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Altered *DREB1A* Gene Expression in *Arabidopsis thaliana* affects Root Growth, Antioxidant Enzymes Activity, and Response to Salinity but Not to Cold

Eisa Kohan-Baghkheirati[1, 2, 3], Mohammad B. Bagherieh-Najjar[1*], Ahmad Abdolzadeh[1] and Jane Geisler-Lee[3]

[1]Department of Biology, Golestan University, Gorgan, Iran
[2]Department of Biology, Hakim Sabzevari University, Sabzevar, Iran
[3]Department of Plant Biology, Southern Illinois University Carbondale. Carbondale, IL 62901, USA

*Corresponding Author: mb.bagherieh@gu.ac.ir

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Abstract

DREB1A (Dehydration Responsive Element Binding 1A) transcription factor is involved in plant responses to abiotic stresses. An *A. thaliana DREB1A* T-DNA insertional mutant (*dreb1a*) alongside previously reported *DREB1A* over-expressing plants (OX28) were detailed in molecular and phenotypic characterizations. The T-DNA of the *dreb1a* line was inserted at position -253, and segregation ratio confirmed a single T-DNA locus in its T0 plant population. The RT-PCR analysis of *dreb1a* seedlings also revealed a null mutant in the *DREB1A* gene. The phenotypes of the *dreb1a* seedlings subjected to cold stress were not different from those of the wild type (WT-Col0), but under salinity *dreb1a* plants showed about 11% less seed germination and the four times less survival rate, compared to WT-Col0 plants. Under normal growth conditions, there was a direct correlation between *DREB1A* expression levels and the root length as the *dreb1a,* in contrast to the OX28 line, showing 29% longer roots than that in the WT-Col0 plants. Interestingly, this root phenotype had an association with the accumulation of reactive oxygen species (ROS) in *dreb1a* by 31% less, and in OX28 by 97% more than that in the control seedlings. In addition, the *dreb1a* plant possessed significantly higher activities in superoxide dismutase, peroxidase, polyphenol oxidase and significantly lower activity in catalase than WT-Col0, but no differences in extracellular peroxidase activity. On the other hand, the OX28 plant possessed a higher extracellular peroxidase activity. Overall, these results suggest that a precise expression level of *DREB1A* gene is required for proper growth and development in *A. thaliana*.

Keywords: *Arabidopsis thaliana*; *DREB1A*; Salinity; Root Growth; Antioxidant Enzymes Activity

Introduction

Plants, as sessile organisms, are constantly exposed to diverse environmental stimuli which may trigger various biochemical, physiological and molecular responses, mostly mediated by transcription factors. DREB1 transcription factors, also called CBF (C-repeat Binding Factors) are conserved in the plant kingdom and play vital roles in plant responses to various abiotic stresses such as cold, drought, and high salinity (Agarwal *et al.,* 2006). All DREB1/CBF transcription factors can bind to the DRE (Dehydration-Responsive Element) *cis*-regulatory conserved CCGAC core DNA sequence and regulate a complex network of between 100 (Park *et al.,* 2015) and 400 genes (Zhao *et al.,* 2016). The *A. thaliana* genome contains three *DREB1/CBF* family members which lie in tandem on chromosome 4 in the order of *DREB1B/CBF1, DREB1A/CBF3* and *DREB1C/CBF2* (Yamaguchi-Shinozaki and Shinozaki, 1994). It seems that *DREB1C* acts as

a negative regulator of *DREB1A* and *DREB1B* (Novillo *et al.,* 2004; Novillo *et al.,* 2007). Among these, the *DREB1B* as a closest homolog of *DREB1A* encodes a protein with 213-amino acids, but two other genes, *DREB1A* and *DREB1C*, encode a protein with 216 amino acids.

In *A. thaliana*, the level of *DREB1A* is up-regulated by ICE1 (Inducer of CBF Expression) transcription factor, which is activated through phosphorylation, when seedlings are grown under cold stress (Chinnusamy *et al.*, 2003). Later studies indicated that the ICE1-DREB1A regulon (*i.e.*, a group of genes that are regulated as a unit) is the main cold tolerance pathway in higher plants that governs about 100 downstream genes modulating diverse stress-inducible metabolic pathways, such as sugar, lipid and osmoprotectant biosynthesis (Seki *et al.,* 2002; Shi *et al.,* 2015). In addition, the ICE1-DREB1A regulon has crosstalk with most plant hormones such as ethylene (Kazan 2015), and Gibberellin (Suo *et al.,* 2012; Tonkinson *et al.*, 1997). For example, elevated levels of *DREB1A* expression in soybean caused a dwarf phenotype with small dark green leaves and delayed flowering, which was associated with decreased endogenous GA levels (Suo *et al.,* 2012). ICE1-DREB1A regulon also contributes in mediating of development through connectivity with ROS status and antioxidant enzyme activity (Jia *et al.,* 2016; Li. X. *et al.,* 2011; Rai *et al.,* 2013; Wan *et al.,* 2014). The complex interaction network of *DREB1A*, hormones, and ROS status modulates plant growth and development under environmental stresses (Bartoli *et al.,* 2013).

A. thaliana plants with elevated levels of *DREB1A* expression show elevated freezing tolerance in non-acclimated plants (Gilmour *et al.,* 2000; Kasuga *et al.,* 1999; Liu *et al.,* 1998). On the other hand, RNAi, antisense, and CRISPR/Cas9 derived mutant lines of *DREB1A* have been applied to curtail expression of *DREB1A* in order to understand their roles in freezing tolerance and to establish functional redundancy among DREBs transcription factors (Novillo *et al.,* 2004; Novillo *et al.,* 2007; Zhao *et al.,* 2016). Despite the aforementioned research, the plants lacking basal levels of *DREB1A* expression has not yet been fully described to establish effects of *DREB1A* in the plant growth and development. In this study, we characterization of a *dreb1a* T-DNA insertional mutant and a *DREB1A* over-expression line in *A. thaliana*. The role of *DREB1A* in root development, ROS status and antioxidant enzymes activity were also investigate as well as the phenotype of *dreb1a* plants under cold stress and salinity.

