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Barley BLZ2, a Seed-specific bZIP Protein That Interacts with BLZ1 *in Vivo* and Activates Transcription from the GCN4-like motif of B-hordein Promoters in Barley Endosperm*

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Luis Oñate‡, Jesús Vicente-Carbajosa, Pilar Lara‡, Isabel Díaz, and Pilar Carbonero§

From the Laboratorio de Bioquímica y Biología Molecular, Departamento de Biotecnología-UPM, ETS Ingenieros Agrónomos, 28040 Madrid, Spain

A barley endosperm cDNA, encoding a DNA-binding protein of the bZIP class of transcription factors, BLZ2, has been characterized. The *Blz2* mRNA expression is restricted to the endosperm, where it precedes that of the hordein genes. BLZ2, expressed in bacteria, binds specifically to the GCN4-like motif (GLM; 5′-GTGAGTCAT-3′) in a 43-base pair oligonucleotide derived from the promoter region of a *Hor-2* gene (B1-hordein). This oligonucleotide also includes the prolamin box (PB; 5′-TGTAAAG-3′). Binding by BLZ2 is prevented when the GLM is mutated to 5′-GTGctTCtc-3′ but not when mutations affect the PB. The BLZ2 protein is a potent transcriptional activator in a yeast two-hybrid system where it dimerizes with BLZ1, a barley bZIP protein encoded by the ubiquitously expressed *Blz1* gene. Transient expression experiments in co-bombarded developing barley endosperms demonstrate that BLZ2 transactivates transcription from the GLM of the *Hor-2* gene promoter and that this activation is also partially dependent on the presence of an intact PB. A drastic decrease in GUS activity is observed in co-bombarded barley endosperms when using as effectors equimolar mixtures of *Blz2* and *Blz1* in antisense constructs. These results strongly implicate the endosperm-specific BLZ2 protein from barley, either as a homodimer or as a heterodimer with BLZ1, as an important transcriptional activator of seed storage protein genes containing the GLM in their promoters.

Hordeins, the major storage proteins of barley seeds, are prolamins specifically synthesized in the starchy endosperm and are classified according to their mobility in SDS-electrophoretic gels into three major classes: B, C, and D, with the B fraction representing ~75% of the total hordein content in most barley cultivars (cv.)[1]. All the hordeins are structurally related, and their genes presumably derive from a common ancestor by gene duplication and subsequent divergent evolution (1). The coordinate expression of all hordein genes suggests common regulatory mechanisms of transcription that should involve both *cis*-acting motifs in their promoters and *trans*-acting transcription factors (2).

A conserved *cis*-acting motif that is found in most storage protein gene promoters of seeds is the endosperm box (EB; Refs. 3 and 4), a bipartite motif located around 300 bp upstream of the translation initiation ATG codon, that contains two distinct nuclear protein binding sites: the prolamin box (PB, 5′-TGTAAAG-3′), also called the endosperm motif (EM), and a GCN4-like motif (GLM, 5′-(G/A)TGA(G/C)TCA(T/C)-3′), which resembles the binding site of the yeast transcription factor GCN4 (5, 6). These two motifs are present in B- and C-hordein promoters, whereas only the PB is present in that of D-hordeins. Functional analysis of a native C-hordein promoter by particle bombardment of developing barley endosperms (7) have demonstrated that the GLM is the dominant *cis*-acting element and that the PB exerts a silencing effect. However, both GLM and PB from the promoter of a *Hor-2* gene (B1-hordein) are essential positive elements conferring a high level of transcriptional activity to the minimal 35S CaMV promoter (Δ35S) in bombarded developing endosperms from barley (8).

Much of what is known about the genetic and molecular mechanisms regulating cereal seed storage genes comes from work on maize, where a bZIP protein, OPAQUE 2 (O2; Refs. 9 and 10), has been shown to bind to and activate transcription from an ACGT core motif adjacent to the PB in the promoter of the 22-kDa class of zein genes (11, 12). In wheat and barley, current knowledge about the transcription factors involved in the regulation of storage protein genes is not as complete as in maize although bZIP proteins, such as SPA from wheat and BLZ1 from barley, have been described in endosperm that are able to interact with the GLM binding site in prolamin genes. Whereas SPA is seed-specific, BLZ1 is also expressed in other tissues and both have been shown to transiently transactivate appropriate reporter genes *in planta* (8, 13). Recently, the O2 protein has been shown to interact *in vitro* with another maize endosperm-specific factor (PBF) of the Dof (DNA-binding with one finger) class that recognizes the PB motif in the −300 region of the 22-kDa zein promoter (14). Its barley orthologue (BPBF) transactivates transcription from the PB element of a native *Hor-2* promoter in co-bombarded barley developing endosperm (15). With the emerging picture of the EB as a refined *cis*-regulatory element whereby several nuclear proteins inter-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) Y10834.

‡ Recipients of Formación Personal Investigator scholarships from MEC (Spain).

§ To whom correspondence should be addressed. Tel.: 3491-3365707/05; Fax: 3491-3365757; E-mail: pcarbonero@bit.etsia.upm.es.

[1] The abbreviations used are: cv., cultivar; *AdhI*, alcohol dehydrogenase I; DAP, days after pollination; Δ35S, −90-bp minimal 35S CaMV promoter; EB, endosperm box; EMSA, electrophoretic mobility shift assay; *Gal1*UAS, *Gal4* responsive elements in a *Gal1* truncated promoter; *Gal4*AD, *Gal4* activation domain; *Gal4*DBD, *Gal4* DNA binding domain; GLM, GCN4-like motif; GUS, β-glucuronidase gene; *HIS3*, imidazole glycerol phosphate dehydratase gene; *LacZ*, β-galactosidase gene; PB, prolamin box; 35S CaMV, cauliflower mosaic virus 35S promoter; 3′-nos, 3′-terminator sequence of the nopaline synthase gene; bp, base pair(s); nt, nucleotide(s); PCR, polymerase chain reaction.

act, it is crucial the identification of the factors that can participate in this complex and coordinate gene regulation in seeds. BZIP factors are particularly interesting because these proteins bind DNA motifs via dimer formation, either as homo- or heterodimers besides interacting with other regulatory proteins (8, 16–19). More than one bZIP factor can bind to the same target sequence. The final regulatory effect of a particular bZIP factor is exerted through the recognition of a particular cis-motif (G-box, C-box, GLM, etc.) in a promoter and through its interaction with other regulatory factors (bZIPs and other types) expressed in a given cell-type at the same time.

We describe here the isolation and characterization of a barley cDNA encoding a novel endosperm-specific bZIP transcriptional activator, BLZ2 (gene *Blz2*, Barley leucine zipper 2), that shows a high degree of sequence similarity to SPA from wheat (13). The BLZ2 protein produced in bacteria binds *in vitro* to the GLM in the EB of a B-hordein promoter and transiently transactivates transcription from the GLM in the homologous system, the barley developing endosperm. For maximum transcriptional activation, BLZ2 requires also an intact PB in the proximity of the GLM site. In addition, BLZ2 activates transcription in the yeast two hybrid-system, where it can form heterodimers with BLZ1.

