Arabidopsis thaliana bZIP44: a transcription factor affecting seed germination and expression of the mannanase-encoding gene AtMAN7

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SUMMARY

Endo-β-mannanases (MAN; EC 3.2.1.78) catalyze the cleavage of β1→4 bonds in mannan polymers and have been associated with the process of weakening the tissues surrounding the embryo during seed germination. In germinating Arabidopsis thaliana seeds, the most highly expressed MAN gene is AtMAN7 and its transcripts are restricted to the micropylar endosperm and to the radicle tip just before radicle emergence. Mutants with a T-DNA insertion in AtMAN7 have a slower germination than the wild type. To gain insight into the transcriptional regulation of the AtMAN7 gene, a bioinformatic search for conserved non-coding cis-elements (phylogenetic shadowing) within the Brassicaceae MAN7 gene promoters has been done, and these conserved motifs have been used as bait to look for their interacting transcription factors (TFs), using as a prey an arrayed yeast library from A. thaliana. The basic-leucine zipper TF AtbZIP44, but not the closely related AtbZIP11, has thus been identified and its transcriptional activation upon AtMAN7 has been validated at the molecular level. In the knock-out lines of AtbZIP44, not only is the expression of the AtMAN7 gene drastically reduced, but these mutants have a significantly slower germination than the wild type, being affected in the two phases of the germination process, both in the rupture of the seed coat and in the breakage of the micropylar endosperm cell walls. In the over-expression lines the opposite phenotype is observed.

Keywords: transcription regulation, AtbZIP44, AtbZIP11, AtMAN7, seeds, germination, endosperm rupture, seed coat rupture, Arabidopsis thaliana.

INTRODUCTION

In mature angiosperm seeds, the diploid embryo and the triploid endosperm are surrounded and protected by the maternal tissue of the seed coat (testa). In Arabidopsis thaliana and other Brassicaceae species, seeds contain an endosperm of one to three cell layers and germination proceeds in two differentially regulated steps, where testa rupture is followed by endosperm breakage that allows the elongating radicle to protrude (germination sensu stricto). Thus, endosperm rupture (ER) and seed coat rupture (SCR) are two distinct limiting factors for germination to occur (Müller et al., 2006; Bentolila and Koornneef, 2006; Holdsworth et al., 2008; Piskurowicz et al., 2009; Iglesias-Fernández and Matilla, 2010). While SCR is not affected by abscisic acid (ABA), this hormone specifically inhibits ER; gibberellic acid (GA), which are ABA antagonists, affect both SCR and ER. Although the upstream GA-signalling components have been identified recently, the cis-elements and trans-acting factors that regulate downstream GA-responsive genes need further investigation (Sun and Gabler, 2004). Other hormones such as ethylene and brassinosteroids also influence germination, but the GA/ABA ratio is the most important factor for the integration of the environmental signals for the occurrence of germination (Kucera et al., 2005; Matilla and Matilla-Vázquez, 2006; Linkies et al., 2009). While the driving force for radicle elongation has been described as the main factor influencing SCR, cell wall (CW)-modifying enzymes, specifically those localized at the micropylar endosperm (ME), are considered main players in ER. These enzymes include cellulases, endo-β(1→4)-mannanases (MANs), chitinases, peroxidases, expansins,
xylglucan endotransglycosylase/hydrolases (XTH), etc. (Potruzzoli et al., 2003; Nonogaki et al., 2007; Iglesias-Fernández et al., 2011a; Martínez-Andújar et al., 2012).

The enzymatic activity of MAN and expression of several members of the MAN gene family have been characterized in many plant species because of their important role in the germination of mannan-rich seeds (Rodriguez-Gacio et al., 2012). In Arabidopsis thaliana, four MAN genes (AtMAN2, AtMAN5, AtMAN6 and AtMAN7) are expressed in germinating seeds, the AtMAN7 transcripts being the most abundantly accumulated through this process. The AtMAN7 mRNAs are restricted to the ME and to the radicle tip, disappearing as soon as the radicle emerges. Moreover, knock-out (KO) mutants in AtMAN7 have a significantly slower germination than wild-type seeds, suggesting a putative role for this gene in this biological process (Iglesias-Fernández et al., 2011a). Since AtMAN genes have predicted signal peptides, and therefore their corresponding proteins could be secreted, it is reasonable to suggest an interaction between the radicle tip of the embryo and the ME MAN enzymes in the dismantling of the mannan-rich seed CWs in order to facilitate radicle protrusion (Iglesias-Fernández et al., 2011b).

Transcriptional regulation of gene expression is a key regulatory process in any living organism that is driven by short DNA sequences (cis-elements) situated in gene promoters and transcription factors (TFs), proteins that recognize and bind to these cis-elements. Identification of the cis-regulatory code and the TFs that interact with them (trans-regulators) is the main challenge in unveiling any combinatorial transcriptional regulatory network (Singh, 1999). Non-coding sequences in gene promoters diverge rapidly during evolution, except for those that are functionally important. In silico comparison of the promoter sequences of orthologous genes from several related species can be used to find conserved non-coding elements. This 'phylogenetic shadowing' process has been described in Saccharomyces (Clifton et al., 2001), in primates (Boffelli et al., 2003) and in Arabidopsis and related Brassicaceae (Hong et al., 2003). Coupling these in silico analyses with screenings of an arrayed library of A. thaliana TFs in yeast (one-hybrid assays: Y1H) has unveiled novel cis–trans interactions (Castrillo et al., 2011, and references therein).

