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

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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 insertion 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/CFB 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/CFB family members which lie in tandem on chromosome 4 in the order of DREB1B/CFB1, DREB1A/CFB3 and DREB1C/CFB2 (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 insertion 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 ddH2O 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 promoter 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.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5'-3')</th>
<th>Product Size (bp)</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>DREB1A-Promoter</td>
<td>P1: TTTCCGCCAAAAACTACCTTTG</td>
<td>486</td>
<td>Mutant Genotyping</td>
</tr>
<tr>
<td></td>
<td>P2: CAAAAGAAAAAGCCGAAGACG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-DNA</td>
<td>P3: ATATTGACCACCATCAGTTTGC</td>
<td>-</td>
<td>Mutant Genotyping</td>
</tr>
<tr>
<td></td>
<td>P4: GTGGATTGTGTGATATCTCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DREB1A-exon</td>
<td>F: GATGACGACGTATCGTTATGGA</td>
<td>144</td>
<td>RT-PCR</td>
</tr>
<tr>
<td></td>
<td>R: TACACTCGTCTCTCAGTTTACAAAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COR15a</td>
<td>F: GCTTCAGATTTGCTGACGGATACCC</td>
<td>333</td>
<td>RT-PCR</td>
</tr>
<tr>
<td></td>
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<td></td>
</tr>
<tr>
<td>18S rRNA</td>
<td>F: ATGATAACTCAGGGATCGCA</td>
<td>169</td>
<td>RT-PCR</td>
</tr>
<tr>
<td></td>
<td>R: CTGGGATGTTGGTACGTTTC</td>
<td></td>
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</tr>
</tbody>
</table>

**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 (dN6) 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 %
Ros et al., analyzed in the ImageJ Java version in Olympus BX51 microscope. The blue color Olympus DP71 camera connected to an blue colors occurred) were taken images from an transpired to slides and their roots (where deep methods. After NBT staining, seedlings were transferred to slides and their roots (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 (NaH2PO4, 2H2O 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 ddH2O 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 H2O2, 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 H2O2, 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 H2O2; 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
The phenotypes of the dreb1a mutant. Our data indicate confidence levels. 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≤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.

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≤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.
**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$_2$O$_2$ accumulation analysis in drebla and OX-28 plants. A) Representative drebla 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$_2$O$_2$ production sites) of root tips in drebla and OX-28 plants alongside their control wild types 20 days after planting on the MS medium. D) Comparison of H$_2$O$_2$ 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-Co10 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-Co10 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; Bilskas-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 drebl1a 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 drebl1a 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 drebl1a 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 drebl1a 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 drebl1a) 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 drebl1a and OX28 plants. This link regulates tolerance of A. thaliana against abiotic stresses.

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