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Protein and gene expression patterns of endo-β-mannanase following germination of rice

Yanfang Ren[1†], J. Derek Bewley[2] and Xiaofeng Wang[1*]

[1]Laboratory of Seed Science and Technology, College of Life Sciences, South China Agricultural University, Guangzhou 510642, China; [2]Department of Molecular and Cellular Biology, University of Guelph, Guelph, Ontario N1G 2W1, Canada

Abstract

The rice (*Oryza sativa* L.) cv. Taichung 65, a *japonica* subspecies, was used to characterize the isoform, protein and gene expression patterns of endo-β-mannanase during and after seed germination. Activity assays and isoform analyses of whole grains or seed parts (scutellum, aleurone layer and starchy endosperm) revealed that seeds began to express endo-β-mannanase activity at 48 h from the start of imbibition at 25°C, after the completion of germination of most seeds. Three isoforms of endo-β-mannanase (pI 8.86, pI 8.92 and pI 8.98) were detected in the aleurone layer and starchy endosperm, but only two (pI 8.86 and pI 8.92) were present in the scutellum. The endo-β-mannanase in the starchy endosperm was mainly from the aleurone layer. Western blot analysis, using a tomato anti-endo-β-mannanase antibody, indicated that an endo-β-mannanase protein was present in an inactive form in dry grains. The amount of this protein decreased in the scutellum, but increased in the aleurone layer during and after germination. Thus, the increase in endo-β-mannanase activity in rice grains may be due to the activation of extant proteins and/or the *de novo* synthesis of the enzyme. Northern blot analysis showed that four putative rice endo-β-mannanase genes (*OsMAN1*, *OsMAN2*, *OsMAN6* and *OsMANP*) were expressed in germinating and germinated rice grains. However, *OsMANP* was not expressed in the scutellum. The amount of *OsMAN6* mRNA decreased after the completion of germination and paralleled the decline in endo-β-mannanase protein. In the aleurone layer, the increase of *OsMAN2*, *OsMAN6* and *OsMANP* mRNA was prior to the increase of endo-β-mannanase protein.

Keywords: aleurone layer, endo-β-mannanase, endosperm, germination, *Oryza sativa*, scutellum

Introduction

Galactomannans, in which a (1–4)-β-mannan backbone is substituted at the 6-carbon with single α-galactosyl residues, are a major component of hemicelluloses deposited in the cell walls of a variety of higher plant species (Bewley *et al.*, 1997). For example, cell walls of the tomato and lettuce endosperm, which completely surrounds the embryo, contain large amounts of galactomannans (Halmer and Bewley, 1979; Groot *et al.*, 1988; Nonogaki *et al.*, 1995). The degradation of galactomannans plays an important role in the germination and/or post-germination of these seeds (Bewley, 1997). Weakening of the tomato micropylar endosperm, as a prerequisite for the completion of seed germination, involves hydrolysis of galactomannans in the cell walls of this tissue (Nonogaki *et al.*, 1992, 1998, 2000; Bewley, 1997). Then, following germination, hydrolysis and mobilization of galactomannans in the cell walls of the lateral endosperm provide a source of nutrients for early seedling growth (Bewley, 1997; Wang *et al.*, 2004).

The complete breakdown of galactomannans requires the cooperation of three enzymes, endo-β-mannanase (EC 3.2.1.78), β-mannosidase (EC 3.2.1.25) and α-galactosidase (EC 3.2.1.22). Of these, endo-β-mannanase, which cleaves the β-1,4 linkages between the mannose residues in the mannan backbone, is the enzyme that initiates the hydrolysis of galactomannans.

Endo-β-mannanase has been investigated extensively in relation to seed germination of some dicotyledonous species. Its activity increases in the micropylar endosperm prior to the completion of seed

*Correspondence
Fax: (86) 20-85282180
Email: xfwang@scau.edu.cn
†Present address: College of Agriculture, Guizhou University, Guiyang 550025, China

germination of *Datura ferox* (Sánchez *et al.*, 1990; Sánchez and de Miguel, 1997) and tomato (Nomaguchi *et al.*, 1995; Toorop *et al.*, 1996). However, endo-β-mannanase activity in seeds of lettuce, celery, date, Chinese senna, pepper and some legume species increases only after the completion of germination, and is regarded as important for the mobilization of galactomannan reserves to support the early growth of seedlings (Bewley, 1997; Gong *et al.*, 2005).

Different isoforms occur in the micropylar and lateral endosperm of tomato seeds (Nonogaki and Morohashi, 1996; Voigt and Bewley, 1996). Endo-β-mannanase has been purified or partially purified from seeds of several species (Dulson and Bewley, 1989; Halmer, 1989; Nonogaki *et al.*, 1995; Marraccini *et al.*, 2001). Gene sequences encoding endo-β-mannanase have been obtained from tomato (Bewley *et al.*, 1997; Nonogaki *et al.*, 2000; Gong and Bewley, 2007), coffee (Marraccini *et al.*, 2001), carrot (Homrichhausen *et al.*, 2003) and lettuce (Wang *et al.*, 2004) seeds.