To gain insight into the transcriptional regulation of the AtMAN7 gene during seed germination, a bioinformatic search for its orthologous genes and corresponding promoters within the Brassicaceae family has been done. In A. thaliana, Arabidopsis lyrata, Capsella rubella, Brassica rapa, Thellungiella halophila and Arabis alpina, a highly conserved non-coding cis-element within the promoter regions of the MAN7 genes was identified by phylogenetic shadowing and shown to be functionally relevant in planta. This cis-element has been used as a bait to look for its interacting TFs (Castrillo et al., 2011). The basic-loucine zipper AtbZIP44, but not its closely related AtbZIP11, has been thus identified and its regulatory function upon AtMAN7 validated by RT-qPCR analyses, mRNA fluorescence in situ hybridization (FISH) experiments and by the germination kinetics of both over-expression (ox) lines and T-DNA insertion mutants in the AtbZIP44 gene. The different ox-hbZIP44 lines show faster germination than the wild type; moreover, KO lines in the AtbZIP44 gene analyzed not only present drastically reduced AtMAN7 transcript content but also display a slower germination than the wild type in the two parameters analyzed: SCR and ER.

RESULTS

The AtMAN7 gene promoter contains evolutionarily conserved cis-elements and responds to GA but not to ABA

The A. thaliana MAN gene family (AtMAN), that is represented by seven members (Iglesias-Fernández et al., 2011a), has been used to search for orthologous genes in related Brassicaceae species (Table S1 in Supporting Information). The corresponding deduced protein sequences have been used to construct an unrooted phylogenetic tree using the neighbor-joining algorithm (Figure S1). The 37 sequences compared are grouped into four major clusters of orthologous genes (MCOGs; A, B, C and D), supported by bootstrap values higher than 70% and by the occurrence of common protein motives shared by members of the same MCOG (Table S2). The MEME analysis indicates that all the MAN proteins annotated share motifs 1, 5, 6, 7 and 8 where amino acids described as critical for enzyme activity are present (Yuan et al., 2007) and that motif 11 spans a putative signal peptide (Figure S1, Tables S1 and S2). The physiological significance of this signal peptide has been validated by the subcellular localization of AtMAN7. For this purpose the AtMAN7 open reading frame (ORF) has been translationally fused to that of green fluorescence protein (GFP) under the control of the constitutive 35S promoter (P35S:AtMAN7-GFP) and this construct used in transient expression experiments in bombarded onion epidermal cells. As shown in Figure S2, the green fluorescent signal appears in the peripheral regions of the cell (cell wall and plasma membrane; arrows), as expected from its signal peptide.

The promoter region of the AtMAN7 gene (At5g68460) has been searched for conserved cis-elements, putatively involved in its transcriptional control, through the promoters of its gene orthologs (Figure 1, Table S3). The length of the DNA sequence compared was 844 bp, considering the intergenic distance between the ATG translation initiation codon of the AtMAN7 (At5g68460) and its preceding gene At5g66470 in the A. thaliana genome (Figure 1a). The pair-wise alignment among the six orthologous gene
promoters compared reveals two highly conserved cis-elements that have been named block A (65 bp) and block B (213 bp; Figure 1b). Block A, spans from -623 to -568 bp upstream of the ATG and block B is situated between -501 and -288 bp and their nucleotide comparison is shown in Figure 1(c).

In order to explore the functional relevance of these conserved motifs, a set of deletion constructs of the AtMAN7 promoter (PMAN7) have been generated, fused to the β-glucuronidase (GUS) reporter gene (uidA). These constructs are PMAN7-1::uidA (844 bp) containing the whole promoter; PMAN7-2::uidA (509 bp) that lacks the conserved motif A and PMAN7-3::uidA (289 bp) that is deprived of blocks A and B (Figure 2a). Homozygous transgenic A. thaliana plants have been generated with these constructs and histochemical GUS expression evaluated (Figure 2b). The transgenic lines with the PMAN7-1::uidA construct shows that uidA is present in leaves (hydathodes) and flowers (anther filaments, stigma, vascular elements of sepals, etc.), it is strongly expressed in the vascular elements of the roots, both in seedlings and in adult plants, and at the base and apical parts of siliques and at the replum (Figures 2b and S3). During germination, GUS activity is restricted to the ME at 24 h of germination. This uidA expression is conserved in transgenic lines where block A has been eliminated (PMAN7-2::uidA; 509 bp) and completely disappears in both germinating seeds and in adult plants where the conserved blocks A and B have been
deleted (PMAN7-3::uidA; 280 bp; Figures 2c and S3). In Figure 2d, the uidA expression in 24-h germinating seeds from the homozygous lines for each one of these three constructs has been quantified. These results have been further corroborated by transient expression assays by agro-infiltration in Nicotiana benthamiana leaves using the same three constructs of the AtMAN7 promoter (Figure 2e). Taken together, these results indicate that block B contains important regulatory cis-elements for the transcriptional regulation of the AtMAN7 gene.

