A Developmental Switch of Gene Expression in the Barley Seed Mediated by HvVP1 (Viviparous-1) and HvGAMYB Interactions[1]

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The accumulation of storage compounds in the starchy endosperm of developing cereal seeds is highly regulated at the transcriptional level. These compounds, mainly starch and proteins, are hydrolyzed upon germination to allow seedling growth. The transcription factor HvGAMYB is a master activator both in the maturation phase of seed development and upon germination, acting in combination with other transcription factors. However, the precise mechanism controlling the switch from maturation to germination programs remains unclear. We report here the identification and molecular characterization of *Hordeum vulgare* VIVIPAROUS1 (HvVP1), orthologous to ABA-INSENSITIVE3 from *Arabidopsis thaliana*. *HvVP1* transcripts accumulate in the endosperm and the embryo of developing seeds at early stages and in the embryo and aleurone of germinating seeds up to 24 h of imbibition. In transient expression assays, HvVP1 controls the activation of *Hor2* and *Amy6.4* promoters exerted by HvGAMYB. HvVP1 interacts with HvGAMYB in *Saccharomyces cerevisiae* and in the plant nuclei, hindering its interaction with other transcription factors involved in seed gene expression programs, like BPBF. Similarly, this interaction leads to a decrease in the DNA binding of HvGAMYB and the Barley Prolamine-Box binding Factor (BPBF) to their target sequences. Our results indicate that the *HvVP1* expression pattern controls the full *Hor2* expression activated by GAMYB and BPBF in the developing endosperm and the *Amy6.4* activation in postgerminative reserve mobilization mediated by GAMYB. All these data demonstrate the participation of HvVP1 in antagonistic gene expression programs and support its central role as a gene expression switch during seed maturation and germination.

Seed development after fertilization has been classically divided into two major phases: zygotic embryogenesis and maturation. The maturation phase is characterized by gene expression programs devoted to the synthesis and accumulation of reserve compounds, among them seed storage proteins (SSPs), and, later, to the acquisition of desiccation tolerance, with an important role for the late embryogenic abundant (LEA) proteins (Vicente-Carbajosa and Carbonero, 2005). In most species of the Spermatophyta, seeds acquire a quiescent state at the end of the maturation phase that is resumed by the germination process, which is generally accepted to comprise the germination sensu stricto from seed imbibition to root emergence that is followed by reserve mobilization. These two distinct phases are independently regulated and influenced by genetic and environmental factors (Bewley, 1997; Finch-Savage and Leubner-Metzger, 2006; Nonogaki et al., 2007; Holdsworth et al., 2008; Iglesias-Fernández et al., 2011b; González-Calle et al., 2015). During early postgermination (reserve mobilization), several genes encoding hydrolytic enzymes (i.e. α-amylases, proteases, and lipases) catalyze the hydrolysis of carbohydrates, proteins, and lipids present in the seed storage tissues, such as the endosperm of monocotyledonous species and the cotyledons of dicotyledonous ones, with the aim of nourishing the growing embryo before attaining its full photosynthetic capacity (Pritchard et al., 2002; González-Calle et al., 2014; Iglesias-Fernández et al., 2014).

The comprehensive molecular and genetic mechanisms controlling seed development and germination still remain elusive, due mainly to the many environmental and

intrinsic parameters influencing it. From a physiological point of view, it is generally accepted that an increased abscisic acid ABA-GA ratio determines the progression of seed maturation by promoting the expression of SSPs, LEAs, heat shock proteins, and anthocyanin biosynthesis genes (Reidt et al., 2000; Koornneef et al., 2002; Vicente-Carbajosa and Carbonero, 2005; Braybrook and Harada, 2008; Finkelstein et al., 2008). By contrast, an increased GA-ABA ratio is the most important factor for the integration of the environmental and intrinsic signals for the occurrence of germination. This enhanced GA-ABA balance raises the expression of hydrolase genes such as those encoding cell wall-remodeling enzymes, involved in the weakening of the embryo-surrounding tissues during germination sensu stricto, and of reserve mobilization enzymes post germination (Gubler et al., 1995, 1999; Mena et al., 2002; Iglesias-Fernández et al., 2011a, 2011c; González-Calle et al., 2015).

In cereal seeds, the transcription factor (TF) of the B3 family VIVIPAROUS1 (VP1), orthologous to ABA-INSENSITIVE3 (ABI3) from Arabidopsis (*Arabidopsis thaliana*), is a key component of ABA signaling and is required for the expression of the maturation program (McCarty et al., 1991; Parcy et al., 1994; Jones et al., 1997; Nambara et al., 2000; Suzuki et al., 2001; Lara et al., 2003; Zeng et al., 2003; To et al., 2006; Swaminathan et al., 2008; Yan et al., 2014). The *VP1* mutants, like *vp1* in maize (*Zea mays*) and *abi3* in Arabidopsis, are characterized by the incapacity of entering the quiescent state at the end of the maturation phase and by displaying premature features of the subsequent germination and postgermination phases, leading to a precocious germination of the seeds still in the mother plant (preharvest sprouting; McCarty et al., 1989; Gubler et al., 2005). It has been described previously that the VP1 TF, through its binding to the specific RY cis-element (5′-CATGCA-3′) and through protein-protein interactions with other TFs of the bZIP family, regulates the expression of seed maturation-specific genes, such as those encoding the Em protein in the wheat (*Triticum aestivum*) caryopsis and the C1 (MYB-like) TF, a key regulator of the anthocyanin biosynthesis pathway in maize (Paz-Ares et al., 1986; Hattori et al., 1992; Vasil et al., 1995). VP1 also has been associated with the repression of postgermination genes, since in the maize *vp1* mutant, α-amylase activity is precociously observed in the kernels while still in the cob, indicating that all necessary factors for its activation are present already during the maturation phase and in support of the idea that the VP1 protein must be preventing this hydrolytic activity (Hoecker et al., 1999; Rodríguez et al., 2015).

In the last 20 years, an important number of barley (*Hordeum vulgare*) TFs, such as GAMYB, BPBF (HvDOF24), SAD (HvDOF23), and BLZ2 (a bZIP orthologous to the maize OPAQUE2), have been characterized as central regulators of gene expression in the seed maturation and postgermination (reserve mobilization) programs (Gubler et al., 1995; Mena et al., 1998, 2002; Oñate et al., 1999; Diaz et al., 2002; Isabel-LaMoneda et al., 2003). However, their possible relationship with barley VP1 has not been investigated, although similar interactions have been reported in other cereal species (Hill et al., 1996; Hobo et al., 1999) and for ABI3 and AtbZIP10/AtbZIP25 (BLZ2 orthologs) with a role in activating the expression of SSP genes upon seed maturation in Arabidopsis (Lara et al., 2003).

In this work, the barley *VP1* gene (*HvVP1*) has been characterized and its expression localized, by mRNA in situ hybridization experiments, to the embryo and the endosperm of barley seeds, both during maturation and upon germination. In transient expression experiments in immature barley endosperms and in aleurone layers of germinating seeds, HvVP1 interferes with the transcriptional activation exerted by GAMYB on the promoters of the genes *Hor2* and *Amy6.4*, encoding a B-hordein (SSP) and a high-pI α-amylase, respectively. By yeast two-hybrid (Y2H) and bimolecular fluorescence complementation assays, the HvVP1-GAMYB protein interaction was confirmed in vivo and in plant nuclei. Interestingly, the presence of HvVP1 not only diminishes the GAMYB-BPBF protein interaction in yeast three-hybrid (Y3H) assays, but HvVP1 also decreases the binding affinities of GAMYB and BPBF for their corresponding cis-elements in the promoters of the *Hor2* and *Amy6.4* genes in electrophoretic mobility shift assays (EMSAs). All these data indicate a central role for HvVP1 as a gene expression switch at key stages of seed maturation and germination.

