The barley cystatin gene (Icy) is regulated by DOF transcription factors in aleurone cells upon germination*

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Abstract

A genomic clone (Icy gene) encoding a barley cystatin has been characterized. The gene contains one intron interrupting its ORF that encodes a protein of 107 amino acid residues. A DNA fragment of −1058 bp upstream of the ATG translation initiation codon has been sequenced and several promoter deletions fused to the β-glucuronidase (GUS; uidA gene) reporter gene obtained. Transient expression assays in different barley tissues have indicated that a −631 bp promoter fragment was sufficient for full activity. In bombarded barley aleurone layers the GUS-driven expression by this promoter is repressed by GA3 incubation, as is the accumulation of the Icy transcripts detected. A spatial and temporal location of cystatin transcripts by in situ hybridization techniques indicates that this gene is ubiquitously expressed and its transcripts are particularly abundant in leaves and roots, and in seeds, both during development and upon germination. Two DOF transcription factors, SAD and BPBF, previously described as involved in gene expression regulation in seeds, interact specifically in vitro with oligonucleotides containing DOF binding-sites derived from the Icy gene promoter. Transient expression experiments in co-bombarded aleurone layers demonstrate that BPBF strongly represses transcription of the native Icy promoter, even when co-transfected with SAD that behaves as an activator in this in vivo system.

Key words: Aleurone cells, barley cystatin, DOF transcription factors, functional gene promoter, promoter analysis.

Introduction

Cystatins are a group of proteins specifically inhibiting cysteine-proteinases that have been identified in vertebrates, invertebrates, and plants. Those from plants, referred to as phytocystatins (PhyCys), comprise more than 70 members (Pfam databank; Bateman et al., 2002), which cluster in a major evolutionary tree branch of the cystatin superfamily of proteins (Margis et al., 1998). Besides the reactive site motif QXVXG, a glycine residue near the NH2-terminus, and the A/PW residues located at the C-terminal part of the protein, common to all plant and animal cystatins, most of the PhyCys are characterized by a molecular mass in the 12–16 kDa range, the absence of disulphide bonds and the presence of a consensus ARFAV sequence in the N-terminal half of the protein. Several cystatin isoforms and cystatin-encoding genes, with different spatial and temporal expression patterns and with different inhibitory activities towards cysteine-proteinases, have been described. The two main functions that have been assigned to PhyCys are: (i) the regulation of endogenous protein turn-over (Arai et al., 2002; Corre-Menguy et al., 2002) and (ii) plant defence (Zhao et al., 1996; Kuroda et al., 1996; Delledonne et al., 2001; Siqueira-Junior et al., 2002; Martinez et al., 2003b).

The cloning and molecular characterization of a cDNA from developing barley endosperm encoding the cystatin Hv-CPI (gene Icy) has previously been reported (Gaddour et al., 2001). This gene, with the molecular characteristics described above, is ubiquitously expressed and its expression in vegetative tissues is affected by light, cold temperatures, and anaerobiosis. The recombinant Hv-CPI protein expressed in E. coli is an efficient inhibitor of papain, chymopapain, ficin, and cathepsin B and also has antifungal...
properties, inhibiting the in vitro growth of Botrytis cinerea and other phytopathogenic fungi (Gaddour et al., 2001; Martínez et al., 2003a, b).

DOF (DNA with One Finger) proteins are a family of plant transcription factors (TFs) that recognize the cis-motif 5′-T/AAAAG-3′, 5′-CTTTT/T-A-3′ (Yanagisawa, 2002) in the promoters of genes regulated by them. These TFs are involved in the expression of genes related to biological processes unique to plants, such as seed development and germination, guard cell function, photosynthesis, phyto-hormone effects, and plant–pathogen interaction (Chen et al., 1996; Vicente-Carbajosa et al., 1997; Mena et al., 1998; Baumann et al., 1999; Plesch et al., 2001; Washio, 2001; Mena et al., 2002; Isabel-LaMoneda et al., 2003). In barley, two DOF TFs, BPBF and SAD, involved in gene regulation during seed development and germination have been characterized. BPBF was first found to be involved in the activation of storage protein genes (Mena et al., 1998) and both TFs have a role in controlling gene expression in aleurone cells upon germination. These DOF proteins recognize the pyrimidine box (5′-CTTTT-3′) in the cathepsin B-like thiol protease (Al21 gene) and the α-amylase (Amy2/32b gene) promoters. In transient expression experiments, BPBF repressed and SAD activated transcription of the Al21 gene promoter in barley aleurone layers (Mena et al., 2002; Isabel-LaMoneda et al., 2003).