In contrast to the extensive investigation of endo-β-mannanase in dicotyledonous seeds, only a few papers have been published about this enzyme in monocotyledonous seeds (DeMason *et al.*, 1985; Dirk *et al.*, 1995; Wang *et al.*, 2005; Hrmova *et al.*, 2006). Endo-β-mannanase activity has been detected in the endosperm of date palm (*Phoenix dactylifera* L.) only after the completion of germination (DeMason *et al.*, 1985; Gong *et al.*, 2005). Several to many isoforms of endo-β-mannanase are present in grains of barley, oats, wheat and rice (Dirk *et al.*, 1995). Previous studies have demonstrated that there is an increase in endo-β-mannanase activity in rice grains, mostly after the completion of germination (Wang *et al.*, 2005). Gibberellic acid (GA) increases and abscisic acid (ABA) decreases this endo-β-mannanase activity. The major site for the production of endo-β-mannanase activity is in the aleurone layer, although there is a requirement for the presence of the embryo. However, GA can increase endo-β-mannanase activity of embryoless half-seeds of rice. Recently endo-β-mannanase was purified from germinated barley grains, and a gene encoding this enzyme was cloned (Hrmova *et al.*, 2006).

Changes in activity of endo-β-mannanase have been investigated in rice (Wang *et al.*, 2005), several isoforms of this enzyme have been reported (Dirk *et al.*, 1995), and nine endo-β-mannanase genes have been identified from its genome by *in silico* sequence analysis (Yuan *et al.*, 2007). Yet the isoform, protein and gene expression patterns of this enzyme during and following rice grain germination are still unclear. Here, changes in the profile of endo-β-mannanase protein and activity during and following germination are reported. Also, because there are four sequences in the genome of rice in GenBank (http://www.ncbi.nlm.nih.gov) that are labelled as putative (1–4)-β-mannan endohydrolases, these genes were partially cloned and their expression patterns determined.

Materials and methods

Germination

Grains of rice (*Oryza sativa* L.) cv. Taichung 65, a *japonica* subspecies, were used. The lemma and palea were removed by hand before imbibition. Triplicate lots of 50 dehulled grains were placed on water-moistened filter paper in Petri dishes and incubated at 25°C in the dark. They were counted every 12 h, and those with protruded radicles were regarded as having germinated.

Enzyme extraction and activity assay

For whole dehulled grains, duplicate lots of five imbibed grains were ground in an ice-cold mortar in 300 μl of 0.1 M Hepes buffer (pH 8.0). For parts of grains, duplicate lots of five imbibed grains were at first dissected into scutellum, starchy endosperm and aleurone layer, then ground separately in 200 μl of 0.1 M Hepes buffer (pH 8.0). The extract was centrifuged at 4°C for 10 min at 13,000 *g*, and the supernatant collected and used to assay endo-β-mannanase activity. Duplicate 2 μl aliquots of the supernatant were loaded into 2-mm-diameter wells in an agarose gel into which locust bean gum (Sigma, St. Louis, Missouri, USA) was added as substrate, and assayed using a gel-diffusion assay (Bourgault and Bewley, 2002). Degradation of galactomannans in the substrate gel, as shown by staining with Congo Red dye (Sigma), was calculated against a serial dilution of *Aspergillus niger* endo-β-mannanase (Megazyme, Bray, Eire) as standard.

Isoform detection

One microlitre aliquots of the enzyme extract and 10 μl pI marker (IEF-MIX 3.6-9.3, Sigma) were pipetted into the slots of an application strip (Sigma) on ultrathin-layer polyacrylamide gels (0.12 mm thick). Isoelectric focusing was carried out with a Desaga (Heidelberg, Germany) horizontal electrophoresis unit connected to a cooling apparatus (Van Der Heijden, Germany) at 10°C. Ultrathin-layer polyacrylamide gels were cast as described previously (Zhao *et al.*, 2005). The polymerization solution for each gel contained 0.16 g taurine, 5 ml acrylamide (T = 6.8%, C = 2.5%), 0.44 ml of pH 7–9 ampholytes (Servalyt, Heidelberg, Germany), 4 μl *N,N,N′,N′*-tetramethylethylenediamine and 30 μl of 20% (w/v) ammonium peroxydisulphate. The power supply of the electro-

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phoresis apparatus was set at 1000 V, and the mA adjusted to give an output voltage of approximately 200 V. After the completion of electrophoresis (*c.* 70 min), the gel was removed from the apparatus; the section of the gel containing the pI marker was cut and stained with 0.015% (w/v) Coomassie Brilliant Blue R 250, 0.045% (w/v) Coomassie Brilliant Blue G250, 11% (v/v) acetic acid, 18% (v/v) ethanol and 71% (v/v) double-distilled water (ddH_2O) for 30 min; and then destained with ethanol solution [30% (v/v) ethanol, 5% (v/v) acetic acid and 65% (v/v) ddH_2O] for 30 min or longer until the background was clear. The remainder of the IEF gel was laid on top of an activity gel as described by Dirk *et al.* (1995). The gel sandwich was incubated in a moist chamber at 25°C for about 30 min before washing in McIlvaine buffer (pH 7.0) for 30 min, staining in 0.5% (w/v) Congo Red dye for 30 min, washing in water for 2 min, fixing in 80% (v/v) ethanol for 10 min, washing in three changes of McIlvaine buffer (pH 7.0) for 20 min each and finally developing in 1 M NaCl overnight, all with gentle agitation. The isoform bands were shown as the clearing zones on the activity gel. The pIs of the isoforms were calculated by comparing the positions of the bands on the activity gel with the pI marker bands.

