the FEBS Journal

Generic normalization method for real-time PCR

Application for the analysis of the mannanase gene expressed in germinating tomato seed

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Keywords
gene expression; mannanase; normalization; real-time polymerase chain reaction; relative quantification

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(Received 26 October 2005, revised 9 December 2005, accepted 19 December 2005)

doi:10.1111/j.1742-4658.2006.05109.x

A generic sample normalization method applicable in relative comparison of mRNAs quantified with real-time polymerase chain reaction (PCR) is proposed. The method was applied in samples obtained from tomato seeds after osmopriming and aging treatments and from untreated seeds at early imbibition stage, when seeds had not completed germination. Normalization in sample variations was accomplished by detecting synthetic DNA sequences tailing cDNA after second strand reverse transcription synthesis, while the use of the common normalizer GAPDH proved unreliable. Results, obtained from the new method and having a standard error less than 10%, verified the expression profile of a germination-specific mannanase gene that was closely recorded at different time intervals in relation to seed germination.

Gene expression studies include quantification of mRNAs being produced under different conditions such as stress, growth, development, and cell and tissue localization or as part of evaluation of the effects of gene transfection. A variety of techniques exist to quantify mRNAs and usually involve northern hybridization, ribonuclease protection or real-time PCR assays [1,2].

In real-time PCR, quantification of mRNA sequences is accomplished by absolute or relative analysis methods [2,3]. Increasingly, the relative method of analysis is being used, as trends in gene expression can be better explained, but results depend on reference genes necessary to normalize sample variations [4–6].

A common technique in relative quantification is the choice of an endogenous control to normalize experimental variations, caused by differences in the amount of the RNA added in the reverse transcription (RT) PCR reactions. Specifications of reliable endogenous controls (i.e. house-keeping genes) are that they need to be abundant, remain constant in proportion to total RNA and be unaffected by the experimental treatments. The best choices proposed to be used as normalizers of isolated mRNA quantities are mainly RNAs produced from glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [7], β-actin [8], tubulin [9] or rRNA [10]. However, in general, depending on the developmental stage or environmental stimuli, the expression of certain reference genes is either up- or down-regulated.

The use of GAPDH mRNA as a normalizer is recommended with caution as it has been shown that its expression may be up-regulated in proliferating cells [3,11]. Usage of 18S RNA as a normalizer is not always appropriate, as it does not have a polyA tail

Abbreviations
EST, expressed sequence tag; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HAS, hours after sowing; RACE, rapid amplification of cDNA ends; RT, reverse transcription.

and thus prohibits synthesis of cDNA with oligo-dT. Additionally 18S RNA, being of ribosomal origin, may not always be representative of the entire cellular mRNA population and is in such overwhelming quantities relative to rare message that competimers must frequently be employed to obtain relevant normalization, making the results more complex. Expression of actin or tubulin often depends on the plant developmental stage [12,13] and is affected upon environmental stresses [14], making their use as normalizers inappropriate. Even in the case of ABI's TaqMan Human Endogenous Control Plate, where a number of available housekeeping genes have been included, it is not certain that the best combination of normalizers will be found. To overcome these problems, the use of multiple controls obtained from microarray data has been suggested [15,16]. However, this method is costly, demands availability of proper instrumentation and is applicable mainly for the genome of the specific species studied, i.e. yeast, human or Arabidopsis genome, where the genomic sequences have been determined.

Here, we propose an alternative method for an internal control that would be applicable across all samples, assuming that one wants to normalize against total mRNA. In the presented methodology, DNA molecules (adapters) to tail cDNA during reverse transcription are used instead of internal reference genes. Tailing sequences are further amplified with polymerase I leading to second strand synthesis of the adapter molecules to be used as indicators of the total mRNA quantity.