Materials and Methods

Plant materials and seed germination

Five different types of *A. thaliana* seeds were used in this study. Two types of the five were Columbia (Col-0) accession seeds including the wild type (hereinafter WT) and the T-DNA transformed line (ID: N413033) in the *DREB1A* gene which was purchased from Nottingham Arabidopsis Seed Center. The wild type Col-0 line (WT-Col0) was used as background control of *DREB1A* mutant line (*dreb1a*). The other three types were Wassilewskija (WS-2) accession seeds including the wild type WS-2 (WT-WS2), the *DREB1A*-overexpressing (OX28), and the empty vector containing plant (Vector), which were kindly provided by Professor M. F. Thomashow (Michigan State University, USA) (Gilmour *et al.,* 2000). The WT-WS2 and Vector lines were used as background controls of *DREB1A* overexpressing line, OX28. Two wild type lines (*i.e.* WT-Col0 and WT-WS2) were applied to normalize background effects of two different accessions in the same treatments.

Seeds were surface sterilized with 15 % (v/v) commercial bleach for 15 min, washed with ddH_2O for five times and sown either on plates containing Murashige and Skoog (MS) (Murashige and Skoog, 1962) medium supplemented with 3 % sucrose and 0.7 % agar, or on Fafard 4M Mix soil (No. 8063028, Conrad Fafard. USA). Plates and pots were incubated for four days at 4 °C and transferred to a growth chamber with a 12-hour light cycle, 22/16 °C and light intensity of 27.20 µmol s^{-1} m^{-2}.

dreb1a mutant isolation and characterization

The T-DNA transformed line (ID: N413033) which contained a T-DNA insertion at the predicted-position of -302 in the prompter region

of the *DREB1A* gene was first genotyped by PCR amplification, using combinations of genomic *DREB1A* and the T-DNA specific primers (first two rows in Table 1). For DNA extraction, 3-5 mg tissue (*i.e.* one rosette leaves) of two-week-old plants were ground in extraction solution according to Kasajima *et al.*, (2004). Residual tissues in solution were left in the tube and 1 µl of the supernatant was added to 20 µl of PCR mixture. Amplification was done using DNA *Taq*-polymerase (Takara, Japan) for 35 cycles: a 3 minutes melt at 94 °C first; then cycles with 30 second melt at 94 °C, 30 second annealing at 55 °C and 30 second extension at 72 °C; finally 5 min of primer extension at 72 °C. Amplified PCR products were run and checked on 1 % agarose gel. The amplified PCR product was sequenced (BioNeer, Korea) to confirm the position of T-DNA in the *DREB1A* promoter. The individual lines were screened to select homozygous lines by sowing seeds on MS medium. After two weeks, seedling was screened by PCR to find homozygous lines. Then, each homozygous seedling was selected and carefully transferred to half strength Hoagland medium (Hoagland and Arnon 1950) to complete its life cycle and to produce seeds. The seeds were allowed to grow and self to get hemizygous *dreb1a* lines for downstream analyses.

Segregation of hemizygous *dreb1a* lines from non-T-DNA lines was performed using sulfadiazine resistance/sensitivity. Fifty seeds per plate were grown on MS medium with or without 7.5 mg/L of sulfadiazine (Sigma, St. Louis, MO, USA), and four plates (*i.e.*, biological replicates) for treatment or control were used; the segregation was repeated three times (*i.e.*, technical replicates). The sulfadiazine resistant/sensitive phenotypes at 20 days after planting (DAP) were presented as yellow plantlets without real first leaves.

Table 1. The list of primers used for T-DNA genotyping and expression analyses.

Name	Sequence (5'-3')	Product Size (bp)	Application
DREB1A-Promoter	P1: TTTCCGCCAAAACTACTTGG P2: CAAAAGAAAAGGCGAAGACG	486	Mutant Genotyping
T-DNA	P3: ATATTGACCATCATACTCATTGC P4: GTGGATTGATGTGATATCTCC	-	Mutant Genotyping
DREB1A-exon	F: GATGACGACGTATCGTTATGGA R: TACACTCGTTTCTCAGTTTTACAAAC	144	RT-PCR
COR15a	F: GCTTCAGATTTCGTGACGGATAAAAC R: GCAAAACATTAAAGAATGTGACGGTG	333	RT-PCR
18SRNA	F: ATGATAACTCGACGGATCGCA R: CTTGGATGTGGTAGCCGTTTC	169	RT-PCR

RNA extraction and RT-PCR analysis

Three to four rosette leaves (*i.e.*, approximately 50 mg of leaf tissues) of 4-week-old plants were grounded in liquid nitrogen using the Trizol reagent (Invitrogen) as described by the supplier to extract total RNA. The extracted RNA was then treated with RNase-free DNaseI (#EN0521, Fermentas, USA) to eliminate any residual genomic DNA.

Semi-quantitative RT-PCR reactions were performed using AccuPower CycleScript RT PerMix (dN$_6$) and PCR PerMix kits (BioNeer, Korea) according to the manufacturer's instructions. gene-specific primers were in Table 1 (bottom three rows). The expression levels of *DREB1A* and its target gene *COLD REGULATED 15 A* (*COR15a*) in *dreb1a* and WT-Col0 were detected after 2-hours incubation at 4 ºC, using 18S rRNA as an internal control. Since *DREB1A* is a cold-responsive gene, there will be no detectable expression by RT-PCR if without cold stress. Thus, 2-hour cold treatment could increase the expression of *DREB1A* if the *dreb1a* mutant were not a null mutant.

Salinity and cold treatments

To further characterize the *dreb1a* mutant, it was subjected to salinity and cold treatments. In the salinity treatment, WT-Col0 and *dreb1a* seeds were sown on MS containing plates with 0.7 % of agar, supplemented with 0, 50, 100 or 150 mM NaCl. Phenotypes of seed germination,

growth Stages 0.5 (*i.e.* radicle emergence), 0.7 (*i.e.* complete emergence of hypocotyl), 1.0 (*i.e.* cotyledons fully opened), 1.02 (*i.e.* two rosette leaves), and 1.04 (*i.e.* four rosette leaves) (Boyes *et al.*, 2001) and survival rates (up to 14 DAP) were recorded and analyzed.

In the cold treatment, the seedlings at 14 DAP were transferred to the dark at 4 °C for 8 hours, 4 °C for 16 hours, and -20 °C for 30 min, followed by 3 days recovery at 22 °C. After the recovery, their phenotypes and gene expression were analyzed. Gene expression analysis followed the aforementioned methods in the sections of RNA Extraction and RT-PCR Analysis.

