Real time quantitative PCR analysis (RT-qPCR) of several DOF, *GAMYb*, and cathepsin B-like encoding *CatB* genes.

(a) Relative mRNA content of *HvDof2*, *HvDof8*, *HvDof17*, *HvDof19*, *HvDof23-Sad* and *HvDof24-Pbf* genes in aleurones of imbibed barley seeds (samples collected at 8, 12 and 18 h after imbibition, hai, were mixed in a ratio 1:1:1) and in aleurones of post-germinating seeds (24, 36 and 48 hai; mixture 1:1:1).

(b) Variation of transcript accumulation of *HvDof17*, *HvDof19*, *Pbf*, *GAMYb* and *CatB* genes in barley aleurones at 8, 16, 24, 48 and 72 hai. Data were standardized to the barley *Actin2* mRNA levels.

To determine the spatial expression of *HvDof17* and *HvDof19* within germinating seeds, mRNA *in situ* hybridization assays were carried out. Samples at 16 hai were hybridized to specific antisense probes for *HvDof17* and *HvDof19*. We detected a strong signal in aleurone cells and in the embryo (Figure 2a). The mRNA label for *HvDof17* was mainly observed in the foliar primordia and in the scutellum, whereas for *HvDof19* it was prominent in the radicle. As expected, when the samples were hybridized with the sense probes, used as negative controls, no signal above background was detected (Figure 2a).

The accumulation pattern and spatial expression of the *HvDof19* and *HvDof17* transcripts upon barley seed imbibition suggested that it might be hormonally regulated. To test this possibility, isolated aleurone layers from imbibed de-embryonated seeds were incubated for 20 h with 1 μ M GA₃, 10 μ M ABA or a mixture of both hormones. Their transcripts were quantified by RT-qPCR. As shown in Figure 2(b), a decrease of 50% in *HvDof19* and *HvDof17* expression levels was detected with the GA treatment, whereas the incubation with ABA produced an increase of approximately sixfold in *HvDof19* mRNAs and it had no effect on the *HvDof17* transcript accumulation. Moreover, the enhancer effect of ABA in *HvDof19* expression was only slightly decreased by the incubation with the mixture of GA + ABA.

As expected from previous data, the cathepsin B-like encoding gene in aleurone layers was induced by GA and repressed by ABA (Martinez *et al.*, 2003). However, *HvDof24-Pbf*, although induced by GA, was not affected by ABA (Mena *et al.*, 2002).

HvDOF19 and HvDOF17 proteins are localized to the aleurone cell nuclei

To investigate the subcellular localization of HvDOF19 and HvDOF17 proteins, translational fusions of their corresponding ORFs to the GFP were made. These constructs were under the control of the 35S promoter followed by the first intron of the maize alcohol dehydrogenase I (*Adhl*) gene. The constructs *P_{35S}-lAdhl::HvDof19-Gfp*, *P_{35S}-lAdhl::HvDof17-Gfp* and the control construct *P_{35S}-lAdhl::Gfp* were used to transiently transform barley aleurone layers by particle bombardment. After 24 h of incubation, samples were observed under a confocal microscope. As shown in Figure 3, when the aleurone layers were transformed with the constructs carrying either HvDOF19 or HvDOF17 proteins fused to the GFP, fluorescence corresponding to the emission spectrum of this protein was detected within the cell nucleus (Figure 3a–f). When the aleurones were transiently transformed with *P_{35S}-lAdhl::Gfp*, the GFP fluorescence

Figure 2. Expression of *HvDof19* and *HvDof17* in germinating barley seeds and their response to hormonal treatments in aleurone cells.

(a) Spatial expression of *HvDof19* and *HvDof17* genes in barley seeds (16 hai) determined by mRNA *in situ* hybridization assays. Upper panels correspond to transversal sections of germinating aleurones and lower panels correspond to longitudinal sections of germinating embryos. Hybridization was performed on the sense and antisense probes as indicated. a, aleurone; en, endosperm; fp, foliar primordia; pe, pericarp; r, radicle; sc, scutellum; t, testa. Scale bars: 100 μ m. (b) Analyses of the mRNA variation (RT-qPCR) of the *HvDof19*, *HvDof17*, *HvDof24-Pbf* and *CatB* genes in aleurone layers after 20 h of incubation in the presence of 1 μ M GA₃, 10 μ M ABA or a mixture of both hormones. Results were referred to the control value (without hormones) in each case, which was arbitrarily set to 1.

Figure 3. Sub-cellular location of HvDOF19 and HvDOF17 in aleurone cells.

Aleurone layers cells were bombarded with the P_{35s} -*Adhl*::*HvDof19*-Gfp construct (a, b, c), the P_{35s} -*Adhl*::*HvDof17*-Gfp construct (d, e, f) or the P_{35s} -*Adhl*::Gfp control constructs (g, h, i) and incubated for 24 h before observation under a confocal ultraspectral microscope LEICA TCS-Sp2.AOBS-UV for the emission spectrum of the GFP (b, e, h) or under Nomarski (a, d, g). The Nomarski and fluorescence photopgraphs were overlapped (c, f, i). Scale bars: 20 μ m.

spread throughout the cell (Figure 3g-i). Nomarski pictures (Figure 3a,d,g) and the overlapping photographs (Figure 3c,f,i) of these and the fluorescence images (Figure 3b,e,h) are also shown.