RESULTS

HvVP1 Sequence Identification and Phylogenetic Dendrogram

The sequence and structure of the predicted gene *HvVP1*, isolated from a barley 'Igri' genomic library (Supplemental Fig. S1), was confirmed by sequencing the corresponding open reading frame (ORF) derived from a PCR-amplified complementary DNA (cDNA) from developing barley seeds (20 d after pollination [dap]). This sequence was compared with other *VP1* sequences from different barley cultivars (cv Igri, cv Morex, and cv Haruna Nijo), with its orthologs in other Gramineae species deposited in public databases, such as wheat (*TaVP1*), *Triticum turgidum* (*TtVP1*), *Triticum monococum* (*TmVP1*), *Brachypodium distachyon* (*BdVP1*), and rice (*Oryza sativa*; *OsVP1*), and with *AtABI3* from Arabidopsis. The *HvVP1* gene is a single-copy gene (Supplemental Fig. S2). The deduced HvVP1 protein sequence from cv Igri is more than 99% identical with those from cv Morex (Mayer et al., 2012) and cv Haruna Nijo (Matsumoto et al., 2011) and with the VP1 partial sequence of cv Himalaya in GenBank (AAO06117.1; Casaretto and Ho, 2003; Supplemental Fig. S3). A phylogenetic tree including these sequences has been constructed (Fig. 1A), and HvVP1 is included on the same branch as those of the Triticeae tribe species, with a bootstrap value of 100%. This phylogenetic tree is

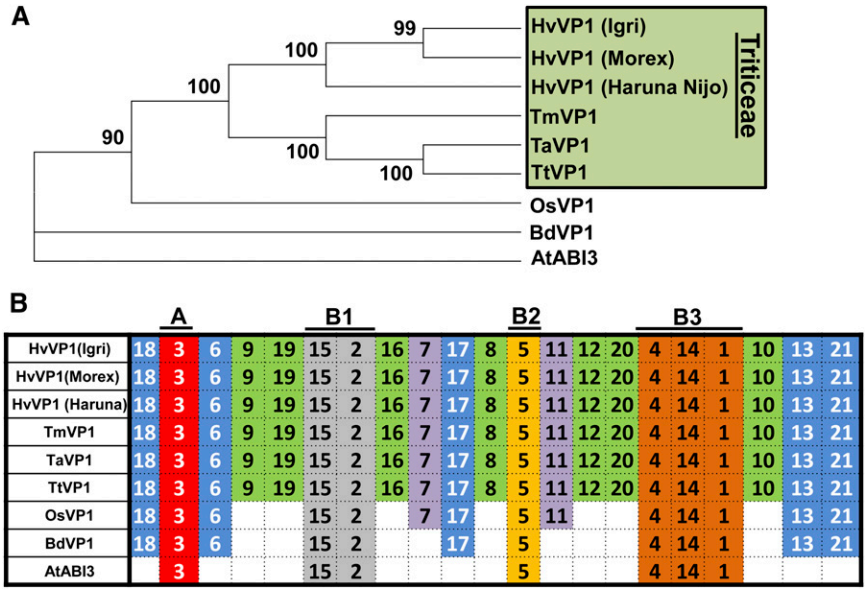

Figure 1. A, Phylogenetic tree with the deduced amino acid sequences of the *VP1* genes from distinct barley cultivars (cv Igri, cv Morex, and cv Haruna Nijo), with wheat (TaVP1), *T. turgidum* (TtVP1), *T. monococum* (TmVP1), *B. distachyon* (BdVP1), and rice (OsVP1), and with AtABI3 from Arabidopsis. Bootstrapping values are specified at the branches. B, Distribution of the conserved motifs among the deduced protein sequences in the dendrogram (A), found by means of MEME analysis. A, B1, B2, and B3 correspond to conserved domains, as described by Nakamura and Toyama (2001) and Marella and Quatrano (2007).

further supported by the occurrence of common motifs (MEME analysis; Fig. 1B; Table I). All sequences share motifs 1, 2, 3, 4, 5, 14, and 15, with the exception of motif 3, which is only partially conserved in AtABI3. According to Nakamura and Toyama (2001), motif 3 corresponds to the activation domain (A), motifs 15 and 2 form the B1 domain, motif 5 matches with the B2 domain, and motifs 1, 4, and 14 integrate the B3 (DNA-binding) domain. VP1 proteins from members of the Triticeae tribe (TaVP1, TtVP1, TmVP1, and HvVP1) share motifs 8, 9, 10, 12, 16, 19, and 20, and all the VP1 proteins in the Poaceae genomes compared share motifs 6, 13, 17, 18, and 21.

The deduced amino acid sequences encoded by these *VP1* genes have nuclear localization signals (RKKR), molecular masses of 72 to 75 kD, and pI between 6.4 and 8.8 (Supplemental Table S1). The *HvVP1* gene contains six exons (Fig. 2A, solid bars) and five introns (Fig. 2A, lines) as determined by comparing the genomic DNA and cDNA clones. While domains A, B1, and B2 (containing the nuclear localization signal RKKR; Graeber et al., 2010) are encoded in the first

Table I. *Sequences of conserved amino acid motifs (MEME; Bailey et al., 2009) of the VP1 orthologous genes from barley, T. monococcum, T. turgidum, wheat, rice, B. distachyon, and Arabidopsis*

The nuclear localization signal RKKR is underlined in boldface.

Motif	*E*	Consensus Sequence
1	4.0e-377	DIGTS[QR]VW[SN]MRYRFWPNNKSRMYLLENTGDFVRSNELQEGDFIV[LI]YSDVK
2	8.8e-310	NNR[DE]CISAEDLRSIRL[RK]RSTIEAAAARLGGGRQGTMQLLKLILTWVQNHH
3	3.3e-175	DDFMFA[QD]DTFPALPDFPCLSSPSSS[TN]FSSSSSSNSSSAF
4	1.1e-150	DKNLRFLLQKVLKQSDVG[TS]LGRIVLPK[KE][EA]
5	3.6e-109	MEP[AS]AT[RK]EA**RKKR**MARQRRLS[CS]
6	1.6e-097	EPSEPAAAGDG[MV]DDL[SA]DID[HQ]LLDFAS[IL][NS]
7	1.1e-149	YEFP[TA]ETGAAAATSWMPYQAFSPT[AG]SYGGEA[MI]YPFQQGCST
8	1.4e-151	DMHAGAWPLQYAAFVPAGATSAGTQTYPMPPPG[AP]VPQPFAAPGFAGQFPQ
9	5.4e-101	DDVPWDDEPLFPDVGMMLEDVISEQQ[QL]QQ
10	5.7e-101	KYLIRGVKVRA[AQ]Q[EG]LAKHKN[AG]SPEKGGAS[DE]VKA
11	2.1e-112	LQQQRSQQLNLSQIQTGGFPQE[PQ]SPRAAHSAPV
12	1.5e-123	[HG]W[SG][PG][PL][AW][VT]Q[AQ][QA][PV][HQ]GQLM[IV]QVPNPLSTKSNSSRQKQQKPSPDAAARPPSGGA
13	5.3e-087	EDGGCKEKSPHGVRRSRQEA[AS]
14	5.0e-080	ETHLPELKT[GR]DGISI
15	2.4e-071	GGG[GS][STG]GSAADDLP[RAL]FFMEWL[TK]
16	8.2e-074	LQKKRPRVGAMDQEAPPAGGQLPSPGANP
17	4.4e-042	SSV[VA]VSSQPFSPPAA
18	8.1e-042	[AH]P[QR][GR][GS][GA]P[HR]RGK[GA]PAVEIR[HQ]GE
19	1.2e-036	[HY]G[AT][AG]GR[TV]AS[DH]AA[AG]GGGEDAFM
20	2.6e-029	[ST][PQ][QH]R[PQ]GQA[AS]AS[DN]KQRQQGA[RS][TR][PT]AAAP[AP]AG
21	1.6e-026	MNQMAVSI

