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RESEARCH PAPER

Mannans and endo-β-mannanases (MAN) in *Brachypodium distachyon*: expression profiling and possible role of the *BdMAN* genes during coleorhiza-limited seed germination

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Abstract

Immunolocalization of mannans in the seeds of *Brachypodium distachyon* reveals the presence of these polysaccharides in the root embryo and in the coleorhiza in the early stages of germination (12 h), decreasing thereafter to the point of being hardly detected at 27 h. Concurrently, the activity of endo-β-mannanases (MANs; EC 3.2.1.78) that catalyse the hydrolysis of β-1,4 bonds in mannan polymers, increases as germination progresses. The *MAN* gene family is represented by six members in the *Brachypodium* genome, and their expression has been explored in different organs and especially in germinating seeds. Transcripts of *BdMAN2*, *BdMAN4* and *BdMAN6* accumulate in embryos, with a maximum at 24–30 h, and are detected in the coleorhiza and in the root by *in situ* hybridization analyses, before root protrusion (germination *sensu stricto*). *BdMAN4* is not only present in the embryo root and coleorhiza, but is abundant in the de-embryonated (endosperm) imbibed seeds, while *BdMAN2* and *BdMAN6* are faintly expressed in endosperm during post-germination (36–42 h). *BdMAN4* and *BdMAN6* transcripts are detected in the aleurone layer. These data indicate that *BdMAN2, BdMAN4* and *BdMAN6* are important for germination *sensu stricto* and that *BdMAN4* and *BdMAN6* may also influence reserve mobilization. Whether the coleorhiza in monocots and the micropylar endosperm in eudicots have similar functions, is discussed.

Key words: *BdMAN* gene family, *Brachypodium distachyon*, coleorhiza, endo-β-mannanases, germination, *MAN* gene expression, mannan immunolocalization, mRNA *in situ* hybridization.

Introduction

Poaceae grains (caryopses) include the seed proper, formed by a triploid endosperm and a diploid embryo, surrounded by the maternal tissues of the seed coat (testa) and the pericarp. The coleorhiza is a non-vascularized multicellular embryonic tissue, covering the seminal roots of Poaceae seeds. The coleorhiza has been thought to have a role in protecting the emerging root (Sargent and Osborne, 1980) and, more recently, it has been also associated with the regulation of dormancy, since abscisic acid (ABA) sensitivity is reduced in this tissue during germination of non-dormant barley seeds and the gene encoding the HvABA8´OH-1 enzyme, that is critical for ABA degradation, is expressed in the coleorhiza. During germination, both in barley and in *Brachypodium* seeds, the coleorhiza is the first structure that protrudes after the pericarp and testa rupture (coleorhiza emergence), followed by the coleorhiza rupture that allows root emergence (root emergence), and indicates the end of germination *sensu stricto* (Millar *et al.*, 2006; Barrero *et al.*, 2009, 2012; Gao and Ayele, 2014).

The germination process can be separated into germination *sensu stricto* and subsequent reserve mobilization (post-germination) and has been more deeply investigated in eudicotyledonous than in monocotyledonous seeds. In

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Arabidopsis thaliana, *Sisymbrium officinale*, *Lepidium sativum* and *Nicotiana tabacum*, the germination *sensu stricto* occurs in two different steps; first, the testa ruptures and, afterwards, the micropylar endosperm breakage takes place, allowing the radicle to emerge (Leubner-Metzger and Meins, 2000; Nonogaki *et al.*, 2000; Müller *et al.*, 2006; Iglesias-Fernández and Matilla, 2010; Iglesias-Fernández *et al.*, 2011*a*, *b*; Weitbrecht *et al.*, 2011; Nonogaki, 2014). Addition of ABA to the imbibition medium specifically blocks endosperm weakening and prevents its rupture (Müller *et al.*, 2006; Piskurewicz *et al.*, 2009; Carrillo-Barral *et al.*, 2014). It is assumed that testa rupture is influenced by the driving force of the imbibed elongating radicle and that the endosperm rupture is mainly produced by the weakening of the endosperm cell walls (CWs) by enzymes, specifically those localized to the micropylar endosperm, such as endo-β-1,4-mannanases (MANs), endo-ß-1,3-glucanases, expansins, xyloglucan-transglycosylases/hydrolases (XTHs) and pectin-methylesterases (Leubner-Metzger, 2005; Nonogaki *et al.*, 2007; Iglesias-Fernández *et al.*, 2011*a*, *b*; Endo *et al.*, 2012; Martínez-Andújar *et al.*, 2012; Rodríguez-Gacio *et al.*, 2012; Scheler *et al.*, 2014).

Since the endosperm CWs of several eudicot seeds are rich in mannans (Lee *et al.*, 2012), the MAN activity and the expression of *MAN* genes upon seed germination have been further characterized and their transcriptional regulation studied. In *A. thaliana*, four *MAN* genes (*AtMAN2*, *AtMAN5*, *AtMAN6* and *AtMAN7)* are expressed in germinating seeds and their transcripts are restricted to the micropylar endosperm and to the radicle, disappearing as soon as the radicle emerges. Moreover, knock-out mutants in the *AtMAN5*, *AtMAN6* and *AtMAN7* genes, as well as, in the *AtbZIP44* gene encoding an important activating transcription factor of *AtMAN7*, have a significantly retarded germination as compared to that of wild-type seeds, indicating a role for these *MAN* genes and their regulators during germination *sensu stricto* (Iglesias-Fernández *et al.*, 2011*a*, *b*, 2013; Rodríguez-Gacio *et al.*, 2012; Yan *et al.*, 2014).

Brachypodium distachyon is being considered a model species for the genetics and molecular genomics of cereals, due in part to its small sequenced genome (~355 Mbp), short life cycle, self-fertility, diploidy and its close phylogenetic relationship with important crop plants of the tribe Triticeae within the Poaceae family, such as wheat and barley (International Brachypodium Initiative, 2010; Mochida and Shinozaki, 2013; Girin *et al.*, 2014). In Poaceae seeds, the β-1,3-1,4-glucans are abundant in the endosperm cell walls (Burton and Fincher, 2009; Guillon *et al.*, 2011) and genes encoding hydrolytic enzymes involved in their degradation, such as endo-β-1,3-glucanases and endo-β-1,3-1,4-glucanases, have been associated with rice post-germination events (2–4 days of imbibition; Akiyama *et al.*, 2004) and with the elongation of barley coleoptiles (Takeda *et al.*, 2010). Although mannan content is lower than glucan content in *Brachypodium* seeds (Rancour *et al.*, 2012), the function of mannans and MANs may be relevant in its germinating seeds.

In this work, mannan polysaccharides were immunolocalized to the root and the coleorhiza of germinating seeds early in imbibition, decreased thereafter at later stages, and the enzymatic activity of endo-β-mannanases increased as germination progressed. The *MAN* gene family of *B. distachyon* was annotated and the expression of its six members explored in vegetative and reproductive organs. Interestingly, genes *BdMAN2*, *BdMAN4* and *BdMAN6* were clearly induced upon seed germination and mRNA *in situ* hybridization analyses demonstrated that these transcripts were found in the coleorhiza and the root during germination *sensu stricto*. *BdMAN4* and *BdMAN6* were also expressed in the aleurone layer, and may also be involved in post-germinative reserve mobilization.

Materials and Methods

Biological material, growth conditions and germination assays

The diploid inbred *Brachypodium distachyon* strain Bd21 (kindly provided by Prof. Garvin from the University of Minnesota, USA; International Brachypodium Initiative, 2010) was used in this work. Seeds were surface-sterilized with 1% NaOCl for 10 min and washed in sterile water, before germinating on Petri dishes, containing two filter papers (Whatman 3) moistened with 8 ml of sterile water, at 22ºC in the dark for 2 d. They were then transferred to pots in the greenhouse under long-day conditions (16h/8h, light/darkness; light intensity 155 µmol photons m^{-2} s^{-1}) for sampling roots (6-week-old plants), young and old leaves (6- and 12-week-old plants) and spikes. For the germination experiments that lasted up to 42 h, seeds were incubated in the dark at 22ºC, in Petri dishes with moistened filter papers, using triplicate lots of 25 non-stratified after-ripened seeds (stored at 22ºC and 30% relative humidity in the dark for 3 months). Seed samples were separated into embryo and endosperm (de-embryonated seeds) at 0, 12, 24, 30, 36 and 42 h of imbibition, and used for RNA quantification and for protein extraction to determine MAN enzymatic activity.

Endo-β-1,4-mannanase (MAN) activity assays

Seed samples, obtained as described above, were homogenized in 100 mM sodium acetate buffer (pH 4.5) containing 1 M NaCl and 0.5% ascorbic acid, at 4ºC for 2 h in an orbital shaker (VWR International Eurolab, Barcelona, Spain). The homogenates were centrifuged at 15,000 × *g* for 45 min, and 80 µl of this supernatant was mixed with 150 µl of 0.25% mannan (1,4-β-D-mannan from carob; Megazyme International Ireland Ltd., Wicklow, Ireland). Incubation was at 30ºC for different periods of time and the enzymatic activity was determined by the increase in reducing sugar production per mg of protein, as determined by the 4-OH-Benzoic Acid Hydrazide (PAH-BAH; Sigma-Aldrich) method (Lever, 1977). The MAN from *Aspergillus niger* (Megazyme) was used to establish the control curve (one unit of MAN activity defined as the amount of enzyme that releases 1 nmol of reducing sugar per minute under the experimental conditions). Protein concentration was determined with the Bradford reagent (Bio-Rad Laboratories, Munich, Germany) using bovine serum albumin (BSA) as a standard.

