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Differential physiological responses of Col-0 and Cvi *Arabidobsis thaliana* seedlings to trehalose feeding

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Abstract

Trehalose-6-phosphate (T6P) has been proposed as an important signaling molecule that controls plant growth and carbon allocation. To gain insight into natural variation in trehalose metabolism, 14-day-old seedlings from eight *Arabidopsis thaliana* accessions were raised on MS medium supplemented with or without 100 mM trehalose and then compared for some physiological and biochemical parameters related to carbohydrate and antioxidant metabolism. Growth arrest occurred in Columbia (Col-0) and all other accessions, but seedlings from the Cape Verde islands (Cvi) accession were relatively resistant to trehalose. Trehalose feeding induced massive anthocyanin, soluble sugar and starch accumulation in Col-0 rather than Cvi cotyledons and the accumulated starch was localized in cotyledons. Trehalose feeding furthermore led to increase activity and transcript levels of trehalase in Col-0 plants while these remained unaltered in Cvi ones, indicating trehalose insensitivity of Cvi plants is not due to greater trehalase activity. Trehalose in addition, suppressed the expression of sucrose transporter *(SUC)*, sucrose phosphate synthase *(SPS*) and invertase (*INV)* genes in Col-0 seedlings whereas it increased the expression of *SPS* and *INV* genes in Cvi seedlings. The trehalose-induced growth arrest of Col-0 seedlings was further accompanied with decreased ascorbate/dehydroascorbate ratio, declined chlorophyll, increased hydrogen peroxide content and enhanced catalase and peroxidase activities. The activity of super oxide dismutase (SOD) and its transcript level were also greater in Cvi seedlings compared to Col-0 ones when supplied with trehalose. It was concluded that trehalose insensitivity of Cvi plants is due to their efficient antioxidant metabolism which by maintaining a greater levels of reduced ascorbate and enhanced SOD activity counteracts the trehalose-induced reactive oxygen species generation. This capacity might be attributed to unaltered and/or optimized carbon metabolism of source-sink tissues in Cvi plants.

Key words: Accession; *Arabidopsis thaliana*; Oxidative metabolism; Sucrose phosphate synthase; Superoxide dismutase; Trehalose insensitivity.

Abbreviations: Cvi_Cape Verde islands; Col-0_Columbia; INV_Invertase; ROS_Reactive Oxygen Species; SOD_Super oxide Dismutase; SPS_Sucrose Phosphate synthase; SUC_Sucrose Phosphate Transporter; T6P_Trehalose-6-Phosphate; TPH_Trehalose-6-phosphate Hydrolase; TPP_Trehalose phosphate Phosphatase; TPS_Trehalose-6-Phosphate Synthase.

Introduction

Trehalose metabolism has recently been recognized to play an important role in carbon signaling in plants (Paul et al., 2008). Trehalose is an alpha, alpha-1,1-linked glucose disaccharide, which is found ubiquitously in the living world and is therefore thought to be ancient. Plants generally contain trace amounts of trehalose (Muller et al., 1995; Zentella et al., 1999), however, some plants which are resistant to extreme drought such as *Selaginella lepidophylla* can accumulate quantitatively high amounts of trehalose (Zentella et al., 1999). Synthesis of trehalose in plants is typically carried out via its phosphorylated intermediate i.e. trehalose-6-phosphate (T6P). In this pathway, trehalose-6-phosphate synthase (TPS) utilizes UDP-glucose and glucose-6-phosphate as substrates to produce T6P. The de-phosphorylation of T6P by trehalose phosphate phosphatase (TPP) then results in the release of trehalose. Using one molecule of water, trehalose can be cleaved off into two molecules of glucose by trehalase. Genes encoding for trehalose metabolism have been reported in all plants. In *Arabidopsis* for example, 11 TPS and 10 TPP orthologues are found (Parmanik and Imai, 2005; Shima et al., 2007). Plant genomes however, only contain one to two trehalase genes (Leyman et al., 2001).

Evidence is thus accumulating that suggests an important regulatory role for T6P i.e. the precursor of trehalose biosynthesis, as a signaling sugar that regulates plant metabolism and development (Paul, 2008; Dellata et al., 2011,Ponnu et al., 2011; Nunes et al., 2013). Minor alterations in the steady state level of T6P lead to dramatic and pleiotropic phenotypic changes in plants (Schluepmann et al., 2003; Pellny et al., 2004; Parmanik and Imai, 2005; Martins et al., 2014). Additionally, *AtTPS1* i.e. the gene for TPS in *Arabidopsis*, is essential for embryo development, plant vegetative growth and transition to flowering (Eastmond et al., 2002; Van Dijken et al., 2004). In *Arabidopsis* seedlings, exogenously supplied trehalose inhibits root growth and emergence of leaves (Wingler et al., 2000; Aghdasi et al., 2010). In addition, it appears that cell wall elasticity and the swelling of cells in the extension zone of roots grown on 100 mM trehalose are altered. The effects of exogenous trehalose application are multiple, yet growth arrest following 100 mM trehalose feeding is due to T6P accumulation, because it can be alleviated in seedlings

