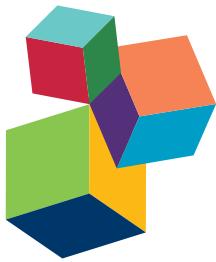


# ROS REGULATION DURING PLANT ABIOTIC STRESS RESPONSES

EDITED BY: Zhulong Chan, Chun-Peng Song, Woe Yeon Kim and Ken Yokawa  
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# ROS REGULATION DURING PLANT ABIOTIC STRESS RESPONSES

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Plants are continuously exposed to a wide range of environmental conditions, including cold, drought, salt, heat, which have major impact on plant growth and development. To survive, plants have evolved complex physiological and biochemical adaptations to cope with a variety of adverse environmental stresses. Among them, reactive oxygen species (ROS) are key regulators and play pivotal roles during plant stress responses, which are thought to function as early signals during plant abiotic stress responses. ROS were long regarded as unwanted and toxic by-products of physiological metabolism. However, ROS are now recognized as central players in the complex signaling network of cells. Therefore, a fine-tuning control between ROS production and scavenging pathways is essential to maintain non-toxic levels in planta under stressful conditions through enzymatic and non-enzymatic antioxidant defense systems.

We focus on the roles of ROS during plant abiotic stress responses in this Research Topic. Plant responses to multiple abiotic stresses and effects of hormones and chemicals on plant stress responses have been carefully studies. Although functions of several stress responsive genes have been characterized and possible interactions between hormones and ROS are discussed, future researches are needed to functionally characterize ROS regulatory and signaling transduction pathways.

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# Editorial: ROS Regulation during Plant Abiotic Stress Responses

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**Keywords:** abiotic stress, hormones, reactive oxygen species, redox

## The Editorial on the Research Topic

### ROS Regulation during Plant Abiotic Stress Responses

Plants frequently encounter a combination of abiotic stresses in their natural habitats. Abiotic stresses, including drought, salt, cold, heat, and heavy metal etc., modulate phytohormone metabolism and enhance expression level of transcription factors which activate stress responsive genes. During plant stress response, reactive oxygen species (ROS) act as important molecules and play pivotal roles in activating downstream metabolic pathways. In this Research Topic, we collected 25 manuscripts related to ROS and redox regulation in plant responses to abiotic stress, including reviews of the role of ROS in plant abiotic stress responses and articles related to gene function analysis, genome-wide gene expression and transcriptomic analysis, and interaction analysis between ROS and phytohormones.

Krieger-Liszka and Feilke reviewed how plastid terminal oxidase (PTOX) interplays with the photosynthetic electron flow and hypothesized that the function of PTOX is dependent of stromal pH. Corpas and Barroso briefly summarized possible roles of reactive sulfur species (RSS) in peroxisomes and hypothesized potential interactions among ROS, reactive nitrogen species (RNS) and sulfur-containing compounds. In crop plants, ROS regulation under abiotic stress condition has been reviewed by You and Chan. In response to abiotic stress, plants have evolved complex signaling pathways to regulate sets of stress responsive genes encoding protein kinases, phosphatases, transcriptional factors, SIMILAR TO RCD ONE (SRO) proteins, ROS-scavenging or detoxification proteins, and proteins involved in hormone pathway and calcium signal (You and Chan). Sewelam et al. summarized that ROS functions as the primary source of the signaling battery in plants under stressed conditions and may interact with other signaling components, e.g., calcium, redox homeostasis, membranes, G-proteins, MAPKs, plant hormones, and transcription factors. Sewelam et al. discussed the interaction between nitric oxide (NO) and ROS which might regulate abscisic acid (ABA) biosynthesis to modulate stomatal closure. Liu et al. reviewed the functions of polyamines (PAs) during plant stress response through modulation of antioxidant systems or suppression of ROS production. Evidences showed that PA catabolism resulted in the production of H<sub>2</sub>O<sub>2</sub>. Exogenous supply of PAs or ectopic expression of PAs biosynthesis related genes increased antioxidant system in several plants (Liu et al.). Dinakar et al. demonstrated the importance of AOX pathway in optimizing photosynthesis in *Pisum sativum* in the presence of osmotic and temperature stress conditions. These articles summarized roles of ROS during plant stress response and possible interaction of ROS with hormones and other chemicals.

Compared to extensive studies on drought, cold, heat, and osmotic stresses, less attention has been paid to heavy metal stress and light stress, which become increasingly important stress factors

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limiting plant growth. Three research articles addressed aluminum (Al), cadmium (Cd) and UV-B stress responses, respectively. Lin et al. observed that  $\text{Al}^{3+}$  stress induced  $\text{O}_2^{\cdot}$  generation in the cell suspension cultures of tobacco and rice, while pretreatment with various concentration of  $\text{Zn}^{2+}$  significantly inhibited the  $\text{Al}^{3+}$ -induced oxidative burst. Moreover,  $\text{Al}^{3+}$ -induced cell death was also inhibited in the presence of  $\text{Zn}^{2+}$ . High concentration of zinc (0.5 mM) effectively lowered the level of  $\text{Al}^{3+}$ -induced  $[\text{Ca}^{2+}]_c$  elevation. In tomato, cadmium (Cd) stress significantly inhibited activities of superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD), while increased the contents of  $\text{H}_2\text{O}_2$  and  $\text{O}_2^{\cdot}$ , resulted in increased malondialdehyde (MDA) and electrolyte leakage (EL) (Hasan et al.). Yokawa et al. found that UV-B promoted the robust generation of ROS which affects endocytic vesicle recycling in *Arabidopsis* root apex. It is well known that many root tropisms require elaborate control of endocytic vesicle recycling in the cells. This finding explains that how light stress situation triggers root negative phototropism through ROS production as a rapid signaling event.

Omic approaches were effectively applied to identify genes involved in plant stress responses. de Abreu Neto and Frei conducted a meta-analysis of microarray experiments in rice. Publicly available microarray transcriptome data were re-analyzed. The results showed that ROS-related genes were overrepresented among the differentially expressed genes (DEGs). After treatments with oxidative stress (ozone and  $\text{H}_2\text{O}_2$ ) and abiotic stresses, 990 and 1727 shared DEGs were identified, respectively. Among them, 311 genes were overlapped by both oxidative and abiotic stresses and 33 were ROS-related genes. Additionally, Mata-Pérez et al. identified genes in response to linolenic acid, a precursor of jasmonic acid (JA) using RNA-seq approach. In total, expression levels of 3034 genes were changed after linolenic acid treatment. This study showed that linolenic acid modulated the expression of genes involved in stress response, particularly those mediated by ROS signaling. Several transcription factors including WRKY, JAZ, MYC were also modified in response to linolenic acid. These data indicated that abiotic stress modulated expression of stress responsive genes as well as ROS related genes. Wei et al. identified 85 WRKY genes in cassava (*Manihot esculenta*) through bioinformatics analysis. RNA-seq data showed that 78 *MeWRKY* genes were differentially expressed in response to drought stress and 9 *MeWRKY* genes were modulated after NaCl, mannitol, cold,  $\text{H}_2\text{O}_2$  and ABA treatments, indicating that *MeWRKY* genes were involved in plant stress response and redox signaling pathway. These analyses provided new clues for identification of genes involved in oxidative stress.

Detailed functions of several stress responsive genes including transcription factors have been characterized. The Universal Stress Protein domain (USP) gene modulated plant response to a wide variety of abiotic stresses. The biochemical function of *AtUSP* (*At3g53990*) was characterized by Jung et al. The results showed that *AtUSP*-OX plants were tolerant to heat shock and oxidative stresses, whereas the knock-out mutants were sensitive to the stress treatments. *AtUSP* exhibited a redox-dependent chaperone function which might contribute

to its protective roles during diverse stress conditions. The *Arabidopsis* sulfotransferase gene *AtSOT12* is a salt inducible gene through transcriptome analysis. Chen et al. found that salt stress affected capping and polyadenylation of *AtSOT12*, but not DNA methylation level in the promoter region. Expression of *AtSOT12* was induced by salt stress is partially through ABA-INSENSITIVE 1 (ABI1)—and SALT OVERLY SENSITIVE 1 (SOS1)-mediated signaling pathways. Mutation of oxidative stress related *oxi1* resulted in increased *AtSOT12* expression, while ROS scavenger treatments also enhanced *AtSOT12* transcript level, indicating that ROS production might be involved in the repression of *AtSOT12* gene. Baek et al. isolated an *Arabidopsis* *ars1* (*aba* and *ros* sensitive 1) mutant which showed hypersensitivity to ABA and methyl viologen (MV). *ARS1* encodes a nuclear protein with one zinc finger domain. Expression level of *CSD3* gene encoding SOD was reduced and ROS was accumulated in *ars1* mutant. Furthermore, *ARS1* inhibited ABA-induced ROS production. Treatment with ABA,  $\text{H}_2\text{O}_2$  and MV modulated localization of *ARS1* protein. Wang et al. assembled 10 WRKY unigenes from ESTs of wheat (*Triticum aestivum*). Among them, *TaWRKY44* was upregulated by various stress treatments, hormones, and  $\text{H}_2\text{O}_2$ . *TaWRKY44* localizes to the nucleus and binds to the core DNA sequences of TTGACC and TTAACC in yeast. *TaWRKY44* transgenic tobacco plants showed increased drought and salt tolerance. Under osmotic stress condition, transgenic lines exhibited lower  $\text{H}_2\text{O}_2$  content and higher SOD, CAT, and POD activities. Overexpression of *TaWRKY44* increased expression of several ROS related genes and stress-responsive genes. The results indicated that several transcription factors might function as ROS upstream regulators.

In response to environmental stresses, plants develop various strategies, including induction of phytohormones. Among the, auxin regulates plant growth and development. *YUCCA6*, a flavin monooxygenase enzyme, converts indole-3-pyruvic acid to auxin. Cha et al. reported that overexpression of *YUCCA6* in *Arabidopsis* reduced the expression of senescence related gene *SAG12* and delayed leaf senescence. *YUCCA6*-OX plants, but not mutated *YUCCA6*-OX<sup>C85S</sup>, a dysfunctional mutation of ROS homeostasis maintained by *YUCCA6*, exhibited reduced ROS accumulation and increased expression of genes encoding NADPH-dependent thioredoxin reductases and *GSH1* involved in redox signaling. Moreover, auxin efflux proteins at both transcriptional and protein level were reduced either by ROS balance or by thiol-reductase activity of *YUCCA6*. Additionally, overexpression of the cytokinin biosynthetic gene *AtIPT8* (adenosine phosphate-isopentenyltransferase 8) in *Arabidopsis thaliana* resulted in increased endogenous cytokinin content (Wang et al.). *AtIPT8* transgenic lines showed increase sensitivity to salt stress and accumulated higher ROS content than the wild type control. Moreover, many genes involving in photosynthesis and abiotic stress responses were differentially expressed in *AtIPT8* transgenic lines (Wang et al.). Therefore, phytohormones affected plant stress responses partially through modulation of ROS levels.

Exogenous application of hormones and chemicals increased plant tolerance to various abiotic stresses. Treatment with spermidine promoted the growth recovery of rice after drainage.

Spermidine treatment decreased ROS generation and improved photosynthesis in submerged rice. Addition of polyamine (PA) alleviated the suppressing effects of osmotic stress in leaves of white clover. Further study showed that PA was involved in regulation of H<sub>2</sub>O<sub>2</sub> and Ca<sup>2+</sup> messenger. PA-induced H<sub>2</sub>O<sub>2</sub> production required Ca<sup>2+</sup> release, while PA-induced Ca<sup>2+</sup> release was also essential for H<sub>2</sub>O<sub>2</sub> production (Li et al.). Pretreatment with spermidine in rice improved submergence tolerance through modulation of ROS production and chlorophyll degradation (Liu et al.). Xu et al. found that exogenous ascorbic acid treatment improved root growth in tall fescue (*Festuca arundinacea*) under water stress condition. Roots in ascorbic acid (ASA)-treated plants had lower ROS and MDA contents, higher non-enzymatic antioxidant accumulation, and increased expression of genes encoding cell-wall loosening proteins. Melatonin (N-acetyl-5-methoxytryptamine) has long been known to be an important animal hormone and identified in various plant species since 1995. In tomato (*Solanum lycopersicum*), cadmium (Cd) stress significantly increased the contents of Cd and melatonin (Hasan et al.). Exogenous application of melatonin increased activities of antioxidant enzymes and H<sup>+</sup>-ATPase, and contents of glutathione (GSH) and phytochelatins. Supplementation with melatonin significantly reduced leaf Cd accumulation. In common wheat (*Triticum aestivum*), application of ABA caused decreased contents of H<sub>2</sub>O<sub>2</sub> and MDA and increased GSH and ASA under osmotic stress condition (Wei et al.). Gene expression analysis showed that ABA treatment regulated transcripts of genes encoding ASA and GSH synthesis-related enzymes. The results shed lights on exogenous application of chemicals to improve plant stress tolerance.

Under abiotic stress condition, metabolites like dehydrin and proline appear to function in stress tolerance by serving as a compatible solute or osmoprotectant. Dehydrin belongs to group II late embryogenesis abundant protein (LEA). Shi et al. characterized functions of *Arabidopsis LOW TEMPERATURE-INDUCED 30* (*LTI30*), encoding a LEA protein, under drought stress condition. *AtLTI30* knockout mutant was less sensitive to ABA and displayed decreased

drought tolerance, whereas *AtLTI30-OX* plants were more sensitive to ABA and showed improved drought tolerance. Manipulation of *AtLTI30* expression increased activities of CAT and decreased drought stress-triggered H<sub>2</sub>O<sub>2</sub> production. In trifoliolate orange (*Poncirus trifoliata*), Peng et al. cloned a hybrid proline-rich protein gene *PtrPRP*. Expression level of *PtrPRP* was progressively induced upon cold stress treatment. *PtrPRP* knock-down lines displayed sensitivity to cold stress as evidenced by higher EL, MDA content, and increased accumulation of ROS. In aged oat seed, Kong et al. suggested that proline and antioxidant enzymes played the main role in adaptation to oxidative stress in seeds with higher (28%) and lower (4%, 16%) moisture contents, respectively. These studies highlighted the protective roles of osmoprotectants and putative functions in modulating of ROS and redox pathways.

In summary, we focus on the roles of ROS during plant abiotic stress responses in this Research Topic. Plant responses to multiple abiotic stresses and effects of hormones and chemicals on plant stress responses have been carefully studies. Although functions of several stress responsive genes have been characterized and possible interactions between hormones and ROS are discussed, future researches are needed to functionally characterize ROS regulatory and signaling transduction pathways.

## AUTHOR CONTRIBUTIONS

ZC and CS wrote the manuscript, KY and WK added notes and revised the manuscript.

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# The Thiol Reductase Activity of YUCCA6 Mediates Delayed Leaf Senescence by Regulating Genes Involved in Auxin Redistribution

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Auxin, a phytohormone that affects almost every aspect of plant growth and development, is biosynthesized from tryptophan via the tryptamine, indole-3-acetamide, indole-3-pyruvic acid, and indole-3-acetaldoxime pathways. YUCCAs (YUCs), flavin monooxygenase enzymes, catalyze the conversion of indole-3-pyruvic acid (IPA) to the auxin (indole acetic acid). *Arabidopsis thaliana* YUC6 also exhibits thiol-reductase and chaperone activity *in vitro*; these activities require the highly conserved Cys-85 and are essential for scavenging of toxic reactive oxygen species (ROS) in the drought tolerance response. Here, we examined whether the YUC6 thiol reductase activity also participates in the delay in senescence observed in YUC6-overexpressing (YUC6-OX) plants. YUC6 overexpression delays leaf senescence in natural and dark-induced senescence conditions by reducing the expression of SENESCENCE-ASSOCIATED GENE 12 (SAG12). ROS accumulation normally occurs during senescence, but was not observed in the leaves of YUC6-OX plants; however, ROS accumulation was observed in YUC6-OX<sup>C85S</sup> plants, which overexpress a mutant YUC6 that lacks thiol reductase activity. We also found that YUC6-OX plants, but not YUC6-OX<sup>C85S</sup> plants, show upregulation of three genes encoding NADPH-dependent thioredoxin reductases (*NTRA*, *NTRB*, and *NTRC*), and GAMMA-GLUTAMYL CYSTEINE SYNTHETASE 1 (*GSH1*), encoding an enzyme involved in redox signaling. We further determined that excess ROS accumulation caused by methyl viologen treatment or decreased glutathione levels caused by buthionine sulfoximine treatment can decrease the levels of auxin efflux proteins such as PIN2-4. The expression of *PINs* is also reduced in YUC6-OX plants. These findings suggest that the thiol reductase activity of YUC6 may play an essential role in delaying senescence via the activation of genes involved in redox signaling and auxin availability.

**Keywords:** auxin, reactive oxygen species, redox signaling, senescence, thiol reductase

## INTRODUCTION

Plants undergo senescence to mobilize nutrients and remove unneeded organs. Senescent leaves break down chlorophyll and degrade macromolecules for nutrient translocation; the reactive oxygen species (ROS)-detoxifying system also breaks down. Various phytohormones and environmental conditions interact to regulate senescence (Lim et al., 2007). Exogenous application of ethylene, abscisic acid (ABA), or jasmonic acid (JA) triggers leaf senescence (Grbić and Bleeker, 1995; Weaver et al., 1998; He et al., 2002). Endogenous salicylic acid (SA) also increases in senescent leaves (Khan et al., 2014). By contrast, treatment with cytokinins or auxin delays senescence (Noodén et al., 1990; Kim et al., 2011). In addition, detoxification systems such as antioxidant enzymes delay senescence (Ye et al., 2000; Lim et al., 2007). The developmental shift to senescence in plants is determined by the induction of Senescence-Associated Genes (SAGs), which are differentially expressed in response to treatment with phytohormones associated with senescence (Weaver et al., 1998; Gepstein et al., 2003).

Auxin regulates diverse aspects of plant growth and development, including apical dominance, tropisms, root, and shoot development, vascular differentiation, and embryo patterning (Vanneste and Friml, 2009). Auxin is synthesized via four distinct tryptophan (Trp)-dependent pathways: the tryptamine (TAM), indole-3-acetamide (IAM), indole-3-pyruvic acid (IPA), and indole-3-acetaldoxime (IAOx) pathways (Quittenden et al., 2009; Zhao, 2012). YUCCA (YUC) proteins, which are members of the plant flavin monooxygenase (FMO) family, catalyze the conversion of IPA to indole acetic acid (IAA) via the action of Trp aminotransferase (TAA1/TAR1/TAR2; Stepanova et al., 2011; Dai et al., 2013). We recently demonstrated that, in addition to its FMO function, YUC6 also acts as a thiol-reductase (TR) involved in auxin biosynthesis and ROS homeostasis (Cha et al., 2015). Independent of the auxin biosynthesis activity of YUC6, its TR activity reduces ROS induction under oxidative and drought stress, thereby increasing stress tolerance. In addition, a cysteine residue (Cys85, based on the YUC6 sequence) that is highly conserved in 11 *Arabidopsis* YUC proteins is essential for TR activity and ROS regulation both *in vitro* and *in vivo*. TR proteins, including NADPH-dependent thioredoxin reductases (NTRs), function as redox proteins involved in the disulfide reduction via dithiol- or monothiol-related mechanisms (Arnér and Holmgren, 2000; Jacquot et al., 2009). Bashandy et al. (2010) demonstrated that mutants affecting NTR (*ntra ntrb*) or glutathione biosynthesis (*cad2*) exhibit disturbed auxin signaling, and *ntra ntrb cad2* triple mutant plants exhibit developmental defects caused by reduced auxin levels and transport. The *Arabidopsis* NADPH-dependent thioredoxin (NTS) and NADPH-dependent glutathione systems (NGS) interact. Recent studies suggest that developmental defects caused by the inhibition of NTS and NGS are due to reduced auxin levels and reduced auxin transport.

Overexpression of *YUC6* in plants delays leaf senescence by increasing auxin levels (Kim et al., 2011). In the current study, we investigated whether delayed leaf senescence caused by *YUC6* overexpression also requires the TR activity of *YUC6*.

Interestingly, reducing TR activity by mutating Cys85 in *YUC6* abolished the delayed leaf senescence of *YUC6*-overexpressing plants by increasing ROS accumulation in senescing leaves. We also found that elevated ROS levels trigger the reduced expression of auxin transporter genes, such as *PINs*, in a TR activity-dependent manner. Therefore, our results suggest that *YUC6* mediates delayed leaf senescence by regulating ROS homeostasis and auxin transporters via its TR activity.

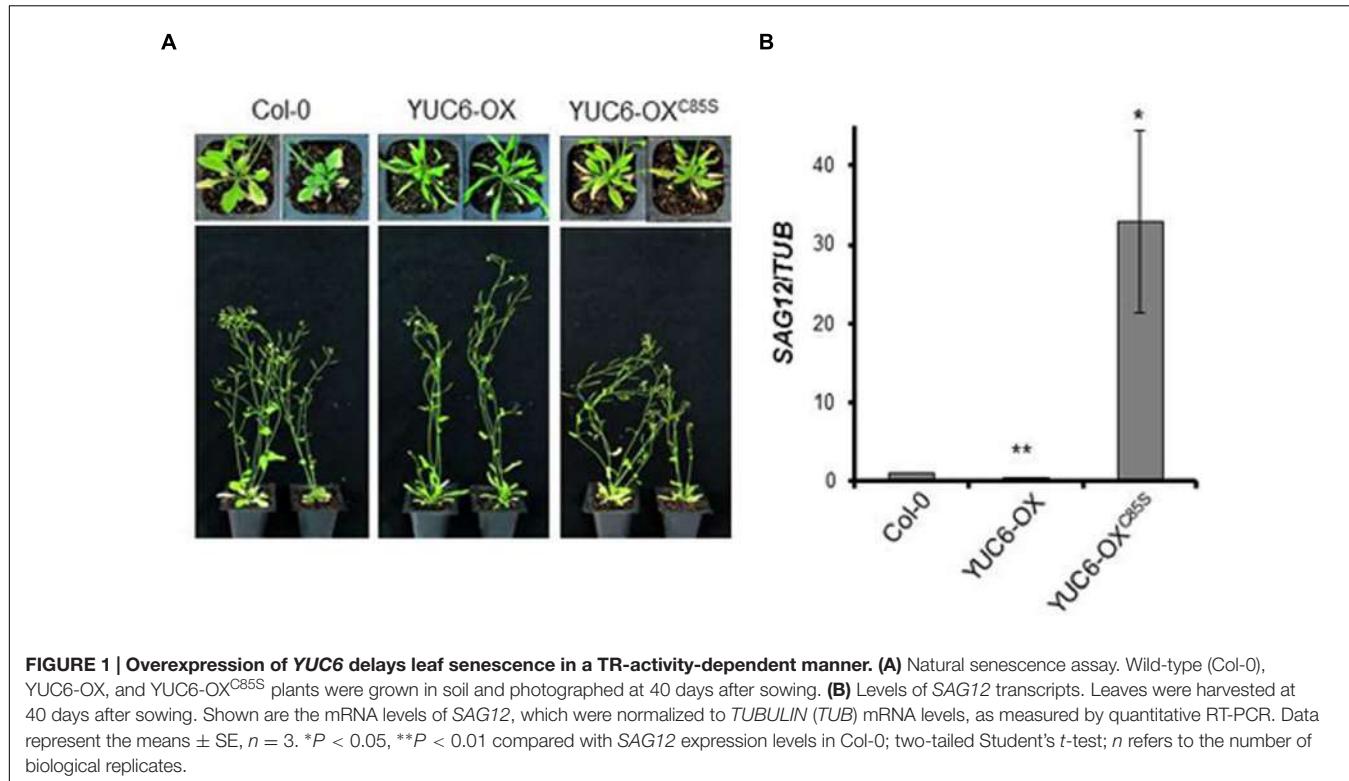
## RESULTS

### The Delay of Natural and Dark-Induced Senescence Requires the Thiol Reductase Activity of YUC6

We previously showed that plants overexpressing *YUC6*, carrying the dominant mutant *yuc6-1D*, or transgenic for a 35S:*YUC6* construct all exhibit delayed leaf senescence, along with extreme longevity (Kim et al., 2011). The delayed senescence in response to *YUC6* overexpression is accompanied by elevated auxin levels. Recently, we also determined that *YUC6* plays a dual role in regulating plant development and reducing stress responses via its FMO and TR activities, respectively (Cha et al., 2015). Therefore, in the present study, we used *YUC6-OX<sup>C85S</sup>* plants to investigate whether the TR activity of *YUC6* is also necessary for delaying senescence. The *YUC6-OX<sup>C85S</sup>* plants overexpress *YUC6* and have a mutation in a conserved cysteine residue (Cys-85); this mutation suppresses the TR activity of *YUC6*, but does not affect its FMO activity *in vitro*. In addition, the overexpressed *YUC6* protein levels and auxin amounts were not significantly different between *YUC6-OX* and *YUC6-OX<sup>C85S</sup>* plants (Cha et al., 2015).

We first examined the *YUC6-OX* and *YUC6-OX<sup>C85S</sup>* plants under natural senescence conditions. At 40 days after sowing, *YUC6-OX* plants had fewer senescent leaves than wild-type (Col-0) plants, which is consistent with a previous report (Kim et al., 2011; **Figure 1A**). However, *YUC6-OX<sup>C85S</sup>* and wild-type plants had more senescent leaves than *YUC6-OX* plants. To confirm that the delayed leaf senescence phenotype was caused by *YUC6* overexpression at the molecular level, we examined the expression of a representative downstream gene, *SENESCENCE-ASSOCIATED GENE 12* (*SAG12*), which is upregulated during senescence. As shown in **Figure 1B**, *SAG12* transcript levels were significantly lower in *YUC6-OX* than in wild-type and *YUC6-OX<sup>C85S</sup>* plants (**Figure 1B**).

To further investigate the importance of the TR activity of *YUC6* during leaf senescence, 2-week-old plants were maintained under dark conditions to artificially induce senescence (Kim et al., 2011). At 7 days after dark treatment (7 DAT), leaves of *YUC6-OX* plants were greener than those of the wild type and *YUC6-OX<sup>C85S</sup>*, whereas *YUC6-OX<sup>C85S</sup>* leaves were either light green or brownish in color, indicating that they were undergoing senescence (**Figure 2A**). We measured the chlorophyll contents in the leaves to monitor the progression of senescence. As shown in **Figure 2B**, chlorophyll contents were significantly higher in *YUC6-OX* plants compared to wild-type and *YUC6-OX<sup>C85S</sup>*



plants, consistent with the phenotypes shown in **Figure 2A**. The leaf senescence phenotypes of dark-treated plant samples were confirmed at the molecular level by measuring the expression of *SAG12*. Consistent with the results under natural senescence conditions, the expression of *SAG12* in YUC6-OX<sup>C85S</sup> plants was significantly higher than in wild-type and YUC6-OX plants under dark-induced senescence (**Figure 2C**). We previously determined that the lack of the TR activity of YUC6 in YUC6-OX<sup>C85S</sup> (due to the mutation of Cys85) does not abolish IAA production *in planta* (Cha et al., 2015). Thus, these data suggest that the TR activity of YUC6 may be also essential for delaying leaf senescence.

## The TR Activity of YUC6 Represses H<sub>2</sub>O<sub>2</sub> Accumulation in Senescence

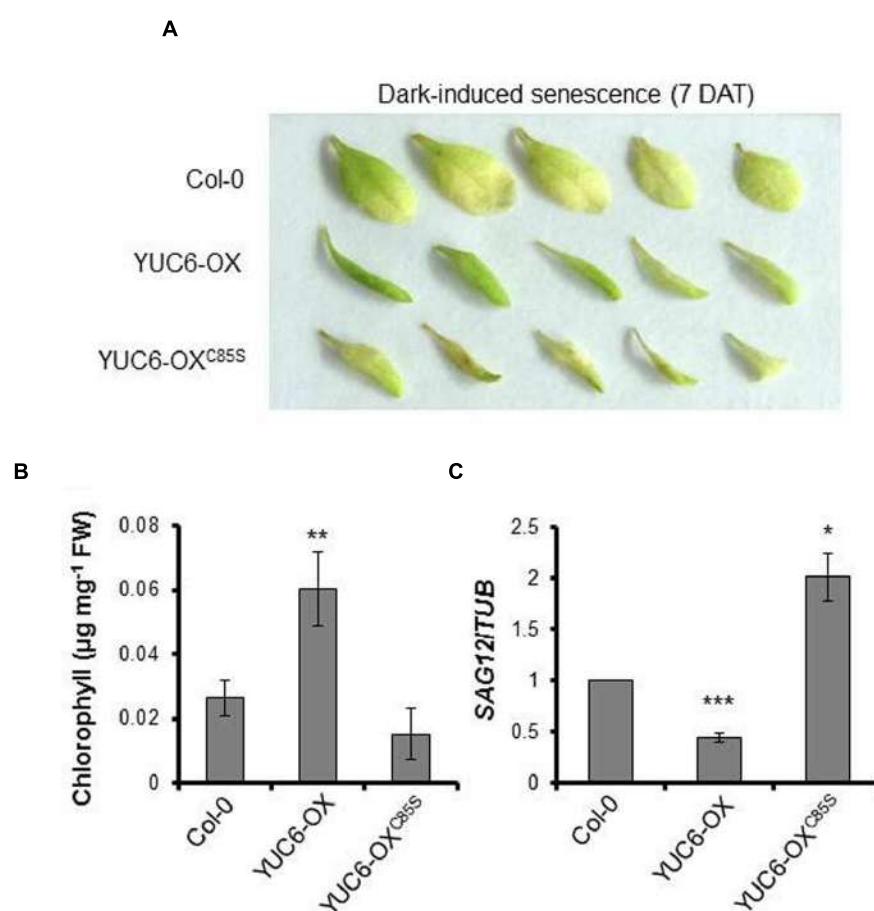
Plant hormones regulate senescence in a complex manner. The production of ROS, such as free radicals and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), also triggers senescence (Strother, 1988; Lim et al., 2007). We recently showed that YUC6 is involved in controlling ROS homeostasis to protect plants from oxidative and drought stress via its TR activity (Cha et al., 2015). To determine whether the regulation of ROS levels by the TR activity of YUC6 affects leaf senescence, we examined H<sub>2</sub>O<sub>2</sub> accumulation in senescent leaves. Naturally senescent leaves (shown in **Figure 1A**) were detached and ROS accumulation was visualized by staining with 3,3'-diaminobenzidine (DAB; **Figure 3A**). Healthy green leaves of YUC6-OX plants exhibited almost no accumulation of H<sub>2</sub>O<sub>2</sub>; by contrast, high levels of H<sub>2</sub>O<sub>2</sub> accumulated in both healthy and senescent leaves of wild-type and YUC6-OX<sup>C85S</sup> plants. ROS

homeostasis is maintained during the vegetative stage, but at the beginning of the reproductive stage, this homeostasis shifts and ROS levels increase, initiating senescence (Jing et al., 2008). This increase occurs in conjunction with a reduction in antioxidant enzyme activity during senescence (Ye et al., 2000). Thus, the disruption of redox homeostasis can lead to leaf senescence. We previously determined that peroxidase activity is enhanced in *yuc6-1D* and YUC6-overexpressing plants in a TR activity-dependent manner, but with no changes in catalase activity (Cha et al., 2015).

In addition, key redox systems such as NTS and NGS are associated with auxin signaling, including regulation of auxin levels and transport (Bashandy et al., 2010). Thus, we examined the transcript levels of three NTR genes, *NTRA*, *NTRB*, and *NTRC*, as well as *GSH1* (**Figures 3B–E**). Interestingly, whereas the transcript levels of all of these genes were elevated in YUC6-OX plants compared to wild type, changes in transcript levels were not observed in YUC6-OX<sup>C85S</sup> plants; rather, the transcript levels in these plants were similar to those of wild type. These results suggest that YUC6 overexpression enhances expression of genes involved in NTS and NGS in a TR activity-dependent manner in YUC6-OX plants and that it also may delay leaf senescence by inhibiting ROS induction.

## ROS Accumulation Reduces Auxin Efflux Transporter Levels

In the absence of NTS and NGS, mutant plants display phenotypes similar to those of auxin-defective mutants. In addition, treatment with buthionine sulfoximine (BSO), an



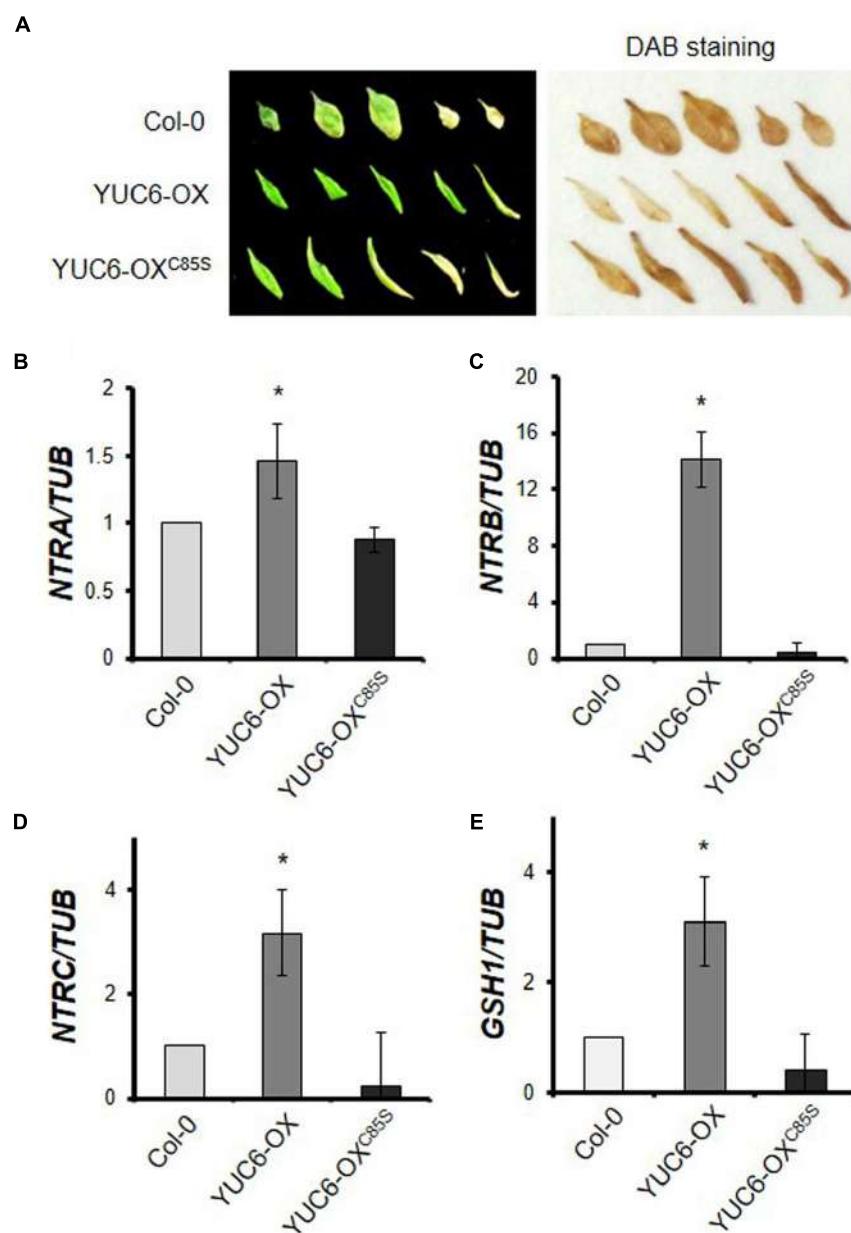
**FIGURE 2 | Dark-induced senescence.** Wild-type (Col-0), YUC6-OX, and YUC6-OX<sup>C85S</sup> plants (3.5 weeks old) were exposed to constant dark conditions for 7 days. The third or fourth leaves were detached and photographed at 7 days after dark treatment (7 DAT), as shown in **(A)**. **(B)** Chlorophyll contents. Leaves from plants treated to induce dark-induced senescence were harvested as described in **(A)** and their chlorophyll contents were measured. Data represent the means  $\pm$  SE,  $n = 3$ . \*\* $P < 0.01$  compared with the chlorophyll contents in Col-0; two-tailed Student's *t*-test;  $n$  refers to the number of biological replicates. **(C)** Expression of SAG12. Shown are the mRNA levels of SAG12, which were relatively normalized to TUBULIN (TUB) mRNA levels, as measured by quantitative RT-PCR. Data represent the means  $\pm$  SE,  $n = 3$ . \* $P < 0.05$ , \*\*\* $P < 0.001$  compared with SAG12 expression levels in Col-0; two-tailed Student's *t*-test;  $n$  refers to number of biological replicates.

inhibitor of glutathione biosynthesis, triggers decreases in the levels of auxin transporters, such as PIN proteins, at the transcriptional and translational levels (Bashandy et al., 2010). Thus, we examined whether the accumulation of ROS induced by methyl viologen (MV), an oxidative stress agent, also causes a decrease in the levels of PIN proteins. Five-day-old transgenic plants expressing *PIN1-GFP*, *PIN2-GFP*, and *PIN3-GFP* (which encode PINs with different polar localizations) driven by their native promoters were subjected to BSO and MV treatment (Figure 4). As shown in Figure 4A, the water-treated control roots showed distinct localization patterns of each PIN-GFP fusion protein, consistent with previous reports (Bashandy et al., 2010; Friml, 2010). However, when the seedlings were subjected to treatment with 5 mM BSO for 12 h, the signals from each PIN-GFP fusion protein were dramatically reduced or absent (Figure 4B). These results support the finding of Bashandy et al. (2010) that glutathione availability is important for the expression of PINs. Moreover, we used MV treatment to investigate whether

ROS accumulation triggered by oxidative damage also mediates the reduced expression of PIN-GFPs (Figure 4C). Consistent with the results of the BSO treatment, all PIN-GFP signals were clearly diminished by MV treatment. These results suggest that excessive ROS accumulation in plant cells reduces the availability of auxin by reducing the levels of auxin efflux transporters.

## YUC6 Regulates the Expression of PINs in a TR Activity-Dependent Manner

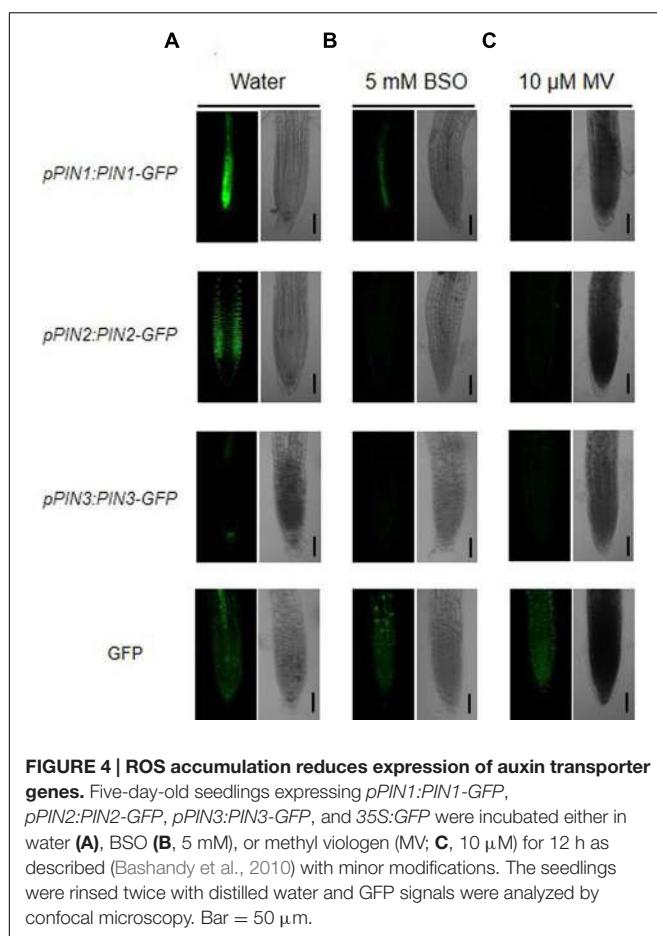
We previously reported that the TR activity of YUC6 functions in activating redox systems to scavenge ROS produced under oxidative and drought stresses (Cha et al., 2015). Auxin transport interacts with the glutathione and thioredoxin systems by influencing transcriptional and translational regulation of PIN proteins, thereby regulating physiological responses in plants (Bashandy et al., 2010; Koprivova et al., 2010). Thus, we



**FIGURE 3 |**  $\text{H}_2\text{O}_2$  accumulation and expression patterns of genes involved in the redox system (NTS/NGS) during senescence. **(A)**  $\text{H}_2\text{O}_2$  accumulation detected by DAB staining. Wild-type (Col-0), YUC6-OX, and YUC6-OX<sup>C85S</sup> plants were grown in soil under natural senescence conditions as shown in **Figure 1**. Leaves from 40-day-old plants were detached and photographed as shown on the left. The leaves were stained with DAB solution for 4 h and  $\text{H}_2\text{O}_2$  accumulation was visualized as dark brown coloring in leaves on the right. **(B–E)** Levels of transcripts of genes involved in redox systems. Third or fourth rosette leaves of 3.5-week-old plants were harvested and the expression of genes involved in the NADPH-dependent thioredoxin system (NTS) and glutathione system (NGS) was quantitatively analyzed using RT-PCR. The mRNA levels of *NTRA* **(B)**, *NTRB* **(C)**, *NTRC* **(D)**, and *GSH1* **(E)** were normalized to *TUB* mRNA levels. Data represent the means  $\pm$  SE,  $n = 3$ . \* $P < 0.05$  compared with each gene expression levels in Col-0; two-tailed Student's *t*-test;  $n$  refers to number of biological replicates.

examined whether the TR activity of YUC6 also influences the expression of auxin transporters by abolishing ROS regulation via the loss of TR activity. Auxin is imported into cells via the *AUX1* carrier and is exported by PIN efflux proteins, which exhibit polar localization in cells (Friml, 2010). The levels of most *PIN* gene transcripts were higher in YUC6-OX plants compared to wild type, while those in YUC6-OX<sup>C85S</sup> plants

were significantly lower than in YUC6-OX plants, but similar to levels in wild type (**Figure 5**). However, *AUX1* and *PIN1* transcript levels did not differ among lines. Although YUC6-OX and YUC6-OX<sup>C85S</sup> plants show no difference in auxin levels (Cha et al., 2015), YUC6 affects the expression of auxin efflux genes for redistribution, such as *PINs*, in a TR activity-dependent manner. As ROS levels are important determinants



of delayed leaf senescence in *YUC6* overexpressing plants, ROS may influence the mobility and availability of biosynthesized auxin.

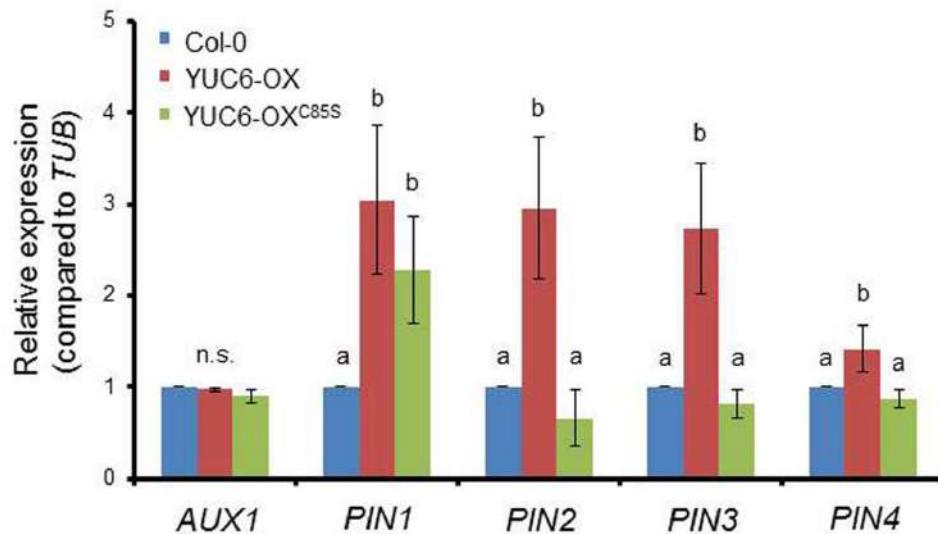
## DISCUSSION

Plants regulate their growth and development in response to numerous external stimuli and internal cues, such as various phytohormones. These external and internal factors interact in a complex manner to activate or repress genetic programs in cells throughout the plant's life (Peleg and Blumwald, 2011). These factors trigger diverse downstream responses depending on the plant's developmental stage, especially during senescence (Lim et al., 2007). Phytohormones play diverse roles in leaf senescence; for example, ethylene, ABA, SA, and JA accelerate senescence, but auxin and cytokinin delay it (Gepstein and Thimann, 1980; Noodén et al., 1990; He et al., 2002; van der Graaff et al., 2006; Kim et al., 2011; Khan et al., 2014). In addition to phytohormones, ROS-detoxification systems regulated by antioxidant enzymes also delay senescence by inhibiting ROS accumulation (Ye et al., 2000). Interestingly, ABA and SA induce ROS production in plant cells and also regulate stomatal closure (Jannat et al., 2011; Khokon et al., 2011). SA induces defense mechanisms (such as the hypersensitive

response) against biotrophic pathogens and also triggers leaf senescence (Khan et al., 2014). Autophagy-defective mutants (*atg* mutants) exhibit hyper accumulation of SA, which accelerates programmed cell death and ROS accumulation in senescence (Yoshimoto et al., 2009). Exogenous auxin treatment also induces transient ROS production (Joo et al., 2001, 2005) and increases the transcript levels of *catalase* in maize (*Zea mays*) root cells (Guan and Scandalios, 2002). ROS not only function as second messengers to regulate signaling cascades, but they are also toxic molecules that induce apoptosis in plant cells in response to environmental stresses. ROS homeostasis, accompanied by the regulation of antioxidant enzyme activity, is also essential for plant development and the regulation of senescence (Ye et al., 2000; Lim et al., 2007; Procházková and Wilhelmová, 2007). Based on these findings, auxin-overproducing mutants may be exposed to high ROS levels induced by auxin and may therefore be sensitive to oxidative stress. However, *YUC6*-overexpressing plants, such as the dominant *yuc6-1D* mutant and *35S:YUC6* plants (both plants constructed under wild-type background), exhibit delayed leaf senescence and lower ROS levels in the presence and absence of oxidative stress (Kim et al., 2011, 2013). The delayed leaf senescence in *YUC6*-overexpressing plants results from the overproduction of auxin (Kim et al., 2011). More recently, we also found that *YUC6* possesses unique TR and chaperone activity *in vitro* and that it exhibits FMO activity, which is necessary for auxin biosynthesis. To confirm these biochemical activities *in planta*, we transformed the *YUC6* or its mutant constructs in a wild-type Col-0 background harboring a *DR5:GUS* reporter gene, because a single loss-of-function mutant of biosynthetic genes did not display auxin deficient phenotypes due to its diversity of auxin biosynthesis pathway in *Arabidopsis* (Stepanova et al., 2011). Interestingly, we found that *YUC6*-overexpressing plants display enhanced drought tolerance caused by alterations in the induction of toxic ROS (Cha et al., 2015). These activities require the highly conserved Cys85 in *YUC6*. Thus, here we examined whether the Cys85-dependent TR activity of *YUC6* also regulates leaf senescence.

Although the delayed senescence in *yuc6-1D* and *YUC6*-overexpressing plants results from their increased auxin levels, the regulation of ROS homeostasis by the TR activity of *YUC6* may also cause this delayed senescence. Moreover, in the current study, we found that plants with a mutation of Cys85 in *YUC6* did not exhibit delayed senescence under either natural or dark-induced senescence conditions (Figures 1 and 2). We previously showed that the *YUC6-OX* and *YUC6-OX<sup>C85S</sup>* plants have similar auxin contents (Cha et al., 2015). However, the ROS contents of these plants differed during senescence; ROS levels were higher in *YUC6-OX<sup>C85S</sup>* than in *YUC6-OX* plants (Figure 3A). These results indicate that the control of ROS levels is also essential for delaying *YUC6*-induced leaf senescence. However, it is still unclear why the *YUC6-OX<sup>C85S</sup>* plants displayed accelerated leaf senescence, as their auxin levels were similar to those of *YUC6-OX* plants.

Based on recent findings, we investigated whether NTS and NGS are inactivated in *YUC6-OX<sup>C85S</sup>* plants. NTS and NGS



**FIGURE 5 | The reduction in expression of auxin transporter genes requires the TR activity of YUC6.** Third or fourth rosette leaves of 3.5-week-old wild-type (Col-0), YUC6-OX, and YUC6-OX<sup>C85S</sup> plants were harvested and analyzed using RT-PCR. The mRNA levels of genes encoding auxin influx carrier (AUX1) and efflux carriers (PIN1, PIN2, PIN3, and PIN4) were monitored and normalized to *TUB* mRNA levels. Data represent the means  $\pm$  SE,  $n = 3$ . Different letters above bars indicate statistically significant differences as determined by one-way ANOVA and Duncan's HSD,  $P < 0.05$ . n.s., no significant differences among plants.

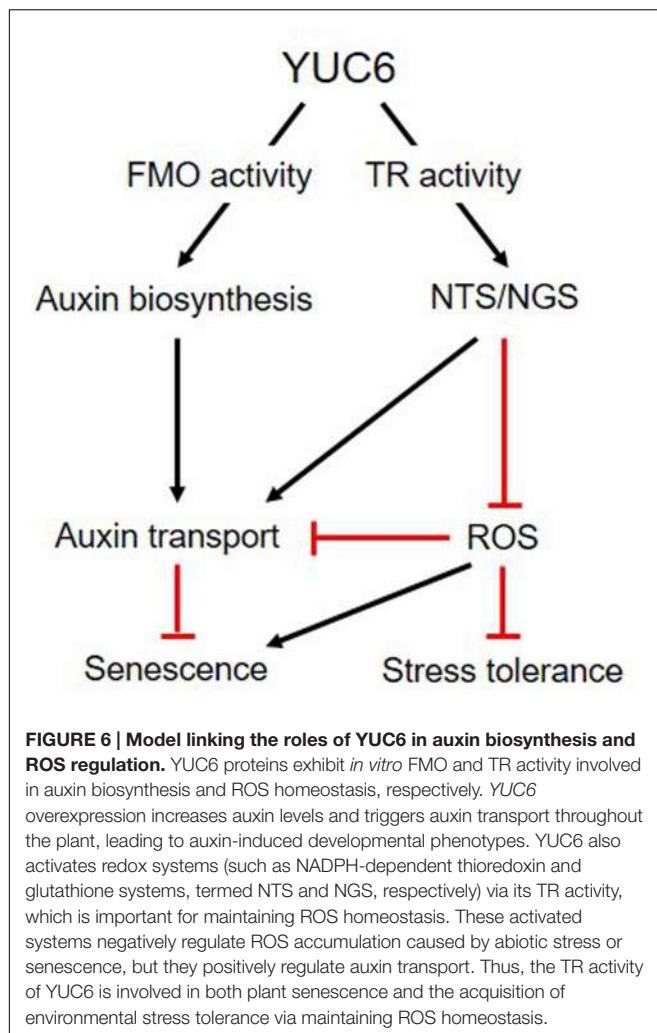
play important roles in redox balance, and a mutant with dysfunctional NTS and NGS pathways (*ntra ntrb cad2* triple mutant) displays a pin-like phenotype (Bashandy et al., 2010). These triple mutant plants are distinctly smaller than wild type at the rosette stage, with abnormal cotyledon and leaf shape and no floral structures. These phenotypes clearly indicate that these plants have reduced auxin transport activity, which is true for NTS and NGS mutant plants such as *cad2*, *ntra ntrb*, and *ntra ntrb cad2* mutants (Bashandy et al., 2010). In the current study, we also found that *NTRA*, *NTRB*, *NTRC*, and *GSH1*, which are involved in the redox system, were highly induced in YUC6-OX plants, and reduced in YUC6-OX<sup>C85S</sup> plants, similar to wild type (Figures 3B–E). Therefore, the increased TR activity due to overexpression of YUC6 activated genes involved in NTS and NGS. In addition, a chloroplastic NTRC mutant (*ntrc*) shows growth retardation with small and pale green leaves and reduced auxin levels under short-day conditions. This indicates that chloroplastic NTRC interacts with auxin to regulate development (Lepistö et al., 2009; Kirchsteiger et al., 2012). Thus, all three *Arabidopsis* NTRs regulating redox systems positively interplay with auxin in plant development. Interestingly, FAD and NADPH cofactor binding sites are well conserved in both YUC6 and TrxR proteins, and fully conserved Gly residues in both binding sites are necessary for the FMO and TR activity of YUC6 protein (Arnér and Holmgren, 2000; Jacquot et al., 2009; Cha et al., 2015). Furthermore, mutations of FAD or NADPH binding sites reduced auxin biosynthesis, delayed leaf senescence and drought tolerance in planta (Kim et al., 2011; Cha et al., 2015).

We previously determined that YUC6 regulates ROS homeostasis via its TR activity, which increases tolerance to

oxidative and drought stresses (Cha et al., 2015). Bashandy et al. (2010) found that reducing thiol activity via BSO treatment (to inhibit GSH biosynthesis) disturbs auxin signaling including auxin levels and transport, indicating that the TR pathway regulates auxin homeostasis. Our findings also suggest that ROS accumulation induced by oxidative stress, like BSO treatment, reduces the expression of PIN genes (Figure 4). Increasing auxin levels via YUC6-overexpression activates auxin transporters, which produces auxin-induced phenotypes. By contrast, disrupting the TR activity of YUC6 via mutation of Cys85 disturbs ROS homeostasis and reduces the expression of auxin transporter genes (Figure 5). However, we did not observe phenotypic differences between YUC6-OX and YUC6-OX<sup>C85S</sup> plants, which exhibit high-auxin phenotypes, although the levels of auxin transporter genes are limited in YUC6-OX<sup>C85S</sup> compared to those in YUC6-OX (Cha et al., 2015). Perhaps the overexpression of YUC6 in YUC6-OX<sup>C85S</sup> plants continuously increases auxin biosynthesis, and spatial accumulation of auxin partly occurs by activation of auxin influx gene, *AUX1*, and main polar auxin efflux gene, *PIN1*. However, reduced TR activity of YUC6<sup>C85S</sup> may perturb the control of ROS and reduce PIN levels to disturb auxin redistribution; this phenomenon requires further study.

## CONCLUSION

Our previous and current results show that YUC6 plays dual roles *in vitro*, displaying FMO and TR activities to activate auxin biosynthesis and NTS/NGS, respectively (Figure 6). Activated NTS/NGS positively regulates auxin transport and



negatively regulates ROS production. Both of these activities, which are enhanced by *YUC6* overexpression, may influence leaf senescence. Taken together, these results suggest that the TR activity of *YUC6* may trigger auxin transport and alter ROS homeostasis, leading to stress tolerance and delayed leaf senescence.

## MATERIALS AND METHODS

### Plant Material and Growth Conditions

The open reading frames (ORF) of *Arabidopsis* *YUC6* (At5g25620) and its point mutation at Cys85 to Ser (*YUC6<sup>C85S</sup>*) were cloned into the pEarleyGate 101 vectors to overexpress *in planta*, introduced into *Agrobacterium tumefaciens* (GV3101), and then the construct re-introduced into a wild-type Col-0 background (harboring a *DR5:GUS* reporter gene) using floral-dip transformation method. Transgenic *YUC6*-overexpressing plants (*YUC6-OX*) and *YUC6-OX<sup>C85S</sup>* *Arabidopsis thaliana* plants were selected using BASTA, and further confirmed their protein expression levels and auxin amounts displaying

no differences among two overexpressing plants as described previously (Cha et al., 2015). Seeds were surface-sterilized with 30% bleach for 5 min, washed five times with sterile distilled water, and incubated for 2 days at 4°C. Plants were grown at 23°C on soil or on Murashige and Skoog (MS) medium containing 0.6% (w/v) agar and 20 g/L sucrose in Petri dishes under a 16 h/8 h light-dark cycle in a growth chamber.

### Senescence Assays

For the natural senescence assay, plants were grown on soil and monitored throughout their lives. For the dark-induced senescence assay, 3.5-week-old soil-grown plants were transferred to constant dark conditions at 23°C. Third and fourth rosette leaves were photographed at 7 days after dark treatment (7 DAT).

### Measuring Chlorophyll Contents

Chlorophyll contents were used as an indicator of senescence (Kim et al., 2011). Plants were subjected to dark-induced senescence as described above. Detached third and fourth rosette leaves were soaked in 80% (v/v) acetone, and total chlorophyll contents were measured by spectrophotometry as previously described (Ni et al., 2009).

### Detection of H<sub>2</sub>O<sub>2</sub> by Histochemical Staining

H<sub>2</sub>O<sub>2</sub> accumulation in plant cells was visualized using 3,3'-diaminobenzidine (DAB). Third and fourth rosette leaves of 40-day-old *Arabidopsis* plants grown in soil were detached and stained with DAB (1 mg mL<sup>-1</sup>, pH 3.8) for 4 h. The chlorophyll in leaves was removed by subsequent incubation in 80% (v/v) ethanol.

### Expression of PIN-GFP

Five-day-old *Arabidopsis* seedlings expressing *pPIN1:PIN1-GFP*, *pPIN2:PIN2-GFP*, *pPIN3:PIN3-GFP*, and *35S:GFP* were incubated in BSO (5 mM) or (10 μM) for 12 h. The plants were rinsed twice using distilled water, and GFP signals were analyzed by confocal microscopy (Olympus).

### Quantitative RT-PCR

For quantitative PCR, the third and fourth rosette leaves were harvested from 40-day-old (for Figure 1), 3.5-week-old (for Figures 3B–E), or 7 DAT plants after dark-induced senescence (for Figure 2). The transcript levels of auxin transporter genes *PIN1*, *PIN2*, *PIN3*, *PIN4*, and *AUX1* (as shown in Figure 5) were measured in 3.5-week-old seedlings. Samples were ground in liquid nitrogen, total RNA was extracted using TRIzol reagent (Qiagen), and cDNA was synthesized using oligo(dT) primer and reverse transcriptase (Solgent). Equal amounts of cDNA were used as templates for PCR amplification. Specific transcripts were amplified with gene-specific forward and reverse primers (Supplementary Table S1) using a step-cycle program and the Quantifast SYBR Green PCR kit (Qiagen). Quantitative PCR analyses were performed on three biological repeats.

Amplification curves and gene expression levels were normalized to the expression of the housekeeping gene *TUBULIN*, which was used as an internal standard.

## Statistical Analysis

Statistical differences were calculated by two-tailed Student's *t*-test, and one-way analysis of variance (ANOVA) followed by Duncan's multiple range test applied for the calculation of confidence level at 95%.

## AUTHOR CONTRIBUTIONS

J-YC, D-JY, and W-YK initiated the project. J-YC, MK, IJ, SK performed the experiments. J-YC, D-JY, and W-YK analyzed the data. J-YC, HP, MK, W-YK wrote the paper with input from other authors. All authors discussed the results and approved the manuscript.

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## SUPPLEMENTARY MATERIAL

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# Global Plant Stress Signaling: Reactive Oxygen Species at the Cross-Road

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Current technologies have changed biology into a data-intensive field and significantly increased our understanding of signal transduction pathways in plants. However, global defense signaling networks in plants have not been established yet. Considering the apparent intricate nature of signaling mechanisms in plants (due to their sessile nature), studying the points at which different signaling pathways converge, rather than the branches, represents a good start to unravel global plant signaling networks. In this regard, growing evidence shows that the generation of reactive oxygen species (ROS) is one of the most common plant responses to different stresses, representing a point at which various signaling pathways come together. In this review, the complex nature of plant stress signaling networks will be discussed. An emphasis on different signaling players with a specific attention to ROS as the primary source of the signaling battery in plants will be presented. The interactions between ROS and other signaling components, e.g., calcium, redox homeostasis, membranes, G-proteins, MAPKs, plant hormones, and transcription factors will be assessed. A better understanding of the vital roles ROS are playing in plant signaling would help innovate new strategies to improve plant productivity under the circumstances of the increasing severity of environmental conditions and the high demand of food and energy worldwide.

**Keywords:** abiotic stress, biotic stress, oxidative stress, plant defense, plant stress signaling, reactive oxygen species

## INTRODUCTION

Plants are increasingly subjected to a variety of environmental stresses which diminish the productivity of various economically important crops. Every year, the world loses a huge amount of crop production through scarcity of water, extreme temperatures, high soil salinity, herbivore attack, and pathogen infection.

The sessile nature of plants has resulted in the evolution of complicated protection mechanisms to survive different environmental challenges. One of the stress tolerance mechanisms is the ability to sense complex stress factors and respond appropriately. Activation of complex signaling pathways helps plants to achieve this. To better understand plant signaling pathways would enable us to modify plants to improve their adaptability. However, this requires reducing the complexity associated with signaling pathways. Focusing on the points at which different signaling pathways

converge, rather than studying the branches of these pathways, would be helpful as a starting point. The rapid generation of reactive oxygen species (ROS) represents a common plant response to different biotic and abiotic stresses (Lamb and Dixon, 1997; Orozco-Cardenas and Ryan, 1999; Kovtun et al., 2000; Kotchoni and Gachomo, 2006; Mittler et al., 2011; Petrov and Van Breusegem, 2012; Noctor et al., 2014; Xia et al., 2015) and thus a basis to unify signaling events.

Recent genomic technologies, especially global gene expression tools, have not only produced new details about plant signaling pathways but also raised many historical questions including the followings: Is there a specific linear signaling pathway for each stress? If so, what about the observed cross-talk? Is there a big common signaling network from which many branches arise for specificity? If so, what about the different receptors? What do represent the points at which different branches of signaling pathways converge? If ROS are at the points of integrating signaling outputs from different signaling pathways, then what are the ROS receptors? What are the upstream and downstream signaling components of ROS? How do ROS set signaling specificity? What about the photosynthetic machinery that generates ROS; does it and its ROS and redox system represent a primary source of the plant signaling battery? As discussed throughout this review, most of these questions have been answered (or are being answered) while we will be in a better shape in providing more definite answers to the remaining ones in the near future.

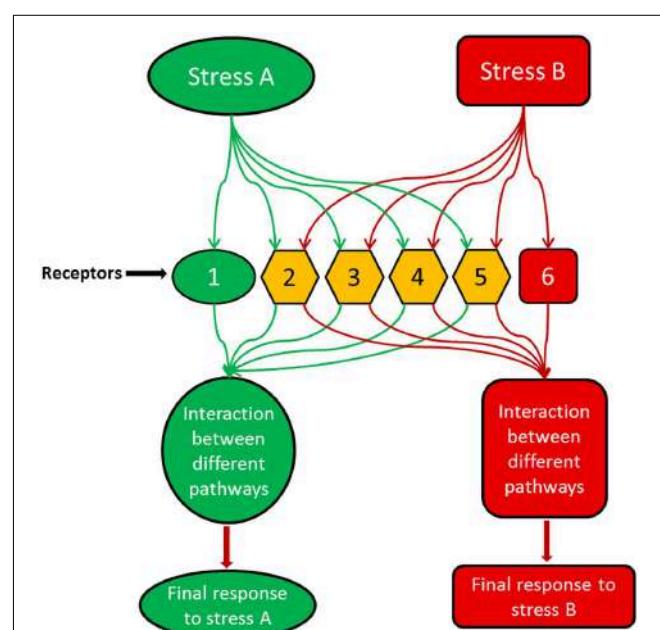
This review presents a discussion about these historical questions by considering the so complex nature of plant stress signaling networks. A special attention will be given on reviewing signaling players and events such as receptors/sensors, secondary messengers, specificity, cross-talk, redundancy, feedback regulations, alternative promoter usage, alternative splicing, nucleo-cytoplasmic trafficking, and epigenetics. Here, an attempt will also be made to indicate the kinds of studies required to fill in the gaps. A specific thought to photosynthetic activities and ROS as the primary source of the signaling battery in plants will be presented. As ROS production represents a common plant response to almost all environmental challenges, a special emphasis will be devoted here to ROS production, scavenging, damaging effects, signaling roles and how they work upstream and downstream of other signaling components, e.g., calcium, redox homeostasis, membranes, G-proteins, MAPKs, plant hormones [such as salicylic acid (SA), jasmonic acid (JA), abscisic acid (ABA), and ethylene] and transcription factors (TFs). We hope to present a holistic summary of various signaling components and concepts that are important for a plant biologist to take into consideration when analyzing signaling events involved in plant response to the ever changing environment. This understanding would help construct comprehensive signaling networks which in turn innovate new strategies to improve plant productivity under the increasing severity of environmental stress conditions and the high global demand for food and energy.

## PLANT SIGNALING NETWORKS

### Signaling Networks are Complex

Recently, our knowledge about signaling mechanisms in plants starting from stimulus sensing to final response has increased. It is obvious that there is a large number of components underlying signaling mechanisms, including a high degree of interconnectivity, many spatio-temporal levels, and a complicated tune of signal transduction pathways. For example, the changes at the expression of certain genes under a definite environmental condition are not necessarily translated into metabolic and structural changes where the interactions between various aspects, including post-transcriptional and post-translational modifications, compartmentalization, metabolite stability, substrate availability may lead to an unexpected response (Krasensky and Jonak, 2012).

Moreover, it is becoming increasingly clear that signaling networks are not linear; rather they are part of a complicated and dynamic network with substantial overlap among their branches (Knight and Knight, 2001). Accordingly, rather than one sensor, there are many sensors that perceive certain stress conditions and control all downstream signals (Figure 1). Each sensor controls a branch of the signaling pathway



**FIGURE 1 |** A model illustrating how different stresses or stimuli could activate overlapping receptors/sensors but produce distinct final outputs which are specific to each stimulus. In the model, stress A activates different receptors, e.g., 1, 2, 3, 4 and 5, while stress B is perceived by receptors 2, 3, 4, 5, and 6. Receptor 1 is activated only by stress A, while receptor 6 is activated only by stress B. The other receptors are shared between both stimuli representing the cross-talk between stress A and B. With stress A, the interaction between the downstream signaling events led by the receptor combination of 1, 2, 3, 4, and 5 produce a final output which can be completely different from the outcome of the receptor combination of 2, 3, 4, 5, and 6 with stress B.

activated by one aspect of the stress condition. For instance, temperature stress is well-known to change the physical state (fluidity) of membranes (Murata and Los, 1997; Königshofer et al., 2008), but this may not be the only condition that elicits signaling events under this stress. Changes of the conformation/activity of some intracellular proteins may also be involved in signaling to cold stress. Therefore, it is likely that the initial stress signal is perceived by multiple primary sensors, and then a cascade of signaling events is initiated by secondary signals such as plant hormones and calcium, which differ from the primary signal in time (coming late) and space (different compartments). Also, these secondary signals may differ in specificity from primary stimuli, may be shared by various stress pathways, and may underlie the interaction among signaling pathways for different insults and stress cross-protection (Figure 1). Consequently, multiple signaling mechanisms may be activated by one stimulus/stress initiating pathways differ in time, space, and outputs. Using shared signaling intermediates, such as phytohormones, these pathways may interconnect or interact with one another producing an intertwined signaling network (Xiong et al., 2002).

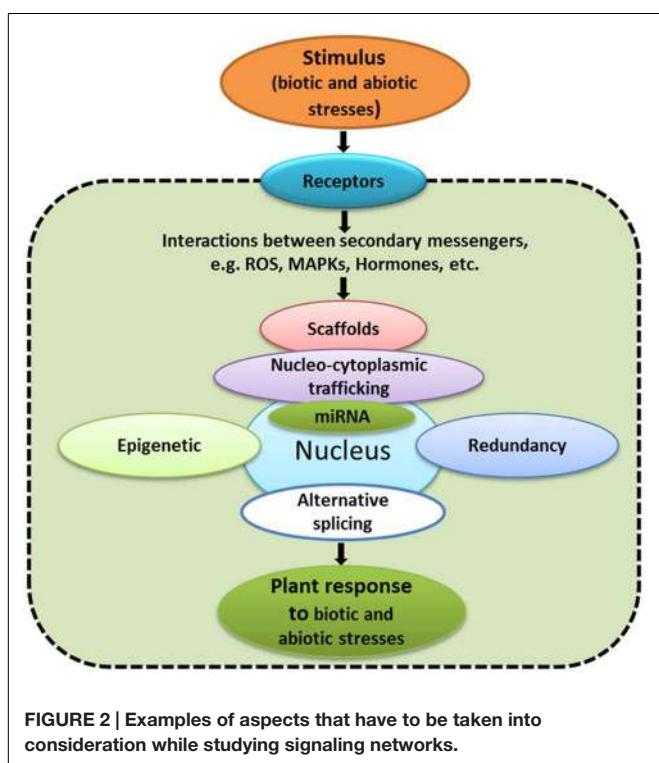
The changes in gene expression do not represent end points for signaling pathways. There are many other aspects, including transcriptional, post-transcriptional and post-translational regulation, redundancy, alternative promoter usage, alternative splicing, protein trafficking, non-coding RNA, and epigenetic effects (Figure 2) that regulate signaling pathways. In the following sections, these latter aspects will be briefly discussed.

## Redundancy and Signaling

During stress, plant signaling networks have a high ability to compensate the effects of disturbances in neighboring nodes and related signaling pathways (Chiwocha et al., 2005). The simple explanation for functional redundancy may be brought about by duplicate genes that eukaryotic genomes contain (Stelling et al., 2004; De Smet and Van de Peer, 2012). It was reported that about one-quarter of functional redundancy in *Saccharomyces cerevisiae* can be explained by compensation by duplicate genes (Gu et al., 2003). However, while sequence redundancy explains some functional redundancy, the ability of networks to compensate the effects of perturbations in neighboring nodes and related pathways could be the main cause (Chiwocha et al., 2005). Of course, this redundancy represents a great advantage to the organism to cope with the ever changing environment. However, this represents a big problem to scientists who are studying signaling transduction pathways, especially when using knockout mutants. They usually prefer an altered phenotype after modulation of single genes (Chiwocha et al., 2005). Therefore, it is imperative, while studying a signaling pathway, to take into consideration the fact that many signaling components can be functionally redundant under a given experimental condition or current phenotypic methods are not sensitive enough to detect the consequences of knocking out single genes. Therefore, gene knockouts should be examined under a variety of experimental conditions, using genome-wide gene expression profiling and other omic technologies wherever possible.

## Alternative Promoter Usage, Alternative Splicing, and Signaling

Many eukaryotic genes have multiple promoter elements. Each one is subjected to different regulatory factors under different situations. Alternative promoter usage is greatly linked to alternative splicing of internal exons and often has physiological implications (Kornblihtt, 2005). Alternative splicing produces multiple transcripts from the same gene and potentially different proteins. In turn, it represents a key post-transcriptional regulatory mechanism for expanding proteomic diversity and functional complexity in higher eukaryotes (Reddy, 2007; Carvalho et al., 2012). At the level of post-transcriptional mRNA processing, alternative splicing represents the primary mechanism to control the number of intracellular components (Bardo et al., 2002). There is substantial evidence that cellular signaling networks control the number and types of network components using alternative splicing. In the human genome, it was reported that 40–60% of the genes are subjected to alternative-splicing, with estimates of an average of 8 exons per gene (Thanaraj, 2004). In plants, alternative splicing has received less attention because this phenomenon was rare to be considered (Reddy, 2007). Others reported that, in plants, alternative splicing is ubiquitous and can mediate a bounty of transcriptome and proteome complexity (Kazan, 2003; Wang and Brendel, 2006). In the model plant *Arabidopsis*, 4,707 genes showed 8,264 alternative splicing events (Wang and Brendel, 2006). It was reported that alternative splicing of introns is involved in the regulation of kinase activity of the MIK



GCK-like MAP4K. Four different mature mRNAs of MIK were found to be accumulated with different expression profiles during maize development (Castells et al., 2006). Under stress conditions alternative splicing of pre-mRNAs dramatically increases (Reddy, 2007). Virus infection led to multiple novel intron-retaining splice variants in *Brachypodium distachyon* (Mandadi et al., 2015). So, while studying signaling networks, it is important to consider not only the signaling events leading to transcriptional changes, but also protein modifications (such as phosphorylation and glycosylation).

## Protein Trafficking and Signaling

The nuclear envelope separates the nuclear compartment containing the genes from the cytoplasm where mRNA translation and protein synthesis occurs. Therefore, all nuclear proteins, including TFs, must be imported to the nucleus. This nucleo-cytoplasmic trafficking is under complex control. The *Arabidopsis* genome, for example, contains at least 17 genes encoding importin B-like nuclear transport receptors (Bollman et al., 2003). TFs and kinases are the main regulatory components in almost all signaling pathways. Hence, it is important, while studying signal transduction pathways, to consider not only the signaling events modulating the expression of regulatory genes and proteins and their downstream interactors, but also to think about how the access of these TFs to the target genes is regulated. In general, control of transcription on both the level of TF activity and the level of nucleo-cytoplasmic partitioning are combined to create a redundant network of regulatory switches to orchestrate different signaling mechanisms (Merkle, 2004; Parry, 2015). During pathogen infection, recent reports have suggested the involvement of the nucleo-cytoplasmic trafficking of plant R proteins to achieve effector-triggered immunity and mediate disease resistance (Shen et al., 2007; Liu and Coaker, 2008). For more intensive discussion on nucleo-cytoplasmic trafficking and signaling, see Merkle (2004) and Parry (2015).

## MicroRNA and Signaling

At the post-transcriptional level, microRNAs (miRNAs) are a class of small non-coding RNAs that are increasingly being recognized as key modulators of gene expression (Covarrubias and Reyes, 2010; van Rooij, 2011; Ding et al., 2013, 2015). miRNAs regulate the expression of relevant genes by binding to reverse complementary sequences, resulting in cleavage or translational inhibition of the target RNAs (Khraiwesh et al., 2012). miRNAs are reported to play important roles in biotic and abiotic stress responses in plants. Through repressing the expression of the respective target genes encoding regulatory and functional proteins, various miRNAs were reported to play crucial roles in drought stress responses, including ABA response, osmoprotection, and antioxidant defense (Ding et al., 2013). It was reported that H<sub>2</sub>O<sub>2</sub> stress led to differential expression of seven miRNA families. The targets of these H<sub>2</sub>O<sub>2</sub>-responsive miRNAs were found to be involved in different cellular responses and metabolic processes including transcriptional regulation, nutrient transport, and programmed cell death (PCD; Li et al.,

2011). The downregulation of miR398 was found to mediate post-transcriptional induction of two Cu/Zn superoxide dismutase (SOD) genes and be important for oxidative stress tolerance in *Arabidopsis* (Sunkar et al., 2006). During biotic stress, miRNAs were found to contribute to antibacterial resistance of *Arabidopsis* against *Pseudomonas syringae* via repressing auxin signaling (Navarro et al., 2006).

## Epigenetic Effects and Signaling

Epigenetics (the study of heritable changes in gene expression that are not due to changes in DNA sequence; Bird, 2007) has become one of the hottest subjects of research in plant functional genomics since it plays an important role in developmental gene regulation, response to environmental stresses, and in natural variation of gene expression levels (Chinnusamy and Zhu, 2009; Sahu et al., 2013; Springer, 2013). Epigenetic effects are ascribed to a variety of molecular mechanisms including stable changes in protein structure, expression of small RNAs, and chromatin modifications. Chromatin modifications include DNA methylation, histone variants, remodeling of chromatin structure, and modification of histones including acetylation, methylation, ubiquitination, and phosphorylation (Springer, 2013). These mechanisms have the ability to regulate almost all genetic functions, including replication, DNA repair, gene transcription, gene transposition, and cell differentiation. For example, modifications in chromatin and generation of small RNAs have been shown to be involved in transcriptional and post-transcriptional control of gene expression during stress responses in plants (Madlung and Comai, 2004; Angers et al., 2010). These modifications are tissue-, species-, organelle-, and age-specific (Vanyushin and Ashapkin, 2011). The changes in hormonal levels that occur during biotic and abiotic stresses can control DNA methylation and other epigenetic effects (Zhang et al., 2012) resulting in plant adaptation (Mirouze and Paszkowski, 2011). Consequently, decoding how epigenetic mechanisms work in developmental gene regulation and during plant response to the environmental stresses is important. In turn, deciphering these mechanisms will also provide valuable information for potential applications, including genetic manipulation of plants toward enhanced tolerance to environmental stresses (Sahu et al., 2013; Springer, 2013).

## Construction of Ever-Larger Signaling Networks is an Urgent Task

Indeed, cellular, genetic, genomic, proteomic, and metabolomic data platforms have resulted in increasingly more detailed descriptions of signaling mechanisms, which have raised the necessity for construction of ever-larger signaling networks (Papin et al., 2005; Baginsky et al., 2010). Understanding the function of these signaling networks through reconstructing the available data about signaling pathways is crucial for studying plant's responses to different diseases and stresses.

Therefore, it is important to somewhat simplify this complexity. The start could be at the points at which the different signaling pathways converge, rather than studying the

branches. Consequently, studying the phenomenon of cross-talk may represent a good point to start to unravel global signaling networks. Additionally, tolerance across different stresses is extremely important for agriculture where plants with tolerance to more than one stress can be produced through breeding as well as transformation (Sewelam et al., 2014b). It was stated that, although different environmental challenges use unique mechanisms to initiate their specific responses, all forms of stresses seem to induce a common set of responses (Levitt, 1972). More recently, it was reported that different stress-induced changes in gene and protein expression include similar fingerprints under various environmental insults in different organisms (Desikan et al., 2001; Scandalios, 2002; Laloi et al., 2004; Polidoros et al., 2005; Walley and Dehesh, 2009; Baena-González, 2010; Atkinson and Urwin, 2012). In this regard, it was found that the accelerated generation of ROS is a common plant response to different biotic and abiotic stresses (Allen et al., 1995; Goulet et al., 1997; Noctor, 1998; Orozco-Cardenas and Ryan, 1999; Asai et al., 2000; Miller et al., 2010; Petrov and Van Breusegem, 2012; Noctor et al., 2014; Perez and Brown, 2014; Hossain et al., 2015; Xia et al., 2015). The remainder of this review will be devoted for studying ROS signaling. ROS production, scavenging, damaging effects, signaling roles and how ROS work upstream or downstream of other signaling components will be discussed.

## REACTIVE OXYGEN SPECIES AT THE CROSS-ROAD

During normal growth and development, ROS are produced in different cellular compartments in living cells with increased production under biotic and abiotic challenges (Figure 3; Møller et al., 2007; Miller et al., 2010). The traditional notion that ROS are toxic by-products of plant metabolism has changed. Substantial experimental data are available assuring that ROS are highly controlled signaling molecules able to transfer the environmental signals, with other signaling intermediates, to the genetic machinery (Polidoros et al., 2005). Here, we present a summary about ROS chemistry and signaling that would help understanding of the next sections. For detailed descriptions, we suggest the following reviews; Mittler (2002), Apel and Hirt (2004), Laloi et al. (2004), Mittler et al. (2004, 2011), Asada (2006), Halliwell (2006), Møller et al. (2007), Heller and Tudzynski (2011), Wrzaczek et al. (2013), Baxter et al. (2014).

## Definition and Chemistry of ROS

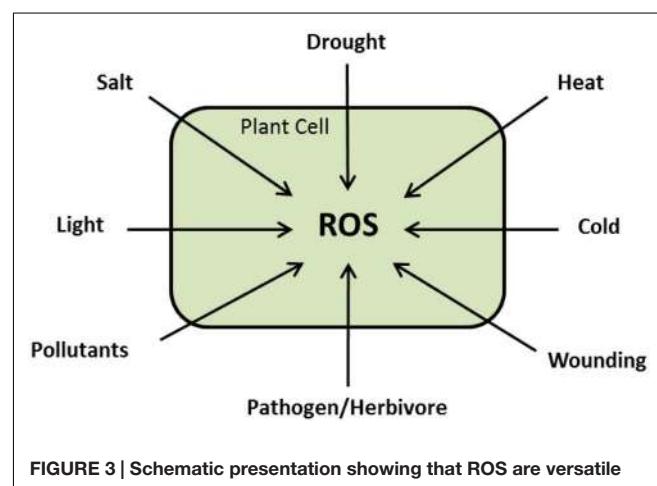
Molecular oxygen, in its ground state, is relatively unreactive. Nevertheless, during normal metabolic activity, and as a result of various environmental stresses,  $O_2$  is capable of giving rise to dangerous reactive states such as free radicals (Polidoros et al., 2005; Phaniendra et al., 2015). Reactive oxygen intermediates may result from the excitation of  $O_2$  to form singlet oxygen ( $^1O_2$ ; Trianthaphylidès and Havaux, 2009) or from the transfer of one, two, or three electrons to  $O_2$  to form, respectively, a

superoxide radical ( $O_2^\bullet-$ ),  $H_2O_2$  or a hydroxyl radical ( $OH^\bullet$ ; Mittler, 2002). The free radical might be defined as any species capable of an independent existence that contains one or more unpaired electrons; an unpaired electron being one that is alone in an orbital (Halliwell, 1991; Figure 4).

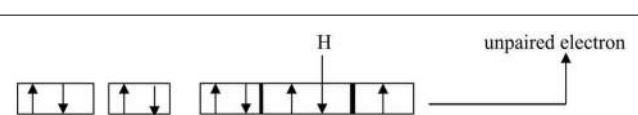
Radicals are generally more reactive than non-radicals because electrons are more stable when paired together in orbitals, but when an electron occupies an orbital by itself it has two possible directions of spin. On the other hand,  $H_2O_2$  and singlet oxygen, themselves, can be quite toxic to cells although they are non-radicals. Accordingly, the term ROS has been introduced to describe collectively, not only free radicals but also other toxic non-radicals (Halliwell, 1991).

## ROS Production *In vivo*

Reactive oxygen species are generated during normal metabolic processes. In addition, they are produced as an inevitable result of electron transport chains in chloroplast and mitochondria. As a result, electrons fall onto  $O_2$ , generating different ROS. Furthermore, abiotic and biotic stresses can further exaggerate the production and accumulation of ROS (Bhattacharjee, 2005). Mittler (2002) mentioned ten sources for production of ROS in plant cells, including, in addition to photosynthetic and respiratory electron transport chains, NADPH oxidase, photorespiration, amine oxidase, and cell wall-bound peroxidases. In chloroplasts, for example, ROS can be produced at photosystem I (PSI) as well as at PSII. During stress conditions the absorbed light energy exceeds the capacity of photosynthesis to use it through photosynthetic electron transport. As a result, various ROS are formed, including singlet oxygen ( $^1O_2$ ) at PSII and superoxide radicals ( $O_2^\bullet-$ ) at PSI and

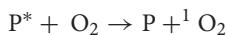
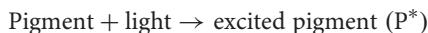


**FIGURE 3 |** Schematic presentation showing that ROS are versatile signaling molecules during plant response to different stresses.



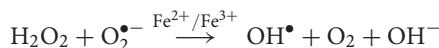
**FIGURE 4 |** Hydroxyl radical ( $OH^\bullet$ ) as an example for ROS.

PSII as byproducts (Pospisil et al., 2004; Asada, 2006; Schmitt et al., 2014). At PSII, the excess energy may be transferred from excited chlorophyll to molecular oxygen (energy is transferred not electrons) forming  $^1\text{O}_2$  as indicated below;



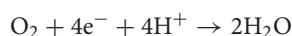
#### Reaction (1)

Under certain conditions (when the transport of photosynthetic products out of the chloroplast or the re-oxidation of NADPH is inhibited as occurs during different stresses) and  $\text{O}_2$  reduction (electron transfer) at PSI, superoxide radical formation takes place (Furbank et al., 1983). Then these  $\text{O}_2^{\bullet-}$  radicals are dismutated into  $\text{H}_2\text{O}_2$  spontaneously as well as through the action of SOD. Later on, inside the chloroplast,  $\text{O}_2^{\bullet-}$  and  $\text{H}_2\text{O}_2$  can react with each other in the presence of soluble metal ions, such as iron, to form the more reactive hydroxyl radicals according to the Haber–Weiss reaction (Bowler et al., 1992).



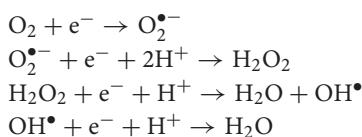
#### Reaction (2)

On the other hand, mitochondria represent a main source for ROS generation in aerobic organisms. It was estimated that from 1 to 5% of the oxygen taken up by isolated mitochondria is used in ROS production (Møller et al., 2007). The complete reduction of  $\text{O}_2$  to water through the respiratory electron transport chain requires four electrons.



#### Reaction (3)

But, as a consequence of spin restrictions,  $\text{O}_2$  cannot accept the four electrons at once, but one at a time. As a result, during  $\text{O}_2$  reduction, stable ROS intermediates such as  $\text{O}_2^{\bullet-}$ ,  $\text{H}_2\text{O}_2$ , and  $\text{OH}^{\bullet}$  are formed in a stepwise fashion as follow;



#### Reaction (4)

In peroxisomes,  $\text{H}_2\text{O}_2$  is produced during the process of photorespiration via the action of the enzyme glycolate oxidase (Møller et al., 2007). Also, the plasma membrane-bound NADPH oxidases make a big contribution to ROS production in plant cells, especially during pathogen infections (Torres and Dangl, 2005).

## Damaging Effects and Scavenging of ROS

Plants are well-adapted for minimizing the damage that could be induced by ROS under natural growth conditions. However,  $\text{O}_2$  toxicity emerges when the production of ROS exceeds the

quenching capacity of the protective systems due to stress conditions (Bowler et al., 1992; Yuasa et al., 2001; Miller et al., 2009; Akter et al., 2015). As a consequence, different ROS, including  $\text{O}_2^{\bullet-}$ ,  $\text{H}_2\text{O}_2$ ,  $\text{OH}^{\bullet}$ , and singlet oxygen, are formed, leading to oxidizing and destroying lipids, proteins, and DNA in the stressed cells (for intense information on this topic see Scandalios, 2005; Møller et al., 2007; Vanderauwera et al., 2011). Thus, plant cells have evolved antioxidant mechanisms to combat the danger posed by the presence of ROS (Baxter et al., 2007; Gill and Tuteja, 2010; Miller et al., 2010; Heller and Tudzynski, 2011; Wrzaczek et al., 2013; Schmitt et al., 2014). Mittler (2002) has reported the presence of ten mechanisms to remove ROS, in addition to five ways to avoid ROS production in plant cells. These include several enzymatic and non-enzymatic mechanisms. The enzymatic mechanisms include antioxidant enzymes, such as SOD (which converts  $\text{O}_2^{\bullet-}$  to  $\text{H}_2\text{O}_2$ ), catalases and peroxidases (which remove  $\text{H}_2\text{O}_2$ ). The non-enzymatic mechanisms of ROS removal include antioxidant molecules, such as ascorbic acid, glutathione, carotenoids, and  $\alpha$ -tocopherol (Noctor, 1998; Asada, 1999; Mittler, 2002). It was reported that there is a network of 152 genes involved in managing the level of ROS in *Arabidopsis* (Mittler et al., 2004).

In addition to these antioxidant mechanisms which scavenge the already formed ROS, plants have evolved smart ways to avoid the production of toxic forms of oxygen. These avoiding mechanisms include anatomical adaptations, such as leaf movement and curling, C4 or CAM (Crassulacean Acid Metabolism), chlorophyll movement, suppression of photosynthesis, and photosystems and antenna modulators (Maxwell et al., 1999; Mittler, 2002).

## ROS Signaling and Specificity

For a signaling molecule to be effective, it needs to be produced quickly and efficiently on demand, to induce distinct effects within the cell, and to be removed rapidly and efficiently when no longer required (Neill et al., 2003). ROS are produced instantly after the onset of the stress. In addition, ROS are very reactive; they can react with membrane lipids, carbohydrates, proteins and DNA. ROS such as  $\text{H}_2\text{O}_2$  can diffuse through the biological membranes through aquaporins (Bienert et al., 2007; D'Autreux and Toledo, 2007; Dynowski et al., 2008; Mubarakshina et al., 2010; Borisova et al., 2012) leading to systemic responses. Moreover, living cells have very efficient antioxidant systems, including enzymatic and non-enzymatic mechanisms, to put ROS under a precise control (Foyer and Noctor, 2005). Collectively, all of these features of ROS render them ideal signaling components.

Levine et al. (1994) have suggested a signaling role for  $\text{H}_2\text{O}_2$ , controlling the hypersensitive response and promoting the expression of glutathione-S-transferase and glutathione peroxidase encoding genes. Many studies have suggested signaling roles for ROS in developmental processes as well as biotic and abiotic responses (Apel and Hirt, 2004; Foyer and Noctor, 2005; Gadjev et al., 2006; Miller et al., 2009; Mittler et al., 2011; Wrzaczek et al., 2013; Perez and Brown, 2014). In an early study, the genomic response of *Escherichia coli*

cells to H<sub>2</sub>O<sub>2</sub> treatment was examined with a DNA microarray composed of 4169 open reading frames (Li et al., 2001). In this study, the mRNA of 140 genes (in wild-type) was considerably induced after H<sub>2</sub>O<sub>2</sub> treatment. On exposure of *S. cerevisiae* cells to H<sub>2</sub>O<sub>2</sub>, expression of about one-third of all yeast genes had changed suggesting that ROS can cause massive alterations in the biology of the oxidative-stressed cells (Gasch et al., 2000). Using cDNA microarray technology from a sample of 11,000 expressed sequence tags (ESTs), 175 non-redundant EST were identified that are regulated by H<sub>2</sub>O<sub>2</sub> in *Arabidopsis* (Desikan et al., 2001).

To this end, it is quite evident that ROS operate as intracellular signaling molecules, but how they can set specific signaling duties is still controversial. This controversy arises from what seems to be a paradox between the reactive nature of ROS that renders them indiscriminate and the specificity that is required for signaling (D'Autreux and Toledano, 2007). In general, the specificity in signaling pathways is mediated via the non-covalent binding of a ligand to its cognate receptor through a shape-complementary fit between macromolecules. On the other side, ROS deliver signaling events via chemical reactions with specific atoms, such as iron (Fe) and sulphur (S), of target proteins that lead to protein modifications (Nathan, 2003). ROS can also react with different target proteins whenever the chemical reaction is possible. The remaining question is how specificity in ROS signaling is managed? By looking into the chemical characteristics and the biological activities of each ROS, including O<sub>2</sub><sup>•-</sup>, H<sub>2</sub>O<sub>2</sub>, OH<sup>•</sup> and singlet oxygen (<sup>1</sup>O<sub>2</sub>), an answer to this question could be revealed.

O<sub>2</sub><sup>•-</sup> is a by-product of electron transport chains of photosynthesis and respiration and is produced by NADPH oxidases and cell wall peroxidases. In *E. coli*, the steady-state concentration of O<sub>2</sub><sup>•-</sup> is very low (~10<sup>-11</sup> M; Halliwell and Gutteridge, 1999), which reflects its instability; this is mainly due to spontaneous and SOD-mediated O<sub>2</sub><sup>•-</sup> dismutation to H<sub>2</sub>O<sub>2</sub>. The instability of O<sub>2</sub><sup>•-</sup> and its inability to diffuse through membranes because of its negative charge make this ROS relatively poor signaling molecule. However, due to high attraction, O<sub>2</sub><sup>•-</sup> oxidizes Fe-S clusters at a rate that is almost diffusion limited (Storz et al., 1990; Storz and Imlay, 1999).

H<sub>2</sub>O<sub>2</sub> is actually a poor oxidant and reacts mildly with [Fe-S] (rate constant of 10<sup>2</sup>-10<sup>3</sup> M<sup>-1</sup> s<sup>-1</sup>), loosely bound metals and, very slowly, with glutathione and free cysteine (Cys) (Imlay, 2003). By contrast, its reactivity toward Cys residues can significantly increase to 10-10<sup>6</sup> M<sup>-1</sup> s<sup>-1</sup>. H<sub>2</sub>O<sub>2</sub> is relatively stable (cellular half-life ~1 ms, steady-state levels ~10<sup>-7</sup> M; D'Autreux and Toledano, 2007), and can diffuse through biological membranes because it is not charged. Its selective reactivity, stability and diffusability make H<sub>2</sub>O<sub>2</sub> fit for signaling. As a second messenger, H<sub>2</sub>O<sub>2</sub> can mediate intracellular signal transduction through chemoselective oxidation of Cys residues in signaling proteins, such as glutathione, thioredoxins, and peroxiredoxins (Paulsen and Carroll, 2010).

OH<sup>•</sup> is the most highly toxic ROS. It has high indiscriminate reactivity, which limits its diffusion to sites of production (half-life 10<sup>-9</sup> s; Halliwell and Gutteridge, 1999), even though OH<sup>•</sup> seems to operate in H<sub>2</sub>O<sub>2</sub> sensing (D'Autreux and Toledano, 2007).

Singlet oxygen (<sup>1</sup>O<sub>2</sub>) is an excited state molecule. The half-life time of <sup>1</sup>O<sub>2</sub> is very short (~100 ns) and it can travel only a very short distance in cells (<100 nm; Moan, 1990; Niedre et al., 2002). This could be because it reacts very rapidly with amino acids, unsaturated lipids, and other cell constituents. As a result, <sup>1</sup>O<sub>2</sub> can react directly only with molecules in close proximity to its production location, i.e., in the chloroplast (Kochevar, 2004; Triantaphylides and Havaux, 2009). This means that <sup>1</sup>O<sub>2</sub> could deliver specific signaling events mainly through spatial aspects of ROS production.

In addition to the previous chemical characteristics that render ROS able to set specificity as signaling molecules, a non-ROS intermediate in a ROS signaling pathway can regulate additional pathways that are physically non-adjacent to the pathway in which it was formed (Nathan, 2003). For instance, a ROS that is produced in a cellular compartment could specifically activate a secondary messenger such as a MAPK or a plant hormone, which in turn activate remote signaling pathways. In *Arabidopsis*, it was suggested that histidine kinase ethylene receptor ETR1 is important for H<sub>2</sub>O<sub>2</sub> perception during stomatal closure (Bright et al., 2006). In addition, it was reported that indirect activation of TFs by ROS may be mediated by some members of MAPK cascades (Asai et al., 2000). Interestingly, it was shown that H<sub>2</sub>O<sub>2</sub> originating in different subcellular sites induces different responses. H<sub>2</sub>O<sub>2</sub> produced in chloroplasts was found to activate early signaling responses, including TFs and biosynthetic genes involved in production of secondary signaling messengers; while H<sub>2</sub>O<sub>2</sub> produced in peroxisomes was found to induce transcripts involved in protein repair responses (Sewelam et al., 2014a). Moreover, ROS-mediated changes in the cellular redox homeostasis could set highly specific signaling roles for ROS. For example, different pathways could sense and weigh the change in cellular redox balance resulting from the change of intracellular ROS concentration, then translate these changes into highly specific cellular signals that direct the cell to produce a relevant adaptive response (Foyer and Noctor, 2005). In simple organisms, such as bacteria and yeast, the enhanced production of ROS is perceived by change in redox homeostasis which in turn is delivered to redox sensitive TFs (Costa and Moradas-Ferreira, 2001; Georgiou, 2002). In addition, it has been proposed that ROS may be perceived indirectly by sensing changes in the cellular redox potential (Price et al., 1994) or by detecting the products of ROS-inflicted damage (Evans et al., 2005). In addition, ROS can generate specific signaling effects through the peptides produced from proteolytic breakdown of oxidatively damaged proteins which act as secondary ROS messengers and contribute to a retrograde ROS signaling during different environmental challenges that generate oxidative stress (Moller and Sweetlove, 2010).

## Components Involved in ROS Signaling

The perceived ROS signals work upstream as well as downstream from many other second messengers in addition to many feedback and feedforward regulations in an interwoven manner to establish specific responses to different developmental and environmental cues. Currently, a major gap exists in our understanding of how ROS induce large-scale and coordinated

expression from many genes. In addition, the big challenge is to identify the upstream sensing and signaling events through which ROS are perceived and delivered to the ROS-induced TFs. Do ROS activate the expression of TFs directly or through another set of signaling intermediates? How could secondary messengers such as G proteins, MAPKs,  $\text{Ca}^{2+}$ , JA, SA, and ABA mediate the ROS signals and which one is upstream or downstream from each other? Does ROS, produced passively during different stresses through their damaging effects on cellular structures, induce signaling events that differ from those signaling events produced actively through activation of cell membrane-bound enzymes, like NADPH oxidases? The discussion below is an overview of the interconnectivity between ROS and other individual components involved in plant signal transduction pathways (Figure 5).

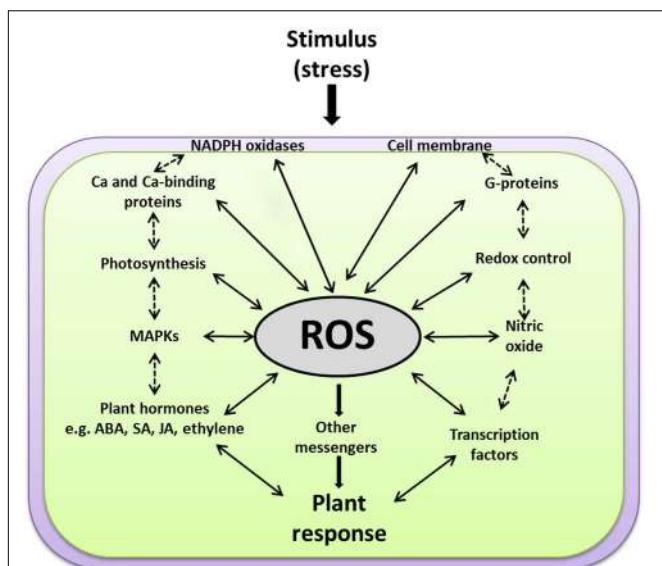
## Photosynthetic Activity

Photosynthesis represents the most peculiar feature that distinguishes plant and animal systems. In photosynthesis, through intersystem electron transport, the light energy captured by photosynthetic pigments is transformed into chemical energy which pumps reductants (NADPH) and ATP into the Calvin cycle (dark reactions) supplying carbon skeletons (sugars) for all major metabolic processes (Kromer, 1995). In addition to this role, plastids synthesize and store a large number of biomolecules, including carbohydrates, amino acids, fatty acids, and plant hormones (Buchanan et al., 2000). Therefore, it is self-evident that any change or imbalance in the function of the chloroplast will affect directly or/and indirectly the other

cellular functions in plant cells. Consequently, environmental challenges can be primarily sensed via production of ROS and the concomitant changes of redox homeostasis of the chloroplast that act synergistically with other signaling pathways inducing then adaptive molecular and physiological responses (Huner et al., 1996). Earlier studies have suggested that the redox state of plastoquinone controls the rate of transcription of the chloroplast genes encoding reaction-center apoproteins of photosystem I and photosystem II (Pfannschmidt et al., 1999, 2009). Recently, it was reported that chloroplasts are able to sense light conditions and generate a remote control to modulate the nuclear gene expression to face the changing environment (Koussevitzky et al., 2007; Pogson et al., 2008; Pfannschmidt et al., 2009; Godoy Herz et al., 2014). As a retrograde signaling pathway, the redox state of components of the photosynthetic electron transport chain can sense the changes in photosynthetic activity and in turn affect the nuclear gene expression (Fey et al., 2005; Nott et al., 2006; Godoy Herz et al., 2014). The plastidial metabolite, methylerythritol cyclodiphosphate (MEcPP), was found to regulate the expression of nuclear stress-response genes through a retrograde signal from the chloroplast to the nucleus (Xiao et al., 2012).

The thylakoid membranes and the involved redox complexes of the photosynthetic apparatus, especially the light energy harvesting PSII, are very sensitive targets to various environmental stress factors. During stress, excess of photosynthetically active light leads to the formation of ROS (e.g.,  $^1\text{O}_2$  and  $\text{OH}^\bullet$ ; Glatz et al., 1999), which can induce membrane damage by attacking double bonds of unsaturated fatty acids. These effects are also expected to feedback signals for stress gene expression via the pathway which senses the physical state of the membrane (Glatz et al., 1999). This observation may reinforce the idea that receptors/sensors at the cell surface or the cell membranes could perceive stimuli, not directly from the stress, but indirectly through the chloroplast stress signals. It was reported that  $\text{H}_2\text{O}_2$  from chloroplasts led to the induced expression of many genes coding for membrane-bound receptor proteins and signaling components (Sewelam et al., 2014a). In ozone-treated *Arabidopsis* leaves, Joo et al. (2005) reported that the chloroplastic ROS signal contributes to activating the membrane associated NADPH oxidases in intercellular signaling during the early component of the oxidative burst. Accordingly, they suggested that signaling from the chloroplast is central for oxidative stress induction by  $\text{O}_3$ . Other studies also found that induction of light and stress response requires chloroplast signaling mediated by ROS (Allen et al., 1999; Fryer et al., 2003; Agrawal et al., 2004; Serrato et al., 2013).

Relatively little attention has been given to the role of photoproduced  $\text{H}_2\text{O}_2$  and other ROS in defense signaling (Delledonne et al., 2001; Parker et al., 2001). This may be because the current models of signaling pathways controlling plant defense against pathogen infection are based mainly on animal models. Recently, new research has led to the development of models incorporating how the signaling pathways that are involved in light perception and in defense could operate and interact to form a complete defense signaling network, which includes systems to sense light and regulate gene expression. In this context, it was suggested that signals from



**FIGURE 5 | A scheme explaining how ROS function at the cross-road of various key signaling events.** ROS work upstream and downstream of the other signaling components, e.g., membranes, NADPH oxidases, G-proteins, calcium, redox homeostasis, photosynthesis, MAPKs, plant hormones [such as salicylic acid (SA), jasmonic acid (JA), abscisic acid (ABA), and ethylene] and transcription factors. Solid arrows for direct ROS interactions with other signaling components, dashed arrows for expected indirect interactions.

the chloroplast and LESION SIMULATING DISEASE1 are integrated to mediate crosstalk between light acclimation and disease resistance in *Arabidopsis* (Mühlenbock et al., 2008). ROS produced from chloroplasts during the infection with *Xanthomonas campestris* play a major role in localized cell death in the non-host interaction between tobacco and this bacterial species (Zurbriggen et al., 2009). It was suggested that the chloroplast protein RPH1, a positive regulator of *Phytophthora brassicae*-induced oxidative burst, plays a role in the defense response of *Arabidopsis* and potato to *P. brassicae* (Belhaj et al., 2009). Based on these observations, it is clear that thylakoid membranes and hence photosynthesis, play a vital and very early role in stress sensing and signaling in plants, an idea that should be considered when constructing plant signaling networks.

## Redox Homeostasis

In plants, the continuous energy conversions, in the chloroplast and mitochondria, and the optimal use of the available light energy are only guaranteed when all reduction–oxidation (redox) processes are under precise control. Information on the redox situation is generated and transferred by various redox components, including various ROS and different antioxidants, that are parts of a robust network that links metabolism with regulation and signaling. Under environmental challenges, the imbalance in the network is sensed, and transformed into redox signals that are transmitted in order to elicit specific responses at various levels of regulation and in different subcellular compartments (Scheibe and Dietz, 2012). Thus, ROS and redox cues, generated under stress conditions, are essential to control the main metabolic processes through which cells convert and distribute the energy and metabolic fluxes, optimize different cell functions, activate acclimation responses through retrograde signaling, and control whole-plant systemic signaling pathways (Noctor, 2006; Suzuki et al., 2012). Redox homeostasis in the plant cell is considered to be an “integrator” of information from the environment controlling plant growth and stress responses, as well as cell death events (Dietz, 2003; Foyer and Noctor, 2009; Potters et al., 2010). The antioxidants, ascorbate, glutathione, carotenoids, and tocopherol, are information-rich redox buffers that affect numerous cellular components. In addition to their vital roles in stress response and as enzyme cofactors, cellular redox components influence plant growth and development by orchestrating processes from cell division to senescence and cell death (de Pinto and De Gara, 2004; Potters et al., 2004; Tokunaga et al., 2005; Halliwell, 2006). Most importantly, antioxidants influence gene expression associated with responses to different environmental cues to maximize defense through tuning cellular ROS levels and redox state (Foyer and Noctor, 2005). Proteins with oxidisable thiols such as glutathione and thioredoxin-1 are crucial for many functions of cell nuclei, including transcription, chromatin stability, nucleo-cytoplasmic trafficking, and DNA replication and repair (Nose, 2005; Go and Jones, 2010; Lukosz et al., 2010). From bacteria to humans, the triplet peptide, glutathione, is involved in protein S-glutathionylation where it regulates a variety of cellular processes by modulating protein function and prevents irreversible oxidation of protein

thiols under unfavorable conditions (Dalle-Donne et al., 2009).

As redox homeostasis is greatly influenced by most, if not all, conditions that affect plant growth and development, the changes of intracellular redox determine various signaling events through their interaction with many other secondary messengers, such as protein kinases and phosphatases, phytohormones and calcium. Intensive current research might confirm that ROS-antioxidant interactions act as a metabolic interface between environmental changes and the concomitant signaling responses (Foyer and Noctor, 2005, 2012). For example, the redox state determined by the ROS-antioxidant interactions could regulate, directly or indirectly, the work of TFs, such as TGA, Athb-9, and RAP2 and hence the regulation of the expression of their downstream genes (Dietz, 2008).

## Membranes

The plasma membrane, as the selective barrier between living cells and their environments, plays a pivotal role in the perception of the changes in the surrounding environment (Guo et al., 2002). As a consequence of their rapid ability to modify their physical state, cellular membranes are not only the primary sites of stress damage, but also able to perceive environmental insults and activate remotely stress-defense genes (Glatz et al., 1999). The microdomain organization and physical state of cell membranes is known to be a very sensitive monitor of different environmental challenges (Horvath et al., 1998). It was stated that heat stress changes the membrane fluidity and H<sub>2</sub>O<sub>2</sub> responds rapidly to this change, leading to the activation of small heat shock protein synthesis (Königshofer et al., 2008).

As a component of cell membranes, ion channels play a vital role in the transduction of environmental and internal signals (Binder et al., 2003). Ion channels are proteins forming hydrophilic pathways through the plasma membranes (Barnes et al., 1997). They function as permeation pores through which the electrically charged species can pass across biological membranes. It was reported that ion channels are directly involved in important cellular processes, such as plant defense responses induced by elicitors (Czempinski et al., 1997), light perception (Ermolayeva et al., 1997), and mechanical signals (Cosgrove and Hedrich, 1991). For example, it was reported that the efficiency of H<sub>2</sub>O<sub>2</sub> signaling between cells is controlled by plasma membrane aquaporin pores where the expression of several plant plasma membrane aquaporins in yeast, such as *Arabidopsis* plasma membrane intrinsic protein PIP2.1, was found to enhance the toxicity of H<sub>2</sub>O<sub>2</sub> when yeast cells were exposed to H<sub>2</sub>O<sub>2</sub> treatment (Dynowski et al., 2008). It was found that the disruption of a cyclic nucleotide-gated calcium channel gene causes a hyper-thermosensitive phenotype in *Arabidopsis* and moss indicating that the plasma membrane cyclic nucleotide-gated calcium channels control plant thermal sensing and acquired thermotolerance (Saidi et al., 2009; Finka et al., 2012).

During exposure to stress, the major role of phospholipids, the backbone of cellular membranes, may be to serve as precursors for the generation of secondary messenger signaling molecules, such as phosphatidylinositol, inositol 1,4,5-triphosphate (IP<sub>3</sub>),

diacylglycerol, and jasmonates. IP<sub>3</sub> and diacylglycerol are secondary messengers that can activate protein kinase and induce Ca<sup>2+</sup> release, respectively. Additionally, IP<sub>3</sub> itself is a signal and may be involved in several processes, such as the recruitment of signaling complexes to specific membrane location and their assembly (Guo et al., 2002). In addition, under oxidative stress, polyunsaturated fatty acids (PUFAs) are attacked by different ROS, specially <sup>1</sup>O<sub>2</sub> and OH<sup>•</sup>. This causes production of lipid hydroperoxides, leading to a decrease of membrane fluidity (Møller et al., 2007). In turn, this change in membrane physical state could activate downstream signaling intermediates.

## NADPH Oxidases

Membrane-bound NADPH oxidases are a group of enzymes that catalyze the production of superoxide radicals (O<sub>2</sub><sup>•-</sup>) in animals and plants (Sagi and Fluhr, 2006). In mammals, NADPH oxidases are also called respiratory burst oxidases (Rbo). Because of their functional homology with mammals, plant NADPH oxidases are known as respiratory burst oxidase homolog (Rboh; Torres and Dangl, 2005). In plants, Rboh enzymes are the source of ROS production under pathogen infection and in many of other processes (Torres and Dangl, 2005). The ability of Rboh enzymes to integrate various signaling players, such as calcium and protein phosphorylation with ROS production, suggests a crucial role for Rboh in many different biological processes in cells, and places them at the core of the ROS signaling network of cells, revealing their important functions in plants (Suzuki et al., 2012; Kadota et al., 2014).

In *Arabidopsis*, there are ten *Rboh* genes (Torres et al., 1998; Dangl and Jones, 2001). Many studies have reported the induction of *Rboh* gene expression by pathogens and fungal elicitors (Simon-Plas et al., 2002; Yoshioka et al., 2003; Wang et al., 2006). In addition, using mutant analysis, it was suggested that RbohD and RbohF are required for ROS production and cell death in *Arabidopsis* plants infected with *P. syringae* or *Peronospora parasitica* (Torres et al., 2002). The same group (Torres et al., 2005) reported that RbohD is required for ROS production but this ROS antagonizes cell death induced by *Pseudomonas* infection. In *Nicotiana benthamiana*, silencing of *NbrbohA* and *NbrbohB* led to reduction of ROS production and reduced resistance to *Phytophthora infestans* infection (Yoshioka et al., 2003). The *Arabidopsis* RbohF was suggested to be a vital player in defense-associated metabolism and a key factor in the interaction between oxidative stress and pathogen infection (Chaouch et al., 2012). Regarding the involvement of Rboh in abiotic interactions, it was reported that the *Arabidopsis* *RbohD* gene is involved in ROS-inducing a rapid systemic signal during various stress factors, such as heat, cold, high light, and salinity (Miller et al., 2009). The abiotic stress-mediating phytohormone ABA was reported to be regulated by the action of RbohD and RbohF in different ROS-ABA signaling pathways (Kwak et al., 2003; Joo et al., 2005; Xue and Seifert, 2015). In a microarray experiment, *RbohD* expression was downregulated by ABA treatment but upregulated by H<sub>2</sub>O<sub>2</sub> treatment in *Arabidopsis* (Barraud et al., 2006). During salt stress, ROS produced by both AtRbohD and AtRbohF seem to function as signal molecules to regulate Na<sup>+</sup>/K<sup>+</sup> homeostasis, where the two *Arabidopsis*

double mutants *atrbohD1/F1* and *atrbohD2/F2* were found to produce less ROS and to be much more sensitive to NaCl treatments than wild-type (Ma et al., 2012). RbohD was found to contribute to the ROS-responsive expression of *ERF6*, a ROS regulator TF in *Arabidopsis* (Sewelam et al., 2013). As NADPH oxidases are physically located at the plasma membrane, they are proposed to play an early and vital signaling role and should be highly considered when constructing plant signaling networks.

## G Proteins

GTP-binding proteins (G proteins) are found in almost all organisms from prokaryotes to humans (Assmann, 2004). G proteins mediate stimulus perception by G-protein-coupled receptors (GPCR), in addition to other regulatory proteins. In humans, there are about 1000 GPCR, representing the largest group of cell surface receptors encoded by mammalian genome (Nagarathnam et al., 2012). It is estimated that about 60% of all drugs currently available target G-protein-based pathways and G protein component disorders have been found to cause various genetic diseases (Assmann, 2004). G proteins are heterotrimeric proteins composed of three monomers;  $\alpha$ ,  $\beta$ , and  $\gamma$ . About 20 G protein  $\alpha$  subunits (G $\alpha$ ), 6 G $\beta$  subunits, and 20 G $\gamma$  subunits have been characterized in mammals (Gutkind, 2000). Controversially, in plants the situation seems to be much simpler than that in animal systems. For example, it was reported that the *Arabidopsis* genome encodes only single G $\alpha$  and G $\beta$  subunits, two G $\gamma$  subunits, just one GPCR protein, and one regulator of G protein signaling (Assmann, 2004).

The involvement of G proteins in plant stress signaling is evident, especially in plant-pathogen interactions (Assmann, 2005; Trusov et al., 2009; Maruta et al., 2015; Xu et al., 2015). Regarding ROS, many studies have suggested a tight relationship between ROS and G proteins in stress-mediated plant signaling. It was reported that, on exposure of *Arabidopsis* leaves to ozone, the first biphasic oxidative burst is greatly attenuated or completely absent in mutant plants lacking G $\alpha$  protein or G $\beta$  protein. This finding suggests that the ROS produced by ozone in the apoplastic fluid do not themselves enter cells to activate intracellular ROS-producing systems. Rather, the extracellular ROS activate the G protein either directly or indirectly (Joo et al., 2005). It is possible that G proteins themselves are directly activated by ROS. In this regard, it has been reported that two mammalian G $\alpha$  proteins, G $\alpha$ i and G $\alpha$ o, are redox-controlled (Fujiki et al., 2000). The membrane-bound NADPH oxidases D and F were suggested to receive initial signals from G proteins to mediate ozone responses in *Arabidopsis* guard cells (Suharsono et al., 2002). The absence of the G $\alpha$  subunit in the *gpa1* mutant disrupts the interplay between ABA perception and ROS production, with a consequent inhibition of Ca<sup>2+</sup>-channel activation (Zhang et al., 2011). The membrane-bound ROS producing enzymes AtRbohD and AtRbohF work in the same pathway with the G $\beta$  subunit of the heterotrimeric G protein for full disease resistance to different *P. syringae* strains (Torres et al., 2013). In plants, further studies are required to unravel the roles of G proteins and their signaling roles.

## Calcium Signaling

The use of calcium ions as a secondary messenger represents an integral part in many signal transduction pathways in all life forms, from vertebrate animals to plants (Berridge et al., 2000; Stael et al., 2012). In contrast to other similar ions, such as Mn<sup>2+</sup>, the Ca<sup>2+</sup> ion has many peculiar features, including a favorable ionic radius and hydration status, an irregular geometry, and flexible coordination chemistry (Jaiswal, 2001). The main calcium stores in plant cells are: the vacuole, the endoplasmic reticulum and the apoplast (Stael et al., 2012). Elevation in cytoplasmic Ca<sup>2+</sup> represents an early response to many different biotic and abiotic stresses, including oxidative (McAinsh and Pittman, 2009; Dodd et al., 2010). As a second messenger in a wide range of signaling pathways in plants, calcium connects the perception of different stimuli and stresses to their downstream cellular responses (Evans et al., 2005). It has been stated that transient cellular calcium elevations are sensed by several Ca<sup>2+</sup> sensors such as calmodulin (CAM), calmodulin-like (CML), calcium-dependent protein kinase (CDPK), and calcineurin B-like protein (CBL; Bouche et al., 2005; McCormack et al., 2005; Das and Pandey, 2010; Asai et al., 2013). A direct interconnection between CBL-CIPK-mediated Ca<sup>2+</sup> and ROS signaling in plants was reported as evidence for a synergistic activation of the NADPH oxidase RbohF by direct Ca<sup>2+</sup>-binding to its EF-hands (Drerup et al., 2013). The *Arabidopsis* CPK5, an isoform of the plant CDPK family, was activated rapidly in response to infection with *P. syringae*, resulting in Rboh-mediated ROS production and enhanced SA-mediated resistance to this bacterial pathogen (Dubiella et al., 2013). In the same study, RbohD was reported to be an *in vivo* phosphorylation target of CPK5. Ca<sup>2+</sup> ions also regulate long-distance root-to-shoot signaling and may also have roles in transmitting ROS signals (Choi et al., 2014).

The concentration of cytosolic Ca<sup>2+</sup>, the expression level of calmodulin 1 (CAM1) gene, the content of CAM proteins and the expression of many antioxidant genes in maize are increased after treatment with ABA or H<sub>2</sub>O<sub>2</sub>. Furthermore, pre-treating plants with CAM inhibitors almost completely blocked the upregulation of many antioxidant enzymes (Hu et al., 2007). These findings show that the increase in cytosolic Ca<sup>2+</sup> requires CAM to deliver its signal to the downstream targets. Ca<sup>2+</sup> elevations have been suggested, in some cases, to be upstream of ROS production; in other cases, Ca<sup>2+</sup> elevations have been reported to be downstream of ROS production (Bowler and Fluhr, 2000). Several workers showed that oxidative stress results in increased cytosolic Ca<sup>2+</sup>. In tobacco seedlings, oxidative stress stimulates cytosolic Ca<sup>2+</sup> increases (Bhattacharjee, 2005). The allelopathic toxin catechin was reported to cause rapid ROS production, followed by ROS-induced Ca<sup>2+</sup> increases in diffuse knapweed (*Centaurea diffusa*) and *Arabidopsis* roots (Bais et al., 2003). It was reported that pre-treatment of *Arabidopsis* plants with the calcium channel blocker lanthanum chloride (LaCl<sub>3</sub>) attenuated the inducing effect of H<sub>2</sub>O<sub>2</sub> on ERF6, suggesting that Ca<sup>2+</sup> is playing a signaling role, which is downstream from ROS, in the induction of this TF by H<sub>2</sub>O<sub>2</sub> (Sewelam et al., 2013). On the contrary, other research groups have reported that Ca<sup>2+</sup> works upstream of ROS. For example, it was reported that inhibitors of Ca<sup>2+</sup> fluxes inhibit both increase in cytosolic Ca<sup>2+</sup> and

H<sub>2</sub>O<sub>2</sub>, whereas inhibitors of NADPH oxidase blocks only the oxidative burst (Abuharbeid et al., 2004). Mechanical forces (e.g., touch) were found to trigger rapid and transient increases in cytosolic Ca<sup>2+</sup> and to stimulate apoplastic ROS production. The production of ROS was inhibited by pre-treatment with Ca<sup>2+</sup> channel blockers (Monshausen et al., 2009), suggesting a role for Ca<sup>2+</sup> as a prerequisite of ROS production under mechanical stimuli. To avoid this ostensible contradiction, future studies should consider the presence of a large number of sources for ROS production as well as a plethora of Ca<sup>2+</sup> subcellular sources, in addition to the expected feedback mechanisms. Nevertheless, these studies, at least, designate a crucial role for ROS-Ca<sup>2+</sup> signaling during plant responses to stresses that should be considered when constructing global plant signaling networks.

## Nitric Oxide (NO)

Nitric oxide (NO) is a small, water-, and lipid-soluble free radical gas with well-characterized signaling roles in mammalian systems (Furchtgott, 1995; Neill et al., 2003; Moreau et al., 2010). Nitric oxide production by plants and its involvement in plant growth were described in the late 1970s (Anderson and Mansfield, 1979; Klepper, 1979). Research on the effects of NO in plants focused on atmospheric pollution by the oxides of nitrogen, NO and NO<sub>2</sub> (nitrogen dioxide; Hufton et al., 1996). It was revealed that plants not only respond to atmospheric NO, but also produce considerable amounts of endogenous NO (Wildt et al., 1997). However, research on NO as a signaling molecule in plants started with the work done by Leshem and Haramaty (1996) and became well-established after the description of its role in plant defense signaling (Delledonne et al., 1998; Durner et al., 1998, 1999). Currently, it is well-known that NO plays an important signaling role in plant growth, development and defense responses (Besson-Bard et al., 2008; Moreau et al., 2010). It was reported that ROS and NO are produced concomitantly under various stresses and can interact with each other to induce a defense response (Neill et al., 2002; Yoshioka et al., 2009; Molassiotis and Fotopoulos, 2011). NO could have toxic or protective effects, depending on its concentration, combination with ROS compounds, and its subcellular localization (Correia-Aragunde et al., 2015).

Many reports have suggested interconnected signaling roles between ROS and NO during plant response to different stresses. Generation of NO at the same time as H<sub>2</sub>O<sub>2</sub> in response to pathogen infection was found to mediate defense responses similar to those seen following H<sub>2</sub>O<sub>2</sub> production (Delledonne et al., 1998; Durner et al., 1998; Asai and Yoshioka, 2009; Del Río, 2015). Delledonne et al. (1998) reported that treatment of soybean cultures with avirulent *P. syringae* induces rapid NO synthesis with kinetics similar to H<sub>2</sub>O<sub>2</sub> generation, indicating an interaction between NO and H<sub>2</sub>O<sub>2</sub> during plant response to pathogen attack. NO biosynthesis was reported to be regulated by H<sub>2</sub>O<sub>2</sub>-mediated activation of MAP Kinase 6 in *Arabidopsis* (Wang et al., 2010). A proteomic study on salt-stressed citrus plants pre-treated with H<sub>2</sub>O<sub>2</sub> or NO has suggested an overlap between H<sub>2</sub>O<sub>2</sub> and NO signaling pathways in acclimation to salinity (Tanou et al., 2009, 2010). Under drought stress, it was suggested that ROS and NO interact to

induce ABA biosynthesis to affect stomatal closure (reviewed by Neill et al., 2003). Regarding the mechanisms by which NO exerts its effects, it is suggested that NO may deliver its signaling roles via modulating the activity of proteins through nitrosylation and probably tyrosine nitration, in addition to the role that NO can act as a Ca-mobilizing messenger (Besson-Bard et al., 2008). ABA signaling in guard cells was found to be negatively regulated by NO through S-nitrosylation-mediated inhibition of the open stomata 1 (OST1)/sucrose non-fermenting 1 (SNF1)-related protein kinase 2.6 (SnRK2.6; Wang et al., 2015). In fact, NO can interact with ROS in different ways and might work as an antioxidant molecule during various stresses (Beligni and Lamattina, 1999; Correa-Aragunde et al., 2015). Moreover, modulation of superoxide formation by NO (Caro and Puntarulo, 1998) and inhibition of lipid peroxidation (Boveris et al., 2000) could illustrate a potential antioxidant role for NO. The oxidative damage in sorghum embryos was found to be alleviated by pre-treatment with sodium nitroprusside and diethylenetriamine NONOate as sources of exogenous NO (Jasid et al., 2008). Alternatively, excess NO can result in nitrosative stress (Hausladen et al., 1998), so a positive balance of ROS/NO is essential.

## Mitogen-Activated Protein Kinases

Mitogen (induces mitotic division)-activated protein kinases (MAPKs) are evolutionary conserved enzymes. In eukaryotes, signaling pathways arbitrated by MAPKs have been considered as a general signal transduction mechanism that links different receptors to their cellular and nuclear targets (Tena et al., 2001). The signaling events mediated by MAPKs are composed of three functionally interlinked protein kinases: a MAP kinase kinase kinase (MAPKKK), a MAP kinase kinase (MAPKK), and a MAP kinase (MAPK; Rodriguez et al., 2010; Sinha et al., 2011). In this phosphorylation module, a MAPKKK phosphorylates and activates a particular MAPKK, which in turn phosphorylates and activates a MAPK by phosphorylation of the tyrosine and threonine residues in the TXY motif (Qi and Elion, 2005).

In the *Arabidopsis* genome, 20 MAPK, 10 MAPKK, and 60 MAPKKK encoding genes were identified (Ichimura et al., 2002). The current functional analysis of MAPK cascades, mainly in *Arabidopsis*, revealed that plants have an overall of 24 MAPK pathways of which only a small set has been investigated so far (Wrzaczek and Hirt, 2001). This may reflect why MAPK signaling cascades are so complicated. The challenge ahead is to describe the elements of plant MAPK cascades and to specify roles of individual MAPK cascade genes, in particular signaling pathways (Wrzaczek and Hirt, 2001). The spatial and temporal expression and interaction characteristics of MAPKs are suggested to define their specificity in different signaling pathways (Dietz et al., 2010). The *Arabidopsis* mitogen-activated protein kinase 8 (MPK8) was reported to connect protein phosphorylation,  $\text{Ca}^{2+}$ , and ROS in wound signaling pathways (Takahashi et al., 2011).

Some of the components of MAPK cascades are elicited by cold, drought,  $\text{H}_2\text{O}_2$ , heat, wounding, pathogens, elicitors, ABA, SA, and ethylene (reviewed by Bowler and Fluhr, 2000). In many

eukaryotes, the transmission of oxidative signals is controlled by protein phosphorylation involving MAPKs (Kyriakis and Avruch, 1996; Gustin et al., 1998; Pitzschke and Hirt, 2006; Xing et al., 2008). On the one hand, MAPKs can be activated by accumulation of  $\text{H}_2\text{O}_2$ , on the other hand they can trigger an  $\text{H}_2\text{O}_2$ -induced oxidative burst (Nakagami et al., 2005; Pitzschke et al., 2009; reviewed by Petrov and Van Breusegem, 2012). In *Nicotiana benthamiana*, the MAPK cascades MEK2-SIPK/NTF4 and MEK1-NTF6 were reported to participate in the regulation of the radical burst induced by the oomycete pathogen *P. infestans* through NO and RbohB-dependant ROS generation (Asai et al., 2008). Using *Arabidopsis* protoplasts, a correlation was revealed between the activation of plant MAPK cascade and  $\text{H}_2\text{O}_2$ , which is generated by various stress factors. In this study, it was observed that  $\text{H}_2\text{O}_2$  activates the MAPKKK, ANP1, which in turn phosphorylates the downstream kinases, AtMPK3 and AtMPK6 (Kovtun et al., 2000). Protein phosphorylation through MAPK cascades was suggested to trigger a positive feedback regulation of  $\text{Ca}^{2+}$  and ROS via the activation of RbohD and RbohF in *Arabidopsis* (Kimura et al., 2012). A maize MAPK, MAP65-1a, was reported to positively control  $\text{H}_2\text{O}_2$  amplification and to enhance the antioxidant enzymes SOD and APX through the brassinosteroid signaling pathway (Zhu et al., 2013). The expression of the *Arabidopsis OXI1* gene, encoding a serine/threonine kinase, was induced in response to a broad range of  $\text{H}_2\text{O}_2$ -producing stimuli and OXI1 kinase activity itself was also induced by  $\text{H}_2\text{O}_2$  *in vivo* (Rentel et al., 2004). Application of bioinformatics and computational analysis would be required to illuminate how different MAPKs coordinate different plant signaling events.

## Abscisic Acid (ABA)

Substantial evidence postulates that ABA plays a vital role in controlling downstream responses essential for adaptation to stress (Leung and Giraudat, 1998; Raghavendra et al., 2010). These responses include changes in stomatal conductance, growth, osmolyte accumulation, and gene expression (Chen et al., 2002; Verslues and Zhu, 2004; Krasensky and Jonak, 2012). In contrast to the positive role of ABA in abiotic stress response, ABA has been considered as a negative regulator of disease resistance. This negative effect appears to be due to the obstruction by ABA of biotic stress signaling pathways that are orchestrated by SA, JA, and ethylene (Coego et al., 2005). ABA can also improve disease resistance by modifying cell wall deposits, such as callose (Mauch-Mani and Mauch, 2005).

A simultaneous enhanced level of ROS and ABA in plant tissues has been monitored under different types of environmental stresses. The concomitant enhancement of ROS and ABA during stress has been suggested to be a node in cross-tolerance to multiple types of stresses (Verslues and Zhu, 2004). It has been indicated that ROS generated by NADPH oxidases work downstream of ABA in mediating stomatal closure during stress (reviewed by Verslues and Zhu, 2004). It was reported that the production of  $\text{H}_2\text{O}_2$  in the chloroplasts, mitochondria and peroxisomes under water stress was abolished in the leaves of maize plants pre-treated with the ABA biosynthesis inhibitor (tungstate) or in an ABA mutant plants, indicating that ABA is

required for H<sub>2</sub>O<sub>2</sub> production in these compartments (Hu et al., 2006). It was demonstrated that a temporal-spatial interaction between ROS and ABA regulates rapid systemic acquired acclimation to environmental challenges in plants (Suzuki et al., 2013). In response to heat and oxidative stresses, it was reported that H<sub>2</sub>O<sub>2</sub> mediates a crosstalk between the plant hormones; brassinosteroid and ABA, via a signaling pathway through which brassinosteroid induces a rapid and transient H<sub>2</sub>O<sub>2</sub> production by NADPH oxidase. The process in turn activates increased ABA levels, leading to further increases in H<sub>2</sub>O<sub>2</sub> production and improved stress tolerance in tomato plants (Zhou et al., 2014).

## Salicylic Acid, Jasmonic Acid, and Ethylene

Various plant developmental and stress responses require a tuned coordination between the phytohormones SA, JA and ethylene. It is thought that, in *Arabidopsis*, a JA-ethylene signaling pathway is important to mediate resistance to necrotrophic pathogens (feed on dead tissues), such as *Botrytis cinerea*. On the other hand, the SA signaling pathway is supposed to mediate resistance to biotrophic pathogens (feed on living tissues), such as *P. syringae* (Thomma et al., 2001; Anderson et al., 2004). However, it has been suggested that many genes are co-regulated by these hormones and there is considerable genetic evidence for crosstalk between these signaling pathways (Schenk et al., 2000; Glazebrook et al., 2003; Leon-Reyes et al., 2009). Regarding ROS signaling, it was suggested that SA, JA, and ethylene work together with ROS and play crucial regulatory roles in plant defense responses (Mur et al., 2006; Mhamdi et al., 2010).

**Salicylic acid (SA)** is well-known to regulate both systemic acquired resistance (SAR) and local disease resistance mechanisms, including host cell death and defense gene expression (Park et al., 2007; Vlot et al., 2008). It was reported that SA elicits an oxidative burst, which in turn promotes SAR (Senaratna et al., 2000). One of the proposed modes of action of SA is the inhibition of catalase, a major enzyme scavenging H<sub>2</sub>O<sub>2</sub>, thereby increasing cellular concentrations of H<sub>2</sub>O<sub>2</sub>, which acts as a second messenger and activates defense-related genes (Ananieva et al., 2002). The extracellular production of ROS was found to enhance SA production inducing stomatal closure in *Arabidopsis* (Khokon et al., 2011). It was reported that SA accumulation in *siz1* mutant plants enhances stomatal closure and drought tolerance through controlling ROS accumulation in *Arabidopsis* guard cells (Miura et al., 2013). It was suggested that SA accumulation and signaling is activated by increased H<sub>2</sub>O<sub>2</sub> levels through changes in the glutathione pool in an *Arabidopsis* catalase 2 (*cat2*) mutant (Han et al., 2013a).

**Jasmonic acid (JA)** and methyl jasmonate (MeJA), are natural products regulating plant development, response to environmental challenges, and gene expression (Bell et al., 1995). A signaling role for JA in defense responses has been suggested in plants (Farmer and Ryan, 1992; Wasternack and Hause, 2013). Currently, there is accumulating evidence suggesting a strong relationship between ROS and JA signaling. For instance, it was suggested that MeJA pretreatment of *Arabidopsis* inhibited O<sub>3</sub>-induced H<sub>2</sub>O<sub>2</sub> production and SA accumulation and completely

abolished O<sub>3</sub> induced cell death (Rao et al., 2000). It was reported that ROS generated by RbohD and RbohF enzymes are important for JA-induced expression of genes regulated by MYC2, a TF involved in the JA-mediated response, where treating *RbohD* and *RbohF* mutant plants with MeJA failed to increase the expression levels of various MYC2 downstream genes (Maruta et al., 2011). A dynamic interaction between JA and ROS was characterized to regulate lignin biosynthesis in response to cell wall damage where ROS produced by RbohD and JA-isoleucine generated by JASMONIC ACID RESISTANT1 were found to form a negative feedback loop that influence lignin accumulation (Denness et al., 2011). It was revealed that the intracellular ROS production in *cat2* mutant *Arabidopsis* plants leads to activating the JA pathway and its related genes with glutathione accumulation as an intermediate (Han et al., 2013b).

**Ethylene (C<sub>2</sub>H<sub>4</sub>)** is one of the simplest organic molecules that have biological activity in plants (Zarembinski and Theologis, 1994). It is well-documented that ethylene is a main player in PCD, either during senescence (Orzaez and Granell, 1997), oxidative stress imposed by ozone (Overmyer et al., 2000), or plant pathogen interactions (Lund et al., 1998). In addition, it was suggested that ethylene is crucial for H<sub>2</sub>O<sub>2</sub> production during PCD in tomato suspension cells (de Jong et al., 2002). It was reported that the ethylene receptor ETR1 mediates H<sub>2</sub>O<sub>2</sub> signaling in guard cells in *Arabidopsis* (Desikan et al., 2005). Together, these findings suggest a cross-talk between ethylene and ROS in plant signaling. Treating *Arabidopsis* plants with the bacterial elicitor flagellin (flg22) enhanced an oxidative burst which was inhibited in ethylene-insensitive mutants, *etr1* and *ein2*, indicating a requirement of ethylene signaling for ROS production (Mersmann et al., 2010). A synergistic biosynthesis of ethylene and ROS production, mediated by the plasma membrane bound enzymes RbohD and RbohF, was reported in tobacco plants infected with the hemibiotrophic *Phytophthora parasitica* (Wi et al., 2012).

## Transcription Factors

Regulation of gene expression at the transcriptional level influences or controls many of the biological processes in a cell or organism, such as progression through the cell cycle, metabolic and physiological balance, and responses to environment (Riechmann et al., 2000). Plant stress responses are regulated by multiple signaling pathways that activate gene transcription and its downstream machinery. Using data from ROS-related microarray studies, Gadjev et al. (2006) examined the expression of 1,500 TFs in *Arabidopsis* in response to different ROS, including singlet oxygen, H<sub>2</sub>O<sub>2</sub> and OH<sup>•</sup>. They reported that different ROS induced or repressed the expression of about 500 of these annotated TFs in *Arabidopsis*. Nevertheless, the transcriptional regulation mechanisms mediating ROS signaling is not fully understood. It is suggested that the regulation of the TFs activity by the most important ROS, H<sub>2</sub>O<sub>2</sub>, is managed at several levels including: (1) upregulation of TF expression or increasing both mRNA stability and translation; (2) increasing the stability of the TF by decreasing its association with the protein degrading ubiquitin E3 ligase complex or by inhibiting this complex; (3) nucleo-cytoplasmic traffic by transferring or

masking nuclear localization signals, or by releasing the TF from partners or from membrane anchors; and (4) DNA binding and nuclear transactivation by adapting TF affinity toward DNA, co-activators or repressors, and by targeting specific regions of chromatin to activate individual genes (Marinho et al., 2014). Many examples of TFs that are regulated by ROS have been revealed. Simple organisms, such as bacteria and yeast, sense the enhanced production of ROS using redox sensitive TFs (Mittler et al., 2004). In bacteria, OxyR (oxygen regulated) and PerR (Peroxide Regulon Repressor) are TFs that are directly activated by H<sub>2</sub>O<sub>2</sub>. The tetrameric OxyR protein is characterized as a regulatory protein that activates nine out of twelve early H<sub>2</sub>O<sub>2</sub>-induced proteins. The OxyR transcription activator exists in two forms, reduced and oxidized; only the oxidized state is able to initiate transcription (Storz et al., 1990; Storz and Imlay, 1999). In yeast, four TFs, namely, Yap1, Maf1, Hsf1, and Msn2/4, were reported to be regulated by ROS. For example, Yap1 is regulated by H<sub>2</sub>O<sub>2</sub> at the level of nucleo-cytoplasmic trafficking. Under oxidative stress, the export of Yap1 to the nucleus is decreased and Yap1 is kept longer in the nucleus where it regulates its target genes (Delaunay et al., 2000). Yap1 has a key role in the oxidative stress response, redox homeostasis and electrophilic response, regulating the transcription of genes encoding antioxidant and detoxification enzymes in yeast cells (Marinho et al., 2014). In multicellular organisms, nine different TFs, namely AP-1, NRF2, CREB, HSF1, HIF-1, TP53, NF-κB, NOTCH, SP1, and SCREB-1, are well-characterized to be regulated by ROS (Marinho et al., 2014).

## CONCLUSION

As reviewed here, plants have evolved complicated protection mechanisms to survive different environmental challenges. The recent functional molecular and physiological studies have produced new details attempting to unravel the complexity of these signaling pathways. It is evident now that there is no a specific linear signaling pathway for each stress, instead, there

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- are interconnected networks including common signaling events that are shared by various pathways represented by what we call cross-talk. From these big signaling networks many branches arise for specificity. As ROS are well-known to be produced by plants in response to different biotic and abiotic stresses, they are designated to work at the cross road within these complex signaling networks. ROS play this central signaling role through their evident interactions, whether upstream or downstream, with other key signaling components, including membranes, NADPH oxidases, G-proteins, calcium, redox homeostasis, MAPKs, plant hormones (such as SA, JA, ABA, and ethylene) and TFs. The recent research implies an early and vital role for photosynthesis in sensing various environmental insults, not only abiotic, but also biotic ones, a concept that needs to be taken into consideration when studying stress signaling pathways in plants. Despite all of these achievements, great efforts are still required to be able to reconstruct larger signaling networks that may include ROS at the convergent points. In this regard, bioinformatics and systems biology approaches are nominated to greatly help in constructing global signaling networks. As a result, these global networks would improve our understanding of plant biology and assist us to develop new strategies for higher plant productivity in the face of increasingly severe environmental conditions and the high demand for food, fiber, and energy crops.

## AUTHOR CONTRIBUTIONS

All authors wrote and approved the final version of the manuscript.

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# Alternative Oxidase Pathway Optimizes Photosynthesis During Osmotic and Temperature Stress by Regulating Cellular ROS, Malate Valve and Antioxidative Systems

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The present study reveals the importance of alternative oxidase (AOX) pathway in optimizing photosynthesis under osmotic and temperature stress conditions in the mesophyll protoplasts of *Pisum sativum*. The responses of photosynthesis and respiration were monitored at saturating light intensity of 1000  $\mu\text{moles m}^{-2} \text{s}^{-1}$  at 25°C under a range of sorbitol concentrations from 0.4 to 1.0 M to induce hyperosmotic stress and by varying the temperature of the thermo-jacketed pre-incubation chamber from 25 to 10°C to impose sub-optimal temperature stress. Compared to controls (0.4 M sorbitol and 25°C), the mesophyll protoplasts showed remarkable decrease in  $\text{NaHCO}_3$ -dependent  $\text{O}_2$  evolution (indicator of photosynthetic carbon assimilation), under both hyper-osmotic (1.0 M sorbitol) and sub-optimal temperature stress conditions (10°C), while the decrease in rates of respiratory  $\text{O}_2$  uptake were marginal. The capacity of AOX pathway increased significantly in parallel to increase in intracellular pyruvate and reactive oxygen species (ROS) levels under both hyperosmotic stress and sub-optimal temperature stress under the background of saturating light. The ratio of redox couple (Malate/OAA) related to malate valve increased in contrast to the ratio of redox couple (GSH/GSSG) related to antioxidative system during hyper-osmotic stress. Further, the ratio of GSH/GSSG decreased in the presence of sub-optimal temperature, while the ratio of Malate/OAA showed no visible changes. Also, the redox ratios of pyridine nucleotides increased under hyper-osmotic (NADH/NAD) and sub-optimal temperature (NADPH/NADP) stresses, respectively. However, upon restriction of AOX pathway by using salicylyhydroxamic acid (SHAM), the observed changes in  $\text{NaHCO}_3$ -dependent  $\text{O}_2$  evolution, cellular ROS, redox ratios of Malate/OAA, NAD(P)H/NAD(P) and GSH/GSSG were further aggravated under stress conditions with concomitant modulations in NADP-MDH and antioxidant enzymes. Taken together, the results indicated the importance of AOX pathway in optimizing photosynthesis under both hyper-osmotic stress and sub-optimal temperatures. Regulation of ROS through redox couples related to malate valve and antioxidant system by AOX pathway to optimize photosynthesis under these stresses are discussed.

**Keywords:** alternative oxidase, cytochrome oxidase, photosynthesis, reactive oxygen species, respiration, salicylyhydroxamic acid

## INTRODUCTION

The mitochondrial oxidative electron transport chain in higher plants is branched at ubiquinone, leading to cyanide sensitive cytochrome oxidase (COX) and cyanide resistant alternative oxidase (AOX) pathways (Millar et al., 2011). The COX pathway transfers electrons from ubiquinone to molecular O<sub>2</sub> through complex III and complex IV and generates a proton gradient which is coupled to ATP synthesis. The electron transport through AOX pathway is mediated by a quinol oxidase and uncoupled from ATP synthesis. However, energy is liberated as heat when the AOX pathway is operative (Day and Wiskich, 1995; Siedow and Umbach, 2000; Schertl and Braun, 2014; Pu et al., 2015). Although, AOX catalyzes the energy-wasteful respiration, its (up) regulation in terms of activity, engagement and expression during development and biotic/abiotic stresses indicates its physiological importance other than thermogenesis (Fung et al., 2006; Matos et al., 2007; Giraud et al., 2008; Arnholdt-Schmitt, 2009; Vanlerberghe et al., 2009; Fu et al., 2010; Florez-Sarasa et al., 2011; Cvetkovska and Vanlerberghe, 2013; Vishwakarma et al., 2014; Garmash et al., 2015; Rogov and Zvyagilskaya, 2015).

Mitochondrial functions contribute to the metabolic flexibility that is essential for plant cells to adjust to highly variable environment (Vanlerberghe, 2013). The functioning of AOX pathway through hand-in-hand cooperation with COX pathway to optimize photosynthetic metabolism (Padmasree and Raghavendra, 1999a,b,c, 2001a,b; Yoshida et al., 2006; Feng et al., 2007; Strodtkötter et al., 2009; Dinakar et al., 2010a,b; Florez-Sarasa et al., 2011; Bailleul et al., 2015; Vishwakarma et al., 2015) and its active participation in balancing carbon/nitrogen availability with sink capacity or antioxidant defense system has added new dimensions to its existence in leaf cells (Parsons et al., 1999; Vanlerberghe et al., 2002; Sieger et al., 2005; Umbach et al., 2005; Yoshida et al., 2007; Gandin et al., 2009, 2014; Dahal et al., 2014). Thus the relative contribution of COX and AOX pathways to total respiration is known to be flexible and dependant on environmental conditions (Gonzalez-Meler et al., 1999; Searle et al., 2011; Liu et al., 2015).

Water stress affects various parameters including stomatal conductance, root growth, leaf number, total leaf area, photosynthetic quantum yield, ATP, NADPH synthesis and the utilization of assimilates (Vandoorne et al., 2012; Cano et al., 2014; Esmailpour et al., 2015) along with the partitioning of electrons between the COX and AOX pathways (Ribas-Carbo et al., 2005). There is a considerable ambiguity in the partitioning of electrons between these pathways. In soyabean and wheat, water stress caused a significant shift of electrons from the COX to the AOX pathway while in leaves of bean and pepper water stress decreased SHAM-resistant respiration, with no effect on cyanide-resistant respiration (Gonzalez-Meler et al., 1997; Ribas-Carbo et al., 2005; Vassileva et al., 2009). Several other studies suggested that changes in electron partitioning between the two respiratory pathways under a given stress were mostly due to the decrease in the activity of the COX pathway rather than an increase in the activity of the AOX pathway (Peñuelas et al., 1996; Lambers et al., 2005; Galle et al., 2010). An increase in the

expression of AOX genes and its activity in photosynthetic tissues has been reported in plants subjected to low/high temperatures (Vanlerberghe and McIntosh, 1992a,b; Fiorani et al., 2005; Wang et al., 2011) or water stress (Bartoli et al., 2005; Ribas-Carbo et al., 2005). Several reports proposed that the AOX pathway maintains electron flow during cold conditions to alleviate the cellular reactive oxygen species (ROS; Purvis et al., 1995; Armstrong et al., 2008; Grabelnych et al., 2014). The ability of AOX pathway to maintain flux in the cold was suggested to be due to (i) its reduced sensitivity to temperature as compared to COX pathway (Kiener and Bramlage, 1981; McNulty and Cummins, 1987; Stewart et al., 1990b) and (ii) an increase in the *de novo* synthesis of AOX protein (Stewart et al., 1990a,b; Vanlerberghe and McIntosh, 1992a; Gonzalez-Meler et al., 1999; Ribas-Carbo et al., 2000). However, the studies of Kühn et al. (2015) suggested that any decrease in electron flux through the COX or AOX pathways trigger common as well as distinct cellular responses which are in-turn dependent on the growth conditions.

Osmotic and temperature stresses are common abiotic stresses to which plants are frequently exposed under conditions of drought and flooding/frost in natural environment. Long term exposure to any biotic or abiotic stress conditions may cause cellular damage and cell death in susceptible plants. However, during short term exposure, the plants adapt or acclimatize to these stress conditions by various mechanisms. Intracellular adjustments like alteration in redox status, ROS and antioxidant levels, particularly mediated by mitochondria are essential for plant cells to acclimatize with changing environmental conditions to maintain redox homeostasis (Foyer and Noctor, 2003, 2005; Baier and Dietz, 2005; Gechev et al., 2006; Noctor, 2006; Navrot et al., 2007; Noctor et al., 2007; Dinakar et al., 2010a; Scheibe and Dietz, 2012; Tripathy and Oelmüller, 2012; Vishwakarma et al., 2014, 2015; Considine et al., 2015; Deng et al., 2015; Sevilla et al., 2015; Zhao et al., 2015). Also, it is intriguing to know that the same parameters were found to be crucial in mediating the beneficial interactions between chloroplasts and mitochondria to optimize photosynthetic carbon assimilation under optimal light and CO<sub>2</sub> (Padmasree and Raghavendra, 1999c; Dinakar et al., 2010a; Yoshida et al., 2011). However, it is not clear which pathway (COX or AOX) of mitochondrial electron transport would play a crucial role in optimizing photosynthesis under hyperosmotic stress or sub-optimal temperature stress. Therefore, the present study was performed using mesophyll protoplasts of pea as the model system to examine the importance of AOX pathway over COX pathway and its coordination with malate valve and glutathione redox system in regulating cellular ROS to optimize photosynthesis under hyper-osmotic and sub-optimal temperature stresses.

## MATERIALS AND METHODS

### Plant Material and Isolation of Mesophyll Protoplasts

Pea plants (*Pisum sativum* L. cv. Arkel; seeds obtained from Pocha seeds, Pune, India) were grown outdoors under

natural photoperiod of approximately 12 h and average daily temperatures of 30°C day/20°C night. The second pair of fully expanded leaves were picked from 8 to 10 days old plants and were used for isolating mesophyll protoplasts. About 10 pairs of leaves were excised from the plants and mesophyll protoplasts were isolated from leaf strips devoid of lower epidermis by enzymatic digestion with 2% (w/v) Cellulase Onozuka R-10 and 0.2% (w/v) Macerozyme R-10 (Yakult Honsha Co. Ltd, Nishinomiya, Japan), under low light intensities of 50–100  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . The protoplasts were collected by filtration through 60  $\mu\text{m}$  nylon filter and purified by centrifugation at 100 g for 5 min, thrice at 4°C. The protoplasts were finally stored on ice in a suspension medium containing 10 mM Hepes-KOH, pH 7.0, 0.4 M sorbitol, 1.0 mM CaCl<sub>2</sub>, and 0.5 mM MgCl<sub>2</sub> until further use and chlorophyll was estimated (Padmasree and Raghavendra, 1999a). The purity of protoplast preparation normally ranged from 90 to 97%.

## Stress Treatments

Mesophyll protoplasts equivalent to 12  $\mu\text{g}$  Chl were subjected to hyper-osmotic stress or sub-optimal temperature stress under a saturating light intensity (1000  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) in the pre-incubation chamber by increasing (step wise) the concentration of sorbitol in the pre-incubation medium from 0.4 M (isotonic) to 1.0 M (hypertonic) or by decreasing the temperature in pre-incubation chamber from 25°C to 10°C using a refrigerated circulatory water bath (Julabo F10) for 10 min, respectively (Dinakar et al., 2010b). Protoplast samples pre-incubated at 1000  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , 25°C temperature and 0.4 M sorbitol were treated as controls (Saradadevi and Raghavendra, 1994). NaHCO<sub>3</sub> (1.0 mM) is added to the pre-incubation media so as to avoid photorespiration and associated O<sub>2</sub> burst. The composition of the pre-incubation medium used were same as that of reaction medium (other than sorbitol) described in Dinakar et al. (2010b).

## Monitoring Total Respiration and Photosynthesis

After hyper-osmotic and temperature stress treatments in the presence or absence of SHAM, mesophyll protoplasts equivalent to 10  $\mu\text{g}$  Chl were transferred from pre-incubation chamber to Clark-type oxygen electrode cuvette and the total rates of respiration and photosynthesis (carbon assimilation/PS II activity) were measured polarographically in a reaction medium as described in Dinakar et al. (2010a). In controls, as the rates of respiratory O<sub>2</sub> uptake and photosynthetic O<sub>2</sub> evolution attained steady state after 3 min in dark and 4 min after switching on light, respectively, we restricted to monitor respiration and photosynthesis during steady state for 5 and 10 min, in the dark and light, respectively, using a Clark type oxygen electrode system, controlled by Hansa-Tech software at 25°C. Saturating light (1000  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) was provided by a 35 mm slide projector (with xenophot [halogen] lamp, 24 V/150 W). The photosynthetic carbon assimilation rates were measured as NaHCO<sub>3</sub> (1.0 mM) dependent O<sub>2</sub> evolution and PS II activity was measured as *p*-BQ-dependent (1.0 mM) O<sub>2</sub> evolution in the presence of an uncoupler (5 mM NH<sub>4</sub>Cl).

Oxygen content (253  $\mu\text{M}$ ) in the electrode chamber was pre-calibrated at 25°C with air saturated water using sodium dithionite.

## Capacity of COX and AOX Pathway

The capacity of AOX pathway was determined as the O<sub>2</sub> uptake sensitive to 10 mM SHAM in the presence of 1 mM KCN (Vanlerberghe et al., 2002), while the capacity of COX pathway was determined as the O<sub>2</sub> uptake sensitive to 1 mM KCN in the presence of both 10 mM SHAM and 1  $\mu\text{M}$  carbonyl cyanide m-chlorophenylhydrazone (CCCP, an uncoupler) as adenylates determine the flux of electrons through COX pathway (Dinakar et al., 2010a,b).

## Protein Extraction and Immunodetection

After stress treatments, mesophyll protoplasts equivalent to 10  $\mu\text{g}$  Chl were withdrawn and centrifuged at 100 g for 1 min. The pelleted protoplasts were snap frozen in liquid nitrogen and homogenized in 125 mM Tris-HCl (pH 6.8) containing 5% (w/v) SDS and 1 mM PMSF. The homogenate was centrifuged at 10,000 g for 10 min. Protein estimation was done according to the method of Lowry et al. (1951). SDS-PAGE of mesophyll protoplast proteins was performed according to Laemmli (1970). The proteins separated on 12.5% SDS-PAGE were transferred electrophoretically from the gel onto polyvinylidene difluoride (PVDF) membranes (Towbin et al., 1979). The blots were probed with 1:100 dilution of D1 antibodies (Agrisera, Vännäs, Sweden) followed by 1:5000 dilution of goat antirabbit IgG alkaline phosphatase conjugate and developed using nitro-blue-tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate as substrates.

## Detection of Reactive Oxygen Species (ROS)

Intracellular production of ROS was measured by using a non polar fluorescent dye 2, 7, -dichlorofluorescein diacetate (H<sub>2</sub>DCF-DA), which is converted to membrane – impermeable polar derivative H<sub>2</sub>DCF by cellular esterases and rapidly oxidized to highly fluorescent DCF by intracellular H<sub>2</sub>O<sub>2</sub> and other peroxides. Mesophyll protoplasts loaded with 5  $\mu\text{M}$  H<sub>2</sub>DCF-DA (Dinakar et al., 2010a) were subjected to hyper-osmotic and sub-optimal temperature stress for 10 min at saturating light intensities (1000  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ). Immediately, after stress treatments, DCF fluorescence of mesophyll protoplasts was measured by using a Hitachi F- 4010 fluorescence spectrophotometer with excitation and emission wavelengths set at 488 and 525 nm, respectively. DCF fluorescence of protoplasts pre-incubated under a saturating light intensity (1000  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) at 25°C and 0.4 M sorbitol were treated as controls.

## Quantification of Pyruvate and Adenylates

After stress treatments at saturating light, the metabolic reactions of mesophyll protoplasts were quenched with HClO<sub>4</sub> as described in Padmasree and Raghavendra (1999a). The samples

neutralized with KOH were centrifuged at 7000  $g$  and the cleared supernatant was used for estimation of pyruvate, ATP and ADP. The intracellular levels of pyruvate were measured spectrophotometrically using enzymatic assay coupled to NADH oxidation as described in Dinakar et al. (2010b). Similarly, the ATP levels were measured using enzymatic assay coupled to NADPH formation while the ADP levels were measured by coupling to NADH utilization (Padmasree and Raghavendra, 1999a).

### Quantification of Malate and OAA

After exposure to stress treatments in the presence and absence of SHAM, at saturating light, aliquots of mesophyll protoplasts equivalent to 100  $\mu\text{g}$  Chl  $\text{ml}^{-1}$  were quenched with  $\text{HClO}_4$  and snap frozen in liquid nitrogen. After neutralization, the samples were centrifuged at 100  $g$  and the supernatant was used for the estimation of intracellular levels of malate and OAA spectrophotometrically. The malate was estimated by incubating the supernatant for 10 min at 25°C in the reaction medium containing 100 mM Tris-HCl, 630 mM hydrazine sulfate, 1.0 mM EDTA pH 9.0, 1.5 mM NAD. The reaction is initiated by the addition of 30U MDH as the concentration of malate is proportional to the amount of NAD reduced at 340 nm (Heineke et al., 1991). Further, the cellular levels of oxaloacetate was calculated from the equation of  $[(\text{oxoglutarate}) \times (\text{aspartate})]/[(\text{glutamate}) \times (6.61)]$ , as suggested by Heineke et al. (1991) based on the equilibrium of glutamate oxaloacetate transaminase (GOT;  $K = 6.61$ , Veech et al., 1969). The levels of oxoglutarate, aspartate and glutamate were determined as described in Bergmeyer (1983) by enzymatic assays coupled to NAD(H) oxidation or reduction.

### Quantification of GSH and GSSG

After stress treatments in the presence and absence of SHAM, protoplast samples equivalent to 100  $\mu\text{g}$  Chl were withdrawn and mixed immediately with 7% sulfosalicylic acid and snap frozen in liquid nitrogen. The samples were thawed and centrifuged for 10 min. 20  $\mu\text{l}$  of 7.5 M triethanolamine was added to the supernatant to neutralize the samples. Total, oxidized, reduced glutathione was determined spectrophotometrically at 412 nm by the cycling method described by Griffith (1980).

### Quantification of NAD(P) and NAD(P)H

Mesophyll protoplasts equivalent to 25  $\mu\text{g}$  Chl were withdrawn from the pre-incubation chamber with and without SHAM after the stress treatments. The samples were centrifuged at 3000  $g$  for 2 min and the pelleted protoplasts were homogenized either in 0.2 N HCl or in 0.2 M NaOH for NAD(P) $^+$  and NAD(P)H extraction, respectively. The homogenate was centrifuged at 10,000  $g$  for 10 min at 4°C. The supernatant was boiled for 1 min and rapidly cooled on ice. For NAD(P) $^+$  measurement the final pH of supernatant was brought between 5.0 and 6.0, while for NAD(P)H measurement the final pH was adjusted between 7.0 and 8.0. Pyridine nucleotides were quantified by monitoring phenazine methosulfate-catalyzed reduction of dichlorophenolindophenol (Queval and Noctor, 2007). For NAD $^+$  and NADH assay, the reaction was started by the addition

of ethanol in presence of alcohol dehydrogenase. On the other hand, for NADP $^+$  and NADPH assay, the reaction was started by addition of Glucose-6-phosphate dehydrogenase in the presence of Glucose-6-phosphate. The decrease in  $A_{600}$  was monitored for 3 min and concentrations of corresponding pyridine nucleotides were calculated using relevant standards (Vishwakarma et al., 2015).

### Assay of NADP-MDH

Mesophyll protoplasts equivalent to 40  $\mu\text{g}$  Chl were withdrawn from the pre-incubation chamber with and without SHAM after the stress treatments. NADP-dependent MDH was extracted and assayed according to Dutilleul et al. (2003). The NADP-MDH was extracted in buffer containing 25 mM Hepes-KOH (pH 7.5), 10 mM MgSO<sub>4</sub>, 1 mM Na<sub>2</sub>EDTA, 5 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 5% (w/v) insoluble polyvinylpyrrolidone, and 0.05% (v/v) Triton X-100. The homogenate was centrifuged for 5 min at 10,000  $g$  (4°C). The actual NADP-MDH activity was measured directly from supernatant (2.5  $\mu\text{g}$  chl) in assay buffer. Assay buffer was comprised of 25 mM Tricine-KOH (pH 8.3), 150 mM KCl, 1 mM EDTA, 5 mM DTT, 0.2 mM NADPH, and 2 mM oxaloacetate, plus sample. To fully activate the enzyme, supernatant (2.5  $\mu\text{l}$  chl) was pre-incubated for 30 min at 25°C in 40 mM Tricine-KOH (pH 9.0), 0.4 mM Na<sub>2</sub>EDTA, 120 mM KCl, 100 mM DTT, and 0.0025% (v/v) Triton X-100. After incubation, 2 mM oxaloacetate and 0.2 mM NADPH were added into total reaction volume and activity was measured at 340 nm.