Root phenotype analysis

Primary phenotypically analysis of the five different lines suggested probable role(s) of *DREB1A* gene in the root development. The root lengths of seeds sowing and growing on agar plates were measured between 10 and 20 DAP. Reactive oxygen species (ROS) accumulation was also localized at the first 5 mm of root tips according to Mellersh *et al.,* (2002). Accumulation of ROS in the first 5 mm of a root to completely cover the elongation zone was measured. Seedlings were placed in 0.005% (v/v) Nitroblue Tetrazolium (NBT) in 100 mM phosphate buffer, pH 7.5 for 10 minutes to detect superoxide radicals. The seedling was, then, washed with the phosphate buffer for at least three times. For negative controls, seedlings were incubated in 10 mM propyl gallate for 60 min prior to NBT staining. Propyl gallate, an antioxidant, was used to verify the method. After NBT staining, seedlings were transferred to slides and their roots (where deep blue colors occurred) were taken images from an Olympus DP71 camera connected to an Olympus BX51 microscope. The blue color intensities of the image were processed and analyzed in the ImageJ Java version (Rueden *et al.,* 2016; Schneider *et al.,* 2012).

ROS-related enzyme assays

Fifty mg of fresh leaves were ground in 2 ml of 100 mM extraction buffer (NaH_2PO_4. $2H_2O$ at pH 7) on the ice bath (Kar and Mishra 1976). Extracts were centrifuged at 13000 g for 15 min at 4 °C. After centrifugation, the supernatant (*i.e.*, top phase) of the extracts was collected and then used to measure the activities of five different ROS-related enzymes: superoxide dismutase, polyphenol oxidase, catalase, ascorbate peroxidase, and peroxidase (Mittler 2002). The remaining sediments (bottom phase) of the extracts were washed with distilled ddH_2O three times and then extracted twice in 1.5 ml of 1 M NaCl. Their supernatants were then used to measure extracellular peroxidase. These enzymes are involved in scavenging of ROS in plants and act as key players of plant response to stress (Mittler 2002). The activity of enzymes was detected spectrophotometrically in kinetic mode (Shimadzu UV-1601, Tokyo, Japan).

For peroxidase and extracellular peroxidase assays, 100 and 300 µl, respectively, of the extract were added to 3 ml of assay mixture containing 40 mM H_2O_2, 20 mM Guaiacol, and 50 mM sodium acetate buffer (pH 5). The absorbance was scanned at 470 nm for 1 min. The molar extinction coefficient of peroxidase is 26.6 L (mmol cm)$^{-1}$.

For catalase assays, 100 µl of the extract was added to 3 ml of assay mixture containing 15 mM H_2O_2, and 50 mM phosphate buffer (pH=7) (Saffar *et al.,* 2009). The absorbance was scanned at 240 nm for 1 min. The molar extinction coefficient of catalase is 40 L (mmol cm)$^{-1}$.

For ascorbate peroxidase assay, 50 µl of the extract was added to 2 ml of assay mixture containing 1.2 mM H_2O_2, 0.5 mM ascorbic acid, 0.1 mM EDTA and 312.5 mM phosphate buffer (pH 7). The absorbance was scanned at 290 nm for 1 min. The molar extinction coefficient of ascorbate peroxidase is 2.8 L (mmol cm)$^{-1}$.

For polyphenol oxidase assay, 100 µl of the extract was added to 3 ml of assay mixture containing 10 mM pyrogallol and 26 mM phosphate buffer (pH 6.8). The absorbance was scanned at 420 nm for 1 min. The molar extinction coefficient of polyphenol oxidase is 2.47 L (mmol cm)$^{-1}$.

Superoxide dismutase was assayed based on Beauchamp et al (Beauchamp and Fridovich 1971). 3 ml of the assay mixture containing 0.05 mM NBT, 0.003 mM riboflavin, 5 mM methionine, 0.66 mM EDTA and 50 mM

phosphate buffer (pH 8) with or without 50 µl of the extract were prepared. The absorbance was scanned at 560 nm for 1 min after 0, 5, 10, and 15 min incubation of reaction tubes in the dark (as control) or in the light. To measure enzyme activity per 15 min, the absorbance at time 15 ($A_{time\ 15}$) minus absorbance at time 0 ($A_{time\ 0}$) was used.

Statistical Analyses

All experiments were performed at least in four biological replicates. Averages and standard deviations were calculated and the significance of the variations was confirmed by the Student's *t*-test, set for two-tailed paired analysis at indicated confidence levels.

Results

The *dreb1a* T-DNA line is a null mutant, mutated at a single locus

The T-DNA of the *dreb1a* line is inserted at position -253, instead of predicted position -302, in the *DREB1A* promoter (Fig. 1A); and the genomic sequences flanking the T-DNA are intact (Fig. 1B). The amplified fragments from both sides of the T-DNA insertion contained a similar left border T-DNA sequence; this suggests an unknown DNA rearrangement in the T-DNA (Fig. 1C). In addition, segregation by the selectable marker - sulfadiazine - in a selfing population (more than 600 seeds/seedlings) of a hemizygous *dreb1a* line revealed a 3:1 ratio (Fig. 1D). This suggests segregation of a single T-DNA locus in its T0 plant population; which corresponded to no expression of the *DREB1A* gene detected in the *dreb1a* seedlings grown in the normal conditions or after cold stress (Fig. 1E). Our data suggest that the *dreb1a* is a null mutant.

dreb1a seedlings are sensitive to salinity but not to the cold stress

The phenotypes of the *dreb1a* seedlings subjected to cold stress were not different from those of the WT-Col0 after treatment with 16 hours of 4 °C or 30 minutes of -20 °C. The expression of the *COR15a* gene - the first downstream gene of the ICE1-DREB1A regulon in the plant response to cold stress - in the *dreb1a* seedlings was comparable to that in the WT-Col0 (Fig. 2A). This indicates that the expression of *COR15a* was not affected by mutation in the *dreb1a* plants, which explains why the *dreb1a* seedlings did not sensitivity to the cold in freezing treatments. When in the normal condition (*i.e.*, no salinity), the germination rate of the *dreb1a* seeds was significantly less (92±2.2) than that of the WT-Col0 seeds (98±0.13; $p \leq 0.01$) (Fig. 2B). As the salinity concentration increased from 0 to 150 mM, the germination rates of the *dreb1a* and WT-Col0 seeds sequentially and significantly declined (Fig. 2B). However, at the 150 mM, there was no difference in the germination rates of the *dreb1a* and WT-Col0 seeds; this concentration can be the toxicity threshold as 100 mM was used in the most salinity studies (Liu *et al.,* 2007; Novillo *et al.,* 2004). These tests demonstrate that *DREB1A* is vital for seed germination of *A. thaliana* in both normal and saline conditions.