expressing *E.coli* trehalose-6 phosphate hydrolase (TPH). The accumulation of T6P in plants is not due to phosphorylation of trehalose as the reaction catalyzed by TPP is irreversible due to the low levels of phosphate in cells (Schluepmann et al., 2003). In the light, growth arrest on 100 mM trehalose is due to T6P accumulation and can be rescued by exogenous supply of metabolizable sugars (Schluepmann et al., 2004). The presence of trehalose in the medium leads to accumulation of large amounts of starch in seedlings source tissues like the cotyledons and to a depletion of starch in the columella cells of the root cap which is a sink tissue (Wingler et al., 2006). Starch depletion in collumella cells of the root tip suggests that T6P accumulation throughout the plant tissues has likely caused starvation of sink tissues such as shoot and root apical meristems, thus restricting their growth. Sink starvation is not caused by sink inability to metabolize carbon since the supplied carbon is utilized and the effects of T6P accumulation are then overcome. Starvation is not due to carbon partitioning into starch in cotyledons, because *pgm1*seedlings defective in starch biosynthesis are also growth arrested on trehalose (Fritzius et al., 2001). Therefore, inhibition is more likely due to problems in carbon loading/ unloading or transport. Genetic variation among plants can be created experimentally or may occur naturally. So far natural variation has attracted increasingly more interest as it has become possible to map quantitative traits, typically traits that are affected quantitatively by several loci. Uncovering natural variation identifies variations that have been selected by environment and thus likely meaningful for a species' adaptation to different environments. The method thus allows identifying key parts of a genetic pathway that are selected upon by environmental adaptation. In our previous work, we characterized the physiological effects of 100 mM trehalose on growth and carbon allocation in Col-0 seedlings (Aghdasi et al., 2010). To determine natural variation in trehalose metabolism, seedlings from eight *Arabidopsis thaliana* accessions were compared after growth on MS medium complemented with 100 mM trehalose. Natural variation of growth inhibition on 100 mM trehalose was found; whilst most accessions of *Arabidopsis* including Col-0 and Ler were sensitive, the Cvi accession was significantly resistant to trehalose in the medium. By further comparative analyses of parameters related to carbohydrate and antioxidant metabolisms, it was tried to understand the underlying mechanism of trehalose induced growth arrested of Col-0 versus Cvi plants.

Results

Differential growth responses of Arabidopsis accessions to trehalose feeding

Exogenous trehalose application inhibits the development of roots and leaves of Arabidiopsis Col-0 seedlings (Wingler et al., 2000). The Arabidopsis accessions identified based on adaptation to varying environmental conditions (Alonso-Blanco and Koornneef, 2000) were evaluated for their seedling resistance to exogenously added trehalose. Significant growth arrest occurred in all accessions of *Arabidopsis* seedling in the presence of 100 mM trehalose however, Cvi seedlings were amongst the least affected ones (Fig. 1). In the presence of 100 mM sorbitol as control, the root length of Cvi seedlings was not significantly different from others but on 100 mM trehalose they produced longer roots compared to all other tested accessions (Fig. 1A- c). The average root length of Col-0 and Cvi was 1.98 and 3.25 cm, respectively (Fig. 1D). Seedlings from Kas appeared partially resistant to trehalose as well, but already produced longer roots on 100 mM sorbitol compared to other accessions (Fig. 1C). Increased trehalose tolerance of Kas is thus likely due to their increased vigor. The relative resistance of Cvi accession seedlings to trehalose was confirmed by inspecting of primary leaf emergence. The primary leaves were emerged after 7 days in Cvi and after 12 days in Kas seedlings on 100 mM trehalose. Because other accessions were sensitive to 100 mM trehalose and had short root, we choose to focus on seedlings of Col-0 and Cvi accession in next experiments. The Col-0 seedlings were different from Cvi seedlings in dry and fresh weights when grown on MS medium supplemented with 100 mM trehalose. The seedlings weights were significantly less in Col-0 in the presence of 100 mM trehalose (Fig. 1E, F).

Trehalose feeding leads to starch accumulation in Col-0 rather than Cvi seedlings

The tissue distribution of starch in Col-0 and Cvi 14d old seedlings was studied after Lugol staining. Trehalose fed Col-0 seedlings displayed large starch granules in cotyledons whereas the columnella cells of root cap were devoid of starch (Fig. 2A). In contrast, seedlings of Cvi growing on MS medium supplemented with 100 mM trehalose displayed starch in columella cells of the root tips and their cotyledons only contained few small starch granules in some area (Fig. 2B). Quantification of starch in whole seedlings of the Col-0 and Cvi seedlings grown on trehalose is shown in Fig. 2C. The starch content of control sorbitol-fed Col-0 seedlings was 11 mg. g^{-1} FW, whereas the figure in trehalose-fed plants increased to about 70 mg .g^{-1} FW. In Cvi seedelings, trehalose feeding could not induce starch accumulation and they contain the same amount of starch as Col-0 ones grown on sorbitol and trehalose (Fig. 2C). The Col-0 seedlings growing on sorbitol had two-folds more soluble sugars than the corresponding Cvi ones (Fig. 2D). Trehalose feeding of Col-0 seedlings increased their soluble sugar contents by about 6-folds compared to control sorbitol-fed plants.