*HvDOF19 and HvDOF17 are transcriptional repressors of the GA-responsive gene *Al21* in aleurone*

The expression patterns and hormonal responses of *HvDof19* and *HvDof17* suggested that they could be regulators of the expression of hydrolase-encoding genes in germinating aleurone cells. Thus, we addressed whether these factors could modulate the transcriptional activity of the promoter of the *Al21* gene, which encodes a cathepsin B-like protease, in transient expression assays by particle bombardment of isolated layers of barley cv. Himalaya. As a reporter (Figure 4a), we used the pCBG13 construct (Cejudo *et al.*, 1992) where the *GUS* gene was under the control of a -480-bp promoter fragment (from the translation start codon) of the *Al21* gene that contained three pyrimidine boxes and two GARE motifs. These two *cis*-motifs were recognized by DOF TFs and GAMYB, respectively (Isabel-LaMoneda *et al.*, 2003; Mena *et al.*, 2002). The effector constructs were the ORFs of *HvDof17*, *HvDof19* and *GAMYb* under the control of the CaMV35S promoter, followed by the first intron of the *Adhl* gene from maize and flanked downstream by the 3' NOS terminator (Figure 4a). Isolated aleurone layers were bombarded either with the reporter alone or in combination with the effectors at a 1:1 molar ratio. The

GUS activity obtained after bombardment of the reporter without effectors was arbitrarily assigned the 100% value. When co-transfection of pCBG13 with *HvDof19*, *HvDof17* or with both effectors was performed either in the presence or in the absence of GA, *GUS* expression was drastically reduced (Figure 4b). Co-transfection with the *GAMYb* construct as effector produced an increase in *GUS* activity of 500%, as previously described (Gubler *et al.*, 1995, 1999). Co-bombardment of pCBG13 with *GAMYb* plus *HvDof17* or plus *HvDof19* (at a 1:1:1 ratio) dramatically decreased *GUS* expression.

In addition, we assayed a shorter version of the *Al21* gene promoter (Cejudo *et al.*, 1992) that only contained one DOF (D2) and two R2R3 MYB (M1 and M2) putative binding sites (Isabel-LaMoneda *et al.*, 2003). Similar patterns of *GUS* activity mediated by *HvDof17* and *HvDof19* transient expression were obtained (data not shown). The *GUS* activity measured for this shorter version of the *Al21* gene promoter was 70% of that measured for the pCBG13 construct. We can thus infer that the *cis*-motif D2 was sufficient for the negative regulation of the *Al21* gene promoter by the HvDOF19 and HvDOF17 TFs.

*Silencing of HvDof19 by dsRNAi prevents the ABA-mediated repression of the *Al21* gene in aleurone*

As RT-qPCR analyses indicated that *HvDof19* was ABA-induced (sixfold), and that HvDOF19 was a transcriptional repressor of the *Al21* gene promoter in transient expression

experiments, we decided to explore whether this TF could have a role in the ABA-mediated repression of *Al21* gene expression in the aleurone. To address this question we transiently expressed, in this tissue, a double-stranded RNAi (ds-RNAi) construct of the *HvDof19* gene together with the pCBG13 reporter described in Figure 4(a). This construct contained a specific fragment (from 502 to 737 bp from the translation start codon) of *HvDof19* in sense and antisense orientations and split by a 110-bp DNA fragment. The response to GA and ABA of the *Al21* gene promoter was studied. Isolated aleurone layers were bombarded with the reporter alone or in combination with the effector at a 1:1 molar ratio, and were incubated for 24 h in saline buffer, used as control, or with 1 μ M GA₃, 10 μ M ABA or a mixture of both hormones. As shown in Figure 4(c), when the reporter was incubated with GA, GUS activity increased by 400% over that obtained when the reporter was used alone without hormones. And when incubated with ABA, GUS activity decreased by 75%. The *HvDof19*-RNAi construct did not have any effect either in the control or upon GA incubation. However, co-bombardment of the reporter with the *HvDof19*-RNAi construct decreased the repressor effect of ABA (from 25 to 65% of GUS activity), and when incubating with ABA + GA, the 60% repression in the absence of the RNAi was transformed into about 200% activation. In addition, the specificity of *HvDof19* RNAi construct was demonstrated in the insert of Figure 4(c). The *HvDof19* repression effect on the GUS activity driven by the *Al21* promoter was reversed by the *HvDof19* RNAi. However, the repression mediated by *HvDof17* was not affected upon bombardment with *HvDof17* + *HvDof19* RNAi. These results indicated that HvDOF19 had a role mediating the ABA response of the *Al21* gene promoter. No effects were found when similar experiments were performed with *HvDof17*-RNAi (data not shown).