The same stable A. thaliana transgenic lines described in Figure 2a have been used to quantify GUS upon seed germination under different hormonal treatments (50 μM GA$_{4+7}$, 1 μM ABA and water as a control; Figure 3a–i). In
PMAN7-1::uidA germinating seeds, the addition of GA4,7 to the imbibition medium produces a significant enhancement of GUS activity, compared to the controls at all time points analyzed (Figure 3a,b,c,d); this increment in GUS activity is particularly remarkable at 18-h (three-fold higher in the GA4,7-treated seeds than in the water controls) and at 36 h, where a peak of activity of one order of magnitude higher than controls is reached. Significantly, in PMAN7-2::uidA (−609 bp) germinating seeds, GA4,7 provokes an earlier (6 h) enhancement of GUS activity (one order of magnitude higher than seeds with the PMAN7-1::uidA construct; Figure 3e,f,g), which suggest that block A contains a negative cis-motif of the AtMAN7 gene GA response. Exogenous addition of ABA (1 μM) to the imbibition medium does not significantly modify GUS activity in any of the transgenic germinating seeds analyzed (Figure 3g,h). The PMAN7-3::uidA germinating seeds do not support any GUS activity (Figure 3i,j).

AtbZIP44, but not its paralog AtbZIP11, binds specifically to the G-box element within conserved block B1 in yeast-one hybrid assays

The conserved cis-element identified in silico as block B has been split into two blocks, B1 (118 bp) and B2 (96 bp), to be used as baits in the screening of the yeast TF library. This library contains a collection of about 1200 A. thaliana TF ORFs, arrayed in a 96-well format (RR library; REGIA + REGULATORS library) and a convenient mating system has been developed, based on the yeast-one hybrid (YIH) screening procedure. An episomal plasmid (pTUY1H) has been used to clone the conserved B1 and B2 elements to be used as baits for the screening of this RR library (Castrillo et al., 2011).

Following this procedure, the B2 element has not been recognized by any TF in our library (data not shown) and the basic-leucine zipper (bZIP) AtbZIP44 (At1g75390) TF has been identified using the conserved B1 element as bait. This result has been further corroborated when using a smaller segment of 48 bp (spanning from −445 to −397 bp) that includes a G-box element (5′-CACGTG-3′; Figure 4a). Diploid yeasts containing the plasmids B1.Wt-pTUY1H and AtbZIP44-pDEST522 are able to grow in an auxotrophic medium lacking histidine, even in the presence of 15 mm 3-amino-1,2,4-triazole (3-AT), an inhibitor of the HIS3 reporter gene, while growth of the negative control (B1.Wt-pTUY1H−GFP-pDEST522) is blocked at 1 mm 3-AT (Figure 4b). To ensure whether the binding of the AtbZIP44 protein is through the G-box element (B1.Wt: 5′-CACGTG-3′), a 2-bp mutation has been produced in such a G-box (B1.G-box mut: 5′-CAGG-3′). Diploid yeasts containing AtbZIP44-pDEST522 and the mutated B1.G-box mut-pTUY1H constructs are not able to grow in 3-AT concentrations higher than 1 mm, as occurs with the negative control with GFP (Figure 4b). All these results indicate that the G-box element of the conserved block B1 is specifically recognized by the AtbZIP44 transcription factor.

Another evidence of this specificity is that other bZIPs present in our library, AtbZIP11 (At4g34590), its putative paralog with 78% similar amino acid residues, and AtbZIP4
(At1g59530), which although it belongs to the same S-group of bZIPs (Jakoby et al., 2002) is more distantly related to AtbZIP44 (only with 21% of identical residues), are not able to bind to block B1 or to its mutated version (Figure 4b).

**AtMAN7 and AtbZIP44 genes are co-expressed in the ME upon seed germination in A. thaliana**

To determine if AtbZIP44 could be a transcriptional regulator of the AtMAN7 gene, transcripts of both genes should be expressed in the same cells at the same time. In order to explore this, we have started by analyzing by RT-quantitative (q)PCR the presence of both mRNAs upon seed germination (Figure 5). Accumulation of AtMAN7 transcript reaches a peak at 12 h and remains constant until 24 h, decreasing sharply at 36 h, and being almost undetectable at 48 h. Accumulation of the AtbZIP44 transcripts starts at 6 h and this expression remains fairly constant at (4-8) × 10^3/18s-RNA until 36 h, diminishing thereafter.

The expression of its closely related AtbZIP11 gene is one order of magnitude lower than that of AtbZIP44.

