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GAMYB and BPBF Transcriptional Factors in the Control of Gene Expression During Development of Barley Endosperm

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Introduction

During cereal seed development, the starchy endosperm cells accumulate proteins, mainly prolamines, as well as several antimicrobial peptides and inhibitors of heterologous hydrolases implicated in plant defence (Shewry *et al.*, 1995; Carbonero *et al.*, 1999). The genes encoding the storage proteins are coordinately expressed in the developing endosperm where they are under spatial and temporal transcriptional control, involving *cis*-acting motifs in their promoters and *trans*-acting transcriptional factors (TFs). A number of consensus sequences in gene promoters have been shown to be involved in conferring endosperm specificity in cereals (Albani *et al.*, 1997; Marzabal *et al.*, 1998; Mena *et al.*, 1998; Vicente-Carbajosa *et al.*, 1998; Oñate *et al.*, 1999; Carbonero *et al.*, 2000; Wu *et al.*, 2000; Díaz *et al.*, 2002). A conserved *cis*-acting motif found in most storage protein gene promoters of seeds is the endosperm-box, a bipartite motif located ~300 bp upstream of the translation initiation codon that contains two distinct binding sites: the GCN4-like motif (GLM) motif (5'-ATGAG/CTCAT-3') that resembles the yeast GCN4 motif and the prolamine box (PB: 5'-TGTAAG-3'). There is a third motif (5'-AACA/TA-3'), which plays a role in the expression of genes encoding glutelins in rice (Suzuki *et al.*, 1998). These three types of motifs are the target sites of different TFs.

In barley, the GLM is recognized by bZIP proteins of the Opaque2 family (BLZ1 and BLZ2) that through dimer formation, as either homo- or heterodimers (Vicente-Carbajosa *et al.*, 1998; Oñate *et al.*, 1999), *trans*-activate the expression of endosperm-specific genes such as the *Hor2* and *Itr1* genes

encoding the B-hordeins and a trypsin inhibitor, respectively. The prolamine box is recognized by a TF of the DOF class, DNA binding with One Finger (BPBF), that activates transcription of a native *Hor2* promoter in co-bombarded barley endosperms through binding to the prolamine box (PB) (Mena *et al.*, 1998; Carbonero *et al.*, 2000). We describe here how the 5'-AACCA/TA-3' motif interacts with a TF of the MYB class, which was previously shown to have a role in the activation of gibberellin (GA)-induced genes upon germination in barley aleurone cells (Gubler *et al.*, 1995, 1999).

Materials and Methods

Plant material

Barley (*Hordeum vulgare*) cv. Bomi was grown in a greenhouse at 18°C under 18/6 h day/night photoperiod. Developing endosperms (10–22 days after flowering), mature embryos and 7-day-old leaves and roots were used for RNA extraction. For transient expression assays, developing endosperms collected at 18 days after flowering (DAF) were used immediately.

Northern blot analysis

RNA was isolated by the Lagrimini *et al.* (1987) procedure, electrophoresed, blotted to Hybond membranes and hybridized according to standard procedures (Sambrook *et al.*, 1989). Specific labelled probes were used to identify the *HvGAMYb*, *Pbf*, *Hor2* and *Itr1* mRNAs (Díaz *et al.*, 1995; Gubler *et al.*, 1995; Mena *et al.*, 1998).

DNA constructs and particle bombardment in developing endosperms

The effector constructs contained the cDNAs of *GAMYB* (Gubler *et al.*, 1995) or *BPBF* (Mena *et al.*, 1998) driven by the 35S promoter plus the first intron of the alcohol dehydrogenase I gene (*Adhl*). The reporter constructs included the GUS reporter gene (*uidA*) fused to the *Itr1* and *Hor2* gene promoter fragments (Díaz *et al.*, 1995; Mena *et al.*, 1998), or their mutated versions (see Fig. 9.3a), which corresponded to base changes in the specific MYB and DOF binding motifs.

Particle bombardment was carried out with a biolistic helium gun device (DuPont PDS-000, BioRad, USA). Gold particle coating and bombardment conditions were performed according to Vicente-Carbajosa *et al.* (1998).