The growth of the *dreb1a* and WT-Col0 seedlings (to Stage 1.04, *i.e.* 14 DAP) in different salinity concentrations was also studied (Fig. 2C). Most of the WT-Col0 seedlings in the normal condition produced the second pair of rosette leaves (Stage 1.04). However, by increase in salinity concentrations, the percentage of the WT-Col0 seedlings with the second pair of rosette leaves decreased as far as at the 150 mM, where all the seedlings were developmentally arrested with only green opened cotyledons (Stage 1.0) and none of them could develop rosette leaves (Fig. 2C). On the other hand, the growth of the *dreb1a* seedlings at different concentrations of salinity was more significantly suppressed than that of the WT-Col0 plants (Fig. 2C). At 14 DAP, the survival rate *dreb1a* seedlings grown at 150 mM salinity (12.22%± 0.618) was about 4 times less than that of WT-Col0 (49.76±2.42)(Fig. 2D-E). Again, this highlighted the importance of the *DREB1A* gene in the *A. thaliana* seed germination and seedling development.

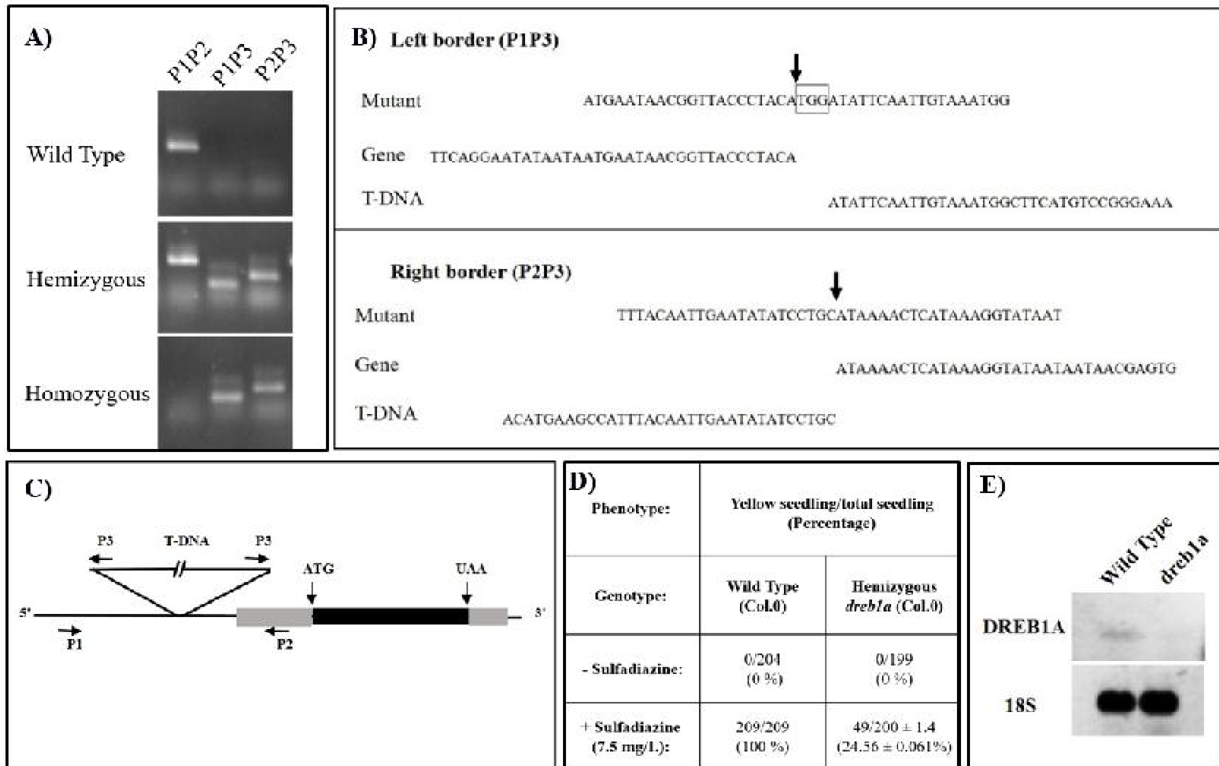

Fig. 1. Molecular identification of the T-DNA insertion in the *dreb1a* mutant: A) PCR amplifications using *DREB1A* gene-specific primers (P1 and P2) and the T-DNA specific primers (P3 and P4); B) The status of the T-DNA junctions in the *dreb1a* plant. Nucleotide sequences of the *dreb1a* plant were aligned with that of the *DREB1A* gene and the T-DNA. Arrows indicate the insertion site. The filler sequences are indicated in the box; C) Schematic diagram of T-DNA position in the promoter of *DREB1A* gene; P, primers used in PCR; Dark Box, coding region; Grey Boxes, untranslated regions (UTRs); ATG, start codon; UAA, stop codon; D) The segregation pattern of sulfadiazine selectable marker in seeds obtained from self-pollination of a hemizygous *dreb1*a plant. E) The transcript level of *DREB1A* gene in wild type and *dreb1a* seedlings exposed to 4 °C for 2h. 18S rRNA served as an internal control.

DREB1A expression alters roots growth

The average root length of the WT-Col0 seedling was 25.03+1.9 mm at the 20 days after planting (Fig. 3A); however, the root of the *dreb1a* seedling grew significantly longer than that of the WT-Col0 seedling (Fig. 3B). The average root length of the WT-WS2 seedlings was 19.46+4.53 mm, slightly shorter than that of the WT-Col0 seedlings (Fig. 3A) at 20 DAP; whereas the root of the OX28 seedlings grew significantly shorter than those of the WT-WS2 seedling (Fig. 3B). Root development and growth is regulated by intrinsic and external (environmental) responses that determine the architecture of the roots and coordinate root development with environmental signals (Malamy 2005). It is interesting to see the opposite effects of null mutation and overexpression of the *DREB1A* gene in the root development.