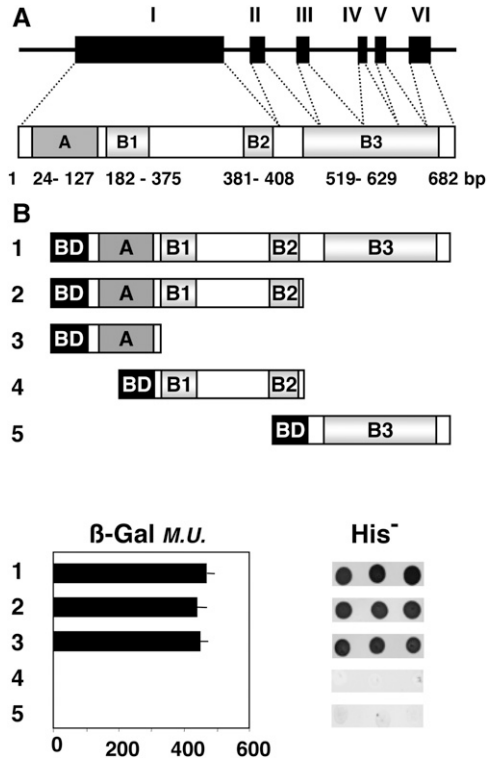

Figure 2. A, *HvVP1* transcript splicing model. Exons I to VI are indicated as black bars. Motifs A, B1, B2, and B3 are as in Figure 1. B, Functional assay in yeast for the activation capacity of the HvVP1 protein. Different constructs (1–5) were generated as fusions to the GAL4 DNA-binding domain (black box at the N terminus), and the activation capacity was assayed in two alternative reporter systems: β-Galactosidase (β-Gal) activity (*LacZ* reporter gene; Miller's units [M.U.] in liquid medium) and *HIS3* (growth capacity in His-depleted [His^-] medium).

exon, the B3 DNA-binding domain is translated from exons II to VI.

Activation Properties of the HvVP1 Protein

To investigate the activation capacity of HvVP1 in yeast (*Saccharomyces cerevisiae*), a series of constructs containing different regions of the *HvVP1* ORF was prepared (Fig. 2B). These regions were cloned as translational fusions to the GAL4 DNA-binding domain into a yeast expression plasmid and tested as effectors for their capacity to transactivate two alternative reporter genes: *LacZ*, encoding a β-Gal assayed in liquid cultured cells; and *HIS3*, which promotes yeast growth in a His-depleted agar medium. Both in qualitative (His depleted) and quantitative (β-Gal; M.U.) assays, HvVP1 acts as a potent activator in yeast (approximately 450 β-Gal M.U.). However, when domain A is deleted, the reporter expression disappears, indicating that this region is essential for HvVP1 being a transcriptional activator in yeast.

HvVP1 Is Expressed in the Embryo and in the Endosperm during Seed Maturation and Germination

Cereal VP1 proteins, such as Arabidopsis ABI3, belong to a subfamily of B3 TFs controlling seed gene expression (Swaminathan et al., 2008). Consequently, it is expected that the *HvVP1* mRNA should be expressed during barley seed maturation and germination. To test this, total RNA was isolated from developing deembrionated seeds (endosperms at 5, 10, 15, and 20 dap), immature (20 dap) and mature dry embryos, and aleurones and embryos of germinating seeds at different times of imbibition (8, 16, 24, 48, and 72 h of imbibition [hoi]). Northern-blot analysis (Fig. 3) shows that the *HvVP1* transcripts are found both in developing endosperms, attaining maximum levels at 5 dap and decreasing thereafter (20 dap), and in immature and mature embryos, being more abundant at the immature stage (Fig. 3A). The *HvVP1* transcripts are expressed upon seed germination in the aleurone layer, reaching a peak at 24 hoi, and also in embryos (Fig. 3B). mRNA in situ hybridization experiments during seed maturation (20 dap) and upon germination (24 hoi) reveal that *HvVP1* transcripts are present throughout the seed (embryo and endosperm) during maturation, while its expression is restricted to the embryo and the aleurone layer upon germination (Fig. 3). The *HvVP1* transcripts are not found in leaves and roots of adult barley plants (data not shown).

HvVP1 Counteracts the HvGAMYB Transactivation Exerted on Endosperm-Specific Genes during Seed Maturation and Germination

HvGAMYB is a barley MYB-R2R3 protein that is a transcriptional activator of seed storage protein genes (B-hordeins: *HvHor2*) during seed maturation and of hydrolase genes (α-amylases: *HvAmy6.4*) needed for reserve mobilization post germination (Gubler et al., 1995, 1999; Diaz et al., 2002).

Taking into account that both *HvVP1* (Fig. 3) and *HvGAMYB* share similar spatial expression patterns in the seed, as occurs with other important seed-specific TFs (Supplemental Fig. S4), transient expression assays were done by cobombardment in barley developing endosperms and in germinating aleurones to investigate their mutual effects. In Figure 4A, a schematic description of the constructs used in the transactivation study is shown. The TFs HvVP1 and HvGAMYB were used as effectors. As reporter constructs, the *HvHor2* promoter (−560 bp upstream of the ATG initiation codon) and the *HvAmy6.4* gene promoter (−174 bp) were fused to the GUS reporter gene (P_{Hor2}:*uidA* and $P_{Amy6.4}$:*uidA*). As shown in Figure 4B, while cobombardment of developing barley endosperms with HvVP1 and P_{Hor2}:*uidA* reduces GUS activity to half that driven by the P_{Hor2}:*uidA* construct alone, cotransfection with HvGAMYB and P_{Hor2}:*uidA* provokes an increment of GUS activity of approximately 5-fold. When both TFs (HvVP1 and HvGAMYB) are cobombarded together with P_{Hor2}:*uidA*, the positive transactivation of P_{Hor2}:*uidA*

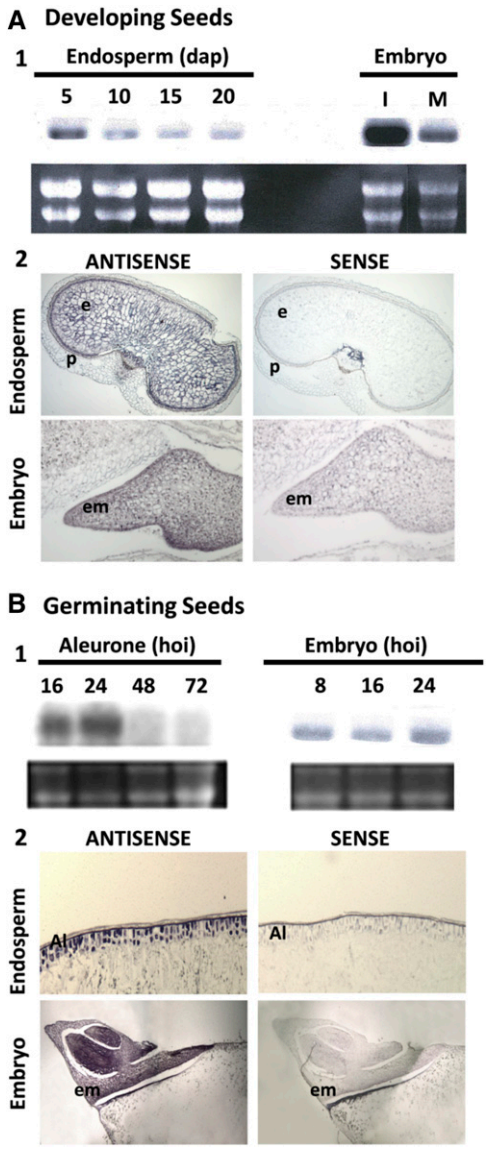

Figure 3. *HvVP1* expression analyses by northern blot and mRNA in situ hybridization analyses in barley developing (A) and germinating (B) seeds. A.1, Northern-blot analysis. Eight micrograms of total RNA from developing endosperms (from 5, 10, 15, and 20 dap) and immature (I; 25 dap) and mature (M) embryos was loaded in each lane and hybridized to an *HvVP1* gene-specific probe. A.2, *HvVP1* transcript localization in longitudinal sections of developing (25 dap) seeds by mRNA in situ hybridization B.1, Northern-blot analysis in germinating aleurone (16, 24, 48, and 72 hoi) and germinating embryo (8, 16, and 24 hoi). B.2, *HvVP1* transcript localization in germinating barley seeds (36 hoi) by mRNA in situ hybridization assay. Left and right images correspond to hybridizations with *HvVP1* antisense and sense probes, respectively. The ethidium bromide-stained ribosomal RNA images are included as loading controls. e, Endosperm; em, embryo; p, pericarp; s, scutellum.

