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Ascorbic acid modulates growth arrest affected by trehalose feeding in *Arabidopsis thaliana* Cvi accession

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Abstract
Trehalose is the alpha, alpha-1, 1-linked glucose disaccharide, which is found in a wide variety of organisms. In here, the interaction of trehalose and ascorbic acid on some biochemical parameters and gene expression level was investigated in Cvi accession of *Arabidopsis* seedlings. Seeds were grown on MS medium supplemented with or without 0.1 mM ascorbic acid, and also MS medium supplemented with 100 mM trehalose, 0.1 mM ascorbic acid and 100 mM trehalose for 14 days. Results showed that the exogenously applied trehalose reduced root length, soluble sugar, chlorophyll a and hydrogen peroxide amount in Cvi seedlings; whereas, the amount of starch, ascorbate, dehydroascorbate, total ascorbate, trehalase activity and antioxidant enzymes activity was increased by trehalose feeding. On the other hands, the combination of trehalose and ascorbic acid treatment increased root length and soluble sugar but decreased starch, ascorbate, total ascorbate, trehalase and antioxidantd enzyme activity. Gene expression profiling showed that trehalose induced *TRE1, SOD, SUS, INV and SUC* expression. While ascorbic acid feeding partially inhibits *TRE1* and *SOD* gene expression, *SPS* and *SUC* gene expression was induced in Cvi accession *Arabidopsis* seedlings. In conclusion, the inhibitory effect of 100 mM trehalose on *Arabidopsis* seedlings growth can be repressed by ascorbic acid treatment; this capacity could be attributed to induced expression of *SPS* and *SUC* genes following exposure to ascorbate.

Keywords**:** *Arabidopsis thaliana*, Ascorbic acid, Cvi, Gene expression, Trehalose, Oxidative metabolism,

Introduction

Metabolism of the alpha, alpha-1,1-glucose disaccharide, trehalose, is indispensable in plants (Elbein et al., 2003). Trehalose metabolites are, however, present at only very low concentrations and their role are not understood (Goddijn and Smeekens, 1998, Rolland et al., 2006). Genes coding for trehalose metabolisms have been found in all plants. Strikingly, extensive radiation is found in the genes for metabolism of the trehalose biosynthetic precursor, trehalose-6-phosphate (T6P), whilst only one or very few genes encode trehalases that hydrolysetrehalose (Leyman et al., 2001; Pramanik et al., 2005).

T6P is essential for plant growth and development, carbon utilization and alters photosynthetic capacity (Schluepman et al., 2004; Dellate et al., 2011; Ponnu et al., 2011; Lunn et al., 2011; Yadav et al., 2014). Additionally, deletion of the T6P synthase (TPS), *AtTPS1*, in Arabidopsis is lethal and can be overcome by complementation with active TPS enzyme (Eastmond et al., 2002; Schluepmann et al., 2004).

Paradoxically, accumulation of T6P in the absence of sufficient metabolisable carbon would limit growth (Schluepmann et al., 2004). This is the case when seedlings are grown on medium containing 100 mM trehalose. Trehalose supplied to the growth medium of seedlings inhibits growth and allocation of carbon to the root and shoot (Wingler et al., 2000; Aghdasi et al., 2010). On this medium, seedlings accumulate T6P and seedlings expressing TPH resist the growth inhibition. Thus, seedlings stop growing as a result of T6P accumulation

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(Schluepman et al., 2004). Several accessions of *A. thaliana* were tested for their ability to grow on 100 mM trehalose. Growth arrest occurs in all accessions of *Arabidopsis* tested but is significantly less in seedlings of Cvi: seedlings from Cvi grow longer roots compared with all other tested accessions on 100 mM trehalose (Aghdasi et al., 2016). *Arabidopsis thaliana* has different accessions that were collected from different geographical regions. Among them, the ecotype Cvi is from the Cape Verde islands (Lobin 1983).

Our previous data from metabolite analysis revealed that T6P levels have a profound effect on metabolite steady states. Metabolite profiling of *tps1* mutants grown on ½ MS at the seedling stage revealed profound changes in metabolite steady states. Interestingly, *tps1* has dramatically changed ascorbate / dehydroascorbate balance, with an accumulation of ascorbate and a reduction of dehydroascorbate. Preliminary measurements suggest that 0.1 mM ascorbate in Col-0 accession seedlings on 100 mM trehalose reduces the T6P steady state (Aghdasi, 2007).

In this research, *Arabidopsis thaliana* Cvi accessions are characterized with respect to biochemical and molecular response to trehalose feeding along with ascorbic acid treatment. In addition, different biochemical factors and genes expression are analyzed to draw a map of the metabolic responses occurring upon feeding trehalose and ascorbic acid.

Materials and methods

Plant materials and growth conditions

Seeds of *Arabidopsis thaliana* accession Cvi were surface sterilized with 70% Ethanol followed by 20% commercial bleach (4% w/v chlorine) for 10 minutes and then washed 5 times with sterile milli-Q water. Sterilized seeds were plated on agar solidified half strength MS medium supplemented with 100 mM trehalose or ascorbic acid and/or 100 mM trehalose plus 0.1 mM ascorbic acid and stratified in darkness at 4ºC for 3 days before transferring to a growth chamber at 25ºC under a 16-h-light/8-h-dark photoperiod. In this experiment, seedlings were grown vertically for 14 days and then they were photographed. The measurement of root length was carried out with ImageJ program (Wayne Rasband, NIH Maryland, USA).