Figure 4. Transient expression assays by bombardment of aleurone layers from de-embryonated barley seeds with the indicated combinations of reporter and effector constructs and subsequent incubation with hormones. (a) Schematic representation of the reporter and effector constructs. The reporter construct pCBG13 is the same as that in Cejudo *et al.* (1992). Blank and black boxes indicate putative *cis* DNA binding sites for DOF and MYBR2R3 transcription factors (TFs), respectively. The effector constructs comprised the ORFs of *HvDof17*, *HvDof19*, *GAMyb* and an RNAi construct for *HvDof19* under the control of the CaMV35S promoter (*P_{35S}*) followed by the first intron of the maize *Adh1* gene (*I-Adh1*). The RNAi construct contained a fragment of the *HvDof19* cDNA from 502 to 737 bp from the translation initiation codon in sense and orientations separated by a stuff DNA of 110 bp. (b) GUS activity under the control of the *Al21* promoter after co-bombardment with different combinations of *HvDof17*, *HvDof19* and *GAMyb* were analysed after being incubated in saline buffer in the absence and in the presence of gibberellins (GA). (c) Expression of GUS activity controlled by the *Al21* promoter after co-bombardment with different combinations of *HvDof17*, *HvDof19* and the RNAi construct for *HvDof19*. GUS activity was analysed after incubation with GA, abscisic acid (ABA) or GA + ABA. GUS activity was expressed as the percentage of GUS activity relative to the control without effectors. In each case, three replicates were performed and sets of five aleurones were bombarded in each experiment. Standard errors are indicated.

HvDOF19 and HvDOF17 interact with GAMBY at plant cell nuclei

The finding that the *trans*-activation of the *Al21* gene promoter mediated by GAMBY in aleurone layers, was reversed by HvDOF17 and HvDOF19 suggested that the two DOF proteins might interact with GAMBY *in vivo*. To this purpose, two different approaches were used: the Bimolecular Fluorescence Complementation (BiFC) assay to detect protein–protein interactions in plant cell nuclei, and the Yeast Two-Hybrid System (Y2HS).

For the BiFC experiments, the ORFs of *HvDof19*, *HvDof17* and *GAMyb* were translationally fused, in all combinations, to two non-fluorescent fragments (N-terminal GFP:GFPNH₂- or C-terminal GFP:GFPCOOH-) of the GFP, as described by Diaz *et al.* (2005). When interaction of two proteins fused to these two GFP fragments take place, the fluorophore is reconstituted and fluorescence is emitted. The co-bombardment of epidermal onion cells with the combinations HvDOF19-GFPNH₂ and GAMBY-GFPCOOH or HvDOF17-GFPNH₂ and GAMBY-GFPCOOH, or in the reverse order, resulted in the GFP reconstitution after incubation for 48 h as observed under a confocal microscope (Figure 5a). This detected green fluorescence was targeted into the onion cell nuclei. To confirm that protein–protein interaction occurred at the cellular nucleus, DAPI staining that specifically marks DNA was used. Co-localization of the green and blue fluorescence when both photographs were overlapped demonstrated a clear spatial location (Figure 5a). In contrast, no reconstitution of the fluorescence was detected when both DOF proteins (HvDOF19 and HvDOF17) were fused to the complementary GFP fragments, or when these GFP fragments were tested alone (data not shown). These results indicated that both DOF19 and DOF17 interacted with

Figure 5. Protein–protein interaction of HvDOF19 and HvDOF17 with GAMBY in *Allium cepa* cell nuclei and in the yeast *Saccharomyces cerevisiae* two-hybrid system (Y2HS).

(a) Nuclear localization of the reconstituted GFP complexes in transiently transformed onion epidermal cells. Fluorescence appeared at the nucleus after the co-bombardment with HvDOF19-GFPNH₂ and GAMBY-GFPCOOH (left panels) or with HvDOF17-GFPCOOH and GAMBY-GFPNH₂ (right panels) when the samples were observed under green fluorescence (GF) in a confocal ultraspectral microscope (LEICA TCS-Sp2-AOBS-UV). Observation under Nomarski bright field (NBF) and under blue fluorescence (BF) showed that reconstituted GFP co-localized with the DAPI-stained nucleus when the pictures were overlapped (O). Scale bars: 50 µm.

(b) Schematic representation of the reporter and effector constructs used for the Y2HS assays. Gal1 UAS, Gal4 responsive element in the Gal1 promoter; Gal4BD, Gal4 DNA-binding domain; Gal4AD, Gal4 DNA activation domain, GAMBY, full-length open reading frame (ORF); the NH₂, COOH-terminal and DOF domain regions of HvDOF19 and HvDOF17 are those indicated in Experimental procedures.

(c) The combinations of constructs 1–5 were used to transform *S. cerevisiae* strain SFY526 and *LacZ* induction was measured to test the interaction between HvDOF19 and GAMBY.

(d) The combinations 1 and 6–9 were used to test the interaction between HvDOF17 and GAMBY. GUS activity in liquid cultures was calculated from six independent replicates in two different assays. Standard errors are indicated.