Bioinformatic tools: BdMANs identification and phylogenetic analysis

The deduced protein sequences of the six *MAN* genes were obtained from the *B. distachyon* genome using the TBLASTN tool at the Phytozome v8.0 Database (Goodstein *et al.*, 2012; www.phytozome.net), using the eight OsMAN proteins from the *Oryza sativa* genome as query sequences (Yuan *et al.*, 2007). The Interpro Program (PFAM database; Bateman *et al.*, 2002; http://pfam.sanger.ac.uk) was used to confirm the presence of the MAN conserved

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domain (glycosyl-hydrolase family 5). The complete amino acid sequences deduced from *B. distachyon*, O. sativa and *Arabidopsis thaliana MAN* genes were aligned by means of the CLUSTAL W program (Thompson *et al.*, 1994) and utilized to construct a phylogenetic dendrogram, using the neighbor-joining algorithm, a bootstrap analysis with 1,000 replicates, complete deletion and the Jones Taylor Thornton matrix, as settings. The MEME program software version 4.0 (Tamura *et al.*, 2007) was used to identify conserved motifs within the deduced MAN proteins and to validate the phylogenetic tree (Table 1). Default parameters were used with the following exceptions: the maximum number of motifs to find was set to 22 and the minimum width was set to eight amino-acid residues (Bailey *et al.*, 2009; http://meme.sdsc.edu/meme4_6_0/intro.htlm). A single capital letter represents a single residue relative frequency, if this is greater than 50% than twice that of the second most frequent residue in the same position. If no single residue matches these criteria, a pair of residues, represented by capital letters in brackets, is given if the sum of their relative frequencies exceeds 75%. If none of these characteristics are satisfied, a lowercase letter is given when the relative frequency of a residue is greater than 40%, if not, x is set.

The major biochemical parameters of the deduced MAN proteins from *B. distachyon* and *O. sativa* are listed in Supplementary Table S1. Both isoeletric point (pI) and molecular weight (MW) were predicted using the Compute pI/MW tool (Gasteiger *et al.*, 2005; http://www.expasy.ch/tools/pi_tool.html) and the putative signal peptide cleavage site and sub-cellular localization were deduced by the SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP) and TargetP 1.1 tools (http://www.cbs.dtu.dk/services/TargetP/), respectively (Emanuelsson *et al.*, 2007).

Real time quantitative PCR (RT-qPCR) analyses

Total RNA was purified from roots (6-week-old plants), young and old leaves (6- and 12–week-old plants) and spikes by the phenol/chloroform method (Lagrimini *et al.*, 1987). For the isolation of RNA from seeds at different stages of development (0–10 d after pollination: dap) and at different time points of germination (12, 24, 30, 36 and 42 h), the protocol described by Oñate-Sánchez and Vicente-Carbajosa (2008) was followed. RNA samples were treated with DNAse I, RNAse-free (Roche Applied Science, Manheim, Germany) to avoid genomic DNA contamination. First-strand cDNA was synthesized with random hexamers using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's recommendations. Samples were stored at −20ºC until used.

PCR-amplification was performed in an Eco Real-Time PCR System (Illumina, San Diego, CA, USA). For each 10 µl reaction, 2 µl of DNA sample was mixed with 5 µl of FastStart SYBR Green Master (Roche Applied Science) and 0.25 µl of each primer (final concentration 500 nM) plus sterile water up to final volume. Samples were subjected to thermal-cycling conditions of 95ºC for 10 min and 40 cycles of 10 s at 95ºC and 30 s at 60ºC for annealing and extension, respectively. The melting curve was designed to increase from 55ºC to 95ºC, and the melting temperatures for each amplicon and primer efficiencies (Supplementary Table S2) were estimated using a calibration dilution curve and slope calculation ($E=10^{(-1/slope)}$). The specific primers used are shown in Supplementary Table S2 and they were designed on the 3′-non-coding region using the Primer3Plus program (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). The *BdGAPDH* gene (encoding glyceraldehyde 3-phospate dehydrogenase; Hong *et al.*, 2008) was used to normalize the data, since the expression of this gene was previously demonstrated to be constant throughout the period studied (Hernando-Amado *et al.*, 2012; González-Calle *et al.*, 2014; Supplementary Fig. S1). Expression levels were calculated as the number of cycles needed for the amplification to reach a cycle threshold fixed in the exponential phase of the PCR (C_t; Pfaffl, 2001). All analyses used three different biological replicates for each time-point and each one was made in triplicate. Means ± standard error (SE) of three independent experiments are indicated in the corresponding figures.

Preparation of embedded material for microscopy

Samples were treated according to a modified version of the protocol described in Ferrandiz *et al.* (2000). After-ripened dry seeds and germinating seeds of *B. distachyon* (12 h, 27 h and 36 h) were collected and, after removal of the lemma and palea, were infiltrated with the FAE solution (formaldehyde: acetic acid: ethanol: water, 3.5:5:50:41.5 by volume) for 40 min in 25 mm Hg vacuum; the seeds were then incubated at 4ºC for 3 d with gentle shaking. The samples were dehydrated through a graded series of aqueous ethanol mixtures and progressively embedded in paraffin after the replacement of ethanol with HistoClear (National Diagnostics, Hessle Hull, England). Thin sections of 8 µm were collected on glass slides and de-waxed.

Heteromannan immunolocalization

The protocol used was a modification of those described in Marcus *et al.* (2010) and Guillon *et al.* (2011). In a pre-immunolabelling step, sections of embedded material as described above, were incubated in phosphate buffer sodium solution (PBS) and treated with 1 mg/ml proteinase-K (Roche Applied Science). In order for the specific antibodies to have access to the heteromannans (mannans, glucomannans, and galactomannans) of the cell walls, β-1,3-1,4- glucans were removed by incubating the sections with a solution of 4 µg/ml lichenase [β-1,3-1,4- glucanase; Megazyme] for 2 h at 37ºC, and then rinsed with de-ionized water. For heteromannan immunodetection, sections were first incubated at room temperature for 30 min in a blocking solution (3% BSA, 1× PBS, and 5mM sodium azide; pH7), and then treated with primary anti-heteromannan antibody LM21 (PlantProbes, Leeds, UK) at a dilution of 1:5 in the same blocking solution but only containing 1% BSA for 2 h. Sections were thoroughly washed in PBS containing 5 mM sodium azide and then incubated for 2 h in the same buffer containing the secondary rabbit antibody Anti-Rat IgG-FITC (Sigma-Aldrich) at a dilution of 1:100. The sections were extensively washed in PBS buffer and in water, mounted and examined in a confocal microscope (absorption 494 nm; emission 521 nm; Leica TCS-SP8, Leica, Wetzlar, Germany).

mRNA in situ *hybridization analyses*

Pre-hybridization was carried out by incubating the sections in 0.2 M HCl, neutralizing them and then treating them with 1 mg/ml proteinase-K (Roche Applied Science). Samples were then dehydrated in an aqueous ethanol dilution series and hybridized with sense and anti-sense digoxigenin (DIG)-labelled RNA probes, corresponding to DNA fragments (200–300 bp) derived from the 3′-non coding regions of the *BdMAN2*, *BdMAN4* and *BdMAN6* genes (Supplementary Table S3), synthesized with the DIG RNA labelling mix according to the manufacturer's specifications (Roche Applied Science). Probes were hybridized at 52ºC overnight followed by two washes in 2× SSC (150 mM NaCl, 15 mM Na_3-citrate) and 50% formamide for 90 min at the same temperature. Incubation with the alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche Applied Science) and colour detection was carried out according to the manufacturer's instructions (Ferrandiz *et al.*, 2000). Sections were dried and examined on a Zeiss Axiophot Microscope (Carl Zeiss, Oberkochen, Germany), and images were captured and processed with the Leica Application Suite 2.8.1 build software (Leica).

Protein and polysaccharide histological determinations

B. distachyon dry and germinating seeds were stained with 5% (w/v) toluidine blue (Merck, Darmstadt, Germany) for checking tissue integrity (Fig. 1A). Samples were stained with PAS reagent (0.5% w/v periodic acid-Schiff reagent) (Merck) to detect polysaccharides and with 1% (w/v) Naphthol Blue Black (Sigma-Aldrich) for proteins (Iglesias-Fernández and Matilla, 2010). Visualization was done

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on a Zeiss Axiophot Microscope (Carl Zeiss) and the images were captured and processed with the Leica Application Suite 2.8.1 build software (Leica).

Results

Enzymatic β-*mannanase activity during* Brachypodium *seed germination*

The time course of *sensu stricto* germination of *B. distachyon* seeds occurs in two different steps: first, the coleorhiza emerges (CE), and in a second step, the root emergence (RE) takes place (Fig. 1A). The enzymatic activity of MAN upon germination has been analysed separately in the embryo and in the de-embryonated seed (endosperm). As shown in Fig. 1B, dried seeds have no detectable MAN activity, but this progressively increases with germination, peaking at 24 h in embryos, containing the coleorhiza (~0.4×10^{-3} units/mg protein), and decreasing to half this value at 42 h (~0.2×10^{-3} units/mg protein). In endosperms, MAN activity is much lower than in embryos, and reaches its maximum level (~0.15×10^{-3} units/mg protein) at 36 h of germination. Data from Fig. 1B indicate that MAN activity is maximum in embryos, just before