Starch staining and measurement

The whole seedlings were first de-stained in 70% and then in 90% (v/v) ethanol. Staining was done with KI/I_2 solution. After washing, the seedlings were photographed with a Normanski microscope (Jena, Germany).

Starch content was measured by an enzymatic method (Boehringer Mannheim, Darmstadt, Germany) as described by the manufacturer. For this purpose, 50 mg fresh weight plant material was frozen and ground in liquid nitrogen. Sugars were extracted with 1 ml 80% ethanol for 10 minutes at 80ºC in ependorf tubes. The tubes were spun for 5 minutes at 13000 rpm and the supernatant was transferred to a clean tube. The pellet was extracted again with 75 µl of milliQ for 10 minutes at 80ºC and the supernatants were pooled. Starch was then extracted from the remaining pellet by incubation in 0.1 mL of 0.5 M NaOH at 60ºC for 30 minutes. After addition of 6 µl acetic acid (96%), starch was digested overnight at 37ºC by addition of amyloglucosidase.

Soluble sugar measurement

The soluble sugars were determined spectrophotometrically with phenolsulfuric acid following Kochert (1978)'s method.

Chlorophyll and anthocyanin measurements

Total chlorophyll was measured spectrophotometrically as described by Jeffery and Humphrey (1975). Seedlings were ground in liquid nitrogen and extracted with 80% (v/v) acetone. Then absorbances at 647, 652 and 664 nm were measured and used to determine chlorophyll content.

The anthocyanin content of seedlings was determined as described by Mita et al., (1997). Frozen, homogenized seedlings (20 mg) were extracted for 1 d at 4°C in 1 ml of 1% (v/v) hydrochloric acid in methanol. The mixture was then centrifuged at 13,000 g for 15 min and the absorbance of the supernatant

was measured 530 and 657 nm. Relative anthocyanin concentratio *A* ns were calculated with the formula [$_{530}$ – (1/4 × A_{657})]. The relative amount of anthocyanin was defined as the product of relative anthocyanin concentration and extraction solution volume. One anthocyanin unit equals to one absorbance unit [A_{530} – (1/4× A_{657})] in 1 ml of extraction solution.

Ascorbate, dehydroascorbate and hydrogen peroxide measurement

Ascorbate, dehydroascorbate and total ascorbate content was determined according to de Pinto et al., (1999). Hydrogene peroxide was determined by the colorimetric method of Jana and Chudhuri (1981).

Enzymes activity measurement

For preparation of the crude enzyme extract, fresh leaves (0.05 g) were ground with 2 ml of 0.1 M cool phosphate buffer (pH 6.8) as described by Kar and Mishra (1976). The obtained homogenate was then centrifuged at 15000 g for 15 min at 4ºC. The clear supernatant was used for assaying the activities of catalase (EC 1.11.1.6) peroxidase (EC 1.11.1.7), polyphenoloxidase (EC 1.10.3.2) and superoxide dismutase (EC 1.15.1.1).

Catalase activity was determined by monitoring the destruction of H_2O_2 at 240 nm (Chance and Maehly 1955). The reaction mixture in a final volume of 3 ml contained 50 mM phosphate buffer (pH 6.8), 100 µl enzyme extract and 15 mM H_2O_2. The decrease in absorbance at 240 nm was recorded with a spectrophotometer (Shimadzu UV-160).

The peroxidase reaction mixture in a final volume of 3ml contained 20 mM guaiacol, 25 mM phosphate buffer (pH 6.8), 40 mM H_2O_2 and 10 µl from the crude enzymes extract. The increase in absorbance at 470 nm due to tetra-guaiacol formation was recorded spectrophotometrically.

Superoxide dismutase activity was determined as described by Beauchamp and Fridovich (Beauchamp and Fridovich 1971).

Trehalase (EC 3.2.1.28) reaction mixture in a final volume of 1000 µl consisted of 50 mM Morpholinoethan sulfonic acid (MES) buffer adjusted to pH 6.3, 10 mM trehalose and aliquots from 13000 *g* supernatant as an enzyme source. The reaction was started by the addition of trehalose and allowed to proceed for at least 60 min at 35° C. At different time intervals from the start of the reaction, aliquots (50 µl) were taken from the reaction mixture and the released glucose molecules were determined spectrophotometrically according to Prado et al., (1998). Changes in the amounts of liberated glucose during 45 min from the initiation of reaction were used for measurement of trehalase activity.

RNA extraction and RT-PCR Analysis

Total RNA was isolated with RNeasy plant mini kit (QIAGEN USA, Valencia, CA). Ten ng RNA was treated with 2 U DNAse I (DNA- free, Ambion, Austin, USA) to remove genomic DNA. The absence of DNA was tested by performing PCR reaction (40 cycles, similar to the real–time PCR program) on the DNaseI-treated RNA using Taq-DNA polymerase.

