HvMCB1, a R1MYB transcription factor from barley with antagonistic regulatory functions during seed development and germination

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Summary

The functional analysis of hydrolase gene promoters induced by gibberellin (GA) in barley aleurone cells upon germination has identified a tripartite GA-response complex (GARC) containing a 5'-TATCCAC-3' box as well as the GA-responsive element (GARE) recognized by GAMYB and the pyrimidine box interacting with the DOF transcription factors BPBF and SAD. We show here that the MCB1 gene encoding a R1MYB protein binds to the 5'-TATCCAC-3' (GATA core) box in vitro and is a transcriptional repressor of a GA-induced amylase (Amy6.4) promoter in bombarded aleurone layers. Northern blot and mRNA in situ hybridization analyses showed that the MCB1 transcripts accumulate in the aleurone cells upon germination, as well as in endosperm tissues during seed development. The HvMCB1 protein expressed in bacteria binds in a specific manner to a 27-mer oligonucleotide containing the 5'-TATCCAC-3' sequence, derived from the promoter region of the Amy6.4 gene. Accumulation of the MCB1 transcript diminished in response to external GA incubation in aleurone cells, and in transient expression experiments HvMCB1 repressed transcription of the Amy6.4 promoter in GA-treated aleurone layers and reversed the GAMYB-mediated activation of this amylase promoter. In contrast, during endosperm maturation HvMCB1 acted as a transcription activator of the seed-specific Itr1 gene promoter through binding to a 5'-GATAAGATA-3' box.

Keywords: antagonistic regulatory roles, barley seeds, developing endosperm, germinating aleurone, HvMCB1, R1MYB SHAQKYF transcription factor.

Introduction

The seed is the plant organ that, by interrupting its life cycle, allows the embryo to survive in the dry state during unfavourable environmental conditions. During maturation of cereal seeds, the endosperm stores carbohydrates, proteins and lipids that will be subsequently used by the germinating seedlings as a source of energy, carbon and nitrogen. The endosperm is differentiated into two predominant tissue types, the starchy endosperm and the peripheral aleurone layer (Berger, 2003). The aleurone layer and the embryo remain viable through desiccation while the starchy endosperm cells die after grain filling is complete (Fath et al., 2000). Seed germination sensu stricto begins with water uptake (imbibition) and ends with the elongation of the embryonic axis inside the seed; the visible sign that germination is completed is the protrusion of the tip of the radicle through the seed coat (Bove et al., 2001). However, early post-germination events in the aleurone cells are crucial for the survival of the seedling until photosynthesis is fully established. Maturation and germination are two phases of development separated by a dormancy period. The release of dormancy, a complex process where environmental and genetic factors interact, remains poorly understood in molecular terms (Alonso-Blanco et al., 2003; Gubler et al., 2005).

The pattern of expression of cereal seed genes during endosperm development is highly regulated in a spatial and temporal manner and much information is available concerning cis motifs in their promoters and the transcription factors (TFs) that interact with them. A survey of protein storage genes (Hor2) and other seed-specific genes, putatively involved in defence, such as that encoding trypsin inhibitor CMe (gene Itr1), shows that several consensus
sequences are highly conserved in their promoter regions (Carbonero et al., 2000; Diaz et al., 1995; Forde et al., 1985; Kreis et al., 1985; Royo et al., 1996). The most prominent of these cis motifs is the bipartite endosperm box, containing two distinct protein-binding sites: the GCN4 like-motif (GLM: 5′-ATGAG/CTCAT-3′) recognized by bZIP proteins of the Opaque2 family (Albani et al., 1997; Ōnate et al., 1999; Vicente-Carbajosa et al., 1998; Wu et al., 1998) and the prolamin box (PB: 5′-TGTAAAG-3′) recognized by TFs of the DNA with one finger (DOF) class (Diaz et al., 2005; Lijavetzky et al., 2003; Mena et al., 1998; Vicente-Carbajosa et al., 1997; Yanagisawa, 2004). Another important motif, 5′-AACA/TA-3′, is recognized by GAMYB, a protein of the R2R3MYB family (Diaz et al., 2002; Suzuki et al., 1998), previously shown to be a regulator of hydrolase genes in post-germinating aleurones (Gubler et al., 1995, 1999).

Upon seed germination, cereal aleurone cells respond to the phytohormone gibberellic acid (GA) synthesized by the embryo, by triggering a signal transduction pathway leading to the transcription activation of a number of hydrolase genes, mainly those for α-amylases (Amy6.4 and others), cathepsin B-like and L-like thiol proteases, β-glucanases and carboxypeptidases (Cejudo et al., 1992; Cercos et al., 1999; Gubler et al., 1999, 2005; McCubbin et al., 2004; Peng and Harberd, 2002; Saleski and Fincher, 1992). Functional analysis of the promoters of these hydrolase genes has allowed the identification of a conserved tripartite cis motif, the GA-response complex (GARC) that contains: (i) the 5′-TAAC-AAA-3′ box or GA-responsive element (GARE); (ii) the pyrimidine box, 5′-CTTTT-3′ and (iii) the 5′-TATCCAC-3′ box, all of them necessary for a full GA response. However, there are differences in the sequences and positions of these cis elements in the studied hydrolase gene promoters that can explain the different temporal pattern of expression of these genes upon seed germination. Gubler et al. (1995) characterized from barley (Hordeum vulgare) a gene encoding the GAMYB protein that was induced by GA in aleurone cells and which through binding to the GARE trans-activated a number of hydrolase gene promoters (Gubler et al., 1995, 1999). Several TFs of the DOF class, able to bind to the pyrimidine box, have been reported in barley and rice. In barley, BPBF, a DOF protein previously shown to be involved in the activation of seed storage protein genes (Mena et al., 1998), has a role as a repressor of transcription of hydrolase genes in aleurone cells (Mena et al., 2002). A second DOF protein from barley, SAD, activates transcription from the pyrimidine box of these promoters (Isabel-LaMoneda et al., 2003). Washio (2001) identified five rice genes (OsDof1–5), encoding DOF proteins, from a rice aleurone cDNA library. Transient expression experiments in co-bombarded aleurone indicated that some of these DOF proteins were activators whilst others were repressors of the GA-inducible activity of a type III carboxypeptidase gene promoter. The 5′-TATCCAC-3′ element (TATC/GATA core) of the GARC complex is the recognition site of a particular class of MYB proteins (Rose et al., 1999) with a single DNA-binding domain (R1MYB-SHAQKYF). R1MYB proteins contain a unique MYB-binding domain, spanning 53 amino acid residues including three equidistant tryptophans (W) that may form a helix-turn-helix structure essential for DNA recognition (Jin and Martin, 1999). The substitution of the third tryptophan (W) by an alanine (A) residue generates the SHAQKYF motif within the MYB domain, characteristic of a new subfamily of R1MYB factors, which recognize the TATC/GATA core (Rose et al., 1999). Recently, several genes belonging to this SHAQKYF subfamily have been described in plants: (i) three genes (OsMYBS1–3) induced in suspension cultures of rice by sugar starvation and GA (Lu et al., 2002); (ii) two maize genes, ZmMRP-1, an activator of transfer cell-specific gene expression in developing seeds (Gomez et al., 2002) and ZmMYBst1 of unknown function (Mercy et al., 2003); (iii) two genes in barley, MCB1 and MCB2, involved in light- and circadian clock-regulated expression in leaves (Churin et al., 2003).

