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RESEARCH ARTICLE

Regulation of α-expansins genes in *Arabidopsis thaliana* seeds during post-osmopriming germination

Alessandra Ferreira Ribas[1] · Nathalia Volpi e Silva[2] · Tiago Benedito dos Santos[1] · Fabiana Lima Abrantes[3] · Ceci Castilho Custódio[3] · Nelson Barbosa Machado-Neto[3] · Luiz Gonzaga Esteves Vieira[1]

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Abstract Seed osmopriming is a pre-sowing treatment that involves limitation of the seed water imbibition, so that pre-germinative metabolic activities proceed without radicular protrusion. This technique is used for improving germination rate, uniformity of seedling growth and hastening the time to start germination. In *Arabidopsis thaliana*, seed germination has been associated with the induction of enzymes involved in cell wall modifications, such as expansins. The α-expansins (EXPAs) are involved in cell wall relaxation and extension during seed germination. We used online tools to identify *AtEXPA* genes with preferential expression during seed germination and RT-qPCR to study the expression of five EXPA genes at different germination stages of non-primed and osmoprimed seeds. In silico promoter analysis of these genes showed that motifs similar to *cis*-acting elements related to abiotic stress, light and phytohormone responses are the most overrepresented in promoters of these *AtEXPA* genes, showing that their expression is likely be regulated by intrinsic developmental and environmental signals during *Arabidopsis* seed germination. The osmopriming conditioning had a decreased time and mean to 50% germination without affecting the percentage of final seed germination. The dried PEG-treated seeds showed noticeable high mRNA levels earlier at the beginning of water imbibition (18 h), showing that transcripts of all five EXPA isoforms were significantly produced during the osmopriming process. The strong up-regulation of these *AtEXPA* genes, mainly *AtEXPA2*, were associated with the earlier germination of the osmoprimed seeds, which qualifies them to monitor osmopriming procedures and the advancement of germination.

Keywords *Arabidopsis thaliana* · α-Expansins · Osmopriming · Seed germination

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Alessandra Ferreira Ribas and Nathalia Volpi e Silva have contributed equally to this article.

✉ Alessandra Ferreira Ribas
alessandra_ribas@hotmail.com

[1] Agronomy Graduate Program, Molecular Genetic Laboratory, Universidade do Oeste Paulista (UNOESTE), Rod. Raposo Tavares, km 572, Limoeiro, Presidente Prudente, SP 19067-175, Brazil

[2] Department of Plant Biology, Institute of Biology, University of Campinas (UNICAMP), Cidade Universitária Zeferina Vaz, Campinas, SP 13083-970, Brazil

[3] Agronomy Graduate Program, Seed Reserach Laboratory, Universidade do Oeste Paulista (UNOESTE), Rod. Raposo Tavares, km 572, Limoeiro, Presidente Prudente, SP 19067-175, Brazil

Introduction

Germination is a complex phenomenon that can be divided into three stages: Phase I—rapid absorption of water, increase in respiratory intensity and energy production and onset of degradation of the reserve substances; Phase II—active transport of substances, a decrease of respiration and water absorption; Phase III—transported substances are reorganized into more complex ones for the beginning of embryonic axis growth (Bewley et al. 2013).

Of the environmental factors that can influence the germination, the water availability is the most determinant factor as it intensifies the metabolic activities, culminating in the growth of the embryonic axis. However,

environmental stresses may also positively influence seed performance as a physiological conditioning (priming) induces improvements in seed quality favoring higher germination rates and more uniform seedling emergence (Chen and Arora 2013; Khadraji et al. 2017; Abid et al. 2018). Seed osmopriming is a pre-sowing treatment which consists of the controlled hydration of the seeds, allowing only the first stages of the germination process (phases I and II) to be completed, with subsequent drying for the seed to return to a quiescent state. In this way, the germination process is initiated without radicular protrusion (phase III) (Bradford 1986). One of the osmopriming methods for controlling the imbibition rate of the seeds uses osmotic solutions of polyethylene glycol (PEG). This solute has desirable characteristics, since it is inert, is not absorbed by the seed and is non-toxic (Heydecker and Gibbins 1978).

There are numerous reports of the positive effect of osmopriming on seed germination ability, uniformity and vigor over non-primed seeds under adverse environmental conditions in different crop species, such as sorghum (Foti et al. 2002) soybean (Zhuo et al. 2009), rice (Sun et al. 2010), chickpeas (Lamichaney and Katiyar 2017), among many others. The beneficial effect of this technique on the germination-related processes has been associated with the accumulation of mRNA and inactive proteins produced during the osmotic conditioning (Bray 1995; Özbingol et al. 1999; Gallardo et al. 2001). For example, Soeda et al. (2005) identified the expression of genes related to stress tolerance during the conditioning of *Brassica oleracea* seeds, such as those coding for superoxide dismutases (SOD), heat shock proteins (HSP) and late embryogenesis abundant proteins (LEA). These authors also identified a group of genes that were up-regulated during germination in normal conditions, but not during priming. The comparative analysis of proteome changes in *Medicago sativa* seeds during germination and osmoconditioning showed some proteins specific to osmopriming, some that varied during germination and there were some proteins found to be commonly present in both the conditions, which were mainly involved in cell structure, metabolism and defense (Yacoubi et al. 2011). In spinach, increased germination of osmoprimed seeds was accompanied by the accumulation of dehydrins, proteins that are normally synthesized in maturating seeds during their desiccation (Chen et al. 2012). Priming-induced modulation of genes and enzymes of the proline metabolism, with a consequent improvement of salinity stress tolerance during post-priming germination, was reported in *Brassica napus* (Kubala et al. 2015).