SDS-PAGE and immunoblotting

Protein content of each enzyme extraction was determined using the Bio-Rad protein assay kit (with bovine serum albumin as the standard). Proteins were separated by SDS-PAGE using 10% (w/v) acrylamide separation gels. After electrophoresis, proteins were transferred to nitrocellulose membranes (Amersham Biosciences, Sweden), which were blocked with 5% (w/v) skimmed milk powder in TTBS (Tween–Tris buffered saline), for 2–4 h at room temperature. The membranes were washed twice with TTBS for 10 min before transfer to the first antibody solution in 3% (w/v) powdered skimmed milk in TTBS for 1–2 h. Antibodies developed against endo-β-mannanase from tomato seeds (kindly provided by Dr Hiro Nonogaki, Oregon State University) were used for immunoblotting at 1 : 1000 dilution. Bound antibody was detected using horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (Sigma). The bands were detected on X-ray film after the reaction with the Detection Reagents 1 and 2 (Amersham Biosciences).

Cloning of cDNA fragments of four putative (1–4)-β-mannan endohydrolases

Four sequences in the rice genome in the GenBank database have been designated as being for (1–4)-β-mannan endohydrolase (BAB91747, BAB56016, BAD61770 and BAD17132). Genes encoding those four proteins have been termed *OsMAN1*, *OsMAN2*, *OsMAN6* and *OsMANP* by Yuan *et al.* (2007). To clone non-homologous cDNA fragments for each of these four genes for use as probes in Northern blot analysis, specific primers were designed and are listed in Table 1.

Total RNA was extracted from the scutellum and aleurone layer of rice grains following the method of Shen *et al.* (2003). First-strand cDNA was synthesized by using oligo (dT) as primer and RevertAid™ M-MuLV reverse transcriptase (Promega, Madison, Wisconsin, USA) according to the manufacturer's instructions. The reverse transcription (RT) product was amplified by primers derived from four rice endo-β-mannanase gene sequences (Table 1) with the following polymerase chain reaction (PCR) conditions: initial denaturation at 94°C (4 min), 25 cycles at 94°C (30 s), at 56°C (30 s), and at 72°C (1 min), followed by extension at 72°C (10 min). The resulting fragments were cloned into the pGEM-T Easy vector (Promega), and sequenced.

Northern hybridization

Total RNA (40 μg) extracted from the scutellum and aleurone layer was subjected to electrophoresis on 1.2% (w/v) agarose gels containing 1.8% (v/v) formaldehyde, transferred to a nitrocellulose membrane (Hybond-N^+, Amersham Biosciences) with a solution

Table 1. Primers used for cloning fragments of four rice endo-β-mannanase genes and the lengths of the PCR products

Genes	Primers	Anticipated length (bp)	Obtained length (bp)
OsMAN1	F: AAGCCGCTGCTGATCGCAG R: TTTCCCGCCGTTGTGTCGG	330	330
OsMAN2	F: TGCCATCCCTCCCGTTCTC R: GGAATCGTTGGAGGCGGTG	446	540
OsMAN6	F: GAAGTAGGCTACCTCCAGC R: GGTGGTATTGAGCATTGCC	291	291
OsMANP	F: CTCCGCGCGACGCTGGTGA R: GGAAGTAGGCGTCCCGCGC	745	778

F, forward primer; R, reverse primer.

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of 0.1 N NaOH/3 M NaCl. cDNA probes were prepared according to the manufacturer's instructions for a Megaprimer DNA Labelling System (Amersham Biosciences). Membranes were prehybridized for 4 h at 42°C in prehybridization solution [3 × SSC, 5 × Denhardt's reagent, 50% (v/v) formamide, 1% (w/v) SDS, 100 mg/ml denatured salmon sperm DNA). Then membranes were hybridized at 42°C for approximately 30 h in hybridization solution [prehybridization solution, 10% (w/v) dextran sulphate, [^{32}P]dCTP]. After hybridization, membranes were briefly rinsed with formamide wash buffer [50% (v/v) formamide, 5 × SSC, 0.2% (w/v) SDS] at 42°C, then washed for 30 min at 42°C with the same buffer, and finally washed 2–3 times for 15 min with blot wash buffer [0.1 × SSC, 0.1% (w/v) SDS] at room temperature, before exposure to X-ray film at −80°C.

Results and discussion

Changes in endo-β-mannanase activity during and after germination

Grains of the rice cv. Taichung 65, a *japonica* subspecies, were used, in contrast to the *indica* subspecies for our previous studies (Wang *et al.*, 2005). Thus, the time courses of germination and endo-β-mannanase activity were first determined for this cultivar.

Emergence of the radicle from dehulled rice grains was first observed at about 16 h from the start of imbibition. All grains completed germination after being imbibed in water for 72 h (Fig. 1A). Activity of endo-β-mannanase in dehulled grains was first detected at 48 h from the start of imbibition, increasing up to 96 h, and then decreasing after 120 h (Fig. 1A). Thus, like the *indica* subspecies cv. Peiza 67, grains of *japonica* cv. Taichung 65 expressed endo-β-mannanase activity only after the completion of germination. To locate the enzyme activity, grains were dissected into the living aleurone layer, non-living starchy endosperm and living scutellum at various times following germination (Fig. 1B). Activity of endo-β-mannanase was first detected in the aleurone layer and starchy endosperm at 48 h from the start of imbibition, and increased thereafter. Enzyme activity in the scutellum was first detected 12 h later than that in the aleurone layer and starchy endosperm, and while it increased, its activity was well below that of the other seed parts.