To test our method, we studied the transient expression pattern of the germination-specific *endo*-β-mannanase gene, in germinating tomato seeds (*Lycopersicon esculentum* Mill.), where there are no documented stably expressed genes to serve as normalizers. *Endo*-β-mannanase is expressed exclusively in the endosperm cap tissue, prior to radicle emergence [17]. It has been shown that its activity develops prior to germination, specifically in the micropylar region of the endosperm opposite the radicle tip [18–23], and is involved in hydrolysis of the mannan-rich cell walls of the tomato endosperm during germination, leading to radicle protrusion [20,21,23].

A common housekeeping gene, *GAPDH*, was tested in parallel to determine its efficiency as a normalizer of the sample variation.

Results

A representation of the proposed methodology is illustrated in Fig. 1. Isolated mRNA molecules were tailed with an adapter molecule of known sequence, which was used in a similar way to an internal standard. For each mRNA sample, reverse transcription was performed using oligo $d(T)_{18}$ extended at the 5′-end with a synthetic DNA sequence of 100 bases length (adapter) forming a molecule of 118 bases. The adapter consisted of a random sequence of 60 bases $RD(dNTP)_{60}$ extended at both its ends with sequences of 20 base lengths, serving as the forward $(PR_F)_{20}$ and the reverse $(PR_R)_{20}$ primer of the adapter. The use of *Escherichia coli* DNA polymerase I ensures that the polyA tract of the mRNA can be used by the enzyme as a primer with which to commence second strand synthesis of the adapter. Only adapter sequences that have tailed cDNA during first strand synthesis result in second strand synthesis of the adapter, thus differentiating samples according to the contained total mRNA quantity. Second strand synthesis of the adapter eliminates the need for sample purification in order to remove excess of the adapter in the sample.

The method was tested in germinating tomato seeds in order to monitor the expression profile of a germi-

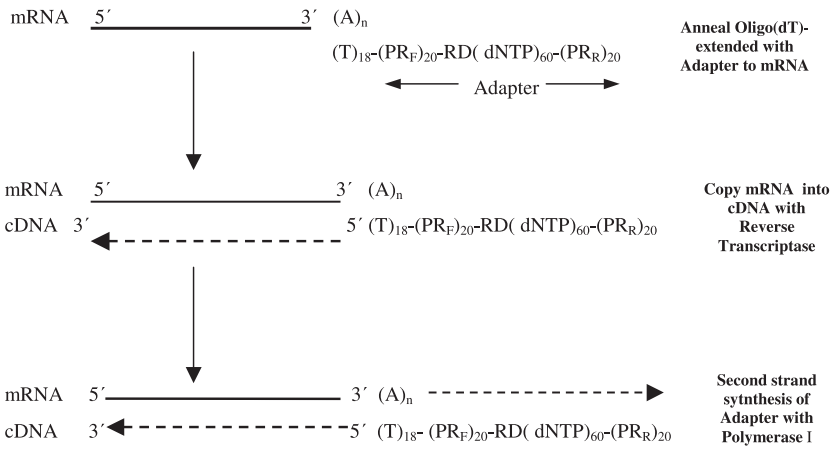

Fig. 1. Representation of second strand synthesis of adapter molecules. Oligo $d(T)_{18}$ are extended with adapter molecules -$(PR_F)_{20}$- RD(dNTP)$_{60}$-$(PR_R)_{20}$ consisting of a random sequence of 60 bases $RD(dNTP)_{60}$ extended at both its ends with sequences of 20 base lengths serving as the forward $(PR_F)_{20}$ and the reverse $(PR_R)_{20}$ primer of the adapter. The formation of the adapter's complementary strands after second strand synthesis is used to differentiate samples according to the contained total mRNA quantity.