In *Arabidopsis thaliana* seeds, the embryo is surrounded by a single layer of cells from the endosperm and a seed coat. The endosperm and its layer cells are responsible for regulating germination, which consists of two sequential events: the testa and endosperm rupture (Holdsworth et al. 2008). One of the main characteristics related to the endosperm rupture by radicle expansion is the weakening of the endosperm, which has been associated with the induction of cell wall loosening by enzymes such as expansins (Kucera et al. 2005). Transcriptomic and proteomic analyses have shown that the expression of several genes and proteins are up- or down-regulated during priming and post-priming germination, including those involved in cell wall loosening and extension (Zhang et al. 2014; Kubala et al. 2015; Sano et al. 2017). However, there is still no specific information on differentially expressed α-expansin (EXPA) isoforms in *Arabidopsis* seeds subjected to osmoconditioning during subsequent germination under normal conditions.

Expansins are proteins capable of inducing cell extension, acting as mediators in response to acidic pH and catalyzing the cell wall expansion (McQueen-Mason et al. 1992). These proteins are assumed to break non-covalent bonds between cell wall polysaccharides, extending these polysaccharides in an irreversible way without causing structural changes in the cell wall (Cosgrove 1997). They are members of a plant-specific superfamily that comprises two major families: α-expansins (EXPA) and β-expansins (EXPB), and two smaller families named expansin-like A (EXLA) and expansin-like B (EXLB) (Cosgrove 2015). Members of EXPA and EXPB families have already been functionally validated, while the functional aspects for the EXPLA and EXPLB families remain uncertain (Cosgrove 2015). The EXPAs are responsible for controlling cell wall extension and developmental processes, including cell dissociation and separation (McQueen-Mason et al. 1992; Cho and Kende 1997).

The role of expansins in elongation and cell expansion has been observed in different cells and tissues during growth and development, such as the hypocotyls (Caderas et al. 2000), roots and root hairs (Cho and Cosgrove 2002; Lin et al. 2001) and leaves (Muller et al. 2007; Goh et al. 2012). These proteins are also expressed during seed developmental stages (Budzinski et al. 2011; Hussain et al. 2016). As seed germination involves cell elongation and division, it is expected that proteins with such a role would be differentially regulated since the onset of this process. Indeed, transcriptional analyses during the germination of *A. thaliana* showed that expansin genes were induced in the endosperm and were involved in the processes that lead to testa rupture and weakening of the micropylar endosperm cap (Morris et al. 2011). Additionally, AtEXPA2 has been considered to be a marker for endosperm tissue in non-dormant imbibed or germinating seeds (Penfield et al. 2006; Yan et al. 2014). In rice, various specific expansins were shown to play important roles in coleoptile elongation under submergence stress (Hussain et al. 2016). These

authors showed that some α-expansin genes were strongly up-regulated due to chemical and hormonal seed priming treatments compared to non-primed seeds, suggesting that expression of α-expansins might have contributed to priming-induced submergence tolerance in rice.

In this work, we aimed to improve our comprehension of gene expression patterns during *A. thaliana* seed germination by studying the expression transcription profile of α-expansins (EXPA) genes associated with seed germination. For this, selected EXPA coding sequences and their upstream regulatory elements were analyzed using experimental data available from transcriptome databases and qPCR analyses were performed to verify the expression of EXPA genes at different germination stages of non-primed and osmoprimed seeds. The data presented here demonstrate that osmopriming *Arabidopsis* seeds with PEG6000 caused differential regulation of α-expansins genes at early stages of germination when compared to non-primed seeds. Understanding the mRNA expression patterns of α-expansins in *A. thaliana* seeds subjected to physiological conditioning (osmopriming) may provide new insights into the mechanisms involved in seed priming-induced enhancement of seed germination, resulting in genetic manipulation approaches to obtain plants more tolerant to abiotic stresses at their initial development stages.

Materials and methods

Selection of α-expansins expressed during seed germination of *A. thaliana*

We initially selected the α-expansins from *A. thaliana* available in the Genevestigator database for heat-map analysis (https://www.genevestigator.com, Zimmermann et al. 2004) (Fig S1). For subsequent analysis, we selected the α-expansins with expression levels greater than or equal to 10 (signal intensity in Affymetrix microarray) and that were expressed preferentially during germination (1–5 days) (Fig S2). Based on these data, only five *AtEXPAs* genes that are preferentially expressed during germination were selected for further study: *AtEXPA2* (AT5G05290), *AtEXPA3* (AT2G37640), *AtEXPA8* (AT2G40610), *AtEXPA9* (AT5G02260) and *AtEXPA20* (AT4G38210) (Fig S2). CLC Main Workbench (CLC bio, Qiagen, Denmark) and MEGA 5.1 (Kumar et al. 2016) software was used for deduced amino acid sequence comparison and alignments. The ScanProsite tool (http://prosite.expasy.org/scanprosite/) were applied to predict the putative motifs and domains.

For the sake of comparison with later RT-qPCR results, we have also analyzed the expression profile of the selected α-expansins in non-primed seeds subjected to various water soaking periods (1, 3, 6, 12 and 24 h at 4 °C) available at the BAR eFP database (Winter et al. 2007; http://bar.utoronto.ca/welcome.htm) (Fig S3).