Our previous data have demonstrated that AtMAN7 gene expression is restricted to the ME and to the radicle tip in the Columbia accession (Col-0) of germinating A. thaliana seeds, this expression disappearing soon after radicle emergence (Iglesias-Fernández et al., 2011a). To determine if the spatial expression of the AtbZIP44 and/or AtbZIP11 mRNAs co-localizes with that of the AtMAN7 gene, FISH experiments have been performed in germinating seeds at 18 h (Figure 6). Samples have been hybridized to specific antisense probes derived from the 3’-non coding sequences of AtMAN7, AtbZIP44 and AtbZIP11 (Table S4). In longitudinal sections, a strong signal for the AtMAN7 transcripts is detected in the ME (Figure 6a,d), while that of the AtbZIP44 mRNA is localized in the ME and in the radicle (Figure 6b,e). The similar localization of both transcripts in the ME is compatible with a possible transcriptional regulation of the AtMAN7 gene by the AtbZIP44 TF in these
cells. However, AtbZIP11 mRNA is faintly expressed at the radicle and it is not detected in any of the cells of the endosperm, thus excluding the possibility of the AtMAN7 gene being regulated by AtbZIP11 at the Me (Figure 6c,f,i). As expected, no signal has been detected in seed sections hybridized with the corresponding sense probes (Figure 6g–i). Microscopic analysis of coarse sections (6 µm) of paraffin samples by differential interference contrast (DIC; Figure 6j–l) reveals that the cellular organization of the seed tissues has been adequately preserved. These data do not exclude other possible functions for AtbZIP44 in the radicle upon germination.

The transcription factor AtbZIP44 affects seed germination and expression of the AtMAN7 gene

The oex lines oex-bZIP44-1 and oex-bZIP44-2 generated in the Col-0 accession have been used to explore not only the AtMAN7 transcript accumulation but also their seed germination kinetics (Figure 7). Accumulation of AtMAN7 transcript is two-fold and three-fold higher at 12 h in oex-bZIP44-1 and oex-bZIP44-2, respectively (Figure 7a). The germination assays performed with the homozygous oex-bZIP44-1-2 seeds appear in Figure 7b–d), where two parameters have been scored: SCR and ER when the radicle protrudes. When establishing the germination kinetics of the two oex-bZIP44 lines, there are significant differences at t50 (time to get 50% of SCR or 50% of ER; see insets of Figure 7c,d) as compared with the s0 values for the wild type (Col-0).

Since KO mutant lines for AtbZIP44 could not be found in the Columbia background, where most of the molecular and physiological MAN gene research has been carried out, other collections of A. thaliana accessions have been searched and two T-DNA insertion lines for AtbZIP44 have been found in the Wassilewskijia (Ws-4) background: Flag-295F02 (KO bZIP44-1) and Flag-270C06 (KO bZIP44-2), at positions -281 and +242 from the ATG translation initiation, respectively (Figure 8a). As a control, a KO mutant for AtMAN7 in the Ws-4 background has also been analyzed (KO MAN7-1: Flag-430B06; Figure 8a). To explore if these T-DNA insertion mutants in AtbZIP44 have an effect on the expression levels of AtMAN7, RT-qPCR analyses have been done in KO bZIP44-1 and KO bZIP44-2 homozygous seeds during germination (0, 36, 42, 60, 72 h; Figure 8b). Accumulation of AtMAN7 transcript decreases to almost 30% in the bZIP44 mutants as compared with the wild type at 36 h, and remains at this level during the rest of the period analyzed (up to 72 h). As expected, the AtMAN7 and AtbZIP44 transcripts are barely detected in their corresponding KO lines and the KO mutant AtMAN7 line is not affected in the expression of the AtbZIP44 transcript (Figure 8b). Germination assays have been performed with the homozygous KO bZIP44-1-2 seeds and SCR and ER have been scored (Figure 8c,d). Germination kinetics of the two KO bZIP44 lines differ significantly at t50 values (see insets of Figure 8c,d) as compared with the wild-type (Ws-4) control and this is particularly remarkable when scoring for the t50 of the SCR.

DISCUSSION

In this study, we have established the phylogeny of the endo-β-mannanase genes among several Brassicaceae species and analyzed the conserved cis-elements in the promoters of the MAN7 orthologous genes with the goal of establishing the cis-trans regulatory code of the AtMAN7 gene; previous studies have demonstrated that the AtMAN7 gene has an important role during germination. We have demonstrated here that the AtbZIP44 TF, but not its closely related AtbZIP11, affects seed germination and expression of the AtMAN7 gene, most probably by specifically interacting with a G-box (5'-CACGTG-3'; -428 bp to -423 bp) within one of the evolutionarily conserved cis-elements (block B1) of the AtMAN7 gene promoter.

Non-coding sequences of orthologous genes diverge rapidly during evolution, except for motifs that are important for function (Vavouri and Elgar, 2005). Through a
phylogenetic shadowing analysis of the promoters of MAN7 orthologous genes from six Brassicaceae species, two highly conserved motifs in these promoter regions have been identified (blocks A and B; Figure 1). In germinating transgenic A. thaliana seeds expressing the *uidA* reporter under the control of the whole AtMAN7 gene promoter (844 bp; PMAN7-1:uidA), GUS activity is restricted to the ME at 24 h (Figure 2), which is in agreement with our previous mRNA in situ analysis of the AtMAN7 gene (Iglesias-Fernández et al., 2011a). Serial deletions of the AtMAN7 promoter constructs reveal the functional relevance of the conserved block B. While absence of block A, such as occurs in the PMAN7-2:uidA construct (509 bp) does not affect substantially the expression of the *uidA* reporter gene, when block B is eliminated no GUS can be detected (PMAN7-3:uidA lines; 280 bp; Figures 2d-e and S3). This indicates that block B contains crucial regulatory elements for the transcriptional regulation of the AtMAN7 gene.