### Assay of Superoxide Dismutase (SOD; E.C. 1.15.1.1), Catalase (CAT; E.C. 1.11.1.6), and Glutathione Reductase (GR; E.C. 1.6.4.2)

Mesophyll protoplasts equivalent to 100  $\mu\text{g}$  Chl in 600  $\mu\text{l}$  were withdrawn from the pre-incubation chamber with and without SHAM after the stress treatments. The samples were centrifuged at 100  $g$  for 1 min and the pelleted protoplasts were snap frozen in liquid nitrogen. The samples were homogenized in 50 mM phosphate buffer pH 7.0 containing 1 mM PMSF and centrifuged at 10,000  $g$  for 10 min. The supernatant was used for enzymatic assays of superoxide dismutase (SOD), catalase (CAT), and glutathione reductase (GR). The protein concentration in the enzyme extracts were determined by Lowry et al. (1951) using defatted BSA as standard. The SOD activity was determined following the method of Beauchamp and Fridovich (1971). CAT activity was measured spectrophotometrically by following the oxidation of H<sub>2</sub>O<sub>2</sub> at 240 nm according to the method of Patterson et al. (1984) and GR activity was determined by modifying the method of Jiang and Zhang (2001). Others details were followed as described in Dinakar et al. (2010a).

### Replications

The data presented are the average values of results ( $\pm$ SE) from atleast four repetitions conducted on different days. The differences between treatments were analyzed by one-way ANOVA, Student-Newman-Keuls method of multiple

comparison analysis using SigmaStat 3.1 software (San Jose, CA, USA).

## RESULTS

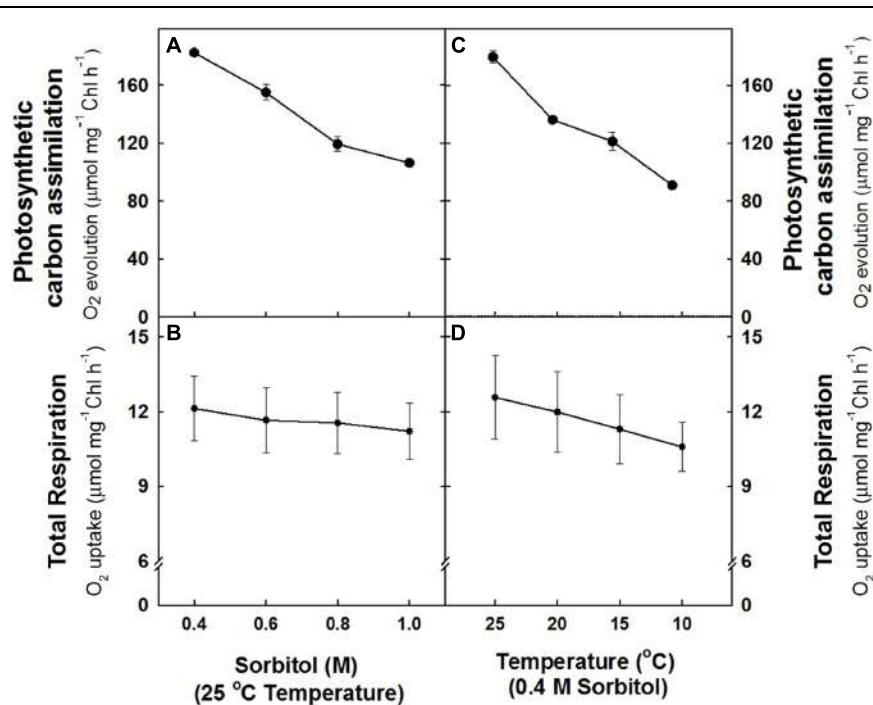
### Photosynthetic Carbon Assimilation and Respiration in Mesophyll Protoplasts Pre-incubated Under Hyper-Osmoticum and Sub-Optimal Temperatures at Saturating Light

In the study, the effect of hyper-osmotic stress and sub-optimal temperature stress on photosynthetic carbon assimilation ( $\text{NaHCO}_3$ -dependent  $\text{O}_2$  evolution) and respiration ( $\text{O}_2$  uptake) was monitored in mesophyll protoplasts under light. The  $\text{O}_2$  evolution rates ( $182.5 \pm 3 \mu\text{mol mg}^{-1} \text{Chl h}^{-1}$ ) observed at 0.4 M sorbitol (isotonic) at 25°C in light (control) declined remarkably up to  $\leq 42\%$  as the concentration of sorbitol was increased to 1.0 M (Figure 1A). In contrast, the rates of  $\text{O}_2$  uptake ( $12.12 \pm 1.3 \mu\text{mol mg}^{-1} \text{Chl h}^{-1}$ ) observed at 0.4 M sorbitol at 25°C temperature (control) decreased marginally by 7% of control with increase in sorbitol concentration to 1.0 M (Figure 1B). Similarly, any decrease in temperature of pre-illumination chamber at 0.4 M sorbitol in light also showed a profound effect on rates of  $\text{O}_2$  evolution as compared to rates of  $\text{O}_2$  uptake. Mesophyll protoplasts pre-incubated at 10°C showed

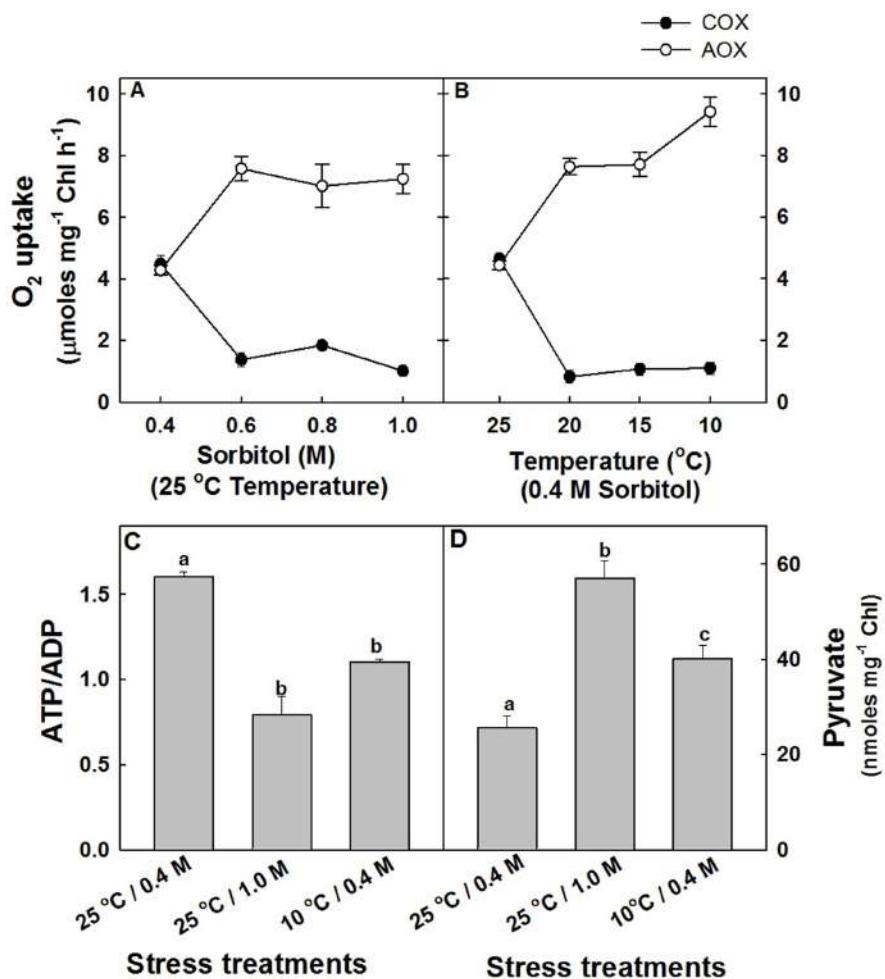
a remarkable decrease in  $\text{O}_2$  evolution rates ( $\leq 49\%$ ) while the decrease in  $\text{O}_2$  uptake rates ( $\leq 13\%$ ) were marginal as compared to control (Figures 1C,D).

### Effects of Hyper-Osmotic Stress and Sub-Optimal Temperature Stress on the Capacity of COX and AOX Pathways

Although the effects of hyper-osmotic stress and sub-optimal temperature stress on total respiratory  $\text{O}_2$  uptake of mesophyll protoplasts were marginal, the *in vivo* rates of COX (COX capacity) and AOX (AOX capacity) pathways were modulated significantly. In mesophyll protoplasts which were exposed to increasing sorbitol concentration at 25°C in saturating light, the capacity of COX pathway was decreased drastically by 77%, while the capacity of AOX pathway was stimulated by 70% at 1.0 M sorbitol as compared to protoplasts in 0.4 M sorbitol at 25°C under saturating light (Figure 2A). A similar trend was observed in response to sub-optimal temperature stress. With decreasing temperature under 0.4 M sorbitol at saturating light, the capacity of COX pathway of mesophyll protoplasts decreased remarkably by  $\leq 76\%$  and the AOX pathway increased significantly by  $\leq 1.2$  fold at 10°C as compared to protoplasts pre-incubated at 25°C under saturating light (Figure 2B). Since the decrease in COX capacity and the increase in AOX capacity were maximum at 1.0 M sorbitol and 10°C temperature, in all further experiments, the stress



**FIGURE 1 |** The rates of photosynthetic  $\text{O}_2$  evolution (A,C) and total respiration (B,D) in mesophyll protoplasts of pea pre-incubated for 10 min at a saturating light intensity of  $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$  under different concentrations of sorbitol (0.4 to 1.0 M) in the reaction media at 25°C and at different temperatures (25–10°C) at 0.4 M sorbitol in the reaction media. After exposing the mesophyll protoplasts to different osmotic and temperature treatments in light, the respiratory rates were measured for 5 min in darkness. The photosynthesis rates were measured as  $\text{NaHCO}_3$ -dependent (1.0 mM)  $\text{O}_2$  evolution for 10 min in light ( $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) using Clark-type oxygen electrode.



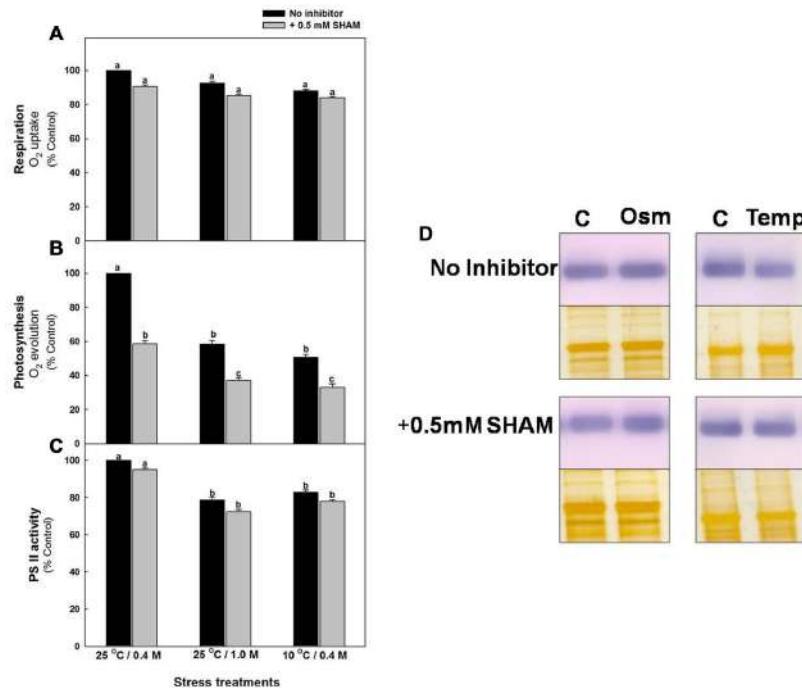
**FIGURE 2 |** The capacity of COX and AOX pathways of mitochondrial electron transport chain in mesophyll protoplasts of pea pre-incubated for 10 min at a saturating light intensity of  $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$  under different concentrations of sorbitol (0.4 to 1.0 M) in the reaction media at 25°C (A) and at different temperatures (25 to 10°C) under 0.4 M sorbitol (B). The open circles indicate the AOX pathway capacity while the closed circles indicate the COX pathway capacity. The changes in intracellular ATP/ADP (C) and pyruvate levels (D) in mesophyll protoplasts pre-incubated under 0.4 M (control), 1.0 M sorbitol (osmotic stress) at 25°C and 0.4 M sorbitol at 10°C (temperature stress) respectively, at a saturating light intensity of  $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$  for 10 min. Values represent the mean ( $\pm \text{SE}$ ) of four experiments and different letters represent values that are statistically different (ANOVA test,  $p \leq 0.05$ ).

treatments were restricted to 1.0 M sorbitol at 25°C to impose hyper-osmotic stress and at 10°C under 0.4 M sorbitol to impose sub-optimal temperature stress under the background of light.

In light, as most of the cellular demands for ATP are met by COX pathway activity, the changes in adenylates (ATP, ADP, and ATP/ADP) which act as a proof of changes in COX pathway capacity were analyzed (Supplementary Figure S1 and Figure 2C). A decrease in ATP/ADP levels at both hyper-osmotic stress (51%) and sub-optimal temperature stress (31%) positively correlated with the decrease in COX pathway capacity (Figure 2C). Similarly, the intracellular concentration of pyruvate which is one among the important factors known to stimulate the activity of AOX are increased significantly by 2.22-fold and 56% at hyper-osmotic stress and sub-optimal temperature stress, respectively (Figure 2D).

### Effect of Restriction of AOX Pathway on Total Respiration, Photosynthetic Carbon Assimilation and PSII Activities Under Osmotic and Temperature Stress in Light

The respiratory O<sub>2</sub> uptake rates of mesophyll protoplasts decreased marginally ( $\leq 13\%$ ) after pre-incubation under hyper-osmotic stress or sub-optimal temperature stress at saturating light as compared to the rates under 0.4 M sorbitol at 25°C in light (control). Pre-incubation of samples in the presence of SHAM further decreased the rates of respiratory O<sub>2</sub> uptake up to 14%, both under hyper-osmotic stress and sub-optimal temperature stress, respectively (Figure 3A). In contrast to respiration, the NaHCO<sub>3</sub>-dependent photosynthetic O<sub>2</sub> evolution rates were decreased remarkably by 42% and 49%, respectively, under hyper-osmotic stress and sub-optimal temperature stress when



**FIGURE 3 |** Effect of 0.5 mM SHAM on respiration (A), photosynthesis (B) and PS II activity (C) measured in mesophyll protoplasts pre-incubated under control, osmotic and temperature stress conditions with or without 0.5 mM SHAM. Different letters represent values that are statistically different (ANOVA test,  $P \leq 0.05$ ). (D) Western blot analysis of D1 protein (32 kDa) from mesophyll protoplasts pre-incubated at a saturating light intensity of  $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$  under 0.4 M sorbitol (control, **C**) 1.0 M sorbitol (osmotic stress; Osm) and 0.4 M sorbitol at  $10^\circ\text{C}$  (temperature stress; Temp) for 10 min in the presence and absence of 0.5 mM SHAM. After the treatments mesophyll protoplasts were homogenized in the extraction buffer and the proteins (8  $\mu\text{g}$ ) were separated on SDS-PAGE. Proteins were transferred to PVDF membranes and were probed with the antibodies raised against D1. Equal loading of protein was confirmed by silver staining of a duplicate gel.

compared with control and the decrease was significantly aggravated to 67% upon addition of SHAM under both osmotic or temperature stress conditions (Figure 3B).

Similar to photosynthetic carbon assimilation, PSII activity of mesophyll protoplasts decreased by <21% of control upon exposure to hyper-osmotic stress or sub-optimal temperature stress and the decrease was aggravated up to <28% with addition of SHAM under both osmotic and temperature stress (Figure 3C). D1 protein, an important component of PS II showed marginal changes under sub-optimal temperature stress as compared to control, while the changes under 1.0 M sorbitol over-lapping with SHAM were negligible (Figure 3D).

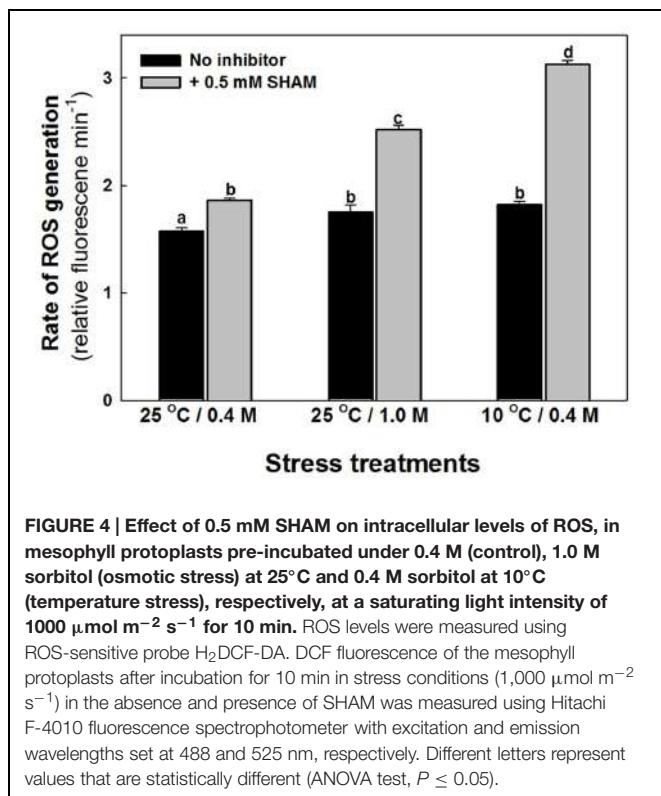
## Effect of Restriction of AOX Pathway on Total Cellular ROS Levels and Redox Ratios Under Hyper-Osmotic Stress and Sub-Optimal Temperature Stress in Light

The intracellular ROS levels of mesophyll protoplasts are increased marginally as compared to control when preincubated under hyper-osmotic stress or sub-optimal temperature stress (Figure 4). Parallel to the effect on photosynthesis, the increase in ROS levels were aggravated significantly on super-imposition of SHAM with hyper-osmotic and sub-optimal temperature stresses (Figure 4).

Any intracellular increase in malate/OAA ratio suggests an imbalance of malate valve, operated to export the photochemically generated reducing equivalents that are in excess of the Calvin cycle requirement (Heineke et al., 1991; Atkin et al., 2000; Scheibe et al., 2005). The malate/OAA ratios of mesophyll protoplasts increased by 79% and 4%, respectively, as compared to control under hyper-osmotic stress or sub-optimal temperature stress and the increase was aggravated significantly upon superimposition with SHAM, under both hyper-osmotic (2.5-fold) and sub-optimal temperature (27%) stresses (Supplementary Figure S2 and Figure 5A).

The changes in the redox state of glutathione (an important component of Ascorbate-glutathione cycle) as indicated by the GSH/GSSG levels were decreased by 47% and 30%, respectively, upon treatment with hyper-osmotic stress or sub-optimal temperature stress (Supplementary Figure S3; Figure 5B). However, the decrease was more pronounced upon superimposition with SHAM in presence of sub-optimal temperature stress when compared with hyper-osmotic stress (Figure 5B). Increase in GSSG levels during stress conditions indicates the oxidation of GSH (Supplementary Figure S3).

The role of AOX pathway in regulating cellular redox homeostasis during hyper-osmotic and sub-optimal temperature stress conditions was also determined by monitoring the changes in the redox couples related to pyridine nucleotides:



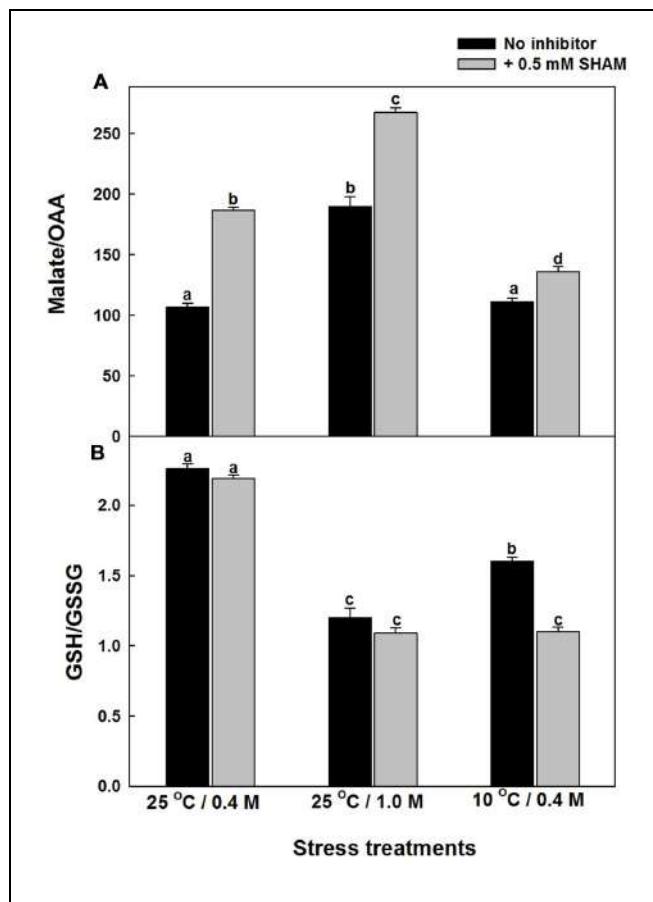
**FIGURE 4 | Effect of 0.5 mM SHAM on intracellular levels of ROS, in mesophyll protoplasts pre-incubated under 0.4 M (control), 1.0 M sorbitol (osmotic stress) at 25°C and 0.4 M sorbitol at 10°C (temperature stress), respectively, at a saturating light intensity of 1000  $\mu\text{mol m}^{-2} \text{s}^{-1}$  for 10 min.** ROS levels were measured using ROS-sensitive probe H<sub>2</sub>DCF-DA. DCF fluorescence of the mesophyll protoplasts after incubation for 10 min in stress conditions (1,000  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) in the absence and presence of SHAM was measured using Hitachi F-4010 fluorescence spectrophotometer with excitation and emission wavelengths set at 488 and 525 nm, respectively. Different letters represent values that are statistically different (ANOVA test,  $P \leq 0.05$ ).

NADH/NAD<sup>+</sup> and NADPH/NADP<sup>+</sup> in the absence and presence of SHAM (Supplementary Figures S4 and S5; Figures 6A,B). Inspite of the significant increase in NADH and NAD<sup>+</sup>, the increase in NADH/NAD<sup>+</sup> were marginal even after treatment with SHAM under both hyper-osmotic stress or sub-optimal temperature stress (Supplementary Figure S4 and Figure 6A). A similar trend in increase of NADPH and NADP<sup>+</sup> was observed with and without SHAM under hyper-osmotic or sub-optimal temperature stress (Supplementary Figure S5). But, in contrast to redox ratio of NADH/NAD<sup>+</sup>, the redox ratio of NADPH/NADP<sup>+</sup> increased significantly under sub-optimal temperature stress and was further aggravated upon treatment with SHAM (Figure 6B).

### Effect of SHAM on the Activities of NADP-MDH and Antioxidant Enzymes During Hyper-Osmotic Stress and Sub-Optimal Temperature Stress in Light

The changes in the actual activity of NADP dependent MDH, associated with malate valve was marginal upon treatment of mesophyll protoplasts with hyper-osmotic stress or sub-optimal temperature stress in the absence and presence of SHAM. But, the maximal activity of NADP-MDH was more pronounced upon treatment with SHAM when compared to samples in the absence of SHAM at both hyper-osmotic and sub-optimal temperature stress (Figures 7A,B).

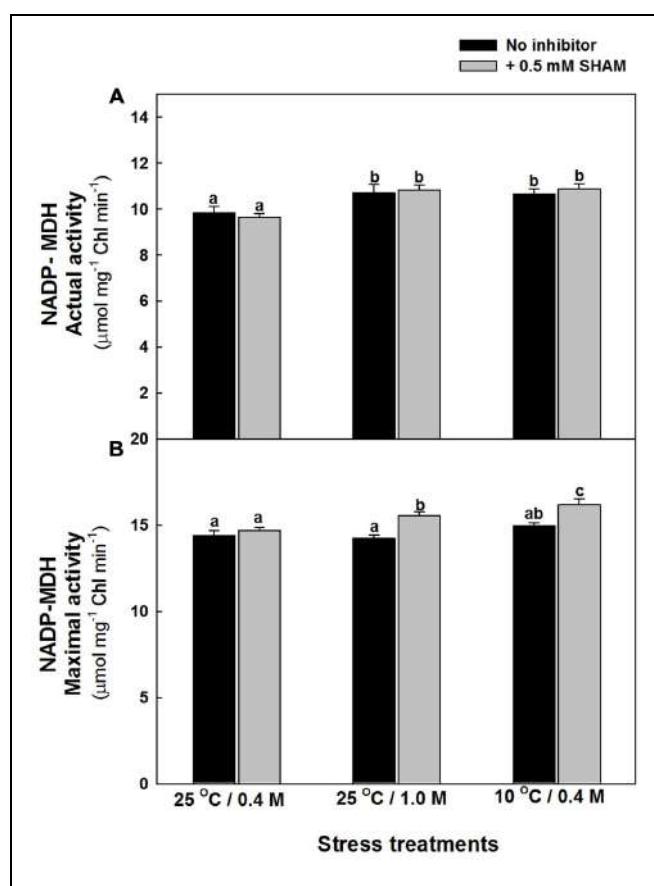
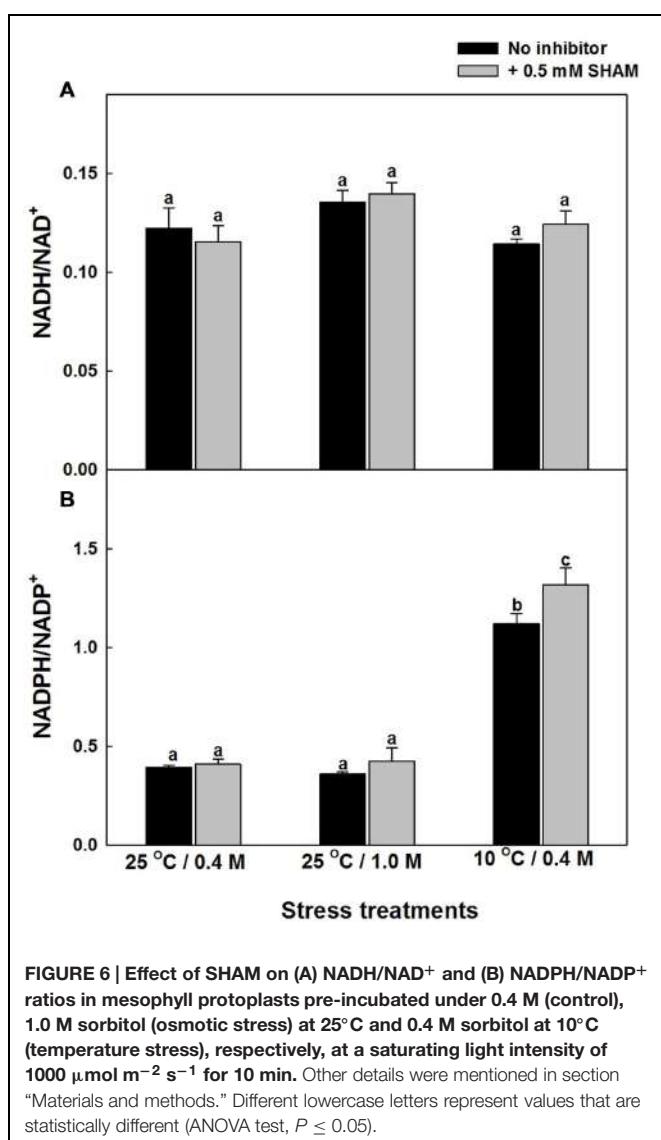
The effect of hyper-osmotic and sub-optimal temperature stresses on the activities of antioxidative system, particularly



**FIGURE 5 | Effect of 0.5 mM SHAM on malate/OAA (A) and GSH/GSSG (B) ratio in mesophyll protoplasts pre-incubated under 0.4 M (control), 1.0 M sorbitol (osmotic stress) at 25°C and 0.4 M sorbitol at 10°C (temperature stress), respectively, at a saturating light intensity of 1000  $\mu\text{mol m}^{-2} \text{s}^{-1}$  for 10 min.** At the end of the stress treatment, HClO<sub>4</sub> was added to the reaction medium and the samples were frozen dry in liquid nitrogen for analysis of malate, oxaloacetate as described in section "Materials and methods." Different letters represent values that are statistically different (ANOVA test,  $P \leq 0.05$ ).

those of ROS generating SOD and ROS scavenging CAT as well as GR, which is involved in ROS scavenging by utilizing redox equivalents were analyzed in the presence and absence of SHAM. The changes in SOD activities were marginal in presence of both stresses examined. However, upon superimposition with SHAM, there was a pronounced increase in the activity of SOD in presence of sub-optimal temperature stress but not under hyper-osmotic stress (Figure 8A). Contrary to SOD activity, the activity of CAT increased significantly by 60% as compared to control under hyper-osmotic stress, while the changes were negligible under 10°C temperature. Also, the superimposition of SHAM increased the activity of catalase furthermore under both stresses (Figure 8B). The activity of GR decreased under 1.0 M sorbitol, while changes were negligible under 10°C temperature. Nevertheless, the changes were marginal on superimposition with SHAM under both the given stresses (Figure 8C).

Taken together, the results from the present study demonstrate that the AOX pathway play a significant role in optimizing



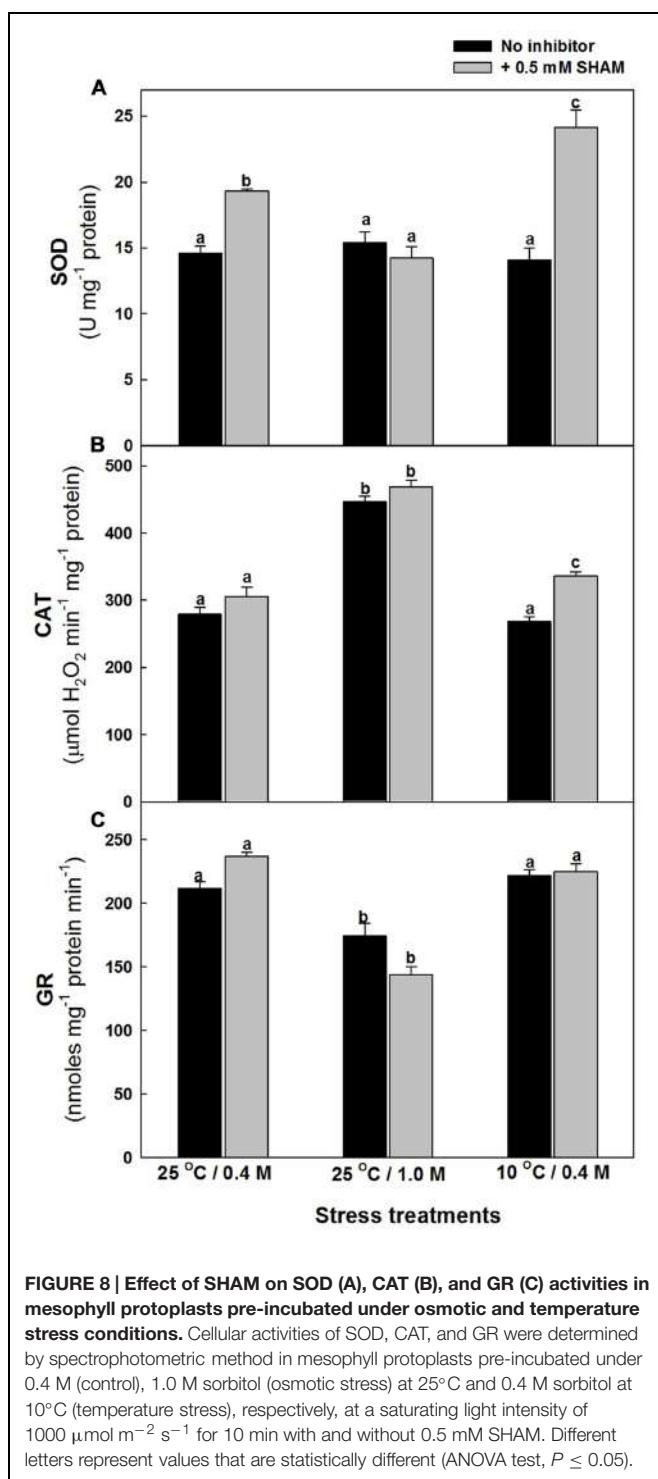
photosynthesis by regulating cellular ROS through redox couples related to malate valve, antioxidative system and pyridine nucleotides.

## DISCUSSION

Chloroplasts and mitochondria are the key organelles that are involved in meeting the energy demands and maintaining the redox homeostasis (Griffin and Turnbull, 2012). Therefore, the metabolic interactions between these organelles through cytosol and/or peroxisomes are mutually beneficial to each other and though reported earlier are still being actively investigated under different biotic and environmental cues (Raghavendra et al., 1994; Krömer, 1995; Padmasree et al., 2002; Raghavendra and Padmasree, 2003; Fernie et al., 2004; Noguchi and Yoshida, 2008; Huang et al., 2013; Sunil et al., 2013; Vanlerberghe, 2013; Shaw and Kundu, 2015). Several of these studies indicated

that a marginal interference in electron transport through COX or AOX pathways of mitochondrial electron transport chain using metabolic inhibitors and transgenic mutants/reverse genetic approaches caused a significant drop in photosynthetic carbon assimilation at optimal/limiting CO<sub>2</sub>, saturating/sub-saturating/highlight and optimal/sub-optimal growth conditions, thereby signifying the importance of mitochondrial electron transport for optimizing photosynthesis (Krömer et al., 1993; Padmasree and Raghavendra, 1999a; Dutilleul et al., 2003; Yoshida et al., 2006; Dinakar et al., 2010a,b; Araújo et al., 2014). The most recent study on diatoms using metabolic inhibitors AA and SHAM as well as knockouts of AOX also demonstrated that the export of reducing power generated in the plastid to mitochondria and the import of mitochondrial ATP into plastid is mandatory for optimized carbon fixation and their growth (Bailleul et al., 2015).

Mesophyll protoplasts can be used as an excellent model system over whole plants, leaves or leaf discs to study beneficial interactions between chloroplasts and mitochondria for the following reasons: (i) allow free diffusion of O<sub>2</sub> and CO<sub>2</sub> which



**FIGURE 8 |** Effect of SHAM on SOD (A), CAT (B), and GR (C) activities in mesophyll protoplasts pre-incubated under osmotic and temperature stress conditions. Cellular activities of SOD, CAT, and GR were determined by spectrophotometric method in mesophyll protoplasts pre-incubated under 0.4 M (control), 1.0 M sorbitol (osmotic stress) at 25°C and 0.4 M sorbitol at 10°C (temperature stress), respectively, at a saturating light intensity of 1000  $\mu\text{mol m}^{-2} \text{s}^{-1}$  for 10 min with and without 0.5 mM SHAM. Different letters represent values that are statistically different (ANOVA test,  $P \leq 0.05$ ).

minimizes the artifacts associated with stomatal patchiness, (ii) devoid of intercellular spaces and cell walls, major hurdles for the passage of metabolic inhibitors/activators and (iii) allow usage of metabolic inhibitors at low concentrations and (iv) allow monitoring of metabolic processes quickly (Padmasree and Raghavendra, 1999a,b,c; Strodtkötter et al., 2009; Dinakar et al., 2010a,b). Under the chosen conditions of isolation, the mesophyll

protoplasts did not show any damage or loss in integrity of plasma membrane when stored on ice for several hours. The oxygen evolution rates were steady up to 30 min at 25°C and 0.4 M sorbitol, under light intensity of 1000  $\mu\text{moles m}^{-2} \text{s}^{-1}$  (data not shown; Saradadevi and Raghavendra, 1994). However, they tend to lose their stability upon prolonged incubation at room temperature. Further, the light intensity applied to attain maximal rates of photosynthesis is known to vary in mesophyll protoplasts isolated from different leaves (Riazunnisa et al., 2007; Dinakar et al., 2010a,b). Considering these factors, we restricted the study to a total time period of <30 min which include: hyper-osmoticum (or) sub-optimal temperature stress treatment in light for '10 min'; followed by a '5 min' respiratory O<sub>2</sub> uptake in darkness and subsequently '10 min' photosynthetic O<sub>2</sub> evolution in light, to monitor the effect of stress on respiration and photosynthesis (Figures 1A–D). Inspite of the known non-specific effects of SHAM, it is frequently used to assess the role of AOX. It is easily permeable through the plasma membrane and at the concentration (0.5 mM) used in the present study, it neither affected photosynthesis nor ROS in isolated chloroplasts (Padmasree et al., 2002; Dinakar et al., 2010a; Bailleul et al., 2015). While our previous studies emphasized on the importance of COX and AOX pathways in optimizing photosynthesis (Padmasree and Raghavendra, 1999a,b,c, 2001a,b; Strodtkötter et al., 2009; Dinakar et al., 2010a; Vishwakarma et al., 2015) and protecting photosynthesis from photoinhibition under high light (Saradadevi and Raghavendra, 1992; Dinakar et al., 2010b; Vishwakarma et al., 2014), the present study demonstrates the importance of AOX pathway in optimizing photosynthesis under hyper-osmotic and sub-optimal temperature stresses.

### Hyper-Osmotic and Sub-Optimal Temperature Treatment Caused Marked Reduction in Photosynthetic Carbon Assimilation but not in Total Respiration

The responses of photosynthesis and respiration in mesophyll protoplasts varied when pre-incubated under hyper-osmoticum or sub-optimal temperature stresses. The results indicated that the optimal conditions to achieve maximum photosynthetic performance (carbon assimilation) and respiratory rates in mesophyll protoplasts as indicated by rates of NaHCO<sub>3</sub>-dependent O<sub>2</sub> evolution and O<sub>2</sub> uptake, respectively, were found to be at an osmoticum of 0.4 M sorbitol and a temperature of 25°C under a light background of 1000  $\mu\text{moles m}^{-2} \text{s}^{-1}$  (data not shown).

Any deviation from the optimized conditions, i.e., increasing the sorbitol concentration from 0.4 to 1.0 M (or) decreasing the temperature from 25 to 10°C, lead to a significant reduction in photosynthetic carbon assimilation while the changes in dark respiration are minimal (Figures 1A–D). Since the plasma membrane of protoplasts was found to be intact after the short-term hyper-osmotic stress and sub-optimal temperature stress treatments (data not shown), the significant decrease in photosynthetic O<sub>2</sub> evolution is considered as a direct effect of stress on photosynthetic performance. Berkowitz and Gibbs (1983) using the system of isolated chloroplasts showed

that hyper-osmotic stress caused inactivation of light activated chloroplastic enzymes like RuBisco and fructose-1,6-bisphatase due to acidification of stroma induced by low osmotic potential. Therefore, the decrease in light activation of the enzymes might be responsible for the decreased photosynthetic O<sub>2</sub> evolution rates observed in the present study under stress conditions.

In cold sensitive hibiscus plants, cold stress treatment (10°C) caused reduction in the light dependent electron transport reactions thereby causing decreased photosynthesis suggesting the sensitivity of the photosynthetic system to cold temperatures (Parades and Quiles, 2015). In another study Krause et al. (1988) showed the impairment of thylakoid membranes along with the inhibition of PS I and PS II in frost damaged leaves thereby affecting photosynthesis. In mesophyll protoplasts isolated from the non-hardened and cold acclimated plants, differential responses were seen. While photosynthetic CO<sub>2</sub> assimilation, chlorophyll fluorescence emission and activities of thylakoids were affected in protoplasts isolated from non-hardened plants, in cold acclimated plants the responses were normal. Inhibition of the light activation of light regulated enzymes fructose-1,6-bisphosphatase, sedoheptulose-1,7-bisphosphatase and ribulose-1,5-bisphosphate carboxylase is also one of the reason for decreased photosynthesis during cold stress (Krause et al., 1988). While the effects of hyper-osmoticum and sub-optimal temperatures on photosynthesis are significant, the effects on total respiration are negligible (Figure 1)

## Flexibility of Mitochondrial Electron Transport During Osmotic and Temperature Stress Conditions

The flexibility of the mitochondrial electron transport chain to divert electrons from phosphorylating to non-phosphorylating pathways decrease the over reduction of the electron transport chain components and ROS generation. This flexibility in mitochondrial electron transport chain is also observed in the present study during hyper-osmotic stress and sub-optimal temperature stress as evident by a significant increase in the capacity of AOX pathway with a concomitant decrease in the capacity of COX pathway (Figures 2A,B). These results corroborated well with the reports of Ribas-Carbo et al. (2005) in soyabean and Dwivedi et al. (2003) in pea, who showed an increase in AOX pathway activity and decrease in COX pathway activity under water and hyper-osmotic stress, respectively. Their results suggested that the increase in AOX pathway activity was due to direct inhibition of the COX pathway activity. Contrary to these results, the COX pathway activity was shown to be increased during water stress in wheat plants, while the leaf discs of *Saxifraga cernua* showed differential responses in COX and AOX pathway activities on exposure to a range of osmotic potentials from 0.0 to 4.0 MPa using sorbitol (Collier and Cummins, 1993; Zagdanska, 1995). The observed variations in the COX and AOX pathway capacity/activities in different studies might be possibly due to variations in the experimental conditions/techniques used to assess them.

Further, the observed decrease in the total cellular ATP/ADP ratios under different stress treatments as compared to controls

(Figure 2C) corroborated well with the studies of Flexas et al. (2004) and Ribas-Carbo et al. (2005). The studies of Tezara et al. (1999) suggested that the decline in leaf ATP concentration during water stress is an indicator of impaired photophosphorylation, which is one of the main factors limiting photosynthesis under water stress. Pyruvate, being a preferential substrate for mitochondrial oxidation is also known to play a significant role in communicating between chloroplasts and mitochondria to activate AOX protein/AOX pathway. The significant increase in the intracellular pyruvate levels under the hyper-osmotic and sub-optimal temperature stresses emphasizes its importance in stimulating the AOX pathway capacity (Figures 2A,B,D). In 10°C grown chick pea plants, application of pyruvate on leaves effectively reduced the oxidative stress by activating the AOX pathway (Erdal et al., 2015). Further, any decrease in the COX pathway activity might generate ROS due to over-reduction of the electron transport chain and AOX pathway is very well known to prevent ROS generation (Wagner and Moore, 1997). Thus, the increased ROS during stress conditions might represent the balance of the COX and AOX pathway capacities in light (Figure 3). The up regulation of AOX pathway capacity during osmotic and temperature stress conditions signifies the importance of AOX pathway during stress conditions and also highlights its role in decreasing the deleterious effects on not only mitochondrial respiration but also on carbon metabolism (Figures 1B,D and 2A,B). Mitochondria also possess several dissipative systems: rotenone (in)sensitive external and internal NAD(P)H dehydrogenases and complex I, COX pathway, uncoupling proteins (UCP) and potassium channel which may cooperate with AOX to prevent oxidative stress and thereby optimize photosynthetic carbon assimilation. Perhaps, these dissipative systems cannot be ignored in light of the heterogeneity of AOX effects on different components examined in the present study, which were found to be essential for efficient functioning of chloroplastic photosynthesis (Krömer et al., 1988; Igamberdiev et al., 1998; Møller, 2001; Dutilleul et al., 2003; Sweetlove et al., 2006; Yoshida et al., 2006, 2007; Noguchi and Yoshida, 2008). The studies of Trono et al. (2013) demonstrated that the hyperosmotic stress activate a mitochondrial PLA2 which in turn activate UCP and potassium channel to control ROS generation (Laus et al., 2011).

## AOX Pathway Plays an Important Role in Optimizing Photosynthesis Under Hyper-Osmotic and Sub-Optimal Temperature Stress in Light

Studies using metabolic inhibitors or transgenic/reverse genetic approaches indicated that any interference in mitochondrial oxidative electron transport components and TCA cycle causes a significant drop in photosynthetic carbon assimilation along with reduction in the rate of transpiration, stomatal and mesophyll conductance to CO<sub>2</sub> (Krömer et al., 1993; Padmasree and Raghavendra, 1999a; Dutilleul et al., 2003; Priault et al., 2006; Yoshida et al., 2006, 2011; Dinakar et al., 2010a; Nunes-Nesi et al., 2010; Florez-Sarasa et al., 2011). The low concentration of SHAM (0.5 mM) used in the present study, neither directly affected the

reduction in bicarbonate dependent oxygen evolution rates in chloroplasts (Padmasree and Raghavendra, 1999a; Dinakar et al., 2010a) nor affected the photochemical activities of mesophyll protoplasts (Padmasree and Raghavendra, 2001a). SHAM also inhibits all the isoforms of AOX as evident from studies with knockouts of AOX1a *Arabidopsis* plants (Strodtkötter et al., 2009). The results from present study demonstrated that while the effect of 0.5 mM SHAM on respiratory rates and PS II activities were marginal, the decrease in photosynthetic carbon assimilation was significant (Figure 3). Since the D1 protein levels were also unchanged in the presence of SHAM under stress conditions, it can be concluded that the marginal interference in AOX pathway under hyper-osmotic stress and sub-optimal temperature stress caused a remarkable decrease in photosynthetic carbon assimilation with marginal effect on photochemical activities, as evident by changes in D1 protein levels (Figures 3B–D). Similar observations were also reported by Saradadevi and Raghavendra (1994), where the photosynthetic rates of mesophyll protoplasts decreased to a significant extent on exposure to solutions of increasing osmolarity. The production of ROS by mitochondria was suggested as the critical factor for the induction of AOX (Clifton et al., 2006; Rhoads et al., 2006) and the respiratory capacities of COX and AOX pathways are known to play a significant role in maintenance of cellular ROS at optimal levels to sustain high photosynthetic rates (Dinakar et al., 2010a). In our studies, although we observed a significant increase in ROS, we did not observe the decrease in D1 protein levels under osmotic as well as temperature stress conditions or even in the presence of SHAM (Figures 3D and 4). These results suggest that the changes observed in ROS during hyper-osmotic stress and sub-optimal temperature stress might be involved in signaling function to activate the cellular defense mechanism, perhaps AOX and ROS scavenging antioxidant system (Figures 2A,B, 4, 5B and 8).

## Role of Malate Valve and ROS Scavenging Antioxidant System in Stimulating the *In Vivo* Activity of AOX Pathway to Optimize Photosynthesis Under Osmotic and Temperature Stress in Light

Decrease in photosynthesis is a primary effect that is observed during stress conditions. Under these conditions chloroplastic electron transport components accumulate reducing equivalents thereby preventing electron transport. Chloroplasts generated reducing equivalents may be transferred to mitochondria through several metabolite shuttles that operate between the two compartments. Malate and OAA are the two most important metabolites that are involved in redox shuttling between the chloroplasts, mitochondria, and cytosol. Malate/OAA shuttle is believed to be mediated by malate dehydrogenase and in equilibrium with the cellular NADH/NAD<sup>+</sup> ratio. The assessment of the total cellular NADH and NAD<sup>+</sup> levels also depends on the other metabolite shuttles and the activity of the mitochondrial oxidative electron transport. Therefore the possibility of change in intracellular malate/OAA ratio

without dramatic changes in NADH/NAD<sup>+</sup> can occur in a cell. The major change in malate/OAA ratio is expected in chloroplasts, while NADH/NAD<sup>+</sup> ratio is mostly in cytosol. This may be partly due to the consumption of reduced equivalents from malate by other metabolic components such as GSH and/or ascorbate. The pronounced increase in malate levels under hyper-osmotic stress conditions in the presence of SHAM indicates the biochemical role of malate in chloroplast-mitochondrial interactions (Supplementary Figure S3; Figure 5A). Biochemically the malate is oxidized to pyruvate via malic enzyme. In isolated mitochondria, malic enzyme activity is correlated with intramitochondrial pyruvate generation and consequent AOX activation (Day et al., 1995). In another study Yoshida et al. (2007) observed an active malic enzyme in AOX1a knockout plants. The COX and AOX pathways were known to play a significant role in oxidizing the malate and regenerating OAA to keep up the chloroplastic electron transport carriers in the oxidized state, which in turn helps to keep the Calvin cycle active for maintaining optimal photosynthesis (Padmasree and Raghavendra, 1999c; Raghavendra and Padmasree, 2003). The pronounced increase in malate/OAA ratio suggests the importance of ‘malate valve’ in mediating the cross talk between chloroplasts and mitochondria to activate AOX pathway under hyper-osmotic stress (Figure 5A). Chloroplastic NADP-dependent malate dehydrogenase (NADP-MDH) is the key enzyme controlling the malate valve, which export reducing equivalents indirectly from chloroplasts. The significant increase in maximal NADP-MDH activity in presence of SHAM corroborate well with the increased NADPH and malate levels, and redox ratios of NADPH/NADP<sup>+</sup> and malate/OAA, respectively, under both hyper-osmotic and temperature stresses (Figures 5A, 6B, and 7B; Supplementary Figure S2A). However, the marginal increase in malate/OAA ratio in the presence of SHAM during sub-optimal temperature stress denotes that a redox modulating factor other than malate might play a role in modulating the ROS to keep up the Calvin cycle activity in chloroplasts.

While the amounts and activities of enzymes involved in ROS scavenging are known to be altered by environmental stresses such as chilling, drought and high salinity (Shao et al., 2008), the reductive detoxification of ROS occurs through the cellular ascorbate and glutathione pools (Smirnoff, 2000; Noctor, 2006). The decrease in photosynthetic carbon assimilation and GSH/GSSG ratio, parallel to a rise in ROS in presence of SHAM under osmotic and temperature stress suggests the role of AOX in optimizing photosynthesis by regulating ROS through glutathione redox couple (Figures 3–5). AOX pathway is known to play a significant role in optimizing photosynthesis by keeping up the light activation of chloroplastic enzymes (Padmasree and Raghavendra, 2001b). As these enzymes are regulated by thioredoxin-glutaredoxins, a remarkable decrease in glutathione redox couple at 10°C in presence of SHAM and increase in AOX pathway capacity provide evidence for the physiological role of AOX pathway in keeping up the light activation of chloroplastic enzymes to sustain photosynthesis under sub-optimal temperature stress

(**Figures 2B, 3B, and 5B**). Further, the marginal changes in NADH/NAD<sup>+</sup> redox couples, in presence of SHAM when superimposed with hyper-osmotic stress and sub-optimal temperature further confirm the tight coupling of AOX pathway with malate/OAA and GSH/GSSG redox couples in regulating cellular ROS to protect photosynthesis from photoinhibition and sustain photosynthetic performance of mesophyll protoplasts under these stresses (**Figures 2A,B, 3B, 5 and 6**). The increase in the redox ratio of NADPH/NADP<sup>+</sup> under sub-optimal temperature stress conditions in the presence of SHAM signifies the importance of AOX in oxidizing excess reducing equivalents (**Figure 6B**). Furthermore, though the changes in SOD and catalase activities were significant in the presence of SHAM during sub-optimal temperature stress, they could not play much role in protecting photosynthesis under hyper-osmotic stress by preventing generation/accumulation of cellular ROS (**Figures 8A,B**). On the other hand, the changes in GR were small but not significant under all conditions examined (**Figure 8C**). The significant increase in NADPH/NADP<sup>+</sup> ratio with concomitant rise in ROS and a decrease in GSH/GSSG ratio while sustaining GR activity in presence of SHAM at 10°C indicated that AOX pathway optimize photosynthesis by regulating antioxidative system at sub-optimal temperature (**Figures 2B, 4, 5B, 6B and 8C**). These results suggest that non-enzymatic antioxidants play a significant role over enzymatic-oxidants in regulating cellular ROS during optimization of photosynthesis by AOX.

## CONCLUSION

The present study demonstrates the importance of AOX pathway in optimizing photosynthesis during hyper-osmotic and temperature stress in light. The increased capacity of AOX pathway during both hyper-osmotic and sub-optimal temperature stress was evident by a parallel modulation in various biochemical factors such as pyruvate, ROS and ATP/ADP levels. Studies using mitochondrial AOX pathway inhibitor SHAM demonstrated that under both osmotic and temperature stress, the AOX pathway optimizes photosynthetic carbon

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assimilation. The results highlight the flexibility of AOX pathway in interacting with different redox couples related to malate valve (malate/OAA) and antioxidative system (GSH/GSSG) to regulate cellular ROS for optimal photosynthetic performance under hyper-osmotic stress and sub-optimal temperature stress. Since the AOX mutants of pea are not available, studies using *Arabidopsis* are required to further understand the underlying molecular mechanisms.

## AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: CD, KP, and AR. Performed the experiments: CD and AV. Analyzed the data: CD, KP, and AR. Contributed reagents/materials/analysis tools: KP and AR. Wrote the paper: CD and KP

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2016.00068>

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# Genome-Wide Identification and Expression Analysis of the WRKY Gene Family in Cassava

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The WRKY family, a large family of transcription factors (TFs) found in higher plants, plays central roles in many aspects of physiological processes and adaption to environment. However, little information is available regarding the WRKY family in cassava (*Manihot esculenta*). In the present study, 85 WRKY genes were identified from the cassava genome and classified into three groups according to conserved WRKY domains and zinc-finger structure. Conserved motif analysis showed that all of the identified MeWRKYS had the conserved WRKY domain. Gene structure analysis suggested that the number of introns in MeWRKY genes varied from 1 to 5, with the majority of MeWRKY genes containing three exons. Expression profiles of MeWRKY genes in different tissues and in response to drought stress were analyzed using the RNA-seq technique. The results showed that 72 MeWRKY genes had differential expression in their transcript abundance and 78 MeWRKY genes were differentially expressed in response to drought stresses in different accessions, indicating their contribution to plant developmental processes and drought stress resistance in cassava. Finally, the expression of 9 WRKY genes was analyzed by qRT-PCR under osmotic, salt, ABA, H<sub>2</sub>O<sub>2</sub>, and cold treatments, indicating that MeWRKYS may be involved in different signaling pathways. Taken together, this systematic analysis identifies some tissue-specific and abiotic stress-responsive candidate MeWRKY genes for further functional assays *in planta*, and provides a solid foundation for understanding of abiotic stress responses and signal transduction mediated by WRKYS in cassava.

**Keywords:** abiotic stress, cassava, gene expression, RNA-seq, WRKY transcription factor

## INTRODUCTION

The WRKY family is a large family of transcription factors (TFs) found in higher plants (Rushton et al., 2010). *SPF1*, the first reported WRKY transcription factors, plays crucial roles in the regulation of gene expression (Ishiguro and Nakamura, 1994). WRKY TFs contain one or two WRKY domains which have a highly conserved WRKYGQK motif at the N-terminus and a zinc-finger structure at the C-terminus (Llorca et al., 2014). Based on the variation in WRKY domain and the pattern of the zinc-finger motif, WRKY proteins can be divided into three major groups (1, 2, and 3) with several subgroups (Eulgem et al., 2000). The group 1 typically contains two

WRKY domains including a C<sub>2</sub>H<sub>2</sub> motif, while group 2 and group 3 are characterized by a single WRKY domain. Group 2 also contains a C<sub>2</sub>H<sub>2</sub> zinc-finger motif and can be further divided into five subgroups (2a–2e) based on the phylogeny of the WRKY domains, whereas group three contains a zinc-finger-like motif ending with C<sub>2</sub>-H-C (Eulgem et al., 2000).

There is considerable evidence showing that WRKY proteins play central roles in various aspects of physiological processes and adaption to the environment (Rushton et al., 2010; Ling et al., 2011), including senescence (Robatzek and Somssich, 2002; Han et al., 2014), trichome development (Johnson et al., 2002), embryogenesis (Lagacé and Matton, 2004), seed dormancy and germination (Xie et al., 2007), root development (Devaiah et al., 2007), and response to biotic stresses including bacterial (Oh et al., 2006; Xu et al., 2006; Zheng et al., 2007; Tao et al., 2009; Hwang et al., 2011; Choi et al., 2014), fungal (Li et al., 2006; Xu et al., 2006; Liu et al., 2014; Ye et al., 2014; Cheng et al., 2015), viral pathogens (Oh et al., 2006; Huh et al., 2015), and insects (Grunewald et al., 2008; Skibbe et al., 2008).

In recent years, accumulated evidence has confirmed that a large number of WRKY genes are induced by abiotic stresses and play important roles in the regulation of plant tolerance to abiotic stress (Seki et al., 2002; Rushton et al., 2010; Li et al., 2011; Scarpeci et al., 2013). In *Arabidopsis*, *AtWRKY30* was induced by abiotic stress including treatments with methyl viologen (MV), H<sub>2</sub>O<sub>2</sub>, arsenic, drought, NaCl, and mannitol, and overexpression of *AtWRKY30* increased plants tolerance to MV and salinity stresses (Scarpeci et al., 2013). WRKY46, another WRKY gene from *Arabidopsis*, was significantly induced by drought, salt, and H<sub>2</sub>O<sub>2</sub>, and *wrky46* mutant was less tolerant to osmotic and salt stress than WT (Ding et al., 2014). WRKY25 and WRKY26 were induced under heat stress and were confirmed to play positive roles thermotolerance in *Arabidopsis* (Li et al., 2011). Additionally, overexpression of WRKY25 or WRKY33 increased plant tolerance to salt stress and sensitivity to ABA (Jiang and Deyholos, 2009). Likewise, 41 out of 103 rice WRKY genes showed significant differences in their transcript abundance under abiotic stress (cold, drought and salinity; Ramamoorthy et al., 2008). Some rice WRKYS have been shown to be positive regulators of abiotic stresses, such as *OsWRKY5* (Berri et al., 2009), *OsWRKY7* (Ramamoorthy et al., 2008), *OsWRKY11* (Wu et al., 2009), *OsWRKY43* (Berri et al., 2009), *OsWRKY45* (Qiu and Yu, 2009), and *OsWRKY47* (Raineri et al., 2015). For example, overexpression of *OsWRKY45* in *Arabidopsis* was found to increase plant tolerance to salt and drought, and to decrease sensitivity to ABA (Qiu and Yu, 2009). Overexpression of *OsWRKY47* increased plant tolerance to drought and yield compared to WT (Raineri et al., 2015). This evidence demonstrated that the WRKY gene family may contain important regulatory factors involved in plant response to abiotic stress.

To date, genome-wide analysis has identified a large number of WRKY family members in several species with 74 WRKY genes in *Arabidopsis* (*Arabidopsis thaliana*; Ulker and Somssich, 2004), 103 in rice (*Oryza sativa* cv. Nipponbare; Ramamoorthy et al., 2008), 45 in barley (*Hordeum vulgare*; Mangelsen et al.,

2008), 55 in cucumber (*Cucumis sativus*; Ling et al., 2011), 119 in maize (*Zea mays*; Wei et al., 2012), 182 in soybean (*Glycine max*; Bencke-Malato et al., 2014), and 109 in cotton (*Gossypium aridum*; Fan et al., 2015). However, there is currently no evidence regarding the WRKY family in the important tropical plant cassava. Cassava (*Manihot esculenta* Crantz) is the third most important crop after rice and maize in Africa, Asia, and Latin America, where it is an important food security crop (Oliveira et al., 2014). Cassava, a major staple crop, has the starchy roots that provide dietary carbohydrate for 800 million people across the tropical and sub-tropical world (International Cassava Genetic Map Consortium, 2014). Due to its high starch production and limited input, cassava is also a major producer of industrial starch and bioethanol (Zidenga et al., 2012; Perera et al., 2014). Cassava is particularly tolerant to drought and low-fertility soils when facing environmental stresses (International Cassava Genetic Map Consortium, 2014; Zeng et al., 2014). However, the mechanisms by which cassava responds to abiotic stress are poorly understood. Thus, understanding of the molecular mechanisms underlying the tolerance of cassava to abiotic stress may provide effective methods for genetic improvement of stress tolerance for cassava and other crops. The high-quality sequencing of cassava wild ancestor and cultivated varieties reported in our previous study have provided an excellent opportunity for genome-wide analysis of cassava genes (Wang et al., 2014a). Based on the significance of WRKYS involved in plant growth, development and adaption to the environment and on the lack of any genome-wide systematic analysis of cassava WRKY genes, the WRKY family was selected for a systematic analysis in cassava. In this study, 85 WRKY genes from the cassava genome were identified and detailed studies of their phylogeny, conserved motifs, gene structure, expression profiles in various tissues, and in response to drought, osmotic, salinity, cold, oxidative stresses, and signaling of ABA were performed. The current results may provide a novel insight into the future work on the function of WRKYS and abiotic stress responses in cassava.

## MATERIALS AND METHODS

### Plant Materials and Treatments

W14 (*M. esculenta* ssp.*flabellifolia*) is an ancestor of the wild cassava subspecies with a strong tolerance to drought stress (Wang et al., 2014a). South China 124 (SC124) is a widely planted cassava cultivar in China (Zeng et al., 2014). Argentina 7 (Arg7) adapts to a geographical high-latitude region of Argentina (Zhao et al., 2014). All plants were grown in a glass house of the Chinese Academy of Tropical Agricultural Sciences (Haikou, China). Stem segments with three nodes were cut from 8 months old cassava plants and inclined into pots with a mixture of soil and vermiculite (1:1) where they were regularly watered (Hu et al., 2015). The plants were grown from April to July 2013 during which time the temperature in the glass house ranged from 20 to 35°C. The transcripts of cassava WRKY genes in different tissues, including stems (90 days after planting), leaves (90 days after planting), and middle storage roots (150 days after planting)

were examined with wild subspecies (W14) and cultivated variety (Arg7) under normal growth conditions. Ninety-days-old leaves and roots were sampled from Arg7, SC124 and W14 to study the transcriptional response of cassava WRKY genes under 12 days drought stress. After 60 days of normal cultivation, the Arg7 seedlings similar in growth vigor were used in the following treatments. For abiotic stress and signal molecule treatments, Arg7 seedlings were subjected to 200 mM mannitol for 14 days, 300 mM NaCl for 14 days, 100  $\mu$ M abscisic acid (ABA) for 24 h, 3.27 M (10%)  $H_2O_2$  for 24 h and low temperature (4°C) for 48 h, respectively. According to Scarpeci et al. (2013) and Ding et al. (2014), 20 mM  $H_2O_2$  can induce oxidative stress in Arabidopsis. In this study, high concentration of  $H_2O_2$  (3.27 M) was used to strongly induce oxidative stress due to the woody feature of cassava.

## Identification and Phylogenetic Analyses of the WRKY Gene Family in Cassava

The whole protein sequence of cassava was obtained from the cassava genome database (<http://www.phytozome.net/cassava.php>). Sequences of the *AtWRKY* and *OsWRKY* genes were downloaded from UniPort (<http://www.uniprot.org/>) and RGAP databases (<http://rice.plantbiology.msu.edu/>), respectively. To identify the cassava WRKY family genes, two different approaches were used as follows: firstly, the local Hidden Markov Model-based searches (HMMER: <http://www.ebi.ac.uk/Tools/hmmer/>) built from known WRKYS to search the cassava genome database (Finn et al., 2011); secondly, BLAST analyses with all the Arabidopsis and rice WRKYS as queries were employed to check the predicted WRKYS in cassava database. With the help of CDD (<http://www.ncbi.nlm.nih.gov/cdd/>) and PFAM databases (<http://pfam.sanger.ac.uk/>), all the potential cassava WRKY genes identified from HMM and BLAST searches were only accepted if they contained the WRKY domain, then using multiple sequence alignments to confirm the conserved domains of predicted WRKY sequences. Additionally, Clustal X 2.0 and MEGA 5.0 were used to constructed a bootstrap neighbor-joining (NJ) phylogenetic tree based on amino acid sequence of WRKY domains of cassava WRKY members and selected Arabidopsis WRKYS with 1000 bootstrap replicates (Larkin et al., 2007; Tamura et al., 2011). Furthermore, to better exhibit the characteristic of MeWRKY gene structure and conserved motifs, a NJ phylogenetic tree was created based on the full amino acids of cassava WRKYS.

## Protein Properties and Sequence Analyses

The online ExPASy proteomics server (<http://expasy.org/>) was used to investigate the molecular weight (MW) and isoelectric points (pi) of presumed WRKY proteins. The conserved motifs in full-length WRKY proteins were identified using the MEME program (<http://meme.nbcr.net/meme/cgi-bin/meme.cgi>). Parameters employed in the analysis were: maximum number of motifs was 10 and the optimum width of motifs was set from 15 to 50 (Tao et al., 2014). Furthermore, all identified motifs were annotated according to InterProScan (<http://www.ebi.ac.uk/Tools/pfa/iprscan/>). The gene structures were identified by gene structure display server program

(GSDS, <http://gsds.cbi.pku.edu.cn/>). Exon/intron organization was further checked by alignment of coding sequence and genomic DNA sequence of each WRKY gene.

## Transcriptomics Analysis

Total RNA was extracted from stems, leaves and storage roots in Arg7 and W14 under normal growth conditions, and was also extracted from leaves and roots of Arg7, SC124 and W14 under normal conditions and 12 days drought treatment. Total RNA was isolated using plant RNeasy extraction kit (TIANGEN, China) following manufacturer's instructions and the concentration and purity were evaluated by NanoDrop 2000c (Thermo Scientific, USA). Three  $\mu$ g total RNA of each sample were used to construct the RNA pools according to the Illumina instructions, and subsequently sequenced by Illumina GAII following Illumina RNA-seq protocol. A total of 610.70 million 51-bp raw reads was generated from the 18 samples. Adapter sequences were removed from raw sequence reads using FASTX-toolkit (version 0.0.13, [http://hannonlab.cshl.edu/fastx\\_toolkit/](http://hannonlab.cshl.edu/fastx_toolkit/)). Sequence quality was examined using FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and low quality sequences (including reads with unknown base pairs "N") were removed, which produced 583.82 million clean reads. Clean reads were mapped to cassava reference genome (version 4.1) derived from the phytozome website (<ftp://ftp.jgi-psf.org/pub/compgen/phytozome/v9.0/Mesculenta/>) using Tophat v.2.0.10 (<http://tophat.cbcb.umd.edu/>) (Trapnell et al., 2009), and 88.7% reads were aligned. The resulting alignment files are provided as input for Cufflinks to generate transcriptome assemblies (Trapnell et al., 2012). Gene expression levels were calculated as FPKM according to the length of the gene and reads count mapped to this gene: FPKM = total exon fragments/[mapped reads (millions)  $\times$  exon length (kb)]. DEGseq was applied to identify differentially expressed genes with a random sampling model based on the read count for each gene (Wang et al., 2010).

## Quantitative RT-PCR Analysis

Expression of *MeWRKY* genes in response to various abiotic stress (osmotic, salt, cold, and oxidative stress) and ABA signaling were examined by qRT-PCR analysis with Stratagene Mx3000P Real-Time PCR system (Stratagene, CA, USA) using SYBR® Premix Ex Taq™ (TaKaRa, Japan) according to the manufacturer's instructions. Total RNA was extracted from leaves of control and treated samples. Two hundred ng Poly(A)<sup>+</sup> mRNA from each treatment was converted into cDNA using AMV Reverse Transcriptase (Promega, Madison, WI, USA) at 42°C in a 20  $\mu$ L reaction volume that subsequently served as the template for qRT-PCR. The amplification conditions used for all PCRs were implemented as follows: 10 min at 95°C, and followed by 40 cycles of 10 s at 95°C, 15 s at 50°C, and 30 s at 72°C. The relative expression of the target genes was determined using the  $2^{-\Delta\Delta Ct}$  method (Livak and Schmittgen, 2001). The specific primers were designed according to the WRKY gene sequences by Primer 5.0 software (Table S1). Subsequently, reaction specificities for each primer pair was tested using qRT-PCR melting curve analysis, agarose gel electrophoresis, and sequencing PCR products. Amplification

efficiencies of gene-specific primers ranged from 90 to 110%.  $\beta$ -tubulin gene (TUB) and elongation factors 1 $\alpha$  gene (EF1) verified to be constitutive expression and suitable as internal controls were used as internal references for all the qRT-PCR analyses (Salcedo et al., 2014). Each treated sample contained a corresponding regularly-watered control and each sample was performed with three independent biological replications. Then, the treated and control plants at each time point were sampled to perform expression analysis. The relative expression levels of *MeWRKY* genes in each treated time point were compared with corresponding regularly-watered control (Wang et al., 2014b). Statistical difference were performed by Duncan's multiple range test ( $n = 3$ ). Means denoted by the same letter do not significantly differ at  $P < 0.05$ .

## RESULTS AND DISCUSSION

### Identification and Phylogenetic Analysis of Cassava WRKYS

To identify the WRKY family members in cassava, both BLAST and HMMER searches were performed to search the cassava genome with Arabidopsis and rice WRKY sequences as queries. After these searches, a total of 85 putative members of the WRKY family were detected in the complete cassava genome. Conserved domain analysis further confirmed that all the WRKYS contain single or double WRKY domains at the N-terminus, which are the basic characteristics of WRKY family. The 85 predicted WRKY proteins ranged from 149 (*MeWRKY22*) to 737 (*MeWRKY64*) amino acids (aa) in length with an average of 369.4 aa, the relative molecular mass varied from 17.19 kDa (*MeWRKY22*) to 79.76 kDa (*MeWRKY64*), and the pIs ranged from 4.91 (*MeWRKY59*) to 9.89 (*MeWRKY1*) with 38 numbers pI > 7 and others pI < 7 (Table S2). cDNAs of all 85 *MeWRKY* genes have been submitted to GenBank and their accession numbers in GenBank are shown in Table S3.

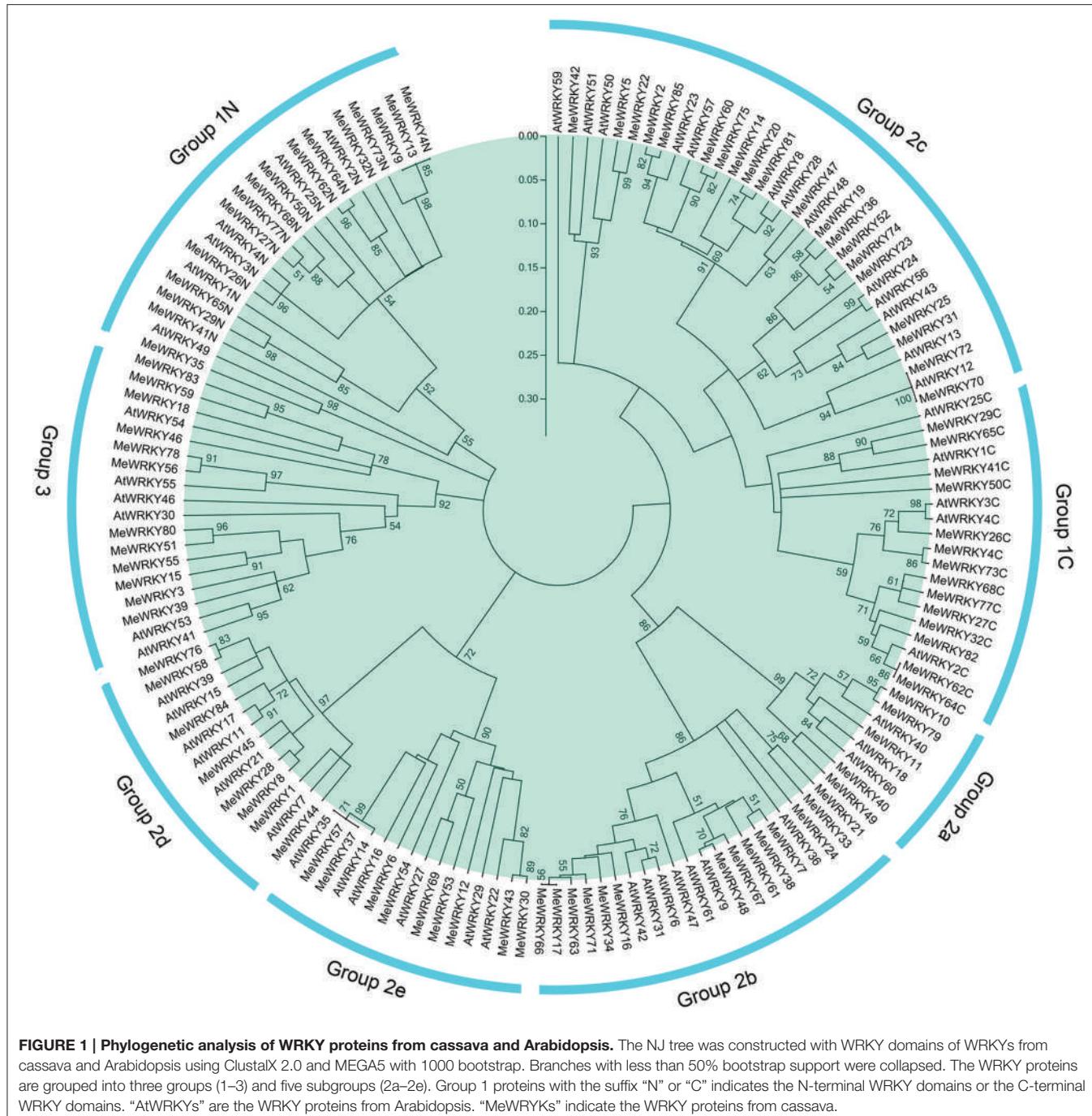
To study the evolutionary relationships between cassava WRKY proteins and known WRKYS from Arabidopsis, an unrooted neighbor-joining phylogenetic tree was created based on multiple alignments of the predicted amino acid sequences of the WRKY domains from cassava and Arabidopsis. As shown in Figure 1, 85 *MeWRKY* proteins were classified into three major groups, among which group 2 was subdivided into five subgroups together with WRKYS from Arabidopsis. This was in accordance with the classification of WRKY family in Arabidopsis (Eulgem et al., 2000), cucumber (Ling et al., 2011), maize (Wei et al., 2012), and soybean (Bencke-Malato et al., 2014). Groups 1, 2, and 3 contained 17, 56, and 12 *MeWRKY* proteins, respectively. A total of 5, 14, 20, 8, and 9 proteins were assigned to subgroups 2a, 2b, 2c, 2d, and 2e, respectively. Generally, group 1 contained two WRKY domains, but there were a few *MeWRKY* proteins that contained only one WRKY domain, such as, *MeWRKY9*, -13, -35, and -82. The same phenomenon was also found in Arabidopsis (Eulgem et al., 2000) and maize (Wei et al., 2012). According to one previous report (Wei et al., 2012), the loss of WRKY domain seems to be more common in monocotyledons than in dicotyledons. It can be deduced that group 1 might

contain the original genes of other groups and that *MeWRKY9*, -13, -35, and -82 emerged later during evolution.

Phylogenetic analysis also showed that there were some closely related orthologous WRKYS between cassava and Arabidopsis (*MeWRKY42* and *AtWRKY51*; *MeWRKY47* and *AtWRKY48*; *MeWRKY48* and *AtWRKY9*; *MeWRKY69* and *AtWRKY27*; *MeWRKY35* and *AtWRKY49*; *MeWRKY70/MeWRKY72* and *AtWRKY12*; *MeWRKY37/MeWRKY57* and *AtWRKY35*; *MeWRKY1/MeWRKY44* and *AtWRKY7*; *MeWRKY45* and *AtWRKY21*; *MeWRKY26* and *AtWRKY3/AtWRKY4*), suggesting that an ancestral set of WRKY genes existed prior to the divergence of cassava and Arabidopsis and that WRKYS from cassava generally have close relationship with the proteins from Arabidopsis. *MeWRKY1* and *MeWRKY44* showed a high degree of similarity with *AtWRKY7*, which was reported to negatively regulate plant defense against bacterial pathogens (Kim et al., 2006). *MeWRKY69* shared considerable similarity with *AtWRKY27* that is also involved in the regulation of plant defense against the bacterial pathogens by regulating the expression of nitrogen metabolism and nitric oxide (NO) generation genes (Mukhtar et al., 2008). *AtWRKY51*, which showed a high degree of similarity with *MeWRKY42*, was reported to mediate jasmonic acid (JA) signaling and partially alter resistance to virulent pathogens (Gao et al., 2011). These results suggested the possible functions of WRKY genes in cassava.

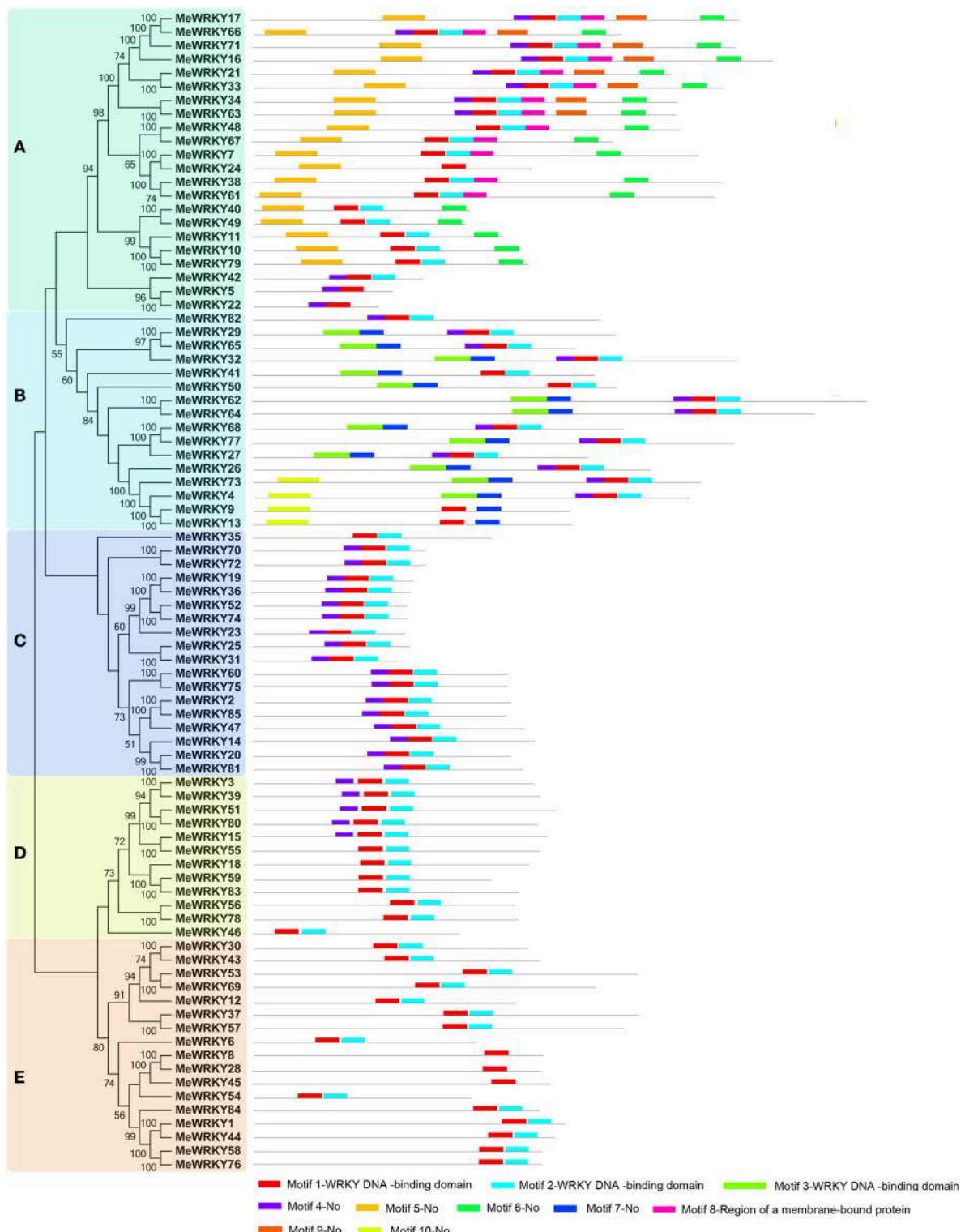
### Conserved Motifs and Gene Structure of Cassava WRKYS

To further detect the structural features of cassava WRKYS, conserved motifs and intron/exon distribution were analyzed according to their phylogenetic relationships. A total of 10 conserved motifs in cassava WRKYS were found using MEME software and further annotated by InterPro Scan 5 (Figure 2; Figure S1). Results showed that three (1–3) of 10 motifs were annotated as WRKY DNA-binding, which is a basic characteristics of the WRKY family. All *MeWRKYs* contained at least one of them, indicating that the cassava WRKYS identified in this study had conserved features of the WRKY family. Notably, all the *MeWRKYs* contain at least two motifs, except for three members (*MeWRKY8*, -28, and -45) only containing motif 2 in cluster E. In cluster A, all the *MeWRKYs*, except for *MeWRKY5*, -22, -24, and -42, contained motifs 1, 2, 6, and 9. Interestingly, most of the *MeWRKY* members in cluster A specially showed motifs 8 and 9 in comparison to *MeWRKYs* in other clusters. In cluster B, all the *MeWRKYs*, except for *MeWRKY82*, contained motifs 1 and 7, and motif 10 was uniquely dispersed in four members (*MeWRKY4*, -9, -13, and -73). In cluster C, all members contained motifs 1, 2, and 4, except for *MeWRKY35* which did not contain the motif 4. In cluster D, all members contained motifs 1 and 2, and five members (*MeWRKY3*, -39, -51, -80, and -15) also contained motif 4 in addition to motifs 1 and 2. In cluster E, all members contained motifs 1 and 2, except for the closely related *MeWRKY8*, -28, and -45, which only contained motif 1. Generally, WRKY members in the same cluster commonly shared similar motif compositions, indicating functional similarity among them.

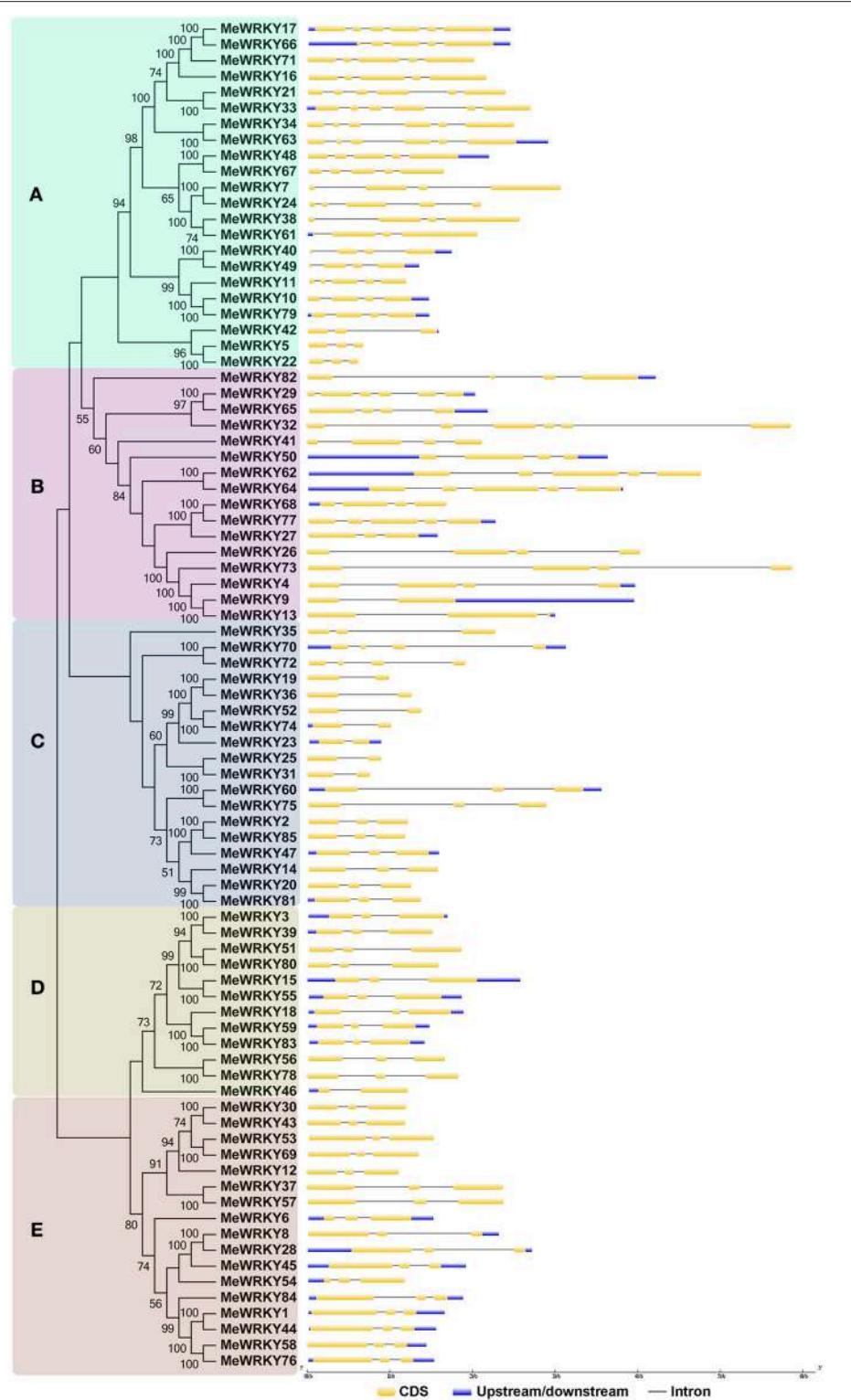


Exon-intron structural diversity, an important part in the evolution of gene families, provides additional evidence supporting phylogenetic groupings (Shiu and Bleeker, 2003; Wang et al., 2014c). Intron/exon distribution was analyzed to better understand phylogenetic relationship and classification of cassava WRKYS. As shown in Figure 3, the number of introns in MeWRKY genes varied from 1 (*MeWRKY9, -19, -23, -25, -31, -36, -46, -52, and -74*) to 5 (*-17, -21, -29, -32, -33, -34, and -63*). However, in rice and rubber tree, the number of introns varied

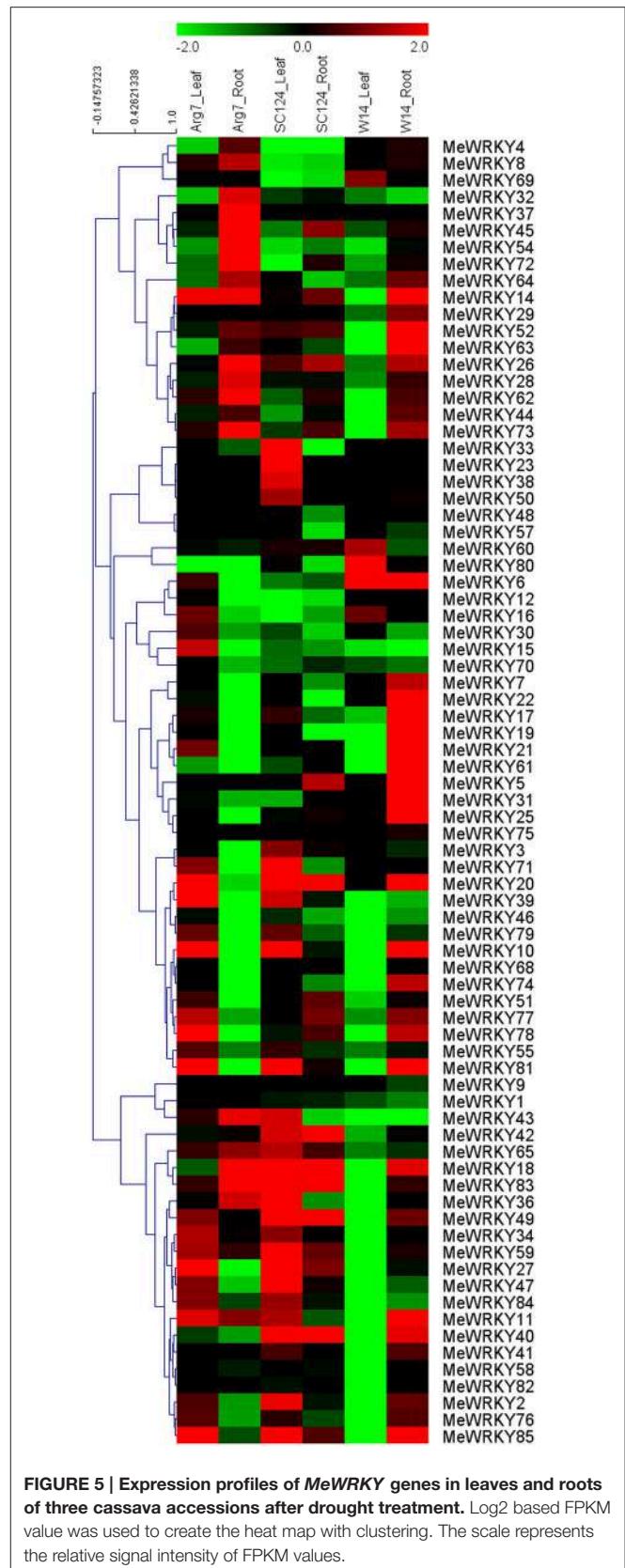
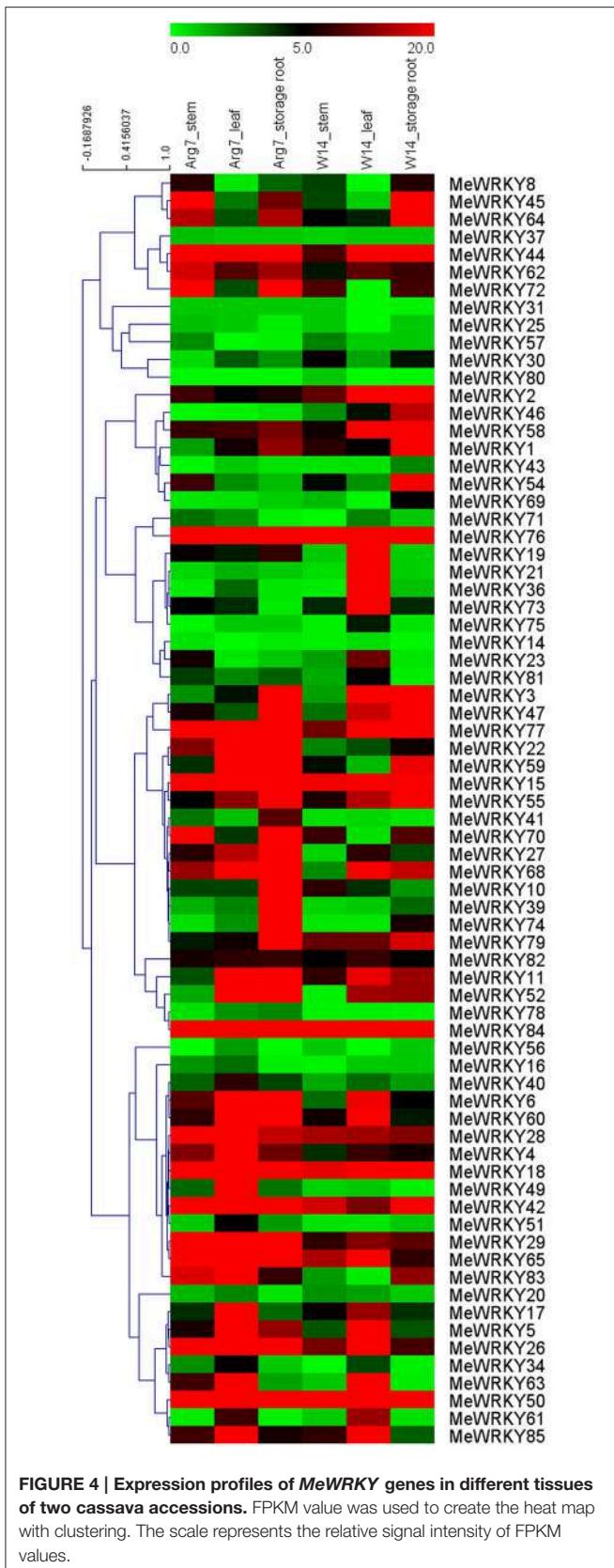
from 0 (*OsWRKY10 and OsWRKY44*) to 8 (*OsWRKY41.D1 and OsWRKY41.D2*) and 1 (*HbWRKY22, -34, -35 and -36*) to 7 (*HbWRKY15*), respectively (Xie et al., 2005; Li et al., 2014). These results indicated that WRKYS in cassava have less gene structure diversity than that in rice and rubber tree. Additionally, 42 out of 85 *MeWRKY* genes each had two introns. The same phenomenon was also observed in rice and rubber tree with 42 of 92 and 40 of 81 WRKY genes containing two introns each, respectively (Xie et al., 2005; Li et al., 2014). Cluster A



**FIGURE 2 | Conserved motifs of MeWRKY proteins according to the phylogenetic relationship.** The NJ tree was constructed with full amino acids of cassava WRKYS using ClustalX 2.0 and MEGA5 with 1000 bootstraps. The conserved motifs in the MeWRKY proteins were identified by MEME. Gray lines represent the non-conserved sequences, and each motif is indicated by a colored box numbered at the bottom. The length of motifs in each protein was exhibited proportionally. **(A–E)** indicates different groups of WRKY family in cassava.



**FIGURE 3 |** The exon-intron structure of *MeWRKY* genes according to the phylogenetic relationship. The unrooted phylogenetic tree was constructed based on the full length sequences of *MeWRKY*s with 1000 bootstraps. Exon-intron structure analyses of *MeWRKY* genes were performed by using the online tool GSDS. Lengths of exons and introns of each *MeWRKY* gene were exhibited proportionally. (A–E) indicates different groups of WRKY family in cassava.



contained 2–5 introns; cluster B contained 1–5 introns; cluster C contained 1–3 introns; all cluster D *MeWRKY*s contained 2 introns, except for *MeWRKY46* with only one intron; and cluster E *MeWRKY*s contained two introns. According to a previous report (Nuruzzaman et al., 2010), the rate of intron loss is faster than the rate of intron gain after segmental duplication in rice. Consequently, it can be concluded that clusters A and B might contain the original genes, from which those in other clusters were derived. Generally, *MeWRKY*s in the same cluster of the phylogenetic tree show similar exon-intron structures.

## Expression Profiles of *MeWRKY* Genes in Different Tissues

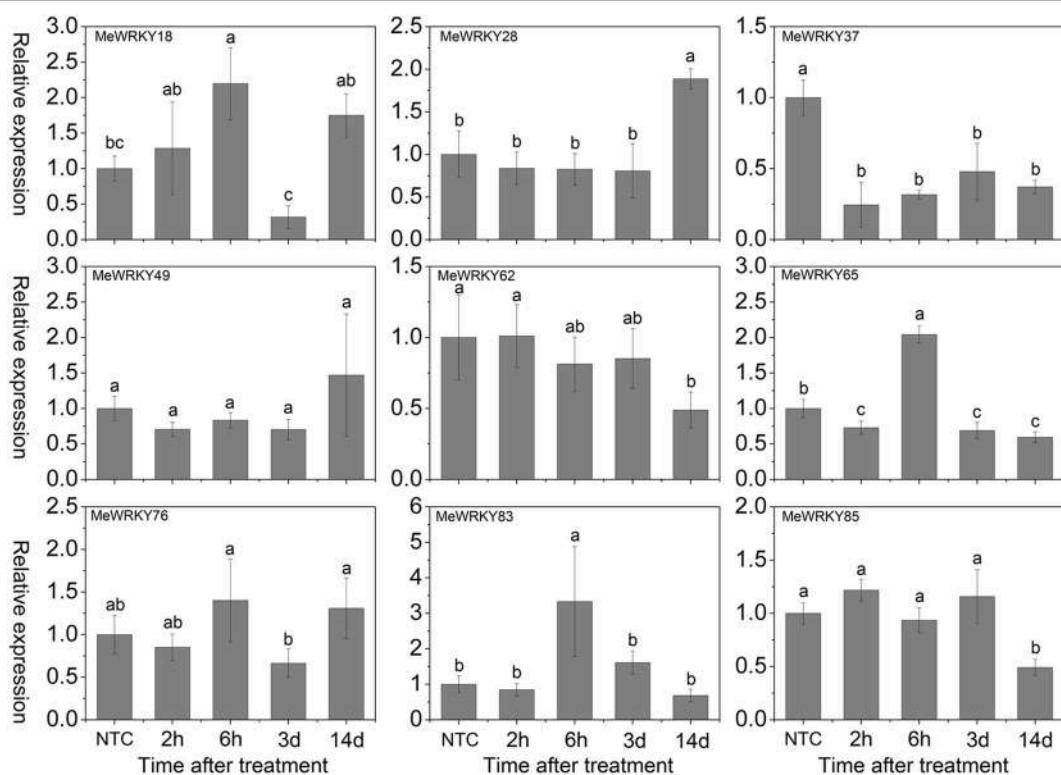
To provide some clues on the roles of *MeWRKY* genes in cassava growth and development, the expression profiles of *MeWRKY* genes from different organs, including stems, leaves and storage roots were tested in a wild subspecies (W14) and cultivated variety (Arg7) using transcriptomic data. W14, a wild cassava subspecies, has a low rate of photosynthesis, tuber root yield, and starch content in root tubers, but strong tolerance to drought stress (Wang et al., 2014a). Arg7, a cultivated variety, can tolerate moderate drought stress (Zhao et al., 2014). Expression analysis of *MeWRKY* genes in these two accessions will provide insight into cassava development between wild subspecies and cultivated

variety. Seventy-two of 85 *MeWRKY* genes were captured from the corresponding transcriptomic data (Figure 4; Table S4).

In the Arg7 variety, 100% (72/72), 94.4% (68/72), and 91.7% (66/72) of *MeWRKY* genes were expressed in stems, leaves, and storage roots, respectively, with 50% (36/72), 55.9% (38/68), and 63.6% (42/66) of *MeWRKY* genes showing high expression levels (value >5) in stems, leaves, and storage roots, respectively. Moreover, 90.3% (65/72) of *MeWRKY* genes were expressed in all organs examined, among which 40% (26/65) showed high expression levels (value >5) in all three organs.

In the W14 subspecies, 91.7% (66/72), 98.6% (71/72), and 88.9% (64/72) of *MeWRKY* genes were found to be expressed in stems, leaves, and storage roots, respectively, with 40.9% (27/66), 54.9% (39/71), and 57.8% (37/64) of *MeWRKY* genes showing high expression levels (value >5) in stems, leaves, and storage roots, respectively. Moreover, 81.9% (59/72) of *MeWRKY* genes were expressed in all organs examined, among which 30.5% (18/59) showed high expression levels (value >5) in all three organs.

About 20.8% (15/72) of *MeWRKY* genes with high expression levels (value >5) in all three tested organs in Arg7 and W14, suggesting that *MeWRKY* genes may be involved in organ development. Transcriptomic data also showed that 56 *MeWRKY* genes had a constitutive expression pattern that expressed in all the tissues of the two accessions, suggesting that these genes



**FIGURE 6 | Expression profiles of *MeWRKY* genes in leaves under salt stress.** The relative expression levels of *MeWRKY* genes in each treated time point were compared with that in each time point at normal conditions. NTC (no treatment control) at each time point was normalized as “1.” Data are means  $\pm$  SE calculated from three biological replicates. Values with the same letter were not significantly different according to Duncan’s multiple range tests ( $P < 0.05$ ,  $n = 3$ ).

might play a role in plant growth, development, and cellular homeostasis. The remaining 16 *MeWRKY* genes exhibited differential expression patterns, with specific to some particular tissues, such as *MeWRKY16*, *MeWRKY20*, and *MeWRKY23*. This phenomenon was also observed in rice (Ramamoorthy et al., 2008), cucumber (Ling et al., 2011), rubber tree (Li et al., 2014) and grape (Wang et al., 2014c), indicating that the functions of the WRKYS are diverse in both monocotyledon and dicotyledon.

There were 33 *MeWRKY* genes that showed higher expression levels in leaf and stem tissues in Arg7 than that in W14. However, 25 *MeWRKY* genes had higher expression levels in storage roots in W14 than that in Arg7. Interestingly, *MeWRKY8*, -18, -34, -45, -54, -80, and -83 showed higher expression levels in Arg7 than in W14 in leaf and stem tissues, but opposite result was observed in storage roots. These *MeWRKY* genes have strong expression levels for special tissues in different accessions, indicating their key roles in tissue development or tissue functions.

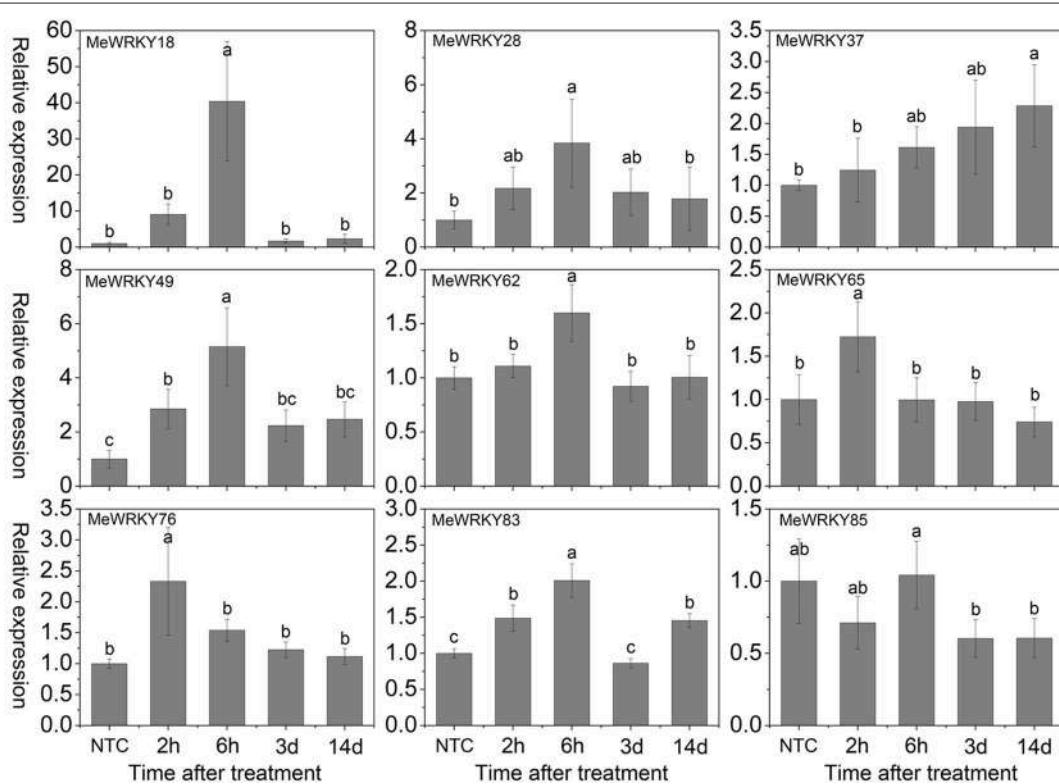
Generally, 15 out of 72 *MeWRKY* genes had high transcript abundance (value >5) in all the tested tissues of the two accessions, including *MeWRKY16*, -29, -50, -65, and -77 in group 1, *MeWRKY2*, and -42 in group 2c, *MeWRKY28*, -44, -58, -76, and -84 in group 2d and *MeWRKY15*, -18, and -55 in group 3. In contrast, 3 *MeWRKY* genes (*MeWRKY14*, and -31 in group 2c, *MeWRKY80* in group 3) showed low expression levels in all the tissues of the two accessions. Overall, the tissue

expression profiles of WRKY genes in different accessions may lay a foundation for further investigation of cassava development.

## Expression of *MeWRKY* Genes in Response to Drought in Different Accessions

Accumulated evidence has suggested that WRKY family genes play a significant role in plants' response to drought or osmotic stress (Ramamoorthy et al., 2008; Ren et al., 2010; Rushton et al., 2010; Ling et al., 2011; Tripathi et al., 2014). Thus, there is need to examine the expression patterns of WRKY genes in response to drought stress, which may provide important clues for further understanding the mechanisms of cassava involved in strong tolerance. For this reason, 3-month-old cassava seedlings (a wild subspecies W14 and two cultivated varieties Arg7 and SC124) were deprived of water for 12 days, and then the leaf and root tissues were collected to extract RNA for subsequent RNA-seq analysis. Heatmap representation of expression profiles of 78 *MeWRKY* genes under drought stress conditions were captured from the corresponding transcriptomic data (Figure 5; Table S5).

In the Arg7 variety, transcripts of 43.6% (34/78) and 33.3% (26/78) of *MeWRKY* genes increased after drought stress in leaves and roots, respectively, and 25.6% (20/78) and 50% (39/78) decreased in leaves and roots, respectively. Significant induction



**FIGURE 7 | Expression profiles of *MeWRKY* genes in leaves under osmotic stress.** The relative expression levels of *MeWRKY* genes in each treated time point were compared with that in each time point at normal conditions. NTC (no treatment control) at each time point was normalized as "1." Data are means  $\pm$  SE calculated from three biological replicates. Values with the same letter were not significantly different according to Duncan's multiple range tests ( $P < 0.05$ ,  $n = 3$ ).

(value >1) of 21.8% (17/78) and 23.1% (18/78) of *MeWRKY* genes was observed after drought stress in leaves and roots, respectively. Eleven genes (14.1%) were upregulated in both leaves and roots, with two genes (*MeWRKY11* and *MeWRKY14*) showing significant induction (value >1).

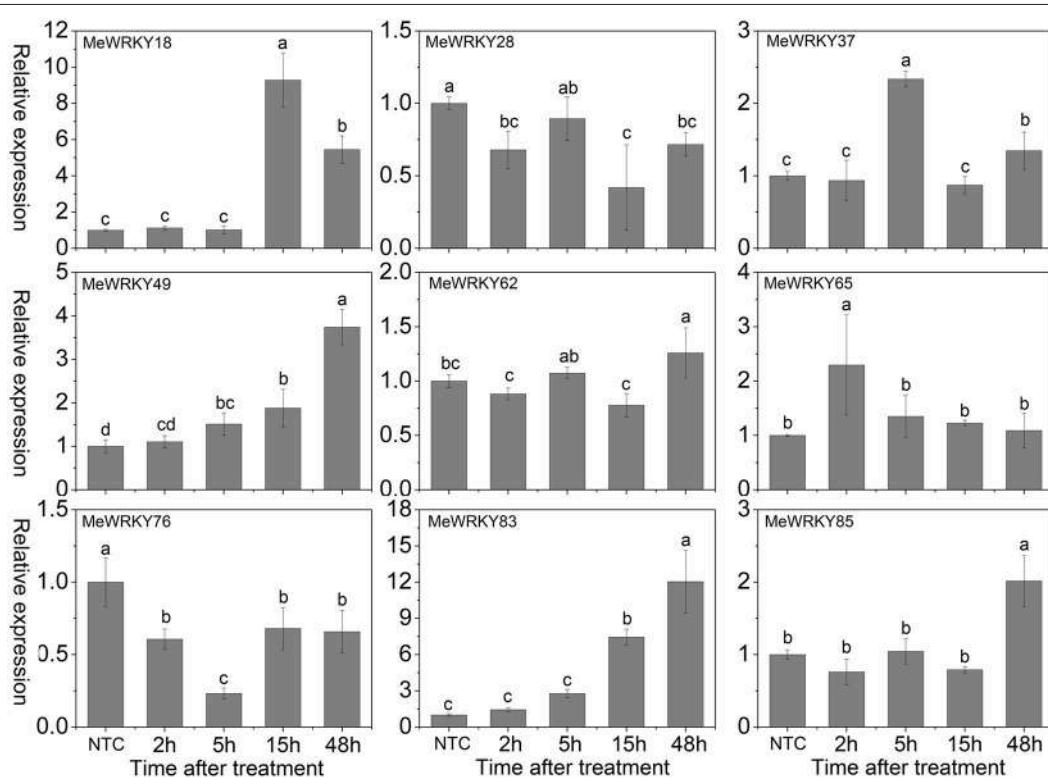
In the SC124 variety, transcripts of 47.4% (37/78) and 33.3% (26/78) of *MeWRKY* genes increased after drought stress in leaves and roots, respectively, and 30.8% (24/78) and 50% (39/78) decreased in leaves and roots, respectively. Significant induction (value >1) of 33.3% (26/78) and 11.5% (9/78) of *MeWRKY* genes was observed after drought stress in leaves and roots, respectively. Eighteen genes (23.1%) were upregulated in both leaves and roots, with six genes (*MeWRKY18*, -20, -40, -42, -49, and -83) showing significant induction (value >1).

In the W14 subspecies, transcripts of 6.4% (5/78) and 55.1% (43/78) of *MeWRKY* genes increased after drought stress in leaves and roots, respectively, and 67.9% (53/78) and 24.4% (19/78) decreased in leaves and roots, respectively. Significant induction (value >1) of 5.1% (4/78) and 32.1% (25/78) of *MeWRKY* genes was observed after drought stress in leaves and roots, respectively. Only *MeWRKY6* was upregulated in both leaves and roots.

The transcriptomic data given above showed that there were significantly more WRKY genes upregulated by drought at the transcription level in roots than in leaves in W14, but there were

fewer in roots than in leaves in Arg7 and SC124. There were also more WRKY genes significantly induced by drought (value >1) in roots than in leaves in W14 but fewer in roots than in leaves in SC124. W14 showed stronger tolerance to drought stress than SC124 and Arg7, two varieties commonly cultivated in China and Southeast Asia, respectively (Wang et al., 2014a). Cassava can form deep root systems (soil depth below 2 m), which is beneficial for penetrating into deeper soil layers and absorbing water stored in the soil (Okogbenin et al., 2013). Moreover, numerous studies have confirmed that the WRKY family genes play a positive role in the drought stress response in various species (Qiu and Yu, 2009; Ren et al., 2010; Jiang et al., 2012; Ding et al., 2014; Raineri et al., 2015). Therefore, these findings indicate that cassava WRKY genes might play an important role in water uptake from soil by roots, and hence maintaining strong tolerance to drought stress in W14 subspecies.

Generally, *MeWRKY* genes showed similar expression profiles in leaves or roots tissues in Arg7 and SC124, which was different from W14. After drought treatment, expression of some *MeWRKY* genes, including *MeWRKY2*, -6, -7, -10, -17, -19, -22, -31, -74, and -76, were upregulated in roots of W14, but downregulated in roots of SC124 and Arg7. The transcripts of some *MeWRKY* genes, including *MeWRKY2*, -10, -11, -14, -17, -27, -34, -39, -43, -47, -49, -55, -59, -65, -76, -77, -81, -83, -84, and -85, increased in leaves of Arg7 and



**FIGURE 8 | Expression profiles of *MeWRKY* genes in leaves under cold treatment.** The relative expression levels of *MeWRKY* genes in each treated time point were compared with that in each time point at normal conditions. NTC (no treatment control) at each time point was normalized as "1." Data are means  $\pm$  SE calculated from three biological replicates. Values with the same letter were not significantly different according to Duncan's multiple range tests ( $P < 0.05$ ,  $n = 3$ ).

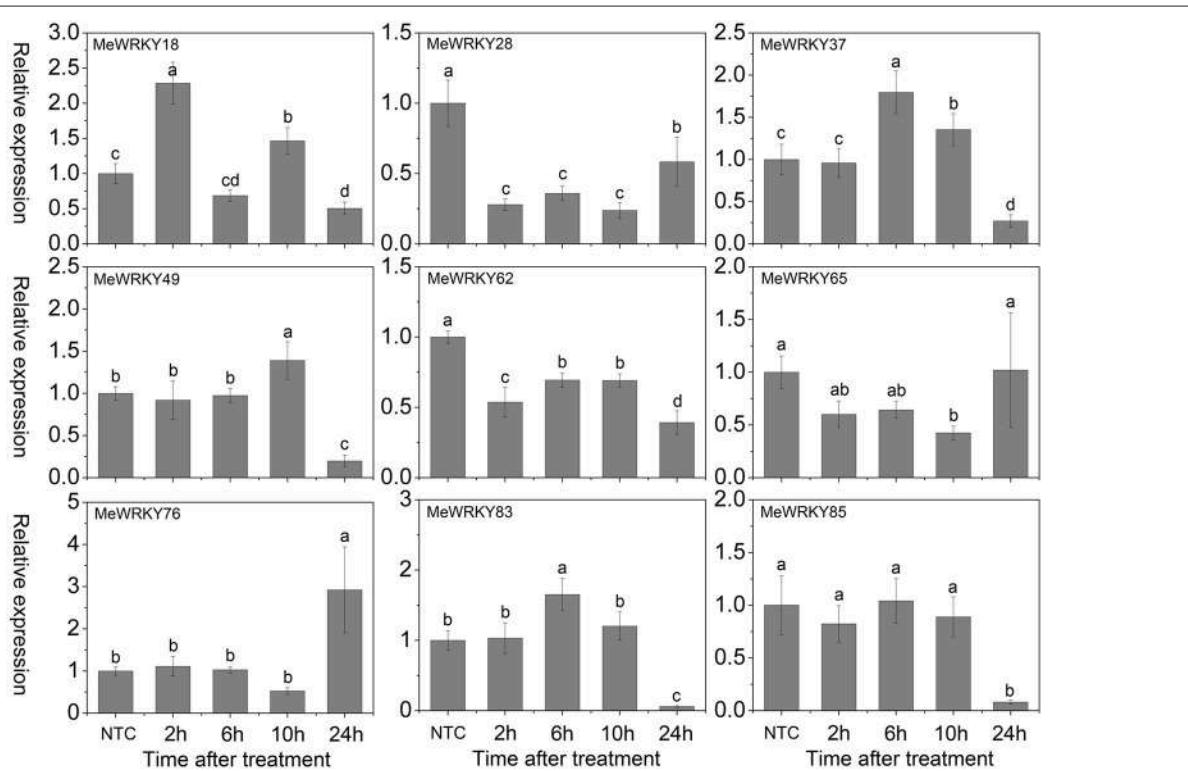
SC124, but decreased in leaves of W14 after drought treatment. WRKY genes in different accessions showed different expression profiles in response to drought, suggesting that the mechanisms of WRKYS involved in drought response differ between wild subspecies and cultivated varieties. Additionally, although some *MeWRKY* genes showed close phylogenetic relationships, their transcriptional levels showed different responses to drought, such as, *MeWRKY4* and -9, *MeWRKY27* and -77, *MeWRKY29* and -65, and *MeWRKY50* and -68 in group 1, *MeWRKY21* and -33 in group 2b, and *MeWRKY70* and -72 in group 2c. Taken together, the transcriptional response of *MeWRKY* genes to drought stress in wild subspecies and cultivated varieties may provide an opportunity for further investigation of the mechanisms underlying strong drought tolerance in cassava.

## Temporal Expression Profiles of *MeWRKY* Genes upon Exposure to Various Stress and Related Signaling

WRKY genes have been reported to play pivotal role in the regulation of plant tolerance to various stress and related signaling transduction in various species (Rushton et al., 2010, 2012; Tripathi et al., 2014; Banerjee and Roychoudhury, 2015). Hence, to investigate the roles of *MeWRKY* genes in response to various environmental stresses and related signaling, the

expression profiles of *MeWRKY* genes under these treatments were analyzed. Nine *MeWRKY* genes (*MeWRKY8*, -28, -37, -49, -62, -65, -76, -83, and -85) distributed in different subgroups and up-regulated by drought stress as indicated by RNA-seq data in different cassava accessions were selected for further examination of their transcriptional response to osmotic, salt, cold, ABA, and H<sub>2</sub>O<sub>2</sub> treatments.

Under NaCl treatment, *MeWRKY18* was induced after 2–6 h and 14 days treatment with significant up-regulation at 6 h. *MeWRKY65* and *MeWRKY83* were significantly induced at 6 h. *MeWRKY28* was significantly induced at 14 days, while *MeWRKY85* was visibly down-regulated at 14 days. *MeWRKY37* showed down-regulation at all the treated time-points. Other three WRKY genes (*MeWRKY49*, -62, and -76) did not display obvious trends during salt treatment (Figure 6). In Arabidopsis, some WRKY genes, including *AtWRKY8* (Hu et al., 2013), *AtWRKY18* (Chen et al., 2010), *AtWRKY25* (Jiang and Deyholos, 2009), *AtWRKY30* (Scarpaci et al., 2013), *AtWRKY33* (Jiang and Deyholos, 2009), *AtWRKY40* (Chen et al., 2010), *AtWRKY46* (Ding et al., 2014), *AtWRKY60* (Chen et al., 2010), and *AtWRKY75* (Yu et al., 2010), were reported to be up-regulated at transcriptional levels after salt treatment. Similarly, about 26 rice WRKY genes showed up-regulation upon salt stress treatment (Ramamoorthy et al., 2008; Yu et al., 2010; Tao et al., 2011). Accumulating evidence has suggested that some WRKY genes play a positive role of in response to salt



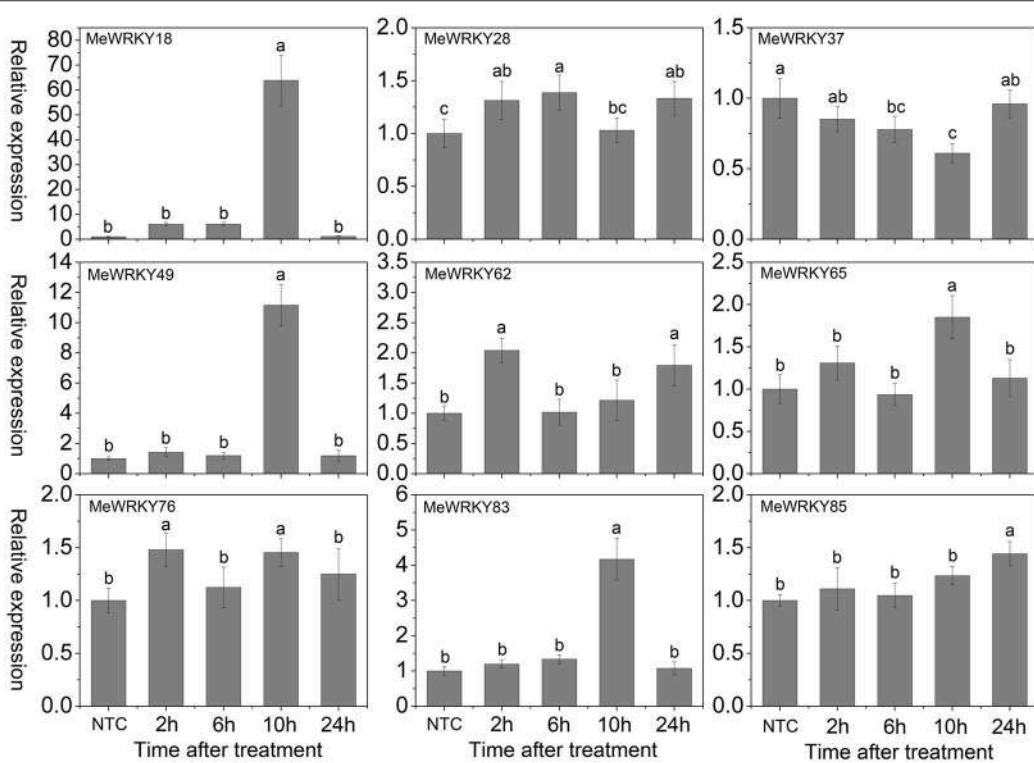
**FIGURE 9 | Expression profiles of *MeWRKY* genes in leaves under H<sub>2</sub>O<sub>2</sub> treatment.** The relative expression levels of *MeWRKY* genes in each treated time point were compared with that in each time point at normal conditions. NTC (no treatment control) at each time point was normalized as “1.” Data are means ± SE calculated from three biological replicates. Values with the same letter were not significantly different according to Duncan’s multiple range tests ( $P < 0.05$ ,  $n = 3$ ).

stress, such as *AtWRKY8* (Hu et al., 2013), *AtWRKY25* (Jiang and Deyholos, 2009), *AtWRKY30* (Scarpeci et al., 2013), and *AtWRKY33* (Jiang and Deyholos, 2009). However, other WRKYS, including *AtWRKY18* (Chen et al., 2010), *OsWRKY45-2* (Tao et al., 2011), *AtWRKY46* (Ding et al., 2014), and *AtWRKY60* (Chen et al., 2010) were found to act as negative regulators in salt stress response in Arabidopsis and rice. These studies indicated that *MeWRKY* genes may be involved in the salt stress response.

As shown in Figure 7, under mannitol treatment, *MeWRKY18*, -28, -37, -49, and -76 were induced during 2 h 14days treatment and showed significant induction at 6 h, 6 h, 14 days, 6 h, and 2 h, respectively. *MeWRKY62* and *MeWRKY83* expression were induced during 2–6 h and 14 days treatment with significant up-regulation at 6 h. *MeWRKY65* showed induction at 2 h treatment. *MeWRKY85* did not show obvious trends during mannitol treatment. Notably, *MeWRKY18* showed up-regulation at all treated points and reached the highest expression level (value >40) at 6 h, indicating its possible roles in osmotic/drought stress responses. In Arabidopsis, some WRKY genes, including *AtWRKY57* (Jiang et al., 2012) and *AtWRKY63/ABO3* (Ren et al., 2010), have been reported to positively regulate drought stress tolerance. However, some WRKY genes, including *AtWRKY18* (Chen et al., 2010), *AtWRKY46* (Ding et al., 2014), *AtWRKY53* (Sun and Yu, 2015), *AtWRKY54* (Li et al., 2013), *AtWRKY60* (Chen et al., 2010), and

*AtWRKY70* (Li et al., 2013), which showed significant induction during drought stress, have been reported to negatively regulate drought stress tolerance. *MeWRKY18*, showing high similarity with *AtWRKY54*, may represent a functional gene involved in drought tolerance in cassava. In rice, 23 WRKY genes have been reported to be induced under drought treatment (Ramamoorthy et al., 2008; Qiu and Yu, 2009; Wu et al., 2009; Shen et al., 2012; Raineri et al., 2015), among which *OsWRKY11* (Wu et al., 2009), *OsWRKY30* (Shen et al., 2012), *OsWRKY45* (Qiu and Yu, 2009), and *OsWRKY47* (Raineri et al., 2015) have been confirmed to function as positive factors in the regulation of plant tolerance to drought/osmotic stress. In cucumber, the expression of 4 WRKY genes (*CsWRKY2*, -14, -18, -21) was found to be upregulated after drought treatment (Ling et al., 2011). In cotton (*Gossypium hirsutum*) roots, 15 out of 26 *GhWRKs* (*GhWRKY9*, -10, -11, -13, -14, -17, -18, -19, -20, -23, -24, -29, -32, -33, and -34) and 7 out of 26 *GhWRKs* (*GhWRKY12*, -15, -21, -22, -26, -27, and -30) were up- and down-regulated, respectively, under dehydration conditions (Zhou et al., 2014). Together, these results indicate the important roles of these WRKY genes in response to osmotic/drought stress.

Cold stress, a common environmental stress, affects plants growth and crop productivity, especially in tropical and sub-tropical origin (Wang et al., 2012). However, little is known about the mechanisms underlying the action of WRKYS in



**FIGURE 10 | Expression profiles of *MeWRKY* genes in leaves under ABA treatment.** The relative expression levels of *MeWRKY* genes in each treated time point were compared with that in each time point at normal conditions. NTC (no treatment control) at each time point was normalized as “1.” Data are means  $\pm$  SE calculated from three biological replicates. Values with the same letter were not significantly different according to Duncan’s multiple range tests ( $P < 0.05$ ,  $n = 3$ ).

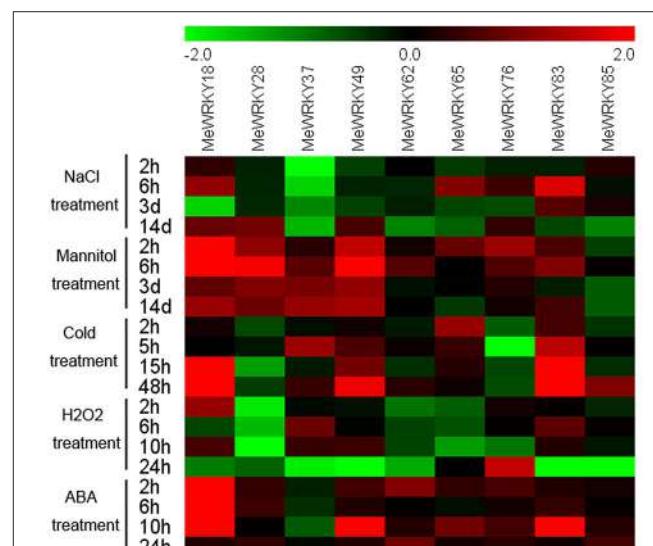
cold stress response. In Arabidopsis, WRKY34 was reported to be significantly induced by cold treatment and act as a negative regulator to cold response (Zou et al., 2010). In rice, 2 and 15 WRKY genes were up- and down-regulated by cold treatment, respectively (Ramamoorthy et al., 2008; Yokotani et al., 2013). Among them, overexpression of *OsWRKY76* increased tolerance to cold stress (Yokotani et al., 2013). Under cold treatment, *MeWRKY18*, -49, -65, and -83 showed up-regulation at all the treated time-points, with significant up-regulation at 15, 48, 2, and 48 h, respectively. *MeWRKY37*, -62, and -85 showed significant up-regulation at 5, 48, and 48 h, respectively. However, *MeWRKY28* and -76 expression was repressed during all the treated time points (Figure 8). The expression levels of *MeWRKY49* and -83 increased as treatment time continued, suggesting their possible function in cold response. They could be used in further functional characterization. Cassava, an important tropical crop, is distributed in tropical areas all over the world. Cold stress significantly restricts plant growth, agricultural productivity, and the development of cassava. Research on WRKY-mediated cold response in cassava may benefit further functional characterization of WRKY genes and investigations of the mechanisms underlying the cold response in cassava.

$H_2O_2$ , a well-known toxic molecule, plays a key role in several biotic and abiotic signaling pathways and its accumulation has been found to be induced by environmental and developmental stimuli (Costa et al., 2010). In Arabidopsis, several WRKYS, including *AtWRKY6*, -22, -28, -30, -46 (Scarpaci et al., 2008), and *AtWRKY25* (Jiang and Deyholos, 2009), are rapidly and highly induced after oxidative stress treatment. Among them, *AtWRKY28* (Babitha et al., 2013) and *AtWRKY30* (Scarpaci et al., 2013) were found to positively regulate oxidative stress tolerance, whereas *AtWRKY25* (Jiang and Deyholos, 2009) acts as a negative regulator of oxidative stress response. In other species, some evidence has suggested that WRKY genes play a positive role in response to oxidative stress; for example, silencing of *SIDRW1*, a WRKY gene from tomato plants (*Solanum lycopersicum*), increased the sensitivity of transgenic plants to  $H_2O_2$  with less chlorophyll content in leaf discs (Liu et al., 2014). Overexpression of *ThWRKY4* in Arabidopsis, a WRKY gene from tamarisk (*Tamarix hispida*), enhanced tolerance to oxidative stress (Zheng et al., 2013). To determine whether cassava WRKY genes play a role in oxidative stress response, the expression of 9 *MeWRKY* genes in response to  $H_2O_2$  was examined. Results suggested that *MeWRKY18* and *MeWRKY37* showed significant up-regulation at 2 and 6 h treatments, respectively. *MeWRKY76* was significantly induced at 24 h, while *MeWRKY49*, -83, and -85 were seriously down-regulated at 24 h. *MeWRKY28* and *MeWRKY62* were strongly repressed at all the treated time-points. *MeWRKY65* did not show obvious trends after  $H_2O_2$  treatment (Figure 9). These results suggest that cassava WRKYS are likely to be involved in oxidative stress response.

The phytohormone ABA mediates plant responses to abiotic stresses, such as salinity, drought, and cold (Rushton et al., 2010; Mittler and Blumwald, 2015). Evidence has suggested that WRKYS play a crucial role in ABA-mediated signal transduction

in plants (Rushton et al., 2010, 2012; Tripathi et al., 2014). In Arabidopsis and rice, several WRKYS, including *AtWRKY18* (Chen et al., 2010), *AtWRKY25* (Jiang and Deyholos, 2009), *AtWRKY33* (Jiang and Deyholos, 2009), *AtWRKY40* (Chen et al., 2010), *AtWRKY60* (Chen et al., 2010), *AtWRKY63/ABO3* (Ren et al., 2010), *OsWRKY24*, -51, -71, and -77 (Xie et al., 2005), *OsWRKY45-1* and -45-2 (Tao et al., 2011), *OsWRKY72* (Yu et al., 2010), and *OsWRKY76* (Yokotani et al., 2013) have been shown to be induced after ABA treatment. Among them, *AtWRKY18* (Chen et al., 2010), *AtWRKY60* (Chen et al., 2010), *AtWRKY63/ABO3* (Ren et al., 2010), and *OsWRKY45-2* (Tao et al., 2011) take part in the positive regulation of ABA signaling. To investigate the response of *MeWRKYs* in ABA signaling, the expression of 9 *MeWRKYs* in response to ABA treatment was examined. Results suggested that *MeWRKY18*, -28, -49, -62, -76, -83, and -85 expression were induced at all the treated time-points, among which *MeWRKY18*, -49, and -83 showed significant up-regulation at 10 h and *MeWRKY28* was significantly induced at 6 h. *MeWRKY37* expression was repressed at all the treated time-points. *MeWRKY65* was significantly up-regulated at 10 h (Figure 10). The expression levels of *MeWRKY18*, -49, and -83 were over four-fold higher at 10 h ABA treatment, indicating their possible function in ABA signaling.

Overall, the patterns in the expression of *MeWRKYs* under various conditions suggest that different *MeWRKY* genes may be involved in different signaling and stress responses, and that a single *MeWRKY* gene also participates in multiple signaling and stress processes. Moreover, most of the cassava WRKY genes can be quickly and significantly induced by multiple stressors, ABA, and  $H_2O_2$  treatments, indicating that WRKY



**FIGURE 11 | Expression profiles of *MeWRKY* genes in leaves under various stresses and ABA treatments.** Log<sub>2</sub> based values from three replicates of qRT-PCR data were used to create the heatmap. The scale represents the relative signal intensity values. Relative expression values for each gene after various treatments are provided in Figures 6–10 and Table S6.

genes may function on multiple transduction pathways in cassava (**Figure 11; Table S6**).

## CONCLUSIONS

In this study, 85 WRKY genes from the cassava genome were identified and their basic classification and evolutionary characteristics were established. This information may provide abundant resources for functional characterization of WRKY genes. The differential expression patterns of *MeWRKYS* in tissues of the wild subspecies and cultivated varieties revealed that they play different roles in cassava development, and a large number of them exhibited tissue-specific expression, thus assisting in understanding the molecular basis for genetic improvement of cassava. In addition, transcriptomic analysis of different cassava accessions associated with drought stress indicated that the majority of *MeWRKYS* in the root of W14 subspecies were activated in response to drought, which may contribute to its strong tolerance to drought. Furthermore, analysis of the expression of *MeWRKY* genes after various treatments suggested that they have a comprehensive response to osmotic, salt, ABA, H<sub>2</sub>O<sub>2</sub>, and cold, implying that cassava WRKYS may represent convergence points of different signaling pathways. These data may facilitate further investigation of WRKY-mediated signaling transduction pathways. Taken together, this work would provide a solid foundation for future functional investigation of the WRKY family in cassava.

## Availability of Supporting Data

The cassava WRKY genes identified in this study was submitted to GenBank and the accession number was shown in **Table S3**. The transcriptomic data was submitted to NCBI and the accession number was shown in **Table S7**.

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## AUTHOR CONTRIBUTIONS

WH, KL, HS, and ZX conceived the study. YW, ZX, WT, ZD, YY, WW performed the experiments and carried out the analysis. WH, YW, and HS designed the experiments and wrote the manuscript. All authors read and approved the final manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2016.00025>

**Figure S1 | Sequence logos for conserved motifs identified in MeWRKYS by MEME analysis.**

**Table S1 | Primers used in qRT-PCR analysis.**

**Table S2 | Characteristics of MeWRKY family in cassava.**

**Table S3 | The accession numbers of WRKYS in cassava and Arabidopsis.**

**Table S4 | The expression data of the cassava WRKY genes in different tissues.**

**Table S5 | The expression data (log2-based values) of the cassava WRKY genes after drought treatment.**

**Table S6 | The expression data (log2-based values) of the cassava WRKY genes after various stresses, ABA and H<sub>2</sub>O<sub>2</sub> treatments.**

**Table S7 | The accession number of transcriptomic data in NCBI.**

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# UV-B Induced Generation of Reactive Oxygen Species Promotes Formation of BFA-Induced Compartments in Cells of *Arabidopsis* Root Apices

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UV-B radiation is an important part of the electromagnetic spectrum emitted by the sun. For much of the period of biological evolution organisms have been exposed to UV radiation, and have developed diverse mechanisms to cope with this potential stress factor. Roots are usually shielded from exposure to UV by the surrounding soil, but may nevertheless be exposed to high energy radiation on the soil surface. Due to their high sensitivity to UV-B radiation, plant roots need to respond rapidly in order to minimize exposure on the surface. In addition to root gravitropism, effective light perception by roots has recently been discovered to be essential for triggering negative root phototropism in *Arabidopsis*. However, it is not fully understood how UV-B affects root growth and phototropism. Here, we report that UV-B induces rapid generation of reactive oxygen species which in turn promotes the formation of BFA-induced compartments in the *Arabidopsis* root apex. During unilateral UV-B irradiation of roots changes in auxin concentration on the illuminated side have been recorded. In conclusion, UV-B-induced and ROS-mediated stimulation of vesicle recycling promotes root growth and induces negative phototropism.

**Keywords:** UV-B, reactive oxygen species, root, negative phototropism, light-escape tropism

## INTRODUCTION

Due to their sessile nature, plants must accommodate changes in the light environment. Light is an essential physical factor in whole plant life cycle for photosynthesis and the regulation of plant development such as seed germination, cell elongation, organ tropisms, and flowering. Many proteins, including photoreceptors and other signaling molecules, are involved in the regulation of many physiological events as well as phenotypic plasticity in response to the light environment. Sunlight penetrating the Earth's atmosphere contains a continuous spectrum of far- red and visible light as well as UV (ultra-violet) radiation. It has been reported that UV radiation affects plant development through various physiological processes (Frohnmeier and Staiger, 2003; Jenkins, 2009). However, it is also well known that an excess amount of UV, containing high energy photons, damages the plant cells (Jansen et al., 1998). Free radicals, especially reactive oxygen species (ROS), are a typical by-product of the photo-excitation of certain compounds (Hidetoshi and Vass, 1996; Allan and Fluhr, 1997; Pristov et al., 2013). ROS directly oxidize many biomolecules such as phospholipids in the plasma membrane, proteins, and nucleic acids, leading to severe damage to

plant cells (Frohnmyer and Staiger, 2003). However, in plant cells ROS also play an important role as signaling molecules in the effective regulation of cellular redox-homeostasis (Green and Fluhr, 1995; Hidetaka et al., 2013), as well as in promoting the biosynthesis of flavonoids which function as a sunscreen (Li et al., 1993; Hectors et al., 2012). In other words, UV-B induced ROS production seems to be involved in plant adaptation to UV radiation.

Since some decades ago, it has been revealed that roots are in fact light sensitive plant organs equipped with a range of photoreceptors and related signaling pathways (Feldman and Briggs, 1987; Kutschera and Briggs, 2012). Only one photoreceptor was known in 1971 whereas fourteen are discovered to date (Briggs and Lin, 2012). The root system possesses the same photoreceptors as the above-ground parts of the plant, which most likely allows roots to respond to light direction, intensity and wavelength (Chen et al., 2004; Briggs and Lin, 2012). It is well known that root-expressed phytochromes are involved in root hair formation, root growth and root gravitropism (De Simone et al., 2000; Correll and Kiss, 2005; Mo et al., 2015). Intriguingly, *Arabidopsis* roots also express the UV-B photoreceptor UVR8 (Rizzini et al., 2011) and a root-specific regulator, ROOT UVB SENSITIVE (RUS1 and 2) (Tong et al., 2008; Leasure et al., 2009); indicating that roots, like shoots, might possess a physiological mechanism for responding to external UV-B radiation.

In 1879, Francis Darwin was the first to discover negative phototropism of plant roots. One year later together with his father, Charles, Francis Darwin published the book, "The Power of Movements in Plants," in which they not only describe both root and shoot tropisms, but also propose that some form of long-distance signaling can link the sensory organ apices with underlying basal tissues (Darwin, 1879, 1880). Since then research in plant physiology has resulted in the discovery of the signaling molecule, auxin, and provided us with insights into plant photo-reception. Specific photoreceptors enable plants to perceive and directionally respond to incoming light and this response is called phototropism. Growth toward a light source, as can be observed in shoots, is called positive phototropism, while bending away from the light source, as can be seen in roots, is called negative phototropism. We have previously reported that brief but strong blue light illumination ( $2 \text{ mW/cm}^2$ ) of *Arabidopsis* roots induces immediate generation of ROS in root tips, resulting in an increase in root growth rate (Yokawa et al., 2011, 2013). This light-induced and ROS-enhanced root growth response is called escape tropism (Xu et al., 2013; Yokawa et al., 2013; for maize roots see Burbach et al., 2012) which, together with negative phototropism (Wan et al., 2012), enables *Arabidopsis* roots to effectively respond by avoiding unfavorable light conditions. It was also reported that PIN2 proteins (PIN-FORMED 2; auxin efflux carrier) expressed in root cells react to the light environment (Laxmi et al., 2008; Kagenishi et al., 2015). Wan et al. (2012) recently demonstrated that phototropin distribution in root cells is altered in response to blue light illumination (Wan et al., 2012). Interestingly, Dyachok et al. (2011) report that when COP1 (CONSTITUTIVE PHOTOMORPHOGENIC1) is activated by light, it dramatically enhances actin polymerization and F-actin

bundling through regulation of the downstream ARP2/3-SCAR pathway in root cells, resulting in enhanced root elongation during the illumination period (Dyachok et al., 2011).

In nature, roots grow underground in the dark soil, to anchor the plant and absorb nutrients and water. Besides positive gravitropism, negative phototropism is essential to maintain appropriate root growth. Phototropism, like other root tropisms, requires polar auxin transport based on a high rate of endocytic vesicle recycling which relocates various membrane proteins including PINs (Blilou et al., 2005; Baluška et al., 2010; Wan et al., 2012; Baluška and Mancuso, 2013). Polar auxin transport, especially PIN2-based auxin transport toward the shoot, is sensitive to blue light which in turn is essential for differential cell growth controlling the underlying negative phototropism of *Arabidopsis* roots (Wan et al., 2012).

However, the mechanism that guides negative phototropism in response to UV-B irradiation is not yet understood. Here, we report that UV-B illumination induces almost immediate ROS generation at the root apex, resulting in the alteration of endocytic vesicle recycling. We have detected a change in the distribution of auxin in root apical cells during UV-B irradiation.

## MATERIALS AND METHODS

### Plant Material

*Arabidopsis* (*Arabidopsis thaliana* L.) seeds were soaked in a sterilizing solution containing 4% sodium hypochlorite and 0.1% Triton X-100 for 15 min and washed at least five times with sterile distilled water. Sterilized seeds were planted on a 0.4% phytogel-fixed half-strength Murashige-Skoog growth medium without vitamin B. Petri dishes were incubated vertically at 23°C in darkness, in order to prevent the roots from acclimating to light.

### UV-B Treatment

For the UV-B treatments, all samples were irradiated with a UV-B 311 nm narrow band lamp (Philips, PL-S 9W/01/2P, Poland). The experimental UV-B intensity was measured and calibrated in each experiment with a UV-B broadband meter (Solarmeter model 6.0, Solartech, MI, USA). The values of UV-B described in the study are un-weighted UV-B intensity. For the UV-B treatments, the seedlings were placed between a microscope slide and a coverslip and kept in a vertical position. All root samples were irradiated from 20 to 30 cm distance of the light source (no heat transmission) with the cover slip in place, entirely blocking potential UV-C radiation emitted by the UV light source. This was confirmed with measurements using a spectrophotometer. For white light treatment, an array of light emitting diode (LED) was used to illuminate, and the irradiance was measured with photometer (HD2302.0, Delta Ohm) with a detector (LP471RAD, Delta Ohm). In the control treatment without UV-B radiation, the same UV-B lamp was covered with a polyester film to block UV-B radiation enabling the effect of any visible plus UV-A background illumination emitted by the lamp to be measured.

## Histochemical ROS Detection

Reactive oxygen species detection was carried out using histochemical staining methods. 3', 3'-Diaminobenzidine (DAB) and Nitroblue tetrazolium (NBT) were used for detecting hydrogen peroxide and superoxide, respectively. For NBT staining, the seedlings were incubated in a solution of 1/10 MS medium for 10 min and then transferred to a 50  $\mu\text{M}$  NBT solution dissolved in 1/10 MS medium for another 5 min at room temperature. Afterwards the samples were rinsed several times and the roots were irradiated with UV-B for 20 min. Likewise, DAB staining was carried out using the following procedure: the seedlings were incubated in a Tris buffer (pH 5.0) for 10 min and then infiltrated with the DAB solution (final concentration 0.7 mg/ml), for 5 min in a vacuum chamber at room temperature. The samples were washed twice with the Tris buffer. The roots were then treated with UV-B radiation for 20 min. The images of roots were captured with  $\times 10$  objective lens of a light microscope Leica DM750 (Solms, Germany). The staining intensity in root apex region was digitized and compared using densitometric method by ImageJ software (ver. 1.43u for Macintosh OSX).

## Confocal Microscopy for Monitoring Endocytic Vesicle Recycling Activity

To monitor the vesicle recycling in root cells, roots were treated with Brefeldin-A (BFA), which binds ARFGNOM; an inhibitor of exocytosis, and FM4-64 fluorescence dye to visualize the plasma membrane. A BFA stock solution was prepared in DMSO at 35 mM concentration. Since the root apical region is very delicate and sensitive to stress, the procedure for monitoring endocytic vesicle recycling was executed very carefully. Firstly, seedlings were placed between a microscope slide and a cover slip and the gap in between was then filled with 1/10 MS medium and kept in a vertical position. The seedlings were incubated for at least 1 h in darkness to reduce any endocytic activity in the root cells. After incubation, the MS medium was removed carefully with small strips of filter paper and replaced by the FM4-64 solution. The roots were then incubated for 10 min to allow proper staining. The FM4-64 solution was then replaced with the BFA solution (final concentration 35  $\mu\text{M}$ ) as described above. The roots were then irradiated with UV-B (UV-C region was completely filtered by the glass cover slip) for 20 min. After the treatment with BFA and UV-B, the seedlings were rinsed with 1/10 MS medium and observed under a confocal laser-scanning microscope (FV-1000, Olympus). Images of FM4-64 fluorescence were captured using excitation and emission wavelengths of 514 and 640-nm, respectively, under  $\times 40$  magnification with an oil-immersed lens.

Auxin distribution altered by unilateral UV-B illumination was visualized using the transgenic *Arabidopsis* line DII-VENUS with a laser-scanning confocal microscope (Brunoud et al., 2012). The concentration of auxin in each cell is reflected by the fluorescence of the nuclei. The samples of the DII-VENUS line were stained with FM4-64 to visualize cell membranes. The excitation and emission wavelengths used to analyze fluorescence in the DII-VENUS line were

515 and 528-nm, respectively (FM4-64 staining carried out as above). Data quantification of the microscope images was performed using ImageJ (ver. 1.43u for Macintosh OSX).

## Image Analysis of BFA-Induced Compartments and DII-VENUS Fluorescence

On computer display, the diameter of BFA-induced compartments visualized with FM4-64 was measured using ImageJ software manually. BFA-induced compartments found in the epidermal cells in transition zone, between meristem and elongation zone, was measured and averaged. Small vesicles (less than 1  $\mu\text{m}$  diameter) were not counted.

For the comparison of DII-VENUS fluorescence, the fluorescence images of green channel (VENUS) taken under the confocal were converted into inverted gray scale using ImageJ. Either left or right side of epidermal cells in root transition zone was selected and total pixels were counted. The ratio of fluorescence intensity between two sides of roots were calculated and averaged to quantify the changes of auxin distributions.

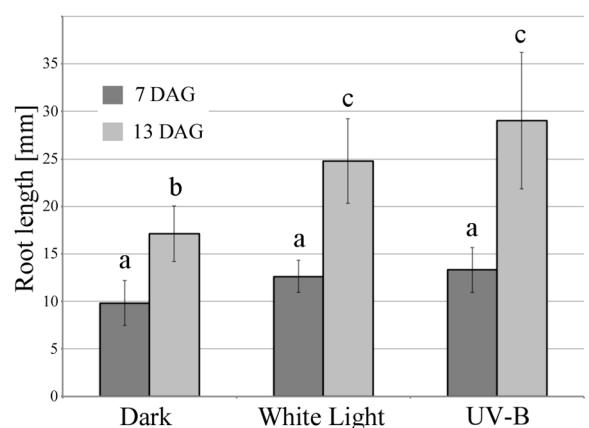
## Statistic Analysis

All numerical data obtained here were analyzed and tested in appropriate statistical methods. Student's *t*-test was applied to test a level of significance using Microsoft Excel 2011, and for the comparison of the effects of either UV-B on BFA-compartment formation in Figure 3 or three different diphenyliodonium (DPI) concentrations in Figure 4, Tukey's HSD (honestly significant difference) was applied to test a level of significance at  $p < 0.05$  using R software (R for Mac OS X Cocoa, <http://www.R-project.org>).

## RESULTS

### *Arabidopsis* Root Growth is Promoted by UV-B Treatment

As described above, the illumination of roots stimulates primary root growth. Figure 1 shows that the growth rate of *Arabidopsis* roots was enhanced by periodic treatment with white light as well as under UV-B irradiation. Starting from 7 days after germination, root growth rate was increased compared to control roots grown in darkness. To assess the effect of low-dose of lights on root growth, periodical irradiation was conducted. UV-B and white light was programmed to irradiate samples incubated in dark chamber at room temperature for 1 min at the beginning of every hour 16 times in a day (8 h were dark cycle). The intensities of UV-B and white light were at 0.3  $\text{mW/cm}^2$  (*c.* 6.7  $\text{kJ/m}^2/\text{day}$ ) and 2  $\text{mW/cm}^2$  (*c.* 19.2  $\text{kJ/m}^2/\text{day}$ ). As indicated previously, *Arabidopsis* roots display escape tropism by bending away from the UV-B source due to an enhanced root growth rate. The UV-B irradiance dose used here is equivalent to that occurring naturally, and it stimulates negative root phototropic responses,



**FIGURE 1 | UV-B and white light-induced primary root elongation.**

Primary root length was measured at 7 and 13 days after germination. The light and dark period was 16 and 8 h, respectively. White light irradiance was  $2 \text{ mW/cm}^2$  (c.  $19.2 \text{ kJ/m}^2/\text{day}$ ) and UV-B was  $0.3 \text{ mW/cm}^2$  (c.  $6.7 \text{ kJ/m}^2/\text{day}$ ). Error bars indicate  $\pm 1$  standard error. ( $n = 14$ ) Different letters on the bars indicate significant difference tested by one-way ANOVA,  $p < 0.05$ .

whereas root growth is strongly inhibited under high UV-B irradiance.

### UV-B Irradiation Triggers Rapid ROS Production in Root Apices

To identify whether ROS function as primary signaling molecules in the UV-B-provoked root response, NBT and DAB histochemical staining methods were applied. NBT and DAB reagents react with superoxide and hydrogen peroxide, respectively. These chemicals immediately form a colored precipitate upon contact with the free radicals, which can then be visualized by microscopy. Strong NBT staining patterns were present in the region of the root apex after 20 min of UV-B irradiation at  $0.3 \text{ mW/cm}^2$  (Figure 2). This illustrates that irradiation with UV-B induced the generation of superoxide in the root apex, which is metabolically very active compared to other parts of the root. In addition, staining intensities reflecting ROS concentration are reduced by treatment with DPI, which is commonly used as an inhibitor of flavo-proteins, including NADPH oxidase (Figure 2A). Although DPI inhibits the function of membrane-associated NADPH oxidase, ROS formation was nevertheless found in *rhd2-4* mutants (NADPH oxidase-deficient mutant, data not shown). Hence, the source of ROS generation must be another light-absorbing molecule containing a flavin group, because DPI also inhibits flavin-mediated electron transfer. Allan and Fluhr (1997) suggested that UV-B-induced ROS generation is likely to originate from specific flavo-proteins. Staining with DAB enabled us to detect hydrogen peroxide production which occurred in the same root region as superoxide production (Figure 2B). This suggests that the superoxide generated by UV-B is immediately converted into hydrogen peroxide by superoxide dismutase (SOD).

### UV-B Activates Endocytic Vesicle Recycling in Cells of *Arabidopsis* Root Apices

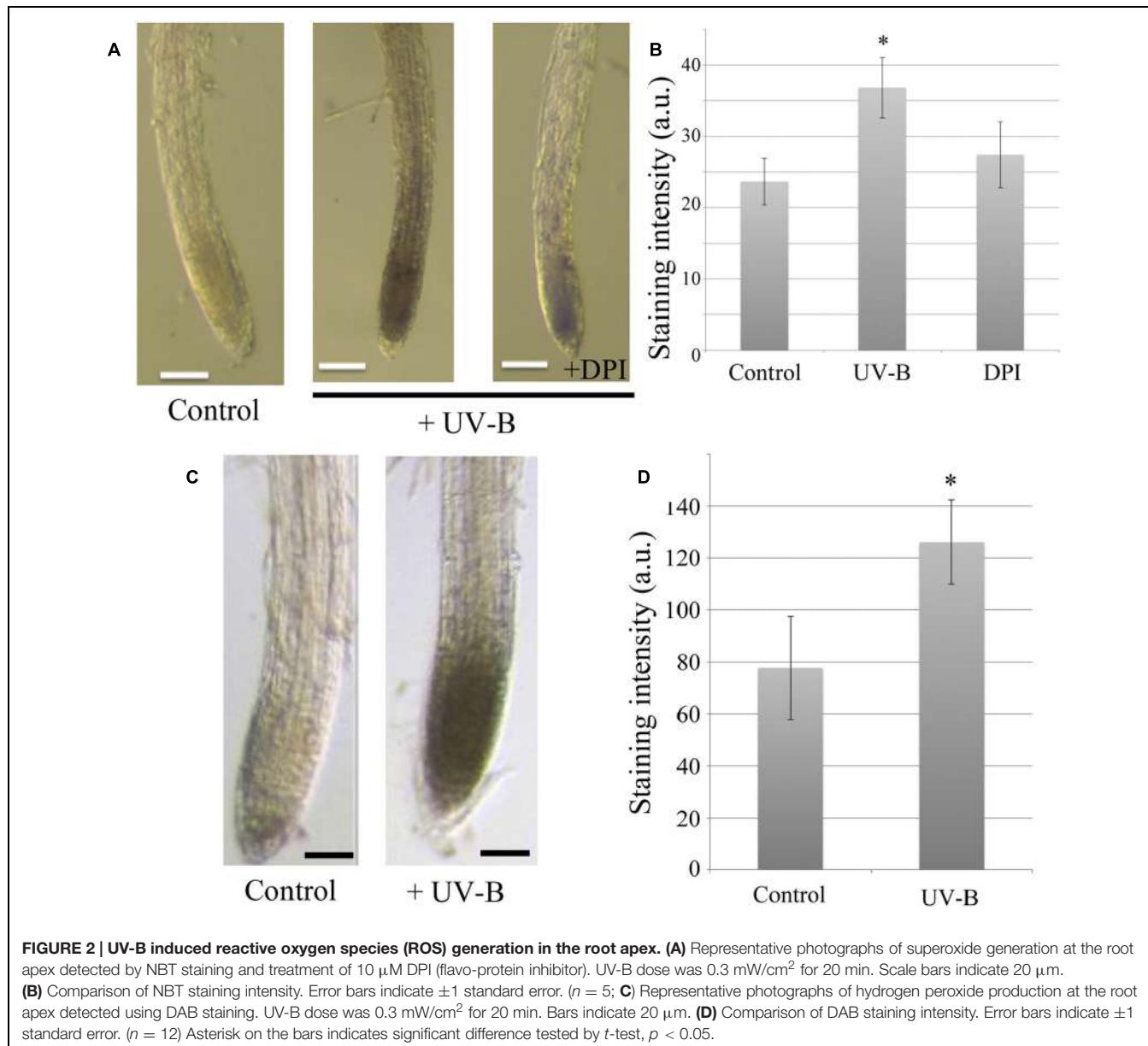
We assessed the effect of UV-B radiation on endocytic vesicle recycling in root cells, because vesicle recycling plays a vital role in polar auxin transport involved in root tropisms. The rate of endocytic vesicle recycling can be visualized using the Brefeldin-A (BFA) reagent, which inhibits exocytosis and leads to the formation of a spherical structure in the cytosolic space called BFA-compartment. After 20 min of UV-B treatment ( $0.3 \text{ mW/cm}^2$ ), BFA-compartments formed in root epidermal cells of the transition zone (Figure 3A), whereas they were not formed in the control plants kept in darkness. Importantly, the endocytic formation of the BFA-induced compartments in response to UV-B did not occur in the UV-B control treatments, when the UV-B lamp was covered with a polyester film. This shows that the background illumination (ranging from UV-A to all visible wavelengths) emitted by the lamp did not alter root cellular responses (Figure 3B). These results indicate that the increase in the rate of vesicle recycling under UV-B radiation is an integral part of negative root tropism to avoid UV-B radiation.

### ROS Enhances Endocytic Vesicle Recycling in Cells of *Arabidopsis* Root Apices

In order to clarify the relationship between ROS and vesicle recycling, the BFA-induced compartments in roots were monitored after the treatment of exogenously applied hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) which induces oxidative stress (Figure 4). The number of BFA compartments was increased by the treatment of  $\text{H}_2\text{O}_2$  at a concentration of  $100 \mu\text{M}$  in the absence of UV-B, indicating that ROS might affect polar auxin transport and root tropism because endocytic vesicle recycling plays important roles in these processes. Additionally, treatment with the flavo-protein inhibitor DPI, illustrated the effect of UV-B radiation on the formation of BFA-induced compartments. The size of these BFA compartments was significantly decreased in root cells treated with  $30 \mu\text{M}$  DPI and UV-B (20 min,  $0.3 \text{ mW/cm}^2$ ) (Figure 5). Based on the results from the DPI treatment, there was an NADPH oxidase- or other flavoprotein-dependency of the UV-B-induced generation of ROS. Alternatively, other physiological mechanisms affected by DPI might alter the formation of BFA-induced compartments. Taken together, UV-B induces ROS production, and ROS promote the formation of BFA-induced compartments in cells of root apices.

### Unilateral UV-B Irradiation Alters the Distribution of Auxin in *Arabidopsis* Root Apex Cells

The transgenic *Arabidopsis* line DII-VENUS (Brunoud et al., 2012) was used to analyze the auxin distribution in root apices. It is a useful tool for visualizing the changes in auxin concentration in single root cells under the laser-scanning confocal microscope.