GAMYB in plant cell nuclei, but that they do not form homo- or hetero-dimers between them.

To ascertain what parts of HvDOF19 and HvDOF17 proteins were responsible for the interaction with GAMYB, Y2HS experiments were performed using the *LacZ* gene as reporter. As represented schematically in Figure 5(b), the GAMYB protein was expressed as a translational fusion to the yeast Gal4 binding domain (BD), and a series of constructs containing either the whole ORFs of HvDOF19 or HvDOF17, or three different fragments derived from them, were fused to the yeast Gal4 activation domain (AD). These constructs were used to transform *Saccharomyces cerevisiae* yeast cells (strain SFY526) and the corresponding *LacZ* reporter activity was quantified in liquid medium. When yeast cells were transformed with the Gal4BD-GAMYB fusion construct, the β -galactosidase activity values were about 40 Miller units, indicating that GAMYB functions as a transcriptional activator in yeast. Combinations of Gal4BD-GAMYB with HvDOF17 or its N-terminal part almost doubled this activity, whereas the fusion of the Gal4AD to its C-terminal part reached a β -galactosidase activity of 100 units (Figure 5b,d). However, no increase above Gal4BD-GAMYB activity was detected when the 52 residues of the DNA-binding domain were fused in frame to the Gal4AD. This indicated that HvDOF17 interacted with GAMYB by both its N- and C-terminal regions, but that the DOF domain did not participate in this interaction. Similar results were obtained

for the HvDOF19-GAMYB interaction. However, in this case the increase in the activity that showed the interaction of GAMYB with the C-terminal region of HvDOF19 was about 1.5-fold higher than when Gal4BD-GAMYB was used alone (Figure 5b,c).

HvDOF19 binds to the three DOF motifs in the Al21 gene promoter in vitro, and HvDOF17 affects the binding affinity of GAMYB for its recognition site CAACG/C

As HvDOF17 and HvDOF19 mediated the repression of the *Al21* gene promoter in transient expression experiments, and as the sequence of this promoter presents three putative DOF-binding motifs (Isabel-LaMoneda *et al.*, 2003), we tested if both proteins were capable of binding specifically to these motifs *in vitro* through electrophoretic mobility shift assays (EMSA).

The HvDOF17 and HvDOF19 proteins were expressed as GST fusions in *Escherichia coli*, and were incubated with the three radioactively-labeled oligonucleotide probes D1, D2 and D3 derived from the *Al21* gene promoter. As shown in Figure 6(a), GST-HvDOF19 bound to the three probes *in vitro*, although the retardation band detected for the D3 probe was considerably fainter. Binding specificities were confirmed by competition titrations, up to 100X molar excess, with the corresponding non-labeled probes D1 and D2. The mutated probes d1, d2 and d3, in which the AAAG

Figure 6. Electrophoretic mobility shift assays (EMSAs) of the recombinant GST-HvDOF17, GST-HvDOF19 and GST-GAMYB proteins with probes derived from the *Al21* gene promoter. (a) EMSA of the recombinant HvDOF19 protein with the ³²P-labeled probes D1, D2 and D3 containing DOF-binding target sequences in bold, or their mutated versions, d1, d2 and d3, in lower letters. Competition experiments were performed using increasing quantities (20X, 50X and 100X) of the indicated unlabeled wild-type or mutated probes. (b) EMSA of serial dilutions of GST-GAMYB with the ³²P-labeled probe M1 that contains a R2R3MYB binding site, and GST-HvDOF17, GST-HvDOF19 or GST, used as control.

core sequence was changed to AgAc, neither produced any shifted complex nor competed with the labeled probe, even when used at 100X molar excess. As expected, binding was not detected when the GST protein expressed in *E. coli* was assayed. No shifted bands were observed in similar experiments when incubation was carried out with the GST-HvDOF17 recombinant protein (data not shown).

Based on the fact that the *trans*-activation exerted by GAMYB in transient expression experiments in aleurone was reversed by HvDOF17 and HvDOF19, and that both DOF factors interacted *in vivo* with GAMYB, we decided to investigate whether these interactions might affect the binding capacity of GAMYB to its specific-target motifs in the *Al21* gene promoter. EMSA experiments were performed using GAMYB expressed as a GST fusion protein, the M1 probe, which included the MYBR2R3 motif located at position -367 bp of the *Al21* promoter (Isabel-LaMoneda *et al.*, 2003), and the recombinant GST-HvDOF17 and GST-HvDOF19 proteins. As shown in Figure 6(b), a clear decrease in the intensity of the retarded band was observed when the GAMYB protein was incubated with the HvDOF17 protein, whereas no effect was observed when the HvDOF19 protein was used in the assay.