Greater impact of trehalose application on chlorophyll and anthocyanin contents of Col-0 versus Cvi seedlings

Chlorophyll a, chlorophyll b and the total chlorophyll content of Cvi seedlings were lower than Col-0 ones raised on MS medium supplemented with 100 mM sorbitol. In the presence of trehalose, these three parameters decreased in both Col-0 and Cvi seedlings. Significant differences in Chla, Chlb and total chlorophyll occurred between Col-0 and Cvi seedlings in response to trehalose feeding. When seedlings were grown on MS medium supplemented with 100 mM trehalose, total chlorophyll content of Col-0 seedlings declined by about 6 folds after 14d growth compared to control sorbitol-fed plants (Fig. 3A-C). The extent of chlorophyll reduction was less in trhalose-fed Cvi seedlings compared to those grown on sorbitol. The anthocyanin level of Col-0 seedlings after 14d growth on trehalose was 10 folds more compared to those grown in the presence of sorbitol (Fig.3D). Cvi seedlings did not show any change in anthocyanin content by trehalose feeding.

Differential responses of antioxidant enzymes of Col-0 and Cvi seedlings to trehalose application

A comparison between the activities of antioxidant enzymes in trehalose-fed and the control sorbitol-fed seedlings reveal-

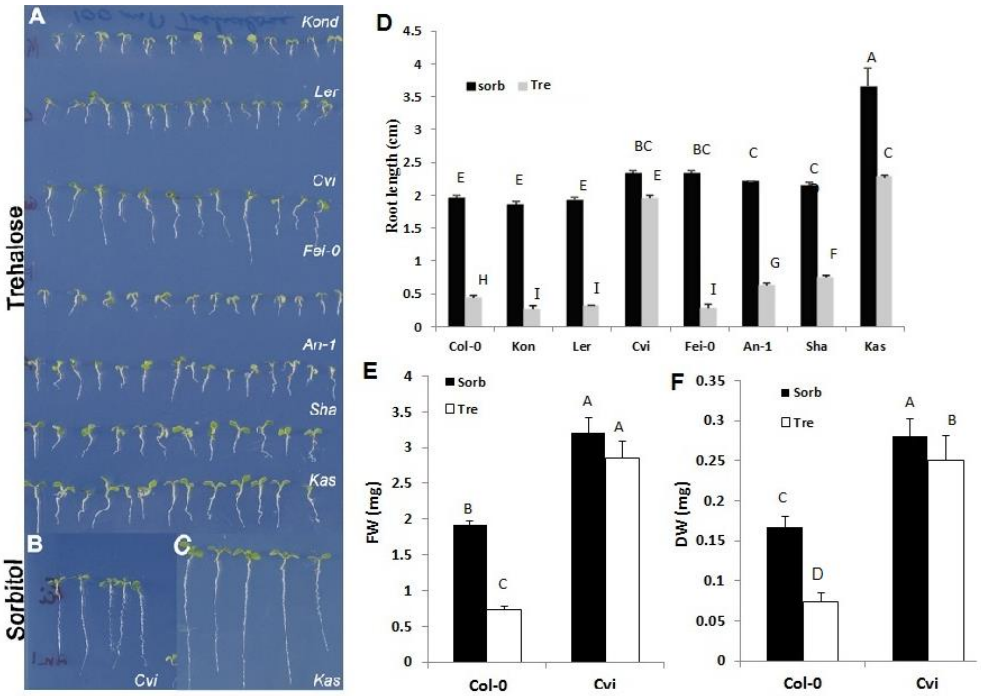

Fig 1. Growth of different accessions of Arabidopsis on 100 mM trehalose. Seedlings from different accessions were grown for 14d in long day conditions on half strength MS supplemented with A) 100 mM of trehalose, B, C) sorbitol. D) Root length, E) Dry weight and F) fresh weight of different accession of Arabidopsis growing on 100 mM trehalose.