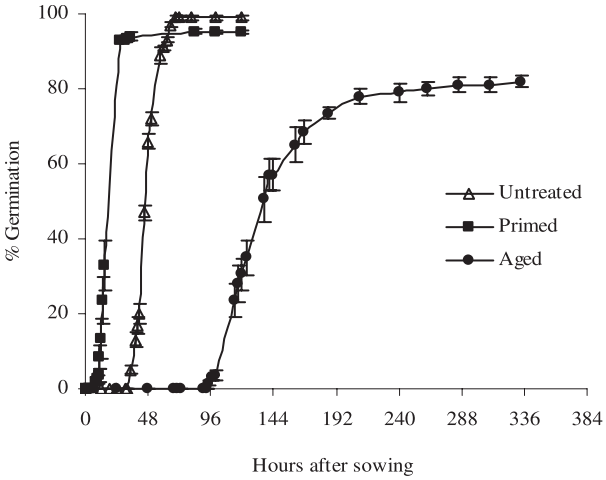

Fig. 2. The percentage germination of tomato seeds. Germination curves of untreated, osmoprimed and aged seeds of tomato cultivar ACE at 25 °C are shown. Values are the means of three replications ± SE. HAS = hours after sowing.

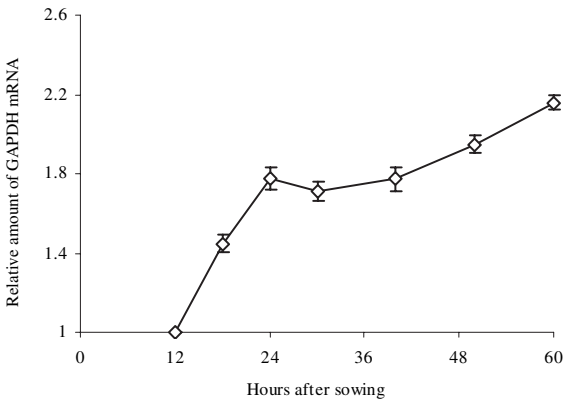

Fig. 3. GAPDH relative to mRNA. Time course of relative amount of GAPDH mRNA to that of 12 HAS during the germination of untreated tomato seeds at 25 °C. Values are the means of six replications ± SE.

nation-specific *endo*-β-mannanase gene. The germination rate and percentage of tomato seeds were studied in untreated, osmoprimed and aged tomato seeds (Fig. 2). It is noticed that, compared with untreated seeds, osmopriming accelerates the germination rate while aging delays it and reduces the final percentage of germination [24].

Figure 3 shows GAPDH expression in untreated germinating tomato seeds during the course of germination. Initially there was a sharp increase, which was followed by a plateau and then a second further increase, showing that GAPDH was not stably expressed during seed germination and could not serve as a normalizer for the expression study of *endo*-β-mannanase.

Table 1 shows a time course of the production of germination-specific mannanase mRNA during seed germination for a period of 50 h after sowing (HAS). The normalized *endo*-β-mannanase mRNA values, obtained at different time intervals after sowing (12, 18, 24, 30, 40 and 50 HAS), as well as the relative expression of these values when compared with those of 12 HAS, are presented in the last two columns. *Endo*-β-mannanase mRNA reached its peak 30 HAS (43-fold higher in comparison with 12 HAS – mannanase mRNA) followed by a sharp reduction to the starting value of 12 HAS.

Relative expression levels of the germination-specific mannanase mRNA and the equivalent enzyme activity during seed germination are shown in Fig. 4. Time course of *endo*-β-mannanase enzyme activity showed its early appearance 12 HAS, long before the start of radicle protrusion (Figs 4 and 2). During the course of germination, mannanase activity increased and started decreasing 35 HAS, when only 5% of the seeds had germinated (Figs 4 and 2).

Endo-β-mannanase mRNA and *endo*-β-mannanase activity, 5 h before the start of radicle emergence, were also examined in untreated (30 HAS), osmoprimed (2 HAS) and aged tomato seeds (91 HAS) (Fig. 5), in

Table 1. Real-time PCR fluorescence intensities. Initial fluorescence intensities of: *endo*-β-mannanase gene ($T_{i,\ Ro}$); adapter ($A_{i,\ Ro}$); normalized $T_{i,\ Ro}$ values ($N_{i,\ Ro}$); and relatively expressed to $N_{12\ h,\ Ro}$ values ($N_{i,\ Ro}/N_{12\ h,\ Ro}$) from the amplification of cDNA with SYBR green in real-time PCR, obtained from untreated tomato seeds germinating for different time periods. Values are the means of six replications ± SE.