exerted by HvGAMYB diminishes drastically (Fig. 4B). Similarly, Figure 4C shows that while cobombardment of HvVP1 with $P_{Amy6.4}$:*uidA* diminishes GUS activity to half that driven by the reporter gene alone ($P_{Amy6.4}$:*uidA*), cotransfection of HvGAMYB and $P_{Amy6.4}$:*uidA* increases GUS activity more than approximately 8-fold, and cobombardment of both TFs reduces the GUS activity of the reporter construct to half that obtained when HvGAMYB is the only transfected TF. This repressor effect of HvVP1 is independent of the presence of GA (1 μM) added to the incubation medium (Supplemental Fig. S5). In summary, HvVP1 is a transcriptional repressor of maturation and germination seed genes.

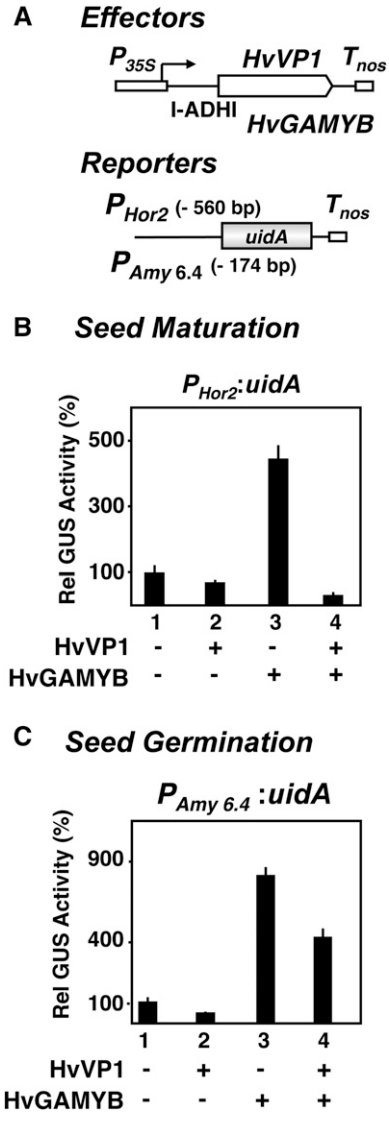

Figure 4. Transactivation assays using as effectors the TFs HvVP1 and HvGAMYB. The *Hor2* and *Amy6.4* gene promoters driving the expression of the *uidA* gene (GUS activity) were used as reporters. A, Schematic representation of the effector and reporter constructs used in the analyses. B, Cobombardment in barley developing endosperms of the effector and reporter combinations indicated. C, The effector and reporter constructs designated were cobombarded in germinating aleurones from barley kernels. The relative amounts of reporter and effector plasmids used in these assays correspond to a 1:1 ratio. Values are means ± SE of three independent replicates.

HvVP1 Interacts with HvGAMYB in the Y2H System and in Plant Nuclei

To explore whether the repression exerted by the HvVP1 TF on the transactivation mediated by HvGAMYB on seed-specific genes during maturation and germination could be due to a direct HvVP1-HvGAMYB protein-protein interaction, Y2H and bimolecular fluorescence complementation assays were done.

LacZ and *HIS3* reporter activities were measured in Y2H assays (Fig. 5). The *HvVP1* N-terminal cDNA (N), encoding 464 amino acid residues, spanning domains A, B1, and B2 (Fig. 2A), was translationally fused to the yeast *GAL4* activation domain (AD), and the full-length cDNA of *HvGAMYB* was fused to the *GAL4* binding domain (BD; Fig. 5B). In Figure 5C, yeast cells (strain SFY526) transformed with the *GAL4BD-HvGAMYB* construct show detectable background levels of *LacZ* reporter activity, and this activity increases sharply (approximately 400 β-Gal M.U.) when this strain is cotransformed with the *GAL4AD-HvVP1* construct. Expression of the *HIS3* gene (strain HF7C), conferring His auxotrophy, was used to confirm the interaction between HvGAMYB and HvVP1N (Fig. 5C). Yeast cells containing *GAL4BD-HvGAMYB* and the *GAL4AD-Ø* constructs are not able to grow in 3-aminotriazole concentrations higher than 30 mM. However, when transformed with *GAL4BD-HvGAMYB* and *GAL4AD-HvVP1N* constructs, yeast cells grow even at 60 mM 3-aminotriazole. All these results support the idea that the HvGAMYB and HvVP1 proteins interact in vivo.

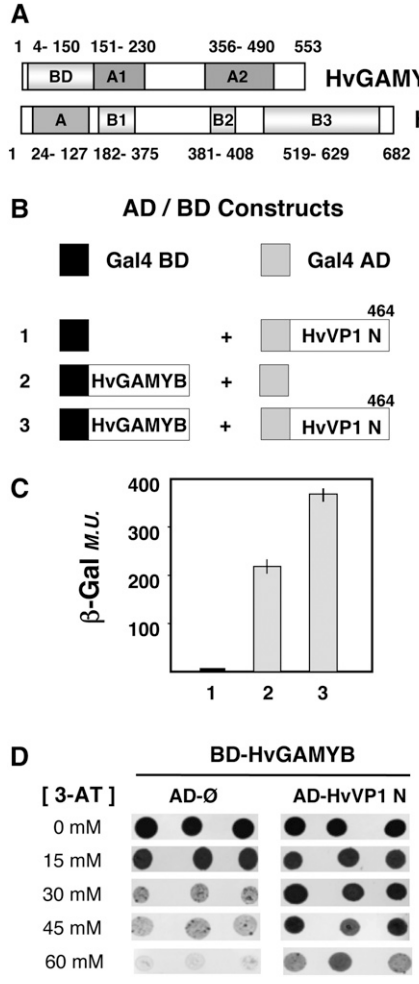

Figure 5. Protein-protein interaction by Y2H assays. A, The structures of HvGAMYB and HvVP1 are depicted, showing the locations of functionally relevant domains within the proteins. B, HvGAMYB (complete ORF) or HvVP1 N^{464} (the 464 N-terminal amino acid residues) expressed as translational fusions to the GAL4 DNA-binding domain (BD) and activation domain (AD), respectively. C and D, Interaction capacity assayed by measuring the β-Gal activity (*LacZ* reporter gene; M.U. in liquid medium; C) and by evaluating the yeast growth capacity in a His-depleted medium (reporter gene *HIS3*) with increasing concentrations of 3-aminotriazole (3-AT; D).

In order to corroborate the HvVP1-HvGAMYB interaction, bimolecular fluorescence complementation experiments were done in planta. With this aim, *HvVP1* and *HvGAMYB* ORFs were translationally fused to the N- and C-terminal fragments, respectively, of the GFP-encoding gene (Diaz et al., 2005) and used for cobombardment experiments in onion (*Allium cepa*) epidermal cells. Microscopic observations show that the GFP fluorescence is reconstituted and targeted to the nucleus, indicating that HvVP1 and HvGAMYB proteins interact in plant nuclei (Fig. 6). As expected, there is no detectable GFP when the GFP fragments are bombarded alone (data not shown).