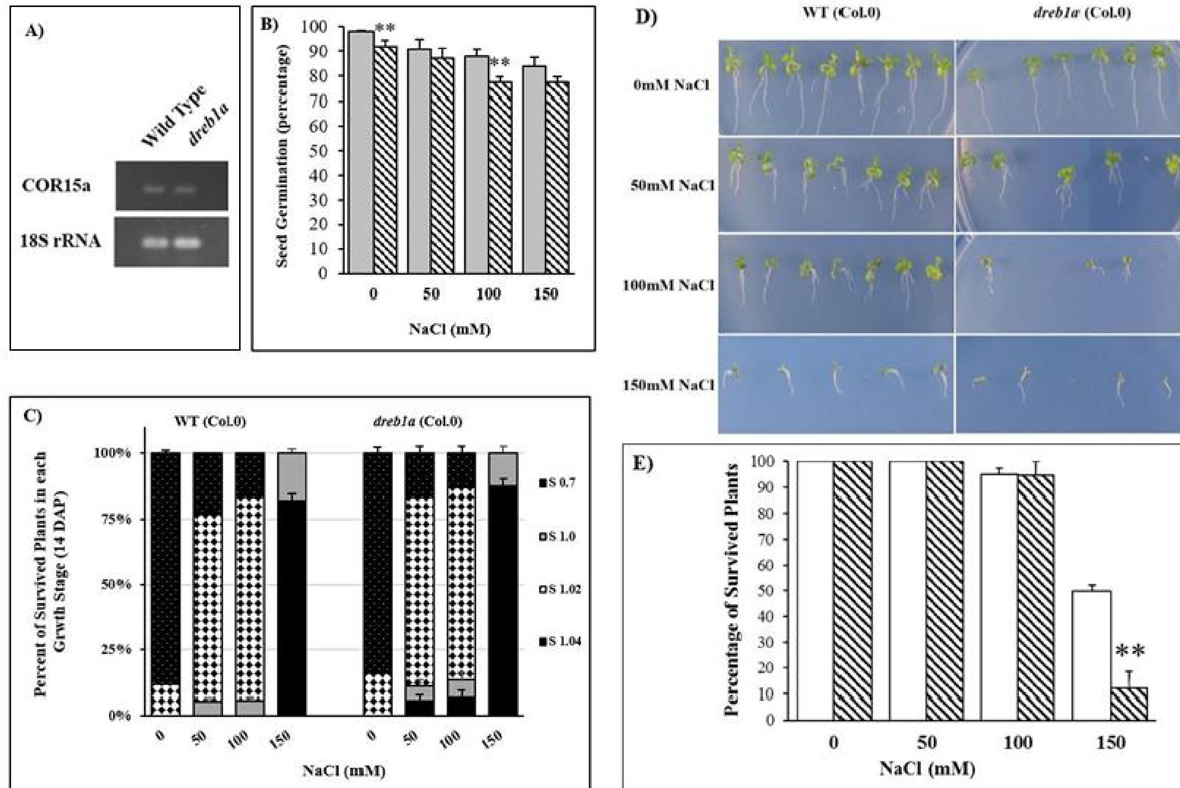

Fig. 2. The sensitivity of the *dreb1a* plants to cold and salinity: A) The transcript level of the *COR15a* gene in WT-Col.0 and *dreb1a* plants exposed to 4 °C for 2h. 18S rRNA served as an internal control; B-E) WT-Col.0 and *dreb1a* seeds were sown on MS medium containing either 0, 50, 100 or 150 mM NaCl and analyzed phenotypically up to the 14th day after sowing; B) Germination percentage of WT-Col.0 and *dreb1a* seeds 7 days after sowing; C) Percentage of WT-Col.0 and *dreb1a* seedlings in each growth stages 14 days after sowing; D) Representative WT-Col.0 and *dreb1a* plant 14 days after sowing on different NaCl concentrations; E) Percentage of survived plant 14 days after sowing on different NaCl concentrations. Bars indicate standard deviations of replicates determined by one-way ANOVA ($P < 0.05$), confirmed by *t*-test at $P<0.05$ (*), $P<0.01$ (**) or $P<0.001$ (***).

Less ROS Accumulation but Increased ROS Scavenger Enzymes in *dreb1a* Mutant Roots

It is well documented that ROS play a central role in the controlling of the root growth in *A. thaliana* (Dunand *et al.,* 2007; Liszkay *et al.,* 2004). The *dreb1a* roots accumulated significantly less reactive oxygen species (ROS) than the WT-Col0; on the other hand, the OX28 roots accumulated significantly more ROS than the WT-WS2 roots, almost a two-fold increase (Fig. 3C-D). Perhaps the aforementioned findings imply that the abundance of ROS accumulation in the OX28 root abolished/dwarfed its growth while the less ROS accumulation in the *dreb1a* root enhanced its growth (Fig. 3B, D). To test this possibility, the activities of several antioxidant enzymes were measured. The *dreb1a* plant possessed significantly higher activities in SOD, peroxidase, polyphenol oxidase while significantly lower activity in catalase than WT-Col0 (Fig. 4A-C, F). There were no enzyme activity differences in extracellular peroxidase and ascorbic peroxidase (Fig. 4D-E) between the two examined plant lines. On the other hand, the OX28 plant possessed a higher extracellular peroxidase activity. The null mutation and overexpression of *DREB1A* in the *dreb1a* plant and OX28 plant possessed a significantly higher activity of peroxidases however only the latter had a higher activity of extracellular peroxidases (Fig. 4B, D).