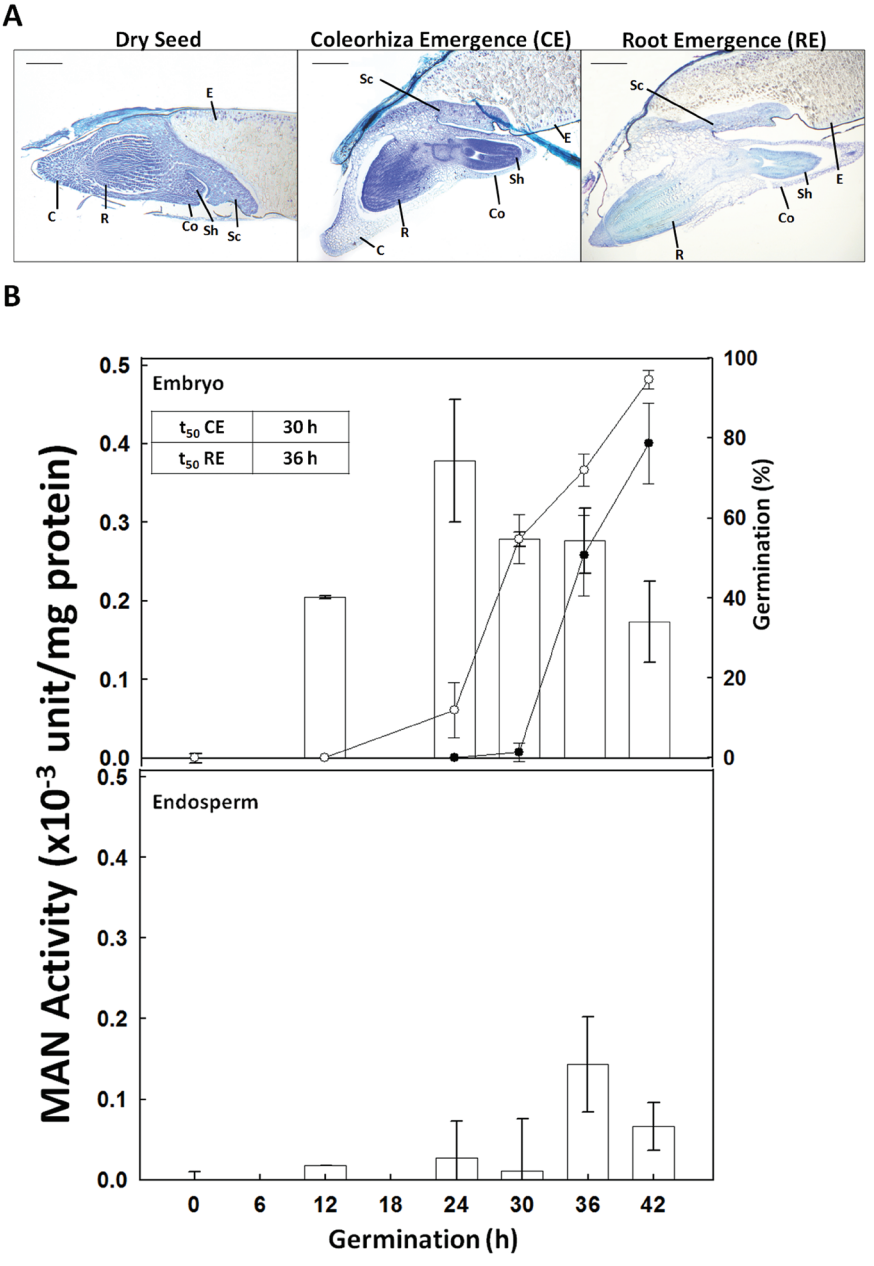

Fig. 1. (A) Longitudinal sections of the different phases of *Brachypodium distachyon* germination *sensu stricto*, stained with toluidine blue. C, coleorhiza; Co, coleoptile; E, endosperm; Sc, scutellum; Sh, shoot; R, root. Scale bar, 200 μm. (B) Endo-β-mannanase activity (white bars) in embryo and endosperm (de-embryonated seed) upon *B. distachyon* seed germination (0–24 h). One unit of MAN activity is defined as the amount of enzyme that releases 1 nmol of reducing sugar per minute and per mg of protein. Percentage germination evaluated as coleorhiza emergence (CE; open circles) and root emergence (RE; close circles) are represented. In the inset, the time needed for 50% of CE (t_{50}CE) and RE (t_{50}RE) is indicated. Data are means ± standard error (SE) of three technical replicates of three biological samples.

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reaching 50% of germination *sensu stricto* (t_{50}CE=30h; t_{50}RE=36h), suggesting that MAN is important for facilitating both coleorhiza and root emergence.

Heteromannans are preferentially localized to the root tip and the coleorhiza in germinating seed embryos

Mannan polymers have been detected in longitudinal sections of *B. distachyon* germinating seeds (at 12 and 27 h of imbibition) by *in situ* immunofluorescence labelling, using the LM21 antibody that specifically recognizes mannan polysaccharides (gluco- and galacto-mannans). To facilitate accession of the antibody to mannans in plant CWs, the seed sections have been previously treated with lichenase (β-1,3-1,4- glucanase; Marcus *et al.*, 2010).

As shown in Fig. 2, at 12 h of seed imbibition, seed mannan polymers are mainly localized to the periphery cells of the coleorhiza (C) and to the epidermis of the root tip (R) (Fig. 2A–C). Interestingly, these mannans are barely detected at later stages of germination (27 h of imbibition; Fig. 2D–F). Differential interference contrast (DIC) images are shown in Fig. 2G–I. This observation together with data of MAN enzymatic activity (Fig. 1B) with a maximum at 24 h in embryos, may suggest that the disappearance of the mannan polymers is due to the hydrolysis catalysed by endo-β-mannanases.

The Brachypodium *endo-β-mannanase gene family*

In order to get a deeper insight into the MAN function upon *B. distachyon* germination, it was decided to annotate and characterize further the BdMAN family. The already described MAN family from *O. sativa* (Yuan *et al.*, 2007) has been used to perform a TBLASTN against the whole *Brachypodium* genome (http://www.phytozome.net). Six predicted non-redundant MAN deduced proteins, with MW 43–52 KDa, and Ip 4.4–8.8, three of them with predicted signal peptides, have been identified and named according to their orthologues in rice (Supplementary Table S1). The MAN protein sequences from *A. thaliana* (AtMAN1-7) and *O. sativa* (OsMAN1-8) together with those from *B. distachyon* (BdMAN1-6) have been used to construct a phylogenetic unrooted tree by using the neighbor-joining algorithm. Four major clusters of orthologous groups (MCOGs) have been defined (A, B, C, D), supported by bootstrapping values higher than 62% (Fig. 3A) and by the occurrence of common motifs (Fig. 3B; MEME).

The search for conserved amino-acid motifs using the MEME software (http://meme.nbcr.net/meme/cgi-bin/

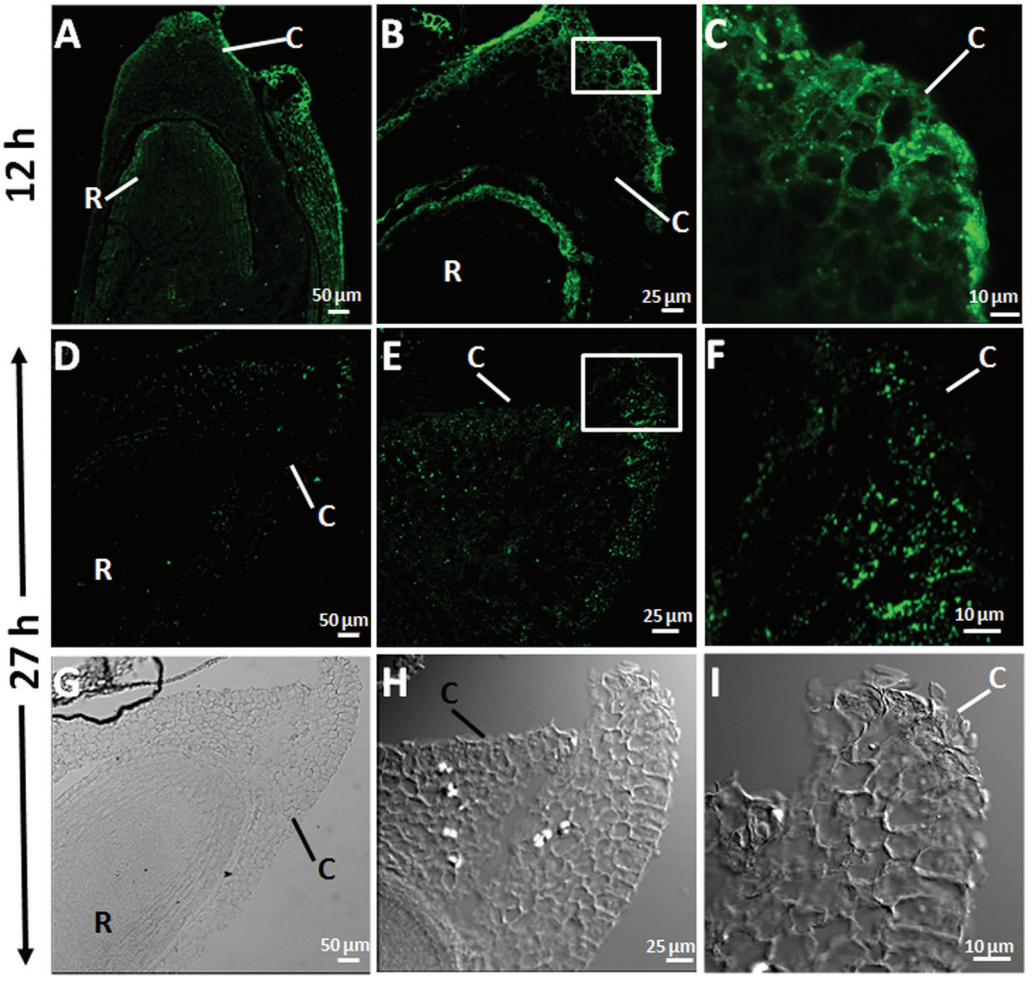

Fig. 2. Mannan polymer immunolocalization at the root tip and the coleorhiza in longitudinal sections of *Brachypodium* germinating embryos at (A–C) 12 h and at (D–F) 27 h. (G–I) DIC images of D, E and F. (C, F, I) Close-up of the coleorhizae. C, coleorhiza; R, root. Scale bars: (A, D, G), 50 µm; (B, E, H), 25 µm; (C, F, I), 10 µm.