HvVP1 Interferes with the HvGAMYB-BPBF Interaction in Vivo and with Their DNA Binding in EMSA

Since we showed that HvVP1 and GAMYB proteins can interact physically, we wanted to determine whether the HvVP1 protein could interfere with the binary complex formed by HvGAMYB and BPBF-HvDOF24, as reported previously (Diaz et al., 2002) For this purpose, a Y3H system was used, based on the pBridge vector, which allows the simultaneous expression of two proteins: in this case, the GAL4BD-BPBF translational fusion and the HvVP1 ORF, the latter conditionally expressed under the control of the *Met-25* promoter ($P_{Met\text{-}25}$) that responds to the Met supply in the medium. The *GAL4AD-HvGAMYB* construct was used as the third component of the system (Fig. 7A). In this Y3H system and in the absence of Met in the yeast growth medium, the joint expression of HvGAMYB-AD and BPBF-BD increases the reporter gene activity (greater than 80 β-Gal M.U.), compared with the expression of BPBF alone (approximately 40 β-Gal M.U.), as expected for a positive interaction between the two proteins. However, this activity diminishes when HvVP1 is present (approximately 60 β-Gal M.U.; Fig. 7B), suggesting that HvVP1 interferes with the interaction between HvGAMYB and BPBF.

The specific DNA-protein interactions between BPBF (HvDOF24) and its corresponding cis-motif (prolamine

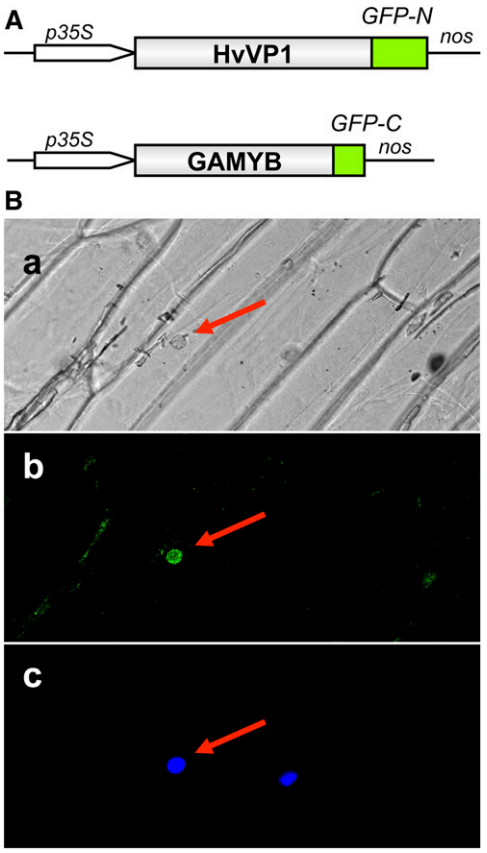

Figure 6. In planta interaction of HvVP1 and HvGAMYB. A, Structures of gene constructs used in bimolecular fluorescence complementation assays using particle gun transformation of onion epidermal cells. B, GFP reconstruction in the nuclei of cells transformed with a combination of the constructs described above (b). Bright-field images (a) and 4′,6-diamidino-2-phenylindole staining (c) was used to reveal nuclei within the cells.

box [PB]; 5′-TGTAAAG-3′) and between HvGAMYB and the MYB-R2R3 box (5′-TAACAAC-3′) at the promoter region of the *HvHor2* gene have been reported previously (Mena et al., 1998; Diaz et al., 2002). The potential effect of HvVP1 in modifying these protein-DNA interactions was investigated by EMSA (Fig. 7C). HvVP1, HvGAMYB, and BPBF proteins produced in *Escherichia coli* were assayed for their binding activities using two ^{32}P-labeled DNA probes containing the MYB-R2R3 and the PB-binding sites, derived from the *HvHor2* promoter (Fig. 7C). While a clear retarded band is observed when HvGAMYB or BPBF protein extracts are incubated with their respective probes, the addition of increasing amounts of HvVP1 protein to the reactions leads to fainter, or even to the absence of, retarded bands. Notably, HvVP1 does not bind to any of the probes used containing the PB and GLM motifs (target-binding sites of BPBF and BLZ2, respectively) or the MYB-R2R3 box (target-binding site of GAMYB), in support of its direct interference with the binding of these proteins. Interestingly, the modification of DNA binding by HvVP1 seems to be specific, since the intensity of the retarded bands in EMSAs for SAD (HvDOF23) was decreased in the presence of HvVP1,

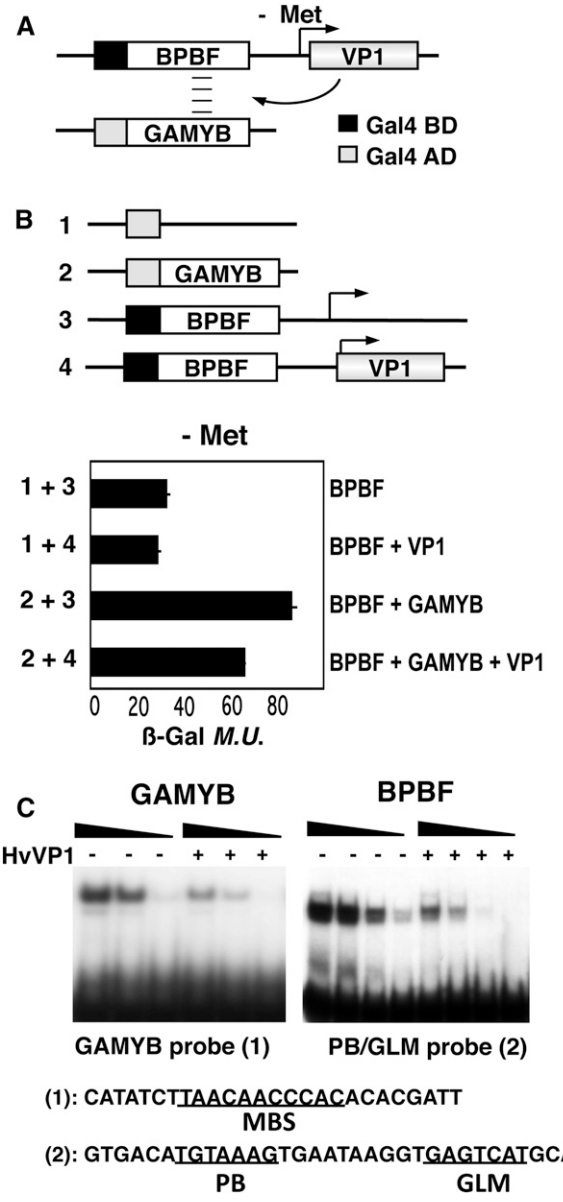

Figure 7. Protein-protein interactions of HvVP1, GAMYB, and BPBF in Y3H experiments and the effect of HvVP1 on GAMYB and BPBF DNA-binding capacities. A, Schematic representation of the constructs used in the Y3H assays, where the BPBF ORF was translationally fused to the GAL4 DNA-binding domain (BD) in the pBridge plasmid harboring HvVP1 under the control of a Met-repressible promoter. The GAMYB ORF was translationally fused to the GAL4 activation domain (AD) in pGAD424. B, Schematic representation of the constructs used for the Y3H assays and quantification of β-Gal activity (expressed as M.U.) in liquid assays using the constructs represented above. The proteins expressed in Met-depleted medium are indicated at right. C, EMSAs of HvGAMYB and BPBF proteins in the absence (−) or presence (+) of the HvVP1 protein. Incubations were done with a specific ^{32}P-labeled probe containing a target site for the corresponding TFs: GLM (G-box-like motif), BLZ2; PB, BPBF; MBS (MYB-binding site), GAMYB.

while the contrary effect (enhancement) was observed for the bZIP protein BLZ2 (Supplemental Fig. S6)

HvVP1 Counteracts the Transcriptional Activation of BPBF on the *HvHor2* Gene Promoter

BPBF is a barley DOF TF that acts as a transcriptional activator of the endosperm-specific gene *HvHor2* during seed maturation (Mena et al., 1998), and the BPBF transcripts are abundant during the seed maturation and germination phases (Supplemental Fig. S4).