The present study describes the characterization of a genomic clone corresponding to the barley cystatin (Icy) gene and the functional analysis of its promoter by transient expression assays. These results are compared with the expression of the Icy mRNAs in barley tissues by in situ hybridization. The data complemented by DNA-protein in vitro interaction and co-expression experiments implicate BPBF and SAD as part of the transcriptional regulation of the Icy gene upon barley seed germination.

Materials and methods

Plant material

Barley (Hordeum vulgare) cv. Bomí seeds were germinated in the dark and grown in a greenhouse at 18 °C under an 18 h day/night photoperiod. Different plant tissues at several stages of development were collected and immediately used for in situ hybridization and for transient expression experiments. Himalaya barley seeds were used as a source of aleurone layers.

Screening of a genomic library

A barley (H. vulgare) cv. Igri genomic library constructed in Lambda FIXII (Stratagene) was screened with a 198 bp specific probe derived from the 3′ non-coding region of the barley cystatin cDNA clone (Gaddour et al., 2001). Library screening was performed under standard hybridization conditions and filters washed at 50 °C in 2× SSC solution (Sambrook et al., 1989). Nucleotide sequences were determined with the ABI PRISM 377 DNA sequence analyser (Perkin Elmer-Applied Biosystems). DNA and deduced protein sequence analyses were carried out with computer services under the European Bioinformatics Institute (http://www.ebi.ac.uk/).

In situ hybridization analysis

Barley seeds were germinated on filter paper soaked with distilled water at 22 °C in the dark and collected after 16 h, 24 h, and 48 h of imbibition. Fixation was in FAEw (ethanol/acetic acid: formaldehyde:water, 50:5.3:5.4:1.5, by vol.) for 2 h at room temperature, dehydrated, and embedded in paraffin, and sectioned at 8 μm. Flowers were anesthetized, developing kernels (18 daf), and 7-d-old leaves and roots were fixed and treated under the same conditions. After de-waxing in histoclear and rehydration, sections were incubated in 0.2 M HCl, neutralized, and treated with 1 mg ml⁻¹ protease K. Finally, tissue sections were dehydrated in an ethanol dilution series and dried under vacuum before applying the hybridization solution (100 mg ml⁻¹ RNA; 6× SSC; 3% formamide) containing approximately 100 ng ml⁻¹ antisense or sense DIG-labelled RNA probes, corresponding with the 198 bp fragment described above. Hybridization was performed overnight at 52 °C followed by two washes in 2× SSC and 50% formamide for 90 min at the same temperature. Antibody incubation and colour detection was carried out according to the manufacturer’s instructions (Boehringer Roche Diagnostics, Germany).

Promoter constructs and transient expression assays

A series of Icy promoter fragments were obtained, spanning from −1058, −631, −582, −512, −472, −356, and −161 bp to the ATG translation start codon. These fragments were amplified using as specific sense primers P1 to P7 that incorporated a BamHI site (underlined) to their 5′-ends: P1: 5′-CGAGGATCCGTA-GTCTCTAGATC-3′; P2: 5′-CGAGGATCCGTTAGAGGTGCTTATGC-3′; P3: 5′-CGAGGATCCGTA-GTCTCTAGATC-3′; P4: 5′-CGAGGATCCGTA-GTCTCTAGATC-3′; P5: 5′-CGAGGATCCGTTAGAGGTGCTTATGC-3′; P6: 5′-CGAGGATCCGTTAGAGGTGCTTATGC-3′; P7: 5′-CGAGGATCCGTTAGAGGTGCTTATGC-3′. As the reverse primer P8: 5′-CGAGGATCCGTA-GTCTCTAGATC-3′ and as the reverse primer P8: 5′-CGAGGATCCGTA-GTCTCTAGATC-3′, that added an NcoI site (underlined) in its 3′-end. The amplified bands were subcloned into the BamHI/NcoI sites of the polylinker in the pH9w, a β-glucuronidase coding region (GUS) containing plasmid (Diaz et al., 1993), producing an in-phase fusion with this reporter gene.

