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# Osmo-priming in tomato seeds down-regulates genes associated with stress response and leads to reduction in longevity

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**Research Paper**

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**Abstract**

Tomato seeds subjected to osmo-priming show fast and more uniform germination. However, osmo-priming reduces seed longevity, which is a complex seed physiological attribute influenced by several mechanisms, including response to stress. Thus, to have new insights as to why osmo-primed tomato seeds show a short life span, we performed a transcript analysis during their priming. For that, we performed gene expression studies of the heat-shock protein family genes that were previously reported to be associated with the enhancement of longevity in primed tomato seeds. Physiological assays of germination, vigour and longevity tests were used to support the data. The results show that the short life span of osmo-primed tomato seeds is related to the decrease in the expression of transcripts associated with response to stress during the priming treatment. These results are important because they add information regarding which seed longevity mechanisms are impacted by the priming treatment. In parallel, it will allow the use of these genes as markers to monitor longevity in osmo-primed tomato seeds.

## Introduction

Seed priming is a broadly used treatment by the seed industry aiming to improve the physiological performance of seed lots. During priming, the hydration of seeds is controlled allowing phases I and II of the germination process to start, and then the treatment is interrupted so that phase III (radicle protrusion) is deterred (Bradford, 1986). Fast and uniform germination are the main benefits of the priming technique. Seeds, such as *Solanum lycopersicum*, may have slow and uneven seed germination due to the mechanical restraint to radicle protrusion imposed by the micropylar endosperm (Toorop et al., 2008; Nonogaki et al., 2000; Bewley et al., 2013). The weakening of micropylar endosperm cells is a prerequisite for radicle protrusion of tomato seeds (Toorop et al., 2008). Thus, priming has been used to increase the speed of germination in tomato seeds (Batista et al., 2020). Apparently, priming improves physiological performance by favouring changes in the embryo and in the micropylar endosperm. For instance, Anese et al. (2011) showed that in *Solanum lycocarpum* seeds, growth of the embryo occurs concomitantly with weakening of the micropylar endosperm during the priming treatment.

Although the priming treatment improves physiological performance as mentioned earlier, it negatively affects seed longevity as was shown by Liu et al. (1996), Bruggink et al. (1999), Buitink et al. (2000) and Batista et al. (2020). Seed longevity is the ability of a seed to remain viable during storage in the dry state (Leprince et al., 2017) and is acquired over seed maturation. Several elements are involved in this process, including the non-reducing sugars, which play a role in membrane protection and form the cytoplasm glassy state, late embryogenesis abundant proteins (LEAs), the seed coat, which protects against oxygen absorption by the embryo, RNA-binding proteins that conserve seed mRNA in the dry state, an array of antioxidant molecules, and heat-shock proteins (HSPs), which are protective proteins that play a role as chaperones under stress conditions, according to the review by Zinsmeister et al. (2020).

According to Batista et al. (2020), some of the mechanisms associated with seed longevity are negatively impacted during the priming treatment. Sano and Seo (2019) proposed that the short life span of primed *Arabidopsis thaliana* seeds is associated with the progress of the cell cycle during the priming treatment. On the other hand, Wang et al. (2018) showed that primed and stored rice seeds presented events associated with the reduction in starch metabolism, the consumption of starch reserves from the endosperm, the accumulation of malondialdehyde and the decrease of antioxidant activities of the enzymes, which could explain the short longevity observed.

Recently, Batista et al. (2020) showed that a heat-shock treatment is able to enhance longevity in primed tomato seeds and preserve the vigour of the seeds during storage. The authors



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showed that transcripts associated with stress response are up-regulated after the heat-shock treatment in primed tomato seeds. They proposed that these transcripts may have a positive influence on the enhancement of longevity. Based on these results and considering the importance of stress response molecules to maintain seed longevity in primed tomato seeds, we hypothesize that the loss of storability observed in osmo-primed tomato seeds is due to the down-regulation of genes associated with stress response that apparently occurs during the priming treatment. To confirm this, we tested this hypothesis by performing gene expression studies of transcripts related to stress response during the osmo-priming treatment in tomato seeds.