In this study we report that the barley gene MCB1 (Churin et al., 2003) is highly expressed in germinating aleurone cells as well as in seed endosperm during development. MCB1, which is located in plant cell nuclei, regulates gene expression through binding to the TATC/GATA core. Transient expression studies in homologous tissues show that HvMCB1 has antagonistic roles in the two seed phases, germination and development. It is a transcriptional repressor of the Amy6.4 gene, induced by GA in germinating aleurones, while it behaves as an activator of Itr1 during endosperm development. The implication of this TF, and probably of other SHAQKYF R1MYB proteins, in the combinatorial regulation of genes expressed specifically in the seed is discussed.

Results

Intron–exon structure of the barley MCB1 gene, encoding a SHAQKYF R1MYB transcription factor

The strong evidence supporting an important role for the 5′-TATCCAC-3′ motif within the tripartite GARC in the promoters of GA-induced hydrolase genes in germinating aleurone cells prompted us to search for genes encoding the SHAQKYF R1MYB proteins binding to this motif. As a first step in the isolation of such genes, a barley probe was generated by PCR. A sense primer, derived from the conserved SHAQKYF motif, and an antisense universal primer (3′-UAL), were used to amplify cDNA from aleurone cells imibed for 16 h. The sequence of the amplified fragment of 670 bp was identical to a fragment of a previously described R1MYB cDNA from barley leaves, MCB1, involved in the regulation of CAB1 gene expression (Churin et al., 2003). To obtain the whole open reading frame (ORF) and to establish
the intron–exon structure spanning the ORF of the MCB1 gene, a PCR amplification strategy, based on the sequence published by Churin et al. (2003), was followed. Aleurone cDNA and barley leaf total DNA were used as templates (Figure 1a,b) and sense and antisense primers, described in Figure 1(b) as #MCB1.1 and #MCB1.2, respectively, were used in the PCR reactions. Five exons and four introns, flanked by typical gt/ag boundaries, were found by comparing the sequence of the amplified genomic fragment with that of the cDNA. A typical nuclear localization signal (NLS) was found at the end of the first exon (Figure 1b).

Figure 1. Genomic structure and sequence of the barley MCB1 gene. (a) Intron–exon disposition spanning the open reading frame (ORF) of the MCB1 gene. The four introns are represented by single lines and the exons (I, II, III, IV, V) by boxes, indicating the corresponding number of nucleotides. (b) Nucleotide and deduced amino acid sequences of the MCB1 gene. The coding region is in upper case and the non-coding in lower case. Amino acid residues corresponding to the R1MYB domain are in bold, and within this domain the SHAQKYF sequence is framed and equidistant tryptophan and alanine residues are circled. The nuclear location signal is indicated by a pointed line. Nucleotide sequence numbers refer to the ATG translation initiation codon (in bold) and those concerning the deduced amino acid positions are in parentheses. The stop codon is indicated with an asterisk. Horizontal arrows indicate the oligonucleotides used as primers to amplify the whole ORF from cDNA (#MCB1.1 and #MCB1.2) and to obtain the specific probe used for Northern blot and in situ hybridization analyses (#MCB1.3 and #MCB1.2). The MCB1 genomic clone has the EMBL accession number AJ965495.
Comparisons of the SHAQKYF motifs among barley MCB1 and other such R1MYB proteins so far characterized in plants are shown in Figure 2(a). When a BLAST with the whole ORF deduced for MCB1 was made in the TIGR rice genome database the most similar sequence corresponded to that deduced from the accession number Os10g41260, that is partially coincident with that reported by Lu et al. (2002) for the rice OsMYBS2 gene (Figure 2b). Barley MCB1 and Os10g41260 are probably orthologous, since they share 90% identical residues over the R1MYB-binding motif (Figure 2a) and 70% similar, 62% identical, residues along the whole protein (Figure 2b). Lu et al. (2002) report as the starting ATG codon what in the Os10g41260 annotation would correspond to methionine 54 in the deduced sequence. The stop codon predicted by these authors is located 64 amino acids downstream of that annotated by us from the TIGR rice genome database that shares the equivalent position to that found by us in the orthologous

(a) Alignment of the deduced amino acid sequence of the MYB-binding domain of barley MCB1 with those of characterized plant SHAQKYF R1MYB proteins: OsMYBS1–3 from rice (Lu et al., 2002), AmDIV (Galego and Almeida, 2002) from Antirrhinum majus, HvMCB2 from barley (Churin et al., 2003), ZmMYBst1 (Mercy et al., 2003) and ZmMRPI (Gomez et al., 2002) from maize, StMyb1 from potato (Baranowskij et al., 1994), LeMybI from tomato (Rose et al., 1999) and AtEPR1 (Kuno et al., 2003), AtCCA1 (Wang et al., 1997) and AtLHY (Schaffer et al., 1998) from Arabidopsis. Percentages of sequence identity with HvMCB1 within the MYB domain are indicated on the right.