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meme.cgi) reveals that all MAN sequences have in common motifs described as critical for the enzymatic activity, such as 1, 3, 5, 6/11 and 7 (Fig. 3B, Table 1). The deduced signature sequence [AWEL(MI)NEPRC] of *Arabidopsis* and rice MANs (Yuan *et al.*, 2007), included in motif 1, is also present in *Brachypodium* MAN. Besides, members in MCOG A, BdMAN2, OsMAN2, BdMAN6 and OsMAN6 share motif 12, and BdMAN6 and OsMAN6 also share motifs14 and 18. In MCOG C, BdMAN1 shares with OsMAN1 motifs 19 and 22, and in MCOG D BdMAN4 shares motifs 17 and 21 with OsMAN4, but lacks motif 15 shared by the rice paralogues OsMAN3 and OsMAN4. Similarly, BdMAN5, OsMAN7 and OsMAN8 (MCOG B) have in common motif 11, but they do not share with OsMAN5 motifs 20 and 13. The

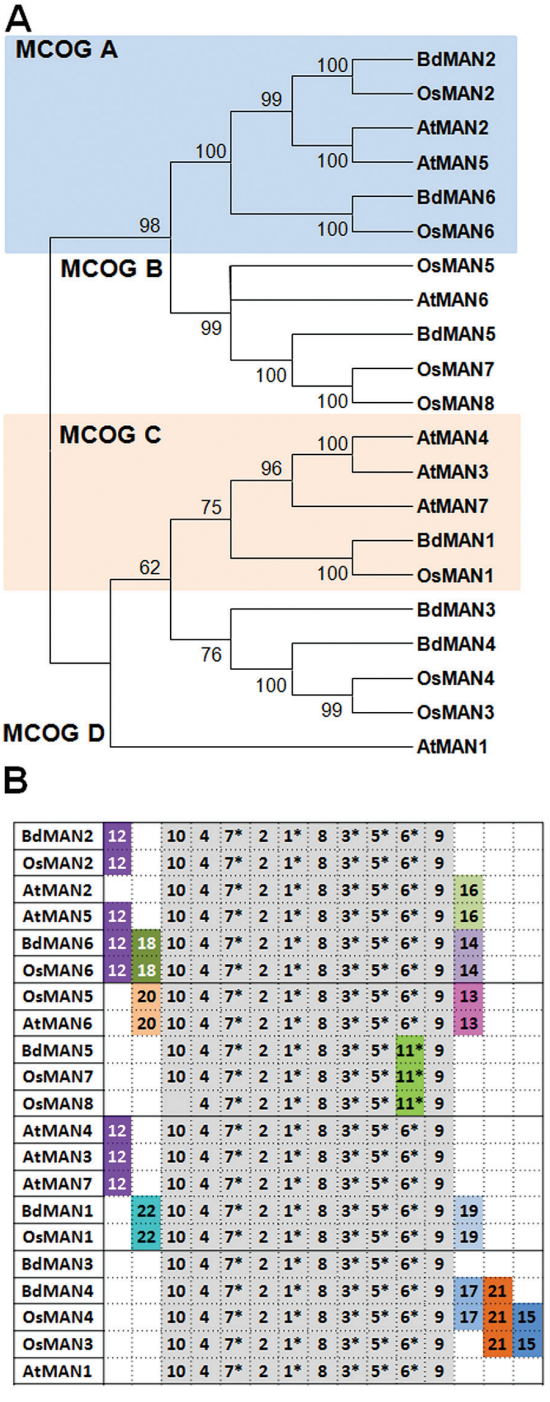

Fig. 3. (A) Phylogenetic dendrogram with deduced protein sequence of the mannanase gene families form *Brachypodium distachyon*, *Oryza sativa* and *Arabidopsis thaliana*; bootstrapping values are indicated in the branches. (B) Schematic distribution of conserved motifs among the deduced protein sequences in the phylogenetic tree (A), identified by means of the MEME analysis. Asterisks indicate those motifs important for enzymatic activity. Motifs in grey share >85% of similar amino acid residues. This figure is available in colour at JXB online.

Table 1. *Conserved amino acid motifs obtained by means of MEME (Bailey et al., 2009) from the analysis of the endo-β-mannanase proteins of* Brachypodium distachyon, Oryza sativa, *and* Arabidopsis thaliana

The conserved amino acids, critical for enzyme activity, are in bold and the signature sequence for endo-β-mannanase enzymes is underlined.

Motif	E value	Consensus sequences
1	5.6e-1050	L[TS]S[DN]D[DS]FF[TS][DN]PT[IV][KR][DS][YF][YF] KN[HY]VK[AT]VLTR[VK]NT[LV]TG[VI]AY[KR]D[DE] PTI[FL] AWEL[MI]NEPRCxSDP[ST]GDTLQAW[IV] EEMAAYVKS[ILV]DP[NK]H
2	6.5e-718	DGGY[RN][AP]LQI[SA]PG[VR][YF][DN]ED[VM]F[QK] [GA]LDFV[IVL][AS]EA[RK][RK]HG[IV][RK]L[IL]L[SC] LVNN [WL][DE][DA][YF]GGK[AK]QYVRWA
3	1.40E-307	x[LY]GTDF[IV][AR]N[HNS]Q[VA]PGIDFA[ST][VI]H[SV] YP**D**xW[LF]P
4	7.90E-213	NGRPFY[VAI]NG[FW]N[AST]YWLMxxA[ASV]DP[AS]T
5	2.80E-202	[KRN]W[ML][DQ][AS]H[IV]ED[AG][AEQ][NA][IE]L[GKR] [KM]P[LVI]L[VFIL][TAG]**E**FG[KL]S
6	3.40E-199	WKxPG[YF][NST]T[AS]QRDA[FL][LFY]R[AT]VYD[KA] IYASAR[RK]GG[APS][GA][AV]G[AG]L[FV]**W**Q[LV]L
7	2.10E-172	[TS][AE][AMV][FL][RQ]QA[AS]A[MH]GL[TN]V[CA] RT**W**AF[SN]
8	2.20E-161	L[LV][ET][VI]GLEGFYGP[SG]SPER[KL]xVN
9	2.50E-146	GM[ED]x[YFM]DDG[YF][ESA][IV][VI][LF][AS]ES[PS] STAS[IL][IL]x[EN][HQ]S[RC]
10	2.90E-64	[PAG][EGS][DGW]G[FM]V[RE]R[NR]GT[QR][FL]V[LV]
11	8.20E-37	KDGKF[GD][NS][EG]FRE[DT]FM[KE]T[VI]Y[RN][IN] FLSSW[KE][EG]GVIGGGCLL**W**QLFP
12	4.40E-11	[YM][KHS][ILC]L[GC][FL][LAF][LSV][LC]LA[IVF][VI][YI] [LAF][SQ][FLSW]
13	4.40E-05	FNS[LR]C[AS]W[RS]CRWGC[KN]K
14	1.00E-02	[HL][KY][GK]EGDPGWQC[ST]IPP
15	2.60E+01	[CR]F[IV]SLSRSISSFI[QV][DQ]NF
16	3.70E+01	[HR]L[KL][DE][KQ]K[LS][IK]E[LM]CSHR[HP]
17	1.50E+02	QLA[AES]L[ND]G[QK][DF][AD][DE][AGV][LV][RC] RR[RA][RS][RS]
18	1.00E+03	[AG][AG][GP]GG[GW][LV]KLPVPWLQ
19	4.30E+04	KAFARA[ER][QR][AE][QR][PS]ARGKG
20	6.60E+04	[AI]VL[CH][ES][AS][SV][FY][IW][EI][LW][RT][CQ]NR
21	2.70E+05	SS[HP]RK[IT][GR][LS][GT]SGG[DS][SW]D
22	3.00E+05	L[GH][AH][GH][AR][AV]ALLVL[AL]CV[HV]

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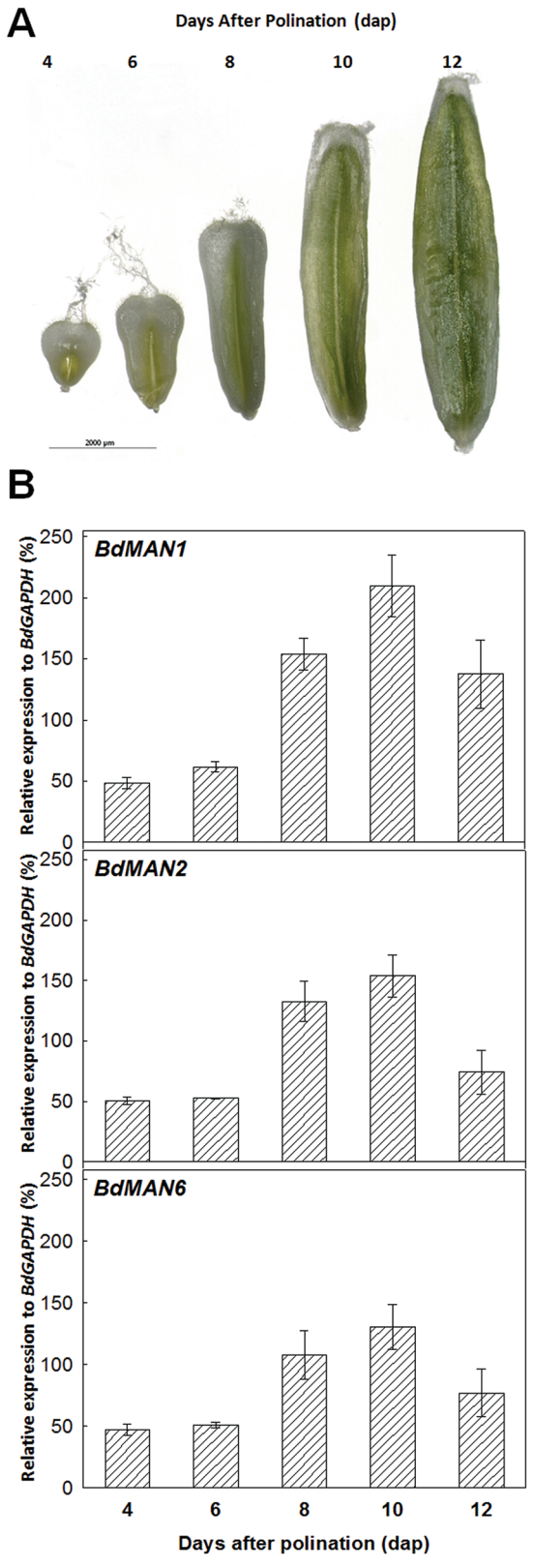

Fig. 4. (A) Different stages of *Brachypodium distachyon* seed development (4, 6, 8, 10, 12 d after pollination; dap). (B) Expression of the *BdMAN1*, *BdMAN2* and *BdMAN6* genes by RT-qPCR during seed development. Data are means ± standard error (SE) of three technical replicates of three biological samples.