Isoform patterns of endo-β-mannanase during and after germination

No isoform band of endo-β-mannanase was detected in whole grains or seed parts imbibed for less than 48 h, as anticipated from the activity data (Fig. 2).

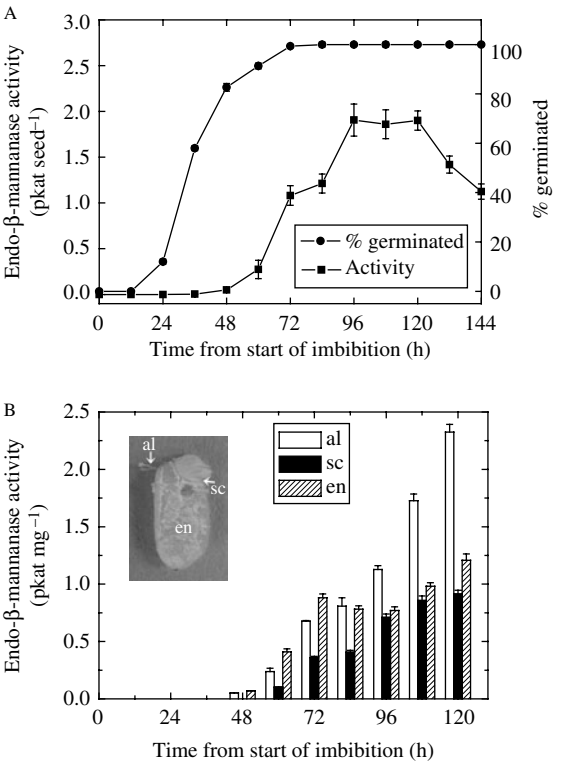

Figure 1. (A) Germination percentage and endo-β-mannanase activity in dehulled rice grains during and following germination at 25°C on water in the dark. Error bars indicate the variation in germination percentage between triplicate lots and enzyme activity between duplicate assays of duplicate extracts. (B) Endo-β-mannanase activity (per mg fresh weight) of different grain parts (aleurone layer, scutellum and starchy endosperm) of dehulled rice during and following germination at 25°C on water in the dark. Error bars indicate the variation in enzyme activity between duplicate assays of duplicate extracts. The inserted image illustrates seed parts: aleurone layer (al), scutellum (sc) and starchy endosperm (en).

At 48 h three isoforms with pI of 8.86, 8.92 and 8.98 were detected in the whole grains; the number of isoform bands remained unchanged up to 144 h. The isoform expression patterns of endo-β-mannanase in the aleurone layer were similar to that in whole grains. However, in the scutellum only two isoforms (pI 8.86 and 8.92), were detected after 60 h from the start of imbibition. In the starchy endosperm, only one isoform band (pI 8.86) was detected at 48 h, but from 60 h, the other two isoforms were present in this tissue. Thus, the isoform pattern of endo-β-mannanase in the scutellum is distinct from that of the aleurone layer, and the pattern in the starchy endosperm reflects that of the aleurone layer, indicating this is the sole source of the enzyme, which is secreted in this non-living tissue.

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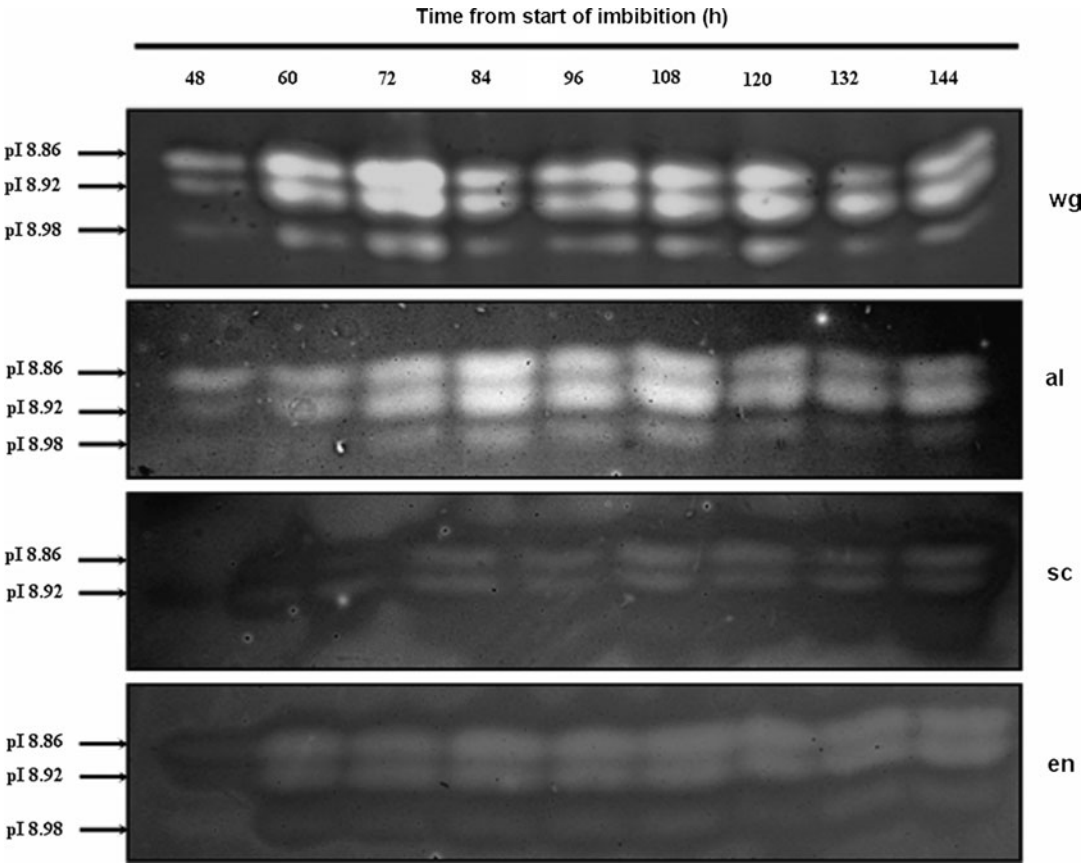

Figure 2. Isoforms of endo-β-mannanase in whole rice grain (wg), aleurone layer (al), scutellum (sc) and starchy endosperm (en).

