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Chromatin-associated regulation of sorbitol synthesis in flower buds of peach

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Key message

PpeS6PDH gene is postulated to mediate sorbitol synthesis in flower buds of peach concomitantly with specific chromatin modifications.

Abstract

Perennial plants have evolved an adaptive mechanism involving protection of meristems within specialized structures named buds in order to survive low temperatures and water deprivation during winter. A seasonal period of dormancy further improves tolerance of buds to environmental stresses through specific mechanisms poorly known at the molecular level. We have shown that peach *PpeS6PDH* gene is down-regulated in flower buds after dormancy release, concomitantly with changes in the methylation level at specific lysine residues of histone H3 (H3K27 and H3K4) in the chromatin around the translation start site of the gene. *PpeS6PDH* encodes a NADPH-dependent sorbitol-6-phosphate dehydrogenase, the key enzyme for biosynthesis of sorbitol. Consistently, sorbitol accumulates in dormant buds showing higher *PpeS6PDH* expression. Moreover, *PpeS6PDH* gene expression is affected by cold and water deficit stress. Particularly, its expression is up-regulated by low temperature in buds and leaves, whereas desiccation treatment induces *PpeS6PDH* in buds and represses the gene in leaves. These data reveal the concurrent participation of chromatin modification mechanisms, transcriptional regulation of *PpeS6PDH* and sorbitol accumulation in flower buds of peach. In addition to its role as a major translocatable photosynthate in Rosaceae species, sorbitol is a widespread compatible solute and cryoprotectant, which suggests its participation in tolerance to environmental stresses in flower buds of peach.

Keywords

Abiotic stress, bud dormancy, chromatin, *Prunus persica* (peach), sorbitol-6-phosphate dehydrogenase (S6PDH), aldose-6-phosphate reductase (Ald6PRase)

Introduction

Adaptation of perennial plants from temperate climates to seasonal fluctuations in temperature relies on bud set-up and dormancy (with the meaning of endodormancy throughout the text) among other adaptive improvements. Dormancy ensures the survival of vegetative and reproductive meristems in a quiescent state, which is released after the fulfilment of bud-intrinsic requirements of chilling, resembling the vernalization process described in *Arabidopsis thaliana* and cereals (Chouard 1960; Horvath et al. 2003). After achievement of chilling requirements and subsequent dormancy release, buds enter in an ecodormant stage requiring of a period of warm temperatures for proper bud-burst and flowering (Couvillon and Erez 1985). Buds undergoing dormancy are more tolerant to low and freezing temperatures and to desiccation, in virtue of physiological and molecular mechanisms that are insufficiently known, and that could partially overlap with those implicated in bud dormancy regulation (Fennell 2014; Wisniewski et al. 2015).

Among other, several epigenetic mechanisms involving genomic DNA methylation (Santamaría et al. 2009; Kumar et al. 2016a), histone modifications (Horvath et al. 2010; Leida et al. 2012; Ríos et al. 2014; Saito et al. 2015) and small RNAs production (Bai et al. 2016; Niu et al. 2016) have been postulated to mediate dormancy-dependent regulation of gene expression. Foremost targets of those regulatory pathways are *DORMANCY-ASSOCIATED MADS-box* (*DAM*) genes, identified as key transcriptional factors modulating bud dormancy in leafy spurge and stone-fruit tree species (Bielenberg et al. 2008; Horvath et al. 2010; Niu et al. 2016). Four from six tandemly arrayed *DAM* genes in peach are specifically modified by trimethylation of histone H3 in lysine 27 residue (H3K27me3) at specific genomic regions (de la Fuente et al. 2015). In spite of such burst of dormancy literature, supporting evidence on the participation of similar epigenetic pathways in the regulation of stress-related genes along bud development is still lacking.

Sorbitol is the primary photosynthetic product and the major phloem-translocated form of carbon in the Rosaceae (Webb and Burley 1962; Bieleski 1969). This sugar alcohol, or polyol, has been proposed to perform a protective role against stresses, acting as cryoprotectant, osmolyte and compatible solute under freezing, osmotic and water stress, respectively (Bieleski 1982; Loescher 1987; Escobar-Gutiérrez and Gaudillère 1996). Drought stress increases sorbitol accumulation in peach, although its participation in osmotic adjustment is a matter of controversy (Escobar-Gutiérrez et al. 1998; Lo Bianco et al. 2000). Sorbitol is produced in source tissues (photosynthetic leaves) via reduction of glucose-6-phosphate to sorbitol-6-phosphate by NADPH-dependent sorbitol-6-phosphate dehydrogenase (S6PDH), and the subsequent dephosphorylation of sorbitol-6-phosphate by a specific phosphatase, whereas sorbitol utilization occurs in sink tissues (Grant and ap Rees 1981; Loescher 1982). Since the first cloning of *S6PDH* gene from apple (Kanayama et al. 1992), many *S6PDH* have been identified based on sequence similarity to this gene, however few of them have been characterized at the enzymatic level. The overexpression of *S6PDH* from apple in transgenic tobacco increases sorbitol content and induces necrotic lesions in some cases (Tao et al. 1995; Sheveleva et al. 1998), whereas *S6PDH* silencing in apple reduces sorbitol accumulation and alters carbon partitioning (Teo et al. 2006). Moreover, the expression of *S6PDH* is up-regulated by abscisic acid, low temperature and NaCl treatments in apple (Kanayama et

al. 2006). Interestingly, expression of *S6PDH* from apple in a *Saccharomyces cerevisiae* mutant deficient in synthesis of the osmolyte glycerol partially restores the tolerance to high NaCl concentrations (Shen et al. 1999). Also, overproduction of sorbitol by the expression of apple *S6PDH* confers NaCl tolerance in transgenic Japanese persimmon (Gao et al. 2001).