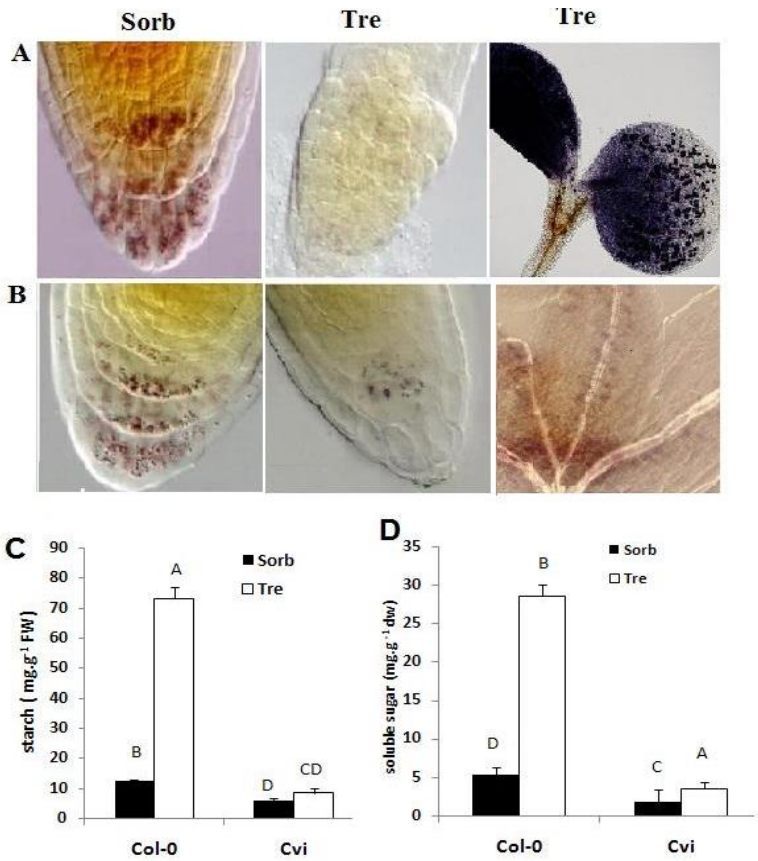

Fig 2. Starch staining and quantification in Col-0 and Cvi accession. Seedlings were grown 14d in long day conditions, then stained with KI/I_2 and studied using Nomarski microscopy. Starch in the columnella and leaf of **A)** Col-0 and B) Cvi grown on 100 mM sorbitol or trehalose **C)** starch quantification in whole seedlings.

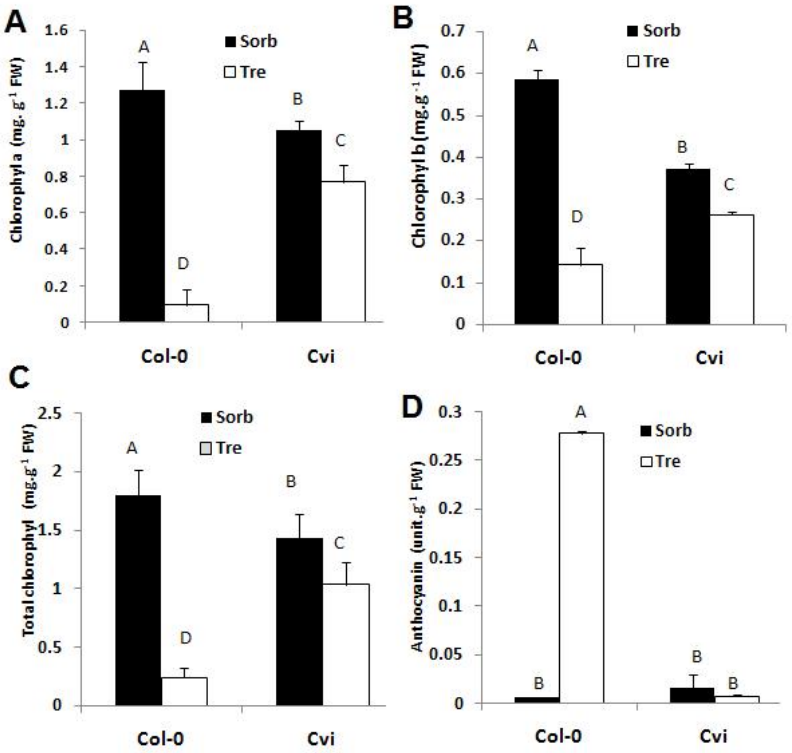

Fig 3. Chlorophyl and anthocyanin content in Col-0 and Cvi accession seedlings growing on 100 mM trehalose or sorbitol. Seedlings were grown 14 d on 100 mM of either sorbitol (Sorb) or trehalose (Tre), then chlorophyll a **(A)**, chlorophyll b (**B),** Total chlorophyll (**C)** and anthocyanin contents **(D)** were determined.

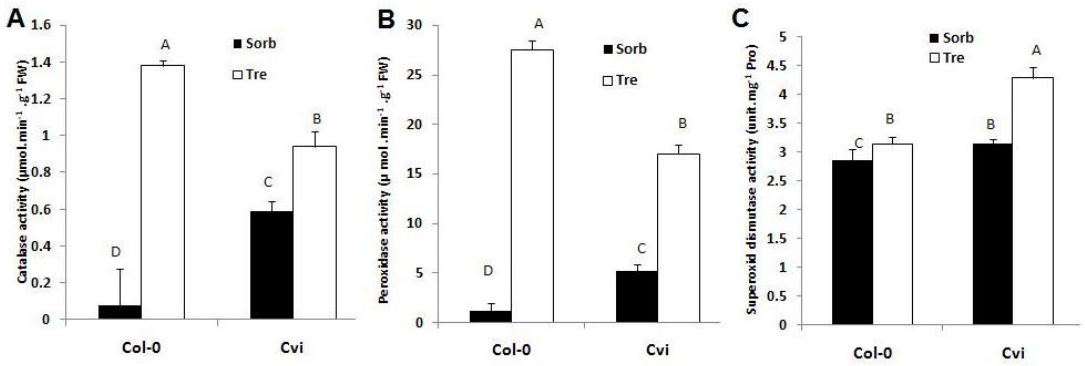

Fig 4. A) Catalase, B) Peroxidase and C) Superoxidase activity in Col-0 and Cvi accession seedlings growing on 100 mM trehalose or sorbitol.

revealed further differences between Col-0 and Cvi accessions. Catalase and peroxidaxe activities of Col-0 seedlings were significantly lower than that of Cvi when seedlings were grown on MS medium containing 100 mM sorbitol (Fig. 4A, B). Upon feeding by 100 mM trehalose, these activities were significantly induced in seedlings compared to those grown on sorbitol. In addition catalase and peroxidaxe activitiy was significantly higher in Col-0 seedlings than that of Cvi seedlings when grown on 100 mM trehalose. The superoxide dismutase activity of Col-0 seedlings remained unchanged after100 mM trehalose application. However, in Cvi seedlings the superoxide dismutase activity was significantly increased on MS medium containing 100 mM trehalose with respect to the control medium (Fig. 4C)