In silico analysis of promoter sequences

A 1000 bp region upstream from translation start codon of *AtEXPA* genes was selected and analyzed using different databases: RegSite (http://softberry.com), PlantCare (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Lescot et al. 2002), *cis*-regulatory DNA Elements PLACE (http://www.dna.affrc.go.jp/PLACE/signalscan.html) (Higo et al. 1999) in order to find regulatory motifs on the putative promoter region. The motifs reported previously in the literature as related to seed germination were selected for further analyses. Pattern Matching in RSA-Tools (http://rsat.scmbb.ulb.ac.be/rsat/) (Thomas-Chollier et al. 2011) was used to map occurrences and frequency of the selected *cis*-elements onto the five *ProAtEXPA* sequences. Pattern matching and image generation were conducted using the default settings.

Plant material and growth conditions

Arabidopsis thaliana ecotype Columbia-0 seeds were germinated in Petri dishes containing moistened qualitative filter paper (Qualy™—J. Prolab, Brazil) at 15 °C in the dark. The seedlings were then transplanted into 5 cm diameter pots containing 80% Bioplant™ substrate with coconut fiber and 20% sphagnum, and fertilized with 1 g/L (w/v) of Osmocote™ and maintained in a growth room under a photoperiod of 8 h of light (120 μE m^2 s^1) at 20 °C. The plants were then allowed to grow until the siliques matured, and then the seeds were collected, dried and stored for the priming treatments.

Osmopriming and germination curve

Priming was carried out for 7 days at 15 °C by incubating seeds on germination paper moistened with a solution of polyethylene glycol 6000 (PEG 6000; Sigma-Aldrich, St. Louis, MO, US) equivalent to osmotic potential of − 0.75 MPa (Gallardo et al. 2001). Subsequently, the seeds were washed to remove the PEG and dried in an airtight mini chamber containing silica gel at 20 °C.

Primed (PEG) and non-primed seeds (control) were placed in 10-cm Petri dishes and distilled water equivalent to 2.5 times the dry weight of the germination paper was added to each Petri dish. The samples were kept in a germination chamber at a constant temperature of 15 °C ± 1 under 8 h of light (Baskin and Baskin 1972) and the germination rates were scored every 6 h during 120 h based on the number of seeds with primary root emission. The

germination experiment consisted of a completely randomized design with 5 replicates with 50 seeds per replicate. The germination indices as maximum germination (GMax, as a percentage), germination times (T10, T50 and mean germination, in hours), uniformity of germination (U75–25: time interval between 25 and 75% of viable seeds to germinate) and area under the curve (AUC) were calculated using the GERMINATOR software (Joosen et al. 2010) for a 120 h period using the mathematical approach described by El-Kassaby et al. (2008). The percentage data, such as MaxG, were transformed to arcsine $\sqrt{x/100}$ (untransformed values are shown in the table to enable comparison).

RNA extraction, primers and RT-qPCR analysis

One gram of non-primed (control treatment) and osmoprimed seeds were sown on distilled water wetted filter paper in Petri dishes at 15 °C in the dark. Samples were collected at 0, 6, 18, 30, 42 and 54 h (20 mg per treatment, approximately 400 seeds) for total RNA extraction using the SV Total RNA Isolation System (Promega, Madison, WI, USA). The extracted RNAs were quantified by the spectrophotometry at A_{260} nm and purity evaluated via the absorbance ratios at A_{260}/A_{280} nm. RNA samples were treated with Turbo DNAse I (Ambion, Austin, TX, USA to eliminate DNA contamination and the quality and integrity of the samples were checked at 1% (w/v) electrophoresis agarose gel dyed with ethidium bromide. The cDNA synthesis was made using SuperScript® IV First-Strand Synthesis SuperMix kit (Invitrogen, Carlsbad, CA, USA) starting from 2 µg of total RNA. cDNAs were subjected to a PCR reaction with the specific primers of each gene to verify whether the primers amplify only the targeted 80–100 base pairs sequence.

Specific primers were designed using Primer Express v. 3.0 software (Applied Biosystems, Foster City, CA, USA) in order to obtain amplicons of 80–100 base pairs in the regions of greatest dissimilarity, mainly from the 3′-untranslated region (3′-UTR). The amplification efficiencies for all primers were calculated using the LinRegPCR software (Ramakers et al. 2003; Table 1). For preventing non-specific annealing, all primers and amplicons were confronted with GenBank sequences using Primer BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) to confirm their specificity. In addition, the primers were evaluated with Primer3Plus (https://primer3plus.com/cgi-bin/dev/primer3plus.cgi) to check for primer dimers and hairpins.

The RT-qPCR was performed on the StepOnePlus™ Real-Time PCR (Thermo Fisher Scientific, Waltham, MA, USA) apparatus using the SYBR Green detection method. The final cDNA products were diluted 10-fold before being used in RT-qPCR. Each reaction contained 5 µl 2× SYBR Green/ROX qPCR Master Mix (Invitrogen, Carlsbad, CA, USA), 0.4 µl of each primer (5 µM), 1 µl of 1:10 diluted cDNA in milli-Q water, with a final volume of 10 µl. The reaction conditions were 2 min at 95 °C, followed by 40 cycles of amplification at 95 °C for 30 s and 60 °C for 60 s. The reactions were performed in triplicate for each of the three biological replicates per sampling time-point of each treatment. In all analyses, transcript levels of the target genes were normalized against the transcriptional profile of the reference gene (At3g25800) reported by Dekkers et al. (2012). The relative expression of the genes was calculated as the expression levels of the target genes minus the At3g2580 gene as the internal reference (ΔCt). Statistical analyses among the different time-points were performed using one-way ANOVA followed by the Tukey test (p value < 0.05). Comparison between non-primed and osmoprimed seeds at each time-point was performed by paired t test for means ($*p < 0.05$).