When the conserved block B1 has been used as bait in Y1H experiments against a TF library of about 1200 Tfs (Castrillo et al., 2011), the AtbZIP44 protein (At1g75390 gene) has been identified as interacting with this cis-element (Figure 4b). The specificity of the binding through the G-box within block B1 has been documented, because when we mutate the G-box (5'-CAAGG-3') AtbZIP44 does not recognize this cis-element in the Y1H experiments. Moreover, neither AtbZIP11 (At4g34590), its putative paralog with 78% similar amino acid residues, nor AtbZIP4 (At1g59530) more distantly related to AtbZIP44 (only with 21% of identical residues), are able to bind to the element (Figure 4b). Homodimerization and heterodimerization are common among bZIFs. In particular, heterodimerization between AtbZIP44 with bZIFs of the C-group (AtbZIP10, AtbZIP25) has been previously described both in the yeast two-hybrid (Y2H) and in Arabidopsis protoplasts (P2H) systems (Ehler et al., 2006). However, in Y2H experiments, we could not find homodimers of AtbZIP44 although we
could find heterodimers of AtbZIP44 with AtbZIP10 and AtbZIP25 (data not shown). AtbZIP10 and AtbZIP25 can form heterodimers between them, and are involved in regulation of seed storage protein genes upon silique development (Lara et al., 2003); transcripts of AtbZIP10 are hardly detected in germinating seeds and those of AtbZIP25, although present, seem not to be induced upon germination (https://www.genevestigator.com/gvw; Figure S4): Whether AtbZIP25 plays a role as co-regulator with AtbZIP44 of the AtMAN7 gene, although improbable, will need further investigation.

As shown in Figure 8, the expression of AtbZIP44 precedes radicle protrusion. Moreover, KO bZIP44 mutants have a slower germination than that of the corresponding wild type and the oex-bZIP44 lines display a faster germination, suggesting a role for the AtbZIP44 TF in the germination process and notably during SCR in the Ws-4 background. Besides, the mRNA in situ localization of the AtbZIP44 and AtMAN7 genes during seed germination is compatible with the transcriptional regulation of the AtMAN7 gene by AtbZIP44; suppression of AtbZIP44 expression, as occurs in the KO bZIP44-1,2 lines, not only lowers AtMAN7 gene expression to 30% of the wild-type control, but these lines have a significantly slower germination. This indicates that AtbZIP44, although not the only TF involved, is not a minor transcriptional regulator of AtMAN7. A similar situation has been described in barley with BLZ2 that is a bZIP associated with a quantitative trait locus for yield that influences the accumulation of protein and carbohydrate in seeds (Oñate et al., 1999; Haseneyer et al., 2010). One possibility could be that AtbZIP44 was redundant with other bZIPs. AtbZIP11 could be a good candidate for that, because of its close phylogenetic relationship with AtbZIP44 (78% similar residues), but AtbZIP11
transcript accumulation is quantitatively one order of magnitude lower than that of AtbZIP44 and AtbZIP11 and is not localized in our FISH experiments to the ME where AtMAN7 is expressed. Moreover, AtbZIP11 does not bind to the G-box contained in block B1 of the AtMAN7 gene promoter and neither bZIP do interacts in Y2H assays (our data not shown). Besides, AtbZIP11 expression is not altered in the T-DNA insertion mutants KO bZIP44,1-2 (our unpublished results), thus discarding a cross-regulation between them. AtbZIP11 is known to regulate the expression of the ASPARAGINE SYNTHETASE1 (ASN1) gene by the interaction with a specific G-box contained in the promoter of ASN1, and as occurs in our case with AtbZIP44 and AtMAN7, AtbZIP11 does not recognize the second G-box present in the ASN1 promoter (Hanson et al., 2008).

There are other bZIPS of the same S-group to which AtbZIP44 belongs, such as AtbZIP1 (At5 g49450), AtbZIP2 (At2 g18160), AtbZIP4 (At1 g59530) and AtbZIP53 (At3 g62420); this last one has been previously described to be a transcription activator of the albumin 252 gene during seed development (Alonso et al., 2009). However, only the AtbZIP2 gene is highly expressed during seed germination, as described in public arrays (Figure S4: https://www.
genevestigator.com/gvl/), but it does not interact with AtbZIP44 (Ehlert et al., 2006). Besides, none of these three TFS have been isolated in the Y1H screening using the B1-element as bait (our data not shown). Taken together, these data indicate that the paralogs of AtbZIP44, AtbZIP11 and AtbZIP2 are not implicated in the transcriptional regulation of AtMAN7 expression upon seed germination.