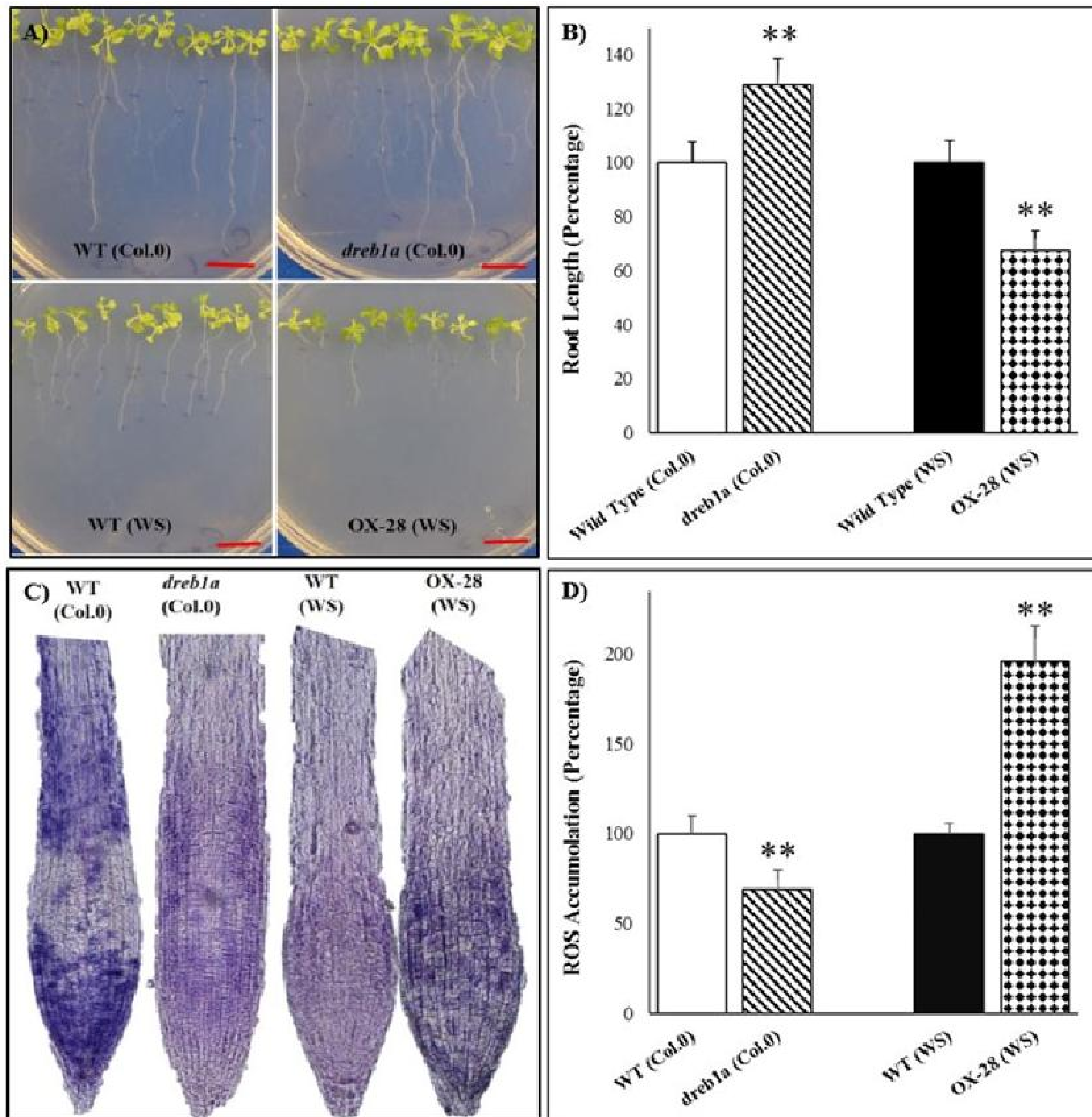

Fig. 3. Root growth and H_2O_2 accumulation analysis in *dreb1a* and OX-28 plants: A) Representative *dreb1a* and OX-28 plants alongside their control wild types 20 days after planting on the MS medium, Bar=1 cm; B) Comparison of root length 20 days after planting on the MS medium; C) NBT staining (indicating H_2O_2 production sites) of root tips in *dreb1a* and OX-28 plants alongside their control wild types 20 days after planting on the MS medium; D) Comparison of H_2O_2 accumulation at the root tips 20 days after planting on the MS medium; Bars indicate standard deviations of replicates determined by one-way ANOVA ($P < 0.05$), confirmed by *t*-test at $P<0.05$ (*), $P<0.01$ (**) or $P<0.001$ (***).