EXPERIMENTAL PROCEDURES

Plant Material—Barley (*Hordeum vulgare*) cv. *Bomi* was germinated in the dark, vernalized at 4 °C for four weeks, and grown in the greenhouse at 18 °C under constant illumination. Developing endosperms (7, 12, 17 and 21 DAP) and 7-day-old leaves and roots were frozen in liquid N_2 and stored at –70 °C until used for RNA extraction. Developing endosperms for particle bombardment experiments were collected from greenhouse plants and used immediately.

Screening of a Barley cDNA Library—A λZAP-II cDNA library from immature barley (cv. *Bomi*) endosperms (15) representing 5×10^6 plaque forming units was plated after infection of the *Escherichia coli* strain XL1-Blue MRF′. The plaques were transferred onto Magna nylon membranes (MSI) by standard procedures (20) and screened using as a probe a 207-bp fragment from the barley *Blz1* cDNA clone (GenBank™/EBI accession number X80068; Ref. 8) spanning most of its bZIP domain. This DNA fragment was ^{32}P-labeled by the random primed method (Boehringer Mannheim). Prehybridization was at 55 °C for 2 h in 5× SSC, 5× Denhardt′s solution, 1% SDS, and 100 μg/ml salmon sperm DNA. Hybridization was done for 16 h at the same conditions. The filters were then washed twice for 15 min at 50 °C in 2× SSC and twice for 30 min at 55 °C in 2× SSC and 0.1% SDS. Using the *in vivo* excision properties of the Uni-ZAP-XR vector system (Stratagene), the selected clones were excised, according to manufacturer instructions, and recovered in the pBluescript SK plasmid. DNA sequences were obtained using the ABI PRISM 377 dye terminator sequencing system and the ABI PRISM 377 DNA sequence analyzer (Perkin Elmer-Applied Biosystems). Analyses of DNA and deduced protein sequences were done with the GeneBee-Net Ver.1.0 computer facilities.

Northern Blots—Total RNA from different barley tissues was isolated essentially as described (21). Blots were probed using the recommended protocols of the manufacturer for Magna nylon membranes (MSI). Hybridization was carried out at 65 °C, following standard procedures (20). A 407-bp fragment (nt 1050–1457, shown in Fig. 1) was used as a specific *Blz2* probe. The Northern blots were subsequently hybridized with a specific probe for a *Hor-2* gene (encoding a B1-hordein; Ref. 22) and with a barley 18 S rDNA-specific probe (23) as a control for sample charge.

Expression of the BLZ2 Protein in E. coli—To produce the barley BLZ2 protein in *E. coli* cells, we used the pT7–7 plasmid (24). To obtain a transcriptional fusion of the *Blz2* full-length coding sequence in the ATG-containing *Nde*I site of the pT7–7 vector, a 1,230-bp *Blz2* cDNA fragment (nt 1–1,230, shown in Fig. 1*B*) was amplified by the polymerase chain reaction (PCR) using the following oligonucleotides as primers: (i) LO2FLs (5′-CCGGGAATTCCAT**ATG**GAGCCCGTG TTC-3′), a forward primer that incorporates *Eco*RI (double underlined) and *Nde*I (single underlined) sites containing the translation initiation codon (bold) of the longest open reading frame of the *Blz2* cDNA; and (ii) LO2FLas (5′-TCGGATCCAAGCTT**C**C**TA**CTGCATCAC-3′), a reverse primer that added *Bam*HI (single underlined) and *Hin*dIII (double underlined) sites at the 3′-end of the amplified fragment (stop codon in bold). This PCR fragment was directionally cloned into an *Nde*I-*Bam*HI digested pT7–7 vector and, after sequencing confirmation, the resulting pT7–7-*Blz2* expression plasmid was introduced into the *E. coli* BL21(DE3)/pLysS strain. Induction of BLZ2 expression and preparation of *E. coli* protein extracts were performed as described previously (8). Cells carrying the pT7–7 vector with no insert were identically processed as negative controls.

Electrophoretic Mobility Shift Assays (EMSAs)—The 43-bp probe containing the EB from the *Hor-2* promoter (HOR) and two mutated versions of it, respectively affecting the GLM (hor1) or the PB (hor2) described in the corresponding figure, were produced by annealing complementary single-stranded oligonucleotides that generate 5′-protruding ends. 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 an 8% polyacrylamide gel electrophoresis (39:1 cross-linking). EMSA experiments were performed essentially as described previously (8), using 0.5 ng of ^{32}P-labeled probes.

Yeast Strains and LacZ Assays—The effector plasmids pGBT9 and pGAD424 (CLONTECH), which contain the alcohol dehydrogenase I (*AdhI*) promoter fused to the *Gal4* DNA binding domain (*Gal4*DBD; pGBT9 vector) or to the *Gal4* DNA activation domain (*Gal4*AD; pGAD424 vector), respectively, were used to generate translational fusions with *Blz2* or *Blz1* cDNAs or with selected fragments derived from them. The haploid strain HF7c of *Saccharomyces cerevisiae* (CLONTECH), carrying *LacZ* (β-galactosidase) and *HIS3* (imidazole glycerol phosphate dehydratase) reporter genes under the control of a truncated *Gal1* promoter that contains *Gal4*-responsive elements (*Gal1*UAS), was used. To investigate if BLZ2 and BLZ1 were able to heterodimerize, the bZIP domain of *Blz2* (nt 538–936, corresponding to amino acids 180–312, shown in Fig. 1*B*), that was flanked with *Eco*RI and *Bam*HI sites by a PCR strategy using as forward oligonucleotide LO2BZs (5′-AGAATTCAGCTCTT CCTCATG-3′; *Eco*RI site underlined) and as reverse primer LO2BZas (5′-GGGATCCACTGAAAT GGGTCC-3′; *Bam*HI site underlined), was subcloned into the *Eco*RI-*Bam*HI sites of the pGBT9 vector ("bait" construction). The resulting construct was introduced into *S. cerevisiae* HF7c cells containing the pGAD424 vector carrying each one of the following *Blz1* inserts ("prey" constructions): (i) the full-length cDNA (amino acids 1–391); (ii) the cDNA region spanning amino acids 195–391; (iii) the region encoding the bZIP domain (amino acids 195–293); and (iv) that of the leucine zipper alone (amino acids 225–293).

To test if BLZ2 were a transcriptional activator in yeast, the full-length *Blz2* cDNA (nt 1–1,230, shown in Fig. 1*B*) was amplified with the LO2FLs and the LO2Flas primers (see above) and inserted into the *Eco*RI and *Bam*HI sites of pGBT9 (pGBT9-*Blz2*) plasmid. The 5′-terminal coding region of *Blz2* (nt 1–612, shown in Fig. 1*B*), was obtained by digestion of the pGBT9-*Blz2* with *Eco*RI and *Ssp*I and inserted into the *Eco*RI and *Sma*I sites of pGBT9. All constructs were checked by restriction digestion and sequencing. Yeast transformation was performed by the polyethylene glycol method (25) and transformants screened for β-galactosidase production (*LacZ*) by the colony filter lift assay (26) and for growth in a histidine-depleted agar medium (His$^-$, BIO101).