Since the KO mutants (bZIP44,1 and bZIP44,2 in theWs-4 background) have a slower germination and the oex-bZIP44 lines show a faster germination than their corresponding wild type, and the AtbZIP44 expression appears in the ME as well as in the radicle, AtbZIP44 TF can influence not only the loosening of the CW in the ME upon germination, but could also be involved in elongation of the radicle cells. Furthermore, the greater difference between the delays of SCR in KO mutants compared with those of the ER suggests a stronger influence of AtbZIP44 in the SCR than in the ER. The SCR is related to the mechanical force exerted by the embryonic axis upon the seed coat when the GA content increases during seed imbibition. The mutant ga1-3, deficient in GA synthesis, has completely blocked both SCR and ER. On the contrary, the addition of ABA to the imbibition medium of wild-type
seeds entirely suppresses ER but only delays SCR. It has been proposed that a low GA level provokes the over-accumulation of three DELLa repressor proteins (GAI, RGA and RGL2) that block testa rupture and eventually avoids ER (Müller et al., 2006; Piskurewicz et al., 2008). A positive GA/ABA balance is critical for germination to occur; GA synthesis starts shortly upon seed imbibition, which is essential for the rupture of both testa and endosperm tissues (Debeaujon and Koornneef, 2000; Wehtbrecht et al., 2011). Gibberellin promotes SCR through the stimulation of radicle growth and reduces the mechanical constraint conferred by the ME; both processes are influenced by the up-regulation of cell wall-modifying enzymes (Bewley, 1997; Leubner-Metzger, 2003). Phenotypic features of KO AtMAN7 lines upon seed imbibition, where both SCR and ER kinetics are slower than those of the wild type, point out for a role for AtMAN7 in both testa rupture and in loosening of the CW endosperm that facilitates protrusion of the radicle during seed germination. Moreover, quantitative determination of GUS activity in germinating seeds of transgenic plants where the uidA reporter gene was under the control of several deletion constructs of the AtMAN7 gene promoter shows a clear response of the AtMAN7 promoter to GA while it did not respond to ABA (Figure 3). GA stimulation occurs earlier in the germinating seeds of plants, with the construct PMAN7-2:uidA, where block A has been eliminated, than in the PMAN7-1:uidA seeds; an indication that block B could be responsible for this GA response and that block A would contain some element interacting with a GA repressor. In Solanum lycopersicum seeds, induction of LmMAN2 and LmMAN1 genes by GA during germination and post-germination, respectively, has been reported (Gong and Bewley, 2008). Transcripts of the orthologous gene of AtMAN7 in Lepidium sativum (LesaMAN7) are accumulated in the endosperm cap and to a lesser extent in the radicle during seed germination, and this induction is not inhibited by ABA (Morris et al., 2011). In A. thaliana and L. sativum, endosperm weakening is mediated, at least in part, by GA-induced genes encoding cell wall remodeling proteins (Voegele et al., 2011). It has been reported that A. thaliana transgenic seeds over-expressing the ortholog of AtbZIP44 of soybean (GmbZIP44) presents a higher germination percentage than those of the wild type when imbibed with 1 μM ABA (Liao et al., 2008). This result supports our view that AtbZIP44 positively affects seed germination, but, in our case, this effect seems to be independent of ABA. However, Glycine max belongs to the Leguminosae family whose seeds, lacking an endosperm, germinate in only one-step and ABA does not inhibit testa rupture but inhibits water uptake in the transition from germination to post-germination growth (Manz et al., 2005). Thus, it could be possible that bZIP44 has a different role during germination of endospermic and non-endospermic seeds. In this work, we have further demonstrated that the protein AtMAN7, with a predicted signal peptide in its deduced amino acid sequence, is exported to the peripheral regions of the cell (cell wall and plasma membrane; Figure S2). This result is in accordance with our previous proposed model for a possible cooperation between radial and endosperm MAN enzymes in the dismantling of the ME CWs, thus facilitating radicle protrusion (Iglesias-Fernández et al., 2011b). Recently, a large amount of heteromannan has been reported to be localized in the mucilage layer but not in the endosperm or in the radicle of germinating (3 h) A. thaliana seeds (Lee et al., 2012). In this context, movement of the AtMAN7 protein through the endoplasmic reticulum to the periphery of the cell acquires a great relevance, since protein produced in the radicle tip and in the ME cells could be secreted to the apoplastic space, and thus be present in the mucilage layer, without discarding that MAN could also be important in softening the CWs at the ME cells.

In summary, the data presented here indicate that AtbZIP44 is a transcriptional regulator affecting seed germination 'sensu stricto' and AtMAN7 gene expression, and this positive regulation involves both loosening of the ME and rupture of the seed coat. The SCR is stimulated by the elongation of the radicle and by the secretion of CW hydrolytic enzymes, such as MAN, to the seed coat to provoke the hydrolysis of the mannan in the CWs. But the question of whether GA is the phytochrome behind the molecular control of AtbZIP44 gene needs further investigation. Moreover, post-transcriptional regulation of AtbZIP44 needs to be considered, since all members of the bZIP ST-group, including AtbZIP44, contain an upstream open reading frame (uORF) that is involved in a post-transcriptional repression by sucrose (Weltmeier et al., 2009). We can’t rule out also the possibility that AtbZIP44 could be participating in the control of other genes in the radicle during seed germination, because their transcripts are also localized in the radicle besides being clearly expressed in the ME.