MAN protein motifs from *A. thaliana* have been included for comparison (Iglesias-Fernández *et al.*, 2011*a*).

Expression kinetics of selected BdMAN *genes during seed maturation and germination*

The expression pattern of the six *BdMAN* genes has been explored by RT-qPCR analysis in different organs: young (6 d) and old (12 d) leaves, roots (6 d) and spikes (mix of different stages; Supplementary Fig. S2). While *BdMAN1*, *2*, *3, 5* genes are not detected in leaves, *BdMAN4* gene expression in old leaves is ~10 times lower than in young leaves, and *BdMAN6* has the same low expression in young and old leaves. In roots, *BdMAN2*, *BdMAN4* and *BdMAN6* are expressed at low levels, and *BdMAN1*, *BdMAN2*, *BdMAN4* and *BdMAN6* transcripts are detected in spikes (Supplementary Fig. S2).

Since our preliminary data indicate that *BdMAN1*, *BdMAN2* and *BdMAN6* transcripts are abundant in developing seeds (see Supplementary Fig. S3A), the expression kinetics of these three genes has been established throughout seed maturation, at 4, 6, 8, 10 and 12 d after pollination (dap) (Fig. 4A). Although the gene *BdMAN1* is the most highly expressed during the late phases of seed development (8, 10, 12 dap), the expression patterns of *BdMAN1*, *BdMAN2* and *BdMAN6* show a progressive increase from 4 to 10 dap, reaching all of them their maximum expression at 10 dap when maturation is almost completed (Fig. 4B).

Data in Supplementary Fig. S3B indicate that genes *BdMAN2*, *BdMAN4* and *BdMAN6* are the most abundantly expressed ones during seed germination, and their expression kinetics has been more thoroughly analysed (Fig. 5). Germinating seeds taken at 12, 24, 30, 36 and 42 h of imbibition, have been sectioned into embryos and de-embryonated seeds (endosperm). In germinating embryos, *BdMAN2*, *BdMAN4* and *BdMAN6* transcripts appear early upon imbibition (12 h), before CE, and their maximum expression is attained between 24–30 h (t_{50}CE=30 h) and it decreases as germination progresses (42 h; Fig. 5A). However, the expression of *BdMAN4* in endosperms is high at early imbibition times (12 h; ~140% relative to *BdGAPDH*), decreasing thereafter. The *BdMAN2* and *BdMAN6* transcripts have low expression in the endosperms of germinating seeds with a maximum at 36 h of imbibition (<10 % for *BdMAN2* and ~25 % for *BdMAN6* relative to *BdGAPDH*, respectively), indicating a possible role in reserve mobilization for these *MAN* genes, and perhaps also for *BdMAN4* post-germination (Fig. 5B).

BdMAN2, BdMAN4 *and* BdMAN6 *transcripts are localized to different seed tissues during* B. distachyon *seed germination*

To determine the spatial expression of *MAN* genes within the *B. distachyon* germinating seeds, *mRNA in situ* hybridization experiments have been done (Fig. 6). Longitudinal sections of seeds at 27 h of imbibition have been hybridized to specific antisense and sense (as negative controls) probes for *BdMAN2, BdMAN4* and *BdMAN6*. The *BdMAN2* transcripts are mainly expressed in the periphery cells of the coleorhiza and are not detected in the aleurone layer (Fig. 6A–D). *BdMAN4* mRNA is localized preferentially to the tip and the apical meristem of the root, to the coleorhiza and to the aleurone layer

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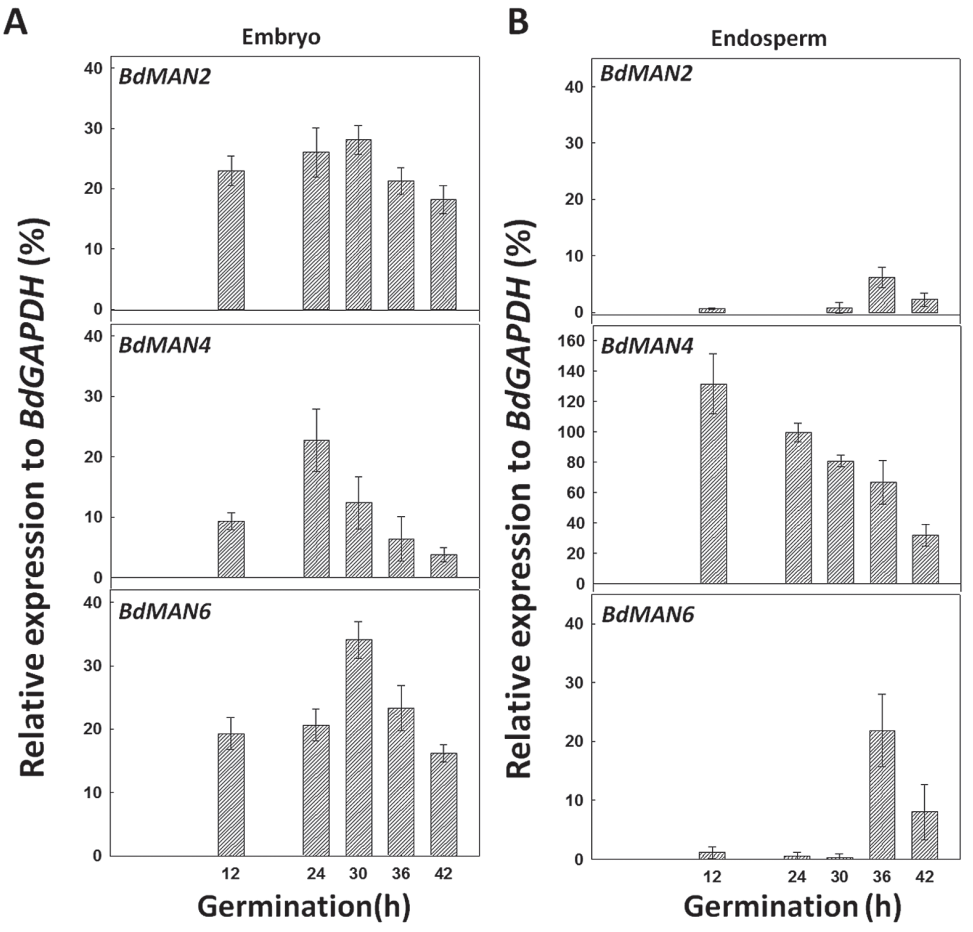

Fig. 5. Transcript accumulation of *BdMAN2*, *BdMAN4* and *BdMAN6* in (A) embryos, and in (B) endosperms (de-embryonated seeds) upon *B. distachyon* seed germination. Data are means ± standard error (SE) of three technical replicates of three biological samples.

(Fig. 6E–H), and the *BdMAN6* transcripts are detected not only at the coleorhiza, but also throughout the embryo and faintly also at the aleurone layer (Fig. 6I–L). *BdMAN6* transcripts are detected in the aleurone layer at 27 h of imbibition by mRNA *in situ* hybridization, but they are scarcely detected in 24–30 h germinating endosperms by RTqPCR (Fig. 5B), indicating that its expression could be diluted by the remaining endosperm during the RNA isolation process. As expected, no signal has been detected when sections have been hybridized with the corresponding sense probes for *BdMAN2*, *BdMAN4* and *BdMAN6* (negative controls; Figs 6D, 6H and 6L).

Other histological observations of germinating B. distachyon *seeds*

Longitudinal sections of *B. distachyon* seeds (dry and water imbibed at 27 and 36 h; including seeds before and after root emergence) have been stained with PAS reagent to detect insoluble polysaccharides (mainly cellulose and starch) and with Naphthol Blue Black for proteins. As shown in Fig. 7A–C, dry seeds have abundant protein bodies (PBs; blue stained) and thick cell walls (CWs; pink stained) in the root, coleorhiza and endosperm cells. When the coleorhiza emerges (27 h of imbibition) the PBs in the coleorhiza (C) and in the mesocotyl (M) cells start to hydrolyze, while the endosperm cells are full of reserves at this stage (Fig. 7D–F). After 36 h, when the seed coat ruptures but before root emergence (RE), the coleorhiza cells start to elongate and its protein bodies (PBs), as well as, those of the mesocotyl (M) are almost completely consumed, while those of the coleoptile (Co) initiate their degradation (Fig. 7G–I) and those of the endosperm remain as in the dry seeds (Fig. 7C, 7I). Finally, when root emergence (RE) takes place, the lateral part of the coleorhiza breaks (≥36 h of imbibition) and its PBs are fully degraded, while those in the endosperm cells are almost intact (Fig. 7J–L).