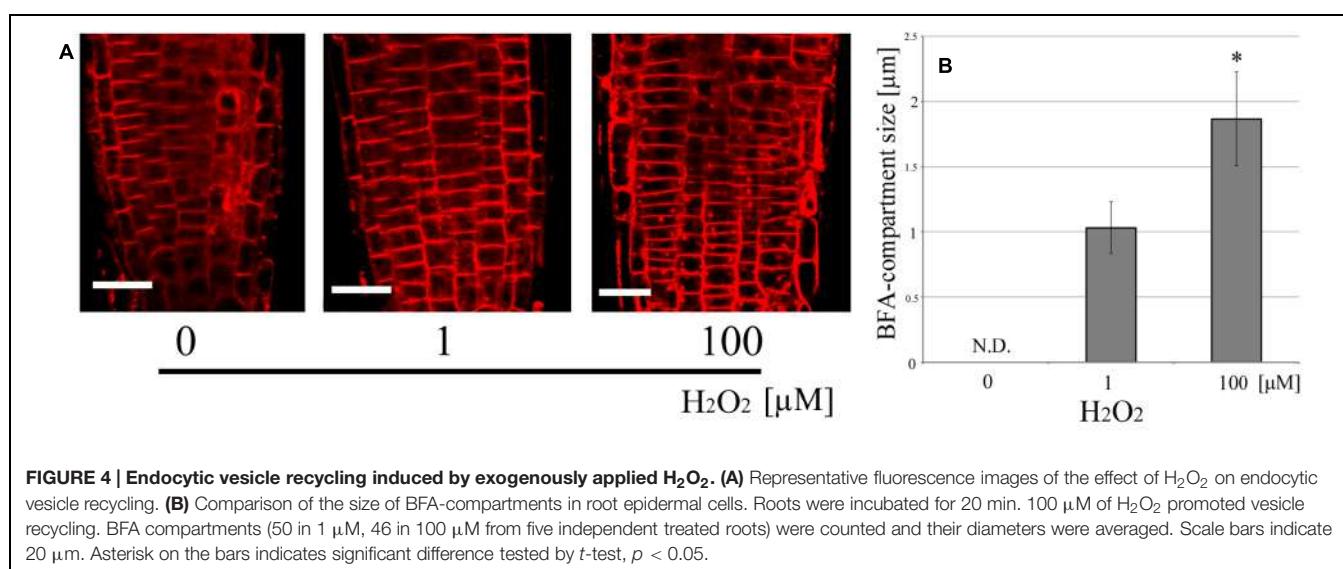
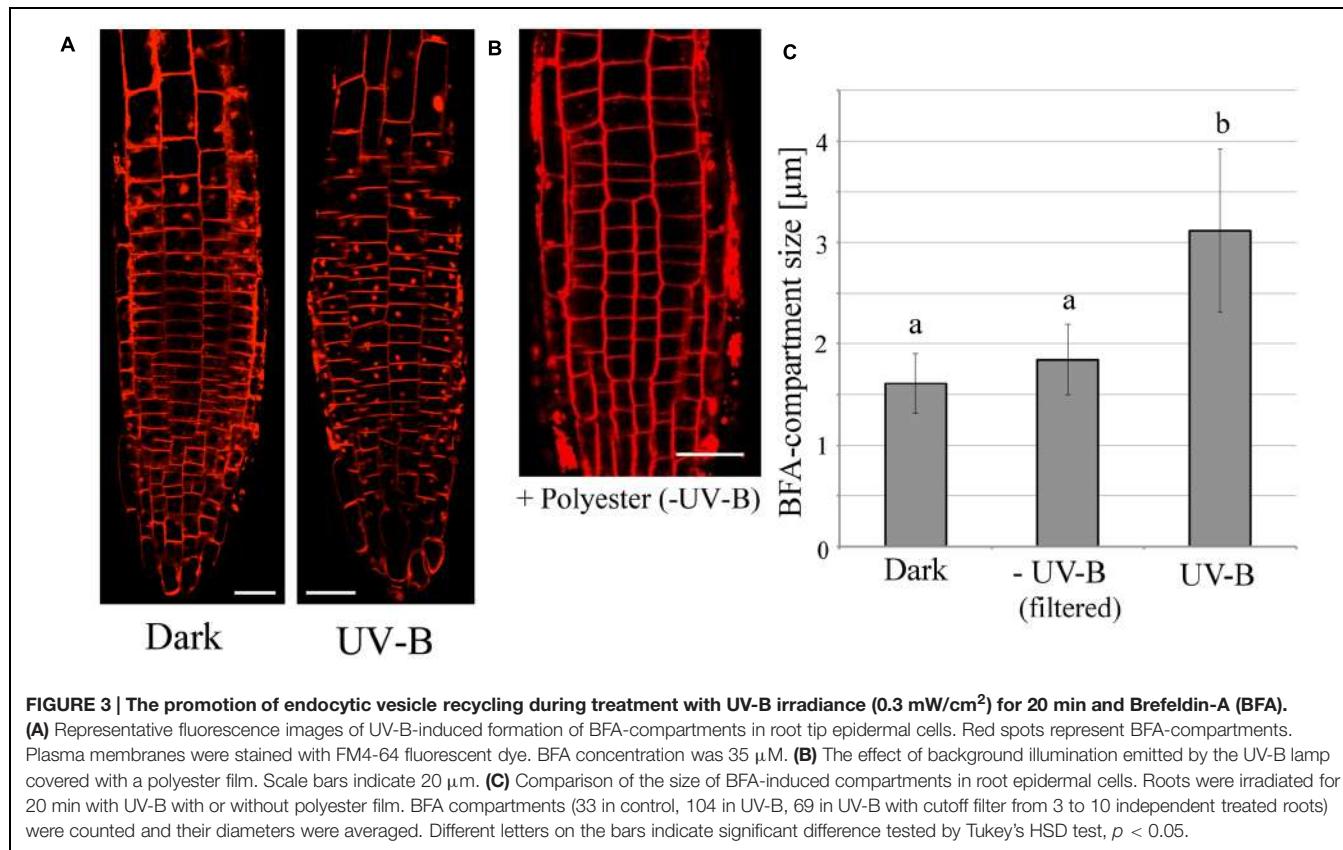
**FIGURE 2 |** UV-B induced reactive oxygen species (ROS) generation in the root apex. **(A)** Representative photographs of superoxide generation at the root apex detected by NBT staining and treatment of 10  $\mu$ M DPI (flavoprotein inhibitor). UV-B dose was 0.3 mW/cm $^2$  for 20 min. Scale bars indicate 20  $\mu$ m. **(B)** Comparison of NBT staining intensity. Error bars indicate  $\pm 1$  standard error. ( $n = 5$ ; **C**) Representative photographs of hydrogen peroxide production at the root apex detected using DAB staining. UV-B dose was 0.3 mW/cm $^2$  for 20 min. Bars indicate 20  $\mu$ m. **(D)** Comparison of DAB staining intensity. Error bars indicate  $\pm 1$  standard error. ( $n = 12$ ) Asterisk on the bars indicates significant difference tested by *t*-test,  $p < 0.05$ .

Fluorescence intensity of nuclei in DII-VENUS root cells decreases with rising auxin concentrations. Figure 6 depicts auxin re-distribution to the shaded side of the root after 20 min of unilateral UV-B irradiation at 0.3 mW/cm $^2$  (light source on the left hand side). Since a high concentration of auxin inhibits root growth, this nicely demonstrates negative phototropism, that is root bending away from the light source. In addition, *Arabidopsis* roots of both the Columbia wild-type and several mutant lines, *uvr8-6* (UVR8 mutant), *phot1/phot2* (PHOT1/2 mutant) and *cop1-4* (COP1 mutant), have produced the same root growth pattern of UV-B avoidance when unilaterally irradiated for 48 h at 0.01 mW/cm $^2$  (data not shown). The results presented here indicate that *Arabidopsis* roots have the ability to detect the direction of incoming UV-B radiation and avoid it, reacting by altering the polar flow of auxin in the root apical region.

## DISCUSSION

### *Arabidopsis* Roots are Extremely Sensitive to Light, Especially to the UV-B Wavelengths

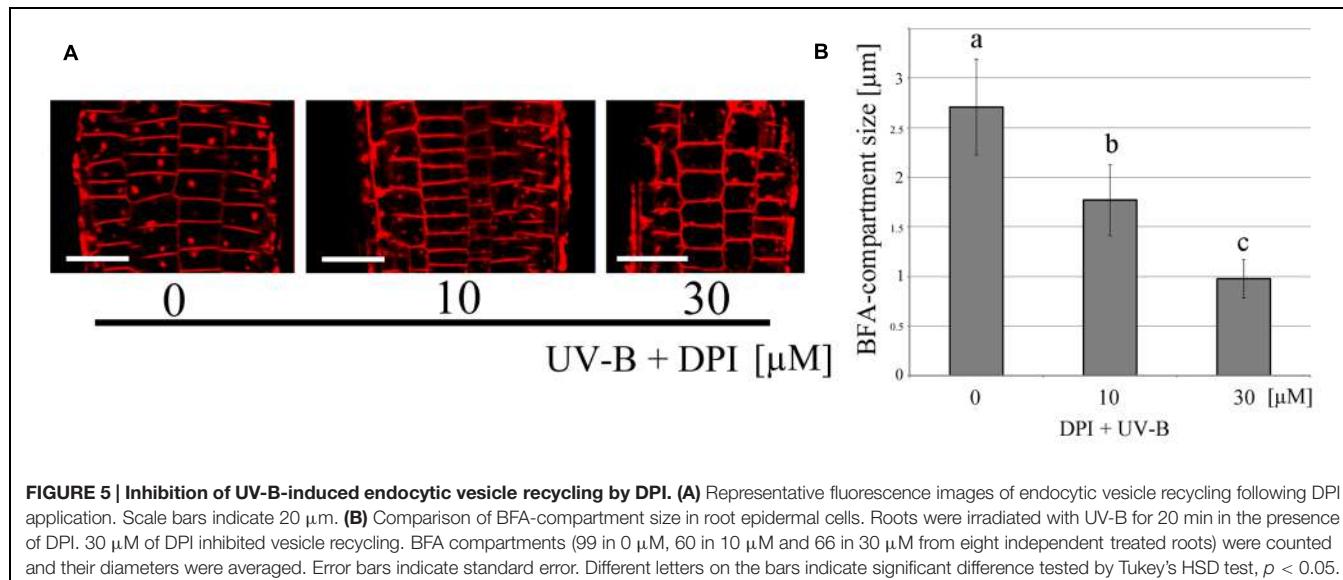
Why should plant roots be so sensitive to light? In nature, root systems are typically in the soil (underground and in darkness) anchoring the plant in the ground, as well as absorbing nutrients and water. Roots possess complex signaling pathways and specific receptors fine-tuned to sense changes in their environment (Malamy, 2005; Forde and Walch-Liu, 2009; Monshausen and Gilroy, 2009; Baluška and Mancuso, 2013). It is well known that germinating roots grow into the soil using positive gravitropism. In addition to the perception of gravity,



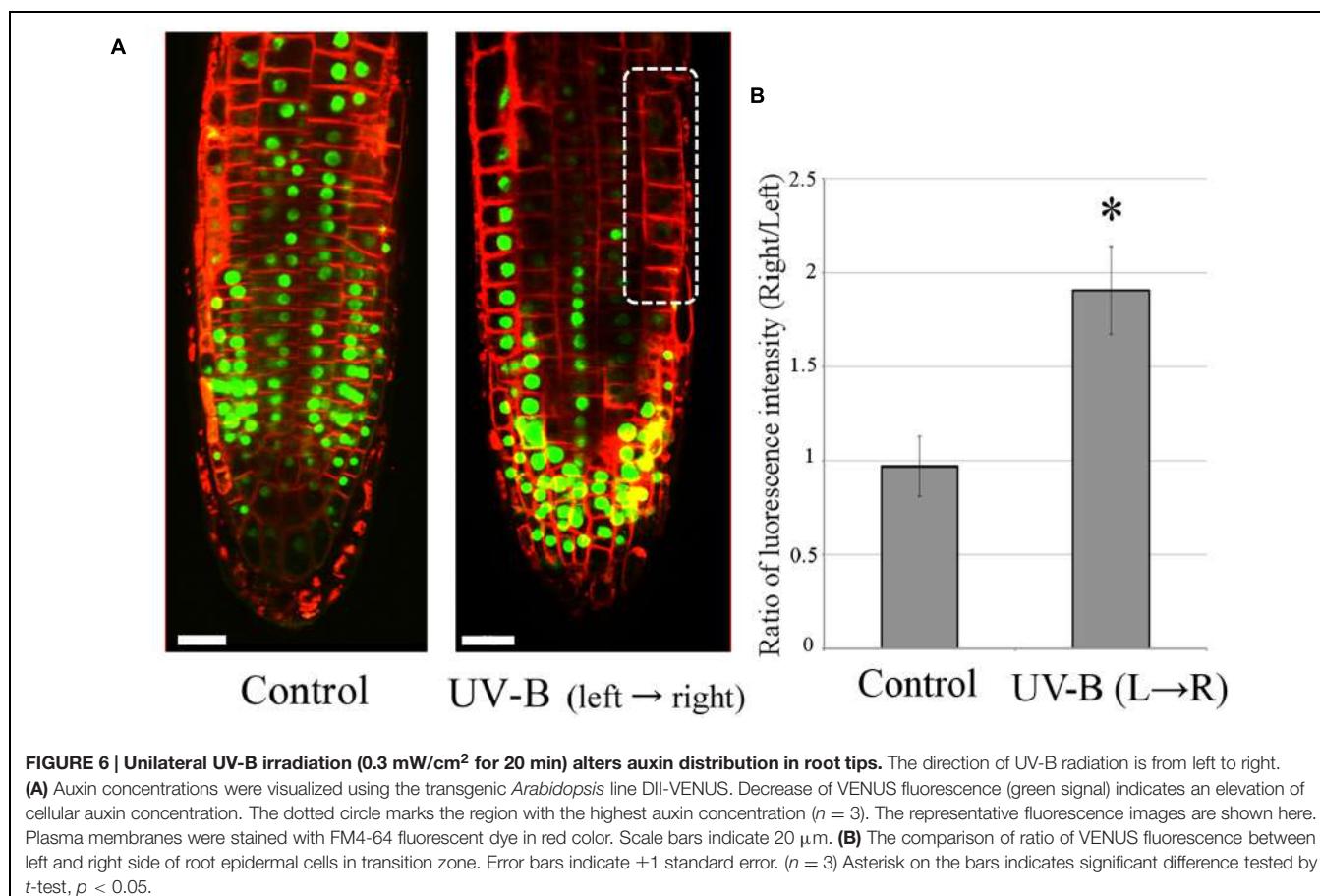
it has recently been shown that roots are also capable of very sensitive photoperception. This is illustrated by the fact that even a few seconds of exposure to light result in a burst of ROS production (Yokawa et al., 2011, 2013). Moreover, root cells have a similar set of photoreceptors to the above-ground parts of the plant (Briggs and Lin, 2012), and these enable them to avoid light.

Besides during seed germination, when the emerging roots are often exposed to light, strong wind or earthquakes may

unearth roots exposing them to sunlight. Hence, it is not surprising that roots are sensitive to light which stimulates a rapid response directed at returning the root to the soil. High energy photons in the UV region of the solar spectrum make it the most damaging to biomolecules, especially DNA, whose nucleotides have an absorption maximum close to the boundary of the UV-B and UV-C wavebands (Björn, 2008). This would explain why plants have developed effective



**FIGURE 5 | Inhibition of UV-B-induced endocytic vesicle recycling by DPI.** **(A)** Representative fluorescence images of endocytic vesicle recycling following DPI application. Scale bars indicate 20  $\mu\text{m}$ . **(B)** Comparison of BFA-compartment size in root epidermal cells. Roots were irradiated with UV-B for 20 min in the presence of DPI. 30  $\mu\text{M}$  of DPI inhibited vesicle recycling. BFA compartments (99 in 0  $\mu\text{M}$ , 60 in 10  $\mu\text{M}$  and 66 in 30  $\mu\text{M}$  from eight independent treated roots) were counted and their diameters were averaged. Error bars indicate standard error. Different letters on the bars indicate significant difference tested by Tukey's HSD test,  $p < 0.05$ .



**FIGURE 6 | Unilateral UV-B irradiation ( $0.3 \text{ mW/cm}^2$  for 20 min) alters auxin distribution in root tips.** The direction of UV-B radiation is from left to right. **(A)** Auxin concentrations were visualized using the transgenic *Arabidopsis* line DII-VENUS. Decrease of VENUS fluorescence (green signal) indicates an elevation of cellular auxin concentration. The dotted circle marks the region with the highest auxin concentration ( $n = 3$ ). The representative fluorescence images are shown here. Plasma membranes were stained with FM4-64 fluorescent dye in red color. Scale bars indicate 20  $\mu\text{m}$ . **(B)** The comparison of ratio of VENUS fluorescence between left and right side of root epidermal cells in transition zone. Error bars indicate  $\pm 1$  standard error. ( $n = 3$ ) Asterisk on the bars indicates significant difference tested by *t*-test,  $p < 0.05$ .

mechanisms for protecting themselves from UV radiation. Furthermore, *Arabidopsis*, and presumably other plants too, possess a root-specific UV-B signaling pathway controlled by RUS1 and RUS2 proteins (Tong et al., 2008; Leasure et al., 2009), strongly suggesting that roots have evolved

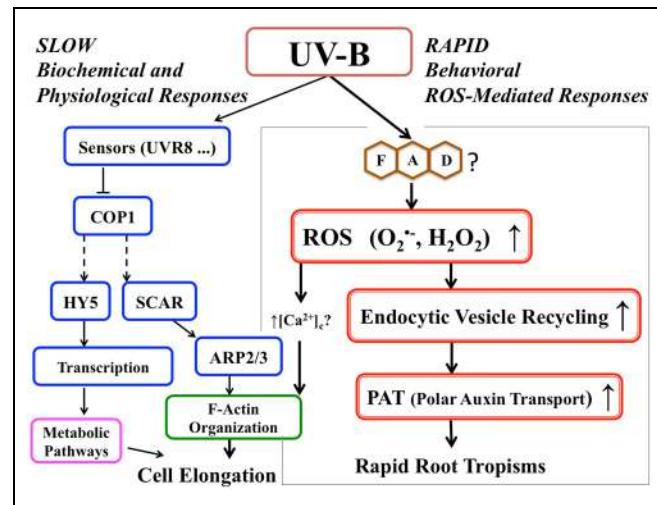
in an environment subjected to UV-B radiation and are able to respond to and avoid UV-B radiation. Interestingly, both RUS1 (Yu et al., 2013) and RUS2 (Ge et al., 2010) are essential for polar auxin transport in root apices of *Arabidopsis*.

## Light-Induced ROS and Auxin Drive Escape and Avoidance Tropisms of *Arabidopsis* Roots

Laxmi et al. (2008) reported that roots of *Arabidopsis* grow faster under illumination which is accompanied by higher PIN expression levels and increased rates of endocytic vesicle recycling. PIN2, which is critical to almost all root tropisms, is especially sensitive to light (Laxmi et al., 2008; Sassi et al., 2012; Wan et al., 2012; Mo et al., 2015). It was also demonstrated that root-localized phytochrome and their precursor PΦB impact on enhanced root elongation under white, blue, and red light conditions (Costigan et al., 2011; Warnasooriya and Montgomery, 2011). We have previously reported that illumination of roots induces an immediate burst of ROS in root apex cells of *Arabidopsis* (Yokawa et al., 2011, 2013). As the experiments shown in Figure 2, root cells irradiated with UV-B induced ROS production especially in apex region, and this pattern is very similar in blue-light illuminated roots (Yokawa et al., 2011). Surprisingly, ROS are also crucial signaling molecules in adaptive stress responses (Green and Fluhr, 1995; Rao et al., 1996; Egert and Tevini, 2003; Hideg et al., 2013; Wrzaczek et al., 2013). In general, ROS are known to induce expression of many genes involved in stress responses allowing plants to cope with a challenging environment. In root apex, the transition zone is localized between the apical meristem and the zone of elongation (Verbelen et al., 2006) and plays an important role in many tropisms which in turn are driven by a high intercellular flux of auxin (Baluška et al., 2005, 2010; Baluška and Mancuso, 2013). Furthermore, it has been reported that ROS are also generated in response to several other stimuli such as gravity and touch (Joo et al., 2001; Chehab et al., 2009), especially in cells of the transition zone. In this study, we showed the acceleration of BFA-induced compartment formation by either UV-B irradiation (Figure 3) or only applying exogenous H<sub>2</sub>O<sub>2</sub> as a ROS source (Figure 4). It indicates that robust UV-B-induced ROS production was evident in cells of the transition zone, suggesting that this region is highly sensitive to external UV-B radiation. However, further studies an additional experiments, such as BFA washout, are required to monitor how UV-B changes entire endo/exocytic vesicle recycling apparatus in cells of the root apex transition zone.

Additionally, the transition zone is particularly sensitive to auxin, which displays particularly high flux rates (Mancuso et al., 2005; Wan et al., 2012). The ROS generated act downstream of auxin (Ivanchenko et al., 2013). Here we report that ROS formation is induced by UV-B radiation, similarly as by blue light, and stimulate endocytic vesicle recycling as well as auxin transport between irradiated and shaded side of the transition zone (Figure 6; for blue light see Wan et al., 2012). Importantly, this specific zone of the root apex determines the direction of root growth through the differential release of auxin vesicles into the region of cell elongation (Baluška et al., 1994, 1996, 2004, 2010).

What is a source of ROS controlling UV-B mediated endocytic vesicle recycling and auxin redistribution? As Figures 2A,B shows, the generation of superoxide and H<sub>2</sub>O<sub>2</sub> were detected



**FIGURE 7 | Proposed signaling pathway for root avoidance responses to UV-B irradiance.** The left-hand side of the scheme depicts the UV-B-specific receptor-dependent signaling pathway ('slow biochemical and physiological response'). The right-hand side shows ROS-driven speeding-up of the endocytic vesicle recycling and polar auxin transport ('rapid response') leading to the escape phototropism.

after 20 min of UV-B irradiation. As a possibility of enzyme-mediating reaction, NADPH oxidase on the plasma membrane would be a candidate as a ROS generator. It is well studied as an important enzyme that produces superoxide in response to many physiological events including abiotic- or biotic- stresses (Miller et al., 2009; Marino et al., 2012). Here we treated DPI, diphenylene iodonium; a NADPH oxidase inhibitor, with UV-B irradiated roots. It reduced superoxide accumulation (Figure 2A) and the rate of vesicle recycling (Figure 5) under UV-B treatment. However, since DPI functions to inhibit an electron transfer of flavoproteins (NADPH oxidase is one of them), we cannot rule out a possibility of other radical generating reaction via flavin-containing biomolecules (O'Donnell et al., 1994). UV-B itself has a high energy compared to visible wavelengths of light and is likely enough to excite many molecules. In blue light region, there are many reported studies indicating that flavin-containing molecules immediately generate radicals and ROS *in vivo* by absorbing light (Massey et al., 1969; Prolla and Mehlhorn, 1990; Hockberger et al., 1999). Intriguingly, it was reported that irradiating blue light to tryptophan solution *in vivo* produced a precursor of auxin, *indole-3-acetaldehyde*, and it might be relevant to rapid phototropism in plants (Koshiba et al., 1993; Yokawa et al., 2014a). We also hypothesized that internalized pectin via endocytosis can also be a factor controlling ROS homeostasis under UV-B environment (Yokawa and Baluška, 2015). ROS is ultimately unstable chemical species. However, it has an advantage to be generated immediately on site, and can trigger downstream signaling through chemical reactions. The mechanism of light-promoted ROS production must be elucidated.

## Roots Exhibit Avoidance to Light via Rapid and Slow Responses

This is the first demonstration that ROS produced by UV-B exposure in the root apical cells are involved in general acceleration of endocytic vesicle recycling. Increased vesicle recycling leads to avoidance tropism in roots presumably caused by differentially increased polar auxin transport. This response can be considered a type of plant tropism that requires rapid responses. Plants cannot wait until proteins are ready to respond to stress situations. They are utilizing dual mode of actions, (1) quick behavior as shown in this report, and (2) slow changes of the physiological conditions which is mainly for preparing for upcoming events. Similar avoidance tropism was also reported for *Arabidopsis* roots exposed to salt stress (Li and Zhang, 2008; Sun et al., 2008; Galvan-Ampudia et al., 2013; Rosquete and Kleine-Vehn, 2013). Moreover, the illumination of roots changes their response to salt stress environment (Yokawa et al., 2014b). In the case of the UV-B escape tropism, this is based on UV-B-induced negative phototropism combined with general root growth acceleration (escape tropism). As summarized in Figure 7, this rapid response of roots to UV-B, mediated by ROS signaling molecules, allows efficient and very rapid root

escape tropism. However, identifying sources of UV-B-induced ROS and the detailed mechanism of how the ROS signal directly stimulates vesicle recycling are important issues which will need to be elucidated in the future.

## AUTHOR CONTRIBUTIONS

KY and TK conducted and analyzed the experiments.

KY, TK, and FB contributed to design of the experiments and composition of the manuscript.

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# Microarray Meta-Analysis Focused on the Response of Genes Involved in Redox Homeostasis to Diverse Abiotic Stresses in Rice

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Plants are exposed to a wide range of abiotic stresses (AS), which often occur in combination. Because physiological investigations typically focus on one stress, our understanding of unspecific stress responses remains limited. The plant redox homeostasis, i.e., the production and removal of reactive oxygen species (ROS), may be involved in many environmental stress conditions. Therefore, this study intended to identify genes, which are activated in diverse AS, focusing on ROS-related pathways. We conducted a meta-analysis (MA) of microarray experiments, focusing on rice. Transcriptome data were mined from public databases and fellow researchers, which represented 36 different experiments and investigated diverse AS, including ozone stress, drought, heat, cold, salinity, and mineral deficiencies/toxicities. To overcome the inherent artifacts of different MA methods, data were processed using Fisher, rOP, REM, and product of rank (GeneSelector), and genes identified by most approaches were considered as shared differentially expressed genes (DEGs). Two MA strategies were adopted: first, datasets were separated into shoot, root, and seedling experiments, and these tissues were analyzed separately to identify shared DEGs. Second, shoot and seedling experiments were classed into oxidative stress (OS), i.e., ozone and hydrogen peroxide treatments directly producing ROS in plant tissue, and other AS, in which ROS production is indirect. In all tissues and stress conditions, genes *a priori* considered as ROS-related were overrepresented among the DEGs, as they represented 4% of all expressed genes but 7–10% of the DEGs. The combined MA approach was substantially more conservative than individual MA methods and identified 1001 shared DEGs in shoots, 837 shared DEGs in root, and 1172 shared DEGs in seedlings. Within the OS and AS groups, 990 and 1727 shared DEGs were identified, respectively. In total, 311 genes were shared between OS and AS, including many regulatory genes. Combined co-expression analysis identified among those a cluster of 42 genes, many involved in the photosynthetic apparatus and responsive to drought, iron deficiency, arsenic toxicity, and ozone. Our data demonstrate the importance of redox homeostasis in plant stress responses and the power of MA to identify candidate genes underlying unspecific signaling pathways.

**Keywords:** microarray, meta-analysis, abiotic stress, rice, redox

## INTRODUCTION

While most animals can move and escape from harmful conditions, plants cannot. Drought, flood, salinity, extremes of temperature, nutrient deficiency, UV radiation, pollutants, herbicides, and pathogens are some of the factors a plant needs to cope with to survive and grow, usually with more than one at time. These factors limit crop yields and quality (Gill and Tuteja, 2010; Miller et al., 2010; Wang and Frei, 2011). Understanding how a plant responds to such stresses and the mechanisms underlying stress tolerance can give us a better view of how to improve the global food production. Investigating the stress responses of rice (*Oryza sativa* L.) is particularly rewarding, because it is both a global staple food of great agronomical importance, and a well-studied model organism, for which many transcriptome profiling studies have been published. About 32% of the rice annual production fluctuations (corresponding to around three million tons) can be attributed abiotic stresses (AS), and specifically variations in climate including precipitation and temperature (Ray et al., 2015).

A common response to different environmental stresses, both abiotic and biotic, is the excessive generation of reactive oxygen species (ROS) including superoxide ( $O_2^-$ ), perhydroxy radical ( $HO_2^-$ ), hydrogen peroxide ( $H_2O_2$ ), hydroxyl radical ( $OH^-$ ), singlet oxygen ( $^1O_2$ ), and organic hydroperoxide (ROOH; Gill and Tuteja, 2010; Bhattacharjee, 2012). Plant cells continuously produce ROS as by-products of various metabolic processes including respiration and photosynthesis. However, these molecules can directly react with DNA, proteins and lipids causing severe damage to individual cells and whole organisms. For this reason, every aerobic organism tightly controls its ROS concentration by ROS-scavenging pathways composed of many enzymatic and non-enzymatic antioxidant components (Gill and Tuteja, 2010).

However, many studies have shown that ROS play important roles in plants' stress signaling processes (Fujita et al., 2006; Miller et al., 2010; Mittler et al., 2011; Kim et al., 2012; Pucciariello et al., 2012). There are many advantages in the use of ROS as signaling molecules. The cell can rapidly produce and scavenge different forms of ROS in a simultaneous manner, enabling rapid and dynamic changes in ROS levels (caused by simply tilting the balance between cellular production and scavenging rates; Bhattacharjee, 2012). Each form of ROS has its own characteristics, such as mobility, process and location of origin and its reactivity with DNA, proteins or lipids (Bhattacharjee, 2012). Another advantage is the tight control over the subcellular localization of ROS signals in cells, with the regulation of enzymes specific of each of the cell compartments (Mittler et al., 2011). ROS may even act as "secondary messengers" modulating the activities of specific proteins or expression of genes by changing the redox balance of the cell. The network of redox signals orchestrates the metabolism for regulating energy production to utilization, interfering with primary signaling agents (hormones) to respond to changing environmental conditions at every stage of plant development (Bhattacharjee, 2012). Oxidative signaling is now considered to be a key in the responses to stress,

involved not only in the defense to these stresses but also in the regulation of the plant growth and development (Noctor et al., 2014).

One limitation in the understanding of ROS homeostasis in AS is that most of the knowledge on molecular mechanisms of stress response was obtained from experiments under controlled laboratory conditions and focused on only one stress at a time. However, plants are often simultaneously exposed to many biotic and AS in their natural or agronomic habitats (Hazen et al., 2003; Rasmussen et al., 2013). By combining the data from different experiments it is possible to identify common and specific elements expressed in response to different stresses (Rabbani et al., 2003; Fujita et al., 2006; Yang et al., 2013).

With the rapid advances in biological high-throughput technology, a large and diverse set of genomic data has become publicly accessible. Combining information from multiple existing studies can increase the reliability and generalizability of results. The use of statistical techniques to combine results from independent but related studies is called "meta-analysis (MA)." Through MA, we can increase the statistical power to obtain a more precise estimate of gene expression differentials (Ramasamy et al., 2008; Tseng et al., 2012). The most common types of Microarray MA are a combination of *p*-values, a combination of effect sizes (fold change) or a combination of ranks, each method with its limitations and advantages (Tseng et al., 2012).

In the present study, instead of choosing one of those, we used an integrative approach to combine the forces of those different microarray MA methods and overcome possible biases, such as a "fishing for significance" effect for preferring one method over the others (Ioannidis, 2005; Ostlund and Sonnhammer, 2014). However, within these three categories of MA, there are many statistical methods to choose from, we selected them based on their evaluation in recent studies (Tseng et al., 2012; Chang et al., 2013; Ostlund and Sonnhammer, 2014). Among the methods of combination of *p*-values, we chose two distinct methods: Fisher's (Rhodes et al., 2002), the most commonly implemented MA method, and rOP (rth ordered *p*-value) that is more restrictive but still flexible (Song and Tseng, 2014). Between the combined effect size methods, the random effect model, REM is the most adequate for a heterogeneous group of samples, as the one processed in the present study (Choi et al., 2003). Furthermore, the MA by ranks was computed by the program GeneSelector that produces a rank combining seven distinct statistic methods (Boulesteix and Slawski, 2009; Ostlund and Sonnhammer, 2014). Only genes elected by the majority of methods (at least three of those four) were further processed in our analyses. Although this strategy may be rather restrictive, it increases the power of our analysis, since each differentially expressed gene (DEG) was confirmed by at least three different statistic methods.

The goal of this MA was to detect DEGs involved in ROS homeostasis, which respond to AS treatments. The transcriptome data from rice plants subjected to different classes of AS were analyzed: drought, submergence, salinity, cold, heat, excess and/or deficiency of essential nutrients, such as phosphorus (P), zinc (Zn), and iron (Fe), and toxicity of heavy metals such as arsenic (As), cadmium (Cd), chrome (Cr), and lead (Pb). Also,

to identify genes specifically involved in the redox homeostasis, data from experiments with ozone ( $O_3$ ) and hydrogen peroxide were included (**Table 1**), because these treatments are presumed to produce direct oxidative stimulus, unlike with the indirect forms of oxidative stress (OS) occurring in other environmental conditions.

More specifically, the following questions were addressed in this study:

- (1) How does a MA integrating several of the MA approaches described above compare to individual MA approaches, and are the results more conservative?
- (2) To what extent do stress responses differ between different stresses and plant tissues, and what is the role of ROS-related genes?

- (3) Through MA, can we nominate possible key genes as major hubs of ROS-related stress signaling, and what are their putative functions?

## MATERIALS AND METHODS

### Data Mining

For this study, expression data of rice plants exposed to diverse AS were combined. The raw expression data of different experiments were obtained from the Rice Oligonucleotide Array Database<sup>1</sup> (Cao et al., 2012), the NCBI's Gene Expression

<sup>1</sup><http://www.ricearray.org/>

**TABLE 1 | Experimental conditions of microarray raw data used for meta-analysis.**

Experiments			Source		
Shoots	Roots	Seedlings	GEO series	Reference	Platform
$O_3$			GSE11157	Cho et al., 2008	GPL892
$O_3$			NA	Frei et al., 2010a	GPL892
Submergence			GSE18930	Mustroph et al., 2010	GPL2025
Drought			GSE21651	Pandit et al., 2011	GPL2025
Salinity			GSE21651	Pandit et al., 2011	GPL2025
-N			GSE66935	Takehisa et al., 2015	GPL6864
-K			GSE66935	Takehisa et al., 2015	GPL6864
-P			GSE66935	Takehisa et al., 2015	GPL6864
-P			GSE17245	Zheng et al., 2009	GPL2025
-Fe			GSE17245	Zheng et al., 2009	GPL2025
-Fe -P			GSE17245	Zheng et al., 2009	GPL2025
-P	-P		GSE6187	Pariasca-Tanaka et al., 2009	GPL892
+Fe	+Fe		NA	Wu et al., unpublished data	GPL19782
-Zn	-Zn		NA	Wu et al., unpublished data	GPL19782
-Zn +Fe	-Zn +Fe		NA	Wu et al., unpublished data	GPL19782
Drought	Drought		GSE26280	Wang et al., 2011	GPL2025
	+As		GSE25206	Dubey et al., 2010	GPL2025
	+Cd		GSE25206	Dubey et al., 2010	GPL2025
	+Cr		GSE25206	Dubey et al., 2010	GPL2025
	+Pb		GSE25206	Dubey et al., 2010	GPL2025
	-K		GSE37161	Ma et al., 2012	GPL2025
	Salinity		GSE14403	Cotsafitis et al., 2011	GPL2025
	$H_2O_2$		GSE19983	Mittal et al., 2012a	GPL9956
	Cold		GSE19983	Mittal et al., 2012a	GPL9956
	Heat		GSE19983	Mittal et al., 2012a	GPL9956
	$H_2O_2$		GSE32704	Mittal et al., 2012b	GPL8852
	$H_2O_2$ + cold		GSE32704	Mittal et al., 2012b	GPL8852
	$H_2O_2$ + heat		GSE32704	Mittal et al., 2012b	GPL8852
	heat		GSE14275	Hu et al., 2009	GPL2025
	Cold		GSE6901	Jain et al., 2007	GPL2025
	Drought		GSE6901	Jain et al., 2007	GPL2025
	Salinity		GSE6901	Jain et al., 2007	GPL2025
	Salinity		GSE16108	Pandit et al., 2011	GPL2025
	+As		GSE4471	Norton et al., 2014	GPL2025
	-K		GSE44250	Shankar et al., 2013	GPL2025
	-Pi		GSE35984	Dai et al., 2012	GPL2025

Omnibus repository<sup>2</sup> (Barrett et al., 2013), and from fellow researchers. The criteria for inclusion of those dataset were: relatively similar genetic background, plants must be from *O. sativa* indica or japonica subgroups; the RNA should have been extracted only from shoots, roots, or seedlings (whole plant), excluding other tissues such as flowers, seeds, or *cali*; the experiments must involve only against AS treatments; the data must originate from Affymetrix or Agilent microarray platforms, and the original study must follow the “Minimal Information About a Microarray Experiment” (MIAME) requirements (Brazma et al., 2001).

## Individual Datasets Analysis

Microarray expression data from each source study was pre-processed separately as individual datasets. Agilent microarray data were processed in the R program using the packages LIMMA (Smyth, 2005), while the package Affy was used for data from the Affymetrix platforms. The raw data from both were treated using Robust Multi-array Average (RMA) background correction and quantile normalization. Non-informative and low-intensity probes were declared following the program standard settings, while duplicated probes were converted into their corresponding genomic locus. The ArrayQualityMetrics package (Kauffmann and Huber, 2013) was used to assess the quality of the normalized datasets. A sample was included or excluded in further analysis based on three different evaluations made by this program: distance between array, array intensity distributions, and individual array quality. The values normalized by RMA were used for the subsequent MA.

A contrast between each treatment and its control was estimated with the LIMMA package. In studies including several genotypes the genotype factor was not considered. After fitting the data into a linear model, the standard errors were corrected using a simple empirical Bayes model. Moderated *t*-statistic and log<sub>2</sub>-fold-change of differential expression were computed for each contrast for each gene. Genes that showed significant *P*-value (*FDR* = 5%) were considered as DEG and log<sub>2</sub>-fold-change values of each experiment/dataset were saved for further analysis. Relative gene expression values corresponding to the same stress in different datasets were averaged for a simplified evaluation of the gene's response to each condition.

## Combined Meta-Analysis

Two different strategies were implemented in the present study. The first (MA.1) investigated the effect of the stresses in different tissues, for which the datasets were separated into shoots, roots and seedling microarrays and processed separately. In the second approach (MA.2), data from shoots and seedlings were combined, while the data set was separated into direct OS and others AS to compare the effects of direct and indirect OS on gene expression. Hydrogen peroxide and ozone stress were considered as OS, because these treatments directly lead to the production of ROS in plant tissue (Uchida et al., 2002; Kangasjärvi et al., 2005). For both approaches,

the normalized expression values were used. Since the probe nomenclature differs between platforms, the MSU-ID was used, and when multiple probes matched to the same gene they were averaged.

The datasets were merged using the packages metaDE (Wang et al., 2012), and this merged dataset was once more filtered, excluding 20% of un-expressed genes (with small expression intensities) and 20% of non-informative genes (genes with small variation). Each independent study sample was processed with a modified two-sample *t*-statistics (modt) contrasting treated and control samples.

In an effort to overcome the inherent artifacts of each MA statistical method, we ran our data through the three common types of MA (i.e., by *p*-value, by effect size and by rank) to identify genes that are considered differentially expressed by distinct methods. Two methods of combination by *p*-value were used: Fisher and rOP. The classical Fisher's method sums the log-transformed *p*-values obtained from individual studies and, under null hypothesis, follows a chi-squared distribution with 2K degrees of freedom, where K is the number of studies combined (Rhodes et al., 2002). However an extremely small *p*-value in only one study can be sufficient to cause statistical significance, even if the same gene are not significant in any other study. A more restrictive but flexible method is rOP, that combines Fisher with a generalized vote counting statistic. It uses the *r*th order statistic among sorted *p*-values of K combined studies, where *r* is a pre-determined minimum number of studies, in which a gene's *p*-values must be small to be significant (Song and Tseng, 2014). In our analysis we implemented K/2 ≤ *r* ≤ K, i.e., each gene must be differentially expressed in at least half of the combined studies to be significant. Taking into consideration the heterogeneity of our cluster of studies and the residual “noise” data derived from technical and biological differences between the studies, a more restrictive approach was not implemented (i.e., *r* = K).

A second way to combine expression data across different microarray studies and platforms is using effect size values. The REM method was implemented because it possesses a random effect element corresponding to the unknown heterogeneities between very distinct studies, such element is not present in the alternative method, the fixed effect model, FEM (Choi et al., 2003). The results of each of these analyses were corrected with the Benjamini and Hochberg procedure with a false discovery rate (FDR) of 5% as threshold.

For a MA based on the rank method, the normalized values were processed using GeneSelector (Boulesteix and Slawski, 2009). This package allows a ranking analysis of the data with seven distinct methods: ordinary *t*-test; Baldi and Long Bayesian *t*-test (Baldi and Long, 2001); Winconxon–Mann–Whitney *U* test; Fox and Dimmic Bayesian *t*-test (Fox and Dimmic, 2006); SAM statistics (Tusher et al., 2001); limma: moderated *t*-statistic based on a Bayesian hierarchical model which is estimated by an empirical Bayes approach (Smyth et al., 2003), and simple fold-change estimation (in log<sub>2</sub>). The obtained gene rankings were then aggregated by the mean value of the rank positions given by each method (AggMean), or aggregated on the basis of a Markov Chain model, AggMC (DeConde et al., 2006).

<sup>2</sup><http://www.ncbi.nlm.nih.gov/geo/>

A combined rank was produced by combining the top genes obtained by each of the seven ranking methods, together with the AggMean and AggMC lists. Genes of this combined list were compared with DEGs obtained by Fisher, rOP, and REM methods.

The DEGs shared by at least three of these four methods were further studied.

## Gene Analysis

The genes elected by the combined meta-analyses MA.1 and MA.2, were further analyzed and characterized. First, the result lists were combined and compared with a list of genes described in the literature as involved in ROS scavenging and signaling processes. This list was curated based on recent studies (Frei et al., 2010a; Liu et al., 2010; Kim et al., 2012; Shaik et al., 2014). This list includes genes involved in the biosynthesis and recycling of enzymatic [such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX), and glutathione reductase (GR)] and non-enzymatic antioxidants (such as ascorbic acid (AsA), reduced glutathione (GSH) and thioredoxin (Trx)], and also transcription factors such as zinc-fingers, MYB (**myeloblastosis**) and WRKY (that contains the WRKYGQK amino acid conserved sequence) families and other elements described as directly or indirectly involved in signaling and response to OS. Co-occurrence of DEG with the resulting list was represented with Venn's diagrams using Venny 2.0 (Oliveiros, 2015).

Gene Ontology (GO) Enrichment analysis of the DEGs obtained by MA.1 and MA.2 was conducted using the AgriGO platform (Du et al., 2010). A Singular Enrichment Analysis was performed using the Rice Gramene Locus set as reference (Jaiswal et al., 2002).

Genes differentially expressed in both OS and AS (MA.2 approach) were studied in depth. The STRING (Search Tool for the Retrieval of Inter-acting Genes/Proteins) database was used to detect functional association between those genes. This database constructs associations based on distinct lines of evidences: Experimental evidence from protein–protein interaction assays; co-expression data based on the expression data stored in the NCBI GEO database; co-occurrence of the genes in the same organisms; available information of other databases; recurring neighborhood of the genes in known genomes; events of fusion between those genes or orthologs; pathway annotation data in other databases such as Gene Ontology or Kyoto Encyclopedia of Genes and Genomes (KEGG), and automated text-mining based on Medline abstracts and a large collection of full-text articles (Szklarczyk et al., 2015). STRING computes a confidence score for those interactions based on the available evidences, from medium (score above 0.4) to highest (above 0.9).

Using the expression values obtained in the individual dataset analysis (a  $\log_2$  fold change difference between control and treated expression values of each gene), an average value of the most relevant conditions was obtained and represented in a heatmap. The heatmap was made using the package gplots in R (Warnes et al., 2015).

## RESULTS

### The Percentage of ROS Related DEGs is Constant in the Response to Different Stresses

After search in the available databases and quality control analysis, raw microarray data from 36 independent experiments were selected. To obtain a global analysis, AS from different categories were chosen: drought, submergence, salinity, cold, heat, excess and/or deficiency of essential nutrients as Phosphorus (P), zinc (Zn), and iron (Fe), heavy metal toxicity (As, Cd, Cr, and Pb), and direct OS ( $O_3$  and  $H_2O_2$ ; Table 1).

Some source data originated from experiments that tested different stressors (Jain et al., 2007; Zheng et al., 2009; Dubey et al., 2010; Mittal et al., 2012a,b); several time points after the exposure to the stressor (Cho et al., 2008; Mustroph et al., 2010; Dai et al., 2012; Ma et al., 2012; Mittal et al., 2012a); several concentrations of a stressor (Takehisa et al., 2015), and/or several genotypes, usually using contrasting lines (Pariasca-Tanaka et al., 2009; Frei et al., 2010a; Cotsaftis et al., 2011; Pandit et al., 2011; Norton et al., 2014; Supplementary Table S1). In these cases, every possible contrast of stress condition versus control was treated as a separate experiment. In total, plants from 21 different genotypes of domestic rice were used, 13 from the *indica* and eight from the *japonica* subspecies (Supplementary Table S1).

In parallel, a list of 1972 genes previously described as involved in ROS scavenging and signaling processes was made by combining information from the literature (Frei et al., 2010a; Liu et al., 2010; Kim et al., 2012; Shaik et al., 2014). These genes correspond to about 4% of the rice genes represented by the microarray probes included in this analysis (Supplementary Table S2) and represent a broad spectrum of functions, ranging from reductase and peroxidase enzymes to transcription factors.

In the different treatments and tissues, 7–10% of the DEGs were included in the list of ROS-related genes, indicating that this category of genes was overrepresented (Table 2). The proportion of DEG considered as ROS-related was similar in OS and AS experiments (Table 3).

### MA.1: DEGs in Response to Diverse Abiotic Stresses Differ Between Tissues

The microarray expression data was processed by two different MA approaches. In the first, MA.1, our objective was to identify DEGs in response to many AS in shoot, root, and seedling samples separately. Using combined MA methods, 1001 DEGs were identified in response to different stresses in shoots, 837 in roots and 1172 in seedlings, although only 14 were identified in all three tissues (Figure 1B, Table 3). From the 2691 genes elected by MA.1, 236 (9%) were included in the ROS-related list, including the coding genes of 42 zinc-finger signaling proteins, 18 MYB transcript factors, nine glutathione S-transferases, a Cu-Zn SOD, a copper chaperone for SOD, 19 peroxidase precursors, APX2, GPX2, and GPX3 (Figure 1B, Supplementary Table S5).

**TABLE 2 |** Differentially expressed genes (DEGs) in response to different abiotic stresses and the proportion of those involved in ROS scavenging or signaling mechanisms (ROS).

	Shoots		Roots		Seedlings			
	DEG	ROS	DEG	ROS	DEG	ROS		
Drought	17682	7%	Drought	11410	7%	Drought	9756	7%
Salinity	7268	8%	Salinity	3625	8%	Salinity	11242	7%
-P	18116	8%	-P	482	10%	-P	15704	7%
-Fe	13548	8%	+Fe	7938	7%	Cold	11347	8%
-Zn +Fe	13679	7%	-K	4243	7%	Heat	14692	8%
O <sub>3</sub>	11979	8%	+Cd	1011	10%	-K	6275	7%
Submergence	14001	7%	+As	8581	7%	H <sub>2</sub> O <sub>2</sub>	15198	7%
			+Pb	285	11%	+As	7599	8%
TOTAL	23197	7%	TOTAL	21273	7%	TOTAL	21533	7%

Total number of DEGs detected in at least one dataset in each sample cluster: shoots, roots, and seedlings (FDR = 5%).

## MA.2: DEGs Detected in Both Oxidative and Others Abiotic Stresses

In a second approach, expression data from seedling and shoot experiments were classed into two groups: OS (direct OS, i.e., O<sub>3</sub> and H<sub>2</sub>O<sub>2</sub>) and AS (all the other AS). It was found that 679 DEGs were exclusive to OS and 1416 DEGs were exclusive to AS (Table 3, Figure 1C). In total 1212 genes were detected by both MA.2 and MA.1, of which 197 coincided with OS, 760 with AS, and 255 with both (Figure 1C). On the other hand, 50 ROS-related genes were only detected by the OS analysis, including APX7 and nine

WRKY proteins, and 47 by the AS analysis (Supplementary Table S5).

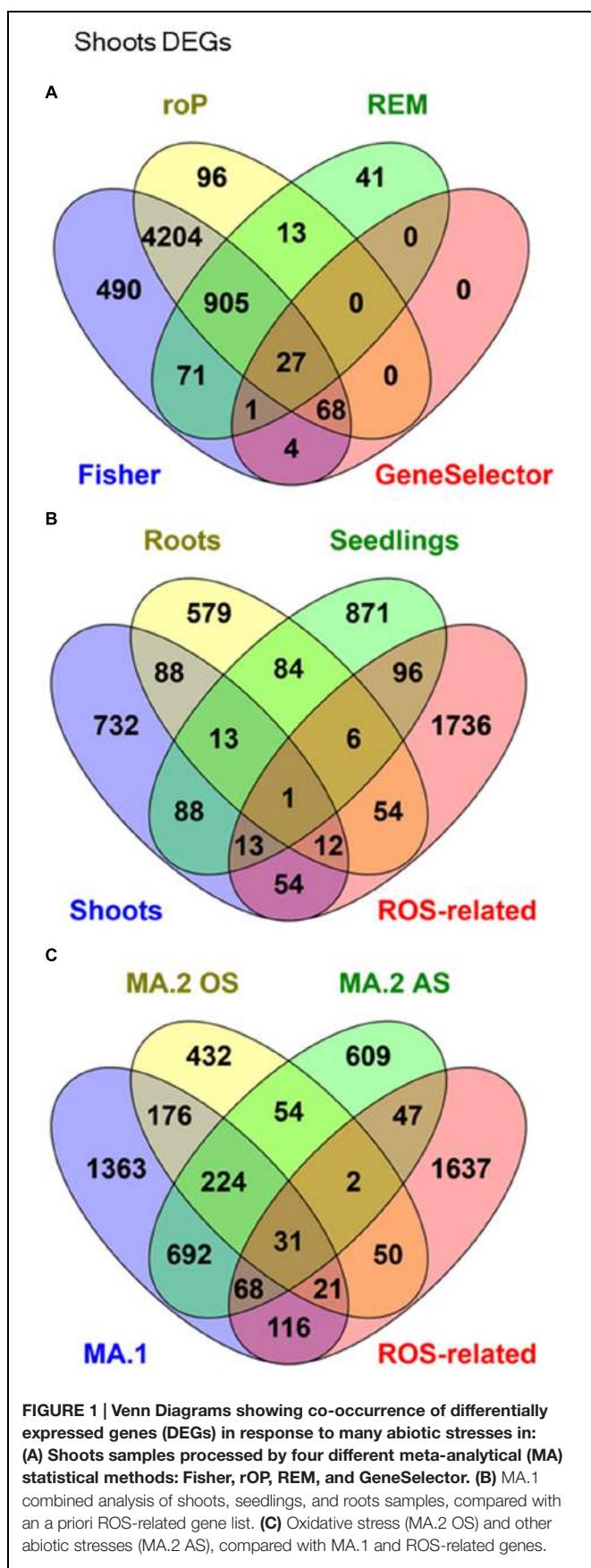
We then focused on the 311 DEGs shared by both OS and AS. Among those, 33 were ROS-related genes, while others belonged to many distinct gene families associated with the response to stress and plant growth, such as Zn fingers, WRKY, and TIFY transcript factors, cytochromes, photosystem subunits, heat shock proteins, HIPPs (Heavy Metal Isoprenylated Plant Proteins), kinases and phosphatases (Supplementary Table S5).

A network analysis using the platform STRING 10.0 detected a cluster between 214 of those genes (Figure 2A). By increasing

**TABLE 3 |** Detected number of genes differentially expressed in response to abiotic stresses.

Meta-analysis	MA.1			MA.2	
	Shoots	Roots	Seedlings	OS	AS
<b>(A) Samples</b>					
Number of studies	9	6	8	4	17
Experiments	32	22	23	10	67
Samples	123	104	87	42	167
DEGs	6336	6657	7988	7370	6966
<b>(B) Number of differentially regulated genes in each MA approach</b>					
Fisher	5770 (91%)	3335 (50%)	6860 (86%)	2972 (40%)	6807 (98%)
rOP	5313 (84%)	3582 (54%)	6610 (83%)	2515 (34%)	6668 (95%)
REM	1058 (17%)	1317 (20%)	1199 (15%)	1320 (18%)	1707 (25%)
GeneSelector	100 (2%)	100 (2%)	100 (1%)	100 (1%)	100 (1%)
Shared DEG	1001 (16%)	837 (13%)	1172 (15%)	990 (13%)	1727 (25%)
<b>(C) ROS related genes differentially expressed in each MA approach</b>					
Total DEGs	468 (7%)	540 (8%)	502 (6%)	548 (7%)	466 (7%)
Fisher	431 (7%)	339 (10%)	500 (7%)	271 (9%)	466 (7%)
rOP	398 (7%)	349 (10%)	499 (8%)	243 (10%)	465 (7%)
REM	79 (7%)	100 (8%)	119 (10%)	129 (10%)	142 (8%)
GeneSelector	7 (7%)	9 (9%)	4 (4%)	11 (11%)	12 (12%)
Shared DEG	72 (7%)	73 (9%)	116 (10%)	97 (10%)	148 (9%)

**(A)** Number of individual studies, experiments in which the contrast between treatment and control was evaluated, biological samples (microarrays) and number of differentially expressed genes (DEGs) in at least one dataset. **(B)** DEGs obtained in each MA statistical approach (FDR = 5%), and the proportion in relation to the total number of DEGs in parenthesis. Shared DEGs are genes elected by the majority of methods. **(C)** Number and proportion of the ROS-related genes in the DEGs obtained by each approach **(B)**. More details in Supplementary Tables S1–S4.



the stringency of this analysis (confidence score  $> 0.9$ ), it was possible to isolate a cluster of 47 protein-coding genes (Figure 2B). Most hub genes of this network code proteins involved in the photosynthetic apparatus, e.g., ATP synthase (LOC\_Os02g51470), oxygen evolving enhancer protein 3 (LOC\_Os07g36080), photosystem I reaction center subunit III (LOC\_Os03g56670), photosystem II core complex proteins psbY (LOC\_Os08g02630), photosystem II reaction center W protein (LOC\_Os05g43310) and many others chloroplastic protein (Supplementary Table S5). Many of these genes showed a similar expression pattern in the samples analyzed in this study (Supplementary Table S7). They were more highly expressed in response to iron deficiency and drought in roots, while suppressed in the samples of submitted to As toxic level, ozone, and submergence (Figure 3).

## Shared DEGs are Involved Many Metabolic, Response to Stimuli, and Regulatory Processes

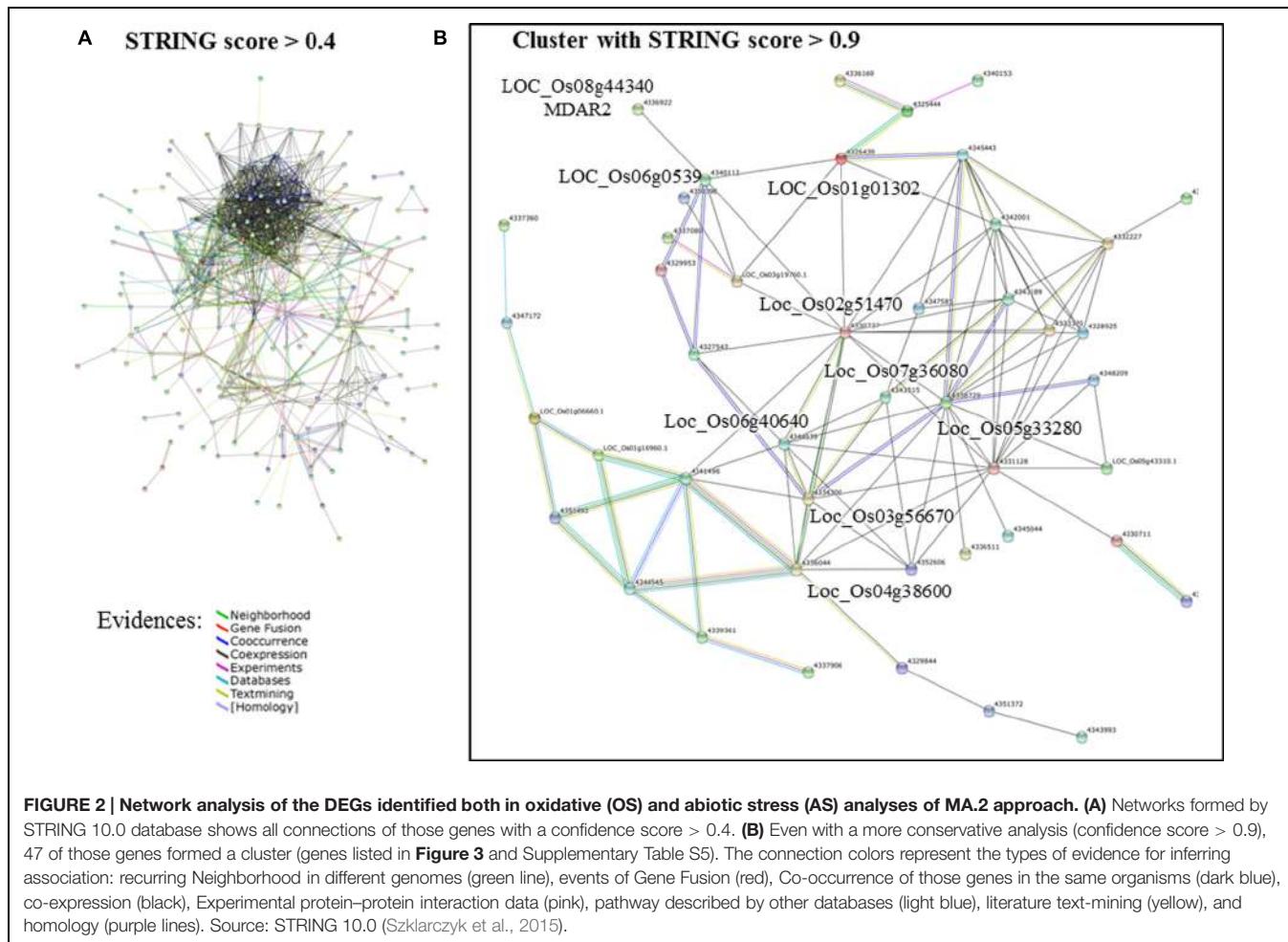
Gene Ontology enrichment analysis was conducted to explore other possible functions of the DEGs detected in the different MA approaches (Supplementary Table S6). The most frequent and significant GO terms associated with DEGs in MA.1, MA.2, AS, and OS are represented in Figure 4. The biological processes terms suggest constitutive roles for those genes, as part of metabolic and biosynthetic processes, but also in the regulation of those processes on different levels (transcription, post-translational protein modification, macromolecule biosynthesis, phosphorylation, signal transduction, transport, and proteolysis). The enrichment of terms such as nucleic acid binding, transcription regulator, kinase activity, transmembrane transporter, and phosphatase activity indicates that many of those shared genes are also involved in signaling processes (Figure 4).

## DISCUSSION

### Advantages of a Combined MA Approach

With the objective of identifying genes involved in the plant response to different AS and direct or indirect OS, the expression data of rice plants exposed to many stress conditions were combined. Instead of using one specific MA approach, we combined the results of different MA statistical methods to obtain the most relevant genes. The stringency of this methodology may exclude many important genes, but also excludes many false positives that could be obtained as artifacts of each statistical method (Tseng et al., 2012; Chang et al., 2013).

While methods such as Fisher's identify a great number of genes, many of those were not identified as differentially expressed by size effect or ranking product methods (e.g., Figure 1A). Only a small fraction of the genes differentially expressed was shared between the independent methods. For example, while with the Fisher's MA method the number of DEGs was between 40 and 98% of the number of DEGs detected



in at least one experiment, our shared DEGs approach reached 13–25% (Table 3B).

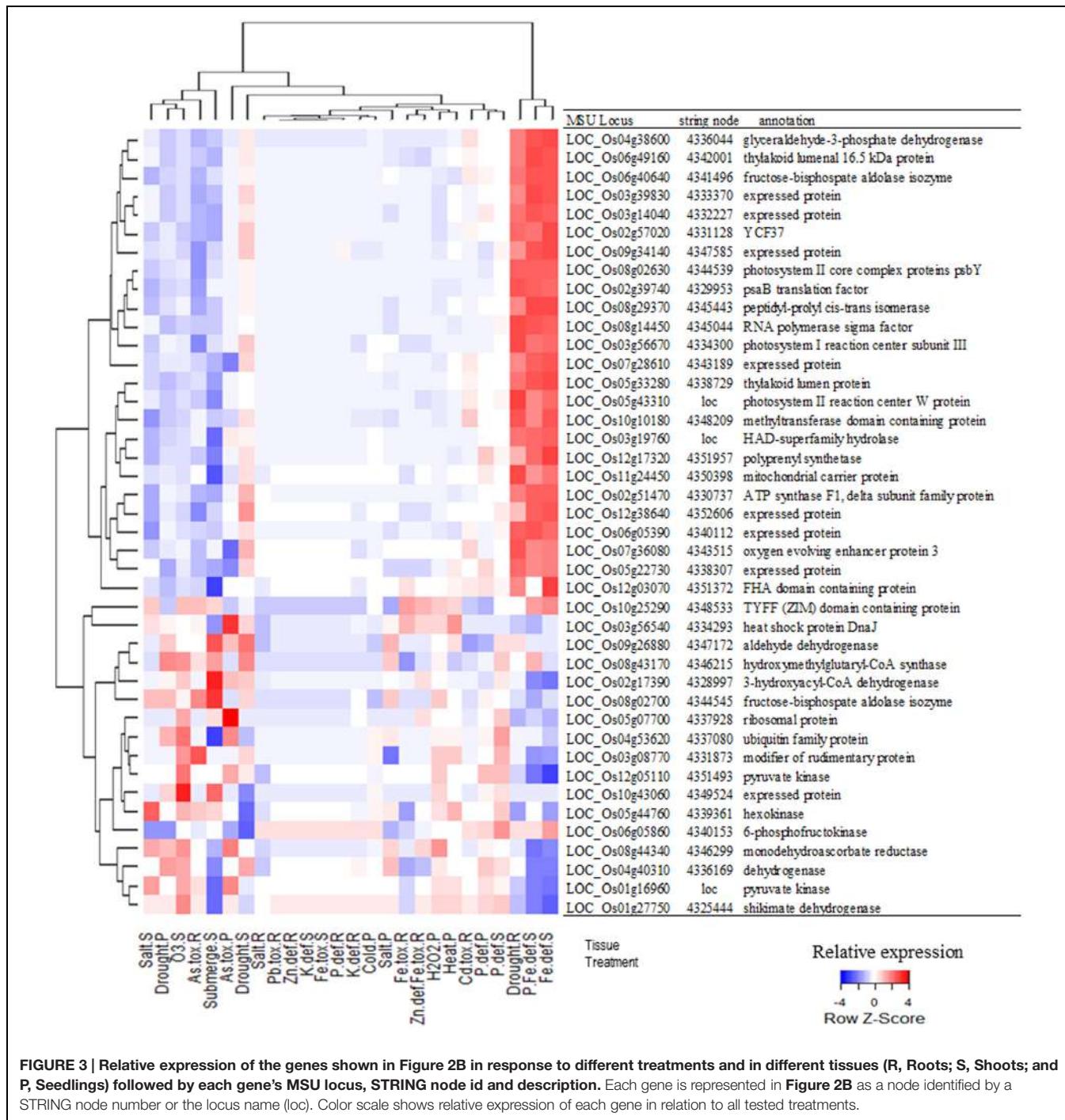
### MA.1: The Response to Stresses Varies Between Tissues

In our first MA approach, microarray data were separated into shoots, roots, and seedlings, and although those groups were composed of samples of similar size (Tables 1 and 2), they showed greatly distinct numbers of DEGs in response to stresses, and only 14 of those were shared between the three groups (Figure 2B). This is in agreement with the concept that the transcriptome and how it responds to a stress differs between tissues or organs of the same organism (Hazen et al., 2003).

### ROS-Related DEGs are Overrepresented Among the DEGs Detected by MA.1 and MA.2

Although the DEGs in response to different stresses varied greatly, the proportion of those that were *a priori* considered as ROS-related was rather constant (Table 2). And while these ROS-related genes correspond to only 4% of the rice expressed genes, this category accounted for up to 10% the shared

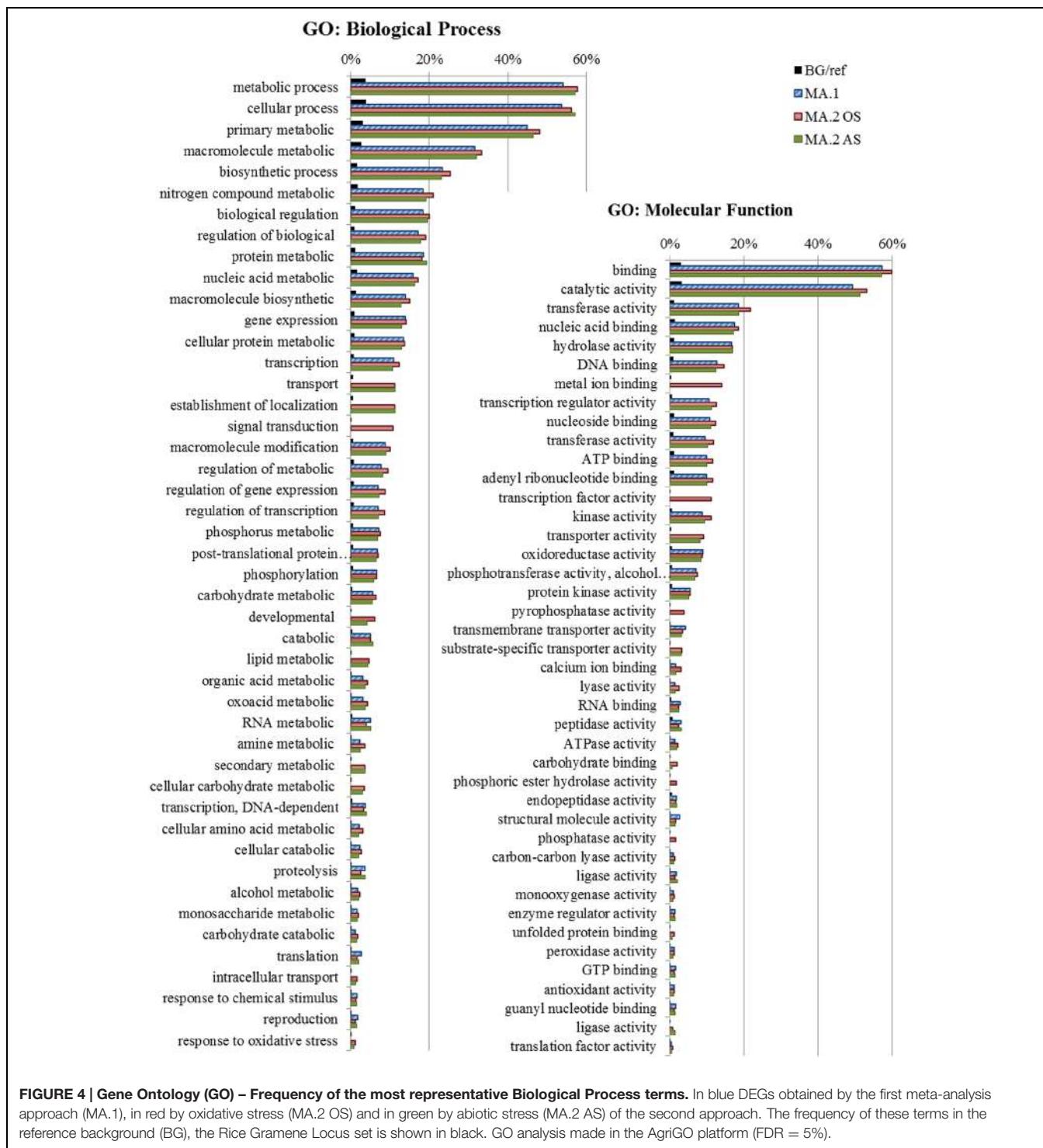
DEGs, highlighting the importance of ROS scavenging and signaling to the response to stress (Table 3C). Interestingly, the proportion was similar in OS and AS experiments. ROS imbalance or oxidative bursts that characterize OS are often indirect consequences of another environmental stresses (Guo et al., 2006). In the MA.2 approach, direct OS were separated and compared with other AS, such as salinity, drought, P deficiency, or Fe toxicity (AS). Ozone and hydrogen peroxide were used to generate the OS in the OS experiments. While hydrogen peroxide is a normal product of the plant metabolism, tropospheric ozone mostly originates from anthropogenic gas emissions (Baier et al., 2005; Wang and Frei, 2011). Most of the damage caused by this air pollutant is caused by its immediate degradation into ROS in the apoplastic space of plant cells, including hydrogen peroxide. Directly and indirectly  $O_3$  induces a cascade of active ROS production and signaling (Vaultier and Jolivet, 2015). Both OS treatments thus induce direct OS, though from opposite starting sites. While the  $O_3$  treatment involved fumigating of leaves (Frei et al., 2010a; Cho et al., 2013), the  $H_2O_2$  treatment was performed by growing seedlings in a solution containing 10 mM  $H_2O_2$ , starting the oxidative reactions from the plant roots (Mittal et al., 2012a,b).



**FIGURE 3 | Relative expression of the genes shown in Figure 2B in response to different treatments and in different tissues (R, Roots; S, Shoots; and P, Seedlings) followed by each gene's MSU locus, STRING node id and description.** Each gene is represented in Figure 2B as a node identified by a STRING node number or the locus name (loc). Color scale shows relative expression of each gene in relation to all tested treatments.

Despite the theoretical differences between OS and AS experiments, the percentage of DEGs considered to be ROS-related was similar (Tables 2 and 3). This data enforces the concept that most AS generate OS or ROS signaling to some degree and highlights the importance of ROS homeostasis in AS response. Diverse studies claimed associations of OS with the stresses included in this MA: drought (Noctor et al., 2014); salinity (Miller et al., 2010; Chawla et al., 2012); temperature extremes (Guo et al., 2006; Mittal et al., 2012a); zinc deficiency

(Frei et al., 2010b; Höller et al., 2014); phosphorous deficiency (Hernandez and Munne-Bosch, 2015); potassium deficiency (Cakmak, 2005; Ma et al., 2012), and iron deficiency (Zheng et al., 2009); iron toxicity (Matthus et al., 2015); cadmium toxicity (Uraguchi et al., 2011; Ogo et al., 2014); arsenic toxicity (Azizur Rahman et al., 2007); and lead toxicity (Li et al., 2012). In agreement with these studies, our results suggest that ROS homeostasis plays a similarly important role in all of these stresses.



## Putative Functions of Multi-Stress-Responsive Genes Detected by Combined MA Approaches

Differentially expressed genes detected by both approaches (i.e., MA.1 and MA.2) shared many GO terms, and even if the individual DEGs detected by each approach diverged

(Figures 1 and 4), their functions were rather conserved. The DEGs identified by MA.1 and MA.2 were mostly related to metabolic and cellular processes, but also involved in the response to stimuli, regulation, transcription, and transport processes. Many of those genes can be associated with signaling pathways possessing functions such as catalytic, transferase, hydrolase, transport activity, DNA binding, and transcript regulator activity

(Figure 3). Interestingly, the terms signal transduction process, metal ion binding activity, transcription factor activity and pyrophosphatase activity were found only among OS DEGs (Figure 4).

As the GO enrichment analysis demonstrated, the DEGs elected by these MA approaches represented different classes of proteins and are involved in many distinct processes. In MA.2, 311 genes were identified in both OS and AS. Between those genes, a great number of distinct transporters were present, such as the calcium transporter ATPases LOC\_Os12g39660 and LOC\_Os05g02940, the citrate transporter protein LOC\_Os02g57620, the inorganic phosphate transporter LOC\_Os02g38020, the sulfate transporter LOC\_Os03g09930, the amino acid transporter LOC\_Os06g36180, the aquaporin LOC\_Os02g41860, and others (Supplementary Table S5). Among those were also four HIPPs, proteins that can act as cytoplasmic transporters of metallic ions and co-factor in the transcription of many stress related genes (de Abreu-Neto et al., 2013).

Using the network tool STRING 10.0, we identified a cluster of 36 DEGs that are mostly involved in the photosynthetic apparatus and its regulation (Figure 2). Photosynthesis is a main source of ROS in plants, which can be enhanced by AS, leading to oxidative damage if not controlled (Foyer and Shigeoka, 2011; Voss et al., 2013). Interestingly, only one of those DEGs is directly involved in the ROS scavenging pathway. The cytosolic monodehydroascorbate reductase 2 (MDAR2), coded by LOC\_Os08g44340, is an enzyme that regenerates ascorbate back from its oxidized form (monodehydroascorbate; Noctor et al., 2014). Most genes identified by this “interactomic approach” did not belong to this group of well-studied antioxidants and antioxidant enzymes, e.g., AsA, GSH, Trx, CAT, APX, and GPX (Foyer and Shigeoka, 2011; Maruta et al., 2012). Instead, the elected genes were directly involved in the photosynthetic apparatus or regulatory elements, such as WRKY, MYB, and TYFF transcription factors (Supplementary Table S5). More than half of these genes showed a similar expression pattern, being highly expressed in response to iron deficiency and drought in roots, while suppressed in As toxicity, ozone, and submergence (Figure 3). Curiously, they were induced in roots and shoots under drought stress, but repressed in seedlings in the same treatments. This apparent contradiction could reflect differences in the age of the samples or the methods used to simulate the stress. In the experiments where roots and shoots were collected, the hydroponic solution in which the plants grew were slowly drained (Wang et al., 2011), while the whole seedlings were dried in tissue paper (Jain et al., 2007). MDAR2 did not represent the same expression pattern as this group of photosynthesis-related genes (Figure 3) and was positioned as a terminal node of the predicted cluster, connected only with LOC\_Os06g0539, that codes a plastid gene of unknown function (Figure 2B). Although we cannot be certain about the involvement of MDAR2 with the other DEGs of this cluster, many studies have shown the importance of MDAR enzymes to the response and tolerance to AS (Sultana et al., 2012; El Airaj et al., 2013).

Among the DEGs shared between OS and AS, which did not fall into that cluster, other genes possessing hub roles (connecting distinct signaling and metabolic pathways) were also identified, for example OsSRO1c (Similar to Radical-Induced Cell Death One 1c, LOC\_Os03g12820). Radical-induced Cell Death1 (*AtRCD1*) received its name due to the ozone hypersensitive phenotype observed in plant knock-outs to this gene (Ahlfors et al., 2004; Miao et al., 2006). *AtRCD1* activity is modulated through oxidation by a GPX (AtGPX3; Ahlfors et al., 2004; Miao et al., 2006). Recent studies have demonstrated that GPX proteins play important roles as redox sensors and connect ROS signaling with hormonal signaling pathways (Fourquet et al., 2008; Passaia et al., 2013; Passaia and Margis-pinheiro, 2015). One way this connection occurs is by SRO proteins (SRO), that were shown to interact with many different transcription factors (e.g., DREB2A and COL10) and are involved in transcription factor regulation and complex formation (Ahlfors et al., 2004; Jaspers et al., 2009). *AtRCD1* plays a role in the plant development and response to stress, mutants experiments show the participation of this protein in ethylene, ROS, salicylic acid, abscisic acid (ABA), and jasmonic acid (JA) signaling pathway (Ahlfors et al., 2004; Jaspers et al., 2009, 2010). A recent study have demonstrated that OsSRO1c is induced in response to multiple stresses and was shown to improve drought and OS tolerance by promoting stomatal closure and H<sub>2</sub>O<sub>2</sub> accumulation (You et al., 2013).

## CONCLUSION

A MA approach integrating different statistical methods allowed us to narrow down shared DEGs to a relatively small number that should be further investigated in detail. The comparison of shared DEGs with a list of genes *a priori* considered to be ROS-related highlighted the importance of redox homeostasis in stress response and signaling. Among the shared DEGs identified in this study are interesting candidates such as OsSRO1c, which regulate a great number of other proteins and connect different signaling pathways.

## ACKNOWLEDGMENTS

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2015.01260>

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# The Dual Role of the Plastid Terminal Oxidase PTOX: Between a Protective and a Pro-oxidant Function

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The plastid terminal oxidase (PTOX) is a non-heme diiron quinol oxidase that oxidizes plastoquinol and reduced O<sub>2</sub> to H<sub>2</sub>O. PTOX was discovered in the so-called *immutans* mutant of *A. thaliana* showing a variegated phenotype (Wetzel et al., 1994; Carol et al., 1999). PTOX is localized in the non-appressed regions of the thylakoid membrane (Lennon et al., 2003) and is involved in carotenoid biosynthesis, plastid development, and chlororespiration. Reviews have focused on the role of PTOX in chlororespiration (Bennoun, 2002; Rumeau et al., 2007), in chloroplast biogenesis (Putarjunan et al., 2013) and in stress responses (McDonald et al., 2011; Sun and Wen, 2011). A recent review by Nawrocki et al. (2015) has addressed the role of PTOX in poising the chloroplast redox potential in darkness. However, its role and interplay with the photosynthetic electron flow remains unclear.

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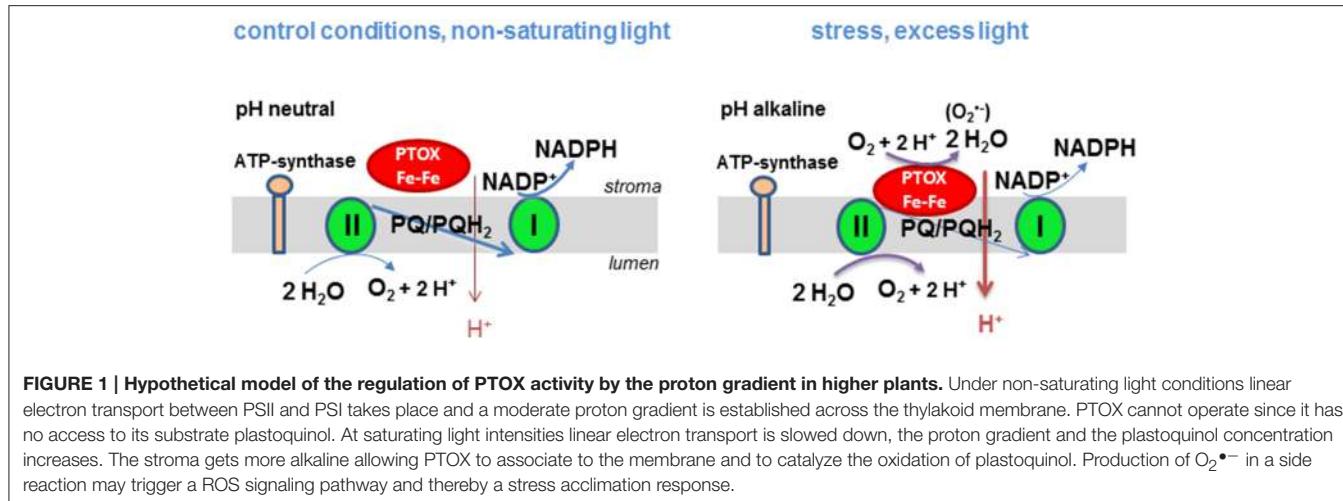
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Plants grown in moderate light under non-stress conditions have low PTOX concentrations (about 1 PTOX protein per 100 PSII; Lennon et al., 2003). By contrast, elevated PTOX levels have been found in plants exposed to abiotic stresses such as high temperatures, high light and drought (Quiles, 2006), salinity (Stepien and Johnson, 2009), low temperatures and high intensities of visible (Ivanov et al., 2012), and UV light (Laureau et al., 2013). PTOX has been proposed to act as a safety valve by protecting the plastoquinone pool from overreduction under abiotic stress. A highly reduced PQ pool hinders forward electron flow and triggers charge recombination in photosystem II (PSII) leading to the generation of triplet chlorophyll and highly toxic singlet oxygen. However, overexpression of PTOX in *A. thaliana* did not protect against light-induced photodamage (Rosso et al., 2006) and even enhanced photo-oxidative stress in tobacco expressing, in addition to its endogenous enzyme, either PTOX from *A. thaliana* (Heyno et al., 2009) or PTOX1 from *C. reinhardtii* (Ahmad et al., 2012). Different to higher plants *C. reinhardtii* possesses two isoforms, PTOX1 and PTOX2. PTOX1 is most likely responsible for regenerating PQ for phytoene desaturation and shows a lower rate of plastoquinol oxidation during photosynthesis than PTOX2 (Houille-Vernes et al., 2011).

Using purified PTOX, Yu and coworkers have recently shown that depending on the quinol concentration PTOX can act as an anti-oxidant or pro-oxidant (Feilke et al., 2014; Yu et al., 2014). PTOX activity was found to be pH insensitive between pH 6.0–8.5 when as substrate decylPQH<sub>2</sub> dissolved in methanol was used (Yu et al., 2014). During the catalysis, peroxide intermediates are formed at the diiron center. Depending on the lifetime of these intermediates, reactive oxygen species (ROS) can be generated as a side reaction. Isolated PTOX generates superoxide radicals at both high, but physiologically relevant, quinol concentrations at pH 8.0 and substrate limiting concentrations at pH 6.0–6.5 (Feilke et al., 2014; Yu et al., 2014). When substrate is limited, the second quinol may not arrive in time leading to superoxide formation directly at the catalytic center. Alternatively, since at pH 8.0 the semiquinone is more stable than at pH 6.0, it is conceivable that the high pH stabilized semiquinone acts as a ROS generator. PTOX in overexpressors has



**FIGURE 1 | Hypothetical model of the regulation of PTOX activity by the proton gradient in higher plants.** Under non-saturating light conditions linear electron transport between PSII and PSI takes place and a moderate proton gradient is established across the thylakoid membrane. PTOX cannot operate since it has no access to its substrate plastoquinol. At saturating light intensities linear electron transport is slowed down, the proton gradient and the plastoquinol concentration increases. The stroma gets more alkaline allowing PTOX to associate to the membrane and to catalyze the oxidation of plastoquinol. Production of O<sub>2</sub><sup>•-</sup> in a side reaction may trigger a ROS signaling pathway and thereby a stress acclimation response.

also been found to generate superoxide in the light (Heyno et al., 2009).

By oxidizing plastoquinol PTOX reduces the number of electrons available for photosynthetic electron flow. It is generally accepted that PTOX has low activity compared to photosynthetic electron flow. The maximum rate of PTOX was reported to be  $5 \text{ e}^- \text{ s}^{-1} \text{ PSII}^{-1}$  for PTOX2 in *C. reinhardtii* and  $0.3 \text{ e}^- \text{ s}^{-1} \text{ PSII}^{-1}$  in tomato while the maximal rate of photosynthesis is approximately  $150 \text{ e}^- \text{ s}^{-1} \text{ PSII}^{-1}$  (Nawrocki et al., 2015). However in plants exposed to stress, PTOX activity can account for 30% of the PSII activity (Stepien and Johnson, 2009). The *in vitro* enzyme activity of PTOX is high when substrate concentrations are saturating (up to  $19.01 \pm 1.1 \mu\text{mol O}_2 \text{ mg protein}^{-1} \text{ min}^{-1}$ ; Yu et al., 2014). This corresponds to a turnover rate of  $320 \text{ e}^- \text{ s}^{-1} \text{ PTOX}^{-1}$  at  $35^\circ\text{C}$ , the optimum temperature for PTOX from rice. The discrepancy between the reported PTOX activities *in planta* and the  $V_{max}$  measured with the purified protein points to a mechanism that allows the regulation of PTOX activity depending on the reduction state of the electron transport chain.

Since PTOX can compete with linear and cyclic electron flow (Feilke et al., 2015) and consequently lowers NADPH, ATP production and CO<sub>2</sub> fixation and potentially generates ROS, its activity must be tightly controlled. High activity is beneficial for the plant to protect the photosynthetic apparatus against photoinhibition when the electron transport chain is in a highly reduced state as it is the case under abiotic stress when the stomata are closed due to water stress or when CO<sub>2</sub> fixation is limited by unfavorable temperatures. However, high PTOX activity is detrimental to high photosynthetic activity when light and CO<sub>2</sub> are not limiting.

These observations have led us to postulate the following hypothesis (Figure 1) that explains the discrepancies in the literature about the safety valve function of PTOX. When stromal pH is alkaline (in high light), PTOX may become associated with the membrane giving it access to its substrate, lipophilic plastoquinol, leading to efficient oxidation of the quinol and

reduction of O<sub>2</sub> to H<sub>2</sub>O. By contrast when stroma pH becomes less alkaline (under non-saturating light conditions) PTOX may be soluble. Soluble PTOX cannot access its substrate plastoquinol that is located in the thylakoid membrane and the enzyme is effectively inactive. Activity of carotenoid biosynthesis enzymes may be regulated in a similar manner. Phytoene desaturase, which catalyzes the reaction of lipophilic phytoene to  $\zeta$ -carotene, is found in the stroma both as a tetrameric membrane-bound form which has access to substrate and a soluble multi-oligomeric form in the stroma that does not (Gemmecker et al., 2015). Another example of an enzyme known to associate with the membrane in a pH-dependent manner is the violaxanthin de-epoxidase (Hager and Holocher, 1994). This enzyme associates with the thylakoid membrane when the luminal pH decreases.

The model of pH-dependent regulation of PTOX activity by membrane association allows us to rationalize how PTOX could act as a safety valve under conditions of stress such as drought, high light and extreme temperatures when the stomata are closed and the CO<sub>2</sub> assimilation rate is low and the stromal pH is alkaline. Its dissociation from the membrane at less alkaline pH would hinder its competition with the photosynthetic electron chain for its substrate plastoquinol. Chlororespiration in the dark requires membrane associated PTOX. In our model, this can only take place when a proton gradient is created in the dark by hydrolysis of ATP that is either present in the chloroplast or delivered to the chloroplast from mitochondria. Additionally, when the plastoquinone pool is highly reduced, PTOX can generate superoxide, a potential signaling mechanism that causes the expression levels of responsive genes to change allowing the plant to acclimate to changes in its environment.

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# Universal Stress Protein Exhibits a Redox-Dependent Chaperone Function in *Arabidopsis* and Enhances Plant Tolerance to Heat Shock and Oxidative Stress

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Although a wide range of physiological information on Universal Stress Proteins (USPs) is available from many organisms, their biochemical, and molecular functions remain unidentified. The biochemical function of AtUSP (At3g53990) from *Arabidopsis thaliana* was therefore investigated. Plants over-expressing AtUSP showed a strong resistance to heat shock and oxidative stress, compared with wild-type and *Atusp* knock-out plants, confirming the crucial role of AtUSP in stress tolerance. AtUSP was present in a variety of structures including monomers, dimers, trimers, and oligomeric complexes, and switched in response to external stresses from low molecular weight (LMW) species to high molecular weight (HMW) complexes. AtUSP exhibited a strong chaperone function under stress conditions in particular, and this activity was significantly increased by heat treatment. Chaperone activity of AtUSP was critically regulated by the redox status of cells and accompanied by structural changes to the protein. Over-expression of AtUSP conferred a strong tolerance to heat shock and oxidative stress upon *Arabidopsis*, primarily via its chaperone function.

**Keywords:** heat shock, high molecular weight (HMW) complex, low molecular weight (LMW) complex, molecular chaperone, oxidative stress, redox status, universal stress protein (USP)

## INTRODUCTION

Because plants are sessile organisms, their growth, development, and survival are significantly affected by a variety of external stresses including cold, heat, water deficit, or drought, flooding, high salinity, and strong winds. These stresses can cause production of reactive oxygen species (ROS) containing hydrogen peroxide ( $H_2O_2$ ), superoxide anion ( $\cdot O_2^-$ ), singlet oxygen ( $^1O_2$ ) and hydroxyl radical ( $\cdot OH$ ) (Baier and Dietz, 2005; D'Autréaux and Toledano, 2007; Schwarzsälder and Finkemeier, 2013). Depending upon the levels of ROS, many downstream signaling systems in cells, such as protein kinases, phosphatases, transcription factors, molecular chaperones, and defense-related proteins, may be activated. ROS thus play dual roles, acting both as useful signaling molecules to sense and activate defense signaling cascades (Møller and Sweetlove, 2010) and as harmful byproducts of aerobic metabolism of plants.

The Universal Stress Protein domain (USP) gene, which encodes a protein containing the 140–160 highly conserved residues of the Universal Stress Protein A domain (USPA, Pfam accession

number PF00582), is a representative stress responsive gene. It has been shown to respond to diverse environmental stresses, such as salt, drought, cold, heat, and oxidative stress (Kerk et al., 2003; Ndimba et al., 2005; Persson et al., 2007). The USP genes are widely distributed across most living organisms, including bacteria, archaea, fungi, protozoa, plants, and mammals.

The C13.5 protein was first identified from bacteria. Its name was changed to USP to reflect its ability to respond to diverse stresses (Zarembinski et al., 1998; Sousa and McKay, 2001). USPs from *Escherichia coli* have been divided into four classes according to their protein structures and amino acid sequences: Class 1 (UspA, UspC, and UspD), Class 2 (UspG and UspF), and Classes 3 and 4 (two domains of UspE) (Nyström and Neidhardt, 1992; Persson et al., 2007). The bacterial USPs are involved in processes including iron scavenging, oxidative stress resistance, cell adhesion, and cell motility (Nachin et al., 2005). The USP domain in MJ0577 (also known as 1MJH) from *Methanocaldococcus jannaschii* contains an ATP-binding motif and may function biochemically as an ATPase or ATP-binding molecular switch. By contrast, the USP domain in *Haemophilus influenzae* has neither ATP-binding residues nor ATP-binding activity (Sousa and McKay, 2001; Kvint et al., 2003; Persson et al., 2007).

Despite this wide range of physiological information on bacterial USP proteins, the biochemical, and molecular mechanisms of USPs have never been identified. This prompted us to investigate the biochemical functions of these proteins in plants. The genome of *Arabidopsis thaliana* contains 44 proteins homologous to bacterial USPs, based on their protein sequences and structural similarities. Sequence analysis of USPs from *Arabidopsis* suggests these proteins evolved from a 1MJH-like ancestor protein (Kerk et al., 2003).

Two USPs from *Arabidopsis*, AtPHOS32 and AtPHOS34, were phosphorylated in response to microbial elicitation and AtPHOS32 was shown to be a substrate of MAP kinases 3 and 6 (Shinozaki and Yamaguchi-Shinozaki, 2007; Coetzer et al., 2010). In rice, OsUSP1 was shown to activate a cellular downstream signaling cascade in response to ethylene, a gaseous hormone in plant, enabling adaptation of plants to hypoxic conditions (Sauter et al., 2002). Similarly, the USP genes of *Gossypium arboreum*, *Astragalus sinicus*, *Solanum pennellii*, and *Salicornia brachiate* are involved in water stress and nodulation, and drought, salt, and osmotic tolerances (Chou et al., 2007; Maqbool et al., 2009; Loukehaich et al., 2012; Udawat et al., 2014).

We examined the biochemical and molecular functions of AtUSP (At3g53990) by analyzing plants over-expressing AtUSP as well as an *Atusp* knock-out mutant, and also recombinant AtUSP. Plants over-expressing AtUSP showed a strong resistance to heat shock and oxidative stress. In particular, we demonstrated that AtUSP exhibited a molecular chaperone function and that chaperone activity was critically regulated in a redox and heat shock-dependent manner, accompanied by structural changes to the protein. The chaperone function of AtUSP thus plays an essential role in protecting plants from heat shock and oxidative stress.

## MATERIALS AND METHODS

### Plants and Growth Conditions

*A. thaliana* (Columbia ecotype) plants were grown under a 16/8 h light/dark cycle at 22°C and 70% humidity. The T-DNA insertion knock-out mutant line (SALK\_146059) was obtained from the Arabidopsis Biological Resource Center (USA). *Arabidopsis* seeds were surface-sterilized and sown either onto solid MS medium containing 0.25% phytagel and 3% sucrose in a Petri dish or onto soil. Seeds were incubated for 3 days at 4°C to synchronize germination. Plants were grown under light conditions of 100–120 μmol m<sup>-2</sup> s<sup>-1</sup> photosynthetic flux.

### RNA Isolation and RT-PCR Analysis

Roots, stems, leaves from 4-week-old wild type plants and flowers from 5-week-old wild type plants were collected. Ten-day-old seedling plants with or without 5 mM H<sub>2</sub>O<sub>2</sub> were collected at 0, 1, 3, 6, and 12 h. Ten-day-old seedling plants with or without heat shock treatment at 37°C were collected at 0, 1, 3, 6, 12, and 24 h. These collected samples were frozen with liquid nitrogen for total RNA isolation and cDNA synthesis. Total RNA was extracted from the collected samples using the MACHEREY-NAGEL RNA kit (Düren, Germany) and reverse-transcribed using RevertAid Reverse Transcriptase (Thermo Scientific RevertAid First Strand cDNA Synthesis Kit, Lithuania) according to the manufacturer instructions. The newly synthesize cDNA was diluted to the 50 ng/μL with ddH<sub>2</sub>O. The PCR program initially started with a 95°C denaturation for 2 min, followed by 24 cycles of 95°C/20 s, 60°C/40 s, 72°C/1 min and finally with elongation step at 72°C for 5 min. Specific PCR primers for genes encoding AtUSP (At3g53990) were used (AtUSP Forward: 5'-GAATTCCATGCCTAAAGACAGGAATATCGG-3', AtUSP Reverse: 5'-ATCGATTATTCTGTTATCCTTGACAACGGT-3'). The gene expression levels of AtUSP were compared with the internal control gene, *Tubulin* (AT5G62690). Specific PCR primers for genes encoding *Tubulin* (AT5G62690) were used (*Tubulin* Forward: 5'-CCAACAACGTGAAATCGACA-3', *Tubulin* Reverse: 5'-TCTTGGTATTGCTGGTACTC-3'). PCR products were observed in 1% agarose gel electrophoresis stained with ethidium bromide.

### Cloning of AtUSP and Preparation of Transgenic Plants Over-Expressing AtUSP

AtUSP was cloned from an *Arabidopsis* cDNA library by the polymerase chain reaction (PCR), as previously described (Bréhelin et al., 2000; Park et al., 2009). After confirmation of the entire coding sequence, the full-length AtUSP cDNA was ligated into *EcoRI/ClaI* sites of the binary vector pCAMBIA1300, which has a FLAG-tag in the N-terminal region (Figure S1C). *Agrobacterium tumefaciens* GV3101 was transformed with the plasmid and used to transfect *Arabidopsis* by the floral dip method (Clough and Bent, 1998). Transformants were selected on MS plates containing 50 μg/ml hygromycin (Duchefa). AtUSP expression was analyzed using western blot analysis with FLAG-tag antibody (Sigma).

## Hydrophobicity Analysis

The ProtScale database (<http://www.expasy.org/tools/protscale.html>) was used to analyze the hydrophobicity of AtUSP. Hydrophobicity plot of AtUSP was generated by the Kyte-Doolittle analysis (Kyte and Doolittle, 1982).

## Purification of AtUSP Recombinant Protein

The full-length cDNA of *AtUSP* was isolated from an *Arabidopsis* cDNA library and ligated into the *Bam*H/*Xba*I sites of the pET28a expression vector (NEB), and the resulting DNA constructs were used to transform *E. coli* BL21 (DE3) cells. The transformants were cultured at 37°C in LB medium containing ampicillin (50 µg/ml) and chloramphenicol (12.5 µg/ml). The culture was diluted 1:50 in LB medium containing 50 µg/ml ampicillin and grown at 30°C until an OD<sub>600</sub> of 0.6–0.8 was reached. Expression of recombinant protein was induced by the addition of 0.5 mM isopropyl-β-D-thiogalacto-pyranoside (IPTG) and the cells were grown for a further 4 h. The cells were then harvested by centrifugation at 5000 g for 10 min, and the pellet was resuspended in PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, and 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.6) with 1 mM PMSF. Cells were stored at –80°C until used. The frozen cells were disrupted by sonication and the soluble extract was loaded onto Ni-NTA agarose columns. Recombinant AtUSP was eluted from the columns by thrombin and dialyzed against 50 mM Hepes-KOH (pH 8.0) at 4°C. The recombinant protein was further purified by FPLC using a Superdex 200 HR 10/30 column. The purity of AtUSP was determined using SDS-PAGE.

## Size Exclusion Chromatography (SEC)

SEC or FPLC (AKTA; Amersham Biosciences, Uppsala, Sweden) was performed using a Superdex 200 HR 10/30 column from GE Healthcare equilibrated with 50 mM Hepes-KOH (pH 8.0) buffer with a flow rate of 0.5 ml/min at 25°C. Fractions corresponding to protein peaks (A<sub>280</sub>) were isolated and concentrated at 4°C using a Centricon YM-30 filter (Millipore Co., Santa Clara, USA) (Park et al., 2009; Jung et al., 2013).

## Analysis of Molecular Chaperone Activity

Chaperone activity of AtUSP was measured using Malate dehydrogenase (MDH) from Sigma-Aldrich (Missouri, USA) as a substrate. MDH was incubated in 50 mM Hepes-KOH (pH 8.0) buffer with various concentrations of AtUSP at 45°C. During the 20 min incubation, thermal aggregation of MDH was determined by monitoring the turbidity at A<sub>340</sub> using a DU800 spectrophotometer equipped with a thermostatic cell holder, as previously described (Lee et al., 2009; Jung et al., 2013).

## Fluorescence Measurement

Fluorescence was measured using a SFM 25 spectrofluorometer (Kontrom, Zurich, Switzerland) was used to measure fluorescence of bis-ANS [1,1-bis(4-anilinonaphthalene-5-sulfonic acid)] obtained from Sigma-Aldrich (Missouri, USA). The excitation wavelength of bis-ANS fluorescence was set to 380 nm, and the emission spectra were scanned between 400 and 600 nm, as described previously (Jung et al., 2013). Reaction mixtures containing 10 µM AtUSP in 50 mM Hepes (pH 8.0) were incubated with 10 µM bis-ANS for 30 min at 25°C.

## Stress Treatments and Measurements of Chlorophyll Content and Electrolyte Leakage

For oxidative stress treatment, leaf discs 7 mm in diameter were collected from the same stages and positions of leaves in wild-type (WT) plants, AtUSP-over-expression lines, and *Atusp* knock-out mutant plants. Discs were immersed abaxial side up in 3 ml of a 0.1% Tween 20 solution containing 10 mM H<sub>2</sub>O<sub>2</sub>. Leaf discs were also subjected to vacuum infiltration for 1 min or incubated at room temperature under light (300 µmol m<sup>-2</sup> s<sup>-1</sup>) conditions for 18–24 h, and the resulting damage was examined. For heat shock treatment, *Arabidopsis* seedlings were grown on MS media containing 3% sucrose in a Petri dish. Heat shock was applied by placing plates containing 10-day-old *Arabidopsis* seedlings in a water bath at 43°C. Chlorophyll content, extracted using 80% (v/v) acetone, was measured, as described (Porra et al., 1989; Park et al., 2009), and electrolyte leakage was analyzed, as previously reported (Sukumaran and Weiser, 1972; Ristic and Ashworth, 1993). For the measurement of ion conductivity, nine leaf discs (10 mm diameter) were placed in a tube test with 25 ml of de-ionized water and treated with heat shock or oxidative stress with overnight shaking. After autoclaving, the tubes containing leaves were cooled to room temperature and conductivity was measured again. The electrolyte leakage was calculated by the percentages of the conductivity before and after autoclaving. The electrolyte leakage assay was performed at least three times, each time with three replicates.

## Statistical Analysis

All values reported in experiments for chlorophyll content, ion leakage, and fresh weight measurements are mean of six replicates. Statistical Analysis were performed using SPSS 12.0.1 software (SPSS Inc., Chicago, IL). One-way ANOVA was used to analyze data (*p* < 0.05) and differences among treatments were performed through Tukey tests.

## RESULTS

### Heat Shock- and Oxidative Stress-Dependent Expression of AtUSP in *Arabidopsis*

Even though a number of USPs have been identified as stress-resistance molecules from diverse organisms, the molecular mechanism of USP action has not been resolved (Nyström and Neidhardt, 1992; Zarembinski et al., 1998; Sousa and McKay, 2001; Kvint et al., 2003; Nachin et al., 2005; Persson et al., 2007). Among 44 USP proteins in *Arabidopsis*, especially AtUSP (At3g53990) was shown to induce various abiotic stresses in *Arabidopsis* eFP Browser (<http://www.bar.utoronto.ca/>) and published papers (Kawamura and Uemura, 2003; Ndimba et al., 2005; Isokpehi et al., 2011). We therefore chose AtUSP (At3g53990) and attempted to unravel the biochemical and molecular functions of a USP associated with stress resistance in *Arabidopsis*. RT-PCR was used to examine the expression levels of *AtUSP* in plants and showed *AtUSP* was widely expressed

in most tissues of *Arabidopsis*, including the root, stem, leaf, and flowers (**Figure 1A**). Since mRNA encoding similar proteins is expressed in various biotic and abiotic stress conditions in other organisms (Nyström and Neidhardt, 1992; Zarembinski et al., 1998; Sousa and McKay, 2001; Kvint et al., 2003; Nachin et al., 2005; Persson et al., 2007), we analyzed expression of *AtUSP* mRNA in *Arabidopsis* after treatment with H<sub>2</sub>O<sub>2</sub> and heat shock. The transcript levels of *AtUSP* in 10-day-old *Arabidopsis* seedlings gradually increased not only after oxidative stress but also under heat shock conditions (**Figure 1B**), suggesting that *AtUSP* plays an important role in the defense system in plant tissues.

## AtUSP Enhances Resistance of Plants to Heat Shock and Oxidative Stress

As the expression of *AtUSP* was significantly increased by heat shock or oxidative stress (**Figure 1**), we investigated its physiological functions using the T-DNA insertion knock-out line of *Atusp* (SALK\_146059; Figure S1A) and transgenic plants over-expressing *AtUSP* driven by the CaMV35S promoter (Figure S1B) under stress conditions. PCR analysis of genomic and cDNA expression levels confirmed that *Atusp* is a homozygous loss-of-function mutant of *Arabidopsis* (Figure S1A). In addition, more than 20 independent transgenic lines of plants over-expressing *AtUSP* were generated. Of the homozygous T3 lines of *Arabidopsis* over-expressing *AtUSP*, we

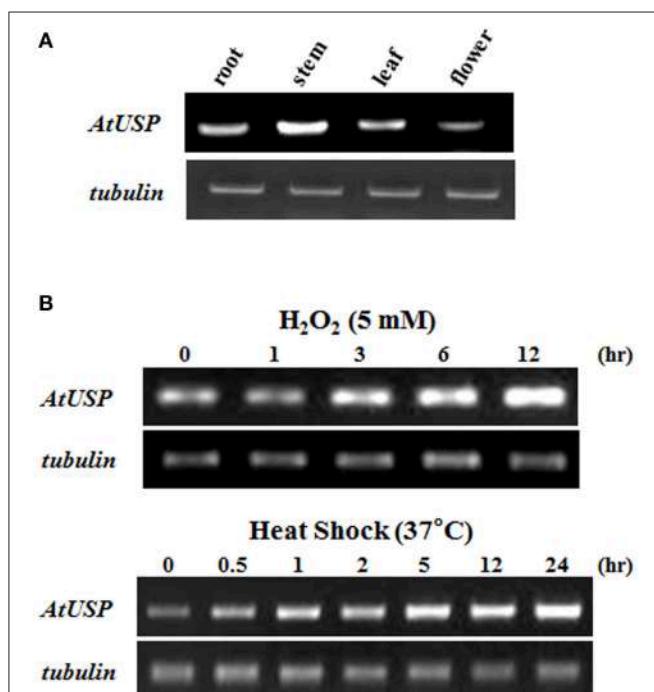
selected lines #12 and #15 for further studies as these showed the strongest expression of *AtUSP* when analyzed using a FLAG-tag antibody (Figure S1B).

To examine the *in vivo* role of *AtUSP* under oxidative stress conditions, leaf discs were collected from 3-week-old *Arabidopsis* seedlings of the WT plants, *Atusp* mutants, and *AtUSP* over-expression lines, and treated with 10 mM H<sub>2</sub>O<sub>2</sub>. Discs from *AtUSP* over-expression lines and *Atusp* mutants showed tolerant and sensitive phenotypes compared with those of the WT plants within 5 days of treatment with 10 mM H<sub>2</sub>O<sub>2</sub>, respectively (**Figure 2A**). Stress tolerance of the over-expression lines and sensitivity of the *Atusp* were confirmed by measuring the total chlorophyll content and electrolyte leakage under stress conditions. The results were consistent with the phenotypic differences (**Figure 2A**), as discs from plants over-expressing *AtUSP* showed three-fold higher chlorophyll content and lower electrolyte leakage than discs from WT plants and discs from *Atusp* plants showed about two-fold lower chlorophyll content and higher electrolyte leakage than discs from WT plants under the same conditions (**Figures 2B,C**).

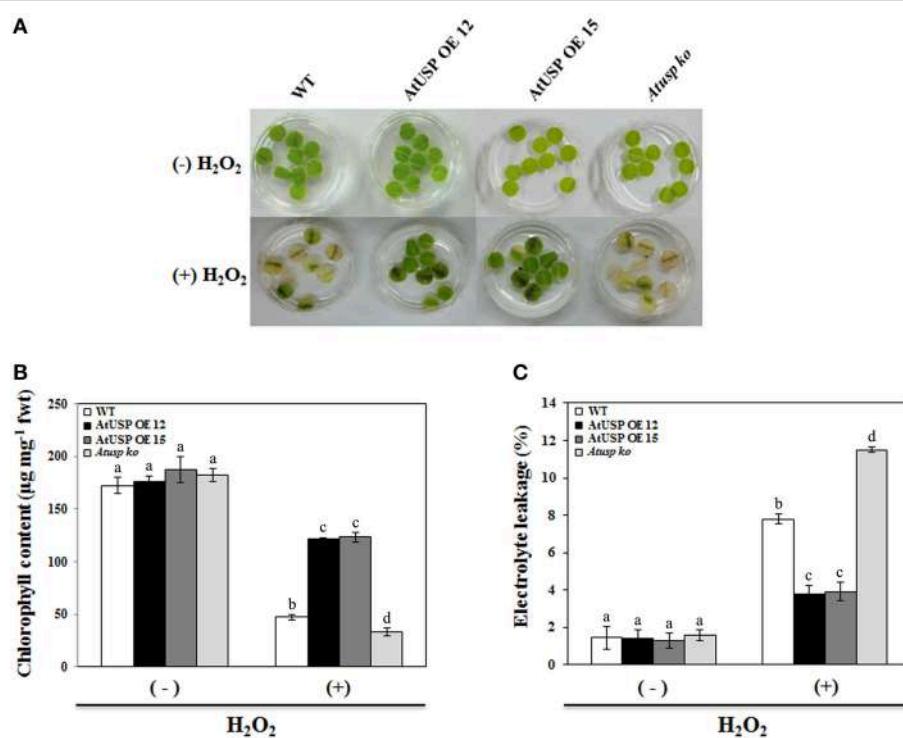
In addition to oxidative stress, the physiological significance of *AtUSP* in *Arabidopsis* under heat shock conditions was examined. A 2 h heat shock (43°C) treatment was applied to 10-day-old *Arabidopsis* seedlings from the WT, *Atusp*, and *AtUSP* over-expressing lines. Following heat shock treatment, all plants were transferred to their optimal growth conditions at 22°C to allow recovery (upper, **Figure 3A**). Most plants from the over-expression lines recovered from heat shock compared with the WT plants and *Atusp* mutants, as their growth and development with greenish pigments were restored. Also the *Atusp* mutants showed white or pale leaves and appeared dead than the WT plants indicating that the *Atusp* mutants were unable to recover from heat stress (**Figure 3A**). Examination of electrolyte leakage, chlorophyll content, and fresh weight also indicated that plants over-expressing *AtUSP* and *Atusp* mutants were strongly resistant and sensitive to heat shock compared with WT plants, respectively (**Figures 3B–D**). This suggests that *AtUSP* plays a very important role in the protection of plants from heat shock and oxidative stress, and is consistent with the data reported for the actions of USPs in microorganisms (Nachin et al., 2005).

## Redox-Dependent Structural and Functional Switching of AtUSP in Response to Oxidative Stress

Structural changes have been reported in many kinds of small heat shock proteins (sHSPs) that have a chaperone function and protect cells from heat shock and oxidative stress (Hendrick and Hartl, 1993; Haley et al., 1998). The structural transformation is induced by changes in their hydrophobicity (Park et al., 2009). In particular, several redox proteins, including AtTrx-h3, AtTDX, and 2-Cys peroxiredoxin (2-Cys Prx), which play crucial roles in oxidative and heat shock tolerance and behave as molecular chaperones, show structural changes from low molecular weight (LMW) to high molecular weight (HMW) structures (Jang et al., 2004; Lee et al., 2009; Park et al., 2009).



**FIGURE 1 |** Expression of *AtUSP* mRNA in different tissues and in response to heat shock and oxidative stresses. **(A)** Expression of *AtUSP* in various tissues of *Arabidopsis*. **(B)** Expression of *AtUSP* following treatment with 5 mM H<sub>2</sub>O<sub>2</sub> or heat shock at 37°C. Time represents the treatment hours of plants with H<sub>2</sub>O<sub>2</sub> and heat shock. *Tubulin* was used as a control.



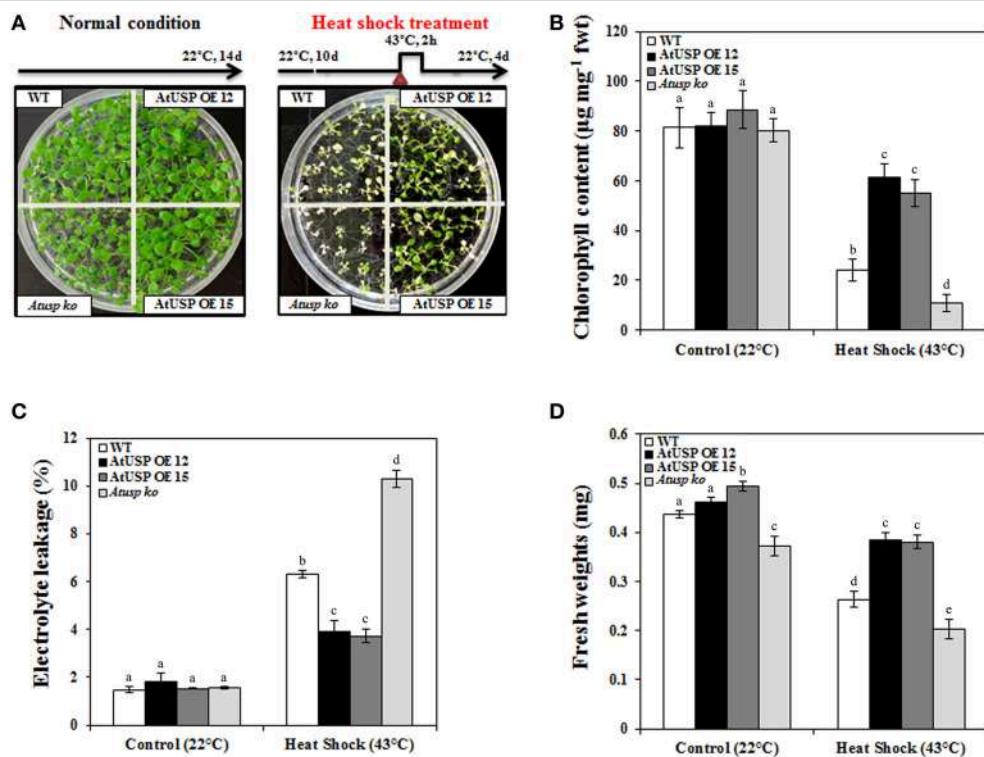
**FIGURE 2 | Comparison of tolerance of oxidative stress in wild-type, AtUSP over-expressing, and Atusp knock-out mutant *Arabidopsis*.** (A) Nine leaf discs isolated from 3-week-old wild-type, AtUSP over-expressing, and Atusp knock-out mutant plants were vacuum infiltrated with water or 10 mM  $\text{H}_2\text{O}_2$  for 1 min and transferred to a growth chamber at 22°C. Samples were collected after 5 days for measurements of chlorophyll content (B) and electrolyte leakages (C). All values are means for six replicates  $\pm$  SE. Data were analyzed using a One-way ANOVA and Tukey test was used to compare the difference between treatments. Different letters indicate the significant differences among the different plant lines ( $p < 0.05$ ).

Because AtUSP was also shown to protect *Arabidopsis* from heat shock and oxidative stress, we investigated its chaperone function and structural changes in response to oxidative stress. First, we analyzed the hydrophobicity of AtUSP using the ProtScale database (<http://www.expasy.org/tools/protscale.html>). This analysis indicated a structure for AtUSP that contained many hydrophobic regions, which may contribute to its protein stability and polymeric structure (Figure S2). USPs stimulated by various kinds of stresses (Nachin et al., 2005; Loukehaich et al., 2012) are known to be target proteins of thioredoxin (Trx), which regulates the redox status of its interaction partners (Mata-Cabana et al., 2007; Montrichard et al., 2009; Meyer et al., 2012). This observation, together with our finding that AtUSP enhanced the tolerance of plants to oxidative stress, suggested that AtUSP might be a redox-regulated protein.

To determine whether, like several redox-related proteins, such as Trx and Prx, AtUSP exhibited redox-dependent structural changes *in vitro*, we expressed recombinant AtUSP in bacteria. The recombinant AtUSP was purified and then its redox-dependent structural changes were tested under reducing and non-reducing SDS-PAGE conditions. AtUSP was sequentially treated with DTT and  $\text{H}_2\text{O}_2$ , electrophoresed using PAGE, and examined for structural changes. Under non-reducing conditions, the protein structure of AtUSP consisted of several oligomeric protein complexes with the monomeric

protein as a major band (Figure 4A, lane 1). However, treatment with 10 mM DTT caused the oligomeric structures of AtUSP to dissociate into the monomer (Figure 4A, lane 2). This suggested that the homo-polymeric structure of AtUSP was produced by linking a varying number of AtUSP subunits with disulfide bonds.

To determine whether this change in AtUSP protein structure showed redox-dependent reversibility, DTT was completely removed from AtUSP by dialysis and then the protein was treated with 10 mM  $\text{H}_2\text{O}_2$  for 30 min to ensure that AtUSP, which had been monomerized by DTT treatment, was repolymerized by the oxidizing agent,  $\text{H}_2\text{O}_2$ . The treated samples were analyzed using non-reducing SDS-PAGE. The result indicated that the reduced thiol groups of AtUSP had been reoxidized to form disulfide bonds and polymeric structures (Figure 4A, lane 3). By contrast, under reducing SDS-PAGE conditions, AtUSP displayed as a single protein band with a MW was estimated at 17.8 kDa, suggesting that treatment with  $\beta$ -mercaptoethanol resulted in the complete dissociation of multimeric AtUSP into its monomers (Figure 4B). The redox-dependent structural changes of AtUSP were independently confirmed using SEC. The protein structure of AtUSP was reversibly shifted from LMW species to HMW complexes by treatment with DTT or  $\text{H}_2\text{O}_2$  (Figure 4C). Next, binding of bis-ANS, a measure of structural changes was determined following treatment of AtUSP with DTT and  $\text{H}_2\text{O}_2$ . The fluorescence intensity of  $\text{H}_2\text{O}_2$ -treated AtUSP



**FIGURE 3 | Comparison of heat shock resistance in wild-type, AtUSP over-expressing, and Atusp knock-out mutant *Arabidopsis*.** (A) Ten-day-old *Arabidopsis* seedlings were heat shocked, as indicated, and then transferred to optimal growth conditions at 22°C. Thermo-tolerance of the plants was examined under optimal conditions after a 4 day recovery period. Ten-day-old *Arabidopsis* seedlings were grown at 22°C for 4 days as a control. Chlorophyll (B), electrolyte leakage (C), and fresh weight (D) were measured. All values are means for six replicates  $\pm$ SE. Data were analyzed using a One-way ANOVA and Tukey test was used to compare the difference between treatments. Different letters indicate the significant differences among the different plant lines ( $p < 0.05$ ).

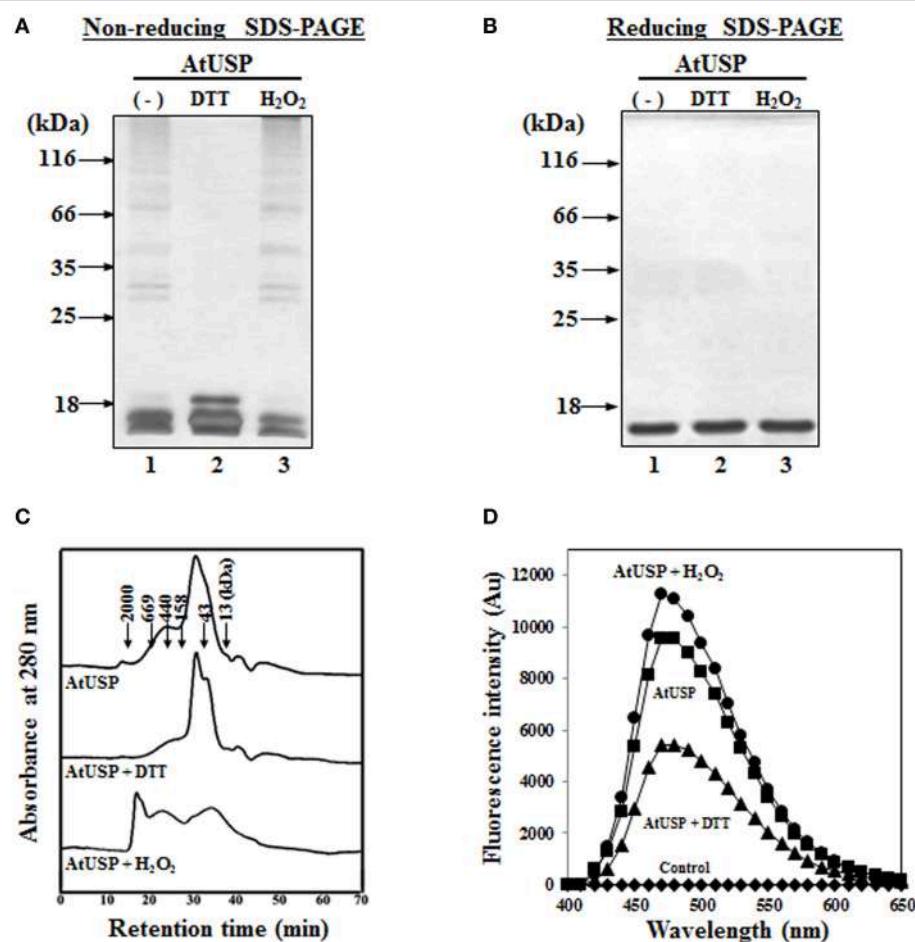
was significantly higher than that of untreated or DTT-treated AtUSP (Figure 4D).

The structural changes analyzed using *in vitro* experiments were confirmed *in vivo* with studies of 2-week-old transgenic *Arabidopsis* plants from over-expression line #12. Proteins were extracted from plants treated with DTT or H<sub>2</sub>O<sub>2</sub> by vacuum infiltration, and subjected to SDS-PAGE under reducing and non-reducing conditions. Under non-reducing conditions, *in vivo* AtUSP showed multiple protein bands, consisting of a number of oligomeric proteins and a monomeric band, despite the presence of an excessive amount of SDS (1%) (Figure 5, lane 1). This indicated that the major factor in oligomerization was the hydrophobic interaction. When, however, AtUSP was incubated with DTT prior to analysis in non-reducing conditions, the oligomeric structures of AtUSP were almost completely dissociated into monomers (Figure 5, lane 2), although treatment of the monomeric form with H<sub>2</sub>O<sub>2</sub> restored the native polymeric structures (Figure 5, lane 3). Taken together, the *in vitro* and *in vivo* results indicate that individual proteins of AtUSP can form polymeric complexes through hydrophobic interactions and disulfide bonds, and the formation of these structures is reversibly regulated in a redox-dependent manner.

## AtUSP Exhibits a Chaperone Function

It is well-known that molecular chaperones not only prevent aggregation of nascent proteins in cells but also facilitate their correct folding by protecting substrate aggregations from stresses (Li et al., 2013). Chaperone proteins exist as multimeric conformations consisting of dimers, trimers, and higher oligomeric complexes (Haley et al., 1998); AtUSP was also shown to form polymeric structures, so we examined whether it had a chaperone function. MDH was used as a substrate to assess the ability of AtUSP to inhibit thermal aggregation of proteins; AtTrx-h3 was a positive control.

Incubation of MDH with an increasing amount of AtUSP gradually prevented the thermal aggregation of substrate, which was significantly blocked at a subunit molar ratio of 1 MDH to 3 AtUSP (Figure 6A). This indicates AtUSP is a novel molecular chaperone that can protect plants from diverse external stresses. In addition, because AtUSP protein structure was reversibly regulated by its redox status, we investigated whether its chaperone function was redox dependent. Chaperone activity was measured using AtUSP treated with DTT or H<sub>2</sub>O<sub>2</sub>. H<sub>2</sub>O<sub>2</sub>-treated AtUSP showed much stronger chaperone activity in protecting MDH from aggregation than protein treated with DTT, suggesting that AtUSP is an efficient molecular



**FIGURE 4 | Redox-dependent structural changes of AtUSP *in vitro*.** Redox-dependent structural changes of recombinant AtUSP were analyzed by silver staining 12% non-reducing SDS-PAGE (**A**) and reducing SDS-PAGE (**B**) gels. (**A,B**) Purified AtUSP (lane 1) and AtUSP treated with 50 mM DTT (lane 2) were loaded onto the PAGE gels. After removal of DTT by dialysis from the sample used in lane 2, AtUSP was treated with 50 mM H<sub>2</sub>O<sub>2</sub> (lane 3) and loaded onto the SDS-PAGE gels. (**C**) Redox-dependent structural changes of AtUSP treated the same way as in (**A**) were analyzed using SEC. (**D**) Comparison of the binding affinity of 10 μM bis-ANS to AtUSP (10 μM). Fluorescence intensities of bis-ANS were measured using an excitation wavelength of 390 nm and emission wavelengths from 400 to 600 nm. (◆) bis-ANS alone, (■) untreated AtUSP, (▲) AtUSP treated with DTT, and (●) AtUSP treated with H<sub>2</sub>O<sub>2</sub>.

chaperone whose activity is regulated in a redox-dependent manner (Figure 6B).

## Effect of Heat Shock on the Protein Structure and Chaperone Function of AtUSP

Since AtUSP exhibited a molecular chaperone function, we analyzed the effect of heat shock on its protein structure using native-PAGE and SEC. Like many other heat shock proteins (HSPs) (Haley et al., 1998), AtUSP associated to form HMW homo-polymeric complexes under heat shock conditions. Structural changes to AtUSP commenced at around 40°C, and almost all proteins associated into HMW oligomeric complexes following a heat shock treatment at 50°C for 20 min (Figure 7A). This suggested that heat shock caused LMW protein complexes to assemble into HMW complexes. The heat shock-mediated

structural changes of AtUSP were confirmed using SEC. As the temperature increased, so did the oligomer peaks of AtUSP, and these changes occurred simultaneously with a decrease in the levels of LMW proteins (Figure 7B). As a measurement of hydrophobicity changes, binding of bis-ANS was analyzed in heat shock-treated AtUSP. The fluorescence intensity of bis-ANS significantly increased at higher temperatures, which indicated that a greater number of hydrophobic regions of AtUSP were exposed at elevated temperatures (Figure 7C). In addition, we measured heat shock-dependent chaperone activity of AtUSP, using MDH as a substrate. The chaperone activity of AtUSP was significantly enhanced by increasing the incubation temperature of AtUSP (Figure 7D). All these results were consistent with the idea that AtUSP plays a critical role in protecting plants from heat shock and oxidative stresses, and its function as a molecular chaperone is accompanied by reversible changes to its protein structure.

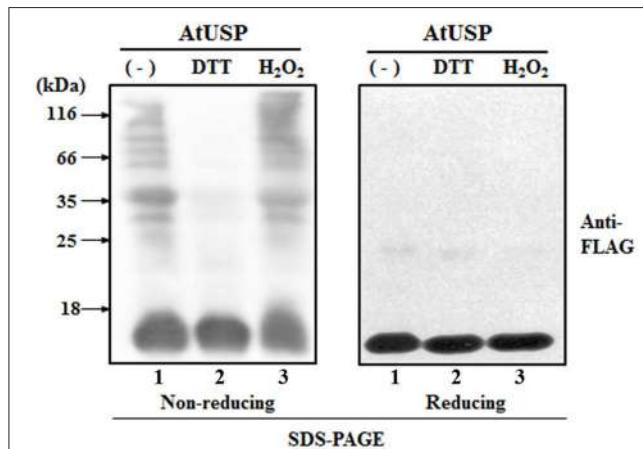
## DISCUSSION

Although the physiological significance of USPs has been well-studied in many organisms, especially in *E. coli*, and expression of USPs is known to respond to various environmental stresses, including salt, drought, cold, heat, oxidative stress, nutrient starvation, and toxic chemicals (Guan and Nothnagel, 2004; Ndimba et al., 2005), the molecular mechanism of USP action has not been identified. USPs are either small polypeptides (14–15 kDa) containing a single USP domain or larger proteins

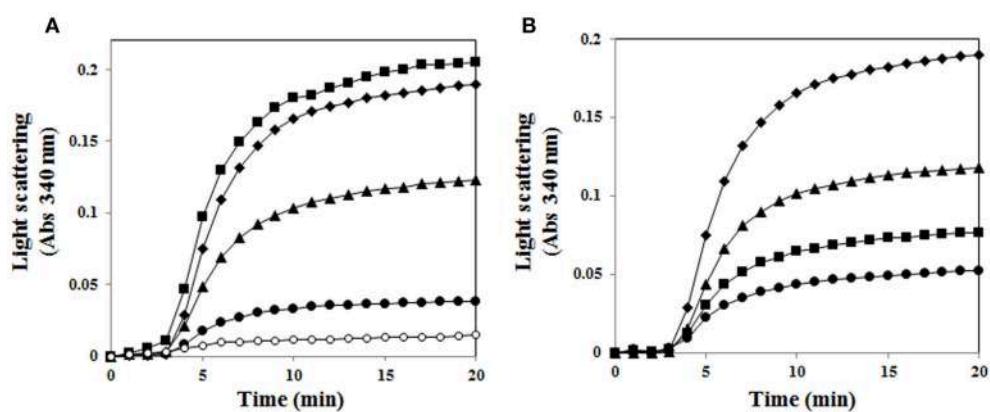
consisting of one or two USP domains together with another functional domain, such as a kinase, permease, or voltage channel domain (Isokpehi et al., 2011). The *Arabidopsis* genome contains 44 USPs that show similarity to the bacterial USP domain and are induced by a variety of stresses (Isokpehi et al., 2011; Loukehaich et al., 2012). AtUSP also shows similarity to genes responsive to ethylene, a plant hormone involved in fruit ripening (Kerk et al., 2003). These observations prompted us to investigate the biochemical functions of USPs in protecting plants from external stresses.

A comparison of the amino acid sequence of AtUSP with USP sequences from other organisms showed a high sequence homology and secondary structural similarity. The USP from *M. jannaschii*, designated as MJ0577, contains five beta strands alternating with four alpha helices (Zarembinski et al., 1998) and a conserved ATP-binding motif, G-2X-G-9X-G-(S/T) (Figure S3), in which three glycine residues are separated by two and nine amino acid residues, followed by a serine or threonine residue. This motif is a typical feature of the USP family (Pfam accession number PF00582) (Kim et al., 2012). Since oxidative stress plays a critical role in all aerobic organisms, the oxidative-associated function of AtUSP may provide much information for understanding the physiological significance of this protein in plant cells (Mayer, 2012).

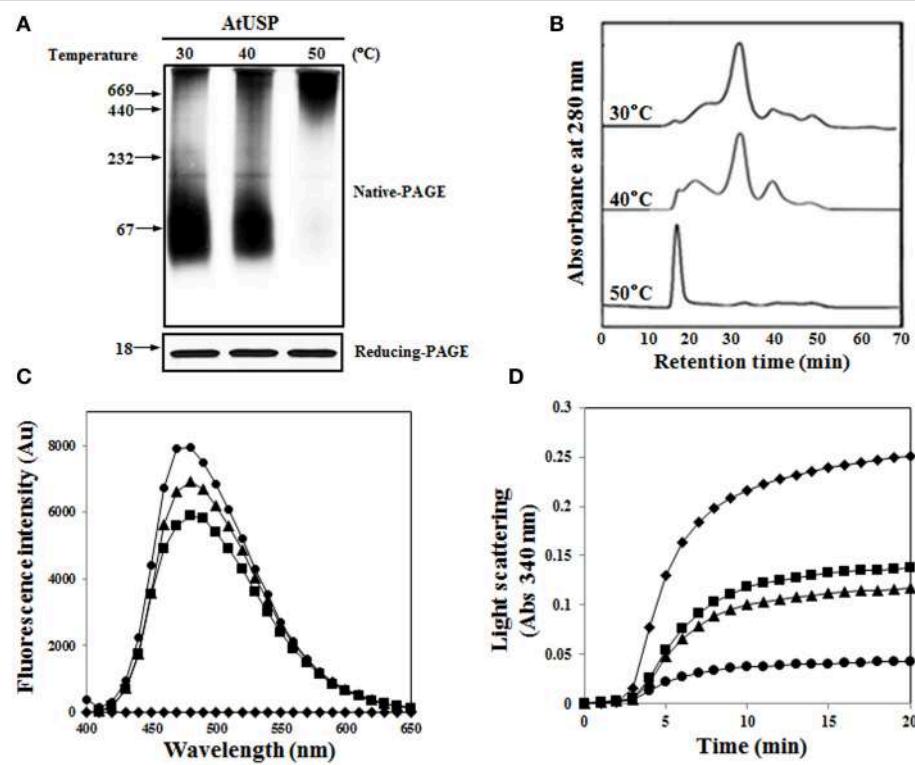
We identified a novel function for AtUSP, showing that it acted as a molecular chaperone under heat shock and oxidative stress conditions, and that this action was accompanied by a switch in protein structure. Both the chaperone function and the changes in protein structure were regulated in a redox-dependent manner. Under normal conditions, AtUSP protein was present in multimeric complexes but treatment with DTT caused these to dissociate completely into monomers; the polymeric structures could be reversibly restored by  $\text{H}_2\text{O}_2$  treatment. Similarly, the protein conformation of NPR1, a pathogen-responsive defense regulator in plants, also changes from an oligomeric structure to a monomer in the presence of a redox protein (Trx) and



**FIGURE 5 | Redox-dependent structural changes of AtUSP *in vivo*.** Samples from 2-week-old transgenic plants over-expressing AtUSP fused to FLAG were prepared and treated with 10 mM  $\text{H}_2\text{O}_2$  and DTT. The proteins were loaded onto non-reducing (left-hand) and reducing (right-hand) SDS-PAGE gels. Protein structures were determined by western blotting using FLAG-tag antibody. Lane 1: Total protein (30  $\mu\text{g}$ ) obtained from an AtUSP over-expression line. Lane 2: Protein sample from an AtUSP over-expression line treated with 10 mM DTT. Lane 3: Protein sample from an AtUSP over-expression line as in lane 2 after removal of DTT by dialysis and treatment with 10 mM  $\text{H}_2\text{O}_2$ .



**FIGURE 6 | Redox-dependent chaperone function of AtUSP.** **(A)** Thermal aggregation of 1.5  $\mu\text{M}$  MDH was examined at 45°C for 20 min in the presence of AtUSP. Molar ratios of AtUSP to MDH were (■) 1:0.5, (▲) 1:1, and (●) 1:3. (◆) Negative control (MDH alone), (○) Positive control (AtTrx-h3). **(B)** Redox-dependent chaperone activity of AtUSP with MDH. (◆) MDH alone, (■) AtUSP, (▲) DTT-treated AtUSP, and (●)  $\text{H}_2\text{O}_2$ -treated AtUSP were used to measure the level of protection against thermal aggregation of MDH.



**FIGURE 7 | Effect of heat shock on structural changes of AtUSP. (A)** Recombinant AtUSP was treated with heat shocks at various temperatures for 20 min and the resulting proteins were separated using native-PAGE (upper) and SDS-PAGE gels (bottom). **(B)** AtUSP treated with heat shock was analyzed using SEC to determine structural changes. **(C)** Heat shock-mediated hydrophobicity changes of AtUSP were measured by bis-ANS binding. AtUSP was incubated at (■) 30°C, (▲) 40°C, or (●) 50°C for 30 min. (♦) Control (bis-ANS alone). **(D)** Heat shock-mediated enhancement of the chaperone function of AtUSP. Thermal aggregation of MDH was examined at 45°C for 20 min in the presence of heat shock-treated AtUSP proteins at (■) 30°C, (▲) 40°C, or (●) 50°C for 20 min. (♦) Control (MDH).

pathogen-induced production of salicylic acid (Tada et al., 2008). These structural changes also induced translocation of NPR1 from the cytosol to the nucleus, thus activating defense-related downstream genes. 2-Cys Prx, which also plays dual roles, acting both as a signaling molecule and as a molecular chaperone, likewise shows changes to its protein structure and function according to oxidative stress and redox status. 2-cys Prx can protect many important intracellular proteins under conditions of oxidative stress (Chuang et al., 2006).

The cysteine residues in the AtTrx-h3, 2-Cys Prx, and NPR1 proteins play an important role in regulation by redox status (Jang et al., 2004; Tada et al., 2008; Park et al., 2009). As USP is one of the targets of Trx in plants (Montrichard et al., 2009), the two cysteine residues of AtUSP may also play a critical role in redox regulation, and this should be investigated in the future. We clearly demonstrated the chaperone function of AtUSP using bacterially expressed recombinant protein with MDH as a substrate. The chaperone function of AtUSP was significantly increased under stress conditions by inducing the formation of HMW complexes. This regulation resembles that of AtTrx-h3 and AtNTRC (Park et al., 2009; Chae et al., 2013). We propose that the molecular switch in AtUSP structure results from its ATP-binding motif forming hydrogen bonds with polar groups of amino acids responsible for the oligomerization

and higher hydrophobic interaction of AtUSP (Isokpehi et al., 2011).

The significance of chaperone function of AtUSP in plants was verified in transgenic plants over-expressing AtUSP. These plants showed significantly increased tolerance to heat shock and oxidative stress, whereas the knock-out mutant, *Atusp*, showed a sensitive-to-stress phenotype with lower chlorophyll content and higher electrolytic leakage compared to the wild type plants under heat shock and oxidative stress. Under normal conditions, however, there were some differences in growth between *Atusp*, WT plants, and AtUSP over-expression lines, suggesting AtUSP may play another role in plant growth and development. In tomato plants, the *SpUSP* transcript is regulated by a number of phytohormones, such as abscisic acid, gibberellin, or ethylene (Loukehaich et al., 2012), and the expression of rice *USP* is also regulated by ethylene; it is probable, therefore, that *AtUSP* expression is also regulated by plant hormones. This study demonstrates novel physiological and molecular functions of AtUSP, showing that it acts to protect plants from heat shock and oxidative stress. Our observation that AtUSP has a redox-dependent chaperone function expands its known roles in plants and thus increases our understanding of the molecular mechanisms underlying its diverse defensive roles.

## AUTHOR CONTRIBUTIONS

YC, DY, and SL designed the experiments and wrote the paper. YJ, SM, EL, JP, CA, HO, and YC performed the experiments and analyzed the data.

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# ROS Regulation During Abiotic Stress Responses in Crop Plants

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Abiotic stresses such as drought, cold, salt and heat cause reduction of plant growth and loss of crop yield worldwide. Reactive oxygen species (ROS) including hydrogen peroxide ( $H_2O_2$ ), superoxide anions ( $O_2^{\bullet-}$ ), hydroxyl radical ( $OH\bullet$ ) and singlet oxygen ( $^1O_2$ ) are by-products of physiological metabolisms, and are precisely controlled by enzymatic and non-enzymatic antioxidant defense systems. ROS are significantly accumulated under abiotic stress conditions, which cause oxidative damage and eventually resulting in cell death. Recently, ROS have been also recognized as key players in the complex signaling network of plants stress responses. The involvement of ROS in signal transduction implies that there must be coordinated function of regulation networks to maintain ROS at non-toxic levels in a delicate balancing act between ROS production, involving ROS generating enzymes and the unavoidable production of ROS during basic cellular metabolism, and ROS-scavenging pathways. Increasing evidence showed that ROS play crucial roles in abiotic stress responses of crop plants for the activation of stress-response and defense pathways. More importantly, manipulating ROS levels provides an opportunity to enhance stress tolerances of crop plants under a variety of unfavorable environmental conditions. This review presents an overview of current knowledge about homeostasis regulation of ROS in crop plants. In particular, we summarize the essential proteins that are involved in abiotic stress tolerance of crop plants through ROS regulation. Finally, the challenges toward the improvement of abiotic stress tolerance through ROS regulation in crops are discussed.

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## INTRODUCTION

Abiotic stress conditions such as drought, heat, or salinity affect plant growth and reduce agricultural production worldwide. These reductions result from climate change and the freshwater-supply shortage as well as the simultaneous occurrence of different abiotic stresses (Mittler and Blumwald, 2010; Hu and Xiong, 2014). To meet the demands of food security in the face of an increasing world population and environmental challenge, scientists envisage a crucial need for a “second green revolution” to enhance crop

**Abbreviations:** ABA, abscisic acid; AOX, alternative oxidases; APX, ascorbate peroxidase; AsA, ascorbic acid; ASR, ABA-, stress-, and ripening-induced; BR, brassinosteroid; CCaMK, calcium/calmodulin-dependent protein kinase; CDPK, calcium-dependent protein kinase; CIPK, calcineurin B-like protein-interacting protein kinase; DHAR, dehydroascorbate reductase; GPX, glutathione peroxidase; GR, glutathione reductase; GRX, glutaredoxin; GSH, reduced glutathione; GST, glutathione S-transferase; MAPK, mitogen-activated protein kinase; MAPKKK, MAPK kinase kinase; MDHAR, monodehydroascorbate reductase; MT, metallothionein; PAs, polyamines; POD, peroxidase; PRX, peroxiredoxin; RBOH, respiratory burst oxidase homolog; RCD, radical-induced cell death; ROS, reactive oxygen species; SOD, superoxide dismutase; SRO, similar to RCD one; TRX, thioredoxin.

yield and yield stability under non-optimal and adverse growing conditions by a combination of approaches based on the recent advances in genomic research (Zhang, 2007; Eckardt et al., 2009).

To cope with adverse conditions, plants have evolved a range of physiological and metabolic responses by activation of a great many of stress-responsive genes and synthesis of diverse functional proteins through a complex signal transduction network, so as to confer tolerance to the environmental stresses (Hirayama and Shinozaki, 2010). Reactive oxygen species (ROS), including hydrogen peroxide ( $H_2O_2$ ), superoxide radical ( $O_2^{\bullet-}$ ), hydroxyl radical ( $OH\bullet$ ) and singlet oxygen ( $^1O_2$ ) etc., resulting from excitation or incomplete reduction of molecular oxygen, are harmful by-products of basic cellular metabolism in aerobic organisms (Apel and Hirt, 2004; Miller et al., 2010). Besides the toxicity of ROS, ROS are also considered to be signaling molecules that regulate plant development, biotic and abiotic stress responses (Apel and Hirt, 2004; Mittler et al., 2004). Many excellent reviews have focused on ROS metabolism (Apel and Hirt, 2004; Noctor et al., 2014), ROS sensory and signaling networks (Miller et al., 2010; Suzuki et al., 2012; Baxter et al., 2014), as well as the cross-talk with other signaling molecules function in developmental and stress response processes (Suzuki et al., 2012; Noctor et al., 2014). However, most of these reviews provided an overall retrospective for model plant *Arabidopsis*. Gill and Tuteja (2010) reviewed enzymatic and non-enzymatic antioxidants and their roles in abiotic stress tolerance of crop plants. However, the regulation mechanism of the antioxidant system and the key components involved in ROS regulation and abiotic stress tolerance have not yet been summarized in crop plants. In this review, we provide an overview of current knowledge about ROS homeostasis regulation in crop plants. In particular, the genes that have been characterized in ROS homeostasis regulation affecting abiotic stress resistance in crop plants were summarized.

## ROS HOMEOSTASIS IN PLANT

The evolution of aerobic metabolic processes such as respiration and photosynthesis unavoidably led to the production of ROS in mitochondria, chloroplast, and peroxisome (Apel and Hirt, 2004; Gill and Tuteja, 2010). Under optimal growth conditions, intracellular ROS are mainly produced at a low level in organelles. However, ROS are dramatically acclimated during stress. Under abiotic stress condition, limitation of  $CO_2$  uptake, caused by stress-induced stomatal closure, favors photorespiratory production of  $H_2O_2$  in the peroxisome and production of superoxide and  $H_2O_2$  or singlet oxygen by the overreduced photosynthetic electron transport chain (Apel and Hirt, 2004; Noctor et al., 2014). In addition to organelles, plasma membrane together with apoplast is the main site for ROS generation in response to endogenous signals and exogenous environmental stimuli. Several types of enzymes, such as NADPH oxidases, amine oxidases, polyamine oxidases, oxalate oxidases, and a large family of class III peroxidases, that localized at the cell surface or apoplast are contributed to production of apoplast ROS (Apel and Hirt, 2004; Cosio and Dunand, 2009; Gill and Tuteja, 2010).

Overproduction of ROS caused by stress conditions in plant cells is highly reactive and toxic to proteins, lipids, and nucleic acid which ultimately results in cellular damage and death (Gill and Tuteja, 2010). On the other hand, the increased production of ROS during stresses also thought to act as signals for the activation of stress response pathways (Baxter et al., 2014). Plants have evolved an efficient enzymatic and non-enzymatic antioxidative system to protect themselves against oxidative damage and fine modulation of low levels of ROS for signal transduction.

ROS-scavenging enzymes of plants include superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR), glutathione S-transferase (GST), and peroxiredoxin (PRX). These antioxidant enzymes are located in different sites of plant cells and work together to detoxify ROS. SOD acts as the first line of defense converting  $O_2^{\bullet-}$  into  $H_2O_2$ . CAT, APX, and GPX then detoxify  $H_2O_2$ . In contrast to CAT, APX requires an ascorbic acid (AsA) and/or a glutathione (GSH) regenerating cycle involved MDHAR, DHAR, and GR. GPX, GST, and PRX reduce  $H_2O_2$  and organic hydroperoxides through ascorbate-independent thiol-mediated pathways using GSH, thioredoxin (TRX) or glutaredoxin (GRX) as nucleophile (Dietz et al., 2006; Meyer et al., 2012; Noctor et al., 2014). Non-enzymatic antioxidants include GSH, AsA, carotenoids, tocopherols, and flavonoids are also crucial for ROS homeostasis in plant (Gill and Tuteja, 2010). Besides traditional enzymatic and non-enzymatic antioxidants, increasing evidences indicated that soluble sugars, including disaccharides, raffinose family oligosaccharides and fructans, have a dual role with respect to ROS (Couee et al., 2006; Keunen et al., 2013). Soluble sugars were directly linked with the production rates of ROS by regulation ROS producing metabolic pathways, such as mitochondrial respiration or photosynthesis. Conversely, they also feed NADPH-producing metabolism to participate in antioxidative processes (Couee et al., 2006).

In addition to the antioxidative system, avoiding ROS production by alleviating the effects of stresses on plant metabolism may also be important for keeping ROS homeostasis. Alternative oxidases (AOX) can prevent the excess generation of ROS in the electron transport chains of mitochondria (Maxwell et al., 1999). By diverting electrons flowing through electron-transport chains, AOX can decrease the possibility of electron leaking to  $O_2$  to generate  $O_2^{\bullet-}$ . Other mechanisms, such as leaf movement and curling, photosynthetic apparatus rearranging, may also represent an attempt to avoid the over-reduction of ROS by balancing the amount of energy absorbed by the plant with the availability of  $CO_2$  (Mittler, 2002).

## REGULATION OF NADPH OXIDASES IN CROP PLANTS

Plant NADPH oxidases, also known as respiratory burst oxidase homologs (RBOHs), are the most studied enzymatic source

of ROS. Plant RBOHs have cytosolic FAD- and NADPH-binding domains in the C-terminal region, and transmembrane domains that correspond to those in mammalian NADPH oxidases (Suzuki et al., 2011). In addition, plant RBOHs have a cytosolic N-terminal extension contains regulatory regions such as calcium-binding EF-hands and phosphorylation target sites that are important for the function and regulation of the plant NADPH oxidases (Oda et al., 2010; Suzuki et al., 2011). Increasing evidence demonstrated NADPH oxidases as key signaling nodes in the ROS regulation network of plants integrating numerous signal transduction pathways with ROS signaling and mediating multiple important biological processes, including cell growth and plant development, abiotic stress response and adaptation, plant-microbe pathogenic and symbiotic interactions (Torres and Dangl, 2005; Suzuki et al., 2011; Marino et al., 2012). Numerous studies have uncovered several regulatory mechanisms of plant NADPH oxidases in *Arabidopsis*, which involved various signaling components including protein phosphorylation,  $\text{Ca}^{2+}$ , CDPKs, and phospholipase D $\alpha$ 1 (PLD $\alpha$ 1) (Baxter et al., 2014).  $\text{Ca}^{2+}$  regulates NADPH oxidase-dependent ROS production by binding directly to the EF-hand motif in the N terminus of RBOH protein and/or regulating  $\text{Ca}^{2+}$ -dependent phosphorylation mediated by CDPK (Ogasawara et al., 2008; Dubiella et al., 2013). RBOHs were also found to be phosphorylated by SnRK2 protein kinase OPEN STOMATA 1 (OST1) during ABA-dependent stomatal closure (Sirichandra et al., 2009).

Functions and regulatory mechanisms of several RBOH proteins were investigated in crops. The activity of NADPH oxidase was increased by drought, and exhibited high-temperature stability and an alkaline-philic feature, suggesting its important role in response to drought stress (Duan et al., 2009). Treatment with ABA and  $\text{Ca}^{2+}$  also considerably induced the activity of NADPH oxidase in leaves of maize seedlings (Jiang and Zhang, 2002a, 2003). Nine NADPH oxidase (RBOH) genes (*OsRBOHA*-*OsRBOHI*) were identified in the rice genome (Wong et al., 2007). Rice RBOH genes exhibited unique patterns of expression changes in response to various environmental stresses (Wang et al., 2013). A small GTPase Rac in rice (*OsRac1*) was identified as a positive regulator of OsRBOHB involved in pathogen defense (Wong et al., 2007). A direct interaction between *OsRac1* and the N-terminal extension of OsRBOHB may be required for NADPH oxidase activity modulated by the cytosolic  $\text{Ca}^{2+}$  concentration in plants (Wong et al., 2007). Further mutation analyses of the regulatory domains of OsRBOHB indicated that not only the EF-hand motif but also the upstream N-terminal region was essential to  $\text{Ca}^{2+}$ -dependent but not phosphorylation-dependent activation (Takahashi et al., 2012). In addition, Liu et al. (2012) found that phosphatidylinositol 3-kinase (PI3K) regulated NADPH oxidase activity by modulating the recruitment of Rac1 to plasma membrane. Rice histidine kinase OsHK3 showed to regulate the expression of NADPH oxidase genes and the production of  $\text{H}_2\text{O}_2$  in ABA signaling (Wen et al., 2015). In potato, two CDPKs, StCDPK4 and StCDPK5, were found to induce the phosphorylation of StRBOHB and regulated the oxidative burst

during pathogen defense (Kobayashi et al., 2007). In tobacco, NbRBOHA and NbRBOHB are in charge of the generation of ROS during the defense response (Yoshioka et al., 2003). Further study indicated that mitogen-activated protein kinase (MAPK) cascades MEK2-SIPK/NTF4 and MEK1-NTF6 were involved in the NbRBOHB-dependent oxidative burst in response to pathogen signals (Asai et al., 2008). Two tomato RBOH genes, *SIRBOHB* (*SlWf1*) and *SIRBOHG* (*SlRBOH1*), have turned out to participate in wounding response and development (Sagi et al., 2004). Other studies revealed that SIRBOHG (*SlRBOH1*) is vital for brassinosteroid (BR)-induced  $\text{H}_2\text{O}_2$  production, ABA accumulation, stomatal closure/opening and oxidative stress tolerance (Xia et al., 2014; Zhou et al., 2014a), while SlRBOHB was found to positively regulate the defense response against *B. cinerea*, the flg22-induced immune response and drought stress response (Li et al., 2015). Lin et al. (2009) observed that the activity of NADPH oxidase is regulated by  $\text{H}_2\text{O}_2$  and ZmMPK5 in maize. Zhu et al. (2013b) identified a BR induced microtubule-associated protein, ZmMAP65-1a, interacts with a MAPK and functions in  $\text{H}_2\text{O}_2$  self-propagation by regulating the expression of NADPH oxidase genes in BR signaling in maize.

## REGULATION OF ANTIOXIDATIVE SYSTEM IN CROP PLANTS

Plant antioxidative system consists of numerous enzymatic and non-enzymatic antioxidative components that work together with ROS-generating pathway to maintain ROS homeostasis. Several studies showed important roles of antioxidative components in ROS homeostasis in crop plants. The rice (*japonica*) genome has eight genes that encode putative SODs, including two cytosolic copper-zinc SODs (*cCuZn-SOD1* and *cCuZn-SOD2*), one putative CuZn-SOD-like (*CuZn-SOD-L*), one plastidic SOD (*pCuZn-SOD*), two iron SODs (*Fe-SOD2* and *Fe-SOD3*), and one manganese SOD (*Mn-SOD1*) (Nath et al., 2014). Transgenic rice plants overexpressing *Mn-SOD1* showed less mitochondrial  $\text{O}_2^{\bullet-}$  under stress and reduced the stress induction of *OsAOX1a/b* specifically (Li et al., 2013). There are eight APX genes in rice, including two cytosolic APXs (*OsAPX1* and *OsAPX2*), two peroxisomal APXs (*OsAPX3* and *OsAPX4*), two mitochondrial APXs (*OsAPX5* and *OsAPX6*) and two chloroplastic APXs (*OsAPX7* and *OsAPX8*) (Teixeira et al., 2004, 2006). Two cytosolic APXs, *OsAPX1* and *OsAPX2*, have crucial roles in abiotic stress resistance in rice (Sato et al., 2011; Zhang et al., 2013). Interestingly, rice mutants double silenced for cytosolic APXs (*APX1/2s*) exhibit significant changes in the redox status indicated by higher  $\text{H}_2\text{O}_2$  levels and increased glutathione and ascorbate redox states, triggering alterations in the ROS signaling networks and making the mutants able to cope with abiotic stress similar to non-transformed plants (Bonifacio et al., 2011). Some of the ROS-scavenging enzymes, such as GST (Dixon and Edwards, 2010), TRX, and GRX (Meyer et al., 2012), have evolved into large multigene families with varied functions that cope with a variety of adverse environmental conditions. Recent mutational and transgenetic plants analyses revealed

special member of multigene enzyme family as a key player in ROS homeostasis regulation in crop plants. *OsTRXh1*, encodes h-type TRX in rice, regulates the redox state of the apoplast and participates in plant development and stress responses (Zhang et al., 2011). *OsTRXh1* protein possesses reduction activity and secreted into the extracellular space. Overexpression of *OsTRXh1* produce less H<sub>2</sub>O<sub>2</sub> under salt stress, reduce the expression of the salt-responsive genes, lead to a salt-sensitive phenotype in rice. In another study, Perez-Ruiz et al. (2006) reported that rice NADPH thioredoxin reductase (NTRC) utilizes NADPH to reduce the chloroplast 2-Cys PRX BAS1, thus protects chloroplast against oxidative damage by reducing H<sub>2</sub>O<sub>2</sub>.

The involvement of ROS in signal transduction implies that there must be coordinated function of regulation networks to maintain ROS at non-toxic levels in a delicate balancing act between ROS production and ROS-scavenging pathways, and to regulate ROS responses and subsequent downstream processes (Mittler et al., 2004). Numerous studies from different plant species observed that the generation of ROS and activity of various antioxidant enzymes increased during abiotic stresses (Damanik et al., 2010; Selote and Khanna-Chopra, 2010; Tang et al., 2010; Turan and Ekmekci, 2011). There is an increasing body of literature concerning the mechanisms by which regulation of antioxidative system response to abiotic stresses in crops. Intrinsic to this regulation is ROS production and signaling that integrated with the action of hormone and small molecules.

The plant hormone ABA is the key regulator of abiotic stress resistance in plants, and regulates large number of stress-responsive genes by a complex regulatory network so as to confer tolerance to the environmental stresses (Cutler et al., 2010; Raghavendra et al., 2010). ABA-induced stress tolerance is partly linked with the activation of antioxidant defense systems, including enzymatic and non-enzymatic constituents, which protects plant cells against oxidative damage (Huang et al., 2012; Zhang et al., 2012a, 2014). Water stress-induced ABA accumulation and exogenous ABA treatment triggers the increased generation of ROS, then leads to the activation of the antioxidant system in crops (Jiang and Zhang, 2002a,b; Ye et al., 2011). Small molecules, such as Ca<sup>2+</sup>, calmodulin (CaM), NO and ROS have been demonstrated to play vital roles in ABA-induced antioxidant defense (Jiang and Zhang, 2003; Hu et al., 2007). In rice, a Ca<sup>2+</sup>/CaM-dependent protein kinase (CCaMK), OsDMI3, is necessary for ABA-induced increases in the expression and the activities of SOD and CAT. ABA-induced H<sub>2</sub>O<sub>2</sub> production activates OsDMI3, and the activation of OsDMI3 also enhances H<sub>2</sub>O<sub>2</sub> production by increasing the expression of NADPH oxidase genes (Shi et al., 2012). Further study indicated that OsDMI3 functions upstream of OsMPK1, to regulate the activities of antioxidant enzymes and the production of H<sub>2</sub>O<sub>2</sub> in rice (Shi et al., 2014). Recent study provides evidence to show that rice histidine kinase OsHK3 functions upstream of OsDMI3 and OsMPK1, and is necessary for ABA-induced antioxidant defense (Wen et al., 2015). Zhang et al. (2012a) reported that C2H2-type ZFP, ZFP182, is involved in ABA-induced antioxidant defense. Another C2H2-type ZFP, ZFP36,

is also necessary for ABA-induced antioxidant defense (Zhang et al., 2014). Moreover, ABA-induced H<sub>2</sub>O<sub>2</sub> production and ABA-induced activation of OsMPKs promote the expression of ZFP36, and ZFP36 also up-regulates the expression of NADPH oxidase and MAPK genes and the production of H<sub>2</sub>O<sub>2</sub> in ABA signaling (Zhang et al., 2014). In maize, ABA and H<sub>2</sub>O<sub>2</sub> increased the expression and the activity of ZmMPK5, which is required for ABA-induced antioxidant defense. The activation of ZmMPK5 also enhances the H<sub>2</sub>O<sub>2</sub> production by increasing the expression and the activity of NADPH oxidase, thus there is a positive feedback loop involving NADPH oxidase, H<sub>2</sub>O<sub>2</sub>, and ZmMPK5 in ABA signaling (Zhang et al., 2006; Hu et al., 2007; Ding et al., 2009; Lin et al., 2009). Subsequent experiments confirmed that ABA-induced H<sub>2</sub>O<sub>2</sub> production mediates NO generation in maize leaves, which, in turn, activates MAPK and increases the expression and the activities of antioxidant enzymes in ABA signaling (Zhang et al., 2007). Moreover, a maize CDPK gene, *ZmCPK11*, acts upstream of ZmMPK5, is essential for ABA-induced up-regulation of the expression and activities of SOD and APX, and the production of H<sub>2</sub>O<sub>2</sub> in maize leaves (Ding et al., 2013). Hu et al. (2007) found that Ca<sup>2+</sup>-CaM is required for ABA-induced antioxidant defense and functions both upstream and downstream of H<sub>2</sub>O<sub>2</sub> production in leaves of maize plants. Afterward, Ca<sup>2+</sup>/CaM-dependent protein kinase, ZmCCaMK, was reported to be essential for ABA-induced antioxidant defense, and H<sub>2</sub>O<sub>2</sub>-induced NO production is involved in the activation of ZmCCaMK in ABA signaling (Ma et al., 2012).

Brassinosteroids are a group of steroid hormones and important for a broad spectrum of plant growth and development processes, as well as responses to biotic and abiotic stresses (Bajguz and Hayat, 2009; Divi and Krishna, 2009; Yang et al., 2011; Zhu et al., 2013a). Numerous studies have shown that BR can activate antioxidant defense systems to improve stress tolerance in crops (Özdemir et al., 2004; Xia et al., 2009). Zhang et al. (2010) reported that ZmMPK5 is required for NADPH oxidase-dependent self-propagation of ROS in BR-induced antioxidant defense systems in maize. Further study founded that a 65 kDa microtubule-associated protein (MAP65), ZmMAP65-1a, directly phosphorylated by ZmMPK5, is required for BR-induced antioxidant defense (Zhu et al., 2013b). Recently, Ca<sup>2+</sup> and maize CCaMK gene, *ZmCCaMK*, was demonstrated to be required for BR-induced antioxidant defense (Yan et al., 2015).