Developing endosperms of 18 daf (days after flowering) and 7-d-old leaves from barley cv. Bomí and aleurone layers from Himalaya barley seeds were prepared for bombardment. Particle coating and bombardment conditions were carried out basically as described previously (Díaz et al., 2002; Isabel-Lamoned, 2003), with a biolistic helium gun device (DuPont PDS-1000). After bombardment, endosperms and leaves were incubated in MS/2 agar-medium at 24 °C in the dark before GUS determination and aleurone layers were placed in Petri dishes with a buffer containing 20 mM CaCl₂ and 20 mM Na succinate, pH 5.2, with 1 μM GA₃ or 10 μM ABA and incubated for 24 h in the dark with gentle shaking.

For particle co-bombardment of aleurone layers, the four plasmids described above were used as reporter constructs that contained −631, −582, −512, and −472 bp fragments upstream of the ATG initiation codon of the Icy promoter, which included respectively, three, two, one, and none binding cis-motifs recognized by DOF transcription factors. The complete PbF and Sad cDNAs (Mena et al., 1998; Isabel-LaMoneda et al., 2003), under the control of the CaMV 35S promoter, followed by the 3′ nos terminator were used as effector constructs.

GUS activity was determined by chemiluminescence (GUS-light kit, Tronix) or histochemically following Jefferson (1987) and calculated as relative luminescence units (RLU) per microgram of protein per hour of reaction, or as the mean value of blue spots per tissue sample. The GUS activity was expressed as a percentage, considering as 100% the GUS value obtained with the longest promoter fragment and, for the co-bombardment experiments, the
value obtained with each reporter construct without effector was considered 100%. The data quantified histochemically and by chemiluminescence were correlated showing a correlation coefficient of 0.96 (data not shown).

RNA preparation, northern blot and RT-PCR analysis

Himalaya barley seeds were de-embryonated, sterilized in 1.7% NaOCl for 10 min, treated with 10 mM HCl for 5 min and, after several washes with distilled water, placed onto filter paper soaked with a solution containing 20 mM Na succinate, pH 5.2 and 20 mM CaCl₂. Aleurone layers were isolated from the imbibed seeds under a dissecting microscope after 8, 16, 24, and 48 h of imbibition at 22 °C in the dark with the buffer described above. For hormonal treatment, aleurone layers were incubated for 16 h with 1 μM GA₃ or 10 μM ABA or no hormones. Aleurone samples were frozen until RNA extraction and total RNA was purified by phenol/chloroform extraction followed by precipitation with 3 M LiCl (Lagrimini et al., 1987).

For northern blot analysis, denatured RNA was electrophoresed in 8% agarose gels containing 7% formaldehyde and blotted onto Hybond N membranes. Hybridization was performed under stringent conditions following standard procedures (Sambrook et al., 1989) using as specific probe the same 198 nt fragment used for the screening of the genomic library.

For RT-PCR analysis, RNA preparations were treated with DNase using the DNA-free system (Ambion Inc Austin, TX, USA). First-strand cDNA synthesis was primed with random hexamers and catalysed by M-MuLV Reverse Transcriptase according to the manufacturer’s recommendations (Amersham Pharmacia Biotech). PCR amplification of the 324 bp portion of the Icy cDNA (nt 1–324 from the ATG initiation codon) was performed using the forward primer 5′-ATGGCCGAAGGCCGCGCATG-3′ and the reverse primer 5′-TTAGCGCCGCGTCTTGAATTC-3′. Fragments of 460 bp and 497 bp of the cDNAs encoding BPBF and SAD transcriptional factors, respectively, were amplified using the primers previously described by Mena et al. (2002) and Isabel-LaMoneda et al. (2003), respectively. The 18S amplicon was used as the internal control using as specific probe the same 198 nt fragment used for the screening of the genomic library.