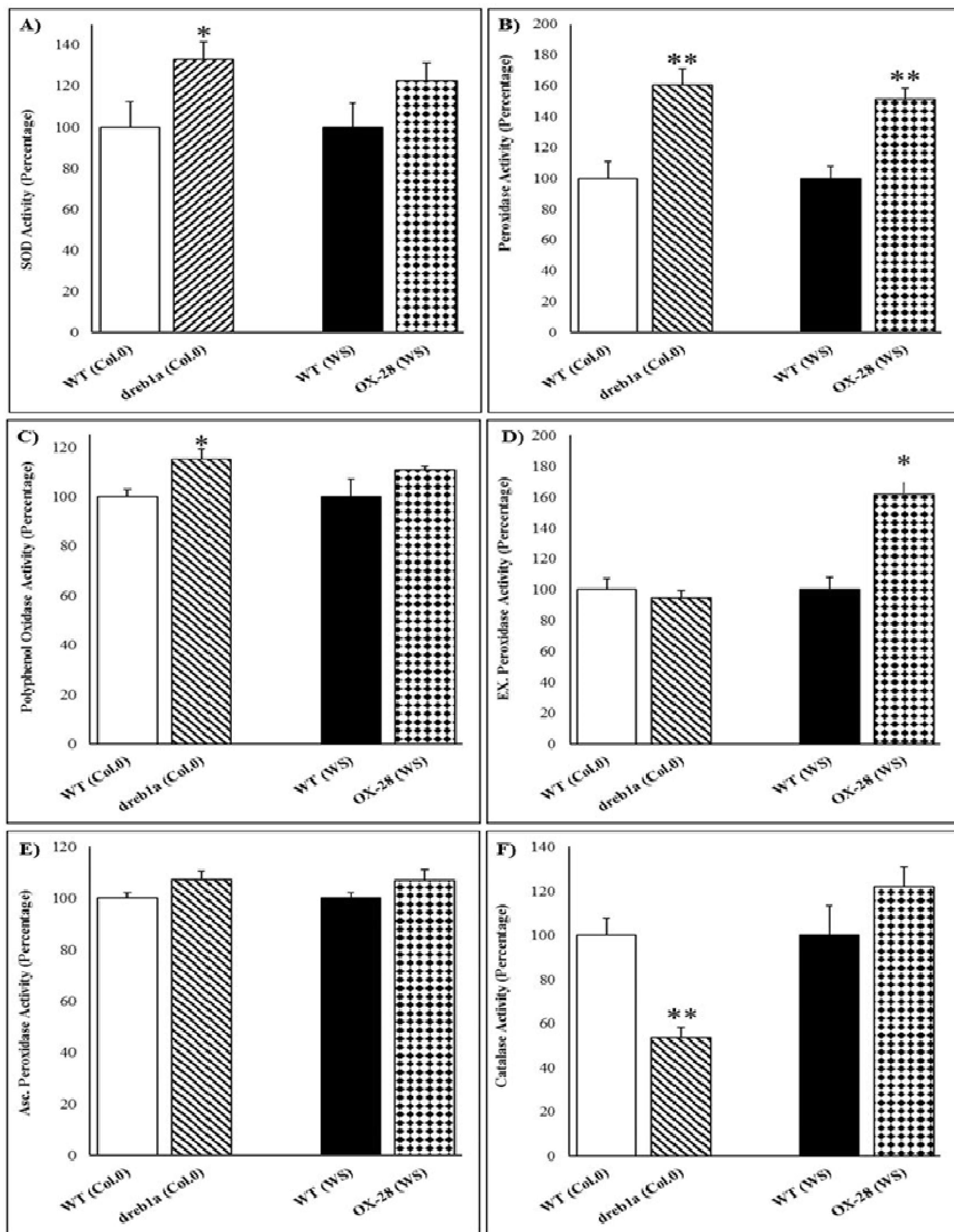

Fig. 4. Antioxidant enzymes activity in *dreb1a* and OX-28 plants, grown on the MS medium under standard growth conditions; Bars indicate standard deviations of replicates determined by one-way ANOVA ($P < 0.05$), confirmed by t-test at $P<0.05$ (*), $P<0.01$ (**) or $P<0.001$ (***).

Discussion

Different responses of the *dreb1a* plants to cold and salinity stresses

The role of DREB1 transcription factors in response to cold and freezing stresses has been confirmed by elevated levels of *DREB1A* expression (Gilmour *et al.,* 2000; Jaglo-Ottosen *et al.,* 1998), and/or by low levels of *DREB1A* expression (Novillo *et al.,* 2004; Novillo *et al.,* 2007; Zhao *et al.,* 2016). However, analysis of the null mutant(s) in *DREB1A* could be more informative to assess its precise role in stress responses. Thus, a *dreb1a* mutant was characterized and was verified as a null mutant of *DREB1A* for downstream studies. The *dreb1a* plants showed no significant difference from the WT-Col0 plants when subjected to cold stress, even when their *COR15a* expression was comparable (Fig. 2A). This suggests that in the absence of a functional *DREB1A* and in response to cold stress, the role of *DREB1A* can be fulfilled/compensated by other members of the DREB1 family, through the shared downstream gene(s) such as *COR15a*. This understanding that DREB1 proteins could be redundant in the regulation of several *COR* genes including *COR15a* has been confirmed by other researchers (Park *et al.,* 2015; Zhao *et al.,* 2016) and *DREB1A*-independent pathways for plant responses to cold stress were proposed. Fowler and Thomashow (2002) report a *DREB1A*-independent pathway which regulates the expression of at least 28 percent of the cold responding genes. *DREB1* genes have prominent roles in plant cold acclimation (Cook *et al.,* 2004; Novillo *et al.,* 2007). In the absence of *DREB1* genes (e.g., triple *cbf* mutants), DREB1-independent pathways could support plant for a proper response to cold stress (Zhao *et al.,* 2016).

Despite no response to cold stress, the *dreb1a* mutants have a strong response to salinity stress leading to lower germination and seedling survival rates. This contrasts to the responses of *dreb1c* (*i.e.*, mutation in *DREB1C*) plants to freezing and salinity; *dreb1c* mutants have a higher tolerance (Novillo *et al.,* 2004). The contrasting phenotypes of *dreb1a* and *dreb1c* may arise from the negative regulatory role of *DREB1C* on *DREB1A* and *DREB1B* (Novillo *et al.,* 2004). Elevated levels of *DREB1A* expression in *dreb1c* plants resulted in salinity tolerance, while *DREB1A* null mutation in *dreb1a* plants (this study) resulted in sensitivity to salinity. Thus, it is possible to conclude that a functional *DREB1A* is necessary for *A. thaliana* plants to respond to salinity stress.

Based on the literature (Agarwal *et al.,* 2006; Huang and Liu 2006; Kasuga *et al.,* 1999; Kim *et al.,* 2008; Kohan-Baghkheirati and Bagherieh-Najjar 2011) and this study (Fig. 2), *DREB1A* could play a crucial role in a complex regulatory network of various abiotic stress responses; but its precise function depends on the stress type and plant developmental stage. In addition, *DREB1A* can perform an indirect inverse role in response to biotic stresses. For example, the expression level of *DREB1A* was decreased in the *ice1* mutant plants, in which SA-dependent genes were up-regulated (Chinnusamy *et al.,* 2003; Rivas-San Vicente and Plasencia 2011). This suggested that a functional *DREB1A* gene may suppress SA-dependent plant responses to biotic stresses. A comprehensive transcriptomic comparison of *dreb1a* and *DREB1A* over-expressing plants under various environmental stresses (abiotic and biotic) can reveal the exact roles of *DREB1A*.

Effect of *DREB1A* on root growth

The *DREB1A* over-expressing plants are dwarf with shorter roots and shoots when grown in the normal condition but they grow better in roots (in terms of root length) under salinity or drought stress (Bhatnagar-Mathur *et al.,* 2007; Cong *et al.,* 2008; Sarkar *et al.,* 2016; Vadez *et al.,* 2012). Similar to the previous studies (Cong *et al.,* 2008; Gilmour *et al.,* 2000), OX28 plants were observed to have dwarf phenotypes, *i.e.*, with shorter root length, in the normal condition. On the other hand, root lengths of *cbf* triple mutants and *cbf2* mutants (*i.e.*, *dreb1c*) were not significantly different from that of wild type plants in the normal condition (Novillo *et al.,* 2004; Zhao *et al.,* 2016). In this study, we showed that the root length of the *dreb1a* plants was significantly longer than that of the WT-Col0 plants; this suggests an inhibitory role of *DREB1A* in root growth in the normal