## GENES INVOLVED IN ROS REGULATION AND ABIOTIC STRESS TOLERANCE IN CROPS

To cope with abiotic stress, plants have evolved multiple and interconnected signaling pathways to regulate different sets of stress-responsive genes for producing various classes of proteins, such as protein kinases, transcriptional factors, enzymes, molecular chaperones, and other functional proteins, resulting in diverse physiological and metabolic response so as to confer tolerance to the environmental stresses. Hundreds or even

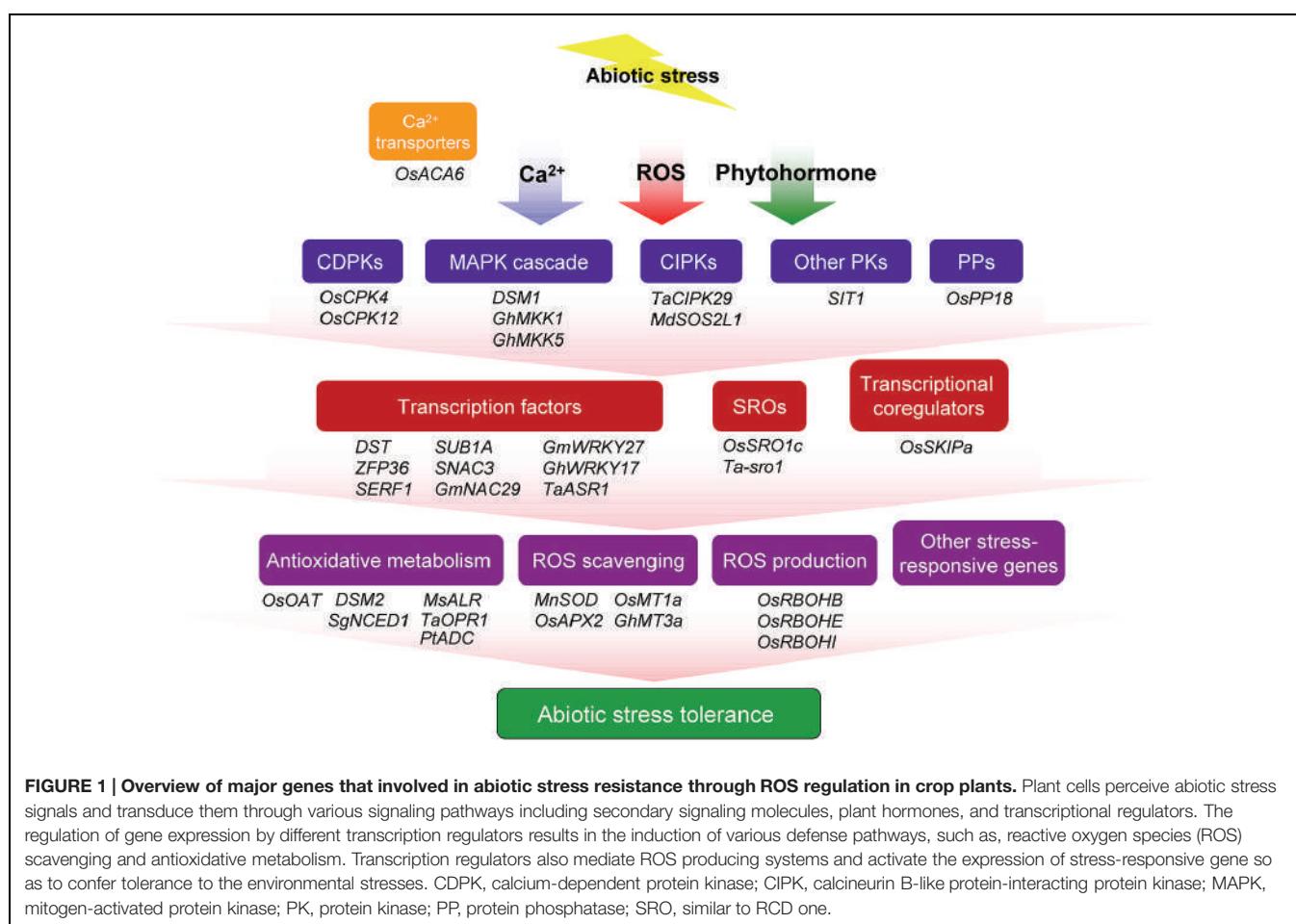
1000s of genes that regulate stress responses have been identified in crop plants by diverse functional genomics approaches (Hu and Xiong, 2014). In parallel to this, the functions of numerous stress-responsive genes involved in ROS homeostasis regulation and abiotic stress resistance have been characterized in transgenic plants (Figure 1; Table 1).

## Protein Kinases and Phosphatases

Mitogen-activated protein kinase cascades are involved in diverse processes from plant growth and development to stress responses. MAPK cascades also play crucial roles in ROS signaling, and several studies in *Arabidopsis* have shown that ROS are not only the trigger, but also the consequence of activation of MAPK signaling (Kovtun et al., 2000; Pitzschke and Hirt, 2006; Pitzschke et al., 2009). However, few MAPK cascades components have been functionally characterized in crops. Two MAPK kinases (MAPKKs), GhMKK1 and GhMKK5 have been characterized to be involved in stress resistance and ROS homeostasis in cotton (Zhang et al., 2012b; Lu et al., 2013). Overexpression of *GhMKK1* in tobacco improved its tolerance to salt and drought stresses, exhibited an enhanced ROS scavenging capability and significantly elevated activities of antioxidant enzymes (Lu et al., 2013). Whereas, overexpression of another cotton MAPKK gene, *GhMKK5*, in tobacco reduced their

tolerance to salt and drought stresses. *GhMKK5*-overexpressing plants showed significantly up-regulated expression of ROS-related and cell death marker genes, and resulted in excessive accumulation of  $H_2O_2$  and hypersensitive response (HR)-like cell death (Zhang et al., 2012b). In another study, a drought-hypersensitive mutant (drought-hypersensitive mutant1 [*dsm1*]) of a putative MAPK kinase gene has been identified in rice (Ning et al., 2010). The *dsm1* mutant was sensitive to oxidative stress with down-regulated expression of two peroxidase (POD) genes and reduced POD activity.

Calcium-dependent protein kinase proteins regulate the downstream components in calcium signaling pathways. A rice CDPK gene, *OsCPK12*, enhances tolerance to salt stress by reducing the accumulation of ROS (Asano et al., 2012). Expression of genes encoding ROS-scavenging enzymes (*OsAPx2* and *OsAPx8*) were up-regulated, whereas the NADPH oxidase gene (*OsRBOH1*) was down-regulated in *OsCPK12*-overexpressing plants compared with wild type plants. Conversely, the *oscpk12* mutant and RNAi plants were more sensitive to high salinity and accumulated more  $H_2O_2$  than wild type plants (Asano et al., 2012). Overexpression of another CDPK gene, *OsCPK4*, results in increased tolerance to salt and drought stresses in rice plants. Transgenic plants exhibited higher expression of numerous genes involved in lipid metabolism and



**TABLE 1 | Representative genes that involved in abiotic stress resistance in major crops through ROS regulation.**

Functional category	Genes	Protein function	Origin	Transformation receptor	ROS regulation	Abiotic stress resistance	Reference
<b>Protein kinase</b>							
MAPKs	GhMKK1 DSM1 OsCPK4	MAPKK MAPKK calcium-dependent protein kinase	<i>G. hirsutum</i> <i>O. sativa</i> <i>O. sativa</i>	<i>N. benthamiana</i> <i>O. sativa</i> <i>O. sativa</i>	ROS scavenging ROS scavenging ROS scavenging	Drought and salt stress Drought stress Drought and salt stress	Lu et al., 2013 Ning et al., 2010 Campo et al., 2014
CDPK	OsCPK12	calcium-dependent protein kinase	<i>O. sativa</i>	<i>N. benthamiana</i>	ROS production and scavenging ROS scavenging	Salt stress salt stress	Asano et al., 2012
CIPK	TaCIPK29	CBL-interacting protein kinase	<i>T. aestivum</i>	<i>N. benthamiana</i>	ROS scavenging	salt stress	Deng et al., 2013
	MdSOS2L1	CBL-interacting protein kinase	<i>Malus × domestica</i>	<i>Malus × domestica</i> <i>S. lycopersicum</i> <i>O. sativa</i>	ROS scavenging; antioxidative metabolism ROS production	Salt stress salt stress	Hu et al., 2015
Other kinase	STT1	Lectin receptor-like kinase	<i>O. sativa</i>	<i>O. sativa</i>	ROS production	Salt stress	Li et al., 2014
Protein phosphatase	OsPPP18	Protein phosphatase 2C	<i>O. sativa</i>	<i>O. sativa</i>	ROS scavenging	Drought and oxidative stress	You et al., 2014
<b>Transcription factors</b>							
Zinc finger	DST ZFP36	C2H2 zinc finger C2H2 zinc finger	<i>O. sativa</i> <i>O. sativa</i>	<i>O. sativa</i> <i>O. sativa</i>	ROS scavenging ABA-induced antioxidant defense	Drought and salt stress Drought and oxidative stress	Huang et al., 2009 Zhang et al., 2014
AP2/ERF	OstTZF1 SERF1 SUB1A JERF3	CCCH zinc finger ERF ERF ERF	<i>O. sativa</i> <i>O. sativa</i> <i>O. sativa</i> <i>S. lycopersicum</i>	<i>O. sativa</i> <i>O. sativa</i> <i>O. sativa</i> <i>N. benthamiana</i>	ROS scavenging ROS signaling ROS scavenging ROS scavenging	Drought, salt and oxidative stress Salt stress Drought, submerge and oxidative stress Drought, salt and freezing stress	Jan et al., 2013 Schmidt et al., 2013 Fukao et al., 2011 Wu et al., 2008
WRKY	GmWRKY27 GmWRKY17	WRKY WRKY	<i>G. max</i> <i>G. hirsutum</i>	<i>G. max</i> <i>N. benthamiana</i>	ROS scavenging ROS production	Drought and salt stress Drought and salt stress	Wang et al., 2015
NAC	GmNAC29 SNAC3	NAC NAC	<i>G. max</i> <i>O. sativa</i>	<i>G. max</i> <i>O. sativa</i>	ROS production ROS scavenging	Drought and salt stress Drought, heat and oxidative stress	Yan et al., 2014 Wang et al., 2015
Other TF	TaASR1	ASR	<i>T. aestivum</i>	<i>N. benthamiana</i>	ROS scavenging	Drought and oxidative stress	Fang et al., 2015 Hu et al., 2013
<b>Other nuclear proteins</b>							
SRO protein	OsSRO1c Ta-sro1	SRO SRO	<i>O. sativa</i> <i>T. aestivum</i>	<i>O. sativa</i> <i>T. aestivum</i> <i>A. thaliana</i>	ROS scavenging ROS production and scavenging	Drought and oxidative stress Osmotic, salt and oxidative stress	You et al., 2013 Liu et al., 2014
ABA metabolism	OsSKIPa DSM2	Ski-interaction protein Carotene hydroxylase	<i>O. sativa</i> <i>O. sativa</i>	<i>O. sativa</i> <i>S. guineensis</i>	ROS scavenging antioxidative metabolism ABA-induced antioxidant defense	Drought stress Drought and oxidative stress Drought and salt stress	Hou et al., 2009 Du et al., 2010
ROS scavenging	SgNCED1 MnSOD OsAPX2	9-cis-epoxycarotenoid MnSOD APX	<i>S. guineensis</i> <i>M. sativa</i> <i>O. sativa</i>	<i>N. plumbeaginifolia</i> <i>M. sativa</i> <i>O. sativa</i>	ROS scavenging ROS scavenging	Drought stress Drought, salt and cold stresses	Zhang et al., 2009 McKersie et al., 1996 Zhang et al., 2013

(Continued)

TABLE 1 | Continued

Functional category	Genes	Protein function	Origin	Transformation receptor	ROS regulation	Abiotic stress resistance	Reference
Detoxification proteins	<i>MsALR</i>	NADPH-dependent aldo/keto-aldehyde reductase	<i>M. sativa</i>	<i>N. benthamiana</i>	antioxidative metabolism	Drought and oxidative stress	Oberschall et al., 2000
	<i>OsMT1a</i>	type 1 metallothionein	<i>O. sativa</i>	<i>O. sativa</i>	ROS scavenging	Drought stress	Yang et al., 2009
	<i>GmMT3a</i>	Type 3 metallothionein	<i>G. hirsutum</i>	<i>N. benthamiana</i>	ROS scavenging	Drought, salt and cold stresses	Xue et al., 2009
	<i>OsACA6</i>	type IIIB Ca <sup>2+</sup> -ATPase	<i>O. sativa</i>	<i>N. benthamiana</i>	ROS scavenging	Drought and salt stress	Huda et al., 2013
	<i>PtADC</i>	Arginine decarboxylase	<i>P. trifoliata</i>	<i>N. benthamiana; L. esculentum</i>	ROS scavenging	Drought stress	Wang et al., 2011
Calcium transporters							
Polyamines metabolism							
Amino acid metabolism	<i>OsOAT</i>	Ornithine δ-aminotransferase	<i>O. sativa</i>	<i>O. sativa</i>	antioxidative metabolism;	Drought and oxidative stress	You et al., 2012
					ROS scavenging	Drought and salt stress	Tuteja et al., 2013
					ROS scavenging	Salt and oxidative stress	Dong et al., 2013
					ABA-induced antioxidant defense		
Helicase	<i>OsSUV3</i>	NTP-dependent RNA/DNA helicase		<i>T. aestivum</i>			
Unknown function		TaOPR1	12-oxo-phytodienoic acid reductases	<i>T. aestivum</i>			

protection against oxidative stress, therefore, reduced levels of membrane lipid peroxidation under stress conditions (Campô et al., 2014).

Calcium-dependent protein kinase proteins also have been found to be responsive to abiotic stress via ROS regulation. Overexpression of wheat CIPK gene *TaCIPK29* in tobacco resulted in increased salt tolerance. Transgenic tobacco seedlings maintained high K<sup>+</sup>/Na<sup>+</sup> ratios and Ca<sup>2+</sup> content by up-regulating the expression of some transporter genes, and also reduced ROS accumulations by increasing the expression and activities of ROS-scavenging enzymes under salt stress (Deng et al., 2013). Overexpression of *MdSOS2L1*, a CIPK gene from apple, also conferred salt tolerance in apple and tomato (Hu et al., 2015). Molecular analysis and functional characterization of *MdSOS2L1* exhibited that it increases the ROS scavenging-enzymes and antioxidant metabolites such as procyanidin and malate, leading to enhanced salt tolerance in apple and tomato (Hu et al., 2015). A rice lectin receptor-like kinase, salt intolerance 1 (SIT1) was demonstrated mediates salt sensitivity by regulating ROS and ethylene homeostasis and signaling (Li et al., 2014). SIT1 phosphorylates MPK3 and 6, and their activation by salt requires SIT1. SIT1 promotes accumulation of ROS, leading to plant death under salt stress, which occurred in an MPK3/6- and ethylene signaling-dependent manner (Li et al., 2014).

The dephosphorylation mediated by protein phosphatase is an important event in the signal transduction process that regulates various cellular activities. A rice protein phosphatase 2C (PP2C) gene, *OsPP18*, was identified as a SNAC1-regulated downstream gene (You et al., 2014). The *ospp18* mutant exhibited sensitive to drought and oxidative stress with reduced activities of ROS-scavenging enzymes. The ABA-induced expression of ABA-responsive genes has not been disrupted in *ospp18* mutant, indicating *OsPP18* mediates drought stress resistance by regulating ROS homeostasis through ABA-independent pathways (You et al., 2014).

## Transcriptional factors

Transcriptional factors (TFs) are one of the important regulatory proteins involved in abiotic stress responses. They play essential roles downstream of stress signaling cascades, which could alter the expression of a subset of stress-responsive genes simultaneously and enhance tolerance to environmental stress in plants. Members of AP2/ERF (APETALA2/ethylene response factor), zinc finger, WRKY, bZIP (basic leucine zipper), and NAC (NAM, ATAF, and CUC) families have been characterized with roles in the regulation of plant abiotic stress responses (Yamaguchi-Shinozaki and Shinozaki, 2006; Ariel et al., 2007; Ciftci-Yilmaz and Mittler, 2008; Fang et al., 2008), and some of them have been demonstrated to be involved in ROS homeostasis regulation and abiotic stress resistance in crops.

Proteins containing zinc finger domain(s) were widely reported to be key players in the regulation of ROS-related defense genes in *Arabidopsis* and other species. For example, the expression of some zinc finger genes in *Arabidopsis*, ZAT7, ZAT10 and ZAT12, is intensely up-regulated by oxidative stress in AtAPX1 knockout plants (Miller et al., 2008). Subsequent experiments showed that these zinc finger proteins were involved

in ROS regulation and multiple abiotic stresses tolerance (Davletova et al., 2005; Mittler et al., 2006; Ciftci-Yilmaz et al., 2007). The zinc finger proteins are divided into several types, such as C2H2, C2C2, C2HC, CCCH and C3HC4, based on the number and the location of characteristic residues (Ciftci-Yilmaz and Mittler, 2008). The signaling pathways participating in stomatal movement were well studied in the model plant *Arabidopsis*, but were largely unknown in crops. Huang et al. (2009) identified a drought and salt tolerance (*dst*) mutant, and the DST was cloned by the map-based cloning. DST encoded a C2H2-type zinc finger transcription factor that negatively regulated stomatal closure by direct regulation of genes related to H<sub>2</sub>O<sub>2</sub> homeostasis, which identified a novel signaling pathway of DST-mediated H<sub>2</sub>O<sub>2</sub>-induced stomatal closure (Huang et al., 2009). Loss of DST function increased the accumulation of H<sub>2</sub>O<sub>2</sub> in guard cell, accordingly, resulted in increased stomatal closure and enhanced drought and salt tolerance in rice. Other two C2H2-type zinc finger proteins, ZFP36 and ZFP179, also play circle role in ROS homeostasis regulation and abiotic stress resistance in rice. *ZFP179* encodes a salt-responsive zinc finger protein with two C2H2-type zinc finger motifs (Sun et al., 2010). The *ZFP179* transgenic rice plants increased ROS-scavenging ability and expression levels of stress-related genes, and exhibited significantly enhanced tolerance to salt and oxidative stress (Sun et al., 2010). *ZFP36* is an ABA and H<sub>2</sub>O<sub>2</sub>-responsive C2H2-type zinc finger protein gene, and plays a important role in ABA-induced antioxidant defense and the tolerance of rice to drought and oxidative stresses (Zhang et al., 2014). Moreover, ZFP36 is a major player in the regulation of the cross-talk involving NADPH oxidase, H<sub>2</sub>O<sub>2</sub>, and MAPK in ABA signaling (Zhang et al., 2014). OsTZF1, a CCCH-tandem zinc finger protein, was identified as a negative regulator of leaf senescence in rice under stress conditions (Jan et al., 2013). Meanwhile, OsTZF1 confers tolerance to oxidative stress in rice by enhancing the expression of redox homeostasis genes and ROS-scavenging enzymes (Jan et al., 2013). A cotton CCCH-type tandem zinc finger gene, *GhTZF1*, also serves as a key player in modulating drought stress resistance and subsequent leaf senescence by mediating ROS homeostasis (Zhou et al., 2014b).

Members of AP2/ERF (APETALA2/ethylene response factor) transcription factor family, including DREB/CBF transcription factors, are especially important as they regulate genes involved in multiple abiotic stress responses (Mizoi et al., 2012). During the initial phase of abiotic stresses, elevated ROS levels might act as a vital acclimation signal. But the key regulatory components of ROS-mediated abiotic stress response signaling are largely unknown. Rice salt- and H<sub>2</sub>O<sub>2</sub>-responsive ERF transcription factor, SERF1, has a critical role in regulating H<sub>2</sub>O<sub>2</sub>-mediated molecular signaling cascade during the initial response to salinity in rice (Schmidt et al., 2013). SERF1 regulates the expression of H<sub>2</sub>O<sub>2</sub>-responsive genes involved in salt stress responses in roots. SERF1 is also a phosphorylation target of a salt-responsive MAPK (MAPK5), and activation the expression of salt-responsive MAPK cascade genes (MAPK5 and MAPKK6), well established salt-responsive TF genes (*ZFP179* and *DREB2A*), and itself through direct interaction with the corresponding promoters in plants (Schmidt et al., 2013). The authors proposed that

SERF1 is essential for the propagation of the initial ROS signal to mediate salt tolerance. SUB1A, an ERF transcription factor found in limited rice accessions, limits ethylene production and gibberellin responsiveness during submergence, economizing carbohydrate reserves and significantly prolonging endurance (Fukao and Xiong, 2013). After floodwaters subside, submerged plants encounter re-exposure to atmospheric oxygen, leading to postanoxic injury and severe leaf desiccation (Setter et al., 2010; Fukao and Xiong, 2013). SUB1A also positively affects postsubmergence responses by restrained accumulation of ROS in aerial tissue during desubmergence (Fukao et al., 2011). Consistently, SUB1A prompts the expression of ROS scavenging enzyme genes, resulting in enhanced tolerance to oxidative stress. On the other hand, SUB1A improves survival of rapid dehydration following desubmergence and water deficit during drought by increasing ABA responses, and activating stress-inducible gene expression (Fukao et al., 2011). A jasmonate and ethylene-responsive ERF gene, JERF3, was isolated from tomato and involved in a ROS-mediated regulatory module in transcriptional networks that govern plant response to stress (Wu et al., 2008). JERF3 modulates the expression of genes involved in osmotic and oxidative stresses responses by binding to the osmotic- and oxidative-responsive related *cis* elements. The expression of these genes leads to reduce accumulation of ROS, resulting in enhanced abiotic stress tolerance in tobacco (Wu et al., 2008).

The WRKY family proteins have one or two conserved WRKY domains comprising a highly conserved WRKYGQK heptapeptide at the N-terminus and a zinc-finger-like motif at the C-terminus (Eulgem et al., 2000). The conserved WRKY domain plays important roles in various physiological processes by binding to the W-box in the promoter regions of target genes (Ulker and Somssich, 2004; Rushton et al., 2010). Wang et al. (2015) reported a multiple stress-responsive WRKY gene, *GmWRKY27*, reduces ROS level and enhances salt and drought tolerance in transgenic soybean hairy roots. *GmWRKY27* interacts with *GmMYB174*, which, in turn, acts in concert to reduce promoter activity and gene expression of *GmNAC29* (Wang et al., 2015). Further experiments showed that *GmNAC29* is a negative factor of stress tolerance for enhancing the ROS production under abiotic stress by directly activating the expression of the gene encoding ROS production enzyme. In another study, overexpression of cotton WRKY gene, *GhWRKY17*, reduced transgenic tobacco plants tolerance to drought and salt stress. Subsequent experiments showed that *GhWRKY17* involved in stress responses by regulating ABA signaling and cellular levels of ROS (Yan et al., 2014). Sun et al. (2015) isolated a WRKY gene, *BdWRKY36*, from *B. distachyon*, and found it functions as a positive regulator of drought stress response by controlling ROS homeostasis and regulating transcription of stress-related genes.

Members of other TF families also functioned in abiotic stress response through ROS regulation. ASR proteins are plant-specific TFs and considered to be important regulators of plant response to various stresses. Wheat ASR gene, *TaASR1*, a positive regulator of plant tolerance to drought/osmotic stress,

is involved in the modulation of ROS homeostasis by activating antioxidant system and transcription of stress-responsive genes (Hu et al., 2013). Soybean NAC TF, GmNAC2, was identified as a negative regulator during abiotic stress, and participates in ROS signaling pathways through modulation of the expression of genes related to ROS-scavenging (Jin et al., 2013). Ramegowda et al. (2012) isolated a stress-responsive NAC gene, *EcNAC1*, from finger millet (*E. coracana*). Transgenic tobacco plants expressing *EcNAC1* increased ROS scavenging activity, up-regulated many stress-responsive genes, and exhibited tolerance to various abiotic stresses and MV-induced oxidative stress (Ramegowda et al., 2012). Recently, a NAC transcription factor gene, SNAC3, functions as a positive regulator under high temperature and drought stress, was identified in rice (Fang et al., 2015). SNAC3 enhances the abiotic stresses tolerance by modulating H<sub>2</sub>O<sub>2</sub> homeostasis state through controlling the expression of ROS-associated enzyme genes (Fang et al., 2015).

In addition to TFs, transcriptional coregulator as well as spliceosome component, OsSKIPa, a rice homolog of human Ski-interacting protein (SKIP), has been studied for effects on drought resistance (Hou et al., 2009). OsSKIPa-overexpressing rice exhibited significantly enhanced drought stress tolerance at both the seedling and reproductive stages by increased ROS-scavenging ability and transcript levels of many stress-related genes (Hou et al., 2009).

## SRO PROTEINS

The SRO (SIMILAR TO RCD ONE) protein family was recently identified as a group of plant-specific proteins, and they are characterized by the plant-specific domain architecture which contains a poly (ADP-ribose) polymerase catalytic (PARP) and a C-terminal RCD1-SRO-TAF4 (RST) domain (Jaspers et al., 2010). In addition to these two domains, some SRO proteins have an N-terminal WWE domain. Our limited knowledge of SRO proteins is mainly from the study in *Arabidopsis* mutant *rcd1* (*radical-induced cell death 1*). *rcd1* exhibits pleiotropic phenotypes related to a wide range of exogenous stimulus responses and developmental processes, including sensitivity to apoplastic ROS and salt stress, resistance to chloroplastic ROS caused by methyl viologen (MV) and UV-B irradiation (Ahlfors et al., 2004; Fujibe et al., 2004; Katiyar-Agarwal et al., 2006). RCD1 interacts with SOS1 and a large number of transcription factors which have been identified or predicted to be involved in both development and stress-related processes (Katiyar-Agarwal et al., 2006; Jaspers et al., 2009). Recent study demonstrated that RCD1 is possibly involved in signaling networks that regulate quantitative changes in gene expression in response to ROS (Brosche et al., 2014).

In rice, an SRO protein, OsSRO1c, was characterized as a direct target of the drought stress-related transcription factor SNAC1 (You et al., 2013). OsSRO1c was induced in guard cells by drought stress. Overexpression of OsSRO1c resulted in accumulated H<sub>2</sub>O<sub>2</sub> in guard cells, which, in turn, decreased stomatal aperture and reduced water loss. Further experiments

indicated that OsSRO1c has dual roles in drought and oxidative stress tolerance of rice by promoting stomatal closure and H<sub>2</sub>O<sub>2</sub> accumulation through a novel pathway involving the SNAC1 and DST regulators (You et al., 2013). Recently, an SRO gene was also identified to be crucial for salinity stress resistance by modulating redox homeostasis in wheat (Liu et al., 2014). *Ta-sro1*, the allele of the salinity-tolerant bread wheat cultivar Shanrong No. 3, is derived from the wheat parent allele via point mutation. Unlike *Arabidopsis* SRO proteins, *Ta-sro1* has PARP activity. Both the overexpression of *Ta-sro1* in wheat and *Arabidopsis* promotes the accumulation of ROS by regulating ROS-associated enzyme. *Ta-sro1* also enhances the activity of AsA-GSH cycle enzymes and GPX cycle enzymes, which regulate ROS content and cellular redox homeostasis (Liu et al., 2014).

## ROS-scavenging or Detoxification Proteins

Reactive oxygen species-scavenging enzymes such as SOD, APX, CAT were properly described its role in ROS-scavenging pathway. The presence of antioxidant enzymes and compounds in almost all cellular compartments suggests the importance of ROS detoxification for protection against various stresses (Mittler et al., 2004). The effect of these ROS-scavenging enzymes in abiotic stress resistance was also investigated in crop plants. Transgenic alfalfa expressing MnSOD cDNA from *Nicotiana plumbaginifolia* improved survival and vigor after exposure to water deficit. Most importantly, transgenic alfalfa showed increased yield and survival rate over three winters in natural field environments (McKersie et al., 1996). A cDNA encoding a cytosolic copper-zinc SOD from the mangrove plant *Avicennia marina* was transformed into rice. The transgenic plants exhibited more tolerant to drought, salinity and oxidative stresses compared with the untransformed control plants (Prashanth et al., 2008). Overexpression of *OsAPX2* increased APX activity and reduced H<sub>2</sub>O<sub>2</sub> and malondialdehyde (MDA) levels in transgenic plants under stress treatments (Zhang et al., 2013). More importantly, *OsAPX2*-overexpressing plants were more tolerant to drought stress than wild-type plants at the booting stage as indicated a significantly increase in spikelet fertility under abiotic stresses (Zhang et al., 2013). Transgenic rice plants that overexpressing another APX gene, *OsAPX1*, also exhibited increased spikelet fertility under cold stress (Sato et al., 2011).

Accumulation of toxic products from ROS with lipids and proteins significantly contributes to the damage of crop plants under biotic and abiotic stresses. A novel plant NADPH-dependent aldose/aldehyde reductase, which has the reduction activity toward toxic products of lipid peroxidation, was isolated from alfalfa. Tobacco plants overproducing the alfalfa aldose/aldehyde reductase showed lower concentrations of reactive aldehydes (products of lipid peroxidation) and tolerance to oxidative and drought stress (Oberschall et al., 2000).

Metallothioneins (MTs) are a group of low molecular weight proteins with the characteristics of high cysteine (Cys) residue content and metal-binding ability. The presence of several Cys residues in MTs suggests their involvement in the detoxification

of ROS or in the maintenance of redox levels. *OsMT1a*, encoding a type 1 MT in rice, was induced by dehydration and Zn<sup>2+</sup> treatment (Yang et al., 2009). Transgenic rice plants overexpressing *OsMT1a* enhanced antioxidant enzyme activities of CAT, POD and APX, and enhanced tolerance to drought. *OsMT1a* also regulates the expression of several zinc finger transcription factors by the modulation of Zn<sup>2+</sup> homeostasis, which leads to enhanced plant stress tolerance (Yang et al., 2009). *GhMT3a* encodes a type 3 plant MT in cotton. Recombinant *GhMT3a* protein showed an ability to bind metal ions and scavenge ROS *in vitro*. Transgenic tobaccos showed more tolerance to multiple abiotic stresses, and lower H<sub>2</sub>O<sub>2</sub> levels when compared with wild-type plants (Xue et al., 2009). The *SbMT-2* gene from a halophyte was also involved in maintaining cellular homeostasis by regulating ROS scavenging during stresses and thus improved tolerance to salt and osmotic stress in transgenic tobacco (Chaturvedi et al., 2014).

## ABA Metabolic-related Proteins

Abscisic acid is a key phytohormone that mediates the adaptive responses to abiotic stresses of plants. ABA-induced antioxidant defense has been well documented in plants. ABA biosynthesis and catabolism also involved in antioxidant defense and abiotic stresses. Du et al. (2010) isolated a rice drought-sensitive mutant *dsm2*, impaired in the gene encoding a putative β-carotene hydroxylase. β-carotene hydroxylase is predicted for the biosynthesis of zeaxanthin, a carotenoid precursor of ABA. Under drought stress, *dsm2* mutants had reduced zeaxanthin and ABA, lower Fv/Fm and non-photochemical quenching (NPQ) capacity than the wild type. Overexpression of *DSM2* in rice increases the xanthophylls and NPQ capacity, stress-related ABA-responsive genes expression, and resulted in enhancing resistance to drought and oxidative stresses (Du et al., 2010). *OsABA8ox3*, encoding ABA 8'-hydroxylase involved in ABA catabolism, is also a key gene regulating ABA accumulation and anti-oxidative stress capability under drought stress (Nguyen et al., 2015). *OsABA8ox3* RNAi plants exhibited significant improvement in drought stress tolerance. Consistent with this, *OsABA8ox3* RNAi plants showed increased SOD and CAT activities and reduced MDA levels during dehydration treatment. In another study, overexpression of the 9-*cis*-epoxycarotenoid dioxygenase gene from *Stylosanthes guianensis* (*SgNCED1*) in the transgenic tobacco increased ABA content and tolerance to drought and salt stresses (Zhang et al., 2009). Moreover, enhanced abiotic stresses tolerance in transgenic plants is associated with ABA-induced production of H<sub>2</sub>O<sub>2</sub> and NO, which, in turn, activate the expression and activities of ROS-scavenging enzymes (Zhang et al., 2009).

## Calcium Transporters and Calcium-binding Proteins

Calcium (Ca<sup>2+</sup>) regulates numerous signaling pathways involved in growth, development and stress tolerance. The influx of Ca<sup>2+</sup> into the cytosol is countered by pumping Ca<sup>2+</sup> out from the cytosol to restore the basal cytosolic level, and this may be achieved either by P-type Ca<sup>2+</sup>ATPases or antiporters.

Huda et al. (2013) report the isolation and characterization of *OsACA6*, which encodes a member of the type IIB Ca<sup>2+</sup>ATPase family from rice. Overexpression of *OsACA6* confers tolerance to salinity and drought stresses in tobacco, which was correlated with reduced accumulation of ROS and enhanced the expression of stress-responsive genes in plants (Huda et al., 2013). In addition, overexpression of *OsACA6* confers Cd<sup>2+</sup> stress tolerance in transgenic lines by maintaining cellular ion homeostasis and modulating ROS-scavenging pathway (Shukla et al., 2014). Annexins are calcium-dependent, phospholipid-binding proteins with suggested functions in response to environmental stresses and signaling during plant growth and development. *OsANN1*, a member of the annexin protein family in rice, has ATPase activity, the ability to bind Ca<sup>2+</sup>, and the ability to bind phospholipids in a Ca<sup>2+</sup>-dependent manner. *OsANN1* confers abiotic stress tolerance by modulating antioxidant accumulation and interacting with *OsCDPK24* (Qiao et al., 2015).

## Other Functional Proteins

Polyamines are low molecular weight aliphatic amines found in all living cells. Because of their cationic nature at physiological pH, PAs have strong binding capacity to negatively charged molecules (DNA, RNA, and protein), thus stabilizing their structure (Alcazar et al., 2010). The PAs biosynthetic pathway has been thoroughly investigated in many organisms, and arginine decarboxylase (ADC) plays a predominant role in the accumulation of PAs under stresses (Capell et al., 2004; Alcazar et al., 2010). Wang et al. (2011) isolated an arginine decarboxylase gene (*PtADC*) from *Poncirus trifoliata*. The transgenic tobacco and tomato plants elevated endogenous PAs level, accumulated less ROS and showed an improvement in drought tolerance. Jang et al. (2012) identified a highly oxidative stress-resistant T-DNA mutant line carried an insertion in *OsLDC-like 1* in rice. The mutant produced much higher levels of PAs compared to the wild type plants. Based on their results, the authors suggested that PAs mediate tolerance to abiotic stresses through their ability to decrease ROS generation and enhance ROS degradation.

The 12-oxo-phytodienoic acid reductases (OPRs) are classified into two subgroups, OPRI and OPRII. OPRII proteins are involved in jasmonic acid synthesis, while the function of OPRI is as yet unclear. Dong et al. (2013) characterized the functions of the wheat OPRI gene *TaOPR1*. Overexpression of *TaOPR1* in wheat and *Arabidopsis* enhanced tolerance to salt stress by regulating of ROS and ABA signaling pathways (Dong et al., 2013).

Helicases are ubiquitous enzymes that catalyze the unwinding of energetically stable duplex DNA or RNA secondary structures, and thereby play an important role in almost all DNA and/or RNA metabolic processes. *OsSUV3*, an NTP-dependent RNA/DNA helicase in rice, exhibits ATPase, RNA and DNA helicase activities (Tuteja et al., 2013). *OsSUV3* sense transgenic rice plants showed lesser lipid peroxidation and H<sub>2</sub>O<sub>2</sub> production, along with higher activities of antioxidant enzymes, consequently resulting in increased tolerance to high salinity (Tuteja et al., 2013).

Ornithine  $\delta$ -aminotransferase ( $\delta$ -OAT) is considered to be an enzyme involved in proline and arginine metabolism. OsOAT-overexpressing rice plants exhibited significantly increased  $\delta$ -OAT activity and proline levels under normal growth conditions, and enhanced drought, osmotic, and oxidative stress tolerance (You et al., 2012).

## CONCLUSION AND PERSPECTIVES

The discovery of the enzymatic activity of SOD 45 years ago (McCord and Fridovich, 1969) ushered in the field of ROS biology. During the last two decades, the major sources and sites of ROS production, and the key antioxidant molecules and enzymes that scavenge ROS have been chartered in plant. However, our current knowledge about ROS homeostasis and signaling remains fragmental. Apoplastic ROS are rapidly produced in plants as a defense response to pathogen attack and abiotic stress. Whereas, in addition to NADPH oxidase, the function and regulation of other apoplastic ROS-associated enzymes, such as class III peroxidases, in stress responses signaling are largely unknown. On the other hand, 100s of genes that encode for ROS-metabolizing enzymes and regulators compose ROS gene network in plants. Thus, more than one enzymatic activity that produces or scavenges ROS exists in certain cellular compartment. How these different enzymes are coordinated within each compartment and between different compartments to adjust a particular ROS at an appropriate level during stresses is an important question needs to be addressed. There is increasing evidence suggesting the vital role of ROS signaling pathway in plant development and stress responses. However, regulatory mechanisms at the biochemical level, the mechanisms of extracellular ROS perception, transduction of ROS-derived signals, and especially the communication and interaction between different subcellular compartments in ROS signaling are still poorly understood. To build comprehensive regulation networks in ROS signaling and responses requires a combination of transcriptomics, proteomics and metabolomics approaches with analysis of mutant as well as protein–protein interactions.

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Plants need diverse responses and adjustment of multiple adaptation mechanisms to cope with the multiple stresses exist in nature. Comparison of transcription profiles of rice in response to multiple stresses suggested the central role of ROS homeostasis in different abiotic stresses (Mittal et al., 2012). Therefore, manipulating endogenous ROS levels provides us with an opportunity to improve common defense mechanisms against different stresses to ensure crop plants growth and survival under adverse growing condition. The functions of numerous stress-responsive genes involved in ROS homeostasis regulation and abiotic stress resistance have been characterized in transgenic plants (Table 1). As expected, transgenic crop plants harbored these genes enhanced tolerance to multiple abiotic stresses (Wu et al., 2008; Fukao et al., 2011; Lu et al., 2013; Campo et al., 2014). However, few studies have reported the abiotic stress tolerance of transgenic plant at the reproductive or flowering stage based on yield and/or setting rate, and very few of these tests were conducted under field conditions. Additionally, most of the reported ROS-associated genes that involved in abiotic stress just have been demonstrated its role in regulation of expression and/or activity of ROS-scavenging enzymes. Thus, network involving in function of these genes in ROS homeostasis to mediate abiotic stress resistance needs to be fully investigated, and the new components need to be integrated into the signaling pathway. With a long-term goal to improve the abiotic stress resistance of crop plants by the utilizing of ROS regulation pathways, more and more key regulators need to be identified. It is also very important to clarify the mechanisms regulating ROS signaling pathways and their interplay during abiotic stresses. This can finally help to incorporate multiple necessary ROS-associated genes into the genetic backgrounds of elite cultivars or hybrids to enhance their abiotic stress resistance under real agricultural field conditions.

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# Zinc-Dependent Protection of Tobacco and Rice Cells From Aluminum-Induced Superoxide-Mediated Cytotoxicity

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$\text{Al}^{3+}$  toxicity in growing plants is considered as one of the major factors limiting the production of crops on acidic soils worldwide. In the last 15 years, it has been proposed that  $\text{Al}^{3+}$  toxicity are mediated with distortion of the cellular signaling mechanisms such as calcium signaling pathways, and production of cytotoxic reactive oxygen species (ROS) causing oxidative damages. On the other hand, zinc is normally present in plants at high concentrations and its deficiency is one of the most widespread micronutrient deficiencies in plants. Earlier studies suggested that lack of zinc often results in ROS-mediated oxidative damage to plant cells. Previously, inhibitory action of  $\text{Zn}^{2+}$  against lanthanide-induced superoxide generation in tobacco cells have been reported, suggesting that  $\text{Zn}^{2+}$  interferes with the cation-induced ROS production via stimulation of NADPH oxidase. In the present study, the effect of  $\text{Zn}^{2+}$  on  $\text{Al}^{3+}$ -induced superoxide generation in the cell suspension cultures of tobacco (*Nicotiana tabacum* L., cell-line, BY-2) and rice (*Oryza sativa* L., cv. Nipponbare), was examined. The  $\text{Zn}^{2+}$ -dependent inhibition of the  $\text{Al}^{3+}$ -induced oxidative burst was observed in both model cells selected from the monocots and dicots (rice and tobacco), suggesting that this phenomenon ( $\text{Al}^{3+}/\text{Zn}^{2+}$  interaction) can be preserved in higher plants. Subsequently induced cell death in tobacco cells was analyzed by lethal cell staining with Evans blue. Obtained results indicated that presence of  $\text{Zn}^{2+}$  at physiological concentrations can protect the cells by preventing the  $\text{Al}^{3+}$ -induced superoxide generation and cell death. Furthermore, the regulation of the  $\text{Ca}^{2+}$  signaling, i.e., change in the cytosolic  $\text{Ca}^{2+}$  ion concentration, and the cross-talks among the elements which participate in the pathway were further explored.

**Keywords:** aluminum, zinc, BY-2, *Nicotiana tabacum* L., *Oryza sativa* L., ROS

## INTRODUCTION

Aluminum is the most abundant metal and the third most abundant chemical element in the Earth's crust. The increase in free aluminum ions (chiefly,  $\text{Al}^{3+}$ ) accompanying soil acidification is considered to be toxic to plants (Poschenrieder et al., 2008) and animals (Markich et al., 2002).  $\text{Al}^{3+}$  toxicity in growing plants is considered as one of the major factors limiting the production

of crops on acidic soils worldwide (Poschenrieder et al., 2008; Panda et al., 2009).

A number of studies documented the toxic impact of Al<sup>3+</sup> especially on roots (Le Van et al., 1994; Lukaszewski and Blevins, 1996; Sanzonowicz et al., 1998). It has been proposed that early effects of Al<sup>3+</sup> toxicity at growing root apex, such as those on cell division, cell extension or nutrient transport, involve the binding to (Ma et al., 1999) or uptake of Al<sup>3+</sup> by plants (Lazof et al., 1994; Babourina and Rengel, 2009). Accordingly, actin cytoskeleton and vesicle trafficking are primary targets for Al<sup>3+</sup> toxicity in the root tips of the sensitive variety (Amenós et al., 2009).

In the last 15 years, it has been proposed that Al<sup>3+</sup> toxicity are mediated with distortion of the cellular signaling mechanisms such as calcium signaling pathways (Kawano et al., 2003a, 2004; Rengel and Zhang, 2003; Lin et al., 2005, 2006a), and production of cytotoxic reactive oxygen species (ROS) causing oxidative damages (Yamamoto et al., 2002; Kawano et al., 2003a). Recently, Al<sup>3+</sup>-induced DNA damages in the root cells of *Allium cepa* was shown to be blocked by calcium channel blockers suggesting that Al<sup>3+</sup>-stimulated influx of extracellular Ca<sup>2+</sup> into cytosol causes the programmed cell death-like decomposition of DNA (Achary et al., 2013).

To date, two independent groups have proposed the likely modes of ROS production in Al<sup>3+</sup>-treated plant cells. While Yamamoto et al. (2002), propounded the role of mitochondria challenged by Al<sup>3+</sup> using the cultured cells of tobacco (*Nicotiana tabacum* L., cell line SL) and the roots of pea (*Pisum sativum* L.); our group (Kawano et al., 2003a) emphasized the involvement of NADPH oxidase, thus sensitive to an inhibitor of NADPH oxidase, diphenylene iodonium (DPI) in tobacco BY-2 cells. While ROS is slowly produced through mitochondrial dysfunction (*ca.* 12 h after Al<sup>3+</sup> treatment; Yamamoto et al., 2002), the NADPH oxidase-mediated production of superoxide anion radical (O<sub>2</sub><sup>•-</sup>) takes place immediately after Al<sup>3+</sup> treatment (Kawano et al., 2003a).

The action of Al<sup>3+</sup> for induction of O<sub>2</sub><sup>•-</sup> generation which is sensitive to DPI was recently confirmed in the cells of *Arabidopsis thaliana* (Kunihiro et al., 2011). Furthermore, the Al<sup>3+</sup>-induced oxidative burst showed biphasic signature consisted with an acute transient spike and a slow but long-lasting wave of O<sub>2</sub><sup>•-</sup> generation. In addition, among six respiratory burst oxidase homologs (*Atrobohs*) coding for plant NADPH oxidase, solely *AtrobohD* was shown to be responsive to Al<sup>3+</sup> in biphasic manner by showing rapid (1 min) and long-lasting (24 h) expression profiles (Kunihiro et al., 2011).

Interestingly, the mechanism of Al<sup>3+</sup>-induced oxidative burst (production of O<sub>2</sub><sup>•-</sup>) is highly analogous to the response of tobacco cell suspension culture to other metal cations, chiefly trivalent cations of lanthanides such as La<sup>3+</sup> and Gd<sup>3+</sup> (Kawano et al., 2001). Therefore, we assume that some known chemical factors reportedly interfere with the lanthanide-induced plant oxidative burst might be active for protection of plant cells from Al<sup>3+</sup>-induced oxidative stress. Such chemicals of interest to be tested include zinc and manganese (Kawano et al., 2002).

Zinc is normally present in plants at high concentrations (Santa-Maria and Cogliatti, 1988) and its deficiency is one of the most widespread micronutrient deficiencies in plants, causing severe reductions in crop production (Cakmak, 2000). Increasing studies indicate that oxidative damage to cellular components caused in plants being challenged by ROS, is highly due to the deficiency of zinc (Pinton et al., 1994; Cakmak, 2000).

Previously, inhibitory action of Zn<sup>2+</sup> against lanthanide-induced O<sub>2</sub><sup>•-</sup> generation in tobacco cells have been reported (Kawano et al., 2002). Pretreatments with Zn<sup>2+</sup> reportedly interferes the La<sup>3+</sup>- and Gd<sup>3+</sup>-induced O<sub>2</sub><sup>•-</sup> generation in tobacco cells. In the tobacco model, Zn<sup>2+</sup> was shown to minimize the earlier phase of lanthanide-induced O<sub>2</sub><sup>•-</sup> production while allowing the release of O<sub>2</sub><sup>•-</sup> in the later phase, thus causing the retardation of the lanthanide actions on O<sub>2</sub><sup>•-</sup> generation.

Although this process is well known, if it is preserved in higher plants and the specific mechanism of action have is not still clear. For this reason, in the present study, effect of Zn<sup>2+</sup> on Al<sup>3+</sup>-induced O<sub>2</sub><sup>•-</sup> generation in the suspension cultures of tobacco BY-2 cells and rice (*Oryza sativa* L., cv. Nipponbare) cells, was examined. Furthermore, the regulation of the Ca<sup>2+</sup> signaling, i.e., change in the cytosolic Ca<sup>2+</sup> ion concentration ([Ca<sup>2+</sup>]<sub>c</sub>), and the cross-talks among the elements which participate in the pathway were further explored. Finally the possible use of Zn<sup>2+</sup> for protection of plant cells from Al<sup>3+</sup> toxicity is discussed.

## MATERIALS AND METHODS

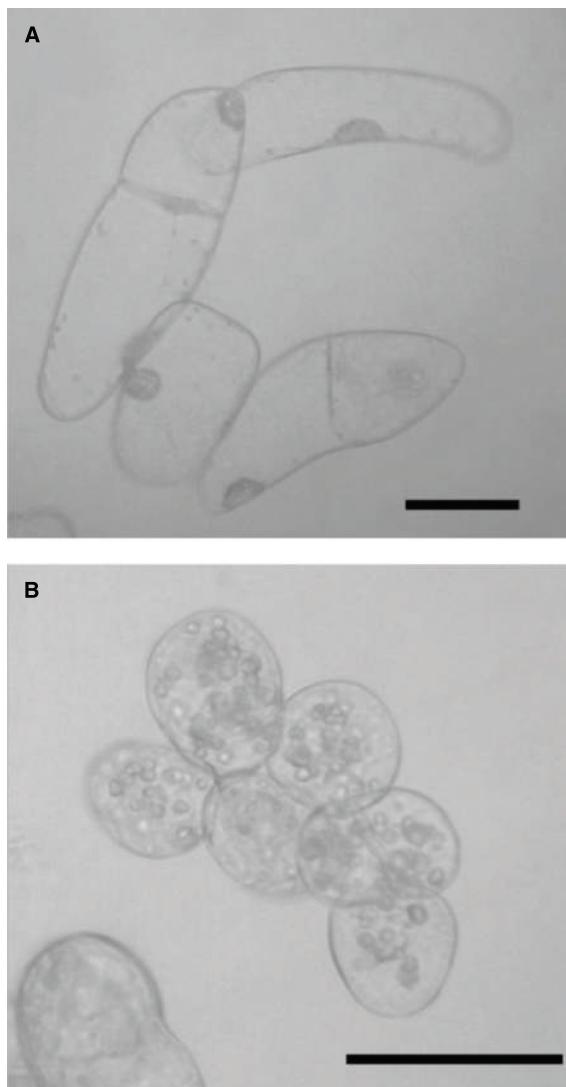
### Chemicals

O<sub>2</sub><sup>•-</sup>-specific chemiluminescence (CL) probe, *Cypridina* luciferin analog (CLA; 2-methyl-6-phenyl-3,7-dihydroimidazo[1,2-a]pyrazin-3-one) designated as CLA was purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Aluminum (III) chloride hexahydrate (AlCl<sub>3</sub>·6H<sub>2</sub>O), zinc sulfate heptahydrate (ZnSO<sub>4</sub>·7H<sub>2</sub>O), gadolinium chloride hexahydrate (GdCl<sub>3</sub>·6H<sub>2</sub>O), and salicylic acid (SA) were from Wako Pure Chemical Industries (Osaka, Japan). Lanthanum chloride heptahydrate (LaCl<sub>3</sub>·7H<sub>2</sub>O) was from Kanto Chemical Co., Inc (Tokyo, Japan). DPI chloride, Evans blue, 4,5-dihydroxy-1,3-benzene-disulfonic acid (Tiron), *N,N'*-dimethylthiourea (DMTU), were from Sigma (St. Louis, MO, USA). Coelenterazine was a gift from Prof. M. Isobe (Nagoya University).

### Plant Cell Culture

Tobacco (*Nicotiana tabacum* L. cv. Bright Yellow-2) suspension-culture cells (cell line, BY-2, expressing the aequorin gene; **Figure 1A**) were propagated as previously described (Kawano et al., 1998). Briefly, the culture was maintained in Murashige-Skoog liquid medium (pH 5.8) supplemented with 3% (w/v) sucrose and 0.2 µg ml<sup>-1</sup> of 2,4-dichlorophenoxyacetic acid. The culture was propagated with shaking on a gyratory shaker in darkness at 23°C. For sub-culturing, 1.0 ml of confluent stationary culture was suspended in 30 ml of fresh culture medium

**Abbreviations:** CL, chemiluminescence; CLA, *Cypridina* luciferin analog; DAI, days after inoculation; DPI, diphenylene iodonium; O<sub>2</sub><sup>•-</sup>, superoxide anion radical; rcu, relative chemiluminescence units; ROS, reactive oxygen species; SA, salicylic acid.



**FIGURE 1 | Microscopic images of plant cells used in this study. (A)** Tobacco BY-2 cells. **(B)** Rice M1 cells. Scale bars, 50  $\mu\text{m}$ .

and incubated at 23°C with shaking at 130 rpm on a gyratory shaker in darkness until used.

Rice callus tissues (*Oryza sativa* L., cv. Nipponbare, cell line, M1; **Figure 1B**) were obtained from root explants derived from young seedlings and transferred in AA liquid medium to develop a suspension-culture. The cells were maintained and propagated at 23°C with shaking at 130 rpm on a gyratory shaker in darkness. For sub-culturing, with 2-week intervals, 10 ml of stationary culture was suspended in 100 ml of fresh culture medium.

### Detection of $\text{O}_2^{\bullet-}$ with CLA

To detect the production of  $\text{O}_2^{\bullet-}$  in plant cells, the 200  $\mu\text{l}$  of plant cell suspension (either of tobacco or rice) was placed in glass cuvettes and CLA was added at final concentration of 2  $\mu\text{M}$  (in tobacco cells) and 4  $\mu\text{M}$  (in rice cells). The glass cuvettes containing 200  $\mu\text{l}$  of plant cell suspension

were placed in luminometers (CHEM-GLOW Photometer, American Instrument Co., Silver Spring, MD, USA; or PSN AB-2200-R Luminescensor, Atto, Tokyo). Generation of  $\text{O}_2^{\bullet-}$  in cell suspension culture was monitored by CLA-CL, and expressed as relative chemiluminescence units (rcu) as previously described (Kawano et al., 1998). CLA-CL specifically indicates the generation of  $\text{O}_2^{\bullet-}$  (and of  ${}^1\text{O}_2$  with a minor extent) but not that of  $\text{O}_3$ ,  $\text{H}_2\text{O}_2$  or hydroxy radicals (Nakano et al., 1986).

### Aequorin $\text{Ca}^{2+}$ Detection

To detect the changes in  $[\text{Ca}^{2+}]_c$  in tobacco cells, 10 mL of plant cell suspension were pre-treated for 8 h with 10  $\mu\text{L}$  of coelenterazine in the dark, then used for the experiments as previously described (Kawano et al., 1998). Also in this case, 200  $\mu\text{l}$  plant of cell suspension was placed in glass cuvettes and placed in luminometers (as above). Increase in  $[\text{Ca}^{2+}]_c$ , reflecting the induced  $\text{Ca}^{2+}$  into cells, was monitored as luminescence derived upon binding of  $\text{Ca}^{2+}$  to aequorin (the recombinant gene over-expressed in the cytosol) and expressed as rcu.

### Treatments with Aluminum, Zinc, and Other Stimuli

Tobacco BY-2 cells were harvested various days after sub-culturing (as indicated), and used for experiments with CLA or aequorin.  $\text{AlCl}_3$  was dissolved in distilled water and diluted with fresh culture media unless indicated, and 10–20  $\mu\text{l}$  of the  $\text{AlCl}_3$  solution was added to 180–190  $\mu\text{l}$  of cell suspension in glass cuvettes, and level of  $[\text{Ca}^{2+}]_c$  (aequorin experiment) or generation of  $\text{O}_2^{\bullet-}$  (CLA experiment) were monitored. For comparison, effects of SA and hypo-osmotic shock (induced by dilution of media giving  $\Delta 100$  mOsmol of hypo-osmolarity difference) on calcium homeostasis with and without zinc was monitored. Inhibition of events induced by  $\text{Al}^{3+}$  and other stimuli, monitored with CLA CL, aequorin luminescence, and cell death staining, was performed by addition of indicated concentration of  $\text{Zn}^{2+}$  to the cells prior to treatments with  $\text{Al}^{3+}$ , SA, and hypo-osmotic shock.

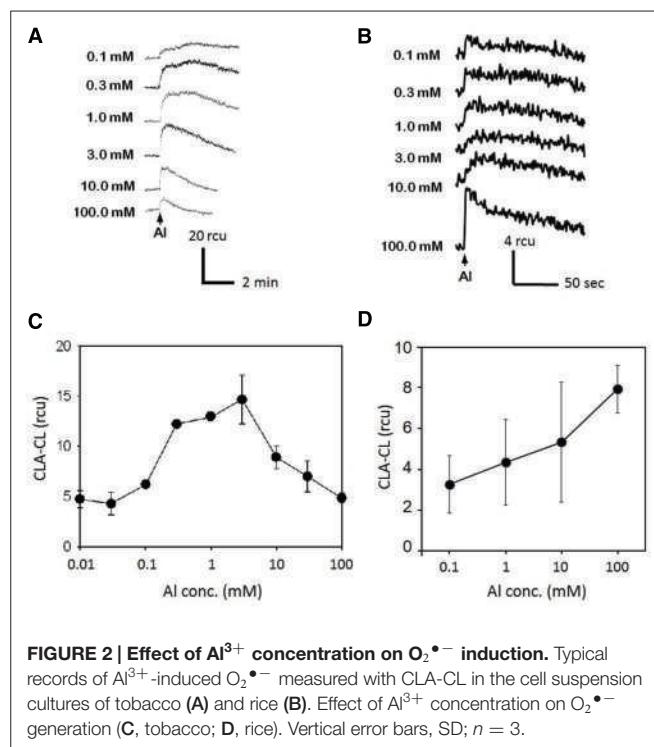
### Monitoring of Cell Death

$\text{Al}^{3+}$ -induced cell death in BY-2 tobacco cell suspension culture was allowed to develop in the presence of Evans blue, a lethal staining dye (0.1%, w/v). Evans blue was added to the cell suspension culture, 6 h after  $\text{Al}^{3+}$  application unless indicated or at the time indicated (0–8 h after Al addition). Then, the cells were further incubated for 1 h for fully developing and detecting the cell death as described (Kadono et al., 2006). After terminating the staining process by washing, stained cells were counted under microscopes. For statistical analyses, four different digital images of cells under the microscope (each covering 50 cells to be counted) were acquired and stained cells were counted.

## RESULTS AND DISCUSSIONS

### Induction of $\text{O}_2^{\bullet-}$

The effect of  $\text{Al}^{3+}$  concentration on induction of  $\text{O}_2^{\bullet-}$  generation has been tested both in tobacco and rice cell suspension cultures



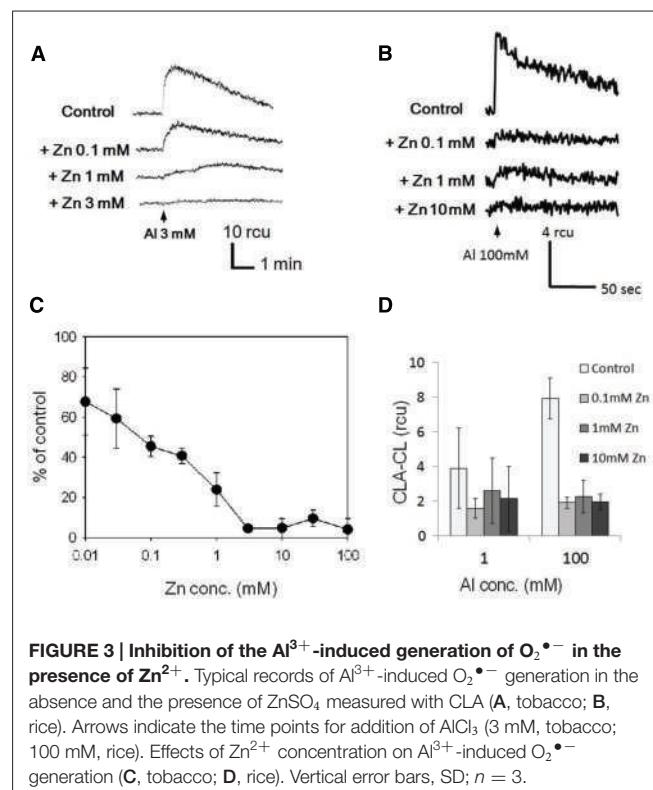
(Figure 2). For this analysis, BY-2 tobacco cells have been tested 4 days after inoculation (DAI) unless indicated whereas the rice cell line M1 suspension culture was used 14 DAI, since the tobacco BY-2 cells grow at faster rate compared to rice M1 cells. In tobacco BY-2 cells, the active  $\text{Al}^{3+}$  concentrations for induction of  $\text{O}_2^{\bullet-}$  generation ranged from 0.1 mM to 30 mM (optimally at 3 mM).

Notably, higher concentration of  $\text{Al}^{3+}$  was shown to be inhibitory to induction of  $\text{O}_2^{\bullet-}$  generation in the tobacco cells (Figures 2A,C), while the rice cells showed only the proportional increase in generation of  $\text{O}_2^{\bullet-}$  with the increase in  $\text{Al}^{3+}$  up to 100 mM (Figures 2B,D). In order to analyze the impact of  $\text{Zn}^{2+}$  against  $\text{Al}^{3+}$ -induced  $\text{O}_2^{\bullet-}$  generation, the concentration of  $\text{Al}^{3+}$  was fixed to at 3 mM for the tobacco cells and 100 mM for rice cells. Different concentrations have been chosen since the tobacco cells showed higher sensitivity to relatively lower concentrations of  $\text{Al}^{3+}$ .

### Effect of Pretreatment with $\text{Zn}^{2+}$

To assess the effect of  $\text{Zn}^{2+}$ , the cells of tobacco and rice were pretreated with various concentration of  $\text{ZnSO}_4$  for 5 min and then  $\text{AlCl}_3$  was added to the cells (Figure 3). In tobacco cell, the  $\text{O}_2^{\bullet-}$  generation induced by 3 mM  $\text{Al}^{3+}$  was significantly inhibited by 1 mM or higher concentrations of  $\text{Zn}^{2+}$ , whilst in rice cell, 0.1 mM of  $\text{Zn}^{2+}$  was high enough to achieve a significant inhibition of  $\text{O}_2^{\bullet-}$  generation induced by 100 mM  $\text{Al}^{3+}$ . Although  $\text{Zn}^{2+}$ -dependent retardation of lanthanide-induced  $\text{O}_2^{\bullet-}$  production has been reported (Kawano et al., 2002), the  $\text{Al}^{3+}$ -induced oxidative burst was simply inhibited without allowing the onset of slower increase in  $\text{O}_2^{\bullet-}$  production.

The  $\text{Zn}^{2+}$ -dependent inhibition was observed in both model cells selected from the monocots and dicots (rice and tobacco),



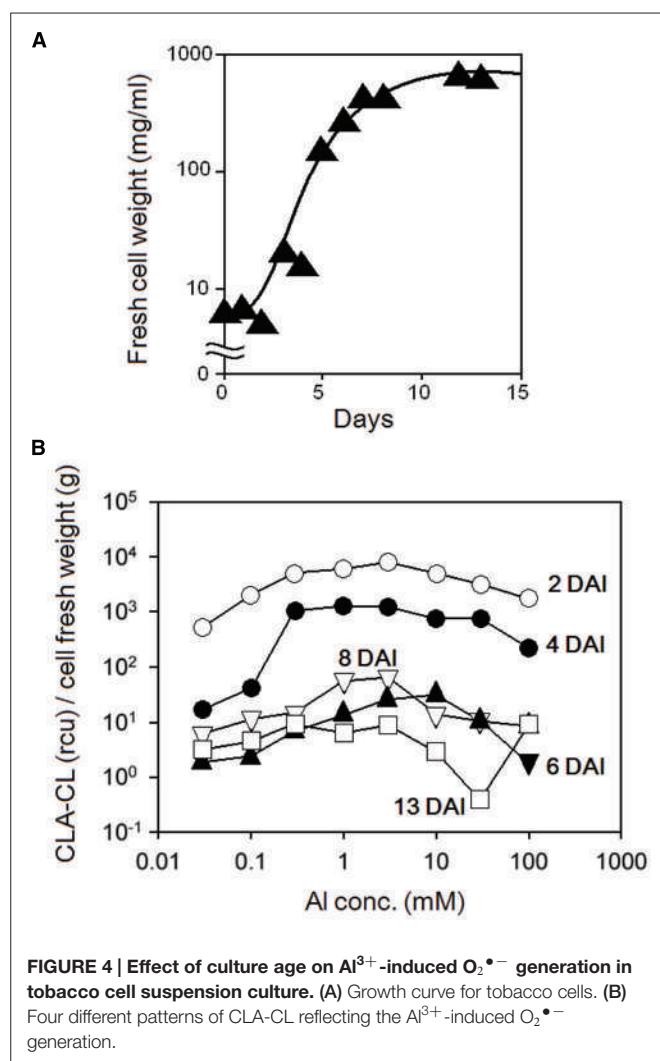
suggesting that this phenomenon ( $\text{Al}^{3+}/\text{Zn}^{2+}$  interaction) can be observed universally in the wide range of higher plants. Since the sensitivity was higher in tobacco BY-2 cells, this cell line was chosen to be used in the further experiments examining the mode of  $\text{Al}^{3+}/\text{Zn}^{2+}$  interaction.

### Effect of Culture Age on $\text{O}_2^{\bullet-}$ Production

Prior to treatment with  $\text{Al}^{3+}$ , tobacco BY-2 cell suspension culture was aged for 1, 3, 5, 8, and 13 DAI of the fresh media (30 ml) with 0.5 ml of confluent culture (at 10 DAI). The cultures at 1 and 3 DAI were smooth and colorless. The 4 and 5 DAI cultures were also smooth but colored slightly yellowish. The 8 and 12 DAI cultures were highly dense and colored yellow. The growth of the culture was assessed by measuring the changes in fresh cell weight at each time point. Figure 4A shows a typical growth curve for tobacco BY-2 cell culture. Effect of culture age of tobacco BY-2 cells on the sensitivity to  $\text{Al}^{3+}$  was examined using the differently aged cultures (Figure 4B), and the high sensitivity to  $\text{Al}^{3+}$  was observed in 2 and 4 DAI of tobacco BY-2 cells.

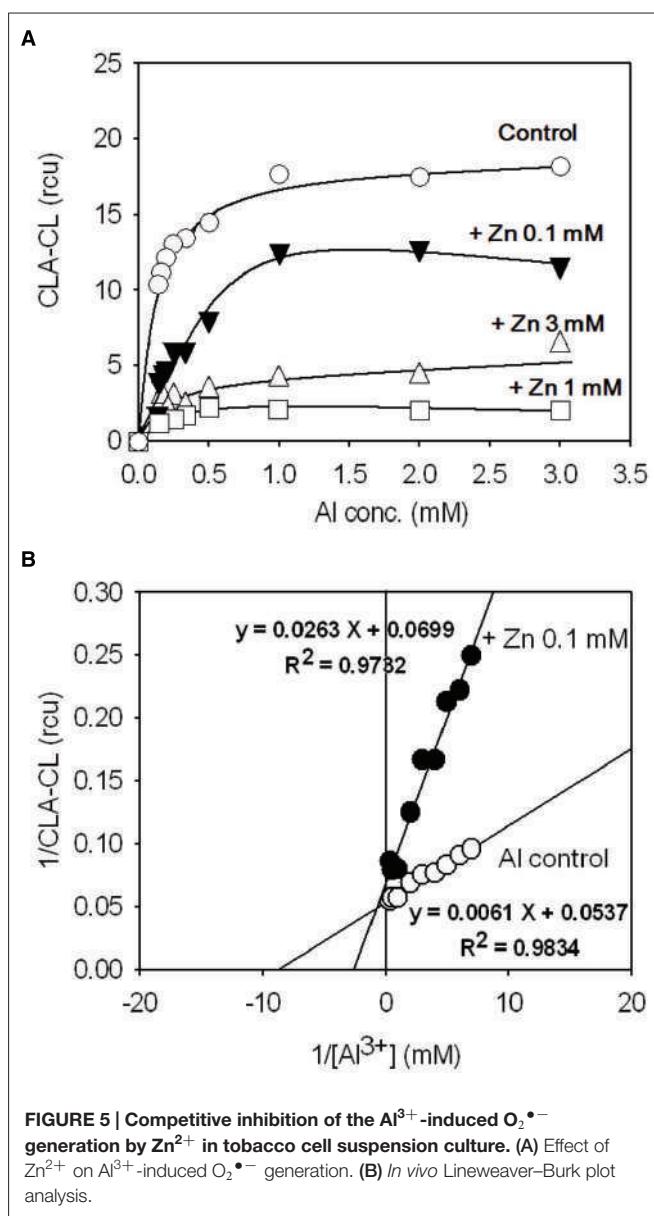
### Competition Between $\text{Zn}^{2+}$ and $\text{Al}^{3+}$

Application of double-reciprocal plot analysis for studying the behavior of living plants or cells, so-called *in vivo* Lineweaver-Burk plot analysis was carried out to assess the mode of  $\text{Al}^{3+}/\text{Zn}^{2+}$  interaction according to the procedure described elsewhere (Kawano et al., 2003b). By making use of linear dose-dependency in the limited range of  $\text{Al}^{3+}$  concentrations (up to 3 mM) in 4 DAI culture of tobacco BY-2 cell, the *in vivo* kinetic analysis was carried out by assuming  $\text{Al}^{3+}$  as a ligand to



the putative  $\text{Al}^{3+}$  receptors on the cells and  $\text{Zn}^{2+}$  as an inhibitor (Figure 5A). The reciprocals of the CLA-CL yields ( $1/\text{CLA-CL}$ ) were plotted against the reciprocals of  $\text{Al}^{3+}$  concentrations ( $1/[\text{Al}^{3+}]$ ). Linear relationship between  $1/\text{CLA-CL}$  and  $1/[\text{Al}^{3+}]$  were obtained both in the presence and absence of  $\text{Zn}^{2+}$  (Figure 5B). In the presence of  $\text{Zn}^{2+}$ , the apparent  $K_m$  for  $\text{Al}^{3+}$  was elevated from  $113 \mu\text{M}$  (control) to  $376 \mu\text{M}$  ( $0.1 \text{ mM Zn}^{2+}$ ; *ca.* 3.3-fold increase), while  $V_{max}$  for  $\text{Al}^{3+}$ -induced CLA-CL was not drastically altered.  $V_{max}$  for  $\text{Al}^{3+}$ -induced response in the absence of  $\text{Zn}^{2+}$  was calculated to be  $14.3 \text{ rcu}$ . In the presence of  $0.1 \text{ mM Zn}^{2+}$ ,  $V_{max}$  was  $18.6 \text{ rcu}$  (*ca.* 30% increase). Therefore, the mode of  $\text{Zn}^{2+}$  action against  $\text{Al}^{3+}$  can be considered as a typical competitive inhibition.

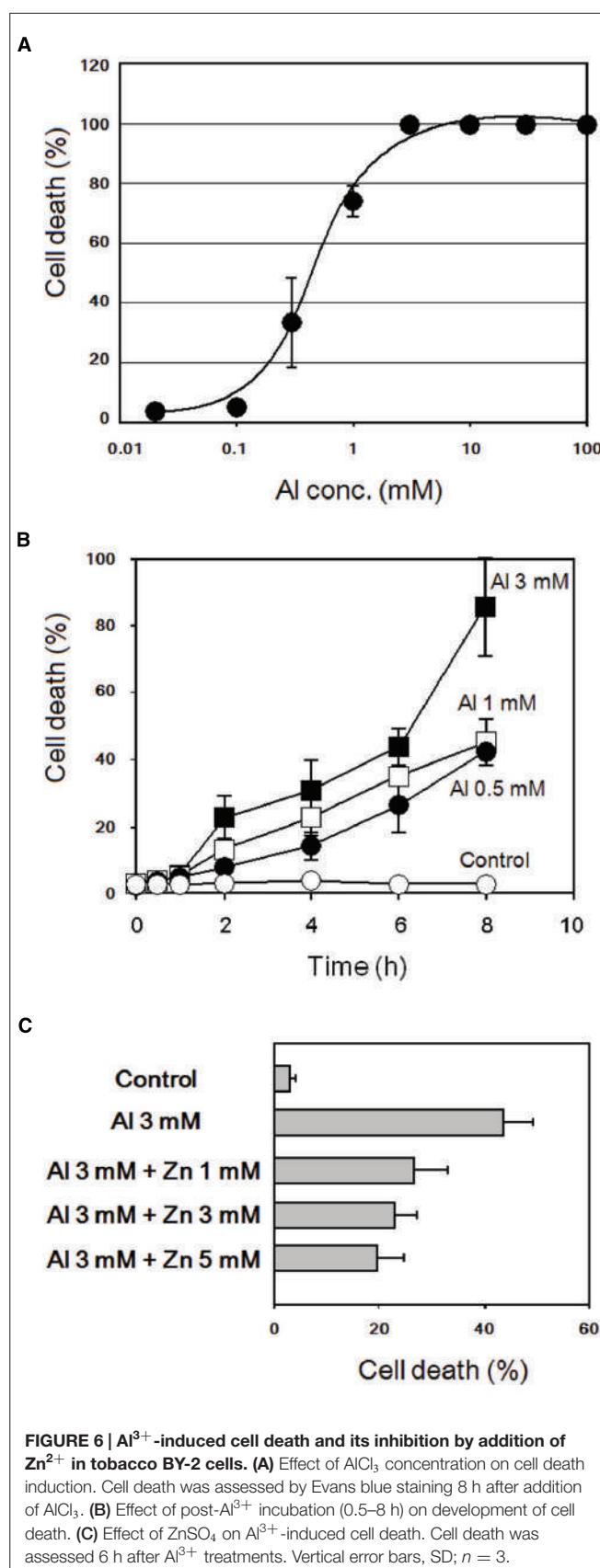
According to Kawano et al. (2003a) the  $\text{Al}^{3+}$ -induced generation of  $\text{O}_2^{\bullet-}$  in tobacco cells is catalyzed by  $\text{Al}^{3+}$ -stimulated NADPH oxidase which is sensitive to DPI. The cation-dependent enhancement in NADPH oxidase-catalyzed  $\text{O}_2^{\bullet-}$  production is also known in human neutrophils in which binding of metal cations possibly results in spontaneous activation of the  $\text{O}_2^{\bullet-}$ -generating activity of the membrane-bound enzyme (Cross et al., 1999). We can assume that



NADPH oxidase itself, localized on the surface of cells (or other factors associated with NADPH oxidase), behaves as the receptor for  $\text{Al}^{3+}$  ions. The competitive mode of  $\text{Zn}^{2+}$  action against the  $\text{Al}^{3+}$ -induced oxidative burst suggests us to consider that the binding site for  $\text{Al}^{3+}$  and  $\text{Zn}^{2+}$  on the NADPH oxidase or on the factors associated nearby must be identical.

### $\text{Al}^{3+}$ -Induced Cell Death and its Inhibition by $\text{Zn}^{2+}$

As shown in Figures 6A,B, treatment of tobacco BY-2 cells with various concentrations of  $\text{AlCl}_3$  resulted in cell death induction. Notably, the presence of  $\text{Zn}^{2+}$  significantly protected the cells from the induction of cell death by  $\text{Al}^{3+}$  (Figure 6C), as predicted by the action of  $\text{Zn}^{2+}$  against  $\text{Al}^{3+}$ -induced oxidative burst.



## Effect of Pretreatment with $\text{Mn}^{2+}$

Manganese is another micronutrient possibly protecting the living cells from oxidative damage (Ledig et al., 1991) and reportedly blocks the lanthanide-induced oxidative burst (Kawano et al., 2002). In fact,  $\text{Mn}^{2+}$  is often employed as a scavenger of  $\text{O}_2^{\bullet-}$  for preventing the biochemical reactions involving  $\text{O}_2^{\bullet-}$  (Momohara et al., 1990).

Therefore, we tested the effect of  $\text{MnSO}_4$  (up to 3 mM) for comparison. The results obtained suggested no inhibitory effect of  $\text{Mn}^{2+}$  against  $\text{Al}^{3+}$ -induced generation of  $\text{O}_2^{\bullet-}$ . Instead, low concentrations of  $\text{Mn}^{2+}$  slightly elevated the level of  $\text{Al}^{3+}$ -induced oxidative burst (data not shown). For inhibition of  $\text{Al}^{3+}$ -induced oxidative burst, much higher concentrations of  $\text{MnSO}_4$  (10–100 mM) were required. Since the range of  $\text{Mn}^{2+}$  concentrations required for lowering the level of  $\text{Al}^{3+}$ -induced generation of  $\text{O}_2^{\bullet-}$  was at phytotoxic range (Caldwell, 1989) and thus inducing cell death even in the absence of  $\text{Al}^{3+}$  in BY-2 cells (*ca.* 40% of cells died in the presence of 30 mM  $\text{MnSO}_4$ ), the use of  $\text{Mn}^{2+}$  is not suitable for preventing the production of  $\text{O}_2^{\bullet-}$  induced by  $\text{Al}^{3+}$ .

## Anti-Oxidative Role for $\text{Zn}^{2+}$

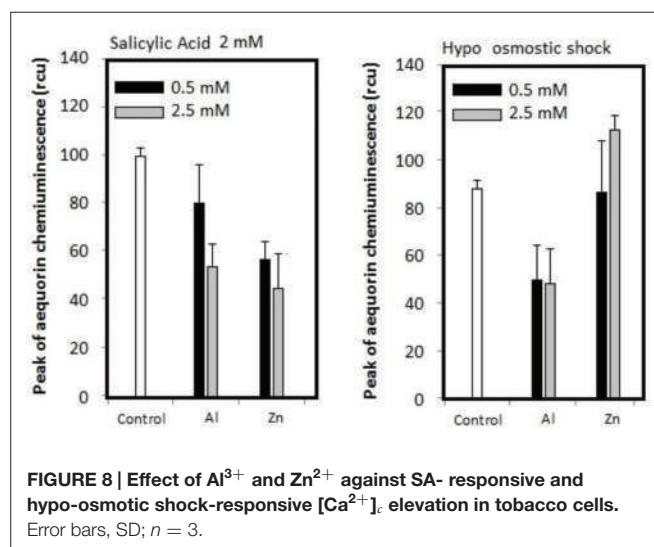
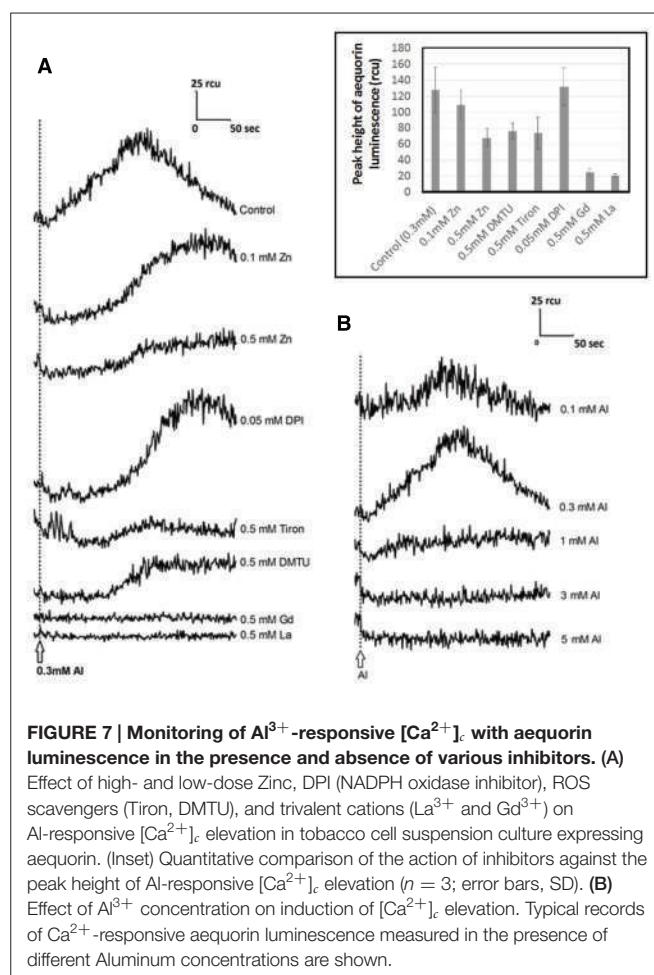
Plants require trace amounts of specific metals known as trace nutrients including  $\text{Zn}^{2+}$ , supporting the essential functions of plant cells ranging from respiration to photosynthesis, and molecular biological studies on the mechanism for uptake of these metals by plants have been documented (Delhaize, 1996). One of the important roles for  $\text{Zn}^{2+}$  in living plants is anti-oxidative action against ROS (Kawano et al., 2002) as the present study successfully demonstrated that extracellular supplementation of  $\text{Zn}^{2+}$  inhibits the generation of  $\text{O}_2^{\bullet-}$  (Figure 4) and cell death (Figure 6C) induced by  $\text{Al}^{3+}$ .

In contrast to manganese, zinc is normally present in plants at high concentrations. For example, in roots of wheat seedlings, the cytoplasmic concentration of total Zn has been estimated to be approximately 0.4 mM (Santa-Maria and Cogliatti, 1988), and Zn-deficiency often results in inhibition of growth, as Zn reportedly protects the plants by preventing the oxidative damages to DNA, membranes, phospholipids, chlorophylls, proteins, SH-containing enzymes, and indole-3-acetic acid (Cakmak, 2000).

Here,  $\text{Zn}^{2+}$  at sub-mM concentrations showed strong inhibitory action against the toxicity of  $\text{Al}^{3+}$  (oxidative burst and cell death). The levels of  $\text{Zn}^{2+}$  naturally present in soil or plant tissues may be contributing to the prevention of  $\text{Al}^{3+}$ -induced cellular damages but further studies on living plants are needed to evaluate this mechanism in living tissue and the possible applications to increase plant tolerance.

## Oxidative and Calcium Crosstalk

$\text{Al}^{3+}$  is known to inhibit plant calcium channels similarly to the action of various lanthanide ions (Lin et al., 2006b). The calcium channels sensitive to  $\text{Al}^{3+}$  could be identical to those involved in responses to ROS (Kawano et al., 2003a, 2004), cold shock (Lin et al., 2005, 2006a, 2007), and heat shock (Lin et al., 2007), but not responsive to osmotic shock (Lin et al., 2005, 2006b, 2007),



as examined in transgenic cell lines of rice (*Oryza sativa* L., cv. Nipponbare) and tobacco (cell-lines, BY-2, Bel-B, and Bel-W3) all expressing aequorin in the cytosolic space.

To support the hypothesis that  $\text{Al}^{3+}$ -induced distortion in  $[\text{Ca}^{2+}]_c$  involves the members of ROS derived from the action

of NADPH oxidase, and calcium channel opening leading to transient  $[\text{Ca}^{2+}]_c$  elevation, the effect of DPI (NADPH oxidase inhibitor), ROS scavengers (Tiron, DMTU), and trivalent cations ( $\text{La}^{3+}$  and  $\text{Gd}^{3+}$ ) have been tested in tobacco cells expressing aequorin and compared with the antagonistic action of zinc protecting the cells (Figure 7A and inset).

As expected, Tiron, DMTU, and high concentration of zinc (0.5 mM) effectively lowered the level of  $\text{Al}^{3+}$ -induced  $[\text{Ca}^{2+}]_c$  elevation. Especially, temporal patterns in which  $\text{Al}^{3+}$  induces an increase in  $[\text{Ca}^{2+}]_c$  was shown to be sensitive to both zinc and DPI. In fact, these chemicals significantly retarded the  $\text{Al}^{3+}$ -responsive calcium influx, thus, time required for attaining the peak of  $\text{Al}^{3+}$ -responsive  $[\text{Ca}^{2+}]_c$  elevation was shown to be longer, suggesting the zinc and DPI might share the common mode of action.

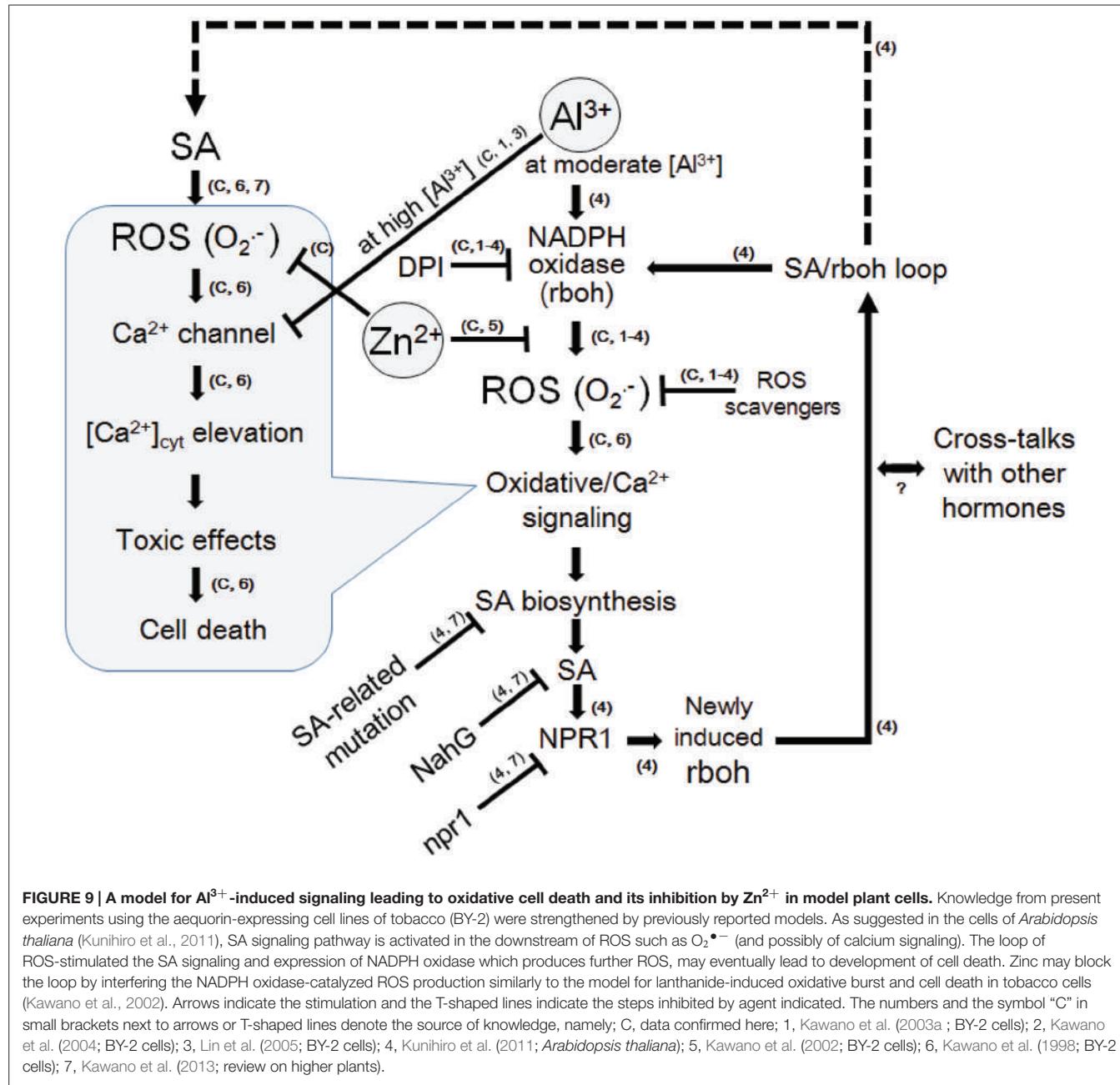
On the other hand,  $\text{La}^{3+}$  and  $\text{Gd}^{3+}$  strongly reduce the signal as we observed for high concentration of  $\text{Al}^{3+}$  (Figure 7A) supporting the view that they can concurrently act inhibiting the  $\text{Ca}^{2+}$  channel.

Previously, we have proposed a model that  $\text{Al}^{3+}$  plays dual roles acting for and against the  $\text{Ca}^{2+}$  influx, by releasing  $\text{O}_2^{\bullet-}$  and by inhibiting the  $\text{Ca}^{2+}$  channel(s), respectively (Kawano et al., 2003a).  $\text{Al}^{3+}$ -dependent distortion in calcium signaling in plant cells can be dissected into two opposing modes of  $\text{Al}^{3+}$  actions, viz., (i) stimulation of ROS-responsive calcium channels via induction of  $\text{O}_2^{\bullet-}$  and (ii) inhibition of calcium channels. At low  $\text{Al}^{3+}$  concentrations, the ROS-responsive  $\text{Ca}^{2+}$  influx potency is high but the driving force (due to ROS) is not sufficient. At high  $\text{Al}^{3+}$  concentrations, the  $\text{Ca}^{2+}$  influx-driving force is at sufficient level but the channel's  $\text{Ca}^{2+}$  permeability is low. This effect is shown in Figure 7B, where  $[\text{Ca}^{2+}]_c$  elevation could be manifested only in the range of  $\text{Al}^{3+}$  concentration in which the two opposing effects eventually compromise (Kawano et al., 2003a).  $\text{Zn}^{2+}$  hardly blocks the calcium influx in model plant cells unless the event of interest is dependent on the ROS generating events (Figure 8). Therefore, we view here that  $\text{Zn}^{2+}$  might target only the earlier phase of  $\text{Al}^{3+}$  action involved in induction of  $\text{O}_2^{\bullet-}$  as illustrated in Figure 9.

## The Likely Signaling Paths

In *Arabidopsis thaliana*,  $\text{Al}^{3+}$ -induced prolonged ROS generation requires the expression of *AtrbohD* coding for NADPH oxidase (Kunihiro et al., 2011). This work suggested that biosynthesis and signal transduction pathway for SA is involved in  $\text{Al}^{3+}$ -mediated oxidative burst since the  $\text{Al}^{3+}$ -induced *AtrbohD* expression and cell death were inhibited in the mutant and transgenic cell lines lacking SA biosynthesis, accumulation of SA, and SA-specific signaling components (*sid2*, *NahG* and *npr1*, respectively). It has been proposed that loop of SA signal transduction, involving the activity and further induction of NADPH oxidase, forms a signaling circuit enabling an amplification of SA-mediated signaling (Figure 9). This type of oxidative signal amplification was designated as SA/rboh loop (Kunihiro et al., 2011).

By analogy, there would be a similar mechanism in response to  $\text{Al}^{3+}$  in the cells of tobacco and rice since both the ROS production and cell death were commonly shown to be induced by  $\text{Al}^{3+}$  in these cells.



**FIGURE 9 |** A model for  $\text{Al}^{3+}$ -induced signaling leading to oxidative cell death and its inhibition by  $\text{Zn}^{2+}$  in model plant cells. Knowledge from present experiments using the aequorin-expressing cell lines of tobacco (BY-2) were strengthened by previously reported models. As suggested in the cells of *Arabidopsis thaliana* (Kunihiro et al., 2011), SA signaling pathway is activated in the downstream of ROS such as  $\text{O}_2^{\bullet-}$  (and possibly of calcium signaling). The loop of ROS-stimulated the SA signaling and expression of NADPH oxidase which produces further ROS, may eventually lead to development of cell death. Zinc may block the loop by interfering the NADPH oxidase-catalyzed ROS production similarly to the model for lanthanide-induced oxidative burst and cell death in tobacco cells (Kawano et al., 2002). Arrows indicate the stimulation and the T-shaped lines indicate the steps inhibited by agent indicated. The numbers and the symbol "C" in small brackets next to arrows or T-shaped lines denote the source of knowledge, namely; C, data confirmed here; 1, Kawano et al. (2003a ; BY-2 cells); 2, Kawano et al. (2004; BY-2 cells); 3, Lin et al. (2005; BY-2 cells); 4, Kunihiro et al. (2011; *Arabidopsis thaliana*); 5, Kawano et al. (2002; BY-2 cells); 6, Kawano et al. (1998; BY-2 cells); 7, Kawano et al. (2013; review on higher plants).

Lastly, we propose a likely mode of  $\text{Zn}^{2+}$  action against  $\text{Al}^{3+}$ -induced cell death.  $\text{Zn}^{2+}$  may competitively antagonize the action of  $\text{Al}^{3+}$  by targeting the NADPH oxidase-catalyzed ROS production at upstream of SA signaling mechanism. As a consequence, activation of SA/rboh loop responsible for long-lasting oxidative burst releasing cytotoxic ROS could be prevented (Figure 9).

By assessing the action of  $\text{Zn}^{2+}$  against SA-induced  $[\text{Ca}^{2+}]_{\text{cyt}}$  elevation which is known to be one of the key events in the SA-induced  $\text{O}_2^{\bullet-}$ -mediated signaling path, involving the activation of  $\text{Ca}^{2+}$  channel identified as TPC1 channel (Kawano et al., 2013; Lin et al., 2005), we understood that target of antioxidant activity of zinc is not limited to the

$\text{Al}^{3+}$ -induced NADPH oxidase-catalyzed mechanism (Figure 8). It is known that SA-induced rapid  $\text{O}_2^{\bullet-}$  is catalyzed by extracellular (cell-wall bound) peroxidase, while SA-induced long-lasting oxidative burst requires the induction of rboh genes coding for NADPH oxidase (Kawano et al., 1998; Yoshioka et al., 2008). In contrast,  $\text{Zn}^{2+}$  failed to block the  $\text{Ca}^{2+}$  influx induced by hypo-osmotic shock possibly involving the mechanosensitive-cation channel (Takahashi et al., 1997).

Taken together, target of  $\text{Zn}^{2+}$  is specifically against the ROS-generating mechanisms (both NADPH oxidase-mediated and peroxidase-mediated) eventually leading to ROS-responsive calcium signaling (Figure 9).

Furthermore, the action of  $\text{Al}^{3+}$  may form a loop of repeated reaction involving the action of SA which further induces specific type of NADPH oxidase (in case of *Arabidopsis thaliana*, only *AtrboHD* is  $\text{Al}^{3+}$ -responsive, Kunihiro et al., 2011).

## AUTHOR CONTRIBUTIONS

TK designed and supervised the experiments and some key data for plant age and ROS production were obtained by him. CL conducted most tobacco experiments (mostly calcium signaling and cell viability tests), AH and DC performed additional

experiments. AH was in charge of rice cell experiments (both ROS detection and calcium signaling). DC and FB contributed on the analysis of data and writing of MS. All authors actively contributed in the discussion.

## ACKNOWLEDGMENT

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# Endogenous Cytokinin Overproduction Modulates ROS Homeostasis and Decreases Salt Stress Resistance in *Arabidopsis Thaliana*

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Cytokinins in plants are crucial for numerous biological processes, including seed germination, cell division and differentiation, floral initiation and adaptation to abiotic stresses. The salt stress can promote reactive oxygen species (ROS) production in plants which are highly toxic and ultimately results in oxidative stress. However, the correlation between endogenous cytokinin production and ROS homeostasis in responding to salt stress is poorly understood. In this study, we analyzed the correlation of overexpressing the cytokinin biosynthetic gene *AtIPT8* (adenosine phosphate-isopentenyl transferase 8) and the response of salt stress in *Arabidopsis*. Overproduction of cytokinins, which was resulted by the inducible overexpression of *AtIPT8*, significantly inhibited the primary root growth and true leaf emergence, especially under the conditions of exogenous salt, glucose and mannitol treatments. Upon cytokinin overproduction, the salt stress resistance was declined, and resulted in less survival rates and chlorophyll content. Interestingly, ROS production was obviously increased with the salt treatment, accompanied by endogenously overproduced cytokinins. The activities of catalase (CAT) and superoxide dismutase (SOD), which are responsible for scavenging ROS, were also affected. Transcription profiling revealed that the differential expressions of ROS-producing and scavenging related genes, the photosynthesis-related genes and stress responsive genes were existed in transgenic plants of overproducing cytokinins. Our results suggested that broken in the homeostasis of cytokinins in plant cells could modulate the salt stress responses through a ROS-mediated regulation in *Arabidopsis*.

**Keywords:** cytokinin overproduction, *AtIPT8*, ROS homeostasis, salt stress, chlorophyll, transcriptomic analysis

## INTRODUCTION

Cytokinins play important and complex roles in plant growth and abiotic stress responses (Wang et al., 2011; Ha et al., 2012; Hwang et al., 2012; Zwack and Rashotte, 2015). Numerous evidences indicate that cytokinins have both positive and negative effects on stress tolerance. Many studies have reported that, in response to extended stress, the concentrations of cytokinins were decreased

in plants (Kudoyarova et al., 2007; Ghanem et al., 2008; Merewitz et al., 2011; Nishiyama et al., 2011). Contrarily, both short-term and sustained increase in cytokinin levels may also occur in plants while encountering severe stress conditions (Pospisilova et al., 2005; Alvarez et al., 2008; Dobra et al., 2010). Cytokinin biosynthesis genes *IPTs* (adenosine phosphate-isopentenyl transferases) can be up-regulated by NaCl treatment, and the deficiency in cytokinin biosynthesis may result in a strong salt-tolerant phenotype (Nishiyama et al., 2011). Many studies have examined the effects of exogenous cytokinin applications in abiotic stress responses. Exogenously supplied cytokinins not only can improve salt tolerance in young wheat seedlings, but also can result in more susceptible phenotype to the salt treatment in beans (Kirkham et al., 1974; Abdullah and Ahmad, 1990). After cytokinin application, the *Arabidopsis* plants are of higher survival ability when they are exposed to freezing or dehydrated conditions (Jones et al., 2010; Kang et al., 2012). The effects of changed endogenous cytokinin levels in transgenic plants overexpressing cytokinin biosynthesis genes (*IPTs*), or cytokinin degraded genes (*CKXs*), are demonstrated. Overproduction of endogenous cytokinins enhances drought stress tolerance. However, decrease in cytokinin levels produce a positive consequence in drought tolerance (Rivero et al., 2007; Werner et al., 2010; Qin et al., 2011; Macková et al., 2013).

The components of cytokinin signaling also play complex roles in responses to abiotic stresses. For instance, *Arabidopsis* AHK1, the histidine kinase 1 of cytokinin signaling, plays as a positive regulator in the responses of drought and salt stresses. The loss-of-function mutations, such as *ahk2*, *ahk3*, and *ahk2 ahk3* are of strong tolerance to drought and salt stresses (Tran et al., 2007; Wohlbach et al., 2008; Kumar et al., 2013). AHPs (histidine phosphotransfer proteins) are involved in regulating the responses to drought stress in a negative and redundant manner (Hutchison et al., 2006; Hwang et al., 2012; Nishiyama et al., 2013). The resistant to salt stress phenotype is reported in studying the quadruple loss-of-function mutant *arr3arr4arr5arr6* (Mason et al., 2010). Collectively, all these studies suggest the impact of cytokinin metabolism and signaling in the stress responses in intricate manners.

The reactive oxygen species (ROS) such as hydrogen peroxide ( $H_2O_2$ ), superoxide radical ( $O_2^-$ ), and hydroxyl radical ( $OH^-$ ), all can be induced by drought, salt, and low temperature conditions (Sharma et al., 2012; Choudhury et al., 2013; Petrov et al., 2015). To detoxify, plants have evolved ROS scavenging systems that involve in enzymic and non-enzymic antioxidants. The major antioxidant enzymes include superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and glutathione peroxidase (GPX). SOD converts superoxide into  $H_2O_2$ , while APX, GPX, and CAT detoxify  $H_2O_2$  (Mittler, 2002; Apel and Hirt, 2004; Das and Roychoudhury, 2014). The cross-talk between the cytokinin signaling and ROS production and scavenging systems is demonstrated in *Arabidopsis*. In cytokinin-deficient mutant *ipt1,3,5,7*, the genes involving in ROS breakdown are greatly affected (Nishiyama et al., 2012). The treatment of N(6)-benzyladenine (6-BA) induces massive production of ROS, eventually, results in a loss of cell viability in tobacco BY-2 cells (Mlejnek et al., 2003). Exogenous applications

of cytokinins lead to increasing in APX and CAT activities during dark-induced senescence (Zavaleta-Mancera et al., 2007), as well as raise of SOD and CAT activities after heat stress (Liu and Huang, 2002). In overexpressing CKX transgenic *Arabidopsis* lines, declined cytokinin levels may cause alterations in activities of antioxidants, while responding to abiotic stresses (Mýtinová et al., 2010; Lubovská et al., 2014). Hence, the impact of cytokinins on ROS homeostasis in plants responding to environmental stresses is imperative.

To in-depth study the correlation between endogenous cytokinin levels and ROS homeostasis in plants responding to abiotic stresses especially to the salt stress, we analyzed the inducible transgenic line overexpressing *AtIPT8*, a cytokinin biosynthesis gene. The results indicated that endogenous cytokinin overproduction, which was promoted by *AtIPT8* overexpression, resulted in enhanced-sensitive phenotype to the salt treatment. Dependent on salt treatment, the ROS contents were strongly increased in plants of overproducing cytokinin; and, the activities of antioxidants and the contents of total chlorophyll were significantly declined with comparing to those in the wild-type (Col). Moreover, many genes involving in photosynthesis and abiotic stress responses were differentially expressed in plants of overexpressing *AtIPT8*. In this study, we provided evidences in that overproduction of endogenous cytokinin could decrease salt resistance, through modulating endogenous ROS homeostasis in *Arabidopsis*.

## MATERIALS AND METHODS

### Plant Materials and Growth Conditions

*Arabidopsis thaliana* ecotype Columbia (Col) was used in this study as wild-type control. Seeds were surface sterilized and sown on Murashige and Skoog (MS) agar plates containing full-strength MS salts, 0.8% (w/v) agar, and 1% (w/v) sucrose. The 17-β-estradiol (Sigma-Aldrich, E8875) was dissolved in DMSO (Dimethyl Sulfoxide) and used in this study. The seeds were stratified in darkness at 4°C for 4 days and then transferred to growth chamber with 16 h/8 h light/dark cycle at 23°C, or were directly sown in soil after stratification under the same conditions. Overexpressing *AtIPT8* transgenic plants (OE) were generated in Col-0 background as described by Wang et al. (2011). The homozygous T4 transgenic lines were used in this study.

### Cytokinins Extraction and Quantification

Cytokinins were extracted and purified from 2 g of 2-week-old seedlings which were induced with 17-β-estradiol (10 μM) for 24 h (Wang et al., 2011). The extraction procedure was performed according to methods described in previous reports (Åstot et al., 2000; Dobrev and Kamínek, 2002; Hoyerová et al., 2006). The internal standards of Deuterium-labeled cytokinin (Olchemim, Czech) were added to the extraction buffer (100 ng per sample). Detection and quantification of cytokinins were performed with HPLC-MS system (Agilent 1200 series HPLC, Agilent Technologies, Palo Alto, CA, USA; AB 3200 Q trap MS/MS, Applied Biosystems, USA).

## Comparisons of Root Growth, Survival Rates and Chlorophyll Contents

To compare the primary root growth under various stress treatments, seeds were respectively sown on MS agar plates supplied with NaCl (100 mM), glucose (300 mM) and D-mannitol (300 mM). 17- $\beta$ -estradiol (10  $\mu$ M) or DMSO (mock) was added to the plates. After stratified at 4°C for 4 days, the plates were transferred to growth chamber and placed vertically. The primary root length was measured at the 10 days after transferring. For salt resistance treatment, 5-day-old seedlings grown on MS plates were transferred to fresh salt-containing MS plates, and then calculated the survival rates after 10 days treatment. The seedlings after survival rates calculation were collected and used for chlorophyll contents determination. Total chlorophyll was extracted in 85% acetone as described by Porra et al. (1989). The contents of chlorophyll were determined at settings of 639 nm and 645 nm, respectively with spectrophotometer. All experiments were performed three times independently.

## Determination of ROS Production and Antioxidant Enzymes Activities

Reactive oxygen species production was detected in roots and cotyledons using dichlorofluorescein (DCF; Foreman et al., 2003). The 5-day-old seedlings were treated with 100 mM NaCl plus or minus 17- $\beta$ -estradiol in plates. After treatment, the seedlings were incubated with 20  $\mu$ M DCF. To detect the DCF fluorescent signals, images were acquired with confocal laser scanning microscopy (TCS SP8, Leica, Germany) under 488 nm excitation and 525 nm emission. Fluorescence intensity was quantified using LAS AF software. Quantification of H<sub>2</sub>O<sub>2</sub> content was determined using the method described by Hu et al. (2012). Ten-day-old seedlings were pre-treated with 100 mM NaCl plus or minus 17- $\beta$ -estradiol in plates. H<sub>2</sub>O<sub>2</sub> content and activity of antioxidant enzymes were measured after salt treatments. The detailed procedure has been described by Wang et al. (2013).

## Gene Expression Analysis by Microarray and Quantitative Real-time RT-PCR (qRT-PCR)

For microarray analysis, 10-day-old plants of Col and *AtIPT8*-OE were pre-treated with 17- $\beta$ -estradiol (10  $\mu$ M) or DMSO for 24 h, respectively. Afterward, the seedlings were collected for total RNA extraction and transcriptomic analysis. The detailed procedure has been described by Wang et al. (2011). To confirm the expression patterns of differentially expressed genes obtained from microarray analysis, qRT-PCR was employed after the seedlings pretreated with or without 17- $\beta$ -estradiol. Total RNA was extracted using a plant RNA purification kit (Tiangen, catalog number #DP432<sup>1</sup>). Equal amounts of RNA were used for reverse transcription with ReverTra Ace- $\alpha$ -TM (TOYOBO, catalog number FSK-100<sup>2</sup>) according to the manufacturer's

instructions. The primers used in real-time quantitative RT-PCR were designed by web tool<sup>3</sup>. The primers used for qRT-PCR experiment are listed in Supplementary Table S1.

## RESULTS

### Induced-overexpression of *AtIPT8* Resulted in Endogenous Cytokinin Overproduction

Due to the lethality caused by constitutively overexpressed *AtIPT8* in plants, we generated transgenic plants with estradiol-inducible overexpression of *AtIPT8*, and the line *AtIPT8*-OE was selected (Wang et al., 2011) for further analysis in this study. First, we examined the relative expression levels of *AtIPT8* in transgenic plants using methods of semi-quantitative RT-PCR and qRT-PCR (Figures 1A,B). The results showed that expression level of *AtIPT8* gene was induced more than 40-fold higher upon estradiol induction (Figure 1B). To examine the effect of *AtIPT8* on the production of endogenous cytokinins, the total cytokinin contents were quantified in plants of Col and *AtIPT8*-OE plants. Upon estradiol induction, the contents of iP and iP9G (iP-type) were increased, more than 100-fold in *AtIPT8*-OE plants than that in Col plants. Moreover, the concentrations of tZ, ZR and ZRMP (Z-type) cytokinins were also elevated more than 10-fold in *AtIPT8*-OE plants (Figure 1C). Thus, the quantitative analysis on cytokinin contents indicated that inducer-dependent activation of *AtIPT8* could lead to elevation of cytokinin contents in *Arabidopsis*.

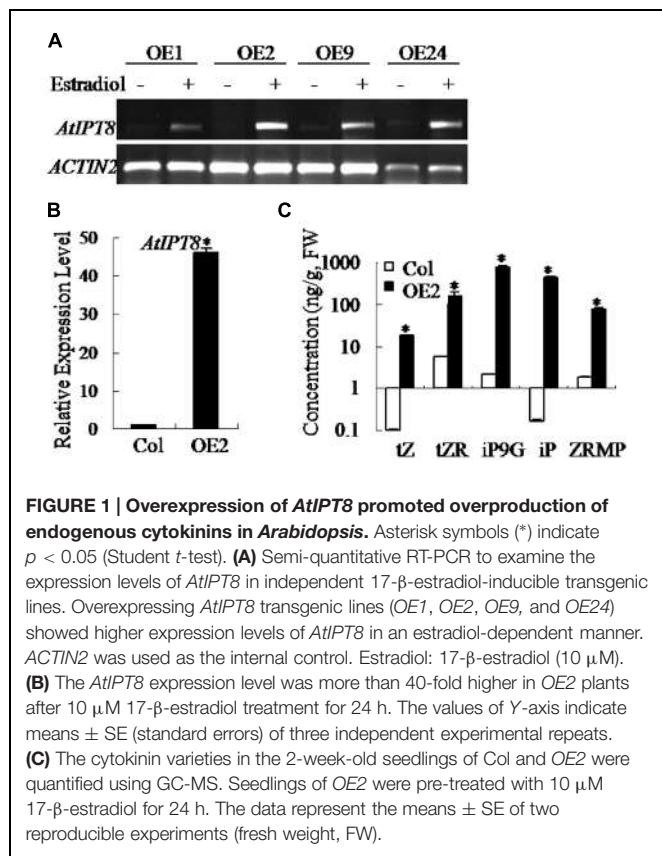
### Endogenous Cytokinin Overproduction Modulated Salt Stress Responses

To determine whether the endogenous cytokinin levels could affect the abiotic stress responses, we compared the stress resistant phenotypes between Col and *AtIPT8*-OE plants in treatments of NaCl, glucose and mannitol. Seeds were germinated on the freshly prepared MS plates containing NaCl, glucose and mannitol, and then, the fresh weight and primary root length were analyzed after 10 days of treatments. The results showed that cytokinin overproduction limited plant growth (Figure 2A, upper panel). Significantly, combining with salt and osmotic stress conditions, the growth of roots and true leaves was inhibited (Figure 2A). The fresh weight of plants was obviously decreased in the same stress treatments (Figure 2B). Furthermore, we examined the primary root length. The severe effect on root growth was observed with *AtIPT8*-OE plants that were treated by estradiol and NaCl or glucose or mannitol (Figure 2C). Interestingly, the most obvious inhibitory effect in the growth of primary roots was showed in the treatment of glucose (300 mM) (Figure 2C). The inhibitory effect in primary root growth by cytokinin overproduction was rescued by exogenously addition of auxin (IAA). Application of 2,4-D could trigger more callus generation in *AtIPT8*-OE plants (Supplementary Figure S1).

<sup>1</sup><http://tiangen.biomart.cn>

<sup>2</sup><http://www.toyobo-global.com>

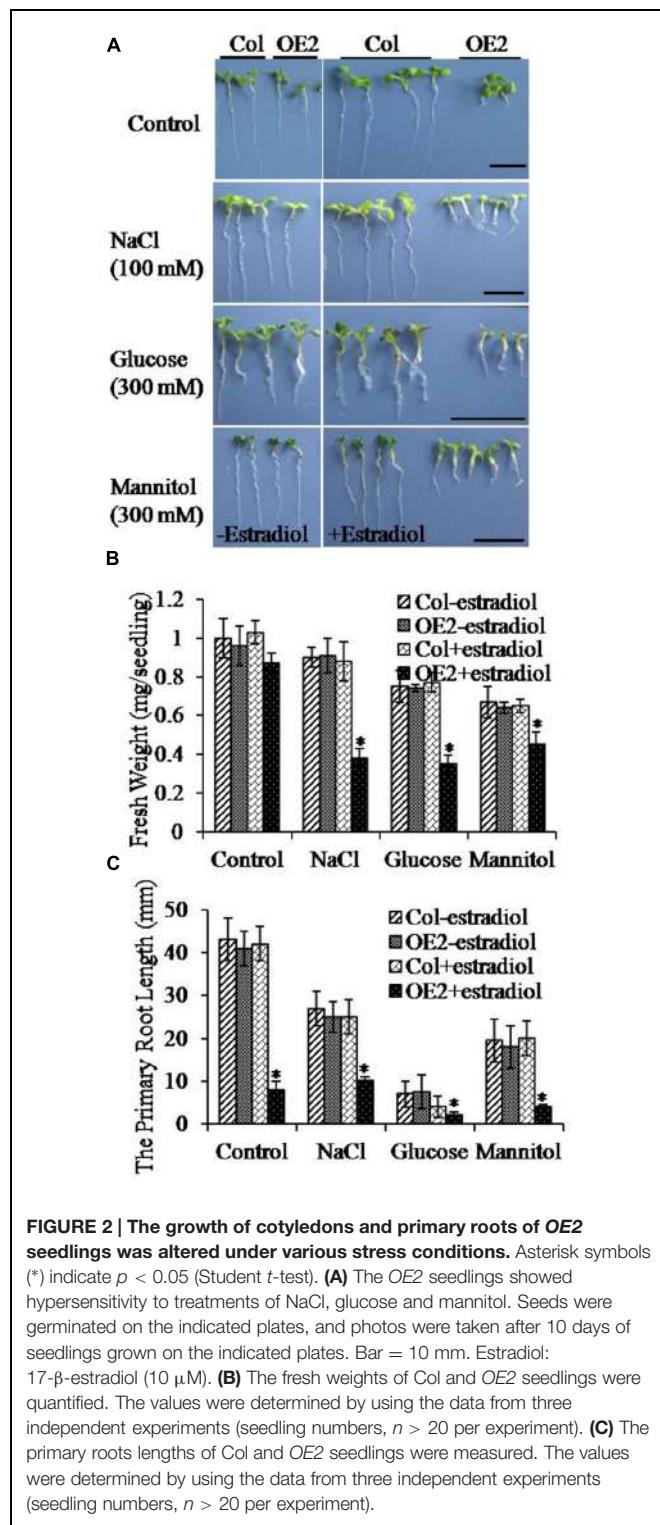
<sup>3</sup><https://www.genscript.com/ssl-bin/app/primer>



Together, these results suggested that cytokinin overproduction may reduce plant's tolerance in salt and osmotic stress conditions.

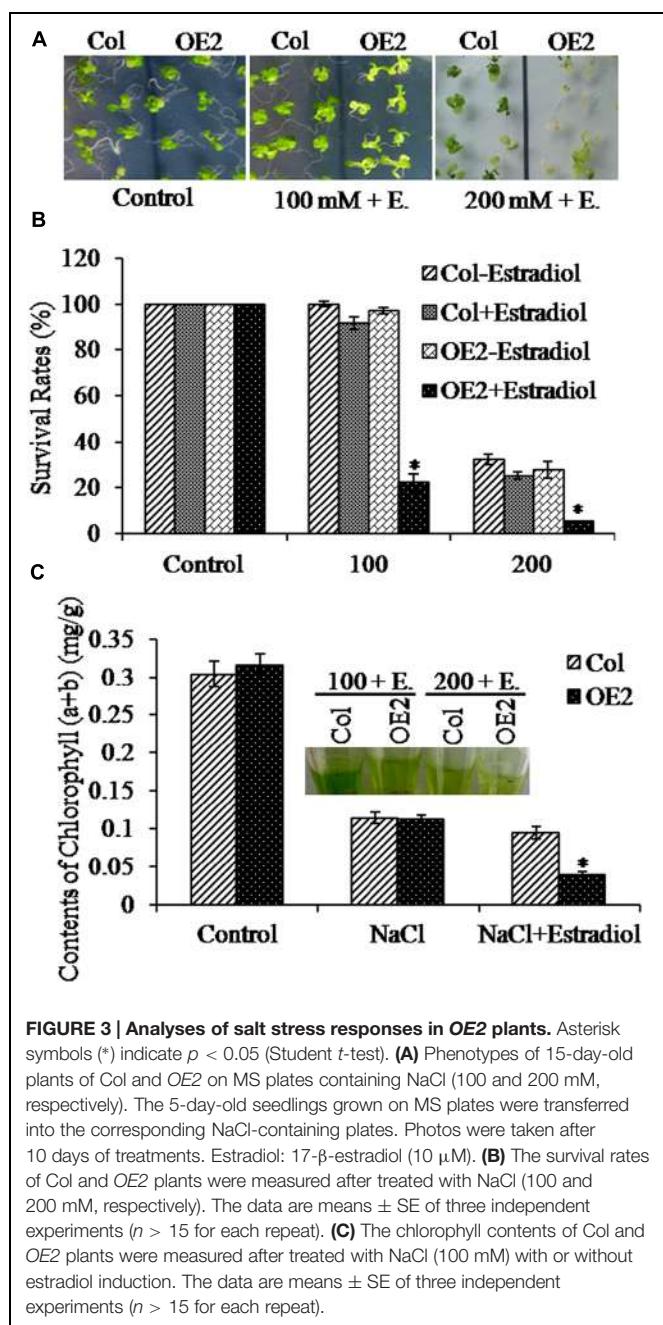
## Endogenous Cytokinin Overproduction Decreased Salt Stress Resistance

To further analyze the salt stress response with overproduced cytokinins in plants, we transferred 5-day-old seedlings to the MS plates containing NaCl, and treated for 10 days. We observed that, after estradiol induction, *AtIPT8-OE* plants appeared more sensitive to the treatments of NaCl (Figure 3A). Then, we measured the relative survival rates under the conditions of the NaCl treatment. Results showed that, lesser survival rates were scored with *AtIPT8-OE* plants which were induced by estradiol and treated with NaCl; without estradiol induction, no obvious differences, in terms of survival rates, were obtained in plants of Col and *AtIPT8-OE* (Figure 3B). The chlorophyll contents are usually used to evaluate the tolerance of plants after stress treatments (Tanaka et al., 2011). Therefore, we measured the total chlorophyll contents in the seedlings. We obtained that decrease in chlorophyll contents caused by NaCl treatments were showing in plants of both Col and *AtIPT8-OE*, more than two-fold decrease in chlorophyll contents was scored with *AtIPT8-OE* plants followed by estradiol induction (Figure 3C). Collectively, these results suggested that overproduction of endogenous cytokinins might play a negative effect on surviving in the salinity condition.

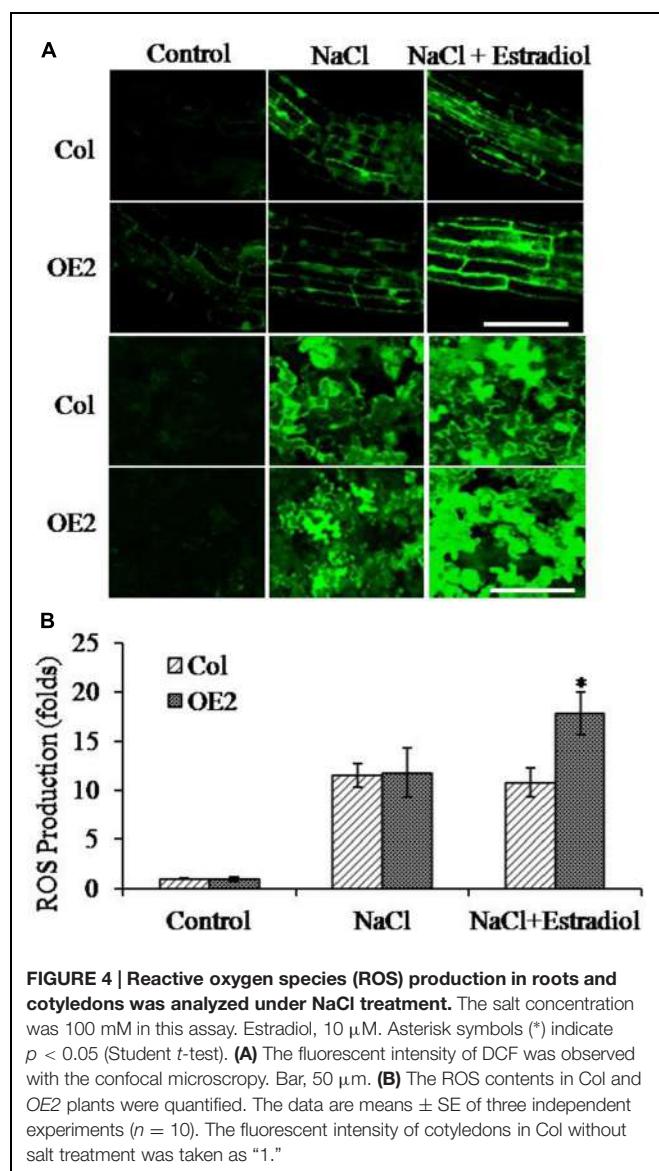


## Endogenous Cytokinin Overproduction Increased ROS Accumulation

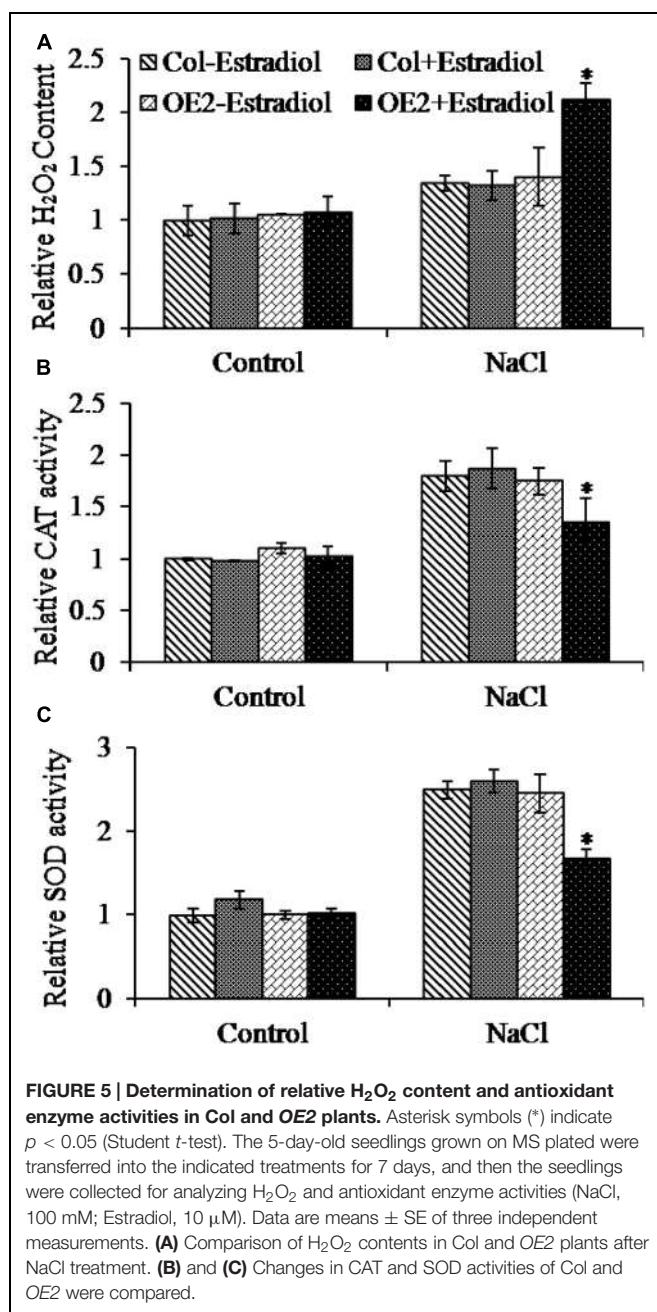
Salt stress triggers the accumulation of intracellular ROS (Das and Roychoudhury, 2014). To investigate the correlation of cytokinin overproduction and ROS homeostasis in plant cells, we



pretreated the seedlings with NaCl and then analyzed the ROS production by quantifying DCF fluorescent intensity. The ROS levels were compared in roots and cotyledons between Col and *AtIPT8-OE* plants. As shown in the results, the NaCl-treatment could promote ROS generation in roots and cotyledons of Col and *AtIPT8-OE* plants (Figure 4A). Moreover, the relative salt-induced ROS levels were significantly increased after estradiol-dependent cytokinin overproduction in all of the detected roots and cotyledons (Figure 4A). The ROS levels increased more than 10-fold without estradiol induction after salt treatment, however, after estradiol induction, the relative ROS contents were extensively increased about 18-fold in *AtIPT8-OE* plants;



whereas there was only 11-fold increased in Col plants when compared with the control treatment (Figure 4B). We also examined the effect of exogenous application of 6-BA on ROS production in Col plants. The results indicated that exogenous cytokinin could promote ROS generation under the condition of salt treatment (Supplementary Figure S2). To further determine the characteristic of ROS, we examined the contents of hydrogen peroxide H<sub>2</sub>O<sub>2</sub>. As shown in the results, H<sub>2</sub>O<sub>2</sub> contents were obviously increased in *AtIPT8-OE* plants under the conditions of estradiol-induction and salt treatment (Figure 5A). To assess the effect of cytokinin overproduction on ROS-scavenging capacity, the major antioxidant enzymes activities of CAT and SOD were compared between Col and *AtIPT8-OE* plants. As the results, the activities of CAT and SOD increased about 1.8-fold and 2.5-fold, respectively, after salt treatment in Col and *AtIPT8-OE* plant without estradiol induction. However, after estradiol application the activities of CAT and SOD showed only 1.2-fold



**FIGURE 5 | Determination of relative H<sub>2</sub>O<sub>2</sub> content and antioxidant enzyme activities in Col and OE2 plants.** Asterisk symbols (\*) indicate  $p < 0.05$  (Student *t*-test). The 5-day-old seedlings grown on MS plated were transferred into the indicated treatments for 7 days, and then the seedlings were collected for analyzing H<sub>2</sub>O<sub>2</sub> and antioxidant enzyme activities (NaCl, 100 mM; Estradiol, 10  $\mu$ M). Data are means  $\pm$  SE of three independent measurements. (A) Comparison of H<sub>2</sub>O<sub>2</sub> contents in Col and OE2 plants after NaCl treatment. (B) and (C) Changes in CAT and SOD activities of Col and OE2 were compared.

and 1.7-fold increase in *AtIPT8-OE* plants (Figures 5B,C). These results suggested that the weakened performance of *AtIPT8-OE* plants against salt stress was due to elevated ROS production and declined SOD and CAT activities.

### Transcriptomic Analysis on the Effect of Endogenous Cytokinin Overproduction

To assess the transcriptomic changes which might have been affected by endogenous cytokinin overproduction in *AtIPT8-OE* plants, we conducted the microarray analysis to analyze the potential genes with differential expression levels in Col and *AtIPT8-OE* plants. Ten-day-old seedlings were

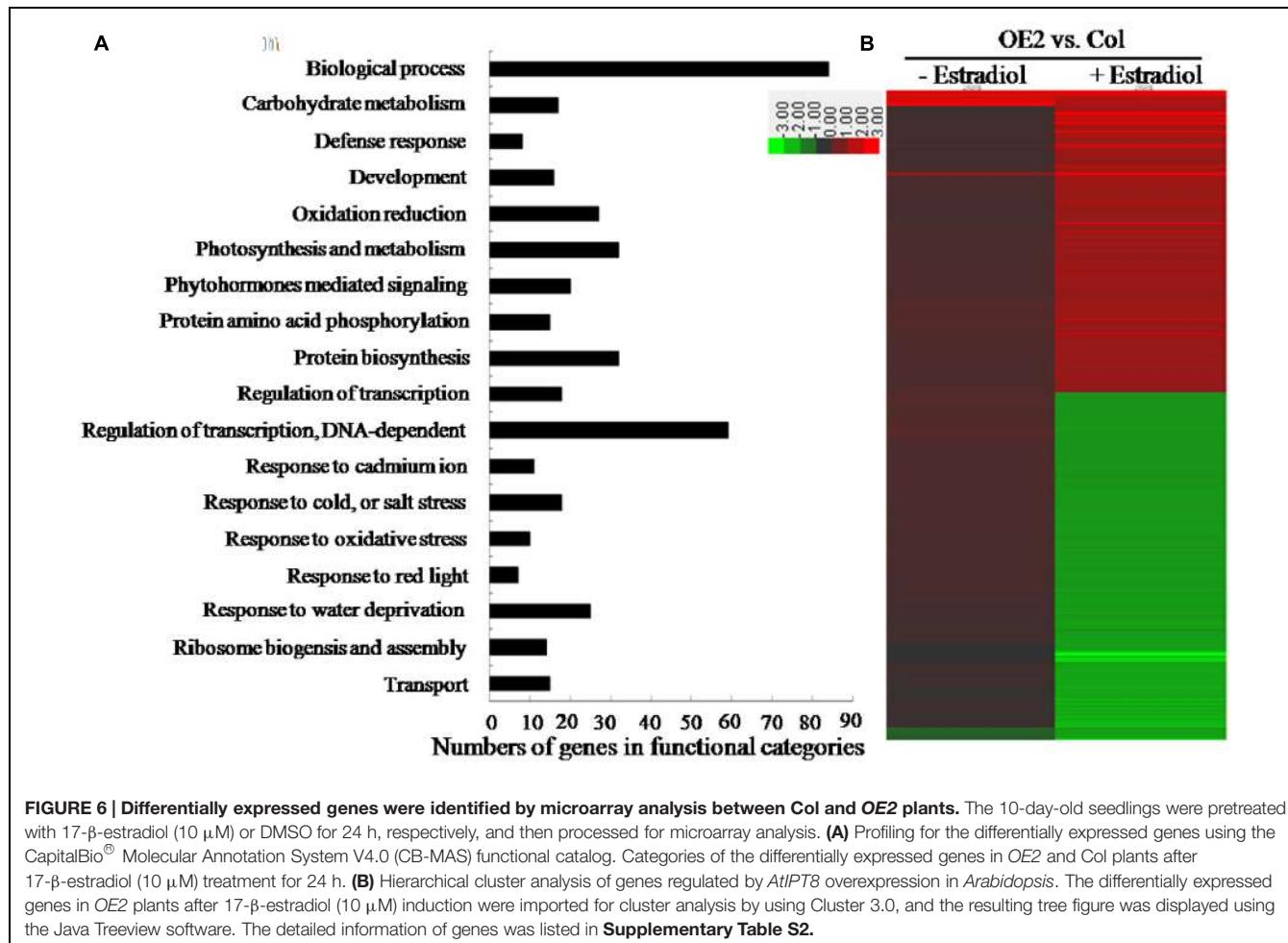
pre-treated with or without estradiol for 24 h, and then total RNA were extracted for microarray analysis. A Two-Way *Arabidopsis* Genome Array (CapitalBio Corp.<sup>4</sup>) was used in this study (Patterson et al., 2006; Wang et al., 2011). Upon estradiol-induction, 425 genes exhibited more than twofold changes in the transcription levels between Col and *AtIPT8-OE* plants (Figure 6A; Supplementary Table S2) (Wang et al., 2011). Functional categorization of the differentially expressed genes revealed that cytokinin overproduction affected the expression of many genes involving in biological process, carbohydrate metabolisms, photosynthesis, transcription regulations and abiotic stress responses (Figure 6A). Detailed functional categorization indicated that many differentially expressed genes were the members which could be involved in responding to various stresses, such as the defense responses, oxidation reductions, cold, salt and water deprivation responses. Hierarchical cluster analysis on genes regulated by cytokinin overproduction in *Arabidopsis* indicated that 197 genes were up-regulated and 228 genes were down-regulated in *AtIPT8-OE* plant, when compared with Col control after estradiol-induction (Figure 6B). Among them, many ABA- and abiotic stress-related genes might be affected by overproducing cytokinin (Wang et al., 2011). To rule out the effect of cytokinin overproduction in ROS generation/signaling and salt stress response, we compared the differentially expressed genes by cytokinin with salt- and oxidative-regulated genes, which were downloaded from the public microarray data<sup>5</sup>. Interestingly, among 425 differentially expressed genes in *AtIPT8-OE* plants dependent upon estradiol induction (Figure 6A; Supplementary Table S2), only 406 genes could be found in the data from the transcriptomic database (Supplementary Figure S3A; Supplementary Table S2). There were 104 genes with significant changes (folds  $\geq 2.0$ ) after treated by cytokinin and salt, respectively; and among of them, 40 genes were up-regulated and 64 genes were down-regulated. Forty-two genes were co-regulated by both of cytokinin and oxidative stress, and among of them 32 genes were up-regulated (Supplementary Figures S3B,C). Only 25 genes have been co-regulated by all of the cytokinin overproduction, salt and oxidative stresses treatment (Supplementary Figure S3B).

### Transcriptional Alterations of ROS-scavenging and -production Related-genes by Endogenous Cytokinin Overproduction and Salt Stress

Next, we selected some genes which were responsible for ROS-production and -scavenging for follow-up qRT-PCR analyses. Ten-day-old seedlings were pretreated with or without estradiol for 24 h, and then treated with NaCl for 3 h. Because *RbohD*, *RbohF*, and *RbohJ* are responsible for fine tuning the control of ROS production, we attested their expression levels. As shown in the results, three examined *Rboh* genes could be up-regulated by NaCl-treatment either in Col or in *AtIPT8-OE* plants; the significantly enhanced expression levels of these three *Rboh* genes

<sup>4</sup><http://www.capitalbio.com>

<sup>5</sup><http://bar.utoronto.ca/welcome.htm>

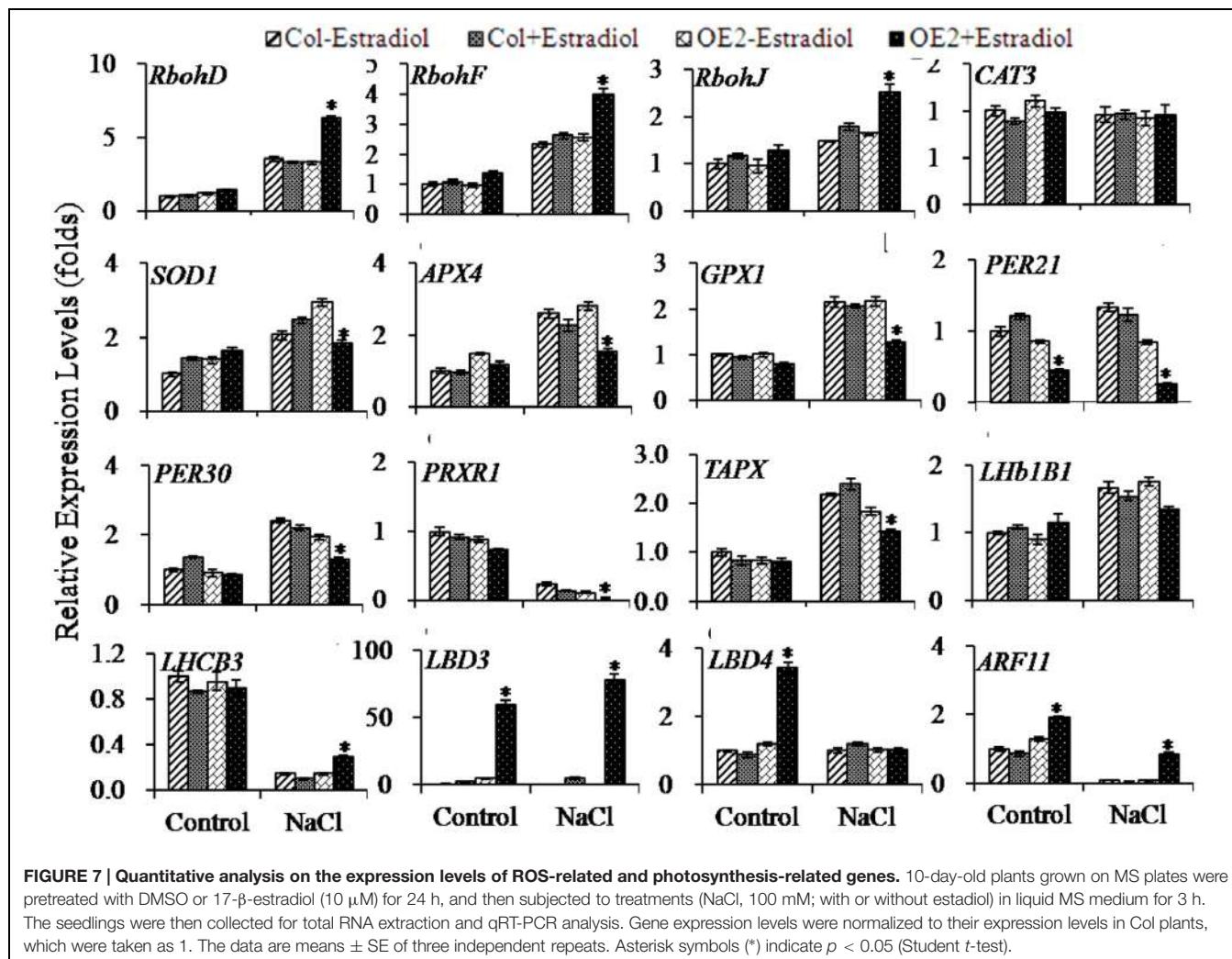


**FIGURE 6 |** Differentially expressed genes were identified by microarray analysis between Col and OE2 plants. The 10-day-old seedlings were pretreated with 17- $\beta$ -estradiol (10  $\mu$ M) or DMSO for 24 h, respectively, and then processed for microarray analysis. **(A)** Profiling for the differentially expressed genes using the CapitalBio® Molecular Annotation System V4.0 (CB-MAS) functional catalog. Categories of the differentially expressed genes in OE2 and Col plants after 17- $\beta$ -estradiol (10  $\mu$ M) treatment for 24 h. **(B)** Hierarchical cluster analysis of genes regulated by AtIPT8 overexpression in *Arabidopsis*. The differentially expressed genes in OE2 plants after 17- $\beta$ -estradiol (10  $\mu$ M) induction were imported for cluster analysis by using Cluster 3.0, and the resulting tree figure was displayed using the Java Treeview software. The detailed information of genes was listed in **Supplementary Table S2**.

were scored with *AtIPT8-OE* plants upon estradiol-induction and salt treatment (**Figure 7**).

The ROS-scavenging related-genes were also compared between Col and *AtIPT8-OE* plants. Under the condition of salt treatment, the promoted expression levels of *SOD1*, *APX4* and *GPX1* were measured in Col and *AtIPT8-OE* plants while estradiol was present or absent. The expression levels of *SOD1*, *APX4* and *GPX1* were obviously lower in *AtIPT8-OE* plants when compared with those in Col under salt treatment (**Figure 7**). Interestingly, the *CAT3* expression in both of Col and *AtIPT8-OE* plants was not affected in all tested conditions. We also compared the expression levels of some genes encoding peroxidases. As shown in **Figure 7**, upon estradiol-induction the expression levels of *PER21* and *PER30* were inhibited despite of the salt treatment. Moreover, *PRXR1* and *TAPX*, which are involved in the hydrogen peroxide catabolic and oxidation-reduction processes, were analyzed. The down-regulated *PRXR1* transcripts showed in the salt treatment, and significant decrease in expression levels of *PRXR1* were scored in the *AtIPT8-OE* plants that were induced by estradiol. In contrast, up-regulated *TAPX* expression was detected after salt treatment, but estradiol-induction weakened the *TAPX* expression (**Figure 7**).

In addition, we examined expressions of those genes which are functional in the photosystem (**Supplementary Table S2**). Thus, expressions of *Lhb1B1* and *LHCb3* were compared under the condition with or without salt treatment. We observed that increased *Lhb1B1* expression level could be triggered by the salt treatment. Notably, the elevated *Lhb1B1* expression level could be retracted in *AtIPT8-OE* plants upon estradiol-induction. As for the expression of *LHCb3*, it was obviously inhibited by the salt treatment in all the examined plants; recovered level of *LHCb3* expression was detected with the estradiol-induction in *AtIPT8-OE* plants (**Figure 7**). Furthermore, we analyzed the expressions of *LBD3* and *LBD4*, known as LOB domain-containing proteins and playing roles in the determination of bilateral symmetry (Shuai et al., 2002). The *LBD3* (ASL9, ASYMMETRIC LEAVES 2 LIKE 9) can be exclusively regulated by the plant hormone cytokinins in a manner of depending on His-Asp phosphorelay signal transduction (Naito et al., 2007). In this study, we found that *LBD3* expression was significantly enhanced in *AtIPT8-OE* plants in the estradiol-dependent manner. When treated with NaCl, the higher level of *LBD3* expression was sustained (**Figure 7; Supplementary Table S2**). *LBD4* showed less than four fold increase in *AtIPT8-OE* plants that were induced by



**FIGURE 7 | Quantitative analysis on the expression levels of ROS-related and photosynthesis-related genes.** 10-day-old plants grown on MS plates were pretreated with DMSO or 17-β-estradiol (10 μM) for 24 h, and then subjected to treatments (NaCl, 100 mM; with or without estradiol) in liquid MS medium for 3 h. The seedlings were then collected for total RNA extraction and qRT-PCR analysis. Gene expression levels were normalized to their expression levels in Col plants, which were taken as 1. The data are means ± SE of three independent repeats. Asterisk symbols (\*) indicate  $p < 0.05$  (Student *t*-test).

estradiol, but the salt treatment revoked the effect of cytokinin overproduction on *LBD4* expression. We also evaluated the auxin responsive factor *ARF11* that could be down-regulated by cytokinin overproduction (Supplementary Table S2). As shown in Figure 7, expression level of *ARF11* was decreased by the salt treatment in Col and *AtIPT8*-OE plants; however, slight rebound of *ARF11* expression was produced with estradiol induction.

## DISCUSSION

Maintaining cytokinin homeostasis is essential for plant growth and development, as well as plant adaptation to environmental stresses. Numerous studies demonstrate that abiotic stresses have both positive and negative effects on the metabolism of endogenous cytokinins (Hansen and Dörfpling, 2003; Kudoyarova et al., 2007; Alvarez et al., 2008). It is usually difficult to define the working concentrations in plant cells for exogenous application of cytokinins. In this study, through analyzing inducible-*AtIPT8* overexpression transgenic

plants we investigated the effects of modulating endogenous cytokinin production in salt treatments. Results in this study demonstrated that inducible *AtIPT8* overexpression could significantly promote endogenous cytokinin overproduction, and affect the responses of *Arabidopsis* plants to salt stresses. The balance of endogenous cytokinin and auxin contents is critical for maintaining primary root growth (Dello Iorio et al., 2007; Müller and Sheen, 2008; Moubayidin et al., 2010; De Rybel et al., 2014; Schaller et al., 2015). In *AtIPT8*-OE plants, the growth of primary roots was significantly inhibited by overproduction of endogenous cytokinin upon estradiol-induction (Figures 1 and 2). We further assessed the effect of cytokinin overproduction on osmotic and salt stress responses. Our results indicated that both of salt and osmotic stress treatments inhibited plant leaf and root growth (Figure 2). Notably, the most significance in inhibitory effect was observed after glucose treatment, which resulted in an extremely shorten roots and etiolated cotyledons (Figure 2). Salt and osmotic stresses have similar effects on water potential, but salinity has additional cytotoxic effects within the cell (Zhu, 2002). When exposed to high salt concentrations, the *AtIPT8*-OE plants showed less

survival rates and the chlorophyll contents were significantly decreased after estradiol application (**Figure 3**). It has been shown that, under normal conditions, exogenous cytokinin (6-benzyladenine) application is able to promote chlorophyll biosynthesis in roots, but, mutations in cytokinins receptors (*ahk2-2ahk3-3* and *cre1-12ahk3-3*) result in lower chlorophyll contents (Kobayashi et al., 2012). In this study, we observed that, if only overproduced endogenous cytokinin in *AtIPT8-OE* plants it had no obvious effects on chlorophyll contents (**Figure 3**). It is likely, the regulations of cytokinins and chlorophyll biosynthesis is much more complicated than we would have expected. Future studies on this point will expand our understanding on the complications of cytokinins and chlorophyll biosynthesis in *Arabidopsis*.

Chlorophyll accumulation is important in abiotic stress responses, because plant cells must strictly regulate their metabolisms to coincide with the machinery of photosynthesis (Tanaka et al., 2011). Interestingly, in our results, we have noticed that many genes, which are involved in the photosynthesis and metabolism, were differentially expressed in the *AtIPT8-OE* plants that were overproducing endogenous cytokinins (**Figures 6 and 7; Supplementary Table S2**). For instance, genes encoding the components of light harvesting protein complexes, such as LHb1B1, LHCBl.2, LHCBl.3, and LHCBl.4 were differentially regulated by overproduced endogenous cytokinins and the salt treatment (**Figure 7; Supplementary Table S2**). Expression levels of the photosystem II subunits including PSAK, PSAN, PSBP, and PSBQ, which are involved in oxygen evolution, were down-regulated by cytokinin overproduction (**Supplementary Table S2**). Nowadays, fewer evidences in the involvement of photosystem II subunits in abiotic stress responses are reported. With altered functions of chlorophyll-binding proteins, the sensitivity of ABA and dehydration conditions may be influenced in plants (Xu et al., 2012). Our results in analyzing the chlorophyll contents and in profiling the photosystem related genes suggested an indispensable mechanism that may involve in modulating endogenous cytokinin levels and responding to abiotic stress conditions.

The expression levels of stress-responsive genes that can be altered at various degrees after cytokinin treatment were revealed by genome-wide transcriptome analyses (Brenner et al., 2012; Bhargava et al., 2013; Brenner and Schmülling, 2015). The effects of salt stress and cytokinin-deficiency on gene expression have been demonstrated, in which a subset of stress-responsive genes are significantly modified in the cytokinin-deficient mutant *ipt1,3,5,7*, under normal and salinity conditions (Nishiyama et al., 2012). Under salinity conditions, cytokinin-deficiency may up-regulate many stress-responsive genes, including DREB-type transcriptional factors, ABA-responsive components, as well as salt-inducible NAC and ZFHD genes (Nishiyama et al., 2012). In agreement with this trend, we demonstrated that cytokinin-overproduction inhibited ABA-signaling downstream targets such as *ABF3*, *RAB18*, *RD29B*, *RD26*, *DREB2A*, as well as homeobox proteins *ATHB5*, *ATHB7*, and *ATHB12* (**Supplementary Table S2**). Thus, cytokinin and ABA are functionally antagonized in the regulation of plant growth and

the adaption of abiotic stresses (Shkolnik-Inbar and Bar-Zvi, 2010; Nishiyama et al., 2011; Wang et al., 2011; Liu et al., 2013; Guan et al., 2014; Yang et al., 2014).

In general, abiotic stress triggers oxidative responses and then stimulates ROS production. In this study, many of the differentially expressed genes, which were triggered by the overproduction of endogenous cytokinins, could be categorized into oxidation reduction and oxidative stress responses (**Figure 6; Supplementary Table S2**). Endogenous cytokinin overproduction enhanced ROS generation and decreased the activities of ROS-scavenging enzymes (**Figures 4 and 5**). In plant cells, ROS production occurs mainly in membrane-enclosed compartments such as chloroplasts, mitochondria and peroxisomes. In chloroplasts, photosystem I and II (PSI and PSII) are the major sites for ROS generation. Emerging evidences have implicated that cytokinin signaling in abiotic stresses lead to photosynthetic dysfunction and ROS production, by affecting genes expression of PSII subunits (Yi et al., 2008; Kobayashi et al., 2012). The enhancement of expressions of *RbohD*, *RbohF*, and *RbohJ* genes, which was triggered by the salt treatment and cytokinin overproduction, suggested the complex network of cytokinin, salt stress and ROS generation in plant cells (**Figure 7**). Overexpression of ROS-scavenging enzymes, such as isoforms of SOD, CAT and APX, can stimulate abiotic stress tolerance in various crop plants (Apel and Hirt, 2004). In this study, expressions of *SOD1*, *APX4*, *GPX1*, *PER21*, *PER30*, *PXRX1*, and *TAPX1* were significantly down-regulated by endogenous cytokinin-overproduction and salt-treatment (**Figure 7; Supplementary Table S2**). In contrast to the complex effects of cytokinin homeostasis to drought stress tolerance, cytokinin deficient mutant *ipt1,3,5,7* resists to salt stress (Nishiyama et al., 2011). Notably, in agreement with this study, we showed that overproduction cytokinin could enhance salt sensitivity in *Arabidopsis*. Thus, under the conditions of endogenous cytokinin overproduction and salt treatment, it is likely that, the lower expression levels of ROS-scavenging related-genes and the promotion of ROS-production were attributed to the decrease in antioxidant enzyme activities and the increase in ROS contents in *AtIPT8-OE* plants.

*LBDs* mainly expressed at the base of lateral organs of shoots and roots. Ectopically overexpressing *LBD* results in smaller organs through limiting the cell division (Shuai et al., 2002). Previous studies indicate that cytokinin is crucial for determining root-meristem size and root stem-cell specification (Dello Ioio et al., 2007; Müller and Sheen, 2008). In this study, significantly up-regulated *LBD3* was linked to the overproduction of endogenous cytokinins and the treatment of salt, which was consistent with a previous study (Naito et al., 2007). Not like *LBD3*, the expression level of *LBD4* was slightly increased by overly produced endogenous cytokinins, and the salt treatment antagonized the effect of cytokinin on *LBD4* expression (**Figure 7; Supplementary Table S2**). The pleiotropic defects in the growth of roots and cotyledons, caused by endogenous cytokinin overproduction, might be achieved by enhancing *LBDs* expression. Collectively, we concluded that endogenous cytokinin overproduction derived by inducible overexpression of *AtIPT8*

shed a negative effect on plant salt tolerance by modulating stress-responsive gene expression, ROS production and chlorophyll homeostasis.

## AUTHOR CONTRIBUTIONS

In this research, YW was responsible for the experimental design, revising and finalizing the manuscript. YW designed and performed most of the experiments, analyzed the data and drafted the manuscript. WS performed physiological, confocal microscopic imaging and gene expression experiments. ZC provided regents and helpful discussions. All the authors in this research read and approved the final manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2015.01004>

### FIGURE S1 | The inhibited elongation of primary roots in OE2 can be rescued by IAA treatment.

**(A)** Exogenously added IAA could rescue estradiol-induced aberrant growth of roots in OE2. Seeds were growing on MS plates containing 17-β-estradiol (10 μM) for 2 weeks, then transferred to medium containing IAA (1.0 nM) for 5 days. (Bar = 5cm). **(B)** The primary root lengths (shown in **A**) were measured. The results represent the means ± SE of three independent experiments (seedling numbers,  $n > 30$  per experiment). **(C)** 17-β-estradiol (10 μM) treatment could induce more callus generation in OE2 plants than that in Col under the same concentrations of exogenous 2,4-D (5 nM). Photos were taken after 7 days growth on MS plates.

### FIGURE S2 | Exogenous application of cytokinin (6-BA) enhanced ROS production under the condition of salt stress.

**(A)** The 5-day-old Col seedlings were pretreated with NaCl (100 mM) plus or minus 6-BA (10 μM) for 3 days. The fluorescent intensity of DCF was observed using the confocal microscope. Bar, 50 μm. **(B)** The ROS contents in Col plants were quantified. The data are means ± SEs of three independent experiments ( $n = 10$ ). The fluorescent intensity of cotyledons in Col without salt treatment was taken as “1.”

### FIGURE S3 | Comparisons of differentially expressed genes in AtPT8-OE plants and salt- and/or oxidative-stress regulated genes downloaded from public microarray database (<http://bar.utoronto.ca/welcome.htm>).

**(A)** Hierarchical cluster analysis of genes affected by AtPT8 overexpression, salt and oxidative stresses in *Arabidopsis*. The differentially expressed genes in OE2 plants after 17-β-estradiol (10 μM) induction, and by the salt and oxidative treatments were analyzed by the method of using Cluster 3.0. The resulting tree figure was displayed using the Java Treeview software. The detailed information of genes was listed in **Supplementary Table S2**. **(B)** Numbers of overlapping transcripts changed by cytokinin overproduction, salt and oxidative treatments. **(C)** Numbers of genes with up- or down-regulated expression levels by cytokinin overproduction, salt and oxidative treatments.

### TABLE S1 | Primers used for qRT-PCR analysis in this study.

### TABLE S2 | The differentially expressed genes in AtPT8-OE plant and ROS-regulated and/or salt-regulated genes from public microarray data (<http://bar.utoronto.ca/welcome.htm>).

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# The role of promoter *cis*-element, mRNA capping, and ROS in the repression and salt-inducible expression of *AtSOT12* in *Arabidopsis*

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Inducible gene expression is a gene regulatory mechanism central to plant response to environmental cues. The inducible genes are often repressed under normal growth conditions while their expression levels are significantly elevated by conditions such as abiotic stresses. Induction of gene expression requires both *cis*-acting DNA elements and *trans*-acting proteins that are modulated through signal transduction pathways. Here we report several molecular events that affect salt induced expression of the *Arabidopsis AtSOT12* gene. Promoter deletion analysis revealed that DNA elements residing in the 5' UTR are required for the salt induced expression of *AtSOT12*. Cytosine methylation in the promoter was low and salt stress slightly increased the DNA methylation level, suggesting that DNA methylation may not contribute to *AtSOT12* gene repression. Co-transcriptional processing of *AtSOT12* mRNA including capping and polyadenylation site selection was also affected by salt stress. The percentage of capped mRNA increased by salt treatment, and the polyadenylation sites were significantly different before and after exposure to salt stress. The expression level of *AtSOT12* under normal growth conditions was markedly higher in the *oxi1* mutant defective of reactive oxygen species (ROS) signaling than in the wild type. Moreover, *AtSOT12* transcript level was elevated by treatments with DPI and DMTU, two chemicals preventing ROS accumulation. These results suggest that repression of *AtSOT12* expression may require physiological level of ROS and ROS signaling.

**Keywords:** *AtSOT12*, salt stress, gene regulation, ROS, promoter analysis, mRNA capping, polyadenylation

## INTRODUCTION

Gene regulation is a fundamental molecular process governing growth, development and environmental response in all living organisms. In eukaryotes, gene expression can be regulated at almost all the steps from chromatin remodeling and transcription to post-transcription, protein translation and protein degradation. DNA methylation and histone modifications are major chromatin regulation mechanisms resulting in alteration of binding affinity of the DNA elements

with their recognition protein factors. These epigenetic regulations have been implicated in human health and diseases (Hamidi et al., 2015; Seidel et al., 2015). In plants, epigenetic control of developmental processes such as embryogenesis and flowering has been well documented (Ahmad et al., 2010). Epigenetic regulation is also an important regulatory node for plant stress response (Chinnusamy and Zhu, 2009; Kim et al., 2015). For example, positive correlation between drought stress intensity and H3K9 histone acetylation of drought inducible genes was observed (Kim et al., 2008); and histone methylation at H3K4, a gene activation marker was also enriched in the drought stress upregulated genes (Ding et al., 2011, 2012; Kim et al., 2012). Implication of epigenetic regulation in salt stress response stemmed from the observation that the *Arabidopsis* mutants defective in histone acetylation and deacetylation enzymes showed hypersensitivity to salt (Chen and Wu, 2010; Kaldis et al., 2011; Luo et al., 2012). Moreover, Sani et al. (2013) showed that the H3K27me3, a histone modification resulting in gene repression, around the *HKT1* gene was decreased along with salt treatment. *HKT1* gene is an important salt tolerance determinant in plants (Zhang and Shi, 2013), and the decrease of histone methylation at H3K27 may lead to a rapid increase in *HKT1* gene transcription. Besides, histone modification has been implicated in inducible gene expression in response to other stress conditions such as cold and heat (Song et al., 2012; Liu et al., 2015). Although DNA methylation was also shown to be altered by stress treatments, the role of DNA methylation in abiotic stress response is still poorly understood (Kim et al., 2015).

Gene expression control at the transcription level is the most studied gene regulation mechanism. By studying abiotic stress induced or associated transcription factors and the promoters of the stress inducible genes, several important transcriptional regulatory networks have been elucidated in plants. The osmotic stress responsive genes are regulated by ABA-dependent and ABA-independent signaling pathways involving transcription factors and their recognition promoter *cis*-acting elements (Yoshida et al., 2014). The ABA-dependent pathway consists of the *cis*-acting element ABA-responsive element (ABRE) and the ABRE binding factors (ABFs), while the ABA-independent pathway includes dehydration-responsive element/C-repeat (DRE/CRT) and the DRE/CRT binding protein 2 (DREB2). Cold stress activates the ICE-CBF/DREB1-CRT/DRE pathway that upregulates cold responsive (COR) genes (Miura and Furumoto, 2013). The ICE1 (inducer of CBF expression 1) is a MYC-type transcription factor that binds to the promoter of CBF3 thus promote the production of this transcription factor (Chinnusamy et al., 2003). The CBF3 binds with the CRT/DRE *cis*-elements residing in the promoters of the COR genes and promotes the transcription of the COR genes (Maruyama et al., 2004). The activation of HSFs (heat stress transcription factors) and its binding with the *cis*-element HSEs (heat shock elements) in the promoters of HSPs (heat shock proteins) is another example of transcriptional regulation of gene expression in response to heat stress in plants (von Koskull-Döring et al., 2007). Besides transcriptional regulation, post-transcriptional regulation of gene expression in response to abiotic stress has

also been considered as important regulatory steps (Mazzucotelli et al., 2008). Alternative splicing has been shown to play crucial roles in plant adaptation to environmental stresses (Filichkin et al., 2015), and increasing number of studies have backed up this notion since the application of RNA-seq in genome-wide transcriptome analysis. In contrast, involvement of the co-transcriptional mRNA capping and polyadenylation processes in plant stress response has been rarely studied. Jiang et al. (2013) reported that the *Arabidopsis* KH domain containing protein SHI1 and its interacting protein FRY2 are involved in repression of stress-inducible gene expression by modulating mRNA capping and polyadenylation site selection. The *Arabidopsis* Cleavage and Polyadenylation Specificity Factor 30 (CPSF30) is a key polyadenylation factor, and CPSF30 has been implicated in oxidative stress and defense response (Chakrabarti and Hunt, 2015). Overall, the role of mRNA capping and polyadenylation regulation in stress response is still largely obscure.

Salt stress induces a number of gene expression in plants. The majority of the salt inducible genes are also induced by osmotic stress treatment, but some salt inducible genes are not shared with osmotic stress (Kreps et al., 2002), suggesting that salt stress imposes plants with not only osmotic stress component but also ion toxicity that is specific to salt stress. The *Arabidopsis* sulfotransferase gene *AtSOT12* is one of the salt inducible genes identified as NaCl specific in the transcriptome analysis by Kreps et al. (2002). However, our further study on the functions of *AtSOT12* revealed that *AtSOT12* gene is induced by multiple stress conditions including salt and osmotic stress (Baek et al., 2010; Chen et al., 2015). This suggests that the induction of *AtSOT12* expression by NaCl treatment may consist of both osmotic and ion stress components. In this study, we focus on the regulation mechanism of *AtSOT12* gene expression. We found that the *cis*-element responsible for salt inducible expression of *AtSOT12* gene resides at the 3' end of the 5'-UTR. Salt stress increases *AtSOT12* mRNA capping and changes the polyadenylation site selection. Furthermore, *AtSOT12* gene repression requires reactive oxygen species (ROS) accumulation and signaling. These results provide novel mechanisms for salt responsive gene regulation.

## MATERIALS AND METHODS

### Plant Materials and Growth Conditions

*Arabidopsis thaliana* Columbia-0 (Col-0), *Landsberg erecta* (Ler), and the *oxi1* mutant seeds were obtained from the *Arabidopsis* Biological Resource Center (ABRC). Seeds were surface sterilized by incubating in 20% Bleach/Clorox with 0.05% Triton X-100 for 15 min with shaking, followed by washing for five times with sterile water. Sterilized seeds were then suspended with 0.1% low melting point agarose (Sigma), and incubated at 4°C for 2–3 days. Seeds were planted on 1/2 MS agar medium (1/2 MS salts, 1.5% sucrose, 0.7% agars, pH 5.7). The plates were placed in a growth room at 22°C under a daily cycle of 16 h light and 8 h dark.

## Promoter Deletion Constructs

The promoter containing approximately 1 kb sequence upstream of the *AtSOT12* coding region, and promoter deletions from 5' or 3' end were PCR-amplified from genomic DNA by using the primers listed in Supplemental Table S1. The PCR fragments were inserted into the plasmid vector pCAMBIA 1318Z-LUC (Jiang et al., 2013) to create transcriptional fusion of *AtSOT12* promoter and the luciferase reporter gene. The resulting constructs were introduced into the *Agrobacterium* strain GV3101, and *Arabidopsis thaliana* Col-0 wild type plants were transformed with these constructs using *Agrobacterium*-mediated floral dip method (Clough and Bent, 1998).

## Luciferase Assay

Luciferase assay were performed by using T2 transgenic plants harboring the promoter deletion constructs. For luminescence imaging, 10-day-old seedlings growing on 1/2 MS agar plates were treated with 200 mM NaCl for 5 h, then sprayed uniformly with 1 mM luciferin in 0.01% Triton X-100 and kept in dark for 5 min before imaging. All images were acquired using a thermoelectrically cooled CCD camera (DU434-BV, Andor Technology, Connecticut). Quantification of luminescence intensity of the individual seedlings was carried out by using the Andor software provided by the camera manufacturer.