(b) Alignment of the deduced amino acid sequences of the whole ORF of HvMCB1, the rice gene of accession number Os10g41260 in the TIGR rice genome database (http://www.tigr.org/) and the rice OsMYBS2 described by Lu et al. (2002). Numbers on the right indicate the number of amino acid residues. The SHAQKYF motif is framed. The tryptophan and alanine are in bold. Strong and weak conservative changes are marked with two dots and one dot, respectively. Asterisks correspond to identical residues.

Figure 2. Comparison of the barley MCB1 with other plant SHAQKYF R1MYB proteins.

(a) Alignment of the deduced amino acid sequence of the MYB-binding domain of barley MCB1 with those of characterized plant SHAQKYF R1MYB proteins: OsMYBS1–3 from rice (Lu et al., 2002), AmDIV (Galego and Almeida, 2002) from Antirrhinum majus, HvMCB2 from barley (Churin et al., 2003), ZmMYBst1 (Mercy et al., 2003) and ZmMRPI (Gomez et al., 2002) from maize, StMyb1 from potato (Baranowskij et al., 1994), LeMybI from tomato (Rose et al., 1999) and AtEPR1 (Kuno et al., 2003), AtCCA1 (Wang et al., 1997) and AtLHY (Schaffer et al., 1998) from Arabidopsis. Percentages of sequence identity with HvMCB1 within the MYB domain are indicated on the right.

(b) Alignment of the deduced amino acid sequences of the whole ORF of HvMCB1, the rice gene of accession number Os10g41260 in the TIGR rice genome database (http://www.tigr.org/) and the rice OsMYBS2 described by Lu et al. (2002). Numbers on the right indicate the number of amino acid residues. The SHAQKYF motif is framed. The tryptophan and alanine are in bold. Strong and weak conservative changes are marked with two dots and one dot, respectively. Asterisks correspond to identical residues.

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barley MCB1 gene (Figure 2b). Since the amino acid residues of the N-terminal part of MCB1 and of rice Os10g41260 are particularly rich in acidic residues that are frequently associated with activation domains and contain a putative cAMP/cGMP-dependent phosphorylation site (KKSS/T), we decided to explore the functional consequence of deleting this part in MCB1 in transient expression assays.

The gene encoding HvMCB1 is expressed in barley seeds

Northern blot analyses were carried out to investigate the expression pattern of the MCB1 gene in barley seeds (Figure 3a,b,c). Total RNA was isolated from aleurone layers at different times after seed rehydration (Figure 3a), from developing endosperm at four stages after flowering and from immature embryos (Figure 3b). Northern blots were hybridized with a MCB1-specific probe, obtained from PCR amplification of the cDNA with primers #MCB1.3 and #MCB1.2, as indicated in Figure 1(b), that produces a single band in Southern blot analysis (data not shown). The barley MCB1 message was already expressed in germinating aleurone cells at 8 h after imbibition and slightly decreased at 48 h (Figure 3a), whereas the expression of the GA-inducible gene Amy6.4 encoding a α-amylase progressively increased with germination up to 48 h, as previously described (Gubler et al., 1995). During endosperm development, MCB1 mRNA was already present by 10 daf and its expression was maintained throughout endosperm development; it was also expressed in immature embryos 22 daf. The mRNA of an important defence gene in developing endosperm, the Itr1 gene, encoding the trypsin inhibitor CMe (Royo et al., 1996), was expressed at 10 daf and its expression increased until 22 daf, being hardly detectable in immature embryos (Figure 3b). The pattern of accumulation of MCB1 transcript was therefore consistent with the possibility of MCB1 being a regulator of the Amy6.4 gene in germinating aleurones and of the Itr1 gene during endosperm development.

The observed pattern of accumulation of the barley MCB1 transcripts upon seed germination and the well-known GA inducibility of the Amy6.4 gene (Gubler et al., 1995), suggested that MCB1 expression may be hormonally regulated. Isolated aleurone layers were used as a system to study the hormonal response of both genes to GA and ABA. These aleurone layers that do not synthesize GA or ABA, but are able to respond to external hormone treatments, were isolated from de-embryonated grains of Himalaya barley after 2 days of imbibition and incubated in the absence and presence of 1 μM GA3 or 10 μM ABA over different periods of time (8, 16, 24 and 48 h). As shown in Figure 3(c), when the aleurone layers were incubated in the buffer containing 1 μM GA3, expression of MCB1 decreased drastically after 24 h, while incubation with 10 μM ABA had practically no effect as compared with controls. As previously described, Amy6.4 mRNA levels increased in response to GA, and no effect of ABA was found (Gubler et al., 1995).

To determine the spatial expression of MCB1 within the seed, mRNA in situ hybridization studies were done. This analysis was performed in developing barley kernels at 20 daf and in germinating seeds 24 h after imbibition. In the developing endosperm, a clear signal with the antisense probe was detected not only in the starchy endosperm but also in the aleurone layer (Figure 3d: A), which is clearly differentiated at this stage of development. In sections of germinating seeds, the accumulation of transcript was observed in aleurone cells at 24 h after imbibition (Figure 3d: C). No signal above background was found when sections of developing and germinating barley seeds were hybridized with the sense probe used as a negative control (Figure 3d: B and D). These in situ hybridization data corroborate the results obtained with Northern blot analysis.