Very recently, an ortholog of *S6PDH* has been cloned in peach, and its encoded protein (PpeAld6PRase) has been purified and extensively characterized at the enzymatic level (Hartman et al. 2017). In this work we refer to the gene encoding PpeAld6PRase as *PpeS6PDH*, following nomenclature suggestions of the Genome Database for Rosaceae (https://www.rosaceae.org/gene_class_listing). *PpeS6PDH* was unexpectedly expressed in dormant flower buds, a sink tissue, in a developmentally regulated manner. We have postulated its participation in the environmental and developmental dependent synthesis of sorbitol in buds.

Materials and methods

Plant material and stress treatments

Peach (*Prunus persica* [L.] Batsch) plants that were employed in this study were grown at Instituto Valenciano de Investigaciones Agrarias (IVIA) located in Moncada (Spain). Chilling requirements for dormancy release of the cultivars under study were previously estimated (Leida et al. 2012), and expressed in chilling hours (CH, hours at < 7º C) required to have a bud break percentage higher than 50 % under forcing conditions. Interval estimates were as follows: 'Red Candem' <278 CH, 'Crimson Baby' 412-511 CH, and 'Big Top' 674-712 CH. Collecting dates, cold accumulation and bud break percentage under forcing conditions of samples used for expression analysis and chromatin studies were respectively the following: 'Big Top' samples BT1 (November 3, 0 CH, 0 %), BT2 (December 29, 276 CH, 0 %), BT3 (January 12, 385 CH, 0 %), BT4 (February 16, 634 CH, 27 %), BT5 (March 2, 684 CH, 97 %), and 'Red Candem' samples RC1 (November 3, 0 CH, 0 %), RC2 (December 1, 50 CH, 0 %), RC3 (December 15, 187 CH, 0 %), and RC4 (December 29, 276 CH, 89 %). As bud break percentages under forcing conditions of BT5 and RC4 were higher than 50 %, chilling requirements for dormancy release in these samples were considered fulfilled (Leida et al. 2012).

Samples required for tissue gene expression analysis (cv. 'Big Top') were obtained from flower buds (collected on January 12, 2010), leaves (November 6, 2012), embryos, flower parts (March 26, 2010) and fruit tissues (June 29, 2010).

For expression analysis in buds under stress conditions, dormant buds (November 3, 2015) and dormancy-released buds (January 25, 2016) of cv. 'Crimson Baby' were collected from three different trees. Six budsticks for each treatment were placed in glass tubes with 25 ml of water at 25 ºC (control) during one and three days. Temperature stress incubations were made at 37 ºC and 4 ºC, salinity stress was made by adding 200 mM NaCl, and desiccation stress was performed without water. Routinely the base of budsticks was cut and the solution replaced with fresh one after two days incubation.

A stress analysis was carried out on leaf discs as described previously (Trotel et al. 1996). Ten discs of 1 cm of diameter per treatment were excised from five different trees of cv. 'Big Top' (June 9, 2015) and were incubated in 5 mM HEPES, 1.5 mM $CaCl_2$, and 10 mM KCl solution at 25 ºC (control). After 4h incubation, discs were transferred to fresh solution with 250 mM NaCl for salt stress treatment, or

incubated at 37 ºC or 4 ºC for temperature stress. Discs were collected at 4 h and 24 h.
For the desiccation assay on leaves, adult leaves from three different trees of cv. 'Red Candem' (April 27, 2015) were placed into glasses with the petiole in contact with water (control) or without water (stressed samples), for one, three and seven days.

Cloning of *PpeS6PDH* in pET-derived vectors

The *PpeS6PDH* gene was cloned into the expression vectors pET302/NT-His and pET303/CT-His (Invitrogen), which facilitates the purification of the recombinant protein. For that, *PpeS6PDH* was amplified using cDNA from peach flower buds collected on January 12 of 2010. The Phusion High-Fidelity DNA Polymerase (Thermo Scientific) was used under the following PCR conditions: 2 min at 94 °C, 5 cycles of 30 s at 94 °C, 30 s at 58 °C, and 1min at 72 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 65 °C and 1 min at 72 °C, and a final step of 5 min at 72 °C. All the primers used in this study are listed in Online Resource Table S1. The PCR product was purified with High Pure PCR Product Purification Kit (Roche) and digested with enzymes *Xho*I and *Bam*HI (Roche) to have an N-terminal His tag or with *Xho*I and *Xba*I (Roche) for a C-terminal His tag. The purified product and corresponding vectors were ligated with T4 DNA ligase (Roche) and cloned into *Escherichia coli* BL21 (DE3) (Novagen). The nucleotide sequence of the inserted gene was confirmed by sequencing.

Phylogenetic analysis of PpeS6PDH protein

For the phylogenetic analysis, PpeS6PDH, S6PDH of *Malus domestica* (Kanayama et al. 1992)*, Pyrus Pyrifolia* (Liu et al. 2013)*, Oryza sativa* (Yadav and Prasad 2014) and other S6PDH-like proteins were used. In addition, mannose-6-phosphate reductase (M6PR) of *Apium graveolens* (Everard et al. 1997) and M6PR-like proteins were also included. M6PRs and S6PDHs belong to the same superfamily of aldo-keto reductases (Hyndman et al. 2003; Yadav and Prasad 2014) and keep high similarity (Bortiri et al. 2002). Sequence alignments were performed by ClustalW (Larkin et al. 2007), and Gblocks (Talavera and Castresana 2007) was used to remove wrong aligned regions. To build a phylogenetic tree, MEGA7 (Kumar et al. 2016b) was used with Maximum Likelihood method and tested using a Bootstrap with 1000 replicates.