Electrophoretic mobility shift assays

The BPBF and SAD proteins were expressed in E. coli (BL21(DE3) pLysS strain) by cloning the cDNAs into the pGEX-2T vector (Amersham Pharmacia Biotech) as a translational fusion to GSTpLysS strain) by cloning the cDNAs into the pGEX-2T vector. Recombinant proteins were induced with 1 mM isopropyl-

Results

Characterization of the Icy genomic clone

A genomic library of barley cv. Igri was screened with a specific barley cystatin probe of 198 nt derived from the 3′ end of the cDNA clone (Gaddour et al., 2001). Several clones were identified and one of them, corresponding to that containing the longest promoter fragment, was further characterized. A 1650 bp fragment of this clone spanning up to −1058 nt upstream of the ATG initiation codon was sequenced (Fig. 1). By comparison with the cDNA clone it was found that the Icy gene comprised two exons and one intron and corroborated that its ORF encoded a protein of 107 amino acid residues, with a calculated molecular mass of 11.7 kDa. The 91 bp intron was flanked by typical gt/ag boundaries. Exon 1 contained the 5′ non-coding region and a DNA fragment encoding the first 48 amino acids of the protein, while the rest of the coding region, the TAA stop codon, and the 3′ non-coding sequence were contained in exon 2. In the 5′-region, −1058 bp upstream of the translation initiation codon ATG, a predicted transcription initiation site was located at position −117, and 28 nt further upstream a typical TATA box was identified.

A computer search for potential cis-regulatory elements in the proximal −631 nt promoter region (Fig. 1) revealed a number of them. (i) Three CCAAT/A boxes, typical sequences recognized by the DOF transcription factors (Yanagisawa, 2002). (ii) Nine C/TACCC core-like sequences, such as those found in the AGCCGCC box and the S box (AGCCACC) that are often found in promoter regions of defence genes (Kirsch et al., 2001; Rushton et al., 2002). (v) Seven GCC core-like sequences, such as those found in the AGCCGCC box and the S box (AGCCACC) that are often found in promoter regions of defence genes (Kirsch et al., 2001; Rushton et al., 2002).

Functional analysis of the Icy gene promoter in barley tissues

A series of deletions of the promoter fused to the reporter uidA gene were constructed and analysed in transient expression assays by particle bombardment of developing endosperms, young leaves, and aleurone layers of barley. As shown in Fig. 2A, the two longest fragments analysed (those spanning to −1058 and −631 nt) supported the highest levels of GUS activity in all barley tissues analysed, indicating that deletion up to position −631 did not affect the expression of the reporter gene. Further deletions gradually reduced the GUS activity down to almost undetectable values when the −161 bp fragment which did contain the TATA box was analysed. These results indicate that the cis-acting elements present between positions −631 and −161 were the most important of the expression of the Icy gene.
Differences in the pattern of expression were found in the different barley tissues, the highest values being obtained in the developing endosperm (see insert of Fig. 2A), which is in agreement with the results of the northern blot analyses previously published by Gaddour et al. (2001).

When the intron of this gene was included in the promoter constructs in transient expression assays in the three different barley tissues, GUS expression was enhanced about 4-fold (data not shown).

Isolated aleurone layers from Himalaya barley seeds that do not synthesize GA$_3$ but are able to respond to it, were bombarded with the $Icy$ promoter fragment corresponding to 631 bp fused to the $uidA$ gene and incubated in the presence of GA$_3$ and ABA. As shown in Fig. 2B, when 1 μM GA$_3$ was added to the culture medium, the GUS activity decreased about 50% whereas a 10 μM ABA treatment did not significantly change the GUS values. The pattern of mRNA expression of this barley gene in aleurone layers analysed by northern blot corroborated the results obtained by transient expression assays. A clear decrease of the $Icy$ transcript is mediated by GA$_3$ while ABA incubation did not alter significantly it (see insert of Fig. 2B).