## Northern Blotting Analysis

Total RNA isolation was carried out according to Shi and Bressan (2006). Northern blotting was performed essentially following Chung et al. (2008). For promoter-LUC analysis, 10-day-old T2 seedlings with the promoter deletion constructs with or without NaCl treatment were collected for RNA extraction and Northern blotting. For *AtSOT12* gene expression analysis, 10-day-old seedlings of wild type and *abi1*, *abi2*, *abi3*, *sos1*, *sos2*, *sos3*, *oxi1* mutants with different treatments indicated in Figure 5 were collected for RNA isolation and Northern blotting. The DNA probes for Northern blotting were PCR-amplified by using the following primers: *LUC*, tggagagcaactgcataagg, and tgacgcaggcaggatctatgc; *AtSOT12*, atgtcatcatcatcatcagtccctg, and tcaagaagaaaatttaagaccagaacc; and  $\beta$ -tubulin gene (AT5G23860.1), cgtggatcacagcaatacagagcc, and ctcctgcacttccacttcgtctc. The DNA probes were labeled with  $^{32}$ P-dCTP by using the Primer-It II Random Primer Labeling Kit (Stratagene).

## Promoter-GUS Analysis

The ~1 kb full promoter sequence of *AtSOT12* gene was PCR-amplified and inserted in front of the GUS reporter gene in the plasmid vector pCAMBIA 1381Z. The resulting construct was transformed into *Arabidopsis thaliana* Col-0 wild type plants by using the *Agrobacterium*-mediated floral dip method (Clough and Bent, 1998). For GUS staining, seedlings, and different plant parts were collected from the T2 transgenic plants and stained with 1 mM X-Gluc in the buffer with 100 mM NaPO<sub>4</sub> pH 7.0, 1 mM EDTA, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 0.1% Triton X-100 at 37°C overnight. The samples were washed

three times with 70% ethanol at 60°C to remove chlorophyll and the GUS-staining images were taken under a dissection microscope.

## Subcellular Localization of *AtSOT12-GFP* Fusion Protein

The *AtSOT12* ORF was PCR-amplified by using the primer pairs, forward, acgcgtcgacatgtcatcatcatcatcagttctgc (*Sall* site is underlined), and reverse, ataagaatgcggccgcccaagaagaaaatttaagaccagaacttaa (*NotI* site is underlined) and inserted in-frame into the Gateway entry vector pENTR1A. The *AtSOT12-GFP* was created through recombination between pENTR1A:*AtSOT12* and the destination vector pMDC43 using Gateway LR Clonase II Enzyme Mix (Invitrogen). The pMDC43-*AtSOT12-GFP* construct was sequenced to confirm the fusion sequences, and then transformed into *Arabidopsis* Col-0 wild type plants by floral dip method (Clough and Bent, 1998). The T2 transgenic lines were selected, and GFP images were taken by using an Olympus IX81 inverted laser scanning confocal microscope system.

## Bisulfite Sequencing

Ten-day-old seedlings of *Arabidopsis* Col-0 wild type grown on 1/2 MS agar medium with or without NaCl treatment were used for genomic DNA isolation. Genomic DNA was isolated using CTAB method. 2  $\mu$ g of purified genomic DNA was used for bisulfite treatment using EpiTech Bisulfite kit (Qiagen) following the manual's instruction. Thermal cycling conditions used for bisulfite conversion were as follow: 99°C for 5 min, 60°C for 25 min, 99°C for 5 min, 60°C for 85 min, 99°C for 5 min, 60°C for 175 min, and 20°C for overnight incubation. Bisulfite treated DNA was purified using clean-up column provided by the kit, and 5  $\mu$ L of the treated DNA samples were used as template for PCR reaction with specific primers. The reverse primer (R1: ttcttttatataatcttcatctccaa) was added to the reaction, and after the primer extension reaction (10 cycles of 95°C for 1 min, 60°C for 3 min, 72°C for 3 min), the forward primer (F1: ggtttgatttttagatttttgttaagaat) was added into the reaction mixture and the second PCR reaction (10 cycles of 95°C for 1 min, 60°C for 1.5 min, and 72°C for 2 min, and 30 cycles of 95°C for 1 min, 50°C for 1.5 min, and 72°C for 2 min, and one cycle of 72°C for 10 min) was followed. The PCR products were used for nested PCR to further enrich the amplified products. The primers used for nested PCR were: nested F2: ttggtaagaatgtttttaattgtt and nested R2: ctcccaaataacaaaactaataataataac. The PCR products from the nested PCR reaction were purified from agarose gel electrophoresis and then cloned into pGEM-T Easy Vector (Promega). Plasmid DNA from about 40 independent clones were isolated and sequenced using Big-Dye Terminator Cycle Sequencing Kit (Applied Biosystem), and ABI3100 Genetic Analyzer (Applied Biosystem). The methylated cytosine residues (CNN) which were not converted to thymine were calculated through alignment of the sequenced DNA sequences with the *AtSOT12* promoter sequence.

## 5' and 3' RACE PCR

RACE PCR was performed essentially following the previous described method by Jiang et al. (2013). Briefly, 10-day-old seedlings of *Arabidopsis* Col-0 wild type grown on  $\frac{1}{2}$  MS agar medium with or without NaCl treatment were used for total RNA isolation using Plant RNA Purification Reagent (Sigma). cDNA was generated from 2  $\mu$ g of total RNA by reverse transcription PCR using AMV reverse transcriptase (Promega) and purified using MinElute PCR Purification Kit (Qiagen). For 5' RACE, the cDNA was incubated with terminal transferase (New England Biolabs) and excess dATP to add poly-A to the 5' end. The poly-A attached cDNA was then used as template for PCR amplification using the primer ctgatcttagaggaccggatcc-dT<sub>(17)</sub> and *AtSOT12*-specific primer tcagactctgttcttgtcaga. The PCR products were used as template for a nested PCR using the adaptor primer ctgatcttagaggaccggatcc and a nested primer ttcatctccaagtaaggcaggaaac. After the nested PCR reaction, final PCR products were cloned into pGEM-T Easy Vector (Promega) and 20–40 positive clones were isolated and sequenced using Big-Dye Terminator Cycle Sequencing Kit (Applied Biosystem), and ABI3100 Genetic Analyzer (Applied Biosystem). The addition of a G at the very end of the cDNA as an indication of a capped mRNA was counted from the sequenced cDNAs.

For 3' RACE, the purified cDNA synthesized by reverse transcription with oligo dT<sub>(17)</sub> was amplified by PCR using the adaptor-dT<sub>(17)</sub> and the *AtSOT12* specific primer ttgccaaatgaaatgagactaaaac. A nested PCR was followed using the adaptor primer and an *AtSOT12* specific nested primer ggagagatacttgagtgtgcattgg. The final PCR products were cloned into pGEM-T Easy Vector (Promega) and plasmids from at least 30 independent colonies were sequenced. The polyadenylation sites were analyzed according to the sequences in conjunction with the poly-A.

## RESULTS

### Salt Inducible Promoter Element Resides in the 5'-UTR of *AtSOT12* Gene

Our previous research indicated that *AtSOT12* gene is a multiple stress inducible gene and can be highly induced by NaCl treatment (Baek et al., 2010; Chen et al., 2015). The approximately 1 kb sequence in front of the initiation codon (ATG) of the *AtSOT12* gene including 208 bp 5'-UTR, 207 bp promoter region, and 624 bp upstream sequence (Figure 1A) was validated to be a salt inducible promoter that could drive salt inducible expression of the luciferase reporter gene in *Arabidopsis* (Jiang et al., 2013). In this study, we attempted to locate the salt inducible element in the *AtSOT12* promoter. A series of deletions from both 5' and 3' end of the promoter were made (Figure 1A), and stable *Arabidopsis* transgenic plants harboring the promoter deletions driving luciferase expression were generated. Luciferase imaging (Figure 1B) and quantification (Figure 1C) showed that all 6 deletions from the 5' end of the promoter did not affect the induced expression of the luciferase gene, while deletion from the 3' end almost entirely abolished the induction of luciferase

expression in response to NaCl treatment. Northern blotting analysis further confirmed these observations; all deletions from the 5' end exhibited clear induced expression of luciferase gene by NaCl treatment, but the deletions from 3' end diminished or abolished the induction (Figure 1D). The deletion 7 (Del-7) from the 3' end still showed a low level of induction, while all other 3' end deletions almost completely abolished the induction (Figure 1D). These results indicate that the DNA element responsible for the salt induced expression is within the ~100 bp region in the 5' UTR in conjunction with the coding region of *AtSOT12* gene.

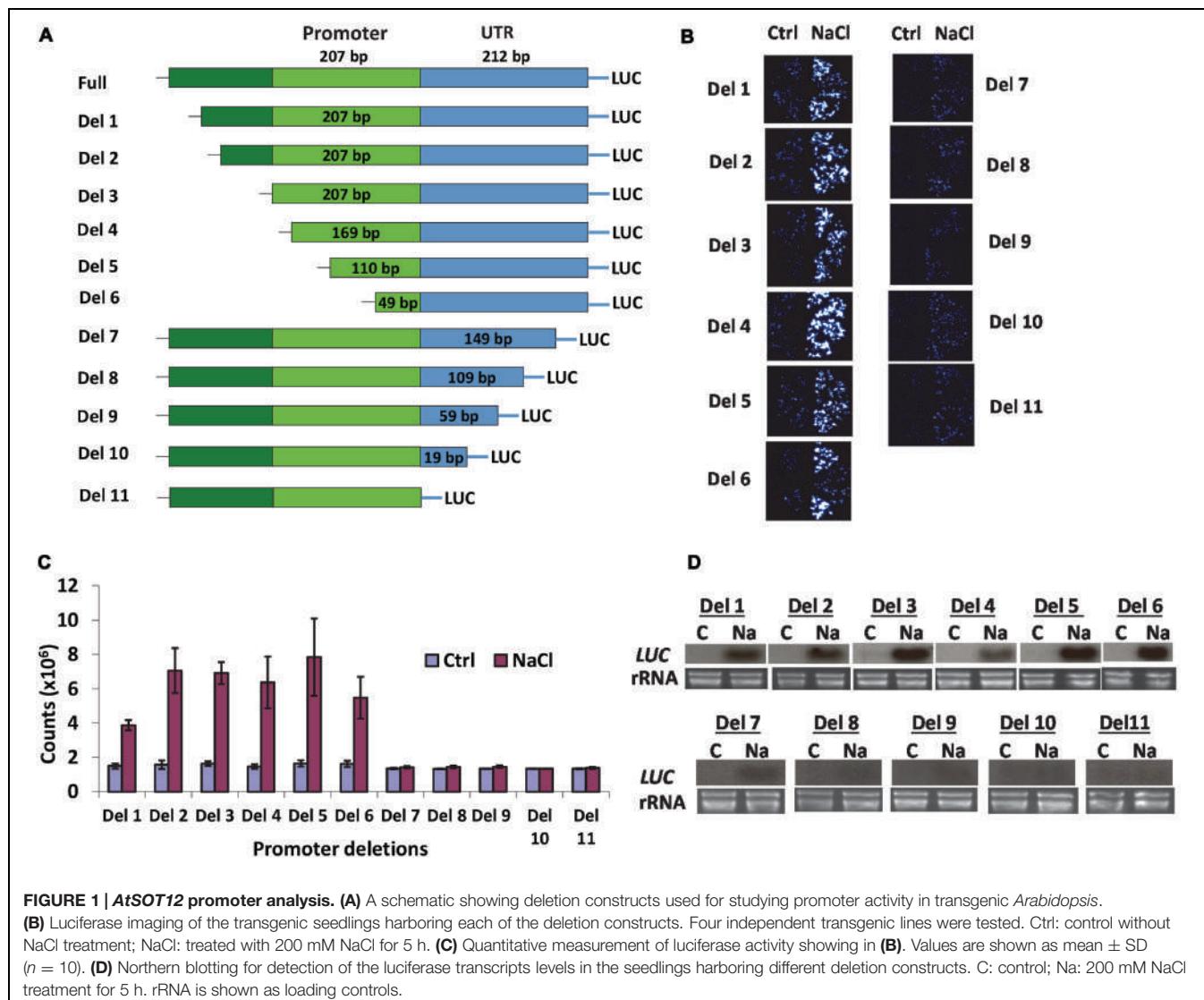
### *AtSOT12* Expression Pattern and Subcellular Localization

Promoter-GUS analysis was used to assess the expression pattern of *AtSOT12* gene. As shown in Figure 2A, GUS expression was detected in most tissues and organs of the plants. In seedling, GUS expression was observed in leaf, hypocotyl, and root with stronger expression in the vascular tissues. In flower, the expression level of GUS was high in sepals, anthers and stigma, and weak in petals, style and ovary of the pistil. GUS expression was also detected in stem and siliques tissues, but not in the seeds within the siliques.

The subcellular localization of *AtSOT12* was visualized by using *AtSOT12*-GFP fusion protein driven by the constitutive 35S promoter. In the transgenic plants harboring this fusion protein, the GFP was mainly localized in the cytosol, which was evidenced by the GFP signal being a thin layer along with the plasma membrane due to a central vacuole in the leave epidermal cells. The cytosolic localization was also observed in the guard cells and root pericycle cells in which vacuoles without GFP are identifiable (Figure 2B). Interestingly, the GFP signal was not uniform in the leaf and root cells. Stronger GFP was detected in the guard cells and the root pericycle cells. This suggests that *AtSOT12* protein level may be modulated in a cell-specific manner via post-transcriptional and/or post-translational regulation.

### DNA Methylation in the Promoter of *AtSOT12* Gene

To determine whether promoter methylation contributes to the repression of *AtSOT12* at normal growth conditions or induced expression by salt stress, bisulfite sequencing was employed to analyze the methylation level of the promoter and the 5' UTR. As shown in Figure 3, the total methylation of cytosine residues (CNN) was very low, 5.0% in the seedlings under normal growth conditions and 7.4% after NaCl treatment. CG methylation was 3.1% in the control seedlings but increased to 10.5% after salt stress treatment. Methylation of cytosine in the form of CHH and CHG was also slightly increased after salt stress treatment. Since DNA methylation generally represses gene expression, it is unlikely that the induced expression of *AtSOT12* by NaCl treatment is through changes in promoter methylation. Low level of cytosine methylation also suggest that promoter methylation may not contribute to the repression of *AtSOT12* expression under normal growth conditions.



**FIGURE 1 | AtSOT12 promoter analysis.** **(A)** A schematic showing deletion constructs used for studying promoter activity in transgenic *Arabidopsis*. **(B)** Luciferase imaging of the transgenic seedlings harboring each of the deletion constructs. Four independent transgenic lines were tested. Ctrl: control without NaCl treatment; NaCl: treated with 200 mM NaCl for 5 h. **(C)** Quantitative measurement of luciferase activity showing in **(B)**. Values are shown as mean  $\pm$  SD ( $n = 10$ ). **(D)** Northern blotting for detection of the luciferase transcripts levels in the seedlings harboring different deletion constructs. C: control; Na: 200 mM NaCl treatment for 5 h. rRNA is shown as loading controls.

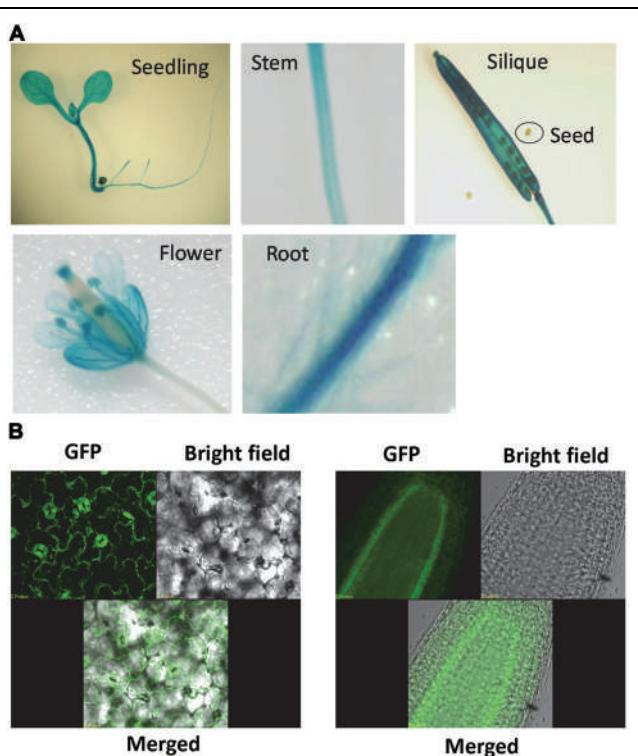
## mRNA 5' Capping and 3' Polyadenylation Site Selection in Response to Salt Stress

mRNA capping and polyadenylation are two crucial co-transcriptional processes in all eukaryotic genes. To determine whether salt stress affects these two processes during *AtSOT12* transcription, *AtSOT12* mRNA capping ratio and polyadenylation sites were analyzed. 5' RACE was used to determine the capping ratio by analyzing the addition of an extra G residue at the very 5' end of the cDNA, which was validated as a reliable method for capping analysis (Ohtake et al., 2004; Jiang et al., 2013). Figure 4A shows that NaCl treatment increased the capping of *AtSOT12* mRNA. Determination of polyadenylation sites revealed multiple sites for *AtSOT12* mRNA polyadenylation (Figure 4B). Although the major polyadenylation site (site 6 shown in Figure 4B) remained unchanged before and after salt treatment, other polyadenylation sites exhibited difference between control and salt treated seedlings. Salt stress caused more disperse distribution of the

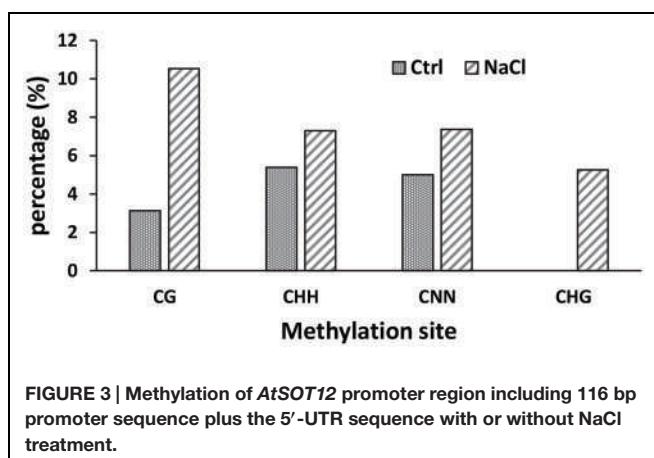
polyadenylation sites (Figure 4C). These results suggest that salt stress not only induces gene expression through the promoter, but also modulate posttranscriptional mRNA modifications which may be a part of gene upregulation mechanism.

## Reactive Oxygen Species Accumulation and Signaling are Required for Repression of *AtSOT12* Gene

The transcript level of *AtSOT12* with or without salt stress was monitored in many genetic mutants to determine the signaling pathways controlling *AtSOT12* regulation. Among the mutants tested, *AtSOT12* expression in the three ABA insensitive mutants, *abi1*, *abi2*, and *abi3*, showed different response to NaCl treatment when compared with the wild type (Figure 5A). The *abi1* mutant displayed clearly diminished induction of *AtSOT12* expression, while the expression levels of *AtSOT12* in *abi2* and *abi3* are comparable with that in the wild type (Figure 5A). This result indicates that the induced



**FIGURE 2 |** AtSOT12 gene expression and protein localization. **(A)** GUS staining of the transgenic plants harboring AtSOT12 promoter-GUS fusion. **(B)** AtSOT12-GFP fusion protein localization visualized using Confocal microscope. Left panel, leaf GFP images; right panel, root GFP images.



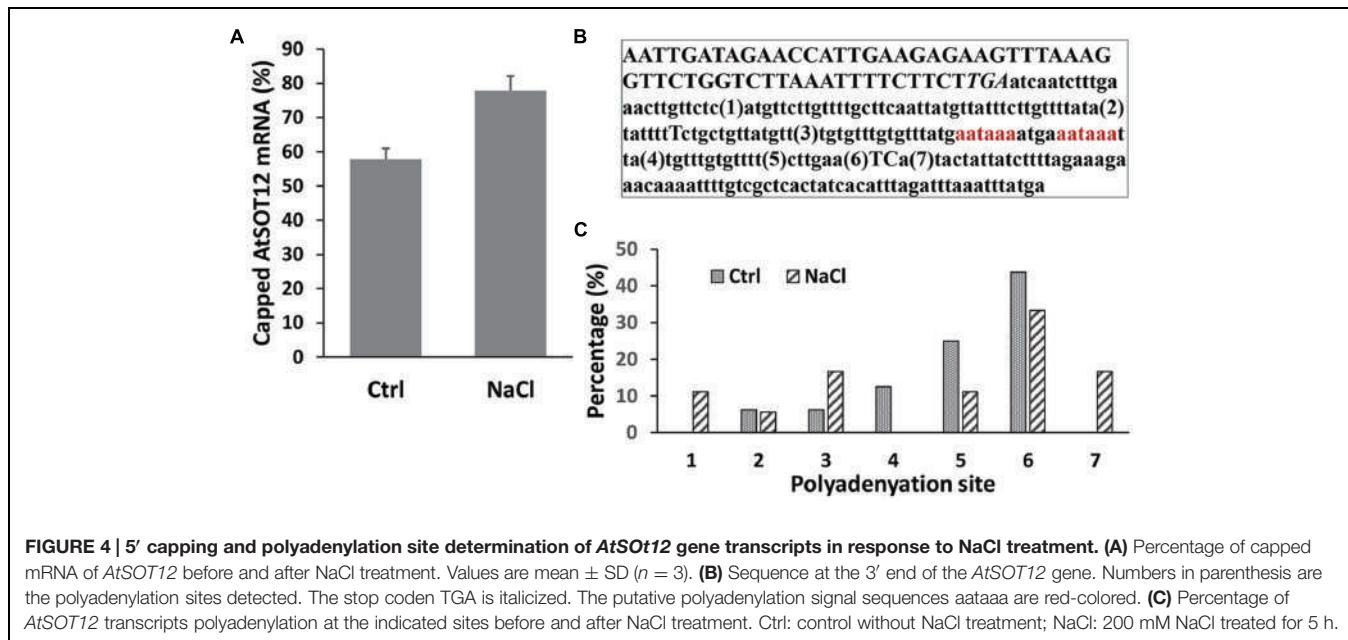
**FIGURE 3 |** Methylation of AtSOT12 promoter region including 116 bp promoter sequence plus the 5'-UTR sequence with or without NaCl treatment.

expression of AtSOT12 by NaCl is partially through ABI1-mediated signaling pathway. In contrast, the salt sensitive mutant *sos1* (Shi et al., 2000, 2002) displayed increased induction of AtSOT12 expression by NaCl treatment, while *sos2*, and *sos3* showed the induction level similar with the wild type (Figure 5B). This suggests that more NaCl accumulation and severe damage by salt stress may be the primary cause of salt induced expression of AtSOT12. Under normal growth conditions, the AtSOT12 expression level in the oxidative stress

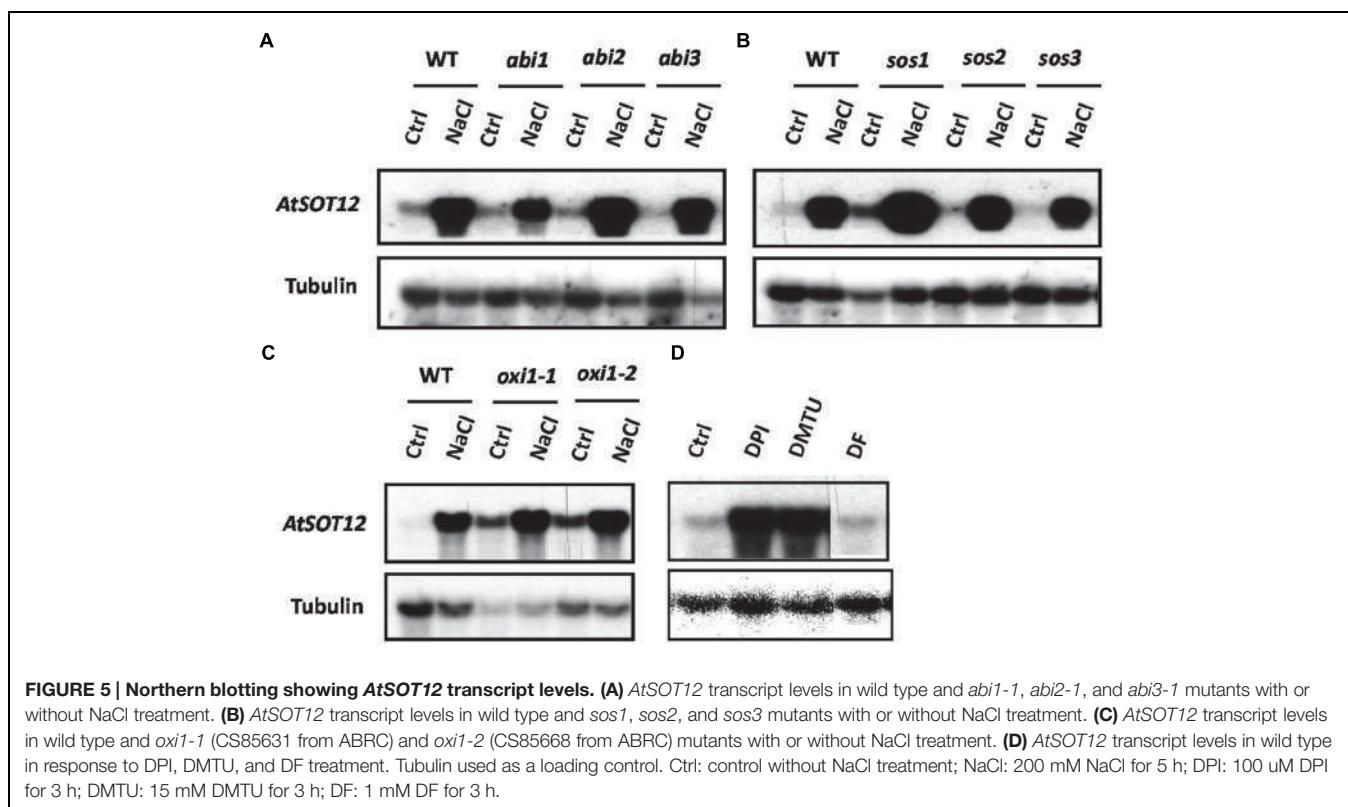
mutant *oxi1* (Rentel et al., 2004) was much higher than that in the wild type (Figure 5C), which indicates that ROS signaling may be required for the maintenance of the repression status of AtSOT12 under normal growth conditions. To further support this notion, two ROS scavengers, dimethylthiourea (DMTU) and deferoxamine (DF), and the NADPH oxidase inhibitor diphenylene iodonium (DPI) were used to study the involvement of ROS in the repression of AtSOT12 gene. DMTU is a potent ROS scavenger and DF is an iron chelator to prevent hydroxyl radical formation from H<sub>2</sub>O<sub>2</sub>. As shown in Figure 5D, DMTU treatment, but not DF treatment, strongly induced the transcript level of AtSOT12, which suggests that repression of AtSOT12 may need specific forms of ROS. DPI treatment also strongly increased the transcript level of AtSOT12, suggesting that ROS production mediated by the plasma membrane-bound NADPH oxidase may be involved in the repression of AtSOT12 gene.

## DISCUSSION

A eukaryotic promoter often contains a core promoter and regulatory sequences that can be either upstream or downstream of the core promoter. These regulatory sequences provide binding sites for transcription factors that either enhance or prevent the binding of RNA polymerase onto the core promoter. The stress responsive *cis*-elements identified in plant promoters are often located upstream of the transcription start site (TSS). For example, the extensively studied ABA responsive element ABRE and drought responsive element DRE/CRT are in the promoter region between -51 and 450 in *Arabidopsis* (Yamaguchi-Shinozaki and Shinozaki, 2005). The DRE/CRT elements in the promoters of drought and COR genes in soybean are also found in the upstream sequence of TSS (Kidokoro et al., 2015). Sequence analysis of the promoters of cold- and dehydration-inducible genes in *Arabidopsis*, rice and soybean revealed that the ABRE and DRE elements are overrepresented in the promoter region between -51 and -100 (Maruyama et al., 2012). Identification of the heat responsive promoter elements also indicated that the HSEs are often located in the promoter between -50 and -200 (Guan et al., 2010; Kurdrid et al., 2010; Mittal et al., 2011; Yoshida et al., 2011). However, our promoter analysis revealed a *cis*-element that is required for salt inducible expression of the reporter gene and located downstream of the TSS. The ~100 bp region in the 5' UTR linking the coding sequence of the AtSOT12 gene was identified to be important for gene upregulation by salt stress (Figure 1). A putative DRE element located ~150 bp from the 3' end of the UTR was predicted using the online program PLACE (<https://sogo.dna.affrc.go.jp/cgi-bin/sogo.cgi?sid=&lang=en&pj=640&action=page&page=newplace>). In addition, many other putative *cis*-elements in the 5' UTR were also identified by this online tool. Whether the putative DRE in combination with other *cis*-elements in the 5' UTR are responsible for the salt induced expression requires further research. Nevertheless, the identification of a salt responsive *cis*-element downstream of the TSS may lead to identifying novel regulatory mechanisms.



**FIGURE 4 |** 5' capping and polyadenylation site determination of *AtSOT12* gene transcripts in response to NaCl treatment. **(A)** Percentage of capped mRNA of *AtSOT12* before and after NaCl treatment. Values are mean  $\pm$  SD ( $n = 3$ ). **(B)** Sequence at the 3' end of the *AtSOT12* gene. Numbers in parenthesis are the polyadenylation sites detected. The stop codon TGA is italicized. The putative polyadenylation signal sequences aataaa are red-colored. **(C)** Percentage of *AtSOT12* transcripts polyadenylation at the indicated sites before and after NaCl treatment. Ctrl: control without NaCl treatment; NaCl: 200 mM NaCl treated for 5 h.



**FIGURE 5 |** Northern blotting showing *AtSOT12* transcript levels. **(A)** *AtSOT12* transcript levels in wild type and *abi1-1*, *abi2-1*, and *abi3-1* mutants with or without NaCl treatment. **(B)** *AtSOT12* transcript levels in wild type and *sos1*, *sos2*, and *sos3* mutants with or without NaCl treatment. **(C)** *AtSOT12* transcript levels in wild type and *oxi1-1* (CS85631 from ABRC) and *oxi1-2* (CS85668 from ABRC) mutants with or without NaCl treatment. **(D)** *AtSOT12* transcript levels in wild type in response to DPI, DMTU, and DF treatment. Tubulin used as a loading control. Ctrl: control without NaCl treatment; NaCl: 200 mM NaCl for 5 h; DPI: 100  $\mu$ M DPI for 3 h; DMTU: 15 mM DMTU for 3 h; DF: 1 mM DF for 3 h.

of salt responsive gene expression. One possible role of the 5' UTR sequence in the *AtSOT12* gene is to provide histone modification sites that regulate the chromatin status in response to stress conditions. It was shown that the gene activation marker H3K4me3 changes dramatically in the dehydration responsive genes, and this modification is predominantly present at the 5'-ends of most transcribed genes (van Dijk et al., 2010). It is

possible that the 5' UTR sequence of *AtSOT12* genes is a hotspot for H3K4me3 modification to de-repress the gene, which might be a pre-requisite for activation of *AtSOT12* gene expression by salt stress.

Many genes are kept inactive at normal growth conditions but activated in response to environmental cues. In contrast to extensive studies on the gene induction mechanisms

involving transcription factors and *cis*-acting elements, the mechanisms of repression of stress inducible genes is still poorly understood. DNA methylation is one of the gene silencing mechanisms, but it seems not to be the mechanism for *AtSOT12* gene repression. This is supported by our analysis of cytosine methylation revealing a very low methylation in the promoter and 5' UTR of *AtSOT12* gene and a slight increase in cytosine methylation after salt stress treatment (**Figure 3**). Repression of *AtSOT12* gene is likely to be an active process involving protein complex including histone deacetylase. A mutant screening for altered expression of the luciferase reporter gene driven by the *AtSOT12* promoter (with 5' UTR) identified a mutation in a histone deacetylase that caused elevated expression of luciferase gene under normal growth conditions (Shi lab unpublished data). This suggests that histone deacetylation in the *AtSOT12* promoter and 5' UTR is important for its repression. The repression complex may also include components such as SHI1 and FRY2 that were also identified in our mutant screening. SHI and FRY2 repress *AtSOT12* gene partly by decreasing mRNA capping (Jiang et al., 2013). In this study, we also found that mRNA capping of *AtSOT12* gene is increased after salt stress treatment (**Figure 4A**). This suggests that salt stress may cause dissociation of the repressor complex including SHI1 and FRY2 thus increase mRNA capping. Salt stress also altered the polyadenylation sites of the *AtSOT12* mRNA (**Figure 4C**), which may be resulted from the transcription complexes that include different components for very low level of *AtSOT12* transcription under normal growth condition and high level transcription under salt stress conditions.

How the repression state of stress inducible genes is maintained under normal growth conditions is another interesting question. Our results suggest that physiological level of ROS and ROS signaling might be involved in the repression of *AtSOT12* gene at normal growth conditions. The basal expression level of *AtSOT12* in the oxidative signaling mutant *oxi1* is elevated significantly (**Figure 5C**), suggesting that ROS signaling is required for *AtSOT12* gene repression. OXI1 is a serine/threonine protein kinase that mediates active oxygen species signaling by activating downstream MAPK kinase cascade and other downstream responses (Rentel et al., 2004). Plants and other living organisms constantly produce ROS in the cells as a result of the most essential biochemical processes such as photosynthesis and respiration, while plant cells also generate apoplastic ROS through the action of plasma membrane-bound NADPH oxidase (Torres and Dangl, 2005). NADPH oxidase converts  $O_2$  into superoxide anion ( $O_2^-$ ) which is dismutated into  $H_2O_2$ .  $H_2O_2$  can generate hydroxyl radical ( $OH^-$ ) in the present of transition metals such as Fe (Sagi and Fluhr, 2006; Kurusu et al., 2015). Under normal growth conditions, ROS homeostasis is maintained through the balance of production and removal, and the physiological level of ROS may serve as an important signal mediating normal levels of gene expression including repression of the stress inducible genes. Stress conditions including salt stress result in increased production of ROS thus reset the cellular redox status, which may signal to a battery of downstream components

such as protein phosphatases and HDACs that trigger changes in chromatin structure. ROS and redox signaling have been implicated in numerous molecular response including histone acetylation, gene transcription, mRNA stability, etc. (Forman and Torres, 2002; Temple et al., 2005; Chung et al., 2008; Miller et al., 2010; Escobar et al., 2012). Our results suggest that the *AtSOT12* repression under normal growth conditions requires ROS and ROS signaling, and the change in redox status after salt stress may be a signal for de-repression of the *AtSOT12* gene through, for example, histone modifications and chromatin remodeling, which could be the prerequisite for the gene activation via the binding of the activator to the *cis* promoter elements. DPI treatment increased the expression of *AtSOT12* (**Figure 5D**), indicating that NADPH oxidase may be the source of ROS production and ROS signaling for *AtSOT12* repression. DMTU but not DF treatment elevated the expression of *AtSOT12* (**Figure 5D**), which suggests that physiological levels of  $O_2^-$  and/or  $H_2O_2$  but not  $OH^-$  radical may be the signaling ROS for maintaining repression status of *AtSOT12* gene.

In addition to the transcriptional regulation of *AtSOT12*, posttranscriptional processes may also be involved in modulating *AtSOT12* protein abundance. Our study on *AtSOT12*-GFP localization revealed a cell-type preferential accumulation of *AtSOT12* protein. Although its expression is controlled by the constitutive 35S promoter, the *AtSOT12*-GFP fusion proteins predominantly accumulated in the guard cells in the leaves and the pericycle cells in the roots. This suggests that *AtSOT12* proteins are modulated at posttranscriptional levels in these cell types through the control of mRNA stability, protein translation, or protein stability. Although we could not pinpoint which posttranscriptional step modulating *AtSOT12* abundance without further research, this result indicated a possible important role of *AtSOT12* in these cell types. The guard cells control stomatal movement in response to environmental changes and ABA plays crucial roles in this process. It has been shown that sulfate availability influences ABA under salt stress (Cao et al., 2014). Perhaps *AtSOT12* is one of the key enzymes regulating sulfur pools in the guard cells due to its strong induction by salt stress and its wide substrates selectivity (Baek et al., 2010; Chen et al., 2015). Through modulating sulfur contents, *AtSOT12* may regulate ABA levels thus play a role in guard cell function. Alternatively, sulfonation of small molecules including hormones such as salicylic acid and brassinosteroids by *AtSOT12* sulfotransferase (Baek et al., 2010) may be a molecular response of the guard cells to stress conditions such as salt stress. Predominant accumulation of *AtSOT12* in the pericycle cells in roots may indicate a role of *AtSOT12* in sulfur assimilation in response to salt stress. Salt stress has been reported to increase sulfur contents and sulfur assimilation (Rodríguez-Hernández et al., 2014; Nazar et al., 2015). Thus, it is conceivable that the pericycle cells are sites for sulfur accumulation, transport, and assimilation and *AtSOT12* functions in these cells for sulfur assimilation by sulfonating a variety of small molecules (Chen et al., 2015). Sulfur assimilation via *AtSOT12* in the pericycle cells may be a molecular response contributing to salt stress tolerance.

## AUTHOR CONTRIBUTIONS

JC, BW, J-SC, HC performed the experiments described in this paper. JC, CL, YR, HS were involved in organizing the data and drafting the manuscript. HS designed the experiments and extensively involved in the writing and finalizing the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2015.00974>

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# The *Arabidopsis* a zinc finger domain protein ARS1 is essential for seed germination and ROS homeostasis in response to ABA and oxidative stress

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The phytohormone abscisic acid (ABA) induces accumulation of reactive oxygen species (ROS), which can disrupt seed dormancy and plant development. Here, we report the isolation and characterization of an *Arabidopsis thaliana* mutant called *ars1* (*aba* and *ros* sensitive 1) that showed hypersensitivity to ABA during seed germination and to methyl viologen (MV) at the seedling stage. ARS1 encodes a nuclear protein with one zinc finger domain, two nuclear localization signal (NLS) domains, and one nuclear export signal (NES). The *ars1* mutants showed reduced expression of a gene for superoxide dismutase (CSD3) and enhanced accumulation of ROS after ABA treatment. Transient expression of ARS1 in *Arabidopsis* protoplasts strongly suppressed ABA-mediated ROS production. Interestingly, nuclear-localized ARS1 translocated to the cytoplasm in response to treatment with ABA, H<sub>2</sub>O<sub>2</sub>, or MV. Taken together, these results suggest that ARS1 modulates seed germination and ROS homeostasis in response to ABA and oxidative stress in plants.

**Keywords:** abiotic stress, abscisic acid, *Arabidopsis*, C2H2 zinc finger, reactive oxygen species, redox

## INTRODUCTION

The phytohormone abscisic acid (ABA) regulates important physiological processes including embryogenesis, seed dormancy, vegetative growth, and abiotic stress responses (Cutler et al., 2010; Raghavendra et al., 2010). ABA signaling is associated with the accumulation of intracellular reactive oxygen species (ROS), which initiates diverse signal transduction processes such as gene expression, enzyme activation, and programmed cell death (Neill et al., 2002; Wasilewska et al., 2008). ROS such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), superoxide anion (O<sub>2</sub><sup>-</sup>), singlet oxygen (<sup>1</sup>O<sub>2</sub>), and hydroxyl radical (OH<sup>-</sup>) form as toxic byproducts of metabolic processes, including photosynthesis, dark respiration, and photorespiration, as well as under abiotic stress conditions; ROS also act as important signaling molecules under optimal growth conditions (Mittler et al., 2004; Kim et al., 2008; Foyer and Shigeoka, 2011).

Reactive oxygen species, as key endogenous messengers, play a crucial role in the complex ABA signaling network (Wang and Song, 2008). This network involves diverse regulators, such as NADPH oxidases, SNF1-related protein kinases (SnRK), type-2C/A protein phosphatases (PP2C),

calcineurin B-like (CBL) interacting protein kinases (CIPK), calcium-dependent protein kinases, and mitogen-activated protein kinases (MAPK). The ABA receptors RCAR/PYR1/PYL (Regulatory Components of ABA-receptor/Pyrabactin resistant Protein/PYR-like protein) perceive ABA, bind to ABA, and interact with a group of PP2Cs (Ma et al., 2009; Park et al., 2009). In the absence of ABA, PP2Cs interact with SnRK2/OST1 (OPEN STOMATA1) and dephosphorylate SnRK2 to inactive its kinase activity. When ABA binds to its receptors, SnRK2 is activated, via the lack of PP2C function (Umezawa et al., 2009). Activated SnRK2 phosphorylates and regulates various downstream target proteins, including guard cell ion channels, NADPH oxidase, and transcription factors (Kwak et al., 2003; Sato et al., 2009; Yoshida et al., 2010; Brandt et al., 2012). Among the SnRK2 targets, NADPH oxidases (respiratory burst oxidase homologues, RBOHs) localize in the plasma membrane and the phosphorylation of RBOHs by SnRK2/OST1 plays a major role in triggering ROS production in plants (Kwak et al., 2003). In addition, RBOHF is phosphorylated by the CBL/CBL9-CIPK26 complex and mediates ROS production (Drerup et al., 2013).

Regulation of ROS-generating and ROS-scavenging systems maintains the delicate balance of ROS (Foyer and Noctor, 2009). Non-enzymatic antioxidants detoxify singlet oxygen and hydroxyl radical; by contrast, antioxidant enzymes including superoxide dismutases (SOD), catalases (CAT), and ascorbate peroxidases (APX) detoxify H<sub>2</sub>O<sub>2</sub> (Op den Camp et al., 2003; Gadjev et al., 2006; Gechev et al., 2006; Foyer and Noctor, 2009). SOD catalyzes the dismutation of O<sub>2</sub><sup>-</sup> to H<sub>2</sub>O<sub>2</sub>; CAT and APX directly react with H<sub>2</sub>O<sub>2</sub> to form water and oxygen (Mittler et al., 2004).

Recently, ROS have been implicated in mediating complex, systemic signaling in plant cells. These ROS signals may function alone or interact with other molecules, including plant hormones, Ca<sup>2+</sup> signals, proteins, and RNA (Karpinski et al., 2013; Shah et al., 2014; Wang et al., 2014). In addition, ABA and ROS may act together in regulating systemic responses to abiotic stress (Suzuki et al., 2013). In this study, we isolated *Arabidopsis ARS1 (ABA AND ROS SENSITIVE 1)* using a genetic screening system and showed that ARS1 is essential for seed germination and maintenance of ROS homeostasis in plants challenged with ABA or oxidative stress. We also demonstrated that ARS1 translocates from the nucleus to the cytoplasm in response to ABA or oxidative stress. We report that ARS1 functions as a positive regulator countering ABA to break seed dormancy and maintaining ROS homeostasis in response to ABA and oxidative stress.

## MATERIALS AND METHODS

### Plant Materials and Growth Conditions

The activation T-DNA vector *pSKI015* was used to generate an insertion mutant population (T1) in the *Arabidopsis thaliana* C24 RD29A::LUC (WT) background, based on Basta herbicide selection. Plants were grouped into 10-line pools, and T2 progenies were screened for mutants that exhibited ABA hypersensitivity compared to WT. T-DNA insertion

mutants of *ARS1* (*At3g02860*), *ars1-2* (SALK\_009596), *ars1-3* (SALK\_030445), and *ars1-4* (SALK\_126300), were obtained from the *Arabidopsis* Biological Resource Center (ABRC). Seeds were surface-sterilized and sown onto MS medium [1/2 Murashige and Skoog (MS) salts, 1.5% sucrose and 0.6% agar, pH 5.7] with or without ABA (as indicated in figures). Plants were grown in a growth chamber with a cycle of 16 h light and 8 h dark at 22°C

### Thermal Asymmetric Interlaced PCR Analysis

DNA flanking the left border of the inserted T-DNA in the *ars1-1* mutant was isolated by thermal asymmetric interlaced PCR (TAIL-PCR). The entire isolated fragment was sequenced. The primers used in the TAIL-PCR were specific primers for the T-DNA left border (LB1, LB2, and LB3) and degenerate primers (DP1, DP2, and DP3; Supplementary Table S1). The nucleotide sequence of the PCR product was determined and subjected to BLASTn analysis.

### Determination of Transcript Levels

Total RNA was isolated from 10-d-old seedlings using the RNeasy kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. Isolated total RNA was treated with DNase I (Qiagen, Valencia, CA, USA) to remove genomic DNA contamination. The first-strand cDNA was synthesized using 2 µg total RNA with a cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA) in a 20-µl reaction volume, and subjected to PCR for examination of gene expression. The specific primers were designed according to the sequence of *ARS1*. *TUBULIN2* was used as a control in the experiment. The primers used for the RT-PCR analysis are listed in Supplementary Table S1.

For quantitative RT-PCR (qRT-PCR) analysis, the first-strand cDNA was synthesized using 2 µg total RNA with a cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA). The QuantiSpeed SYBR No-Rox Mix (PhileKorea, Seoul, Korea) was used for qRT-PCR as follows: 50°C for 10 min, 95°C for 2 min, and 40 cycles of 95°C for 5 s, and 60°C for 30 s. *TUBULIN2* was used for RNA normalization. The relative expression levels of all samples were automatically calculated using CFX Manager program (Bio-Rad, Hercules, CA, USA) and carried out in three biological replicates. The primers used for the qRT-PCR analysis are listed in Supplementary Table S1.

### Protoplast Transient Expression Analysis

The cDNA encoding *ARS1* was isolated from a cDNA library by PCR. The PCR product was confirmed by nucleotide sequencing and was inserted into *Xba*I and *Bam*H I sites of the *sGFP* vector (kindly provided by Inhwan Hwang, POSTECH, Korea) to create chimeric GFP-fusion constructs under the control of the 35S promoter (Supplementary Table S1). The *sGFP* plasmid vector is a *pUC*-based vector containing *CaMV35S-sGFP-NOS3* for protoplast expression.

Protoplast isolation from *Arabidopsis* leaves and transformation into protoplasts was as described in Jin et al. (2001). Expression of the fusion constructs was monitored

at various time points after transformation and images were captured with a Zeiss Axioplan fluorescence microscope (Carl Zeiss Co., Jena, Germany). The filter sets used were: XF116 (exciter, 474AF20; dichroic, 500DRLP; and emitter, 605DF50) and XF137 (exciter, 540AF30; dichroic, 500DRLP; and emitter, 585ALP; Omega, Inc., Brattleboro, VT) for GFP and RFP, respectively. Data were then processed using Adobe Photoshop software (Adobe System, Mountainview, CA, USA) and presented in pseudo-color format.

## Detection of ROS in Protoplasts

To measure intracellular ROS levels, an aliquot of protoplast suspension ( $\sim 2 \times 10^5 \cdot \text{ml}^{-1}$ ) was incubated with 5  $\mu\text{M}$  2,7-dichlorohydrofluoroscein diacetate (DCFH-DA, Molecular Probes, Eugene, OR, USA) for 5 min and 20  $\mu\text{M}$  dihydrorhodamine123 (Rh123, Molecular Probes, Eugene, OR, USA) for 15 min and were observed under a Zeiss Axioplan fluorescence microscope using XF116 (DCFH-DA; exciter, 474AF20; dichroic, 500DRLP; and emitter, 605DF50) and XF33/E (Rh123; exciter, 535DF35; dichroic, 570DRLP; emitter, 605DF50; Omega, Inc., Brattleboro, VT, USA).

## Histochemical Detection of $\text{O}_2^-$

NBT (nitro blue tetrazolium; Sigma-Aldrich, Saint Louis, MO, USA) staining was used to detect  $\text{O}_2^-$  accumulation in tissues.  $\text{O}_2^-$  was visualized as a dark blue formazan compound within tissues. Seven-day-old seedlings were immersed in 50 mM potassium phosphate buffer (pH 7.8) containing 0.1% NBT and 10 mM sodium azide and incubated for 2 h in the dark. Chlorophyll was removed from the seedlings prior to imaging by infiltrating them with lacto-glycerol-ethanol (1: 1: 4 volume) and boiling for 5 min (Bournonville and Díaz-Ricci, 2011).

## Measurement of $\text{H}_2\text{O}_2$

$\text{H}_2\text{O}_2$  was measured in tissues using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen/Molecular Probes, Eugene, OR, USA) following the manufacturer's instructions. Fluorescence was determined by excitation at 530 nm and emission at 590 nm.  $\text{H}_2\text{O}_2$  concentration was calculated based on a standard curve and expressed as  $\text{H}_2\text{O}_2$  per fresh weight (Bournonville and Díaz-Ricci, 2011).

## RESULTS

### Isolation and Identification of the ABA-Hypersensitive *ars1* Mutant

The *RD29Apro::LUC* transgene has been widely adapted to screen for ABA- or abiotic stress-responsive mutants from large populations of *Arabidopsis* C24 ecotype plants with T-DNA insertions (Ishitani et al., 1997; Zhu et al., 2008). Here, the  $T_2$  progeny were screened for enhanced sensitivity of seed germination to ABA compared to wild type (WT, C24 ecotype), and the phenotypes were further identified in  $T_3$  progeny. The *ars1-1* mutant displayed hypersensitivity to ABA with reduced seed germination and retarded emergence of green cotyledons

compared to WT (Supplementary Figure S1). Using TAIL-PCR, we found that the *ars1-1* mutant had a T-DNA insertion in *ARS1* (At3g02860), located in the second exon, 701 bp upstream of the ATG translation start site. Genotypic analysis showed the T-DNA insertion in *ARS1* segregated with the *ars1-1* mutant phenotype, as all tested  $F_2$  homozygotes exhibited identical phenotypes ( $n = 100$ , data not shown).

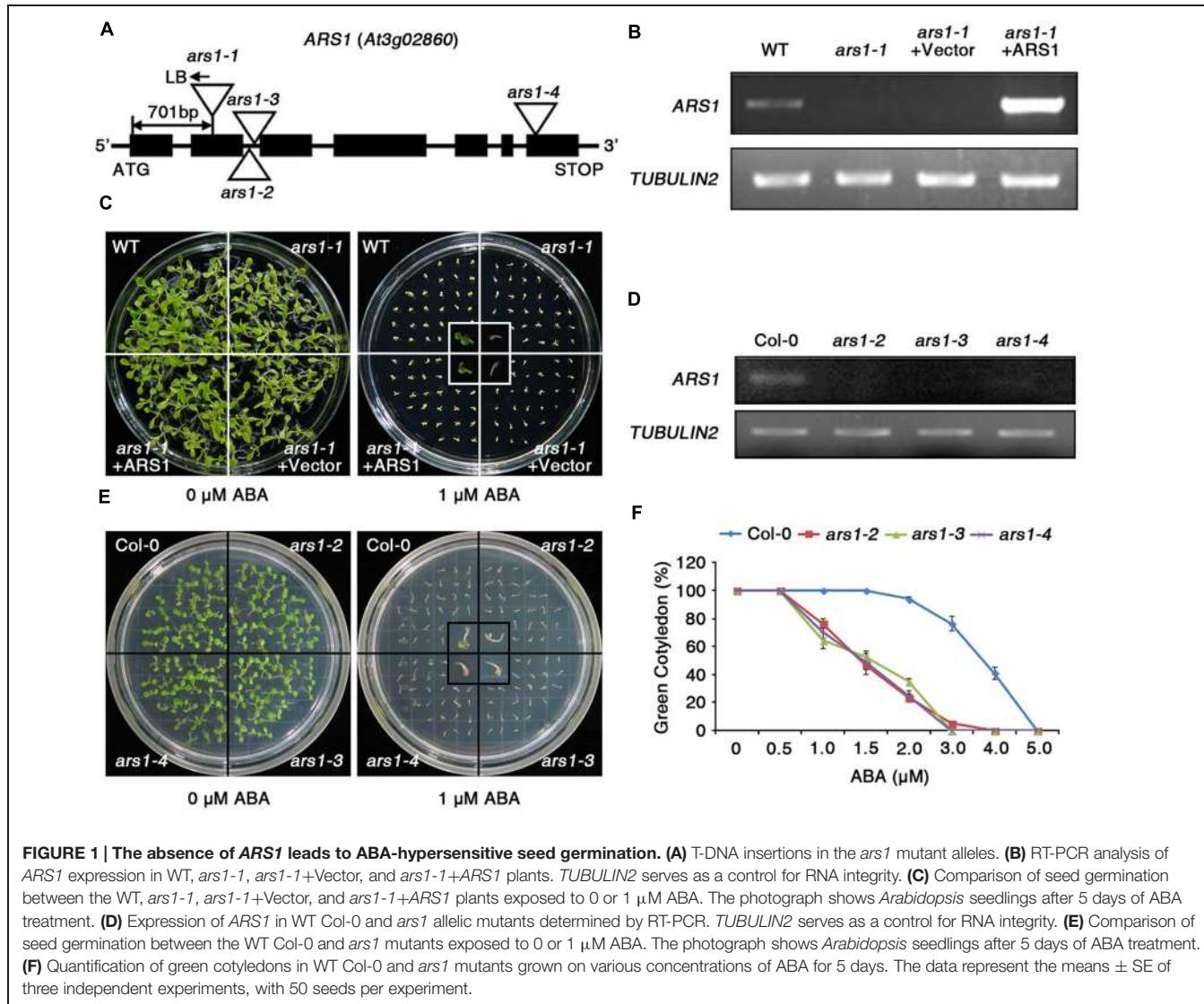
*ARS1* encodes a zinc ion-binding protein (Figure 1A), but its biological functions have not been reported yet. To elucidate the functional roles of *ARS1* in the ABA response, we generated a construct overexpressing *ARS1* under the control of the cauliflower mosaic virus 35S promoter, and transformed this construct into the *ars1-1* mutant (*ars1-1+ARS1*). RT-PCR analysis revealed that the *ars1-1+ARS1* plants had high levels of *ARS1* transcripts compared to WT, but *ARS1* transcripts were absent in both *ars1-1* and *ars1-1* harboring the empty vector (*ars1-1+Vector*) (Figure 1B). In addition, *ars1-1+ARS1* plants showed rescue of the ABA hypersensitivity of *ars1-1* and displayed WT phenotypes in seed germination, while *ars1-1+Vector* showed germination defects similar to those of *ars1-1* (Figure 1C).

To identify the roles of *ARS1* in response to ABA in multiple alleles, we obtained three additional, different T-DNA alleles, *ars1-2*, *ars1-3*, and *ars1-4*, in the *Arabidopsis* Col-0 background (Figure 1A). *ARS1* transcripts were absent in *ars1-2* and *ars1-3* plants compared to Col-0 plants, but were barely detectable in *ars1-4* (Figure 1D). All *ars1* allelic mutants in the Col-0 background also showed an ABA-sensitive phenotype with retarded emergence of green cotyledons compared to Col-0 plants (Figures 1E,F). Root length also provided a measure of ABA sensitivity (Supplementary Figure S2). Four-day-old plants grown on MS medium were transferred to MS medium containing ABA, and root growth was monitored 11 days later. At 30 and 40  $\mu\text{M}$  ABA, root growth in *ars1-2* and *ars1-3* plants lacking *ARS1* transcripts was significantly lower compared to that in Col-0 plant; however *ars1-4* mutants showed no significant differences from Col-0. Although the ABA-dependent root growth phenotypes of *ars1-4* marginally differed from the germination phenotypes, the data are consistent with the differences in expression of *ARS1* in those mutants, suggesting that *ARS1* may also affect post-germination plant growth (Figure 1D, Supplementary Figure S2). These results indicate that *ARS1* is essential for breaking seed dormancy in germination, which is inhibited mainly by ABA.

### Characterization of *ARS1*

To predict the potential function of *ARS1* in plants, we compared the protein sequences of *Arabidopsis* *ARS1* and its homologs in other plants, retrieved by BLAST-P search. Phylogenetic analysis using the BAR Expressolog Tree program<sup>1</sup> revealed that *ARS1* had the highest sequence similarity (53.1%) to a zinc finger protein in *Medicago truncatula*, and also shared 32.4–49.9% similarity with its homologs in plant species such as *Glycine max*, *Solanum tuberosum*, *Lycopersicon esculentum*, *Oryza sativa*, and *Zea mays* (Figure 2A). The tree clearly

<sup>1</sup><http://bar.utoronto.ca/expressologtreeviewer/>



**FIGURE 1 |** The absence of *ARS1* leads to ABA-hypersensitive seed germination. **(A)** T-DNA insertions in the *ars1* mutant alleles. **(B)** RT-PCR analysis of *ARS1* expression in WT, *ars1-1*, *ars1-1+Vector*, and *ars1-1+ARS1* plants. *TUBULIN2* serves as a control for RNA integrity. **(C)** Comparison of seed germination between the WT, *ars1-1*, *ars1-1+Vector*, and *ars1-1+ARS1* plants exposed to 0 or 1 μM ABA. The photograph shows *Arabidopsis* seedlings after 5 days of ABA treatment. **(D)** Expression of *ARS1* in WT Col-0 and *ars1* allelic mutants determined by RT-PCR. *TUBULIN2* serves as a control for RNA integrity. **(E)** Comparison of seed germination between the WT Col-0 and *ars1* mutants exposed to 0 or 1 μM ABA. The photograph shows *Arabidopsis* seedlings after 5 days of ABA treatment. **(F)** Quantification of green cotyledons in WT Col-0 and *ars1* mutants grown on various concentrations of ABA for 5 days. The data represent the means ± SE of three independent experiments, with 50 seeds per experiment.

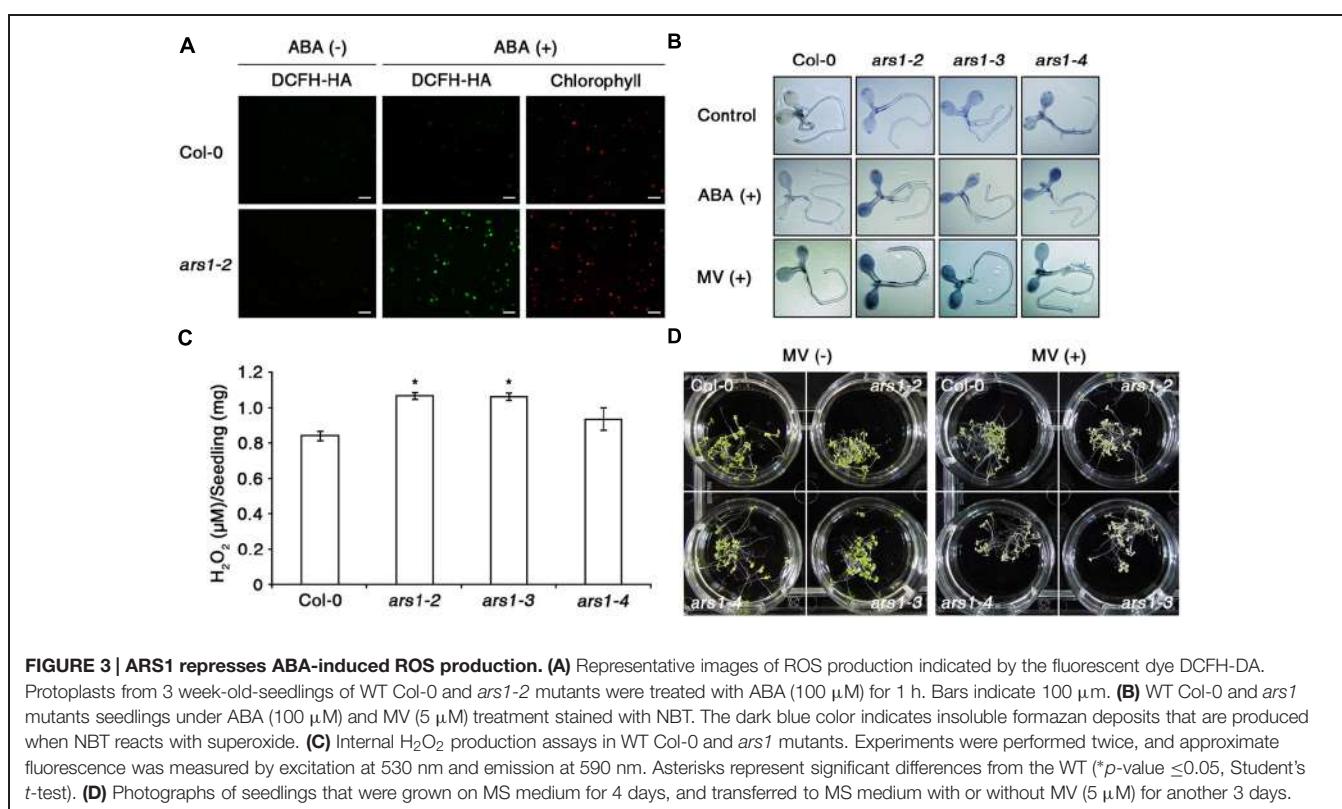
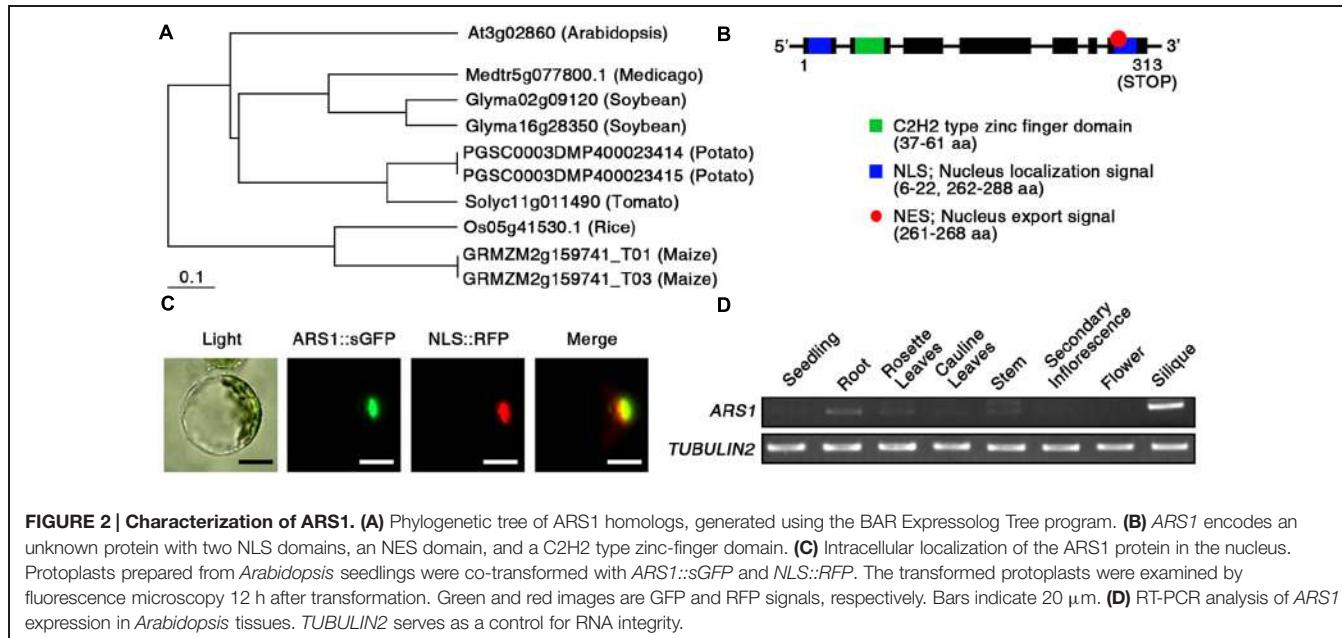
divided ARS1 homologs from dicots or monocots into different branches. However, their biological functions remain elusive in plants. *ARS1* encoded a 313-amino acid protein with predicted molecular mass of 35.2 kDa<sup>2</sup> (Figure 2B). *ARS1* protein possesses a nuclear localization signal (NLS; 6–22 a.a.), a C2H2-type zinc finger domain (37–61 a.a.) in the N-terminal region, a nuclear export signal (NES; 261–268 a.a.), and another NLS (262–288 a.a.) in the C-terminus. C2H2 zinc finger proteins are classified three sets (A, B, and C), and set C is sub-classified into three distinguishable subsets such as C1, C2, and C3 (Englbrecht et al., 2004). We found that *ARS1* belongs to subfamily of C3 subset (Supplementary Figure S3). It indicates that *ARS1* as a C2H2 zinc finger protein may be located either in the nucleus or cytoplasm and may translocate under certain conditions. To reveal the intracellular localization of *ARS1*, we used protoplast transient expression co-transforming an *ARS1::sGFP* construct

and a chimeric construct containing the NLS domain fused to RFP (*NLS::RFP*) as a nuclear marker (Lee et al., 2001). As shown in Figure 2C, the intracellular distribution of green and red fluorescent signals overlapped, indicating that *ARS1* localizes to the nucleus. We also examined the expression of *ARS1* transcripts in different organs by RT-PCR analysis (Figure 2D). Interestingly, *ARS1* transcripts accumulated to high levels in siliques and root, which show strong effects of ABA. *ARS1* expression was slightly higher in rosette leaves and stem than in highly proliferating organs such as seedlings, secondary inflorescences, and flowers.

## ARS1 Represses ABA-Induced ROS Production

Abscisic acid shows a strong relationship with abiotic stress tolerance, especially with drought tolerance via regulation of stomatal closure to reduce water loss (Lim et al., 2015). Thus, we first examined water loss assays

<sup>2</sup><https://www.arabidopsis.org/>



showed no significant differences between Col-0 and *ars1*-2 plants in the absence or presence of ABA (Supplementary Figure S4).

Next, we examined ABA-induced ROS production in Col-0 and *ars1*-2 protoplasts using an ROS-sensitive, cell-permeable fluorescent dye, 2',7'-dichlorofluorescin diacetate (DCFH-DA) (Wang and Joseph, 1999; Pei et al., 2000). As shown in Figure 3A,

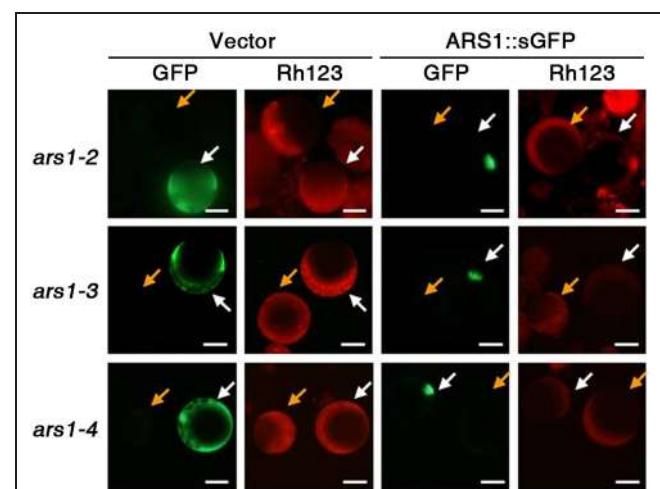
the fluorescent signals increased slightly in response to ABA treatment in Col-0 plants, consistent with previous reports (Wang and Song, 2008). Interestingly, the signals increased dramatically in *ars1*-2 plants exposed to ABA. In addition, ABA-induced ROS production in *ars1*-3 and *ars1*-4 was similar to that in *ars1*-2 mutants (data not shown). We further examined ABA- or MV- induced ROS induction *in planta*

by staining the superoxide anion with nitroblue tetrazolium (NBT) (Figure 3B). Both ABA and MV increased the dark purple staining, indicating increased superoxide levels in all *ars1* mutant plants, while those in Col-0 plants remained consistent in the absence or presence of ABA or MV. Interestingly, *ars1* mutants showed more staining than Col-0 plants, even in the absence of treatments, indicating that the absence of ARS1 may increase ROS levels in plants. To confirm the ROS accumulation in *ars1* mutants, we compared hydrogen peroxide contents of Col-0 and *ars1* mutants (Figure 3C). As shown in Figure 3B, hydrogen peroxide contents in all *ars1* mutants were higher than those in Col-0 plants, even in the absence of treatments. The *ars1-2* and *ars1-3* mutants showed significantly higher ROS than *ars1-4*, which is also consistent with the different levels of ARS1 transcripts among *ars1* mutants (Figure 1D). Furthermore, *ars1* mutants displayed MV-sensitive phenotypes, showing bleaching of leaves compared to Col-0 (Figure 3D).

To determine whether ARS1 directly affects ABA-induced ROS production, we carried out complementation tests in protoplasts using the fluorescent dye dihydrorhodamine123 (Rh123) to monitor ROS production. Rh123 becomes the fluorescent chromophore Rh123 upon oxidation by ROS (Schulz et al., 1996). Empty GFP vector and *ARS1::sGFP* constructs were independently transformed into protoplasts isolated from the *ars1* mutants, and approximately 40% of the protoplasts showed expression of both constructs (data not shown). After treatment with ABA for 1 h and then with Rh123 for 15 min, we found that *ARS1::sGFP* clearly targeted to the nucleus but the empty vector produced a GFP signal in the cytoplasm of protoplasts from all *ars1* mutants (Figure 4). In addition, the fluorescent signals from Rh123 were greatly reduced in all protoplasts transformed with *ARS1::sGFP* compared to those transformed with empty vector (Figure 4). These results suggest that ARS1 acts positively in repressing ABA-induced ROS production.

## ARS1 Deficiency Reduces Expression of SOD

To identify how ARS1 regulates ABA responses, we first used qRT-PCR to analyze the transcript levels of *ARS1* in response to ABA. *ARS1* transcripts slightly increased (1.2-fold induction) in response to ABA treatments for 1 and 3 h (Supplementary Figure S5A). Englbrecht et al. (2004) found that C2H2 zinc finger proteins act as transcriptional regulators in conserved biological processes in response to stress. Accordingly, we examined the expression of *RD29A* genes in *ars1* mutants to explore the possible role of *ARS1* in the ABA signaling pathway. *RD29A* expression is highly induced by ABA as a stress-responsive marker gene but does not change in response to H<sub>2</sub>O<sub>2</sub> treatment (Trouverie et al., 2008). However, *RD29A* transcript accumulation did not show any significant differences in the *ars1* mutants compared to that in Col-0 plants either in the absence or presence of ABA (Supplementary Figure S5B). Thus, ARS1 activity may not be necessary for regulation at the transcriptional level in the ABA signaling pathway.

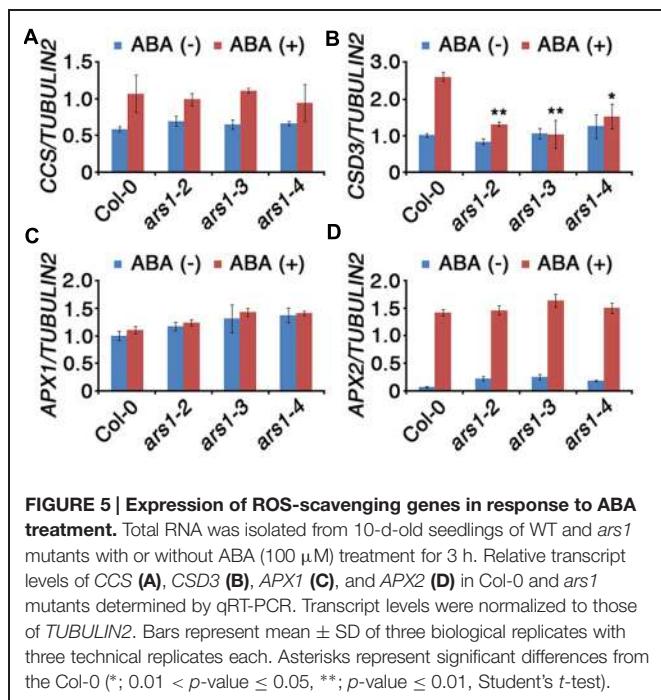


**FIGURE 4 | ARS1 inhibits ABA-induced ROS production.** Protoplasts were isolated from *ars1* mutants seedlings transformed with the empty sGFP vector (Vector) or *ARS1::sGFP*. Twelve hours after transformation, protoplasts were treated with ABA (100  $\mu$ M) for 1 h and stained with dihydrorhodamine123 (Rh123) for 15 min. The images are green (GFP) and red (Rh123) fluorescence images of one aliquot of protoplasts. Bars indicate 20  $\mu$ m. Yellow arrows point to the non-expressing protoplasts, white arrows point to the protoplasts expressing sGFP and *ARS1::sGFP*.

Based on the effect of ARS1 on ABA-induced ROS production, we investigated the transcripts encoding ROS-scavenging enzymes in *ars1* mutants (Figure 5). SOD catalyze the dismutation of O<sub>2</sub><sup>-</sup> to O<sub>2</sub> and H<sub>2</sub>O<sub>2</sub>, which is subsequently reduced to water by CAT and APX (Mittler et al., 2004). We used qRT-PCR to measure the transcript levels of two SOD genes (*CCS* and *CSD3*) and two APX genes (*APX1* and *APX2*) in the absence or presence of ABA. Transcripts of *CCS* (COPPER/ZINC SUPEROXIDE DISMUTASE), *APX1*, and *APX2* did not show any significant differences between Col-0 and *ars1* mutants and even in response to ABA treatment (Figures 5A,C,D). However, transcripts of *CSD3*, encoding a copper/zinc superoxide dismutase 3, significantly decreased in all *ars1* mutants in response to ABA treatment (Figure 5B). These results indicate that ARS1 represses ABA-induced ROS accumulation via inhibiting SOD transcripts, especially *CSD3*.

## ARS1 Translocates from the Nucleus to the Cytoplasm in response to ABA and Oxidative Stress

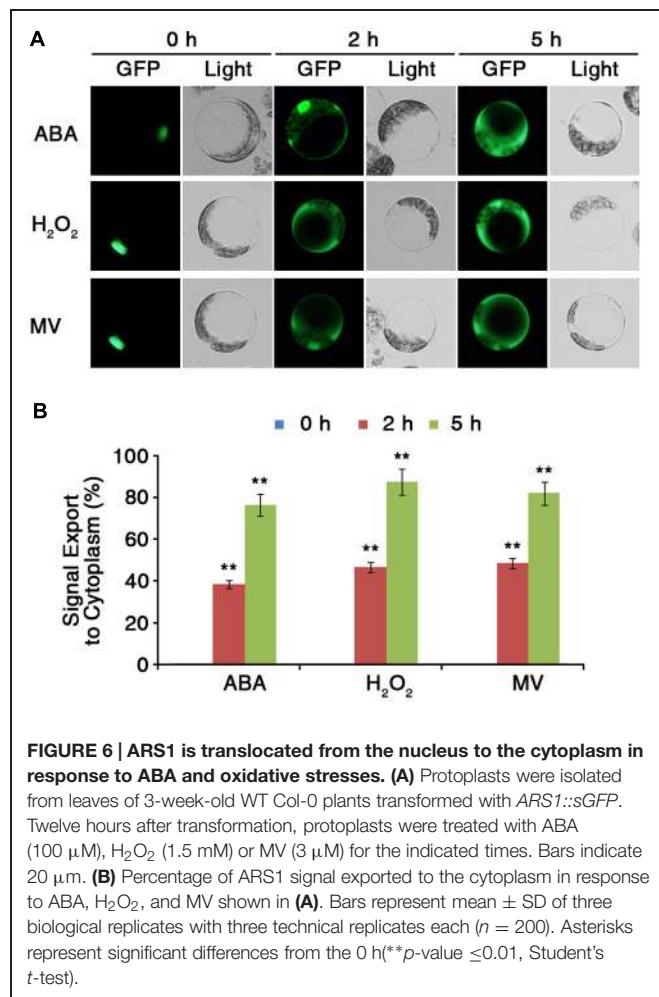
Protein sequence analysis revealed that ARS1 has a putative NES motif at its C terminus (Figure 2B). The presence of the putative NES signal suggested that ARS1 could be exported from the nucleus in response to certain stress conditions and led us to investigate the changes of subcellular localization of ARS1 in *Arabidopsis* cells. To analyze the subcellular localization of ARS1 under stress conditions, we first transiently expressed *ARS1::sGFP* in *Arabidopsis* (Col-0) protoplast cells. Twelve hours after transformation of *ARS1::sGFP* into protoplasts, we treated the protoplasts with ABA, H<sub>2</sub>O<sub>2</sub>, or methyl viologen (MV)



as ROS triggers. In the absence of stressors, ARS1 clearly localized to the nucleus (**Figures 2C** and **6A**, Supplementary Figure S5). Surprisingly, nuclear-localized ARS1 was abundantly translocated to the cytoplasm in response to ABA,  $H_2O_2$ , and MV. (**Figure 6**, Supplementary Figure S6). These translocation patterns of ARS1 in protoplasts markedly increased as the stress duration increased up to 5 h (**Figure 6B**). These results indicate that the putative NES motif is likely important for ARS1 function in stress responses. Together, this evidence suggests that ARS1 exists as an inactive form in the nucleus, but changes its localization to the cytoplasm as a result of ROS signals induced by ABA and other stresses to repress ABA/stress-induced ROS production in plant cells.

## DISCUSSION

Seed germination involves extensive crosstalk between phytohormones and secondary messengers. ABA and gibberellin (GA) act antagonistically in germinating seeds, and secondary messengers such as ROS and  $Ca^{2+}$  might be also responding differently in germination. In dormant seeds, high levels of ABA in seed coats repress germination via expression of the DELLA gene *RGL2* and the ABA biosynthesis gene *ABA1*, thus triggering induction of *ABI5* (Lee et al., 2010). Seed dormancy can be broken by environmental cues such as exposure to cold, oxygen, and water (Finch-Savage and Leubner-Metzger, 2006). In germinating seeds, endogenous ROS and cytosolic  $Ca^{2+}$  concentrations increase to promote germination, counteracting the effects of ABA. Exogenous ROS and  $Ca^{2+}$  treatments also enhance seed germination (El-Maarouf-Bouteau and Bailly, 2008; Kong et al., 2015).



However, either low or excess ROS delay or inhibit germination, indicating that ROS homeostasis, termed the “oxidative window of germination,” is essential for breaking seed dormancy (Bailly et al., 2008). In this study, we identified ARS1, encoding an uncharacterized zinc finger domain protein, as essential for seed germination to escape ABA-induced dormancy in *Arabidopsis* (**Figures 1** and **2**, Supplementary Figure S1). Interestingly, *ars1* mutants displayed higher ROS accumulation either in the absence or presence of ABA, indicating that ARS1 may act to repress ROS production (**Figures 3** and **4**). Furthermore, this finding also suggests that ARS1 may regulate the “oxidative window of germination” to prevent excess ROS accumulation during the period of breaking seed dormancy. Using *in silico* analysis, we found 211 C2H2-type zinc finger proteins that constitute the most abundant family of putative transcriptional regulators in *Arabidopsis*<sup>3</sup>. Several of these proteins act in abiotic stress responses, in particular ABA or oxidative stress signaling. SAZ (SA- AND ABA-DOWNREGULATED ZINC FINGER GENE), ZFP3 (ZINC FINGER PROTEIN 3), AZF1 (ARABIDOPSIS C2H2 ZINC

<sup>3</sup><https://www.arabidopsis.org/>

FINGER PROTEIN 1), and AZF2 act as negative regulators in ABA signaling during seed germination and early seedling development (Jiang et al., 2008; Kodaira et al., 2011; Joseph et al., 2014). SAP12 (STRESS-ASSOCIATED PROTEIN 12) contains two AN1 zinc fingers and conformational changes due to redox states of cysteine residues located between the zinc finger structures modulate its activity (Ströher et al., 2009). ZAT10 (ZINC-FINGER OF ARABIDOPSIS 10) is phosphorylated by MPK3 and MPK6 and is involved in ROS-dependent ABA signaling (Mittler et al., 2006). The high conservation of functional motifs among the C2H2 zinc finger proteins suggests that these proteins may have similar molecular functions with respect to transcriptional regulation in diverse biological processes.

Reactive oxygen species act as important signaling molecules and control various biological processes including germination, growth, development, and abiotic stress responses. Diverse abiotic stresses lead to production of toxic levels of ROS, which causes oxidative damage to organelles such as chloroplasts and mitochondria in plant cells (Bailey-Serres and Mittler, 2006; Foyer and Noctor, 2009). To balance the accumulation of toxic ROS, plants have efficient, well-conserved mechanisms for the removal of ROS from cells, including both enzymatic and non-enzymatic ROS scavenging antioxidant systems (Mittler et al., 2004; Bailey-Serres and Mittler, 2006; Foyer and Noctor, 2009). For instance, SOD, CAT, and APX serve as ROS-scavenging enzymes. Abiotic stresses as well as ABA induce production of ROS such as oxygen radicals and hydrogen peroxide via plasma membrane-localized NADPH oxidases (Kwak et al., 2003). ROS accumulation in the *ars1* mutant plants may be caused by repressed expression of the SOD gene *CDS3* (Figure 5). Our results indicate that the function of ARS1 in stress tolerance may be associated with the regulation of antioxidant ability. However, the direct mechanisms by which ARS1 regulates *CDS3* expression are still elusive. To better understand the mechanisms, future research should examine the interaction between ARS1, ROS scavenging systems, and antioxidant enzyme activity.

Reactive oxygen species are generated in various subcellular organelles including chloroplasts, mitochondria, and peroxisomes, and they trigger changes in the nuclear transcriptome during stress (Apel and Hirt, 2004; Suzuki et al., 2012). ROS signaling occurs via interlinked exchanges between two distinct pathways: retrograde (organelle-to-nucleus) and anterograde (nucleus-to-organelle) signaling, which might be involved in acclimation, adaptation, or resistance against stresses (Suzuki et al., 2012). Disruption of ROS homeostasis can occur in chloroplasts or mitochondria, from which signals are transmitted to the nucleus via retrograde signaling cascades. We found that ARS1 in the nucleus was translocated to the cytoplasm upon exposure to ABA or oxidative stress, presumably in response to ROS signals (Figure 6). We cannot conclude that a protein is involved in retrograde regulation just because it exists in two different locations or relocates to another subcellular compartment depending on conditions. However,

ARS1 translocates from the nucleus to cytoplasm in an ROS-dependent manner (Figure 6). This suggests that ARS1 may affect ROS-dependent anterograde signaling between the nucleus and cytoplasm under stress conditions or in response to ABA.

## CONCLUSION

The phytohormone ABA regulates important physiological processes, and is closely related with the accumulation of intracellular ROS to transfer signals triggered in diverse physiological and environmental cues. In this study, we isolated and designated an *ars1* mutant from large populations of *Arabidopsis* with T-DNA insertions as an ABA hypersensitive mutant. We identified that *ARS1* encodes a C2H2 type zinc finger domain protein and may play as a positive regulator for seed germination and maintenance of ROS homeostasis in response to ABA and oxidative stress, which trigger the induction of toxic ROS in cells, via the regulation of a gene for superoxide dismutase (*CSD3*). Interestingly, we also demonstrated that nuclear-localized ARS1 is translocated to the cytoplasm in response to ABA or oxidative stress. Translocation of ARS1 induced by ROS signals may help clarify the role of ROS-dependent anterograde signaling pathways that underlie plant stress responses. Taken together, our results suggest that ARS1 is essential to modulate seed germination and ROS homeostasis in response to ABA and oxidative stress in *Arabidopsis*.

## AUTHOR CONTRIBUTIONS

DB, J-YC, and D-JY designed the experiments. DB performed most of the experiments, and J-YC and D-JY wrote the manuscript. DK, SL, and MK discussed and commented on the results and manuscripts. SK, BP, H-JL, HH, and HC performed some of the experiments. D-JY provided funding for research work as corresponding author.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2015.00963>

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# Exogenous spermidine alleviates oxidative damage and reduce yield loss in rice submerged at tillering stage

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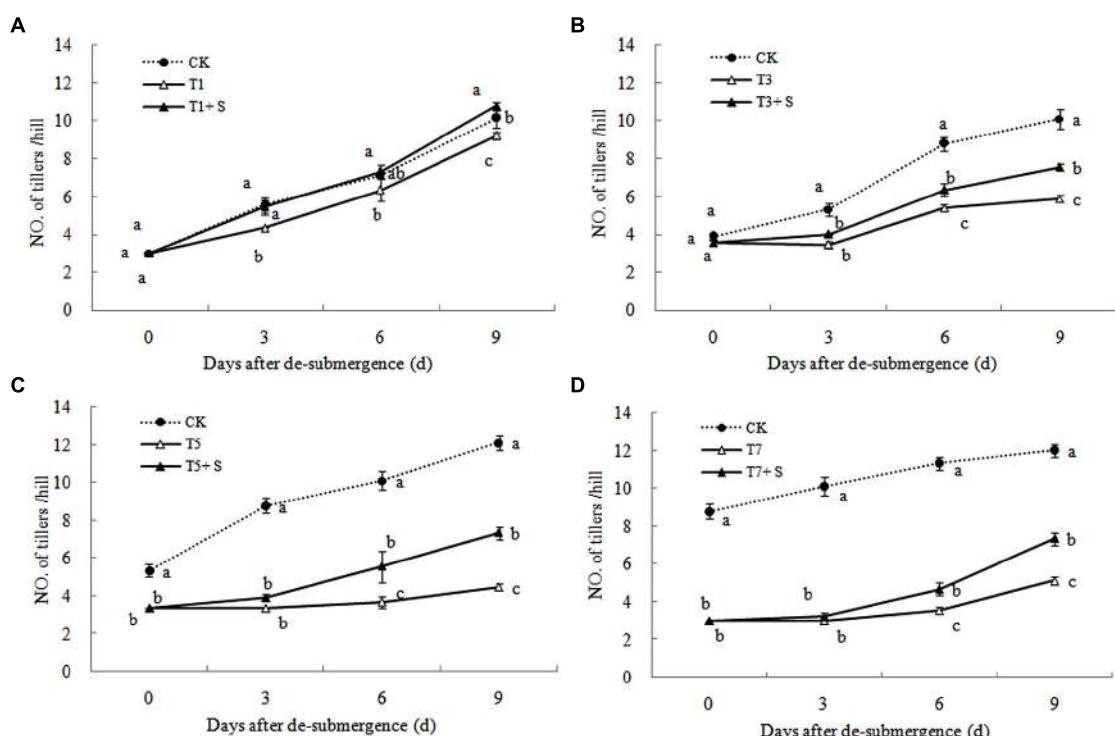
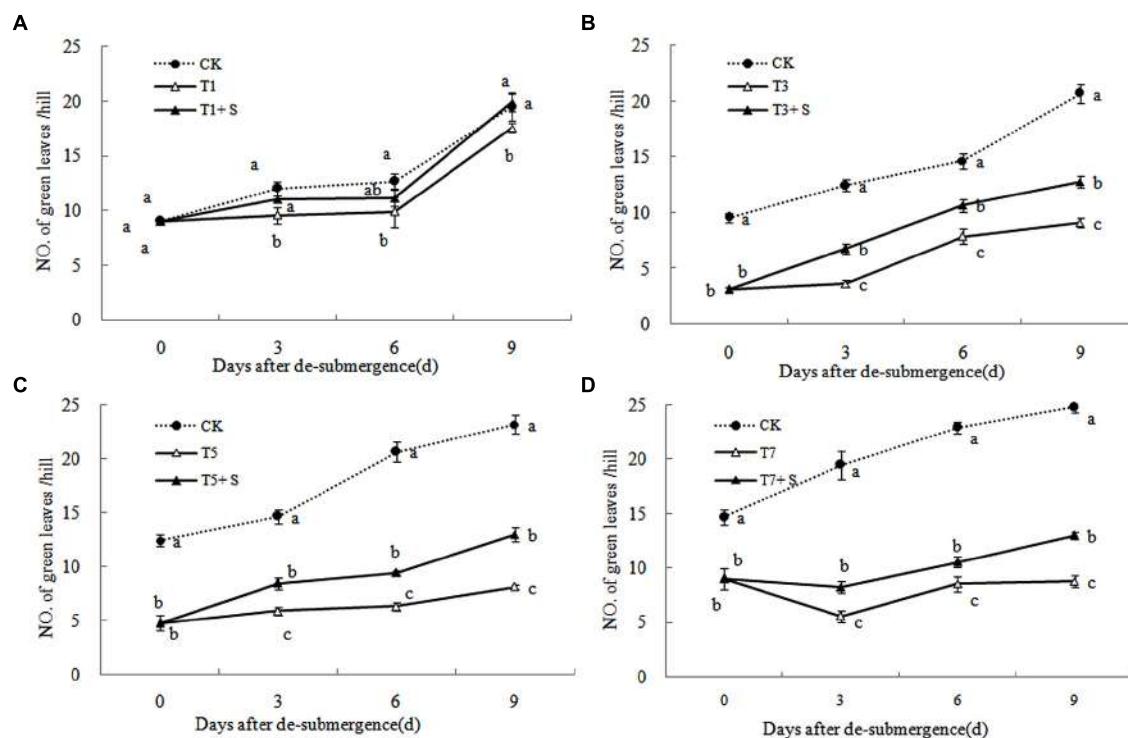
To figure out whether spermidine (Spd) can alleviate oxidative damage on rice (*Oryza sativa* L.) caused by submergence stress, Ningjing 3 was used in this study. The results showed that, spraying Spd on rice leaves at a concentration of 0.5 mM promoted the growth recovery of rice after drainage, such as green leaves, tillers, and aboveground dry mass. According to physiological analysis, Spd accelerate restored chlorophylls damage by submergence, and decreased the rate of  $O_2^-$  generation and  $H_2O_2$  content, inhibited submergence-induced lipid peroxidation. Spd also helped to maintain antioxidant enzyme activities after drainage, such as superoxide dismutase, peroxidase, and GR, which ultimately improved the recovery ability of submerged rice. With the effect of Spd, the rice yields increased by 12.1, 17.9, 13.5, and 18.0%, of which submerged for 1, 3, 5, 7 days, respectively. It is supposed that exogenous Spd really has an alleviate effect on submergence damage and reduce yield loss of rice.

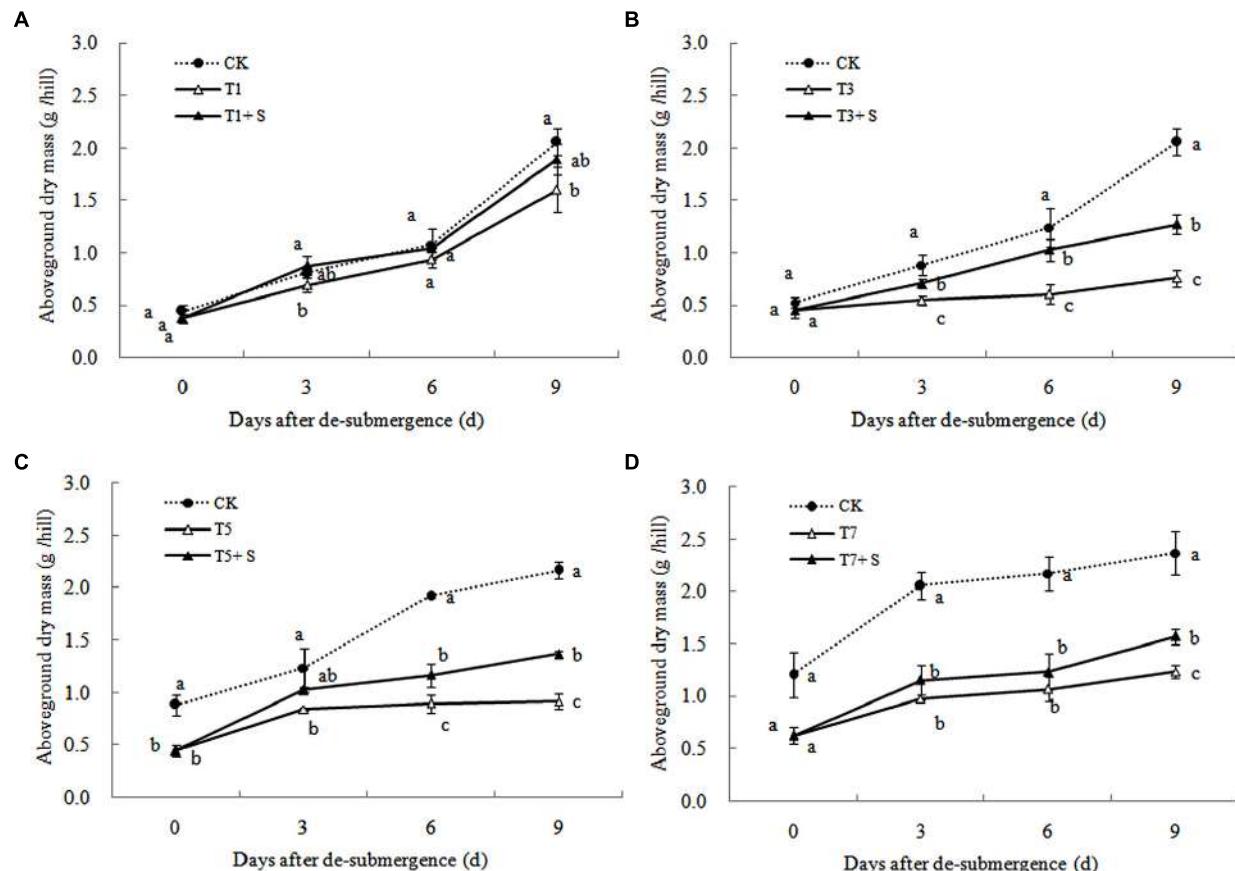
**Keywords:** rice, submergence stress, exogenous spermidine, tillering stage, physiological characteristics

## INTRODUCTION

Paddy flood disaster is one of China's major agricultural disasters. The monsoon climate has a strong impact on the Yangtze River Basin and causes frequent flood disasters, which has brought serious threats to rice production safety. Therefore, the flood disaster has become one of the main obstacle factors limiting rice production in this area (Qin and Jiang, 2005; Huang and Qian, 2007). In the midstream and downstream of Yangtze River, panicles of rice is determined at tillering stage, when paddy fields often encounter flood disasters (Tian et al., 2005). As flood brings serious harm to the growth of rice in this period, effective measures should be took to reduce disaster losses.

Application of exogenous growth regulators is one of the effective ways to improve plant resistance of waterlogging. Studies have shown that paclobutrazol (Lin et al., 2006), proline and betaine (Hoque et al., 2007), brassinolide (BR; Li and Luo, 1999) and other exogenous regulators can effectively reduce the plant injury and yield loss caused by waterlogging. In recent years, polyamine (PAs) has been regarded as a new class of growth substances in improving plant stress resistance ability (Walters, 2003; Yuan et al., 2008). PAs are low-molecular-weight aliphatic amines that are ubiquitous in all organisms with high biological activity coming from biological metabolism. Common natural PAs include the higher PAs, spermine (Spm) and spermidine (Spd),

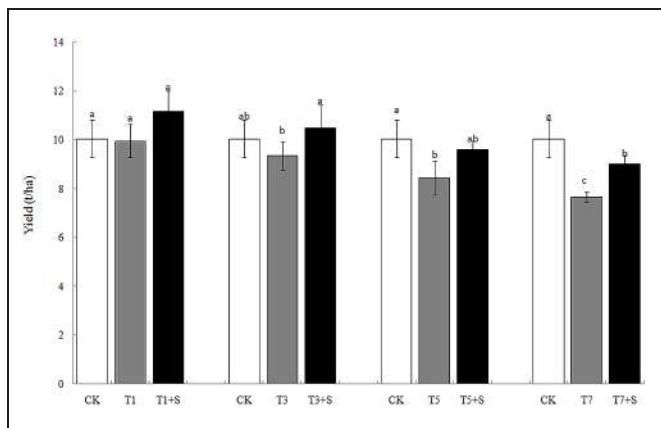




**FIGURE 3 |** Effects of Spd on shoot dry weight per hill of rice after submergence.

and their diamine obligate precursor putrescine (Put). Among the three major PAs, Spd is most closely associated with stress tolerance in plants (Shen et al., 2000). Spd cannot only be used as a stress protective substance directly, but also be used as a signal molecule in stress signal transduction, constructing a stress resistant mechanism (Kasukabe et al., 2004). Spraying different concentrations of Spd on *Typha latifolia* L. could effectively improve the AsA and GSH content, GR and APX activity, and also reduce the production of active oxygen and MDA level in leaves under Cd<sup>2+</sup> stress (Tang et al., 2005). Spd application to salinized nutrient solution resulted in an increase in PA and proline contents and antioxidant enzyme activities in cucumber seedlings, which contributed to osmotic adjustment during salinity (Duan et al., 2008).

It has been proved that exogenous Spd can improve plant resistance to drought (Németh et al., 2002), chilling (Zheng et al., 2008), aging (Wang et al., 2000), and heat (Tian et al., 2009), but whether exogenous Spd can be used to reduce submergence damage in rice has not been shown. To answer this question, whole plant of rice were harvested from both treated and untreated plants to measure growth, yield, and antioxidant enzyme activity. The present study aims to assess the possible effect of exogenous Spd application to alleviate the damage caused by submergence stress.

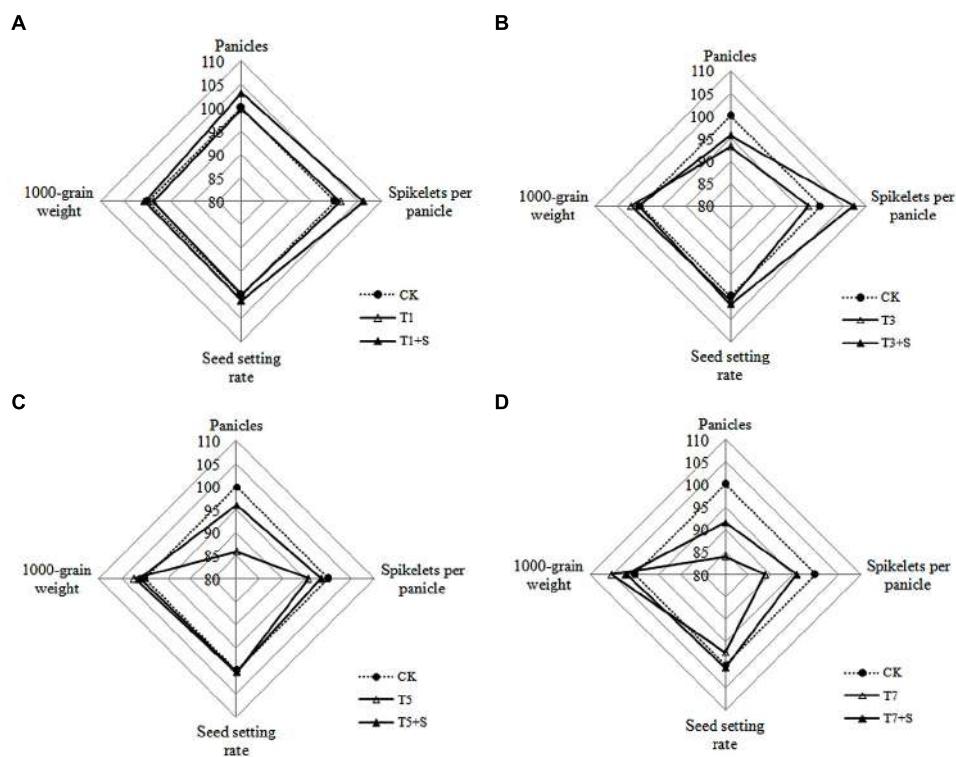


**FIGURE 4 |** Effects of Spd on yield of rice after submergence.

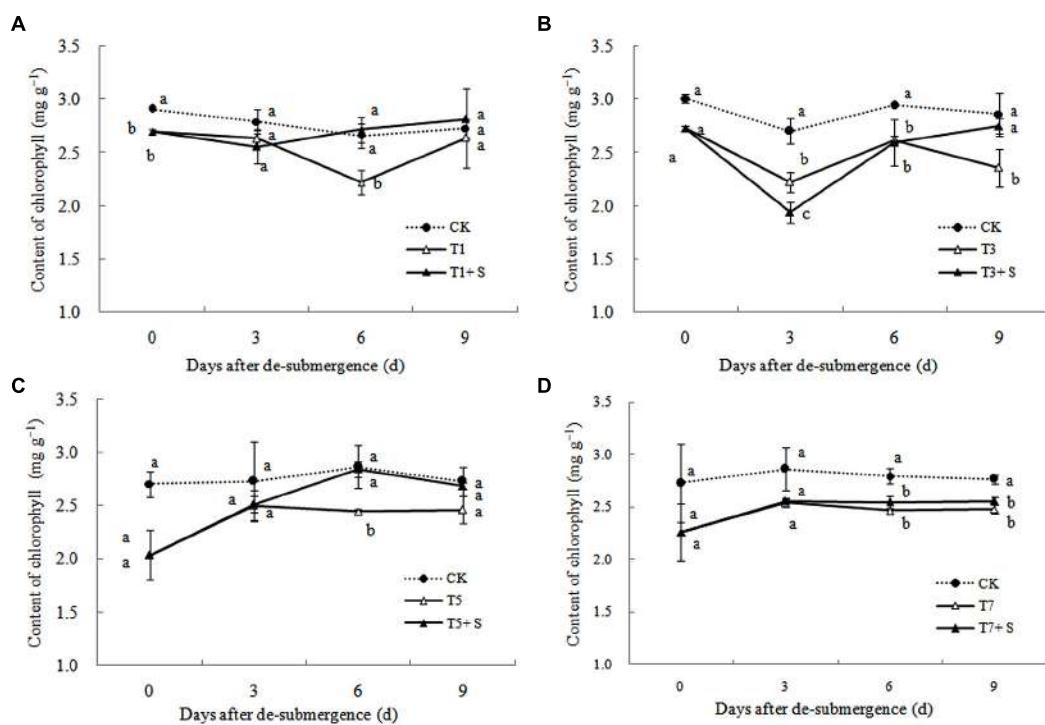
## MATERIALS AND METHODS

### Experimental Design

The experiment was performed in 2014 growing seasons in Baolin village, Danyang County, Jiangsu province (31°54'N, 119°28'E). A japonica rice cultivar (Ningjing 3) popularized



**FIGURE 5 | Effects of Spd on yield traits of rice after submergence.** Each data point in the spider plots represents the percentage of the mean values of the agronomic traits. The mean measurements from the CK were assigned a 100% reference value.



**FIGURE 6 | Effects of Spd on chlorophyll content of rice after submergence.**

in lower reaches of the Yangtze River were grown on a farm during the rice growing seasons, which occurs from later-May to early-November. During the last year, the species was identified as sensitive to submergence. The soil was an Orthic Acrisol, with a total nitrogen content of  $1.1 \text{ g kg}^{-1}$ , total phosphorus content of  $0.48 \text{ g kg}^{-1}$ , total potassium content of  $1.96 \text{ g kg}^{-1}$ . The experiment was arranged in a completely randomized block design with three replicates. The area of a plot was  $4 \text{ m} \times 5 \text{ m} = 20 \text{ m}^2$ .

## Crop Management

Seedlings 20-day-old raised in the seedling disk were transplanted in 10th June, with hill spacing of  $0.3 \text{ m} \times 0.13 \text{ m}$  and three seedlings per hill at both sites. Nitrogen ( $135 \text{ kg ha}^{-1}$  N as urea), phosphorus ( $247 \text{ kg ha}^{-1}$   $\text{P}_2\text{O}_5$  as single superphosphate), potassium ( $450 \text{ kg ha}^{-1}$   $\text{K}_2\text{O}$  as KCl), were incorporated in plots 1 day before transplanting, additional N was applied 7 days after transplanting ( $135 \text{ kg ha}^{-1}$ ), panicle initial (PI;  $135 \text{ kg ha}^{-1}$ ), and the stage of the second leaf from the top extension ( $135 \text{ kg ha}^{-1}$ ).

The waterproof wall above 1 m was built around both sites, laying plastic film to prevent leakage. After 10 days transplanting, flooding treatment was started. The submerged water came from nearly river. Set 1, 3, 5, 7 days submergence treatments in four sites, respectively, and set a control site (CK) with

no submergence. The submerged sites set a daily supplement of water to ensure complete submergence. At the end of the submergence treatments, excess water was discharged, and converted to normal field management.

Each submerged site was equally divided into two parts, separated by a ridge wrapped in plastic film. One part of each submerged site sprayed with Spd on rice leaves, which were signed as T1 + S, T3 + S, T5 + S, T7 + S, and the other part sprayed with equally water, signed as T1, T3, T5, T7, respectively. We used nebulizer to sprayed 50 ml Spd per square meter (0.5 mM, Sigma-Aldrich Chemical Co., St. Louis, MO, USA) on rice leaves at 8:00 and 18:00 in the day of drainage. Tween-20 (0.5%, v/v; Haijiechem, Zibo, China) was used both in Spd solution(T + S) and water(T) as a surfactant to increase adsorption, ensuring both side of the leaves were all stained with solution.

## Parameter Measurements

Four plants were sampled in each sites immediately at the day flooding over (0 day), and 3, 6, 9 days after spraying Spd. The fully expanded three leaves of the two plants immediately snap-frozen in liquid nitrogen, and stored at  $-40^\circ\text{C}$  until required for analysis. The remaining two plants were oven-dried at  $105^\circ\text{C}$  for 30 min followed by  $80^\circ\text{C}$  for 72 h to constant weight. The tillers

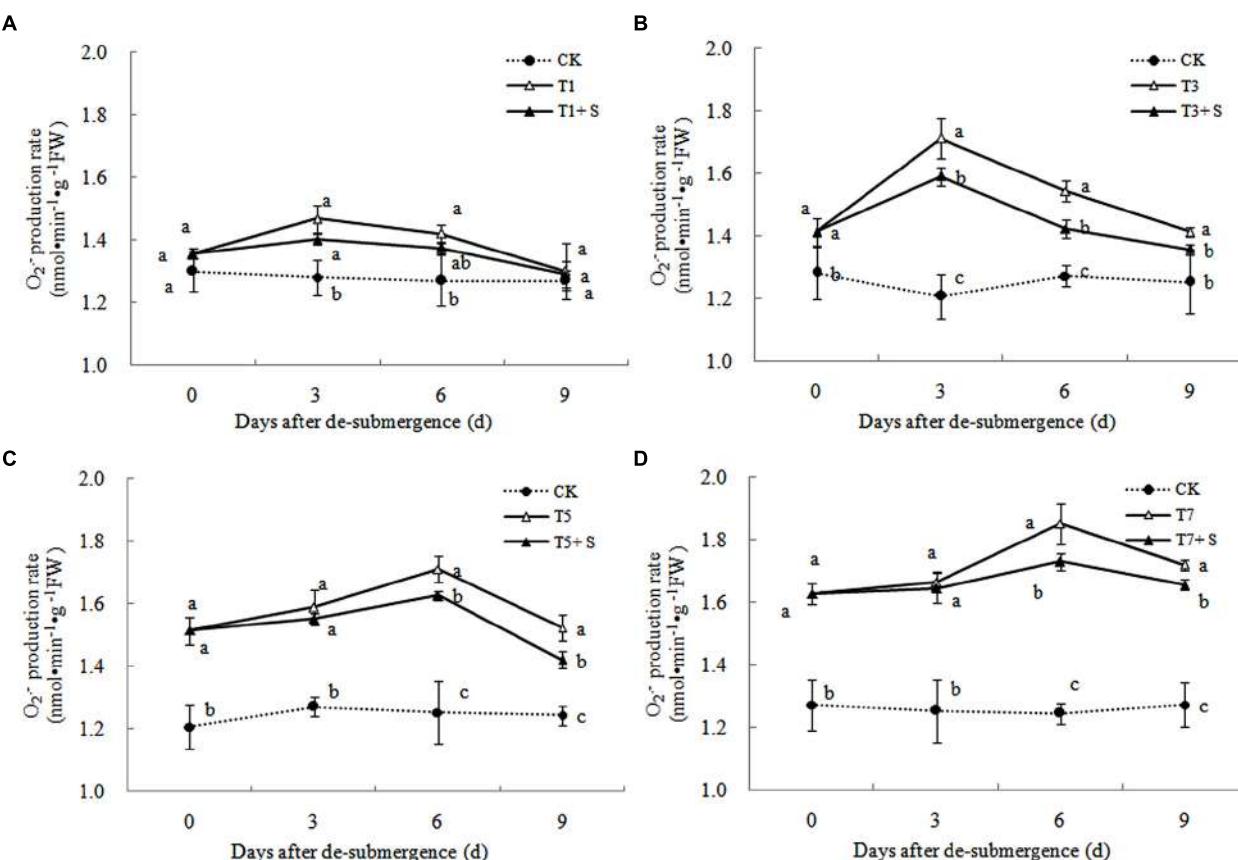


FIGURE 7 | Effects of Spd on  $\text{O}_2^-$  production rate of rice after submergence.

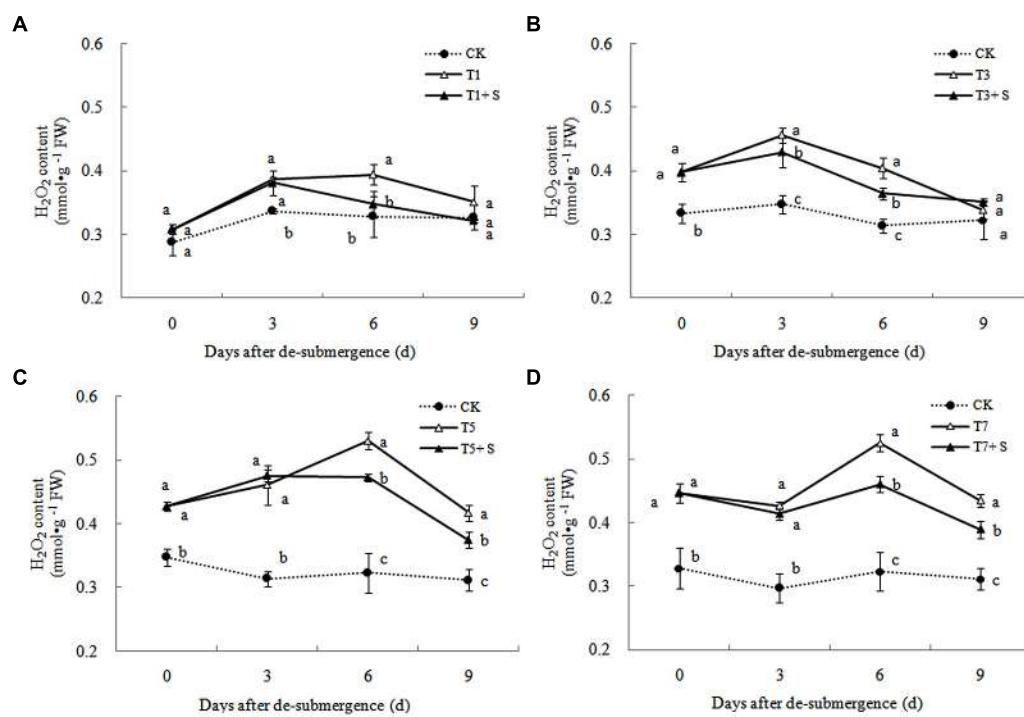
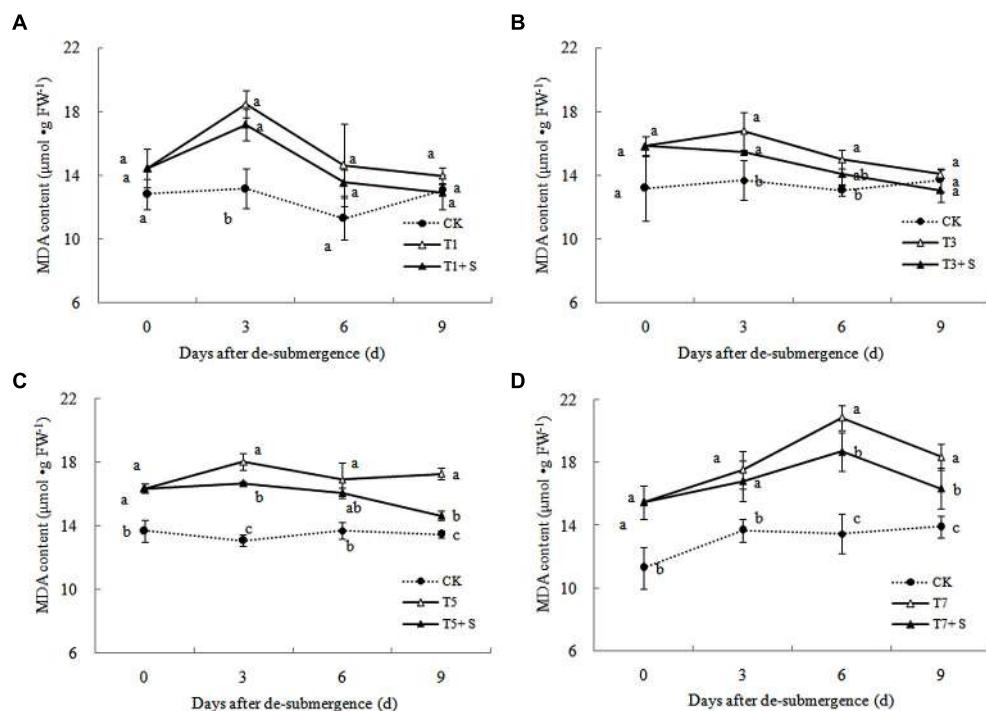
FIGURE 8 | Effects of Spd on  $\text{H}_2\text{O}_2$  content of rice after submergence.

FIGURE 9 | Effects of Spd on malondialdehyde (MDA) content of rice after submergence.

number and green leaves number per hill was counted by manual, as well as yield traits in mature stage.

Chlorophyll content was measured by the method according to a previous study (Li, 2000). The measurement of  $O_2^-$  production rate has been described previously (Wang and Luo, 1990).  $H_2O_2$  content was measured by the kit provided by Nanjing Jiancheng Biology Company. The leave sample (0.3 g) was homogenized with a mortar and pestle in 5 mL ice-cold phosphate buffer (50 mM, pH 7.8) containing 1% (w/v) insoluble polyvinylpolypyrrolidone (PVPP; Jiang and Zhang, 2002). The extract was centrifuged at  $16,000 \times g_n$  for 20 min at 4°C. The supernatant was used to measure enzyme activity. Superoxide dismutase (SOD) activity, peroxidase (POD) activity and GR activity was measured according to the method of Li (2000). Malondialdehyde (MDA) content was determined by the thiobarbituric acid reaction following the method described previously (Zhao et al., 1994).

## Data Analysis

All data were analyzed by SPSS (IBM SPSS statistics 20), and the results were presented as the means  $\pm$  SD. Statistically analyzed using Duncan's multiple range test at a level of significance of 0.05. Figures were manufactured by Microsoft Excel 2007 software.

## RESULTS

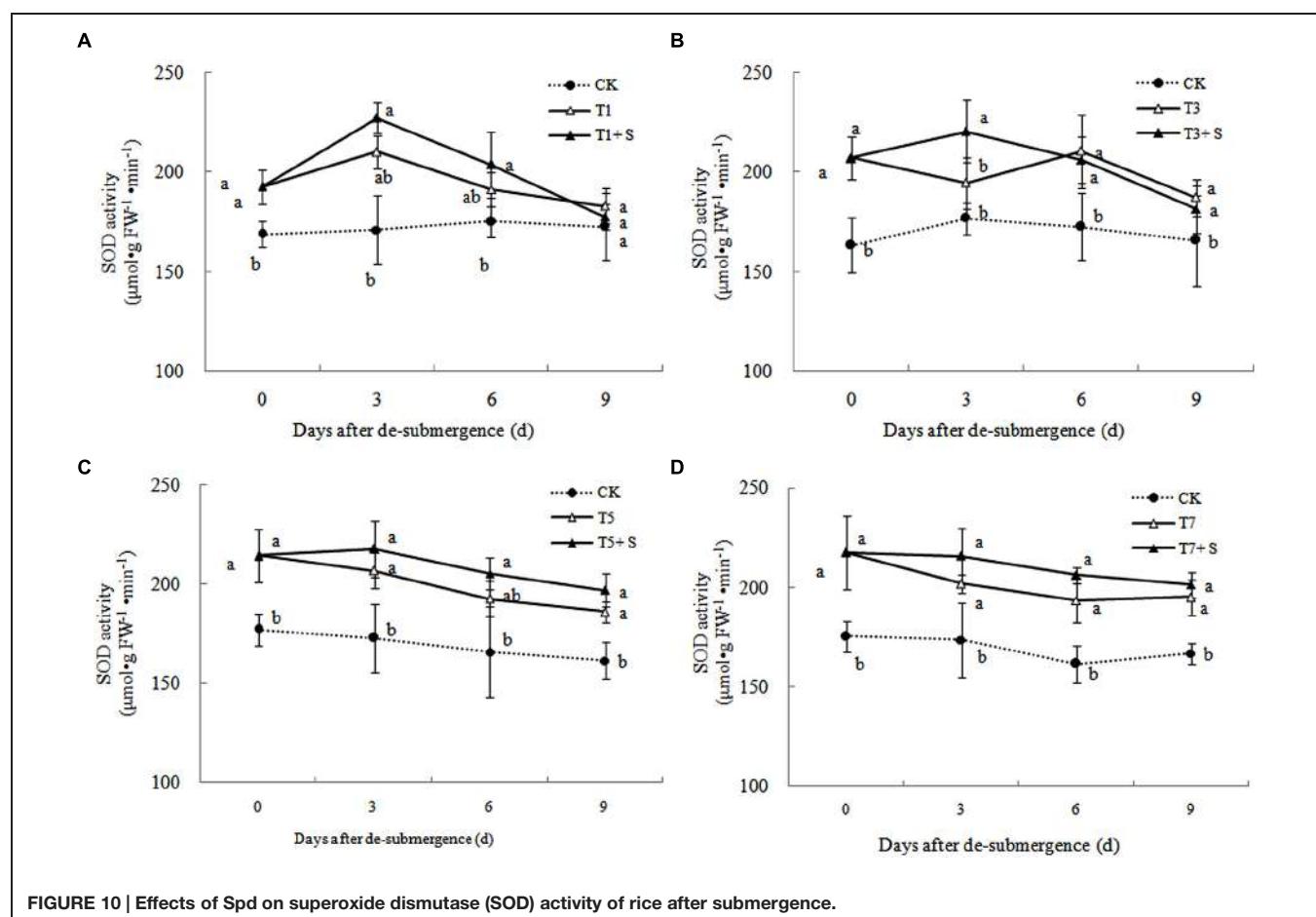
### Effects of Exogenous Spd on Growth and Yield of Submerged Rice

#### Growth

Except for T1 treatment, the green leaves number (Figure 1), tillers number (Figure 2) and aboveground dry mass (Figure 3) of rice were significantly decreased after submergence stress ( $P < 0.05$ ) compared with the control, and resumed slowly after drainage. Application of exogenous SPD significantly increased the green leaves number, tillers number, and aboveground dry mass of submerged rice, effectively alleviating the submergence-mediated growth reduction.

#### Yield

The rice yields decreased gradually with the increase of the days under submergence stress (Figure 4), especially the yields of T5 and T7 significantly decreased compared with the control (CK). Exogenous Spd apparently elevated rice yields after submergence stresses. 12.1 and 17.9% of yields were increased, respectively, compared with T1 and T3, and returned to the CK level. Exogenous Spd also increased the yields of T5 + S and T7 + S by 13.5 and 18.0%, respectively.



**FIGURE 10 |** Effects of Spd on superoxide dismutase (SOD) activity of rice after submergence.

As for yield traits (Figure 5), panicle number and spikelet number per panicle decreased due to submergence stresses, while the seed setting rates and 1000-grain weights were less affected. Application of exogenous Spd obviously improved the panicle number and spikelet number per panicle, not the seed setting rates and 1000-grain weights.

## Effects of Exogenous Spd on Physiological Characteristics of Submerged Rice Chlorophyll Content

After different days of submergence treatment, the chlorophyll content of leaves under submergence conditions was significantly lower than that of leaves under CK conditions (Figure 6). The plants applied with exogenous Spd, had an accelerate effect on the process of chlorophyll recovery, but not apparently for T7 + S.

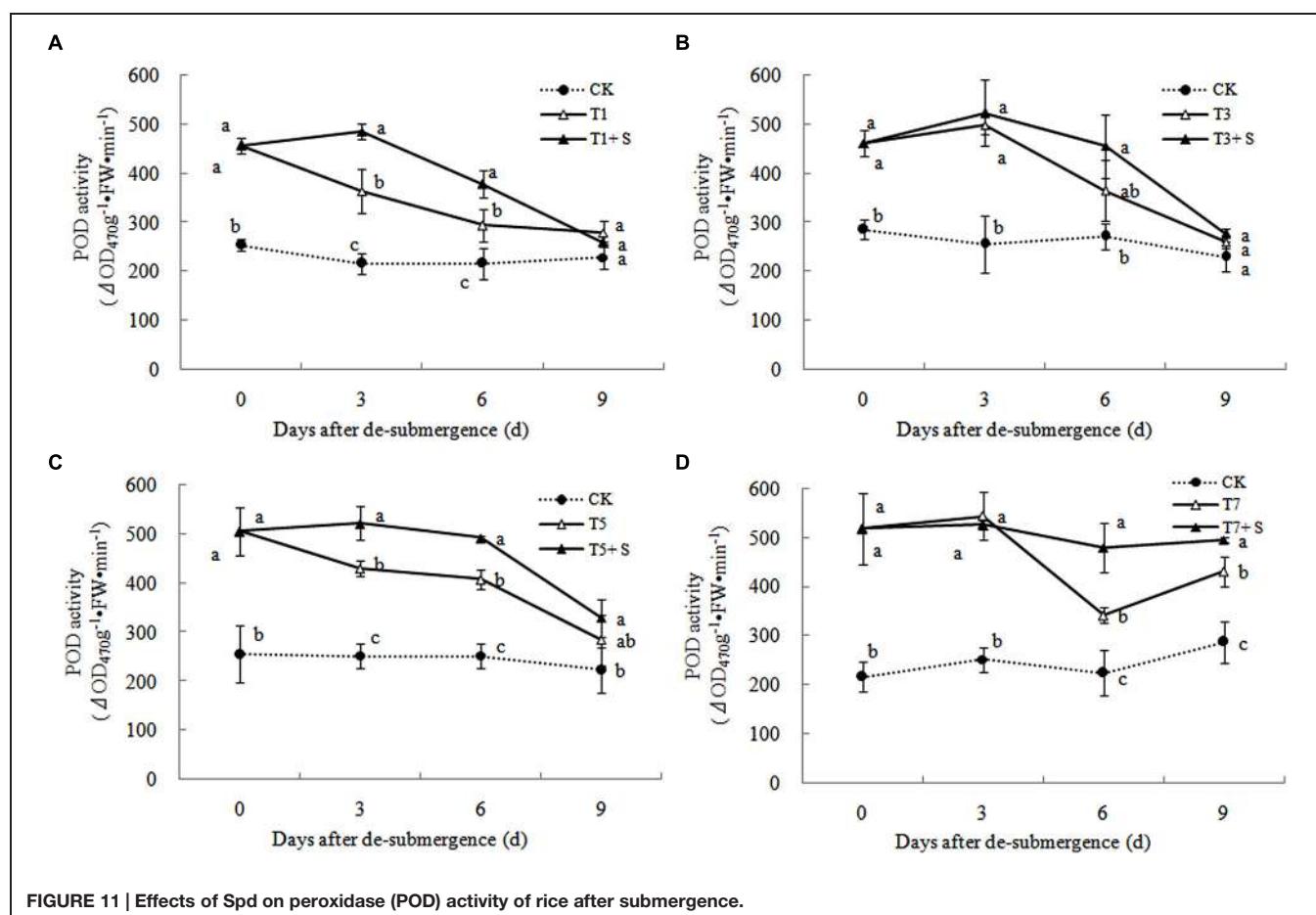
### Free Radical Production and Membrane Damage

In plants subjected to submergence stress for 3 days or longer, the  $\text{O}_2^-$  production rate (Figure 7) and  $\text{H}_2\text{O}_2$  content (Figure 8) significantly increased compared with CK plants. After 9 days of recovery, the  $\text{O}_2^-$  production rate and  $\text{H}_2\text{O}_2$  content of T1 and T3 reduced to the CK level, exogenous Spd accelerated this process.

This phenomenon could also found in T5 + S and T7 + S, the  $\text{O}_2^-$  production rate were 6.7 and 3.8% lower than T5 and T7 at ninth day after drainage, and  $\text{H}_2\text{O}_2$  content was 10.2 and 10.4% lower, respectively. Lipid peroxidation of membranes can be estimated from the MDA content. In submerged plants, MDA levels were significantly higher than those in the CK plants (Figure 9) at third day after drainage. The MDA contents, respectively, decreased by 7.5, 7.1, 15.2, and 11.0% than water sprayed plants (T), after 9 days of Spd treatments.

### Antioxidant Enzyme Activity

Superoxide dismutase, POD, and GR were the enzymes selected to evaluate the oxidative damage caused by flooding on the antioxidant defense system. SOD activity was significantly enhanced after submergence stresses (Figure 10), and similar phenomenon were observed in POD (Figure 11) and GR (Figure 12). Whereas the activities of these enzymes turned to decline rapidly after drainage. We observed that exogenous Spd further increased the activity of SOD, 8.16, 13.31, 5.29, and 6.98% higher than T1, T3, T5 and T7 in third day after spraying, respectively. Similarly, POD and GR activities were also apparently elevated compared with the plants sprayed with water. In addition, exogenous Spd slowed down the decline of the activities of antioxidant enzymes which maintain high levels consistently.



**FIGURE 11 |** Effects of Spd on peroxidase (POD) activity of rice after submergence.

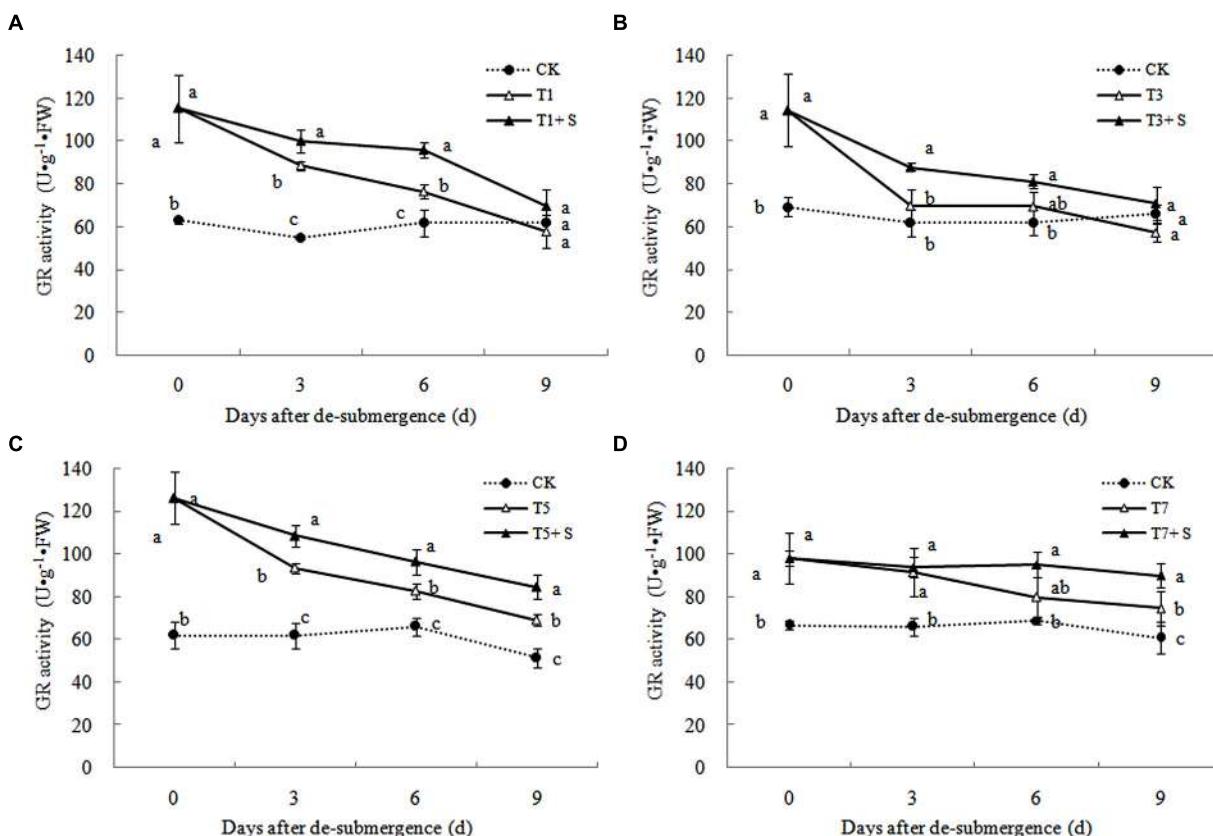
## DISCUSSION

Under natural conditions, plants maintain a balance between producing and scavenging ROS through a well-coordinated and rapidly responsive antioxidant system (Bowler et al., 1992). However, diverse environmental stresses differentially affect cellular homeostasis and induce the formation of ROS, such as submergence, which causes oxidative damage to membrane and lipids (Srivalli et al., 2003). MDA content can reflect the damage degree of plants, because lipid peroxidation of membranes can be estimated from the MDA content (Liao et al., 2005). In this study, submergence stress significantly increased the levels of  $O_2^-$  (Figure 7) and  $H_2O_2$  (Figure 8), as well as the MDA contents (Figure 9). These indicators increased at first and then decreased after drainage, showing that submergence stress caused a delayed stress on rice, which is similar to the research on the rapeseed (Tao, 2013). Rely on the recovery abilities themselves, the MDA content,  $O_2^-$  production rate and  $H_2O_2$  content in the plants of T1 and T3 can return back to the normal levels after drainage. However, it was not showed in T5 and T7, probably the activities of antioxidant enzymes was stronger affected (Han et al., 2011). It is well documented that PAs can counteract oxidative damage in plants by acting as direct free radical scavengers (Drolet et al., 1986). On the other hand, Spd may act as a protectant for the plasma

membrane against stress damage by maintaining membrane integrity (Tadolini et al., 1984; Tadolini, 1988). Our results showed that exogenous Spd promoted the reduction of the severe lipid peroxidation under submergence stress (Figures 7–9), which is in agreement with the previous report (Yiu et al., 2009).

It has been reported that a major role of oxygen radicals in chlorophyll destruction by waterlogging in mung bean leaves is indicated (Ahmed et al., 2002). It was also found in this study that chlorophyll significantly decreased in rice leaves under submergence stress (Figure 6). Lower chlorophyll would turn the leaves yellow, resulting in a decline in photosynthesis and photosynthetic products, thus affects plant physiological metabolism (Zahed et al., 2009). Our results showed that chlorophyll losses were effectively reversed by the exogenous Spd (Figure 6), which was consistent with other study (Wang et al., 2000). The increased chlorophyll content in leaves perhaps has an important role to promote the growth of tillers and leaves and increase dry matter of rice (Figures 1–3).

The major ROS-scavenging enzymes of plants include SOD, POD, CAT, APX, and GR. SOD converts  $O_2^-$  to  $H_2O_2$  (Alscher et al., 2002), whereas POD, CAT, and GR help to minimize the damaging effects of  $H_2O_2$  by converting it into oxygen and water (Peters et al., 1989; Liu et al., 2009). With the action of the enzymes, the MDA content,  $O_2^-$  production rate



**FIGURE 12 |** Effects of Spd on GR activity of rice after submergence.

and H<sub>2</sub>O<sub>2</sub> content showed a trend of decline (**Figures 7–9**). Previous research indicated that exogenous Spd could increase antioxidant enzymes activities in plant under stress (Xu et al., 2001; Kasukabe et al., 2004; Tang and Newton, 2005). Our study found that exogenous Spd not only promoted the activities of SOD, POD, and GR, but also delayed the rate of decline in these enzymes activities (**Figures 10–12**). Maintaining a high level of activities can ensure the efficient removal of ROS (Huang et al., 1990; Jiang et al., 1992; Xie et al., 1998). The probably reason for Spd acting as an inhibitor of ROS production is that Spd can scavenge ROS directly (Drolet et al., 1986) or indirectly by improving antioxidant enzyme activities, through combining with antioxidant enzymes molecule (Slocum et al., 1984; Mehta et al., 1991). In this study, how Spd scavenge ROS is unclear, which may need further research to verify it.

Submergence stress causes adverse effects on plant growth and productivity (Boru et al., 2001; Wang et al., 2007). Growth analysis is widely used as a tool to characterize plant growth. Our results showed that exogenous Spd sprayed on rice leaves significantly alleviated the growth inhibition by submergence stress, increasing green leaves (**Figure 1**), tillers (**Figure 2**), and biomass accumulation (**Figure 3**).

Damage of submergence stress on rice was ultimately reflected on yield. Our results showed that the panicle number and the spikelet number per panicle of rice significantly decreased under submergence stress (**Figure 5**), leading to a decline of yield (**Figure 4**). The longer under submergence, the more yield decreased (Lin et al., 1997). Submergence stress seriously affected

the growth of tillers (**Figure 2**), thus led to the significant decrease of the panicle number. The spikelet number per panicle are closely associated with nutrient levels at tillering stage (Mae, 1997), which is the reason why the spikelet number per panicle declined resulted from the decrease of biomass accumulation. The exogenous Spd significantly improved the panicle number, the spikelet number per panicle and grain yields of rice (**Figures 4** and **5**), probably resulting from the raising of the green leaves, tillers, and biomass accumulation (**Figures 1–3**). We found that spraying Spd increased rice yields by more than 10%, indicating that the exogenous Spd plays an important role in reducing the yield loss.

## AUTHOR CONTRIBUTIONS

GL conceived and designed the experiments. YD and SW performed the experiments. ML analyzed the data. ML and MC wrote the paper.

## FUNDING

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Low Temperature-Induced 30 (LTI30) positively regulates drought stress resistance in *Arabidopsis*: effect on abscisic acid sensitivity and hydrogen peroxide accumulation

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As a dehydrin belonging to group II late embryogenesis abundant protein (LEA) family, *Arabidopsis* Low Temperature-Induced 30 (LTI30)/XERO2 has been shown to be involved in plant freezing stress resistance. However, the other roles of *AtLTI30* remain unknown. In this study, we found that the expression of *AtLTI30* was largely induced by drought stress and abscisic acid (ABA) treatments. Thereafter, *AtLTI30* knockout mutants and overexpressing plants were isolated to investigate the possible involvement of *AtLTI30* in ABA and drought stress responses. *AtLTI30* knockout mutants were less sensitive to ABA-mediated seed germination, while *AtLTI30* overexpressing plants were more sensitive to ABA compared with wild type (WT). Consistently, the *AtLTI30* knockout mutants displayed decreased drought stress resistance, while the *AtLTI30* overexpressing plants showed improved drought stress resistance compared with WT, as evidenced by a higher survival rate and lower leaf water loss than WT after drought stress. Moreover, manipulation of *AtLTI30* expression positively regulated the activities of catalases (CATs) and endogenous proline content, as a result, negatively regulated drought stress-triggered hydrogen peroxide ( $H_2O_2$ ) accumulation. All these results indicate that *AtLTI30* is a positive regulator of plant drought stress resistance, partially through the modulation of ABA sensitivity,  $H_2O_2$  and proline accumulation.

**Keywords:** Low Temperature-Induced 30, abscisic acid, hydrogen peroxide, drought stress, reactive oxygen species, *Arabidopsis*

## INTRODUCTION

Plants are exposed to various environmental conditions, however, plants can not change their location to avoid unfavorable circumstance (Shi et al., 2013a,b, 2014a,b). Among multiple stresses, drought stress is one of the most harsh environmental stresses (Seki et al., 2007; Harb et al., 2010; Chan and Shi, 2015). To date, plants have developed sophisticated strategies to counteract sudden environmental changes. Many secondary messengers, including abscisic acid (ABA), and hydrogen peroxide ( $H_2O_2$ ), are involved in plant stress transduction (Seki et al., 2007; Yu et al., 2008; Fujii et al., 2009; Cutler et al., 2010; Qin et al., 2011). Both endogenous concentrations and

the underlying signaling pathways of ABA and H<sub>2</sub>O<sub>2</sub> play essential roles in plant drought stress responses (Fujii et al., 2009; Cutler et al., 2010; Harb et al., 2010; Qin et al., 2011; Munemasa et al., 2013).

*Arabidopsis Low Temperature-Induced 30* (LTI30)/XERO2 belongs to the group II late embryogenes abundant protein (LEA)/dehydrin family (Rouse et al., 1996). The transcript level of *AtLTI30/XERO2* can be induced by ABA, cold, dehydration, wounding, and salt stresses (Welin et al., 1994; Rouse et al., 1996; Nylander et al., 2001; Chung and Parish, 2008). Overexpression of *AtLTI30/XERO2* enhances freezing stress resistance in *Arabidopsis* (Puhalainen et al., 2004). AtCBF1, AtCBF2, and AtCBF3 (also known as AtDREB1b, AtDREB1c, and AtDREB1a, respectively) are important transcription factors in plant abiotic stress responses. To date, many stress-responsive genes with C-repeat (CRT)/dehydration-responsive element (DRE) in the promoters have been identified as the direct targets of AtCBFs. These genes include *COR* (*cold regulated*), *ERD* (*early responsive to dehydration*), *KIN* (*cold inducible*), *LTI* (*low-temperature induced*), and *RD* (*responsive to dehydration*) (Gilmour et al., 1998; Zarka et al., 2003; Cook et al., 2004; Thomashow, 2010). Using multiple combinations of mutations in the promoter of *AtLTI30/XERO2*, Chung and Parish (2008) found that two of the ACGT and DRE/CRT elements in the promoter of *AtLTI30/XERO2* were essential for cold and ABA transcriptional induction of *AtLTI30/XERO2*. Mouillon et al. (2006) found that the lysine-rich segment of *AtLTI30/XERO2* showed sequence similarity with the animal chaperone heat shock protein 90 (HSP90). The conserved segments of *AtLTI30* exerted its biological function more locally upon interaction with specific biological targets. Moreover, Eriksson et al. (2011) identified three factors that regulate the lipid interaction of LTI30 *in vitro*, including the pH dependent His on/off switch, reversal of membrane binding by proteolytic digestion, and phosphorylation by protein kinase C.

Although *AtLTI30* has been shown to be involved in plant freezing stress resistance, the other roles of *AtLTI30* and the underlying mechanisms remain unknown. In this study, the expression and function of *AtLTI30* were characterized in response to drought stress treatment. We investigated the effects of manipulation of *AtLTI30* expression on drought stress resistance, as well as the underlying mechanisms. The results indicate that *AtLTI30* is a positive regulator of drought stress resistance in *Arabidopsis*.

## MATERIALS AND METHODS

### Plant Materials and Growth Conditions

After stratification at 4°C for 3 days in darkness, *Arabidopsis thaliana* seeds were sown in soil in a growth chamber, and watered with a nutrient solution twice per week. The growth chamber was controlled at 23°C, with an irradiance of about 150 μmol quanta m<sup>-2</sup> s<sup>-1</sup>, under 65% relative humidity and 16-h light and 8-h dark cycles. The *lti30-1* (SALK\_114915) and *lti30-2* (SALK\_016819) mutants were obtained from the *Arabidopsis* Biological Resource Center (ABRC).

### RNA Isolation, Semi-quantitative RT-PCR and Quantitative Real-time PCR

Total RNA was extracted and purified using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and RQ1 RNase-free DNase (Promega, Madison, WI, USA). First-strand cDNA was synthesized from total RNA using reverse transcriptase (TOYOBO, Osaka city, Japan) as (Shi et al., 2013a,b, 2014a,b, 2015) previously described. Semi-quantitative RT-PCR was performed as Shi et al. (2013a) described using *ubiquitin 10* (*UBQ10*) as the internal control. Quantitative real-time PCR was performed using the CFX96™ Real-Time System (BIO-RAD, Hercules, CA, USA) and the comparative ΔΔCT method with *UBQ10* as a reference gene following (Shi et al., 2013a,b, 2014a,b, 2015). The primers of *UBQ10* (At4g05320) and *LTI30* (At3g50970) are shown in Supplementary Table S1.

### Construction of Vectors and Generation of Transgenic Lines

For the *pLTI30::β-glucuronidase (GUS)* transgenic construction, the promoter region of *AtLTI30* was amplified by PCR and inserted into the *BamHI* site of the pBI101.2 vector with kanamycin resistance. For *AtLTI30* overexpressing transgenic construction, the coding region of *AtLTI30* was amplified by PCR and inserted into the *SmaI/XhoI* sites of the pBIM vector with kanamycin resistance under the control of the cauliflower mosaic virus (CaMV) 35S promoter (Yang et al., 2005). The responsible primers for the above vector constructions are shown in Supplementary Table S2. The recombinant constructions were confirmed by DNA sequencing and introduced into wild type (WT) plants of Columbia-0 (Col-0) using *Agrobacterium tumefaciens* strain GV3101-mediated transformation and the floral dip method (Clough and Bent, 1998). Thereafter the transgenic plants were selected on MS medium using kanamycin resistance, and further confirmed by PCR analysis.

### GUS Staining and Quantification of GUS Activity

Glucuronidase staining and quantification of GUS activity were performed as Jefferson et al. (1987) previously described. For GUS staining, *proLTI30::GUS* transgenic plants were incubated in staining solution (100 mM sodium phosphate buffer, pH 7.5, 0.5 mM K<sub>3</sub>[Fe(CN)<sub>6</sub>], and 0.5 mM K<sub>4</sub>[Fe(CN)<sub>6</sub>], 10 mM EDTA, 1.0 mM 5-bromo-chloro-3-indolyl-β-D-glucuronide and 0.1% Triton X-100) at 37°C for 8 h, after which the plants were incubated in 70% ethanol to remove chlorophyll. GUS activity was quantified by detecting the conversion of 4-methylumbelliferyl-β-glucuronide in the same concentration of protein extract, as Jefferson et al. (1987) previously described.

### Determination of ABA Sensitivity

For ABA sensitivity assay, different genotypes of *Arabidopsis* seeds were sterilized with 70% (v/v) ethyl alcohol, 5% (w/v) NaClO, and deionized water. The seeds were stratified at 4°C for 3 days, thereafter were sown on Murashige and Skoog (MS) medium plates containing different concentrations of ABA.

Germination ratios as seen with emerged radicals and green cotyledons were scored after 10 days in the growth chamber. Stomatal aperture in *Arabidopsis* leaves was determined as Shi et al. (2013a) described.

## Plant Drought Stress Treatment and Drought Stress Resistance Assay

For drought stress, 14-day-old *Arabidopsis* plants in the soil were subjected to control (well-watered) and drought stress (withheld water) conditions for another 21 days. More than three pots of each variety (27 plants) were used in each biological repeat, and all these pots with plants were rotated daily during drought stress to minimize the environment effect. The survival rate of stressed plants was recorded after re-watered for 4 days later than 21 days of drought stress treatment. Relative *in vitro* leaf water loss rate was expressed as percent change in leaf fresh weight (FW) as (Shi et al., 2013a,b, 2014a,b, 2015) described.

## Quantification of Hydrogen Peroxide ( $H_2O_2$ ) and Catalase (CAT) Activity

The concentration of  $H_2O_2$  in plant leaves was determined using the titanium sulfate method, and CAT (EC 1.11.1.6) activity was determined using the enzyme assay kit as (Shi et al., 2013a,b, 2014a,b, 2015) previously reported.

## The Determination of Proline Content

Quantification of endogenous proline content was performed as Shi et al. (2013a,b) previously described. Briefly, endogenous proline in plant leaves was extracted using 3% (w/v) sulfosalicylic acid, and the red solution at the absorbance of 520 nm was determined by adding the mixtures of ninhydrin reagent and glacial acetic acid to the extractions.

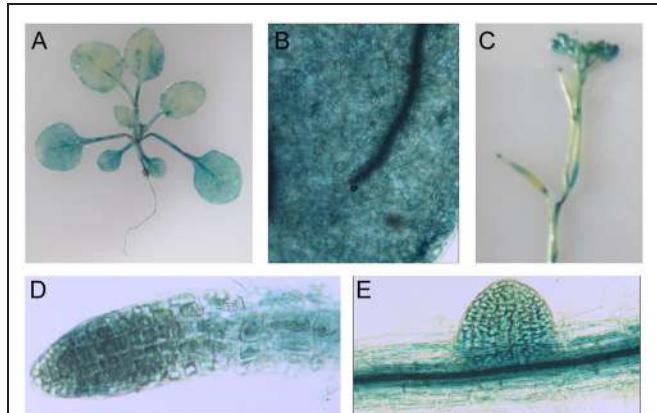
## Statistical Analysis

All experiments were performed with at least three biological repeats, and plant leave samples in each biological repeat were mixture samples of at least 10 plants per genotype. Student's *t*-test and analysis of variance (ANOVA) were used to analysis the significant difference, and asterisk symbols (\*) indicate the significant differences of  $p < 0.05$  in comparison to WT.

## RESULTS

### The Expression Pattern of AtLTI30

Using *proLTI30::GUS* transgenic plants, we found that *AtLTI30* was widely expressed in leaves, stems, flowers, primary roots and lateral roots (Figures 1A–E). Moreover, we found that the GUS activities of *proLTI30::GUS* transgenic plants were significantly increased after dehydration stress and ABA treatments for 1, 3, and 6 h through GUS activity assay (Figure 2A). The GUS result is consistent with previous studies that ABA and dehydration induced the transcript level of *AtLTI30* using northern blot (Welin et al., 1994; Rouse et al., 1996; Nylander et al., 2001; Chung and Parish, 2008). Moreover, we also found that the transcript levels of *AtLTI30* are induced by both dehydration



**FIGURE 1 |** Glucuronidase staining of *proLTI30::GUS* transgenic plants in different organs. (A) GUS staining of 21-day-old of *proLTI30::GUS* transgenic plants. (B–E) GUS staining of *proLTI30::GUS* transgenic plants in leaves (B), flowers (C), primary root (D), and lateral root (E).

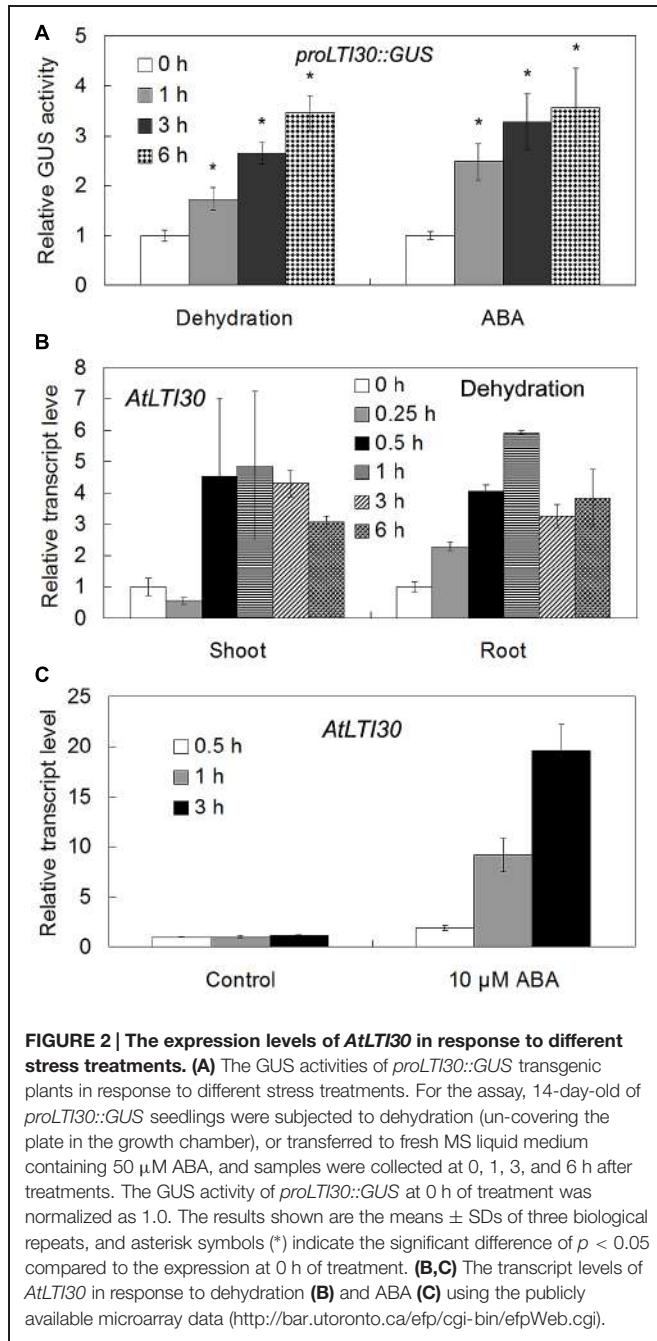
and ABA treatments (Figures 2B,C) using the publicly available microarray data (<http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi>) (Winter et al., 2007). These results indicate the possible link between *AtLTI30* and these stress treatments, and suggest the possible involvement of *AtLTI30* in drought stress responses in *Arabidopsis*.

### Isolation of AtLTI30 Knockout Mutants and Overexpressing Plants

To further reveal the *in vivo* role of *AtLTI30*, we isolated the T-DNA mutants with T-DNA insertion in the exon of *AtLTI30* [*lti30-1* (SALK\_114915) and *lti30-2* (SALK\_016819)] (Figures 3A–C), and constructed *AtLTI30* overexpressing transgenic plants (Figures 3B,C). Using semi-quantitative RT-PCR analysis, the transcript of *AtLTI30* was un-detectable in *lti30-1* and *lti30-2* mutants (Figure 3B). Consistently, quantitative real-time PCR analysis also showed that the transcript level of *AtLTI30* was largely inhibited in *lti30-1* and *lti30-2* mutants, with about 30% of *AtLTI30* transcripts compared with WT plants (Figure 3C). Moreover, the *AtLTI30* overexpressing transgenic plants displayed significantly higher *AtLTI30* transcripts than WT plants, with 15–25-folds higher *AtLTI30* transcripts compared with WT, and the homozygous transgenic plants (OX-2 and OX-3) were chosen for further analysis (Figures 3B,C).

### AtLTI30 Positively Regulates Plant Sensitivity to ABA

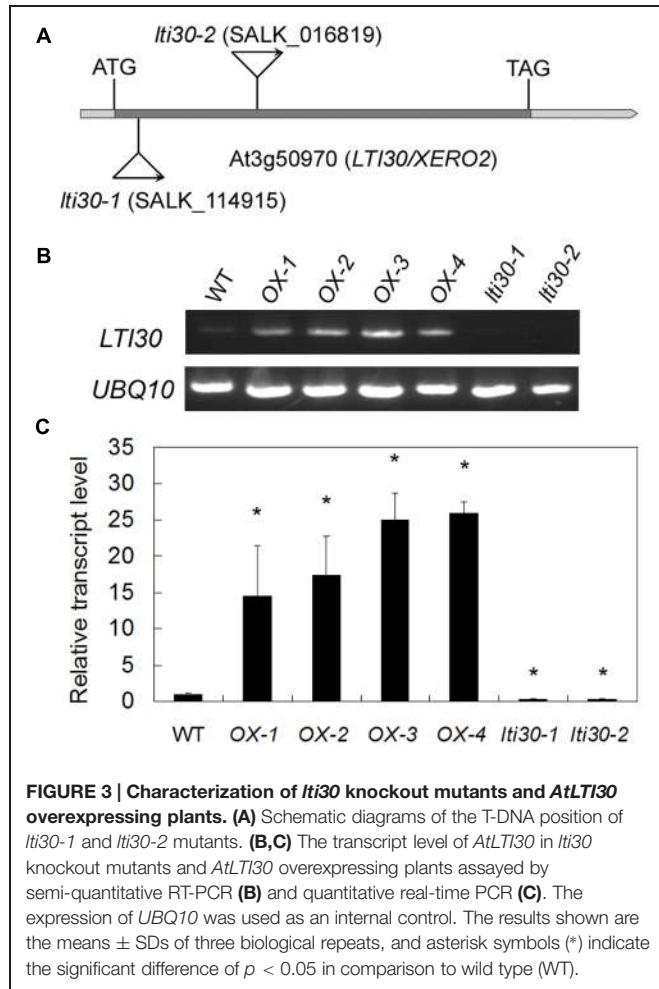
Since the expression of *AtLTI30* was increased after ABA treatment, the responses of the WT, *lti30* knockout mutants, and *AtLTI30* overexpressing plants to ABA were further compared. Germination of *AtLTI30* overexpressing plant seeds was severely inhibited after ABA treatment, as shown with less emerged radical, less green cotyledon compared with those of WT (Figures 4A–C). On the contrary, *lti30-1* and *lti30-2* mutants showed more emerged radicals and more green cotyledons



than those of WT (Figures 4A–C). These results indicate that modulation of *AtLT130* expression positively affects ABA sensitivity in seed germination stage.

### AtLT130 Positively Regulates Drought Stress Resistance

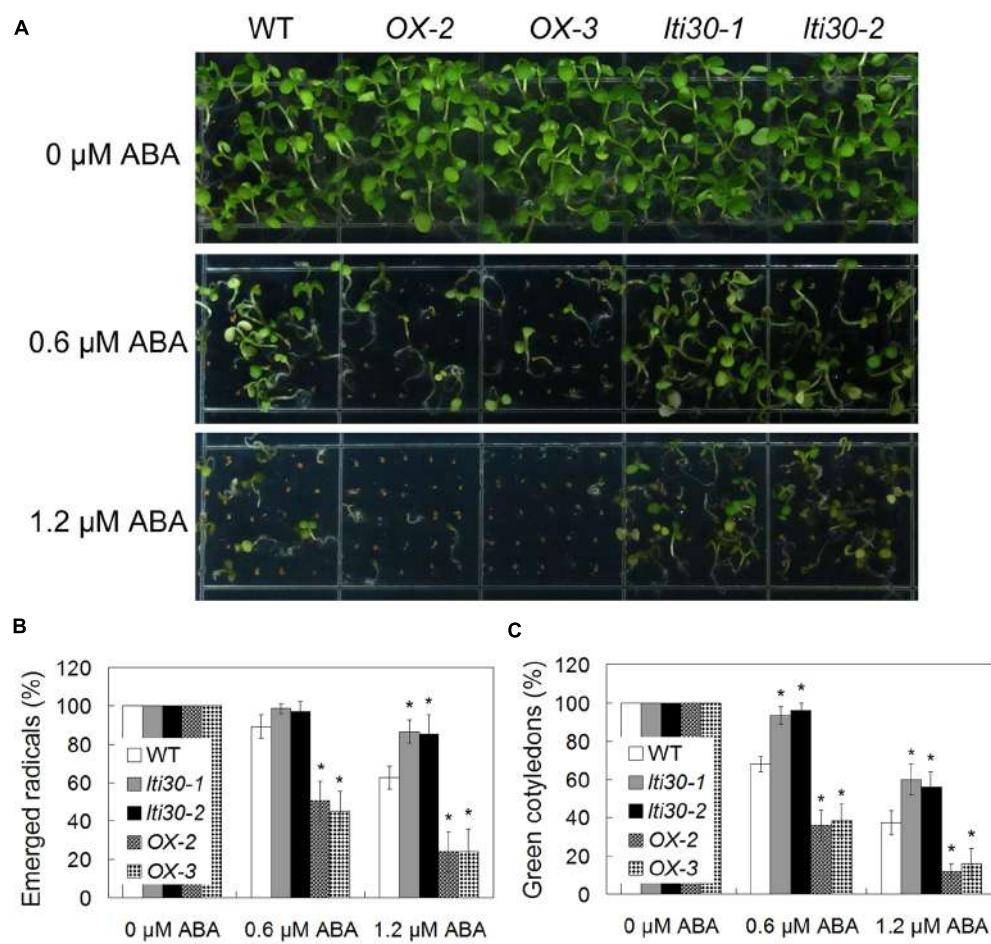
To test whether *AtLT130* regulates drought stress resistance, 14-day-old WT, *lti30* mutants, and *AtLT130* overexpressing plants in pots were subjected to drought stress by withholding water for 21 days and then re-watering the plants for 4 days. After the drought stress treatment, the *lti30-1* and *lti30-2*



mutants displayed significantly lower survival rate, while *AtLT130* overexpressing plants exhibited higher survival rate compared with WT (Figures 5A,B). Consistently, the *lti30-1* and *lti30-2* mutants displayed significantly higher leave water loss rate, while *AtLT130* overexpressing plants showed significantly lower leaf water loss rate from 2 to 8 h after detachment in comparison to WT plants (Figure 5C). These results indicate that *AtLT130* positively regulates drought stress resistance.

### Modulation of AtLT130 Expression Affects H<sub>2</sub>O<sub>2</sub> Accumulation

Oxidative burst especially H<sub>2</sub>O<sub>2</sub> accumulation occurs following drought stress in plants. We further investigated the effects of *AtLT130* expression on H<sub>2</sub>O<sub>2</sub> accumulation and associated antioxidant defense enzyme activity during the drought stress treatments. During the period between 0 and 15 days of drought stress, H<sub>2</sub>O<sub>2</sub> burst was significantly displayed in WT, the *AtLT130* knockout mutants and overexpressing plants (Figure 6A). In comparison to WT plants, the *AtLT130* knockout mutants showed higher levels of H<sub>2</sub>O<sub>2</sub> at 0, 5, 10, and 15 days of drought stress, while the *AtLT130* overexpressing plants displayed relatively lower levels of H<sub>2</sub>O<sub>2</sub> at these periods (Figure 6A).



**FIGURE 4 | Modulation of *AtLTI30* expression positively affects plant sensitivity to ABA.** **(A)** The photograph showing 10-day-old of *Arabidopsis* plant seedlings after grown on MS plate containing different concentrations of ABA. **(B,C)** Germination rates of plant seeds on MS plate containing different concentrations of ABA that were determined as emerged radicals **(B)** and green cotyledons **(C)**. The results shown are the means  $\pm$  SDs of three biological repeats, and asterisk symbols (\*) indicate the significant difference of  $p < 0.05$  in comparison to WT.