Expression and purification of recombinant protein

In order to express the His-tagged PpeS6PDH, 50mL of LB medium supplemented with antibiotic was inoculated with 1/20 of an overnight culture of transformed *E. coli* BL21 (DE3) cells and incubated at 37 ºC in an orbital shaker until the OD_{600}~0.6. Then the expression of recombinant protein was induced by adding 1mM isopropyl-β-D-thiogalactoside (IPTG; Roche) and incubated for 2h at 37ºC. Induced BL21 (DE3) cells were harvested by centrifugation at 10,000 x g during 10 min at 4 ºC. The enzyme was extracted using Bugbuster Plus Lysonase Kit (Novagen) and purified with PureProteome Nickel Magnetic Beads (Novagen), according to manufacture's instructions in both cases. Protein concentration was then measured with the Protein Quantification Kit-Rapid (Fluka) using bovine serum albumin as a standard. Protein size was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 15 % resolving gel and 3.5 % stacking gel (Laemmli 1970). Protein bands were stained using

Coomassie brilliant blue R-250.

Enzymatic activity assay

The enzymatic activity of PpeS6PDH was calculated as described previously (Yadav and Prasad 2014) with minor modifications. The assay solution contained 100 mM Tris-HCl (pH 8), 0.2 mM NADPH, 50 mM of substrate (glucose-6-phosphate or mannose-6-phosphate), and 2.5 µg/mL of recombinant protein in a final volume of 0.8 mL. The decrease of absorbance at 340 nm was measured. The assay was repeated three times for each condition.

Expression analysis by real-time quantitative PCR

For *PpeS6PDH* gene expression analysis, total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen) and purified with the RNase-Free DNase Set (Qiagen), according to manufacturer's instructions with minor modifications. Polyvinylpyrrolidone (PVP-40) 1 % (w/v) was added to the kit extraction buffer before use. Then, RNA (500 ng) was reverse transcribed with PrimeScript RT reagent kit (Takara Bio) and cDNA was analyzed by quantitative real-time PCR on a StepOnePlus Real-Time PCR System (Life Technologies), utilizing SYBR premix Ex Taq (Tli RNaseH plus) (Takara Bio). Cycling conditions were 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, and 1 min at 60 °C. Specificity of the amplification was evaluated by the presence of a single peak in the dissociation curve after PCR and by size estimation of the amplified product in agarose gel. Relative expression was measured using a relative standard curve. Bestkeeper (Pfaffl et al. 2004), NormFinder (Andersen et al. 2004) and ΔCt (Silver et al. 2006) methods were used in a previous study in order to determine the most stable housekeeping genes (Lloret et al. 2017). According to this study, actin-like and tubulin-like genes were used as references for tissue-dependent expression, actin-like and SAND-like for bud development samples, and SAND-like for stress treatments (Online Resource Table S1). When two reference genes were required for the analysis, the normalization factor was calculated by the geometric mean of the values of both genes. Results were the average of three independent biological replicates.

Chromatin immunoprecipitation (ChIP) assays

The chromatin immunoprecipitation method and 'Big Top' samples collected along bud development have been described previously (Leida et al. 2012). Those preceding immunoprecipitated samples were employed in real-time quantitative PCR assays using primers listed in Online Resource Table S1 and following PCR conditions shown above. The enrichment in H3K27me3 and H3K4me3 modifications present in *PpeS6PDH* gene were made relative to H3 values. Results were the average of three independent biological replicates with three technical replicates.

Measurement of sugars and sorbitol content in buds

Soluble sugars and sorbitol were analyzed as previously described (Eshghi et al. 2007) with minor modifications. Ground and dried buds of cv 'Red Candem' (100 mg) were mixed with 5 mL of petrolium ether (40-60º) and centrifuged at 1,109 x g for 10 min at 4 ºC. Then, petrolium ether containing lipids, chlorophyll and other contaminants was removed and 4 mL of ethanol 80 % and 0.1 mL of mannitol (60

mg/mL) were added to the pellet. Mannitol was used as an internal control. The samples were incubated during 20 min at 65 ºC. After centrifugation for 5 min at 1,109 x g at 4 °C, the supernatant was recovered and the remaining plant material was then re-extracted with ethanol twice. All supernatants were mixed and dried again. For dried residue purification, 4 mL of H_2O MiliQ and 20 µg of activated charcoal were added and then, after another centrifugation, the supernatant was recovered and filtered through a nylon membrane (0.45 µm). Finally, the samples were diluted and injected in a HPLC (High Performance Liquid Cromatography Spectra System) with CarboSep COREGEL-87 (Transgenomic) and a refractive index detector. The mobile phase (ultrapure water) was supplied at a flow rate of 0.6 mL/min. Standard solutions containing glucose, fructose, sorbitol, sucrose and mannitol at different concentrations were injected into the column and their peaks were used to construct calibration curves for each compound. The concentration of individual sugars and sugar alcohols in each tissue sample was then calculated using peak areas and the calibration curves. Results were the average of two independent replicates assayed twice.

Statistical analysis

Statistical analyses were performed using the Statgraphics XVI.I package 324 (Statpoint Technologies). The means of two samples were compared using non-parametric Man-Whitney U test and comparisons of multiple samples were evaluated by non-parametric Klustal-Wallis test with a confidence level of 95 %. Significant differences between samples were labelled with asterisks or different letters.

Results

Characterization of S6PDH protein in peach

In previous transcriptomic studies we have identified some expressed sequence tags (GenBank accessions GR410685 and JK006377) corresponding to a S6PDH-like gene with differential expression along the development of flower buds of peach (Leida et al. 2010, 2012). The International Peach Genome Initiative (Verde et al. 2013) named this gene ppa009007m (v1.0) and Prupe.8G083400 (v2.1). In this study we will refer to ppa009007m gene as *PpeS6PDH*, according to the standard gene nomenclature in the Rosaceae (Jung et al. 2015) and suggestions of the Genome Database for Rosaceae (https://www.rosaceae.org/gene_class_listing).