As *BPBF* shares similar spatial expression patterns during seed development with *HvVP1* and the binding of its encoded protein for the PB box is decreased drastically by HvVP1 (EMSA in Fig. 7C), we decided to perform transient expression assays by particle bombardment in developing barley endosperms to explore the in planta effects of the coexpression of both TFs. Figure 8A schematically shows the constructs used in the transactivation study, where BPBF and HvVP1 were tested as effectors of GUS reporter activity driven by the P_{Hor2}:*uidA* construct. As shown in Figure 8B, cobombardment of barley developing endosperms with the HvVP1 effector and the P_{Hor2}:*uidA* reporter diminishes GUS activity to half that of P_{Hor2}:*uidA* alone, whereas cotransfection with BPBF and P_{Hor2}:*uidA* provokes an increment of GUS activity of approximately 7-fold. When both HvVP1 and BPBF are cobombarded, the positive P_{Hor2}:*uidA* transactivation exerted by BPBF is reduced to approximately one-half. This result demonstrates that HvVP1 counteracts the activation of BPBF upon the *HvHor2* gene in developing barley endosperms.

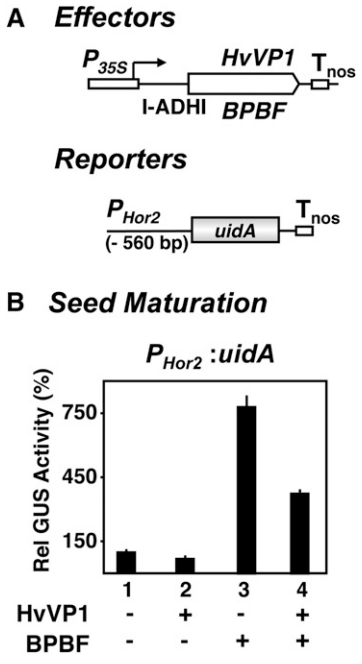

Figure 8. Transactivation assays using, as effectors, the TFs HvVP1 and BPBF and, as reporter, the *Hor2* gene promoter driving the expression of the *uidA* gene (GUS activity). A, Schematic representation of the effector and reporter constructs used in the analyses. B, Cobombardment experiments on barley developing endosperms using the indicated combinations of effector and reporter constructs. The relative amount of reporter-to-effector plasmids used in these assays corresponds to a 1:1 ratio. Values are means ± SE of three independent replicates.

DISCUSSION

In this work, we have identified the *HvVP1* gene of cv Igri and explored its function in the control of gene expression programs during seed development and germination. In particular, we address the role of HvVP1 through its interaction with HvGAMYB, a key TF that participates in the regulation of both maturation and germination genes in the barley seed. Our work unveils important features of HvVP1 action derived from its precise spatiotemporal expression patterns in the seed and its capacity to modulate regulatory complexes in which HvGAMYB participates. We propose a general model (Fig. 9) that integrates the data presented here with current knowledge derived from previous studies in the cereal seed.

HvVP1 Effects during Barley Seed Development

In barley, several TFs such as HvGAMYB, BPBF, and the bZIPs BLZ1 and BLZ2 (Vicente-Carbajosa et al., 1997, 1998; Mena et al., 1998; Oñate et al., 1999; Diaz et al., 2002), acting as part of regulatory complexes, have been reported to participate in the regulation of maturation genes like SSPs (B-hordein; gene *HvHor2*). Among them, the HvGAMYB protein activates the transcription of the *HvHor2* gene by binding to the corresponding MYB-R2R3 box (5′-TAACAAC-3′) in its promoter. Moreover, HvGAMYB interacts physically with BPBF (Diaz et al., 2002), and together, they significantly increase the transactivation of the *HvHor2* promoter in developing barley endosperms as compared with the transactivation of each TF considered separately.

In this work, we have performed a detailed expression analysis of HvVP1 during seed development to define its precise expression pattern. Since HvVP1 is a known regulator of seed maturation genes, we wanted to survey its potential effect on key regulatory factors controlling this process. As a first step, we explored HvVP1 action on the transactivation of the *HvHor2* promoter mediated by HvGAMYB and demonstrate that HvVP1 interferes with its activation capacity (Fig. 4B). Furthermore, our work shows that HvVP1 and HvGAMYB interact in vivo both in yeast (Y2H) and in plant nuclei, in support of the idea that a direct HvVP1-HvGAMYB protein-protein interaction takes place. Thus, we considered whether the observed negative effects on the HvGAMYB-mediated transactivation are derived from the HvVP1-HvGAMYB interaction. Interestingly, our results show that HvVP1 interferes with the HvGAMYB-BPBF interaction (Y3H assays; Fig. 7,

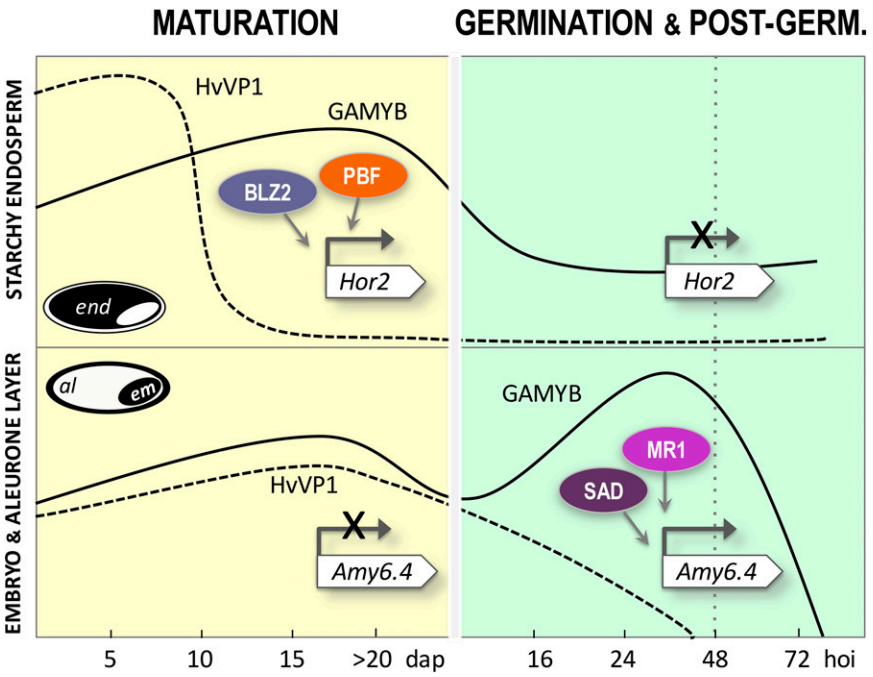

Figure 9. Proposed model of the transcriptional regulation of SSP (*Hor2*) and hydrolase (*Amy6.4*) genes in barley seeds mediated by HvVP1 interacting with GAMYB, DOF (BPBF and SAD), BLZ2, and MR1 (MYBR1) TFs during the maturation and postgermination phases. The starchy endosperm and the embryo-plus-aleurone layer are considered separately. Relative gene expression levels are indicated on the *y* axis. The dotted vertical lines indicate the time of root emergence.

A and B) and also reduces the binding affinity of both HvGAMYB and BPBF for their corresponding cis-motifs in the *HvHor2* promoter (Fig. 7C). However, as reflected in our proposed model (Fig. 9), this repressing activity of HvVP1 is not displayed on maturation genes (e.g. *Hor2*) in the developing starchy endosperm, due to a nonoverlapping temporal pattern of expression. Notably, *HvVP1* transcript levels decrease as maturation progresses while the expression of *HvGAMYB* and *BPBF* increases, hindering the possibility of HvGAMYB being kidnapped by HvVP1 and rendering a fully operative activation complex.

Besides the *HvVP1* expression pattern in the starchy endosperm, its persistence in the developing aleurone and immature embryos would favor the repression of the α-amylase and other germination-related genes necessary for the acquisition of primary dormancy and for the avoidance of preharvest sprouting, as has been documented previously for its orthologs in maize and Arabidopsis (Parcy et al., 1994; Hoecker et al., 1995).