Particle Bombardment in Barley Developing Endosperm—The reporter vector was a pUC19-derived plasmid containing the β-glucuronidase reporter gene (27) under the control of a Δ35S promoter and fused to the 3′-terminator of the nopaline synthase gene (3′-nos). In the *Eco*RI and *Bam*HI sites upstream of this promoter, the following oligonucleotides were fused: (i) HOR, composed of the 43-bp sequence of the endosperm box from the *Hor-2* promoter (HOR-Δ35S); (ii) hor1, containing the HOR element mutated in the GLM (hor1-Δ35S); and (iii) hor2, containing the HOR element mutated in the PB (hor2-Δ35S). The Δ35S promoter alone was used as a control. The effector constructs corresponding to *Blz2* were prepared by cloning its cDNA in the sense or antisense orientation under the control of the 35S CaMV promoter fused to the first intron of the maize *AdhI* gene and followed by the 3′-nos (35S-I; Ref. 28): for the sense construct, the *Blz2* cDNA, obtained by PCR with the LO2FLs and the LO2Flas primers, was digested with *Hin*dIII and blunted and then *Eco*RI-digested and cloned into the 35S-I plasmid; for the antisense construct, the PCR-amplified fragment was digested with *Eco*RI and *Bam*HI and inserted into a 35S-I plasmid restricted with the same enzymes. The sense and antisense constructs for *Blz1* were prepared as described previously (8).

Particle bombardment was carried out with a biolistic helium gun device (DuPont PSD-1000) according to Kikkert (29). Gold particles (1.0 μm in size) were prepared essentially as described by Taylor and Vasil (30) by mixing 18 μl of gold suspension (60 mg ml^{-1}) with 2 μl (2 μg) of Qiagen-prepared plasmid, 25 μl of 2.5 M $CaCl_2$, and 10 μl of 0.1 M

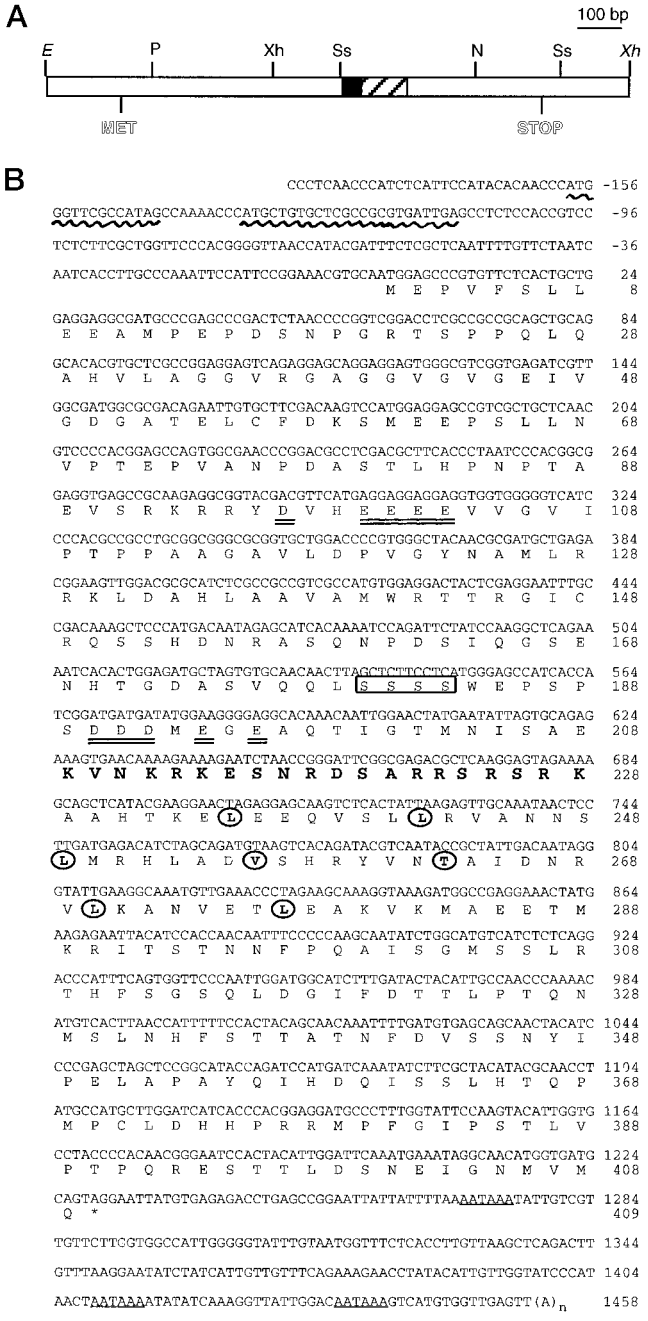

FIG. 1. **Restriction map and nucleotide and deduced amino acid sequence of the barley *Blz2* cDNA.** *A*, restriction map of the *Blz2* cDNA. The initiation and termination translation codons are indicated as MET and STOP, respectively. *E*, *Eco*RI; *N*, *Nhe*I; *P*, *Pst*I; *Ss*, *Ssp*I; *Xh*, *Xho*I. The *Eco*RI and the last *Xho*I restriction sites derive from the polylinker of the Uni-ZAP-XR vector. The *black box* and its *adjacent box* with *diagonal lines* represent the basic and leucine zipper regions, respectively. *B*, nucleotide sequence and deduced amino acid sequence of the *Blz2* cDNA. Amino acid residues of the basic DNA-binding domain are in *bold*, the leucine heptad repeats are *circled*, and a presumptive serine-rich phosphorylation site is *boxed*. Acidic amino acid residues, putatively involved in activation, are *double underlined*. Nucleotide sequence numbers refer to the ATG translation initiation codon. The stop codon is indicated with an *asterisk*. The AATAAA polyadenylation signals are *single underlined*. Two upstream open reading frames in the mRNA leader sequence are indicated with a *wavy line*.

spermidine. In all cases, 150 or 250 ng of the reporter plasmids were used and the appropriate concentrations of effector plasmids at the indicated molar ratio. After vortexing for 1 min, the mixture was incubated on ice for at least 2 min, washed twice with ethanol, and finally resuspended in 50 μl of ethanol. For bombardment, rupture disks of 1,100 pounds per square inch (psi) were used and 7 μl of particles, briefly sonicated, were spotted onto macrocarriers. At a distance of 7.5 cm from the macroprojectile stopper, developing barley endosperms (15 DAP) were placed on half-strength MS medium containing 15 g/liter of sucrose and 0.4% phytagel. After bombardment with 154 ng of gold particles, the endosperms were incubated at 25 °C for 24 h according to Jefferson (27). Blue spots were counted under a dissecting microscope, and the GUS activity in each assay was expressed as the mean value of blue spots per endosperm. The histochemical data were directly correlated with the fluorometrically quantified GUS activity per mg of protein with a correlation coefficient of 0.96 (data not shown).

TABLE I
Comparison between related plant bZIPs

Percentage of identical (similar) amino acid residues between the BLZ2 protein and other related plant bZIP transcription factors.[a]

Transcription factors	Whole protein	bZIP domain
BLZ2 (barley)	100	100
SPA (wheat)	77.5 (81.8)	94.8 (94.8)
CLJO2 (Coix)	36.4 (49.4)	61.0 (77.9)
O2 (maize)	31.5 (42.7)	59.7 (79.2)
SBO2 (sorghum)	33.5 (46.3)	61.0 (79.2)
REB (rice)	35.2 (44.5)	70.1 (85.7)
BLZ1 (barley)	34.3 (42.5)	70.1 (84.4)
OHP1 (maize)	31.3 (41.0)	67.5 (83.1)
CPRF2 (parsley)	27.7 (38.0)	63.6 (79.2)
RITA1 (rice)	20.5 (27.4)	51.9 (63.6)

[a] Calculations were done using the Clustal W alignment program.