Western blot analysis of endo-β-mannanase protein during and following germination

In some tissues, endo-β-mannanase protein can be detected antigenically, even though there is no enzyme activity. No antibody to the rice enzyme has been produced, so tomato seed enzyme antibody was used here. This is known to detect endo-β-mannanase in seeds and fruits of tomato (Bewley *et al.*, 2000), as well as in seeds of other species, e.g. fenugreek (Malek and Bewley, 1991; Gong, 2007). A clear band of the appropriate kDa was detected in extracts from tomato fruit and those from dry, germinating and germinated rice grains (Fig. 3A). The kDa values of different isozymes of endo-β-mannanases in tomato, as derived from their cDNA sequences, varies from 42 to 44 kDa (Gong and Bewley, 2007), and that obtained from lettuce is almost 44 kDa (Wang *et al.*, 2004). However, in the latter species, the kDa of active enzyme proteins in the seed itself, as detected using Western blot analysis, varies from 39 to 43 kDa (Nonogaki and Morohashi, 1999). This could be attributed to limited hydrolysis of the protein to make it smaller, without loss of activity, which would also account for the large number of isoforms that are detectable (Nonogaki and Morohashi, 1996). The size of the protein detected in rice grains by the antibody to tomato endo-β-mannanase is clearly within the range of those already known. Endo-β-mannanase proteins existed in dry (0 h) and early imbibed whole grains (24 h), in which no endo-β-mannanase activity could be detected (Fig. 1A, B), and the amount of enzyme protein did not change greatly up to 96 h from the start of imbibition, other than weaker detection at 72 h.

Compared with the whole grains, endo-β-mannanase protein amounts in the scutellum and aleurone layer changed greatly during and following germination (Fig. 3B). In the latter, enzyme protein was low in dry grains and in those imbibed until 60 h from the start of imbibition; then it increased. In marked contrast, endo-β-mannanase proteins in the scutellum of dry (0 h) and early germinating grains (up to 24 h) were at a higher level and decreased after the completion of germination. Thus, the initial amount of enzyme protein in the grain is presumably that which is synthesized during development, and stored

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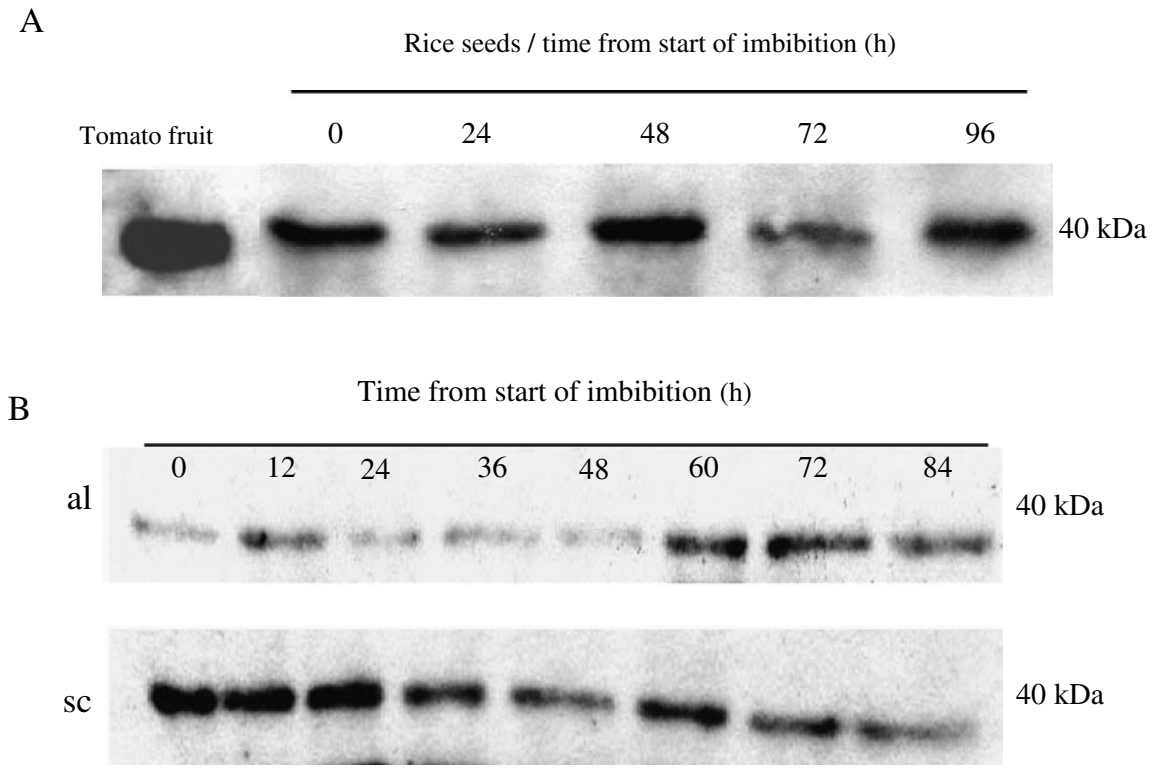