## Material and methods

### Seed production

Tomato seeds from the LA1509 accession, donated by the Tomato Genetics Resource Center (https://tgrc.ucdavis.edu/), were produced as described in Batista et al. (2020). Red fruits without the presence of green colour were harvested. The fruits were cut with the aid of a knife and the seeds were extracted by hand. The seeds were then treated with a sodium hypochlorite solution at an initial concentration of 9% at a ratio of 1:1 of seeds for a period of 30 min to remove the remnants of the fruit. Seeds were stored at 12°C and 60% ±2% relative humidity (RH) until the beginning of the experiment. The seed lot was mixed and empty seeds were discarded.

### Priming, drying and heat-shock treatment

The priming treatment was performed according to Batista et al. (2020), which consisted of placing the seeds into tubes containing a polyethylene glycol (PEG) 6000 solution with an osmotic potential of −1.0 MPa at 20°C, for 60 h in the dark. The tubes were placed over a mixer to shake the solution. The PEG solutions were renewed three times to avoid changes in the osmotic potential. After the priming treatment, seeds were washed in running water, placed over paper towels, and kept for 24 h at 20°C and 60% ±2% RH for drying. In parallel, another group of seeds were placed over paper towels and exposed to 38°C and 32% RH, in an oven with air circulation for 2 h following the heat-shock treatment protocol as defined by Batista et al. (2020). After that, these seeds were maintained in the drying conditions described above. The unprimed seeds were used as a control group.

### Physiological assays

After treatment, the seeds were stored in hermetic glass pots and placed in a cold chamber at 12°C and 60% RH ±2% which resulted in a moisture content of $0.08 \pm 0.01$ g $H_2O$/g $DW^{-1}$. After that, the following tests were performed:

*Seed germination and vigour* – Seven replications of 50 seeds each were germinated in plastic boxes ($11 \times 11 \times 3.5$ cm) with a substrate of paper towel moistened with distilled water equal to 2.5 times the weight of the substrate at 25°C, in an 8 h light and 16 h dark cycle. A radicle length of 2 mm or more was used as a germination criterion. We determined seed vigour by first germination count on the 5th day and the calculation of t50 (time to 50% of germination) through the analysis of cumulative germination data. The curve fitting module of the Germinator software package was used to calculate t50 (Joosen et al., 2010). Total germination was determined on the 14th day.

*Longevity* – This test was performed by placing the seeds on a support over a saturated solution of NaCl (75% RH) at 35°C in hermetically sealed glass bottles. At different time intervals, seed viability was assessed by placing the seeds under the same conditions as described for the germination assay. The viability data were transformed into probits to determine the moment when the germination was reduced to half (p50), by using the equation: $v = Ki - p/\sigma$, according to Ellis and Roberts (1980) where $v$ is viability in days, $Ki$ is the initial germination in probit values, $p$ is expected death over time and $\sigma$ is the slope of the curve.

### RNA extraction and quantitative real-time PCR

Three biological samples of 100 seeds each were collected from seeds that had been primed, unprimed and primed plus heat shock. At the same time, we collected three biological samples of 100 seeds each at 3, 12, 42 and 60 h during priming (in phase II, data not shown). All seeds were stored at −80°C. Total RNA was extracted using the NucleoSpin® RNA plant commercial Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. The purity and quantity of total RNA was checked in a Nanodrop-2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The integrity of the RNA was checked in agarose gel at 1%.

cDNA was synthesized by using a High-Capacity cDNA Reverse Transcription commercial kit (Applied Biosystems, Victoria, Australia) following the manufacturer's instructions. For a 20 µl reaction, we used 2.0 µl 10× RT Buffer, 0.8 µl 25× dNTP, 2.0 µl 10× RT Primer, 1 µl Reverse Transcriptase, 10 µl of the extracted RNA and 4.2 µl Nuclease-free water. The reaction was incubated in a thermocycler following these steps: 5 min at 25°C, 2 h at 37°C, followed by 5 min at 85°C and the synthesis was ended at a constant temperature of 4°C.