Discussion

Seed germination begins with water imbibition, proceeds with the elongation of the radicle tip inside the seed and ends when the radicle protrudes from the seed coat (Bove *et al.*, 2001). This process and the subsequent post-germination events are mediated by hormones, particularly GA and ABA. GA activation and ABA repression of hydrolase genes in the cereal aleurone are key processes that regulate the proper supply of nutrients from the endosperm to the embryo until the photosynthetic capability is completely acquired. In barley, GA promotes the degradation of the DELLA protein SLN1 that finally induces the expression of GAMYB, which is the principal transcriptional activator of hydrolase gene expression (Fu *et al.*, 2002; Kaneko *et al.*, 2004; Sun and Gubler, 2004). However, the ABA-mediated repression of these genes is a mechanism not yet well understood. This article provides novel insights into the ABA regulation of genes encoding hydrolases, and proposes a role for HvDOF19 in the ABA-dependent pathway. HvDOF17 and the previously reported BPBF (Mena *et al.*, 2002) would also contribute to the negative regulation of hydrolase gene expression in germinating aleurone, but in an ABA-independent pathway.

Evidence of the ABA negative regulation of hydrolase gene expression put forward the existence of various mechanisms of regulation. Firstly, ABA would act through the GA-positive regulator GAMYB, as its mRNA levels decrease by the addition of ABA (Gubler *et al.*, 1995, 2002). However, the ABA repression of hydrolase gene expression is independent

of the GA-signaling transduction pathway and acts downstream of the barley SLN1 protein because: (1) ABA suppresses the derepression of α -amylase genes that occurs in the absence of GA when RNAi technology is used to inhibit the SLN1 synthesis (Zentella *et al.*, 2002); and (2) the GA-induced degradation of SLN1 is not blocked by ABA (Gubler *et al.*, 2002). In addition, ABA only partially counteracts the GA-induced expression of GAMYB (Gubler *et al.*, 2002), so there must be other components/factors implicated in the ABA repression of hydrolase genes. The ABA-induced protein kinase PKABA1 inhibits the GA induction of both *GAMYB* and hydrolase gene expression when this hormone is externally added (Gomez-Cadenas *et al.*, 1999, 2001b; Shen *et al.*, 2001). However, PKABA1 has no effect on GAMYB-transactivated hydrolase gene expression, and the use of RNAi to block its synthesis does not affect the ABA-mediated repression of α -amylase genes (Gomez-Cadenas *et al.*, 2001b; Zentella *et al.*, 2002). This suggests that an alternative pathway independent of this kinase must exist. Our results indicate that HvDOF19, the mRNA levels of which are highly induced by ABA in aleurone layers (sixfold, Figure 2b), represses the expression of the *Al21* gene promoter both in the presence and in the absence of GA in transient expression experiments (Figure 4b). In addition, when we simultaneously use HvDOF19 and GAMYB as effectors, we observe that HvDOF19 suppresses the GAMYB-induced expression of the GUS gene under the control of the *Al21* gene promoter. As the mRNA levels of *HvDof19* are still induced in aleurones incubated with ABA plus GA, the DOF19 transcription factor could mediate the ABA repression of hydrolase genes both in the presence of GA and in its absence. This was further supported by RNAi experiments silencing the *HvDof19* gene transcripts. As shown in Figure 4(c), the ABA-mediated repression of the *Al21* gene decreased by at least 60% when *HvDof19* was silenced and both ABA alone and the mixture of ABA and GA were added to the aleurone incubation medium. HvDOF17 also represses the expression of the *Al21* gene promoter in transient expression experiments (Figure 4b). However, the expression of the *HvDof17* gene is not affected by incubation with ABA (Figure 2b), and RNAi experiments silencing *HvDof17* do not support a role for this gene in the ABA-mediated regulation of hydrolase genes (data not shown). Therefore, HvDOF17 would negatively regulate *Al21* gene expression independently of the ABA pathway.

Other genes from cereals participating in the ABA-mediated repression of hydrolase genes in the aleurone are *OsWrky51* and *OsWrky71* from rice. Both genes are induced by ABA, and their corresponding proteins repress the GA-induced expression of hydrolase genes through binding to the 5'-TGAC-3' sequence within the O2S box (Xie *et al.*, 2006; Zhang *et al.*, 2004). Although this sequence is present in low-pl α -amylase gene promoters, it seems to be absent in the high-pl α -amylase gene promoters (Lanahan *et al.*, 1992). In addition, the developmental regulator VIVIPAROUS (VP1)

from maize, which is required for ABA-induced gene expression during seed maturation (Rock and Quatrano, 1995 and references cited therein), has been described to specifically repress the induction of α -amylase genes by GA in aleurone of germinating barley seeds (Hoecker *et al.*, 1995). However, no direct interaction with hydrolase gene promoters has been reported.

Our results also show that HvDOF19 specifically binds to the pyrimidine boxes in the promoter of the *Al21* gene. On the contrary, HvDOF17 does not seem to bind directly to any of these putative DOF-binding motifs *in vitro* (EMSA). However, it is able to repress the expression of the *Al21* gene, probably by decreasing the binding affinity of the GAMYB protein to its target site in this promoter (Figure 6b). In addition, protein–protein interactions between GAMYB and HvDOF19 and HvDOF17 in plant nuclei have been observed, pointing to their participation in a combinatorial regulatory complex with GAMYB and other DOF TFs, such as BPBF and SAD (Figure 7).