Figure 3. Western blot detection of endo-β-mannanase proteins extracted from (A) whole grains and (B) the aleurone layer (al) and scutellum (sc) of rice during and following germination. In (A), the lane on the left was loaded with an extract from tomato fruit. The amount of protein loaded in each lane was 30 μg.

in the dry grain, albeit in an inactive form. Later, the enzyme protein that is present is due to its increase in the aleurone layer. There is a poor coincidence between endo-β-mannanase protein and activity of this enzyme during germination and early seedling growth. It is not known if later activity is due to an increase due to *de novo* synthesis, or to activation of existing protein, or both. In an effort to resolve this, Northern blot analysis of endo-β-mannanase transcripts was carried out.

Northern blot analysis of four rice endo-β-mannanase transcripts during and following germination

To clone partial cDNA fragments of *OsMAN1*, *OsMAN2*, *OsMAN6* and *OsMANP* as probes for Northern blot analysis, four pairs of primers were designed (Table 1) based on the gene sequences recorded in GenBank. After RT-PCR amplification, fragments of 330, 540, 291 and 778 bp were obtained for *OsMAN1*, *OsMAN2*, *OsMAN6* and *OsMANP*, respectively (Table 1). The obtained partial cDNA sequences of *OsMAN1* and *OsMAN6* were identical to the corresponding putative sequences in GenBank, but the obtained partial cDNA sequences of *OsMAN2* and *OsMANP* contained 94 and 33 bp more bases than those recorded in GenBank (Fig. 4, Table 1). This might be because the gene sequences for *OsMAN1*, *OsMAN2*, *OsMAN6* and *OsMANP* published in GenBank were the predicted sequences obtained by performing comparative genomic studies, and were not experimental results; therefore, parts of the exon sequences may be those of the intron, and were omitted.

Northern blot analysis showed that the four genes were expressed differently in the scutellum and aleurone layer during and following germination (Fig. 5). *OsMAN1*, *OsMAN2* and *OsMAN6* were expressed in both the scutellum and aleurone layer, but *OsMANP* only in the aleurone layer. High amounts of *OsMAN1* and *OsMAN2* mRNA were detectable in the scutellum as early as 24 h from the start of imbibition and changed little following germination. Although a high content of *OsMAN6* mRNA was also detectable in scutellum at 24 h from the start of imbibition, its abundance decreased

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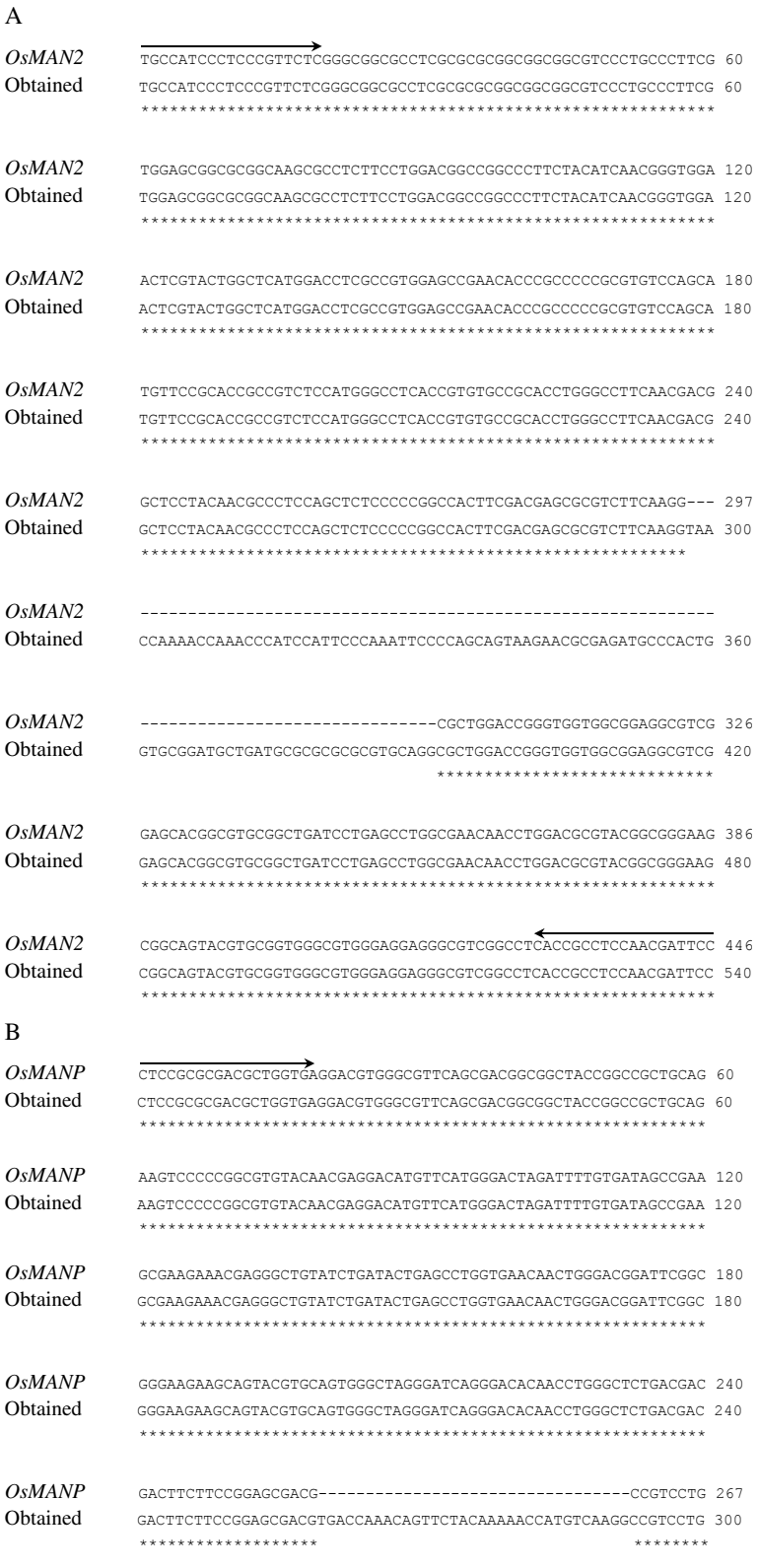