The genes used for Real-Time PCR were selected based on the work done by Batista et al. (2020). In order to confirm the effect of priming, we selected expansin which is a gene related to germination (Chen and Bradford, 2000; Chen et al., 2001). Thus, the following genes were studied: *HEAT STRESS TRANSCRIPTION FACTOR B-2B (HSFB2b)*, *DNAJ PROTEIN HOMOLOG*, *HEAT SHOCK PROTEIN 70 (HSP70)*, *SMALL HEAT SHOCK PROTEIN PRECURSOR (er-HSP)*, *15.7 KDA HEAT SHOCK PROTEIN (HSP15.7)* and *EXPANSIN (EXP2)*. The primers used were efficient above 1.8 and $r^2$ of approximately 1.0, according to the parameters of the LinRegPCR program (Ruijter et al., 2013). The primers used are listed in Table 1.

We performed the gene expression analysis in a thermocycler Eco Real-Time (Illumina, San Diego, USA) with SYBR Green qPCR ReadyMix (Sigma-Aldrich, St. Louis, USA), using two technical samples for each biological sample. For a reaction of 10 µl, 5 µl of SYBR Green, 1 µl of cDNA and 0.25 µl of each primer were used and the volume was adjusted with Nuclease-free water. The amplification consisted of 2 min at 50°C, 2 min at 95°C; then 45 cycles of 10 s at 95°C and 1 min at 60°C. At the end of the process, the melting curve was performed following these steps: 15 s at 95, 65 and 95°C, respectively. The normalized expression (NE) was assessed using a geometric means of three reference genes, *UBIQUITIN CONJUGATING ENZYME 21*

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**Table 1.** Primer sequences used as target and reference genes (mRNAs) in RT-qPCR reactions

| Gene name | Forward (5′–3′) | Reverse (5′–3′) | bp |
|---|---|---|---|
| UBC21[a] | GGACGGCTCTTGTTAAAGG | TGGATACTGCTCTGGAACTG | 86 |
| MTP[a] | CTACACCGAAAGCAGCAC | CAGCCATTCTCAGCAACAG | 110 |
| ASAR1[a] | TAGCGACTGTTCCCTTCC | TTACCCTTGCCAGTAGTGAC | 114 |
| HSFB2b | ATGAAGATATGAGCCCACGG | GCGGTTGTACTTGATCCTG | 102 |
| DnaJ protein homolog | TCTCCAACAGAAAGATCACCC | CCAAGTGTGCCAATATGAACTG | 116 |
| HSP 70 | AATCCCTCCAGCTCCCAG | GCCGTGACAGAAAGAATACC | 81 |
| HSP 15.7 | GACGAATTCCACGGTAAAGAG | TGATCCACCTTCACATCTTCC | 116 |
| er-HSP | ACCAAATGATAAGCAGCAATCC | TTCGCCGTCTCTTTCCAG | 121 |
| EXP | ACTTGTGGTGCTTGTTATGAG | TGTTAGGTAGAGACGGGTTC | 115 |

bp, basepairs.
[a]Reference genes.

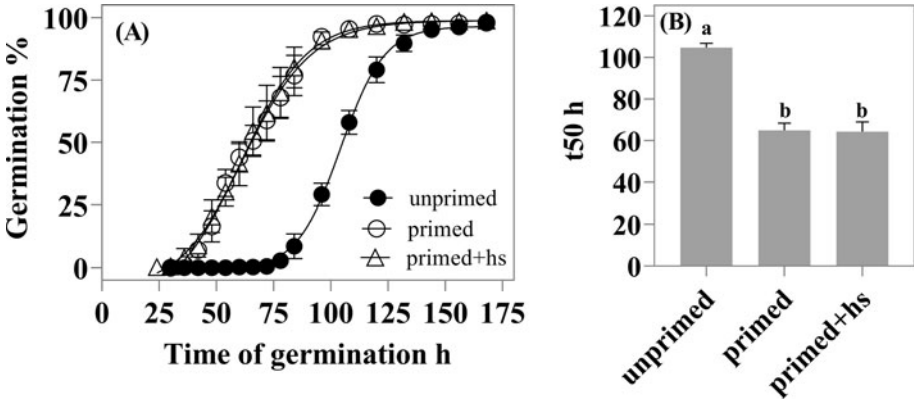


**Fig. 1.** Effect of the priming and heat-shock treatments on seed germination and vigour in *S. lycopersicum* seeds over time. Seed germination time curve (A). Time to 50% of germination – t50 (B) in unprimed, primed and primed plus heat shock seeds. Different letters indicate significant difference ($P \leq 0.05$) by Fisher's LSD test. n.s., not significant. Error bars show standard deviation from seven samples.