RT-PCR experiments were performed using M-MLV Reverse Transcriptase (promega, Madison, WI), odT16v (custom oligo from invitrogen, Carlsbad, CA) and random hexamer (Invitrogen). The gene specific primers used were:

5'- GCTGCACCACGAACCAGTAGA-3' and 5'-TTCTTCGTTCTCCACGTTGGA-3' for *TRE1*, 5'-ACATTTCAACCCCGATGGTA-3' and 5'-CCAGTAGCCAGGTGAGTTC-3' for *CDS1*, 5'-GAAGAAACGCAGCAGAAACC- 3' and 5'-GTGTGCTTGTCACCACCATC-3' for *SPS1*, 5'-CTCTGCCCAAATCAGTTGATCACG-3' and 5'-GACAACCAAACAAAGTGGACC-3' for *INV*, 5'-ACAGTTCGGTTGGGCTTTACAGTTATCTC-3 and 5'-TTGGAGGCTTTTCCATCGGCTGTTGGCTCT G-3' for *SUC* and 5'-GACCCAAAGACGGAGACTCTT-3' and 5'-GCCAAGT GATTGTGGAGACTC for *AtACTIN2* as the reference gene.

Statistical analysis

The reported values were means of three replicates. Means were compared by the

Duncan's multiple range tests at $\rho \leq 0.05$ significant level using SAS software (*Version 9.1*).

Results

Ascorbic acid partially suppress the effect of trehalose

Trehalose supplied to the growth medium of Col-0 *Arabidopsis thaliana* seedlings inhibits root growth, emergence of priming leaves and accumulation of large amounts of starch in cotyledone and to a depletion of starch in the collumella cells of the root (Wingler et al., 2000; Fritzius et al., 2001; Rolland et al., 2006; Aghdasi et al., 2010).

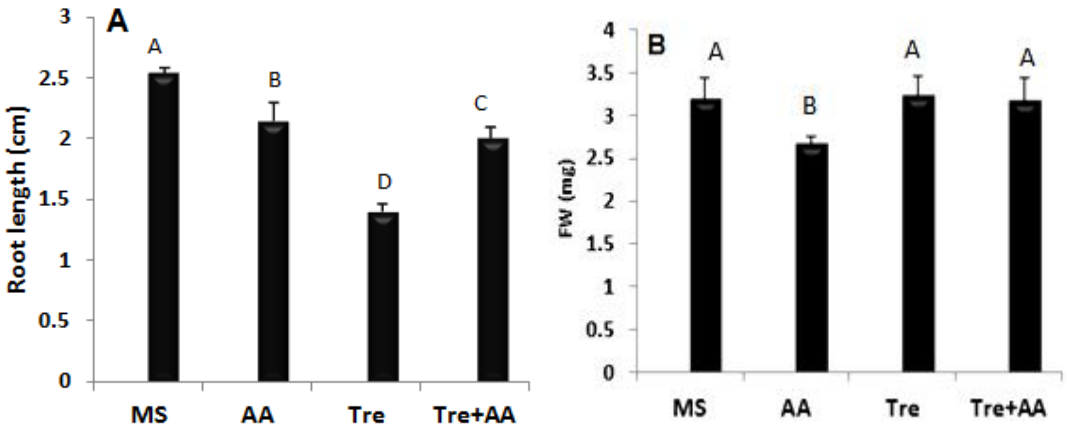
To show that ascorbate can affect *Arabidopsis* seedlings growth and development on 100 mM trehalose, we sown Cvi accession seeds on ½ MS with 100 mM trehalose supplemented with 0.1 mM ascorbate. By using 0.1mM ascorbate with 100 mM trehalose, whole seeds were germinated on ½ MS medium. While root length of Cvi seedlings was 2.5 cm on MS medium, it was significantly decreased by 0.1 mM ascorbic acid treatment. In the presence of 100 mM trehalose, the root length of Cvi seedlings was significantly shorter than seedlings when grown on MS medium. The current results showed that 0.1 mM ascorbate can rescue growth arrest effect by 100 mM trehlaose. By adding ascorbic acid to MS medium supplemented with 100 mM trehalose, the average root length of Cvi was significantly higher compared with seedlings when grown on 100 mM trehalose (Figure 1A).

There was significant difference in the fresh weight of seedlings when grown on MS medium supplemented with or without ascorbic acid. The seedlings weights were significantly less in the presence of ascorbic acid (Figure 1B).

The distribution of starch in the 14 d old-Cvi seedlings was studied following Lugol staining. Seedlings of Cvi, growing on MS medium supplemented with 100 mM trehalose, displayed starch in columella cells of the root tips, and their cotyledons contained only a few small starch granules in some areas (Figure 1C).

Starch measurement of Cvi seedlings at 14 days old-showed that by adding 0.1 mM ascorbate, the amount of starch reduced to 20.34 mg/g, compare with 52.33 mg/g in seedlings when grown on ½ MS medium (Figure 2A). Although starch content in trehalose-fed seedlings was increased to 63.17 $mg.g^{-1}$ FW, it was decreased in seedlings when grown on MS medium supplemented with 100 mM trehalose and ascorbic acid.