RESULTS

Isolation of a Barley bZIP cDNA Encoding the Homeologue of Wheat SPA—The presence in wheat and maize of endosperm-specific bZIP proteins, SPA and O2 (9, 10, 13), that activate transcription through interaction with the GLM or ACGT core, respectively, of the EB in storage protein gene promoters, led us to search for their barley counterpart. To isolate such a gene, we used the bZIP coding region of the ubiquitously expressed barley *Blz1* cDNA (8) as a probe to screen, at moderate stringency, a λZAPII cDNA library (15) from early developing endosperm (10–15 DAP).

Among ten positive clones purified, after screening 5×10^6 plaque forming units, seven were different from *Blz1*, and one of them, hereafter *Blz2*, containing the longest insert, was selected for further characterization. The restriction map and the DNA sequence of the insert (1,647 nt) in this clone appear in Fig. 1, *A* and *B*, respectively. *Blz2* is a single copy gene (data not shown) and encodes a bZIP protein (hereafter BLZ2) that contains 409 amino acid residues and a deduced molecular mass of 44,600 Da. Two short open reading frames are found upstream of the ATG start codon. This feature, shared by *O2*, *Blz1*, and some other transcription factor genes, may have a role in translation regulation (8, 31).

The BLZ2 protein has a typical bZIP domain (Fig. 1*B*) with a basic region followed by leucine heptad repeats. A serine-rich motif putatively involved in phosphorylation (32), two short stretches rich in acidic residues that could be involved in transcription activation, and a putative nuclear localization signal that spans the complete basic region of the bZIP domain (33) are also found.

The BLZ2 protein is probably the homeologue of wheat SPA (13) because they share 77.5% identical residues along the whole protein, and 94.8% in their bZIP domains. BLZ2 is also related to the barley BLZ1 protein (34.3% identity over the whole protein; 70.1% in the bZIP domain), to O2 from maize, coix, and sorghum, to REB from rice, and to OHP1 from maize and has limited but significant homology with CPRF2 from parsley and RITA1 from rice (see Table I and references of Fig. 2). The phylogenetic dendrogram based on the comparison of the whole proteins and the sequence alignments that appear in Fig. 2, clearly indicate that these proteins form a well defined

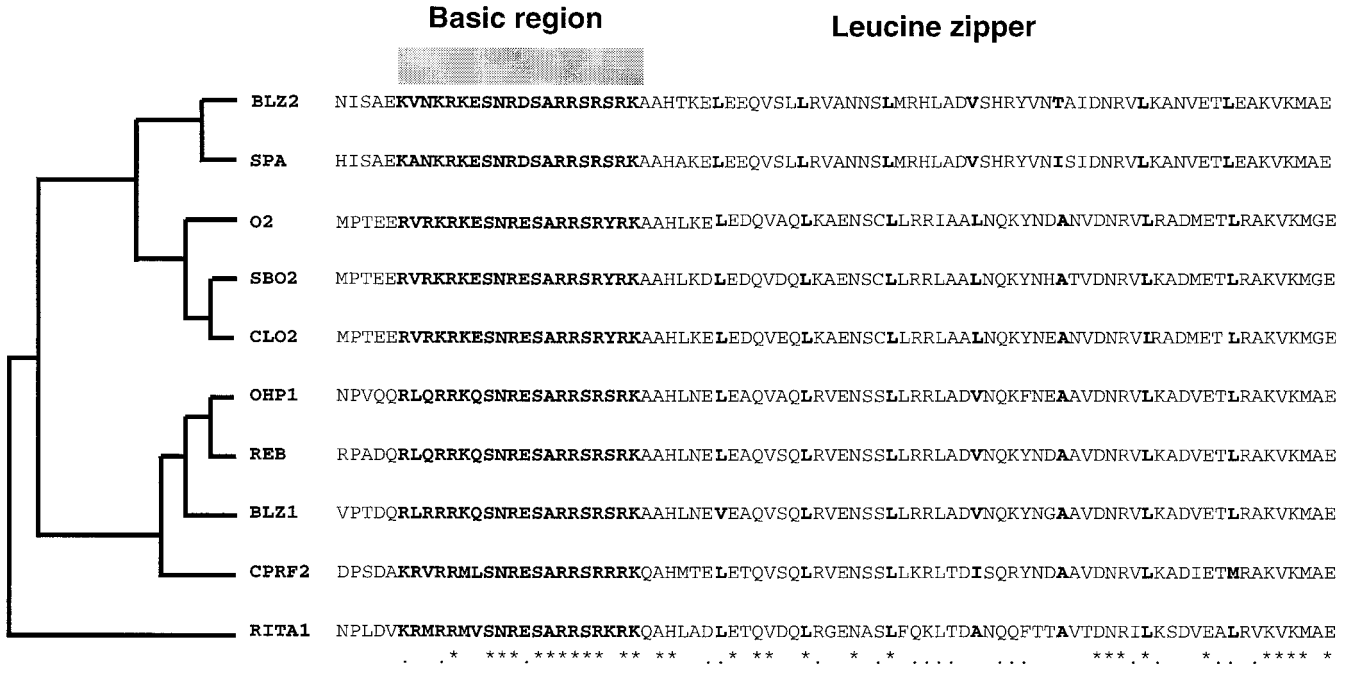

FIG. 2. **Dendrogram and alignment of the deduced amino acid sequence of the bZIP domain of *Blz2* with those of related plant bZIP proteins.** The basic region is indicated with a *shaded bar*, and both the basic region and the leucine repeats are in *bold*. *Asterisks* correspond to identical residues in the ten sequences compared, and *dots* represent conserved substitutions: SPA from wheat (13), O2 from maize (9, 10), sorghum (SBO2; Ref. 51) and coix (CLO2; GenBank™/EBI accession number X78287), OHP1 from maize (18), REB from rice (52), BLZ1 from barley (8), CPRF2 from parsley (48), and RITA1 from rice (53).

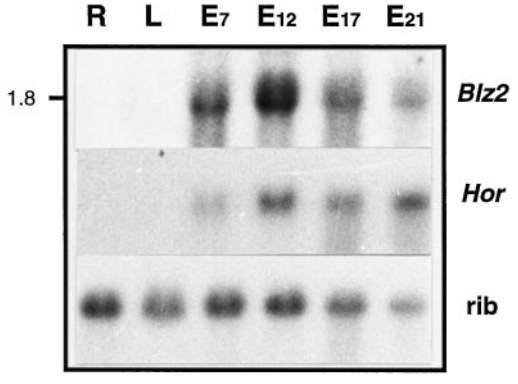

FIG. 3. **Northern blot analysis of *Blz2* in different barley tissues.** Total RNA (15 μg) from 7-day-old roots (*R*), 7-day-old green leaves (*L*), and from 7, 12, 17, and 21 DAP endosperms (E_7, E_{12}, E_{17}, and E_{21}) were electrophoresed in formaldehyde-agarose gels, blotted, and hybridized with a *Blz2*-specific probe under stringent conditions. The same filter was subsequently hybridized with a specific probe for the *Hor2* gene (*Hor*) and with an 18 S rDNA probe (*rib*) as a loading control.

subfamily of plant bZIPs.