Discussion

In this work, mannans and endo-β-mannanases (MAN) in *Brachypodium distachyon* have been investigated in order to establish whether they are important in the germination of these monocotyledonous seeds. Mannans have been immunolocalized in the embryo root and the coleorhiza in the early stages of germination and these polymers decrease upon imbibition while the enzymatic activity of MAN increases. The *MAN* gene family in *B. distachyon* has been annotated and the gene expression of the six members of this family has been explored in different vegetative and reproductive organs, and, more specifically, in germinating seeds. Three of these genes, *BdMAN2*, *BdMAN4* and *BdMAN6*, are highly induced in

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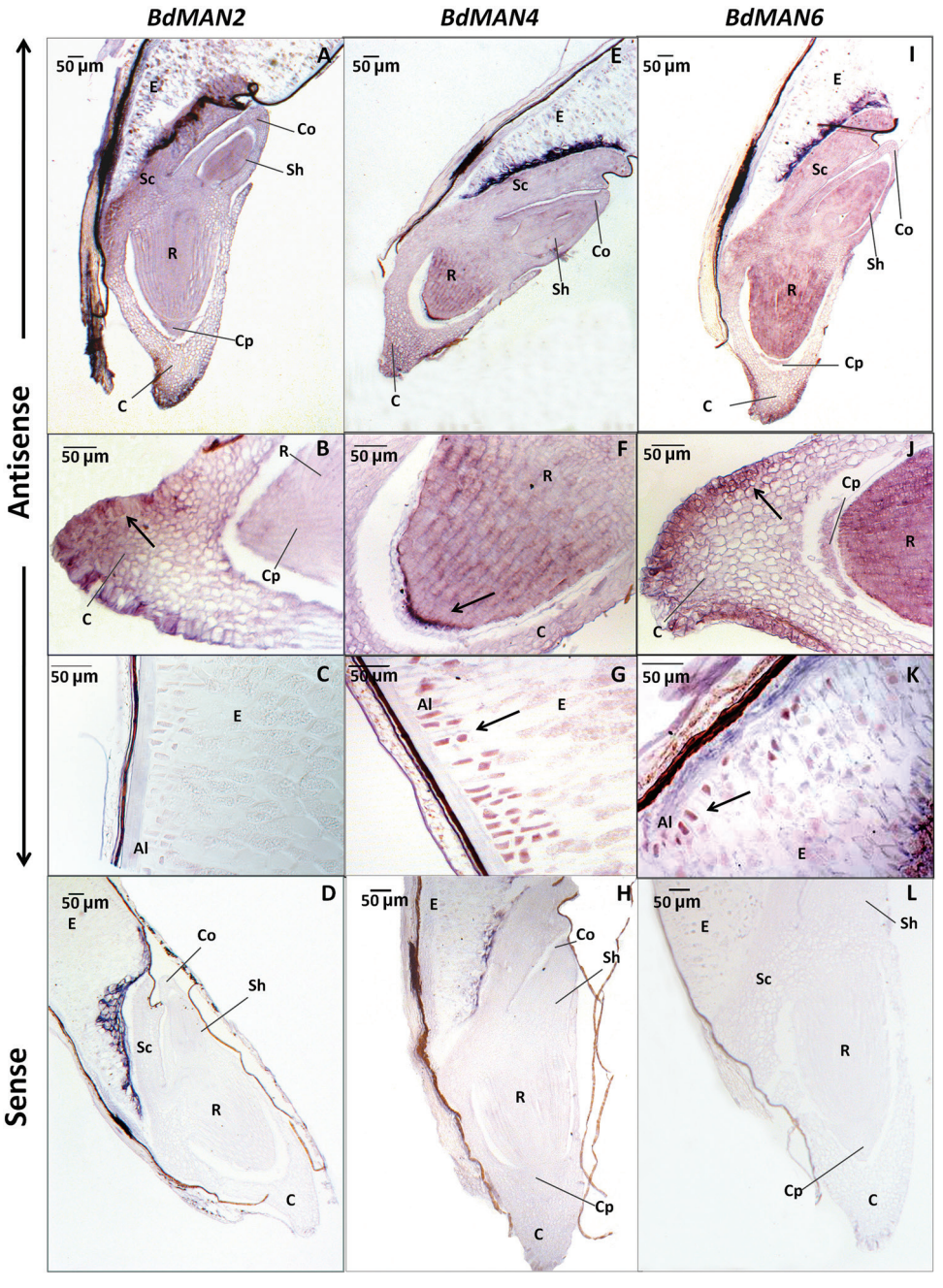

Fig. 6. *In situ* mRNA hybridization analysis of *BdMAN2*, *BdMAN4* and *BdMAN6* in 27 h germinating *Brachypodium* seeds. (A–D) *BdMAN2,* (E–H) *BdMAN4*, (I–L) *BdMAN6.* (A, E, I) Longitudinal sections of germinating embryos. (B, F, J) Close-up of the coleorhiza and the root tip. (C, G, K) Close-up of the endosperm and the aleurone. (D, H, L) Control sense probes. Al, aleurone layer; C, coleorhiza; Co, coleoptile; Cp, calyptra; E, endosperm; Sc, scutellum; Sh, shoot; R, root. The black arrow indicates the localization of transcripts. Scale bar, 50 µm.

germinating embryos and their transcripts are localized to the coleorhiza and the root, and *BdMAN4* and *BdMAN6* appear also in the aleurone layer. These facts indicate that the BdMAN enzymes should be spatially distributed in the seed in the vicinity of their putative substrates, thus contributing to the mannan hydrolysis and to the loosening of the coleorhiza cell walls, thereby facilitating root protrusion (germination *sensu stricto*).

During seed development, *BdMAN1*, *BdMAN2* and *BdMAN6* genes are expressed, and their mRNAs are abundant at the middle and late maturation stages. Upon cereal seed maturation, several tissues undergo a progressive enlargement of their cells, a process that involves nutrient remobilization and CW softening to allow cell expansion; to this aim, the participation of a complex set of hydrolytic enzymes have been described (Domínguez and Cejudo, 2014). These data indicate that the BdMAN1, BdMAN2 and BdMAN6 proteins could contribute to such a process during *Brachypodium* seed development.

The enzymatic analysis in the embryos of germinating seeds shows a maximum of MAN activity at 24 h of imbibition, just before the coleorhiza emergence (CE_{50}=30 h), and this enzymatic activity progressively decreases to 50% at 42 h. However, MAN activity in de-embryonated seeds

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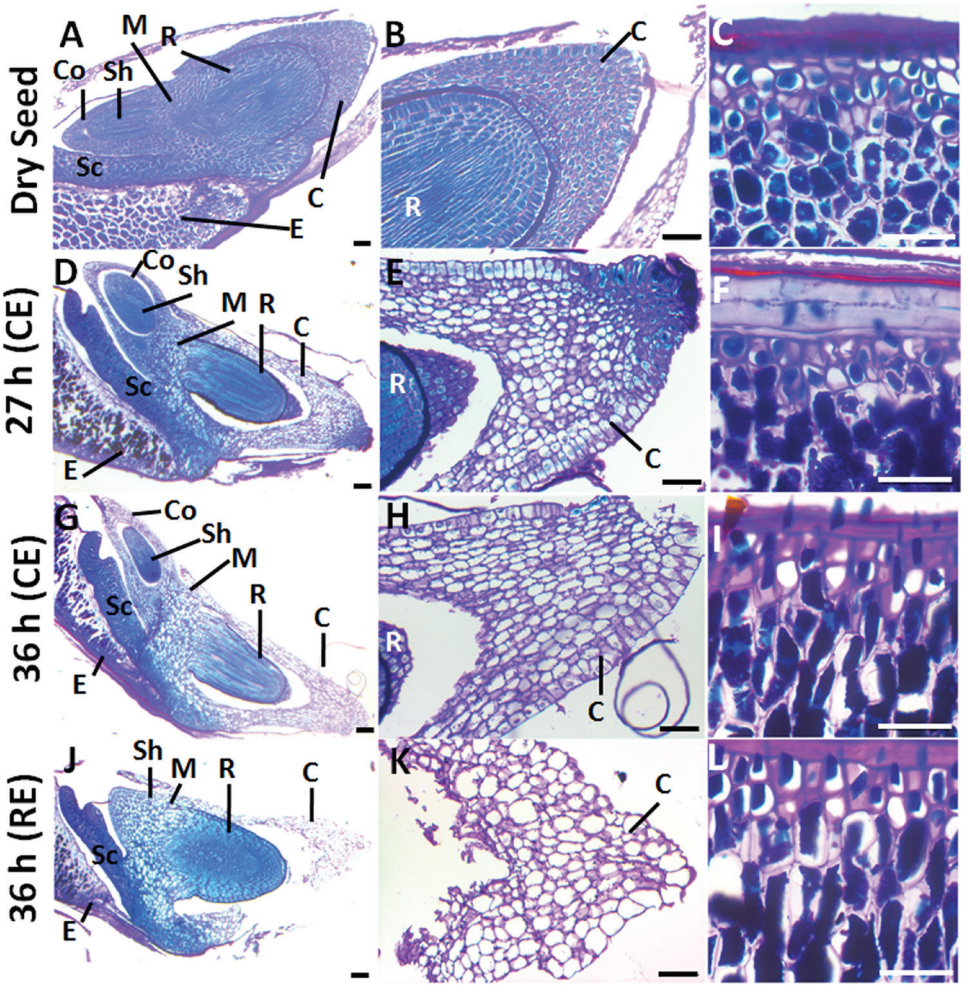

Fig. 7. Polysaccharide and protein mobilization upon *B. distachyon* seed germination. Bright field microscopy of longitudinal seed sections stained with PAS-Naphthol Blue Black. (A, D, G, J) Longitudinal sections from dry and water-imbibed seeds at 27 h and 36 h. (B, E, H, K) Close-up of the coleorhiza in A, D, G, J and (C, F, I, L) close-up of the endosperm in A, D, G, J, respectively. Proteins stain in blue and polysaccharide-rich cell walls in pink. C, coleorhiza; Co, coleoptile; E, endosperm; M, mesocotile; Sc, scutellum; Sh, shoot; R, root. Scale bar: 50 μm.