*(UBC21)*, *METALLOTHIONEIN (MTP)* and *ARF-LIKE GTPASE FAMILY PROTEIN (ASAR1)*, calculated according to: $NE = 2^{\Delta Ct}$, where ΔCt is the geometric mean of the Ct reference genes − Ct target gene.

### Statistical analysis

We checked the normality of the data by the Shapiro–Wilk test. The data fulfilled the assumption for normality. Thus, a one-way analysis of variance (ANOVA) was performed and the significant physiological characteristics or transcript levels were separated using Fisher's Least Significant Difference (LSD) test at 0.05-confidence level. The sigmoidal behaviour during storage was adjusted using the Boltzmann equation parameters.

## Results

### Physiological characterization

Unprimed seeds started to germinate after 60 h of imbibition and reached 50% of germination (t50) in 104 h, while primed and primed plus heat shock seeds started to germinate after 36 h, and t50 was reached in 64 h (Fig. 1A). Therefore, primed and primed plus heat shock seeds showed an increase in the speed of germination with a significant reduction ($P < 0.001$) in t50 of 40 h in relation to unprimed seeds (Fig. 1B).

Consequently, there was an increase ($P < 0.001$) greater than 17% in the first germination count in primed and primed plus heat shock seeds in relation to unprimed seeds (Fig. 2A). Unprimed, primed and primed plus heat shock seeds reached their maximum germination rate (≥98%) on the 7th day (Fig. 1A). Thus, the priming and heat-shock treatment did not affect the final germination (Fig. 2B).

At the beginning of storage, unprimed, primed and primed plus heat shock seeds displayed 100% germination. Nevertheless, the germination of primed seeds was reduced to 50% on the 26th day, while primed plus heat shock seed germination was reduced to 50% on the 42nd day, and unprimed seeds reached p50 after 112 d (Fig. 3A). Thus, there was a significant reduction ($P < 0.001$) of 83 d in longevity (p50, days) of primed seeds in relation to unprimed seeds (Fig. 3B). The heat-shock treatment promoted an increase in longevity of 16 d compared to primed seeds ($P < 0.001$).

Through controlled storage, it was possible to verify the reduction in storability for primed and primed plus heat shock seeds, which lost germination capacity completely after 100 d, while more than 85% of unprimed seeds remained viable (Fig. 3A).

### The pattern of Gene expression profile during priming

We found that for primed seeds without the heat-shock treatment, there was a significant reduction ($P < 0.001$) in the transcript levels of *HSFB2b*; *HSP 70*; *HSP 15.7*; *DNAJ PROTEIN HOMOLG*; *ER-HSP* and *HSP 15.7* over the process of priming, mainly from 12 h of the priming treatment, which was not

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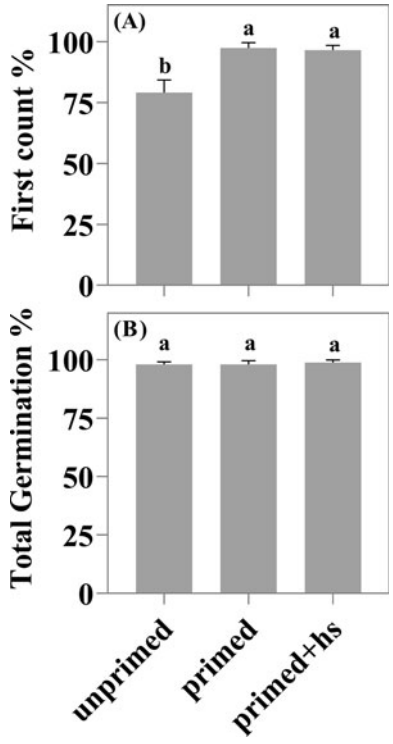


**Fig. 2.** Effect of the priming and heat-shock treatments on seed germination and vigour in *S. lycopersicum* seeds. First germination count on the 5th day (A) and total germination (B) in unprimed, primed and primed plus heat shock seeds. Different letters indicate a significant difference ($P \leq 0.05$) by Fisher's LSD test. n.s., not significant. Error bars show standard deviation from seven samples.

re-established after drying (Fig. 4A–E). The expression of *HSP 15.7* increased at 3 h of priming in relation to the unprimed seeds, however this decreased after 12 h, similar to the other genes after this period (Fig. 4E).