Figure 4. *(continued)*.

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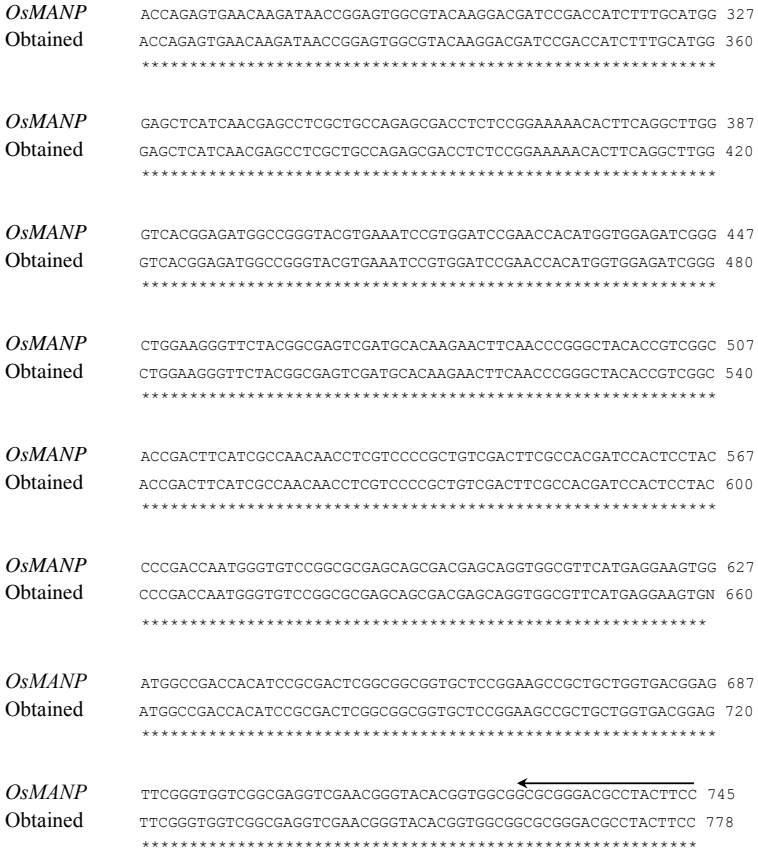

Figure 4. Alignment of sequences obtained by reverse transcriptase-polymerase chain reaction (RT-PCR) using the primers listed in Table 1 (Obtained) and sequences present in GenBank for (A) *OsMAN2* and (B) *OsMANP*. Primer sequences are indicated by horizontal arrows. Asterisks indicate matches.

after 48 h. In the aleurone layer, only transcripts of *OsMAN1* could be detected abundantly as early as 24 h; expression increased up to 72 h and then decreased slightly. Little *OsMAN2* and *OsMAN6* mRNA was detectable in the aleurone layer throughout the experimental period. Transcripts of *OsMANP* increased markedly after germination and remained high (Fig. 5).

Yuan *et al.* (2007) identified nine endo-β-mannanase genes (*OsMAN1–8* and *OsMANP*) from the rice genome by *in silico* sequence analysis and investigated their expression patterns by RT-PCR in grain, stem, root and leaf of rice. They found that *OsMAN1*, *OsMAN2*, *OsMAN3*, *OsMAN4*, *OsMAN6* and *OsMAN8* were expressed in grains, but mRNA for *OsMAN5*, *OsMAN7* and *OsMANP* were not detectable. However, Yuan *et al.* (2007) only analysed grains imbibed for 18 h, whereas the data presented here show that the transcripts do not appear until some 30 h later.