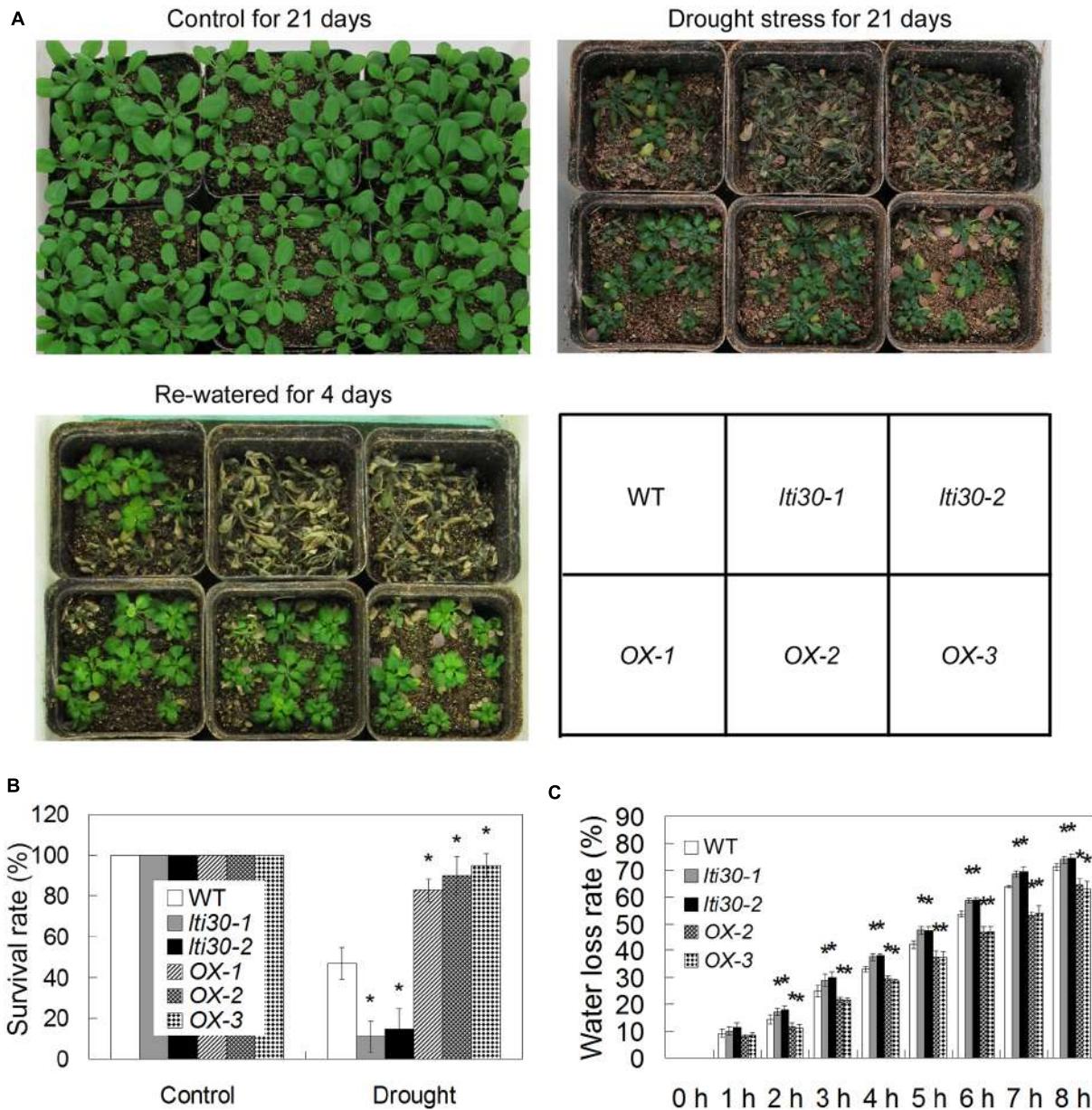
In accordance with the  $H_2O_2$  burst, the *AtLTI30* knockout mutants exhibited lower activities of AtCATs under both control and drought stress conditions, while the *AtLTI30* overexpressing plants showed relatively higher activities of AtCATs in comparison to WT (Figure 6B). Therefore, these results indicate that the *AtLTI30* positively regulates the activities of AtCATs, and negatively regulates  $H_2O_2$  accumulation during drought stress treatment.

## AtLTI30 Positively Regulates Drought Stress Resistance

During the period between 0 and 15 days of drought stress, the endogenous proline content gradually increased in WT, *AtLTI30* knockout mutants and overexpressing plants (Figure 7). In comparison to WT plants, the *AtLTI30* knockout mutants exhibited lower proline contents at 0, 5, 10, and 15 days of drought stress, while the *AtLTI30* overexpressing plants displayed higher levels of proline at these periods (Figure 7).

## DISCUSSION

As a dehydrin belonging to the group II LEA family, *AtLTI30/XERO2* is widely involved in plant freezing stress resistance (Rouse et al., 1996; Nylander et al., 2001). Not only is its transcription induced under cold stress treatment, but its overexpression confers improved freezing stress resistance in *Arabidopsis* (Rouse et al., 1996; Nylander et al., 2001; Puhakainen et al., 2004). Moreover, the common expression between *CBF1/2/3* and *AtLTI30/XERO2*, and between *ABA insensitive 5 (ABI5)* and *AtLTI30/XERO2*, indicated that *AtLTI30/XERO2* may be a direct target of both AtCBFs and AtABI5 (Chung and Parish, 2008). Using multiple combinations of mutations in the promoter of *AtLTI30/XERO2*, they also found that two of the ACGT and CRT/DRE elements are essential for both ABA and cold transcriptional induction of *AtLTI30/XERO2* (Chung and Parish, 2008). However, the possible involvement of *AtLTI30* in ABA signaling and the *in vivo* role of *AtLTI30* in plant drought stress response remains unknown.

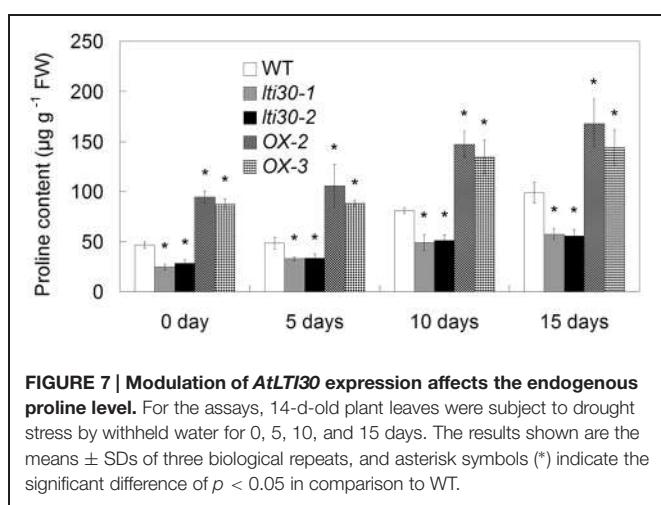
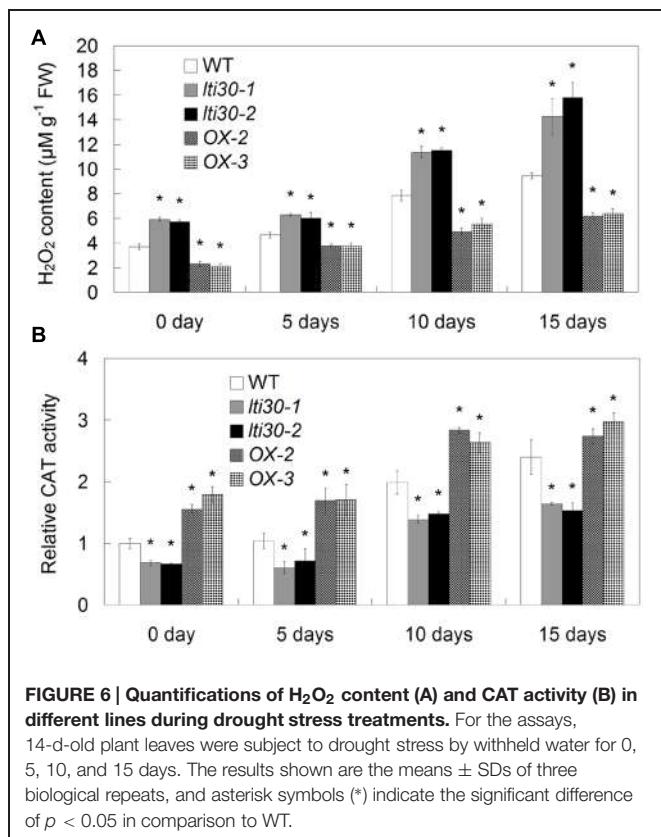


**FIGURE 5 |** AtLTI30 positively regulates drought stress resistance in *Arabidopsis*. **(A)** The photograph showing 14-day-old *Arabidopsis* plants with well-watered for 21 days, withheld water for 21 days, and re-watered for 4 days after withheld water for 21 days. **(B)** The survival rate of different genotypes after recovery for 4 days after 21 days of drought stress treatment. **(C)** Relative leaf water loss rate *in vitro* of different lines. The results shown are the means  $\pm$  SDs of three biological repeats, and asterisk symbols (\*) indicate the significant difference of  $p < 0.05$  in comparison to WT.

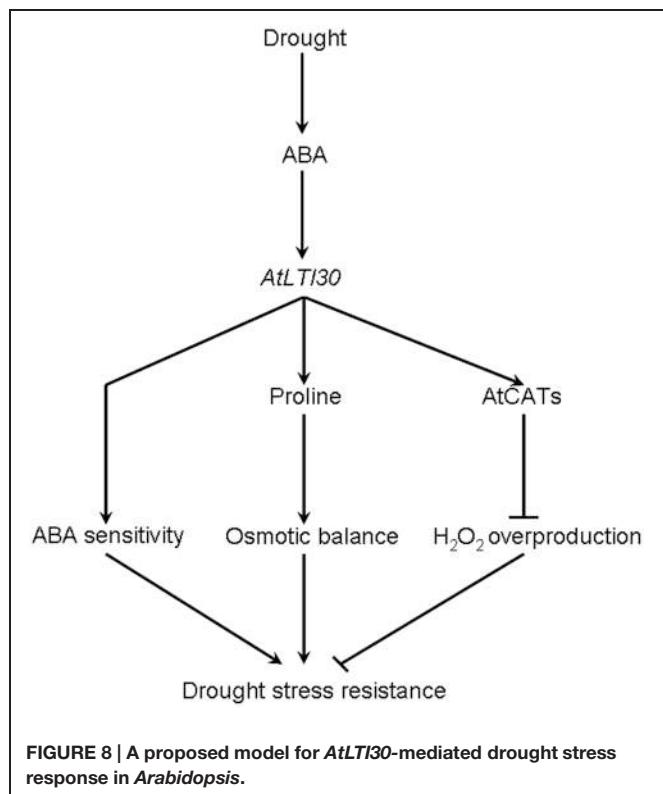
To date, there are three effective methods to improve plant drought stress resistance. The first is to screen and identify drought tolerant varieties, and the second method is the exogenous application of multiple small molecules such as ABA, nitric oxide (NO), polyamines, hydrogen sulfide ( $H_2S$ ), and melatonin (Shi et al., 2013a, 2014a, 2015; Chan and Shi, 2015). The third method is the investigation of new genes that confer plant drought stress resistance and genetic breeding (Miao et al., 2006; Yu et al., 2008; Qin et al., 2011; Zhang et al., 2013). In this study, the new roles of AtLTI30 in drought stress resistance may

provide a useful candidate gene for drought tolerant crop genetic breeding.

In response to drought stress, plant endogenous ABA synthesis and the following ABA-responsive genes are rapidly and largely activated. Briefly, with an increase in the endogenous ABA level, ABA receptors (Pyrabactin Resistance (PYR)/PYR1-Like (PYL)/Regulatory Components of ABA Receptor (RCAR)) disrupt the interaction between type 2C protein phosphatases (PP2Cs) and sucrose non-fermenting 1 (SNF1)-related protein kinases 2 (SnRK2s) by competitively interacting with



PP2Cs. Thereafter, these interaction prevent PP2Cs-mediated dephosphorylation of SnRK2s and the activation of the SnRK2s, leading to the transcriptional activation of ABA-responsive genes (Fujii et al., 2009; Cutler et al., 2010; Harb et al., 2010). Moreover, ABA also induces the accumulation of  $\text{H}_2\text{O}_2$ , and both ABA and ABA-induced  $\text{H}_2\text{O}_2$  play important roles in plant drought stress response, especially in the modulation of stomatal closure (Zhang et al., 2001; Miao et al., 2006; Munemasa et al., 2013). In accordance with previous studies, which showed that ABA and dehydration induced the transcription level of *AtLTI30*, as



evidenced by northern blot analysis (Welin et al., 1994; Rouse et al., 1996; Nylander et al., 2001; Chung and Parish, 2008), the expression of *AtLTI30* was significantly increased after ABA and drought stress treatments using *proLTI30::GUS* transgenic plants and the publicly available microarray data (<http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi>) (Figure 2). Together with the association among ABA,  $\text{H}_2\text{O}_2$  and drought stress, these results indicated the possible role of the *AtLTI30* in the process. After identifying the *AtLTI30* knockout mutants and the *AtLTI30* overexpressing plants (Figure 3), we found that the *AtLTI30* positively regulated plant sensitivity to ABA (Figure 4). In accordance with the ABA insensitive phenotype, the *lti30-1* and *lti30-2* mutants showed decreased drought stress resistance, as evidenced by a higher water loss rate and lower survival rate in comparison to WT (Figure 5). On the contrary, the *AtLTI30* overexpressing plants were more sensitive to ABA and exhibited improved drought stress resistance (Figure 5). These results suggest that *AtLTI30* may function in drought stress response in an ABA-dependent pathway. However, the stomatal response of the *AtLTI30* knockout mutants and the *AtLTI30* overexpressing plants displayed no significant difference in comparison to WT plants under mock, ABA and  $\text{H}_2\text{O}_2$  conditions (Supplementary Figure S1). This result indicated that modulation of *AtLTI30* expression has no significant effect on stomatal response in *Arabidopsis*.

Reactive oxygen species (ROS) including  $\text{H}_2\text{O}_2$ , superoxide anions ( $\text{O}_2^-$ ), singlet oxygen ( ${}^1\text{O}_2$ ) and hydroxyl radical ( $\text{OH}^-$ ) plays pivotal roles in plant drought stress responses. On one hand,  $\text{H}_2\text{O}_2$  is key secondary messenger in drought stress

perception and transduction (Zhang et al., 2001; Miao et al., 2006; Munemasa et al., 2013; Wang et al., 2013). On the other hand, as toxic by-products of physiological metabolism, H<sub>2</sub>O<sub>2</sub> accumulation is rapidly and largely increased under drought stress conditions, and overproduction of H<sub>2</sub>O<sub>2</sub> triggers serious oxidative damage and decreased drought resistance (Miller et al., 2010; Mittler et al., 2011). To cope with stress-triggered ROS overproduction and oxidative stress, plants have developed complex defense systems including both enzymatic and non-enzymatic antioxidants. Among the enzymatic enzymes, CAT catalyzes the decomposition of H<sub>2</sub>O<sub>2</sub> into H<sub>2</sub>O and O<sub>2</sub> and plays an essential role in controlling ROS homeostasis. In *Arabidopsis*, the AtCATs transcripts can be largely induced by various stress treatments including ABA, drought, salt, cold, and oxidative stresses (Du et al., 2008; Mhamdi et al., 2010; Hu et al., 2011). The interactions among nucleoside diphosphate kinase 2 (NDPK2), CAT2 or CAT3 and Salt Overly Sensitive 2 (SOS2) indicate the relationship between H<sub>2</sub>O<sub>2</sub> and abiotic stress response (Verslues et al., 2007). Together with previous studies showing the interaction of SOS2 and other SnRK3s with ABI1 and ABI2 (Guo et al., 2002; Ohta et al., 2003) and the importance of H<sub>2</sub>O<sub>2</sub>-dependent inactivation of ABI1 and ABI2 in ABA signaling (Miao et al., 2006), CAT2 and CAT3 may occur in the same protein complex as ABI1 and ABI2, indicating the possible involvement of AtCATs in the ABA signaling pathway. In this study, the positive effect of *AtLTI30* expression on the activities of AtCATs may be directly related to drought stress-induced ROS accumulation (**Figure 6**), as well as *AtLTI30*-mediated drought stress resistance. Additionally, H<sub>2</sub>O<sub>2</sub> is an important secondary messenger in ABA signal transduction (Miao et al., 2006; Cutler et al., 2010). Thus, the effects of *AtLTI30* expression on ABA sensitivity, H<sub>2</sub>O<sub>2</sub> accumulation and drought resistance further suggest the dual cross-talks among these pathways.

Based on these results, a model for *AtLTI30*-mediated drought stress response is proposed in this study (**Figure 8**). In response to drought stress, the endogenous ABA level is rapidly and largely induced, and thereafter induces the expression of *AtLTI30*. Firstly, overexpression of *AtLTI30* conferred ABA sensitivity, which is directly linked with ABA-mediated stress responses. Secondly, overexpression of *AtLTI30* up-regulated the activities of AtCATs, leading to less H<sub>2</sub>O<sub>2</sub> accumulation as well as less oxidative damage under the drought stress condition. Moreover,

*AtLTI30* positively regulated the endogenous level of proline, which functions as an important osmolyte in alleviating osmotic pressure under drought stress conditions (Shi et al., 2013b, 2014a), thereafter resulting in less osmotic pressure in response to drought stress. Thus, the ABA sensitivity, lower H<sub>2</sub>O<sub>2</sub> accumulation and more proline content resulted in enhanced drought stress resistance of *AtLTI30* overexpressing plants.

Taken together, these results indicate the possible novel role of *AtLTI30* in ABA signaling, ROS accumulation and drought stress resistance. *AtLTI30* confers enhanced drought stress resistance in *Arabidopsis*, by positively regulating ABA sensitivity, CAT activity and proline accumulation, at least partially.

## AUTHOR CONTRIBUTORS

HS conceived and directed this study, designed and performed the experiments, analyzed the data, wrote and revised the manuscript; YC and YQ performed the experiments, analyzed the data and revised the manuscript; ZC provided suggestions and revised the manuscript. All authors approved the manuscript and the version to be published, and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2015.00893>

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# Polyamines function in stress tolerance: from synthesis to regulation

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Plants are challenged by a variety of biotic or abiotic stresses, which can affect their growth and development, productivity, and geographic distribution. In order to survive adverse environmental conditions, plants have evolved various adaptive strategies, among which is the accumulation of metabolites that play protective roles. A well-established example of the metabolites that are involved in stress responses, or stress tolerance, is the low-molecular-weight aliphatic polyamines, including putrescine, spermidine, and spermine. The critical role of polyamines in stress tolerance is suggested by several lines of evidence: firstly, the transcript levels of polyamine biosynthetic genes, as well as the activities of the corresponding enzymes, are induced by stresses; secondly, elevation of endogenous polyamine levels by exogenous supply of polyamines, or overexpression of polyamine biosynthetic genes, results in enhanced stress tolerance; and thirdly, a reduction of endogenous polyamines is accompanied by compromised stress tolerance. A number of studies have demonstrated that polyamines function in stress tolerance largely by modulating the homeostasis of reactive oxygen species (ROS) due to their direct, or indirect, roles in regulating antioxidant systems or suppressing ROS production. The transcriptional regulation of polyamine synthesis by transcription factors is also reviewed here. Meanwhile, future perspectives on polyamine research are also suggested.

**Keywords:** abiotic stress, antioxidant, polyamine, polyamine biosynthesis, ROS, transcriptional regulation

## INTRODUCTION

As sessile organisms, plants are frequently challenged by a variety of adverse biotic or abiotic environmental factors. Since, unlike animals, plants cannot escape from unfavorable environments, harsh stresses constitute major factors that limit growth and development, and severely restrict the production of high-quality agricultural crops. Exposure to the stressful conditions can therefore lead to a substantial difference in potential and actual crop yields, the size of which largely depends on the severity and duration of the environmental stresses in question. Abiotic stresses, such as drought, flooding, extreme temperatures, high salinity, chemical toxicity, nutrient deficiency and others, are regarded as the predominant causes of crop loss and may account for more than 50% reduction of the yield of the major annual and perennial crops worldwide (Wang et al., 2003). In this regard, understanding how plants adapt to, and survive, the abiotic stresses is important for the efficient exploitation of genetic resources with desirable stress tolerance, and for developing new approaches to enhance stress tolerance.

Plant evolution has been accompanied by the development of complex and highly coordinated systems that allow adaptation to the stresses, involving signaling cascades that start with signal perception, and result in a variety of stress responses. Recently, significant progresses have been made in elucidating the molecular and genetic pathways involved in stress responses, and a number of key components in the stress signaling cascade events have been identified (Yuan et al., 2013; Wisniewski et al., 2014; Gehan et al., 2015; Shi et al., 2015). In the signal transduction pathway, stress signals are perceived by sensors that are primarily located at the plasma membrane, resulting in the release or activation of various secondary messengers, such as calcium (Ca), ROS (reactive oxygen species), and inositol phosphates, which relay the stress signals and activate downstream components, such as protein kinases and protein phosphatases (Nakashima et al., 2009; Danquah et al., 2014; Liu et al., 2014a; Ma et al., 2015). These proteins orchestrate the balance of protein phosphorylation and play a key role in the regulation of transcription factors (TFs), which bind to *cis*-acting elements in the promoters of their downstream target genes, thereby activating their transcription. This signaling cascade has been shown to be conserved in various plants and allows the plants to survive under the harsh environments (Liu et al., 2014a).

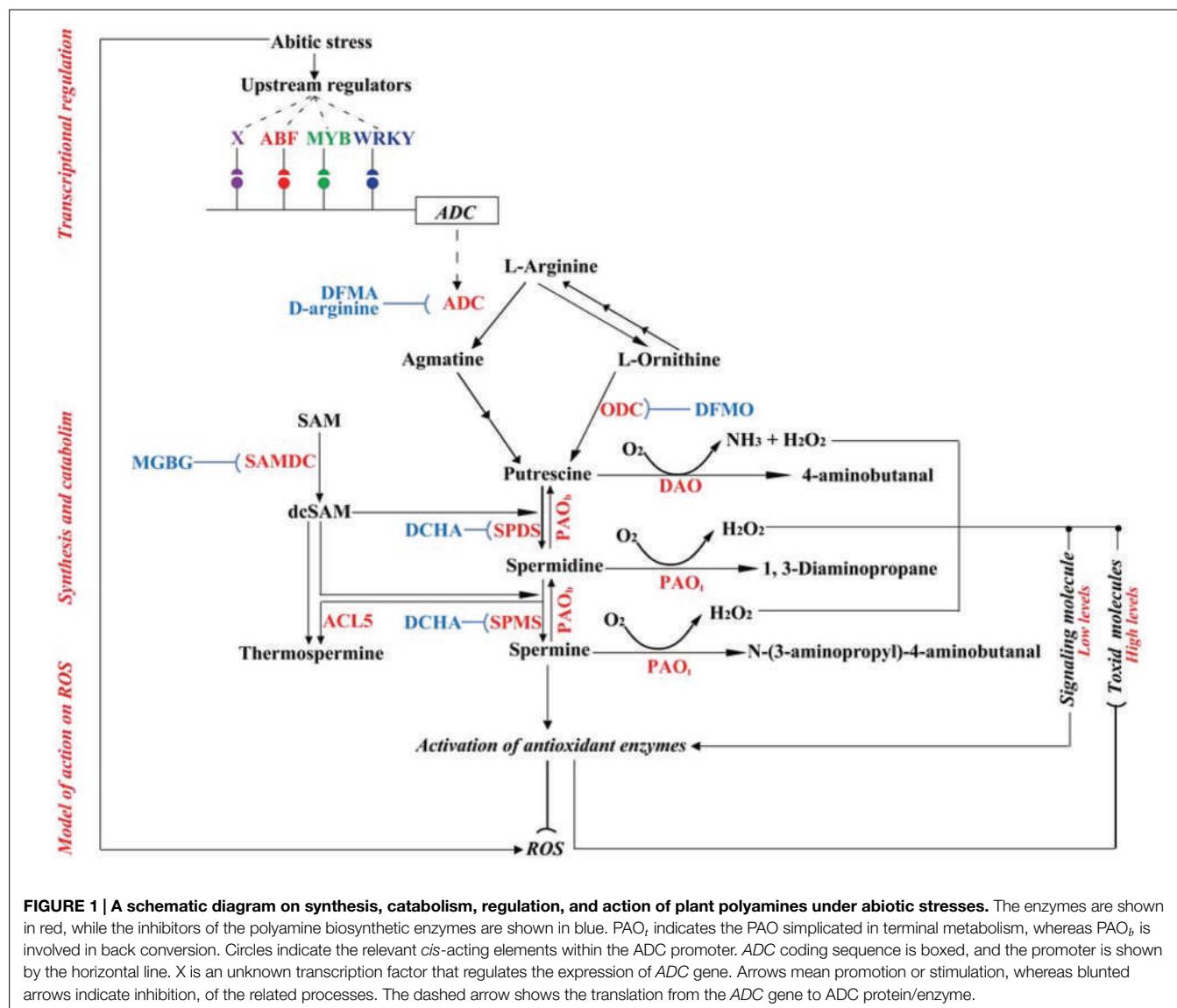
Stress responses are manifested by a range of morphological, physiological, biochemical, and molecular changes. Among these, molecular reprogramming plays a pivotal role, and a large number of studies have described the up- or down-regulation of a wide spectrum of stress-responsive genes (Seki et al., 2002; Thomashow, 2010; Liu et al., 2014a). These genes are generally classified into regulatory or functional types, based on the function of their products. Regulatory genes, encoding protein kinases, phospholipases, and TFs, act as master switches involved in hierarchical signaling cascades, thereby playing vital roles in transcriptional control of downstream stress-responsive genes. The functional genes act directly to mitigate stress-derived injuries via their products, which include a diverse set of metabolites (Shinozaki and Yamaguchi-Shinozaki, 2007). These protective approaches include the stabilization of membranes and macromolecules, alleviation of oxidative stresses, and maintenance of water status. One well studied group of metabolites comprises the polyamines, which have long been suggested to protect and maintain the function and structure of cellular components in response to stresses. Since the first report describing the accumulation of the polyamine putrescine as a result of potassium deficiency (Richards and Coleman, 1952), a large number of studies have implicated polyamines in plant responses to a myriad of abiotic stresses, and these have been reviewed elsewhere (Liu et al., 2007; Kusano et al., 2008; Alcázar et al., 2010a; Hussain et al., 2011; Minocha et al., 2014; Shi and Chan, 2014; Tiburcio et al., 2014). Here, we review recent progress in understanding the association between polyamines and stress responses, with an emphasis on their role in maintenance of ROS homeostasis. In addition, recent advances in identifying and characterizing upstream regulatory genes involved in the stress-induced transcriptional regulation of polyamine metabolism are also discussed.

## POLYAMINE SYNTHESIS AND CATABOLISM: CURRENT STATUS

Polyamines (PAs) are low-molecular-weight, aliphatic polycations that are ubiquitously distributed in all living organisms, including bacteria, animals, and plants (Hussain et al., 2011). There are three major PAs in plants, putrescine (Put), spermidine (Spd), and spermine (Spm), although other types of PAs, such as cadaverine, can also be present. The plant PA biosynthetic pathway has been extensively studied (Kusano et al., 2008; Vera-Sirera et al., 2010; Pegg and Casero, 2011; Gupta et al., 2013) and differs from that of animals in that it involves two precursors, l-ornithine and l-arginine, to generate putrescine, while only l-ornithine is used in animals. In plants, Put is produced via the catalytic actions of ornithine decarboxylase (ODC, EC 4.1.1.17) and arginine decarboxylase (ADC, EC 4.1.1.19) in three steps. Put is then converted into Spd by Spd synthase (SPDS, EC 2.5.1.16), with the addition of an aminopropyl moiety donated by decarboxylated S-adenosylmethionine (dcSAM). dcSAM is synthesized from methionine via two sequential reactions that are catalyzed by methionine adenosyltransferase (EC 2.5.1.6) and S-adenosylmethionine decarboxylase (SAMDC, EC 4.1.1.50), respectively. Spd is then converted into Spm or thermospermine, again using dcSAM as an aminopropyl donor, in a reaction catalyzed by Spm synthase (SPMS, EC 2.5.1.22) and thermospermine synthase (ACL5, EC 2.5.1.79), respectively (Figure 1). It should be noted that there is no known gene encoding ODC in the sequenced genome of model plant *Arabidopsis thaliana* (Hanfrey et al., 2001), suggesting that this species may only produce Put via the ADC pathway. Finally, PA synthesis may vary between tissues/organs, one example being that the shoot apical meristem of tobacco (*Nicotiana tabacum*) serves as the predominant site of Spd and Spm synthesis, while Put is mostly synthesized in roots (Moschou et al., 2008).

Apart from their *de novo* synthesis, PAs have been shown to undergo catabolism (Figure 1), catalyzed by two classes of enzymes, copper-containing diamine oxidases (CuAOs) and FAD-containing polyamine oxidases (PAOs; Cona et al., 2006). CuAOs mainly catalyze the oxidation of Put and cadaverine (Cad) at the primary amino groups, producing 4-aminobutanal, peroxide ( $H_2O_2$ ) and ammonia (Alcázar et al., 2010a; Moschou et al., 2012). Generally, CuAO proteins exhibit high affinity for Put and Cad than for Spd and Spm (Moschou et al., 2012), although it has been demonstrated that *A. thaliana* CuAO enzymes can also use Spd as a substrate (Planas-Portell et al., 2013). Plant CuAO proteins are present at high level in dicots, especially in Fabaceous species, such as pea, chickpea, lentil, and soybean seedlings (Cona et al., 2006). Until now, CuAO genes have been identified in only a few plant species, such as *A. thaliana* (Møller and McPherson, 1998; Planas-Portell et al., 2013) and chickpea (Rea et al., 1998). In *A. thaliana* there are at least 10 putative CuAO genes, and four of these (AtAO1, AtCuAO1, AtCuAO2, and AtCuAO3) have also been identified (Møller and McPherson, 1998; Planas-Portell et al., 2013).

In contrast to CuAOs, PAOs are present at high levels in monocots and have a high affinity for Spd, Spm, and their derivatives (Alcázar et al., 2010a). Plant PAOs are divided into



**FIGURE 1 | A schematic diagram on synthesis, catabolism, regulation, and action of plant polyamines under abiotic stresses.** The enzymes are shown in red, while the inhibitors of the polyamine biosynthetic enzymes are shown in blue. PAO<sub>t</sub> indicates the PAO implicated in terminal metabolism, whereas PAO<sub>b</sub> is involved in back conversion. Circles indicate the relevant cis-acting elements within the ADC promoter. ADC coding sequence is boxed, and the promoter is shown by the horizontal line. X is an unknown transcription factor that regulates the expression of ADC gene. Arrows mean promotion or stimulation, whereas blunted arrows indicate inhibition, of the related processes. The dashed arrow shows the translation from the ADC gene to ADC protein/enzyme.

two major groups, depending on their potential functions in polyamine catabolism. The first group catalyzes the terminal catabolism of Spd and Spm to produce 1,3-diaminopropane (DAP), H<sub>2</sub>O<sub>2</sub>, and *N*-(3-aminopropyl)-4-aminobutanal (Spm catabolism), or 4-aminobutanal (Spd catabolism; Cona et al., 2006; Angelini et al., 2010; Moschou et al., 2012). The second group is responsible for PA conversions, in which Spm is converted back to Spd, and Spd to Put (Moschou et al., 2012; Mo et al., 2015). To date, PAO genes have been identified in several plant species, including *A. thaliana* (Fincato et al., 2011), tobacco (Yoda et al., 2006), rice (*Oryza sativa*; Ono et al., 2012), barley (*Hordeum vulgare*; Cervelli et al., 2001), maize (*Zea mays*; Cervelli et al., 2000), poplar (Tuskan et al., 2006), apple (*Malus domestica*; Kitashiba et al., 2006), cotton (*Gossypium barbadense*; Mo et al., 2015), and sweet orange (*Citrus sinensis*; Wang and Liu, 2015). Plant PAO proteins are encoded by small gene families, as revealed by analyses of fully sequenced genomes. There are five, seven, and six PAO genes in *A. thaliana*, rice, and sweet orange,

respectively. However, only few of the PAO genes belonging to the first group have been characterized, and to date only *ZmPAO* and *OsPAO7*, from maize and rice, respectively, have been reported to be involved in PA terminal catabolism (Cona et al., 2006; Liu et al., 2014b). In contrast, many PAO genes belonging to the second group have been identified. For example, all five PAO genes from *A. thaliana* (*AtPAO1*–*AtPAO5*) and four PAO genes from rice (*OsPAO1*, *OsPAO3*, *OsPAO4*, and *OsPAO5*) have been shown to be involved in the back conversion of polyamines (Tavladoraki et al., 2006; Kamada-Nobusada et al., 2008; Moschou et al., 2008; Fincato et al., 2011; Ono et al., 2012; Ahou et al., 2014; Liu et al., 2014b), and recently, a sweet orange PAO gene (*CsPAO3*) was added to the list of identified genes from this group (Wang and Liu, 2015). As PA catabolism gives rise to the production of H<sub>2</sub>O<sub>2</sub>, which may act either as a signaling molecule at low levels or as a toxic compound when the level is high, the ratio of PA catabolism to biosynthesis has been considered as a crucial factor for induction of tolerance responses or plant cell death under

abiotic stress (Moschou et al., 2008). This suggests that PA-derived H<sub>2</sub>O<sub>2</sub> may play a key role in the maintenance of ROS homeostasis, depending on its cellular levels.

## INVOLVEMENT OF POLYAMINE METABOLISM IN STRESS RESPONSE

### Change in PA Levels and Transcript Levels of Genes Involved in Polyamine Metabolism Under Abiotic Stress Conditions

Polyamines have been shown to be involved in various processes associated with plant growth and development, such as embryogenic competence (Silveira et al., 2013), programmed cell death (Kim et al., 2013), fruit ripening (Gil-Amado and Gomez-Jimenez, 2012), xylem differentiation (Tisi et al., 2011), as well as biofilm formation (Lee et al., 2009). Accumulating evidences suggest that plant PAs function in adaptive responses to various environmental stresses, and this is supported by the extensive variation in polyamine levels under stress conditions. Since the first report described Put accumulation under potassium deficiency decades ago (Richards and Coleman, 1952), changes in PA levels have been universally observed in various plant species subjected to a range of abiotic stresses, including drought, high salinity, low and high temperatures, nutrient deficiency, and others (Liu et al., 2007). In some cases, it has been observed that the three most abundant PAs, Put, Spd and Spm, show substantial increases in abundance following abiotic stress (Yang et al., 2007). However, in most cases, only one type of the three PAs shows a significant increase. For example, when apple callus was treated with salt, Put levels increased, while those of Spd and Spm underwent only minor changes (Liu et al., 2006). In contrast, sweet orange callus was reported to show predominant increases in Spd content when exposed to salt and cold stress conditions (Wang and Liu, 2009), and grape (*Vitis vinifera*) plants showed a dramatic accumulation of Spd and Spm following salt stress (Iqbal et al., 2014). In another study, it was reported that 18 rice varieties exhibited notable changes in Spm levels when grown under long-term drought stress (Do et al., 2014). These findings suggest that PA accumulation is influenced by different factors, such as plant species in question, stress tolerance capacity, stress types and conditions, and the physiological status of the examined tissues/organs. It also indicates the existence of complicated PA dynamics under abiotic stress, which may explain why differing or contradictory results have been reported. The size of PA pool can be correlated with the stress tolerance capacity, further underlining the significance of PAs in providing protection against stresses. Generally, tolerant genotypes accumulate greater amounts of PAs than sensitive genotypes (Hatmi et al., 2015); however, genotypes with contrasting stress tolerance have been shown to display different patterns of PA accumulation under some abiotic stresses. In several studies tolerant genotypes accumulated more Spd and Spm, while the sensitive genotypes from the same plants species accumulated more Put under the same types of stresses (Krishnamurthy and Bhagwat, 1989; Santa-Cruz et al., 1998; Liu et al., 2004). Although

whether Spd and Spm play more important roles in counteracting abiotic stress remains to be determined, it is a reasonable hypothesis, since Spd and Spm contain one and two additional primary amino groups (–NH<sub>2</sub>), respectively, compared to Put, allowing them to be more efficient for executing protective functions.

The accumulation of PAs under abiotic stress conditions is largely due to the increased *de novo* synthesis of free PAs. Since their synthesis is primarily regulated at the transcriptional level, an understanding of the expression patterns of the biosynthetic genes is important for understanding the regulation of PA levels. To this end, a myriad of studies have been carried out to investigate the steady-state transcript levels of PA biosynthetic genes. Available data to date indicate that most of the PA biosynthetic genes, including *ADC*, *SPDS*, *SPMS*, and *SAMDC*, are up-regulated by stresses, despite a difference in the timing and degree of induction (Liu et al., 2006, 2008, 2009, 2011; Wang et al., 2011b). Of these genes, *ADC* is most widely characterized in different plants and has been demonstrated to be a crucial stress-responsive gene (Urano et al., 2004; Liu et al., 2006; Wang et al., 2011b). Increased transcript levels of the PA biosynthetic genes coincide with the accumulation of free PA in some studies, but inconsistent in others (Liu et al., 2006; Wang and Liu, 2009). One reason for the disparity between gene expression profiles and PA accumulation is likely due to PA catabolism. Notably, the expression patterns of PA biosynthetic genes have also been shown to be correlated with stress tolerance (Pillai and Akiyama, 2004). For instance, citrus genotypes with better salt and cold tolerance displayed earlier and/or greater induction of *SAMDC* transcript at the initial stages of stress treatment (Wang et al., 2010).

### Effects of Modulating PA Content on Stress Tolerance

Polyamine accumulation is usually considered to be a general plant response to abiotic stresses, but the cause-effect relationship between PA accumulation and protection remains unclear. An effective strategy for understanding the roles of PAs in stress tolerance is to modulate their cellular levels, which has been accomplished using three approaches, including their exogenous application, overexpression of their biosynthetic genes and the use of PA synthesis inhibitors.

Exogenous application of Put, Spd, or Spm at different concentrations has been shown to confer enhanced tolerance to various stresses in different plants (Duan et al., 2008). For example, exogenous application of Put considerably enhanced salt tolerance in apple callus and thermotolerance of wheat (Liu et al., 2006; Kumar et al., 2014). A recent study by Zhang et al. (2015b) demonstrated that damage caused by saline-alkaline stress to tomato (*Solanum lycopersicum*) plants was substantially alleviated when 0.25 mM Spd was applied, and exogenous Spd supplementation can also alleviate salt stress in sorghum (*Sorghum bicolor*) seedlings (Yin et al., 2015). In another study, Harindra Champa et al. (2015) demonstrated that exogenous application of 1 mM Spm reduced chilling injury during low temperature storage of grape berries, leading to maintenance of fruit quality and shelf life.

Apart from exogenous PA application, several elegant studies have shown that the overexpression of PA biosynthetic genes is an effective strategy to elevate the endogenous PA pool and to modify stress tolerance. For example, overexpression of *ADC* genes from oat (*Avena sativa*) and *Datura stramonium* resulted in greater accumulation of Put in the transgenic plants, which displayed enhanced drought tolerance when compared with the wild type (WT) genotypes (Roy and Wu, 2001; Capell et al., 2004). Recently, it was shown that constitutive overexpression of *ADC2* in *A. thaliana*, oat *ADC* in *Lotus tenuis* and *PtADC* of *Poncirus trifoliata* in tobacco and tomato noticeably increased drought tolerance in the transgenic plants (Alcázar et al., 2010b; Wang et al., 2011a; Espasandin et al., 2014). In addition, other PA biosynthetic genes, such as *ODC*, *SAMDC*, and *SPDS*, have also been overexpressed in transgenic plants, resulting in enhanced tolerance to specific stresses, such as drought and salt (Roy and Wu, 2002; Waie and Rajam, 2003). In summary, overexpression of a PA biosynthetic gene has been demonstrated to confer tolerance to various abiotic stresses (Kasukabe et al., 2004, 2006; Wi et al., 2006; Wang et al., 2011a,b), indicating that changes in the endogenous PA pool has a profound influences on stress tolerance.

Several inhibitors have been identified that repress different PA biosynthetic enzymes, thereby inhibiting endogenous PA synthesis. Their use has provided useful insights into the role of PAs in stress tolerance. Specific or non-specific inhibitors have been used in order to elucidate the role of different PAs. d-arginine, an inhibitor of *ADC*, was shown to be effective in reducing Put synthesis and its application to apple callus compromised salt tolerance; an effect that was reversed when exogenous Put was applied, suggesting a role for Put in combating salt stress (Liu et al., 2006). Moreover, treatment of grape plants with methylglyoxal-bis(guanylhydrazone) (MGBG), an inhibitor of *SAMDC*, which is involved in synthesis of Spd and Spm, led to a greater deterioration of plant growth under salt stress than those without MGBG treatment (Ikbal et al., 2014). Recently, it was shown that treatment of sorghum plants with dicyclohexylammonium sulphate (DCHA), an inhibitor of *SPDS* and *SPMS*, ameliorated the silicon-induced salt tolerance, implying the positive role of PA in this process (Yin et al., 2015).

## ROLE OF POLYAMINES IN STRESS TOLERANCE: FROM AN ANTIOXIDANT PERSPECTIVE

As mentioned above, elevation of endogenous PA levels is one of the metabolic hallmarks of plants exposed to abiotic stresses (Kusano et al., 2008), implying that they are important for protecting plants against harsh environmental conditions. Nevertheless, in spite of many observations of changes in PA levels under stresses, the precise physiological and molecular mechanisms by which they confer protection remain elusive (Marco et al., 2011). The biological function of the polycationic PAs were initially associated with their capacity to bind anionic macromolecules, such as nucleic acids and proteins, a characteristic that allows PAs to play a role in the regulation of transcription and translation (Bachrach, 2010; Gill and Tuteja,

2010; Igarashi and Kashiwagi, 2010; Tiburcio et al., 2014). They have also been suggested to function in maintaining membrane stability under adverse conditions (Liu et al., 2007; Tiburcio et al., 2014); however, besides other than these mechanisms, there is increasing evidence that their role in stress tolerance is associated with modulating antioxidant systems.

Reactive oxygen species are produced under normal growth conditions, but their homeostasis is a highly coordinated balance between generation and detoxification. Under abiotic stresses, ROS production is elevated, causing excessive ROS accumulation and oxidative stress, which is toxic to living cells due to lipid peroxidation and membrane damage, and can finally result in cell death (Biswas and Mano, 2015). PAs are thought to play a role in modulating ROS homeostasis in two ways. Firstly, they may inhibit the auto-oxidation of metals, which in turn impairs the supply of electrons for the generation of ROS (Shi et al., 2010). They may also directly act as antioxidants and scavenge ROS, although there is no evidence for this mechanism at present. Secondly, PAs may affect antioxidant systems, and a number of studies have demonstrated that priming of plants with polyamines led to increases in endogenous PA contents and concomitant enhanced tolerance to abiotic stresses, such as drought, heat, and cold. The elevation of stress tolerance is concurrent with the activation of antioxidant enzymes. For example, exogenous application of Spm to *P. trifoliata* led to an elevation of POD, SOD, and CAT activities, accompanied by a remarkable decrease in ROS levels under dehydration (Shi et al., 2010). Exogenous supply of Spd to rice seedlings mitigated heat-induced damages, and increased activities of antioxidant enzymes and levels of antioxidant, accompanied by reduced accumulation of H<sub>2</sub>O<sub>2</sub> (Mostofa et al., 2014). Similar findings have been observed using other plants, such as tobacco, soybean, cucumber, and pistachio (Xu et al., 2011; Radhakrishnan and Lee, 2013; Shu et al., 2013; Kamiab et al., 2014). On the other hand, genetic manipulation of PA biosynthetic genes has been demonstrated to promote stress tolerance via modulation of antioxidant machineries. Overexpression of *MdSPDS1* in European pear (*Pyrus communis*) resulted in an enhanced tolerance to heavy metals, which was largely ascribed to the activation of antioxidant enzymes (Wen et al., 2009). Ectopic expression of *PtADC* in tobacco and tomato also conferred enhanced dehydration and drought tolerance, coincident with a substantial repression of ROS generation in the transgenic plants (Wang et al., 2011a). Another line of evidence supporting the role of PAs in modulating ROS homeostasis is the use of inhibitors of PA biosynthetic enzymes. As an example, it was shown that the use of D-arginine resulted in a decrease in endogenous PA levels and a consequent increase in ROS accumulation (Wang et al., 2011b; Zhang et al., 2015a). These studies demonstrate that PAs may alleviate the oxidative stress of the stressed plants through regulation of antioxidant systems, along with changes in the ROS production and redox status (Shu et al., 2013; Tanou et al., 2014).

However, a direct link between increased PA levels and antioxidant enzyme activity has yet to be proven. One possibility is that the PAs may function as signaling molecules that can activate the antioxidant enzymes, and indeed Spm has been suggested to act as a signaling molecule (Mitsuya et al., 2009). Another link

may be the production of H<sub>2</sub>O<sub>2</sub> by PAO-mediated PA catabolism. An increase in the endogenous PA levels to a certain threshold may promote their degradation, generating H<sub>2</sub>O<sub>2</sub>. It is known that H<sub>2</sub>O<sub>2</sub> plays dual roles in plant responses to abiotic stresses, one being to act as a regulator of signaling cascades at a low cytosolic concentration, which may contribute to the induction of antioxidant enzymes (Moschou et al., 2008; Zhang et al., 2015a; Figure 1). On the other hand, the PAs may influence various antioxidant enzymes through regulation of their expression. Higher transcript levels of antioxidant enzyme-encoding genes have been detected in tissues treated with exogenous PAs or in the transgenic plants overexpressing PA biosynthetic genes (Tanou et al., 2014; Zhang et al., 2015b).

## TRANSCRIPTIONAL REGULATION OF POLYAMINE SYNTHESIS UNDER ABIOTIC STRESS

Earlier studies demonstrated that the PA biosynthetic genes display disparate expression profiles under abiotic stresses. For example, *PpADC* of peach was up-regulated by dehydration, salt, cold, and cadmium (Liu et al., 2009), but repressed by high temperature. It has to be pointed out that the PA biosynthetic genes can be differentially influenced by a particular stress, as exemplified by *MdADC1*, which is more responsive to salt stress than other PA biosynthetic genes (Liu et al., 2006). In addition, the transcript levels of PA biosynthetic genes can vary significantly between stress tolerant and stress sensitive genotypes. Such findings suggest that the PA biosynthetic genes may be under tight transcriptional regulation during abiotic stress responses, and so identification and characterization of the associated upstream transcriptional regulators will likely be important in connecting stress responses with PA metabolism.

It has been suggested that *ADC* acts as an important polyamine biosynthetic gene in response to abiotic stresses, and it has been more extensively characterized than other genes in the pathway. The expression of *ADC* genes from a number of plant species has been described; in particular those from several species, such as *A. thaliana*, *P. trifoliata*, have been functionally characterized (Urano et al., 2004; Wang et al., 2011a,b). Thus, *ADC* genes are promising candidates used for identifying potential transcriptional regulators, such as TFs or protein kinases. The identification and bioinformatics analysis of promoter sequences are common first steps toward identifying potential TFs that regulate a PA biosynthetic gene, prior to the use of yeast one-hybrid screening of cDNA libraries. This generally involves characterizing putative *cis*-acting elements that are present within the promoters. Recently, Basu et al. (2014) reported that *in silico* analysis of the promoter region of rice *SamDC* gene revealed the presence of several putative *cis*-acting elements, such as ABRE, LTRE, MYBR, and W-box, which have been shown to be closely associated with various environmental factors, such as drought, cold, and abscisic acid (ABA) signaling. These findings suggest that the PA biosynthetic genes may be controlled by a common set of TFs, or that a given TF may control different genes involved in PA biosynthesis. This idea is congruent with earlier reports that PA biosynthetic genes, such as *PpADC* (Liu et al., 2009) or *PtADC*

(Wang et al., 2011b), are responsive to different stresses. In addition, it also suggests that the endogenous PA levels may be modulated by altering the expression of TFs, either through overexpression or repression (Huang et al., 2010; Chen et al., 2015).

MYB proteins are TFs that play important roles in plant development and stress responses (Dubos et al., 2010). Sun et al. (2014) reported that a stress-responsive R2R3-type MYB gene of *P. trifoliata*, *PtsrMYB*, regulated its *ADC* gene, *PtADC*. Yeast one-hybrid assay demonstrated that *PtsrMYB* predominantly interacts with two regions of the *PtADC* promoter, indicating the *PtADC* may be a target gene of *PtsrMYB*. Moreover, overexpression of *PtsrMYB* led to an increase in mRNA levels of *ADC* genes in the transgenic lines when compared with WT plants, concurrent with increased PA levels. In a recent study, Chen et al. (2015) showed that overexpression of a cotton MYB TF, *GbMYB5*, also led to up-regulation of three polyamine biosynthetic genes in the transgenic lines. These findings suggest that MYBs might be likely to govern polyamine synthesis under abiotic stresses through regulating the relevant genes.

ABF is a key TF involved in the transduction of signals associated with drought and osmotic stress (Yoshida et al., 2010, 2015). It is known that under abiotic stress conditions, synthesis of ABA is typically increased, which in turn triggers signaling through a network that includes components such as ABA receptors, protein phosphatases, and SnRK proteins. The activated SnRK proteins can in turn phosphorylate ABF TFs, which then regulate downstream target genes (Danquah et al., 2014; Zhang et al., 2015a). In addition, PA biosynthetic genes, such as those encoding ADC, SAMDC, SPDS, have been shown to be induced under drought stress or following ABA treatment, and this is accompanied by increase in the endogenous PAs. However, whether the induction of PA genes or the accumulation of PAs is directly associated with ABA signaling cascades has not yet been addressed. Recently, *PtrABF* from *P. trifoliata*, an ABF4 homolog, was shown to regulate the expression of an *ADC* gene by interacting with the ABRE elements within the *ADC* promoter. Overexpression of *PtrABF* greatly increased the mRNA levels of *ADC*, and resulted in an increase in endogenous Put levels, whereas treatment of the *PtrABF*-overexpressing lines with an *ADC* inhibitor resulted in a decrease of Put contents, and compromised dehydration tolerance (Huang et al., 2010; Zhang et al., 2015a). These results provide convincing evidence that *ADC* is a candidate target gene of ABF. Characterization of this regulatory cascade may elucidate the transcriptional regulation of *ADC* genes and the associated accumulation of PAs under drought or osmotic stresses.

WRKY proteins comprise a large family of TFs in plants and play important roles in regulating the synthesis of several metabolites, such as lignin, phytoalexins, terpenoid indole alkaloid (Suttipanta et al., 2011). Recently, Gong et al. (2015) reported that overexpression of *FcWRKY70*, a WRKY70 homolog from *Fortunella crassifolia*, led to the increased expression of *ADC* genes, whereas suppression of *FcWRKY70*, using an RNAi approach, down-regulated *ADC* expression. Put levels were prominently increased in *FcWRKY70*-overexpressing lines, but decreased in the RNAi lines when compared to the WT.

In addition, FcWRKY70 can interact with the W-box elements in the *ADC* promoter. Taken together, these results indicate that FcWRKY70 may also act as a positive regulator of *ADC* expression and regulate Put synthesis under abiotic stresses.

The above data suggests that *ADC* genes may be under the control of members from different TF families, including ABF, WRKY, and MYB proteins (Figure 1). However, TFs from other families may also be involved in the regulation of *ADC* gene expression (Huang et al., 2015). Of note, all of the TFs mentioned above are proposed as positive regulators of PA biosynthesis and *ADC* expression, while TFs that may negatively regulate PA gene expression have not been characterized. In addition, it is not known whether other PA biosynthetic genes, such as those encoding SAMDC, SPDS and SPMS proteins, are regulated by the same TFs mentioned above.

Apart from the TFs, other regulatory proteins may also control PA biosynthetic gene expression and PA accumulation. One of these is mitogen-activated protein kinase (MAPK), which has been shown to play roles in various signaling pathways related to plant development and stress responses. Huang et al. (2011) reported that the expression of *NtADC1* and *NtSAMDC*, two tobacco genes involved in PA biosynthesis, was induced in transgenic tobacco plants overexpressing *PtrMAPK* of trifoliate orange. It can be inferred from this result that MAPK may phosphorylate the two corresponding proteins, but this needs to be verified in the future.

## PERSPECTIVES AND CONCLUDING REMARKS

Polyamines are considered to play important roles in protecting plant cells from stress-associated damages. To date, tremendous progresses have been made in understanding the significance of PAs in stress responses. There is accumulating evidence that PA levels undergo extensive changes in response to a range of abiotic stresses, and physiological, molecular and genetic approaches have been used to identify and functionally characterize PA biosynthetic genes in various plant species. These efforts underpin our understanding of the role of PAs in counteracting adverse environmental cues, and provide valuable information for enhancing stress tolerance through the modulation of cellular PA levels via exogenous PA application,

or the transgenic manipulation of PA biosynthetic genes. Nevertheless, many key questions remain unanswered. First, the causal relationship between PA accumulation and stress tolerance has not been determined, despite numerous observations of changes in PA levels in response to abiotic stresses. Second, the cellular compartmentation and transportation of PAs is not well understood, although a few PA transporters have been identified (Fujita et al., 2012; Mulangi et al., 2012). In addition, the mode of action of PAs in enhancing stress tolerance has not been definitely established, although several possible models have been proposed. One example is the scarcity of direct evidence confirming the involvement of PAs in the activation of antioxidant enzymes for ROS detoxification. Last, the signaling cascades linking stress responses and PA genes are still far from being well defined. To date, TFs regulating *ADC* genes have been identified, but those that regulate other PA biosynthetic genes are unknown. In keeping with these unanswered questions, there are several promising areas of future study. First, the sites of PA production and actions in plant cells need to be identified and to this end, the cellular localization of PAs and their transporters should be determined. Second, the physiological and molecular mechanisms concerning the roles of PAs in stress tolerance need to be elucidated, and in particular, how PAs contribute to the activation of antioxidant enzymes and ROS removal should be clearly deciphered. Last but not the least, the molecular mechanisms underlying the accumulation of PAs in response to abiotic stresses, including the PA biosynthetic genes and the transcriptional regulation network associated with those genes, must be defined. This information will advance our understanding of PA accumulation and gene expression, and can be incorporated with physiological, biochemical, molecular and genetic approaches to better understand the complex regulation of PA synthesis under abiotic stresses, as well as the cross talk between different TF-mediated signaling pathways.

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# Polyamine regulates tolerance to water stress in leaves of white clover associated with antioxidant defense and dehydrin genes via involvement in calcium messenger system and hydrogen peroxide signaling

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Endogenous polyamine (PA) may play a critical role in tolerance to water stress in plants acting as a signaling molecule activator. Water stress caused increases in endogenous PA content in leaves, including putrescine (Put), spermidine (Spd), and spermine (Spm). Exogenous application of Spd could induce the instantaneous H<sub>2</sub>O<sub>2</sub> burst and accumulation of cytosolic free Ca<sup>2+</sup>, and activate NADPH oxidase and CDPK gene expression in cells. To a great extent, PA biosynthetic inhibitor reduced the water stress-induced H<sub>2</sub>O<sub>2</sub> accumulation, free cytosolic Ca<sup>2+</sup> release, antioxidant enzyme activities and genes expression leading to aggravate water stress-induced oxidative damage, while these suppressing effects were alleviated by the addition of exogenous Spd, indicating PA was involved in water stress-induced H<sub>2</sub>O<sub>2</sub> and cytosolic free Ca<sup>2+</sup> production as well as stress tolerance. Dehydrin genes (*Y<sub>2</sub>SK*, *Y<sub>2</sub>K*, and *SK<sub>2</sub>*) were showed to be highly responsive to exogenous Spd. PA-induced antioxidant defense and dehydrin genes expression could be blocked by the scavenger of H<sub>2</sub>O<sub>2</sub> and the inhibitors of H<sub>2</sub>O<sub>2</sub> generation or Ca<sup>2+</sup> channels blockers, a calmodulin antagonist, as well as the inhibitor of CDPK. These findings suggested that PA regulated tolerance to water stress in white clover associated with antioxidant defenses and dehydrins via involvement in the calcium messenger system and H<sub>2</sub>O<sub>2</sub> signaling pathways. PA-induced H<sub>2</sub>O<sub>2</sub> production required Ca<sup>2+</sup> release, while PA-induced Ca<sup>2+</sup> release was also essential for H<sub>2</sub>O<sub>2</sub> production, suggesting an interaction between PA-induced H<sub>2</sub>O<sub>2</sub> and Ca<sup>2+</sup> signaling.

**Keywords:** antioxidant, dehydrin, gene expression, oxidative damage, polyamine, regulation, white clover (*Trifolium repens*)

## Introduction

Water deficit leads to decreases in crop yields which are associated with changes of various physiological and molecular factors. Recently, increasing evidence indicates that polyamine (PA) is closely involved in growth and development (Krasuska et al., 2014; Pottosin et al., 2014), as well as stress tolerance in plants (Yiu et al., 2009; Do et al., 2014). Maintenance of PA levels and metabolism, whether through exogenously applied putrescine (Put), spermidine (Spd) and spermine (Spd), or transgenic approaches with PA biosynthesis genes, has been found to promote plant stress tolerance due to their roles in protecting membranes, maintaining osmotic adjustment, and promoting tolerance-related gene expression and protein levels (Tang and Newton, 2005; Shi et al., 2013; Li et al., 2014a). It has been reported that PA synthesis and oxidation could improve  $\text{H}_2\text{O}_2$ -induced antioxidant protection in *Medicago sativa* (Guo et al., 2014). Diamine oxidase (CuAO) catalyzed the degradation of Put to produce  $\text{H}_2\text{O}_2$ , thereby elevating the  $\text{Ca}^{2+}$  level in guard cells of *Vicia faba* (An et al., 2008). All of the above suggest that PA plays an important role in signaling pathways and may activate multiple signal factors to cope with abiotic stresses.

The production of reactive oxygen species (ROS), such as superoxide and  $\text{H}_2\text{O}_2$ , is regarded as the most common response in plants under abiotic stress. High levels of ROS cause protein and lipid peroxidation, degradation of chlorophyll, and programmed cell death (Imlay and Linn, 1988; Yan et al., 2011; Wang et al., 2012), while low levels of ROS produced during early phases of stress response have a regulatory role acting as intermediate signaling molecules in plant cells (Bolwell et al., 1998; Zhang et al., 2006). Application of abscisic acid (ABA), brassinosteroid (BR), and salicylic acid (SA) all resulted in a temporary accumulation of  $\text{H}_2\text{O}_2$  followed by a gain in stress tolerance in different plant species (Agarwal et al., 2005; Lu et al., 2009; Xia et al., 2009). PA has also been implicated in the regulation of  $\text{H}_2\text{O}_2$  generation in plants, since  $\text{H}_2\text{O}_2$  is generally one of by-products from Put oxidation catalyzed by CuAO (An et al., 2008). It has been shown that PA-derived  $\text{H}_2\text{O}_2$  participated in stress-induced cell wall stiffening and maturation (Angelini et al., 2010). Spm oxidase acted as a mediator of ROS production in HIV-induced neuronal toxicity (Capone et al., 2013).  $\text{H}_2\text{O}_2$  involvement in PA-induced cell death has been demonstrated in

tobacco (*Nicotiana tabacum*) (Iannone et al., 2013). In spite of these studies, it's still unclear whether  $\text{H}_2\text{O}_2$  signaling is involved in PA-induced tolerance to water stress and whether PA could induce other  $\text{H}_2\text{O}_2$  generation pathways in plants under water stress.

ROS-related stress signaling may involve the calcium messenger system, which plays a critical role in coupling a wide range of extracellular stimuli with intracellular responses in plant cells. Calcium-signal-encoding elements such as  $\text{Ca}^{2+}/\text{H}^+$  antiporters or  $\text{Ca}^{2+}$ -ATPases regulate  $\text{Ca}^{2+}$  transport leading to the change of cytosolic  $\text{Ca}^{2+}$  concentrations (Shao et al., 2008).  $\text{Ca}^{2+}$  binds to different calcium sensors such as calmodulin (CaM) and  $\text{Ca}^{2+}$ -dependent protein kinase (CDPK) to pass calcium signals as a result of transient cytosolic  $\text{Ca}^{2+}$  or oscillating  $\text{Ca}^{2+}$  levels (Sanders et al., 2002). The raising of cytosolic  $\text{Ca}^{2+}$  levels in guard cells can trigger stomatal closure, which demonstrates the importance of  $\text{Ca}^{2+}$  signaling for drought tolerance (Fu and Lu, 2007). ROS-induced  $\text{Ca}^{2+}$  influx across plasma membranes of *Arabidopsis* was important for the growth of root hairs (Demidchik et al., 2007), and cytosolic  $\text{Ca}^{2+}$  could activate NOX to regulate plant adaptive responses (Pottosin et al., 2014). These studies indicate that there may be positive feedback between  $\text{H}_2\text{O}_2$  and  $\text{Ca}^{2+}$  signaling in plants. PA has been found to induce stomatal closure by inducing  $\text{Ca}^{2+}$  release in *Arabidopsis* (Yamaguchi et al., 2007). Exogenous  $\text{Ca}^{2+}$  application elevated endogenous PA content, alleviating hypoxia damage in cucumber seedlings (*Cucumis sativus*) (Jiao et al., 2007). However, the cross-talk among PA,  $\text{H}_2\text{O}_2$ , and  $\text{Ca}^{2+}$  for water stress responses is not well-documented.

The PA-induced tolerance to water stress could be associated with the interaction between  $\text{H}_2\text{O}_2$  and  $\text{Ca}^{2+}$  for the regulation of antioxidant systems. The objective of this study was to elucidate whether PA-induced stress tolerance involves the calcium messenger system and  $\text{H}_2\text{O}_2$  signaling, the roles of PA signal transduction in antioxidant defense and dehydrins, and the interaction between  $\text{H}_2\text{O}_2$  and  $\text{Ca}^{2+}$  induced by PA in plant cells.

## Materials and Methods

### Plant Materials

The white clover cultivar "Ladino" was used as plant material. All seeds were immersed in 0.1% mercuric chloride for 4 min

**TABLE 1 |** Primer sequences and their corresponding GeneBank accession numbers of the analyzed genes.

Target gene	Accession no.	Forward primer (5'-3')	Reverse primer (5'-3')
SOD	FY461274	TCACCTTCCACTTCAAACCTCTC	TGTTGGACCTTCGTCTTCTTGAGT
CAT	FY464988	GTCTTCTTGTTCACGATGGATG	GAAAGTGGAGAAGAAGTCAGGAT
GPOX	AJ011939	TCTAGGGCAACGGTTAACCATCCT	GGTACGGATTCCCATTCCTTG
APX	FY460674	GCAGCATCAGTGGCAAGACC	GGCAAACCTGAGACTAAATACACGA
CDPK	FY465549.1	CAAACCGGCATCCAATGACTT	GCATTCGTTCAAGGAGGCATT
Dehydrin, $Y_2SK$	GU443965.1	GTGCGATGGAGATGCTGTTG	CCTAACCTAACCTCAGGTTCAAC
DHN1, $Y_2K$	JF748410.1	AGCCCACGCAACAAGGTTCTAA	TTGAGGATAACGGGATGGGTG
Dhn b, $SK_2$	GU443960.1	TGGAACAGGAGTAACAAACAGGTGGA	TGCCAGTTGAGAAAGTTGAGGTTGT
$\beta$ -Actin	JF968419	TTACAATGAATTGCGTGTG	AGAGGACAGCCTGAATGG

and rinsed three times with distilled water. 0.1 g sterilized seeds were sown in trays (24 cm length, 20 cm width, and 15 cm deep) filled with sterilized quartz sand and distilled water, and then put in a controlled growth chamber (12 h photoperiod, 75% relative humidity, 23/19°C day/night temperature, and 500  $\mu\text{mol}\cdot\text{m}^{-2}\text{s}^{-1}$  photosynthetically active radiation). After 7 days of germination in distilled water solution, the seedlings of white clover grew in full-strength Hoagland's solution (Hoagland and Arnon, 1950) for another 23 days (replacing the solution every other day). The second leaves were collected for all investigations.

### Treatments with Chemicals

The detached leaves were pre-treated with distilled  $\text{H}_2\text{O}$  for 1 h to eliminate wound stress and then placed in 50 ml centrifuge tubes containing distilled  $\text{H}_2\text{O}$ , spermidine (Spd) or polyethyleneglycol (PEG) 6000 solution to induce water stress for various time periods under the same growth chamber conditions as mentioned above. In order to investigate the effects of different inhibitors or scavengers, the detached leaves were incubated in 20  $\mu\text{M}$  Spd or 15% PEG solution separately containing 100  $\mu\text{M}$  dicyclohexylamine (DCHA), an inhibitor of polyamine biosynthesis (Meskaoui and Trembaly, 2009); 5 mM dimethylthiourea (DMTU), a  $\text{H}_2\text{O}_2$  scavenger (Lu et al., 2009); 100  $\mu\text{M}$  Diphenyleneiodonium (DPI), an inhibitor of NADPH oxidase (Jiang and Zhang, 2002); 5 mM salicyhydroxamic (SHAM), an inhibitor of cell wall-localized peroxidase (Kiba et al., 1997); 100  $\mu\text{M}$  quinacrine (QC), an inhibitor of amine oxidase (Kiba et al., 1997); 1 mM lanthanum (III) ( $\text{LaCl}_3$ ), a plasma membrane calcium channel blocker (Knight et al., 1992); 50  $\mu\text{M}$  ruthenium red (RR), a mitochondrial, and endoplasmic reticulum calcium channel blocker (Knight et al., 1992); 150  $\mu\text{M}$  N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7), a calmodulin antagonist (Gonzalez et al., 2012) or 30  $\mu\text{M}$  trifluoperazine (TFP), a inhibitor of  $\text{Ca}^{2+}$ -dependent protein kinase (CDPK) (Lanteri et al., 2006). Detached leaves were incubated in distilled  $\text{H}_2\text{O}$  as control under the same condition. All treatments were repeated at least 4–6 times (specific number of replicates and length of time about treatments with chemicals please see each figure legend).

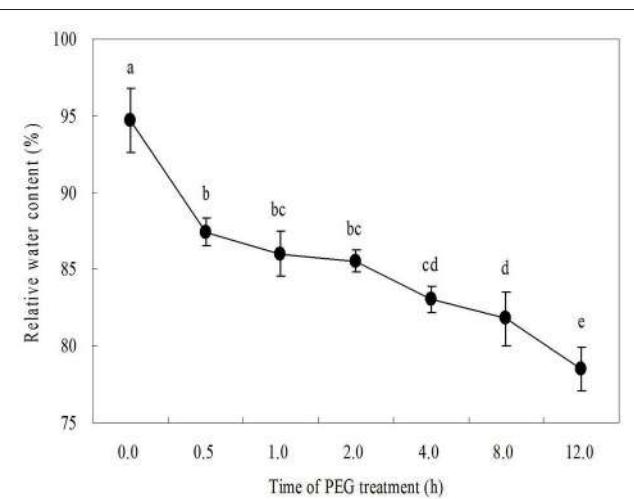
### Endogenous Polyamine Determination by HPLC

Polyamines were extracted using the method of Duan et al. (2008). Detached leaves (0.25 g) were ground in 1 ml cold perchloric acid (5%, v: v) and the homogenate was incubated at 4°C for 1 h and then centrifuged for 30 min (12,000 rpm, 4°C). The supernatant was benzoylated as follows. Two milliliters NaOH (2 M) and 10  $\mu\text{l}$  benzoyl chlorides were added into 500  $\mu\text{l}$  supernatant and then they were incubated at 37°C for 30 min. In order to terminate the reaction, 2 ml saturated NaCl solution was added into the mixed solution. After that, 2 ml cold diethyl was used for extracting benzoyl polyamine. Finally, 1 ml of the diethyl ether phase was evaporated to dryness and re-dissolved in 1 ml methanol for determination of endogenous polyamines. High performance liquid chromatography (Agilent-1200, Agilent Technologies, USA) was used for analyzing endogenous PA content. 20  $\mu\text{l}$  of benzoyl PA was injected

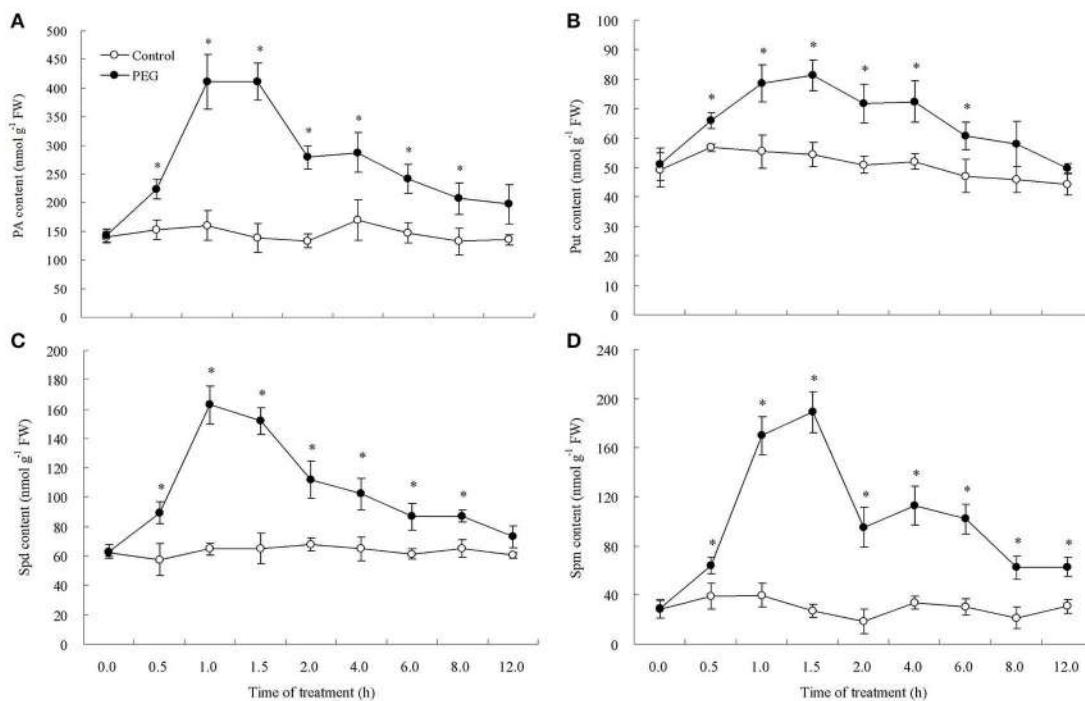
into loop and then loaded onto a reverse-phase Tigerkin® C18 column (150 × 4.6 mm, 5  $\mu\text{m}$  particle size). Column temperature was maintained at 25°C. Mobile phase was methanol- $\text{H}_2\text{O}$  (64: 36, v: v). PA peak was detected at a flow rate of 1 ml  $\text{min}^{-1}$  at 254 nm.

### Determination of Water Deficit and Oxidative Damage

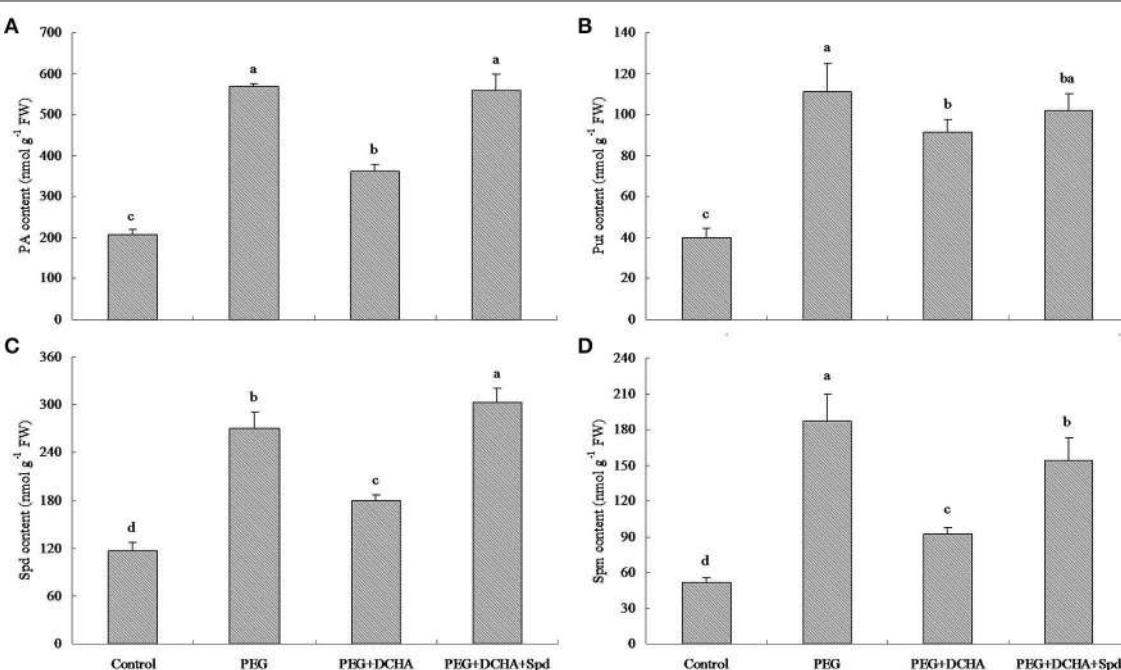
The leaf relative water content (RWC) was measured as an indicator of water deficit by using the formula  $\text{RWC} (\%) = [(\text{FW-DW})/(\text{TW-DW})] \times 100$  (FW, fresh weights; TW, turgid weights; and DW, dry weights). Treated leaves (0.1 g) in different chemical solutions were collected, gently blotted dry, and weighed for FW. After immersing these leaves in deionized water for 16 h at 4°C in the dark, TW was measured. Samples were then dried in an 80°C oven for more than 72 h for weighing DW (Barrs and Weatherley, 1962). For extraction of malondialdehyde (MDA), the leaves (0.1 g) were ground with 2 ml PBS (50 mM, pH 7.8) and then centrifuged for 30 min (12,000 rpm, 4°C). The 0.5 ml supernatant was added to 1 ml reaction solution (20% trichloroacetic acid, 0.5% thiobarbituric acid) and then heated for 15 min at 95°C. After cooling quickly, the mixture was centrifuged for 10 min at 8000 rpm. The absorbance of the supernatant was calculated by subtraction of  $\text{OD}_{600}$  from  $\text{OD}_{532}$  (Dhindsa et al., 1981). Electrolyte leakage (EL) was measured to evaluate plasma membrane stability. Leaves (0.1 g) were placed in centrifuge tubes containing 35 ml of distilled  $\text{H}_2\text{O}$  for 24 h. The initial conductance ( $C_i$ ) was measured using a conductivity meter (YSI Model 32, Yellow Springs, OH). Tubes were then put in an autoclave for 20 min at 140°C and the maximal conductance ( $C_{\max}$ ) was measured. Finally, EL was calculated according to the formula  $(\%) = C_i/C_{\max} \times 100$  (Blum and Ebercon, 1981). Protein carbonyl content was analyzed by using



**FIGURE 1 |** Time course of PEG 6000-induced the change of leaf relative water content (%) as an indicator of water deficit. The detached leaves were pre-treated with distilled water for 1 h to eliminate wound stress and then exposed to 15% PEG solution for 12 h. Means of six independent samples are presented. Bars represent standard errors. The same letter indicates no significant difference (LSD) at  $P < 0.05$ .



**FIGURE 2 | Time course of PEG 6000-induced polyamine (PA) (A), putrescine (Put) (B), spermidine (Spd) (C), and spermine (Spm) accumulation (D) in leaves.** The detached leaves were pre-treated with distilled water for 1 h to eliminate wound stress and then exposed to distilled water (control) or 15% PEG solution for 12 h. Means of four independent samples are presented. Bars represent standard error. \*\*\* indicate LSD values where significant differences were detected ( $P < 0.05$ ) between two treatments at a given time.



**FIGURE 3 | Effects of spermidine (Spd) and inhibitor (DCHA) of aminopropyl transferase on PEG 6000-induced polyamine (PA) (A), putrescine (Put) (B), spermidine (Spd) (C), and spermine (Spm) accumulation (D) in leaves.** The detached leaves of white clover were pre-treated with distilled water for 1 h to eliminate wound stress and then treated for 1 h as follows: 1, distilled water (control); 2, 15% PEG; 3, 15% PEG + 100  $\mu\text{M}$  dicyclohexylamine (DCHA); 4, 15% PEG + 100  $\mu\text{M}$  DCHA + 20  $\mu\text{M}$  Spd. Means of four independent samples are presented. Bars represent standard errors. The same letter above columns indicates no significant difference (LSD) at  $P < 0.05$ .

a Protein Carbonyl Colorimetric Assay Kit (Cayman Chemical, USA) and the absorbency of reaction solution was determined on a microplate reader (Synergy HTX, Bio Tek, USA).

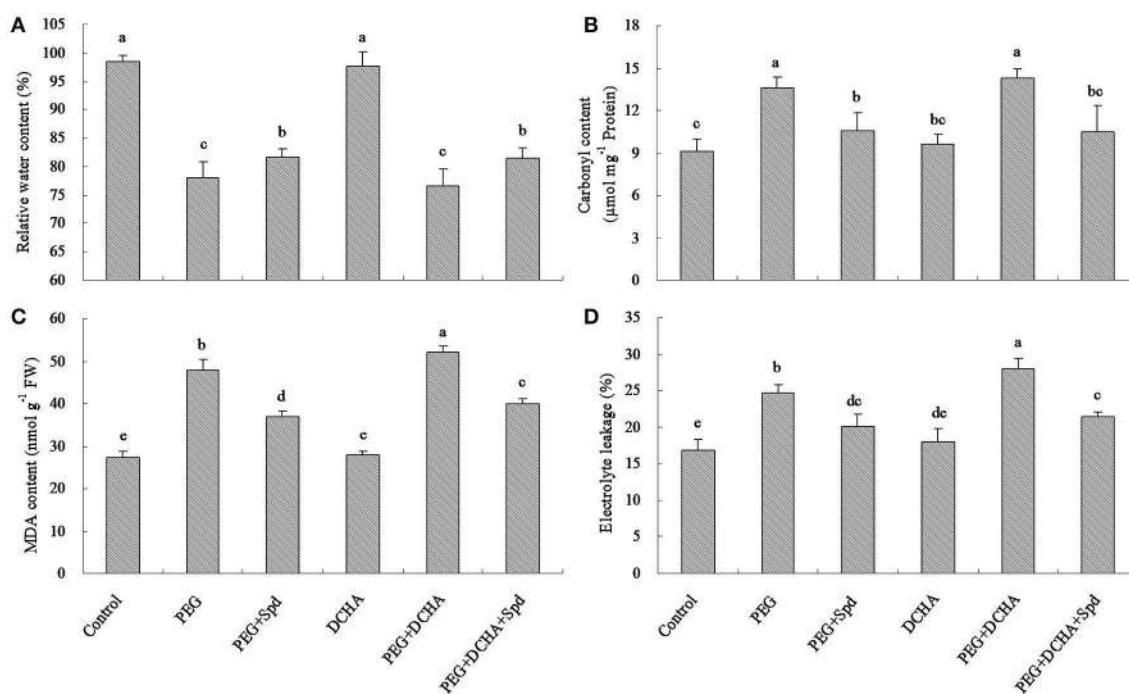
### Cytosolic Free $\text{Ca}^{2+}$ and $\text{H}_2\text{O}_2$ Detection by CLSM

$\text{H}_2\text{O}_2$  and cytosolic free  $\text{Ca}^{2+}$  were detected by using a  $\text{H}_2\text{O}_2$ -sensitive fluorescent probe H<sub>2</sub>DCFDA (Sigma, USA) and free cytosolic  $\text{Ca}^{2+}$ -specific fluorescent Fluo-3 AM probe (Beyotime, China). In order to eliminate wound stress, the detached leaves were pre-treated with distilled  $\text{H}_2\text{O}$  for 1 h and then incubated in distilled  $\text{H}_2\text{O}$  to act as a control or 20  $\mu\text{M}$  Spd or 15% PEG for different times or chemical treatments (specifically described in each figure legend) in growth chamber conditions as mentioned above, followed by loading with 25  $\mu\text{M}$  H<sub>2</sub>DCFDA or 2.5  $\mu\text{M}$  Fluo-3 AM for 30 min in the dark. After that, detached leaves were rinsed three times in Tris-KCl buffer (pH 7.2) until all fluorescent probes were removed from the foliar surface. A confocal laser scanning microscope (Nikon A1, Nikon, Japan) was used for visualizing all images (excitation 488, emission 525). For measurement of  $\text{H}_2\text{O}_2$  content, 0.2 g fresh leaves were ground with 4 ml of 5% TCA and 0.15 g activated charcoal and then centrifuged at 10000 rpm for 20 min at 4°C. The 3 ml supernatant was adjusted to pH 8.4 with 17 M ammonia solution and then filtered. One milliliter of colorimetric reagent (10 mg of 4-aminoantipyrine, 10 mg of phenol, and 5 mg of peroxidase) was added into the filtrate with and without catalase. The reaction

solution was incubated for 10 min at 30°C. Absorbance was determined at 505 nm on a microplate reader (Zhou et al., 2006).

### Determination of Antioxidant Enzyme and NADPH Oxidase Activity

For antioxidant enzyme activity assays, the supernatant was collected according to the procedure for MDA extraction as mentioned above. For determination of superoxide dismutase (SOD) activity, 1.5 ml reaction solution (50 mM PBS containing 195 mM methionine, 60  $\mu\text{M}$  riboflavin, and 1.125 mM NBT) was added into 0.05 ml enzyme extract and the change of absorbance was recorded at 560 nm (Giannopolitis and Ries, 1977). The activities of guaiacol peroxidase (GPOX) and catalase (CAT) were measured based on the methods of Chance and Maehly (1955). Briefly, 0.05 ml enzyme extract was mixed with 1.5 ml reaction solution (0.05 ml of 0.75%  $\text{H}_2\text{O}_2$ , 0.5 ml of 0.25% guaiacol solution, and 0.995 ml of 100 mM PBS, pH 5.0 for the assay of GPOX or 0.5 ml of 45 mM  $\text{H}_2\text{O}_2$  and 1 ml of 50 mM PBS, pH 7.0 for the assay of CAT). The changes in absorbance were monitored at 460 or 240 nm every 10 s for 1 min for GPOX and CAT, respectively. For measurement of ascorbate peroxidase (APX) activity, 0.05 ml of enzyme extract was added into 1.5 ml of reaction solution containing 10 mM ascorbic acid, 0.003 mM EDTA, 5 mM  $\text{H}_2\text{O}_2$  and 100 mM PBS (pH 5.8) and the change of absorbance was recorded every 10 s for 1 min at 290 nm (Nakano and Asada, 1981). The method of two-phase aqueous polymer partition system was used for obtaining of leaf plasma



**FIGURE 4 |** Effects of spermidine (Spd) and inhibitor (DCHA) of aminopropyl transferase on PEG 6000-induced relative water content (A), carbonyl content (B), malondialdehyde content (C) and electrolyte leakage (D). The detached leaves of white clover were pre-treated with distilled water for 1 h to eliminate wound stress and then treated for 12 h as follows: 1, distilled water (control); 2, 15% PEG; 3, 15% PEG + 20  $\mu\text{M}$  Spd; 4, 100  $\mu\text{M}$  dicyclohexylamine (DCHA); 5, 15% PEG + 100  $\mu\text{M}$  DCHA + 20  $\mu\text{M}$  Spd. Means of six independent samples are presented. Bars represent standard errors. The same letter indicates no significant difference (LSD) at  $P < 0.05$ .

membrane (Larsson et al., 1987). One milliliter reaction solution contains 20  $\mu\text{l}$  NADPH (50  $\mu\text{M}$ ), 100  $\mu\text{l}$  XTT (0.5 mM), 780  $\mu\text{l}$  Tris-HCl (pH 7.5) and 20  $\mu\text{g}$  plasma membrane protein. The blank was added with 20  $\mu\text{l}$  SOD (100 U  $\text{mg}^{-1}$ ). After 5 min at 25°C, the reaction solution was used for spectrophotometric analysis at A470.  $\Delta\text{A470}$  represents the difference in XTT (3'-[1-[phenylamino-carbonyl]-3,4-tetrazolium]-bis (4-methoxy-6-naitro)) absorbance in the presence and absence of 100 units of SOD at 470 nm (Jiang and Zhang, 2002). Bradford's (1976) method was used for determining protein content.

### Total RNA Extraction and qRT-PCR Analysis

Transcript levels of antioxidant enzyme genes were performed using real-time quantitative polymerase chain reaction (qRT-PCR). For total RNA extraction, the detached leaves of white clover were extracted by using RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. A cDNA Synthesis Kit (Fermentas, Lithuania) was used for reverse-transcribing RNA to cDNA. Primer sequences for the genes SOD, GPOX, CAT, and APX, CDPK,  $Y_2\text{SK}$ ,  $Y_2\text{K}$ , SK<sub>2</sub> (Vaseva et al., 2011) and  $\beta\text{-Actin}$  (internal control) are shown in Table 1. Gene expression levels were determined using an iCycler iQ

qRT-PCR detection system with SYBR Green Supermix (Bio-Rad). The conditions of the PCR protocol for all genes were as follows: 5 min at 95°C and 40 repeats of denaturation at 95°C for 15 s, annealing at 58°C (SOD, CAT, GPOX, and SK<sub>2</sub> gene), or 60°C ( $Y_2\text{SK}$ , and  $Y_2\text{K}$ ) or 63°C (APX and CDPK gene) for 45 s, following by heating the amplicon from 60 to 95°C to obtain the melting curve. At the end of PCR cycle, the transcript level of all genes was calculated according to the formula  $2^{-\Delta\Delta\text{Ct}}$  described by Xia et al. (2009).

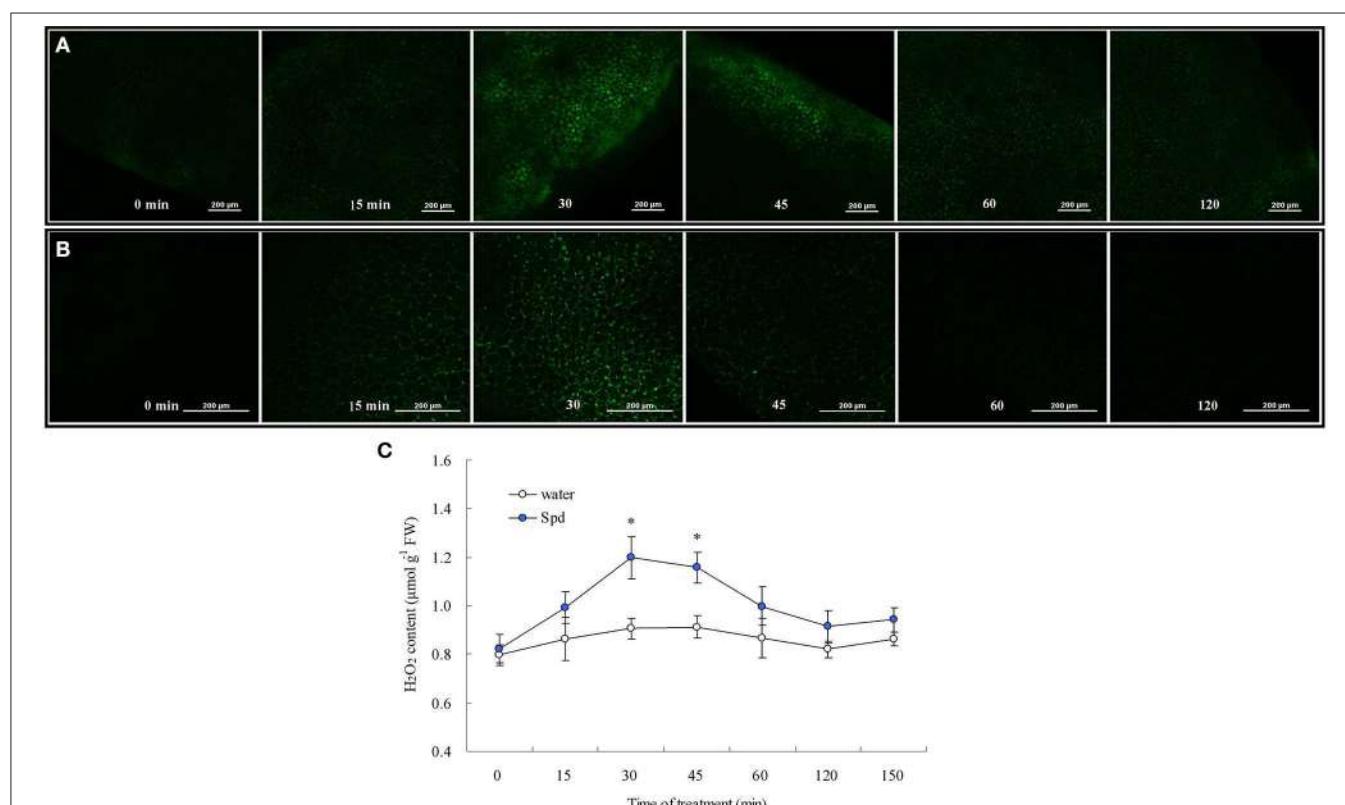
### Statistical Analysis

The data was analyzed by using SAS 9.1 (SAS Institute, Cary, NC). Significant differences among treatments were determined using Fischer's least significance difference (LSD) at the 0.05 probability level.

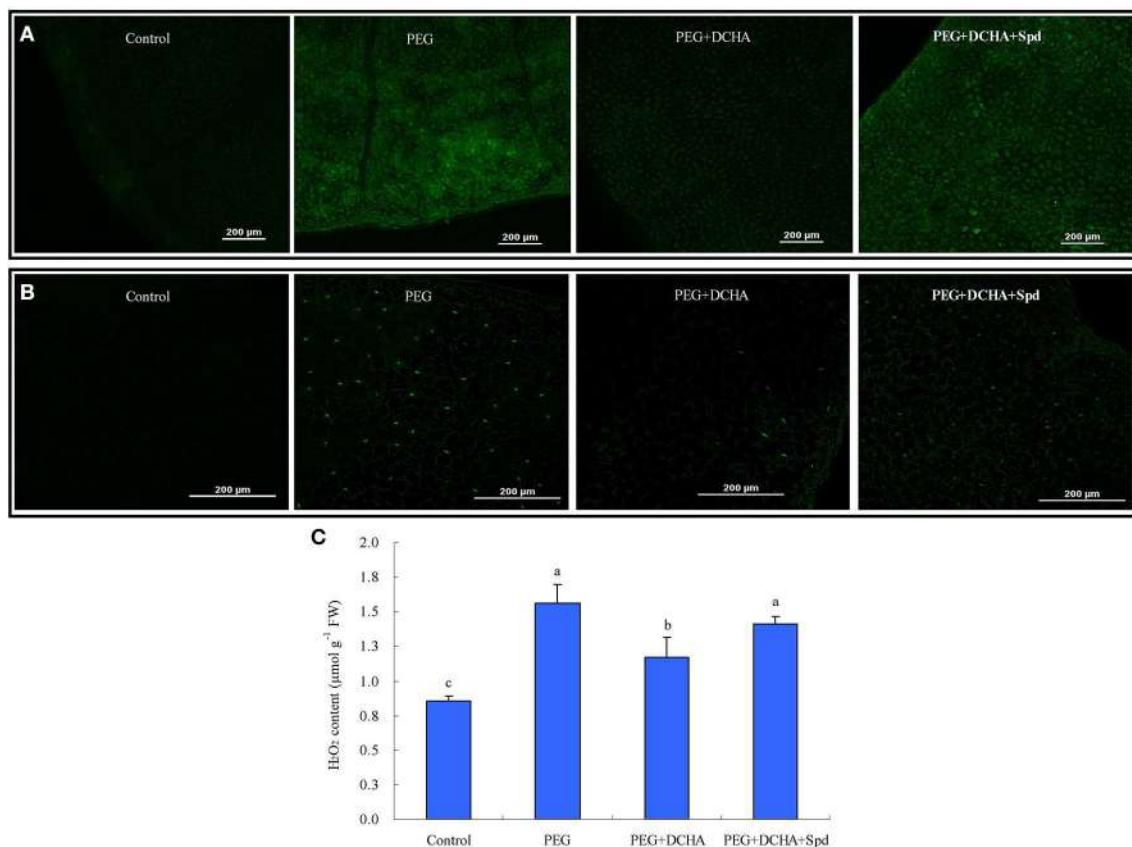
## Results

### PA is Involved in Tolerance to Water Stress in Leaves of White Clover

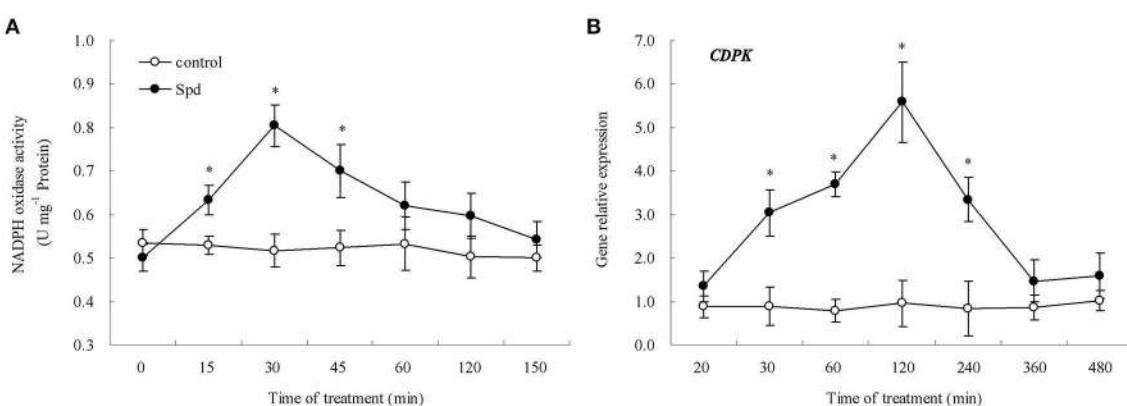
As shown in Figure 1, 15% PEG caused the decline in leaf relative water content (RWC). Only after 0.5 h, leaf RWC decreased to a significant lower degree. When treated in 15%



**FIGURE 5 | Cytosolic free  $\text{Ca}^{2+}$  (A) and  $\text{H}_2\text{O}_2$  production (B,C) as affected by spermidine (Spd) at different times.** The detached leaves were pre-treated with distilled water for 1 h to eliminate wound stress and then exposed to 20  $\mu\text{M}$  Spd for various time, followed by incubation with  $\text{Ca}^{2+}$ -sensitive fluorescent probe Fluo-3-AM or  $\text{H}_2\text{O}_2$ -sensitive fluorescent probe  $\text{H}_2\text{DCFDA}$  for 30 min. Images are visualized using confocal laser scanning microscopy (CLSM). Means of four independent samples are presented. Bars represent standard error. \*\* indicate LSD values where significant differences were detected ( $P < 0.05$ ) between two treatments at a given time.



**FIGURE 6 | Effects of polyamine (PA) on PEG-induced cytosolic free  $\text{Ca}^{2+}$  (A) and  $\text{H}_2\text{O}_2$  production (B,C).** The detached leaves of white clover were pre-treated with distilled water for 1 h to eliminate wound stress and then treated for 45 min as follows: 1, distilled water (control); 2, 15% PEG; 3, 15% PEG + 100  $\mu\text{M}$  dicyclohexylamine (DCHA); 4, 15% PEG + 100  $\mu\text{M}$  DCHA + 20  $\mu\text{M}$  Spd and followed by incubation with  $\text{Ca}^{2+}$ -sensitive fluorescent probe Fluo-3-AM or  $\text{H}_2\text{O}_2$ -sensitive fluorescent probe  $\text{H}_2\text{DCFDA}$  for 30 min. Images are visualized using confocal laser scanning microscopy (CLSM). Means of four independent samples are presented. Bars represent standard errors. The same letter indicates no significant difference (LSD) at  $P < 0.05$ .



**FIGURE 7 | Time course of spermidine (Spd)-induced NADPH oxidase activity (A), and CDPK gene relative expression (B) in leaves.** The detached leaves were pre-treated with distilled water for 1 h to eliminate wound stress and then exposed to distilled water (control) or 20  $\mu\text{M}$  Spd solution for 480 min. Means of four independent samples are presented. Bars represent standard error. \*\* indicate LSD values where significant differences were detected ( $P < 0.05$ ) between two treatments at a given time.

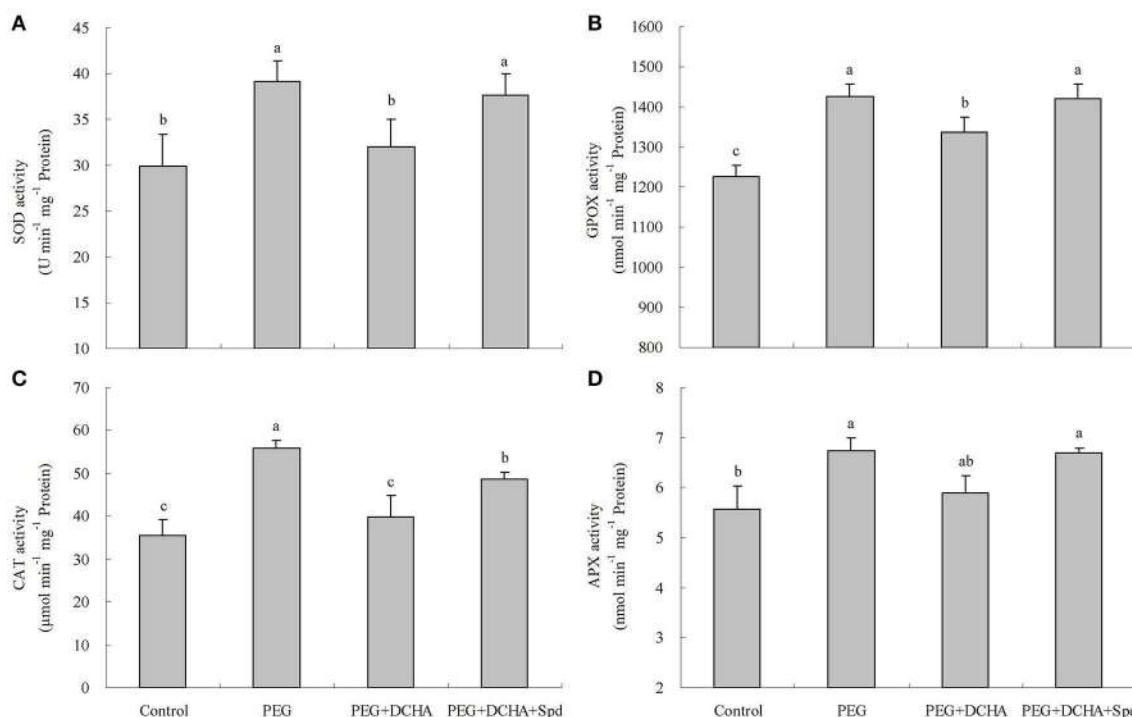
PEG solution for 12 h, leaf RWC fell from almost 95% to only 78% (**Figure 1**). Water deficit induced changes in endogenous PA content in leaves of white clover (**Figure 2**). Endogenous PA, Put, Spd, and Spm content increased significantly at 0.5 h of water stress caused by 15% PEG and then reached a peak value at 1 h as compared with the control (treated with water). After 2 h of water stress, PA began to decline gradually in leaves (**Figure 2**). As shown in **Figure 3**, the inhibitor of aminopropyl transferase “DCHA” effectively inhibited the PEG-induced increase in endogenous PA, including Put, Spd, and Spm. However, exogenously applied Spd could reverse DCHA-induced decrease in PA content under water stress (**Figure 3**). In order to investigate whether PA was involved in tolerance to water stress in leaves, the effects of exogenous Spd and DCHA on leaf RWC, carbonyl content, MDA content, and EL level were tested in detached leaves under water stress (**Figure 4**). Exogenous Spd significantly improved leaf RWC under water stress. In addition, water stress caused oxidative damage in detached leaves, as reflected by increasing in protein oxidation (carbonyl content), lipid peroxidation level (MDA content), and membrane leakage (EL). These three indicators significantly declined with the application of exogenous Spd in response to water stress. However, DCHA caused further increase in PEG-induced carbonyl content, MDA content, and EL levels, but these negative effects of DCHA under water stress were alleviated by addition of 20  $\mu\text{M}$  Spd.

## PA is Involved in PEG-induced Cytosolic Free $\text{Ca}^{2+}$ and $\text{H}_2\text{O}_2$ Production

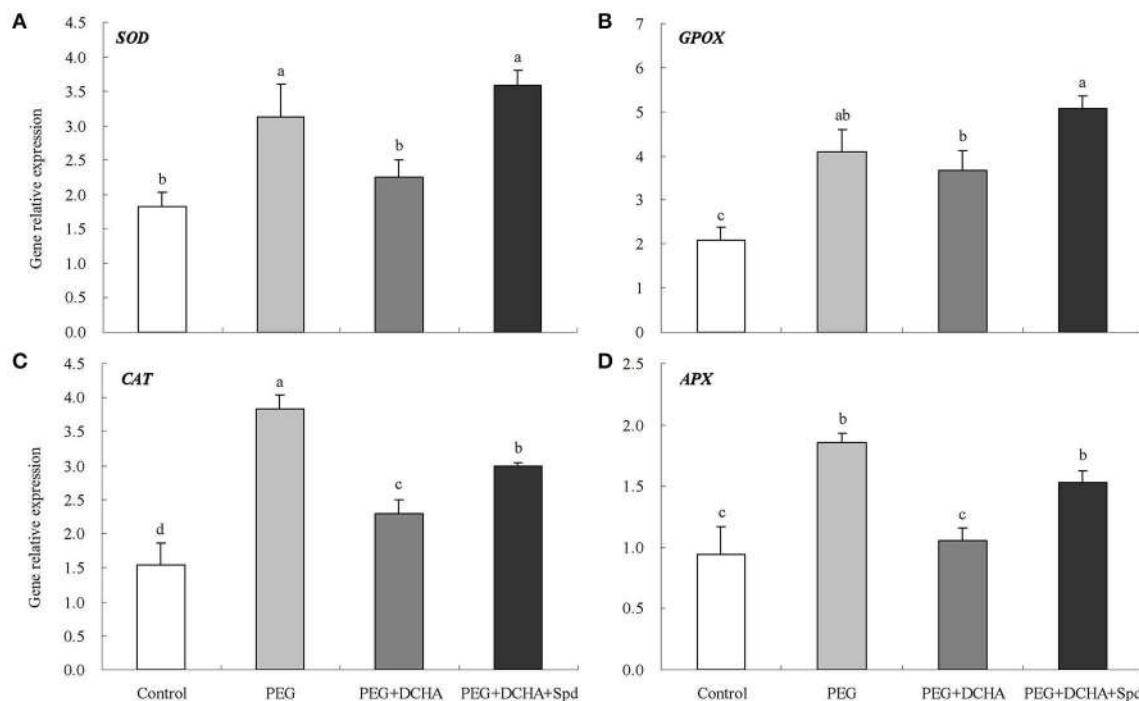
A rapid change of cytosolic free  $\text{Ca}^{2+}$  was caused by exogenous Spd in detached leaves. The Fluo-3 AM fluorescent probe signal reached a maximum at 30 min, and remained high at 45 min. After that, cytosolic free  $\text{Ca}^{2+}$  subsided to the normal level almost as quickly as it increased (**Figure 5A**). After 15 min treatment in Spd solution, cytosolic  $\text{H}_2\text{O}_2$  generation in detached leaves was also observed (**Figure 5B**). Cytosolic  $\text{H}_2\text{O}_2$  content reached a maximum at 30 min and then declined, which suggested that Spd induced instantaneous  $\text{H}_2\text{O}_2$  burst in cells (**Figures 5B,C**). In **Figure 6**, it's shown that the treatment with 15% PEG for 45 min resulted in a significant accumulation of cytosolic free  $\text{Ca}^{2+}$  and  $\text{H}_2\text{O}_2$ , whereas 100  $\mu\text{M}$  DCHA effectively blocked the accumulation of  $\text{Ca}^{2+}$  and  $\text{H}_2\text{O}_2$  induced by 15% PEG. The application of exogenous Spd markedly restored the increase of cytosolic free  $\text{Ca}^{2+}$  and  $\text{H}_2\text{O}_2$  blocked by DCHA in detached leaves exposed to 15% PEG (**Figure 6**). Exogenous Spd affected the time-course changes of NADPH oxidase activity and CDPK gene expression (**Figure 7**).

## PA is Involved in PEG-Induced Antioxidant Enzyme Activities and Genes Expression

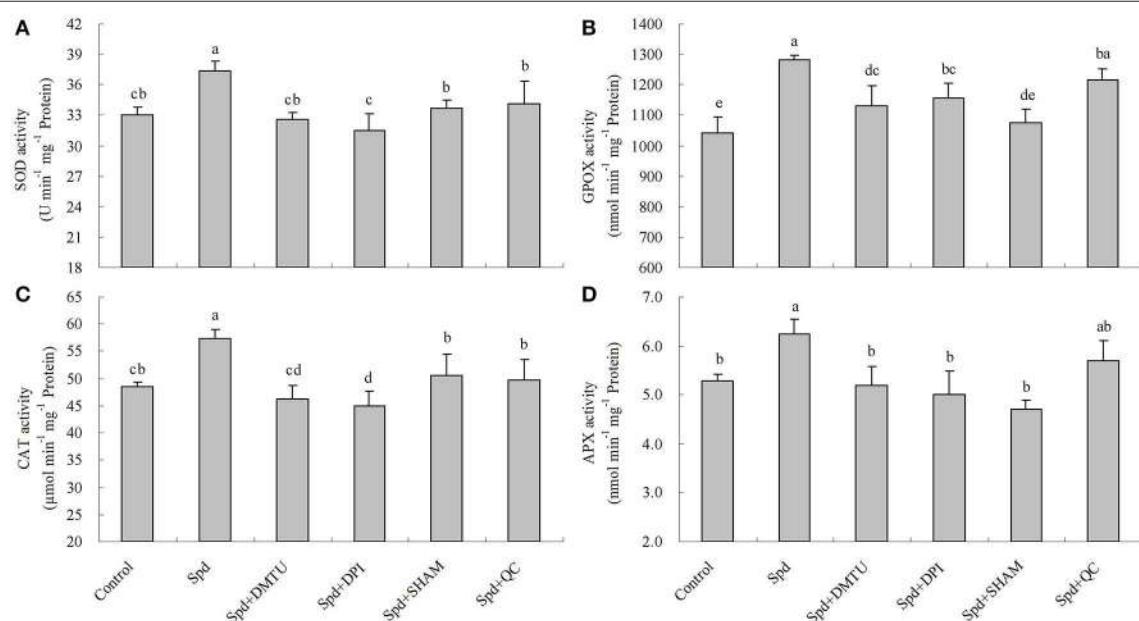
Different PEG concentrations affected antioxidant enzyme activities (SOD, GPOX, CAT, and APX) in detached leaves as shown in Figure S1 and lower PEG concentrations (10



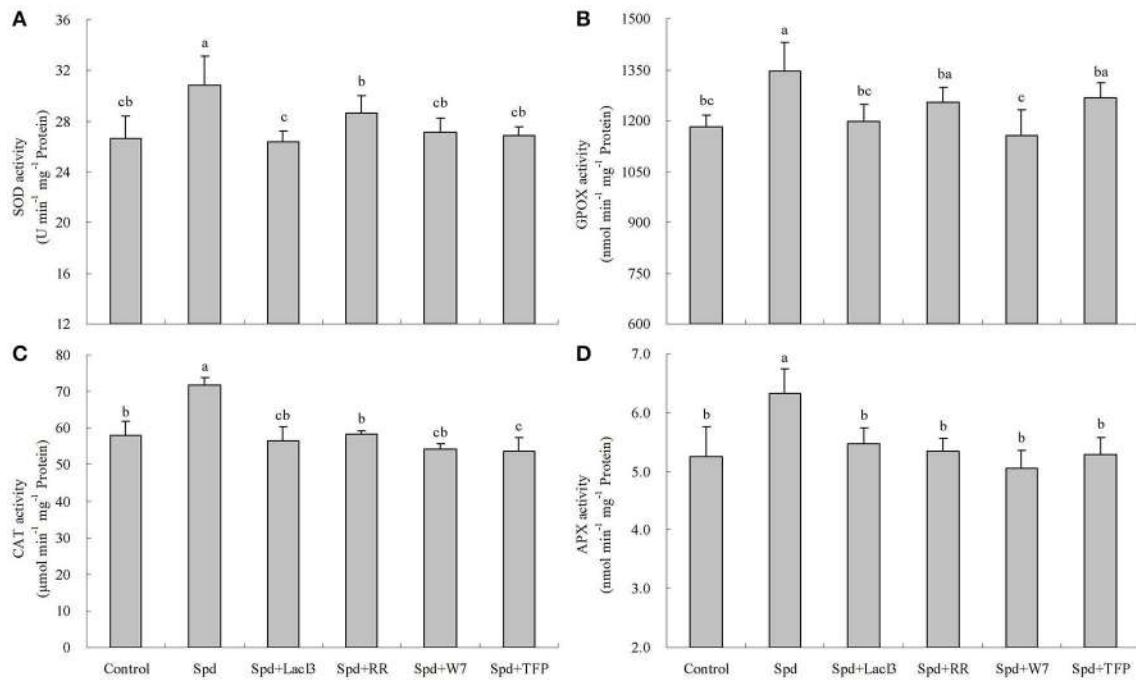
**FIGURE 8 |** Antioxidant activities as affected by polyamines (PA) under PEG-induced water stress. SOD (**A**), GPOX (**B**), CAT (**C**), and APX (**D**) The detached leaves of white clover were pre-treated with distilled water for 1 h to eliminate wound stress and then treated for 8 h as follows: 1, distilled water (control); 2, 15% PEG; 3, 15% PEG + 100  $\mu\text{M}$  dicyclohexylamine (DCHA); 4, 15% PEG + 100  $\mu\text{M}$  DCHA + 20  $\mu\text{M}$  Spd. Means of six independent samples are presented. Bars represent standard errors. The same letter indicates no significant difference (LSD) at  $P < 0.05$ .



**FIGURE 9 | Gene transcript levels encoding antioxidant enzymes as affected by polyamines (PA) under PEG-induced water stress.** *SOD* gene (A), *GPOX* gene (B), *CAT* gene (C), and *APX* gene (D). The detached leaves of white clover were pre-treated with distilled water for 1 h to eliminate wound stress and then treated for 2 h as follows: 1, distilled water (control); 2, 15% PEG; 3, 15% PEG + 100  $\mu\text{M}$  dicyclohexylamine (DCHA); 4, 15% PEG + 100  $\mu\text{M}$  DCHA + 20  $\mu\text{M}$  Spd. Means of four independent samples are presented. Bars represent standard errors. The same letter indicates no significant difference (LSD) at  $P < 0.05$ .



**FIGURE 10 | Spd-induced SOD (A), GPOX (B), CAT (C), and APX (D) activities were dependent on hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) signaling.** The detached leaves of white clover were pre-treated with distilled water for 1 h to eliminate wound stress and then treated for 8 h as follows: 1, distilled water (control); 2, 20  $\mu\text{M}$  Spd; 3, 20  $\mu\text{M}$  Spd + 5 mM dimethylthiourea (DMTU); 4, 20  $\mu\text{M}$  Spd + 100  $\mu\text{M}$  Diphenyleneiodonium (DPI); 5, 20  $\mu\text{M}$  Spd + 5 mM salicyhydroxamic (SHAM); 6, 20  $\mu\text{M}$  Spd + 100  $\mu\text{M}$  quinacrine (QC). Means of six independent samples are presented. Bars represent standard errors. The same letter indicates no significant difference (LSD) at  $P < 0.05$ .



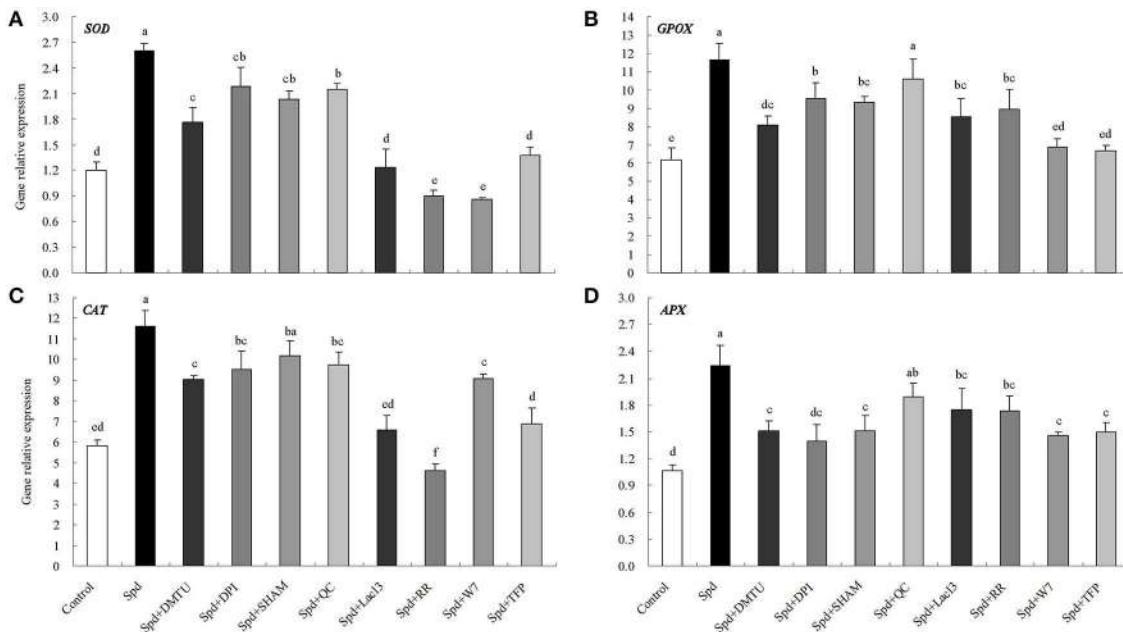
**FIGURE 11 | Spd-induced SOD (A), GPOX (B), CAT (C), and APX (D) activities were dependent on calcium ( $\text{Ca}^{2+}$ ) messenger system.** The detached leaves of white clover were pre-treated with distilled water for 1 h to eliminate wound stress and then treated for 8 h as follows: 1, distilled water (control); 2, 20  $\mu\text{M}$  Spd; 3, 20  $\mu\text{M}$  Spd + 1 mM  $\text{LaCl}_3$ ; 4, 20  $\mu\text{M}$  Spd + 50  $\mu\text{M}$  ruthenium red (RR); 5, 20  $\mu\text{M}$  Spd + 150  $\mu\text{M}$  N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7); 6, 20  $\mu\text{M}$  Spd + 30  $\mu\text{M}$  trifluoperazine (TFP). Means of six independent samples are presented. Bars represent standard errors. The same letter indicates no significant difference (LSD) at  $P < 0.05$ .

and 15%) significantly improved four enzyme activities. With increases in PEG concentration, antioxidant enzyme activities decreased gradually. Higher PEG concentrations (25%) failed to improve the four enzyme activities and even suppressed APX activity compared with the control (0% PEG) after 8 h of treatment (Figure S1D). Transcript levels of four genes encoding antioxidant enzymes were also up-regulated under 15% PEG (Figure S3). SOD, GPOX, CAT, and APX activities were enhanced by 31, 16, 57, and 21%, respectively after 15% PEG treatment for 8 h (Figure 8). To a certain extent, the PEG-induced effects on antioxidant enzyme activities were nullified when DCHA was added into 15% PEG solution, but these effects were reversed by the addition of Spd into 15% PEG and DCHA solution (Figure 8). PEG, DCHA, and Spd had similar effects on SOD, GPOX, CAT, and APX gene transcript levels (Figure 9).

### H<sub>2</sub>O<sub>2</sub> and Ca<sup>2+</sup> Messenger Systems Are Involved in Spd-induced Antioxidant Defense and Dehydrin Genes Expression

Figure S2 showed the effects of Spd concentrations on elevating the enzyme activities of SOD, GPOX, CAT, and APX. Three concentrations of Spd (15, 20, and 25  $\mu\text{M}$ ) significantly enhanced antioxidant enzyme activities after 8 h of treatment. Among these concentrations, 20  $\mu\text{M}$  Spd exhibited the highest induction of activity on the four enzymes (Figure S2). Treatment with 20  $\mu\text{M}$

Spd also led to significant up-regulation of gene transcript levels for SOD, GPOX, CAT, and APX within 8 h of treatment (Figure S3). To investigate whether H<sub>2</sub>O<sub>2</sub> signaling and the calcium messenger system were involved in Spd-induced antioxidant enzyme activities and genes expression, a H<sub>2</sub>O<sub>2</sub> scavenger, enzyme inhibitors, and calcium channel blockers were tested. Spd-induced enzyme activities and genes expression (SOD, GPOX, CAT, and APX) were suppressed to a certain extent when H<sub>2</sub>O<sub>2</sub> scavenger (DMTU), NADPH oxidase inhibitor (DPI), cell wall-localized peroxidase inhibitor (SHAM), or amine oxidase inhibitor (QC) was added to the Spd solution (Figures 10, 12). Moreover, DMTU and DPI showed the greatest effects on reducing Spd-induced antioxidant enzyme activities and genes expression, while the effect of QC was the least. Similarly, treatment with  $\text{Ca}^{2+}$  channel blockers (plasma membrane calcium channel blocker,  $\text{LaCl}_3$ ; inhibitor of mitochondrial and endoplasmic reticulum calcium channels, RR), calmodulin antagonist (W7), or an inhibitor of CDPK (TFP) significantly blocked the increases in antioxidant enzyme activities and gene transcript levels induced by Spd (Figures 11, 12). Detached leaves treated with 20  $\mu\text{M}$  Spd led to significant changes in three dehydrin gene transcript levels relative to the control at different times (Figures 13A–C). However, DMTU, DPI, and TFP significantly reduced Spd-induced dehydrin genes expression including *Y<sub>2</sub>SK*, *Y<sub>2</sub>K*, and *SK<sub>2</sub>* (Figures 13D–F).



**FIGURE 12 | Spd-induced SOD (A), GPOX (B), CAT (C), and APX (D) gene transcript levels were dependent on hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) signaling and calcium ( $\text{Ca}^{2+}$ ) messenger system.** The detached leaves of white clover were pre-treated with distilled water for 1 h to eliminate wound stress and then treated for 2 h as follows: 1, distilled water (control); 2, 20  $\mu\text{M}$  Spd; 3, 20  $\mu\text{M}$  Spd + 5 mM dimethylthiourea (DMTU); 4, 20  $\mu\text{M}$  Spd + 100  $\mu\text{M}$  Diphenyleneiodonium (DPI); 5, 20  $\mu\text{M}$  Spd + 5 mM salicylyhydroxamic (SHAM); 6, 20  $\mu\text{M}$  Spd + 100  $\mu\text{M}$  quinacrine (QC); 7, 20  $\mu\text{M}$  Spd + 1 mM LaCl<sub>3</sub>; 8, 20  $\mu\text{M}$  Spd + 50  $\mu\text{M}$  ruthenium red (RR); 9, 20  $\mu\text{M}$  Spd + 150  $\mu\text{M}$  N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7); 10, 20  $\mu\text{M}$  Spd + 30  $\mu\text{M}$  trifluoperazine (TFP). Means of four independent samples are presented. Bars represent standard errors. The same letter indicates no significant difference (LSD) at  $P < 0.05$ .

## The Interaction of Spd-induced $\text{H}_2\text{O}_2$ and $\text{Ca}^{2+}$

The interaction between Spd-induced  $\text{H}_2\text{O}_2$  and  $\text{Ca}^{2+}$  was further investigated (Figure 14). Cytosolic free  $\text{Ca}^{2+}$  content increased to higher levels after treatment with 20  $\mu\text{M}$  Spd for 30 min (Figure 14A). However, the change in free  $\text{Ca}^{2+}$  content was not detected in the control after the same length of time. The enhanced  $\text{Ca}^{2+}$  fluorescence was inhibited by calcium channel blockers LaCl<sub>3</sub> and RR in detached leaves, and was also eliminated by  $\text{H}_2\text{O}_2$  scavenger (DMTU), indicating that Spd-induced cytosolic free  $\text{Ca}^{2+}$  accumulation was dependent upon  $\text{H}_2\text{O}_2$  signaling (Figure 14A). Similarly, a Spd-induced  $\text{H}_2\text{O}_2$  increase in cells was significantly blocked by application of LaCl<sub>3</sub>, RR, and DMTU, which showed that Spd-induced  $\text{H}_2\text{O}_2$  accumulation was involved in  $\text{Ca}^{2+}$  signaling (Figures 14B,C). Based on all above findings, we could deduce a model for PA regulation of  $\text{Ca}^{2+}$  and  $\text{H}_2\text{O}_2$  signaling in control of antioxidant defense involves in tolerance to water stress in leaves of white clover (Figure 15).

## Discussion

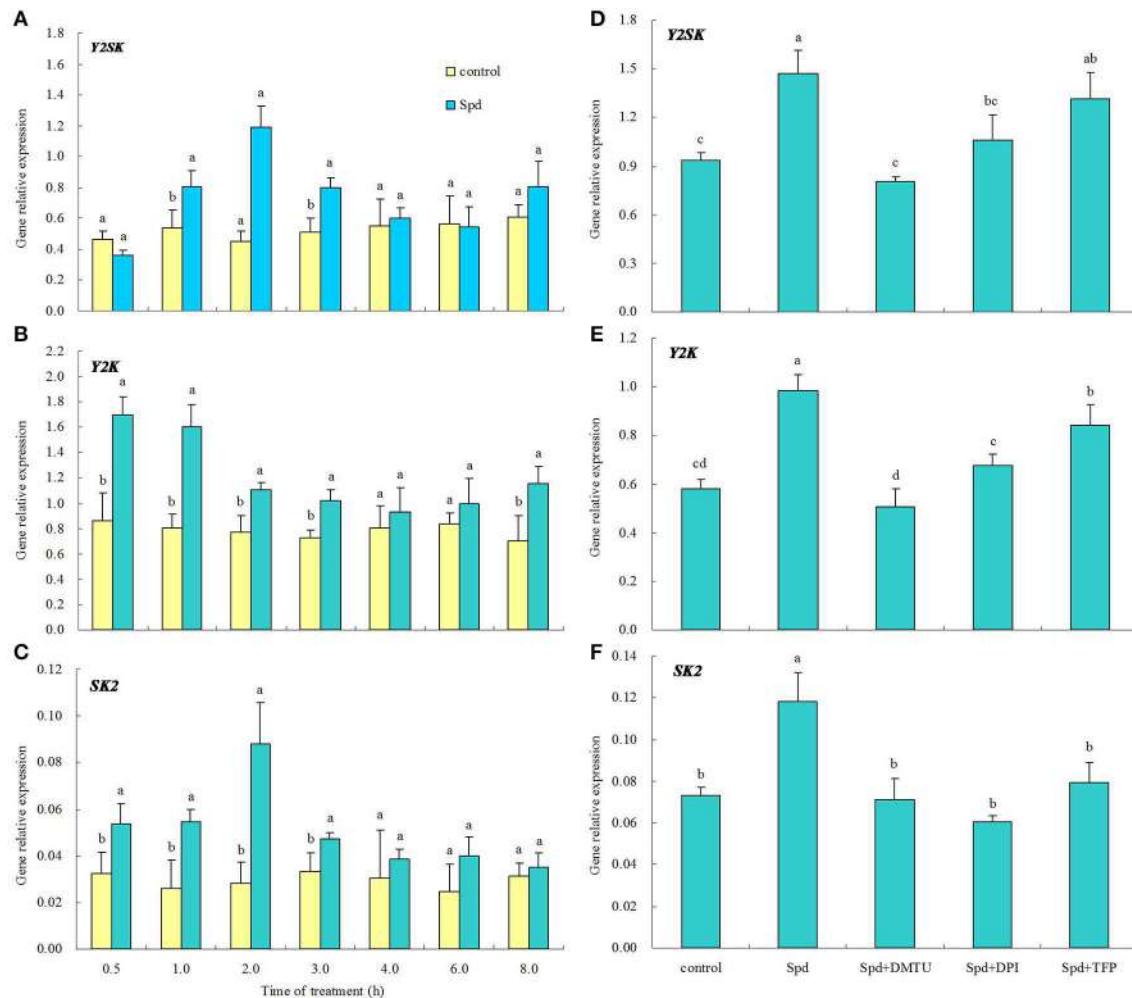
### PA is Involved in Tolerance to Water Stress in White Clover

It has been observed that abiotic stress can induce increases in endogenous PA content in various plant species, which is important for adaptation to drought stress (Kusano et al., 2008; Zhou and Yu, 2010; Hu et al., 2012). Elevated endogenous

PA content through exogenously applied Spd or Spm led to enhancement in drought tolerance in *Arabidopsis thaliana* (Kusano et al., 2007), rice (*Oryza sativa*) (Farooq et al., 2009), and creeping bentgrass (*Agrostis stolonifera*) (Li et al., 2015). In the current study, our results showed that endogenous PA content increased significantly in detached clover leaves under water stress. However, DCHA, an inhibitor of PA biosynthesis, suppressed water stress-induced increases in endogenous PA levels, in agreement with the early research of Meskaoui and Trembaly (2009). Deficiencies in endogenous PA induced by DCHA further aggravated oxidative damage in white clover under water stress, as demonstrated by significantly higher carbonyl content, MDA content, and EL levels, but it could be rescued by exogenously applied Spd. Previous finding has also demonstrated that the inhibition of PA biosynthesis in pear shoots resulted in a considerable reduction in stress tolerance (Wen et al., 2011). These data indicate that PA plays a positive role in white clover tolerance to water stress, and it could be an adaptive response that increasing endogenous PA levels confer white clover a certain stress tolerance when subjected to water stress.

### PA is Involved in Water Stress-induced Cytosolic Free $\text{Ca}^{2+}$ and $\text{H}_2\text{O}_2$ Production

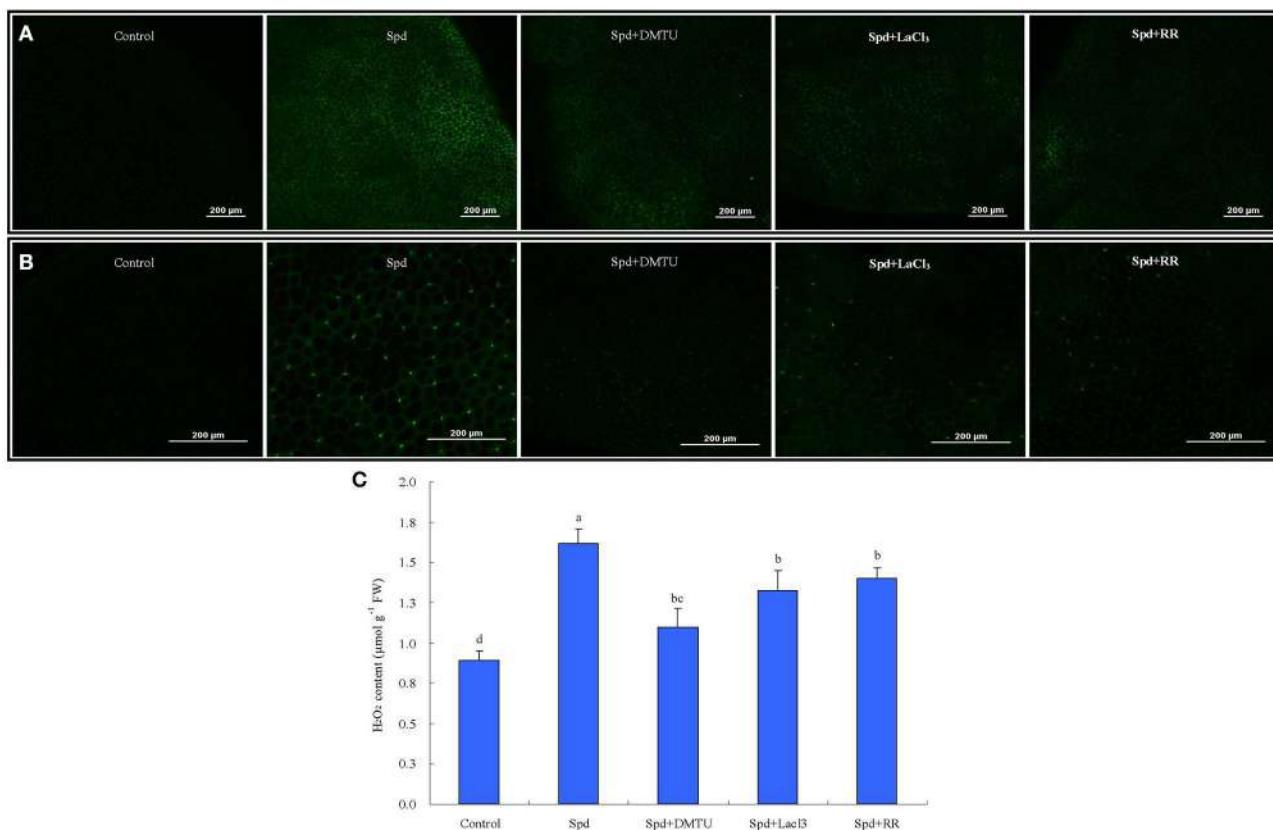
Despite the knowledge of PA's involvement in stress tolerance, PA-regulated signal transduction pathways and molecular mechanisms associated with stress tolerance still remain unclear.



**FIGURE 13 |** Spd-induced dehydrins *Y2SK*, *Y2K* and *SK2* genes relative expression were dependent on hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and CDPK. The detached leaves of white clover were pre-treated with distilled water for 1 h to eliminate wound stress and then treated for different times (A–C) or 2 h (D–F) as follows: 1, distilled water (control); 2, 20  $\mu\text{M}$  Spd; 3, 20  $\mu\text{M}$  Spd + 5 mM dimethylthiourea (DMTU); 4, 20  $\mu\text{M}$  Spd + 100  $\mu\text{M}$  Diphenyleneiodonium (DPI); 5, 20  $\mu\text{M}$  Spd + 30  $\mu\text{M}$  trifluoperazine (TFP). Means of four independent samples and standard errors are presented. The different letter above the columns between two treatments at a given time indicates significant difference at  $P < 0.05$  (A–C). The same letter above the columns indicates no significant difference at  $P < 0.05$  (D–F).

It has been well-documented that the production of  $\text{H}_2\text{O}_2$  or  $\text{Ca}^{2+}$  signaling molecules induce plant defense mechanisms under abiotic stress (Zhou and Guo, 2009; Gonzalez et al., 2012). A growing number of findings indicate that the interactions among PA,  $\text{H}_2\text{O}_2$ , and  $\text{Ca}^{2+}$  are related to plant growth and development or biotic stress such as herbivore-induced volatiles in bean (*Phaseolus lunatus*) (Ozawa et al., 2009), pollen tube growth in *Pyrus pyrifolia nakai* (Wu et al., 2010), ethylene-induced stomatal closure in *Arabidopsis thaliana* (Hou et al., 2013), and cell death in tobacco (Iannone et al., 2013). In this study, the data showed exogenous Spd could induce an instantaneous increase in  $\text{H}_2\text{O}_2$  or cytosolic free  $\text{Ca}^{2+}$  in cells, and activate NADPH oxidase and CDPK gene expression. To a great extent, DCHA could reduce the water stress-induced  $\text{H}_2\text{O}_2$  or cytosolic free  $\text{Ca}^{2+}$  accumulation, while the depressive effects of DCHA were alleviated by the application of exogenous Spd

under water stress. An earlier study in *Vicia faba* showed that Put exhibited similar effects with ABA on regulation of  $\text{H}_2\text{O}_2$  production as well as cytosolic free  $\text{Ca}^{2+}$  level, which resulted in stomatal closure (An et al., 2008). These findings suggest that, at least in part, PA is involved in water stress-induced production of  $\text{H}_2\text{O}_2$  and  $\text{Ca}^{2+}$  signaling. Furthermore, it's shown that  $\text{Ca}^{2+}$  activated  $\text{H}_2\text{O}_2$  production by increasing NADH, and accordingly,  $\text{H}_2\text{O}_2$  induced  $\text{Ca}^{2+}$  release via oxidation of cysteine in human cells (Camello-Almaraz et al., 2006; Hidalgo and Donoso, 2008). According to obtained results in this study, Spd-induced  $\text{H}_2\text{O}_2$  production required  $\text{Ca}^{2+}$  release, but at the same time, Spd-induced  $\text{Ca}^{2+}$  release was also essential for  $\text{H}_2\text{O}_2$  production, which meant that there was a potential mutual influence between PA-induced  $\text{H}_2\text{O}_2$  and  $\text{Ca}^{2+}$  signaling. Gonzalez et al. (2012) also found that cross talk between calcium message systems and  $\text{H}_2\text{O}_2$  was involved in copper-induced



**FIGURE 14 | Cytosolic free  $\text{Ca}^{2+}$  (A) and  $\text{H}_2\text{O}_2$  accumulation (B–C) in response to spermidine (Spd),  $\text{H}_2\text{O}_2$ scavenger or calcium ( $\text{Ca}^{2+}$ ) channels blockers.**

The detached leaves of white clover were pre-treated with distilled water for 1 h to eliminate wound stress and then treated for 30 min as follows: 1, distilled water (control); 2, 20  $\mu\text{M}$  Spd; 3, 20  $\mu\text{M}$  Spd + 5 mM dimethylthiourea (DMTU); 4, 20  $\mu\text{M}$  Spd + 1 mM LaCl<sub>3</sub>; 5, 20  $\mu\text{M}$  Spd + 50  $\mu\text{M}$  ruthenium red (RR) and followed by incubation with  $\text{Ca}^{2+}$ -sensitive fluorescent probe Fluo-3-AM or  $\text{H}_2\text{O}_2$ -sensitive fluorescent probe H<sub>2</sub>DCFDA for 30 min. Images are visualized using confocal laser scanning microscopy (CLSM). Means of four independent samples are presented. Bars represent standard errors. The same letter indicates no significant difference (LSD) at  $P < 0.05$ .

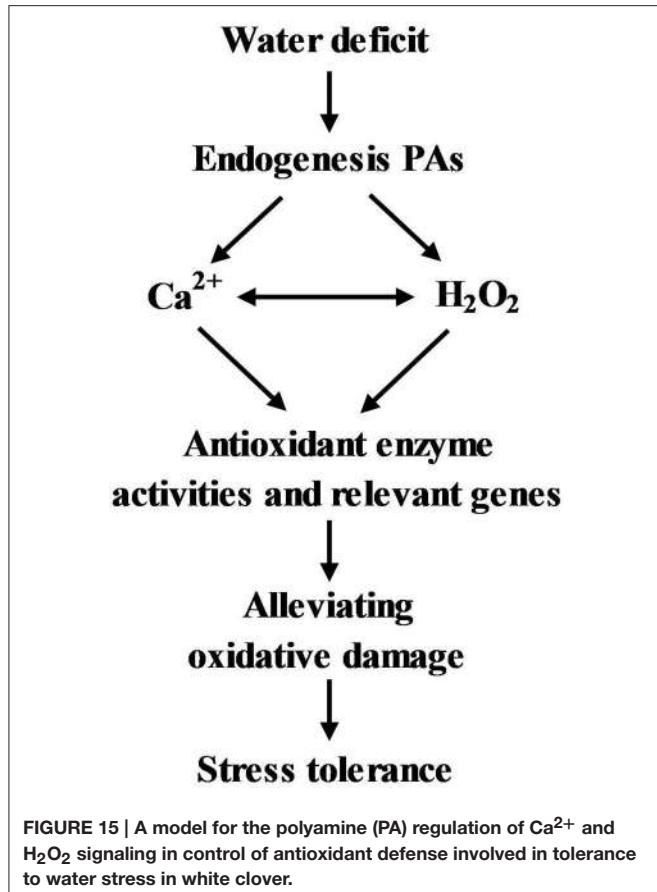
antioxidant enzyme gene transcripts such as APX and GST. Results from the present study suggest that PA could be involved in water stress-induced  $\text{H}_2\text{O}_2$  and cytosolic free  $\text{Ca}^{2+}$  production, contributing to improved stress tolerance in white clover. Previous studies have proved that ABA-induced  $\text{H}_2\text{O}_2$ , as signaling molecule, accumulated in stoma associated with stoma closure (Zhang et al., 2001; Bright et al., 2006). Interestingly, similar result was observed in this study.  $\text{H}_2\text{O}_2$  accumulation increased rapidly in stomas of treated leaves, which may imply that Spd-induced  $\text{H}_2\text{O}_2$  was associated with stoma closure under water stress, but accurate mechanisms still need to be further studied.

### PA, $\text{H}_2\text{O}_2$ , and $\text{Ca}^{2+}$ Messenger Systems Are Involved in Water Stress-induced Antioxidant Defense and Dehydrin Genes Expression

Antioxidant defense systems play a fundamental role in plant tolerance of oxidative damage. Under drought stress, a drought-resistant white clover cultivar exhibited significantly elevated antioxidant defenses relative to a sensitive one (Li et al., 2013a). Drought preconditioning improved drought tolerance

of white clover associated with enhancing SOD activity and gene transcript level (Li et al., 2013b). Our previous studies also showed that exogenous PA improved antioxidant enzyme activities and relevant genes expression such as SOD, GPOX, CAT, and APX in white clover during drought conditions (Li et al., 2014a,b). From the results of the present study, it was obvious that the treatment with PA biosynthesis inhibitor “DCHA” effectively inhibited water stress-induced antioxidant enzyme activities and gene expression, while the effects were reversed with the exogenously applied Spd to a great extent. This indicates that water stress-induced antioxidant defense involves PA biosynthesis.

However, whether PA is involved in water stress-induced antioxidant defenses through regulating  $\text{H}_2\text{O}_2$  and  $\text{Ca}^{2+}$  signaling in white clover, thereby gaining drought tolerance is not clear. The up-regulation of PA oxidation induced the release of  $\text{H}_2\text{O}_2$  in tobacco, which was related to changes in antioxidant enzyme activities (Iannone et al., 2013; Guo et al., 2014). However, at least two more pathways could explain the accumulation of  $\text{H}_2\text{O}_2$  in plant cells besides PA oxidation: one is NADPH oxidase, the other is cell wall-localized peroxidase.



**FIGURE 15 |** A model for the polyamine (PA) regulation of  $\text{Ca}^{2+}$  and  $\text{H}_2\text{O}_2$  signaling in control of antioxidant defense involved in tolerance to water stress in white clover.

Specific  $\text{H}_2\text{O}_2$ -generating pathways will be activated when plants suffer from different stimuli (Neill et al., 2002; Guo et al., 2014). Apart from the inhibitor of amine oxidase “QC,” both inhibitors of NADPH oxidase “DPI” and cell wall-localized peroxidase “SHAM” inhibited Spd-induced antioxidant enzyme activities as well as gene transcript levels including *SOD*, *GPOX*, *CAT*, and *APX* in leaves of white clover, demonstrating that both cell wall-localized peroxidase and NADPH oxidase were related to Spd-induced antioxidant defense. This further supports previous studies which PA-induced antioxidant defense is related to  $\text{H}_2\text{O}_2$  in plants under water stress.  $\text{H}_2\text{O}_2$  generation induced by PA during early phases of stress response has a regulatory role in activate antioxidant enzymes and genes expression resulting in improvement of stress tolerance via maintaining the balance between the generation and quenching of ROS in plant cells. It has been shown that the interaction between  $\text{Ca}^{2+}$  and PA was relative to calcium-sensing receptors in cardiac tissues (Wang et al., 2003). Despite this, little is known about effects of PA on the induction of calcium sensors to regulate antioxidant defenses in plants. In this study, not only two calcium channel

blockers but also a CaM antagonist and an inhibitor of CDPK effectively inhibited the Spd-induced increases in antioxidant enzyme activities as well. These changes in enzyme activities were in accordance with gene transcript levels encoding antioxidant enzymes. Thereby, obtained findings imply that both  $\text{Ca}^{2+}$  and calcium sensors could be involved in PA-induced antioxidant defense.

The abundance and gene transcript level of dehydrins are altered by water stress associated with drought tolerance in plants (Bian et al., 2002; Hu et al., 2010). Various phytohormones or physiological activators could induce the expression of dehydrins such as ABA, cytokinin, and proline, which means there are a potential interaction between dehydrins and multiple stresses signaling in plants (Wang et al., 2002; Khedr et al., 2003; Cerny et al., 2011). A drought resistant white clover cultivar maintained higher dehydrin gene expression and content than the sensitive one under drought stress (Vaseva et al., 2011). Our recent study also showed that exogenous Spm-induced dehydrins synthesis in two different white clover cultivars was responsible for improved drought tolerance (2015b). It's obvious at present that dehydrin genes *Y<sub>2</sub>SK*, *Y<sub>2</sub>K*, and *SK<sub>2</sub>* were shown to be highly responsive to exogenous Spd. But Spd-induced enhancement of these gene transcript levels was inhibited by application of  $\text{H}_2\text{O}_2$  scavenger or the inhibitors of NADPH oxidase and CDPK. It is suggested that PA-regulated  $\text{H}_2\text{O}_2$  and CDPK signaling are involved in dehydrin genes expression in white clover. The result from this study highlights the function of PA on improvement of tolerance to water stress.

In summary, our results reveal that PA involvement in the regulation of  $\text{H}_2\text{O}_2$  and  $\text{Ca}^{2+}$  messenger systems results in tolerance to water stress associated with antioxidant defense and dehydrins in leaves of white clover. In addition, it's worth pointing out that further investigations are needed for PA-induced stress tolerance associated with  $\text{H}_2\text{O}_2$  and  $\text{Ca}^{2+}$  signaling in different metabolic pathways, plant species and stress conditions.

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## Supplementary Material

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fphys.2015.00280>

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# Ascorbic acid mitigation of water stress-inhibition of root growth in association with oxidative defense in tall fescue (*Festuca arundinacea* Schreb.)

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Root growth inhibition by water stress may be related to oxidative damages. The objectives of this study were to determine whether exogenous application of ascorbic acid (ASA) could mitigate root growth decline due to water stress and whether ASA effects on root growth could be regulated through activating non-enzymatic or enzymatic antioxidant systems in perennial grass species. Tall fescue (*Festuca arundinacea* Schreb. cv. "K-31") plants were grown in nutrient solution, and polyethylene glycol (PEG)-8000 was added into the solution to induce water stress. For exogenous ASA treatment, ASA (5 mM) was added into the solution with or without PEG-8000. Plants treated with ASA under water stress showed significantly increased root growth rate, and those roots had significantly lower content of reactive oxygen species (ROS) ( $H_2O_2$  and  $O_2^-$  content) than those without ASA treatment. Malondialdehyde content in root tips treated with ASA under water stress was also significantly reduced compared with those under water stress alone. In addition, free ascorbate and total ascorbate content were significantly higher in roots treated with ASA under water stress than those without ASA treatment. The enzymatic activities for ROS scavenging-related genes were not significantly altered by ASA treatment under water stress, while transcript abundances of genes encoding superoxide dismutase, catalase, ascorbate peroxidase, glutathione reductase, dehydroascorbate reductase, and monohydroascorbate reductase showed significant decreases in the root elongation zone and significant increases in the root maturation zone treated with ASA under water stress. Transcripts of genes for expansins and xyloglucan endotransglycosylases showed increased abundances in ASA-treated root maturation zone under water stress, indicating that ASA could accelerate cell wall loosening and cell expansion. The results suggested that exogenous treatment of roots with ASA enhanced root elongation under water stress, which could be attributed by increasing non-enzymatic antioxidant production, suppressing ROS toxicity and up-regulating gene expression of cell-wall loosening proteins controlling cell expansion.

**Keywords:** turfgrass, water stress, root growth, reactive oxygen species, cell wall, gene expression

## Introduction

Cell growth is one of the most sensitive processes to water stress, with a restriction of cell expansion occurring at a water potential of  $-0.5\text{ MPa}$  (Nilsen and Orcutt, 1996). Therefore, water stress is one of major limiting factors for root growth (Morgan, 1984; Sharp et al., 2004; Bengough et al., 2011). Maintaining active root growth is therefore critically important for plants efficiently utilizing water under water stress conditions.

Many metabolic factors could contribute to the inhibitory effects of water stress on root growth (Chaves et al., 2003). It is well known that water stress induces the production of reactive oxygen species (ROS), such as superoxide ( $\text{O}_2^-$ ) and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), causing oxidative damages, which could be related to root growth restriction due to water stress (Wu et al., 2006; Bian and Jiang, 2009; Selote and Khanna-Chopra, 2010). Plants develop non-enzymatic antioxidants and the enzymatic scavenging systems to detoxifying ROS (Mittler, 2002; Mittler et al., 2004). In the enzymatic pathways, the damaging  $\text{O}_2^-$  produced from electron transfer chain would be first catalyzed by superoxide dismutase (SOD) into  $\text{H}_2\text{O}_2$ . The  $\text{H}_2\text{O}_2$  is further reduced into water by catalase (CAT), peroxidase (POD), or by ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate reductase (MR), and dehydroascorbate reductase (DR) into  $\text{H}_2\text{O}$ . Non-enzymatic antioxidant compounds, including ascorbate (ASA), possess intrinsic antioxidant properties, serving as electron donors to directly reduce ROS accumulation (Noctor and Foyer, 1998) and also as reaction substrates within the enzymatic cycle (Mittler, 2002). In addition, ASA also play roles in stress signaling and other physiological processes (Smirnoff, 1996; Wolucka et al., 2005). The accumulation of free ASA in plant cells is of great importance for ROS scavenging since it is involved in both non-enzymatic and enzymatic systems, as well as the other important functions. The physiological function of ascorbic acid under water deficit stress has been studied in leaf tissues (Al-Hakimi and Hamada, 2001; Shalata and Neumann, 2001; Athar et al., 2008; Beltagi, 2008; Dolatabadian and Saleh Jouneghani, 2009; Dolatabadian et al., 2009), and in roots (Al-Hakimi and Hamada, 2001; Shalata and Neumann, 2001; Afzal et al., 2006). Moreover, the *Arabidopsis* ascorbate-deficient mutants were also obtained to study the physiological role of ASA *in vivo* (Conklin et al., 1996, 1999; Huang et al., 2005). The benefits of exogenous ASA application in order to attenuate water deficit stress include increased nutrient uptake, improved leaf and root growth, reduced lipid peroxidation, and relieved oxidative stress (Khan et al., 2011). A lot of efforts have been taken into the study of applying ASA to foliar part of plant in order to promote shoot growth under environmental stresses (Khan et al., 2006; Farahat et al., 2007; Dolatabadian et al., 2008, 2009). However, there is

little information available to link ASA and ROS scavenging with its impact on root growth under water stress conditions.

Cell expansion is controlled by cell-wall loosening proteins that modify the linkage of cellulose microfibrils and thus cellular matrix, such as expansin (EXP) and xyloglucan endotransglycosylase (XET) (Cosgrove, 2000). The expansins is a gene family that contains multiple gene members, mainly grouped as  $\alpha$ -,  $\alpha$ -like,  $\beta$ - and  $\beta$ -like expansins, which share conserved protein domains (Li et al., 2003; Choi et al., 2008). Expansins are believed to bind to the surface of cell wall microfibrils and disrupt hydrogen bonds between cellulose microfibrils and crosslinking glycans, although lacking hydrolytic activity (Cosgrove, 2000, 2005; Li et al., 2003). XETs also belong to a class of cell wall enzymes that are responsible for the cleavage of xyloglucan chains and the reconnection of their reducing ends to non-reducing ends, thus enable microfibril relaxation and cell expansion (Fry et al., 1992; Rose and Bennett, 1999). Evidences have been found in both expansins (Yang et al., 2004; Buchanan et al., 2005; Sabirzhanova et al., 2005; Li et al., 2011) and XETs (Xu et al., 1996; Bray, 2004; Cho et al., 2006) that they are also induced by drought stress. However, whether ASA-mediated root growth responses to water stress is associated with expansin and XET genes remains unknown.

The objectives of this study were to determine whether treatment of roots with ASA could mitigate root growth decline due to water stress and whether ASA effects on root growth could be regulated through activating non-enzymatic or enzymatic antioxidant systems and changes in gene expression controlling cell expansion in a perennial grass species, tall fescue (*Festuca arundinacea* Schreb.). Tall fescue is a widely-used forage and turf grass and known for superior drought avoidance due to deep rooting characteristics (Qian et al., 1997; Huang and Fry, 1998; Huang and Gao, 2000). Understanding how antioxidants and oxidative defense may be involved in root responses to water stress would provide further insights into strategies for genetic modification or chemical-priming to promote root growth of plants that exposed to prolonged periods of water stress in water-limiting environments.

## Materials and Methods

### Plant Materials and Growth Conditions

The experiment was conducted in a hydroponic system for convenience of non-destructive monitoring of root growth and minimizing damages of roots during root treatment and sampling. Seedlings of tall fescue "K-31" were initially established from seeds planted in fritted clay medium (Profile Products, Deerfield, IL) for about 3 weeks. Seedlings of similar sizes and developmental stages were then carefully rinsed with water and roots were washed free of soil medium, and transferred to the hydroponic growing medium. About three to five seedlings were wrapped together at the base part of tillers using a foam cube, inserted in a polystyrene panel which was pre-drilled with  $9 \times 7$  holes, and floated in a plastic container ( $50.8\text{ cm} \times 36.8\text{ cm} \times 15.2\text{ cm}$ ) filled with half-strength Hoagland's nutrition solution (Hoagland and Arnon, 1950). To ensure the appropriate aeration, the nutrition solution was continuously aerated using

**Abbreviations:** APX, ascorbate peroxidase; ASA, ascorbic acid; CAT, catalase; DR, dehydroascorbate reductase; EXP, expansin; GR, glutathione reductase; MDA, malondialdehyde; MR, monodehydroascorbate reductase; PBS, phosphate-buffered saline; PEG, polyethylene glycol; POD, peroxidase; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; ROS, reactive oxygen species; TCA, trichloroacetic acid; XET, xyloglucan endotransglycosylase.

an air pump. The experiment was carried out in a controlled environment room (Environmental Growth Chamber, Chagrin Falls, Ohio), which was set to maintain 22/18°C (day/night), 60% relative humidity, 12-h photoperiod, and 650  $\mu\text{mol m}^{-2} \text{s}^{-1}$  photosynthetically active radiation (PAR) at the canopy level.

## Treatments and Experimental Design

Seedlings of 3-week-old were cultured in the half-strength Hoagland's nutrition solution for additional 7 days before exposure to water stress. Water stress was induced by adding polyethylene glycol (PEG)-8000 with incremental concentration

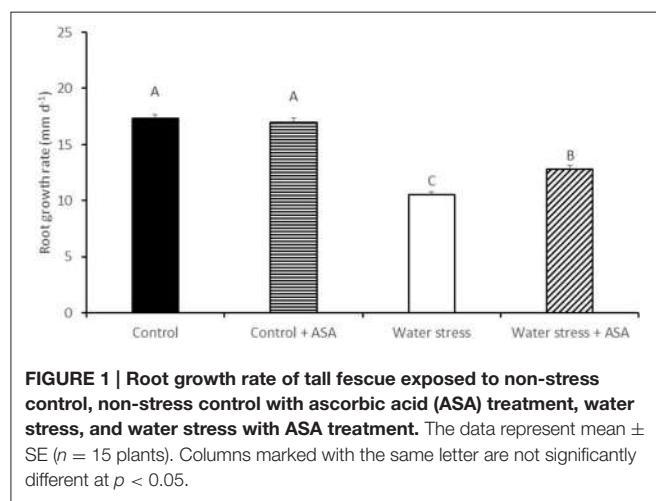
**TABLE 1 |** Primer sequences used in qRT-PCR.

Gene	Accession number	Best BLAST hit	E-value	Identity (%)		Primer sequence (5'-3')	Tm (°C)	Size (bp)
CuZn-SOD	DT712833.1	XM_003562436.2 ( <i>Brachypodium distachyon</i> )	0	93	Forward	TATCCCCCTACTGGACCACAT	61.7	85
					Reverse	GTTGCCACCCCTTGCCAAGAT	61.6	
Mn-SOD	DT694762.1	XM_010233228.1 ( <i>Brachypodium distachyon</i> )	1e-162	91	Forward	GGCGCCATCAAGTCAA	59.6	85
					Reverse	ACCCCCACCCCTCATTAGCA	62.1	
POD	GT036635.1	XM_003566650.2 ( <i>Brachypodium distachyon</i> )	0	90	Forward	CACATGCCACAAGCTGATG	60.8	85
					Reverse	CAGAAGCGAAGCGGCAAT	59.8	
CAT-A	DT680104.1	XM_003573193.2 ( <i>Brachypodium distachyon</i> )	0	90	Forward	CTGCTGGCAACAACTTC	57.8	89
					Reverse	GACTTGGGTTGGGCTTG	57.6	
CAT-B	DT704412.1	NM_001065170.1 ( <i>Oryza sativa</i> )	2e-171	87	Forward	TCCTACGCTGATAACCAAAG	58.1	93
					Reverse	GTGATGGTTGTTGTGGTGA	58	
CAT-C	AJ634002.1	XM_003558844.2 ( <i>Brachypodium distachyon</i> )	0	91	Forward	GACCCACATCCAGGAGAAC	58.5	85
					Reverse	GTCGAAGAGGAAGGTGAAC	58.4	
APX2	DT702685.1	XM_010235217.1 ( <i>Brachypodium distachyon</i> )	0	93	Forward	TTTGAGCGACCAGGACATTG	59.6	85
					Reverse	GGCTCCCTCAAAGCCAGATC	61.6	
APX4	DT714958.1	XM_003574845.2 ( <i>Brachypodium distachyon</i> )	0	91	Forward	TGGTTTGAAAGGTGCATGGA	59.5	85
					Reverse	CCCCTCAGATTCTCCCTCAG	60.5	
DR	DT684182.1	XM_003568966.2 ( <i>Brachypodium distachyon</i> )	0	93	Forward	GTCACCCCTCCTGAGTATGCA	58.2	80
					Reverse	GTGGCATCCTGCTCTTCAAG	57.8	
GR	GT036447.1	XM_003558703.2 ( <i>Brachypodium distachyon</i> )	0	93	Forward	GCTGCACTAGACCTGCCTCA	63.3	80
					Reverse	ATGCCAGCAAACCTCAAAGC	60.8	
EXP-A3	DT686661.1	AY692477.1 ( <i>Triticum aestivum</i> )	4e-112	91	Forward	TGCCGTGCCGGAAAGTC	61	72
					Reverse	TGATCAGCACCGAGTTGAAGTAG	61.4	
XET1	DT683504.1	AJ295943.1 ( <i>Festuca pratensis</i> )	1e-173	92	Forward	GCACCGTCACAGCCTACTACCT	64.8	80
					Reverse	GGTCTCGTGCCTCAGGAA	60.7	
XET2	DT707331.1	AJ295944.1 ( <i>Festuca pratensis</i> )	0	99	Forward	GCCCTACGTGATGAACACCAA	61.6	80
					Reverse	AGGGATCGAACCGAGGAGGTAGAAC	62.3	
XET3	AJ295945.1	Self			Forward	CGTTGATTCCGGTGCTAGCT	61.4	80
					Reverse	GTCGCAATCGTCGTTGAAGTT	60.4	
Actin	AY194227.1	Self			Forward	TCTTACCGAGAGAGGTTACTCC	59.3	107
					Reverse	CCAGCTCCTGTTCATAGTCAG	59.5	

Proposed gene names, GenBank accession numbers, best BLAST hit names, E-values, sequence identity scores, melting temperatures, and amplicon sizes are also listed.

into the nutrition solution which is a widely used osmotic regulator in hydroponic system imposing water deficit in plants (Lagerwerff et al., 1961; Janes, 1974). The osmotic potential of the solution was first adjusted to  $-0.25$  MPa with PEG for 3 days, and then more PEG was added to bring the osmotic potential to  $-0.5$  MPa to induce water stress. The non-stress control plants were grown in the half-strength Hoagland's nutrition solution without adding PEG.

For ascorbate (ASA) treatment, root systems of seedlings with or without PEG treatments were first incubated in half-strength Hoagland's nutrition solution without ASA (ASA-untreated control) or containing 5 mM ASA for 10 h (ASA treatment), and then transferred back into the original growth conditions as described above. The optimal concentration of ASA used here was chosen after a preliminary experiment with 0.25, 0.5, 1, 5, and 10 mM ASA, which has showed that 5 mM was the most effective in improving root growth.