The RT-qPCR experiment followed the "Minimum Information for Publication of Quantitative Real-Time PCR Experiments" MIQE (Bustin et al. 2009).

Results

In silico analysis of *cis*-elements in *AtEXPAs* gene promoters

From all α-expansins in *A. thaliana*, we selected those whose expression levels were greater than or equal to 10 (signal intensity in Affymetrix microarray) in the Genevestigator database (Fig S1 and Fig S2). Although *AtEXPA10* presented high expression during the germination, it was excluded from analysis due to its similar and constant expression profile in the remaining stages of plant development (Fig S2). *AtEXPA11* and *AtEXPA13* were also excluded from analysis because their higher expression peaks were in other developmental stage than seed germination, such as in rosette phase for *AtEXPA11*, and mature seed and for *AtEXPA13* (Fig S2). Therefore, five *AtEXPA* that were preferentially expressed during germination (*AtEXPA2*, *AtEXPA3*, *AtEXPA8*, *AtEXPA9* and *AtEXPA20*) were chosen for further analysis.

In silico promoter analyses of the 1 kb region upstream from the start codon of these five α-expansin genes (*ProAtEXPAs*), which are located at different positions on chromosomes 2, 4 and 5 of *A. thaliana* (Fig S4) were performed to support the choice of these isoforms for the mRNA expression experiment. More than 300 *cis*-element motifs were identified in the putative regulatory regions. Based on this initial analysis, we selected the *cis*-elements that have been previously reported in literature to be

Table 1 Genes and primers of *A. thaliana* α-expansin genes used for RT-qPCR

Gene	Primer forward (5′–3′)	Primer reverse (5′–3′)	Amplicon (bp)	Efficiency[b]
AtEXPA2 (AT5G05290)	AGCCTTTCTTTCCAAGTCACTGA	TCTGACCGAACTGCCAATCA	85	1.99
AtEXPA3 (AT2G37640)	CGTTGAGCACTGCTTTGTTCA	ACACACCATCTCGGATCATCAG	85	1.94
AtEXPA8 (AT2G40610)	CAAAGCCTTTCCTTCCAGGTAA	GGTTTGTCCGAACTGCCAAT	90	1.96
AtEXPA9 (AT5G02260)	TTACGGCCAACGCCAAAAT	GCGTCAGCTTCACCGTAGAAG	85	1.89
AtEXPA10 (AT1G26770)	CCTTGCTCAGCCTGTTTTTCA	TCTCCTGCAAGGAACCCTTCT	85	1.93
AtEXPA20 (AT4G38210)	ACTCTCAAAGGCGGCAAAAC	GTGGAACTGCTTTCCTTGGTATG	90	1.98
At3g25800[a]	AATCGGTTGTGGAGAAGACG	GCGAAAAACCTGACATCAACAT	79	1.99

[a]Reference gene according to Dekkers et al. (2012)

[b]Efficiency values calculated from LinRegPCR software (Ramakers et al. 2003)

related to seed germination. Most of the motifs found are related to the response to GA, ABA, light and seed specific (Table 2). We mapped the frequencies of identified putative *cis*-elements related to seed germination onto the selected *ProAtEXPAs* using Pattern Matching in RSA-Tools (Fig. S5). The most frequent seed specific elements were DOF in *ProAtEXPA9* (21 copies), CAATBOX1 in *ProAtEXPA20* (11 copies), POLASIG1 in *ProAtEXPA9* (7 copies) and EBOXBNNAPA in *ProAtEXPA2* (7 copies), even though being found in all *ProAtEXPAs* studied in this work.

AtEXPAs gene expression patterns in non-primed and osmoprimed seeds during germination

Our results are in agreement to what is usually expected for seeds of various plant species after osmoconditioning (Fig. 1). Time needed for the primary root emission decreased from 36 to 6 h in osmoprimed seeds as compared to non-primed seeds (Fig. 1). From day 6, the germination of primed seeds progressed gradually and linearly for 54 h and plateau at approximately 66 h after the seeds were placed on wetted filter paper. Moreover, ∼ 14-fold more primed seeds than non-primed ones were germinated after 42 h of imbibition (Fig. 1). On the other hand, the radical protrusion of the non-primed seeds was only detected after 36 h, followed by a rapid increase in the germination rate, almost leveling at approximately 72 h after the beginning of the experiment (Fig. 1).

The results presented in Table 3 show that the osmopriming treatment had no effect on the maximum percentage germination as the values of this parameter were similar in seeds subjected to the PEG treatment and dry non-primed seeds (control). However, the time required for germination of 10% and 50% of seeds was ∼ 3.0 and 1.7 times shorter in PEG treated seeds compared with the non-primed seeds and the mean germination time was ∼ 50% higher in response to osmopriming (Table 3). The time interval between 25 and 75% of viable seeds to germinate (U75–25) was 2.5-fold longer for osmoprimed seeds compared to the control seeds, showing that osmopriming caused less uniform seed germination (Table 3). Overall, the AUC measurements, which integrates the parameters Gmax, t50 and U7525, demonstrated that PEG-treated seeds had better performance (∼ 23% higher AUC values) than non-primed seeds.