Yeast two-hybrid system assays

Translational fusions with the *GAMYB* and *BPBF* (or derived fragments of it) to the alcohol dehydrogenase I (*Adhl*) promoter fused to the Gal4 DNA-binding and DNA-activation domains (BD- and AD fusions, respectively) were done (see Fig. 9.4a). *Saccharomyces cerevisiae* transformation was per-

Fig. 9.1. Schematic representation of *cis*-motifs in the barley *Hor2* gene promoter and their putative interaction with transcriptional factors (TF) of the bZIP (BLZ1 and BLZ2), DOF (BPBF) and MYB (GAMYB) classes. GLM: GCN4-like motif; PB: Prolamine Box; M: MYB binding site; TATA: TATA box.

formed by the polyethyleneglycol method and transformants were screened for β -galactosidase production (*LacZ*).

Results and Discussion

Functional analyses of the promoters of several genes expressed in barley endosperm, such as those encoding B-hordeins (*Hor2*) and trypsin inhibitor CMe (*Itr1*), had shown the presence of putative bZIP, MYB and DOF binding motifs in their promoters (Fig. 9.1), which led us to investigate the possible involvement of the HvGAMYB and the BPBF protein factors in their regulation.

The HvGAMYB is a TF of the MYB class that binds specifically to the GA-responsive element (GARE) present in the promoters of α -amylases, β -glucanases and cysteine-proteases of the cathepsin B and cathepsin L types (Cercos *et al.*, 1999; Gubler *et al.*, 1999). Mutations in the GARE abolished both binding and *trans*-activation. The BPBF is a TF of the DOF class that we had previously shown to be expressed in the developing barley endosperm. The BPBF protein expressed in bacteria was able to bind specifically to the PB motif in a *Hor2* gene promoter and through direct interaction with this *cis*-element did transiently *trans*-activate transcription from the *Hor2* promoter in microprojectile-bombarded developing barley endosperms (Mena *et al.*, 1998; Carbonero *et al.*, 2000).

The expression of the encoding gene GAMYB was analysed by Northern blot and these experiments demonstrated that this gene was already present in developing endosperms at 10 DAF and could be detected throughout all stages analysed, as occurred with the DOF transcription factor BPBF (*Pbf* gene) (Fig. 9.2). The transcripts of B-hordein (*Hor2* gene) and trypsin inhibitor CMe (*Itr1* gene) were detectable at 10 DAF and peaked at 22 DAF (Fig. 9.2). The pattern of GAMYB accumulation was consistent with its being a regulator of the *Hor2* and *Itr1* genes, whose temporal mRNA expression overlaps with that of the HvGAMYB. *In situ* hybridization studies have localized the spatial expression of the HvGAMYB within the developing

Fig. 9.2. Northern blot analysis of *GAMYB* and *BPBF* mRNA expression in different barley tissues. Total RNA (8 μ g) from leaves (L), roots (R), mature embryo (E) and developing endosperm (End) at 10–22 DAF were electrophoresed and blotted. The filter was hybridized with specific probes from *GAMYB*, *BPBF*, *Hor2* and *Itr1* cDNAs.

seed in the endosperms, aleurone layers, vascular tissues and embryo (Díaz *et al.*, 2002).

The capability of *in vitro* binding of the GAMYB protein to the 5'-C/TAACT/AACC/A-3' motif in the promoters of *Hor2* and *Itr1* genes was shown by electrophoretic mobility shift assays (EMSA), using two oligonucleotide probes whose sequences were deduced from the corresponding regions of the *Hor2* and *Itr1* gene promoters and protein extracts derived from *Escherichia coli* expressing GAMYB as a fusion protein. Retarded bands were produced only when the probes were incubated with the transformed bacterial protein extracts (data not shown). Binding specificity was demonstrated by competition titration up to 100 $\times$ concentration with the same unlabelled probe, but not by the mutated probes which differed only in two base changes at the binding MYB motif. The formation of the shifted DNA-protein complexes was not observed with the mutated variants.