conditions. Phenotypes of the *dreb1a* roots were different from those of *cbf* mutant roots (Novillo *et al.,* 2004; Zhao *et al.,* 2016); these differences may depend on *DREB1C* (*CBF2*) mutation in those *cbf* mutant lines. *DREB1A* and *DREB1B* perform different functions due to their different expression patterns and possess different effects on different subsets of downstream genes from *DREB1C* does (Novillo *et al.,* 2007). The growth of the *A. thaliana* root follows two stages - first determinate growth with 17-cell proliferation as root primordium and then indeterminate growth after primordia reactivation by forming root apical meristem (Seeley *et al.,* 2000). The inhibitory role of *DREB1A* in root growth is proposed at the second stage, based on two models. The first model is that *DREB1A* might act on the cell wall to strengthen and increase cellular osmolarity in the elongation zone of roots. It was evidenced by up-regulation genes and metabolites (such as *CORs*, Proline, and soluble sugars) by over-expression of *DREB1A,* which led to the accumulation of sugars and increase of cellular osmolarity (Gilmour *et al.,* 2000). Recently, both sugar metabolism and cell wall modification genes were reported to be regulated by *DREB1s* transcription factors; these indicate loosening and extension of cell wall might be affected by *DREB1s* expression levels. Increase of cellular osmolarity and strengthening of cell walls helps efflux of water from the cells and cold stress responses (Afzal *et al.,* 2016; Bilska-Kos *et al.,* 2016); thus, the combination of these three processes (*i.e.*, cellular osmolarity, cell wall strengthen, and efflux of water) inhibits rapid cell wall loosening and extension and prevents root growth in the root elongation zone. It is known that the accumulation of ROS in the root tips can induce rapid loosening of the cell wall (Dunand *et al.,* 2007; Liszkay *et al.,* 2004). Our study showed two-fold accumulation of ROS at the root tips of the OX28 plant but a 30% reduction of ROS at the root tips of the *dreb1a* plant. It is also known that the increase of ROS at the root tip leads to an increase in the root length by fostering a discontinuity of the cell wall polysaccharides (Dunand *et al.,* 2007; Liszkay *et al.,* 2004). Rapid loosening of the cell wall can be triggered by ROS accumulation in the root tips (Dunand *et al.,* 2007; Liszkay *et al.,* 2004). This processes of wall loosening is promoted by extracellular peroxidases through an oxidative cleavage by the hydroxyl radical (•OH) (Daudi *et al.,* 2012; Kelij *et al.,* 2015). Likewise, the accumulation of ROS in the OX28 roots could help cleave cell wall polysaccharides.

The second model is that the negative effects of *DREB1A* on root growth might be mediated by ethylene. Recently, one of the ethylene signaling transcription factors (Ethylene Insensitive 3, *EIN3*) was found to bind the promoter of *DREB1A* and to down-regulate *DREB1A* expression during cold acclimation (Shi *et al.,* 2012). The pattern of *EIN3* expression was reverse to that of *DREB1A* expression under cold stress. During the first three hours of cold treatment, accumulation of *DREB1A* transcripts was high while that of *EIN3* was low. After six hours of cold treatment, *EIN3* transcripts accumulated but *DREB1A* transcripts dropped. This suggests that accumulation of the *DREB1A* transcripts is controlled by both cold and ethylene but in an antagonistic manner (Shi *et al.,* 2015; Shi *et al.,* 2012). Thus, accumulation of the *DREB1A* transcripts in the OX28 roots may trigger accumulation of ethylene and in turn, it may inhibit cell wall elongation at the root tips. In agreement with this scenario, it is predicted that the slow growth rate of roots in the OX28 line can be complemented by silver nanoparticles treatment which inhibits ethylene production and signaling (Kumar *et al.,* 2009). In the future, a detailed study of the *ein3/dreb1a* double mutant under various stress conditions may provide new insights on how *DREB1A* affects root tip growth.

DREB1A expression affects antioxidant enzyme activity

Most of biochemical/physiological changes in altered *DREB1A* gene expression were in sugar metabolism and osmoprotectants (e.g., proline) (Cong *et al.,* 2008; Gilmour *et al.,* 2000; Maruyama *et al.,* 2009; Zhao *et al.,* 2016). Plant responses to abiotic stresses, oxidative response through ROS production and scavenging have been recently investigated in *DREB1A* overexpressing plants (Jia *et al.,* 2016; Rai *et al.,* 2013; Wan *et al.,* 2014). In this study,

differential accumulation of ROS at the root tips of the *dreb1a* and OX28 plants suggests a potential link between *DREB1A* expression levels and ROS regulation. ROS levels in response to stresses are controlled precisely by a complex regulatory network; this network includes enzymatic and non-enzymatic antioxidant defense systems (Ghorbannejad and Amooaghaie 2017; Gill and Tuteja 2010; Jaspers and Kangasjärvi 2010). Our result showed that the enzymatic activity of most members of antioxidants defense systems were affected by altered expression (*i.e.*, either null mutation or overexpression) of the *DREB1A* gene. In both *dreb1a* and OX28 plants, peroxidase and superoxide dismutase showed a significant increase (to reduce ROS). This is in agreement with previous reports that activities of peroxidase and superoxide dismutase showed significant increase in *DREB1A* overexpressing plants and improved plant resistance to abiotic stresses (Jia *et al.,* 2016; Li. X. *et al.,* 2011; Rai *et al.,* 2013; Wan *et al.,* 2014). Polyphenol oxidase is involved in oxygen chemistry and oxidizing phenolic compounds (Mayer 2006; Vaughn and Duke 1984); but its activity increased only in the *dreb1a* plants. Extracellular peroxidase contributes to superoxide (O_2^-) production for cell wall extension (Mika *et al.,* 2004); and its activity increased only in the OX28 plants.

Elevated levels of polyphenol oxidase activity, by oxidizing some cell wall compounds, may result in in the phenotype of longer roots in the *dreb1a* plants while elevated levels of the extracellular peroxidase activity, by producing superoxide for cell wall extension, may result in the phenotype of shorter roots in the OX28 plants. However, transcriptomic analysis of *DREB1s* over-expressing plants (Park *et al.,* 2015) and/or *DREB1s* mutant plants (Zhao *et al.,* 2016) didn't revealed enrichment of antioxidant enzyme-coding genes. Despite the changes of antioxidant enzyme activities in altered *DREB1A* expression plants (OX28 and *dreb1a*) and the transcriptomic studies, the molecular mechanisms of antioxidant enzymes are yet to be understood (Jia *et al.,* 2016; Li. X. *et al.,* 2011; Rai *et al.,* 2013; Wan *et al.,* 2014). In sum, there is an indirect, but important, link between *DREB1A* expression levels and ROS generating/scavenging systems in modulating root elongation enzymatic activities of the *dreb1a* and OX28 plants. This link regulates tolerance of *A. thaliana* against abiotic stresses.

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