(endosperms) is low, with a maximum at 36 h of imbibition, when germination *sensu stricto* is almost completed [~100% coleorhiza emergence (CE); ~80% root emergence (RE) at 42 h]. These data point out to a more important role of the MAN activity in the embryo than in the endosperm during germination *sensu stricto*. In rice, MAN activity and expression of the *OsMAN1*, *OsMAN2* and *OsMAN6* genes have been detected in the aleurone layer only after 48 h of imbibition when 100% of germination has been achieved. This MAN activity is associated with reserve mobilization, a clear post-germinative event (Ren *et al.*, 2008). In barley, the HvMAN1 enzyme has been purified from 10-day-old seedlings and its catalytic parameters established, although its physiological role has not been investigated (Hrmova *et al.*, 2006).

The function of the coleorhiza tissue in the grasses has been classically associated to a protective function of the growing root during germination, but other physiological functions are being uncovered, such as our observations of the hydrolysis of proteins (disappearance of PBs) or the decrease in mannan content detected within the coleorhiza cells during *Brachypodium* germination *sensu stricto*. Nowadays, and similarly to what has been proposed for the endosperm of eudicot seeds (Piskurewicz *et al.*, 2009), the coleorhiza is being considered to be a key tissue preventing root emergence in dormant barley seeds (Millar *et al.*, 2006; Barrero *et al.*, 2009). These authors have hypothesized that root emergence may not depend only on the softening of the coleorhiza, driven by CW remodelling enzymes, but also by the expansive force of the imbibing root cells. Important transcriptional changes in the barley coleorhiza associated to the dormancy degree have been found and these differences affect mainly the expression of CW modifying genes (mannanases among them), nitrate and nitrite reductase genes etc. (Barrero *et al.*, 2009). The cytosolic nitrate reductase is an important source of the hormone nitric oxide (NO) that is involved in promoting seed germination (Arc *et al.*, 2013). Therefore, the coexistence at the coleorhiza of NO and mannanases, and perharps proteases of the CatepsinB3 type (Iglesias-Fernández *et al.*, 2014), should have an influence in the seed germination of the grasses.

The *Arabidopsis* radicle tip has been described as the primary location of growth-promoting genes and its

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surrounding-cells the centre for CW expansion (Bassel *et al.*, 2014). Moreover, a dual enzymatic activity for MAN (hydrolase and transglycosylase activities) has been described; the transglycosylase activity being more related to cell expansion, as occurs in the radicle before protrusion, and the hydrolytic activity could be relevant for weakening of the CWs of the embryo-surrounding tissues (Schröder *et al.*, 2009; Iglesias-Fernández *et al.*, 2011*a*, *b*). It is remarkable that the *BdMAN4* transcripts are localized to the aleurone layer during imbibition (27 h), when practically no MAN activity is detected in the de-embryonated (endosperm) seed, suggesting a possible accumulation of these transcripts and their corresponding proteins as inactive forms in the aleurone cells. Interestingly, the BdMAN4 deduced protein sequence has a predicted signal peptide for the secretory pathway and it is possible that the BdMAN4 isozyme could be transported later on during post-germinative reserve mobilization from the aleurone to the endosperm cells through the apoplastic space. In *Arabidopsis* the AtMAN7 and in poplar the PtrMAN6 proteins also contain signal peptides and have been localized to the apoplast, indicating that the mature MANs could be mobilized to the outer space (Iglesias-Fernández *et al.*, 2013; Zhao *et al.*, 2013).

Several authors have proposed that polysaccharides (β-1,3-1,4-glucans, mannans and others) present at the endosperm CWs of the Poaceae grains, not only have a structural function, but also a storage role (Guillón *et al.*, 2012). Heteromannans, although globally less abundant in these seeds than the β-glucans, are concentrated not only in the coleorhiza and in the root but also these polymers are found in the aleurone layer and storage endosperm of *B. distachyon* (Guillón *et al.*, 2011). In this context, our data demonstrate that MAN activity is important for the weakening of the coleorhiza cell walls and for the expansion of the root cells, thus facilitating germination *sensu stricto*, but it may be also important for the mannan hydrolysis in endosperm during post-germinative reserve mobilization.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Transcription levels of the housekeeping (*BdGAPDH* gene), presented as Ct mean values, in different organs, in developing seeds and during seed germination of *B. distachyon*.

Supplementary Fig. S2. Transcripts analysis by RTqPCR of the *BdMAN1-6* genes in different organs.

Supplementary Fig. S3. Expression analysis by RT-qPCR of *BdMAN1-6* genes in developing seeds and germinating seeds.

Supplementary Table S1. Major biochemical characteristics of *Brachypodium distachyon* and *Oryza sativa* endo-β-mannanase proteins.

Supplementary Table S2. Oligonucleotide sequences, amplicon length and PCR efficiency of the primers used for RT-qPCR analyses.

Supplementary Table S3. Primers used for the synthesis of the *in situ* mRNA hybridization probes.

Acknowledgments

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References

Akiyama T, Pillai MA, Sentoku N. 2004. Cloning, characterization and expression of OsGLN2, a rice endo-1,3-beta-glucanase gene regulated developmentally in flowers and hormonally in germinating seeds. *Planta* **220,** 129–139.

Arc E, Galland M, Godin B, Cueff G, Rajjou L. 2013. Nitric oxide implication in the control of seed dormancy and germination. *Frontiers in Plant Science* **4,** 346.

Bailey TL, Boden M, Buske FA, Frith M, Grant CE, Clementi L, Ren J, Li WW, Noble WS. 2009. MEME SUITE: tools for motifs discovery and searching. *Nucleic Acids Research* **37,** W202–W208.

Barrero JM, Jacobsen JV, Talbot MJ, White RG, Swain SM, Garvin DF, Gubler F. 2012. Grain dormancy and light quality effects on germination in the model grass Brachypodium distachyon. *New Phytologist* **93,** 376–386.

Barrero JM, Talbot MJ, White RG, Jacobsen JV, Gubler F. 2009. Anatomical and transcriptomic studies of the coleorhiza reveal the importance of this tissue in regulating dormancy in barley. *Plant Physiology* **150,** 1006–1021.

Bassel GW, Stamm P, Mosca G, Barbier de Reuille P, Gibbs DJ, Winter R, Janka A, Holdsworth MJ, Smith RS. 2014. Mechanical constraints imposed by 3D cellular geometry and arrangement modulate growth patterns in the *Arabidopsis* embryo. *Proceedings of the National Academy of Sciences USA* **111,** 8685–8690.

Bateman A, Birney E, Cerruti L, Durbin R, Etwiller L, Eddy SR, Griffiths-Jones S, Howe KL, Marshall M, Sonnhammer EL. 2002. The Pfam protein families database. *Nucleic Acids Research* **30,** 276–280.

Burton RA, Fincher GB. 2009. (1,3;1,4)-beta-D-glucans in cell walls of the poaceae, lower plants, and fungi: a tale of two linkages. *Molecular Plant* **2,** 873–882.

Carrillo-Barral N, Matilla AJ, Rodríguez-Gacio Mdel C, Iglesias-Fernández R. 2014. Nitrate affects *sensu-stricto* germination of after-ripened *Sisymbrium officinale* seeds by modifying expression of *SoNCED5*, *SoCYP707A2* and *SoGA3ox2* genes. *Plant Science* **217–218,** 99–108.

Domínguez F, Cejudo FJ. 2014. Programmed cell death (PCD): an essential process of cereal seed development and germination. *Frontiers in Plant Science* **5,** 366.

Emanuelsson O, Brunnak S, von Heijne G, Nielsen H. 2007. Locating proteins in the cell using TargetP, SignalP and related tools. *Nature Protocols* **2,** 643–652.

Endo A, Tatematsu K, Hanada K, Duermeyer L, Okamoto M, Yonekura-Sakakibara K, Saito K, Toyoda T, Kawakami N, Kamiya Y, Seki M, Nambara E. 2012. Tissue-specific transcriptome analysis reveals cell wall metabolism, flavonol biosynthesis and defense responses are activated in the endosperm of germinating Arabidopsis thaliana seeds. *Plant Cell Physiology* **53,** 16–27.

Ferrandiz C, Liljegren S, Yanofsky M. 2000. FRUITFULL negatively regulates the SHATTERPROOF genes during *Arabidopsis* fruit development. *Science* **289,** 436–438.

Gao F, Ayele BT. 2014. Functional genomics of seed dormancy in wheat: advances and prospects. *Frontiers in Plant Science* **5,** 458.

Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkins MR, Appel RD, Bairoch A. 2005. Protein identification and analysis tools on the ExPASy server. In: Walker JK, ed. *The Proteomics Protocols Handbook* . New York: Humana Press, 571–607.

Girin T, David LC, Chardin C, Sibout R, Krapp A, Ferrario-Méry S, Daniel-Vedele F. 2014. *Brachypodium*: a promising hub between model species and cereals. *Journal of Experimental Botany* **65,** 5683–5696.