Trehalose feeding affect ascorbate metabolism

To examine whether trehalose feeding affects system that produce antioxidant, we proffered to measure ascorbate, dehydroascorbate and total ascorbate amount. The ascorbate, dehydroascorbate and total ascorbate level was lower in Cvi seedlings compared to Col-o seedlings when grown on 100 mM sorbitol (Fig. 5A-C). By adding 100 mM trehalose to the medium, ascorbate, dehydroascorbate and total ascorbate amount was significantly increased in Col-0 seedlings. These three parameters have also been increased in Cvi seedlings growing on 100 mM trehalose, but it was as same as Col-0 seedlings growing on 100 mM sorbitol. The ascorbate /dehydroascorbate ratio in Col-0 seedlings was higher than Cvi when grown on 100 mM sorbitol. By feeding 100 mM

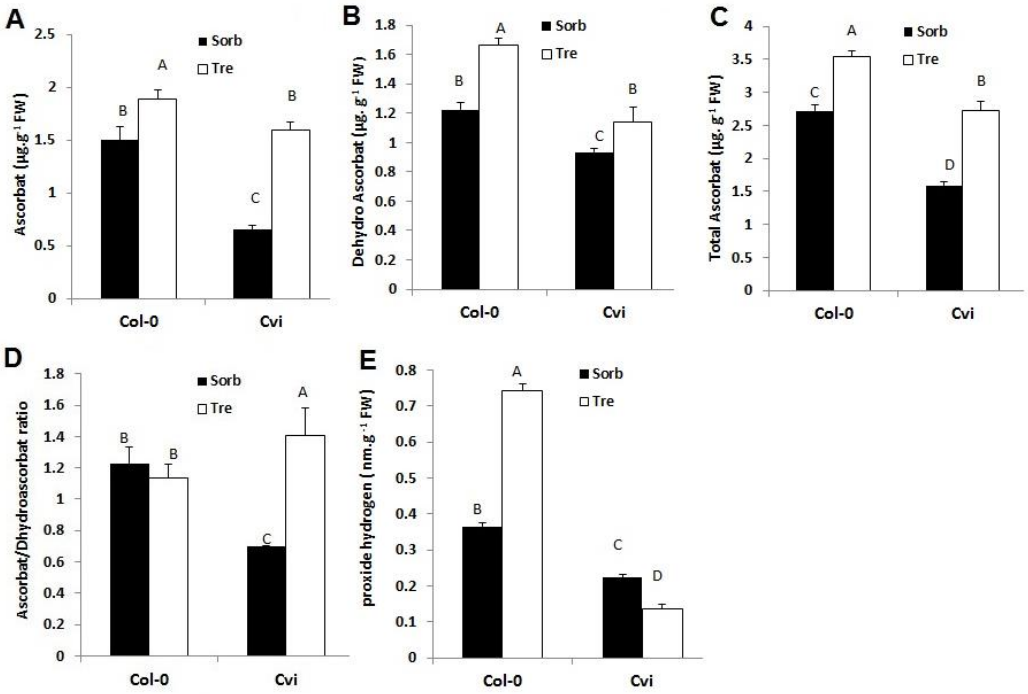

Fig 5. A) Ascorbate, B) Dehydroascorbate, C) total ascorbate, D) ascorbate/dehydroascorbate ratio and D) hydrogen peroxide level in Col-0 and Cvi accession seedlings growing on 100 mM trehalose or sorbitol.

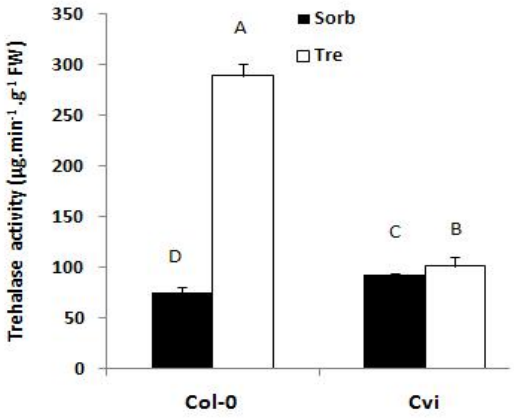

Fig 6. Trhalase activity in Col-0 and Cvi accession seedlings growing on 100 mM trehalose or sorbitol.

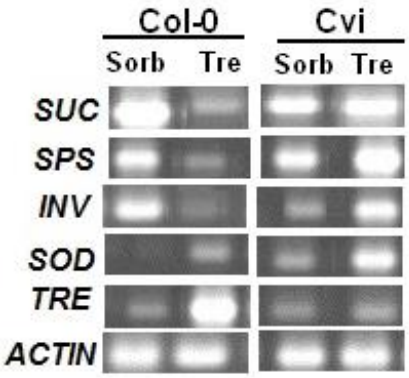

Fig 7. Gene expression in Col-0 and Cvi accession seedlings growing on 100 mM trehalose or sorbitol. Seedlings were grown on agar solidified half strength MS supplemented with 100 mM trehalose or sorbitol for 10 days before RNA extraction and RT-PCR analysis of gene-expression. Levels of gene expression were determined with reference to *AtACTIN2 (n=3)*.

trehalose, this ratio was increased in Cvi seedlings, but it was reduced in Col-0 seedlings (Fig. 5D)

Trehalose feeding induced hydrogen peroxide accumulation up to 2 folds more in Col-o seedlings. But hydrogen peroxide level was significantly decreased in Cvi seedlings grown on 100 mM trehalose (Fig. 5E).