Over the past few years information regarding variation of ABA and GA content in germinating barley seeds has been reported (Jacobsen *et al.*, 2002; Millar *et al.*, 2006). After imbibition, ABA mainly decreases in non-dormant grains, and once its levels are reduced (which seems to occur around 18 hai), biologically active GA begins to accumulate. In Arabidopsis, the GA requirements to promote germination seem to be determined by embryonic ABA (Ali-Rachedi *et al.*, 2004; Debeaujon and Koornneef, 2000), which would be *de novo* synthesized to maintain dormancy in imbibed seeds (Koornneef *et al.*, 2002). Our results show that in aleurone layers *HvDof19* mRNA levels peak at 16 hai, just before radicle emergence (18 hai), only to fall drastically thereafter. According to this GA/ABA profile, *HvDof19* mRNAs could be induced by ABA before it is catabolized, and before the GA synthesized by the embryo diffuses to the aleurone. Later on, when these cells change to a high GA/ABA ratio, a decrease in the transcript levels of the *HvDof19* gene would take place. Interestingly, *HvDof17*, *HvDof19* and the barley *Pbf* present similar expression patterns, although neither *HvDof17* nor *Pbf* are induced by ABA. GA would contribute to the decrease in the *HvDof17* and *HvDof19* mRNA levels, whereas for the *HvDof24-Pbf* gene, which is GA induced (Figure 2b and Mena *et al.*, 2002), the 50% detected decrease at 24 hai might be motivated by the disappearance of some other unknown factor(s) that would have caused its induction up to fourfold at 16 hai.

We propose a model of negative regulation of hydrolase gene expression mediated by DOF TFs in aleurone layers. In this model, when the ABA level is still high, as occurs in the early germinative phase, HvDOF19 would mediate the ABA repression of hydrolase genes. HvDOF17 and BPBF would help in this process independently of the ABA pathway (Figure 7b). In the post-germination, when the ABA level has decreased and the GA diffusion from the scutellum has been

Figure 7. Proposed model of transcriptional regulation of abscisic acid repressed/gibberellins induced expression of hydrolase genes in barley aleurone layers mediated by DOF TFs and GAMYB.

(a) The diagram summarizes the proposed role of HvDOF17 and HvDOF19 in the suppression of hydrolytic enzymes expression in barley aleurone.

(b) In an early phase of germination (before 18 hai) when the ABA level is high, negative regulation would be exerted by HvDOF17, HvDOF19 and BPBF on the promoter of the *Al21* gene.

(c) In a later phase of germination (post-germination, after 18 hai), when the GA/ABA ratio is high, GAMYB and other transcriptional activators would trigger the GA-mediated transcriptional activation of the *Al21* gene.

Transcription factors (TFs) are indicated as jigsaw puzzle pieces (HvDOF17, HvDOF19, BPBF, GAMYB and SAD) and DNA target sequences for each class of TF are boxed.

produced, the quantity of these TFs would be insignificant compared with that of the positive effectors such as GAMYB, SAD and other DOF and R1MYB activators (Rubio-Somoza *et al.*, 2006a,b). Therefore, transcription of aleurone hydrolase genes would be carried out (Figure 7c).

Experimental procedures

Cloning of HvDof19 and HvDof17

The ORFs of *HvDof19* and *HvDof17* (Moreno-Risueno *et al.*, 2007) were amplified from first-strand cDNA of germinating barley seeds cv. Bomi by PCR using the primers 5'-ATGCTCTCCACGTC-3' and 5'-TCAGAACCGGGGGTC-3' for *HvDof17* and 5'-ATGGTGTCTCACTCTC-3' and 5'-TCATGCGTATTGGTTG-3' for *HvDof19*. The resultant amplified fragments were cloned in pGEM T-easy (Promega, <http://www.promega.com>) and further used for their molecular and functional characterization.