Goodstein DM, Shu S, Howson R, Fazo J, Mitros T, Dirks W, Hellsten U, Putnam N, Rokhsar DS. 2012. Phytozome: a comparartive platform for green plant genomics. *Nucleic Acids Research* **40,** D1178–D1186.

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González-Calle V, Iglesias-Fernández R, Carbonero P, Barrero-Sicilia C. 2014. The BdGAMYB protein from Brachypodium distachyon interacts with BdDOF24 and regulates transcription of the BdCathB gene upon seed germination. *Planta* **240,** 539–552.

Guillon F, Bouchet B, Jamme F, Robert P, Quemener B, Barron C, Larre C, Dumas P, Saulnier L. 2011. *Brachypodium distachyon* grain: characterization of endosperm cell walls. *Journal of Experimental Botany* **62,** 1001–1015.

Guillon F, Larré C, Petipas F, Berger A, Moussawi J, Rogniaux H, Santoni A, Saulnier L, Jamme F, Miquel M, Lepiniec L, Dubreucq B. 2012. A comprehensive overview of grain development in *Brachypodium distachyon* variety Bd21. *Journal of Experimental Botany* **63,** 739–755.

Hernando-Amado S, González-Calle V, Carbonero P, Barrero-Sicilia C. 2012. The family of DOF transcription factors in *Brachypodium distachyon*: phylogenetic comparison with rice and barley DOFs and expression profiling. *BMC Plant Biology* **12,** 202.

Hong SY, Seo PJ, Ynag MS, Xiang F, Park CM. 2008. Exploring valid reference genes for gene expression studies in *Brachypodium distachyon* by real-time PCR. *BMC Plant Biology* **8,** 112.

Hrmova M, Burton RA, Biely P, Lahnstein J, Fincher GB. 2006. Hydrolysis of (1,4)-β-D-mannans in barley (*Hordeum vulgare* L.) is mediated by the concerted action of (1,4)- β-D-mannan endohydrolase and β-D-mannosidase. *Biochemical Journal* **399,** 77–90.

Iglesias-Fernández R, Matilla AJ. 2010. Genes involved in ethylene and gibberellins metabolism are required for endosperm-limited germination of *Sisymbrium officinale* L. seeds. *Planta* **231,** 653–664.

Iglesias-Fernández R, Barrero-Sicilia C, Carrillo-Barral N, Oñate-Sanchez L, Carbonero P. 2013. *Arabidopsis thaliana* bZIP44: a transcription factor affecting seed germination and expression of the mannanase-encoding gene *AtMAN7*. *The Plant Journal* **74,** 767–780.

Iglesias-Fernández R, Rodríguez-Gacio MC, Barrero-Sicilia C, Carbonero P, Matilla AJ. 2011*a*. Three endo-β-mannanase genes expressed in the micropilar endosperm and in the radicle influence germination of *Arabidopsis thaliana* seeds. *Planta* **233,** 25–36.

Iglesias-Fernández R, Rodríguez-Gacio MC, Barrero-Sicilia C, Carbonero P, Matilla A. 2011*b*. Molecular analysis of endo-β-mannanase genes upon seed imbibition suggest a cross-talk between radicle and micropylar endosperm during germination of *Arabidopsis thaliana*. *Plant Signaling & Behavior* **6,** 80–82.

Iglesias-Fernández R, Wozny D, Iriondo-de Hond M, Oñate-Sánchez L, Carbonero P, Barrero-Sicilia C. 2014. The AtCathB3 gene, encoding a cathepsin B-like protease, is expressed during germination of Arabidopsis thaliana and transcriptionally repressed by the basic leucine zipper protein GBF1. *Journal of Experimental Botany* **65,** 2009–2021.

International Brachypodium Initiative. 2010. Genome sequencing and analysis of the model grass *Brachypodium distachyon*. *Nature* **463,** 736–768.

Lagrimini LM, Burkhart W, Moyer M, Rothstein S. 1987. Molecular cloning of complementary DNA encoding the lignin-forming peroxidase from tobacco: molecular analysis and tissue-specific expression. *Proceedings of the National Academy of Sciences USA* **84,** 7542–7546.

Lee KJ, Dekkers BJ, Steinbrecher T, Walsh CT, Bacic A, Bentsink L, Leubner-Metzger G, Knox JP. 2012. Distinct cell wall architectures in seed endosperms in representatives of the Brassicaceae and Solanaceae. *Plant Physiology* **160,** 1551–1566.

Leubner-Metzger G. 2005. Beta-1,3-glucanase gene expression in low-hydrated seeds as a mechanism for dormancy release during tobacco after-ripening. *The Plant Journal* **41,** 133–145.

Leubner-Metzger G, Meins F Jr. 2000. Sense transformation reveals a novel role for class I beta-1, 3-glucanase in tobacco seed germination. *The Plant Journal* **23,** 215–221.

Lever, M. 1977. Carbohydrate determination with 4-hydroxybenzoic acid hydrazide (PAH-BAH): Effect of bismuth on the reaction. *Analytical Biochemistry* **81,** 21–27.

Marcus SE, Blake AW, Benians TA, *et al.* 2010. Restricted access of proteins to mannan polysaccharides in intact plant cell walls. *The Plant Journal* **64,** 191–203.

Martínez-Andújar C, Pluskota WE, Bassel GW, *et al.* 2012. Mechanisms of hormonal regulation of endosperm cap-specific gene expression in tomato seeds. *The Plant Journal* **71,** 575–586.

Millar AA, Jacobsen JV, Ross JJ, Helliwell CA, Poole AT, Scofield G, Reid JB, Gubler F. 2006. Seed dormancy and ABA metabolism in *Arabidopsis* and barley: the role of ABA 8'-hydroxylase. *The Plant Journal* **45,** 942–954.

Mochida K, Shinozaki K. 2013. Unlocking Triticeae genomics to sustainably feed the future. *Plant Cell Physiology* **54,** 1931–1950.

Müller K, Tintelnot S, Leubner-Metzger G. 2006. Endosperm-limited *Brassicacae* seed germination: abscisic acid inhibits embryo-induced endosperm weakening of *Lepidium sativum* (cress) and endosperm rupture of cress and *Arabidopsis thaliana*. *Plant Cell Physiology* **47,** 864–977.

Nonogaki H. 2014. Seed dormancy and germination-emerging mechanisms and new hypotheses. *Frontiers in Plant Science* **5,** 233.

Nonogaki H, Chen F, Bradford K. 2007. Mechanisms and genes involved in germination *sensu stricto*. In: Bradford K, Nonogaki H, eds. *Seed Development, Dormancy and Germination* . Oxford: Blackwell, 264–304.

Nonogaki H, Gee OH, Bradford KJ. 2000. A germination-specific endo-beta-mannanase gene is expressed in the micropylar endosperm cap of tomato seeds. *Plant Physiology* **123,** 1235–1245.

Oñate-Sanchez L, Vicente-Carbajosa J. 2008. DNA-free RNA isolation protocols for *Arabidopsis thaliana*, including sedes and siliques. *BMC Research Notes* **1,** 93.

Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research* **29,** e45.

Piskurewicz U, Tureckova V, Lacombe E, Lopez-Molina L. 2009. Far-red light inhibits germination through DELLA-dependent stimulation of ABA synthesis and ABI3 activity. *EMBO Journal* **28,** 2259–2271.

Rancour DM, Marita JM, Hatfield RD. 2012. Cell wall composition throughout development for the model grass *Brachypodium distachyon*. *Frontiers in Plant Science* **3,** 266.

Ren YF, Bewley JD, Wang XF. 2008. Protein and gene expression patterns of endo-ß-mannanase following germination of rice. *Seed Science Research* **8,** 139–149.

Rodríguez-Gacio MC, Iglesias-Fernández R, Carbonero P, Matilla AJ. 2012. Softening-up mannan-rich cell walls. *Journal of Experimental Botany* **63,** 3975–3988.

Sargent JA, Osborne DJ. 1980. A comparative study of the fine structure of coleorhiza and root cells during the early hours of germination of rye embryos. *Protoplasma* **104,** 91–103.

Scheler C, Weitbrecht K, Pearce SP, *et al.* 2014. Promotion of testa rupture during *Lepidium sativum* germination involves seed compartment-specific expression and activity of pectin methylesterases. *Plant Physiology* pp.114.247429.

Schröder R, Atkinson RG, Redgwell RJ. 2009. Re-interpreting the role of endo-β-mannanases as mannan endotransglycosylase/hydrolases in the plant cell wall. *Annals of Botany* **104,** 197–204.

Takeda H, Sugahara T, Kotake T, Nakagawa N, Sakurai N. 2010. Sugar treatment inhibits IAA-induced expression of *endo-1,3:1,4-beta-glucanase EI* transcripts in barley coleoptile segments. *Physiologia Plantarum* **139,** 413–420.

Tamura K, Dudley J, Nei M, Kumar C. 2007. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24,** 1596–1599.

Thompson J, Higgins D, Gibson T. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22,** 4673–4680.

Weitbrecht K, Muller K, Leubner-Metzger G. 2011. First off the mark: early seed germination. *Journal of Experimental Botany* **62,** 3289–3309.

Yan D, Duermeyer L, Leoveanu C, Nambara E. 2014. The functions of the endosperm during seed germination. *Plant Cell and Physiology* **55,** 1521–1533.

Yuan JS, Yang X, Lai J, Lin H, Cheng Z-M, Nonogaki H, Chen F. 2007. The endo-β mannanase gene families in *Arabidopsis*, rice, and poplar. *Functional and Integrative Genomics* **7,** 1–16.

Zhao Y, Song D, Sun J, Li L. 2013. *Populus* endo-beta-mannanase PtrMAN6 plays a role in coordinating cell wall remodeling with suppression of secondary wall thickening through generation of oligosaccharide signals. *The Plant Journal* **74,** 473–485.

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