The functional relevance of the interaction *in vitro* was further tested *in planta* by transient expression assays in co-bombarded developing endosperms (Fig. 9.3). When a reporter gene under the control of the -343 bp or -560 bp promoter regions from the *Hor2* and *Itr1* genes, respectively, containing intact MYB and DOF binding sites (pItr1 and pBhor in Fig. 9.3A), were co-transfected with *GAMYB* or *BPBF* as effectors, a threefold to tenfold increase in GUS activity was observed (Fig. 9.3B,C). As expected, mutations in the MYB binding motif (pItr1* and pBhor** in Fig. 9.3A) that prevent *in vitro* binding by the GAMYB protein in EMSA assays abolished GUS transactivation (Fig. 9.3B,C). In contrast, when the reporter construct mutated in the DOF binding site was used (pBhor* in Fig. 9.3C), GAMYB failed to transactivate GUS expression, although the MYB site was intact, while the BPBF protein, as effector, was still able to increase the GUS activity over the control without effector (Fig. 9.3C), probably because there are two DOF binding sites in the promoter (see Fig. 9.1) participating in the regulation process.

Fig. 9.3. *Trans*-activation of the *ltr1* and *Hor2* promoters by HvGAMYB and BPBF in developing endosperm. (A) Schematic representation of the effector and reporter constructs. The effectors contained the *GAMYB* or *BPBF* cDNAs driven by the CaMV 35S promoter plus the first intron of the *Adhl* gene. The reporter constructs consisted of the GUS gene (*uidA*) under the control of the *ltr1* gene promoter (pltr1), the *Hor2* promoter (pBhor), or under the mutated promoters with the indicated base changes (pltr1*, pBhor* and pBhor**). (B), (C) Transient expression assays by co-bombardment of developing barley endosperms with the indicated combinations of reporter and effector constructs. GUS activity was expressed as percentage relative to the control plasmids without effector. Transient expression driven by the promoter mutants under study relative to their respective wild types are shown in the inserts.

Mutation at the MYB and DOF binding sites in the reporter plasmids resulted in the loss of the basal transcriptional activity of the native pBhor and pltr1 promoter constructs (inserts of Fig. 9.3B,C).

Taken together, all these results indicate that the GAMYB mediates the *trans*-activation of the *ltr1* and *Hor2* gene promoters in developing barley endosperm through binding to the MYB motif, and that an interaction with the DOF factor BPBF is necessary for the full *trans*-activation of *Hor2*. This

hypothesis was checked, *in vivo*, by the yeast two-hybrid system, using the effector constructs schematically represented in Fig. 9.4A. As shown in Fig. 9.4B (lane 2), yeast cells co-transformed with the full-length cDNA fragments of both proteins activated the expression of a *Lac* reporter gene, indicating interaction between these two TFs in this system. This interaction took place between HvGAMYB and the C-terminal part of the BPBF (lane 6 in Fig. 9.4B). In addition, we observed that BPBF is a transcriptional activator in yeast and this protein factor devoid of the N-terminal DOF domain was a stronger activator than the full-length BPBF (lanes 1 and 5 in Fig. 9.4B), an observation previously reported by Kang and Singh (2000) with other DOF transcriptional factors.

These results strongly implicate the GAMYB protein from barley as a TF involved in the combinatorial regulation of genes specifically expressed in the endosperm during development. Recent data from our laboratory have demonstrated that BPBF also has a role in the control of gene expression upon germination (Mena *et al.*, 2002), which suggests that involvement of the same TFs in the processes of seed development and germination may be a more general phenomenon than previously suspected.

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Fig. 9.4. Interaction between HvGAMYB and BPBF in the yeast *S. cerevisiae* two-hybrid system. (A) GAL4-BD and GAL4-AD: GAL4-binding and -activation domains; BPBF: full-length BPBF cDNA (nt 1–999); DOF: BPBF N-terminal region (nt 1–273); C-term: BPBF C-terminal region (nt 274–999); GAMYB: full-length GAMYB cDNA (nt 1–1719). (B) Quantitative evaluation of the β -galactosidase (*LacZ*) reporter activity in liquid medium, expressed as Miller's units. Standard errors of the mean of four isolates were 10%. The reporter *LacZ* was driven by GAL4 truncated promoter under the control of Gal4-responsive elements.

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