The Cvi seedlings growing on MS medium had two-folds less soluble sugars than the seedlings grown on MS medium supplemented with ascorbic acid (Figure 2B). The soluble sugar was significantly decreased by trehalose feeding compared with seedlings grown on MS medium contained 0.1 mM ascorbic acid.



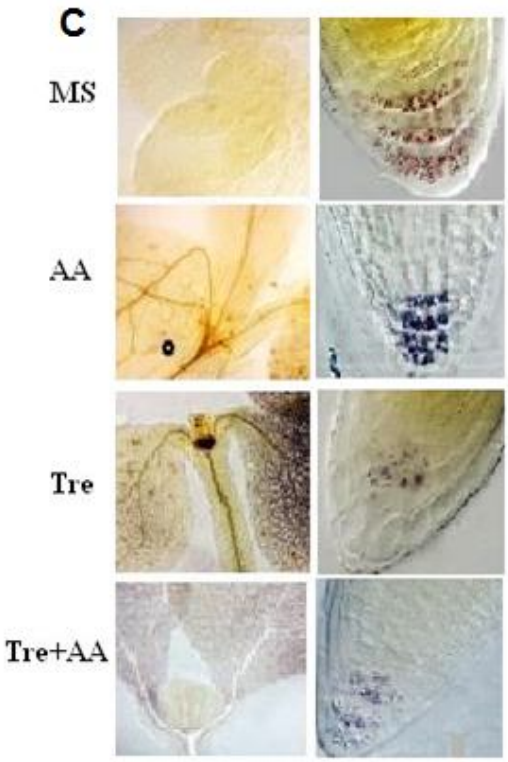


Figure 1. A) Root length, B) fresh weight and C) starch staining in Cvi *Arabidopsis* accession. Seedlings were grown on MS medium (MS), MS medium with 0.1 mM ascorbic acid (AA), MS medium with 100 mM trehalose (Tre) and MS medium with 100 mM trehalose plus 0.1 mM ascorbic acid (Tre+AA) in long day conditions, then root length and fresh weight were measured. Starch stained with KI/I_2 and studied using Nomarski microscopy.

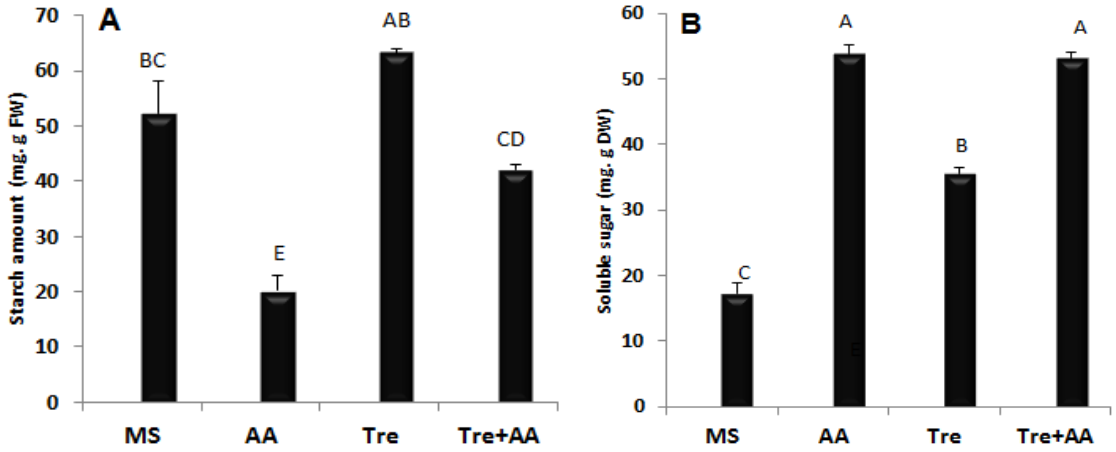


Figure 2. A) Starch and B) insoluble sugar level in Cvi *Arabidopsis* accession. Seedlings were grown on MS medium (MS), MS medium with 0.1 mM ascorbic acid (AA), MS medium with 100 mM trehalose (Tre) and MS medium with 100 mM trehalose plus 0.1 mM ascorbic acid (Tre+AA) in long day conditions, then starch and insoluble sugar were determined

Anthocyanin content is not affected by ascorbate feeding

Chlorophyll a, chlorophyll b and the total chlorophyll content were highest in seedlings grown on MS medium. In the presence of trehalose or ascorbic acid, these parameters were significantly decreased compared with seedlings grown on MS

medium (Figure 3A and 3B). By adding 0.1 mM ascorbic acid to MS medium supplemented with 100 mM trehalose, the total chlorophyll and the chla/Chlb ratio were significantly increased (Figure 3C and 3D). The Cvi seedlings did not show any change in anthocyanin content follwing trehalose or ascorbic acid feeding (Figure 3E).