Sample (h)	$T_{i,\ Ro}$	$A_{i,\ Ro}$	$N_{i,\ Ro}$	$N_{i,\ Ro}/N_{12\ h,\ Ro}$
12	0.005 ± 0.0006	0.98 ± 0.08	0.006 ± 0.00004	1.00 ± 0.00
18	0.018 ± 0.0017	0.99 ± 0.07	0.022 ± 0.00004	3.58 ± 0.01
24	0.072 ± 0.0079	0.98 ± 0.07	0.088 ± 0.00114	13.97 ± 0.08
30	0.176 ± 0.0207	0.78 ± 0.05	0.269 ± 0.00662	42.67 ± 0.74
40	0.061 ± 0.0062	0.92 ± 0.06	0.077 ± 0.00095	12.31 ± 0.06
50	0.004 ± 0.0003	0.85 ± 0.06	0.006 ± 0.00012	1.06 ± 0.02

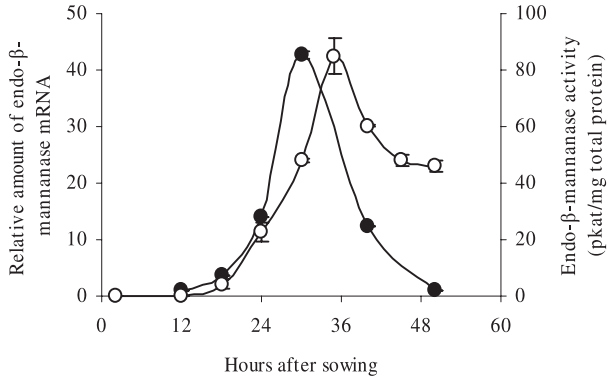

Fig. 4. Relative *endo*-β-mannanase mRNA amount and the corresponding endo-β-mannanase activity. Time course of relative amount of *endo*-β-mannanase mRNA to that of 12 HAS (●) and time course of *endo*-β-mannanase activity (○) during germination of untreated tomato seeds at 25 °C. Values are the means of the six replications ± SE.

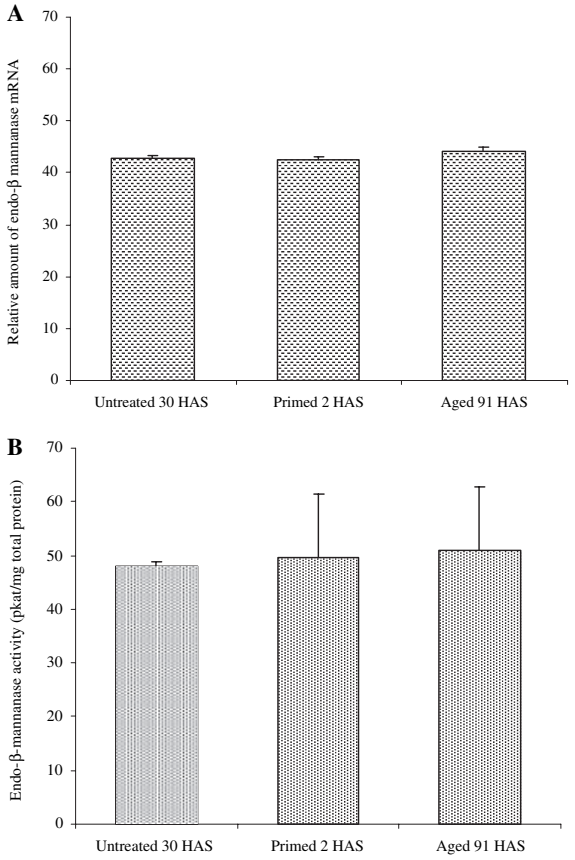

Fig. 5. Relative amount of *endo*-β-mannanase mRNA and enzyme activity 5 h before germination. (A) *Endo*-β-mannanase mRNA amount of untreated, osmoprimed and aged tomato seeds of variety ACE, 5 h before radicle protrusion, relative to that of 12 HAS untreated seeds. (B) *Endo*-β-mannanase activity (pkat·mg total protein) of untreated, osmoprimed and aged tomato seeds of variety ACE, 5 h before radicle protrusion. Values are the means of the six replications ± SE.