In situ hybridization analysis

To define more precisely the spatial and temporal pattern of expression of the $Icy$ gene, mRNA in situ hybridization studies were done. In transverse sections of vegetative tissues such as 7-d-old leaves and roots, a clear signal with the antisense probe was mainly detected in the leaf mesophyl and in root epidermal cells, as well as in the stomas and bundle sheaths of the leaf, and in the cortex and vascular cylinder of the root (Fig. 3A, B). A strong signal was also observed in flowers before anthesis. Expression was prominent in the pollen grains, inside the locules as assessed in longitudinal sections (Fig. 3C). Transverse
sections of the developing spike were done at three different levels. In the basal part of the floret, the mRNA label was very strong in the two distinctive bracts, lemma and palea, as well as in the base of the two lodicules and the pistil complex (Fig. 3G). At the middle level, the expression was maintained in the lemma and palea, but decreased in the lodicules and pistil complex and was also observed in the three filaments of the stamens (Fig. 3H). In the upper section of the floret, transcripts were accumulated in the lemma, palea, pollen sacs, and pollen grains (Fig. 3I).

During seed development, Icy messages were detected in the starchy endosperm, aleurone layers, pericarp, and vascular tissues (Fig. 3M, N) and a weaker signal was also distributed throughout the embryos (Fig. 3O). Upon seed germination, a clear signal was detected in the aleurone layer and pericarp at 16 h of rehydration (Fig. 3S) and in longitudinal sections of embryos, mRNAs were detected after 16 h of imbibition in the region corresponding to the scutellum, coleorhiza, and foliar primordia (Fig. 3T), where it gradually increased up to 48 h of imbibition (Fig. 3U). No signal above background was detected when sections were hybridized with the sense probe, used as negative control (Fig. 3D–F, J–L, P–R, V–X).

**Pattern of expression of barley cystatin and DOF factors in aleurone**

The accumulation pattern of the barley cystatin transcripts (Gaddour et al., 2001) together with the presence of binding DOF motifs in the corresponding Icy gene promoter, suggested that its expression could be regulated by the two DOF transcriptional factors, BPBF and SAD (genes Pbf and Sad), previously characterized by our group (Mena et al., 1998; Isabel-LaMoneda et al., 2003). To explore the
Fig. 3. Expression of the *Icy* mRNA in different barley tissues by *in situ* hybridization. Transverse sections of 7-d-old leaves (A, D) and roots (B, E). Longitudinal (C, F) and transverse (G–L) sections of flowers before anthesis, sectioned at different levels. Transverse sections of 18 daf developing endosperm (M,N,P,Q). Longitudinal sections of 18 daf developing embryo (O, R), 1-d-old germinating aleurones 16 h after hydration (S, V) and embryos collected at 16 h (T, W) and 48 h (U, X) of seed imbibition. Hybridization was done with the antisense *Icy* probe (A–C, G–I, M–O, S–U) or with the control sense probe (D–F, J–L, P–R, V–X). a, aleurone; bs, bundle sheath; c, cortex; co, coleorhiza; e, epidermis; em, embryo; en, endosperm; f, filaments; fp, foliar primordia; l, locule; lm, lemma; lo, lodicule; m, mesophyll; p, pollen; pa, palea; pc, pistil complex; pe, pericarp; ps, pollen sac; r, root; s, stoma; sc, scutellum; vc, vascular cylinder; vt, vascular tissues. Scale bars: (M, P) 200 μm; (O, R, T–X) 100 μm (A–L, S, V); 50 μm; (N, Q) 25 μm.
time-course of mRNA accumulation of *Icy* gene in germinating aleurone cells, compared with the expression of the *Pbf* and *Sad* genes, total RNA was prepared from isolated aleurone layers at different imbibition times and analysed using the relative reverse transcriptase (RT)-PCR technique. As shown in Fig. 4, the *Icy* transcripts were abundantly expressed in aleurone cells at 8 h of imbibition, but this expression decreased to the point of being almost undetectable at 24 h. Meanwhile, the *Pbf* and *Sad* increased, although it was shown by previous data that *Pbf* was induced by GA and repressed by ABA while *Sad* did not respond to these hormones in aleurone cells (Mena *et al.*, 2002; Isabel-LaMoneda *et al.*, 2003). The co-expression of the two DOF factors and the cystatin was consistent with the possibility of BPBF and SAD being regulators of this *Icy* gene.