In conclusion, seeds of the cv. Taichung 65, a *japonica* rice, exhibit endo-β-mannanase activity from 48 h after the start of imbibition, following germination, similar to the *indica* cv. Peiza 67 (Wang *et al.*, 2005). There are three major isoforms of the enzyme, one of which is exclusive to the aleurone layer, and is the major, or sole source of endo-β-mannanase in the non-living starchy endosperm, where it presumably degrades cell walls as a source of sugars for the embryo, and/or to permit faster penetration of the starch degrading enzymes known to be released there for both the scutellum and aleurone layer (Thévenot *et al.*, 1991). The tomato anti-endo-β-mannanase antibody detected an antigenic band of approximately 40 kDa in rice grains. This antigen was already present in dry grains, its abundance decreased in the scutellum and increased in the aleurone layer during and after seed germination. A possible interpretation of this finding is that inactive endo-β-mannanase protein is present in dry grains, and the accumulation of endo-β-mannanase activity is due to the activation of extant proteins, *de novo* synthesis of enzyme proteins or both. Transcripts from three *OsMAN* genes are present prior to germination, three in the

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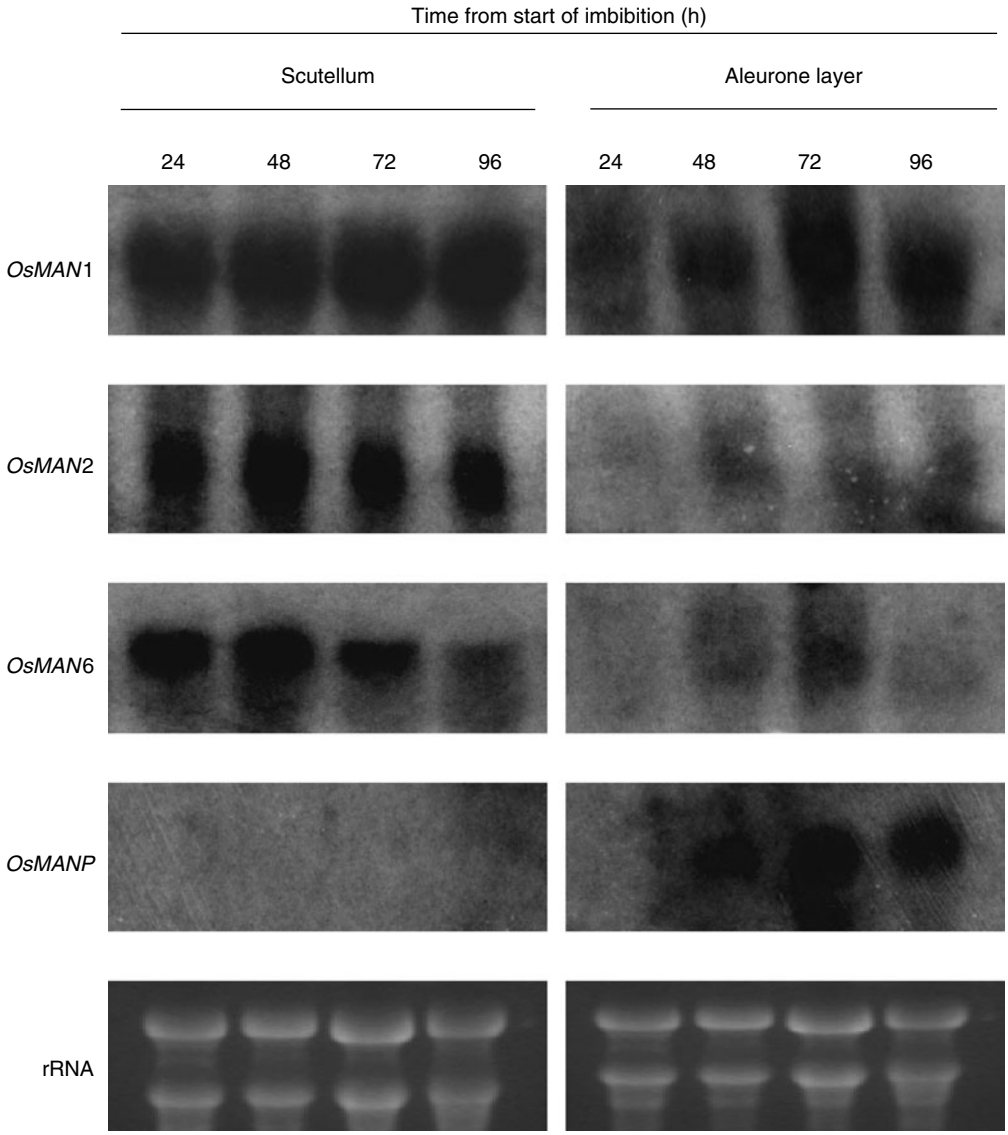

Figure 5. Northern blot analysis of *OsMAN1*, *OsMAN2*, *OsMAN6* and *OsMANP* in germinating and germinated rice grains imbibed on water at 25°C in the dark. Each lane was loaded with 40 μg total RNA extracted from the scutellum or aleurone layer.

scutellum (*OsMAN1*, *2* and *6*) and two major ones in the aleurone layer (*OsMAN1* and *OsMANP*). Thus, there is no obvious relationship between the presence of active enzyme, its protein or transcripts during germination. As in coffee, lettuce and tomato seeds, the peak of accumulation of mRNA for endo-β-mannanase was prior to the maximum enzyme activity (Nonogaki *et al.*, 2000; Marraccini *et al.*, 2001; Wang *et al.*, 2004). After germination, paradoxically, there are two distinct isoforms of the enzyme in the scutellum but three transcripts, whereas in the aleurone layer, three isoforms exist, but only two transcripts are abundantly present. However, there are two additional transcripts in lower abundance. It is therefore difficult to correlate activity of the enzyme with either the presence of the protein or its transcripts. To what extent the post-germinative increase in endo-β-mannanase activity is due to *de novo* synthesis, or to activation of extant protein, is not known.

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