The PCR-amplified coding DNA sequence of *PpeS6PDH* was identical to ppa009007m and to the mRNA coding for an Ald6PRase enzyme recently characterized (Hartman et al. 2017). The 310 amino acids long PpeS6PDH protein shared 76 % identity with NADPH-dependent sorbitol-6-phosphate dehydrogenase from *Malus domestica* and 62% identity with NADPH-dependent sorbitol-6-phosphate dehydrogenase from *Oryza sativa*, both enzymes well characterized. The phylogenetic analysis showed that PpeS6PDH is very close to S6PDH-like proteins from the *Prunus* genus and to other well characterized S6PDH proteins from the Rosaceae family (*Malus domestica* and *Pyrus pyrifolia*) (Fig. 1; Hartman et al. 2017). The gene has been recently cloned with an N-terminal His-tag fusion into pET19b vector, and the recombinant protein purified and characterized at the enzymatic level, showing a NADPH-dependent reductase activity on glucose-6-phosphate to produce sorbitol-6-phosphate (Hartman et al. 2017). We independently amplified *PpeS6PDH* gene and cloned it into pET302/NT-His vector inserting a 6xHis tag

at the N-terminal end of the protein and pET303/CT-His leading to a C-terminal 6xHis tag. The SDS-PAGE analysis of the purified recombinant protein showed a band between 29 kDa and 47 kDa markers, which was in concordance with the expected molecular mass of 36.7 kDa (Online Resource Fig. S1). In a specific enzymatic assay, purified His-PpeS6PDH reduced glucose-6-phosphate to sorbitol-6-phosphate using NADPH as electron donor with a specific activity of 2.95 U/mg (Table 1), slightly higher than the activity described for related S6PDHs fused to N-terminal His tags from rice and apple (Figueroa and Iglesias 2010; Yadav and Prasad 2014), and very similar to the Vmax parameter found by Hartman et al. (2017) in their particular His-PpeS6PDH preparations. Reduction of mannose-6-phosphate occurred at a much lower specific activity of 0.07 U/mg. We could not detect the enzymatic activity of PpeS6PDH with a His tag at the C-terminus, as similarly observed in apple S6PDH (Figueroa and Iglesias 2010). Taken together, these results confirmed that PpeS6PDH is able to perform sorbitol-6-phosphate dehydrogenase activity *in vitro* and that short fusions at the C terminus abolish PpeS6PDH activity.

PpeS6PDH expression is developmentally regulated in buds

To properly characterize *PpeS6PDH* at the molecular level, its relative expression was studied in different peach tissues. *PpeS6PDH* showed higher expression in leaves, flower buds, petals and sepals (Fig. 2). *PpeS6PDH* expression was also appreciable in other flower organs (stamens and carpels), but insignificant in fruit (skin and flesh) and embryo samples.

We estimated the expression profile of *PpeS6PDH* along bud development in two cultivars with different chilling requirements for bud dormancy. Results confirmed the down-regulation of *PpeS6PDH* in flower buds after dormancy release (Fig. 3d), as revealed by previous transcriptomic studies (Leida et al. 2010, 2012). Interestingly, *PpeS6PDH* expression peaked at different times in dormant samples of 'Red Candem' and 'Big Top' cultivars, in concordance with their different chilling requirements. The early cultivar 'Red Candem' showed maximal expression on December 1 (RC2 sample), while the medium cultivar 'Big Top' reached maximal levels of expression on January 12 (BT3 sample). The expression decreased drastically in dormancy released buds, on December 29 (RC4) and March 2 (BT5) in 'Red Candem' and 'Big Top' cultivars, respectively (Fig. 3d).

Interestingly, the 'Big Top' sample collected on December 29 (BT2) increased *PpeS6PDH* expression with respect to the sample collected on November 3 (BT1), contrarily to the strong reduction observed in 'Red Candem' in the same period of time. These data argue for a dormancy-dependent regulation of *PpeS6PDH* expression in flower buds, acting independently of putative environmental effects.

Changes in _PpeS6PDH_ expression associate with chromatin modifications

We performed ChIP analysis in order to identify chromatin modifications at *PpeS6PDH* during dormancy release. A previous genome-wide study of H3K27me3 enrichment in buds was used to identify this specific modification in *PpeS6PDH* locus (de la Fuente et al. 2015). We found that H3K27me3 was significantly enriched on the translation start region of *PpeS6PDH* locus in dormancy released buds (Fig. 3a). H3K27me3 modification has been found associated with silencing of gene expression in peach and other plant and animal species, in close agreement with the repression of *PpeS6PDH* expression in dormancy released buds (Fig. 3d). H3K27 trimethylated region in *PpeS6PDH* contained a repetitive GA

dinucleotide motif (marked with asterisks in Fig. 3a), which has been found associated with H3K27me3 stretches in peach and *Arabidopsis* (Deng et al. 2013; de la Fuente et al. 2015). In order to confirm these data, we amplified by quantitative real-time PCR a fragment contained into the H3K27 trimethylated region in H3K27me3- and H3K4me3-immunoprecipitated samples along bud development. A concomitant increase in H3K27me3 and decrease in H3K4me3 enrichment was observed in BT5 sample containing buds after dormancy release (Fig. 3b,c). Differences in H3K27me3 were not statistically significant despite BT5 showed a higher value in the three biological replicates, due to the high variability found in this sample. H3K27me3 modification in the region around the ATG of *PpeS6PDH* was paralleled by a slight decrease in trimethylation of H3K4, a modification usually associated with transcriptional activation.

The gene model ppa008399m has been proposed to overlap *PpeS6PDH* in their common 3'-UTRs and the last exon of *PpeS6PDH* (Fig. 3a). We measured ppa008399m gene expression along development of flower buds of 'Red Candem' and 'Big Top' (Fig. 3e). The expression of ppa008399m increased slightly during bud development, but transcript accumulation did not correlate inversely with *PpeS6PDH* expression (Fig. 3d). Consequently we did not obtain evidences of co-regulated expression.