Blz2 Expression Is Restricted to the Endosperm—The temporal and spatial pattern of expression of the *Blz2* gene was examined by Northern blot analysis. Total RNA was isolated from endosperms at different developmental stages, ranging from 7 to 21 DAP, and from young leaves and roots (7 days after germination). After hybridization with a *Blz2*-specific probe, the mRNA was only detected in the endosperm (Fig. 3). If BLZ2 were a regulator of hordein gene expression, then one would expect the occurrence of *Blz2* expression prior to and during the synthesis of the hordein message. To establish this correlation, the same blot was subsequently hybridized with a probe spanning the coding sequence of a *Hor-2* gene. The *Blz2* transcript was already present at 7 DAP, peaking at 12 DAP and decreasing thereafter (Fig. 3). In contrast, the B-hordein transcripts were barely detectable at 7 DAP and were still expressed in late endosperm development (21 DAP). The patterns of *Blz2* and hordein message accumulation are therefore consistent with the possibility that *Blz2* was an activator of the hordein genes.

BLZ2 Binds to the GLM from Hordein Gene Promoters—To investigate the involvement of BLZ2 in the regulation of hordein gene expression, we tested if BLZ2 was capable of binding specifically to the GLM within the EB sequence from the promoter of the *Hor-2* gene (HOR in Fig. 4*A*). This DNA fragment was used because it represents a highly conserved sequence in the −300 promoter region of many genes that are exclusively expressed in the endosperm (34).

Protein extracts of *E. coli*, expressing the BLZ2 protein, were incubated with the ^{32}P-labeled HOR sequence, and its ability to bind to this probe was tested by EMSAs. As shown in Fig. 4*A*, two shifted bands appeared when BLZ2 was incubated with the HOR probe. To further define the motifs within this probe that were recognized by BLZ2, HOR derivatives containing mutations in the GLM (hor1) or in the PB (hor2) were tested in EMSAs. Although the BLZ2 was able to bind to the HOR oligonucleotide, the interaction was abolished when the GLM 5′-GTGAGTCAT-3′ was mutated to 5′-GTGctTCtc-3′, as occurs when using the hor1 oligonucleotide (Fig. 4*A*). However, an intact PB, 5′-CATGTAAAGTG-3′, was not required for the *in vitro* binding because mutations in this motif, 5′-gAgGTAAAtTt-3′ (hor2), did not affect the retarded complex formation (Fig. 4*B*). Specificity of all interactions were assessed by competition experiments employing 50, 100, and 200-fold molar excess of cold oligonucleotide probes. In these experiments, HOR and hor2 were effective competitors, whereas hor1 was unable to compete the BLZ2 binding to HOR or hor2 (Fig. 4, *A* and *B*). These results indicate that BLZ2 binds specifically to the GLM sequence within the EB of hordein promoters.

BLZ2 Activates Transcription from the GLM of Hordein Promoters in Bombarded Barley Endosperms—The functional relevance of the interaction observed *in vitro* between BLZ2 and the GLM was further investigated *in vivo* by assessing the effect of BLZ2 on transient expression assays in co-bombarded barley endosperms. As shown in Fig. 5*A*, GUS reporter con-

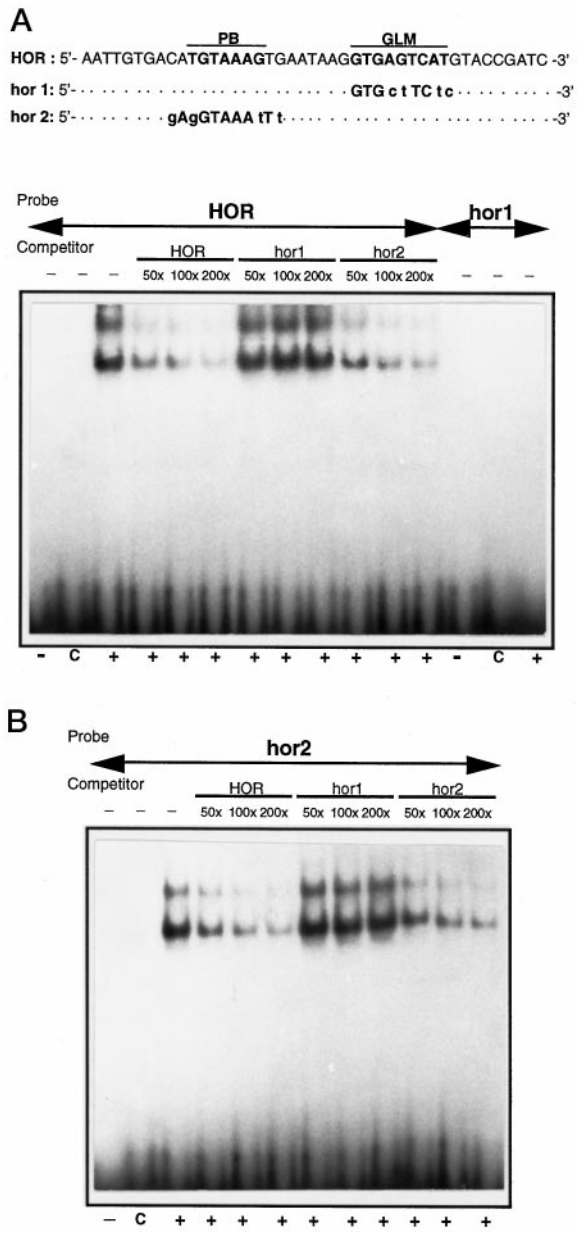

FIG. 4. **EMSAs of the BLZ2 protein with the 43-bp endosperm box element (*HOR*) of the promoter of the *Hor2* gene and with its mutated derivatives affected in the GLM (*hor1*) or in the PB (*hor2*).** *A*, EMSAs with the ^{32}P-labeled HOR and hor1 oligonucleotide probes. *B*, EMSAs with the ^{32}P-labeled hor2 oligonucleotide probe. In the *two panels*, ^{32}P-labeled probe without protein (−); probe incubated with 2 μg of protein extracts from bacterial cells transformed with the pT7–7 plasmid without the *Blz2* cDNA insert (*C*); probe incubated with 2 μg of protein extract from bacterial cells transformed with the pT7–7-*Blz2* construction (+). 0.5 ng of probes were used in all cases. Competition experiments were performed by using increasing amounts (50, 100, and 200×) of the indicated unlabeled HOR, hor1, and hor2. Sequences of the three oligonucleotides, used as probes, are shown at the *top* of the panel, with the GLM and PB in *bold*; identical residues as in HOR in hor1 and hor2 are represented by *dots*, and base mutations are written in *lowercase*.