We next examined the effect of osmopriming on the expression pattern of α-expansins, RT-qPCR analyses were performed using germinating osmoprimed and non-primed *A. thaliana* seeds collected at the same germination time-points. We first analyzed the expression profile of the five selected α-expansins in seeds subjected to the germination process following different water soaking periods using the in silico data available in the BAR eFP database (Fig S3). The peak of transcript accumulation for the five selected *AtEXPAs* was observed when the seeds were soaked in water for at least 12 h. Among all α-expansins studied, *AtEXPA8* and *AtEXPA9* showed the highest mRNA expression level at 12 and 24 h after imbibition, respectively (Fig S3).

Corroborating the data obtained from in silico analysis, the RT-qPCR analysis in different time-points post-germination demonstrated that the *AtEXPAs* displayed high levels of expression during germination of dry non-primed seeds. Here, the peak of transcription activity for the majority of the *AtEXPAs* was observed at later time (30 h) than reported by microarray analysis (Fig S3). This difference is probably due to the different temperatures used during seed imbibition (in our case 15 °C). From the five *AtEXPA* selected in our study, the *AtEXPA2* reached the highest expression at 30 h, exhibiting a fold change of ∼ 690 compared with 0 h time point (Fig. 2a). After this sampling-point, all *AtEXPAs* were progressively down-regulated, with exception of *AtEXPA20* (Fig. 2d). *AtEXPA3* showed the highest decline after 30 h of water imbibition (Fig. 2b).

Table 2 *Cis*-elements in five selected α-expansins promoters from *A. thaliana* based on *cis*-regulatory DNA elements PLACE and used for DNA pattern matching

Function	Motif identity	Motif sequence	*EXPA2*	*EXPA3*	*EXPA8*	*EXPA9*	*EXPA20*
GA regulated	CAREOSREP1	CAACTC	−	−	−	+	−
	GARE-motif	TAACAGA	+	+	−	−	−
	MYBGAHV	TAACAAA	+	−	−	−	+
	PYRIMIDINE BOX	CCTTTT	+	+	+	+	+
ABA/abiotic stress regulated	ABRE	YACGTGGC	+	−	−	+	−
	ACGTABREMOTIFA2OSEM	ACGTGKC	+	−	−	+	−
	DPBFCOREDCDC3	ACACNNG	+	+	+	+	+
	EMBP1TAEM	CACGTGGC	+	−	−	−	−
	LTRECOREATCOR15	CCGAC	−	−	−	+	−
	MYBCORE	CNGTTR	+	+	+	+	+
	WBOX	TGAC	+	+	+	+	+
	CBFHV	RYCGAC	−	+	−	+	+
	MYCCONSENSUSAT	CANNTG	+	+	+	+	+
	RAV1AAT	CAACA	−	+	+	+	+
Light regulated	CIACADIANLELHC	CAANNNNATC	+	+	−	−	+
	GATA-motif	WGATAR	+	+	+	+	+
	GT1	GGTTAA	+	+	+	+	+
	IBOX	GATAAG	+	−	+	+	+
	SORLIP1AT	GCCAC	+	+	+	+	+
	BOXIIPCCHS	ACGTGGC	+	−	−	+	+
	2SSEEDPROTBANAPA	CAAACAC	−	+	−	−	+
Seed-specific element	-300ELEMENT	TGHAAARK	+	+	+	+	+
	AACACOREOSGLUB1	AACAAAC	+	+	−	−	−
	ACGTABOX	ACGTA/ACGT	+	+	+	+	+
	AMYBOX1	TAACARA	+	−	−	−	+
	AMYBOX2	TATCCAT	−	−	−	−	+
	CAATBOX1	CAAT	+	+	+	+	+
	CANBNNAPA	CNAACAC	−	+	−	−	+
	CGACGOSAMY3	CGACG	−	−	−	+	+
	DOF	AAAG	+	+	+	+	+
	EBOXBNNAPA	CANNTG	+	+	+	+	+
	POLASIG1	AATAAA	+	+	+	+	+
	RYREPEAT (RY-box)	CATGCY/CATGCA	+	+	+	+	−
	SEF1MOTIF	ATATTTAWW	−	+	+	+	−
	SEF4MOTIFGM7S	RTTTTTR	+	+	+	+	−
	TGACGTVMAMY	TATCCA	+	−	−	+	−

(−) Motif absent; (+) motif present

As observed in Fig. 2, the most striking difference between osmoprimed and non-primed seeds was the level of the *AtEXPAs* transcripts at the 18-h time point of water soaking initiation. At this point, a significant amount of *AtEXPA* mRNAs were already detected in the PEG-treated seeds. In the case of the *AtEXPA2*, the highest-expressed isoform, a further increase in the number of transcripts was observed at 30 h post-imbibition, with expression levels rapidly decreasing in subsequent time points. Similar to the non-primed seeds, the mRNA levels of genes *AtEXPA8* and *AtEXPA9* were gradually decreased throughout the germination period, while the *AtEXPA20* was the least expressed gene in all sampling points.