Sorbitol content increases along bud development

The content of some sugars (sucrose, glucose and fructose) and the sugar alcohol sorbitol was determined at different stages of bud development. Glucose and fructose contents were not altered in the assay. In contrast, sucrose and sorbitol amounts increased along bud development until the sample previous to dormancy release. After dormancy release their content remained stable or decreased slightly (Fig. 4). The increase in sorbitol level was concordant with changes in *PpeS6PDH* expression during the first stages of bud development (Fig. 3d). After that point, the transcriptional repression of *PpeS6PDH* explained the interrupted accumulation of sorbitol.

***PpeS6PDH* shows cold-inducible expression**

The response of *PpeS6PDH* expression to abiotic stresses was assayed in buds and leaves, since certain previous studies propose a protective role of sorbitol and S6PDH against environmental stresses. Firstly, flower buds were exposed to temperature and water stresses during one and three days treatments. *PpeS6PDH* expression was highly up-regulated after desiccation and cold (4 ºC) stresses in both dormant and non-dormant buds (Fig. 5a,b). In addition, transcript accumulation slightly increased with the duration of the treatment in both cases. On the other hand, *PpeS6PDH* expression showed a complex behaviour under heat treatment (37 ºC), being down-regulated in dormant buds and up-regulated in dormancy released buds (Fig. 5b). Finally, we could notice a decreased *PpeS6PDH* expression following saline stress in dormant buds (Fig. 5a).

In leaf discs, cold exposure induced *PpeS6PDH* expression at a level similar to buds (Fig. 5d). However, in excised leaves desiccation caused a drastic down-regulation of *PpeS6PDH* (Fig. 5e), in contrast to the opposite behaviour observed in buds. Neither saline nor heat treatments affected significantly *PpeS6PDH* expression in leaves (Fig. 5c,d).

Discussion

A recent study by Hartman et al. (2017) has shown by phylogenetic and enzymatic analysis that peach *PpeS6PDH* encodes a NADPH-dependent aldose-6-phosphate reductase with specificity for the reduction of glucose-6-phosphate to sorbitol-6-phosphate (also referred as S6PDH). The enzyme was inhibited by several hexose-phosphates, orthophosphate and oxidizing agents, offering alternative pathways for enzyme regulation. In our study, we have obtained similar activity values of the His-PpeS6PDH recombinant protein, and have confirmed the inhibitory effect of C-terminal His fusions on S6PDH activity, as also observed in apple S6PDH (Figueroa and Iglesias 2010).

In many Rosaceae, S6PDH enzymes are involved in sorbitol synthesis in source tissues (photosynthetic leaves). Subsequently, sorbitol is translocated to sink tissues and converted to fructose by sorbitol dehydrogenase (SDH) enzymes (Loescher 1987). Thus *S6PDH* genes are expected to be mainly expressed in fully developed leaves, where photosynthesis takes place. However we have found that *PpeS6PDH* is highly expressed in dormant flower buds, a sink tissue, in line with sorbitol accumulation data (Fig. 3d; Fig. 4). These results suggest that the active biosynthesis of sorbitol in flower buds is mediated by the transcriptional activity of *PpeS6PDH* gene, however biochemical studies showing a concomitant accumulation of PpeS6PDH protein are not currently available. In pear, as a response to artificial chilling exposure, sorbitol, sucrose and hexoses accumulated in flower and vegetative buds concomitantly with starch hydrolysis, suggesting the utilization of starch reserves to synthesize soluble sugars and sorbitol during bud dormancy (Hussain et al. 2015). In another study, sucrose and stachyose/raffinose carbohydrates accumulated in vegetative buds of peach instead of sorbitol, but no data about flower buds were presented (Marquat et al. 1999). A high increase in sorbitol content was observed in xylem sap of Japanese pear in late December, around bud dormancy release date, which prompted the authors to postulate a role of soluble sugars and sorbitol in flower bud dormancy regulation (Ito et al. 2012). An independent increase of sorbitol and carbohydrates occurred in xylem sap under 0 ºC treatment, suggesting a role of sugar accumulation in acquisition of freezing tolerance (Ito et al. 2013). Similarly, sorbitol and sucrose accumulation observed in this study could perform a protective role against water deficit and low temperature stresses in flower buds. In that case, *PpeS6PDH* could exert a key regulatory role in seasonal tolerance of buds to abiotic stresses through sorbitol production.

Gene expression analyses provide further insight into *PpeS6PDH* function. *PpeS6PDH* is up-regulated in dormant buds and subsequently repressed in dormancy-released samples, while a fragment including the translation start codon and a (GA)n repeated stretch undergoes H3K27me3 chromatin modification (Fig. 3a). This modification, associated with gene expression silencing at specific loci, has been also proposed to mediate stable silencing of several *DAM* genes, leading to bud dormancy release after the accomplishment of chilling requirements (Leida et al. 2012; de la Fuente et al. 2015). Interestingly, (GA)n repeat elements have been found enriched in H3K27me3 modified and FIE-binding regions in *Arabidopsis* (Deng et al. 2013), suggesting its participation in chromatin regulatory circuits.

In addition to H3K27me3 modification, *PpeS6PDH* chromatin associates with a decreased trimethylation of H3K4 along bud development and dormancy release, as similarly described at *DAM6* locus (Leida et al. 2012). Both, H3K27me3 and H3K4me3 changes could account for specific and sequential steps of gene expression down-regulation, as formerly postulated in *DAM6* (Ríos et al. 2014). Moreover,

additional epigenetic modifications such as DNA methylation and others are expected to act coordinately with the observed histone changes. In fact, the occurrence of specific DNA methylation events has been reported in other tree species during bud dormancy and development (Kumar et al. 2016a, Conde et al. 2017).

Expression analysis of the gene adjacent to *PpeS6PDH* (ppa008399m) served to conclude that H3K27me3-associated silencing affected locally to *PpeS6PDH* instead of being sprayed to near genes, as was previously proposed in a genome-wide analysis studying H3K27me3 enrichment in buds (de la Fuente et al. 2015).