**DNA–protein interactions**

To evaluate this possibility further, it was tested whether BPBF and SAD were capable of recognizing and interacting *in vitro* with the three putative DOF-binding motifs (5'-GAAAGG-3'; 5'-GCTTTT-3'; and 5'-AAAAGG-3') in the promoter of the *Icy* gene. The SAD and BPBF proteins were expressed as a GST-fusion in *E. coli* (Mena *et al.*, 2002; Isabel-LaMoneda *et al.*, 2003), and their binding ability was tested in electrophoretic mobility-shift assays (EMSA), using three different oligonucleotide probes. These probes, D1, D2, and D3 in Fig. 5, were deduced from the corresponding region of the *Icy* gene promoter (Fig. 1). As shown in lanes 2, 3, and 4 of Fig. 5, the wild-type probes were shifted when incubated with the GST-BPBF and GST-SAD-enriched extracts (+). No retardation bands (lanes 1, 5, and 6 of Fig. 5) were detected when the control empty GST vector extracts (-) or when the mutated probes (d1, d2, and d3) were used in the assay. These results indicate that BPBF and SAD proteins bind *in vitro* in a sequence-specific manner to the three DOF motifs in the *Icy* gene promoter.

**Expression of the Icy promoter in co-bombarded barley aleurones with BPBF and SAD**

The functional relevance of the *in vitro* DNA-protein interaction between SAD and BPBF and the DOF motifs was further tested *in planta* by transient expression assays in co-bombarded barley aleurone layers. Figure 6A shows...
schematically the deletion constructs of the *Icy* gene promoter fused to the GUS reporter gene. The longest promoter fragment, −631 bp from the ATG translation initiation codon, included three putative DOF-binding cores: D1 (5′-GAAAGG-3′), D2 (5′-GCTTTT-3′), and D3 (5′-AAAA-GG-3′) at positions −586, −517, and −475, respectively. The three promoter deletions (constructs p-582, p-512, and p-472) contained two, one, and no DOF motifs, respectively. The effector constructs expressed the whole cDNAs from the *Sad* and *Pbf* genes under the control of the CaMV 35S promoter followed by the first intron of the maize *AdhI* gene (Mena et al., 1998; Isabel-LaMoneda et al., 2003). Isolated barley aleurone layers were transiently transformed with the reporter constructs alone or in combination with the effectors at a 1:1 molar ratio. As shown in Fig. 6B, the co-transfection of the reporter constructs p-631, p-582, and p-512 with SAD as effector resulted in 2–4-fold enhancement of the GUS expression over that directed by the reporter alone, although the basal GUS activities were about 60% of that supported by the longest p-631 construct. However, the co-bombardment of these three reporter constructs with BPBF produced about a 50% reduction in GUS activity, with respect to the values obtained with the reporter alone. The co-expression of the reporter constructs containing DOF motifs with an equimolar mixture of both effectors, SAD and BPBF, drastically reduced the basal GUS levels, indicating that BPBF reverted the trans-activation mediated by SAD. When the reporter p-472 construct was used, no effect was observed, which is in agreement with the absence of DOF motifs in this promoter fragment.

**Discussion**

Although more than 70 plant cystatin cDNAs have already been reported, only a few genomic clones have been characterized. This is the case of those isolated from maize, rice, and potato (Kondo et al., 1991; Waldron et al., 1993; Abe et al., 1996). However, in none of them has the functional analysis of their promoters been determined.

The comparison of the sequence of the *Icy* genomic clone and its corresponding cDNA clone (Gaddour et al., 2001) showed that this barley gene contained one intron and two exons. The position of the intron was identical to that of the first intron in the rice and maize cystatin genes, although a second intron described in the 3′-non-coding region next to the stop codon could not be found in this study’s barley gene (Kondo et al., 1991; Abe et al., 1996; Arai et al., 2002).