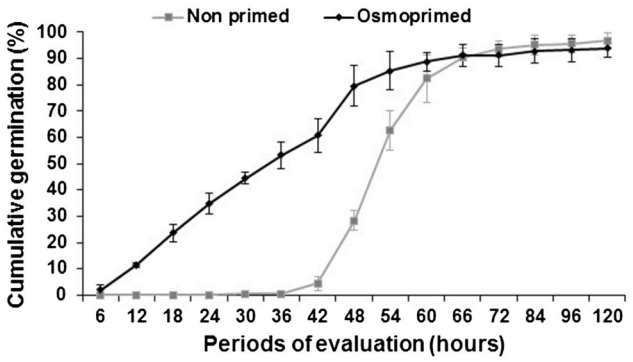

Fig. 1 Germination curve of non-primed (control) and osmoprimed (PEG) *A. thaliana* seeds. Primed seeds were exposed to − 0.75 MPa PEG soaking during 7 days. Each value is the mean of 5 replicates (50 seeds each) and vertical bars are standard deviations

Discussion

Analysis of *cis*-elements in *AtEXPA* promoters

The regulation of gene expression of various biological processes, including germination, usually depends on the type, number, position and combination of regulatory elements present in and around the promoter (Hernandez-Garcia and Finer 2014). Therefore, the identification of such *cis*-elements in *EXPA* genes help us to assess the specificity of the selected isoforms prior to experimental verification.

Among the *cis*-elements implicated in control of seed germination found in the *ProAtEXPAs*, EBOXBNNAPA (Stålberg et al. 1996), DOF (Yanagisawa and Schmidt 1999), POLASIG1 (O'Neill et al. 1990), A-box motif (ACGTABOX) (Toyofuku et al. 1998), CAATBOX1 (Wenkel et al. 2006) and − 300 element (Colot et al. 1987) were present in all sequences analyzed. DOF (AAAG) was the most frequent motif in the α-expansin promoters. A strong enrichment of motifs associated with DOF-type transcription factors was observed in the promoters coding for the DELLA protein RGL2 (one of the major negative regulators of gibberellin signaling), which inhibits seed germination by hindering the transcription of *EXPA3* and *EXPA8* (Stamm et al. 2012).

Several light-responsive *cis*-elements are present in the *AtEXPA* promoters analyzed. Among them, the elements GATA (Gilmartin et al. 1990), GT1 (Terzaghi and Cashmore 1995) and SORLIP1AT (Jiao et al. 2005) were found in all five *ProAtEXPAs*. These results can be explained by the fact that the luminosity acidifies the cell wall (Elzenga et al. 2000) and that cellular acidification is a pre-requisite for the activity of the expansins (Cosgrove 1997).

Expansin isoforms exhibit different expression patterns according to external regulatory stimuli and response to plant growth regulators (Karaaslan and Hrazdina 2010; Cosgrove 2015; Lamichaney and Katiyar 2017). Motifs related to gibberellin were identified in all analyzed EXPA promoters. For example, GAREOSREP1, which is expressed in the aleurone cells of rice seeds (Sutoh and Yamauchi 2003), was found in *ProAtEXPA9*. The GARE motif (TAACAGA) (Ogawa et al. 2003) was present in *ProAtEXPA2* and *3*, and the element MYBGAHV in *ProAtEXPA2* which is a central component in the gibberellin response complex (GARC) (Olszewski et al. 2002).

The presence of several ABA motifs in all the *AtEXPAs* promoters analyzed might be related to the secondary responses of the seeds to light and desiccation stress tolerance. The *cis*-elements W box (Xie et al. 2005) and PYRIMIDINE box (GA repressor) (Sutoh and Yamauchi 2003) were identified in all five *ProAtEXPAs* analyzed. Various motifs related to this phytoregulator were also present in *ProAtEXPAs*, as for example, the CBFHV (Xue 2002) and YACGTGGC (Hattori et al. 2002), related to dehydration and the ABA response, the DPBFCOREDCDC3, usually embryo-specific (Finkelstein and Lynch 2000) and the binding site for the transcription factor MYB (CNGTTR) (Abe et al. 2003).

ABA synthesis in imbibed seeds is required for maintaining seed dormancy and preventing precocious germination (Nambara et al. 2010). On the other hand, during germination, GA acts as an inducer of transcription of cell wall loosening protein genes, such as expansins and pectin methyl esterases (PMEs) (Ogawa et al. 2003). Thus, the balance of the antagonistic effects of GA/ABA might be involved in the expression patterns of the α-expansins during the osmopriming treatment. Indeed, Seo et al. (2006) showed that the balance of these two hormones affects the expression of *AtEXPA2*.

Table 3 Effects of osmopriming on germination performance of *A. thaliana* seeds

Treatment	T10 (h)*	T50 (h)*	MGT (h)*	U75–25 (h)*	AUC*	G max %
Non-primed (control)	43.4 ± 0.5	51.5 ± 0.5	52.1 ± 0.6	8.9 ± 0.9	64.9 ± 0.9	97 ± 1
Osmoprimed (PEG)	14.1 ± 0,3	29.7 ± 0.8	33.1 ± 0.8	22.6 ± 1.2	79.9 ± 1.7	94 ± 2

T10—time to reach 10% of germination (± SE); T50—time to reach 50% of germination (± SE); MGT—mean germination time (± SE); GMax—maximum germination (± SE); U75–25—uniformity of germination between 25 and 75% of viable germinated seeds (± SE); AUC 120 h—Area under the curve after 120 h of imbibition (± SE). * Mean statistically different by Student *t* test ($p < 0.05$). The asterisk indicates that the two means are statistically different by Student test