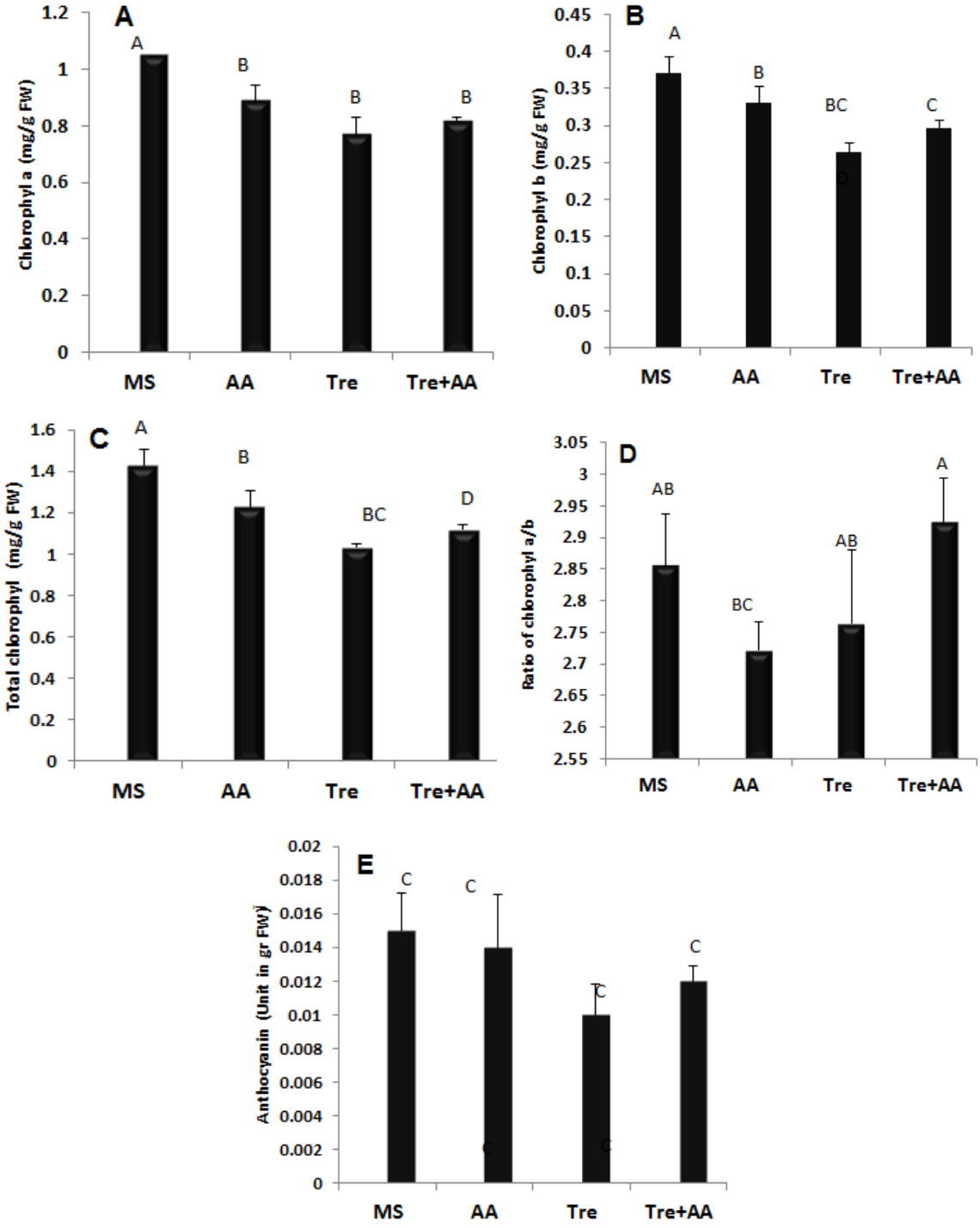


Figure 3. Chlorophyll and anthocyanin content in Cvi *Arabidopsis* accession. Seedlings were grown on MS medium (MS), MS medium with 0.1 mM ascorbic acid (AA), MS medium with 100 mM trehalose (Tre) and MS medium with 100 mM trehalose plus 0.1 mM ascorbic acid (Tre+AA) in long day conditions, then chlorophyll a (A), chlorophyll b (B), Total chlorophyll (C), ratio of chlorophyll a/b (D) and anthocyanin contents (D) were determined.

Trehalose feeding change ascorbate steady state

To find out if trehalose feeding can affect ascorbate metabolism, we analyzed endogenous ascorbate, dehydroascorbate and total ascorbate levels. By adding ascorbate to the MS medium, ascorbate, dehydroascorbate and total ascorbate levels were lower in seedlings when compared with those grown on MS medium without ascorbate. However, trehalose feeding induced the level of these parameters. By adding ascorbate to MS medium supplemented with 100 mM trehalose, these three parameters were decreased (Figure 4A-C).

Meanwhile, ascorbate feeding significantly reduced hydrogen peroxide accumulation in Cvi seedlings. The level of hydrogen peroxide was also significantly decreased in Cvi seedlings grown on 100 mM trehalose, compared with seedlings when grown on MS medium (Figure 4D).