RT-qPCR analyses

RT-qPCR analyses were performed to study variation in the rate of transcription of the genes *HvDof2*, *HvDof8*, *HvDof17*, *HvDof19*, *HvDof23-Sad*, *HvDof24-Pbf* (encoding DOF factors), *GAMYb* (encoding a R2R3MYB factor) and *CatB* (encoding a cathepsin B-like protease) in aleurone cells and in embryos upon germination, as well as in isolated aleurone layers in response to GA and ABA treatments. Seeds of barley cv. Himalaya were sterilized in 1.7% NaOCl for 10 min, washed in distilled water and incubated on water imbibed filter paper at 22°C in the dark. Aleurone layers and embryos were collected at different incubation times. For hormone treatments, seeds of barley cv. Himalaya were de-embryonated, sterilized and incubated for 48 h in the dark at 22°C on filter paper soaked with calcium succinate buffer (20 mM Na-succinate, 20 mM CaCl₂, pH 5.2). Then, aleurone layers were isolated under a dissecting microscope and incubated for 20 h in the same buffer containing 1 µM GA₃, 10 µM ABA or a mixture of both hormones, or with no hormones as controls. 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.com>). These samples (20–50 µg) were used to isolate mRNA (Quick-Prep™ Micro mRNA Purification Kit; GE Healthcare, <http://www.gehealthcare.com>), and duplicate first-strand cDNA samples were synthesized in the presence of a 17xT oligonucleotide according to the manufacturer's instructions (first-strand cDNA Synthesis Kit; GE Healthcare). RT-qPCR analyses were performed for each of the duplicate samples by means of a 7300 Real Time PCR System (Applied Biosystems, <http://www.appliedbiosystems.com>) using an SYBR-green detection system for *HvDof2*, *HvDof8*, *HvDof23-Sad*, *HvDof24-Pbf*, *GAMYb* and *CatB* genes, and for *HvDof17* and *HvDof19* genes only in the analysis shown in Figure 1(a), and a TaqMan MGB probe detection system for *HvDof17* and *HvDof19* in the rest of the analyses. Quantification was standardized to the barley *Actin2* mRNA levels, and when indicated were referred to the control value that was arbitrarily set to 1. The primers used for PCR amplification were as follows: barley *Actin2* (SYBR-green detection system: forward, 5'-TCCAGCTATCGTCCACAGGAA-3'; reverse, 5'-TGTTCAACAAACCACACCT-3'; TaqMan MGB probe detection system: forward, 5'-TCCACGGAGAGGAAGTACAGT-3'; reverse, 5'-AATGTGCTCAGAGATGCAAGGA-3'), *HvDof17* (SYBR-green detection system: forward, 5'-TGTTTCCTCCCCCTGGTTTCTT-3'; reverse,

5'-TTGATGCTACTACCGCGCAAC-3'; TaqMan MGB probe detection system: forward, 5'-ACAGACTCGGGCTTGAAGA-3'; reverse, 5'-CCGTCGCGTCACGATTC-3'), *HvDof19* (SYBR-green detection system: forward, 5'-TGGCTCGTTCATCAGTACCCA-3'; reverse, 5'-CGGCAAGAAATGCAAGTTGG-3'; TaqMan MGB probe detection system: forward, 5'-CTCGGCCACGGAGAGATG-3'; reverse, 5'-TTGGGCTGGTACTGATGAAC-3'), *HvDof2* (forward, 5'-ATATGCATGGCTCATGCGATC-3'; reverse, 5'-CCAATGCCACCACCATCTTATT-3'), *HvDof8* (forward, 5'-CACACCCACAAAGCCACTGATA-3'; reverse, 5'-TGGACTGGAAGTCTTGCATGG-3'), *HvDof23-Sad* (forward, 5'-TGCTCTAGCCCCGAGGACATAAA-3'; reverse, 5'-GGAATGTCTTGTGGATGACCGT-3'), *HvDof24-Pbf* (forward, 5'-GGAAATGGAATAGGTGGCACA-3'; reverse, 5'-TACCAACATGTGGCTGCCACT-3'), *GAMYb* (forward, 5'-TGTGATACCACGTTGGTTAGCC-3'; reverse, 5'-GGATCCAGTGAGAGAATTGCCA-3') and *CatB* (forward, 5'-TCGATGAAGAATCGCCG-3'; reverse, 5'-TGCCCCGTTAATTTGACAGG-3'). The sequences of the TaqMan MGB probes were: *Actin2* (5'-TCTGGATCGGAGGTC-3'), *HvDof17* (5'-AGAGTTTGGTTTGACCCG-3') and *HvDof19* (5'-AGCAGTGCTGGTGGC-3').

In situ hybridization

Seeds of barley cv. Bomi were sterilized in 1.7% NaOCl for 10 min, washed in distilled water and germinated at 22°C in the dark for 16 h. Aleurone layers and embryos were collected, fixed in 4% formaldehyde in PBS, dehydrated, embedded in paraffin and sectioned to 10 µm. After de-waxing in HistoClear, the samples were rehydrated, treated with cellulase 2% in PBS, with 0.25% acetic anhydride in 0.1 M triethanolamine and dehydrated. Hybridization was performed overnight at 50°C in hybridization solution (100 µg ml⁻¹ tRNA, 6× SSC, 3% SDS and 50% formamide) plus approximately 100 ng µl⁻¹ of the antisense or sense DIG-labelled RNA probes corresponding to *HvDof17* (from 670 to 984 bp from the ATG initiation codon) and *HvDof19* (from 401 to 770 bp from the ATG initiation codon). Washes of 2× SSC and 0.2× SSC for 15 min at 50°C were then carried out twice. Antibody incubation and colour detection were performed according to the manufacturer's instructions (Roche Diagnostics).