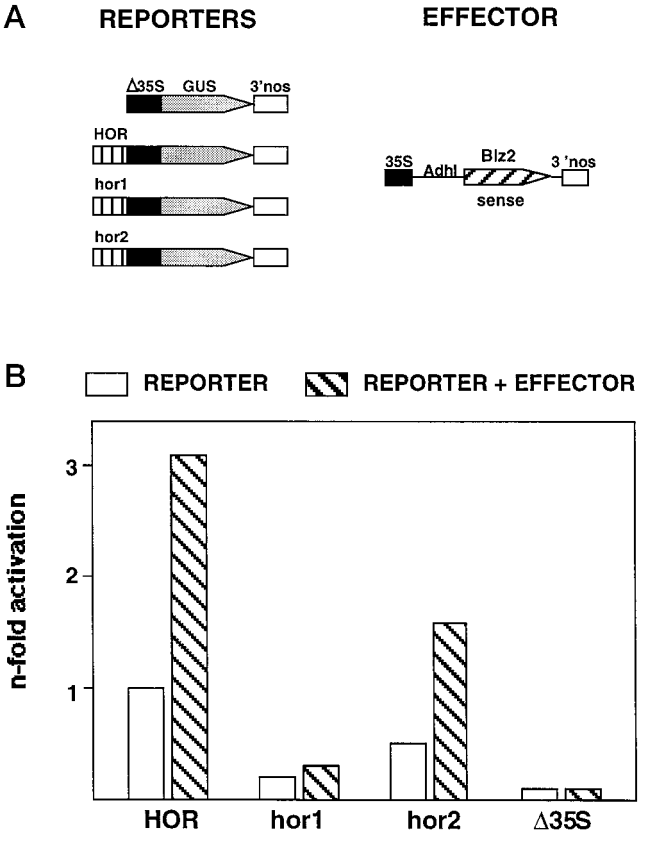

FIG. 5. **Transactivation by BLZ2 in co-bombarded developing barley endosperms.** *A*, schematic representation of the reporter and effector constructs used in the transient expression assays. The effector construct was the *Blz2* cDNA under the control of the 35S CaMV promoter fused to the first intron of the maize *AdhI* gene. The reporter constructs consisted of the GUS gene under the control of the Δ35S promoter alone or under the control of synthetic promoters containing the HOR, hor1, and hor2 oligonucleotides (sequences shown in Fig. 4*A*) fused at the 5′-end of the Δ35S. *B*, transient expression assays by particle bombardment of developing barley endosperms (15 DAP) with 150 ng of the indicated reporter plasmids with or without the *Blz2* effector at a 1:0.5 ratio. GUS activity was detected by biochemical staining and subsequent counting of blue dots per endosperm and was expressed as *n*-fold activation relative to controls without effector. Standard error of the mean for triplicate independent bombardments, with the same particle to plasmid suspension ratio, was <15%.

structs were generated containing in their promoters EB variants affected either in the GLM or in the PB, respectively. Developing endosperms (~15 DAP) were transiently transformed by particle bombardment with these reporters alone or in combination with the *Blz2* as effector at a 1:0.5 molar ratio. As represented in Fig. 5*B*, co-transfection of HOR-Δ35S-GUS and the *Blz2* effector resulted in an increase of about 3-fold in the GUS activity compared with that driven by the HOR-Δ35S-GUS alone. The HOR-Δ35S-GUS construct gives by itself a higher GUS expression than that of the control Δ35S-GUS, probably by the transactivation elicited by the endogenous barley endosperm factors that bind to the bipartite EB element. The promoter bearing the mutation in the GLM of the HOR element (hor1-Δ35S) renders a lower GUS activity (~20%) than the HOR-Δ35S and is not transactivated by the co-bombarded BLZ2. The hor2-Δ35S promoter, mutated in the PB, directed a GUS expression of ~50% of that controlled by the HOR-Δ35S promoter, both with or without the BLZ2 effector. These results clearly demonstrate that BLZ2 activates transcription through the GLM in barley endosperm, and that this activation is also partially dependent on the presence of an intact PB in the promoter.

BLZ2 Functions as a Transcriptional Activator That Can Form Heterodimers with BLZ1 in Yeast—The coexistence of *Blz2* and *Blz1* mRNAs in the barley endosperm prompted us to test if BLZ2 and BLZ1 may form heterodimers *in vivo*. We explored this possibility by the yeast two-hybrid system, using as a "bait" the bZIP domain of BLZ2, fused to the GAL4DBD and as a "prey" the whole BLZ1 or different fragments of it fused to the GAL4AD. If these two proteins expressed in yeast

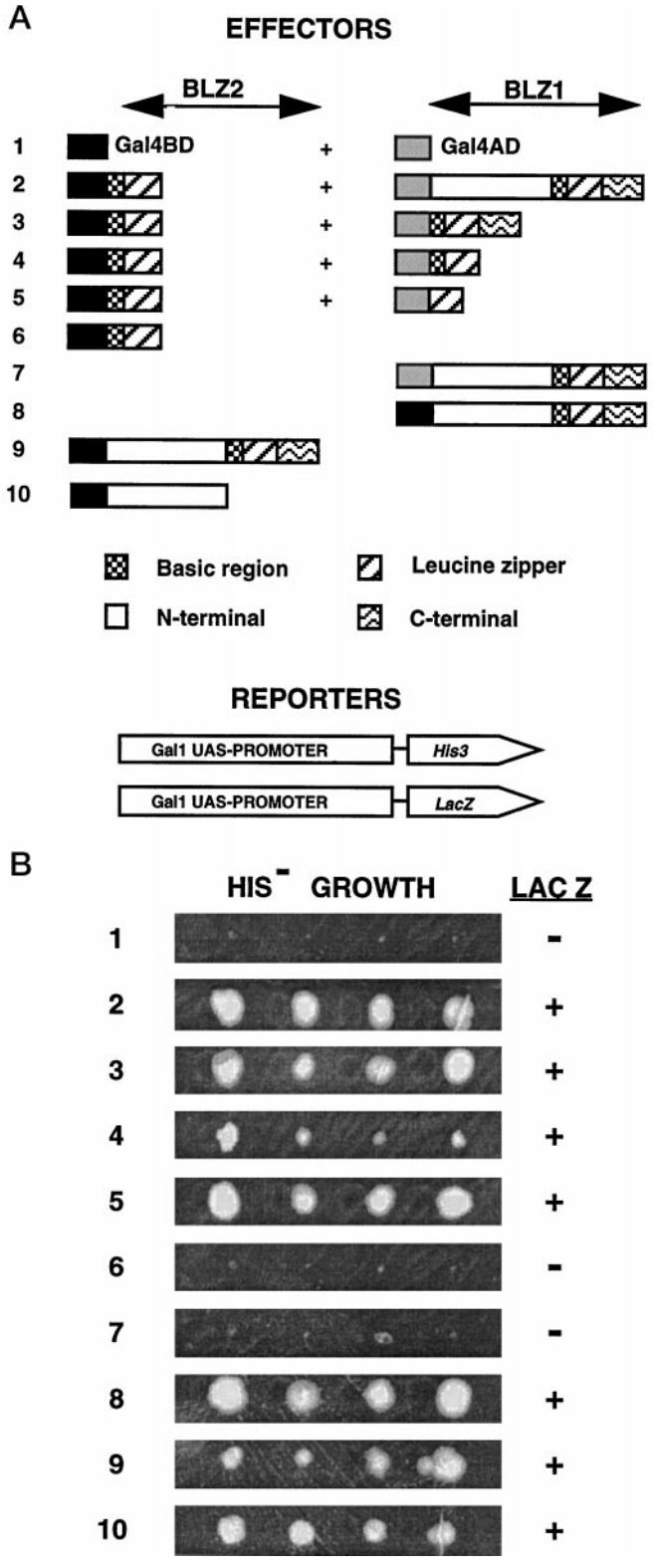

FIG. 6. **Transactivation by BLZ2 and interaction between BLZ2 and BLZ1 in the yeast two-hybrid system.** *A*, schematic structures of *S. cerevisiae* reporter genes and effector constructions used. *Gal4DBD*, *Gal4* DNA binding domain; *Gal4AD*, *Gal4* activation domain; *Gal1UAS*, *Gal4* responsive elements in a *Gal1* truncated promoter. *B*, growth of yeast cells containing the corresponding constructions indicated in Fig. 7*A* on a minimal His^- medium and induction of *LacZ* (colony lift filter assay). Only cells carrying "prey" and "bait" interacting proteins (*lanes 2, 3, 4*, and *5*) or activation domains fused to Gal4BD (*lanes 8, 9*, and *10*) were able to grow without histidine and to turn blue in the colony lift filter assay for *LacZ* induction (+).

do interact, then the complex becomes a functional transcription factor, which is capable of binding to the *Gal1*UAS in the promoters of reporter genes *HIS3* and *LacZ*, thus activating their transcription.