EXPERIMENTAL PROCEDURES

Plant material, growth conditions and germination assays

Seeds of the A. thaliana ecotypes Col-0 and Ws-4 and T-DNA insertion mutants in this latter ecotype obtained from the French National Institute for Agricultural Research (INRA), were identified from INP5-INA (http://www.ipbs.versailles.inra.fr) and they are: Flag-295F02 (KO bZIP44-1), Flag-270C06 (KO bZIP44-2), Flag-406B06 (KO MAN7-1). Hormogynous plants for the T-DNA insertions were selected by PCR using a gene-specific primer and a primer derived from the left border (LBA) of the T-DNA (https://signal.salk.edu/t-dnaprime2.html); Table S4).

Plant growth conditions and germination assays, in non-cold-stratified seeds, were performed as described (Iglesias-Fernández et al., 2011a). Statistical analyses were done using the GERMINATOR package program (http://www.ppb.wur.nl/UK/seedlab/resources/germinator; Jooosen et al., 2010).
Generation of transgenic lines

The promoter (~844 bp) of the AtMAN7 gene was amplified from A. thaliana genomic DNA by nested PCR using oligonucleotide pairs containing attB sites (Table S4) for cloning into the pDONR®221 by the Gateway® BP recombination and then transferred by Gateway LR recombination (Invitrogen; http://www.invitrogen.com) into the destination vector pMD163, containing the uidd reporter gene (Curtis and Grossniklaus, 2003). The same strategy was used to obtain the over-expression lines in the Columbia background. The pRSs:bsZIP44 was cloned into the pMD43, using the plasmid pDEST22® containing the bsZIP44 ORF, as entry clone. All constructs were introduced into Agrobacterium tumefaciens strain C58C1 GV3101 by electroporation and these were used to transform A. thaliana (Col-0) by the floral dip method (Clough and Bent, 1998).

Histochemical GUS assays

Qualitative GUS staining assays were performed using the protocol described by Jefferson et al. (1987). Samples were cleared using Hoyer’s light medium as described by Stangeland and Salehian (2002).

For quantitative GUS assays, seeds of two transgenic independent lines (representative of a total of 12 independent transgenic lines) were imbibed at different time periods in 3 ml of sterile water or with different hormonal treatments 50 μM GA3, and 1 μM α-ABA (Duchefa Biochemie, http://www.duchefa-biochemie.nl).

In Nigella damascena leaves, suspensions of A. tumefaciens strain C58C1 GV3101 were infiltrated containing constructs described in Figure 2a), the plasmid pMD43:35s:β-LUC (Luciferase; Gateway technology) was used to normalize the data and pBIN1:35s::βT19 to avoid genome silencing (Vilain et al., 2003). Quantitative GUS assays were performed according to Barrero et al. (2009) and for quantification of luciferase activity a high-sensitivity Luciferase Reporter Gene Assay kit (Roche Diagnostics, http://www.roche.com) was used, following the manufacturer’s instructions. Fluorescence/luminescence were determined using Genios Pro 96/384 multifunction microplate reader (TECAN®; http://www.tecan.com/). For statistical analysis of quantitative GUS activity data, a Student’s t-test was performed.

Subcellular localization

Translational fusion of AtMAN7 to the GFP reporter was generated by cloning the ORFs into the pBariH1-fire restriction sites of a psmRS-GFP plasmid, that was obtained from a pUC18 vector (U07643) in which the 35s-psmRS-GFP-Nos was previously cloned into the HindIII-EcoRI sites (Davis and Vierstra, 1998). The generated constructs were: PSSs::AtMAN7-GFP and PSSs:: GFP that was used as a control. Gold particles (1 μm), coated with the appropriate DNA constructs, were bombarded into freshly prepared onion epidermal peels with a biologic helium gun device (DuPont FDS-1000; Bio-Rad Laboratories, http://www.bio-rad.com). The expression of the fluorescent proteins in onion epidermal cells was observed after 24 h of incubation at 22°C in the dark (Barrero-Sicilia et al., 2011). Images were captured with a Zeiss Axiohot fluorescence microscope (Leica, http://www.leica.com/).

Bioinformatic tools: dendrogram and phylogenetic shadowing

All the sequences, from five different species within the Brassicaceae family, used in this work, were obtained from the Phytozome v8.0 Database (Goodstein et al., 2012; http://www.phytozome.net), except for those of the Arabis alpina genome that were produced by the TRANSESNET KBBE consortium (Prof. P. Carbonero, Universidad Politécnica de Madrid, Madrid, Spain, personal communication). Major characteristics of the predicted endo-β-mannanase proteins are listed in Table S1 and promoter sequences of MAN7 orthologous genes are found in Table S3.