order to elucidate the relation between mannanase mRNA and mannanase activity with germination ability of tomato seeds. It was noticed that mannanase mRNA and enzyme activity 5 h before the radicle protrusion were at the same level, independent of the seed treatment.

Representative final products from the real-time PCR, amplicons of (a) the germination-specific mannanase gene and (b) the adapter, are presented in Fig. 6.

Discussion

The current study formed a basis with which to test and establish a generic methodology with real-time PCR for sample normalization, a common problem in studies of gene expression. First, identification of the proper housekeeping gene to normalize sample variations was required. Then the proper set of primers needed to be found, which is a laborious process because there are no universal sets of primers and information on genomic sequences with which to design them is not always available.

In the present work, the housekeeping gene *GAPDH*, commonly used in RNA normalization, was tested. However, the time course of GAPDH mRNA (Fig. 3) indicated that *GAPDH* was not stably expressed and followed the pattern of O_2 consumption observed in the respiratory process during seed germination [25]. This is in accordance with the fact that GAPDH is a dehydrogenase participating in respiration. Thus, it was not a suitable housekeeping gene for normalization in our case. Similarly, research with the model plant Arabidopsis showed that *GAPDH* was not stably expressed in seed and pollen samples [13]. Additionally, in previous studies with animal and human samples, it has also been reported that GAPDH mRNA levels are not constant [2,15]. GAPDH participates in diverse cellular functions and, although it might be suitable for normalization for some cases, for most experimental conditions its use is inappropriate and should be discontinued [3].

Specifically in tomato plants, expressed sequence tag (EST) libraries have been previously used for the investigation of the stability of expression of commonly used reference genes, but no genes were identified that showed stable expression, as judged by relative EST abundance, across a wide range of developmental and environmental conditions [26]. It must be noted, that in many cases, the search for the proper housekeeping gene to use for normalization of mRNA amounts is an unlimited, costly process, subject to failure.