The constructs prepared are schematically represented in Fig. 6*A*. The cDNA encoding the bZIP domain of *Blz2* was fused to the *Gal4*DBD in the pGBT9 plasmid ("bait" construction) and introduced into *S. cerevisiae* HF7c strain. Subsequently, the *Blz1* fragments fused to the *Gal4*AD in the pGAD424 plasmid ("prey"constructions) were introduced into the yeast cells. As shown in Fig. 6*B* (*lanes 2–5*), all strains co-transformed with cDNA fragments containing the leucine zipper encoding regions of both proteins activated expression of the *HIS3* and *LacZ* reporter genes, indicating that BLZ2 interacts through its bZIP domain with BLZ1 *in vivo*. As expected, no activation of the reporters was obtained when yeast was co-transformed with the two pGBT9 and pGAD424 plasmids without inserts (Fig. 6*B*, *lane 1*) or when transformed only with the "bait" or with the "prey" constructions alone (Fig. 6*B*, *lanes 6* and *7*). As a positive control, we used the *Blz1* cDNA in the pGBT9 plasmid (Fig. 6*B*, *lane 8*) that had been previously shown to be an activator in the yeast system (8). The *Gal4*AD constructs in *lanes 3–5* by themselves did not activate the *Gal1*UAS promoter (data not shown).

To assess whether BLZ2 also functions in yeast as a transcriptional activator, we cloned the full-length or 5′-terminal *Blz2* cDNA in the pGBT9 vector and introduced it into *S. cerevisiae* HF7c strain. As shown in Fig. 6*B* (*lanes 9* and *10*), BLZ2 is a transcriptional activator, and the N-terminal region is sufficient to activate transcription of the reporter genes.

Do BLZ2 and BLZ1 Interact in Bombarded Barley Endosperm?—Having established that BLZ2 heterodimerizes with BLZ1 in yeast and that BLZ2, as did BLZ1 (8), mediates transcriptional activation in barley endosperm from the GLM of hordein promoters, we decided to evaluate the contribution of BLZ2 and BLZ1 to such activation in developing barley endosperms. For this purpose, experiments of co-bombardment were done using the HOR-Δ35S-GUS construct as reporter and the *Blz2* and/or the *Blz1* as effectors, both in the sense and antisense orientations (Fig. 7*A*).

As shown in Fig. 7B, BLZ2 and BLZ1, at 0.5:1 ratio to the GUS reporter, were able to transactivate approximately 3-fold the GUS expression from the HOR-Δ35S promoter, and no differences in the level of activation were observed when endosperms were co-bombarded with either one of the two effectors or with the equimolar mixture of *Blz2* plus *Blz1* in the sense orientation. Co-transfections with *Blz2* or with *Blz1* antisense constructs decreased the GUS activity obtained with the HOR-Δ35S-GUS reporter (Fig. 7*B*). In addition, co-bombardment of endosperms with the equimolar mixture of both *Blz2* and *Blz1* antisense constructs, produced a synergistic effect that resulted in almost the complete loss of detectable GUS activity, even at the lowest concentration assayed (Fig. 7*B*). The GUS enhancement observed when the effectors were used in the sense orientation and the drastic decrease when using them in antisense was not an unspecific effect because GUS fusions to the promoters of the barley sucrose synthase encoding genes *Ss1* and *Ss2* (GenBank™/EBI accession numbers X73221 and X92354; Ref. 35), as well as to the Δ35S promoter, did not respond either to *Blz2* (Table II) or to *Blz1* (8).

DISCUSSION

We have characterized a cDNA clone from barley that encodes an endosperm-specific bZIP transcription factor (BLZ2) that activates transcription in the homologous tissue (developing barley endosperm) through interaction with the GLM se-

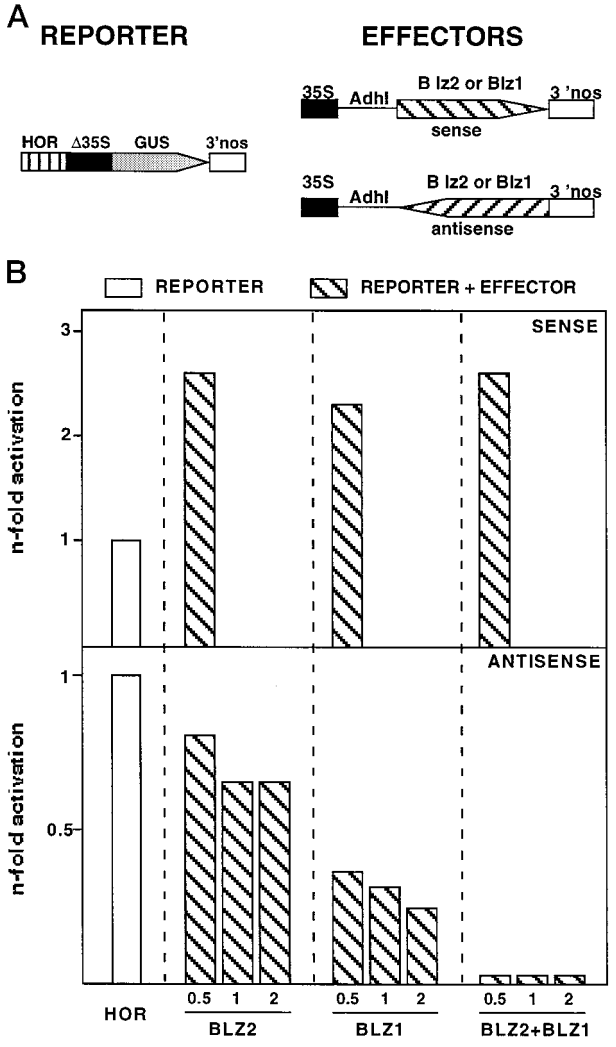

FIG. 7. **Transient expression assays by co-bombardment of developing barley endosperms (15 DAP) using as effectors both the sense and antisense *Blz2* and *Blz1* cDNAs.** *A*, schematic representation of the reporter and effector constructs used. The effector constructs were the *Blz2* or *Blz1* cDNAs in the sense or antisense orientation under the control of the 35S CaMV promoter fused to the first intron of the maize *AdhI* gene. The reporter construct consisted of the GUS gene under the control of the HOR-Δ35S and followed by the 3′-nos. *B*, transient expression assays by co-transfection of developing barley endosperms (15 DAP) with 150 ng of the reporter construct and 1:0.5 ratio of effector in the sense orientation. For the antisense experiments 250 ng of the reporter and the indicated reporter:effector ratios (1:0.5, 1:1, and 1:2) were used. GUS activity was detected by histochemical staining and subsequent counting of blue dots per endosperm and expressed as *n*-fold activation relative to the HOR-Δ35S control construct without effector. Standard error of the mean for triplicate independent bombardments, with the same particle to plasmid suspension ratio, was <15%.

quence from prolamin gene promoters. In addition, BLZ2 can function as an activator in yeast where it is capable of heterodimerizing with BLZ1.