Trehalase activity is not necessary for Cvi trehlose resistance

To find out whether resistance to trehalose feeding of Cvi seedlings is simply due to higher trehalase (EC 3.2.1.28) activity, the enzyme activity was assayed and compared in Col-0 and Cvi seedlings. Trehalase activity in Col-0 seedlings was a little lower than Cvi seedlings when grown on 100 mM sorbitol (Fig.6). But exogenous trehalose strongly induced trehalase activity 4 times more in Col-0 seedlings.

Gene expression analysis in Col-0 and Cvi accessions

To examine the transcriptional regulation of the genes related to carbon allocation, we preferred RT-PCR analysis of the key genes like: sucrose transporter (*SUC*), sucrose phosphate synthase (*SPS*) and invertase (*INV*). Gene expression analysis was performed using mRNA from 10 d old seedlings grown on MS medium with 100 mM sorbitol or trehalose, as described in Materials and Methods. A high level of *SUC* gene expression occurred in the Col-0 seedlings growing on 100 mM sorbitol. The results showed that trehalose feeding suppresses the expression of *SUC*, *SPS* and *INV* genes in Col-0 accession; however it induced *SPS* and *INV* gene expression in Cvi seedlings. The expression of *SUC* was almost unchanged by trehalose feeding (Fig. 7).mThe gene expression analysis showed that *SOD* expression was very low in Col-0 seedlings growing on MS medium supplemented with 100 mM sorbitol. Trehalose feeding induced *SOD* gene expression in both Col-0 and Cvi seedlings. Expression analysis of the *AtTRE1*, the only trehalase gene in *Arabidopsis*, showed that trehalase expression level in Cvi seedlings is as same as Col-0 seedlings when grown on 100 mM sorbitol. Trehalose feeding induced trehalase expression level in Col-0 seedlings, but *AtTRE1* expression in Cvi seedlings remained unchanged (Fig. 7).

Discussion

Trehalose has been shown to hold several biological functions as both carbon reserve and stress protection. To gain insight into the role of trehalose on growth of different accessions of *Arabidopsis* plants they were grown on 100 mM trehalose. The growth arrest further occurred in all ecotypes of *Arabidopsis* tested but was significantly less in seedlings of Cvi. Trehalose feeding decreased seedling dry and fresh weights of other accessions, whereas it did not show any significant effect on Cvi seedlings. Because of distinct phenotype of Cvi plants, the seedlings of this accession was compared with those of Col-0 ones under trehalose feeding in more details. Trehalose feeding induced massive starch accumulation in source tissue and inhibited starch accumulation in the collumnella of root tips of Col-0 seedlings (Fig. 2A and Wingler et al., 2000). Seedlings from Cvi accession however, displayed a few amount of starch in cotyledons. Unlike Col-0 seedlings, starch staining was observed in the columnella cells of the root tips of Cvi accession. Quantitative analyses revealed that trehalose feeding induced cotyledonary starch synthesis up to 6 fold in Col-0 seedlings whereas there was not a significant effect on the starch content of Cvi seedlings grown on 100 mM trehalose. The effects of exogenous trehalose is believed to be mediated by in vivo formation of its phosphorylated form i.e. T6P (Schluepmanne et al., 2004). T6P mediated inhibition of growth in Col-0 accession is likely due to starvation of sink tissues important for growth, such as shoot and root apical meristems. Because the *pgm1* starchless mutant seedlings are also growth arrested on trehalose (Fritzius et al., 2001; Dellate et al., 2011). T6P inhibition of growth is not due to carbon partitioning into starch in the cotyledons.mTrehalose feeding caused the accumulation of soluble sugars and anthocyanin and reduction of chlorophyll in Col-0 seedlings. These might be indicative of oxidative stress being observed by the Col-0 seedlings under this condition. Anthocyanins are secondary metabolites that protect plants against reactive oxygen species (Gould, 2002). It has been shown that exogenous sugars can induce anthocyanin accumulation in plants (Teng et al., 2005). Interestingly, trehalose feeding increased soluble sugar content in Col-0 seedlings and there are several reports that sugar accumulation in leaf tissues accompanies with increased anthocyanin content (Teng et al., 2005). Accumulation of soluble sugars is also known as an adaptation mechanism in response to oxidative stress (Rotisch, 1999). Thus, trehalose feeding indirectly affects anthocyanin biosynthesis through increasing sugar content. The suppression of anthocyanin and sugar accumulation and chlorophyll reduction in Cvi seedlings grown on trehalose along their unaltered growth are more evidence for the above contention. Oxidative stress also induces apoptosis (Palma et al., 2002). The accumulation of soluble protein and reduction of total protein in trehalose-fed Col-0 seedlings might be because of proteases biosynthesis upon apoptosis. Exogenous trehalose increased ascorbate, dehydroascorbate and total ascorbate in both col-0 and Cvi seedlings after 14 days. Ascorbic acid is strong antioxidant that scavenger ROS in the plants (Smirnoff, 1996). But ascorbate/dehydroascorbate ratio was increased in Cvi seedlings when grown on 100 mM trehalose, compared to Col-0 seedlings. It was reported that in the ascorbate-deficient Arabidopsis thaliana mutant (*vtc1*) the ratio of dehydroascorbate/total ascorbate was increased when exposure to Ultraviolet-B (UV-B) radiation, compared to wild type plant (Gao and Zang, 2008). This data suggest that increased ratio of ascorbate/dehydroascorbate might be main reason for trehalose insensitivity phenotype of Cvi plants. Thus one way these plants remain more efficient in counteracting oxidative stress might be due to maintenance of a more reduced pool of ascorbate after trehalose application. Accordingly, the hydrogen peroxide content of Cvi plants even reduced after trehalose application whereas it did increased to a significant level in Col-0 ones. Our previous data from microarray expression profile revealed that trehalose elicits gene expression responses consistent with ROS and secondary metabolism activation. One of these genes is Calcium binding EF hand that induces hydrogene peroxide production under stress condition (Aghdasi et al., 2008). Further evidence for the predominance of oxidative stress in trehalose-fed Col-0 seedlings was provided by studying the activities of some ROS scavenging enzymes. Thus catalase and peroxidase activity were increased in both Col-0 and Cvi seedlings by trehalose treatment. But catalase and peroxidase activities were higher in trehalos-fed Col-0 than Cvi seedlings. These findings are consistent with our former study which suggested provocation of oxidative stress in *Arabidopsis* plants after 100 mM trehalose application, in which case enzymatic and non-enzymatic antioxidant