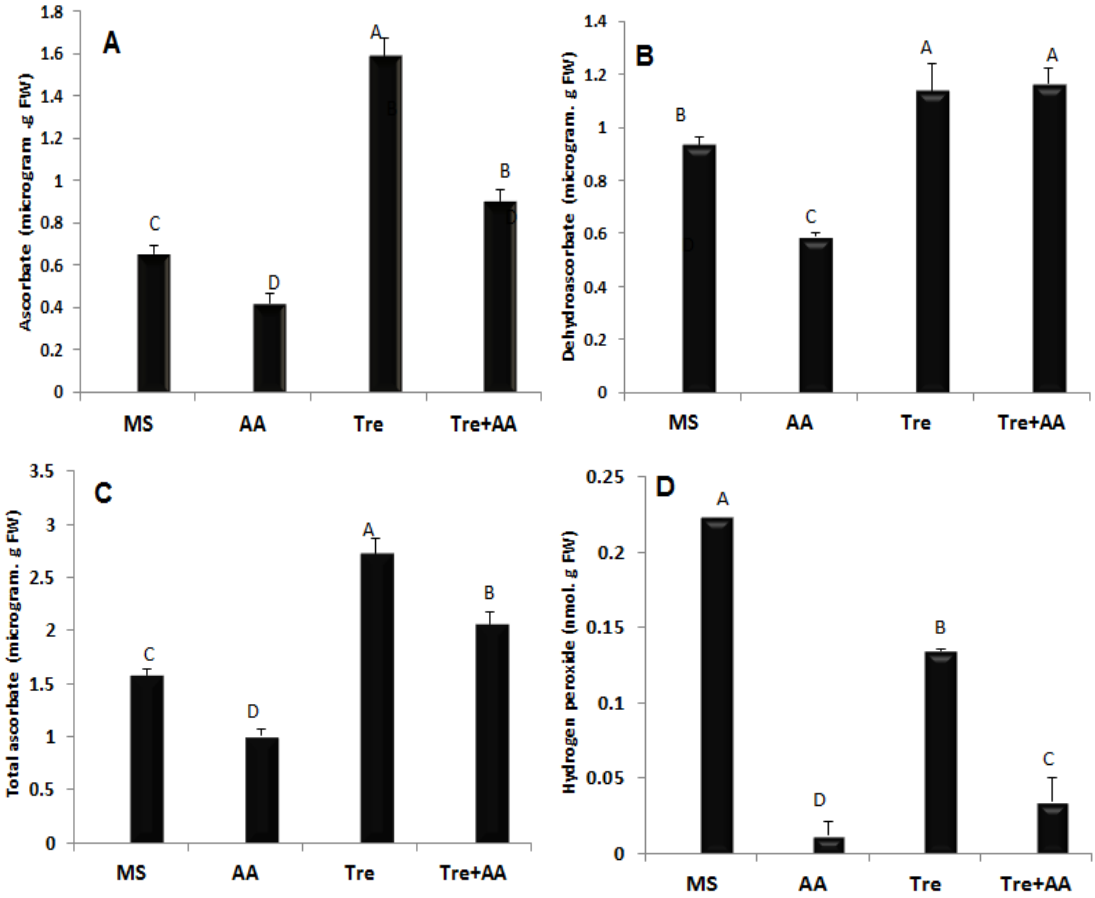


Figure 4. A) Ascorbate, B) Dehydroascorbate, C) total ascorbate and D) hydrogen peroxide level in Cvi *Arabidopsis* accession. Seedlings were grown on MS medium (MS), MS medium with 0.1 mM ascorbic acid (AA), MS medium with 100 mM trehalose (Tre) and MS medium with 100 mM trehalose plus 0.1 mM ascorbic acid (Tre+AA) in long day conditions.

Ascorbate feeding modulate antioxidant enzymes activity in respond to trehalose feeding

Upon feeding by 100 mM trehalose, peroxidase, catalase and superoxide dismutase (SOD) activities were significantly induced in seedlings compared with those grown on MS medium with or without 0.1 mM ascorbate. However, antioxidant enzymes activity was significantly decreased after adding exogenous ascorbate to MS medium supplemented with 100 mM trehalose, (Figure 5A, 5B and 5C).

Ascorbate feedings affect trehalase activity in Cvi accession Arabidopsis seedlings

To assess whether resistance to trehalose in the seedlings is due to high endogenous trehalse activity, we measured trehalase activity. The current results showed that ascorbate feeding reduced trehalase activity compared with seedlings grown on MS medium. However, exogenous trehalose significantly induced trehalase activity. Trehalase activity was also decreased after adding exogenous ascorbate to MS medium supplemented with 100 mM trehalose (Figure 5D).