The pattern of expression of *Blz2* would be consistent with a putative role in the transcription regulation of those endosperm-specific genes whose temporal mRNA expression overlap with that of *Blz2*. Genes encoding B-hordeins (such as *Hor-2*), which account for the major fraction of storage proteins in barley seeds, follow this expression profile.

The EB of the promoter of a *Hor-2* gene is made of two *cis*-motifs, PB and GLM, that are well conserved in the promoters of prolamin genes of the *Pooideae* grasses, such as

TABLE II
Transient expression analysis in barley endosperm

Transient GUS expression under the control of several promoters in co-bombarded barley endosperms using as effector the *Blz2* gene in sense or in antisense orientations. Fifteen developing endosperms in three independent experiments were co-bombarded with 150 ng of the reporter plasmids and a 1:0.5 ratio to the *Blz2* effector, in sense (S) or in antisense (A) orientations as indicated.

Reporters (Promoter::GUS)	Effectors (35S::Gene)	GUS activity[a]	
		mean ± S.E.	%
Δ35S		2.0 ± 0.2	100
	S	1.9 ± 0.1	95
	A	2.0 ± 0.2	100
HOR-Δ35S		9.2 ± 1.3	100
	S	23.5 ± 2.2	255
	A	6.2 ± 1.4	68
Ss1		48.4 ± 3.2	100
	S	46.6 ± 3.0	95
	A	51.2 ± 3.0	106
Ss2		41.0 ± 3.5	100
	S	42.4 ± 3.2	102
	A	38.7 ± 3.5	93

[a] GUS activity was expressed as number of blue spots per endosperm (mean ± S.E.) and as percentage over controls without effector.

those encoding B- and C-hordeins in barley (22, 36, 37), α/β gliadins, and LMW-glutenins in wheat (38, 39) and ω-secalins in rye (40). Our results show that BLZ2 binds *in vitro* in a sequence-specific manner to the GLM within the EB of a *Hor-2* gene promoter and that mutations altering the sequence of the GLM disrupt the interaction.

To investigate the role of BLZ2 *in planta*, we used transient expression assays in the barley endosperm tissue where it is naturally expressed. It is well known that in dicotyledonous hosts the quantitative or qualitative expression patterns controlled by monocotyledonous seed-specific gene promoters are frequently altered (38, 41–43). To avoid these problems, we used a system based on microparticle bombardment of the homologous developing endosperm that has been successfully experimented by us and other groups (7, 8, 15, 44). When a reporter gene controlled by the EB from the *Hor-2* promoter fused to the 5′-end of the minimal Δ35S promoter (8) was co-transfected with *Blz2* as effector, a 3-fold increase in GUS activity was observed at a 1:0.5 ratio. As expected, mutations in the GLM that avoided *in vitro* binding by the BLZ2 protein, abolished GUS activation. Mutations in the contiguous PB sequence that did not interfere with the BLZ2 binding *in vitro*, supported lower levels of BLZ2 activation *in planta* (~50%). These results indicate that BLZ2 mediates transcriptional activation in barley endosperm through specific interaction with the GLM sequence. An intact PB, recently reported by us to be recognized by a transcription factor of the DOF class (BPBF; Ref. 15), is also essential for full transactivation. Moreover, in absence of the exogenous BLZ2 effector, the reporters with mutations, either in the GLM or in the PB promoter sequences, display a much lower basal GUS activity compared with that sustained by the HOR-Δ35S promoter. Thus, our results also suggest that a positive relationship between bZIP and DOF transcription factors is necessary for high expression levels to be obtained from the EB of hordein promoters in barley endosperm. It should be noted that transient expression data indicate that BLZ2 must not be saturating in barley endosperm or we would not have seen stimulus upon adding the *Blz2* effector plasmid together with the HOR-Δ35S-GUS reporter. This suggests that overexpression of BLZ2 in transgenic barley might lead to increased levels of storage protein gene expression, an agronomic important goal.

BLZ2 also behaves as a transcriptional activator in yeast and the N-terminal region of the protein is sufficient for that acti-

vation. The possibility of protein-protein interactions between BLZ2 and BLZ1, has also been investigated in the yeast two-hybrid system. These bZIP proteins are able to interact and, similarly to the vast majority of factors belonging to this group, the bZIP domain is sufficient to sustain the dimerization between BLZ2 and BLZ1 (17–19, 45–47). Considering the potential of BLZ2 to heterodimerize with BLZ1 *in vivo*, it is worth noting that such interactions, which are common in the bZIP family of transcription factors, allow the elaboration of complex regulatory networks based on the different properties of homo- and heterodimers in terms of DNA binding and transcription regulation. Extensively documented examples of such mechanisms exist both in the animal kingdom and in plants where the presence of different family members in a given tissue can modify substantially the final regulatory effect (17, 19, 47, 48).

Transient expression assays were conducted with effector plasmids carrying *Blz2* and/or *Blz1* constructs in antisense orientation to investigate the effects of their depletion. The mRNA expression of *Blz2* in developing endosperm was only partially counteracted by the antisense approach, and a more effective GUS reduction was obtained with the *Blz1* antisense construct. However, it was remarkable the effect achieved by co-transforming with both *Blz2* and *Blz1* antisense effectors, which resulted in a dramatic reduction of the basal GUS activity even at the lowest effector/reporter ratio tested. This suggests a synergistic effect by a possible BLZ2/BLZ1 heterodimer in barley endosperm. It is worth noting that the GLM sequences present in most hordein promoters are of the AP-1 type (AC/GT cores), this being a constraint for the binding by other plant bZIP factors belonging to the ATF/CREB group that recognize the ACGT core (49, 50). This observation together with the data concerning the transcriptional properties *in planta* of BLZ2 and BLZ1, strongly support the fact that both proteins are significant, if not the unique, components of the machinery that mediates transcription activation through the GLM. However, we cannot rule out the contribution of other factors to the endosperm box complex. In this context, interactions with the barley PB-binding factor (15) could account for major differences in their mode of action as compared with homo- or heterodimer bZIP formation. We are currently investigating this possibility.

Barley BLZ2 and wheat SPA show a relevant sequence homology that is not restricted to the bZIP domain, similar endosperm-specific expression pattern and similar DNA binding specificities, which suggest that these genes may be homologous. Thus, our results endorse and substantiate the observations about the importance of the GLM in the regulation of storage protein genes and argue for a general conservation of the endosperm-specific BLZ2/SPA type of bZIP proteins as transcriptional regulators in cereal seeds of the *Pooideae* subfamily.

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Barley BLZ2, a Seed-specific bZIP Protein That Interacts with BLZ1 *in Vivo* and Activates Transcription from the GCN4-like motif of B-hordein Promoters in Barley Endosperm

Luis Oñate, Jesús Vicente-Carbajosa, Pilar Lara, Isabel Di?az and Pilar Carbonero

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