The barley HvMCB1 protein is targeted to the nucleus

To investigate the subcellular location of the HvMCB1 protein, the full-size ORF of the MCB1 gene was fused in frame to the GFP reporter gene and this construct was driven by the CaMV 35S promoter or under this promoter plus the first intron of the maize Adh1 gene. The fusion construct 35S::MCB1–GFP and the control construct 35S::GFP were introduced into onion epidermal layers by particle bombardment and the constructs 35S-Adh1::MCB1–GFP and the control construct 35S-Adh1::GFP were bombarded into barley aleurone layers. Microscopic observations indicated that the fluorescence of the GFP was targeted to the nuclei of cells bombarded with the constructs carrying the HvMCB1 protein (Figure 4b,f,g). The nuclear location of HvMCB1 was as expected for a transcription factor carrying an NLS (Figure 1b). In contrast, in the onion epidermal layers and in barley aleurone cells transiently transformed with the 35S::GFP or 35S-Adh1::GFP constructs, respectively, GFP fluorescence was scattered throughout the whole cells (Figure 4d,i,j). Bright field controls in onion epidermal cells (Figure 4a,c) and Nomarski images in barley aleurone layers (Figure 4e,h) are also shown, as well as the overlapping photographs of fluorescence and Nomarski images (Figure 4g,j).

HvMCB1 binds in vitro to the 5′-TATCCAC-3′ motif from the Amy6.4 gene promoter

To evaluate further the possibility that HvMCB1 is a transcription regulator of hydrolase genes in germinating aleurone cells of barley, we tested the capability of binding in vitro to the TATCCAC motif in the promoter of the Amy6.4 gene by electrophoretic mobility shift assays (EMSA). The HvMCB1 protein was expressed as a Glutathione S-transferase (GST) fusion in Escherichia coli purified from bacterial extracts and...
Figure 3. Expression analysis of MCB1 during seed germination and maturation.
(a) Northern blot analysis of MCB1 during seed germination. Eight micrograms of total RNA from aleurone layers (Al) isolated from Himalaya seeds 8, 16, 24 and 48 h after water imbibition (hai) was electrophoresed and blotted. The filter was first hybridized with the specific probe for MCB1 and subsequently with a probe derived from the cDNA of the Amy6.4 gene. The pattern of Amy6.4 mRNA accumulation was used as a control. Total RNA (RNA) stained with ethidium bromide is shown as a loading control.
(b) Northern blot analysis of MCB1 during seed development. Eight micrograms of total RNA from endosperm (End) at 10, 14, 18 and 22 days after flowering (daf) and from immature embryos (iEm) at 18 daf, was processed in a similar to (a). The filter was first hybridized with the specific probe for MCB1 and subsequently with a probe derived from the cDNA of the Itr1 gene. The pattern of Itr1 mRNA accumulation was used as a control. Total RNA (RNA) stained with ethidium bromide is shown as a loading control.
(c) Northern blot analysis of MCB1 during seed germination in response to hormones. Eight micrograms of total RNA from aleurone layers isolated from 2-day imbibed Himalaya de-embryonated seeds 8, 16, 24 and 48 h after incubation with 1 μM GA or 10 μM ABA. No hormones were added in the control. Probes were the same as described in (a). The pattern of Amy6.4 mRNA accumulation was used as a control.
(d) Spatial expression of the MCB1 mRNA in developing and germinating barley seeds determined by in situ hybridization. A and B, transverse sections of 20 daf developing seeds. C and D, transverse sections of germinating seeds after 24 h of water imbibition. Hybridization was done with an antisense MCB1 probe (A, C) or with the negative control sense probe (B, D): al, aleurone; e, endosperm; p, pericarp. Scale bars: [A, B] 100 μm; [C, D] 50 μm.
incubated with the appropriate radiolabelled DNA probes. The α-amylase probe (AmyM in Figure 5) was a 27-mer oligonucleotide deduced from the promoter sequence of the high-pI α-amylase gene Amy6.4 (Gubler et al., 1995), which contains the canonical element 5′-TATCCAC-3′, previously described as the motif recognized by SHAQKYF R1MYB factors (Rose et al., 1999). When this probe was incubated with the GST-HvMCB1 protein, a retarded band was observed that was competed out when a molar excess of the corresponding unlabelled oligonucleotide was added to this assay. As expected, this shifted complex was not produced when the control GST protein or a mutated variant of the probe (TAG-

Figure 4. HvMCB1 protein is targeted to the nuclei of onion epidermal cells and of barley aleurone cells. Epidermal onion layers (a,b) and barley aleurone cells (e–g) were transiently transformed with the 35S::MCB1–GFP construct. As controls, onion layers (c,d) and barley aleurones (h–j) were transformed with the 35S::GFP. After incubation for 24 h, onion cells were observed under a Zeiss Axiophot microscope under bright field (a,c) and under fluorescence (b,d) using BP450/90 FT 510 LP 520 filters. Aleurone layers were observed under a confocal Leica TCS-Sp2-AOBS-UV ultraspectral microscope under Nomarski (e,h) and under fluorescence (f,i) conditions. The overlap of the Nomarski and fluorescence photographs is shown (g,j). Arrows point to the location of the nucleus.
aleurone layers were incubated in a solution with or without reporter to effector molar ratio was 1:1. Upon bombardment, and negative effector controls, respectively (Figure 6a). The et al. (Gubler et al., 1995; Mena et al., 1998) were used as positive promoters of hydrolase genes expressed in the aleurone upon germination but also in the promoters of genes expressed during development of cereal endosperm, such as Hor2 and Itr1. This, together with the fact that the MCB1 transcripts were abundantly expressed during endosperm development, led us to find out whether R1MYB factors of the SHAQKYF subfamily present in germinating aleurone are also expressed during endosperm development of barley seeds and to explore their potential involvement in the regulation of the endosperm-specific gene Itr1 that was investigated both by EMSA and by transient co-bombardment of developing barley endosperm with appropriate constructs.