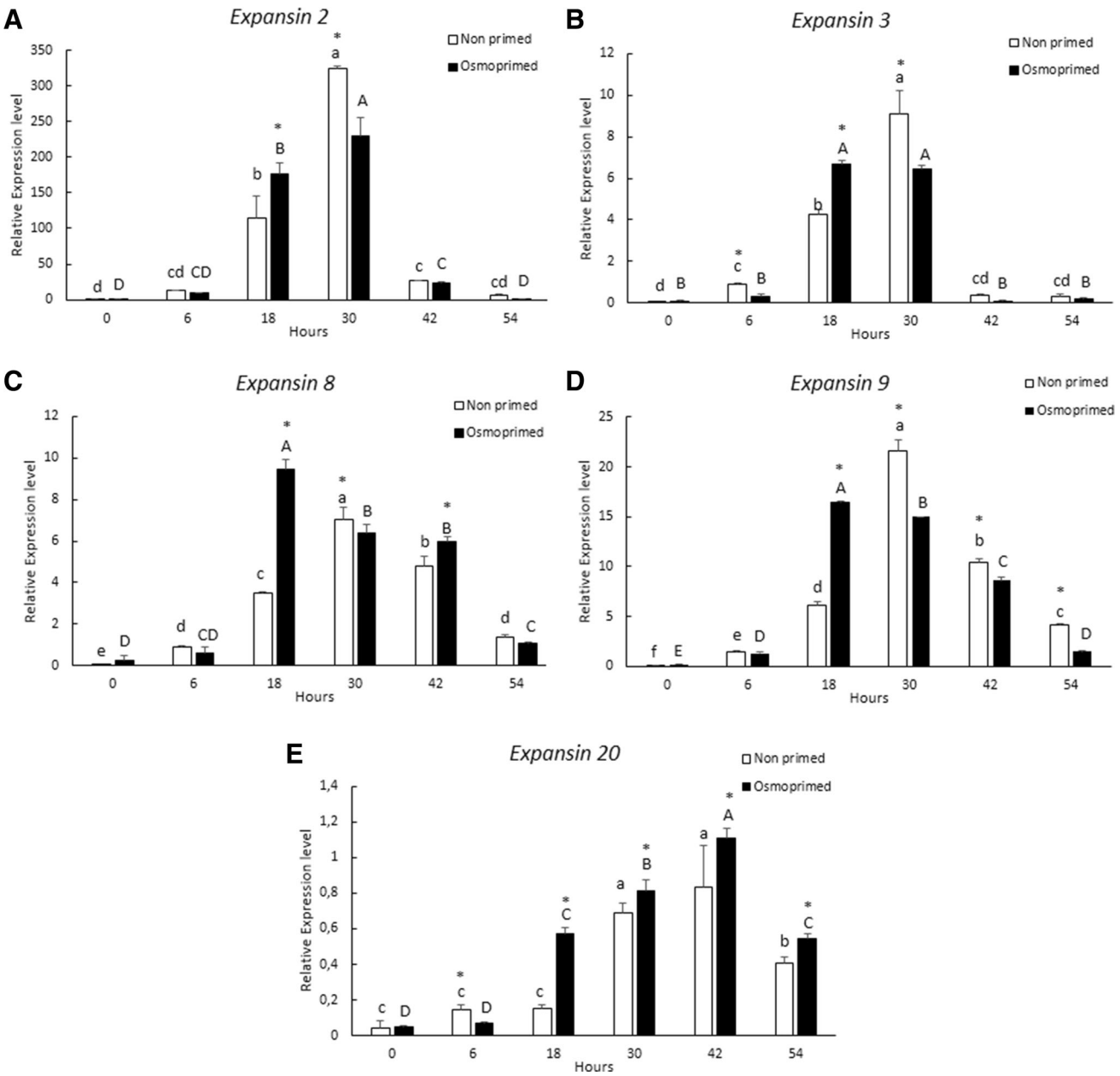

Fig. 2 Relative expression of *AtEXPA* genes in non-primed and osmoprimed seeds of *A. thaliana* collected at different germination time-points. The results are expressed as relative expression levels of the target genes with At3g2580 as the internal reference (ΔCt). Values represent mean (± SD) calculated from three biological replicates per sampling time-point. Statistical analysis among the different time-points was performed using one-way ANOVA followed by the Tukey test ($p < 0.05$). Lower- and upper-case letters indicate comparisons at different time-points in non-primed and osmoprimed seeds, respectively. Comparison between non-primed and osmoprimed seeds at each time-point was performed by paired *t* test for means (*$p < 0.05$)

Expression of *EXPAs* genes in seeds *A. thaliana* during post-priming

Seed priming often involves restriction of the imbibition time and is used to improve seed performance including germinability, vigor and stress tolerance. In this study, osmopriming induced earlier (without an apparent lag phase) and faster germination, but less uniform, compared with the non-primed seeds at 15 °C (Fig. 1; Table 3). The higher values for AUC of the PEG-treated seeds indicates that the earlier germination and higher germination rate compensate for the asynchronous germination, which can be associated with changes in thermal time requirement and length of the priming treatment (Elkoca et al. 2007). As it has been pointed out, modifications of mRNA levels and protein accumulation, occur during osmopriming and