The functional analysis of the *Icy* promoter by transient expression in bombarded barley tissues showed that this promoter is stronger in developing endosperm (3–4-fold) than in leaves or in post-imbibition aleurone layers, and that the −631 bp upstream of the ATG initiation codon contained all the information required for full activity, whereas the region further upstream (up to −1058) seems...
to be of small relevance for promoter function. Promoter activity gradually decreased in all barley tissues tested, with the extent of the 5' deletions down to the −161 promoter fragment that supported only around 5% of the maximum activity encountered. A computer analysis of the −631 bp sequence revealed the presence of several cis-elements that could be the interaction sites of TFs of different families: MYBR1, bZIP, MYBR2R3, DOF, etc. Although this paper is focused only on the role of certain DOF proteins (BPBF and SAD) in the regulation of the Icy gene in aleurone cells, the importance of other TFs in the regulation of this gene in other tissues is not excluded since the accumulation of a given transcript is the result of a complex combinatorial regulation process.

Upon germination, the cystatin mRNA levels in aleurone cells, that were quite abundant 8 h after imbibition, drastically decreased until they were almost undetectable at 24 h and, in isolated aleurone layers incubated with 1 μM GA3, the Icy transcripts also markedly decreased. The GUS expression driven by the Icy promoter in transient assays in barley aleurone layers also decreased on incubation in GA. Meanwhile most of the hydrolytic enzyme-encoding genes expressed in post-germinating aleurone are induced by GA and repressed by ABA (Gubler et al., 1999; Martínez et al., 2003a).

The pattern of mRNA accumulation detected by RT-PCR and in situ hybridization assays is in line with the observed transient expression results in bombarded barley tissues. Besides the Icy expression in seeds, the cystatin transcripts accumulate in barley florets and in pollen. In these organs the presence of other proteinase inhibitors: (i) serine-proteinases (Domoney et al., 2002), (ii) metallo-carboxypeptidases (Martínez et al., 1991), and (iii) aspartyl-proteinases (Ishikawa et al., 1994) have also been described. The physiological function of cystatins in pollen grains is still unclear, but it may play a role by regulating the enzymes responsible for the germination of the pollen tube (Raven et al., 1992).

The success of seed germination depends on the regulatory complex controlling expression at the transcriptional level of genes encoding hydrolytic enzymes, but it is still unknown how the regulation of genes encoding inhibitors of these enzymes affects the germination process. The regulation of the Icy gene, encoding a barley cysteine-protease inhibitor, by two DOF proteins, SAD and BPBF, is described here. The Icy gene expression in barley aleurone may be explained by the possibility that both factors compete for binding to the same cis-motives. BPBF behaves as a strong transcriptional repressor of this gene in transient expression assays in aleurone layers, while SAD functions as an activator. Moreover, BPBF counteracts strongly the trans-activation of the Icy gene promoter mediated by Sad when present in equimolar amounts.

The repressor activity of BPBF might also operate indirectly through the interaction with other TFs putatively associated with the Icy promoter, for example, bZIP and MYB R2R3 proteins. Previous results from this laboratory have demonstrated that SAD and BPBF do interact in the yeast two hybrid assays with GAMYB, a MYB R2R3 transcriptional activator of several hydrolase-encoding genes in barley aleurone cells following germination (Gubler et al., 1999; Isabel-LaMoneda et al., 2003) and of endosperm protein genes during seed development (Diaz et al., 2002). Experiments focused on the putative role of bZIP factors (Vicente-Carbajosa et al., 1998; Oñate et al., 1999) and their interaction with the DOF described here in the expression control of this gene, not only in post-germinating aleurone but also during the endosperm development, are underway, since the Icy transcripts are particularly abundant in the developing seeds.

These data suggest that the two DOF factors, SAD and BPBF, are an important part of the transcription combinatorial complex mediated by hormones, GA, and ABA, responsible of the fine-tuning expression of genes encoding inhibitors of hydrolases that trigger the mobilization of storage compounds for providing nutrients for seedling growth.

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