Up-regulation of *PpeS6PDH* expression by low temperature treatments in buds and leaves (Fig. 5b,d) confirmed its participation in the chilling response. Induction of *S6PDH* expression by low temperature has been also observed in apple leaves (Kanayama et al. 2006; Liang et al. 2012). Interestingly, high temperature and desiccation treatments produced antagonistic effects in different samples. Particularly, desiccation induced *PpeS6PDH* expression in buds, whereas *PpeS6PDH* was strongly down-regulated in leaves (Fig. 5a,e). These differences reveal tissue-specific mechanisms of regulation that could respond to distinct source/sink roles. Different degrees of drought stress also caused a reduction in S6PDH enzymatic activity in leaves of peach, whereas SDH enzymatic activity in shoot tips (a sink tissue) decreased (Lo Bianco et al. 2000).

In our opinion, developmental and environmental issues affecting *PpeS6PDH* expression, in addition to sorbitol accumulation data, suggests a role of this gene in protection against abiotic stresses, particularly chilling and desiccation, in flower buds of peach. Moreover, down-regulation of *PpeS6PDH* in dormancy-released buds associates with chromatin modification mechanisms similarly to *DAM6* gene, suggesting the participation of common regulatory factors in *PpeS6PDH* and bud dormancy regulation.

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Author contributions

MLB and GR conceived the study. AL, AMF and GR performed the experimental work. MA provided supervision and designed the method for sugars measurements. AL and GR prepared the manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Figure legends

Fig 1 Phylogenetic tree of S6PDH and related proteins. The tree was constructed using the Maximum Likelihood method and bootstrapped with 1000 replicates. The scale bar indicates the branch length that corresponds to the number of substitutions per amino acid position

Fig 2 Relative expression of *PpeS6PDH* by qRT-PCR. Samples included different plant tissues and organs obtained from 'Big Top' cultivar: leaf (Le), fruit skin, fruit flesh, sepal (Se), petal (Pe), stamen (St), carpel (Ca), embryo (Em) and dormant flower bud (FB, sample BT3). Tubulin-like and actin-like genes were used as reference genes. An expression value of one is assigned to the leaf sample. Data are means from three biological replicates, with error bars representing standard deviation. Different letters (a–e) indicate significant difference between samples with a confidence level of 95%

Fig 3 Chromatin immunoprecipitation (ChIP) analysis of *PpeS6PDH* gene during bud development. (**a)** Local H3K27me3 enrichment in a genomic region including *PpeS6PDH* and the adjacent gene ppa008399m by ChIP-Seq analysis in dormant (BT1) and non-dormant (BT5) buds of 'Big Top', compared with input sample. A peak on the first exon of *PpeS6PDH* in the non-dormant sample indicates the presence of a differentially methylated region. Predicted *PpeS6PDH* and ppa008399m transcripts are shown (peach genome v1.0) with their respective coding sequences (white rectangles) and untranslated 5' and 3' regions (grey rectangles). Repeated (GA)n elements are labelled with asterisks. The fragment amplified by qRT-PCR with specific primers is labelled with a striped rectangle. H3K27me3 (**b**) and H3K4me3 (**c**) modifications around the translation start site in the first exon of *PpeS6PDH* have been confirmed by qRT-PCR of ChIP samples at different bud development stages ('Big Top' BT1-5, see below). H3K27me3 and H3K4me3 enrichment has been made relative to histone H3 immunoprecipitated samples. The relative expression level of *PpeS6PDH* (**d**) and ppa008399m (**e**) during bud development has been analyzed in cultivars 'Big Top' and 'Red Candem'. Bud samples from 'Big Top' and 'Red Candem' were collected in autumn and winter (2009/2010) at different dates: November 3 (BT1 and RC1), December 1 (RC2), December 15 (RC3), December 29 (BT2 and RC4), January 12 (BT3), February 16 (BT4) and March 2 (BT5). In BT5 and RC4 samples (dark bars in the graphs) dormancy was already released. SAND-like and actin-like genes were used as reference genes. An expression value of one is assigned to the first sample (BT1 or RC1). Data are means from three biological samples with three technical replicates each, with error bars representing standard deviation. Different letters (a–d) indicate significant difference between samples with a confidence level of 95%

Fig 4 Sugar and sorbitol accumulation in buds. Sucrose, glucose, fructose and sorbitol were measured in flower bud samples RC1 (1), RC2 (2), RC3 (3) and RC4 (4) of 'Red Candem'. Sample code is explained in Figure 3. Dormancy has been released in RC4 (black bars). Different letters (a–c) indicate significant difference between samples with a confidence level of 95%

Fig 5 Effect of abiotic stresses on *PpeS6PDH* relative expression by qRT-PCR. Dormant and non-

dormant flower buds of peach (cv. ‘Crimson Baby’) were treated with 200 mM NaCl or desiccated (**a**), incubated at 4 ºC or 37 ºC (**b**), for one (white bars) or three days (grey bars). Leaf discs from ‘Big Top’ cultivar were treated with 250 mM NaCl (**c**), incubated at 4 ºC or 37 ºC (**d**), for 4 h (white bars) or 24 h (grey bars). In a last experiment (**e),** excised leaves from ‘Red Candem’ cultivar were desiccated for one (white bars), three (grey bars) or seven days (black bars). SAND-like gene was used as reference. An expression value of one is assigned to the untreated sample at the first time point, that is one day (panels **a**, **b** and **e)** and 4 hours (panels **c** and **d)**. Data are means from three biological samples with two technical replicates each, with error bars representing standard deviation. An asterisk indicates significant difference with the untreated control at a confidence level of 95%

Tables

Table 1. Enzymatic activity of recombinant PpeS6PDH

	Substrate	Activity (mU)	Protein (μg)	Specific activity (U/mg)
His-PpeS6PDH	Glucose-6-phosphate	8.85 ± 0.81	3.0	2.95 ± 0.27
His-PpeS6PDH	Mannose-6-phosphate	0.25 ± 0.09	3.0	0.07 ± 0.03
PpeS6PDH-His	Glucose-6-phosphate	ND	1.5	-