For EMSAs, the HvMCB1 protein was expressed in bacteria, as described above, and the DNA used as a probe (litrM in Figure 7a) was a 23-mer oligonucleotide containing the sequence 5’-GATAAGATA-3’/5’-TATCTTATC-3’ derived from the Itr1 gene promoter (Royo et al., 1996); three mutated versions of litrM (m1, m2 and m1/m2 in Figure 7a) were also used in these experiments. When the core GATA sequence, proximal to the 5’ end of the wild-type probe derived from the Itr1 promoter, was changed to GcTA (m1 probe), binding of the HvMCB1 protein was drastically reduced (Figure 7a). The same single base substitution introduced in the second GATA core (probe m2) or

As represented in Figure 6(b), co-expression of the reporter with the 35S-Adh-I::MCB1 effector construct lowered the GUS activity under the control of the Amy6.4 promoter to less than half of that found with the reporter alone, either in the absence or in the presence of GA, while the MCB1 truncated version of this effector did not significantly alter GUS activity. As previously described, the Amy6.4 gene promoter was activated by co-bombardment with GAMYB (Gubler et al., 1995, 1999) and was repressed by PBF (Mena et al., 2002). When binary combinations of effectors were studied, MCB1 reversed the GAMYB trans-activation, independently of the GA treatment, while the MCB1 truncated effector again did not produce any effect. The exogenous GA treatment resulted in an almost eight-fold enhancement of the GUS expression controlled by the Amy6.4 promoter over that found with GA-untreated controls, and this was decreased by half when co-bombardment was done with MCB1. These data indicate that MCB1 functions as a negative regulator of the GA-dependent or GAMYB-inducible GUS activity driven by the Amy6.4 promoter and that the 41 amino acid residues at its N-terminal sequence are essential for this repression since its truncated version lacking them, MCB1tr, has no effect.

**The Itr1 promoter is activated by HvMCB1 in developing barley endosperm**

In a sequence exploration through the data banks, we found the presence of the TATC/GATA core not only in the promoters of hydrolase genes expressed in the aleurone upon germination but also in the promoters of genes expressed during development of cereal endosperm, such as Hor2 and Itr1. This, together with the fact that the MCB1 transcripts were abundantly expressed during endosperm development, led us to find out whether R1MYB factors of the SHAQKYF subfamily present in germinating aleurone are also expressed during endosperm development of barley seeds and to explore their potential involvement in the regulation of the endosperm-specific gene Itr1 that was investigated both by EMSA and by transient co-bombardment of developing barley endosperm with appropriate constructs.

CCA; Amy probe in Figure 5) were used in the assays. Neither binding to the HvMCB1 protein nor binding competition with the mutated probe were observed (Figure 5).

**HvMCB1 negatively regulates the expression of the Amy6.4 promoter in co-bombarded aleurones**

Because promoters containing the TATCCAC motif are likely to be the targets for R1MYB proteins, we addressed the question of whether MCB1 modulates the transcriptional activity of the Amy6.4 promoters by transient expression experiments, using particle bombardment into aleurone layers prepared from cv. Himalaya barley.

As the reporter construct, a 174-bp promoter fragment of the Amy6.4 gene (Gubler et al., 1995), which includes the tripartite GARC motif, was fused to the GUS reporter gene (Figure 6a). Two constructs were used as effectors: (i) the whole ORF of the MCB1 gene under the control of the CaMV 35S promoter followed by the first intron of the maize Adh1 gene and the 3’ nos terminator and (ii) a truncated version of the MCB1 lacking the first 41 amino acid residues (MCB1tr).

Similar constructs with barley GAMYB and PBF cDNAs (Gubler et al., 1995; Mena et al., 1998) were used as positive and negative effector controls, respectively (Figure 6a). The reporter to effector molar ratio was 1:1. Upon bombardment, aleurone layers were incubated in a solution with or without 1 µM GA3.

**Figure 5.** Electrophoretic mobility shift assays (EMSA) of the recombinant MCB1 protein with the 27 bp 32P-labelled (AmyM probe) derived from the Amy6.4 gene promoter, and with its mutated version affected in the MYBR1 binding motif (Amym probe, TATCCAC). Competition experiments were performed using increasing molar amounts (20×, 100×, 200×) of the indicated unlabelled probe. The sequences of the oligonucleotides used as probes are shown at the bottom of the panel. The putative binding site is underlined.
simultaneously in both cores (m1/m2 probe) completely abolished the formation of the retarded band. Besides, the mutated probes (m1, m2, m1/m2) were unable to compete the binding of protein MCB1 to the wild-type ItrM probe (Figure 7a and data not shown).

The functional relevance of the interaction observed in vitro between MCB1 and the 5'-GATA-3' motif in the Itr1 gene promoter was further corroborated in planta by transient expression assays in co-bombarded barley endosperm. Figure 7b shows schematically the reporter and effector constructs used in the transient assays. As reporters, serial deletions of the Itr1 gene promoter fused to the GUS reporter gene described in a previous study (Royo et al., 1996) were chosen. These consisted of four promoter fragments spanning to positions −343, −211, −179 and −113 bp, respectively, from the translation initiation codon. Sequence analysis of the −343 bp promoter region in the pltr1.343 showed that it contained a putative binding motif for R1MYB proteins (5'-GATAAGATA-3') at position −306 as well as a canonical pyrimidine box (5'-AAAAGG-3') recognized by DOF factors at position −176 and the GAMYB-binding site (5'-CAACTAAC-3') at position −120 (Diaz et al., 2002). The deletion pltr1.211 still contained the DOF- and GAMYB-binding motifs and the pltr1.179 included just the latter motif. The pltr1.113 that contains the TATA box has none of the three binding sites (Figure 7b).