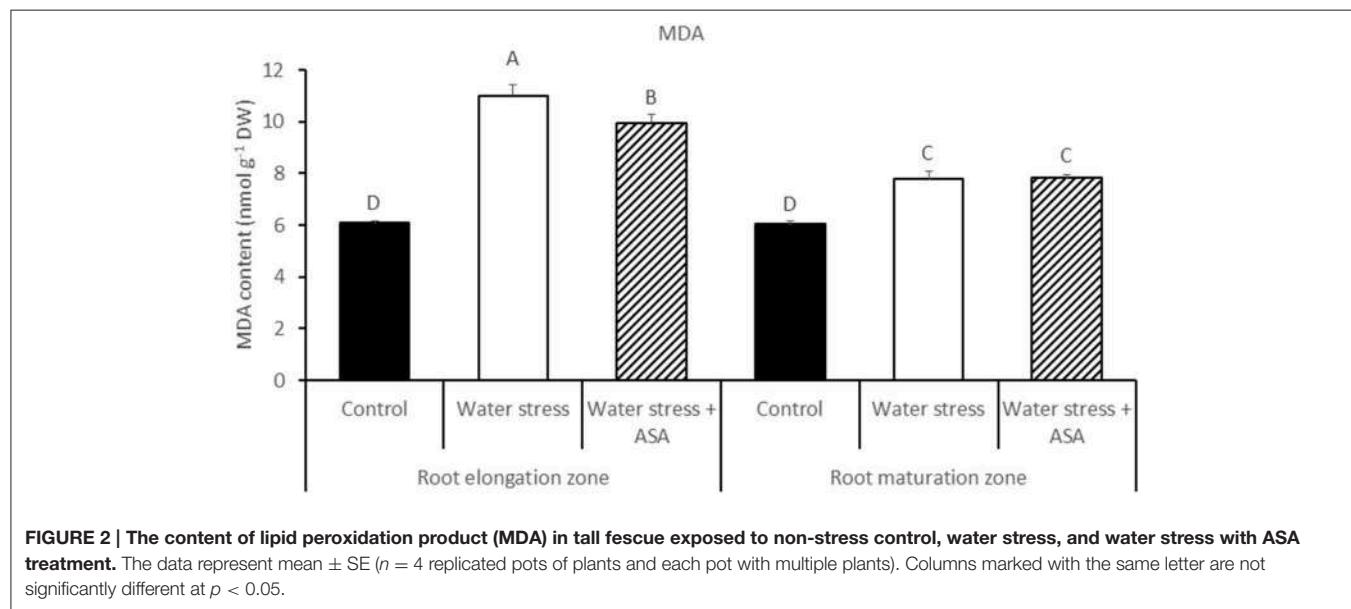
Water stress and ASA treatment were arranged in a split-plot design with four containers of plants (or four replicates) exposed to either water stress or non-stress condition as main plots, and approximately 120 plants with either ASA-treated or -untreated plants randomly placed inside each water-stress or non-stress container as subplots.

### Analysis of Root Elongation Rate

Newly-formed roots approximately 1-cm long were selected from 15 plants in each replicate or container for water stress or ASA treatment for the analysis of root elongation rate. Roots attached to the plant were carefully taken out of the culture solution, the length of roots was measured quickly using a plastic ruler, and then the plants were returned to the culture solution. Root length was measured daily for a period of 7 days. Root elongation rate was calculated as the daily average increase of root length per root.

### Analysis of Malondialdehyde (MDA) Content in Roots

Malondialdehyde is the final product of lipid peroxidation in plant tissues and was quantified according to the procedure described by Zhang and Kirkham (1996) with slight modifications. The MDA content was measured for tissues from the root elongation zone (apical 1 cm) and for the maturation zone (basipetal 1–5 cm). Root tissues (0.5 g) of the tip or the base were washed free of culture solution and ground to powder in liquid nitrogen, and then homogenized in 6 mL 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 10,000 g for 10 min, and 1 mL supernatant was added to 4 mL 10% TCA containing 0.5% thiobarbituric acid. The mixture was incubated at 95°C for 30 min, quickly cooled on ice, and centrifuged at 10,000 g for 10 min at 4°C. The absorbance of supernatant was measured at 532 and 600 nm using a spectrophotometer (Spectronic Instruments, Rochester, NY).

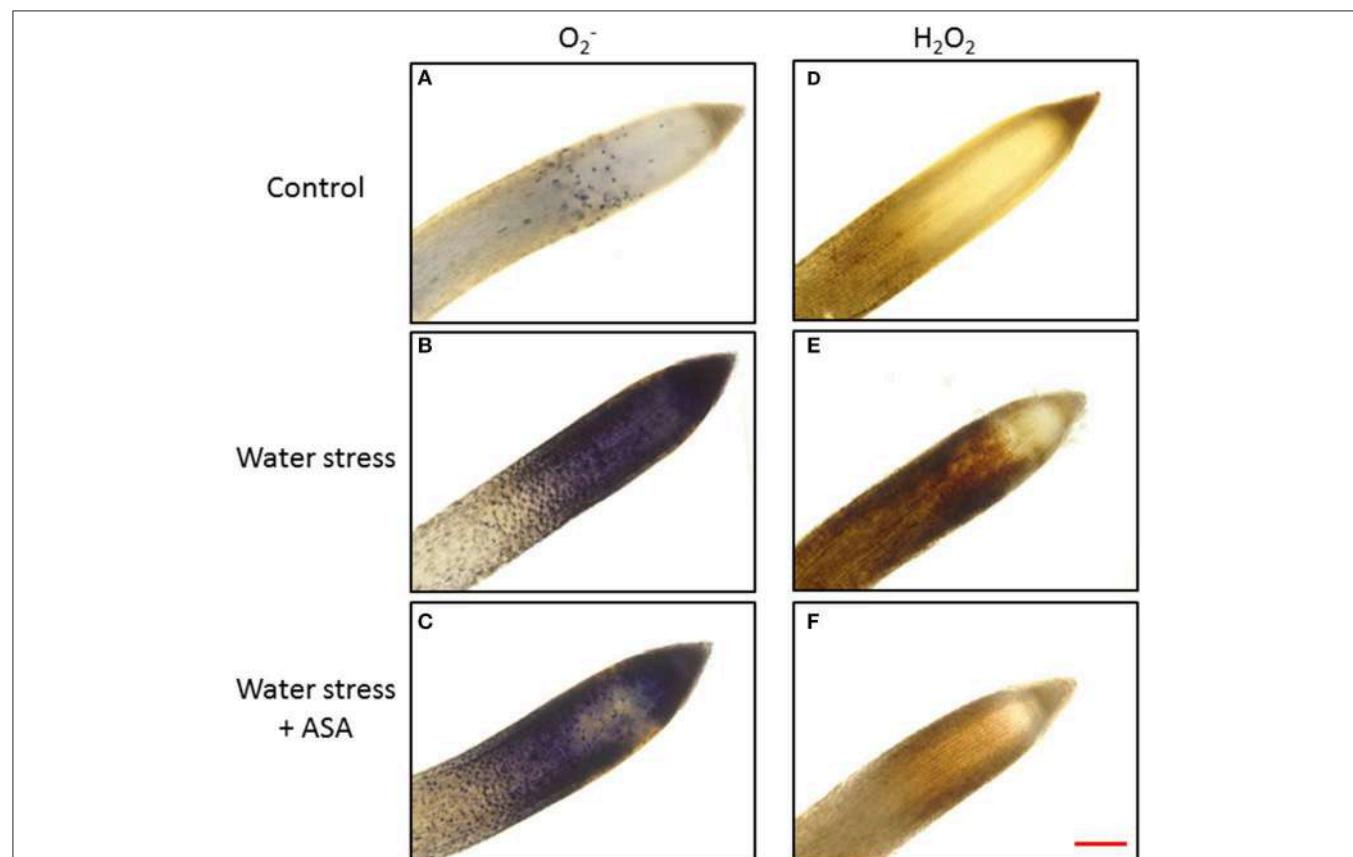
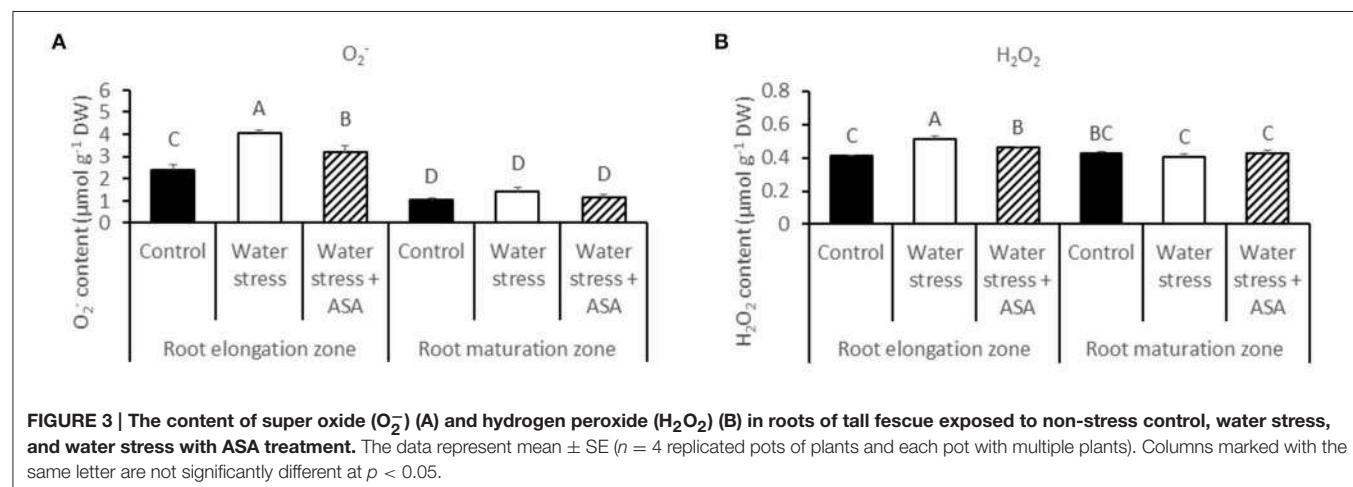


The concentration of MDA was calculated using an extinction coefficient of  $155 \text{ mM}^{-1} \text{ cm}^{-1}$  (Heath and Packer, 1968).

### Histochemical Staining for Hydrogen Peroxide and Superoxide in Roots

Histochemical staining for the presence of hydrogen peroxide and superoxide was performed following treatments according

to the procedures described in Thordal-Christensen et al. (1997) and Dunand et al. (2007), respectively, with slight modifications for each of them. To evaluate the presence of  $\text{H}_2\text{O}_2$ , roots were stained with 1% (w/v) 3-diaminobenzidine (DAB; pH 3.8) for 1 h and subsequently rinsed with deionized water. To evaluate the presence of  $\text{O}_2^-$ , roots were stained with 2 mM nitroblue tetrozolium (NBT) in 20 mM phosphate-buffered



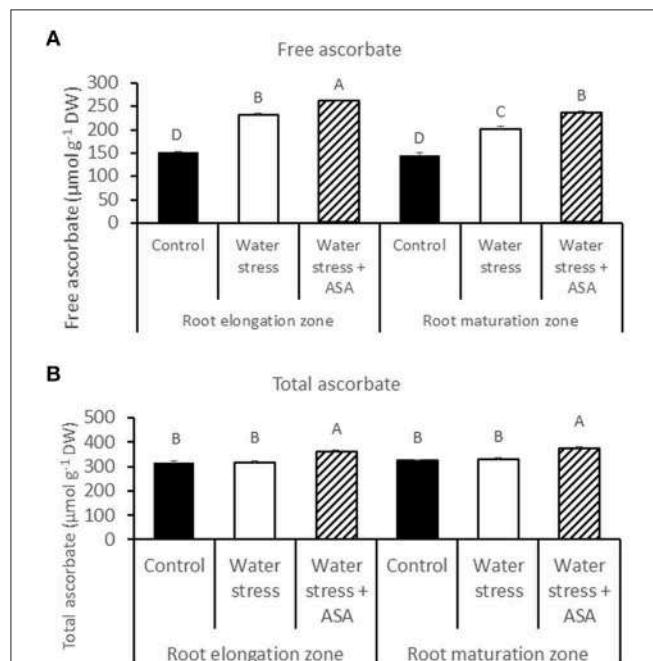
**FIGURE 4 |** Histochemical staining of tall fescue roots for visual localization of super oxide ( $\text{O}_2^-$ ) (A for non-stress control, B for water stress, and C for water stress with ASA treatment) and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) (D for non-stress control, E for water stress, and F for water stress with ASA treatment). Bar represents for 100  $\mu\text{m}$ .

saline (PBS; pH 6.8) for 15 min and subsequently rinsed with deionized water. DAB- or NBT-stained roots were visually observed using an Olympus FSX100 Bio-imaging navigator (Olympus America, Central Valley, PA) and pictures were captured using bright-field single-shot mode at 4.2x magnification.

### Quantification of Reactive Oxygen Species in Roots

The production rate of  $O_2^-$  and content of  $H_2O_2$  were measured for tissues from the root elongation zone (apical 1 cm) and for the maturation zone (basipetal 1–5 cm). The production rate of  $O_2^-$  was measured according to the procedure described by Bian and Jiang (2009) with slight modifications. Root tissues (0.2 g) were ground to powder in liquid nitrogen, homogenized in 1 mL 50 mM Tris-HCl (pH 7.5), and centrifuged at 5000 g for 10 min at 4°C. 200  $\mu$ L supernatant was added to 800  $\mu$ L 0.5 mM 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt (XTT). XTT reduction was recorded once per minute for 3 min at 470 nm and the background absorbance was corrected with 50 units of superoxide dismutase (SOD). The  $O_2^-$  production rate was calculated using a  $2.16 \times 10^4 M^{-1} cm^{-1}$  extinction coefficient and expressed as  $\mu\text{mol } O_2^- \text{ min}^{-1} \text{ g}^{-1}$  dry weight (DW) (Sutherland and Learmonth, 1997).

The content of  $H_2O_2$  was measured according to the procedure described by Zhou et al. (2006) with slight

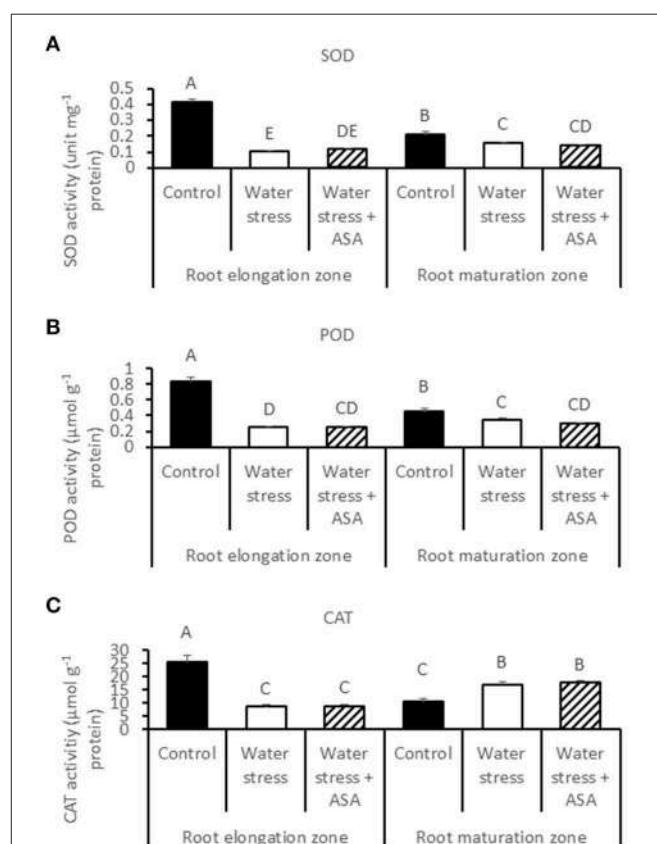


**FIGURE 5 |** Free (A) and total (B) ascorbate content in tall fescue roots exposed to non-stress control, water stress, and water stress with ASA treatment. The data represent mean  $\pm$  SE ( $n = 4$  replicated pots of plants and each pot with multiple plants). Columns marked with the same letter are not significantly different at  $p < 0.05$ .

modifications. Ground root tissues (0.5 g) were homogenized in 5 mL 5% (w/v) TCA and the homogenate was centrifuged at 10,000 g for 20 min at 4°C. The supernatant was adjusted to pH 8.4 with 17 M ammonia solution, briefly centrifuged to remove large particles, and divided into 1 mL aliquots. 8  $\mu\text{g}$  catalase was added to one aliquot to serve as the blank. 1 mL colorimetric reagent solution containing 10 mg 4-aminoantipyrine, 10 mg phenol, and 5 mg peroxidase in 100 mM acetic acid buffer (pH 5.6) was added to each aliquot and the color reaction was incubated for 10 min at 30°C. Following incubation, the absorbance was measured at 505 nm and  $H_2O_2$  content determined based on standard curve generated with known  $H_2O_2$  concentrations.

### Quantification of Non-enzymatic Antioxidant Content in Roots

Endogenous content of free ascorbate and total ascorbate content were quantified for tissues from the root elongation zone (apical 1 cm) and for the maturation zone (basipetal 1–5 cm) according to the procedure described in Ma et al. (2008) with slight modifications. Frozen root powder (0.5 g)



**FIGURE 6 |** Activity of superoxide dismutase (SOD) (A), peroxidase (POD) (B), and catalase (CAT) (C) in tall fescue roots exposed to non-stress control, water stress, and water stress with ASA treatment. The data represent mean  $\pm$  SE ( $n = 4$  replicated pots of plants and each pot with multiple plants). Columns marked with the same letter are not significantly different at  $p < 0.05$ .

was homogenized in 8 ml 5% (w/v) TCA on ice, centrifuged at 10,000 g for 10 min at 4°C, and the supernatant was used immediately for analysis. For total ascorbate quantification, the supernatant was incubated in 200 mM PBS (pH 7.4) and 1.5 mM dithiothreitol (DTT) for 50 min to reduce all dehydroascorbic acid to ASA. Following incubation, 200 µL 0.5% (w/v) N-ethylmaleimide (NEM) was added to remove excess DTT. The resulting solution (0.8 ml) was then added to a reaction mixture containing 1 mL 10% (w/v) TCA, 800 µL 42% (w/v) o-phosphoric acid, 800 µL 65 mM 2,2'-dipyridyl in 70% (v/v) ethanol, and 400 µL 3% (w/v) iron (III) chloride. The reaction was incubated at 42°C for 1 h, and the absorbance was measured at 525 nm. Free ascorbate was measured using the procedure described above, while DTT and NEM were substituted with 400 µL deionized water. Free and total ascorbate content were determined based on a standard curve generated with known ASA concentrations.

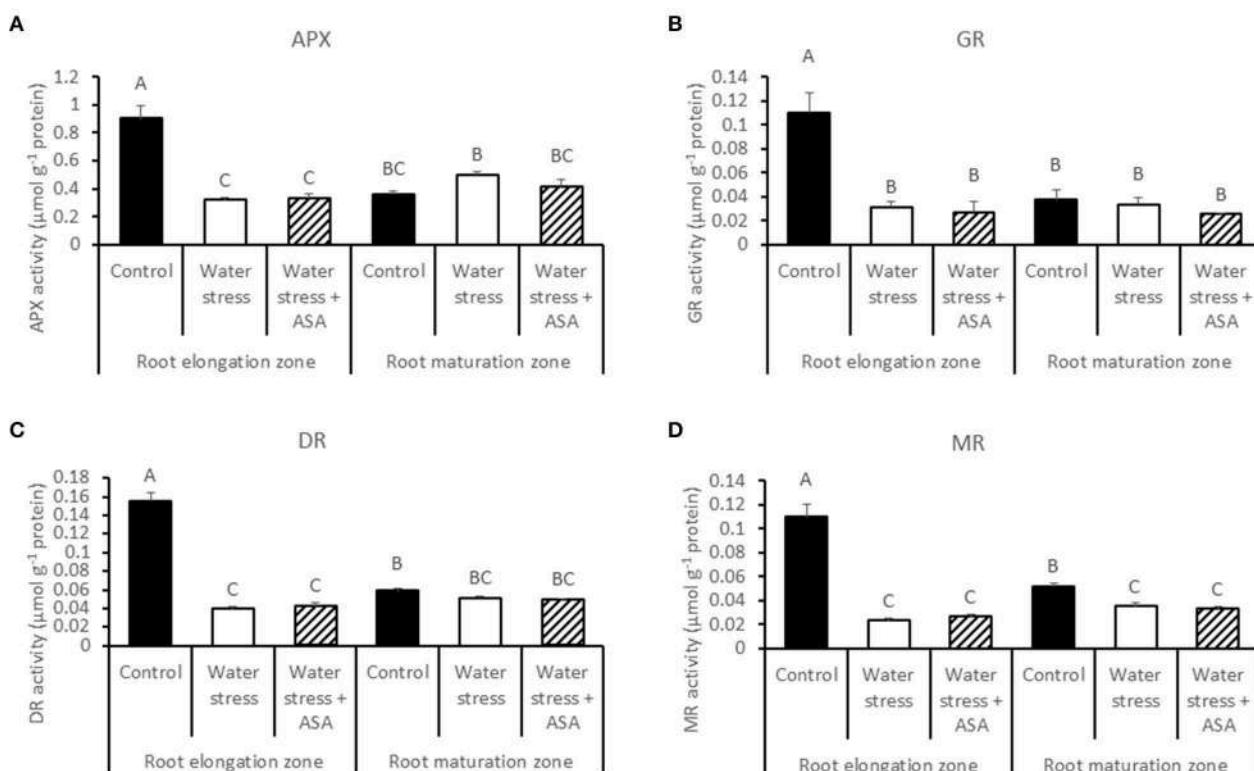
### Quantification of Enzymatic Antioxidant Activity in Roots

Enzyme activities of CAT, POD, SOD, APX, DR, MR, and GR were measured for tissues from the root elongation zone (apical 1 cm) and for the maturation zone (basipetal 1–5 cm) according to the procedures described by Zhang and Kirkham

(1996). For CAT, POD, and SOD assays, 0.5 g of root tissues were homogenized in 6 ml 50 mM PBS (pH 7.0) containing 0.2 mM ethylenediaminetetraacetic acid (EDTA) and 1% (w/v) polyvinylpyrrolidone (PVP) on ice, and the homogenates were centrifuged at 15,000 g for 20 min at 4°C. The absorbances were measured at 240, 470, and 560 nm for CAT, POD, and SOD, respectively. For APX, DR, MR, and GR assays, 0.5 g ground root tissues were homogenized in 6 ml 25 mM PBS (pH 7.8) containing 0.2 mM EDTA and 1% (w/v) PVP, and the homogenates were centrifuged at 15,000 g for 20 min at 4°C. The absorbance was measured at 290, 265, 340, and 340 nm for APX, DR, MR, and GR, respectively.

### Gene Expression Analysis of Enzymatic Antioxidants in Roots

Gene expression analysis in the root elongation zone (apical 1 cm) and for the maturation zone (basipetal 1–5 cm) was performed by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). Total RNA was isolated from root tissue using TRIzol reagent (Life Technologies, Grand Island, NY) and treated with DNase (TURBO DNA-free kit; Life Technologies, Grand Island, NY) to remove contaminating genomic DNA. After that, 2 µg total RNA was reverse-transcribed using a high-capacity cDNA reverse transcription kit (Life Technologies,



**FIGURE 7 |** Activity of ascorbate peroxidase (APX) (A), glutathione reductase (GR) (B), dehydroascorbate reductase (DR) (C), and monodehydroascorbate reductase (MR) (D) in tall fescue roots exposed to non-stress control, water stress, and water stress with ASA treatment. The data represent mean  $\pm$  SE ( $n = 4$  replicated pots of plants and each pot with multiple plants). Columns marked with the same letter are not significantly different at  $p < 0.05$ .

Grand Island, NY) and the synthesized cDNA was amplified in a StepOnePlus Real-Time PCR system (Life Technologies, Grand Island, NY) using the following parameters: pre-heat cycle of 95°C for 3 min, 40 cycles of 95°C denaturation for 30 s, and 60°C annealing/extension for 60 s. Power SYBR Green PCR Master Mix (Life Technologies, Grand Island, NY) was the intercalating dye used to detect gene expression level. Gene names, accession numbers, forward, and reverse primer sequences are provided in **Table 1**. A melting curve analysis was performed for each primer pair to confirm its binding specificity. *Actin* was used as the reference gene, since its expression was constant throughout treatments. A  $\Delta\Delta Ct$  method was used to calculate the relative expression level between genes of interest and reference gene, respectively.

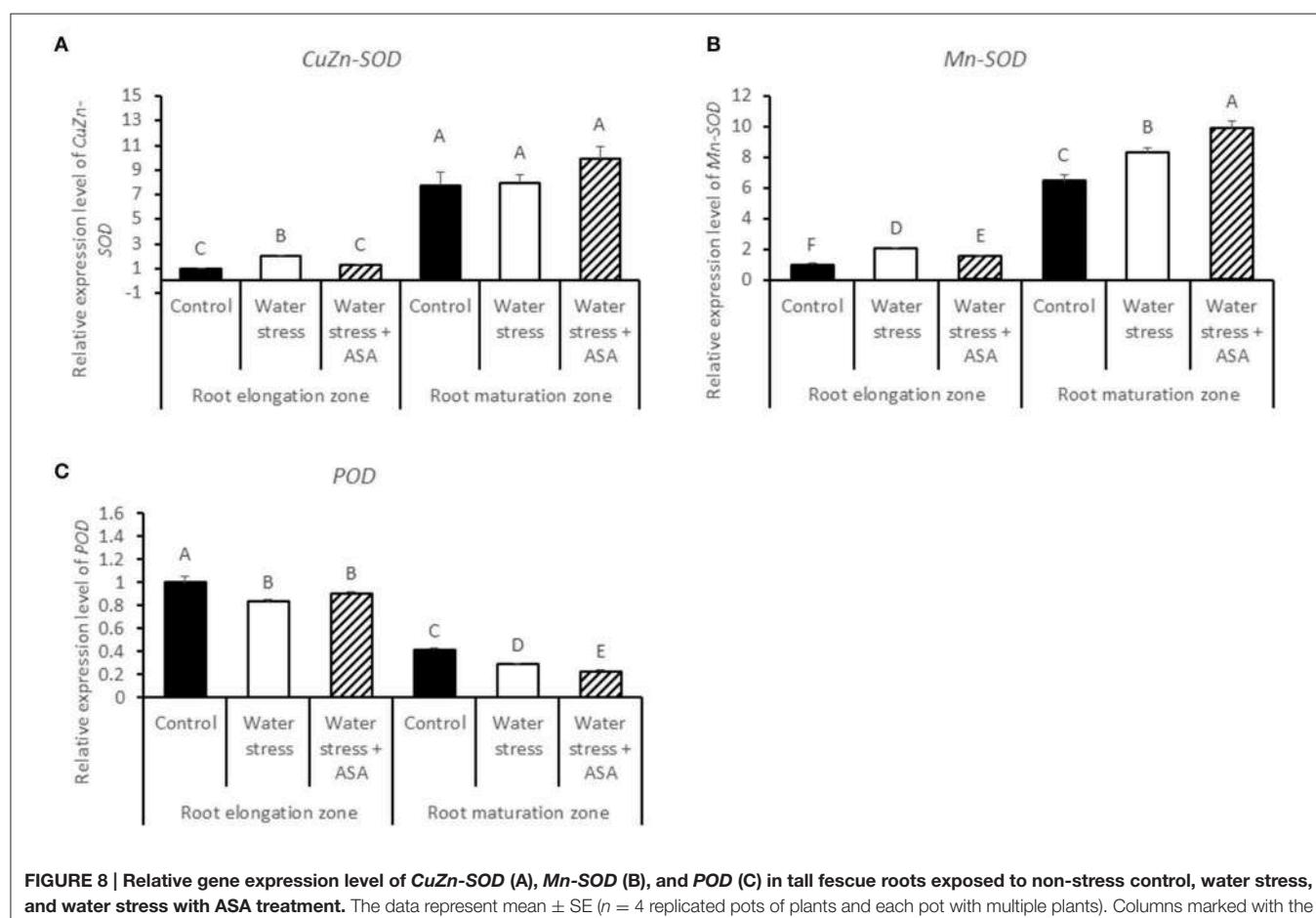
### Statistical Analysis

Effects of water stress and ASA treatments on all parameters were determined using the Two-Way analysis of variance (ANOVA) using a statistical program (JMP11, SAS Institute, Cary, NC). Differences between mean values for each parameter were distinguished by student's *t*-test at the 0.05 probability level.

## Results

### Root Elongation Rate and Membrane Lipid Peroxidation as Affected by Water Stress Alone and with Additional ASA Treatment

Water stress induced by PEG caused significant reduction (by 59%) in root elongation rate, from 17.8 mm d<sup>-1</sup> of the non-stress control plants to 10.6 mm d<sup>-1</sup> of the water-stressed plants (**Figure 1**). The exogenous treatment of roots with ASA in the nutrition solution ameliorated the inhibitory effects of water stress on root elongation, causing a 72% increase in root elongation rate compared to ASA-untreated plants exposed to water stress (**Figure 1**). The exogenous treatment of roots with ASA under control condition did not have significant effect on root elongation rate. Further analysis of MDA content, ROS production, antioxidant enzyme activities, and gene expression were performed only in roots treated with or without ASA under water stress, but not conducted for roots treated with ASA under the non-stress control conditions due to the lack of ASA effects on root elongation under the non-stress conditions. The MDA content of both root elongation and maturation zones in PEG-treated plants was significantly (1.81-fold in elongation zone and 1.29-fold in maturation zone) higher than that in the



non-stress control, indicating the induction of oxidative damages in the entire roots by PEG-induced water stress (Figure 2). Exogenous ASA treatment resulted in significantly lower (1.64-fold) MDA content in the elongation zone of roots exposed to water stress, compared to the water stress treatment alone, but had no significant effects on MDA content of the maturation zone.

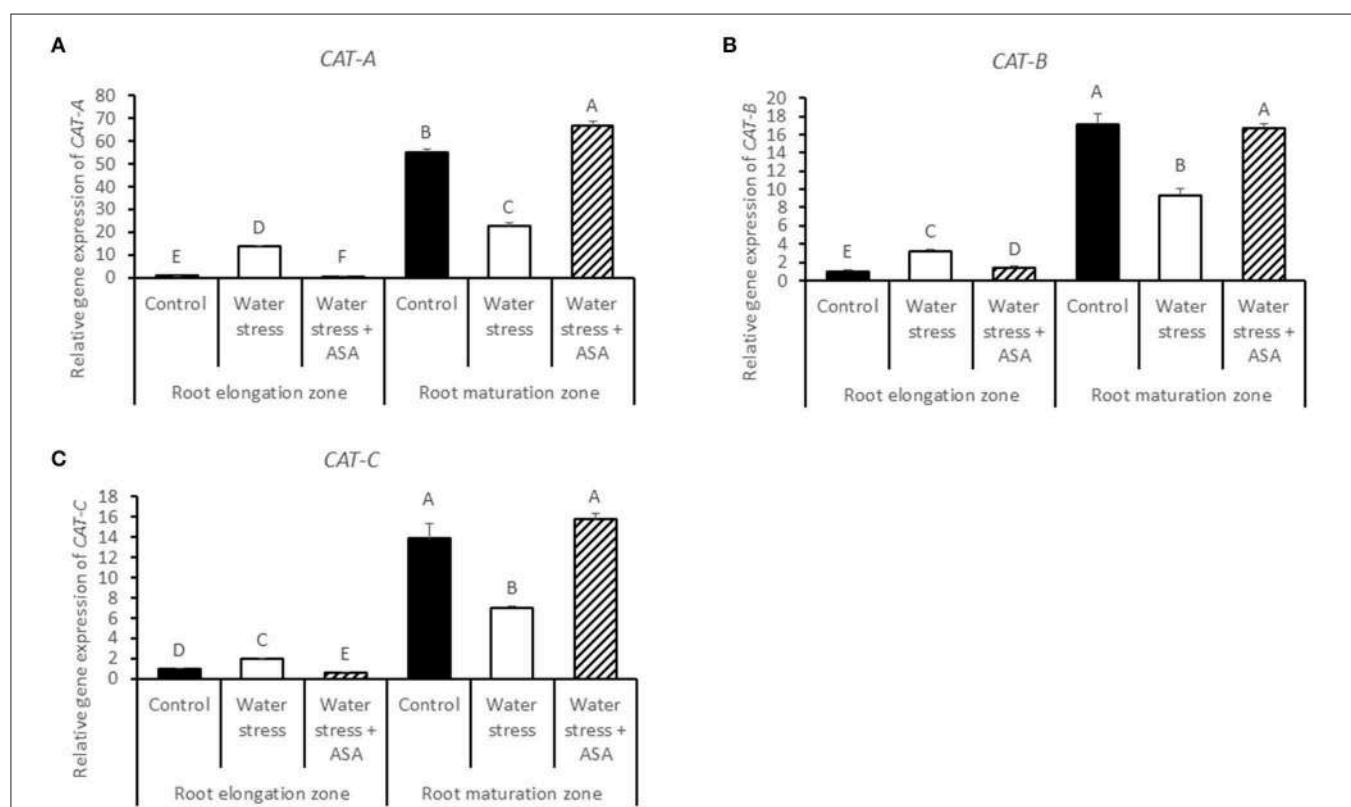
### Production of ROS in Roots as Affected by Water Stress Alone and with Additional ASA Treatment

The production of  $O_2^-$  and  $H_2O_2$  was examined in both root elongation and maturation zones using quantitative measurement (Figure 3) and histochemical staining (Figure 4) to determine level of oxidative stress induced by water stress and effectiveness of ASA treatment for oxidative scavenging in roots. Water stress significantly increased both  $O_2^-$  (1.68-fold) and  $H_2O_2$  content (1.25-fold) in the root elongation zone, while it did not alter either  $O_2^-$  or  $H_2O_2$  production in the maturation zone (Figures 3A,B). The histochemical staining also showed increased staining density for both  $O_2^-$  and  $H_2O_2$  in the root elongation zone under water stress (Figures 4B,E). The ASA treatment caused significantly lower  $O_2^-$  (1.32-fold) and  $H_2O_2$  (1.13-fold) content in the elongation zone than those without ASA treatment under water stress, but had no effects on

either  $O_2^-$  or  $H_2O_2$  in the maturation zone (Figures 3A,B). The histochemical staining pattern for ASA-treated roots also have less staining density for both  $O_2^-$  and  $H_2O_2$  in the elongation zone, indicating ASA-mitigation of oxidative stress in the root elongation zone (Figures 4C,F).

### Production of Antioxidant Compounds in Roots as Affected by Water Stress Alone and with Additional ASA Treatment

The endogenous content of free ascorbate and total ascorbate was quantified in both root elongation and maturation zones in order to evaluate whether ASA-mitigation of oxidative damages in roots due to water stress was associated with changes in the non-enzymatic antioxidant production. The content of free ascorbate increased under water stress in both root elongation and maturation zones, with 1.53- and 1.38-fold greater than the non-stress control, respectively (Figure 5). The exogenous ASA treatment caused significant higher content of endogenous free ascorbate, with 1.72-fold increase for the root elongation zone and 1.61-fold increase in the maturation zone under water stress (Figure 5A). The content of the total ascorbate content did not change under water stress, while exogenous ASA treatment significantly increased the total ascorbate content in both root elongation and maturation zones under water stress (Figure 5B).



**FIGURE 9 |** Relative gene expression level of CAT-A (A), CAT-B (B), and CAT-C (C) in tall fescue roots exposed to non-stress control, water stress, and water stress with ASA treatment. The data represent mean  $\pm$  SE ( $n = 4$  replicated pots of plants and each pot with multiple plants). Columns marked with the same letter are not significantly different at  $p < 0.05$ .

## Antioxidant Enzyme Activities and Transcript Levels as Affected by Water Stress Alone and with Additional ASA Treatment

ROS-scavenging enzyme activities and their respective transcript levels were examined in order to evaluate whether ASA mitigation of oxidative damages in roots due to water stress was associated with the enzymatic ROS-scavenging system. The activities of all antioxidant enzymes examined in this study (SOD, POD, CAT, APX, GR, MR, and DR) decreased significantly in the root elongation zone under water stress compared to the non-stress control (Figures 6, 7). The activities of SOD, POD, and MR in the root maturation zone also decreased under water stress, but those of other enzymes in the root maturation zone did not differ between the non-stress control and water stress. Exogenous ASA treatment had no significant effects on the activities of antioxidant enzymes in either root elongation or maturation zone.

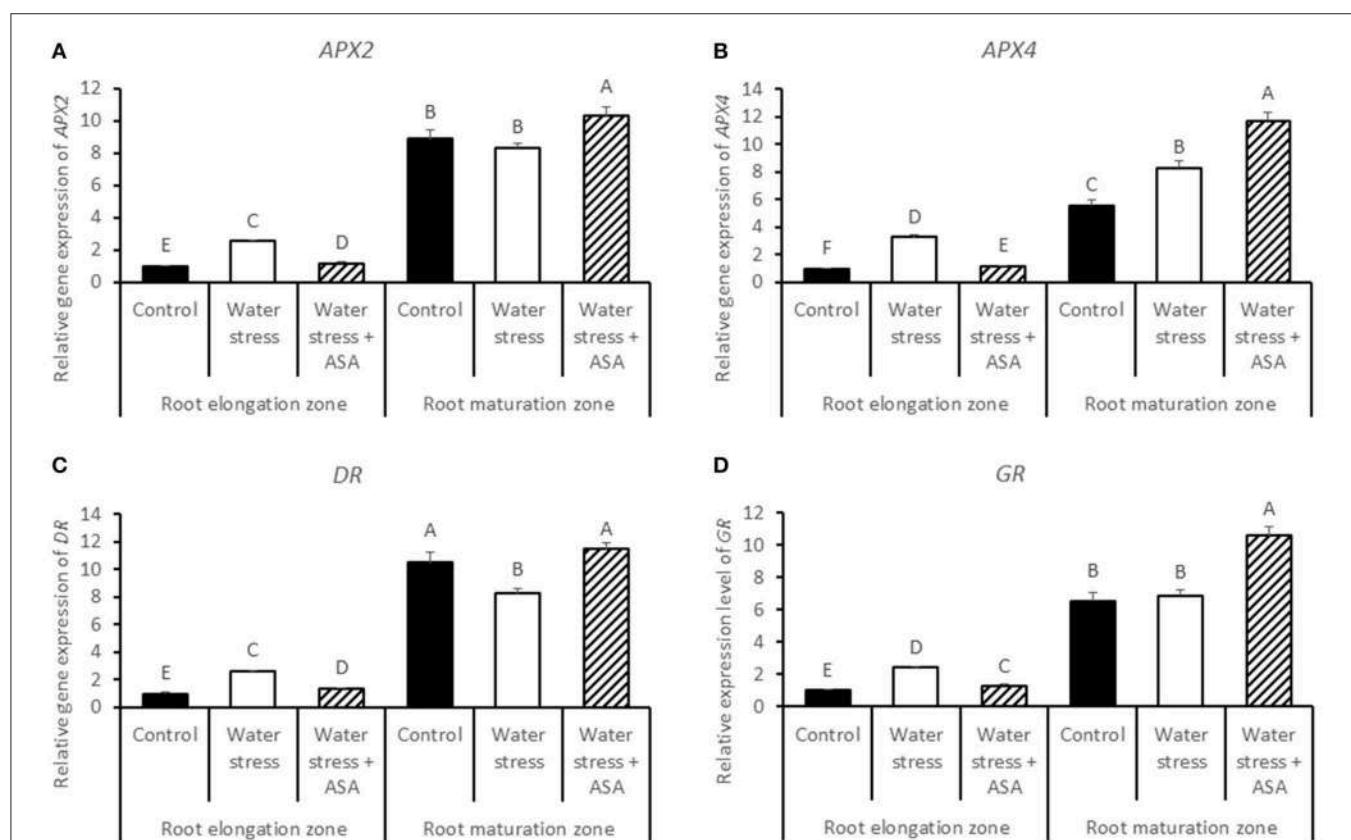
The transcript abundances of ROS-scavenging enzymes and cell-expansion-related proteins were detected by qRT-PCR. The *CuZn-SOD* and *Mn-SOD* transcript level in the root elongation zone were significantly higher in roots exposed to water stress with or without exogenous ASA treatment (Figures 8A,B). The transcript level of *CuZn-SOD* in the root maturation zone was not affected by either water stress or ASA treatment while *Mn-SOD* transcript level significantly increased under water stress

without ASA treatment (1.28-fold) or with ASA (1.52-fold), and was significantly higher for ASA-treated than untreated roots exposed to water stress (Figure 8B). The *POD* transcript level was significantly decreased in both root elongation and maturation zones under water stress with or without ASA treatment (Figure 8C).

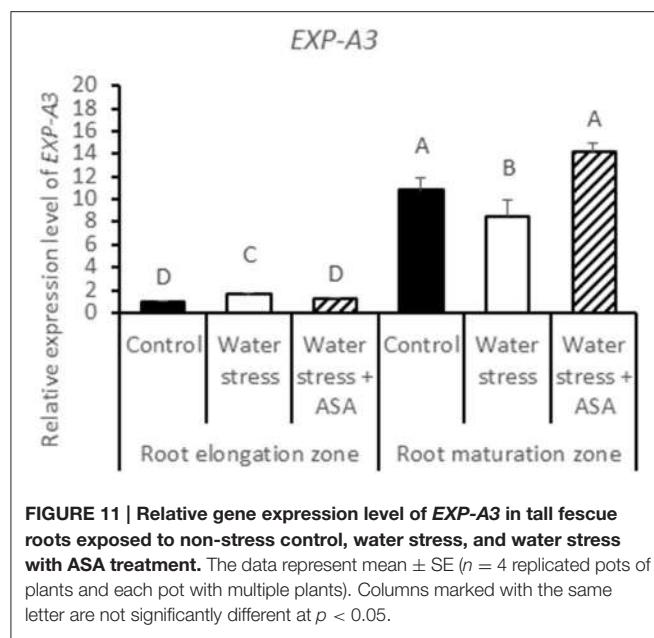
The transcript levels of *CAT-A*, *CAT-B*, and *CAT-C* in the root elongation zone were all significantly increased under water stress alone, which were significantly higher than the non-stress control or water stress with ASA treatment. The transcript levels of *CAT-A*, *CAT-B*, and *CAT-C* in the root maturation zone were all significantly decreased under water stress without ASA treatment while ASA treatment increased the transcript levels compared to the untreated plants (Figures 9A–C).

Transcript levels of *APX2* (Figure 10A), *APX4* (Figure 10B), *DR* (Figure 10C), and *GR* (Figure 10D) in the root elongation zone were significantly higher under water stress alone than those of the non-stress control or water stress with ASA treatment. Transcript levels of all four genes in the maturation zone were significantly greater in roots with ASA treatment than under water stress alone.

The expression levels of five expansin genes (*EXP-A3*, *EXP-A4*, *EXP-A5*, *EXP-B4*, and *EXP-B7*) and three XET genes (*XET1*, *XET2*, and *XET3*) regulating cell-wall loosening that were



**FIGURE 10 |** Relative gene expression level of *APX2* (A), *APX4* (B), *DR* (C), and *GR* (D) in tall fescue roots exposed to non-stress control, water stress, and water stress with ASA treatment. The data represent mean  $\pm$  SE ( $n = 4$  replicated pots of plants and each pot with multiple plants). Columns marked with the same letter are not significantly different at  $p < 0.05$ .



**FIGURE 11 |** Relative gene expression level of *EXP-A3* in tall fescue roots exposed to non-stress control, water stress, and water stress with ASA treatment. The data represent mean  $\pm$  SE ( $n = 4$  replicated pots of plants and each pot with multiple plants). Columns marked with the same letter are not significantly different at  $p < 0.05$ .

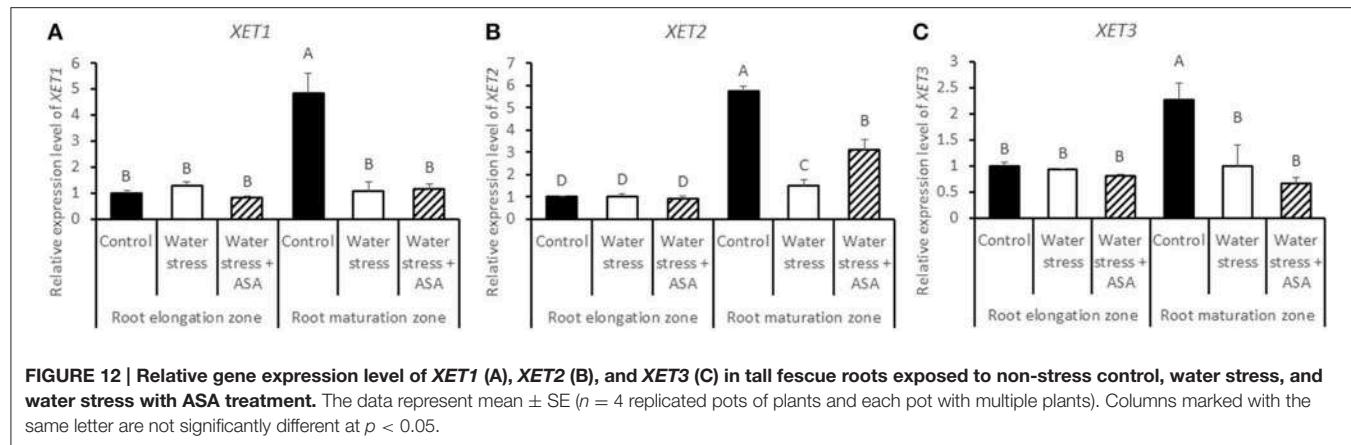
available from the tall fescue EST database, were examined to determine whether effects of water stress and ASA treatment on root elongation involve changes in different *expansin* and *XET* genes. Among five *expansin* genes, only *EXP-A3* exhibited differential expression in the root elongation and maturation zones among the non-stress control water stress and ASA treatment (Figure 11), while others did not show clear patterns in response to either water stress or ASA treatment in both root elongation and maturation zones (data not shown). Transcript level of *EXP-A3* significantly increased in the root elongation zone, but decreased in the maturation zone under water stress alone, while ASA treatment reversed *EXP-A3* responses to water stress, with a reduction and increase in the transcript level in the root elongation zone and maturation zone, respectively, compared to water stress alone (Figure 11). *XET1*, *XET2*, and *XET3* transcript levels in the root elongation zone were not significantly changed with water stress or ASA treatment (Figures 12A–C). In the root maturation zone, water stress led to significant decrease for *XET1* (22.0%), *XET2* (26.0%), and *XET3* (43.5%); exogenous ASA treatment of roots under water stress increased the transcript level of *XET2* compared to with water stress alone, although it did not have significant effects on *XET1* and *XET3* (Figures 12A,C).

## Discussion

Cell growth is one of the most sensitive responses to water stress (Nilsen and Orcutt, 1996). Many metabolic factors could be involved in growth inhibition by water stress, such as excessive production of ROS, as mostly found that limit leaf growth (Hernández and Almansa, 2002; Nayyar and Gupta, 2006). The beneficial effects on plant survival rate, biomass, shoot, and root growth by exogenous ASA have been reported under either water (Shalata and Neumann, 2001; Athar et al.,

2008) or heat stress (Kumar et al., 2011). In this study, root growth inhibition by water stress in tall fescue was accompanied by increasing  $O_2^-$  and  $H_2O_2$  production mainly in the root elongation zone, which could lead to the increases in MDA content that reflect the extent of oxidative damages of cellular membranes. Our data is in accordance with previous research results in alfalfa (*Medicago sativa*, Wang et al., 2009), thale cress (*Arabidopsis thaliana*, Duan et al., 2010), and maize (*Zea mays*, Zhu et al., 2007; Yamaguchi and Sharp, 2010) that also showed ROS accumulation in association with root elongation inhibition under water stress. Antioxidants such as ASA, which are found at high concentrations mainly in chloroplasts in leaf tissues and other cellular compartments in roots, are known to play important roles in cellular defense against oxidative stress (Noctor and Foyer, 1998). Unlike more extensive studies in ASA protecting leaves from oxidative damages in, only a few reports of exogenous ASA application and its effect on root tissues were documented, either under non-stressed (Hidalgo et al., 1991; Cordoba-Pedregosa et al., 1996; Aroca, 2006; Tyburski et al., 2006), or salt stress condition (Shalata and Neumann, 2001; Afzal et al., 2006). In this study, exogenous ASA treatment mitigated root growth decline due to water stress, although it could not completely reverse the adverse effects of water stress. In addition, ASA treatment effectively suppressed the production of both  $O_2^-$  and  $H_2O_2$ , as well as MDA accumulation in the root elongation zone. These results suggested that the inhibitory effects of water stress on root elongation was associated with oxidative damages, which could be mediated by ASA mainly in the root elongation zone. The inhibitory effect of exogenous ASA on lipid peroxidation was also found in leaves (Zhang and Kirkham, 1996; Dolatabadian et al., 2008) and roots (Shalata and Neumann, 2001) in a few other plant species. The endogenous free and total ASA content indeed was increased with exogenous ASA treatment of roots exposed to water stress. The additional ASA could suppress ROS production and membrane lipid peroxidation serving directly as an antioxidant molecule, mitigating water stress-inhibition of root elongation in tall fescue.

Plant ROS scavenging system also involves the enzymatic pathways (Mittler, 2002). Water stress led to significant reduction in the activities of antioxidant enzymes, particularly in the root elongation zone, indicating that water stress weakened enzymatic antioxidant systems in roots, particularly in the root elongation zone, thereby inhibiting contribute to the inhibitory to root elongation. However, ASA mitigation of stress-inhibition of root elongation in tall fescue seemed not to be related to changes in the activities of antioxidant enzymes, as exogenous ASA treatment did not have significant effects on any of the enzymes in either root elongation zone or maturation zone. Therefore, water stress-induced oxidative stress in roots of tall fescue could be quenched directly by the non-enzymatic pathway without involving the enzymatic activities, since ASA is not only serves as the substrate for the enzymatic reaction catalyzing by APX, DR, or GR, but also is a strong reductant (Noctor and Foyer, 1998). In addition, exogenous application of ASA has been found to enhance cell proliferation in root primordia in several plant species (Citterio et al., 1994; de Cabo et al., 1996; Arrigoni et al., 1997), possibly



**FIGURE 12 |** Relative gene expression level of *XET1* (A), *XET2* (B), and *XET3* (C) in tall fescue roots exposed to non-stress control, water stress, and water stress with ASA treatment. The data represent mean  $\pm$  SE ( $n = 4$  replicated pots of plants and each pot with multiple plants). Columns marked with the same letter are not significantly different at  $p < 0.05$ .

due to their induced G1 to S progression in quiescent center cells (Liso et al., 1988; Navas and Gomez-Diaz, 1995). The transcript abundances of two antioxidant-enzyme genes, *CAT* (-A, -B, and -C) and *DR* in the root maturation zone decreased with water stress, but increased with exogenous ASA treatment in roots exposed to water stress, which corresponded to the water stress-inhibition and the ASA mitigation effects on root growth in tall fescue, suggesting the importance of *CAT* and *DR* could be transcriptionally involved in antioxidant protection from stress damages in root growth in tall fescue. The changes in the transcript levels of other antioxidant-enzyme genes exhibited variable responses to water stress and ASA in the root elongation or maturation zone. The enzymatic data and transcript levels are not always directly corresponded with each other, due to post-transcriptional and post-translational modifications, and due to the existence of multiple gene family for an enzyme protein. We have obtained all the transcript sequences available in GenBank database of tall fescue, but there may still be gene family members not discovered. For those reasons, the discrepancy between enzymatic activities and transcript levels was possible. However, the changes in transcript levels without changing enzymatic activities at least suggested transcriptional regulation of root growth could have occurred due to the application of ASA. The antioxidant mechanisms by which water stress and ASA affect root growth is complex and deserves further investigation.

Cell expansin and elongation are directly controlled by cell-wall loosening proteins, including expansins and XETs. Our data suggested that *EXP-A3* have increased transcript abundance in the root elongation zone, and decreased transcript abundance in the root maturation zone under water stress. Similarly, Wu et al. (2001) also reported that three expansins have increased transcript level in the apical region and decreased transcript level in the basal region of the root elongation zone under water stress. The transcript level of *XET2* was significantly decreased in the root maturation zone, corresponding with decreased root elongation rate under water stress. Furthermore, the effect of

water stress on the transcript levels of *EXP-A3* and *XET2* could be reversed after the exogenous application of ASA, while the other expansin and XET family genes examined in this study exhibited variable responses to water stress and ASA in the root elongation or maturation zone. Nevertheless, these results indicated that *EXP-A3* and *XET2* could be involved in water stress-inhibition of root elongation and ASA-mediated promoting effects on root growth under water stress. Our results also suggested potential interactive roles of the two cell-wall loosening genes (*expansin* and *XET*) and antioxidant metabolism controlling root responses to water stress, but this postulation is worthy of further research.

In summary, tall fescue root growth could be enhanced by the exogenous application of ASA under water stress. The inhibition of root elongation by water stress was accompanied by increased production of  $O_2^-$  and  $H_2O_2$ , which was suppressed by the addition of ASA. The positive effects of ASA on root elongation under water stress were mainly due to the direct antioxidant effects of this strong reductant. The transcript levels of *CATs* and *DR* in root maturation zone were increased after ASA application under drought stress, compared with drought stress alone, although their enzymatic activities were not changed. Moreover, The *EXP-A3* and *XET2* transcript levels were also increased in root maturation zone with ASA treatment, which could be associated with the increased root growth under water stress. The direct association of *EXP-A3* and *XET2* with cell division and cell elongation controlling root elongation rate are unknown, which requires further investigation. This study also suggests potential interactions of antioxidant defense and cell-expansion genes controlling root elongation in responses to environmental stresses, which deserves further investigation.

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# RNAi-based functional elucidation of *PtrPRP*, a gene encoding a hybrid proline rich protein, in cold tolerance of *Poncirus trifoliata*

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Hybrid proline-rich proteins (HyPRPs) have been suggested to play important roles in various plant development and stress response. In this study, we report the cloning and functional analysis of *PtrPRP*, a HyPRP-encoding gene of *Poncirus trifoliata*. *PtrPRP* contains 176 amino acids, among which 21% are proline residues, and has an 8-cysteine motif (8 CM) domain at the C terminal, a signal peptide and a proline-rich region at the N terminal. *PtrPRP* is constitutively expressed in root, stem and leaf, with the highest expression levels in leaf. It was progressively induced by cold, but transiently upregulated by salt and ABA. Transgenic *P. trifoliata* plants with knock-down *PtrPRP* by RNA interference (RNAi) were generated to investigate the role of *PtrPRP* in cold tolerance. When challenged by low temperature, the *PtrPRP*-RNAi plants displayed more sensitive performance compared with wild type (WT), as shown by higher electrolyte leakage and malondialdehyde content. In addition, the RNAi lines accumulated more reactive oxygen species (ROS) and lower levels of proline relative to WT. These results suggested that *PtrPRP* might be positively involved in cold tolerance by maintaining membrane integrity and ROS homeostasis.

**Keywords:** cold stress, *Poncirus trifoliata*, hybrid proline-rich protein, RNA interference, ROS

## Introduction

Plants are frequently challenged with a variety of abiotic stresses, among which cold, at either freezing, or chilling regimes, constitutes an important factor leading to adverse impacts on plant growth, development and yield potential. Chilling and freezing stresses lead to physiological or structural alterations, such as elevation of reactive oxygen species (ROS), imbalance of osmotic pressure, and formation of ice crystals. All of these unfavorable situations will result in deterioration of membrane integrity, impairment of cell viability and eventually lead to cell death when they are not precisely coped with (Xiong and Zhu, 2002; Einset et al., 2007; Peng et al., 2012).

It has been well known that to survive under cold plants experience a cascade of physiological and biochemical changes, such as accumulation of various proteins or soluble compounds, and alteration of a series of metabolic reactions (Wilson and Cooper, 1994; Knight et al., 1996; Fowler et al., 1999; Welling and Palva, 2006; Huang et al., 2011).

In addition, accumulating evidences show that extensive reprogramming of a cohort of cold-responsive genes is an elegant strategy for the plants to adapt to the harsh environments (Stockinger et al., 1997; Zarka et al., 2003; Nakashima and Yamaguchi-Shinozaki, 2006; Shi et al., 2015). These genes are generally classified into two major categories, functional proteins, and regulatory proteins, which play either direct protective or regulatory roles in stress tolerance (Yamaguchi-Shinozaki and Shinozaki, 2005). For example, the functional proteins participate in stabilization of membrane integrity, maintenance of enzyme activity, physical structures of cellular components, which are critical factors contributing to enhance stress tolerance in plants (Walker et al., 2010). Genetic manipulation of the cold-responsive genes has been suggested to serve as an alternative approach for generating transgenic plants with enhanced stress tolerance (Huang et al., 2013). However, it is worth mentioning that functional characterization of a stress-responsive gene is required before it can be efficiently manipulated.

Hybrid proline-rich proteins (HyPRPs) are a subset of proline-rich proteins (PRPs) specific to seed plants; however, they do not contain the domain typical of PRPs (Josè-Estanyol et al., 2004; Priyanka et al., 2010; Xu et al., 2011). Based on the position and number of cysteine residues at C terminal, HrPRPs can be categorized into two major groups, in which Class A has 4–6 cysteine residues, while Class B contains a conserved eight-cysteine-motif (8 CM) at C terminal, a repetitive proline-rich N-terminal domain (PRD) and a signal peptide in front of the PRD (Josè-Estanyol and Puigdomènech, 2000; Josè-Estanyol et al., 2004; Battaglia et al., 2007; Dvořáková et al., 2007; Neto et al., 2013). HyPRPs have been suggested to play important biological roles in various processes, including plant cell elongation, ontogeny, and morphogenesis of different organs, defenses against viral or fungal pathogens (He et al., 2002; Holk et al., 2002; Dvořáková et al., 2012). In addition, HyPRPs of *Arabidopsis thaliana*, *Brassica napus*, *Medicago sativa*, *Glycine max*, and *M. truncatula* have been also suggested to be involved in responses to biotic and abiotic stresses (Deutch and Winicov, 1995; He et al., 2002; Bouton et al., 2005; Zhang and Schläppi, 2007). Nevertheless, so far little knowledge is available on the role of HyPRPs in stress tolerance of perennial plants, such as *Poncirus trifoliata*.

*Poncirus trifoliata* (L.) Raf. is extremely cold hardy when it is fully cold acclimated. In a previous work, suppression subtractive hybridization was employed to unravel cold-responsive genes of valuable significance for engineering cold tolerance (Peng et al., 2012). One of the ESTs draws our attention as its expression level was elevated over 70-folds under cold; the EST was later annotated to encode a HyPRP. However, whether it plays a role in cold tolerance remains undetermined. As a follow-up and continuum of our earlier work, in this study we report the isolation and functional analysis of this gene, designated as *PtrPRP*, in cold tolerance by RNA interference (RNAi). Transcript levels of *PtrPRP* were enhanced by abiotic stresses, but the response to cold was extremely dramatic. Knockdown of *PtrPRP* in trifoliate orange by RNAi led to enhanced cold sensitivity. We further demonstrated that the RNAi lines accumulated more ROS and malondialdehyde (MDA), but lower

levels of proline. Our data indicate that *PtrPRP* is a cold-responsive gene that plays an essential role in cold tolerance.

## Materials and Methods

### Plant Materials and Stress Treatments

Three-month-old trifoliate orange seedlings were grown in a growth chamber (25°C) with a photoperiod regime of 8 h dark/16 h light (light intensity is about 100 μmol m<sup>-2</sup> s), and an relative humidity of 60–70%. The seedlings were well watered before they were subjected to various stresses. For cold treatment the seedlings were placed in the chamber set at 4°C for 6 d, followed by transfer to 25°C for recovery. The leaves were sampled at 0, 6, 24, 72, and 144 h after cold treatment and 6 h after recovery. Dehydration was imposed by placing the seedlings on a clean bench under ambient environment for 6 h and then shifted to water for 0.5 h. The leaves were collected at 0, 0.5, 1, 3, and 6 h after dehydration and 6 h after rehydration. For salt stress, the seedlings were treated with 200 mM NaCl solution for up to 144 h and then shifted to water for another 6 h; the leaves were sampled at 0, 6, 24, 72, and 144 h of salt treatment and after 6 h of recovery in water. In addition, the seedlings were treated with 100 μM ABA for 0, 6, 12, 24 h, and 48 h, followed by transfer to water for another 6 h; the leaves were sampled at the designated time points. The samples were immediately frozen in liquid nitrogen and stored at –80°C until further use.

### Gene Isolation and Sequence Analysis

Rapid amplification of cDNA ends (RACEs) was employed to obtain full-length cDNA of *PtrPRP*. For this purpose, gene-specific primers for 5'-RACE and 3'-RACE (Table 1) were designed based on the EST sequence identified in the previous work (Peng et al., 2012). RACE-PCR was carried out using the SMART™ RACE cDNA Kit (Clontech, USA). Total RNA was

**TABLE 1 | Oligonucleotide primers used in this study.**

Name	Primer sequences (5' – 3')
5'-RACE	CGCCAAACCGAAATGTGCTCTGATA
3'-RACE	TAGTGTGAGATACCCACGCC
PtrPRP-S	ATGGGAAAATATCAATTAGC
PtrPRP-A	TTAACGAGGACACTGAAATCC
PtrActin-S	CATCCCTCAGCACCTTCC
PtrActin-A	CCAACCTTAGCAGTCTCC
PtrPRP-q-S	ACCGATTGAAAGACGCCAC
PtrPRP-q-A	CACCGAGTTGAGAGCATCA
PtrPRP-L-S	GAAGATCTATGGAAAATATCAATTAGC
PtrPRP-L-A	GAATGAGTACAGGACACTGAAATCC
PtrPRP-attB-S	GGGGACAAGTTGTACAAAAAAGCAG
	GCTATGGGAAAATATCAATTAGC
PtrPRP-attB-A	GGGGACCACCTTGACAAGAAAAGCT
	GGGTCTAGGGCTTGGTCGTTA
NPTII-S	AGACAATCGGCTGCTCTGAT
NPT II-A	TCATTTCGAACCCAGAGTC
PtrPRP-s-S	ATGGGAAAATATCAATTAGC
PtrPRP-s-A	TTAACGAGGACACTGAAATCC

extracted with RNAiso Plus (TaKaRa, Japan) according to the manufacturer's instructions. First strand cDNA was synthesized via PrimeScript® RT Reagent Kit With gDNA Eraser (TaKaRa) following the user's manual. The 5'-RACE and 3'-RACE PCR products were sequenced and analyzed, and then merged with the original EST to get a single sequence, which was verified using RT-PCR with a pair of full-length primers (PtrPRP-S/PtrPRP-A, **Table 1**). Molecular weight (MW) and isoelectric point (pI) of the protein were predicted on ExPASy<sup>1</sup>. Phylogenetic analysis was constructed by Phylogeny.fr online software<sup>2</sup> (Dereeper et al., 2008) and MEGA4.0. Multiple alignments of the HyPRPs were performed by ClustalX program with defaulted settings and displayed by Jalview<sup>3</sup>. Signal peptide was predicted with SignalP4.1 Server<sup>4</sup> and iPSORT<sup>5</sup> (Bannai et al., 2002).

### Gene Expression Analysis by Quantitative Real-time RT-PCR (qPCR)

Total RNA extraction and cDNA synthesis were performed as mentioned above. Expression of *PtrPRP* under the stresses and in different tissues was assessed by qPCR, which was carried out with the SYBR® Green PCR kit (TaKaRa) on a LightCycler 480 Real-Time System (Roche). PCR solution, in a total volume of 10  $\mu$ L, contained 5  $\mu$ L of 2  $\times$  SYBR Premix Ex Taq (Tli RNaseH Plus), 50 ng of cDNA, 0.25  $\mu$ M of each primer (PtrPRP-q-S/A). The reaction cycles were 95°C for 30 s, and 40 cycles of 95°C for 5 s, 56°C for 10 s, and 72°C for 15 s. Each reaction was repeated at least three times, and  $\Delta\Delta$ CT method was applied to calculate relative expression levels. The *Actin* gene of trifoliate orange was used as a reference control and analyzed in parallel with specific primers (**Table 1**) to normalize the expression levels.

### Subcellular Localization of PtrPRP

To determine subcellular localization of PtrPRP, the full-length *PtrPRP* cDNA without stop codon was amplified using primers (PtrPRP-L-S/A) containing restriction sites of *Bgl*II and *Spe*I. The PCR products were purified with AxyPrep™ DNA Gel Extraction Kit (Axygen scientific, USA), digested with *Bgl*II and *Spe*I and subcloned into the pCAMBIA1302 vector containing a GFP reporter gene, under the control of *CaMV* 35S promoter. The resultant fusion construct PtrPRP::GFP and the control vector (GFP) were separately introduced into onion epidermis via *Agrobacterium*-mediated transformation as described by Peng et al. (2014), followed by visualization of green fluorescence under a confocal microscope (FV1000; Olympus, Tokyo, Japan) or a fluorescence microscope (Nikon 90i).

### Generation and Identification of RNAi Plants

To generate *PtrPRP*-RNAi plants, a 253-bp cDNA fragment of *PtrPRP* was amplified using a pair of primers (PtrPRP-attB-S/A) and introduced into pHETSGATE2 through BP recombination reactions (Invitrogen, Japan). The RNAi

vector was introduced into *A. tumefaciens* strain GV3101. *Agrobacterium*-mediated transformation of trifoliate orange was performed according to Fu et al. (2011). Kanamycin-resistant shoots were identified by genomic PCR using primers specific to neomycin phosphotransferase II (NPTII-S/A) and the 253-bp sequence (**Table 1**). Examination of *PtrPRP* expression was carried out by semi-quantitative RT-PCR according to Shi et al. (2010) except using specific primers (PtrPRP-s-S/A, **Table 1**). QRT-PCR was also used to confirm the expression of one transgenic line, as done mentioned above. *Actin* gene was used as the reference gene. The positive transgenic plants were vegetatively propagated to obtain enough plants that were used for the subsequent experiments.

### Cold Tolerance Assessment and Physiological Measurements

Uniform and healthy 3-month-old plants of wild type (WT) and two RNAi lines were used for cold treatment. Two days after sufficient watering, the plants were placed in a growth chamber set at 0°C and kept for 48 h without light in order to avoid light-induced oxidative stress under cold treatment. The leaves were sampled after completion of the chilling treatment and used for measurement of electrolyte leakage (EL), MDA, ROS, and proline.

Measurement of EL was conducted according to Peng et al. (2012). In brief, the sampled leaves were immersed in 20 mL of double distilled water (ddH<sub>2</sub>O), while the control tube contained only 20 mL of ddH<sub>2</sub>O. The tubes were gently shaken for 2 h on a shaker (QB-206, Qilinbeier, China) at room temperature; the EL of sample (C<sub>1</sub>) and control (CK<sub>1</sub>) were then measured on a DSS-307 conductivity meter (SPSIC, China). The tubes were then boiled for 10 min, and cooled down at room temperature before measurement of the EL (C<sub>2</sub> and CK<sub>2</sub>). A relative conductance was calculated by C (%) = (C<sub>1</sub> - CK<sub>1</sub>)/(C<sub>2</sub> - CK<sub>2</sub>) × 100.

Proline content was determined according to Zhao et al. (2009) with minor modification. The leaf tissues (about 0.5 g) were extracted in 5 mL of 3% sulphosalicylic acid at 95°C for 10 min. After cooling down, the homogenate was filtered and 2 mL of supernatant was transferred to a new tube containing 2 mL of acetic acid and 2 mL of acidified ninhydrin reagent. After 30 min of incubation in boiling water, 4 mL of toluene was added to the tubes and vortexed for 30 s. The absorbance of the toluene layer was colorimetrically determined at 520 nm. Protein concentration was determined based on the method reported by Bradford (1976).

Accumulation of H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>-</sup>, two major types of ROS, was assayed using histochemical staining with 3, 3'-diaminobenzidine (DAB) and nitrotetrazolium blue chloride (NBT), respectively. The leaves sampled after 48 h of chilling treatment were immediately immersed in 1 mg mL<sup>-1</sup> freshly prepared NBT or DAB solution at ambient temperature, until blue or brown precipitates were observed. The stained leaves were then bleached in concentrated ethanol, and kept in 70% ethanol. Quantitative measurement of H<sub>2</sub>O<sub>2</sub> was also carried out using a specific detection kit based on the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, China).

<sup>1</sup><http://us.expasy.org>

<sup>2</sup>[http://phylogeny.lirmm.fr/phylo\\_cgi/index.cgi](http://phylogeny.lirmm.fr/phylo_cgi/index.cgi)

<sup>3</sup><http://www.jalview.org/>

<sup>4</sup><http://www.cbs.dtu.dk/services/SignalP/>

<sup>5</sup><http://hc.ims.u-tokyo.ac.jp/iPSORT/>

Malondialdehyde content, expressed as nmol/mg protein, was measured using a detection kit specifically designed for MDA quantification (Nanjing Jiancheng Bioengineering Institute, China) based on the manufacturer's instructions. Protein concentration was determined based on the method reported by Bradford (1976).

## Statistical Analysis

The data were analyzed using analysis of variance (ANOVA), and statistical difference between WT and transgenic lines was compared, taking  $P < 0.05$  as significant.

## Results

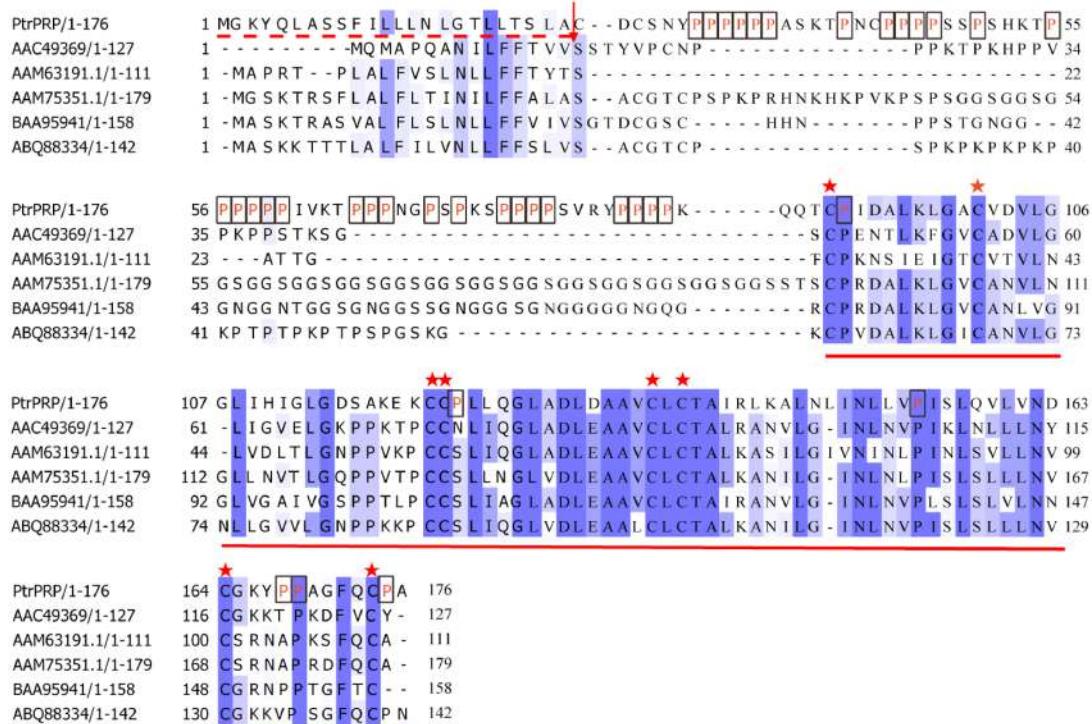
### Isolation of PtrPRP from *P. trifoliata*

In a previous work, an EST annotated as PRP was fished out by SSH-based screening of a cold-treated cDNA library of *P. trifoliata* (Peng et al., 2012). As the whole genome sequence information of *P. trifoliata* is unavailable at this time, we employed RACE to isolate the full-length cDNA of this gene. For this purpose, 5'-RACE and 3'-RACE PCR were carried out using primers designed base on the original EST, leading to amplification of two fragments of 554 and 802 bp, respectively, which were shown to be homologous to known PRP genes by

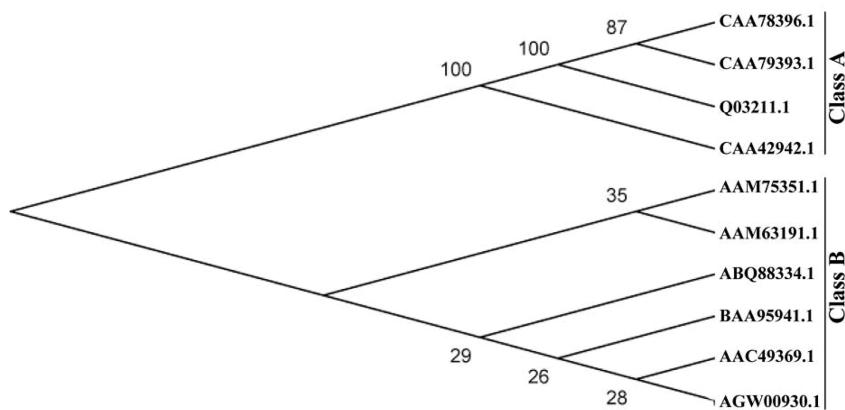
Blastn against NCBI. Assembly of the two RACE sequences and the original EST resulted in generation of a full-length sequence of 849 bp in size, which was further verified by PCR to be correct in the sequence. The sequence displayed 74% of identity to PRP gene of *Solanum palustre*; so the gene was designated as *PtrPRP* (*Poncirus trifoliata Proline-Rich Protein*). The gene has been deposited in the NCBI database under the accession number of KF171887.1.

### Sequence Analysis of PtrPRP

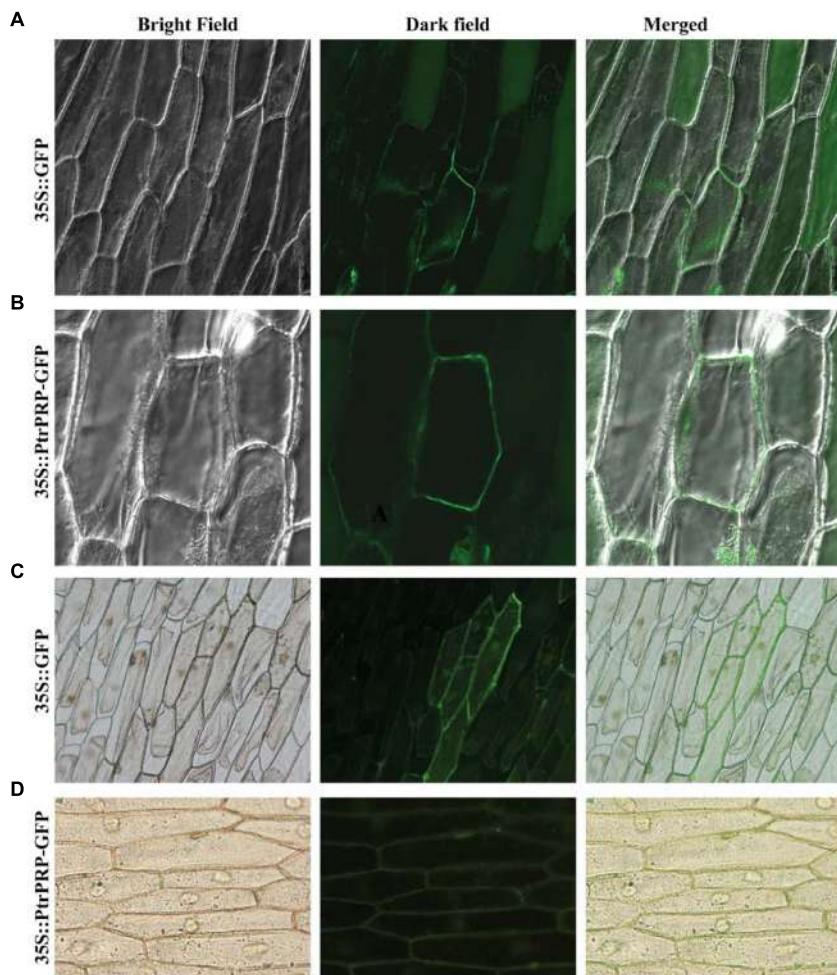
The full-length *PtrPRP* cDNA contained a 531-bp open reading frame (ORF) encoding 176 amino acids, 21% of which were proline residues (Figure 1). *PtrPRP* had a predicted molecular mass of 18,242 kD and an isoelectric point of 8.65. In addition, a signal peptide composed of 24 amino acids was observed at the N terminal. In order to reveal the relationship between *PtrPRP* and PRPs from other plants, we constructed a phylogenetic tree using deduced amino acid sequences of *PtrPRP* and PRPs from other plants. PRPs were evolutionarily divergent in plant. However, *PtrPRP* was not clustered into the five groups that have been established by earlier studies (data not shown). Therefore, we assume that *PtrPRP* might be a HyPRP. Alignment with *PtrPRP* and HyPRPs from *Phaseolous vulgaris*, *A. thaliana*, *G. max*, *Nicotiana tabacum*, and *Capsicum annuum* revealed that *PtrPRP* has the conserved 8 CM domain at the C



**FIGURE 1 | Sequence alignment between PtrPRP and HyPRPs from other plants.** Prolines (P) residues in PtrPRP are marked in red letter and boxed. The signal peptide is indicated with dotted red line at the N-terminal, and the arrow indicates the cleavage site. Identical and highly conserved residues are shaded in dark and light blue, respectively. The conserved 8 CM domain at the C-terminal is underlined, while the cysteine residues are marked by asterisks. GenBank accession numbers for the HyPRPs are AGW00930.1 (PtrPRP, *Poncirus trifoliata*), AAC49369 (*Phaseolous vulgaris*), AAM63191.1 (*Arabidopsis thaliana*), AAM75351.1 (*Glycine max*), BAA95941 (*Nicotiana tabacum*), ABQ88334 (*C. annuum*).



**FIGURE 2 |** A phylogenetic tree constructed using proline-rich proteins (PRPs) of trifoliate orange and other plants, including CAA78396.1 (*N. tabacum*), CAA78393.1 (*N. tabacum*), Q03211.1 (*N. tabacum*), CAA42942.1 (*Phaseolus vulgaris*), AGW00930.1 (PtrPRP, *Poncirus trifoliata*), AAC49369 (*Phaseolus vulgaris*), AAM63191.1 (*A. thaliana*), AAM75351.1 (*G. max*), BAA95941 (*N. tabacum*), and ABQ88334 (*C. annuum*). The neighbor-joining tree was generated with MEGA4.0 from 1000 bootstrap replicates.



**FIGURE 3 |** Subcellular localization analysis of PtrPRP. GFP (A,C) or PtrPRP-GFP (B,D) was transiently expressed in onion epidermal cells by Agrobacterium-mediated transfection, and the onion cells were observed under a confocal microscope (A,B) or fluorescence microscope (C,D). Images taken under bright (left) or dark (middle) field were shown, while the merged images are shown on the right.

terminal (**Figure 1**), which is an important signature for a HyPRP, indicating that PtrPRP was actually a typical HyPRP. Besides, the phylogenetic tree indicated that PtrPRP was a Class B HyPRP (**Figure 2**).

### Subcellular Localization of PtrPRP

Subcellular localization of PtrPRP was investigated by constructing a fusion protein of PtrPRP without the stop codon and GFP, driven by *CaMV 35S* promoter, using the GFP vector as a control. Microscopic observation showed that green fluorescence could be observed in the whole cells when the control plasmid was transiently expressed (**Figures 3A,C**). However, when the fusion protein was expressed in the onion epidermis, green fluorescence was predominantly observed on the outer surface of the cells (**Figures 3B,D**), which may include the plasma membrane (PM), internal membranes and PM/cell wall interphase. However, the exact localization to a certain position of *PtrPRP* remains to be determined.

### Expression Profiles of PtrPRP in Different Tissues and Under Various Treatments

Spatial expression of *PtrPRP* in three tissues, leaf, root, and stem, was assessed by qPCR. *PtrPRP* was constitutively expressed in the three tissues, but their transcript levels varied among each other (**Figure 4**). The highest mRNA abundance was detected in the leaf, whereas root showed the lowest expression level among the three tissues. In addition, expression patterns of *PtrPRP* in response to cold, dehydration, salt, and ABA treatments were examined. Under exposure to cold, transcript level of *PtrPRP* was quickly induced within 6 h, and then progressively accumulated to reach the highest level at 144 h, when the expression level was elevated by more than 30-folds compared with that at 0 h. However, when the cold treatment was removed, transcript level of *PtrPRP* was sharply decreased (**Figure 5A**). Dehydration treatment led to gradual reduction of *PtrPRP* mRNA levels up to the lowest value at 3 h, followed by an accretion to the basal level at 6 h. Surprisingly, rehydration for 6 h resulted in a notable induction of *PtrPRP* (**Figure 5B**). Steady-state mRNA level of *PtrPRP* was up-regulated by nearly 14-folds within 6 h of salt treatment, and continued to rise until reaching the peak value at 24 h, followed by a sharp decrease to basal level at 72 and 144 h. Relief of the salt stress for 6 h led to a sevenfolds elevation of the transcript level (**Figure 5C**). Expression pattern of *PtrPRP* in response to exogenous ABA application was similar to that under salt treatment, except the greater induction within the first two time points. Removal of ABA also led to an up-regulation of *PtrPRP* transcript (**Figure 5D**).

### Production of Trifoliate Orange RNAi Lines with Knock-down of PtrPRP

To elucidate the role of *PtrPRP* in cold tolerance, RNAi strategy was used to suppress *PtrPRP* in trifoliate orange. For this purpose, a 253-bp cDNA fragment displaying lower degree of sequence conservation among C-terminal ends of the PRPs was used to construct *PtrPRP*-RNAi vector, which was then transferred into trifoliate orange via *Agrobacterium*-mediated transformation (**Figures 6A–D**). Kanamycin-resistant plants were confirmed to

be positive using genomic PCR (**Figure 6E**). Semi-quantitative RT-PCR assay indicated that among the positive lines *PtrPRP* was successfully down-regulated in three lines, and the greatest suppression was observed in lines #51 and #52, which were hereafter designated as RNAi-51 and RNAi-52, respectively (**Figure 6F**). Expression of *PtrPRP* in RNAi-51 was also checked using qRT-PCR, and the results confirmed that PtrPRP is truly knocked down, purporting the semi-quantitative RT-PCR data (**Figure 6G**). The two RNAi lines, RNAi-51 and RNAi-52, showed no difference in plant morphology in comparison with the WT.

### Knock-down of PtrPRP Confers Sensitivity to Chilling Stress

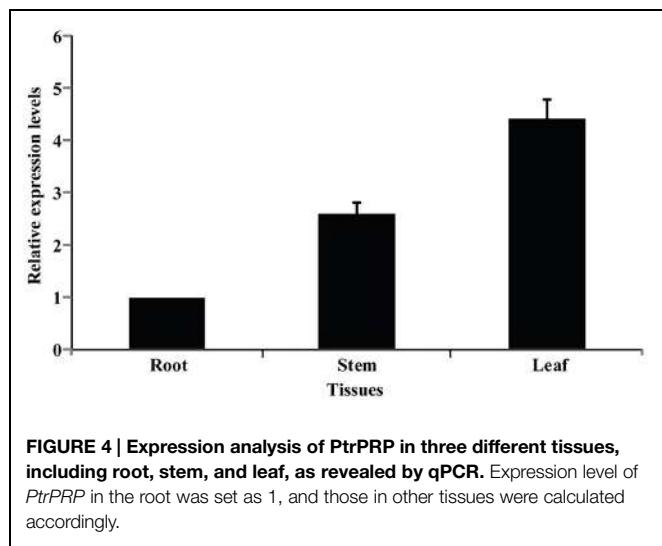
The two RNAi lines and WT were subjected to a chilling temperature at 0°C for 48 h so as to investigate the impact of silencing *PtrPRP* on cold tolerance. We examined EL and MDA, two critical parameters that have been widely used for evaluating the stress tolerance in earlier studies (Peng et al., 2012, 2014). After the chilling treatment, EL and MDA of both RNAi lines were significantly higher than those of WT, indicating that more severe damages have been imposed on the RNAi lines compared to the WT (**Figures 7A,B**).

### The RNAi Lines Accumulate More ROS but Less Proline

Electrolyte leakage and MDL are indirect indices for oxidative stress that is primarily caused by excessive accumulation of ROS. The drastic difference in EL and MDA levels between RNAi lines and WT prompted us to check ROS status of the tested lines after cold treatment. We first used histochemical staining with DAB and NBT to reveal *in situ* accumulation of H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>−</sup>, respectively, in the cold treated leaves. This method is valid as the ROS levels can be directly disclosed based on the color of reaction. As shown in **Figure 8A**, conspicuous difference in the staining patterns was observed between WT and the RNAi lines. The leaves of RNAi lines were stained by both DAB and NBT in deeper manner or the areas of staining were larger, indicating that the RNAi lines produced more ROS after cold treatment compared with the WT. The staining was partly confirmed by quantitative measurement of H<sub>2</sub>O<sub>2</sub> using a specific kit designed for it (**Figure 8B**). We also measured proline contents in the RNAi lines and WT after cold treatment, as this compound has been considered as an important metabolite indicating the relevance to stress tolerance. As shown in **Figure 9**, the WT accumulated more proline in comparison with the two RNAi lines.

## Discussion

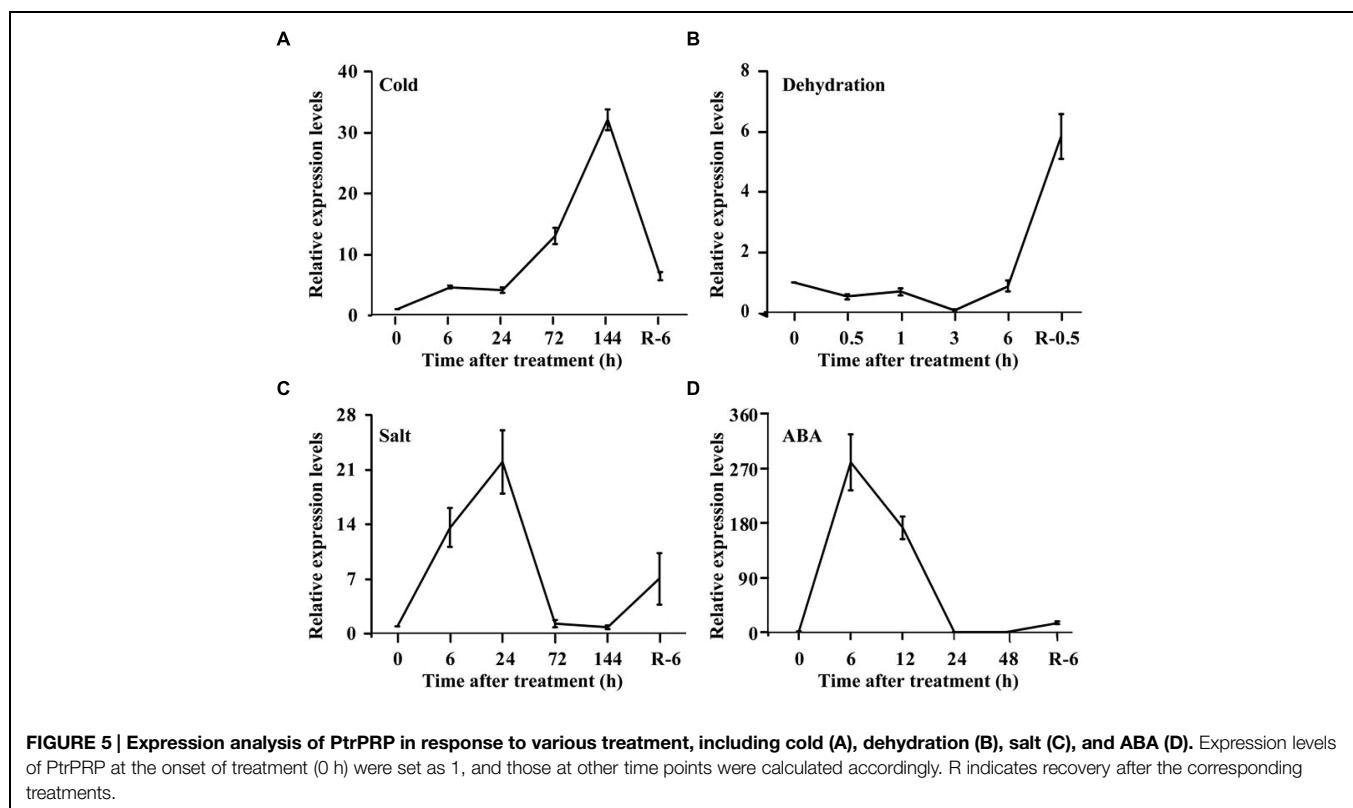
Although HyPRPs have been reported in many plants, the precise functions of HyPRPs were still poorly elucidated, especially in woody plants. Here we report molecular cloning and functional characterization of *PtrPRP*, a *HyPRP* gene from *Poncirus trifoliata*. Since genome of *P. trifoliata* has not been sequenced, it remains to be determined whether *P. trifoliata* has more PRP members at this stage. By search against the whole

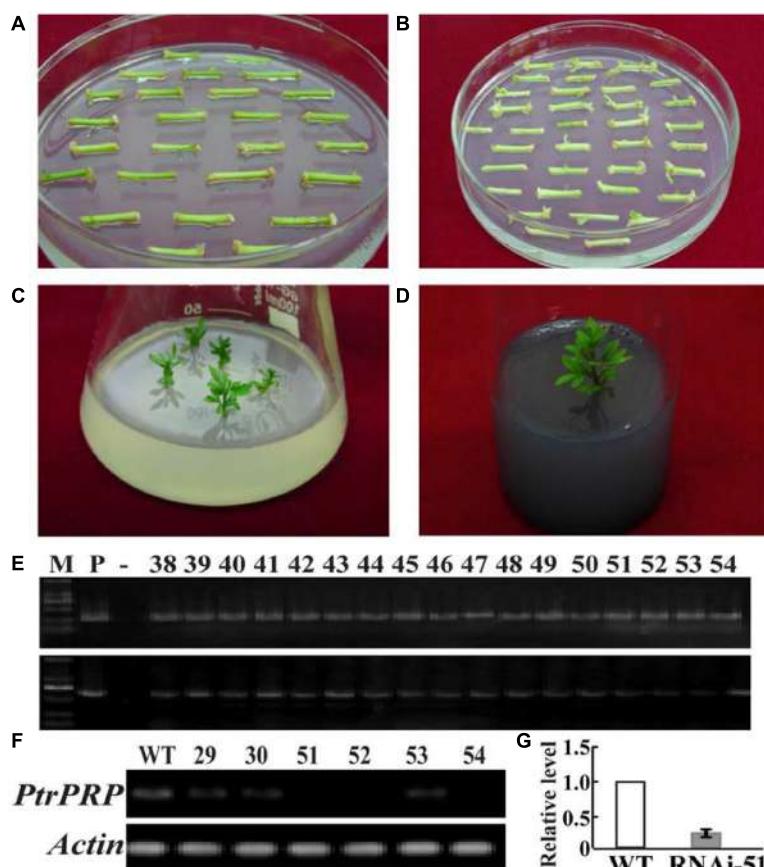


genome sequences of *Citrus sinensis*, a closely related species of *P. trifoliata* we found four putative PRPs (CsPRPs), among which CsPRP1 shares the highest similarity with *PtrPRP*. It suggests that *P. trifoliata* may possibly contain other PRPs, but this assumption needs to be verified in the future. In *PtrPRP*, proline residues account for 21% of the total amino acids of *PtrPRP*, allowing us to believe that *PtrPRP* is a PRP. Of note, the prominent abundance of proline residues at the N terminus may be possibly associated with the targeting of this protein, as the presence of hydrophobic

proline-rich (HPR) motif has been suggested to be necessary and sufficient for the intracellular targeting of a temperature-induced lipocalin in *Arabidopsis* (*AtTIL*; Hernández-Gras and Boronat, 2015). So far, PRPs have been previously categorized into five groups based on signature motifs (Gothandam et al., 2010), but *PtrPRP* belongs to none of them. However, sequence alignments between *PtrPRP* and HyPRPs from several plants revealed that *PtrPRP* has the conserved 8 CM domain, which is a typical signature of HyPRPs, but not present in PRPs (José-Estanyol et al., 2004), which indicates that *PtrPRP* is possibly considered as a HyPRP. In addition, since *PtrPRP* contains the 8 CM domain it should be categorized into the Class B HyPRPs.

Expression of *PRPs* was shown to be associated with development of various tissues and exhibited temporal and spatial expression patterns (Vignols et al., 1999; Gothandam et al., 2010; Neto et al., 2013). Transcripts of *ZmHyPRP* from maize were specifically observed in the immature embryos (José-Estanyol et al., 1992), while a *HyPRP* gene of strawberry was exclusively found in mature fruits (Blanco-Portales et al., 2004). Hong et al. (1989) reported that soybean contained three PRPs, *SbPRP1*, *SbPRP2*, and *SbPRP3*. *SbPRP1* was highly expressed in mature hypocotyl, root, and immature seed coat. *SbPRP2* was the predominant form of transcripts in the apical hypocotyl and young suspension culture cells, while the transcripts of *SbPRP3* accumulated mainly in aerial parts, especially in leaves. In a recent study, Neto et al. (2013) expanded the soybean HyPRP proteins to 35 members, which exhibited variable expression patterns in six vegetative organs, root, and root tip, nodule, leaves, green pods, flower, and apical meristem. Some of them





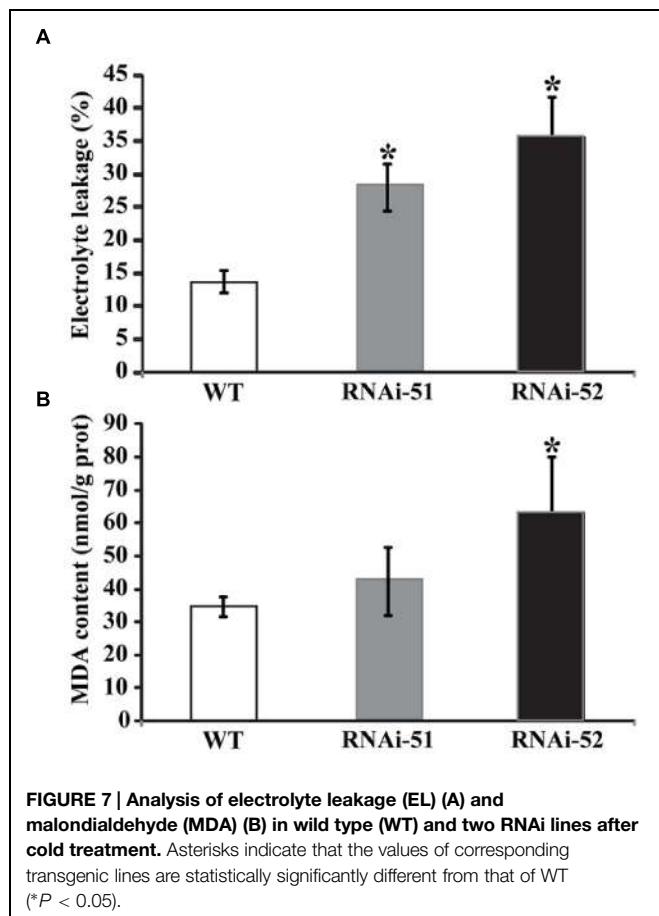
**FIGURE 6 | Transformation, regeneration, and characterization of trifoliate orange transgenic plants. (A,B)** Culture of the stem segments on selection medium for 30 d (A) and 60 d (B), respectively. (C) Regeneration of kanamycin -resistant shoots on the selection medium. (D) A rooting plant on the root-inducing medium. (E) Genomic PCR of the kanamycin-resistant plants using designed primers specific to *PtrPRP* (upper) and *NPTII* (bottom), respectively. (F) Expression analysis of *PtrPRP* in six positive transgenic plants, as revealed by RT-PCR. Actin gene was used as an internal control. (G) Analysis of *PtrPRP* expression level in RNAi-51 using qRT-PCR.

were not detected in any tissue, while others were expressed in specific organs. Interestingly, four soybean *HyPRPs* were almost exclusively highly expressed in leaves, consistent with our finding on *PtrPRP*, which displayed the highest expression level in leaf. These findings suggest that plant *PRPs* may exhibit spatial expression patterns, which implies that the PRPs may function in different ways among various tissues.

In this study, we also noticed that *PtrPRP* was remarkably induced by cold and salt, but underwent minor change under dehydration. Of note, when the abiotic stresses were removed, *PtrPRP* showed opposite expression patterns in comparison with those under the corresponding stresses. These results indicate that *PtrPRP* was sensitively responsive to the stresses, but the response is different among various environmental stimuli. This phenomenon has been also observed in other PRP gene. For example, *OsPRP1* exhibited different or even reverse expression patterns under various stress factors (Wang et al., 2006). In another work, *CcHyPRP* transcripts of pigeonpea (*Cajanus cajan* L.) were shown to be enhanced in response to treatments with PEG, NaCl, heat (42°C), and cold (Priyanka et al., 2010). These

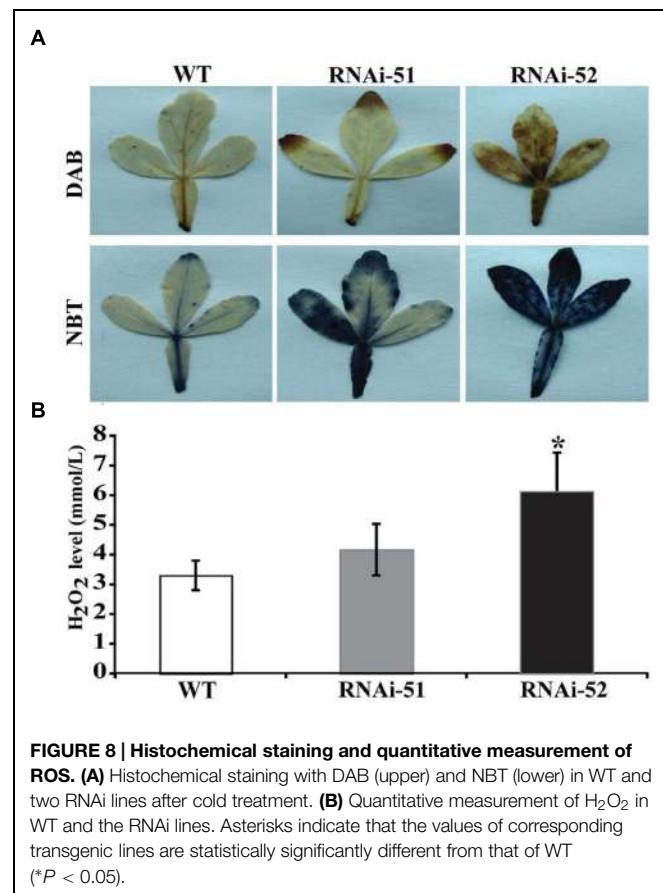
findings suggest that plant PRP genes are differentially regulated under abiotic stresses and that different member of the PRP family may possibly play specific roles in mediating abiotic stress tolerance. In addition, *PtrPRP* was quickly and sharply induced by ABA, implying that *PtrPRP* is an ABA-responsive gene. However, expression of *PtrPRP* under ABA treatment was consistent with *RePRP* of rice and *CcHyPRP* of pigeonpea, but it was contradictory to *OsPRP*, which was repressed by ABA (Akiyama and Pillai, 2003; Priyanka et al., 2010; Tseng et al., 2013). However, whether *PtrPRP* functions in an ABA-dependent manner needs to be elucidated.

Down-regulation of gene expression via RNAi at post-transcriptional level has been widely used as an alternative approach for functionally characterizing genes involved in biotic and abiotic stress tolerance, because RNAi plants may clearly display altered phenotype or metabolic disorders (Mao et al., 2007; Tardieu and Tuberosa, 2010; Duan et al., 2012; Huang et al., 2013; Saurabh et al., 2014). In keeping with this, herein we employed RNAi approach to knock down *PtrPRP* in trifoliate orange so as to elucidate the function of this gene in cold

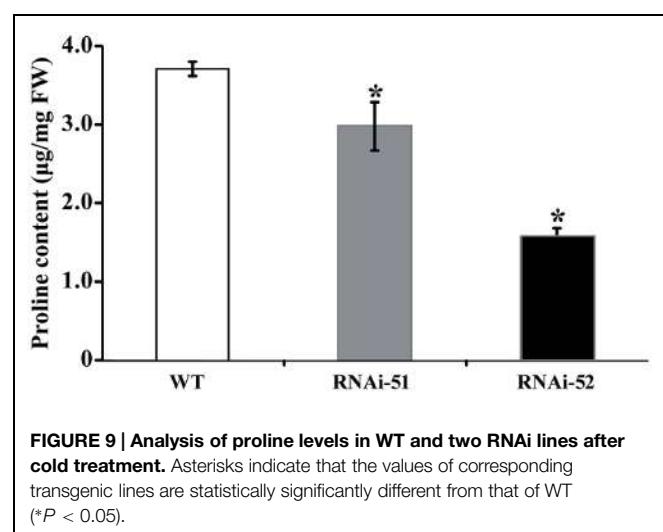


**FIGURE 7 |** Analysis of electrolyte leakage (EL) (A) and malondialdehyde (MDA) (B) in wild type (WT) and two RNAi lines after cold treatment. Asterisks indicate that the values of corresponding transgenic lines are statistically significantly different from that of WT (\*P < 0.05).

stress tolerance. After 48 h of cold treatment, EL and MDA content, two parameters for membrane integrity, in the RNAi lines were higher than in the control, suggesting that knock-down of *PtrPRP* led to severer membrane damage. It was reasonable because *PtrPRP* was localized in the membrane. In higher plant, cell membrane encloses cytoplasm and various organelles; thus maintenance of cell membrane integrity is critical for plant to overcome the physiological and biochemical changes induced by cold stress. The well-documented injuries caused by cold are largely due to osmotic and oxidative stresses that pose serious threat to membrane integrity (Welti et al., 2002; Peng et al., 2014). Therefore, when challenged by low temperature stress, biosynthesis and degradation of some related proteins might be expedited to stabilize the integrity of cellular membranes against cold injury (Achard et al., 2008). PRPs are proposed to play an integral role in consolidation of extracellular matrix structure of plant cells, which is an important approach for elevating mechanical strength to the cell wall. In this case, the PRPs may confer the integrity of plant membranes and promote the structure maintenance of organs (Gothandam et al., 2010). Therefore, it is reasonable to assume that when this protein accumulation was suppressed by RNAi, the protective roles of *PtrPRP* in membrane integrity might be impaired, which in turn leads to greater membrane damage, as revealed by higher EL and MDA levels.



**FIGURE 8 |** Histochemical staining and quantitative measurement of ROS. (A) Histochemical staining with DAB (upper) and NBT (lower) in WT and two RNAi lines after cold treatment. (B) Quantitative measurement of H<sub>2</sub>O<sub>2</sub> in WT and the RNAi lines. Asterisks indicate that the values of corresponding transgenic lines are statistically significantly different from that of WT (\*P < 0.05).



**FIGURE 9 |** Analysis of proline levels in WT and two RNAi lines after cold treatment. Asterisks indicate that the values of corresponding transgenic lines are statistically significantly different from that of WT (\*P < 0.05).

Proline has been documented to act as an important compounds involved in stress tolerance as they can serve as an osmolyte to optimize the physical structure of cell membrane for proper cellular function (Duncan and Widholm, 1987; Akiyama and Pillai, 2003; Rodriguez and Redman, 2005; Battaglia et al., 2007). The proline level has been shown to be associated with the magnitude of a plant to alleviate membrane damages and enhance

cell viability via mitigation of osmotic stress and reduction of MDA production (Ozden et al., 2009), which agrees with our data that MDA content in the two RNAi lines was higher than in the WT. These illustrations suggest that accumulation of less proline may lead to disordered osmotic adjustment, which constitutes a mechanism underlying the cold sensitivity of the RNAi lines. Except acting as an osmolyte, PtrPRP may also contribute to scavenging of ROS, which are by-products of various metabolic processes (Mittler, 2002; Finkel, 2011). Excessive accumulation of ROS is known to cause oxidative damages to cells and thus impairs normal physiological or biological processes. Plant has developed either enzymatic or non-enzymatic antioxidant defense systems to maintain ROS homeostasis and to alleviate the burst of ROS. The non-enzymatic system is composed of a number of compounds that are generally known as low MW antioxidants, such as betaine and proline (Rodriguez and Redman, 2005; Fu et al., 2011; Hayat et al., 2012; Keunen et al., 2013; Peng et al., 2014). In earlier studies, exogenous application of proline has been shown to mitigate ROS production and confer enhanced stress tolerance, implying that proline plays a significant role in ROS scavenging (Hoque et al., 2007; Kaul et al., 2008). Hong et al. (2000) suggested that the role of proline as a ROS scavenger is more important than its role as an osmolyte under stress conditions, further supporting the crucial value of proline in detoxifying ROS. In this work, we found that suppression of *PtrPRP* in the RNAi lines was accompanied by a noticeable reduction of proline, concurrent with the prominent

elevation of ROS levels. These findings indicate that *PtrPRP* may function in cold tolerance by influencing ROS homeostasis due to, at least in part, the role of proline as an efficient antioxidant for ROS detoxification. Nevertheless, why and how knock-down of *PtrPRP* caused a decrease of proline in the RNAi lines remained elusive and needs to be investigated in the future.

## Conclusion

Our data demonstrated that *PtrPRP* of *P. trifoliata* is a hybrid-PRP. *PtrPRP* expresses in various tissues and responds to abiotic stimuli, such as cold, salt, and ABA. RNAi-based knock-down of *PtrPRP* conferred enhanced cold sensitivity of the RNAi lines at chilling temperature, as manifested by severer membrane damage, accumulation of more ROS and less production of proline under cold stress. Therefore, *PtrPRP* may contribute to cold tolerance via modulation of proline, an important osmolyte and ROS scavenger.

## Acknowledgments

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# Melatonin mitigates cadmium phytotoxicity through modulation of phytochelatins biosynthesis, vacuolar sequestration, and antioxidant potential in *Solanum lycopersicum* L.

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Melatonin is a ubiquitous signal molecule, playing crucial roles in plant growth and stress tolerance. Recently, toxic metal cadmium (Cd) has been reported to regulate melatonin content in rice; however, the function of melatonin under Cd stress, particularly in higher plants, still remains elusive. Here, we show that optimal dose of melatonin could effectively ameliorate Cd-induced phytotoxicity in tomato. The contents of Cd and melatonin were gradually increased over time under Cd stress. However, such increase in endogenous melatonin was incapable to reverse detrimental effects of Cd. Meanwhile, supplementation with melatonin conferred Cd tolerance as evident by plant biomass and photosynthesis. In addition to notable increase in antioxidant enzymes activity, melatonin-induced Cd stress mitigation was closely associated with enhanced H<sup>+</sup>-ATPase activity and the contents of glutathione and phytochelatins. Although exogenous melatonin had no effect on root Cd content, it significantly reduced leaf Cd content, indicating its role in Cd transport. Analysis of Cd in different subcellular compartments revealed that melatonin increased cell wall and vacuolar fractions of Cd. Our results suggest that melatonin-induced enhancements in antioxidant potential, phytochelatins biosynthesis and subsequent Cd sequestration might play a critical role in plant tolerance to Cd. Such a mechanism may have potential implication in safe food production.

**Keywords:** cadmium, food safety, melatonin, phytochelatins, tomato, vacuolar sequestration

## Introduction

The rising concern on cadmium (Cd) as a toxic heavy metal, is not only due to its detrimental effects on crop production, but also for potential health hazards associated with food chain contamination (Hall, 2002; Mobin and Khan, 2007; Ajijmaporn et al., 2012). Given that Cd is not an essential nutrient, but easily assimilated in plants due to flexible specificity of ion channels and divalent metal

transporters (Liu et al., 2003). Upon accumulation, Cd disrupts normal metabolism leading to diverse morphological, physiological, biochemical and cellular changes (Llamas et al., 2000; Pietrini et al., 2003). Inhibition of Calvin cycle enzymes, photosynthesis, and carbohydrate metabolism by Cd results in low biomass accumulation in plants (Mobin and Khan, 2007). Although Cd does not participate in Fenton-reaction, it induces reactive oxygen species (ROS) accumulation and eventually causes oxidative stress in plant cells (Romero-Puertas et al., 2002; Pietrini et al., 2003; Cui et al., 2012). Moreover, Cd stress alters plasma membrane permeability by inhibiting H<sup>+</sup>-ATPase, which plays important roles in ionic balance and substance transport (Janicka-Russak et al., 2012). At cellular level, Cd alters plant cell cycle and division, and increases chromosomal aberrations and malformed embryos (DalCorso et al., 2010). Cd contamination severely limits crop production particularly in marginal areas of developing countries, where cultivation is being injudiciously expanded to meet rising demand of food. On the other hand, Cd accumulation in edible plant parts ultimately poses threat to human health as Cd toxicity is closely associated with various health hazards including kidney damage, bladder and lung cancer (Ajijimaporn et al., 2012). Therefore, the enhancement of Cd tolerance in plant to boost crop yield in marginal area as well as reduction of Cd content in edible plant parts for food safety, are ongoing endeavors in plant science and food production.

Plants have intrinsically evolved sophisticated cellular mechanisms for Cd detoxification and tolerance such as immobilization, exclusion, chelation, compartmentalization of metal ions and the repair of cell structural alteration (di Toppi and Gabbrielli, 1999; Hall, 2002). The root cell wall, which is in direct contact with heavy metals in soil, can first release chelating compounds, such as phytosiderophores, nicotianamine and organic acids, to bind Cd and other heavy metals in the epidermis (Xiong et al., 2009). Plant cells can increase the leakage of solutes and reduce influx across plasma membranes to exclude Cd and other heavy metals (Meharg, 1993). Moreover, heat shock proteins and metallothioneins have been reported to repair and protect plasma membranes under heavy metal stress (Neumann et al., 1994; Hall, 2002). The chelation of heavy metals with high-affinity ligands in the cytoplasm and its subsequent compartmentalization in the vacuole are generally considered as the “first line” defense mechanisms in plant Cd tolerance (Hall, 2002; Park et al., 2012). Phytochelatins (PCs) comprise a family of peptides ( $\gamma$ -Glu-Cys)n-Gly ( $n = 2-11$ ) that are synthesized from glutathione (GSH). PCs synthesis is rapidly induced by Cd treatment that chelate Cd and sequester the PC-Cd conjugates into the vacuoles (Rauser, 1995). Besides, GSH activates antioxidant enzymes such as superoxide dismutase (SOD), ascorbic acid peroxidase (APX), and catalase (CAT) that neutralize Cd-induced ROS for cellular redox homeostasis (Jozefczak et al., 2014).

In the past decade, the complex molecular mechanisms of Cd stress response have been extensively elucidated in plants (DalCorso et al., 2010). Involvement of several phytohormones such as abscisic acid (ABA), brassinosteroids (BRs), jasmonic acid (JA), ethylene (EHT), and salicylic acid (SA) was found

to be associated with Cd stress response (Metwally et al., 2003; Maksymiec, 2007; Masood et al., 2012; Ahammed et al., 2013; Stroinski et al., 2013). Previously, we found that exogenous 24-epibrassinolide (EBR, a biologically active BR) could not only alleviate Cd-induced photosynthetic inhibition and oxidative stress, but also could reduce Cd content in tomato plants (Ahammed et al., 2013). Considering the hazardous effects of Cd on plants and animals, extensive researches are still underway to explore new natural compounds which can improve plant tolerance to Cd and/or minimize toxic Cd content in edible plant parts for food safety purpose.

Most recently, Cd has been reported to regulate melatonin content in rice (*Oryza sativa*), a ubiquitous signal molecule, well-accepted as a new plant growth regulator and/or biostimulator rather than plant hormone (Arnao and Hernández-Ruiz, 2014; Byeon et al., 2015). Melatonin (*N*-acetyl-5-methoxytryptamine) has long been known to be an important animal hormone involved in multiple biological processes (Escames et al., 2012; Calvo et al., 2013). Although its function as a hormone has been well-characterized in animals, our knowledge in plant kingdom is still fragmentary (Arnao and Hernández-Ruiz, 2014). In recent years, melatonin appeared as a research focus in plant biology. Melatonin is considered as a plant growth regulator because of its specific physiological functions such as regulation of the growth of roots, shoots and explants, seed germination, rhizogenesis and delay in leaf senescence (Arnao and Hernández-Ruiz, 2014). In addition, melatonin has been found to fortify plant tolerance to a range of abiotic stresses such as drought, cold, heat, salinity, chemical pollutants, herbicides, and UV radiation (Zhang et al., 2015). The mechanisms of melatonin-mediated stress tolerance involve the promotion of antioxidant biosynthesis, the activation of related enzymes and the direct scavenging of ROS following the exposure of plants to harsh environments (Li et al., 2012; Tan et al., 2012; Park et al., 2013; Zhang et al., 2013; Bajwa et al., 2014). Like any other abiotic stress, heavy metals such as Cd significantly induces melatonin content in lower as well as higher plant species. Strikingly, exogenous melatonin can ameliorate Cd-induced stress in green micro algae; however, its role in higher plants still remains elusive. Although PCs are well known to bind many toxic metals including Cd to promote metal detoxification and subsequent stress tolerance in plants, information relating to melatonin-induced PC biosynthesis and/or stress tolerance in higher plants is missing.

To better understand the mechanistic basis of melatonin in plant stress responses, we sought to identify and functionally analyze melatonin under Cd stress. We hypothesize that apart from its well-known antioxidative and chelating properties, melatonin might have a protective role in Cd tolerance involving other mechanisms. We consider PC biosynthesis as a novel target for melatonin-mediated Cd stress response. To testify this hypothesis, we carried out a set of experiments in tomato (*Solanum lycopersicum* L.) taking photosynthetic function and ROS generation as biomarkers. This study explored crucial mechanisms involved in melatonin-mediated Cd stress response which might have potential implication for ensuring food safety and food security in marginal agriculture.

## Materials and Methods

### Plant Materials and Experimental Design

Tomato seeds (*Solanum lycopersicum* L. cv. Ailsa Craig) were germinated in a growth medium containing a mixture of vermiculite and perlite (3:1, v/v) in a greenhouse. When the first true leaves were fully expanded, a group of six uniform seedlings were transferred into a container (40 cm × 25 cm × 15 cm) filled with Hoagland's nutrient solution for hydroponics culture. The growth conditions were as follows: a temperature of 25/17°C (day/night), a mean relative humidity of 80%, a photosynthetic photon flux density (PPFD) of 800  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , and a photoperiod of 14/10 h (day/night).

To investigate the influence of Cd on endogenous melatonin content, the roots of tomato seedlings, at the four-leaf stage were treated with 25 and 100  $\mu\text{M}$  Cd in hydroponics. Cd was supplied in the form of CdCl<sub>2</sub> (analytical grade). Based on our preliminary experiments, 100  $\mu\text{M}$  Cd was selected for studying effects of exogenous melatonin on Cd stress response. Foliar portions of tomato plants were sprayed with 0, 25, 50, 100, 250, and 500  $\mu\text{M}$  melatonin. Hoagland's nutrient solution with or without Cd, were changed at every 5 days followed by foliar spray of melatonin. The experiment was terminated 2 weeks after initial Cd treatment. Four replicates were used for each treatment, and each replicate consisted of 12 plants.

### Gas Exchange and Chlorophyll Fluorescence Measurements

Net photosynthetic rate (*Pn*) and the maximum quantum efficiency of photosystem II photochemistry (*Fv/Fm*) were determined in the second fully expanded leaves from the plant tops. The *Pn* was determined using an infrared gas analyzer (IRGA) portable photosynthesis system (LI-COR 6400, Lincoln, NE, USA). The air temperature, air relative humidity, CO<sub>2</sub> concentration, and PPFD for *Pn* measurement were 25°C, 85%, 400  $\mu\text{mol mol}^{-1}$ , and 1000  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , respectively. The *Fv/Fm* was determined after 30 min of dark adaptation using an imaging pulse amplitude-modulated (PAM) fluorimeter (IMAG-MAXI; Heinz Walz, Effeltrich, Germany) as described (Zhou et al., 2014).

### Determination of H<sub>2</sub>O<sub>2</sub>, Lipid Peroxidation and Electrolyte Leakage

The H<sub>2</sub>O<sub>2</sub> and MDA concentrations in leaves and roots were assessed spectrophotometrically. To determine the H<sub>2</sub>O<sub>2</sub> concentration, 0.3 g of fresh sample was homogenized in 3 mL of pre-cooled HClO<sub>4</sub> (1.0 M) using a pre-chilled mortar and pestle (Willekens et al., 1997). The homogenates were then transferred to 10-mL plastic tubes and centrifuged at 6000 g for 5 min at 4°C. The pH of resulting supernatant's was adjusted to 6.0 with 4 M KOH and centrifuged at 6000 g for 1 min at 4°C. The resulting supernatant was passed through an AG1.8 prepak column (Bio-Rad, Hercules, CA, USA) and H<sub>2</sub>O<sub>2</sub> was eluted with double-distilled H<sub>2</sub>O. The sample (800  $\mu\text{L}$ ) was mixed with 400  $\mu\text{L}$  reaction buffer containing 4 mM 2,2'-azino-di(3-ethylbenzthiazoline-6-sulfonic acid) and 100 mM potassium acetate at pH 4.4, 400  $\mu\text{L}$  deionized water, and 0.25 U

of horseradish peroxidase. Equal recovery from the different samples was checked by analyzing duplicate samples. The lipid peroxidation level was determined by quantifying the MDA equivalents using 2-thiobarbituric acid (TBA), as described by Hodges et al. (1999). In brief, samples (0.5 g) were homogenized in 5 mL 0.1% (w/v) trichloroacetic acid (TCA). The homogenates were centrifuged at 10,000 g for 10 min, and 4 mL 20% TCA containing 0.65% (w/v) TBA was added to 1 mL of the supernatants. The mixtures were heated in a hot water bath at 95°C for 25 min and immediately placed in an ice bath to stop the reaction. They were then centrifuged at 3000 g for 10 min, and the absorbance of the supernatants was recorded at 440, 532, and 600 nm. The MDA equivalents were calculated as described previously (Hodges et al., 1999). To the measurements of electrolyte leakage (EL), plant samples were cut into small pieces with razor blade, rinsed briefly with deionized water, and immediately placed in a tube with 5 mL of deionized water. EL was then measured before and after 4 h of rehydration and ultimately after autoclaving according to the method described by Bajji et al. (2002).

### Histochemical Staining of H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>-</sup> in Leaves

H<sub>2</sub>O<sub>2</sub> accumulation in leaves was visually detected by staining with 3,3-diaminobenzidine (DAB) using the method as described (ThordalChristensen et al., 1997). Leaves were immediately removed from plants, submerged in DAB solution (1 mg mL<sup>-1</sup>, pH 3.8) and incubated under light for 6 h at 25°C. O<sub>2</sub><sup>-</sup> was detected with the nitroblue tetrazolium (NBT) staining procedure, which involved the incubation of the leaves with NBT (0.5 mg mL<sup>-1</sup>, pH 7.8) solution in the dark (Jabs et al., 1996).

### Extraction and Assay of Antioxidant Enzymes

For enzyme extraction, 0.3 g of fresh sample was homogenized in 50 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA and 1% polyvinylpyrrolidone (w/v). The homogenate was centrifuged at 12,000 g for 15 min at 4°C, and the supernatant was collected. This supernatant was used to measure the activities of SOD, guaiacol peroxidase (G-POD), APX, CAT, and glutathione reductase (GR) enzymes. SOD activity was assayed by determining its ability to inhibit the photochemical reduction of NBT as described (Beauchamp and Fridovic, 1971). G-POD activity was assessed using guaiacol as a substrate (Cakmak and Marschner, 1992). APX activity in the tomato leaves was determined as described (Nakano and Asada, 1981). CAT activity was measured according to the method as described (Cakmak and Marschner, 1992). GR activity was measured and calculated as described (Foyer and Halliwell, 1976). For the assessment of H<sup>+</sup>-ATPase activity, 0.3 g of fresh sample was homogenized in 50 mM Tris-HCl buffer. Then, the release of Pi was measured using an activity assay kit (Jiancheng Bio Co., Nanjing, Jiangsu, China) as described (Ahmed et al., 2013).

### Extraction and Quantification of Endogenous Melatonin by HPLC

To measure the endogenous plant melatonin concentration, a direct sampling extraction procedure was used as described

(Arnao and Hernandez-Ruiz, 2009a) with modifications. Fresh leaf or root samples (0.3 g) were cut into small sections and placed into a 15 mL tube containing 6 mL of chloroform, which was shaken overnight at 4°C in the dark. The sections were extracted again using 2 mL of chloroform. The supernatant was transferred to a C<sup>18</sup> solid-phase extraction (SPE) cartridge (Waters, Milford, MA, USA) for the purification of melatonin. The extract was then concentrated to dryness under nitrogen. The residue was dissolved in 2 mL of the mobile phase for HPLC analysis as described (Korkmaz et al., 2014).

### Extraction, Derivatization, and Separation of Thiol Compounds by HPLC

For thiol extraction, fresh leaf, or root samples (1 g) were homogenized with liquid nitrogen, and then 1 mL of extraction buffer containing 6.3 mM diethylene triamine pentaacetic acid (DTPA) and 0.1% (v/v) trifluoroacetic acid (TFA) was added. The supernatant was collected by centrifugation at 12,000 g for 10 min at 4°C. The derivatization of the thiol compounds GSH and PCs was analyzed with monobromobimane (mBBr) according to the method as described (Minocha et al., 2008). The derivative samples were filtered with 0.45 μm nylon syringe filters and stored at -20°C for up to 1 week for HPLC analyses. The thiol compounds were separated by HPLC using a fluorescence detector and a Phenomenex Synergi Hydro-RP C<sup>18</sup> column (4 μm particle size, 100 mm × 4.6 mm) and a C<sup>18</sup> SecurityGuard<sup>TM</sup> (5 μm, 4 mm × 3 mm) cartridge guard column. The excitation and emission wavelengths were set at 380 and 470 nm, respectively, as described (Minocha et al., 2008).

### Histochemical Localization of Cd

The histochemical detection of Cd in both the leaf and root tissues was performed using a staining-based method (Seregin and Ivanov, 1997). This method involves staining with dithizone, a reagent that produces an insoluble red salt (Cd-dithizonate) in the presence of Cd. Plant samples were removed from the glutaraldehyde solution, and cross sections were taken from the root tips or the leaf veins. The sections were reacted with 2–3 drops of dithizone solution for 15 min, and then they were examined under a light microscope (BX61; Olympus Co., Tokyo, Japan).

### Separation of Tissue Fractionations

To observe the subcellular compartmentalization of Cd, the tissues were separated into the following four fractions using differential centrifugation techniques: the cell wall (CW), vacuole (V), organelle (O), and soluble (S) fractions. All of the fractions except for the V fraction were separated as previously described (Wu et al., 2005). Cell vacuoles were separated according to the method as described (Marty and Branton, 1980). In brief, fresh (5 g) root and leaf samples were homogenized in pre-chilled extraction buffer containing 50 mM Tris-HCl (pH 7.5), 250 mM sucrose, 1.0 mM DTE (C<sub>4</sub>H<sub>10</sub>O<sub>2</sub>S<sub>2</sub>), 5.0 mM ascorbic acid and 1.0% w/v Polyclar AT PVPP, pH 7.5. The homogenate was sieved through a nylon cloth (80 μM), and the residue on the nylon cloth was washed twice with the same homogenization buffer and designated as the CW fraction. This first filtrate was centrifuged

at 2,000 g for 10 min, and the precipitate was designated as the V fraction. The resulting supernatant solution was further centrifuged at 15,000 g for 30 min. The resulting pellet and supernatant were referred to as the O and S fractions, respectively. All steps were performed at 4°C.

### Determination of Cd

The different separated cell fractions or 0.20 g of homogenized dry powdered sample were digested with an HClO<sub>4</sub> and HNO<sub>3</sub> mixture (v/v = 1:3) at 180°C. The digested colorless liquids were washed with 2 mL of diluted HNO<sub>3</sub> (distilled water/concentrated HNO<sub>3</sub> = 1:1) and were then washed three times with distilled water. The liquid was collected and transferred to 50 mL volumetric flasks and diluted to a constant volume. The Cd concentrations in the different fractions and the total Cd were analyzed using an atomic absorption spectrophotometer (AA-6300; Shimadzu Co., Kyoto, Japan; Wu et al., 2005).

### Extraction of Total RNA and Quantitative Real-Time PCR (qRT-PCR) Analyses

Total RNA extraction from the tomato leaf and root tissues was performed with an RNA extraction kit (Tiangen, Shanghai, China). A 1 μg aliquot of total RNA was reverse-transcribed to generate cDNA using a ReverTra Ace qPCR RT Kit (Toyobo, Japan), following the manufacturer's instructions. qRT-PCR was performed with SYBR Green PCR Master Mix (Takara, Tokyo, Japan) using an ABI Step One Plus real-time machine (Eppendorf, USA) under the default thermal cycling conditions with an added melting curve. The gene-specific primers used for qRT-PCR were designed based on the mRNA or expressed sequence tag (EST) sequences of the corresponding genes (Supplementary Table S1). The PCR conditions consisted of denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s. Relative transcript levels were calculated as described by Livak and Schmittgen (2001).

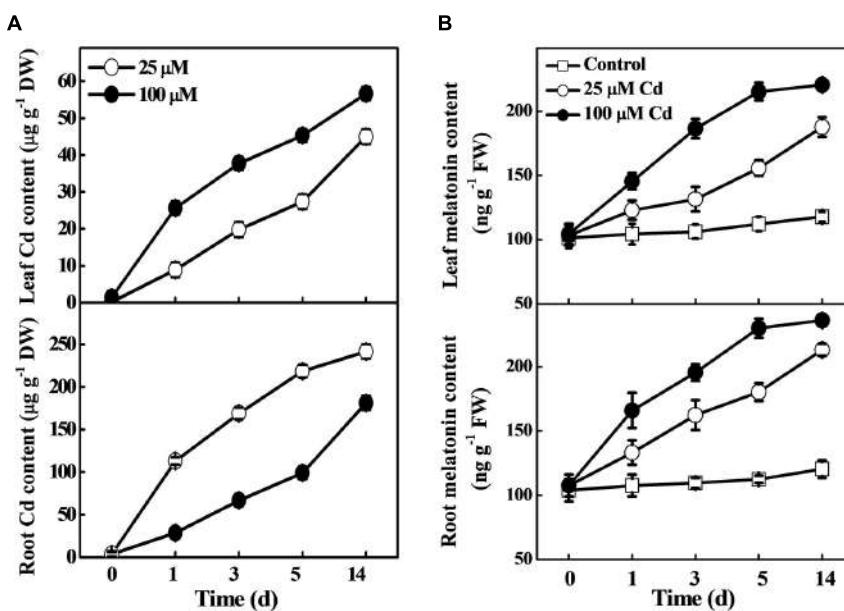
### Statistical Analysis

Data were subjected to ANOVA and presented as the mean value for each treatment. Statistical analyses were performed using Data Processing System (DPS) statistical software package. A Tukey's test (*P* < 0.05) was performed to evaluate the treatment effects. Each treatment value is the average of four to six replicates.

## Results

### Treatment with Cd Induces Melatonin Biosynthesis

To examine the effect of ecotoxic Cd on melatonin biosynthesis, Cd uptake and translocation, we determined the concentrations of melatonin and Cd in roots and leaves of tomato plants at various time-points. As shown in **Figure 1A**, both the low (25 μM) and high (100 μM) concentration of Cd induced a substantial increase in Cd accumulation in tomato leaves and



**FIGURE 1 |** Accumulation of cadmium (Cd) and melatonin in tomato leaves and roots as influenced by exogenous Cd treatment. Tomato seedlings at the four-leaf stage were treated with 25 and 100  $\mu\text{M}$  Cd in hydroponics. Endogenous contents

of Cd (**A**) and melatonin (**B**) were determined at the indicated time points. The data are the means of four replicates, where vertical bars indicate standard errors. DW, dry weight; FW, fresh weight.

roots. In parallel with the Cd content, melatonin accumulation was gradually increased over time after Cd treatment. Notably, high Cd treatment induced high accumulation of melatonin as compared with low Cd treatment. Meanwhile, under optimum conditions, the melatonin concentration remained almost stable in both leaves and roots (**Figure 1B**). Eventually, the Cd-treated plants displayed approximately 59.6/77.2% and 87.4/96.3% increased melatonin concentration in leaves/roots than those in control under the low and high Cd treatments, respectively at 14 days (**Figure 1B**). These results confirmed that exogenous Cd could induce endogenous melatonin accumulation in leaves and roots of tomato plants.

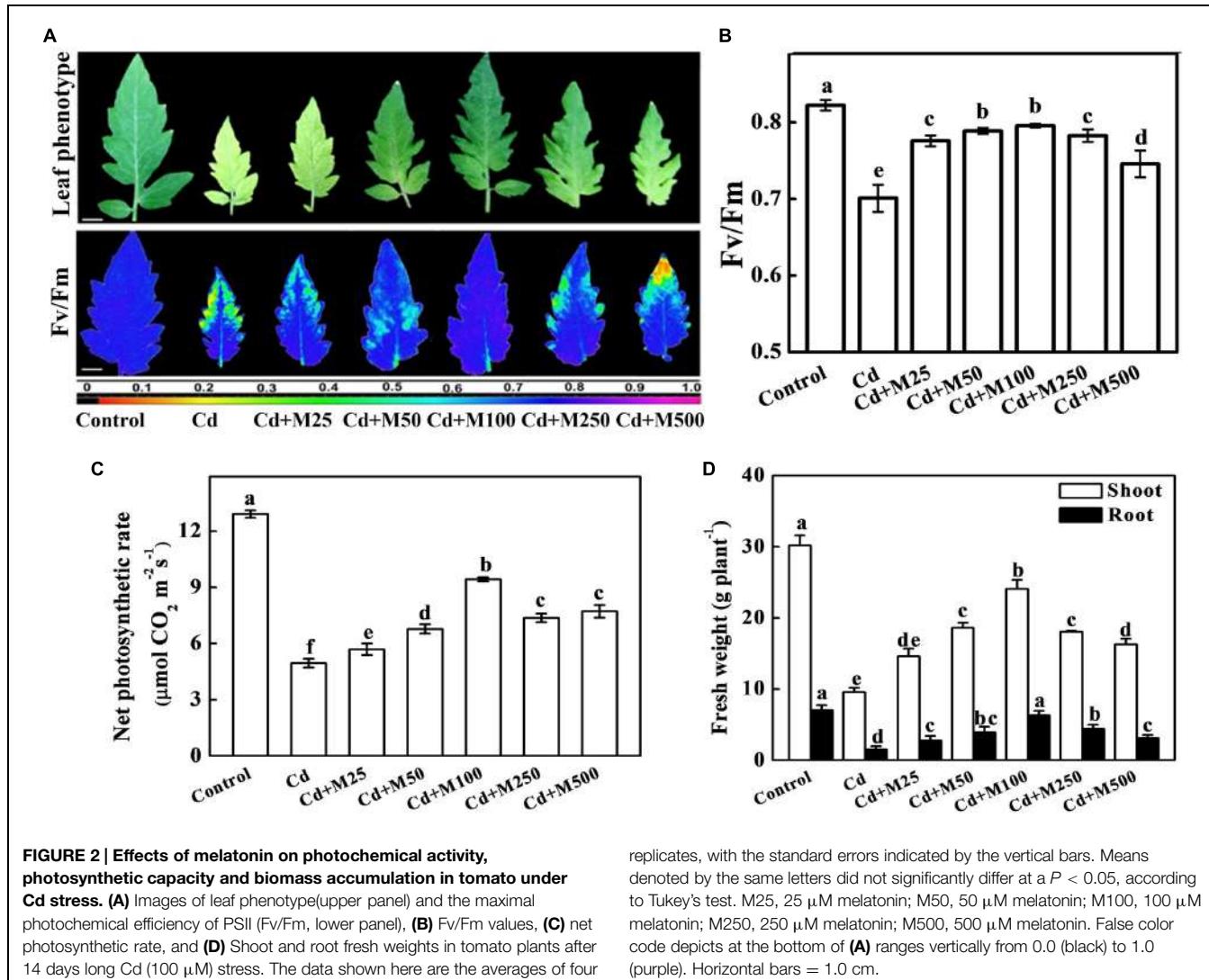
### Effects of Exogenous Melatonin on Plant Growth, Photosynthesis, and Biomass Accumulation under Cd Stress

To investigate the role of exogenous melatonin in Cd tolerance, we first focused on the phenotypic changes that occurred in the tomato plants. As shown in **Figure 2**, plants treated with only Cd displayed severe leaf chlorosis, and decreased  $Fv/Fm$  (18.6%),  $Pn$  (62.1%), and fresh biomass (67.2%) when compared with that in control. In contrast, almost all doses of melatonin significantly reversed deleterious effects of Cd. Among the tested concentrations of melatonin, moderate concentration particularly 100  $\mu\text{M}$  melatonin showed profound ameliorative effect as evident by 13.5 and 90.4% increased  $Fv/Fm$  and  $Pn$ , respectively compared with Cd alone treatment (**Figures 2B,C**). Similarly, Cd-induced reductions in the fresh weights of both shoot and root were significantly alleviated by the treatments with moderate concentrations of exogenous

melatonin (**Figure 2D**). Considering the studied phenotypes, the order of efficiency for the melatonin concentrations in achieving positive effects against Cd was as follows: 100 > 50 > 250 > 500 > 25  $\mu\text{M}$ .

### Melatonin Protects Plants from Cd-Induced Oxidative Damage

Results from histochemical study showed that the ROS ( $\text{H}_2\text{O}_2$  and  $\text{O}_2^-$ ) concentrations remained almost constant in leaves under control conditions; however, Cd treatment caused substantial increases in ROS accumulation (**Figures 3A,B**). Strikingly, melatonin application decreased the excess ROS accumulation under Cd stress (**Figures 3A,B**). The 100  $\mu\text{M}$  melatonin treatment caused the greatest reduction in ROS accumulation resulting in 43.1/35.9% decreased  $\text{H}_2\text{O}_2$  in the tomato leaves/roots under Cd stress (**Figure 3B**). The MDA concentrations and EL in leaves/roots were increased by approximately 112/139.2% and 118.5/159.3% in the leaf/root of Cd-treated plants compared with the control plants (**Figures 3C,D**). In line with the effect of melatonin on ROS accumulation, melatonin treatments prevented the Cd-induced increases in EL and MDA to a varying degree (**Figures 3C,D**). We also quantified the endogenous content of melatonin under different treatments (Supplementary Figure S1). It appears that exogenous melatonin has strong effect on endogenous melatonin accumulation under Cd stress, while greatest effect accounts for 100  $\mu\text{M}$  melatonin. These results suggest that the application of melatonin induces endogenous melatonin content to mitigate Cd phytotoxicity through minimizing Cd-induced oxidative stress in the tomato plants.



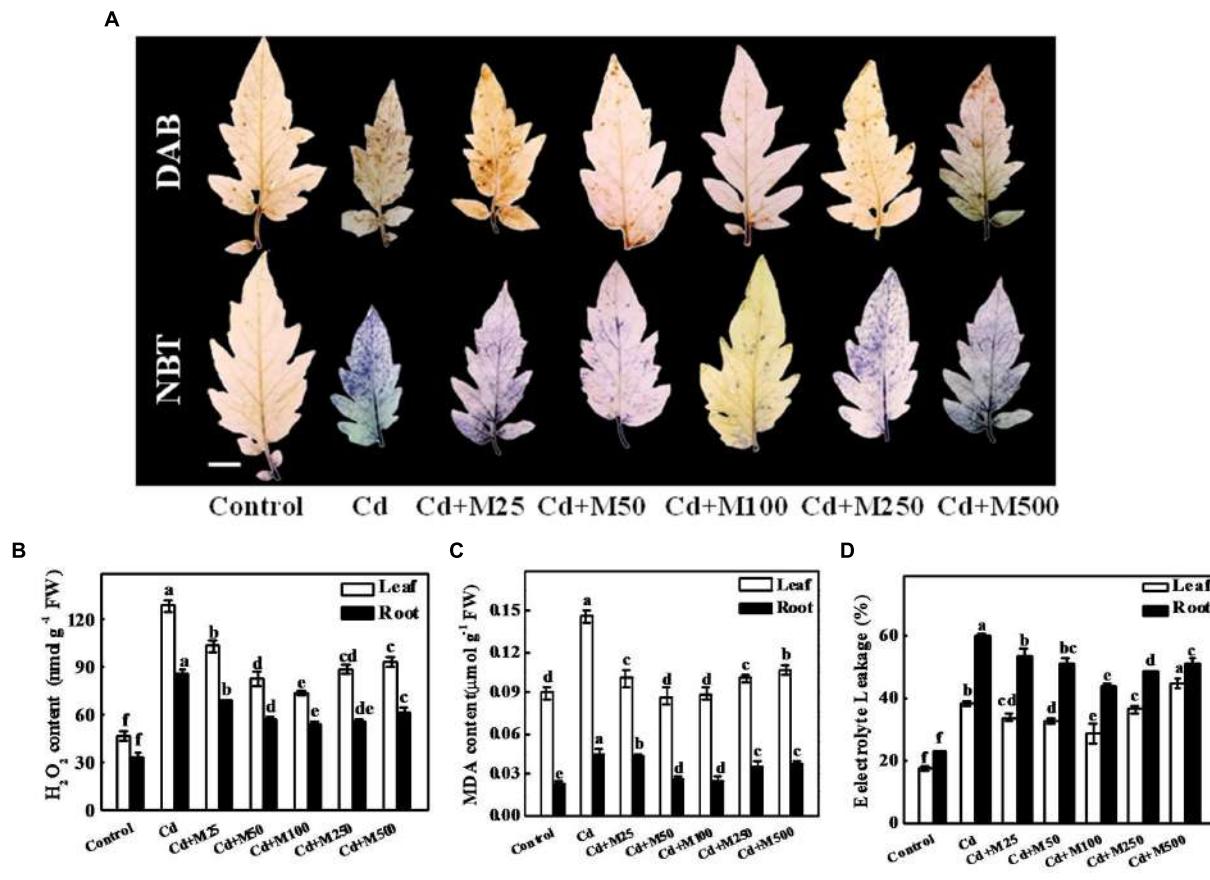
## Melatonin Stimulates Activities of Antioxidant Enzyme and H<sup>+</sup>-ATPase to Confer Cd Tolerance

To investigate the role of melatonin in the regulation of the plant antioxidative system under Cd stress, we assessed the activities of SOD, G-POD, CAT, and APX. Cd treatment induced the activities of antioxidant enzymes both in leaves and roots (Figure 4). Interestingly, the exogenous application of different concentrations of melatonin induced further increases in the activities of these enzymes at various degrees. Melatonin treatment at 100  $\mu\text{M}$  concentration showed the best efficiency in this regard. The activities of SOD, G-POD, CAT, APX, and GR were increased by approximately 19.5/15.5%, 84.5/42.8%, 66.6/91.4%, 86.5/82.6%, and 80.2/52.3%, respectively, in leaves/roots under Cd+M100 treatment compared with Cd alone (Figure 4). Likewise, melatonin treatment induced the activity of plasma membrane H<sup>+</sup>-ATPase which acts as a primary transporter for pumping protons out of the cell (Morsomme and Boutry, 2000). Although Cd induced a 7.1-fold increase in

H<sup>+</sup>-ATPase activity in roots, there was no significant difference in H<sup>+</sup>-ATPase activity in leaves between the Cd-treated and control plants (Figure 4). Meanwhile, compared with Cd alone treatment, H<sup>+</sup>-ATPase activity was further induced by combined treatment of melatonin and Cd (Figure 4). These results provide evidence that melatonin-induced alleviation of oxidative stress is associated with the up-regulation of the activities of antioxidant enzymes and H<sup>+</sup>-ATPase in the leaves and roots of tomato plants.

## Melatonin Promotes Cd Sequestration in Tomato Plants

To determine the role of melatonin in the biosynthesis of intracellular chelating compounds, we examined the concentrations of various PCs (PC<sub>2</sub>, PC<sub>3</sub>, and PC<sub>4</sub>) and GSH (precursor of PCs synthesis) under Cd stress (Figure 5). Cd treatment significantly increased the endogenous concentrations of PC<sub>2</sub>, PC<sub>3</sub>, PC<sub>4</sub>, and GSH by 9.4/8.9-fold, 7.9/10.0-fold, 14.8/28.7-fold, and 1.2/3.0-fold in leaves/roots, respectively (Figure 5). Interestingly, the treatments with the different



**FIGURE 3 |** Effects of melatonin on ROS accumulation, lipid peroxidation, and membrane integrity after 14 days long Cd stress.

(A) The *in situ* detection of H<sub>2</sub>O<sub>2</sub> (upper panel) and O<sub>2</sub><sup>-</sup> (lower panel) in tomato leaves. Bar = 1.0 cm, (B) H<sub>2</sub>O<sub>2</sub> concentrations in tomato leaves and roots, (C) MDA concentrations in tomato leaves and roots, and (D) Electrolyte leakage from tomato leaves and roots. Accumulation of H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>-</sup> in leaves was visually detected by staining with 3,

3-diaminobenzidine (DAB) and nitroblue tetrazolium (NBT), respectively. The data shown are the averages of four replicates, with the standard errors indicated by the vertical bars. The means denoted by the same letter within the same color histograms did not significantly differ at a *P* < 0.05, according to Tukey's test. Cd, 100  $\mu$ M cadmium; M25, 25  $\mu$ M melatonin; M50, 50  $\mu$ M melatonin; M100, 100  $\mu$ M melatonin; M250, 250  $\mu$ M melatonin; M500, 500  $\mu$ M melatonin; FW, fresh weight.

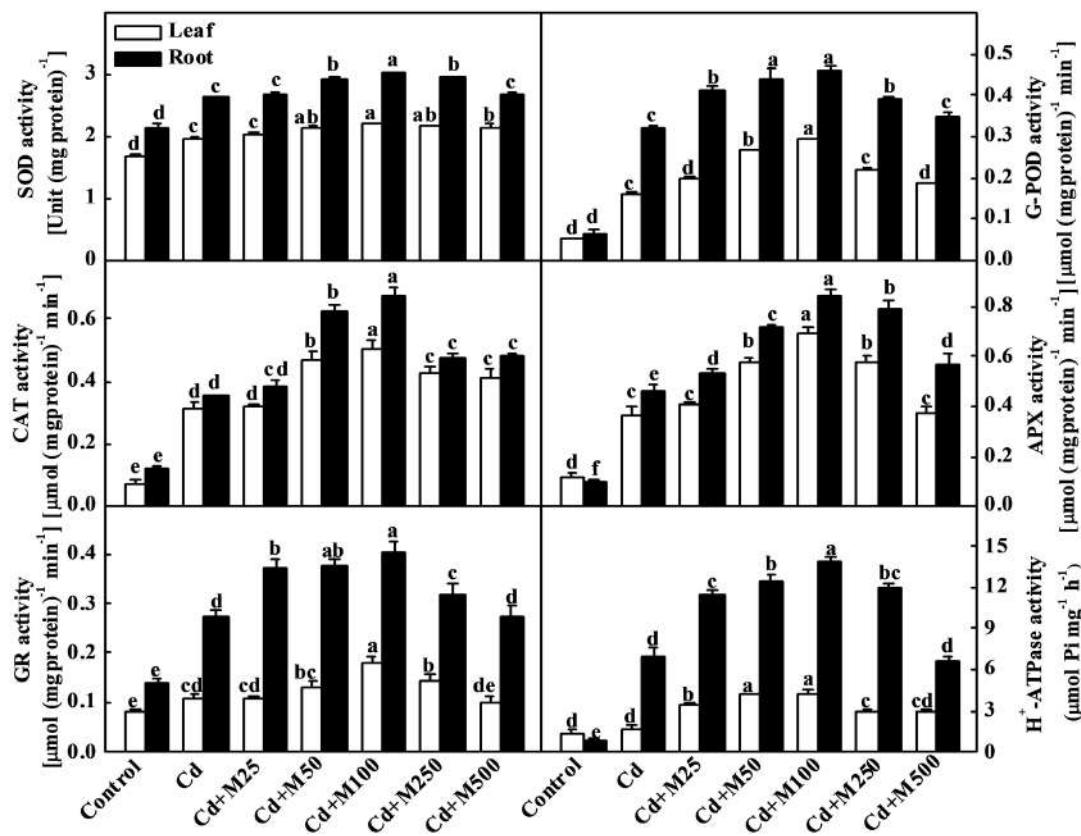
concentrations of exogenous melatonin further increased the levels of these thiol compounds in roots under Cd stress (Figure 5). The levels of GSH, PC<sub>2</sub>, PC<sub>3</sub>, and PC<sub>4</sub> were increased by 46.0, 67.8, 80.7, and 66.5%, preferentially in roots following the 100  $\mu$ M melatonin treatment under Cd stress (Figure 5).

To further understand the molecular mechanism of melatonin-induced Cd detoxification, we analyzed the expression of the *SIGSH1*, *SIPCS*, *SIMT2*, and *SIABC1* genes, which are responsible for the synthesis of GSH, PCs, and metallothionein (Whitelaw et al., 1997; Wang et al., 2010; Ahammed et al., 2013; Patade et al., 2013), and ABC transporter that transports Cd-PC complex to vacuole (Hall, 2002; Park et al., 2012). The transcript levels of *SIGSH1*, *SIPCS*, *SIMT2*, and *SIABC1* were all induced by Cd stress (Figure 6). Importantly, exogenous melatonin further increased the transcript levels of those genes, and the 100  $\mu$ M melatonin treatment was the most effective in this regard. The expression levels of *SIGSH1*, *SIPCS*, *SIMT2*, and *SIABC1* were increased by approximately 1.4/1.7-fold, 1.3/1.5-fold, 1.2/1.7-fold, and 1.5/1.4-fold in the leaves/roots of the plants treated

with 100  $\mu$ M melatonin compared with those in the leaves/roots of the non-melatonin-treated plants under Cd stress (Figure 6). These observations suggest that melatonin might be involved in the biosynthesis of metal chelating compounds and transport of Cd during Cd stress.

### Role of Melatonin in the Uptake, Subcellular Distribution, and Localization of Cd in Plant Cells

To understand the effects of melatonin on Cd uptake and its cellular distribution in tomato cells, we assessed the total Cd content, subcellular distribution and localization in leaf and root tissues. The Cd concentrations were approximately 224.19 and 54.16  $\mu$ g (g DW)<sup>-1</sup> in roots and leaves, respectively, after 14-days long Cd treatment (Figure 7A). Interestingly, Cd accumulation was not altered in roots but was significantly reduced in leaves with the melatonin treatments (Figure 7A). Treatment with 100  $\mu$ M melatonin resulted in the greatest reduction in the leaf Cd concentration which was about 39.15% below the Cd



**FIGURE 4 | Activities of antioxidant enzymes and  $\text{H}^+$ -ATPase in tomato leaves and roots as influenced by melatonin and Cd treatments.** Tomato seedlings at the four-leaf stage were subjected to 100  $\mu\text{M}$  Cd in hydroponics for 14 days. Meanwhile, foliar portion was sprayed with various concentrations of melatonin at every 5 days. The data shown here are the averages of four

replicates, with the standard errors indicated by the vertical bars. The means denoted by the same letter did not significantly differ at a  $P < 0.05$ , according to Tukey's test. Cd, 100  $\mu\text{M}$  cadmium; M25, 25  $\mu\text{M}$  melatonin; M50, 50  $\mu\text{M}$  melatonin; M100, 100  $\mu\text{M}$  melatonin; M250, 250  $\mu\text{M}$  melatonin; M500, 500  $\mu\text{M}$  melatonin.

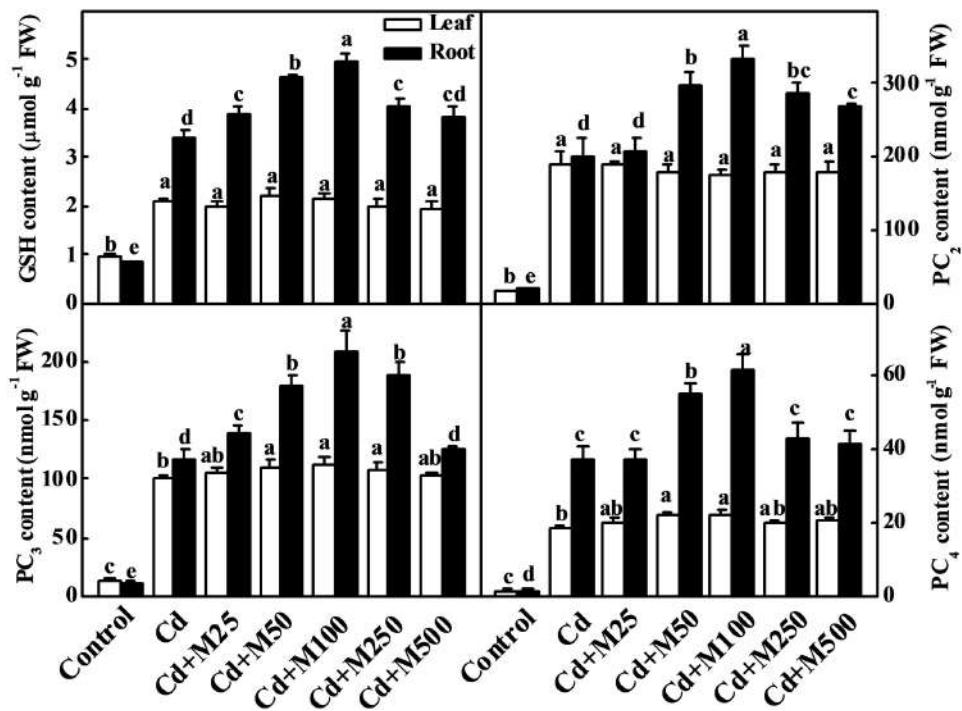
alone treatment (**Figure 7A**). We also visualized the localization of Cd in root and leaf tissues using a dithizone staining-based histochemical method. As shown in **Figure 7B**, compared with leaf tissue, intensive Cd accumulation was observed in root steles regardless of the melatonin treatment. Similarly, the less intensive brown color deposits in leaf tissues indicated lower Cd content in leaf as compared with root. In line with quantitative data, visualization of Cd in Cd + 100  $\mu\text{M}$  melatonin treatment showed less Cd accumulation in leaf tissues when compared with Cd alone treatment (**Figure 7B**).

To examine the subcellular distribution of Cd as influenced by exogenous melatonin treatments, we quantified the Cd content in various cellular compartments of leaf and root tissues. We found that under Cd alone treatment, the Cd levels in the cell wall (CW), vacuole (V), organelle (O), and soluble (S) fractions were approximately 10.6/11.3%, 15.3/13.1%, 42.1/40.2%, 32.0/35.4% of total Cd content in the root/leaf cells, respectively (**Figure 7C**). Importantly, treatment with 100  $\mu\text{M}$  melatonin altered the subcellular distributions of Cd in both root and leaf cells. The Cd levels in the CW and V fractions, where Cd is preferentially sequestered to minimize cellular toxicity, were increased by 25.9/24.3% and 42.1/44.1% in the root/leaf cells, respectively after

melatonin treatment (**Figure 7C**). Meanwhile, the Cd levels in the O and S fractions, where Cd is mostly free and highly toxic, were decreased by 74.1/75.6% and 57.8/55.9% in the root/leaf cells, respectively after melatonin treatment (**Figure 7C**). These findings indicate a critical role of melatonin in promoting Cd immobilization for minimizing cellular toxicity.

## Discussion

In the present work, we elucidated some unique functions of melatonin in response to Cd stress in tomato, an important horticultural crop and model plant system as well. We noticed that endogenous melatonin could be induced by both low and high concentrations of Cd (**Figure 1**). Notably, exogenous application of melatonin significantly improved plant tolerance to Cd by enhancing the antioxidant capacity, PCs biosynthesis, and compartmentalization of Cd in cell walls and vacuoles (**Figures 2–7**). Besides, melatonin restricted Cd transport from root to shoot in order to protect photosynthetic apparatus from Cd-induced damages. We propose that, in addition to direct function of melatonin as antioxidant and metal



**FIGURE 5 | Effects of melatonin on GSH and PCs concentrations in tomato leaves and roots after 14 days long cadmium stress.** The data shown here are the averages of four replicates, with the standard errors indicated by the vertical bars. The means denoted by the same letter within the

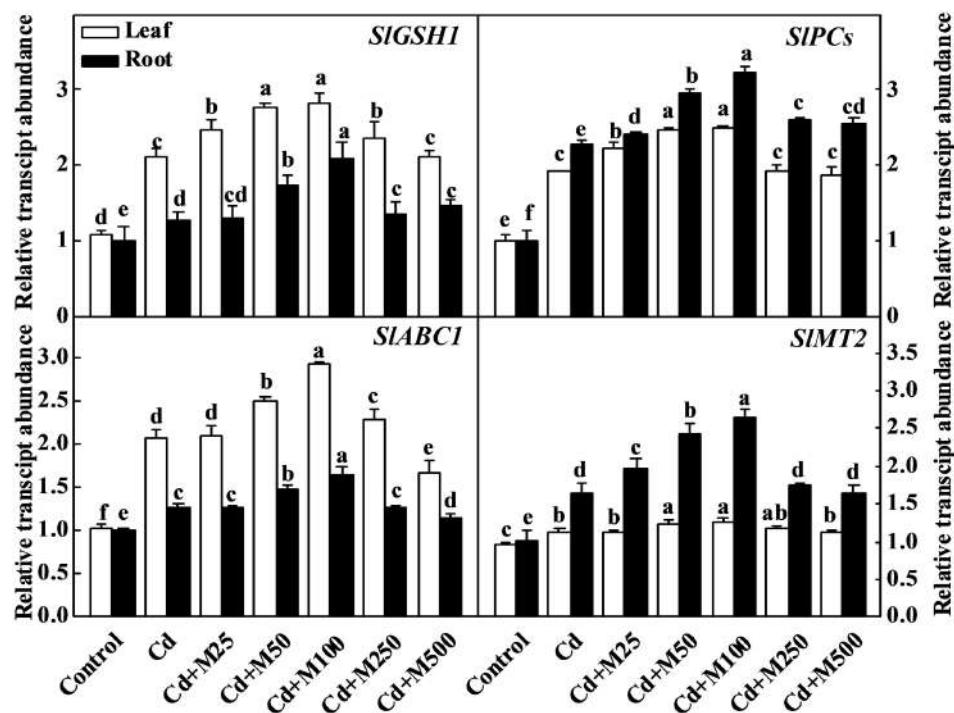
same color histograms did not significantly differ at a  $P < 0.05$ , according to Tukey's test. Cd, 100  $\mu\text{M}$  cadmium; M25, 25  $\mu\text{M}$  melatonin; M50, 50  $\mu\text{M}$  melatonin; M100, 100  $\mu\text{M}$  melatonin; M250, 250  $\mu\text{M}$  melatonin; M500, 500  $\mu\text{M}$  melatonin; FW, fresh weight.

chelator, melatonin-induced biosynthesis of phytochelatins and subsequent compartmentalization of Cd in cell wall and vacuole might play a critical role to confer Cd tolerance in tomato.

Melatonin is a major animal hormone involved in regulating seasonal reproductive function and modulating circadian rhythms. In recent years, a growing body of evidence has suggested that it plays a significant role in plant responses to environmental stimuli, including biotic and abiotic stresses (Park et al., 2013; Zhang et al., 2013; Bajwa et al., 2014; Lee et al., 2014). Oxidative stress resulted from various forms of environmental extremes has been reported to affect the endogenous level of melatonin in a range of plant species (Arnao and Hernandez-Ruiz, 2009b). Additionally, exogenous application of melatonin was found effective to protect plant cell from oxidative damage (Zhang et al., 2015). In the present study, exposure of tomato seedlings to low and high concentrations of Cd induced substantial increases in endogenous melatonin concentrations gradually over time. However, such increase in endogenous melatonin was unable to ameliorate oxidative stress possibly due to insufficient ROS scavenging by melatonin in parallel with Cd-induced ROS generation. Nevertheless, induction of endogenous melatonin by Cd may indicate an adaptive response in plants as melatonin is well-recognized as a ubiquitous signal molecule (Arnao and Hernández-Ruiz, 2014; Byeon et al., 2015). In contrast, exogenous application of melatonin efficiently ameliorate Cd-induced oxidative stress as characterized by low ROS accumulation, reduced MDA content and decreased EL

percentage. We also noticed that exogenous melatonin greatly induced endogenous melatonin content in a concentration-dependent manner. The highest level of melatonin was recorded in 100  $\mu\text{M}$  melatonin + Cd treatment followed by a decreasing trend with the increase in exogenous melatonin concentration (Supplementary Figure S1). This observation confirmed that exogenous melatonin could stimulate endogenous melatonin level in a dose dependent manner to confer Cd tolerance. It is worth mentioning that low and high concentration of melatonin showed significantly different roles in regulating plant growth and development even in the same species, which support our current observation (Zhang et al., 2015). By virtue, melatonin can cross cell membrane easily and enter subcellular compartments. Given that melatonin is a ubiquitous antioxidant and thus it might be plausible that exogenous melatonin functions as an antioxidant at the initial stage, which is strictly supply dependent (Poeggeler et al., 1996; Tan et al., 2000). As the frequency of melatonin application was sparse in our current study, exogenous melatonin might not suffice proper neutralization of ROS generated from continuous Cd exposure, indicating some other mechanisms involved in melatonin-mediated Cd stress tolerance. It is quite possible that exogenous melatonin may trigger endogenous melatonin biosynthesis which then serves as antioxidant and also regulates other defense pathways for environmental adaptation.