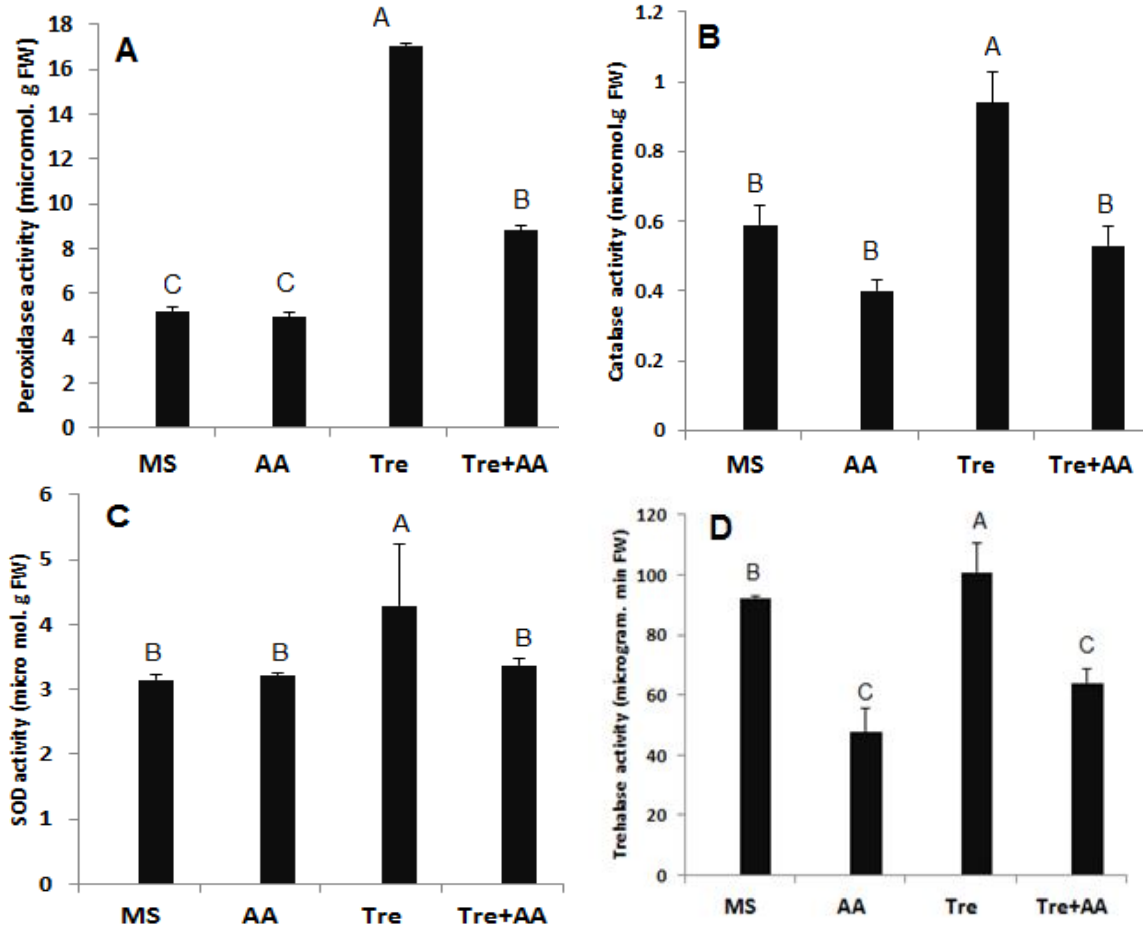


Figure 5. A) Catalase, B) Peroxidase and C) Superoxidase and D) Trehalase activity in Cvi *Arabidopsis* accession**.** Seedlings were grown on MS medium (MS), MS medium with 0.1 mM ascorbic acid (AA), MS medium with 100 mM trehalose (Tre) and MS medium with 100 mM trehalose plus 0.1 mM ascorbic acid (Tre+AA) in long day conditions.

Ascorbate feeding changed gene expression pattern

To analyse the pattern of gene expression related to carbon allocation, we performed RT-PCR analysis of the key genes, including sucrose transporter (*SUC*), sucrose phosphate synthase (*SPS*) and invertase (*INV*).

Cvi accession Arabidopsis seedlings showed a high level of *SPS and SUC* genes expression when grown on MS medium; whereas ascorbate feeding suppressed the expression of the two genes. Meanwhile, trehalose feeding induced *SPS and SUC* genes expression. While the expression of *SUC* gene was almost unchanged by trehalose plus ascorbate feeding, the expression of *SPS* gene was increased when seedlings were grown on MS medium plus trehalose and ascorbate (Figure 6).

The *SOD* gene expression was typically found very low in seedlings growing on MS

medium. However, the expression was induced in seedlings when grown on MS medium supplemented with 100 mM trehalose or trehalose plus ascorbic acid. The expression of *INV* gene was induced following trehalose feeding; however, it remained unchanged following ascorbate feeding. The expression of *AtTRE1* was typically suppressed by ascorbate feeding (Figure 6).

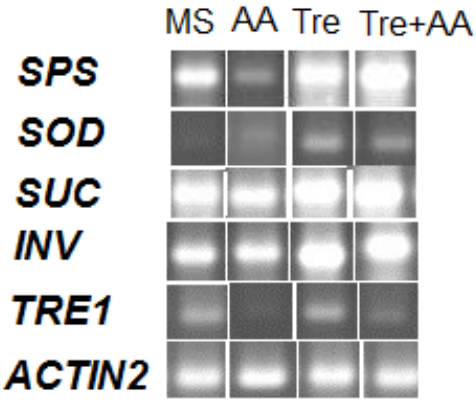


Figure 6. Gene expression in Cvi *Arabidopsis* accession seedlings. Seedlings were grown on MS medium (MS), MS medium with 0.1 mM ascorbic acid (AA), MS medium with 100 mM trehalose (Tre) and MS medium with 100 mM trehalose plus 0.1 mM ascorbic acid (Tre+AA)for 10 days before RNA extraction and RT-PCR analysis of gene-expression. Levels of gene expression were determined with reference to *AtACTIN2 (n=3)*.