Developing endosperm, at approximately 18 daf, was transiently transformed by particle bombardment with the Itr1::GUS reporter alone or in combination with the MCB1 effector at a 1:1 molar ratio. As effector controls we used also GAMYB and BPBF that both behaved as transcriptional activators (Diaz et al., 2002; Mena et al., 1998).

As shown in Figure 7(c), the co-transfection of the pltr1.343 with the HvMCB1 effector construct resulted in about a two-fold increase in GUS activity over that directed by the reporter alone, while co-transfections with GAMYB and/or with BPBF increased this activity three-fold (Diaz et al., 2002). The MCB1 truncated version did not alter the GUS activity controlled by the Itr1 promoter (data not shown).

When the three derived reporter deletions Itr1.211, Itr1.179 and Itr1.113 were used, no trans-activation was exerted by HvMCB1, which is in agreement with the absence of the MYBR1-binding motif in these constructs. Meanwhile, 300% increases over background levels in GUS activation mediated by GAMYB and BPBF were obtained when the pltr1.211 reporter was used. As expected, only GAMYB was able to trans-activate the reporter expression of pltr1.179 plasmid (Diaz et al., 2002). Finally, the complete deletion of
the three binding motifs in the minimal Itr1 promoter, as occurs in the pltr1.113 construct, resulted in a lack of trans-
activation by any of the three tested TFs in bombarded
developing barley endosperm.

Discussion

The two phases of the life cycle of cereal seeds, development
and germination, are associated with the expression of dif-
ferent sets of genes and the ratio of GA to ABA plays an
essential role in the regulatory network in both phases.

The success of germination depends on the responses of
aleurone cells to GA, which activates the expression of
genes encoding hydrolases, an effect that is counteracted by
ABA (Lovegrove and Hooley, 2000). Analysis of GA-respon-
sive gene promoters has identified a conserved cis-acting
tripartite GARC (Jacobsen et al., 1995). So far, two proteins
interacting with the GARE motif within the tripartite complex
have been identified in barley. One of them is the R2R3MYB
factor GAMYB that is induced by GA and is a strong
transcription activator (Gubler et al., 1995, 1999), the other
is HRT, a TF with three unusual fingers that is a repressor of
transcription (Raventos et al., 1998). The pyrimidine box of
the GARC complex is recognized by the DOF proteins SAD
and BPBF, with opposite effects upon hydrolase gene
expression: transcription activation (SAD; Isabel-LaMoneda
et al., 2003) and transcription repression (BPBF; Mena et al.,
2002). Three rice proteins (OsMYBS1, -S2, -S3) of the R1MYB
class able to recognize the third element, TATCCAC, of the
GARC complex have only recently been characterized, all of
them acting as positive regulators of the hydrolase genes
expressed upon seed germination (Lu et al., 2002).

Here we show that the barley HvMCB1 (MCB1 gene), a
transcription factor of the R1MYB class previously shown to
regulate transcription of the CAB gene in barley leaves
(Churin et al., 2003), also functions as a transcriptional
repressor of the Amy6.4 gene encoding an α-amylase gene
upon seed germination, through interaction with the third

![Figure 7. Electrophoretic mobility shift assays (EMSA) of the recombinant
MCB1 protein fusions to GST, with oligonucleotides derived from the Itr1
gene promoter and transient expression assays in particle-bombarded
developing barley endosperm.](image-url)

(a) EMSA with the 23 bp 32P-labelled (ItrM) probe derived from the Itr1
gene promoter and its mutated versions affected in the MYB1 binding motifs (m1,
m2 and m1/m2 probes). Competition experiments were performed using
increasing molar amounts (20x, 100x, 200x) of the indicated unlabelled probe.
Sequences of the oligonucleotides used as probes are shown at the bottom of
the panel. The putative binding sites are underlined.

(b) Schematic representation of reporter and effector constructs used in
transient expression assays. The reporter constructs were pltr1.343, pltr1.211,
pltr1.179 and pltr1.113, as described by Royo et al. (1996). Boxes with the
putative binding sites for TFs of the MYB and DOF classes, as well as the TATA
box, are indicated. The effector constructs are those described in the legend to
Figure 6b.

(c) Transient expression assays by co-bombardment of developing barley
endosperm (18 daf) with the indicated combination of reporter and effector
plasmids at a 1:1 molar ratio. β-Glucuronidase (GUS) activity was expressed
as in Figure 6. Transient GUS expression driven by the longest Itr1 promoter
fragment assayed (pltr1.343) and by several deletions derived from it
(pltr1.211, pltr1.179, pltr1.113) are shown in the inset. In each experiment,
sets of five endosperms were bombarded and four replicates of each
experiment were done. Standard errors are indicated.
motif of the GARC (5'-TATCCAC-3'). In contrast, HvMCB1 trans-activates transcription of an endosperm gene, ltr1, expressed during development of the barley endosperm, by interacting with the 5'-GATAAGGATA-3' motif in its promoter (5'-TATCTTATC-3' in the antisense strand).

As shown by Northern blot and in situ hybridization analyses, MCB1 is expressed in the aleurone cells following germination in such a way that its mRNA is downregulated by GA. This pattern of expression, together with the GA upregulated Amy6.4 gene expression and with in vitro binding assays of MCB1 to the TATCCAC motif in the context of the z-amylase promoter, is compatible with HvMCB1 being a transcriptional repressor of this Amy6.4 gene in aleurone cells upon germination. This is corroborated by transient expression assays where MCB1 behaves as a transcriptional repressor of the GAMYB-trans-activated or GA-inducible GUS reporter activity controlled by the z-amylase Amy6.4 gene promoter, in a similar way to which the DOF transcription factor BPBF acts in repression of the cathepsin B-like A121 gene (Mena et al., 2002).