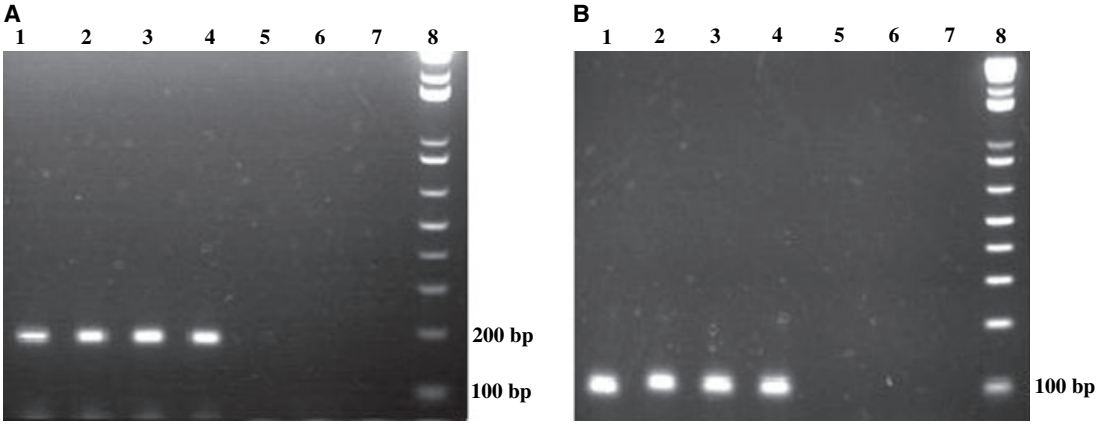

Fig. 6. Agarose gel electrophoresis of real-time PCR products. (A) Real-time PCR product (200 bp) after amplification of cDNA, obtained from germinating tomato seeds, with primers specific for the germination-specific mannanase. (B) Real-time PCR product (100 bp) after amplification of cDNA tailed with adapter, obtained from germinating tomato seeds, with primers for the adapter sequence. Lanes are as follows: Lane 1, untreated 12 HAS; lane 2, untreated 30 HAS; lane 3, primed 2 HAS; lane 4, aged 91 HAS; lane 5, NTC; lane 6, NAC_1 (absence of primers); lane 7, NAC_2 (absence of polymerase); lane 8, 1 kb + DNA marker.

Alleviating the problem of sample normalization by extending cDNA with adapter sequences, in a manner similar to the rapid amplification of cDNA ends (RACE) technique, it was possible to relatively quantify mRNA. Without prior knowledge of established internal standards, constantly expressed during the seed germination process, the proposed methodology for real-time PCR offers a valuable tool to study gene expression, possibly applicable across kingdoms.

Limitations of the proposed technique are: (a) preliminary PCR experiments are needed in order to define that designed adapter sequences have no homologies with the primers for the genes under investigation or the genome in order to avoid nonspecific PCR products; (b) the adapter is designed with its ends to serve as primers in order to result in the total length of the adapter during real-time PCR; (c) the adapter is designed so that its primers have similar annealing temperatures with the primers for the genes under investigation in order to avoid different PCR runs; and (d) samples tailed with adapter molecules need to be diluted in cases where the produced fluorescence signal exceeds the detection limits of the instrumentation.

Seed germination forms an ideal environment in which to study events like transient gene expression and cell differentiation. Expression levels of the germination-specific mannanase gene and its protein product have been studied as possible indicators of successful seed germination, and also to confirm the validity of the presented normalization methodology.

Using this method, the transient expression of the germination-specific mannanase gene was verified, with mannanase mRNA reaching a maximum of 30 HAS, followed by a steep reduction until, by 50 HAS, it was again in low abundance (Table 1 and Fig. 4). Accordingly, *endo*-β-mannanase activity had a similar profile and reached the highest value of 35 HAS. Similar observations have also been reported (based on fewer sampling points), regarding the timing of the accumulation of *LeMAN2* message during germination and a corresponding increase in mannanase activity in the endosperm cap prior to radicle emergence [17,24]. The observed delay between the peaks of mRNA and enzyme activity was probably caused by differences between the half-life of the mRNA and that of the mannanase, leading to enzyme accumulation. This contention is verified from the fact that when mRNA amounts were reduced, enzyme activity declined at a slower rate (Fig. 4). Similar time delays between the peaks of mRNA accumulation and the corresponding maximum enzyme activity in the micropylar region of tomato seeds have also been reported for *endo*-β-mannanase for the cultivar Moneymaker [17] and for β-mannosidase for the cultivar Glamour [27].

In conclusion, the proposed method with which to analyze mRNA helped to obtain, in comparison with previously published data, a well-defined profile of the transiently expressed mannanase gene in tomato germinating seeds. Furthermore, it was shown that mannanase mRNA accumulated, in parallel with mannanase activity, to the same extent in seeds 5 h prior to the completion of germination, irrespective of seed treatment, suggesting a close relationship between *endo*-β-mannanase accumulation, activity and tomato seed germination.

Finally, the observed similarities between the expression patterns of mannanase mRNA and the

corresponding enzyme activity, as well as the low standard error of the mRNA measurements, confirm the validity of the proposed methodology.

Experimental procedures

Plant material and seed treatments

Seeds from the commercial tomato variety ACE were subjected to osmopriming and aging.

Osmopriming was carried out for 5 days, at 20 °C, in solutions of 120 mol·L^{-1} K_2HPO_4 + 150 mol·L^{-1} KNO_3 at -1.25 MPa under fluorescent light [28]. Seeds were then rinsed thoroughly in distilled water and dried with forced air at 30 °C to moisture content of 6% (on a dry-weight basis).