However, in primed plus heat shock seeds, there was an increase in the expression level of genes related to the response to stress in relation to seeds collected at 42 and 60, and primed seeds (Fig. 4A until E) when the reduction of transcript levels was drastic. Nonetheless, the heat-shock treatment re-established the transcript levels of HSFB2b, *DNAJ PROTEIN HOMOLG* and *ER-HSP* genes to the same level as found at 12 h (Fig. 4A, B, D).

To confirm the effect of the priming treatment at the molecular level, we studied the pattern of *EXP* expression levels. Thus, we verified that the expression increased after 42 h during the priming treatment, and in primed seeds, it was higher than in unprimed seeds (Fig. 4F).

## Discussion

Seed priming is an important technique used by the seed industry. Faster and more uniform seed germination are considered the main benefits promoted by the priming treatment. However, primed seeds have shorter longevity. Seed longevity maintains the life span of seeds during storage, which is important to ensure the propagation of the seeds over the time. Here, we demonstrated that during osmo-priming of tomato seeds, the genes that code for proteins associated with longevity in primed seeds have their expression pattern affected.

In our study, the priming protocol increased the performance of *S. lycopersicum* seeds and the heat-shock treatment did not interfere with this, which was verified through the speed of germination and consequently germination rate in the first count (Figs 1 and 2A). However, there was a notable reduction in seed longevity in the primed seeds (Fig. 3), confirming previous studies by Gurusinghe et al. (2002) and Batista et al. (2020). This reduction in longevity is less pronounced when using the heat-shock treatment after priming (Fig. 3) as previously reported by Batista et al. (2020).

Previous studies were performed to comprehend the mechanism associated with the loss of seed longevity in primed seeds. In *Impatiens* and pepper seeds, the reduced longevity after priming was not related to an increase in molecular mobility in the cytoplasm (Buitink et al., 2000). In primed *Arabidopsis* seeds, the short longevity was correlated with the advancement of the cell cycle (Sano and Seo, 2019).

Nevertheless, there is still a knowledge gap about how exactly this loss of longevity is controlled. Recently, Batista et al. (2020) demonstrated that the transcripts associated with protection to stress are involved in the enhancement of longevity in primed tomato seeds. Thus, considering the role of the protection mechanism in seed longevity as reviewed by Zinsmeister et al. (2020), we investigated the expression pattern of transcripts associated with stress response during osmo-priming to confirm whether there is a significant change in the expression level of these genes that could be responsible for the short longevity of osmo-primed tomato seeds.

Our hypothesis proved correct since our results demonstrated that there is down-regulation of *HSFB2b* (Fig. 4A), which leads to a reduction in transcripts that code for HSPs (Fig. 4B until E) which are associated with stress protection (Guo et al., 2016). The heat-shock protein family is important to seeds due to their chaperone role in protecting against cellular damage under stress conditions. The HSPs are present during seed development and are always related to the acquisition of germinability, desiccation tolerance and consequently longevity (Kaur et al., 2016). It was demonstrated that heat shock factor A9 acts against deterioration in transgenic tobacco seeds (Prieto-Dapena et al., 2006), which implies its role in storability. HSFA6B and sHSPs were correlated with the increase in seed longevity in soybean (Lima et al., 2017). Kaur et al. (2015) reported that *OsHSP18.2* in *A. thaliana* is an ageing responsive protein that possibly protects and stabilizes the cellular proteins during maturation drying, desiccation and ageing in seeds by restricting reactive oxygen species accumulation and thereby improving seed vigour, longevity and seedling establishment. According to Zhang et al. (2018), a cytosolic class II small heat-shock protein, *PfHSP17.2*, confers resistance to heat stress in transgenic *Arabidopsis*, which may result in resistance to deterioration. The DnaJ proteins resemble HSPs and act as chaperones in response to heat stress and plant growth and development (Fan et al., 2017); the DnaJ protein homologous gene was associated with the enhanced longevity of primed tomato seeds, as shown by Batista et al. (2020).