HvVP1 Effects during Barley Germination and Postgermination Phases

After root protrusion (postgermination), different genes encoding hydrolytic enzymes, such as α-amylases and proteases that mobilize storage reserves, are expressed in the aleurone. In the promoters of these genes, a conserved tripartite cis-acting element, the GA-responsive complex, has been described to contain a GA-responsive element (5′-TAACAAA-3′), the pyrimidine box (5′-CCTTTT-3′), and the 5′-TATCCAC-3′ box (Sun and Gubler, 2004). In barley, the interaction of these three cis-elements with TFs belonging to the MYB-R2R3 (HvGAMYB), DOF (BPBF and three others), and MYBR1-SHAQKYF (HvMCB1 and HvMYBS3) families has been demonstrated (Gubler, et al., 1999; Mena et al., 2002; Isabel-LaMoneda et al., 2003; Rubio-Somoza et al., 2006a, 2006b; Moreno-Risueno et al., 2007). HvGAMYB is considered the master regulator of hydrolase gene expression and is highly induced by GA in the aleurone of germinating seeds (Gubler et al., 1999, 2002). In agreement with our observations during seed maturation, our results show that HvVP1 also interferes with the HvGAMYB transactivation of the *Amy6.4* promoter in germinating aleurone cells (Fig. 4C). Again, this effect is sustained by our disclosure that the interaction with HvVP1 leads to a decreased affinity of GAMYB and other TFs (SAD [HvDOF23]) for their DNA targets as well as to the interference in their protein complex formation. Accordingly, the HvGAMYB-dependent activation of postgermination genes can be achieved only once *HvVP1* expression disappears from the germinating aleurone.

As reflected in our model (Fig. 9), our results show that, upon seed imbibition, *HvVP1* is highly expressed in the mature embryo and in the aleurone up to 24 hoi (the time at which root protrusion occurs) and then decays sharply, in contrast to *HvGAMYB*, whose expression rises up to 48 hoi (Diaz et al., 2002). Taken together, these data indicate that HvVP1 not only prevents the premature expression of postgermination genes (encoding α-amylases, proteases, etc.) but also that its disappearance during postgermination is required for the mobilization of reserves to take place. The transcriptional repression of hydrolase genes mediated by VP1/ABI3 during germination has been reported previously in monocot and dicot seeds, in species like maize, tomato (*Solanum lycopersicum*), and

Arabidopsis (Hoecker et al., 1999; Nambara et al., 2000; Bassel et al., 2006).

In conclusion, the precise spatiotemporal expression of *HvVP1* allows fine-tuning of the regulation of the maturation and germination programs both within distinct seed compartments and at different developmental stages. As disclosed here, HvVP1 action occurs mainly by modulating the DNA-binding capacity and protein-protein interactions of TFs, like HvGAMYB and BPBF, that participate in different regulatory complexes.

The HvVP1 effects described here rely mostly on its repressing activity or the alternative activation derived from its release. In addition, an intrinsic activation capacity also has been reported for the VP1/ABI3 proteins. In particular, in nonendosperm seeds, commonly found in dicot plants like Arabidopsis, ABI3 has been described as an activator of maturation (SSP) and LEA genes, as reflected by their impaired expression in *abi3* mutants (Parcy et al., 1994). Moreover, the ectopic overexpression of *ABI3* (*35S::ABI3*) in conjunction with bZIP factors (*AtbZIP10*, *AtbZIP25*, and *AtbZIP53*) induces some of the *SSP* genes in vegetative tissues, in support of the idea that ABI3 is a positive regulator of *SSP* gene expression (Lara et al., 2003; Alonso et al., 2009). In this respect, the observation that HvVP1 functions as a transcriptional repressor of the *HvHor2* promoter upon seed maturation is not contradictory, since, in Arabidopsis, SSP and LEA proteins are expressed in the embryo, in contrast to their accumulation in the cereal endosperm. Moreover, globulin storage proteins similar to those of nonendosperm seeds are accumulated in the embryos of cereal seeds and display a similar positive regulation under VP1, in support of a conserved function of VP1/ABI3 in the control of *SSP* gene expression. Hence, the molecular mechanisms underlying the regulatory properties of VP1/ABI3 proteins seem to be preserved but displayed differentially in the context of endosperm and embryo, where interactions with a different set of transcription factors occur.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Barley (*Hordeum vulgare* 'Bomi') seeds were germinated in the dark, stratified at 4°C for 4 d, and then grown in the greenhouse. Developing endosperms (5, 10, 15, and 20 dap) and germinating seeds (16, 24, 48, and 72 hoi) were frozen in liquid N_2 and stored at −80°C until used for RNA extraction.

Developing endosperms from cv Bomi (15 dap) and aleurone layers peeled off from cv Himalaya germinating seeds (48 hoi) were collected for particle bombardment experiments and used immediately.

Screening of a Barley Genomic Library

A Lambda phage FIX II genomic library from 8-d-old barley seedlings (cv Igri; Agilent Technologies), representing 16×10^8 plaque-forming units mL^{-1}, was plated after infection with the *Escherichia coli* strain XL1-Blue MRA (Agilent Technologies). The plaques were transferred onto nylon membranes that were hybridized with a specific probe of a 465-bp fragment obtained by PCR with primers derived from conserved sequences of previously described *VP1* genes in other cereals (GenBank accession numbers as follows: *Avena fatua*, AJ001140; rice [*Oryza sativa japonica*], D16640.1; *Sorghum bicolor*, AF249881.1; wheat [*Triticum aestivum*], AB047554.1; and maize [*Zea mays*], M60214.1; for primers, see Supplemental Table S2). This fragment spanned the B3 domain and a 3′ end conserved region and was α-^{32}P labeled following standard procedures. Prehybridization, hybridization, and membrane washing were done as described (Vicente-Carbajosa et al., 1998). The in vivo excision properties of the Lambda phage FIX II vector system allowed the recovery of selected clones in the pBluescript SK plasmid for sequencing (Agilent Technologies).

The *HvVP1* ORF was obtained by PCR from cDNA isolated from developing barley seeds (20 dap; Supplemental Table S2); its corresponding deduced protein appears in Supplemental Figure S1.

Bioinformatic Resources: HvVP1 Identification, Phylogenetic Dendrogram, and Transcriptomic Analysis

Identification of the *HvVP1* genomic sequence (AJ431703.1) and its corresponding deduced protein (CAD24413.1) was done using the bioinformatic tools at the European Molecular Biology Laboratory and the National Center for Biotechnology Information databases. The Interpro Program (Pfam database; Bateman et al., 2002; http://pfam.sanger.ac.uk) was used to confirm the presence of the B3 DNA-binding domain.

The deduced protein sequences of *VP1* genes were obtained from public databases: those of cv Morex (Mayer et al., 2012); those of cv Himalaya and cv Haruna Nijo (HvVP1), wheat (TaVP1), *Triticum turgidum* (TtVP1), and *Triticum monococcum* (TmVP1) from GenBank (http://www.ncbi.nlm.nih.gov/genbank/); and those of *Brachypodium distachyon* (BdVP1), rice (OsVP1), and Arabidopsis (AtABI3) from Phytozome version 8.0 (Goodstein et al., 2012; www.phytozome.net). These sequences were aligned by means of the ClustalW program (Thompson et al., 1994) and utilized to construct a phylogenetic dendrogram using the neighbor-joining algorithm, a bootstrap analysis with 1,000 replicates, complete deletion, and the Jones-Taylor-Thornton matrix as settings. The MEME program version 6.0 (Tamura et al., 2013) was used to identify conserved motifs within the deduced VP1 proteins and to validate the phylogenetic tree (Table I). Default parameters were used, except that the maximum number of motifs to find was set to 21 and the minimum width was set to six amino acid residues (Bailey et al., 2009; http://meme-suite.org/). A single uppercase letter is given when the frequency of a residue is greater than twice that of the second most frequent one at the same position. A pair of uppercase letters in brackets represents two residues with a relative frequency sum of greater than 75%. When these criteria are not satisfied, a lowercase letter is set when the relative frequency of a residue is greater than 40%; if not, X is given.