Subcellular localization in aleurone cells

Translational fusions of the ORFs of the *HvDof17* and *HvDof19* genes to the GFP reporter gene were made by cloning both ORFs into the *Bam*HI restriction site of a pBlueScript vector (Stratagene, <http://www.stratagene.com>) in which the GFP gene had been previously cloned into the *Bam*HI-*Eco*RI sites. This plasmid already contained the CaMV35S promoter followed by the first intron of the maize *Adhl* gene and the 3' NOS terminator. The generated constructs were: P_{35S}-*Adhl*::*HvDof17-Gfp*, P_{35S}-*Adhl*::*HvDof19-Gfp* and P_{35S}-*Adhl*::*Gfp* as a control. Preparation of aleurone layers, gold particle coating and bombardment with a biolistic Helium gun device (DuPont PDS-1000; BioRad, <http://www.biorad.com>) were performed as described in (Rubio-Somoza *et al.*, 2006b). After 24 h of incubation at 22°C in the dark, aleurone layers were observed and photographed under a confocal ultraspectral microscope LEICA TCS-Sp2-AOBS-UV (Leica, <http://www.leica.com>) using the appropriated filters.

Transient expression assays in aleurone layers

The reporter constructs pCBG13 and pCBG13.1 containing two fragments of the wheat cathepsin B-like promoter of the *Al21* gene

were the same as those used in Cejudo *et al.* (1992). The effector constructs were made by cloning the whole ORFs of *HvDof17* and *HvDof19*, or a fragment of the *HvDof19* gene (from 502 to 737 bp from the translation start codon), in sense and antisense, and separated by a 110-bp length DNA using an *NcoI* and an *XbaI* site, into the *BamHI-EcoRI* restriction sites of the pBlueScript vector (Stratagene) under the control of the CaMV35S promoter followed by the first intron of the maize *Adhl* gene and the 3' NOS terminator. The effector construct carrying the ORF of *GAMYb* and preparation of aleurone layers, gold particle coating and bombardment with a biolistic Helium gun device (DuPont PDS-1000; BioRad) were performed as described previously (Diaz *et al.*, 2002; Isabel-LaMoneda *et al.*, 2003). GUS expression was determined histochemically following the method of Jefferson (1987). GUS activity was calculated as the mean value of blue spots per aleurone in each assay and expressed as a percentage, considering 100% as the value obtained with the reporter constructs without effectors. The histochemical data were directly correlated with the GUS expression quantified by chemiluminescence (GUS light Kit; Tropix, Bedford, MA, USA) per milligram protein with a correlation coefficient of 0.98 (data not shown).

Bimolecular fluorescence complementation

HvDof19 and *HvDof17* were fused in-frame with both the NH₂- and COOH-terminal encoding fragments of *Gfp*, by cloning both ORFs into the *BamHI* restriction site of the psmRS-GFP plasmids carrying the corresponding GFP fragments. The constructs generated were 35S::HvDOF19-GFP NH₂, 35S::HvDOF19-GFP COOH, 35S::HvDOF17-GFP NH₂, 35S::HvDOF17-GFP COOH. The constructs carrying *GAMYb* were the same as those in Diaz *et al.* (2005). Inner epidermal layers of fresh local purchased onions (*Allium cepa*) were prepared and co-bombarded as previously described (Diaz *et al.*, 2005). The fluorescence emission was observed after 48 h of incubation at 22°C in the dark under a confocal ultraspectral microscope LEICA TCS-Sp2-AOBS-UV (Leica). As a control of nuclei localization, tissue samples were stained with DAPI (Serva, <http://www.serva.de>).

Yeast transformation and LacZ assays

Plasmids pGBT9 and pGAD424 (Clontech, <http://www.clontech.com>) were used to generate translational fusions. The complete ORF of *GAMYb* fused to the Gal4 DNA binding domain in the pGBT9 vector is the same as that in Diaz *et al.* (2002). The whole *HvDof19* and *HvDof17* ORFs and derived fragments from both genes were cloned into the *EcoRI-Sall* restriction sites of the pGAD424 plasmid. The derived fragments from *HvDof17* and *HvDof19* were: (1) the NH₂-terminal region of 1–396 nt and 1–462 nt from the ATG initiation codon of *HvDof19* and *HvDof17*, respectively; (2) the Dof domain regions of 231–396 nt and 297–462 nt of *HvDof19* and *HvDof17*, respectively; (3) the combination of N-terminal region plus Dof domain, and (4) the COOH-terminal regions of 396–648 nt and 462–609 nt from *HvDof19* and *HvDof17*, respectively. Yeast transformation was performed by the polyethylene glycol method and quantification of β -galactosidase production (*LacZ*) for the different transformants was calculated using Miller's formulas as described by Ausubel *et al.* (1990).

Electrophoretic mobility shift assays

The HvDOF19, HvDOF17 and GAMYB proteins were expressed in *E. coli* BL21-CodonPlus(DE3)-RP strain (Stratagene) by cloning their

ORFs into the *BamHI* 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-thio-galacto-pyranoside (IPTG) for 2 h and bacterial extracts were obtained by sonication in 8 mM DTT in PBS. Cells carrying the pGEX-2T vector with no insert were identically processed as negative controls. The end-labeling of the probes containing the putative consensus DOF (D1, D2 and D3) or GAMYB (M1 and M2) DNA binding sites from the *At21* gene promoter and mutated versions (d1, d2 and d3), and DNA-protein binding reactions were performed as described by Isabel-LaMoneda *et al.* (2003).

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