The studies reported earlier showed an important role of the HSP family in seed quality, especially seed storability, and as demonstrated here, the expression of these genes was reduced in tomato seeds by the priming treatment (Fig. 4A until E). According to Batista et al. (2020), these genes are important to maintain viability in the dry state during storage in primed tomato seeds.

Our experiments in primed and primed plus heat-shock treatment seeds confirmed the importance of these transcripts to enhance longevity in primed tomato seeds as mentioned earlier, since it was demonstrated that there was an increase in the gene

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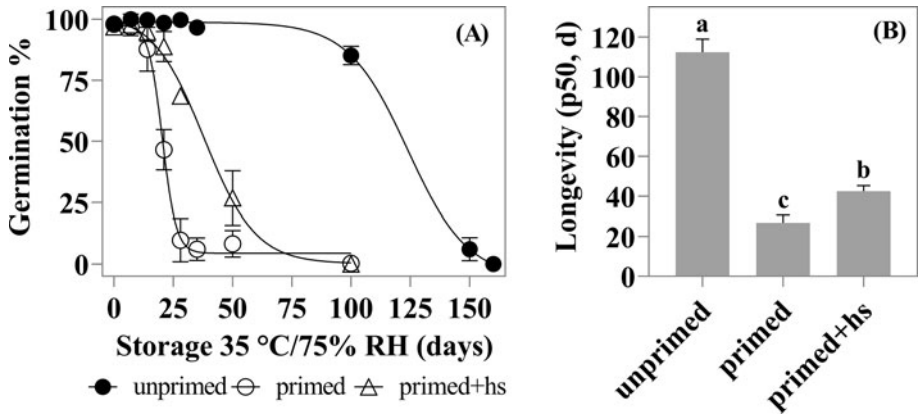


**Fig. 3.** Germination during storage and seed longevity in primed, primed plus heat shock and unprimed *S. lycopersicum* seeds. Germinability during storage (A) and longevity expressed in p50 (the number of days in which the seed lot has lost 50% of viability during storage) (B) from unprimed, primed and primed plus heat shock seeds. Germination during storage was fitted using Boltzmann sigmoid [$y = \text{top} + (\text{bottom-top})/1 + [v50 - (x/\text{slope})]$]. Different letters indicate a significant difference ($P \leq 0.05$) by Fisher's LSD test. Error bars show standard deviation from seven samples.