The activity of HvMCB1 as a transcriptional repressor of the Amy6.4 gene promoter is dependent on the integrity of the N-terminal portion of MCB1, since its truncated version lacking the first 41 amino acid residues, although retaining its binding affinity towards the TATCCCA motif (data not shown), was inactive in transient expression assays (Figure 6). The difference in activity between HvMCB1 and HvMCB1tr, observed by us, is probably responsible for the different function (activator of GA-inducible genes) assigned by Lu et al. (2002) to OsMYBS2. This protein is a truncated version of the rice gene product corresponding to the accession number Os10g41260, that is the orthologous to barley MCB1, and lacks the N-terminal part containing acidic residues and the KKSS phosphorylation site.

Since the 5'-TATCCAC-3' motif (GATA core) of the tripartite GARC is a positive promoter element of the Amy6.4 gene and its deletion or mutation leads to a lower promoter activity (Jacobsen et al., 1995), the regulation of this gene by MCB1 may be modelled under a possible scenario where this protein can compete with the transcription activator of the same SHAQKYF class for binding to the same TATCCAC motif. According to this model, the relative concentrations of MCB1 and of this putative activator(s) and their relative binding affinities would direct the overall effect upon expression of Amy6.4. Efforts in our group are now focused in the isolation and characterization of the hypothetical SHAQKYF R1MYB transcription activator(s).

In the course of this study we have also determined the presence of similar R1MYB SHAQKYF binding sites (GATA core): in the promoters of barley genes specifically expressed in developing endosperm such as the ltr1 gene, encoding a trypsin inhibitor, used in this work, and the Hor2 gene encoding a B-hordein (data not shown). The pattern of expression of MCB1 in the developing endosperm was consistent with its putative role as a transcription regulator. Antagonistic activator and repressor functions for the same TF, during maturation and germination of cereal seeds, such is the case for the barley HvMCB1 described here, have been documented in a number of cases. Two relevant examples are the antagonistic roles displayed by VP1 (viviparous), a transcription factor of the B3 class, characterized for the first time in maize (Hoecker et al., 1995, 1999; McCarty et al., 1991) and by the DOF protein BPBF (Mena et al., 1998, 2002). Both transcription factors act as activators of maturation-specific genes and as repressors of z-amylase genes normally expressed after germination. However, other TFs such as GAMYB and the DOF protein SAD are transcriptional activators in both phases of seed development, maturation and germination (Diaz et al., 2002; Diaz et al., 2005; Gubler et al., 1995, 1999; Isabel-LaMoneda et al., 2003).

The hormone GA is crucial for seed germination; it promotes the degradation of the DELLA protein SLN1 of barley that is a repressor of GAMYB, thus activating this R2R3MYB that is a strong inducer of hydrolase genes in germinating aleurone cells (Fleet and Sun, 2005). How DELLA proteins affect HvMCB1 and other R1MYB proteins should be explored in the future. In all possible scenarios, the combined interactions among all the TFs (and co-factors) expressed at a given time of seed development would result in diverse programmes of gene regulation.

**Experimental procedures**

**Plant material**

Seeds of barley (cv. Bomi) were germinated at 22°C in the dark, and were used to collect samples at different times of imbibition. Developing endosperm (10–22 daf) and immature embryos (18 daf) were obtained from plants grown in a greenhouse at 18°C under an 18/6 h day/night photoperiod and frozen in liquid nitrogen and stored at −70°C until used for nucleic acid extraction. Seeds of barley cv. Himalaya were used for the preparation of aleurone layers for GA/ABA induction experiments. Developing endosperm (18 daf) and germinating seeds from barley cv. Bomi were used for mRNA in situ hybridization. Transient expression experiments were done using fresh developing endosperm (18 daf) from cv. Bomi and isolated aleurone layers from cv. Himalaya. Onions (Allium cepa) were purchased locally and stored in the dark until used for subcellular locations.

**Isolation of MCB1 from barley**

A primer derived from the conserved region SHAQKYF of this class of plant R1MYBs was designed (5'-GCCAGCCACGCCGAGATAA-3'). Using this primer as sense and a primer derived from the poly(A) region as antisense, a 670 bp fragment was amplified from germinating barley aleurone cDNA obtained after imbibing for 16 h. Nucleotide sequences were determined on both strands using the automated sequencer (ABI PRISM TM 3100, Applied Biosystems, Bedford, MA, USA). The SHAQKYF R1MYB deduced sequence was identified using the NCBI site (http://www.ncbi.nlm.nih.gov), and
RNA extracted. Isolated aleurone layers from barley Himalaya dehydration imbibition from 8 to 48 h, aleurone layers were separated and corresponding to the 423 bp specific probe of the antisense or sense digoxigenin (DIG)-labelled RNA probes, sterilized as described by Isabel-LaMoneda et al. (2002). For the isolation of RNA from aleurones, Himalaya seeds were sterilized as described by Royo et al. (2002). Total RNA was purified following Chang (2003), before RNA extraction. For aleurone samples, total RNA was purified following Chang et al. (1993). Denatured RNA was electrophoresed in 7% formaldehyde, 1.2% agarose gels and blotted onto Hybond N-membranes. Hybridizations and washings were performed under stringent conditions according to standard procedures (Sambrook and Russell, 2001). Blots were subsequently hybridized with a 423 bp fragment of the barley MCB1 cDNA clone, spanning nucleotides 389-812, that are amplified using the oligonucleotides, indicated below, to generate 5’-protuberating regions. All probes were end-labelled with 32P-dATP by fill-in reaction with the Klenow exo-free DNA polymerase (United States Biochemicals, Cleveland, OH, USA) and purified from an 8% polyacylamide gel (28:1 cross-linking).

mRNA in situ hybridization

Developing (20 da) and germinating (8–48 h) barley seeds of cv. Bomi were fixed, treated and sectioned as described by Diaz et al. (2002). Hybridization was done at 52°C in a solution (100 µg ml−1 tRNA; 6% SSC; 3% formamide) containing approximately 100 ng µl-1 antisense or sense digoxigenin (DIG)-labelled RNA probes, corresponding to the 423 bp specific probe of the MCB1 gene that was used for Northern blot assays. Washes were done in 2x SSC and 50% formamide for 90 min at 52°C. Antibody incubation and colour detection were carried out according to the manufacturer’s instructions (Boehringer Roche Diagnostics, Mannheim, Germany).