The complete deduced amino acid sequences of the 37 MAN genes from the six Brassicaceae species were used to construct a phylogenetic dendrogram; signal peptide, molecular weight and isoelectric point predictions were done as described by Iglesias-Fernández et al. (2011a).

The Brassicaceae MAN7 promoter sequences were analyzed by the mVista Shuffle-LAGAN program that was used to create pairwise alignments of these promoters (Frazer et al., 2004; http://genome.jbliowlaviesta). Sequences of conserved regions within promoters were analyzed with T-coffee (Notredame et al., 2000; http://www.ebi.ac.uk/Tools/msa/tcoffee). Plant cis-acting regulatory DNA elements were searched through the following databases: MotifFinder (http://www.genome.jptools/motif) and PlantCare (Lescot et al., 2002; http://bioinformatics.psb.ugent.be/webtools/plantcare/html).

Yeast one-hybrid assays

To amplify by PCR the conserved block E1 within the AtMAN7 promoter and its mutated version (E1.G-box mut) we used the primers AS-PAMAN7.B1-HYBS-PAMAN7.E1-HYB and AS-PAMAN7.B1-HYBS-PAMAN7.E1-mut-HYB, respectively (Table S4). Yeast one-hybrid screenings were performed as described by Castrillo et al. (2011).

Real-time quantitative PCR assays

Total RNA was purified from A. thaliana seeds at different time points of germination (up to 72 h) as described by Osate-Sanchez and Vicente-Carbayosa (2008). The cDNA was synthesized from 1 μg of total RNA using the First-Strand Synthesis kit for RT-PCR (Roche Diagnostics) following the manufacturer’s instructions. Samples were stored at −20°C until use.

The specific primers used in the RT-qPCR analysis appear in Table S5. We used 18s-RNA to normalize the data (ΔCt; Figures S5–S7). The RT-qPCR was performed in an Eco® Real-Time PCR System (illumina, http://www.illumina.com). For each 10-μl reaction, a 1 μl cDNA sample was mixed with 5 μl of FastStart Universal SYBR Green Master (ROX; Roche Diagnostics), 0.25 μl of each primer (final concentration 500 nm), plus sterile water up to the final volume. Samples were subjected to thermal-cycling conditions of 95°C for 10 min, 40 cycles of (95°C, 10 sec at 95°C, 30 sec at 60°C of annealing and extension). The melting curve was designed to increase from 55 to 95°C; melting temperatures for each amplification and primer efficiencies, estimated via a calibration dilution curve and slope calculation, are shown in Table S6. This analysis was performed with three different biological samples for each time-point and each one was duplicated. Expression levels were determined as the number of cycles needed for the amplification to reach a threshold fixed in the exponential phase of the PCR reaction (Ct; Pfaffl, 2001).

Messenger RNA FISH experiments

The protocol followed here was described by Iglesias-Fernández et al. (2011b) and by Testillano and Riusheo (2009). Fragments of DNA (200–300 bp) derived from the 3′-non coding regions of the Arabidopsis genes analyzed have been used as probes (Table S4).
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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Phylogenetic tree and schematic distribution of conserved motifs among the deduced protein sequences of theendo-β-1,3mannanase genes of the Brassicaceae species used in this study.

Figure S2. Subcellular localization of the AtMAN7 protein.

Figure S3. Histochemical localization of GUS in 24-hr transgenic adult roots of PMAN7-1::GUS; PMAN7-2::GUS; PMAN7-3::GUS.

Figure S4. Heatmap representing expression levels of AtbZIP44 (At1 g76390), AtbZIP11 (At4 g34590) AtbZIP1 (At5 g8455), AtbZIP2 (At2 g18460), AtbZIP4 (At1 g95530) AtbZIP53 (At5 g52420), AtbZIP10 (At4 g02640) and AtbZIP25 (At3 g54620) during seed germination.

Figure S5. Transcription levels of housekeeping gene (18s-RNA), presented as Ct mean values, during seed germination in Arabidopsis thaliana Col-0 seeds and during silique development of A. thaliana Col-0.

Figure S6. Transcription levels of housekeeping gene (18s-RNA), presented as Ct mean values, during seed germination in Arabidopsis thaliana Ws-4, knock-out (KO) bZIP44-1, KO bZIP44-2 and KO MAN7-1 seeds.

Figure S7. Transcription levels of housekeeping gene (18s-RNA), presented as Ct mean values, during seed germination of A. thaliana Col-0, oex-bZIP44-1, and oex-bZIP44-2 seeds.

Table S1. Major characteristics of Brassicaceae predictedendo-β-1,3mannanase proteins.

Table S2. Sequences of conserved amino acids motifs (MEME; Bailey et al., 2006) of theendo-β-1,3mannanases from the Brassicaceae species in Figure S1.

Table S3. Promoter sequences of MAN7 genes of the Brassicaceae used in the phylogenetic shadowing analysis.

Table S4. List of primers used for cloning, for probe synthesis in fluorescent in situ hybridization and for genotype T-DNA insertion mutagenesis.

Table S5. Characteristics and sequences of the primers used in the RT-qPCR analysis.

REFERENCES