Aging was performed by adjusting the seed water content to 13% with addition of water and thorough mixing, followed by incubation for 24 h at 4 °C to allow moisture equilibration within the lot [28]. The seeds were then sealed in a container and immersed in a water bath at 50 °C for 24 h. The seeds were then dried to 6% moisture content at 30 °C with forced air.

Seed germination

Seeds (untreated, osmoprimed and aged) were surface sterilized in 1% sodium hypochlorite solution, washed three times with sterile distilled water and placed in 9-cm Petri dishes on two layers of Whatman no. 1 filter paper moistened with 5 mL of sterile water. Petri dishes contained 50 tomato seeds each and were incubated at 25 °C in the dark. Radicle protrusion to 1 mm was scored as germination and counts were made until no further germination was observed.

Enzyme extraction and assay

Nontreated tomato seeds were imbibed in water for different time intervals (12, 24, 30, 40, 50 and 60 h). Osmoprimed and aged seeds were imbibed for 2 and 91 h, respectively, 5 h before the first seeds started to complete germination.

Extracts from tomato seeds mentioned above were obtained in triplicate by grinding 50 seeds in a mortar with 500 μL MacIlvaine buffer (pH 5.0). The samples were then centrifuged for 10 min at 18 000 ***g*** and the supernatant was used for enzyme assays.

A gel diffusion assay was used to determine *endo*-β-mannanase activity with locust bean gum (Sigma-Aldrich, St. Louis, MO, USA) as substrate [29]. Sample volumes of 2 μL, from the isolated supernatant, were loaded in duplicate in 2-mm diameter wells in the gels. After incubation of the gels for 14–16 h at 25 °C, gels were stained with Congo Red (Sigma) and the clearing zone around each well, indicative of enzyme activity, was measured. Calculation of mannanase activity was according to Downie *et al.* [30], while a standard curve was established by using commercial *endo*-β-mannanase from *Aspergillus niger* (EC, Megazyme, North Rocks, Sydney, Australia).

Total protein assay

Total protein content in sample extraction isolates, which were described above, was determined with Bradford reagent (Sigma) according to Sigma's guidelines, using bovine serum albumin as a standard.

Extraction of total RNA

Extraction of total RNA was performed in triplicate with samples obtained from untreated nongerminated tomato seeds imbibed for different time periods (12, 18, 24, 30, 40, 50 and 60 h). For the isolation of total RNA the NucleoSpin® RNA Plant kit (Macherey-Nagel, Dureu, Germany) was used for a sample size of 50 tomato seeds per replication.

Extraction of mRNA

Extraction of mRNA was performed in triplicate with samples obtained from untreated nongerminated tomato seeds imbibed for different time periods (12, 18, 24, 30, 40 and 50 h), and for osmoprimed and aged seeds, imbibed for 2 and 91 h, respectively. For the isolation of mRNA the PolyATtract® System 1000 (Promega, Madison, WI, USA) was used for a sample size of 50 tomato seeds per replication [31].

cDNA preparation

First strand synthesis of cDNA from total RNA or mRNA was carried out according to Applied Biosystems general guidelines. Reactions were set according to the instructions in the TaqMan® RT Reagents kit (P/N N808-0234 for Applied Biosystems by Roche) on a GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA, USA). Sample volume was 100 μL and consisted of 10 μL TaqMan® RT buffer (10·), 15 μL $MgCl_2$ (25 mM), 15 μL dNTPs mixture (2.5 mM dATP, 2.5 mM dCTP, 2.5 mM dGTP, 2.5 mM dTTP), 6 μL Random Hexamers (50 μM) for total RNA or oligo $d(T)_{18}$ (50 μM) for mRNA, 1 μL RNase inhibitor (20 U·μL^{-1}), 2 μL MultiScribe Reverse Transcriptase (Applied Biosystems) (50 U·μL^{-1}) and 50 μL of isolated total RNA or mRNA, containing 2% dimethyl sulfoxide, that was preheated at 95 °C for 30 s. The thermal cycling parameters were as follows: Stage 1: Primer incubation at 25 °C for 10 min; stage 2: reverse transcription at 48 °C for 60 min; stage 3: reverse transcriptase inactivation at 95 °C for 5 min.