Constructions and particle bombardment for subcellular location of MCB1

To create the 35S::MCB1–GFP and the 35S-AdhI::MCB1–GFP constructs, for particle bombardment of onion and barley cells, respectively, an in-frame translational fusion of the MCB1 ORF with the GFP reporter gene was done in the plasmid psmRS–GFP, containing the CaMV 35S promoter alone or followed by the first intron of the AdhI gene from maize. A soluble modified red-shifted GFP (Davis and Vierstra, 1998) was used. As controls, we used plasmids without MCB1; these were constructs 35S::GFP and 35S-AdhI::GFP.

Inner epidermal layers of onion bulbs (A. cepa), locally purchased, were peeled and placed onto MS/2 agar medium as described by Borrell et al. (2002). Aleurone layers were prepared as described by Isabel-LaMoneda et al. (2003). Particle bombardment was performed with a biolistic helium gun device (PSP-1000; DuPont, Hercules CA, USA) basically as described by Diaz et al. (2002) with the following modifications. For bombardment of onion cells, each shot delivered 140 ng of DNA using a rupture disc of 7500 kPa and a distance between the macrocarrier and the sample of 9 cm. After 24 h of bombardment, the green fluorescence was observed under a BP450/90 FT 510 LP 520 Zeiss filter and photographed in a Zeiss Axiopt (Zeiss, Oberkochen, Germany) microscope for onion cells. Aleurone layers were observed and photographed under a confocal ultraspectral Leica microscope TCS-Sp2-AOBS-UV (Easley, SC, USA).

Electrophoretic mobility shift assays

The HvMCB1 protein was expressed in Echerichia coli (BL21DE3 pLYs strain) by cloning the cDNA into the pGEX-2T vector (Amer sham Pharmacia Biotech, Freiburg, Germany) as a translational fusion to GST. Expression of the recombinant protein was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 1 h and bacterial crude proteins were extracted. Cells carrying the pGEX-2T vector with no insert were identically processed as negative controls. The probes containing the putative consensus MYBR1 sites (M) from the Itr1 and Amy6.4 gene promoters and their mutated versions (m) were produced by annealing complementary single-strand oligonucleotides, indicated below, to generate 5’-protuberating reactions. All probes were end-labelled with 32P-dATP by fill-in reaction with the Klenow exo-free DNA polymerase (United States Biochemicals, Cleveland, OH, USA) and purified from an 8% polyacylamide gel (28:1 cross-linking).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>#Itr-M</td>
<td>5’-TAGATGATAAGATAACCTAGC-3’</td>
</tr>
<tr>
<td>#Itr-m1</td>
<td>5’-TAGATGATGCTAGATAACCTAGCTGC-3’</td>
</tr>
<tr>
<td>#Itr-m2</td>
<td>5’-TAGATGATGCTAGATAACCTAGC-3’</td>
</tr>
<tr>
<td>#Amy-M</td>
<td>5’-TAACA-AACTCCGGCGCATATCACC-3’</td>
</tr>
<tr>
<td>#Amy-m</td>
<td>5’-TAACAAACTCCGGCGCATATCACC-3’</td>
</tr>
</tbody>
</table>

DNA constructs and particle bombardment of barley tissues

To transiently transform barley aleurone layers we used as a reporter construct the pAmy6.4 reporter plasmid, containing the −174 bp from the ATG translation start site of the high-π α-amylase Amy6.4 gene promoter (Gubler et al., 1995). This promoter fragment includes three binding motifs putatively recognized by Dof, R1MYB SHAQKYF and R2R3MYB factors, respectively. For bombardment of developing endosperm we used the ptrl1.343 plasmid containing the −343 bp portion upstream of the ATG initiation codon of the barley endosperm-specific Itr1 gene promoter and three deletions derived from it (ptrl1.211, ptrl1.179 and ptrl1.113), spanning to positions −211, −179 and −113 of the ATG, respectively.
HvMCB1 regulates seed development and germination

(Cercos et al., 1996). In all constructs, the promoter was fused to the β-glucuronidase reporter gene (uidA) followed by the 3’ nos terminator. As effectors, the pHvMCB1 plasmid included the complete MCB1 cDNA cloned in the Bluescript vector under the control of the CaMV 35S promoter, followed by the first intron of the maize Adhl gene and the 3’ nos terminator. The GAMYB and BPBF plasmids were described by Gubler et al. (1995) and Mena et al. (1998). Isolated aleurone layers from Himalaya barley and developing endosperm (18 df) from Bomi barley seeds were prepared as described by Isabel-LaMoneda et al. (2003) and by Diaz et al. (2002), respectively. Particle coating and bombardment were carried out with a biolistic helium gun device (DuPont PDS-1000, Bio-Rad, Hercules, CA, USA), as described (Diaz et al., 2002; Isabel-LaMoneda et al., 2003). GUS expression was determined histochemically (Jefferson, 1987) 24 h after bombardment, and calculated as the mean value of blue spots per tissue sample in each assay and expressed as percentage, taking as 100% the value obtained with each reporter construct without effectors. The histochemical data were directly correlated with the GUS expression quantified by chemiluminescence (GUS light kit, Tropix, Bedford, MA, USA) per milligram protein with a correlation coefficient of 0.94 and 0.97 per aleurone and per endosperm assay, respectively (data not shown).

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References