ND, not detected

Fig. 1

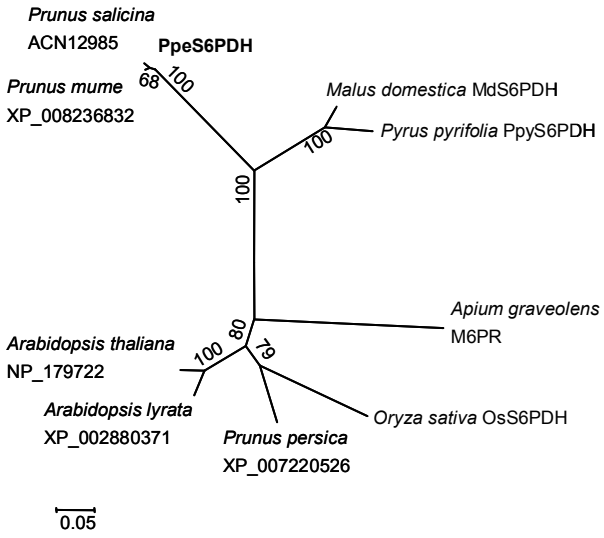


Fig. 2

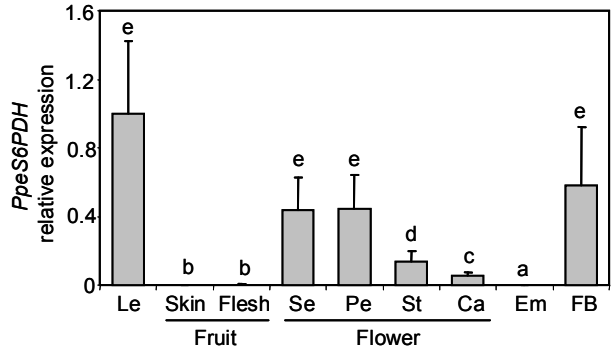


Fig. 3

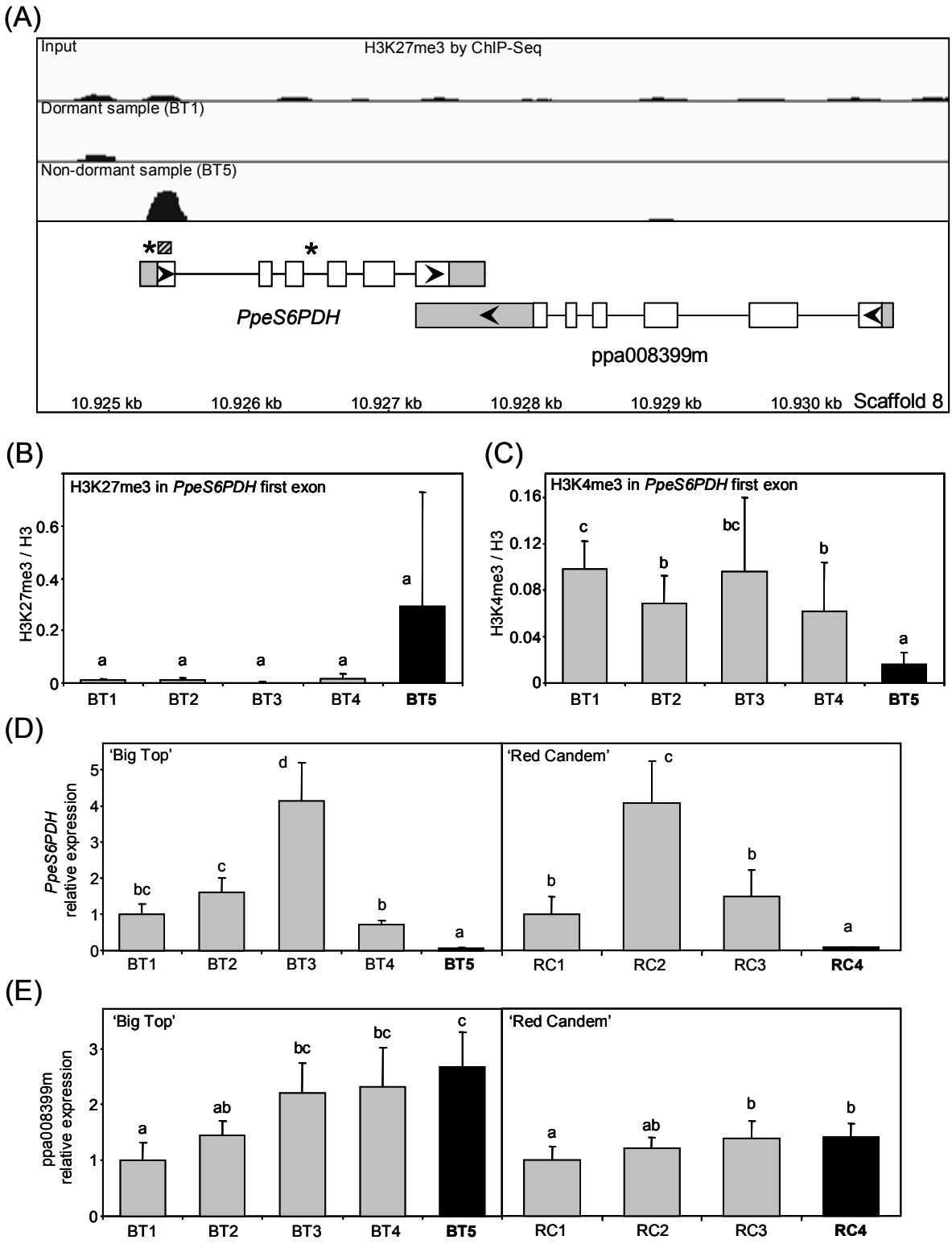

(A)
Input
H3K27me3 by ChIP-Seq
Dormant sample (BT1)
Non-dormant sample (BT5)
PpeS6PDH
ppa008399m
10.925 kb
10.926 kb
10.927 kb
10.928 kb
10.929 kb
10.930 kb
Scaffold 8
(B)
H3K27me3 in PpeS6PDH first exon
H3K27me3 / H3
BT1
BT2
BT3
BT4
BT5
(C)
H3K4me3 in PpeS6PDH first exon
H3K4me3 / H3
(D)
'Big Top'
'Red Candem'
PpeS6PDH relative expression
RC1
RC2
RC3
RC4
(E)
ppa008399m relative expression