For each mRNA sample, a second reverse transcription was performed with the same PCR conditions as mentioned above, with the exception of using oligo $d(T)_{18}$ extended at the 5′-end with a synthetic DNA sequence of 100 base lengths (adapter), forming a molecule of 118 bases. The whole molecule, consisting of 118 bases, as well as the adapter primers, was ordered from MWG-Biotech AG (Ebersberg, Germany). At the end of the second stage of reverse transcription cycle, second strand DNA synthesis was performed by adding 10 units of DNA polymerase I from *E. coli* (Takara Bio Inc., Otsu, Shiga, Japan). Samples were incubated for 30 min at 37 °C followed by enzyme inactivation at 70 °C for 10 min.

Real-time PCR

GAPDH

Duplicates of all cDNA samples obtained from every total RNA extraction (in total six replications per sample case), were analyzed in a GeneAmp® 5700 Sequence Detection System (Applied Biosystems). Real-time PCR was performed with the SYBR® Green PCR Core Reagents kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instructions and substituting AmpliTaq Gold Polymerase with Taq DNA Polymerase from Bioron GmbH (Ludwigshafen, Germany) using a pair of specific primers (5′-gcaatgcatcttgcactaccaactgtcttgc-3′ forward, 5′-ctgtgagtaaccccattcattatcataccaagc-3′ reverse) (MWG Biotech, Ebersberg, Germany) for the detection of the *GAPDH* gene. The thermal cycling parameters for real-time PCR reactions were: 95 °C, 6 min (95 °C, 25 s; 60 °C, 1 min) × 35 cycles.

Germination-specific mannanase

Duplicates of all cDNA samples obtained from every mRNA extraction (in total six replications per sample case) were analyzed with real-time PCR as previously described using a pair of specific primers (5′-caagtcattggcaagacgcaa-3′ forward, 5′-aacttcctcccatcgttcc-3′ reverse) for the detection of the germination-specific mannanase gene and a pair of primers based on the adapter sequence for the estimation of total mRNA. The thermal cycling parameters for real-time PCR reactions were: 95 °C, 5 min (95 °C, 30 s; 69 °C, 1 min) × 40 cycles [31].

Evaluation of real-time PCR data

Raw data obtained from the GeneAmp® 5700 Sequence Detection System were exported to an EXCEL spreadsheet with fluorescence intensities (R_n, fluorescence intensity at PCR cycle n) as logarithmic values on the Y axis and their corresponding n-values on the X axis. From the data, the linear stage of each PCR reaction was identified and a fitting the linear data equation $Y = (A*X) + B$ was determined. The resulting equations were of the type $\log(R_n) = [n*\log(E)] + \log(R_o)$, where the slope corresponds to the logarithm of (E) and the intercept corresponds to the logarithm of the fluorescence intensity (R_o), proportional to the initially loaded cDNA amount in the PCR. Values of (R_o) and (E) were calculated by raising, to the base 10, the logarithmic value [31].

GAPDH

Normalization between samples of GAPDH was based on total RNA concentration estimated spectrophotometrically [2].

Pre-germinative mannanase

Normalization of sample variation was based on the formula:

$$N_{i,Ro} = T_{i,Ro}/A_{i,Ro}$$

where $N_{i,\ Ro}$ is the normalized R_o value of sample i, proportional to the initially loaded cDNA amount in the PCR; $T_{i,\ Ro}$ is the R_o value of target gene (*endo*-β-mannanase) of sample i; and $A_{i,\ Ro}$ is the R_o value of adapter, indicative of total mRNA, of sample i.

All normalized samples were expressed relative to 12 h normalized sample value ($N_{i,\ Ro}/N_{12\ h,\ Ro}$).

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