Discussion

In Col-0 accession *Arabidopsis* seedlings, exogenously supplied trehalose inhibits root growth and emergence of leaves (Wingler et al., 2000; Aghdasi et al., 2010). Supplied trehalose is transported through plant tissue and enters plant cells since plants expressing trehalase in the cytosol thrive on medium with trehalose. Exogenously applied trehalose leads to root growth arrest. Concurrently, cells in the extension zone behind the meristem swell and lyse, promote the accumulation of starch and anthocyanin while decreasing the levels of chlorophyll in Col-0 accession (Wingler et al., 2000, Kolbe et al., 2005). We have observed natural variation of growth inhibition on 100 mM trehalose: whilst the most accessions of *Arabidopsis thaliana* are sensitive, the Cvi accession is significantly more resistant to trehalose in the medium (Aghdasi et al., 2016). Quantitative analyses reveal that trehalose feeding do not have a significant effect on the fresh weight, anthocyanin and starch content of Cvi seedlings grown on 100 mM trehalose. The Cvi accession is from Cape Verde islands, somewhere with an average monthly precipitation around 9.4 mm during rainy seasons. In fact, Cvi accession is adapted to high temperature and irradiance. So far, different reports have showed that Cvi accession is resistance to different stress conditions such as ozone and freezing, compared with other accessions (Alonso-Blanco and Kornneef, 2000).

In current research, ascorbic acid supply is shown to partially suppress growth inhibition and starch accumulation mediated by T6P. Preliminary measurements of T6P levels in seedlings on ascorbic acid and trehalose suggest that ascorbic acid does not respond to T6P accumulation, but T6P steady state not clear (Aghdasi 2007). T6P controls AGPase redox regulation, and it may also control starch remobilization (Michalska et al., 2009; Kolbe et al., 2005, Ramon et al., 2007). The pathway controlling growth inhibition has not been studied previously except that plants with reduced T6P and pale green leaves grow at a slower pace and have a lower photosynthetic capacity per leaf area than WT (Pellny et al.,, 2004, Schluepmann et al., 2003).

Ascorbate feeding showed it can rescue both growth and inhibits starch

accumulation. Previous reports have showed that ascorbate is involved in the regulation of cell elongation and progression through the cell cycle. Ascorbate promotes G1 to S progression of cells within onion (*Allium cepa*) root meristem and pericycle (Smirnoff and Pallanca 1996). It seems that ascorbate does have an effect on root growth and that is not dependent on trehalose inhibitory effect. Then, why ascorbate feeding has different effect on root length of Cvi accession? It is possible that trehalose can affect root tip mersitem cell division, which warrants further studies. On the other hands, ascorbate can inhibit starch accumulation that is induced by 100 mM trehalose. Our results also showed that, in Cvi seedlings, 0.1 mM ascorbate feeding reduced starch levels, about % of that seen in other seedlings. Other data showed ascorbate does not directly affect AGPase redox activation of isolated chloroplasts *in vitro* (data not shown). That means ascorbate target should be somewhere in the early pathway.

The current data showed that exogenous trehalose induced the enzymatic and non-enzymatic antioxidant defense system responsible for scavenging of ROS. Trehalose feeding induced Ascorbate, dehydroascorbare and total ascorbate accumulation in Cvi seedlings. These findings revealed that trehalose feeding change ascorbate steady state in *Arabidopsis* plants. Oligosaccharides such as glucose and sucrose have an important role in plants response to oxidative stress (31). Attempts to produce trehalose in plants by over expressing *TPS* in Tobacco yielded drought and oxidative stress resistant plant (Nounjan and Theerakulpisut 2012, Martins et al., 2014). Our previous data from gene-expression profiling of Col-0 accession *Arabidopsis* seedlings after 24h on 100 mM trehalose reveals changes in specific combination of genes known to be involved in biotic stress responses (Aghdasi et al., 2008). This links trehalose metabolism with a specific stress response. The stress response could result from carbon starvation of the sinks linked to T6P accumulation (Dellata et al., 2011). A genetic dissection of the effects after feeding 100 mM trehalose is necessary to establish causal relations between trehalose / T6P and stress and carbon allocation.

Plant genomes only contain 1 or 2 trehalse genes. Trehalse enzyme cleaves trehalose into two molecules of glucose (Leyman et al., 2001; Pramanik and Imai 2005). The trehalase activity has increased following trehalose feeding in Cvi seedlings. Interestingly, trehalase gene expression has not changed in Cvi seedlings when grown in the presence of trehalose. However, ascorbate feeding would reduce both trehalase activity and gene expression.

Ascorbate feeding changed the expression levels of marker genes involved in phloem loading/unloading (Roitsch et al., 2003) such as sucrose phosphate synthase (*SPS*) and sucrose transporter (*SUC*). Further research is needed to determine how ascorbate can induce *SPS* gene expression level in seedlings grown on 100 mM trehalose.

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