The major biochemical parameters of the deduced VP1 proteins are listed in Supplemental Table S1. Both pI and molecular weight were predicted using the Compute pI/MW tool (Gasteiger et al., 2005; http://www.expasy.ch/tools/pi_tool.html).

The in silico transcriptomic analysis of genes *HvVP1*, *HvGAMYB*, *BPBF*, and *BLZ2* in cv Morex (Supplemental Fig. S4) was done using the Hordeum eFP browser at the Bio-Analytic Resource for Plant Biology (http://bar.utoronto.ca/efpbarley/cgi-bin/efpWeb.cgi; Druka et al., 2006). For identification of the probe sets for *HvVP1*, *HvGAMYB*, *BPBF*, and *BLZ2* transcription analysis, the PLEXdb BLAST was used (http://www.plexdb.org/modules/tools/plexdb_blast.php).

Total RNA Isolation and Northern-Blot Analysis

Total RNA was isolated from 1 to 2 g of barley developing deembrionated seeds (endosperms at 5, 10, 15, and 20 dap), immature (20 dap) and mature dry embryos, germinating aleurones, and germinating embryos (8, 16, 24, 48, and 72 hoi). After electrophoresis and transfer to nylon membranes, hybridization was carried out at 68°C, following standard procedures (Moreno-Risueno et al., 2007). A 280-bp fragment at the 3′ untranslated region (nucleotides 3,528–3,808) was used as a specific *HvVP1* probe (Supplemental Table S2). The 18S RNA gene was used as a control for sample charge.

mRNA in Situ Hybridization Experiments

The protocol described here is a modification of that described by Lara et al. (2003). Barley developing (20 dap) and germinating (24 hoi) seeds were collected and fixed in 4% (w/v) paraformaldehyde, embedded in paraffin, sectioned to 8 μm, and dewaxed. An antisense or sense digoxigenin-labeled RNA probe,

corresponding to the same DNA fragment (280 bp) used for northern blots (Supplemental Table S2), was synthesized with the digoxigenin RNA labeling mix, and hybridization was at 56°C overnight. Antibody incubation and color detection were done according to the manufacturer's instructions (Boehringer Ingelheim).

Yeast One-Hybrid, Y2H, and Y3H Assays

For Y2H assays, the effector plasmids pGBT9 and pGAD424 (Clontech), which contain the alcohol dehydrogenase I (*AdhI*) promoter fused to the *GAL4* DNA-binding domain (GAL4BD; pGBT9 bait vector) and the *GAL4* DNA activation domain (GAL4AD; pGAD424 prey vector), respectively, were used to generate translational fusions with *HvVP1*, *HvGAMYB*, and *BPBF* ORFs or with selected fragments derived from *HvVP1* that were cloned into the *Eco*RI-*Bam*HI sites by a PCR strategy. The haploid strains SFY526 and HF7c of *Saccharomyces cerevisiae* (Clontech), carrying the *LacZ* (β-Gal) and *HIS3* (imidazole glycerol phosphate dehydratase) reporter genes, under the control of a truncated *Gal1* promoter that contains *GAL4*-responsive elements (Gal1UAS), were used.

For Y3H assays, the *BPBF* and *HvVP1* ORFs were cloned into the pBridge vector (Clontech) in Multiple Cloning Sites I and II, respectively. The *BPBF* ORF was cloned into the *Eco*RI-*Bam*HI sites of Multiple Cloning Site I fused to the GAL4 binding domain, and that of *HvVP1* was cloned into the *Not*I-*Bgl*II sites (Multiple Cloning Site II) by a PCR strategy (for primer sequences, see Supplemental Table S2). *GAL4AD-HvGAMYB* (pGAD424 vector), used previously for Y2H assays, was used as the third component. The pBridge vector allows the conditional expression of a second gene under the control of the *Met-25* promoter ($P_{Met\text{-}25}$) in response to Met levels in the medium.

All Y2H and Y3H assays were done following the manufacturer's instructions (Clontech).

Bimolecular Fluorescence Complementation

Essentially, *HvVP1* and *HvGAMYB* ORFs were translationally fused to the N- or C-terminal encoding fragment of the *GFP* gene ORF using a PCR strategy and subsequent subcloning into the two versions of the *psmRS-GFP* plasmid (*psmRS-N-GFP* and *psmRS-C-GFP*). The final constructs were used to cobombard inner epidermal cell layers of fresh onion (*Allium cepa*) using a biolistic helium gun device (DuPont PDS-1000; Bio-Rad Laboratories), as described previously (Diaz et al., 2005). The fluorescence emission was observed after 36 h of incubation at 22°C in the dark with a Carl Zeiss Axiophot fluorescence microscope (filter parameters, excitation at 450–490 nm and emission at 520 nm). As a control for nuclei localization, the onion cell layers were stained with 4′,6-diamidino-2-phenylindole (Serva).

Transient Expression Assays in Barley Developing Endosperms and in Germinating Aleurone Layers

The reporter vectors containing the promoters of genes *Hor2* (encoding a B-hordein) and *Amy6.4* (encoding a high-pI barley α-amylase), P_{Hor2} and $P_{Amy6.4}$, fused to the GUS reporter gene were described previously (Mena et al., 1998, 2002). The effector constructs corresponding to *HvVP1*, *HvGAMYB*, and *BPBF* were prepared by cloning the corresponding ORFs in the pMF6 plasmid under the control of the 35S cauliflower mosaic virus promoter followed by the first intron of the maize *AdhI* gene (35S-I) using a PCR strategy, followed by subcloning at the *Eco*RI-*Bam*HI sites of this plasmid. Particle bombardment and subsequent GUS evaluation were carried out with a biolistic helium gun device (DuPont PSD-1000) as described previously (Mena et al., 1998, 2002; Diaz et al., 2002).

EMSAs

Translational fusions of *HvVP1*, *HvGAMYB*, *BPBF*, *SAD*, and *BLZ2* ORFs to the glutathione *S*-transferase gene were prepared by cloning their ORFs into the expression vector pGEX-2T (Pharmacia). For this purpose, a PCR strategy was used followed by the subcloning of these ORFs into the *Bam*HI-*Eco*RI sites of the pGEX-2T plasmid (Supplemental Table S2). Induction of recombinant proteins with 0.1 mM isopropyl-β-D-thiogalatopyranoside and preparation of the *E. coli* protein extracts were performed as described previously (Vicente-Carbajosa et al., 1997).

Two probes, one containing the binding sites for HvVP1 besides those for BLZ2 and BPBF (PB/GLM; Fig. 7C) and the other containing that for GAMYB (5′-TAACAAC-3′) within the *Hor2* promoter (P_{Hor2}), were produced by annealing complementary single-stranded oligonucleotides that generate protruding ends (Supplemental Table S2). These probes were end labeled with [^{32}P] dATP by the fill-in reaction (Klenow *exo*-free DNA polymerase; United States Biochemical) and purified from 8% PAGE (39:1 cross linking). The DNA-protein binding reactions and the EMSA experiments were done, as described previously, using 1 ng of ^{32}P-labeled probes (Moreno-Risueno et al., 2008).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Genomic, cDNA, and deduced amino-acid sequences of HvVP1 cv. Igri.

Supplemental Figure S2. Gene copy number of *HvVP1*.

Supplemental Figure S3. Comparison of the HvVP1 deduced sequences.

Supplemental Figure S4. Pictographic representation of *HvVP1*, *GAMYB*, *BPBF*, and BLZ2 transcript accumulation during seed development.

Supplemental Figure S5. Transactivation assays using as effectors HvVP1 and BPBF, and as reporter the *Amy6.4* gene promoter.

Supplemental Figure S6. Electrophoretic Mobility Shift Assays (EMSA) of the recombinant proteins BLZ2 (bZIP family) and SAD (DOF family) with the PB/GLM probe derived.

Supplemental Table S1. Major characteristics of VP1/ABI3 predicted proteins.

Supplemental Table S2. Oligonucleotide sequences of primers used.

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