systems (such as ascorbate) scavenge ROS. Superoxide dismutase is a key protective enzyme that inter-converts highly toxic superoxide radicals into a weaker ROS i.e. hydrogen peroxide (Bowler et al., 1994). In Col-0 seedlings, SOD activity did not changed significantly after trehalose application. In contrast in Cvi seedlings trehalose application induced SOD activation. It has been reported that Cvi accession has a new chloroplastic Cu/Zn superoxide dismutase isoenzyme which shows an increased tolerance to photo-oxidative stress (Abarca et al., 2001). Trehalose treatment resulted in higher SOD activity in Cvi seedlings compared with Col-0 seedlings. Analysis of gene expression showed that *Cu/Zn SOD (SOD)* transcript level in Cvi seedlings is much higher than Col-0 seedlings when grown in the presence of 100 mM trehalose (Fig. 7). These findings suggest that *SOD* transcription or its transcript stability might be higher in Cvi than Col-0 accession. Cvi accession was initially identified from Cape Verde islands, a dry region close to equator (Lobin, 1983) with low level of precipitation (9.4 mm per month) during rainy season. This means that Cvi is more adapted to environmental stress conditions. So far many reports showed capability of Cvi adapting to different abiotic and biotic stresses, such as ozone, freezing, UV radiation, photo-oxidative and drought (Alonso- Blanco and Kornneef, 2000; Rao et al., 2000; Abarca et al., 2001; Bouchabke et al., 2008; Perchepied et al., 2010). The current data revealed that Cvi is more resistant to 100 mM trehalose than Col-0 accession. This might have been resulted partly to greater gene expression and enzyme activity of SOD in Cvi compared to Col-0. Trehalase is the unique enzyme that cleaves trehalose into two molecules of glucose. Both trehalase activity and its gene expression were increased by trehalose feeding in Col-0 seedlings. Notably, there were no detectable changes in trehalase gene expression or activity in Cvi seedlings when grown in the presence of trehalose. Thus, the relative trehalose resistance of Cvi accession is independent of trehalase activity or its gene expression. It seems amino acids sequence analysis of the enzymatic domain of trehalase protein is necessary to be investigated to find out any natural variation between Col-0 and Cvi accessions. In Arabidopsis seedlings, exogenously supplied trehalose has a strong inhibitory effect on growth and carbon allocation (Wingler et al., 2000, Aghdasi et al., 2010). This inhibitory effect is not because of starch accumulation in source organs and a depletion of starch in sink organs. Since starchless *pgm1* mutants shows the same phenotype as Col-0 seedlings when grown on 100 mM trehalose (Delatte et al., 2011) In light, growth arrest on 100 mM trehalose can be rescued by exogenous supply of some sugars which can be metabolized (Schluepmann et al., 2004). Therefore inhibition is more likely due to carbon loading/unloading or transport. T6P has been recently introduced as an important regulator of metabolism and transcription which trigger plant growth, carbon assimilation and sugar availability (Paul, 2008; Yavada et al., 2014). The expression levels of marker genes involved in phloem loading/unloading (Roitsch et al., 2003) such as invertase (*INV*), sucrose phosphate synthase (*SPS*) and sucrose transporter (*SUC*) were altered by trehalose feeding. While transcript levels of these genes were suppressed in Col-0 seedlings growing on 100 mM trehalose, they were induced in Cvi seedlings. Sucrose is the major transport sugar in phloem of higher plants. Sucrose metabolism is very important for hexose utilization. Its catabolism in sink tissues needs two enzymes i.e. invertase and sucrose synthase. Invertase irreversibly hydrolyzes sucrose into hexoses. But, sucrose synthase catalyzes the reversible hydrolysis of sucrose into UDP- glucose and fructose (Koch, 1996). Suppression of *SPS* leads to increased amount of UDP-Glc and the consequence accumulation of starch in cotyledons. Meanwhile it has been demonstrated that T6P regulatory effect on growth and carbon utilization is via inhibition of SnRK1 (Dellata et al., 2011), which acts on SPS as a non-authentic substrate (McMichael et al., 1995). Accordingly, starvation of sink organs is likely due to accumulation of soluble sugars in source tissues resulting from inactivation of phloem transport (by suppression of sucrose transporter). It remained to be known how the expression level of *SUC, INV* and *SPS* were induced in Cvi seedlings after trehalose feeding. Further research such as the characterization of QTLs by genetic mapping is needed to isolate novel genes or alleles involved in resistance of Cvi seedlings to trehalose. The partial resistance is not due to increased trehalase expression or the altered MYB75/PAP1 allele found in this ecotype. The current results imply environmental pressure on trehalose metabolism control over plant metabolism.