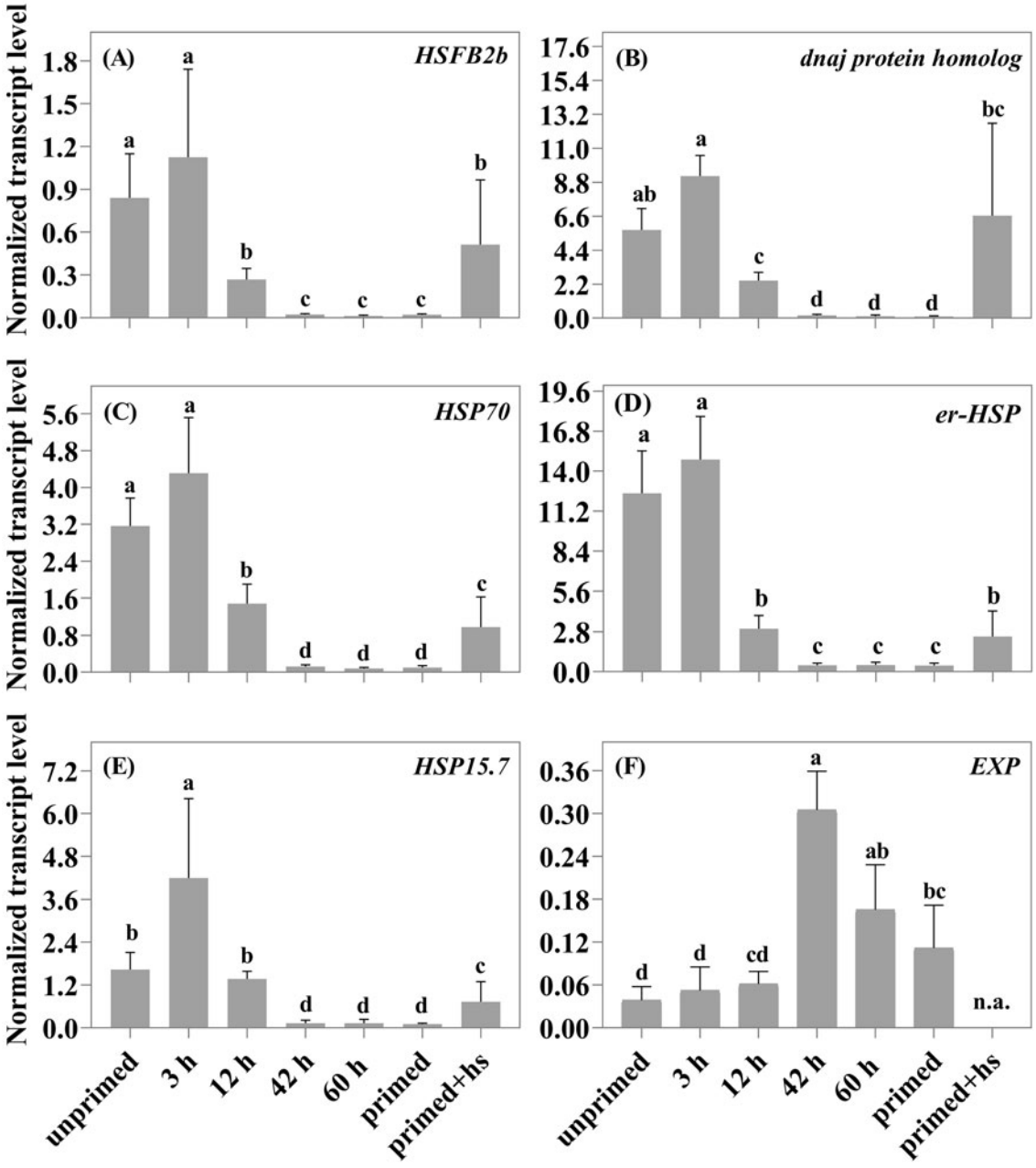


**Fig. 4.** Gene expression before (unprimed seeds), during and after the priming treatment and plus heat-shock treatment of *S. lycopersicum* seeds. Different letters indicate a significant difference ($P \leq 0.05$) by Fisher' LSD test. n.a., not available. Error bars indicate standard deviation from six technique samples.

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expression of transcripts related to seed longevity, and consequently, an enhancement of storability in the primed tomato seeds subjected to heat-shock treatment in comparison to seeds that were only primed (Figs 3 and 4A until E).

In addition, during seed germination, there is an activation of the protection system that protects the seeds against damage and reduces the effects of ageing (Bewley et al., 2013). In our study, the expression of *HSP 15.7* increased at the beginning of the treatment while other genes maintained their initial level, and was gradually reduced with the advance of the treatment (Fig. 4A until E). As a result, after drying (primed seeds), the expression of genes related to stress response was lower than the initial level (Fig. 4A until E), which is associated with the shorter longevity found in osmo-primed tomato seeds (Fig. 3). Apparently, the deterioration process may begin already during the treatment.

Thus, transcripts that code for molecules associated with stress response are compromised during priming. It is true that several other mechanisms are associated with longevity as mentioned earlier. However, the findings of Batista et al. (2020) have shown that the stress response genes are involved in enhancing the longevity in primed tomato seeds. This led us to explore these genes during priming and better understand how the reduction in the expression of these transcripts by priming caused the shorter life span in primed tomato seeds (Figs 3 and 4A until E). In addition, this research opens the possibility to use some of these transcripts as markers to monitor longevity of primed tomato seeds during storage.

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