post-priming germination (Gallardo et al. 2001; Soeda et al. 2005; Galland et al. 2014; Kubala et al. 2015). Therefore, it is expected that mRNA levels of several genes, including those involved in the relaxation of primary cell walls (such as the *EXPAs*), should be distinctively modulated in primed seeds during the germination. Previous studies have demonstrated that EXPAs play distinct tissue-specific and temporal functions during seed germination (Chen and Bradford 2000; Chen et al. 2001). The in silico expression analysis of the five selected *AtEXPAs* gene using the *Arabidopsis* eFP browser (Winter et al. 2007; Fig S3) suggests enhanced expression in seeds after water imbibition. Here, we were able to compare the transcript levels of *AtEXPAs* in dried osmoprimed seeds with non-primed seeds during the initial stages of germination by RT-qPCR. This analysis confirmed the increased expression levels of all five *AtEXPAs* during the early phases of seed germination and, in general, that the transcripts abundance mostly increased until circa 30 h of water imbibition for both non-primed and osmoprimed seeds (Fig. 2). Consistent with the microarray data (Winter et al. 2007; Fig S3), *AtEXPA 2*, *3*, *8* and *9*, with emphasis for the first isoforms, were the most expressed genes in non-primed germinating seeds (Fig. 2). In fact, based on gene expression studies, it has been proposed the use of *AtEXPA2* as a molecular marker for specific tissues of non-dormant or germinating *A. thaliana* seeds (Penfield et al. 2006; Yan et al. 2014).

The primed seeds presented high *AtEXPAs* mRNA levels at 18 h of water imbibition for all *AtEXPAs* but *AtEXP20*, showing that transcripts of all five EXPA isoforms are produced in more quantities earlier than non-primed seeds due to the osmopriming treatment. Specifically, the higher fold changes comparatively to non-primed seeds were observed for *AtEXPA8* and *9* at 18 h when elevated levels of transcripts (almost 2.7-fold) were already detected (Fig. 2). Together with *AtEXPA2*, the mRNAs of these EXPA were highly detected in the endosperm cap prior to the onset of endosperm weakening process that leads to testa rupture (Penfield et al. 2006, Holdsworth et al. 2008; Morris et al. 2011). The *AtEXPA3*, reported to be specifically expressed in the radicle (Morris et al. 2011), showed a great decline in expression after 30 h imbibition ($\sim$ 60-fold) with progression of the germination process (late germination phase and endosperm rupture) (Fig. 2b).

Despite the great difference in mRNA abundance at the 18 h time point, the osmoprimed and non-primed seeds presented similar gene expression levels of *AtEXPAs* at 0 and 6 h post-germination (Fig. 2). This equalization in expression indicates that the de novo transcription of the studied EXPAs at the very beginning of water imbibition does not reflect in a faster germination rate of the primed seeds (Fig. 1). In addition to the *AtEXPA* mRNAs triggered by the applied osmopriming procedure (PEG treatment and post-priming drying), we cannot discard the possibility that active α-expansins accumulated during the 7 days of osmopriming treatment may have had some effect on the earlier germination times observed in the osmoprimed seeds (Fig. 1). Seed treatment with a low osmotic potential PEG-solution during several days, as our case, can be considered a water stress situation. It is well known that EXPAs are up-regulated in response to water deficit condition (Cosgrove 2015; Marowa et al. 2016); hence, the accumulation of *AtEXPAs* may have occurred under water restriction conditions caused by the PEG treatment during the osmotic stress or even during the post-priming drying. In cucumber hypocotyl cell walls, expansins were detected at very low concentrations under normal conditions (McQueen-Mason and Cosgrove 1995). It is well known that transcriptional regulation of gene expression is a major mechanism used by plants for adaptation in changing environments. However, complex processes exert profound influence on the modulation of the final protein level. As such, further detailed studies on post-transcriptional and post-transductional regulation from a more specific perspective are necessary to fully assess the role of EXPAs during short or long treatments with different PEG concentration, either during and after water imbibition in osmoprimed seeds compared to the non-primed ones. Finally, our results are consistent with the knowledge that the osmopriming process (PEG treatment and post-priming drying) acts as a strong activator in the germination process (Heydecker and Gibbins 1978; Bray 1995) evoking stronger and earlier expression of the *AtEXPA* genes associated with the acquisition of germination capacity. Given the high mRNA levels of the α-expansin isoforms (*AtEXPA2*, *3*, *8* and *9*), this observation suggests that these genes may serve as markers of the earlier stages of germination and priming efficiency in *Arabidopsis*. Also, as plant promoters tend to respond to several transcription factors and are consequently activated by more than one pathway, the biological significance of the identified *cis*-acting elements occurring onto the promoters of up-regulated *AtEXPAs* and their effective involvement in different stages and tissues should be further investigated in non-primed and osmoprimed seeds.

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Author contributions LGEV and AFR conceived and designed the experiments. FLA, CCC and NBMN performed the germination and priming experiments. AFR and NVS carried out the bioinformatics analysis. AFR and TBS carried out the RT-qPCR assays and analyzed the data. LGEV, AFR and NVC wrote the manuscript.

Compliance with ethical standards

Conflict of interest NVS has received a grant from São Paulo Research Foundation (FAPESP) and Coordination for the Improvement of Higher Education Personnel (CAPES) for the fellowship during her doctor degree and National Council for Scientific and Technological Development—CNPq for the fellowship during her master degree. TBS has received a grant from Coordination for the Improvement of Higher Education Personnel (CAPES) for postdoctoral fellowship. LGEV has received a grant from National Council for Scientific and Technological Development—CNPq for the research fellowship. AFR, FLA, CCC, NBM declare that they have no conflict of interest.

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