Materials and Methods

Plant materials and growth conditions

Seeds from different *Arabidopsis thaliana* accessions were sterilized 5 minutes with 70% Ethanol followed by 10 minutes in 20 % commercial bleach (4% w/v chlorine) and 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 sorbitol and stratified in darkness at 4ºC for 3 days before they were transferred 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 the Image j program (Wayne Rasband, NIH Maryland, USA).

Starch staining and measurement

For analysis of tissue starch distribution, whole seedlings were taken and de-stained in 70% and then 90% (v/v) ethanol. Staining was done with KI/I_2 solution and after washing the seedlings they were photographed with a Normanski microscope (Jena, Germany).

For starch quantification, plant material (50 mg) was frozen and ground in liquid nitrogen. Soluble sugars were extracted with 1 ml 80% ethanol for 10 minutes at 80ºC in ependorff tubes. The tubes were then centrifuged at 13000 g for 5 minutes and the clear supernatant was transferred into a clean tube. The 13000 g pellet was re-extracted with 75 µl of milliQ for 10 minutes at 80ºC and after centrifugation the supernatants were pooled. Starch was then extracted from the remaining pellet by the addition of 0.5 M NaOH (0.1 ml) and subsequent incubation at 60ºC for 30 minutes. After the addition of 6 µl acetic acid (96%), starch was digested overnight at 37ºC by addition of Amyloglucosidase and quantified as described by manufacturer (Boehringer Mannheim, Darmstadt, Germany).

Soluble sugar and protein determination

The soluble sugars were determined spectrophotometrically by the phenol sulfuric acid method (Kochert, 1985).

Chlorophyll and anthocyanin measurements

Total chlorophyll was measured spectrophotometrically as described by Jeffery and Humphrey (1975). In brief, 14 days old 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 according to Mita et al. (1997).

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

The crude enzyme extract was prepared 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 the enzyme activity measurements.
The activity of catalase (EC 1.11.1.6) was determined according to Chance and Maehly (1955). The activity of peroxidase (EC 1.11.1.7) was recorded spectrophotometrically at 470 nm as described by Kar and Mishra (1976). The superoxide dismutase (EC 1.15.1.1) activity was determined as described by 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

Seeds of *Arabidopsis thaliana* accession Columbia-0 and Cvi were grown on half strength MS medium for 10 days. Plant material was snap frozen in liquid nitrogen and pulverized with glass beads for 2 minutes at 2800 rpm in a dismembrator (Braun, Germany). Total RNA was isolated with RNeasy plant mini kit (QIAGEN USA, Valencia, CA). RNA concentration and purity were determined by measuring absorbance at 260 nm. Ten ng RNA was treated with 2 U DNAse I (DNA- free, Ambion, Austin, USA) to remove genomic DNA. The absence of DNA was attested 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 1 ng of total RNA extracted and used for first-strand cDNA synthesis with sixty units M-MLV Reverse Transcriptase (Promega, Madison, WI), 0.5 µg of odT16v (custom oligo from Invitrogen, Carlsbad, CA) and 0.5 µg 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'- ACAACCAAACAAAGTGGACC-3' for *INV*,
5'- ACAGTTCGGTTGGGCTTTACAGTTATCTC-3 and
5'- TTGGAGGCTTTTCCATCGGCTGTTGGCTCTG-3' for *SUC* and 5'- GACCCAAAGACGGAGACTCTT-3' and
5'- GCCAAGT GATTGTGGAGACTC for *AtACTIN2* as the reference gene.

Statistical analysis

Data from all experiments were processed by statistical SAS package (version 9). The reported values were means of three replicates. Means were compared for significance using the Duncan's test ($P<0.01$).

Conclusion

In this study, we characterized several accessions of *Arabidopsis thaliana* for their ability to grow on 100 mM trehaloae. Growth arrest occurred in all accessions of *Arabidopsis* tested except for Cvi seedlings. The partial resistance of Cvi seedlings to trehalose feeding is not due to the altered trehalase enzyme activity. In conclusion this capacity might be attributed to unaltered and/or optimized carbon metabolism of source-sink tissues in Cvi plants as evidenced by their unchanged and/ or induced expression of *SPS*, *SUC* and *INV* genes following exposure to trehalose.

Conflict of interest statement

We declare that we have no conflict of interest

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