Fig. 4

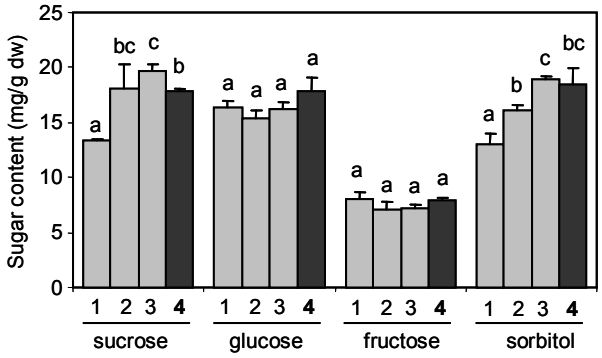
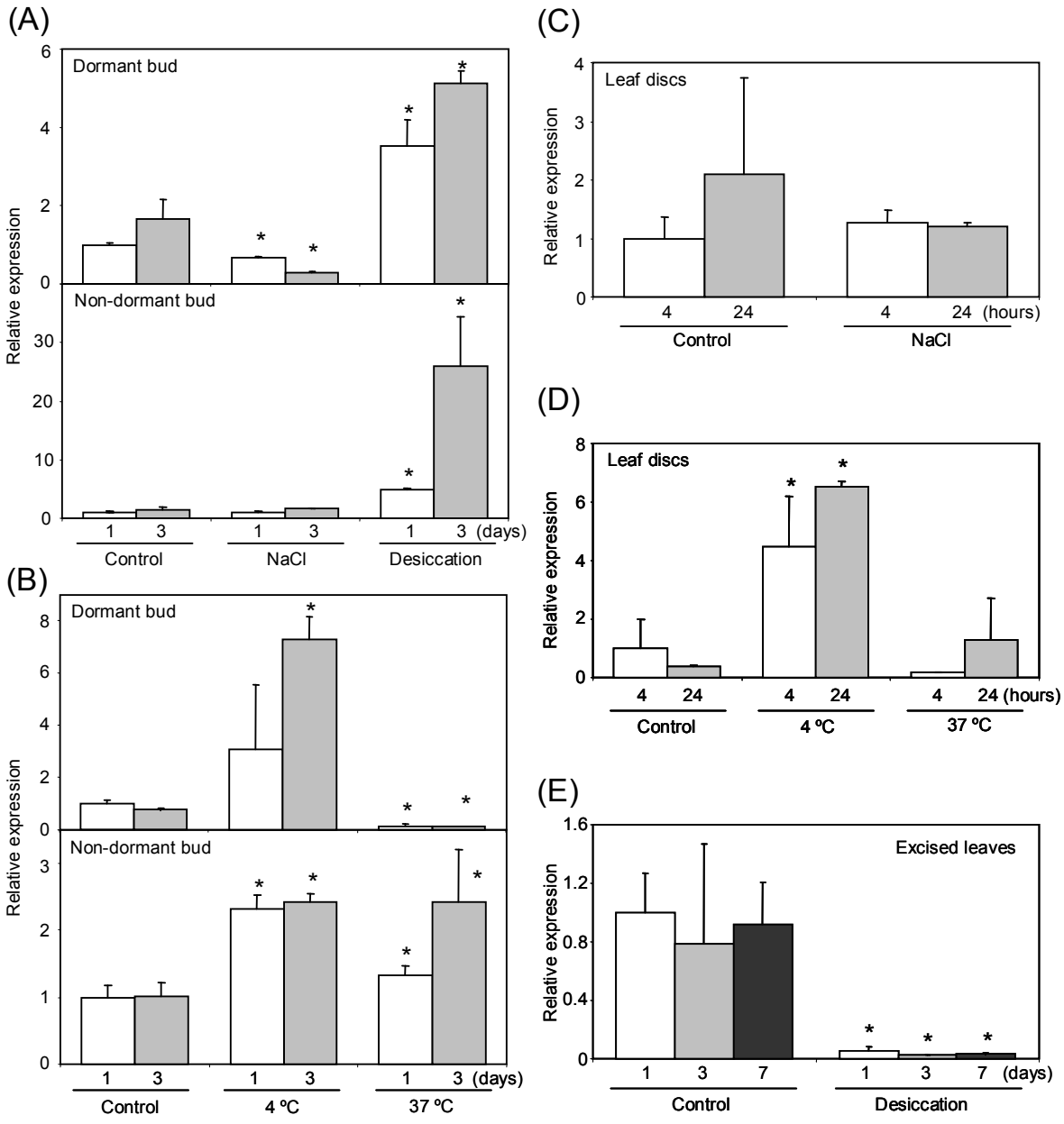

Sugar content (mg/g dw)
25
20
15
10
5
0
a
bc
c
b
a
a
a
a
a
a
a
a
a
b
c
bc
1 2 3 4
sucrose
glucose
fructose
sorbitol

Fig. 5


(A)
Dormant bud
Non-dormant bud
Relative expression
1 3 (days)
Control
NaCl
Desiccation
(B)
Dormant bud
Non-dormant bud
Relative expression
1 3 (days)
Control
4 ºC
37 ºC
(C)
Leaf discs
Relative expression
4 24 (hours)
Control
NaCl
(D)
Leaf discs
Relative expression
4 24 (hours)
Control
4 ºC
37 ºC
(E)
Excised leaves
Relative expression
1 3 7 (days)
Control
Desiccation