Recent studies have revealed that pre-sowing seed treatment with melatonin protects red cabbage seedlings against toxic Cu<sup>2+</sup>



**FIGURE 6 | Expression genes related to thiol compound biosynthesis in tomato leaves and roots following 14 days cadmium stress as influenced by melatonin treatments.** The data shown here are the averages of four replicates, with the standard errors indicated by the vertical bars. The means denoted by the

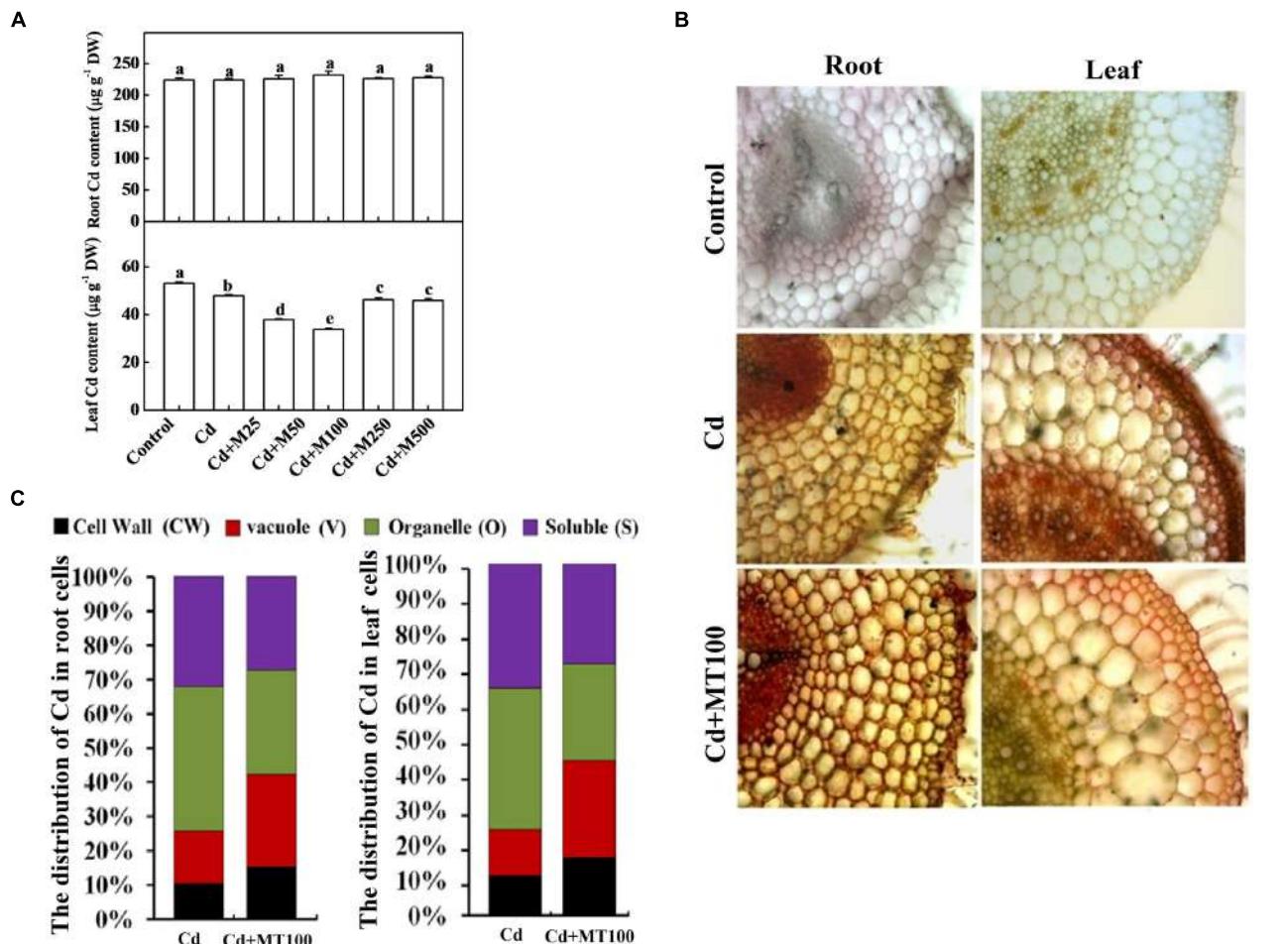
same letter within the same color histograms did not significantly differ at a  $P < 0.05$ , according to Tukey's test. Cd, 100  $\mu\text{M}$  cadmium; M25, 25  $\mu\text{M}$  melatonin; M50, 50  $\mu\text{M}$  melatonin; M100, 100  $\mu\text{M}$  melatonin; M250, 250  $\mu\text{M}$  melatonin; M500, 500  $\mu\text{M}$  melatonin; FW, fresh weight.

(Posmyk et al., 2008), and the supplementation with exogenous melatonin alleviates Cd-induced stress in algae (Tal et al., 2011). In cucumber seedlings, melatonin treatment decreased chlorophyll degradation and enhanced the photosynthetic rate and antioxidant potential, and thereby ameliorating the deleterious effects of water stress (Zhang et al., 2013). Consistent with these findings, we also found that melatonin increased the chlorophylls contents (data not shown), photosynthetic capacity and biomass accumulation in both non-stressed and Cd-stressed plants. (Figure 2; Supplementary Figure S2). It is speculated that melatonin increases photosynthetic efficiency through an unusual biostimulatory pathway by improving the efficiency of photosystem II under both dark and light conditions in plants (Wang et al., 2013). Serving as a signal, melatonin can activate the antioxidant system to promote the scavenging of stress-induced ROS (Tal et al., 2011; Park et al., 2013). Accordingly, melatonin activated SOD, G-POD, APX, CAT, and GR in the present study, which might partially contribute to ROS scavenging under Cd stress (Figure 4). Taken together, it is quite likely that melatonin acts as a signal molecule to trigger defense systems, such as the antioxidant system, resulting in the alleviation of oxidative stress.

Phytochelatins are synthesized from GSH through an enzymatic pathway, and they chelate metal ions with the -SH groups of cysteine residues (Hall, 2002). The current study showed that thiol compounds, including GSH and PCs, accumulated under Cd stress in tomato leaves and roots, were

further induced in roots by exogenous melatonin treatment (Figure 5). Melatonin also increased GR activity required to maintain the GSH/GSSG (oxidized GSH) ratio in tomato plants under Cd stress (Figure 4). Recently, Wang et al. (2012) have found that melatonin increases the GSH concentration to delay the senescence of apple leaves by stimulating the activity of  $\gamma$ -glutamylcysteine. The expression levels of the *SIGSH1* and *SIPCs* genes, which are responsible for the biosyntheses of GSH and PCs in tomato plants, respectively, were increased by Cd. Exogenous melatonin treatment further upregulated expression of *SIGSH1* and *SIPCs* (Figure 6). Although expression of those genes and GR activity were increased in the melatonin-treated leaves under Cd stress (Figure 6), the concentration of GSH in leaves remained unaltered (Figure 5). Ben Ammar et al. (2008) reported that a high level of PC synthesis in roots was coupled to a decrease in the GSH concentration in leaves which might partially support our current observation. It seems that post-transcriptional events might be involved in maintaining GSH and PCs concentration in tomato leaves.

In plant cells, the toxicity of free metal ions can be decreased by their reactions with metal ligands, including proteins, polysaccharides, and organic acids (Hall, 2002). Nevertheless, the fraction of free metal ions that are not chelated can be transferred to metabolic organelles, such as chloroplasts, mitochondria, and nuclei. These free metal ions cause malfunctions of cellular organelles and cell damage (Zeng et al., 2011). Several studies



**FIGURE 7 |** The accumulation of cadmium in tomato plants and its subcellular distribution following 14 days long Cd stress as influenced by melatonin treatments. **(A)** Cd concentrations in tomato roots and leaves. **(B)** The *in situ* detection of Cd in tomato roots and leaves using a dithizone staining-based histochemical method. **(C)** The distribution of Cd in different subcellular compartments. The data shown

in **(A)**, are the averages of four replicates, with the standard errors indicated by the vertical bars. The means denoted by the same letter did not significantly differ at a  $P < 0.05$ , according to Tukey's test. Cd, 100  $\mu\text{M}$  cadmium; M25, 25  $\mu\text{M}$  melatonin; M50, 50  $\mu\text{M}$  melatonin; M100, 100  $\mu\text{M}$  melatonin; M250, 250  $\mu\text{M}$  melatonin; M500, 500  $\mu\text{M}$  melatonin; DW, dry weight.

have revealed that decreased translocation of Cd from roots to shoots is an effective barrier which promotes Cd sequestration in the cortex and endodermis of roots (Akhter et al., 2012, 2014). In our study, although melatonin did not affect the Cd content in root, it (50 and 100  $\mu\text{M}$ ) significantly reduced the leaf Cd content, suggesting that melatonin might induce barrier for preventing translocation of Cd from root to shoot (Figures 7A,B). We also noticed that exogenous melatonin treatment greatly improved immobilization of Cd in the cell wall and vacuoles that substantially minimized Cd toxicity (Figure 7C). Cd immobilization may be correlated with the melatonin-induced biosynthesis of thiol compounds (Figure 5; Ben Ammar et al., 2008). Given that melatonin restricted Cd translocation from root to shoot, it might facilitate healthy cellular atmosphere for normal functioning in leaves. As leaves accumulated less Cd following melatonin treatment, demand for GSH and PCs in leaves was comparatively low for metal

detoxification, while roots appeared as major site for Cd detoxification staying in direct contact. It seemed logical that the higher thiol-peptide levels in roots of melatonin-treated plants might act as a sink for thiol-reactive metal and thus these plants could sequester higher levels of Cd. All of these results indicate that exogenous melatonin increases the proportion of immobilized Cd through cell wall binding and vacuole sequestration, thereby increasing the detoxification of this metal (Zeng et al., 2011).

Cadmium competes for the same transport systems with mineral nutrients, and it alters plasma membrane stability and the ionic balance (Llamas et al., 2000). Plasma membrane H<sup>+</sup>-ATPase transports ions and organic compounds across the plasma membrane (Morsomme and Boutry, 2000). Melatonin can be transformed into 5-methoxytryptamine and thus stimulating H<sup>+</sup>-ATPase activity in plants (Hardeland et al., 1995). Nevertheless, melatonin also protects the plasma

membrane lipid environment by preventing Cd-induced ROS generation (Tan et al., 2012) and maintaining increased levels of antioxidants (Zhang et al., 2013). In line with previous reports, the current study showed that melatonin induced H<sup>+</sup>-ATPase activity under Cd stress (**Figure 4**), which might have significant role in maintaining plasma membrane stability and EL to confer Cd tolerance (**Figure 3D**).

Survival of plants under polluted environments largely depends on plants' ability to sequester and/or detoxify toxic pollutants such as Cd. This study showed that both exogenous melatonin and Cd stress could induce endogenous melatonin accumulation. However, exogenous melatonin had complex and influential effects on plant growth, ROS scavenging, antioxidant potential, thiol compound biosynthesis and Cd immobilization in cell walls and vacuoles under Cd stress. Even though, exogenous melatonin had no effect on root Cd content, it significantly decreased leaf Cd content, indicating its potential role in regulating Cd translocation. Therefore, these findings can be implicated for developing new strategies to produce safe food in

an eco-friendly manner particularly in marginal areas where Cd contamination is a limiting factor for crop production. Further studies are needed to provide genetic evidence in support of the involvement of melatonin in Cd detoxification and to elucidate the subsequent signaling cascades in plants.

## Acknowledgments

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## Supplementary Material

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2015.00601>

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# Expression of *TaWRKY44*, a wheat WRKY gene, in transgenic tobacco confers multiple abiotic stress tolerances

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The WRKY transcription factors have been reported to be involved in various plant physiological and biochemical processes. In this study, we successfully assembled 10 unigenes from expressed sequence tags (ESTs) of wheat and designated them as *TaWRKY44–TaWRKY53*, respectively. Among these genes, a subgroup I gene, *TaWRKY44*, was found to be upregulated by treatments with PEG6000, NaCl, 4°C, abscisic acid (ABA), H<sub>2</sub>O<sub>2</sub> and gibberellin (GA). The *TaWRKY44*-GFP fusion protein was localized to the nucleus of onion epidermal cells, and *TaWRKY44* was able to bind to the core DNA sequences of TTGACC and TTAACC in yeast. The N-terminal of *TaWRKY44* showed transcriptional activation activity. Expression of *TaWRKY44* in tobacco plants conferred drought and salt tolerance and transgenic tobacco exhibited a higher survival rate, relative water content (RWC), soluble sugar, proline and superoxide dismutase (SOD) content, as well as higher activities of catalase (CAT) and peroxidase (POD), but less ion leakage (IL), lower contents of malondialdehyde (MDA), and H<sub>2</sub>O<sub>2</sub>. In addition, expression of *TaWRKY44* also increased the seed germination rate in the transgenic lines under osmotic stress conditions while exhibiting a lower H<sub>2</sub>O<sub>2</sub> content and higher SOD, CAT, and POD activities. Expression of *TaWRKY44* upregulated the expression of some reactive oxygen species (ROS)-related genes and stress-responsive genes in tobacco under osmotic stresses. These data demonstrate that *TaWRKY44* may act as a positive regulator in drought/salt/osmotic stress responses by either efficient ROS elimination through direct or indirect activation of the cellular antioxidant systems or activation of stress-associated gene expression.

**Keywords:** abiotic stress tolerance, antioxidant systems, ROS elimination, stress-associated gene, wheat, WRKY

**Abbreviations:** 3-AT, 3-Amino-1,2,4-triazole; ABA, Abscisic acid; CaMV, *Cauliflower mosaic virus*; CAT, Catalase; DAPI, 4,6-diamidino-2-phenylindole; GA, Gibberellin; GFP, Green fluorescent protein; IL, Ion leakage; LEA, Late embryogenesis abundant; MDA, Malonaldehyde; MS, Murashige and Skoog; OE, Overexpression; ORF, Open reading frame; POD, Peroxidase; QRT-PCR, Real-time quantitative PCR; ROS, Reactive oxygen species; RT-PCR, Reverse transcription-PCR; RWC, Relative water content; SOD, Superoxide dismutase; VC, Vector control; WT, Wild type.

## Introduction

As sessile organisms, plants are easily affected by various environmental stresses, including biotic and abiotic stresses. Abiotic stress conditions cause a devastating influence on plant yield under field conditions (Suzuki et al., 2014), with drought, salinity and low temperature as three of the most vital problems for agriculture because these abiotic stresses limit the plant from exhibiting its full genetic potential (Zhu, 2002). Plants have evolved complex mechanisms to increase their tolerance to these abiotic stresses through both physical adaptations and molecular and cellular changes in response to these stresses (Knight and Knight, 2001). To date, extensive research has focused on identifying key regulation components, including signal transduction receptors or effectors (Shen et al., 2006; Park et al., 2009), transcription factors (Dubos et al., 2010; Lata and Prasad, 2011; Chen et al., 2012) and various functional proteins related to physiological and metabolic responses under drought or salt stresses (Yonamine et al., 2004; Wang et al., 2011). Through binding to the *cis*-acting elements in the promoters of stress-related genes, transcription factors play vital roles in stress responses (Nakashima et al., 2009). Among them, plant WRKY transcription factors, one of the largest families of transcriptional regulators in plants, have been proven to play crucial roles in response to various stresses. After the initial reports on WRKY transcription factors (Ishiguro and Nakamura, 1994), three members (WRKY1, WRKY2, and WRKY3) were identified from parsley (*Petroselinum crispum*) and the name WRKY (pronounced “worky”) was coined (Rushton et al., 1996).

WRKY family proteins are transcription factors that are characterized by a conserved DNA-binding WRKY domain. The WRKY domain consists of 60 amino acid residues at the N-terminal and an atypical zinc-finger structure at the C-terminal. It was reported that the conserved WRKY amino acid sequences have variants, including WRRY, WSKY, WKRY, WVKY, or WKKY, in some WRKY proteins (Xie et al., 2005). The zinc-finger structure is either Cx<sub>4–5</sub>Cx<sub>22–23</sub>HxH or Cx<sub>7</sub>Cx<sub>23</sub>HxC (Rushton et al., 2010). The WRKY transcription factors were further divided into three groups based on the number of WRKY domains (two domains in Group I proteins and one in the others) and the structure of their zinc fingers (C2H2 in Group II proteins and C2HC in Group III proteins) (Eulgem et al., 2000). The WRKY factors have high binding affinity to the TTGACC/T promoter element sequence called the W-box sequence, which is common to numerous defense-associated genes. The TGAC core sequence of the W-box elements is important for WRKY binding, as demonstrated by numerous binding experiments (Ulker and Somssich, 2004; Ciolkowski et al., 2008).

Major advances in WRKY transcription factor function research have occurred over the past 20 years (Rushton et al., 2010). A massive amount of evidence has demonstrated that WRKY transcription factors participate in numerous physiological processes, including pathogen defense (Dong et al., 2003), sugar signaling (Sun et al., 2003), senescence (Zhou et al., 2011), trichome development (Johnson et al., 2002), root growth (Ren et al., 2010), and hormone signaling (Mao et al., 2007; Jiang et al., 2014). In recent years, the physiological

functions of WRKYS in abiotic stress have also been reported (Zhou et al., 2008; Luo et al., 2013). However, compared with the extensive progress on biotic stresses, the functional understanding of WRKY proteins in abiotic stress is limited (Niu et al., 2012). As a staple crop, wheat production is constrained by multi-environmental stresses, such as drought, salinity and extreme temperature (Hu et al., 2012). Therefore, to improve the stress tolerance of wheat through genetic engineering, the detailed mechanisms of abiotic stress responses in wheat must be clarified. As a hexaploid plant, some studies have indicated that there are at least 200 WRKY genes in wheat (Okay et al., 2014; Satapathy et al., 2014), but to date, less than one-third of WRKY genes have been cloned and only a few of them have been functionally analyzed. Therefore, identification and functional analysis of WRKYS in wheat remain a challenge. Overexpression of some WRKY genes conferring tolerance to abiotic stresses through activating the antioxidant system has been reported in other species, such as rice and *Arabidopsis*, but there have been limited studies in wheat due to its complex and large genome. In this work, we successfully assembled 10 new unigenes from the ESTs of wheat. Previous work has identified 43 genes named *TaWRKY1-TaWRKY43* in wheat (Niu et al., 2012), in order to build a systematic naming system of WRKY genes in wheat, we designated these 10 genes as *TaWRKY44-TaWRKY53*, respectively. Among these genes, the expression of *TaWRKY44* in transgenic tobacco plants was shown to confer drought/salt/osmotic tolerance through direct or indirect activation of cellular antioxidant systems or stress-associated genes to eliminate ROS accumulation.

## Materials and Methods

### Plant Materials and Stress Treatments

Wheat (*Triticum aestivum* L. cv. Chinese Spring) seeds were sterilized and then germinated in sterile water and cultured in growth chambers (16 h light/8 h dark cycle at 25°C) for 10 days. For stress and signaling molecule treatments, uniform and healthy 10-day-old seedlings were steeped in and sprayed with sterile water, a 100 mM NaCl solution, a 20% PEG6000 solution, 100 μM ABA, 10 mM H<sub>2</sub>O<sub>2</sub> and 5 μM GA and incubated under light for 24 h. Leaves from sterile water treatment were taken as a control. For organ expression analysis, roots, stems and leaves were collected from sterile seedlings, while pistils and stamens were collected from wheat plants in the growth chamber. Leaves were independently harvested at 0, 1, 3, 6, 12, and 24 h; immediately frozen in liquid nitrogen; and stored at –80°C until RNA extraction.

### Cloning and Bioinformatic Analysis of *TaWRKYS*

Total RNA from wheat seedlings was extracted using a RNAprep pure Plant Kit (DP432, Tiangen), and after the removal of genomic DNA by DNase I (Code No. 2212, Takara), 1 μg of total RNA was used to synthesize first-strand cDNA using a RevertAid First Strand cDNA Synthesis Kit (# K1691, Fermentas). The wheat ESTs were obtained from the DFCI wheat gene index database and NCBI to assemble 10 unigenes that were then amplified from wheat cDNA by PCR using specific primer

pairs (**Supplementary Table 1**). The PCR products were purified by a TIANgel Midi Purification Kit (DP209, Tiangen), ligated to the pMD-18T plasmid (TakaRa) and then sequenced. After confirmation of the full-length sequence of *TaWRKY44*, a homology search was done in NCBI using BLASTp.

### Subcellular Localization of *TaWRKY44*

The complete coding sequence of *TaWRKY44* without the stop codon was ligated into the pBI121-GFP vector after it was amplified by PCR using primer P1 (**Supplementary Table 2**) with *Xba*I and *Bam*H I restriction sites to create a fusion construct (pBI121-TaWRKY44-GFP). After confirmation by sequencing, pBI121-TaWRKY44-GFP was introduced into onion (*Allium cepa* L.) epidermal cells by particle bombardment (PDS-1000, Bio-Rad). pBI121-GFP was used as a control. After incubation at 25°C for 24 h, the tissue was stained with DNA-specific nuclear stain 4',6-diamidino-2-phenylindole (DAPI) for 10 min. Fluorescence microscopy images were observed using a fluorescence microscope (Olympus FV500, <http://www.olympus-global.com/>).

### Analysis of Transcriptional Activation in Yeast Cells

A transcription activation assay was performed in yeast strain AH109 according to the Yeast Protocols Handbook (Clontech). The full length coding region and truncated fragments of *TaWRKY44* were generated by PCR using primers P2-P7 (**Supplementary Table 2**). The PCR products were cloned into the pGBKT7 vector using *Eco*RI and *Pst*I sites and were named pBD-TaWRKY44-N, pBD-TaWRKY44-NW1, pBD-TaWRKY44-NW2, pBD-TaWRKY44, pBD-TaWRKY44-W2C, and pBD-TaWRKY44-C, respectively. Plasmid pGBKT7 (pBD) was used as a negative control. These constructs were transformed into yeast strain AH109 by the lithium acetate-method. After confirmation by screening on selective medium plates without tryptophan (SD/-Trp) and colony PCR, the positive colonies were transferred onto the SD/-His plates with or without X- $\alpha$ -D-Galactosidase (X- $\alpha$ -gal), and the growth status of the yeast cells were photographed after incubating the plates for 3 d to evaluate the transcription activation activities.

### W-box Binding Assay of *TaWRKY44* in Yeast

A yeast one-hybrid assay was used to investigate whether *TaWRKY44* binds to the W-box element. The full length coding region of *TaWRKY44* was generated by PCR using primer P8 (**Supplementary Table 2**). The PCR products were cloned into the pGADT7 vector using *Eco*RI and *Bam*H I sites to obtain pGADT7-TaWRKY44. A 18-bp oligonucleotide sequence containing three tandem repeat copies of the W-box element (5'-TTGACC-3', the core sequence is underlined) was cloned into the pHIS2.0 vector using *Eco*RI and *Sac*I sites to obtain the reporter vector pHIS2-W-box. Similarly, vectors pHIS2-mW-box1-5 (pHIS2-mW1-5) were obtained by mutating the core TGAC sequence of the W-box elements to TGAT, CGAC, TAAC, TGGC, or AAAA (the mutated sites are underlined). Oligonucleotides were obtained by direct annealing using primers P9-P14 (**Supplementary Table 2**). Using the lithium

acetate-method, pGAD-TaWRKY44 and pHIS2-(m) W-box were co-transformed into yeast strain Y187, while pHIS2/pGAD-TaWRKY44, pHIS2/pGADT7 and pHIS2-W-box/pGADT7 were also co-transformed as negative controls. The DNA-protein interaction was evaluated according to the growth status of yeast cells cultured on SD/-His/-Leu/-Trp plates with 0, 30, and 60 mM 3-amino-1,2,4-triazole (3-AT) for 3 d.

### RT-PCR

After stress and signaling molecule treatments, the transcription levels of these 10 WRKY genes were monitored for 24 h using semi-quantitative RT-PCR. The specificity of the primers (**Supplementary Table 1**) used in RT-PCR was confirmed by agarose gel electrophoresis and sequencing. The cDNA was obtained following the procedures mentioned above. All of the reactions were performed for 30 cycles using TaKaRa DNA polymerase; *TaActin* or *NtActin* were used as internal controls.

### Real-time Quantitative PCR (qRT-PCR)

To investigate the expression levels of *TaWRKY44* in response to various treatments in different wheat tissues, qRT-PCR was applied. Three biological replicates of cDNA prepared as mentioned above were used as the template for amplification. The qRT-PCR was carried out following the SuperReal PreMix Plus (SYBR Green, FP205, Tiangen) on a CFX Connect™ Optics Module (Bio-Rad) Real-Time PCR System. The PCR conditions were 95°C for 15 min followed by 40 cycles at 95°C for 10 s and 60°C for 30 s and 72°C for 32 s. The primers (**Supplementary Table 1**) used in qRT-PCR were designed based on sequence characterization to avoid the highly conserved WRKY domains, and the efficiency and specificity of the primers were first confirmed by agarose gel electrophoresis, validated by melting curve analysis using CFX Manager Software and sequencing of the amplified PCR products. The expression of the *TaActin* gene was used as an internal control. The  $2^{-\Delta\Delta Ct}$  method was used to calculate the relative gene expression (Livak and Schmittgen, 2001).

### Plant Transformation and Generation of Transgenic Plants

To generate transgenic tobacco plants expressing *TaWRKY44*-GFP fusion protein, plasmids pBI121-TaWRKY44-GFP, under control of the *Cauliflower mosaic virus* 35S (*CaMV* 35S) promoter, and pBI121-GFP (VC) were transferred into *Agrobacterium tumefaciens* strain LBA4404, respectively. Transgenic tobacco plants were generated using the leaf disc transformation method according to Horsch et al. (1985). The seeds from  $T_0$  transgenic plants were harvested and sown on MS medium containing kanamycin (100 mg L<sup>-1</sup>), and the kanamycin-resistant  $T_1$  seedlings were confirmed by amplification of the *TaWRKY44* and *GFP* genes using primers P15-P16 (**Supplementary Table 2**). Three independent transgenic  $T_2$  line seedlings (OE-1, OE-7, and OE-35), almost all survived on MS medium containing 100 mg L<sup>-1</sup> of kanamycin. The pBI121-GFP vector control line was used in the following experiments. Expression of *TaWRKY44* in three of the selected putative transgenic plants was examined by semi-quantitative

RT-PCR using primers P16. Similarly, semi-quantitative RT-PCR expression analysis of *NtActin* using primer P17 (**Supplementary Table 2**), was used as an internal control.

### Stress Tolerance Assays of the Wild-type (WT), Vector Control (VC), and *TaWRKY44* Transgenic Plants

Seed of WT, VC and three transgenic lines (OE-1, OE-7, OE-35) were surface sterilized with 75% (v/v) ethanol for 10 s and 10% (v/v) H<sub>2</sub>O<sub>2</sub> for 10 min before they were sown on MS medium under a 16 h light/8 h dark cycle at 25°C for 2 weeks and then were transplanted into containers filled with a mixture of soil and sand (3:1) for 3 weeks with regular watering. Five-week-old plants with similar growth state were used in the following experiments. For drought/salt stress tolerance assays, 30 seedlings from each line were withheld from watering for 3 weeks before re-watering for 1 week or were irrigated with 400 mM NaCl for 3 weeks. After 3 weeks of drought/salt stresses, the survival rates were calculated and the leaves were collected to measure the RWC; IL; MDA, proline and soluble sugar contents; H<sub>2</sub>O<sub>2</sub> accumulation; and antioxidant enzymes (SOD, POD, CAT) activities. For the osmotic stress tolerance assay, 2-week-old seedlings were transplanted to MS with mannitol (300 mM) and NaCl (200 mM) for 1 week, as described above. Leaves were used to measure ROS accumulation and CAT, SOD and POD activities. Fifty sterilized seeds from each line were sown on MS with mannitol (0, 150, or 300 mM) and NaCl (0, 100 or 200 mM) for 2 week, and the germination rates were scored daily.

### Analysis of Proline, RWC, IL, MDA, Soluble Sugar, H<sub>2</sub>O<sub>2</sub> Accumulation and Antioxidant Enzyme Activities

The proline content was measured by the ninhydrin reaction method. Proline was extracted from approximately 0.5 g of fresh leaves homogenized in 5 mL of 3% suphosalicylic acid and heated at 100°C for 10 min, and then, 2 mL of the extracted solution was added into 2 mL of acetic acid and 2 mL of 2.5% acid ninhydrin reagent and heated at 100°C for 30 min; the color of the solution turned to red. After cooling to room temperature, 4 mL of methylbenzene was added to the solution and incubated for 10 min after 30 s of shaking. The methylbenzene solution was used as a control to determine the optical density of the supernatant organic phase at 520 nm. The RWC and IL were measured according to previous reports (Deng et al., 2013; Hu et al., 2013), and the MDA content was determined according to the thiobarbituric acid (TBA)-based colorimetric method as described by Draper et al. (1993), with slight modifications. The soluble sugar content was examined by the phenol reaction method according to a previous study (Kong et al., 2011), with little modification. Approximately 0.2 g of fresh leaves were boiled in 5 mL of distilled water for 30 min for extraction and then diluted with distilled water to 10 mL. Two milliliters of the diluted solution was mixed with 1 mL of 9% phenol and 5 mL of concentrated sulfuric acid. After standing for 30 min, distilled water was used as a control to determine the optical density of the aqueous extract at 485 nm. A standard curve was drawn to calculate the soluble sugar

content. The activity of three antioxidant enzymes, CAT, POD, and SOD, and the content of H<sub>2</sub>O<sub>2</sub> were spectrophotometrically measured using four detection kits (A001, A007, A084, and A064, Jiancheng Bioengineering Institute) following the manufacturer's instructions.

### Analysis of the Downstream Genes Regulated By *TaWRKY44*

The control lines (WT and VC) and the transgenic lines cultured on MS medium were transplanted to MS medium with mannitol (300 mM) and NaCl (200 mM) for 1 week. The total RNA of the leaves was extracted to synthesize cDNA. The expressions of the 14 selected stress-related genes were detected using qRT-PCR. The *NtActin* gene (P17) was used as the internal control. The sequences of the qPCR primers are listed in **Supplementary Table 2** (P18–P31).

### Sequence and Statistical Analysis

Amino acid sequences were aligned by DNAMAN 8, and a phylogenetic tree was constructed using Mega 5.0. The data were analyzed in Excel, and the mean values ± SD were calculated from three independent experiments. Student's *t*-test was applied for the significant difference statistical analysis.

## Results

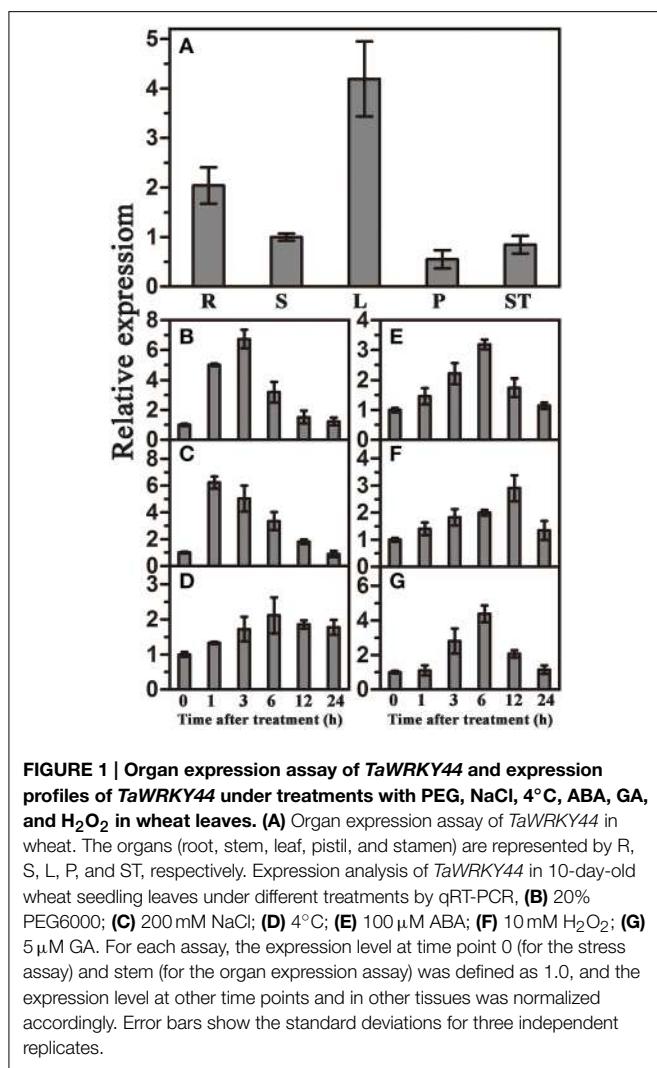
### Identification of WRKY Genes in Wheat

In this work, ten new WRKY genes were identified from wheat and designated as *TaWRKY44*-*TaWRKY53*, respectively according to previous studies, and the characteristics and GenBank accession numbers of the WRKYS are provided in **Supplementary Tables 3, 6**. Multiple alignments of the deduced amino acid sequences of *TaWRKY44*-*TaWRKY53* clearly showed that these proteins contained the conserved WRKY domain (**Supplementary Figure 1**). Phylogenetic analysis of these 10 *TaWRKY* genes compared to other WRKYS (The GenBank accession numbers showed in **Supplementary Table 5**) from various plants demonstrated that these 10 WRKY genes could be divided into three subgroups (**Supplementary Figure 2**). The expression patterns of the 10 WRKYs under abiotic stresses and plant hormone treatments were analyzed by RT-PCR (**Supplementary Table 4**). The results showed that *TaWRKY44* was upregulated by multiple stress treatments, and *TaWRKY44* was thus chosen for the further analysis of its role in abiotic stress responses. *TaWRKY44* cDNA is 1212 bp, with an 1113-bp open reading frame (ORF), and the deduced *TaWRKY44* protein contains 370 amino acid residues with a predicted relative molecular mass of 40.39 kDa and isoelectric point of 8.43. Evolutionary relationship analysis showed that the *TaWRKY44* protein contains two conserved DNA-binding domains (WRKY domain) and a zinc finger region, indicating that it belongs to Group I. BLASTp analysis revealed that the amino acid sequence of *TaWRKY44* had an 90% sequence identity with the putative WRKY transcription factor 4 (EMS63397.1) from *Triticum urartu* and an 87% sequence identity with putative WRKY transcription factor 4 (EMT16145.1) from *Aegilops*

*tauschii*. These results indicated that *TaWRKY44* is a member of the WRKY family from wheat.

### Expression Pattern of *TaWRKY44* under Various Stress Conditions

qRT-PCR was used to investigate the expression patterns of *TaWRKY44* in different tissues and under various abiotic stresses and signaling molecule treatments. The results showed that *TaWRKY44* was expressed in all tissues, with higher expression levels in leaves and roots and lower expression levels in stems, pistils and stamens (Figure 1A). *TaWRKY44* was obviously up-regulated after treatments with PEG and NaCl. During ABA and H<sub>2</sub>O<sub>2</sub> treatments, the expression of *TaWRKY44* was gradually increased by 3.2-fold at 6 h and 2.9-fold at 12 h, respectively. Low temperature treatment led to a slight up-regulation, and GA treatment distinctly increased the expression of *TaWRKY44* (Figures 1B–G). On the other hand, with no treatment, the expression level of *TaWRKY44* had no obvious change (data not shown). These results demonstrated that the expression of *TaWRKY44* was up-regulated by PEG, NaCl, ABA H<sub>2</sub>O<sub>2</sub>, and GA treatments.



**FIGURE 1 |** Organ expression assay of *TaWRKY44* and expression profiles of *TaWRKY44* under treatments with PEG, NaCl, 4°C, ABA, GA, and H<sub>2</sub>O<sub>2</sub> in wheat leaves. (A) Organ expression assay of *TaWRKY44* in wheat. The organs (root, stem, leaf, pistil, and stamen) are represented by R, S, L, P, and ST, respectively. Expression analysis of *TaWRKY44* in 10-day-old wheat seedling leaves under different treatments by qRT-PCR. (B) 20% PEG6000; (C) 200 mM NaCl; (D) 4°C; (E) 100 μM ABA; (F) 10 mM H<sub>2</sub>O<sub>2</sub>; (G) 5 μM GA. For each assay, the expression level at time point 0 (for the stress assay) and stem (for the organ expression assay) was defined as 1.0, and the expression level at other time points and in other tissues was normalized accordingly. Error bars show the standard deviations for three independent replicates.

### Transcription Activation Activity of *TaWRKY44*

The yeast expression system was used to investigate whether *TaWRKY44* possesses transcription activation activity. Yeast strain AH109 was transformed with fusion plasmids pGBK7-TaWRKY44-N, pGBK7-TaWRKY44-NW1, pGBK7-TaWRKY44-NW2, pGBK7-TaWRKY44, pGBK7-TaWRKY44-W2C and pGBK7-TaWRKY44-C, and pGBK7 as a control. As shown in Figure 2A, the yeast cells transformed with pGBK7-TaWRKY44-N grew well in His<sup>-</sup> medium. Meanwhile, yeast cells transformed with other plasmids could only survive on SD/-Trp medium. The result of LacZ staining showed that the yeast cells transformed with pGBK7-TaWRKY44-N turned blue in the presence of X-α-gal. These results indicated that the N-terminal region of *TaWRKY44* has transcription activation activity, whereas the full-length *TaWRKY44* appears to lack this activity.

### The *TaWRKY44*-GFP Fusion Protein Is Localized to the Nucleus

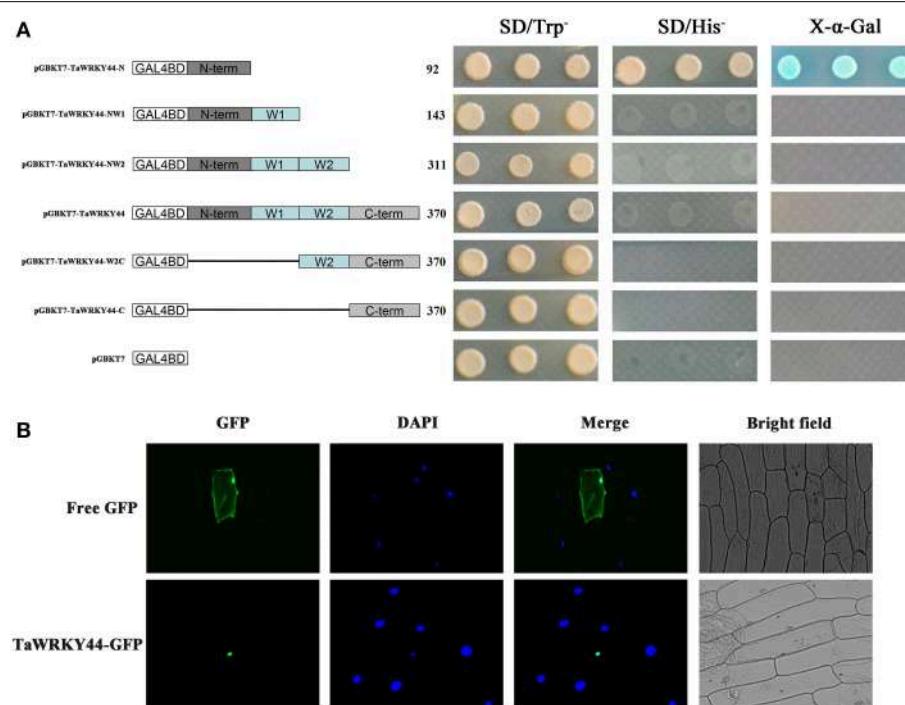
The 35S-*TaWRKY44*-GFP plasmid was generated using the pBI121-GFP vector to investigate its subcellular distribution. Fluorescence imaging showed that the *TaWRKY44*-GFP fusion protein was localized exclusively to the nuclei of onion epidermal cells in a transient expression assay (Figure 2B). The control GFP was distributed throughout the cell. DAPI staining was used as a nuclear marker. The nuclear localization of *TaWRKY44*-GFP is consistent with its predicted function as a transcription factor.

### *TaWRKY44* Binds to the TGAC and TAAC Core Sequence

Plant WRKY proteins have high binding affinity to various W-box elements with the TGAC core sequence in the promoters of numerous defense-associated genes (Yu et al., 2001). A yeast one-hybrid system was used to evaluate the binding specificity between *TaWRKY44* and the W-box (TTGACC/T) element. The full-length ORF of *TaWRKY44* was fused to the GAL4 activation domain of vector pGADT7, and the fused construct was co-transformed with pHIS2-W-box or the pHIS2-mW-box1-5 construct containing triple tandem repeats of the W-box and mutated W-box into yeast strain Y187 (Figure 3A). As shown in Figure 3B, all of the yeast cells transformed with the different combination of constructs described above could grow on SD/-Leu/-Trp/-His medium without 3-AT. However in the presence of 60 mM 3-AT, only the cells co-transformed with pGADT7-*TaWRKY44* and pHIS2-W-box grew well, and the cells co-transformed with pGADT7-*TaWRKY44* and pHIS2-mW-box3 (TTAACCC) grew poorly, while others were completely inhibited. These results suggest that *TaWRKY44* could strongly bind with the W-box core sequence TTGACC, even when the core sequence TTGACC was mutated to TTAACCC, it could still weakly bind with it and activate the reporter gene in yeast.

### Generation of Transgenic Tobacco Plants Expressing *TaWRKY44*

As *TaWRKY44* was up-regulated by NaCl and PEG treatment, transgenic tobacco plants expressing *TaWRKY44*-GFP fusion protein were generated to examine the role of *TaWRKY44*.



**FIGURE 2 | Analysis of the transactivation activity and subcellular localization of *TaWRKY44*. (A)** Transactivation activity of the *TaWRKY44* protein in yeast. Schematic diagrams of fused vectors illustrating the different portions of *TaWRKY44* that were fused to the yeast vector pGBKT7. Yeast strain AH109 was used in the transactivation activity analysis of *TaWRKY44*. The transformants were incubated on the SD-/Trp or SD-/His medium and subjected to X- $\alpha$ -gal assay. Three biological experiments produced similar

results. **(B)** Subcellular localization of the *TaWRKY44* protein in onion epidermal cells. The fusion protein *TaWRKY44*-GFP (pBI121-*TaWRKY44*-GFP) and GFP (pBI121-GFP) were transiently expressed in the onion epidermis using the bombardment method. Pictures were taken in bright and fluorescence fields after DAPI staining with fluorescence microscopy 24 h after bombardment. Three biological experiments were carried out, which produced similar results.

in salt and drought stress response, while the empty vector transformed into tobacco was served as a negative control. The ORF of *TaWRKY44* was ligated into the modified pBI121-GFP expression vector under the control of the *CaMV* 35S promoter. In total, 39 putative transgenic lines were confirmed by PCR. RT-PCR analysis showed that *TaWRKY44* mRNA was detected in transgenic plants, but not in the WT and VC, and *NtActin* was used as an internal control (Figure 4A). Three transgenic T<sub>2</sub> lines (OE-1, OE-7, and OE-35) had a nearly 100% germination rate on MS medium containing 100 mg L<sup>-1</sup> of kanamycin, which were thought to be homozygous transgenic lines, were used for further stress tolerance test.

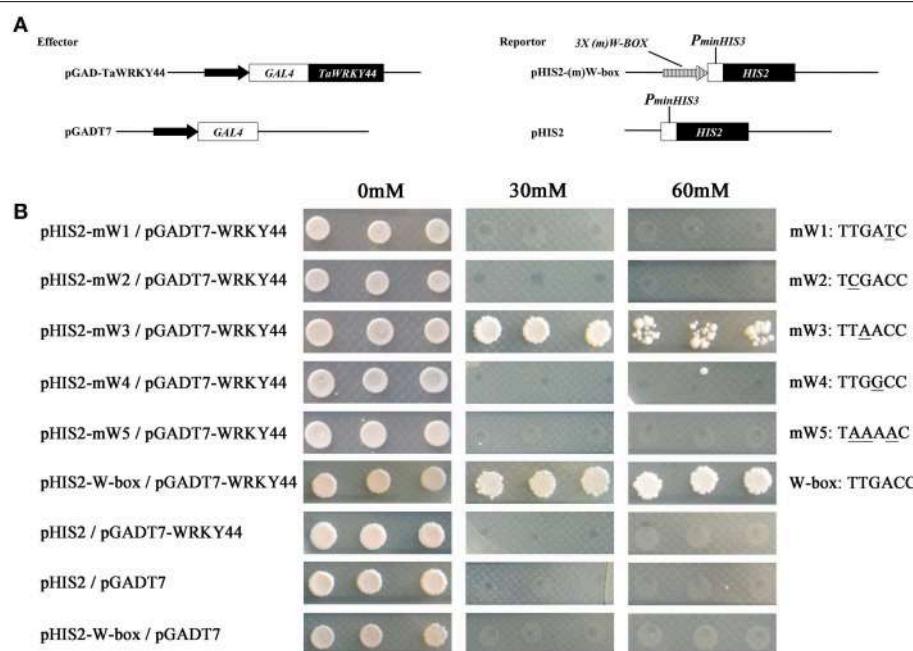
### Expression of *TaWRKY44* Enhances Drought/Salt Tolerance in Transgenic Tobacco Plants

To investigate whether transgenic expression of *TaWRKY44* was correlated with stress tolerance, the 5-week-old control lines (WT and VC) and transgenic line plants were subjected to drought/salt stress assays. For drought tolerance analysis, the 5-week-old plants were deprived of water for 3 weeks, followed by re-watering for 1 week. Leaf wilting was more evident in the WT and VC plants relative to the three transgenic lines after 3 weeks without watering (Figure 4B). For salt tolerance analysis, the 5-week-old plants were exposed to salt treatment (400 mM NaCl)

for 3 weeks (Figure 4B). The survival rates of the transgenic lines were significantly higher than those of the control lines after drought/salt stresses (Figure 4C). These results indicated that the expression of *TaWRKY44* could enhance drought/ salt tolerance in transgenic tobacco.

### Expression of *TaWRKY44* Increases RWC, Proline and Soluble Sugar Accumulation and Decreases MDA and IL under Drought/Salt Stresses

That expression of *TaWRKY44* enhanced drought and salt tolerance led us to determine the effects of the physiological status caused by *TaWRKY44* expression. Result of RWC analysis, a credible evaluation of the plant water status, indicated that the activity of the plants under various environmental conditions was less reduced in transgenic lines after 3 weeks of drought/salt stress (Figure 5A). Proline is thought to play an important role as an osmotic-regulatory solute in plants subjected to hyperosmotic stresses, primarily through drought and soil salinity (Delauney and Verma, 1993). The proline content was higher in the transgenic lines after 3 weeks of drought/salt stress (Figure 5B). In addition, the soluble sugar levels exhibited a profile similar to that of proline (Figure 5C). These results showed that the transgenic lines possess more powerful resistance to hyperosmotic stresses compared to control



**FIGURE 3 | Analysis of the W-box binding activity of *TaWRKY44* using a yeast one-hybrid system. (A)** Schematic diagrams of the effector plasmids (pGAD-TaWRKY44 and pGADT7) and reporter plasmids (pHIS2-(m)W-box and pHIS2) used for the yeast one-hybrid assay. **(B)** Schematic

diagrams of vector transformation combinations and the growth of yeast cells on SD/-His/-Leu/-Trp supplemented with (30 and 60 mM) or without 3-AT. The mW1-5 and W-box sequence indicated the core sequence of each vector. Three biological experiments produced similar results.

lines. The MDA and IL levels, important indicators of membrane injury, were significantly lower in the transgenic lines relative to the control lines (Figures 5D,E), indicating that the WT and VC lines suffered from more severe membrane damage after drought/salt stress. Moreover, the transgenic lines showed no obvious difference with the control lines in these physiological indicators without stress treatment. These results demonstrated that the transgenic lines possess more powerful resistance to drought and salt stress.

### Expression of *TaWRKY44* Increases Antioxidant Enzyme Activity and Decreases the H<sub>2</sub>O<sub>2</sub> Content under Drought/Salt Stresses

Results of the MDA and IL levels indicated that the WT and VC lines suffered from more severe oxidative membrane damage after drought/salt stresses. Because enzymatic antioxidants could affect cellular ROS levels, we detected the levels of ROS and the activity of three significant antioxidant enzymes activities (SOD, POD, and CAT) in the leaves from the plants described above to further understand the relationships between enzymatic antioxidants and the influence of *TaWRKY44* expression on drought and salt stress tolerance. The results showed that after drought/salt treatments, the SOD, POD, and CAT activities in transgenic plants were significantly higher than those in the control plants; meanwhile, the H<sub>2</sub>O<sub>2</sub> levels were lower in the transgenic plants (Figure 7). In addition, under normal growth condition, the SOD, POD, and CAT activities in transgenic plants were slightly higher than in the control plants, but there is no obvious difference in the H<sub>2</sub>O<sub>2</sub> levels between the

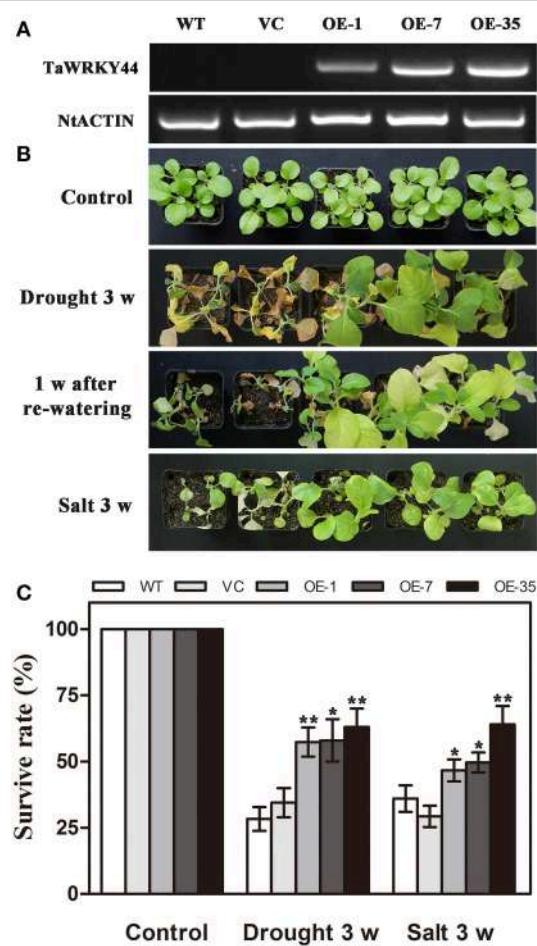
transgenic plants and the control plants. These results indicated that expression of *TaWRKY44* could influence the ROS levels by enhancing three significant antioxidant enzyme activities in the antioxidant system under drought/salt stress.

### Expression of *TaWRKY44* Enhances Osmotic Tolerance in Transgenic Tobacco Plants

To examine the osmotic stress tolerance of the transgenic plants, the control and transgenic lines were sown on MS medium containing mannitol (0, 150, or 300 mM) and NaCl (0, 100, or 200 mM) and the germination rate was monitored for 2 weeks before taking photographs. The results showed that the germination rates of the transgenic plants were obviously higher than those of the control plants grown on MS medium containing mannitol (150 or 300 mM) and NaCl (100 or 200 mM), while the plants grown on the MS medium without mannitol and NaCl had no significant difference between the control lines and transgenic lines (Figure 6). These results indicated that expression of *TaWRKY44* enhanced osmotic tolerance during seed germination in transgenic tobacco plants.

### Expression of *TaWRKY44* Decreases ROS Accumulation and Improves SOD and Cat Activities under Osmotic Stress

To further confirm the ability of transgenic tobacco plants to scavenge ROS, we detected the levels of ROS and three significant antioxidant activities in the leaves under osmotic stress. Two-week-old seedlings cultured on MS medium were transplanted to MS with mannitol (300 mM) and NaCl (200 mM) for 1 week. The



**FIGURE 4 | Phenotype and survival rate of transgenic tobacco under drought and salt stress. (A)** Expression of *TaWRKY44* in the transgenic lines. **(B)** The photographs of WT, VC and transgenic lines after water withholding for 3 weeks, re-watering for 1 week and exposure to salt treatment (400 mM NaCl) for 3 weeks. **(C)** The survival rate of WT, VC and transgenic lines after drought and salt stress treatments. Data are the means  $\pm$  SD calculated from three replicates. \* $P < 0.05$  and \*\* $P < 0.01$  indicate that the value in the transgenic lines is significantly different from that of the WT. Three biological experiments produced similar results.

results showed that without osmotic stress, there was no obvious difference in H<sub>2</sub>O<sub>2</sub> accumulation and SOD, POD, and CAT activities between the control and transgenic plant seedlings; however, after mannitol and NaCl treatments, the SOD, POD, and CAT activities in the transgenic plants were significantly higher than in the control plants, but, the H<sub>2</sub>O<sub>2</sub> levels were lower in the transgenic plants (Figure 7). These results indicated that the expression of *TaWRKY44* could influence the ROS levels by enhancing the activity of three significant antioxidant enzymes in the antioxidant system under osmotic stresses.

### TaWRKY44 Regulates Stress-responsive Gene Expressions under Osmotic Treatments

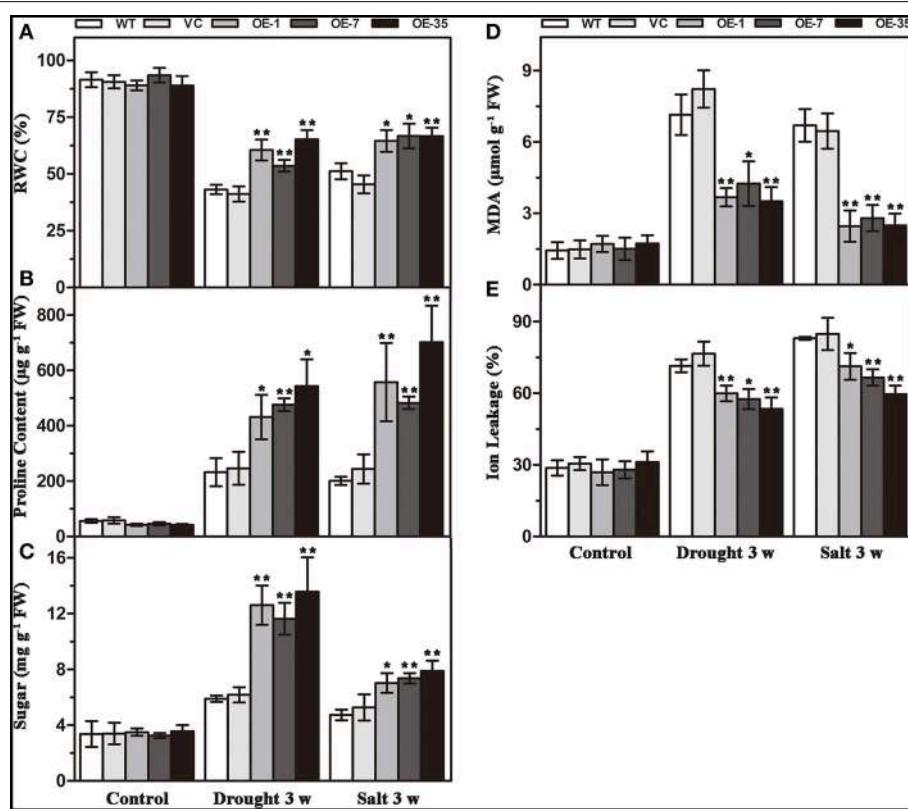
To gain further insights into the molecular mechanism underlying the enhanced drought/salt and osmotic resistance in

transgenic tobacco plants, the expression levels of 14 ROS-related and stress-responsive genes were examined in the 2-week control and transgenic lines (OE-35) with or without 1 week of osmotic stress. We selected 14 genes listed below for this experiment: the genes encoding enzymes involved in ROS detoxification (*NtSOD*, *NtAPX*, *NtCAT*, *NtPOX*, and *NtGST*), enzyme genes for biosynthesis of polyamine (*NtADC1* and *NtSAMDC*), sucrose (*NtSPSA*), or abscisic acid (ABA; *NtNCED1*), stress response proteins (*NtERD10C*, *NtERD10D*, and *NtLEA5*) and lipid-transfer protein genes (*NtLTP1* and *TobLTP1*). Three-week-old seedlings, after 1 week of mannitol (300 mM) and NaCl (200 mM) treatment as described above, were used in this assay. Compared to the control plants, all of the stress-responsive genes analyzed were significantly upregulated in the transgenic line either when exposed to mannitol treatment, NaCl treatment or both, with the exception of *NtERD10D* and *NtLEA5* (Figure 8). These results demonstrated that the expression of *TaWRKY44* in tobacco enhances drought/salt/osmotic tolerance by inducing the expression of some ROS-related and stress-responsive genes.

### Discussion

Although it has been well documented that WRKY transcription factors are tightly connected with various plant defense mechanisms and respond to various adverse environmental conditions, functional studies have only been performed for a few WRKYS in wheat, and evidence for the relationships between WRKY proteins and abiotic stresses remains limited compared to biotic stresses. Meanwhile, the mechanisms of non-WRKY genes that enhance plant drought and salt stresses by either efficient ROS elimination through the activation of the cellular antioxidant systems or the activation of the stress-associated genes have been extensively reported in *Arabidopsis* (Moon et al., 2003; Luo et al., 2009), *Oryza sativa* (Ning et al., 2010; Kumar et al., 2012), *Poncirus trifoliata* (Huang et al., 2010, 2011), and wheat (Hu et al., 2012, 2013), but there is limited documentation of the function of WRKY proteins in wheat transcription processes.

In our present study, 10 new *TaWRKY* genes were identified from wheat. Phylogenetic analysis of these 10 *TaWRKY* genes compared to WRKYS from various plants demonstrated that these 10 WRKY genes could be divided into three subgroups (Supplementary Figure 2). Previous studies indicated that the Group I and group III WRKY genes were mainly involved in the abiotic stress response, while Group II WRKY genes were mainly involved in the response to biotic stresses, such as senescence, and a few studies have reported that an abiotic stress response, such as low Pi, was involved. BLASTp analysis revealed that *TaWRKY44* had 90% sequence identity with the putative WRKY transcription factor 4 (EMS63397.1) from *Triticum urartu* and 87% sequence identity with putative WRKY transcription factor 4 (EMT16145.1) from *Aegilops tauschii*. However, no functional analyses of these two genes have been published to date. *TaWRKY44* was upregulated by PEG, NaCl, cold (4°C), ABA, H<sub>2</sub>O<sub>2</sub>, and GA (Figure 1), which implied that *TaWRKY44* might play important roles in plant abiotic stress response. Transcriptional activation analysis

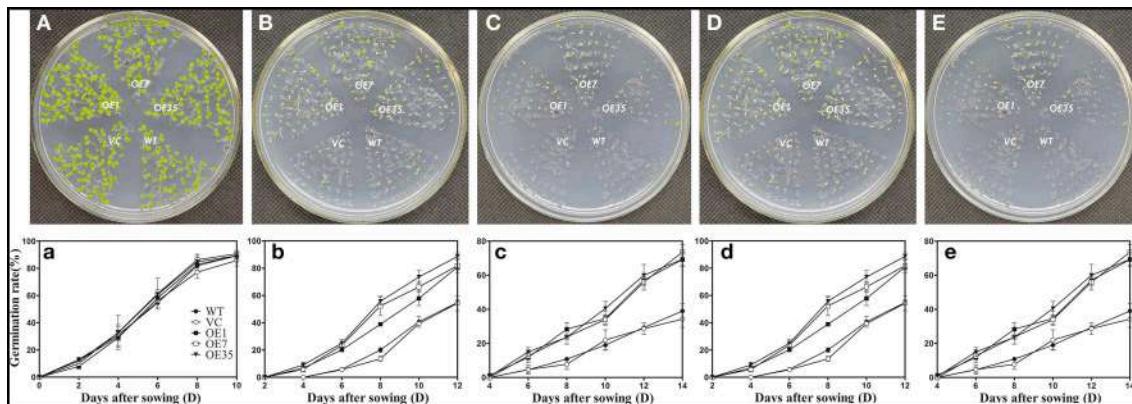


**FIGURE 5 | Analysis of the physiological indices in the control (WT and VC) and transgenic lines (OE-1, OE-7 and OE-35) under normal and drought/salt conditions.** Five-week-old plants were subjected to drought and salt treatments for 3 weeks, then the leaves were sampled to assess the value of RWC (A), proline content (B),

soluble sugar content (C), MDA content (D), and IL (E). Data are the means  $\pm$  SD calculated from three replicates. \* $(P < 0.05)$  and \*\* $(P < 0.01)$  indicate that the value in the transgenic lines is significantly different from that of the WT. Three biological experiments produced similar results.

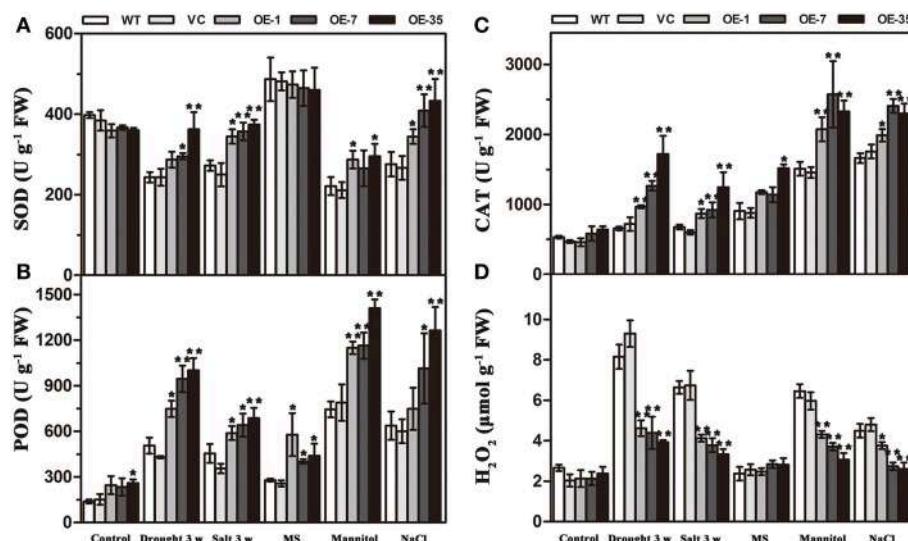
demonstrated that only the N-terminal region of *TaWRKY44* has transcriptional activation activity, whereas the full-length and C-terminal region of *TaWRKY44* appears lacking this activity (Figure 2A). It is possible that *TaWRKY44* needs additional posttranslational modifications to exhibit its full function. Additional modifications of transcription factors for stress tolerance have rarely been reported in crops (Tang et al., 2012). In a previous study, the soybean NAC transcription factor GmNAC20 appears to function as both a transcriptional repressor and transcriptional activator, and its activity as an activator or a repressor depends on a conformational change or its interactions with other regulatory proteins (Hao et al., 2011). The *Arabidopsis* transcription factor AREB1 activates expression of ABRE-dependent downstream genes through an ABA-induced modification of the AREB1 protein (Fujita et al., 2005), which is consistent with our results. Previous research indicated that phosphorylation is an important way to activate WRKY proteins (Ishihama et al., 2011), and potential phosphorylation sites of the *TaWRKY44* protein were found using the NetPhos 2.0 Server (<http://www.cbs.dtu.dk/services/NetPhos/>, data not shown). This result implies a possible regulation of *TaWRKY44* activity by phosphorylation via different protein kinases. Consistent with

its putative role as a transcription factor, the *TaWRKY44*-GFP fusion protein was exclusively localized to the nuclei of onion epidermal cells in a transient expression assay similar to previous studies on other WRKY transcription factors (Figure 2B) (Lai et al., 2008). A yeast one-hybrid system was used to evaluate the binding specificity between *TaWRKY44* and the W-box element (TTGACC/T), as in previous studies (Liu et al., 2013a; Zheng et al., 2013), and the results showed that *TaWRKY44* had high affinity to the W-box core sequence TTGACC. Even when the core sequence was mutated to TTAACC, *TaWRKY44* could still weakly bind to it and activate the reporter gene in yeast (Figure 3), suggesting that the G in TTGACC is not necessary for *TaWRKY44* recognition. The induction of *TaWRKY44* expression under abiotic stresses promoted us to further clarify the function of *TaWRKY44* in abiotic stress tolerance. Transgenic tobacco plants expressing *TaWRKY44*-GFP fusion protein were subjected to drought and salt stress treatments to examine the role of *TaWRKY44* in salt and drought stress responses like some previous studies (Hu et al., 2013; Xu et al., 2014). The results indicated that the expression of *TaWRKY44* increased the survival rate under drought and salt stresses in transgenic tobacco (Figure 4). This result is consistent with previous work on other



**FIGURE 6 | Expression of *TaWRKY44* in tobacco increases the germination rate under osmotic stress on MS medium containing mannitol and NaCl.** The control (WT and VC) and transgenic lines (OE-1, OE-7 and OE-35) were sown on MS medium containing no mannitol and NaCl (**A,a**), 150 mM (**B,b**), 300 mM (**C,c**) mannitol, and 100 mM (**D,d**),

200 mM (**E,e**) NaCl, the germination rates were monitored for 2 weeks before taking photographs. Panels (**A–E**) are the photos of germination status on media after 2 weeks; (**a–e**) are the chart of germination rate calculated for 2 weeks. Data are the means  $\pm$  SD calculated from three replicates. Three biological experiments produced similar results.

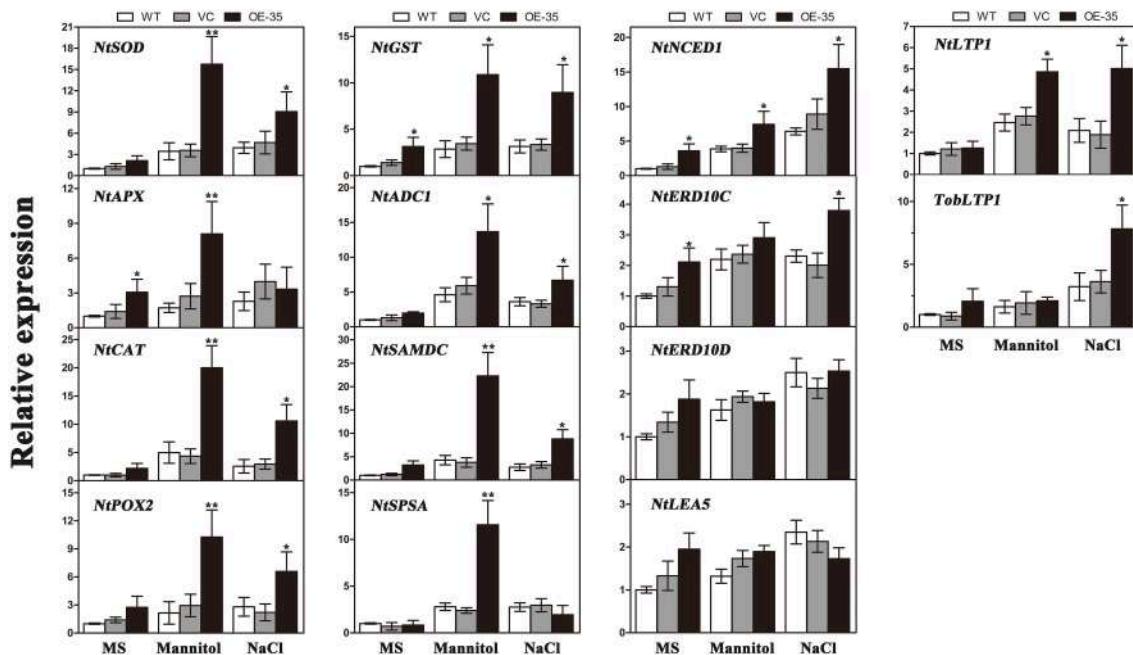


**FIGURE 7 | Analysis of three antioxidant enzyme activities and H<sub>2</sub>O<sub>2</sub> accumulation in the control (WT and VC) and transgenic lines (OE-1, OE-7 and OE-35) under normal and drought/salt/osmotic conditions.** Five-week-old plants were subjected to drought and salt treatments for 3 weeks. Two-week-old seedlings cultured on MS medium were transplanted to MS with

mannitol (300 mM) and NaCl (200 mM) for 1 week, then the leaves were sampled to assess the SOD (**A**), POD (**B**), and CAT (**C**) activities and the H<sub>2</sub>O<sub>2</sub> content (**D**). Data are the means  $\pm$  SD calculated from three replicates. \*( $P < 0.05$ ) and \*\*( $P < 0.01$ ) indicate that the value in the transgenic lines is significantly different from that of the WT. Three biological experiments produced similar results.

WRKYS. For instance, *TaWRKY10* confers drought and salt tolerance in transgenic tobacco (Wang et al., 2013), expressing *TaWRKY2* confers salt and drought tolerance, and expression of *TaWRKY19* confers salt, drought and freezing tolerance in transgenic plants (Niu et al., 2012). The *abo3* mutant lost drought tolerance compared to the wild type and was hypersensitive to ABA in both seedling establishment and seedling growth in *Arabidopsis* (Ren et al., 2010). Constitutive expression of *BcWRKY46* in tobacco under the control of the *CaMV* 35S promoter reduced the susceptibility of transgenic tobacco to

freezing, ABA, salt and dehydration stresses (Wang et al., 2012). On the basis of the phenotype analysis results, physiological and biochemical analysis was performed, and the results showed that the expression of *TaWRKY44* increased RWC, proline and soluble sugar accumulation and decreased MDA and IL under drought/salt stresses (Figures 5A–E). Because IL is an indicator of the severity of a membrane injury and MDA is a product of oxidative attack on membrane lipids (Moore and Roberts, 1998), we concluded that less oxidative damage occurred in transgenic plants under drought and salt stress. We detected



**FIGURE 8 | Expression levels of ROS-related and stress-responsive genes in the WT, VC and the transgenic lines (OE-35) under normal and osmotic conditions analyzed qRT-PCR.**

Two-week-old seedlings cultured on MS medium were transplanted to MS with mannitol (300 mM) and NaCl (200 mM) for 1 week. The tobacco leaves were then sampled to extract the total RNA to

generate cDNA. Fourteen gene expression levels were detected; the *NtActin* gene was used as the internal control for normalization. Data are means  $\pm$  SD calculated from three replicates.  $^{*}(P < 0.05)$  and  $^{**}(P < 0.01)$  indicate that the value in the transgenic line is significantly different from that of the WT. Three biological experiments produced similar results.

the  $H_2O_2$  levels in transgenic and control lines before and after drought and salt stress, and the results showed that the  $H_2O_2$  levels in the transgenic lines were obviously lower than in the control lines after drought and salt stress (Figure 7D). These results indicated that the oxidative damage scavenging systems in transgenic plants might work more effectively compared with WT and VC. To detoxify stress-induced ROS, plants have evolved a complex antioxidant system (Miller et al., 2010). Plants possess very efficient enzymatic antioxidant defense systems to protect plant cells from oxidative damage by scavenging ROS (Gill and Tuteja, 2010). SOD provides the first line of defense against ROS by catalyzing the dismutation of  $O_2^-$  to oxygen and  $H_2O_2$ , which is then scavenged by the coordinated action of CAT and POD (Blokhina et al., 2003). The activities of three significant antioxidant enzymes (SOD, CAT, and POD) were assessed in transgenic lines and control lines before and after drought and salt stresses. The results indicated that the activities of three significant antioxidant enzymes (SOD, POD, and CAT) were higher than those in the control lines (Figures 6A–C). These results were similar to previous studies (Huang et al., 2010, 2011; Kong et al., 2011) and demonstrated that the antioxidant enzyme systems were activated to reduce the ROS levels in transgenic lines after drought and salt stresses. Moreover, germination assay was performed to examine the osmotic stress tolerance of the transgenic plants, and the germination rates of the transgenic plants were significantly higher than those of the control plants

grown on MS medium containing 150/300 mM mannitol and 100/200 mM NaCl (Figure 6) and had lower  $H_2O_2$  levels and improved antioxidant enzyme systems compared to controls under osmotic stress (Figure 7).

To gain further insights into the mechanisms of action of *TaWRKY44* in drought/salt/osmotic stresses at the molecular level, the expression levels of 14 ROS-related and stress-responsive genes were tested under osmotic stress; these include genes encoding enzymes for ROS detoxification (*NtSOD*, *NtAPX*, *NtCAT*, *NtPOX*, and *NtGST*); enzymes involved in the biosynthesis of polyamine (*NtADC1* and *NtSAMDC*), sucrose (*NtSPSA*) or ABA (*NtNCED1*); stress-defensive proteins (*NtERD10C*, *NtERD10D*, and *NtLEA5*); and lipid-transfer protein genes (*NtLTP1* and *TobLTP1*). It was found that all of the stress-responsive genes analyzed were significantly upregulated in the transgenic lines either when exposed to mannitol (300 mM) or NaCl (200 mM) treatment or both, with the exception of *NtERD10D* and *NtLEA5*, compared to the control plants (Figure 8). The expression levels of the genes encoding three antioxidant enzymes were upregulated in the *TaWRKY44*-expressing lines with or without stress treatments (Figure 8, *NtSOD*, *NtCAT*, and *NtPOX2*), which is consistent with the results for the antioxidant enzyme activities described above. This could be an explanation for the antioxidant enzymes activities and the  $H_2O_2$  contents in the transgenic lines and controls under drought/salt/osmotic stresses. Although the elaborate

mechanism underlying the up-regulation of these antioxidant genes has not been clearly understood, previous studies have shown that the WRKY transcription factors could regulate the expressions of ROS-related genes in *Tamarix hispida* (Zheng et al., 2013), wheat (Niu et al., 2012; Wang et al., 2013), soybean (Luo et al., 2013), and cotton (Yan et al., 2014) under various stresses. On the other hand, two genes (*NtADC1* and *NtSAMDC*) related to the synthesis of polyamines, which are low-molecular-weight polycations that have been proven to be important stress molecules (Groppa and Benavides, 2008; Jang et al., 2009), were also induced in the transgenic lines relative to the control lines (Figure 8, *NtADC1* and *NtSAMDC*). This polyamine function (osmotic regulator or membrane stabilizer) in stress response could provide another explanation for the enhanced tolerance seen in the transgenic lines. Previous studies demonstrated that SPSA is critical in the synthesis of sucrose in plants and plays an essential role in plant osmotic pressure; *NtNECD1* plays an essential role in ABA biosynthesis regulation (Huang et al., 2010); *NtLEA5* and *NtERD10* (C/D) belong to the LEA protein family that protects and stabilizes macromolecules and/or cellular structures during plant stress responses (Xiong and Zhu, 2002; Liu et al., 2013b); and *NtLTP1* and *TobLTP1* encode the lipid-transfer proteins, which are involved in plant response to ABA, cold, drought and salt stresses (Torres-Schumann et al., 1992; Hu et al., 2013). The up-regulation of these genes implies that these proteins may act as the intermediates between *TaWRKY44* and the phenotype under drought/salt/osmotic stresses. These results demonstrated that the mechanism of enhanced drought/salt/osmotic tolerance in transgenic tobacco plants is the increase of the expression levels of some ROS-related and stress-responsive genes.

In conclusion, a wheat Group I WRKY gene, *TaWRKY44*, was upregulated by PEG, NaCl, ABA, and H<sub>2</sub>O<sub>2</sub> treatments. Expression of *TaWRKY44* enhanced tolerance to drought, salt and osmotic in transgenic tobacco with increased RWC, proline and soluble sugar accumulation, decreased MDA and IL, improved antioxidant system and up-regulated transcription levels of ROS-related and stress responsive genes under various stresses. However, the elaborate mechanisms underlying these phenomena need to be clarified. Although whether these phenomena (compounds accumulation, changes in enzyme activity, genes expression etc.) were caused directly or indirectly by overexpressing *TaWRKY44* cannot be concluded in this study, but these changes were absolutely produced by overexpressing *TaWRKY44*. In our study, we found that full-length of *TaWRKY44* has no transcriptional activation activity, it is suggested that *TaWRKY44* needs additional posttranslational modifications or interactions with its cofactors to exhibit

its full function. Therefore, it is possible that the genes encoding cofactors of *TaWRKY44* were also induced by stress conditions. The induced expression of these genes, together with constitutive expression of *TaWRKY44*, resulted in up-regulation of these stress-related genes, and the increased tolerance of transgenic tobacco to drought/salt/osmotic stresses. In the future, it is necessary to identify if *TaWRKY44* could function directly as transcription factor through binding to the upstream sequence of these genes, result of which will shed light on the mechanisms of *TaWRKY44*-mediated stress tolerance.

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## Supplementary Material

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2015.00615>

**Supplementary Table 1 |** Primer sequences used for cloning wheat WRKY genes and expression analysis.

**Supplementary Table 2 |** Primer sequences used for subcellular localization, vector construction, transgenic confirmation and expression analysis.

**Supplementary Table 3 |** Characteristics of *TaWRKY44-TaWRKY53* in wheat (*T. aestivum* L. cv. Chinese Spring).

**Supplementary Table 4 |** *TaWRKY44-TaWRKY53* expression patterns under abiotic stresses and signaling molecule treatments in wheat (*T. aestivum* L. cv. Chinese Spring).

**Supplementary Table 5 |** The GenBank accession numbers of the WRKY proteins used for drawing phylogenetic tree.

**Supplementary Table 6 |** The GenBank accession numbers of the *TaWRKY44-TaWRKY53*.

**Supplementary Figure 1 |** Multiple alignments of the deduced amino acid sequences of *TaWRKY44-TaWRKY53*.

**Supplementary Figure 2 |** Phylogenetic analysis of TaWRKY domains from various plants.

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# Abscisic acid enhances tolerance of wheat seedlings to drought and regulates transcript levels of genes encoding ascorbate-glutathione biosynthesis

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Glutathione (GSH) and ascorbate (ASA) are associated with the abscisic acid (ABA)-induced abiotic tolerance in higher plant, however, its molecular mechanism remains obscure. In this study, exogenous application ( $10\text{ }\mu\text{M}$ ) of ABA significantly increased the tolerance of seedlings of common wheat (*Triticum aestivum* L.) suffering from 5 days of 15% polyethylene glycol (PEG)-stimulated drought stress, as demonstrated by increased shoot lengths and shoot and root dry weights, while showing decreased content of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and malondialdehyde (MDA). Under drought stress conditions, ABA markedly increased content of GSH and ASA in both leaves and roots of ABA-treated plants. Temporal and spatial expression patterns of eight genes encoding ASA and GSH synthesis-related enzymes were measured using quantitative real-time reverse transcription polymerase chain reaction (qPCR). The results showed that ABA temporally regulated the transcript levels of genes encoding ASA-GSH cycle enzymes. Moreover, these genes exhibited differential expression patterns between the root and leaf organs of ABA-treated wheat seedlings during drought stress. These results implied that exogenous ABA increased the levels of GSH and ASA in drought-stressed wheat seedlings in time- and organ-specific manners. Moreover, the transcriptional profiles of ASA-GSH synthesis-related enzyme genes in the leaf tissue were compared between ABA- and salicylic acid (SA)-treated wheat seedlings under PEG-stimulated drought stress, suggesting that they increased the content of ASA and GSH by differentially regulating expression levels of ASA-GSH synthesis enzyme genes. Our results increase our understanding of the molecular mechanism of ABA-induced drought tolerance in higher plants.

**Keywords:** abscisic acid, ascorbate, drought stress, glutathione, transcription level, *Triticum aestivum* L.

## Introduction

It is known that biotic and abiotic stresses (including drought) induces the generation of reactive oxygen species (ROS), such as the superoxide radical ( $O_2^-$ ) and hydrogen peroxide ( $H_2O_2$ ) (Liu et al., 2009; Borges et al., 2014). The accumulation of ROS damages lipids and proteins, results in cell death, and inhibits plant growth (Li et al., 2011). To alleviate ROS accumulation under stress conditions, both enzymatic and non-enzymatic antioxidants are present in plants. The enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), and ascorbate peroxidase (APX) (Asada, 1992), while the non-enzymatic antioxidants include glutathione (GSH) and ascorbate (ASA) (Li et al., 2011). The SOD catalyzes the dismutation of  $O_2^-$  to  $O_2$  and  $H_2O_2$ , which is subsequently reduced to  $H_2O$  and  $O_2$  by CAT, APX, GR, etc. (Asada, 1992). ASA and GSH function as cofactors of enzymes of the antioxidant pathways, both can also directly quench ROS (Hernandez et al., 2001; Hossain et al., 2012).

The plant hormone abscisic acid (ABA) regulates many important plant developmental processes and is known to induce tolerance to various abiotic stresses; e.g., drought, salt, and low temperature, suggesting that it has significant agronomic potential (Giraudat et al., 1994). The involvement of ABA in mediating drought stress has been extensively explored, and many studies have examined the mechanism of ABA action at the physiological and molecular levels (Ferrandino and Lovisolo, 2014), and their findings have already been reviewed (Verslues and Zhu, 2007; Chinnusamy et al., 2008; Mehrotra et al., 2014). Increased levels of endogenous ABA have been reported in many plant species under abiotic stress, such as drought stress (Aimar et al., 2014), and exogenous ABA decreases ROS accumulation by inducing activities or expression levels of many antioxidative enzymes, resulting in enhanced abiotic tolerance, although the data supporting this hypothesis remain inconsistent. For instance, application of exogenous ABA in drought-stressed kiwifruit plant significantly enhances the activities of guaiacol peroxidase (POD), CAT, SOD, and APX (Wang et al., 2011). In drought-stressed *Cotinus coggygria*, however, ABA decreases activities of CAT, although it also markedly increases activities of SOD and POD (Li et al., 2010).

Moreover, exogenous ABA can increase content of ASA and GSH and enhance plant tolerance to abiotic stresses (Jiang and Zhang, 2002; Liu et al., 2011). To our knowledge, the molecular mechanism of ASA and GSH biosynthesis regulated by exogenous ABA application has not been reported. Wheat is an important drought-sensitive cereal crop whose growth and grain yield are severely affected by drought stress (Doyle and Fischer, 1979; Gao et al., 2011). Polyethylene glycol (PEG) 6000 is often used to stimulate drought stress in higher plants (Xiong et al., 2010; Benesova et al., 2012). In this study, transcript levels of the genes encoding ASA-GSH cycle enzymes were measured

**Abbreviations:** ABA, abscisic acid; ASA, ascorbate; DHAR, dehydroascorbate reductase; GPX, glutathione peroxidase; GR, glutathione reductase; GS, glutathione synthetase; GSH, glutathione; GST, glutathione-S-transferase;  $H_2O_2$ , hydrogen peroxide; MDA, malondialdehyde; MDHAR, monodehydroascorbate reductase; PEG, polyethylene-glycol; SA, salicylic acid.

using qPCR in ABA-treated wheat seedlings suffering from PEG-stimulated drought stress to help us further understand the molecular mechanism of ABA-enhanced drought tolerance in higher plants.

## Materials and Methods

### Plant Materials and Growth Conditions

Seeds of the common wheat (*Triticum aestivum* L.) cv. Yumai 34 were sterilized with 0.01% (m/v)  $HgCl_2$  followed by washing with distilled water. Sterilized seeds were grown hydroponically in full-strength Hoagland's solution (Elberse et al., 2003) in glass dishes (diameter 15 cm) in a FPG-300C-30D incubator (Ningbo Laifu Technology Co., Beijing, China) under a 14-h photoperiod, irradiance of  $250 \mu\text{mol m}^{-2} \text{s}^{-1}$ , day/night temperature of  $25/15^\circ\text{C}$ , and relative humidity of 60/75%. Each dish contained approximately 60 seedlings. After 2 weeks seedlings showed approximately three leaves, and two dishes were grown under the above conditions with fresh Hoagland medium (control, CK), two dishes were incubated with fresh Hoagland medium supplemented with PEG-6000 (15%) solution for drought treatment, and another two dishes were transferred to Hoagland solution supplemented with PEG-6000 (15%) plus 10  $\mu\text{M}$  ABA (Wei et al., 2009) (PEG and ABA treatment, PEG + ABA). Solution (100 mL) was exchanged every day in all treatments. The uppermost fully expanded leaves and the longest roots of wheat seedlings were separately collected at 0, 1, 2, 3, 4, and 5 days after initiating drought stress, and were immediately frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  before assessment of physiological parameters and gene expression profiles.

### Plant Measurements and Analysis

Growth parameters (plant height, root length, fresh and dry weight of root and leaf tissues) were recorded every day during the stress period. Ten individual wheat seedlings were randomly harvested from each dish. Plant heights and dry and fresh weights of roots and leaves were measured and calculated.

### Assays of Malondialdehyde (MDA) and $H_2O_2$ Content in Leaves and Roots of Wheat Seedlings

Lipid peroxidation was determined by estimating the MDA content using the method described by Zheng et al. (2008). The content of  $H_2O_2$  was measured by monitoring the absorbance of the titanium-peroxide complex at 390 nm, following the method of Jessup et al. (1994).

### Assays of GSH and ASA Content

The content of GSH and ASA was measured according to the methods of Kampfenkel et al. (1995) and Smith (1985), respectively.

### Determination of the Transcript Levels of the Eight Genes Encoding ASA-GSH Cycle Enzymes by qPCR

The genes encoding ASA-GSH synthesis-related enzymes are illustrated in Supplementary Figure S1. These genes are glutathione-S-transferase 1 (GST1), glutathione-S-transferase

2 (*GST2*), glutathione peroxidase 1 (*GPX1*), phospholipid hydroperoxide glutathione peroxidase 2 (*GPX2*), glutathione reductase (*GR*), dehydroascorbate reductase (*DHAR*), monodehydroascorbate reductase (*MDHAR*), and glutathione synthetase (*GS*) (Chen et al., 2011), and the genes encoding these enzymes were previously isolated from common wheat in our laboratory (Li et al., 2013). Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and treated with RNase-free DNase I (Takara Biotechnology [Dalian] Co., Ltd., Dalian, China) to remove contaminating genomic DNA. First-strand cDNAs were synthesized from 2 µg of total RNA using Super-Script II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). qPCR was performed using a SYBR Premix Ex Taq (Perfect Real Time) kit (Takara Biotechnology [Dalian] Co., Ltd.) on a Light Cycler 480 Real-Time PCR System (Roche Diagnostics Ltd., West Sussex, UK) according to the manufacturer's instructions. Each reaction (20 µL) comprised 10 µL of SYBR Green Supermix (2×), 1 µL of diluted cDNA, and 0.5 µL of forward and reserve primers. The relative transcript levels were calculated using the  $2^{-\Delta\Delta Ct}$  method, with the wheat *β-actin* (GenBank Accession no. AB181991) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (GenBank Accession no. EF592180) genes as two internal controls. All primers are listed in Supplementary Table S1. Each data point was expressed as the average ± SD of three independent replicates.

### Statistical Analysis

Data were analyzed statistically using one-way analysis of variance and Duncan's multiple range tests to determine significant differences among group means. Significant differences from the control values were determined at  $P < 0.05$ . All recorded values represent the means of the results of three replicates.

## Results

### Effects of Exogenous ABA on Phenotypes and Growth Parameters of Wheat Seedlings Exposed to PEG-stimulated Drought-stress Conditions

In the present study, wheat seedlings suffering from 15% PEG-stimulated drought stress showed time-dependent characteristics (Figure 1). Wheat seedlings suffering from drought stress showed no visible changes before 3 days, and then exhibited significant and deleterious phenotypes, such as curled and wilted leaves, shorter plant heights, and inhibited growth compared to control plants (Figure 1). These qualitative phenotypic effects were confirmed by quantitative analysis (Table 1). However, in the presence of exogenous 10 µM ABA, the effect of drought stress on wheat seedling growth was significantly abrogated. This was also demonstrated by the significantly increased plant heights, fresh and dry shoot weights, and fresh and dry root weights (Table 1). After 5 days, plant height, shoot fresh weight, shoot dry weight, root fresh weight, and root dry weight of PEG + ABA treated wheat seedlings were significantly higher than those of sole PEG-treated wheat seedlings by 11.4, 21.8, 21.4, 34.9, and

23.1%, respectively. These results showed that ABA improved the drought tolerance of wheat seedlings.

In this study, we also found that growth of wheat seedlings was significantly inhibited by 10 µM ABA under normal conditions, which was also confirmed by qualitative and quantitative data (Figure 1, Table 1, Supplementary Figure S2). We speculated that changes in transcriptional levels in this treatment may be associated with wheat growth, and not stress tolerance. In this study, wheat seedlings treated with 10 µM ABA under normal conditions were not used in further experiments.

### MDA and H<sub>2</sub>O<sub>2</sub> Content in Leaves and Roots of Wheat Seedlings Exposed to PEG-stimulated Drought Stress Conditions in Response to Exogenous ABA

MDA and H<sub>2</sub>O<sub>2</sub> content in drought and PEG + ABA treatments also increased in a time-dependent manner (Table 2). However, the MDA and H<sub>2</sub>O<sub>2</sub> content in root and leaf tissues of PEG + ABA-treated wheat seedlings was significantly lower than those of solely PEG-treated wheat seedlings after 3 days of drought (Table 2). After 5 days of drought stress, the MDA and H<sub>2</sub>O<sub>2</sub> content in root and leaf tissues of PEG + ABA-treated wheat seedlings were markedly lower than those of sole PEG-treated wheat seedlings by 8.7 and 31.0%, and 13.3 and 22.6%, respectively (Table 2). These results indicated that exogenous ABA application alleviated the accumulation of MDA and H<sub>2</sub>O<sub>2</sub> induced by drought.

### Effects of Exogenous ABA on GSH and ASA Content in Root and Leaf Tissues of Wheat Seedlings Exposed to PEG-stimulated Drought Stress Conditions

GSH content increased gradually in both leaf and root tissues of PEG-treated wheat seedlings after drought stress, and exogenous ABA application significantly accelerated this increase (Figures 2A,B). After 5 days of drought stress, GSH content in both root and leaf tissues of PEG + ABA treated wheat seedlings was 29.9 and 33.3% higher, respectively, than those in the tissues of solely PEG-treated wheat seedlings. In contrast, ASA content in root and leaf tissues of solely PEG-treated wheat seedlings decreased rapidly with prolonged drought stress, but ABA application inhibited this effect (Figures 2C,D). After 5 days of drought stress, ASA content of the root and leaf of PEG + ABA-treated wheat seedlings was 68.5 and 49.7% higher, respectively, than those in the tissues of solely PEG-treated wheat seedlings.

### Transcript Levels of Genes Encoding Enzymes Involved in the ASA-GSH Cycle in Leaves and Roots of Wheat Seedlings Exposed to PEG-stimulated Drought Stress

*GST1*, *GST2*, *GPX1*, *GPX2*, *GR*, *DHAR*, *MDHAR*, and *GS* transcript levels were measured using qPCR with the *Actin* gene as the internal control in leaf and root tissues of wheat seedlings (Figures 3, 4). Similar results were obtained using *GAPDH*



**FIGURE 1 | Phenotypic changes of wheat seedlings with exogenous application of 10  $\mu\text{M}$  ABA under 15% PEG-stimulated drought stress for 5 days.** CK, control; PEG, 15% PEG-6000; PEG + ABA, 15% PEG and

10  $\mu\text{M}$  ABA treatment. Three independent biological replications were performed with two dishes each, and about 60 wheat seedlings were planted in a dish.

gene as another internal control, as indicated in Supplementary Figures S3, S4.

In root and leaf tissues of control wheat seedlings, the expression levels of the above mentioned eight genes encoding ASA-GSH synthesis-related enzymes remained almost constant (**Figures 3, 4**). Under PEG-stimulated drought-stress conditions, the expression patterns of these eight genes varied in the root

and leaf tissues of wheat seedlings. In roots of PEG-treated wheat seedlings, *GST1* expression was significantly induced at 1 day of stress, decreased rapidly, peaked again at 4 days, and then slowly decreased after 5 days of PEG-stimulated drought stress (**Figure 3A**). *GST2*, *GPX1*, *GPX2*, *GR*, *MDHAR*, and *GS* genes exhibited similar expression patterns in roots of wheat seedlings exposed to PEG-stimulated drought stress. Transcript

**TABLE 1 | Effect of exogenous ABA application on growth characteristics of wheat seedlings suffering from PEG-stimulated drought stress for 5 days.**

Growth parameters	Treatments	0 day	1 day	2 days	3 days	4 days	5 days
Plant height (cm)	Control	15.06 ± 0.49	16.14 ± 0.54	17.02 ± 0.35	18.41 ± 0.49	19.35 ± 0.49 <sup>a</sup>	22.15 ± 0.55 <sup>a</sup>
	ABA		15.69 ± 0.55	16.34 ± 0.43	17.11 ± 0.39	17.90 ± 0.41 <sup>b</sup>	18.98 ± 0.34 <sup>b</sup>
	PEG		15.32 ± 0.58	15.46 ± 0.44	15.94 ± 0.35	16.16 ± 0.40 <sup>c</sup>	16.71 ± 0.38 <sup>c</sup>
	PEG + ABA		15.54 ± 0.56	16.01 ± 0.51	16.79 ± 0.38	17.09 ± 0.45 <sup>b</sup>	18.61 ± 0.63 <sup>b</sup>
Shoot fresh weight (g·plant <sup>-1</sup> )	Control	0.161 ± 0.019	0.217 ± 0.016	0.257 ± 0.012	0.262 ± 0.014	0.291 ± 0.014 <sup>a</sup>	0.381 ± 0.014 <sup>a</sup>
	ABA		0.190 ± 0.013	0.217 ± 0.012	0.230 ± 0.014	0.241 ± 0.011 <sup>b</sup>	0.298 ± 0.013 <sup>b</sup>
	PEG		0.175 ± 0.015	0.188 ± 0.018	0.194 ± 0.016	0.212 ± 0.012 <sup>b</sup>	0.234 ± 0.017 <sup>b</sup>
	PEG + ABA		0.184 ± 0.014	0.205 ± 0.013	0.213 ± 0.018	0.224 ± 0.013 <sup>b</sup>	0.285 ± 0.013 <sup>b</sup>
Shoot dry weight (g·plant <sup>-1</sup> )	Control	0.019 ± 0.002	0.022 ± 0.002	0.025 ± 0.002	0.033 ± 0.001 <sup>a</sup>	0.037 ± 0.001 <sup>a</sup>	0.041 ± 0.001 <sup>a</sup>
	ABA		0.021 ± 0.001	0.023 ± 0.001	0.028 ± 0.001 <sup>b</sup>	0.033 ± 0.001 <sup>b</sup>	0.036 ± 0.000 <sup>b</sup>
	PEG		0.021 ± 0.002	0.022 ± 0.000	0.024 ± 0.002 <sup>b</sup>	0.026 ± 0.001 <sup>c</sup>	0.028 ± 0.001 <sup>c</sup>
	PEG + ABA		0.021 ± 0.001	0.023 ± 0.002	0.027 ± 0.001 <sup>b</sup>	0.032 ± 0.001 <sup>b</sup>	0.034 ± 0.001 <sup>b</sup>
Root fresh weight (g·plant <sup>-1</sup> )	Control	0.091 ± 0.011	0.098 ± 0.014	0.124 ± 0.010	0.148 ± 0.011 <sup>a</sup>	0.194 ± 0.010 <sup>a</sup>	0.233 ± 0.011 <sup>a</sup>
	ABA		0.094 ± 0.011	0.119 ± 0.011	0.129 ± 0.099 <sup>b</sup>	0.141 ± 0.011 <sup>b</sup>	0.157 ± 0.009 <sup>b</sup>
	PEG		0.093 ± 0.012	0.097 ± 0.011	0.099 ± 0.010 <sup>b</sup>	0.101 ± 0.007 <sup>c</sup>	0.106 ± 0.009 <sup>c</sup>
	PEG + ABA		0.092 ± 0.011	0.112 ± 0.013	0.120 ± 0.107 <sup>b</sup>	0.130 ± 0.012 <sup>b</sup>	0.143 ± 0.010 <sup>b</sup>
Root dry weight (g·plant <sup>-1</sup> )	Control	0.010 ± 0.001	0.011 ± 0.002	0.015 ± 0.001	0.017 ± 0.000 <sup>a</sup>	0.018 ± 0.000 <sup>a</sup>	0.022 ± 0.001 <sup>a</sup>
	ABA		0.010 ± 0.001	0.011 ± 0.001	0.014 ± 0.000 <sup>b</sup>	0.016 ± 0.000 <sup>b</sup>	0.017 ± 0.000 <sup>b</sup>
	PEG		0.010 ± 0.001	0.011 ± 0.001	0.012 ± 0.000 <sup>b</sup>	0.012 ± 0.000 <sup>c</sup>	0.013 ± 0.000 <sup>c</sup>
	PEG + ABA		0.010 ± 0.002	0.011 ± 0.001	0.013 ± 0.001 <sup>b</sup>	0.015 ± 0.000 <sup>b</sup>	0.016 ± 0.001 <sup>b</sup>

At 0 day of PEG-stimulated drought stress (before stress), the growth parameters of wheat seedlings are same, because PEG or ABA are not immersed in Hoagland's solution at this time point, and sizes and heights of seedlings in all treatments are uniform at this time point. Average values of 10 plants from one dish were considered as one replication and three independent biological replications were performed. Different letters indicate a significant difference at  $P < 0.05$ .

levels of these six genes were enhanced or inhibited in root tissue within 1–2 days after initiation of PEG-stimulated drought stress, peaked at 3 days, and slowly or rapidly decreased thereafter (**Figures 3B–E,G,H**). *DHAR* expression in roots was significantly induced at 1 day after PEG-stimulated drought stress, whereas it decreased rapidly thereafter (**Figure 3F**). During the PEG-treatment period, exogenous ABA significantly increased *GST1* transcript levels at day 2 (**Figure 3A**), those of *GPX1* at day 1 (**Figure 3C**), *GPX2* at days 1 and 5 (**Figure 3D**), *GR* at days 1 and 2 (**Figure 3E**), *DHAR* at days 3, 4, and 5 (**Figure 3F**), and *MDHAR* and *GS* at days 1, 4, and 5 (**Figures 3G,H**) after PEG treatment.

During PEG-stimulated drought stress, transcript levels of eight genes encoding ASA-GSH synthesis-related enzymes in leaf differed significantly compared to those in root tissue. At all time points after PEG treatment, *GST1* and *GPX1* transcript levels were markedly inhibited (**Figures 4A,C**). *GST2* and *DHAR* genes were induced early, peaked at 2 days after PEG treatment, and then rapidly decreased (**Figures 4B,F**). *GPX2*, *GR*, and *GS* expression levels were strongly inhibited at 1 day after PEG treatment, increased and peaked separately at days 2 and 3, then increased, and rapidly decreased at subsequent time points (**Figures 4D,E,H**). *MDHAR* transcript levels were enhanced at 1 day after PEG treatment, whereas they decreased rapidly at subsequent time points (**Figure 4G**). During the PEG-treatment period, exogenous ABA significantly increased the transcript

levels of *GST1* at days 1, 4, and 5 (**Figure 4A**), *GST2* at days 3, 4, and 5 (**Figure 4B**), *GPX1*, *GPX2* and *GS* at days 2, 3, and 4 (**Figures 4C,D,H**), *GR* at days 1, 3, and 4 (**Figure 4E**), *DHAR* at days 1 and 4 (**Figure 4F**), and *MDHAR* at days 1 and 3 (**Figure 4G**) after PEG treatment.

## Discussion

### Exogenous ABA Enhances the Tolerance of Wheat Seedlings Suffering to PEG-stimulated Drought Stress

Actual soil drought stress is rarely used, because components of soil are very complicated, and it is difficult to control all soil components. In addition, it is also very difficult to discriminate water stress from other abiotic stresses in soil system. However, it is important for water stress experiment to establish a stable and controlled condition (Zhang et al., 2004). PEG have been used extensively to induce plant water deficit in a relatively controlled manner, appropriate to experimental protocols because it is a very low chronic toxicity, molecules with mol wt greater than 3000 are apparently not absorbed at all, and plant water relations can be similar whether the plants are growing in soil or in a PEG solution having an equal water potential (Kaufmann and Eckard, 1971; Mexal et al., 1975; Carpita et al., 1979).

**TABLE 2 | Effect of exogenous ABA on content of MDA and H<sub>2</sub>O<sub>2</sub> in root and leaf tissues of wheat seedlings suffering from PEG-stimulated drought stress for 5 days.**

Parameters	Treatments	0 day	1 day	2 days	3 days	4 days	5 days
MDA content in leaf	Control	5.06 ± 0.10	4.99 ± 0.21	4.95 ± 0.17	5.00 ± 0.12	5.09 ± 0.14 <sup>c</sup>	5.17 ± 0.14 <sup>c</sup>
	PEG		5.25 ± 0.14	5.83 ± 0.13	6.38 ± 0.16	6.66 ± 0.13 <sup>a</sup>	7.22 ± 0.13 <sup>a</sup>
	PEG + ABA		5.16 ± 0.16	5.58 ± 0.12	5.91 ± 0.12	6.12 ± 0.14 <sup>b</sup>	6.59 ± 0.14 <sup>b</sup>
MDA content in root	Control	5.59 ± 0.31	5.55 ± 0.28	5.52 ± 0.24	5.56 ± 0.27 <sup>c</sup>	5.45 ± 0.25 <sup>c</sup>	5.60 ± 0.23 <sup>c</sup>
	PEG		5.88 ± 0.35	6.40 ± 0.27	7.86 ± 0.30 <sup>a</sup>	9.11 ± 0.28 <sup>a</sup>	11.39 ± 0.29 <sup>a</sup>
	PEG + ABA		5.75 ± 0.28	6.08 ± 0.35	6.39 ± 0.36 <sup>b</sup>	7.12 ± 0.32 <sup>b</sup>	7.86 ± 0.20 <sup>b</sup>
H <sub>2</sub> O <sub>2</sub> content in leaf	Control	1.07 ± 0.09	1.04 ± 0.07	1.04 ± 0.05	1.08 ± 0.07 <sup>c</sup>	1.08 ± 0.07 <sup>c</sup>	1.02 ± 0.07 <sup>c</sup>
	PEG		1.27 ± 0.03	1.42 ± 0.10	1.66 ± 0.09 <sup>a</sup>	1.88 ± 0.08 <sup>a</sup>	1.96 ± 0.11 <sup>a</sup>
	PEG + ABA		1.18 ± 0.08	1.26 ± 0.06	1.39 ± 0.05 <sup>b</sup>	1.55 ± 0.08 <sup>b</sup>	1.70 ± 0.08 <sup>b</sup>
H <sub>2</sub> O <sub>2</sub> content in leaf	Control	1.19 ± 0.07	1.19 ± 0.01	1.19 ± 0.08	1.21 ± 0.08 <sup>c</sup>	1.19 ± 0.10 <sup>c</sup>	1.22 ± 0.07 <sup>c</sup>
	PEG		1.28 ± 0.08	1.46 ± 0.05	1.75 ± 0.07 <sup>a</sup>	2.31 ± 0.07 <sup>a</sup>	2.57 ± 0.07 <sup>a</sup>
	PEG + ABA		1.23 ± 0.05	1.28 ± 0.08	1.44 ± 0.05 <sup>b</sup>	1.62 ± 0.07 <sup>b</sup>	1.99 ± 0.06 <sup>b</sup>

At 0 day of PEG-stimulated drought stress (before stress), the physiological parameters of wheat seedlings are same, because PEG or ABA are not immersed in Hoagland's solution at this timepoint, and sizes and heights of seedlings in all treatments are uniform at this time point. The uppermost fully expanded leaves and longest roots of three seedlings were separately collected in one replication and three independent biological replications were performed. Different letters indicate a significant difference at  $P < 0.05$ . Units of MDA and H<sub>2</sub>O<sub>2</sub> content,  $\mu\text{mol.g}^{-1}$  FW.

In this study, application of exogenous 10  $\mu\text{M}$  ABA decreased the growth inhibition caused by 15% PEG 6000-stimulated drought stress, as manifested by increased growth parameters (plant height, shoot and root fresh weights, and shoot and root dry weights), and decreased MDA and H<sub>2</sub>O<sub>2</sub> content (Tables 1, 2). These suggest that exogenous ABA enhances the tolerance of wheat seedlings to drought stress, similar to the previous reports in maize, bermudagrass, and grapevine (Todorov et al., 1998; Lu et al., 2009; Ferrandino and Lovisolo, 2014). However, ABA-enhanced drought tolerance might also be related to its protective roles, such as closing stomata, and decreasing evapotranspiration and solute uptake (Kirkham, 1983). In addition, PEG has some disadvantages in stimulating water stress, including its uptake by plants, hypoxia, and mineral contamination (Lawlor, 1970; Janes, 1974; Reid, 1978; Yaniv and Werker, 1983; Jacomini et al., 1988; Verslues et al., 1998; Blum, 2013). Thus, ABA-induced drought tolerance in wheat plant could need to be further measured under actual soil drought stress conditions.

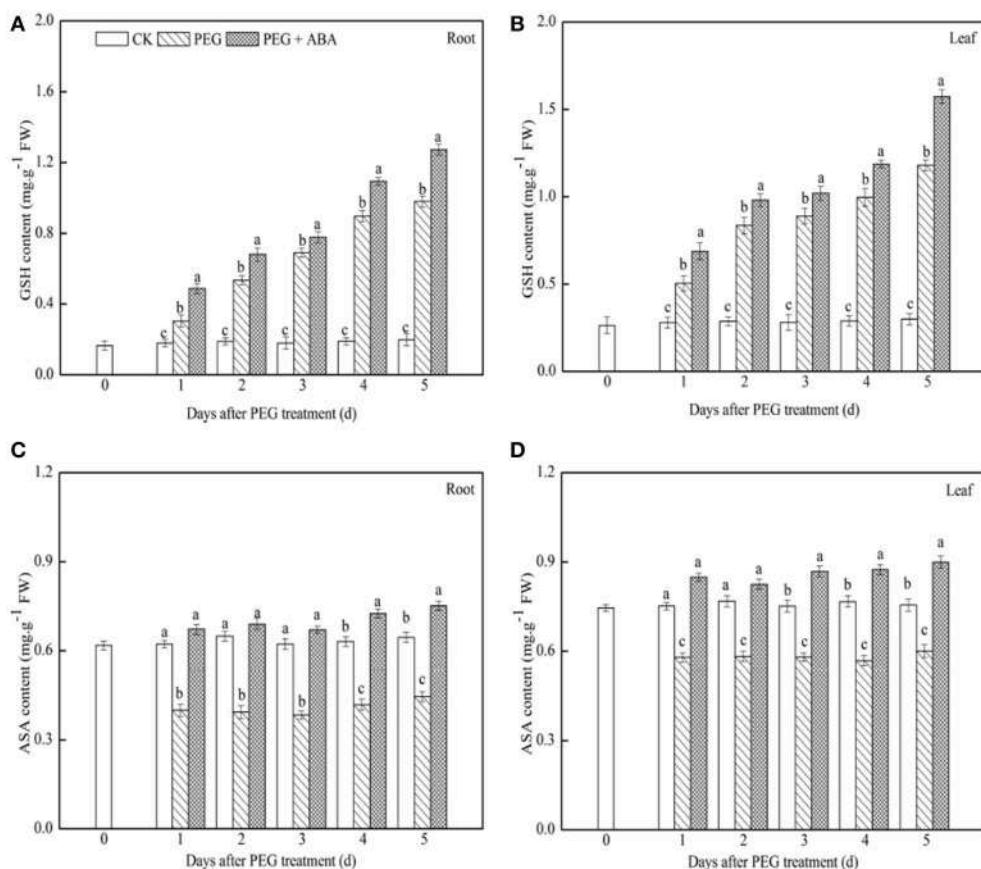
### ABA Temporally Regulates the Transcriptional Levels of the Genes Encoding ASA-GSH Cycle Enzymes in Wheat Seedlings, Resulting in Increased GSH and ASA Content

GSH and ASA are major non-enzymatic antioxidants, and the enzymes and antioxidants in the ASA-GSH cycle play important roles in scavenging of ROS (Shan and Liang, 2010; Liu et al., 2012; Rakić et al., 2014). GSH and ASA content in abiotic-tolerant plant varieties are significantly higher than those in abiotic-sensitive varieties (Vaidyanathan et al., 2003). Overexpression of the genes encoding ASA-GSH cycle enzymes in higher plants confers enhanced tolerance to abiotic stresses (e.g., salt, low temperature) by maintaining higher content of GSH and ASA (Eltayeb et al.,

2006; Duan et al., 2012; Sultana et al., 2012). Our findings indicated that ASA content declined in solely PEG-treated wheat seedlings (Figure 2), indicating that PEG-stimulated drought stress could disturb synthesis of ASA. However, content of GSH increased in this treatment (Figure 2), possibly combating the oxidative stress generated due to drought stresses. This suggested that drought stress had the differential effects on between ASA and GSH synthesis. Under abiotic stresses, various expression profiles of different antioxidative enzymes and antioxidants have also been reported in previous studies (Li et al., 2010; Wang et al., 2011; Hossain et al., 2012).

In the present study, much higher content of ASA and GSH was observed in both root and leaf tissues of PEG + ABA-treated wheat seedlings (Figure 2), implying that the drought tolerance enhanced by exogenous ABA application in wheat seedlings may be related to increased content of GSH and ASA. It has been reported that there may be no post-transcriptional, translational, or post-translational regulations of the genes encoding ASA-GSH cycle enzymes (Shan and Liang, 2010; Chen et al., 2011; Liu et al., 2012). Transcriptional analysis enables quantification of changes in transcript levels of genes. Therefore, transcriptional analysis can facilitate identification of genes involved in the regulation of metabolism and provide valuable insight into the molecular mechanisms of many biosynthetic pathways (Ohdan et al., 2005). In this study, the expression levels of eight genes encoding ASA-GSH cycle enzymes were determined in PEG-stimulated drought-stressed wheat seedlings to identify their associations with the increased GSH and ASA content of wheat seedlings after exogenous ABA application.

Our results showed that, in root and leaf tissues of PEG-treated wheat seedlings to which ABA had been applied, the transcript profiles of ASA-GSH synthesis-related genes varied in a time-dependent manner, and the transcript levels of at least



**FIGURE 2 | Effects of exogenous ABA on content of GSH and ASA in roots and leaves of wheat seedlings suffered from PEG-stimulated drought stress. (A,C),** GSH and ASA content in root of wheat seedling suffering from PEG-stimulated drought stress, respectively; **(B,D),** GSH and ASA content in leaf of wheat seedling

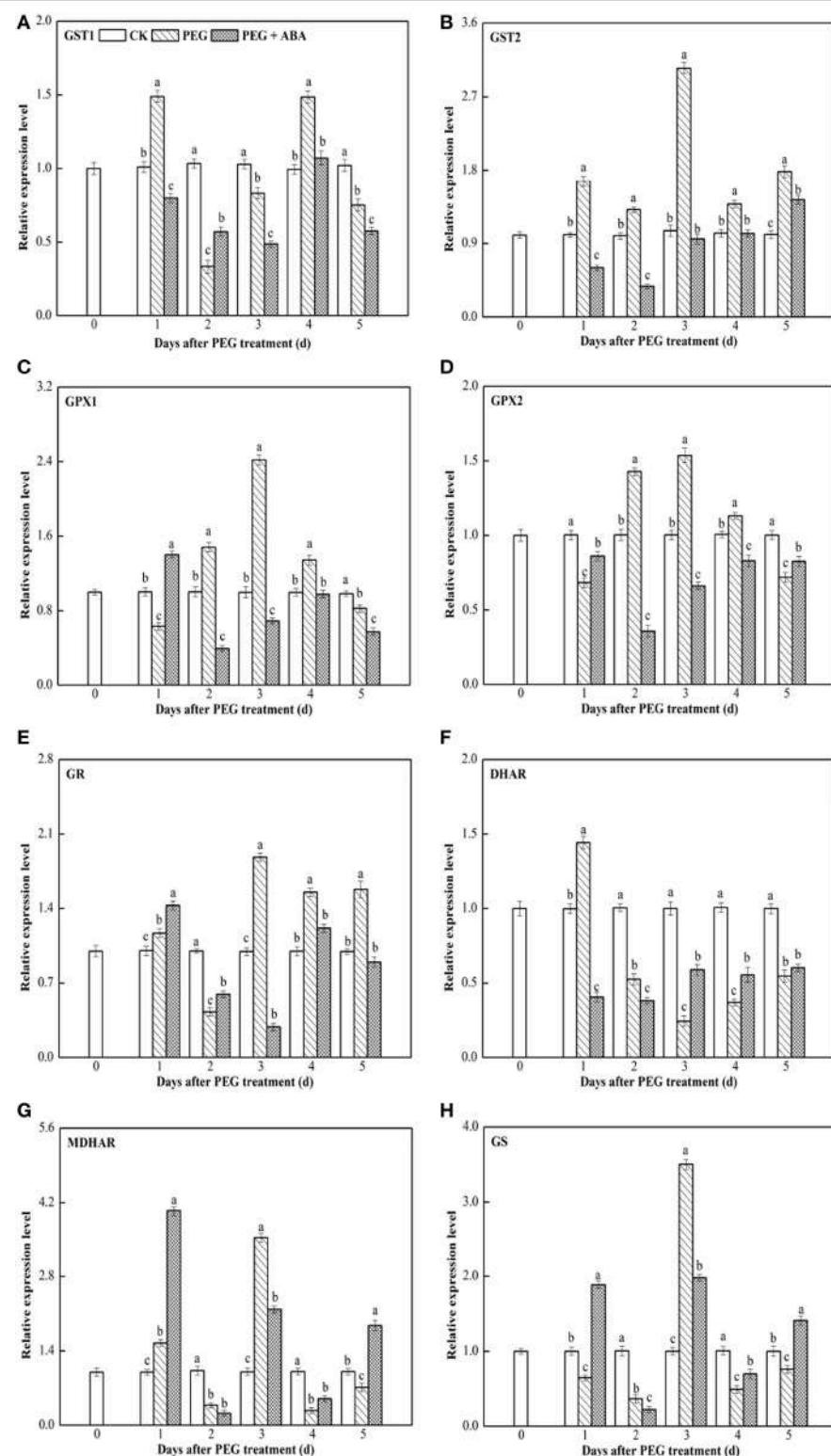
suffering from PEG-stimulated drought stress, respectively. The uppermost fully expanded leaves and longest roots of three seedlings were separately collected in one replication and three independent biological replications were performed. Different letters indicates significant differences ( $P < 0.05$ ).

one of the genes were markedly enhanced by ABA application at each time point (Figures 3, 4, Supplementary Figures S3, S4). This may be associated with the increased content of GSH and ASA during the PEG-stimulated drought-stress period in these two tissues, as well as the enhanced drought tolerance of PEG + ABA-treated wheat plants (Figure 1). These results are also supported by several previous studies of maize and wheat, in which the transcript levels of diverse genes encoding ASA-GSH cycle enzymes were temporally regulated by SA under cold- and salt-stress conditions (Liu et al., 2012; Li et al., 2013).

### Transcriptional Patterns of ASA-GSH Synthesis-related Genes in Root and Leaf Tissues of PEG-treated Wheat Seedlings Treated with Exogenous ABA

Figures 3, 4 and Supplementary Figures S3, S4 show that the root and leaf tissues of drought-stressed wheat seedlings exhibited similar transcriptional patterns of ASA-GSH synthesis genes after pretreatment with ABA. Transcript levels of several genes at various time points, such as *GR* at day 1, *DHAR* at day 4, *MDHAR* at day 1, and *GS* at day 4, were increased by ABA in both the

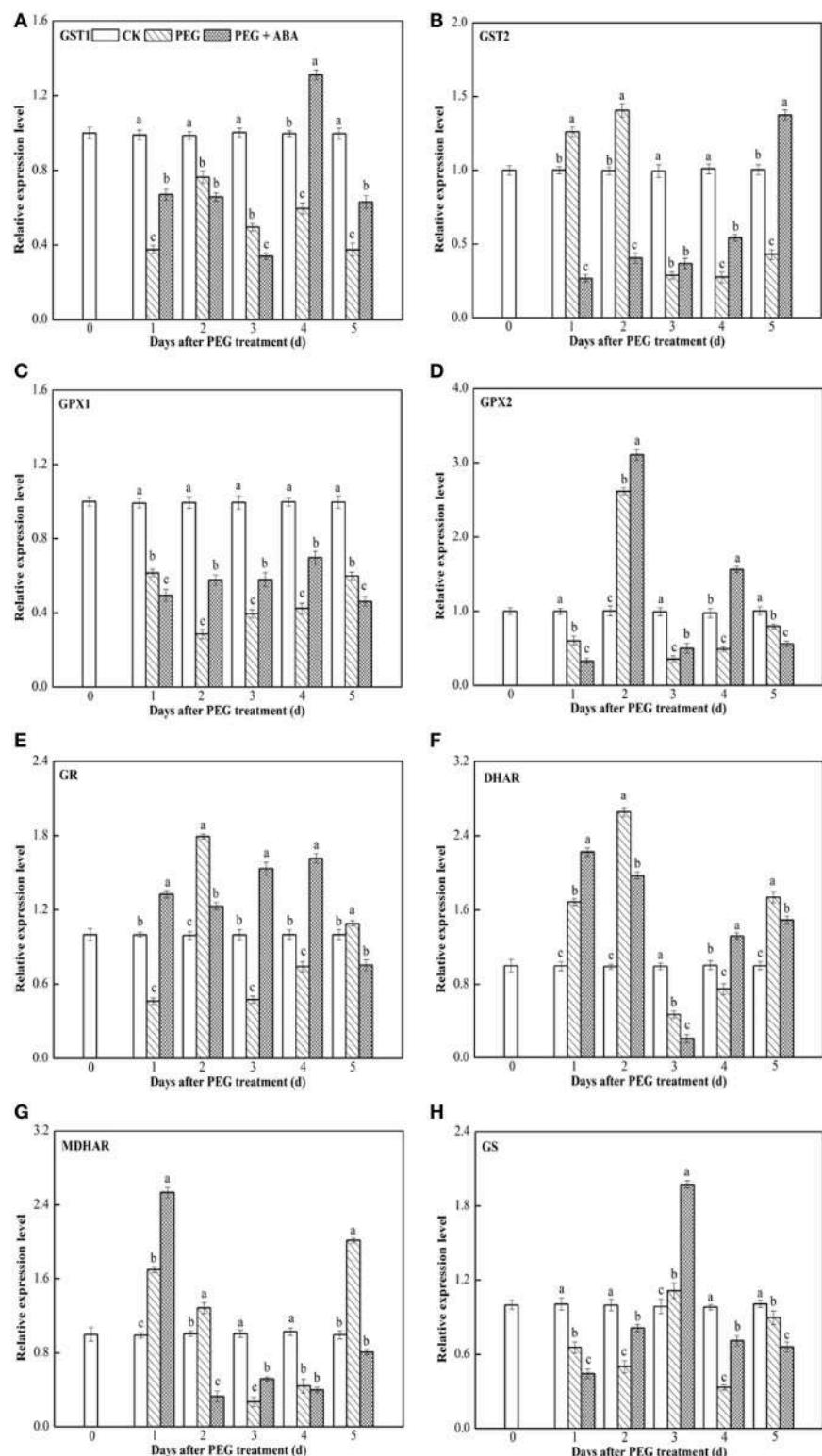
roots and leaves of PEG-treated wheat seedlings (Supplementary Table S2). These results suggested that root and leaf tissues may show similar responses to exogenous ABA application. However, differences in the transcriptional patterns in root and leaf tissues were also observed. For example, transcript levels of *GST1* at days 1 and 4, *GST2* at days 3, 4, and 5, *GPX1* at days 2, 3, 4, *GR* at days 3 and 4, and *GS* at days 2 and 3 significantly increased in the leaves of wheat seedlings subjected to PEG + ABA treatment, whereas transcript levels of these genes markedly decreased at the above time points in root tissue (Supplementary Table S2). These suggest that the mechanisms of action of ABA differ between root and leaf tissues of wheat seedlings. The differences in the transcript levels of ASA-GSH synthesis-related genes identified between root and leaf tissues may be related to differences in GSH and ASA content in the two tissues (Figure 2), or to the different functions, growth environments, and sensitivities of roots and leaves to PEG-stimulated drought stress and ABA. The difference in the transcriptional profiles of root and leaf tissues further suggests that exogenous ABA application may have profound and distinct effects on these two tissues.



**FIGURE 3 | Effects of exogenous ABA on transcript levels of the eight genes encoding ASA-GSH cycle enzymes in root of wheat seedlings suffering from PEG-stimulated drought stress.**

Transcripts were analyzed by qPCR using Actin gene as internal control. **(A–H)**, transcript levels of *GST1*, *GST2*, *GPX1*, *GPX2*, *GR*,

*DHAR*, *MDHAR*, and *GS* genes, respectively. The three seedlings were collected in one replication and three independent biological replications were performed. Each value is the mean  $\pm$  standard deviation of three independent measurements. Different letters indicate significant differences ( $P < 0.05$ ).



**FIGURE 4 | Effects of exogenous ABA on transcript levels of the eight genes encoding ASA-GSH cycle enzymes in leaf of wheat seedlings suffering from PEG-stimulated drought stress.** Transcripts were analyzed by qPCR using *Actin* gene as internal control. **(A–H)**, transcript levels of *GST1*, *GST2*, *GPX1*, *GPX2*, *GR*, *DHAR*, *MDHAR*, and *GS* genes,

respectively. The uppermost fully expanded leaves and longest roots of three seedlings were collected in one replication and three independent biological replications were performed. Each value is the mean  $\pm$  standard deviation of three independent measurements. Different letters indicate significant differences ( $P < 0.05$ ).

## Effects of ABA and Other Plant Hormones on the Transcriptional Profiles of Genes Encoding ASA-GSH Synthesis-related Enzymes under PEG-stimulated Drought Stress

qPCR has been used previously to examine the transcriptional profiles of genes encoding ASA-GSH synthesis-related enzymes regulated by SA in drought- and salt-stressed wheat seedlings, and cold-stressed eggplant (Chen et al., 2011; Kang et al., 2013; Li et al., 2013). The transcriptional profiles of genes encoding ASA-GSH synthesis-related enzymes in the leaf tissue of PEG-treated wheat seedlings in the present study were compared to our previous findings on genes regulated by SA (Kang et al., 2013), because there are many similarities between the two studies; e.g., use of identical materials (leaf tissue of wheat seedlings), experimental conditions (15% PEG-stimulated drought stress), and several sampling time-points (1 and 3 days after initiation of PEG-stimulated drought stress). Expression of *GST* at day 1, *GST2* at day 3, *GPX1* at day 2, *GR* at days 1 and 3, *DHAR* at days 2 and 3, *MDHAR* at day 1, and *GS* at day 2 were induced by both ABA and SA in the leaf tissue of wheat seedlings under PEG-stimulated drought stress (Supplementary Table S3). However, the expression profiles of the majority of genes encoding ASA-GSH synthesis enzymes were differentially regulated by ABA and SA under PEG-stimulated drought conditions (Supplementary Table S3). These results suggest that multiple plant hormones may increase the GSH and ASA content, possibly by differentially regulating the expression of genes encoding ASA-GSH synthesis enzymes.

## Conclusions

Exogenous application of 10  $\mu$ M ABA significantly enhanced the tolerance of wheat seedlings to PEG-stimulated drought stress, as shown by alleviated growth inhibition, reduced content of MDA and  $H_2O_2$ , and increased content of GSH and ASA in the root and leaf tissues. The increased GSH and ASA content may

be associated with upregulated expression levels of ASA-GSH synthesis enzyme genes in time- and organ-specific manners. The transcriptional profiles of eight genes encoding ASA-GSH synthesis enzymes regulated by ABA differed between the root and leaf tissues of PEG-treated wheat seedlings. Comparison of our results with the findings of previous studies showed that, under PEG-stimulated drought-stress conditions, ABA and SA induced differential transcriptional profiles of genes encoding ASA-GSH synthesis enzymes in wheat seedlings. Our findings can provide specific information on the molecular mechanisms of the ASA and GSH synthesis regulated by ABA in drought-stressed plants.

## Author Contributions

Liting Wei and Lina Wang performed the main experimental work, Liting Wei wrote the manuscript, Yang Yang and Pengfei Wang were responsible for viability tests and statistical analysis, Tiancai Guo designed the experiments, and Guozhang Kang provided the financial support and revised the manuscript.

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## Supplementary Material

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2015.00458>

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Antioxidant response and related gene expression in aged oat seed

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To evaluate deterioration of oat seeds during storage, we analyzed oxygen radicals, antioxidant enzyme activity, proline content, and gene transcript levels in oat seeds with different moisture contents (MCs; 4, 16, and 28% w/w) during storage for 0, 6, and 12 months (CK, LT-6, and LT-12 treatments, respectively) at 4°C. The germination percentage decreased significantly with higher seed MCs and longer storage duration. The concentrations of superoxide radical and hydrogen peroxide increased with seed MC increasing. The activities of catalase (CAT), ascorbate peroxidase (APX), and superoxide dismutase (SOD) may have had a complementary or interacting role to scavenge reactive oxygen species. As the storage duration extended, the proline content decreased in seeds with 4 and 16% MC and increased in 28%. These findings suggest that proline played the main role in adaptation to oxidative stress in seeds with higher MC (28%), while antioxidant enzymes played the main role in seeds with lower MCs (4%, 16%). In the gene transcript analyses, SOD1 transcript levels were not consistent with total SOD activity. The transcript levels of APX1 and CAT1 showed similar trends to those of APX and CAT activity. The transcript levels of P5CS1, which encodes a proline biosynthetic enzyme, increased with seed MC increasing in CK. Compared with changing of proline content in seeds stored 12 months, PDH1 transcript levels showed the opposite trend and maintained the lower levels in seeds of 16 and 28% MCs. The transcript level of P5CS1 was significantly affected by MC, and PDH1 could improve stress resistance for seed aging and maintain seed vigor during long-term storage.

**Keywords:** oat seed, antioxidant enzymes, proline, storage duration, moisture content, gene expression regulation

## Introduction

Deterioration of seeds is a major problem in agricultural production. Seed deterioration depends on the temperature, seed moisture content (MC), and duration of storage (Priestley, 1986; Spanò et al., 2004). The vigor of seeds is reduced or lost during long-term storage, even as stored under low-temperature and low-moisture conditions. The reduction in seed vigor leads to commercial losses and decreased genetic diversity (Lu et al., 2005). Seed aging is associated with certain changes in cellular metabolism and biochemistry, including lipid peroxidation, enzyme inactivation, disruption of membrane integrity, and damage to DNA (McDonald, 1999; Hu et al., 2012). Although the exact mechanisms of seed aging are unknown, the accumulation of reactive oxygen species (ROS), including the superoxide radical ( $O_2^-$ ) and hydrogen peroxide ( $H_2O_2$ ), has been suggested to be the major cause of seed deterioration (Lehner et al., 2008; Yao et al., 2012). It has been hypothesized that seeds germinate completely only when the ROS content is maintained below

a critical threshold (Bailly et al., 2008). Malondialdehyde (MDA) is a product of ROS, and is a biomarker of oxidative damage (Bailly et al., 1996). To minimize the damaging effects of ROS, antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) scavenge ROS (Moller et al., 2007). The amount of ROS is linked to the rate of their production and the capacity of the antioxidant system (Esfandiari et al., 2007). Overexpression of genes encoding SOD, APX, and CAT in tobacco, wheat, and *Arabidopsis* resulted in enhanced seed longevity (Melchiorre et al., 2009; Li and Yi, 2012). Proline has also been shown to scavenge ROS (Smirnoff and Cumbes, 1989). In many plant species, proline accumulation is one of the main metabolic responses to abiotic stress (Evers et al., 2010; Irina et al., 2012). Early studies on proline established a model whereby stress caused the transcriptional up-regulation of the gene encoding  $\Delta$ -1-pyrroline-5-carboxylate synthetase1 (*P5CS1*), which catalyzes the first two steps of proline biosynthesis (Armengaud et al., 2004), and down-regulation of the gene encoding proline dehydrogenase (*PDH1*), which catalyzes the first step of proline catabolism (Kiyosue et al., 1996; Miller et al., 2005). Both genes were necessary and sufficient for stress-induced proline accumulation.

Oat (*Avena sativa* L.) is the fifth largest cereal crop in the world, with an annual yield of approximately 700 000 tons. Compared with other cereals, oat seed has higher concentrations of soluble fiber, vitamins, minerals, antioxidants, and high quality protein. Therefore, it is an important cereal in terms of its nutritional value (Klose and Arendt, 2012). In recent years, oat has been cultivated more widely, and has become an important forage grass in alpine regions where other grasses cannot grow. The lager deterioration occurs in oat seeds with 10 years storage (Magdalena et al., 1999). The oat seed deterioration results in greater losses in seed vigor, causing great economic losses (Price, 1975; Heneen et al., 2008). The oat seeds have higher lipid content than other cereals such as wheat, maize, rice, and barley. This high lipid content results in faster deterioration of oat seeds (Pekka et al., 2003). The seed MC is another key factor affecting seed vigor during storage. The germinability lost in untreated oat seed was found to depend on its temperature and water content at storage condition (Machacer et al., 1961; Kong et al., 2014). However, the physiological and transcriptional changes in oat seeds with different MCs during storage are unknown.

In this study, we examined the dynamics of ROS, antioxidant enzyme activity, proline content, and gene transcript levels of *APX1*, *CAT1*, *SOD1*, *P5CS1*, and *PDH1* in deteriorated oat seeds with different MCs during storage. The aims were to investigate whether storage treatments at low temperature (4°C) affected ROS levels via its effects on the enzymatic system and proline content, and to evaluate the transcript level of genes encoding antioxidant enzymes during seed aging.

## Materials and Methods

### Plant Material

Oat seeds (Lot#P708O2498) were purchased from the Lockwood Seed and Grain Company (Woodland, CA, USA). The

experiments were initiated in May, 2012. The seeds were initially at 8.8% MC and germinated 98% (ISTA, 2012).

### Determination of Seed MC

Seed MC was measured according to the ISTA Procedure (2012). Briefly, approximately 4.5 g seeds were placed in a sample container, weighed, dried at 130–133°C for 1 h, and then reweighed. Four replicates were evaluated for each sample.

### Adjustment of Seed MC

To adjust the MC of the oat seeds to 4, 16, and 28% (w/w) before storage, seeds (~25 g) with 8.8% MC were placed in an aluminum foil bag and then subjected to rehydration or dehydration to achieve the desired water content. The seed MCs were adjusted to 16 and 28% by adding appropriate amounts of distilled water into the foil bags and incubating the seeds at 5°C for 48 h. The seeds were adjusted to 4% water content by desiccation.

### Storage and Germination Assay

Seeds with different MCs were stored at 4°C for 0 months (CK), 6 months (LT-6), and 12 months (LT-12). The seed germination percentage was determined by standard germination tests according to the ISTA protocol (ISTA, 2012). Four replicates of 100 seeds each were germinated in 150-mm Petri dishes on filter paper hydrated with 14 ml water. Germination tests were conducted in a growth chamber (Bio Chamber-Enconair, Winnipeg, MB, Canada) at 20°C under an 8-h light/16-h dark photoperiod. The number of normal seedlings was recorded after 10 days. The seed germination percentage was expressed as the percentage of normal seedlings determined as described in the ISTA protocol (ISTA, 2012).

### Determination of Superoxide Anion ( $O_2^-$ ) Production Rate

The  $O_2^-$  production rate was measured as described elsewhere (Elstner and Heupel, 1976). Seed embryos (1 g) were ground in liquid nitrogen, homogenized in 7 ml phosphate buffer (50 mM, pH 7.8), and then centrifuged at 16 000 rpm for 10 min. The supernatant was centrifuged again. Then, 1 ml supernatant was mixed with 0.9 ml phosphate buffer (50 mM) and 0.1 ml hydroxylamine hydrochloride. The mixture was incubated at 25°C for 30 min, and then mixed with 1 ml sulfanilic acid (17 mM) and 1 ml  $\alpha$ -naphthylamine (7 mM); the mixture was incubated at 25°C for 20 min and then the absorbance at 530 nm was recorded.

### Determination of Hydrogen Peroxide ( $H_2O_2$ ) Content

To measure the  $H_2O_2$  content, embryos (200 mg) were ground in liquid nitrogen, homogenized in 2.0 ml cold acetone, and then centrifuged at 16 000 rpm for 10 min. The supernatant (1.0 ml) was mixed with 100  $\mu$ l 10% (w/v) titanium tetrachloride and 200  $\mu$ l ammonia water, and then mixed well. The mixture was centrifuged at 3000 rpm for 10 min, and the supernatant was discarded. The pellet was dissolved in concentrated sulfuric acid, and then absorbance at 415 nm was recorded. A standard curve was prepared by diluting a 100  $\mu$ mol/L  $H_2O_2$  stock solution to 10, 20, 40, 60, 80, 100  $\mu$ mol/L.

## Determination of Antioxidant Enzyme Activities

To extract enzymes, embryos (200 mg) were ground in liquid nitrogen, homogenized in 2 ml phosphate buffer (50 mM, pH 7.0), and then centrifuged at 15 000 rpm for 20 min at 4°C. The supernatant was used in the antioxidant enzyme assays.

The activity of SOD was determined according to the method of (Beaucham and Fridovic, 1971). The 3 ml reaction mixture contained 13 mM methionine, 1.3 µM riboflavin, 63 µM nitroblue tetrazolium (NBT) in 50 mM phosphate buffer (pH 7.8), and 25 µl enzyme extract. The enzyme extract was replaced with phosphate buffer in two controls. The reaction mixtures were incubated in a growth chamber (LRH-250-GII, Ningbo, China) at 25°C under illumination. Identical tubes that were not illuminated served as blanks. After illumination for 17 min, absorbance was measured at 560 nm.

The activity of APX was measured in the presence of 0.25 mM ascorbic acid and 10 mM H<sub>2</sub>O<sub>2</sub> by monitoring the decrease in absorption at 290 nm. The reaction mixture consisted of 200 µl supernatant, 3.4 ml phosphate buffer, 200 µl ascorbic acid, and 200 µl H<sub>2</sub>O<sub>2</sub>. Enzyme activity was determined by measuring the change in absorbance at 290 nm every minute.

To measure CAT activity, 50 µl supernatant was mixed with 3.4 ml phosphate buffer (25 mM, pH 7.0, containing 0.1 mM EDTA), and 200 µl H<sub>2</sub>O<sub>2</sub>. Enzyme activity was determined by measuring the change in absorbance at 240 nm after 1 min.

## Determination of Proline Content

To measure proline content, embryos (200 mg) were ground in liquid nitrogen, homogenized in 2 ml 3% sulfosalicylic acid, and then centrifuged at 4 000 rpm for 5 min. The supernatant (200 µl) was mixed with 200 µl glacial acetic acid and 200 µl acidic ninhydrin, and then mixed well. The mixture was incubated at 100°C for 60 min. The reaction was terminated by placing the mixture on ice, and then extracting the sample with 1.0 ml toluene. The absorbance of the mixture was measured at 520 nm. To prepare the standard curve, an L-proline stock solution (100 µg/ml) was diluted to 1, 3, 5, 7, 9, 11 µg/ml.

## Gene Transcript Analyses

Total RNA was isolated from seed embryos using the phenol-chloroform method (Cooley et al., 1999). Briefly, seed embryos (100 mg) were ground in liquid nitrogen and homogenized in 2 ml RNA extraction buffer (15 ml TLE buffer, 15 ml phenol, 3 ml chloroform) with 30 µl β-mercaptoethanol. The mixture was shaken for 30 min at 300 rpm at 4°C, and then centrifuged at

10 000 rpm for 2 min. The supernatant was collected and mixed with an equal volume of phenol–chloroform–isoamylalcohol (25:24:1), then shaken for 10 min at 4°C and centrifuged for 2 min at 10 000 rpm at 4°C. The supernatant was collected and mixed with an equal volume of chloroform–isoamyl alcohol (24:1), then shaken for 10 min at 4°C and centrifuged for 5 min at 10 000 rpm at 4°C. The supernatant was collected and mixed with a one-third volume of 8 M LiCl, mixed thoroughly, and then incubated at –20°C overnight. The next day, the sample was thawed and then centrifuged at 10 000 rpm for 3 min. The pellet was washed with 1.0 ml 80% (v/v) ethanol. The RNA was dried completely, and then dissolved in 50 µl diethyl pyrocarbonate water. The RNA quality was assessed by 1% agarose gel electrophoresis.

Candidate and reference gene sequences corresponding to the top BLAST hits were identified at the Compositae Genome Project EST database based on sequence homology to known candidates in *Arabidopsis*, and from existing oat sequence data in GenBank. Primer sequences for qRT-PCR analyses were designed using Primer Express (Applied Biosystems, Foster City, CA, USA). The genes and primers used for qRT-PCR analyses are shown in **Table 1**.

Total RNA was reverse-transcribed and DNase-treated using a QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). The cDNAs of housekeeping genes and genes of interest were PCR-amplified with an Applied Biosystems 7300 Real-Time PCR System using SYBR Green detection. For each sample, the change in fluorescence was analyzed using DART PCR 1.0 software.

## Statistical Analyses

Data were subjected to analysis of variance (ANOVA) using SPSS for Windows ver. 13.0. Duncan's multiple range tests ( $P = 0.05$ ) were used to compare treatment means of germination and physiological indicators.

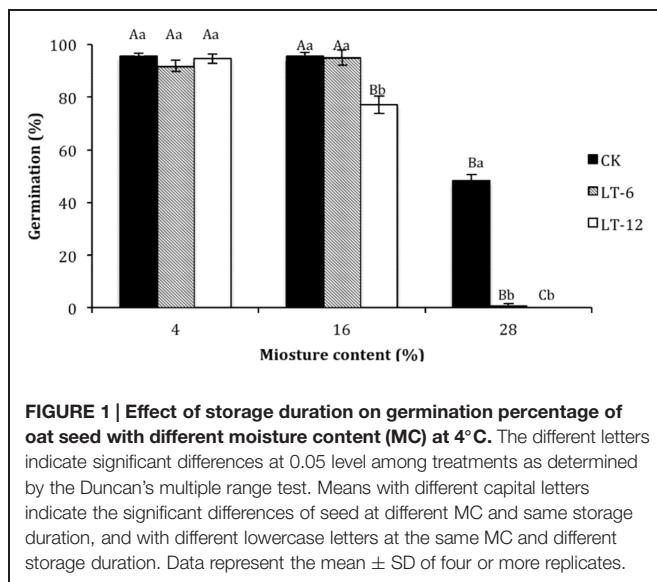
## Results

### Changes in Germination Percentage in Oat Seeds during Storage

The germination percentage of oat seeds decreased with seed MCs increasing (**Figure 1**). In the storage treatments of CK and LT-6, the germination percentage decreased significantly ( $P < 0.05$ ) at 28% MC. In LT-12, the germination percentage decreased significantly ( $P < 0.05$ ) with MC increasing from 4 to 28%.

**TABLE 1 |** Primers used in real-time PCR.

Accession no.	Gene name	Gene description	Forward primers	Reverse primers
AT2G39800.1	P5CS1	Biosynthesis of proline	TGTCCTCTGGGTGTTCTTGTAT	CGAATGGCTAAAGACGCAATC
AT3G30775	PDH1	Proline oxidase	CCCCGTGGAGCACATCAT	AAGGTTGAAGCAGAGAGCAATCC
AT1G20630	CAT1	Catalase 1	CAGGCTGGCGAGAGATTCC	AGGATCCGTGAATGCATCAA
AT1G07890	APX1	Ascorbate peroxidase 1 (APX1)	GCTCCGTGAAGTAAGTGTATCAAAC	CCTGGGAAGGTGCCACAA
AT1G12520	SOD1	Copper–Zinc Superoxide dismutase 1 (SOD1)	CACAAGCACTTCACAGGAACAGT	TGCCACTCTGAACATTTCATCAC
AT2G37620	ACTIN1	Actin	GCTATTCAAGCCGTGCTTC	AGCATGTGGAAGGGCATAAC



**FIGURE 1 | Effect of storage duration on germination percentage of oat seed with different moisture content (MC) at 4°C.** The different letters indicate significant differences at 0.05 level among treatments as determined by the Duncan's multiple range test. Means with different capital letters indicate the significant differences of seed at different MC and same storage duration, and with different lowercase letters at the same MC and different storage duration. Data represent the mean  $\pm$  SD of four or more replicates.

The germination percentage of oat seeds with 4% MC did not change significantly ( $P > 0.05$ ) during storage (Figure 1). At 16% MC, the germination percentage of seeds in LT-12 decreased significantly ( $P < 0.05$ ). The germination percentage of seeds in LT-6 and LT-12 was zero at 28% MC (Figure 1).

### Changes in Superoxide Anion ( $O_2^-$ ) Production Rate in Oat Seeds during Storage

In the storage treatments of CK, the  $O_2^-$  production rate increased significantly ( $P < 0.05$ ) at 28% MC. The  $O_2^-$  production rate in the LT-6 treatment increased significantly ( $P < 0.05$ ) with MC increasing, but not in the LT-12 treatment (Figure 2A).

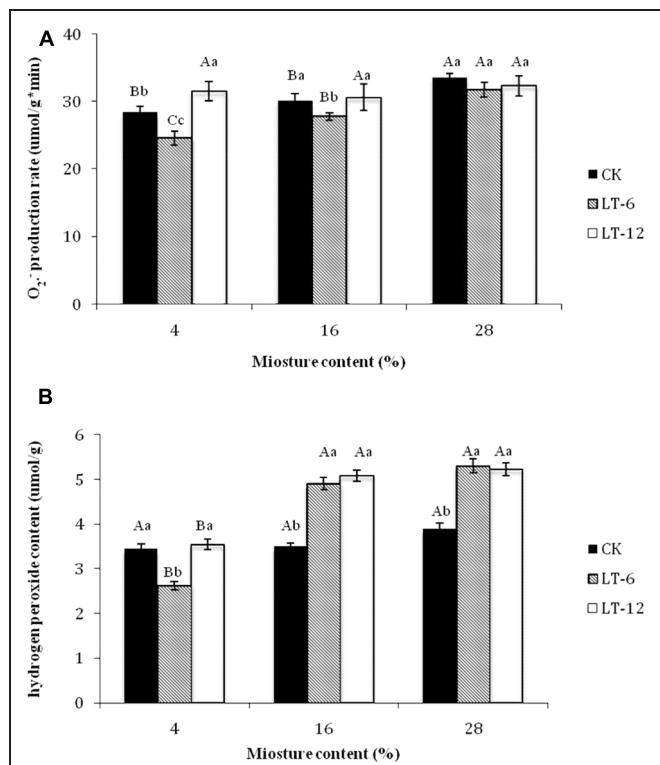
In the seeds with 4 and 16% MC, the  $O_2^-$  production rate decreased and then increased during storage; the lowest rate was after the stored 6 months (Figure 2A). For seeds with 28% MC, there were no significant ( $P > 0.05$ ) differences for  $O_2^-$  production rate among CK, LT-6, and LT-12 treatments.

### Changes in Hydrogen Peroxide ( $H_2O_2$ ) Contents in Oat Seeds during Storage

In the storage treatments of CK, there was not significantly ( $P > 0.05$ ) different for  $H_2O_2$  content of oat seeds among different MCs (Figure 2B). In the LT-6 and LT-12 treatments, the  $H_2O_2$  contents of seeds with 16 and 28% MC were significantly higher ( $P < 0.05$ ) than those with 4% MC. For seeds with 4% MC, the lowest of  $H_2O_2$  content presented after stored 6 months. For 16 and 28% MCs, the  $H_2O_2$  content in LT-6 and LT-12 were significantly higher ( $P < 0.05$ ) than those in CK (Figure 2B).

### Changes in Activities of Antioxidant Enzymes in Oat Seeds during Storage

In the storage treatments of CK, there was no significant difference ( $P > 0.05$ ) for SOD activity of seeds among different MC (Figure 3A). In LT-6 and LT-12, the SOD activity significantly ( $P < 0.05$ ) decreased with MC increasing from 4 to 28%. For

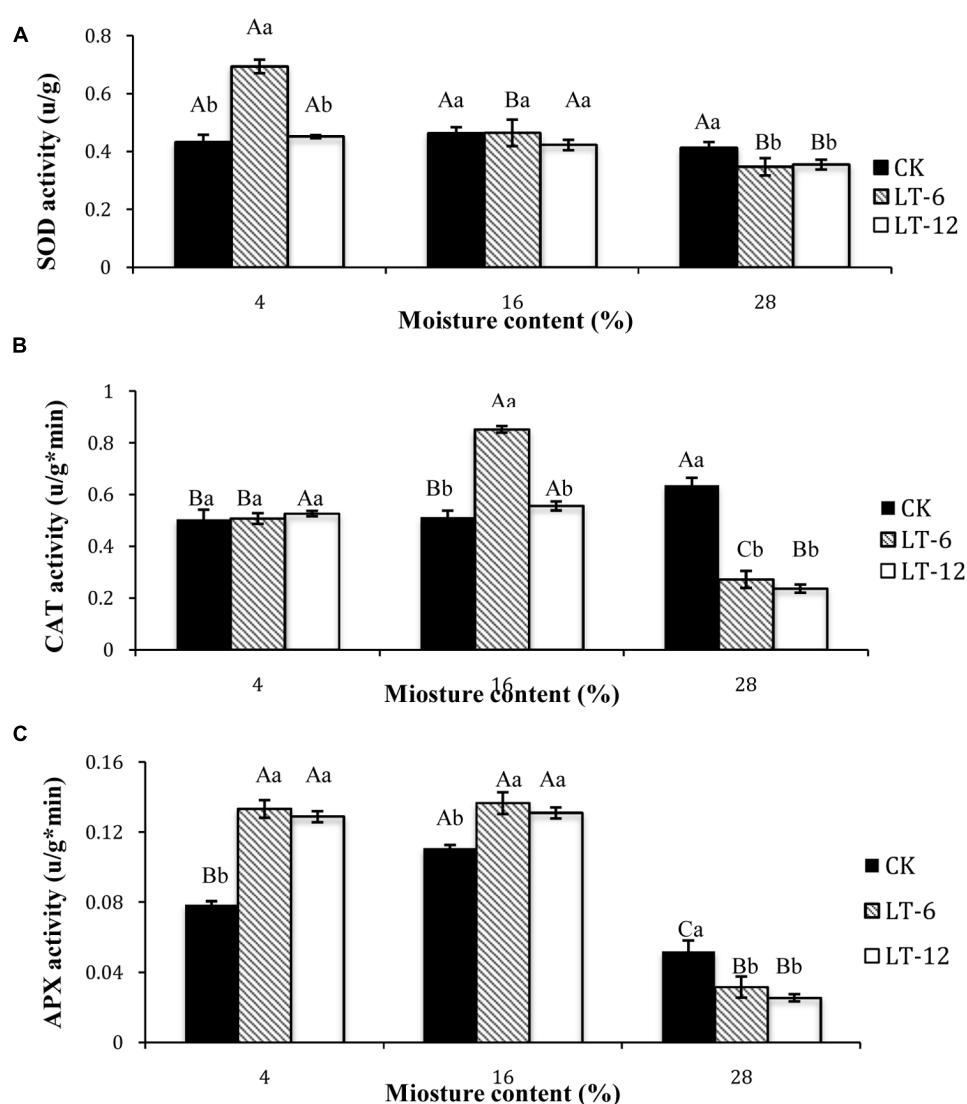


**FIGURE 2 | Effect of storage duration on  $O_2^-$  production rate and  $H_2O_2$  content of oat seed with different MC at 4°C.** (A)  $O_2^-$  production rate; (B)  $H_2O_2$  content. The different letters indicate significant differences at 0.05 level among treatments as determined by the Duncan's multiple range test. Means with different capital letters indicate the significant differences of seed at different MC and same storage duration, and with different lowercase letters at the same MC and different storage duration. Data represent the mean  $\pm$  SD of four or more replicates.

seeds with 4% MC, SOD activities in CK and LT-12 were significantly ( $P < 0.05$ ) lower than that in LT-6. For seeds of 16% MC, there were no significant ( $P < 0.05$ ) differences for SOD activity among storage treatments. For seeds with 28% MC, SOD activity in LT-6 or LT-12 was significantly ( $P < 0.05$ ) lower than that in CK.

In the storage treatments of CK, the CAT activity of seeds with 28% MC was significantly ( $P < 0.05$ ) higher than that with 4 or 16%. In LT-6, the highest CAT activity occurred in seeds with 16% MC, and the lowest was in 28% MC. In LT-12, CAT activity in seeds of 28% MC decreased significantly ( $P < 0.05$ ). For 4% moisture seeds, there were no significant ( $P > 0.05$ ) differences for CAT activity among CK, LT-6, and LT-12. For 16% moisture seeds, the maximum CAT activity appeared after stored 6 months. For 28% moisture seeds, CAT activity in CK was significantly ( $P < 0.05$ ) higher than other treatments (Figure 3B).

The changes of APX activity in oat seeds during storage showed that the highest APX activity in CK occurred in 16% moisture seeds, and the lowest was in those with 28% MC (Figure 3C). In LT-6 and LT-12, APX activities decreased significantly ( $P < 0.05$ ) in 28% moisture seeds. For seeds with 4 and 16% MC, APX activities in LT-6 and LT-12 were significantly higher



**FIGURE 3 |** Effect of storage duration on SOD, CAT, and APX activity of oat seed with different MC at 4°C. **(A)** SOD; **(B)** CAT; **(C)** APX. The different letters indicate significant differences at 0.05 level among treatments as determined by the Duncan's multiple range test. Means with different capital

letters indicate the significant differences of seed at different MC and same storage duration, and with different lowercase letters at the same MC and different storage duration. Data represent the mean  $\pm$  SD of four or more replicates.

( $P < 0.05$ ) than that in CK. In seeds with 28% MC, changing of APX activity was opposite (Figure 3C).

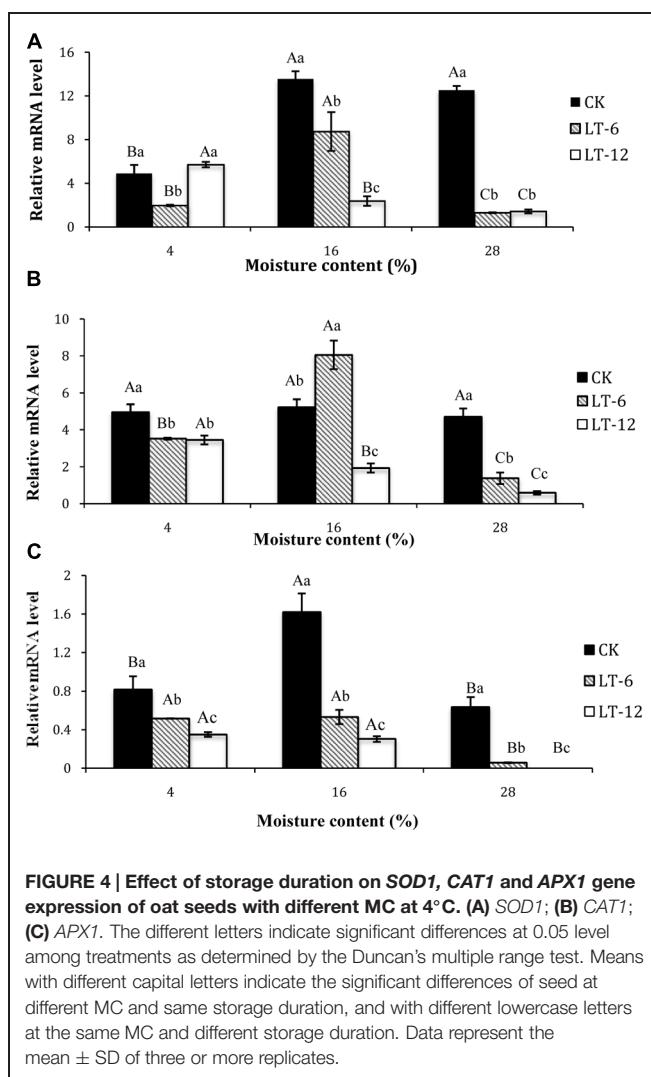
### Changes in Transcript Levels of Genes Encoding Antioxidant Enzymes in Oat Seeds During Storage

According to the results of transcript levels in oat seeds with different MC (Figure 4A), the *SOD1* transcript level in CK was lowest in 4% moisture seeds. In LT-6, the highest *SOD1* transcript level presented in seeds of 16% MC. In LT-12, the *SOD1* transcript level were down-regulated significantly ( $P < 0.05$ ) with MC increased from 4% to 28%. For seeds with 4% MC, *SOD1* in LT-6 was down-regulated and up-regulated in LT-12. In seeds with

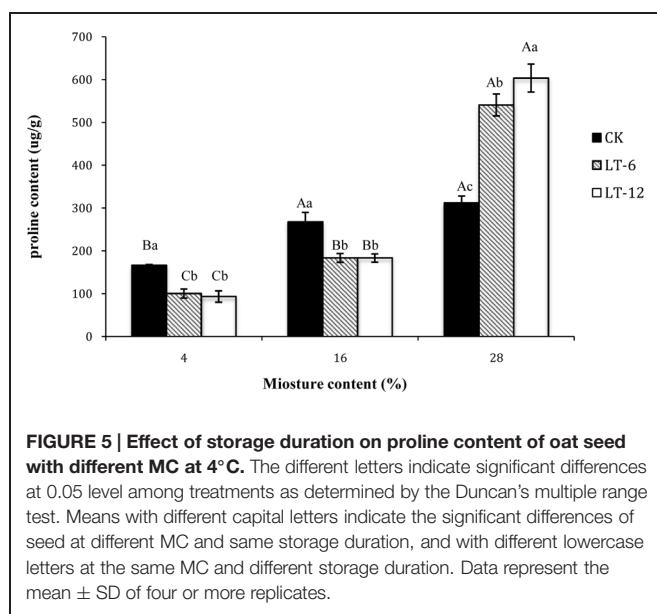
16 and 28% MC, the levels of *SOD1* were down-regulated as the storage duration prolonged.

The *CAT1* transcript levels of seeds were similar among different MC in CK (Figure 4B). In LT-6 and LT-12, the changing of *CAT1* transcript level was similar with *SOD1*. The *CAT1* transcript levels tended to decrease with increasing storage duration in seeds with 4 and 28% MCs (Figure 4B). The transcript level of *CAT1* in seeds with 16% MC was up-regulated in LT-6, but down-regulated in LT-12.

The changes of *APX1* indicated that the highest *APX1* level in CK occurred in seeds of 16% MC, and the lowest was of 28% MC. The transcript levels of *APX1* in seeds with 4, 16, and 28% MC all decreased significantly ( $P < 0.05$ ) during storage (Figure 4C).



**FIGURE 4 | Effect of storage duration on *SOD1*, *CAT1* and *APX1* gene expression of oat seeds with different MC at 4°C. (A) *SOD1*; (B) *CAT1*; (C) *APX1*.** The different letters indicate significant differences at 0.05 level among treatments as determined by the Duncan's multiple range test. Means with different capital letters indicate the significant differences of seed at different MC and same storage duration, and with different lowercase letters at the same MC and different storage duration. Data represent the mean  $\pm$  SD of four or more replicates.



**FIGURE 5 | Effect of storage duration on proline content of oat seed with different MC at 4°C.** The different letters indicate significant differences at 0.05 level among treatments as determined by the Duncan's multiple range test. Means with different capital letters indicate the significant differences of seed at different MC and same storage duration, and with different lowercase letters at the same MC and different storage duration. Data represent the mean  $\pm$  SD of four or more replicates.

significant ( $P > 0.05$ ) differences for *P5CS1* transcript levels in seeds with 4% MC during storage. For seeds with 16% MC, the transcript levels of *P5CS1* in LT-6 and LT-12 were higher than in CK. For seeds with 28% MC, the transcript levels of *P5CS1* reached the minimum level after stored 12 months.

The changing of transcript levels of *PDH1* during seed storage indicated that *PDH1* levels in CK and LT-6 both increased with MC increasing from 4 to 28% (Figure 6B), but there was opposite changing for the transcript levels of *PDH1* in LT-12. For seeds with 4% MC, there were no significant ( $P > 0.05$ ) differences for transcript levels of *PDH1* among three storage treatments. For seeds with 28% MC, the transcript levels of *PDH1* decreased with storage duration prolonged, and reached the minimum after stored 12 months.

## Changes in Proline Contents in Oat Seeds during Storage

The proline contents in LT-6 and LT-12 increased significantly ( $P < 0.05$ ) with seed MC increasing from 4 to 28% (Figure 5). In CK, the proline contents of seeds with 4% MC was significantly ( $P < 0.05$ ) lower than that with 16 and 28% MC. For seeds with 4 and 16% MC, there were no significant ( $P > 0.05$ ) differences in proline content between LT-6 and LT-12, but both showed significantly ( $P < 0.05$ ) lower proline contents than those in CK (Figure 5). For seeds with 28% MC, the proline content increased significantly ( $P < 0.05$ ) with storage duration prolonged.

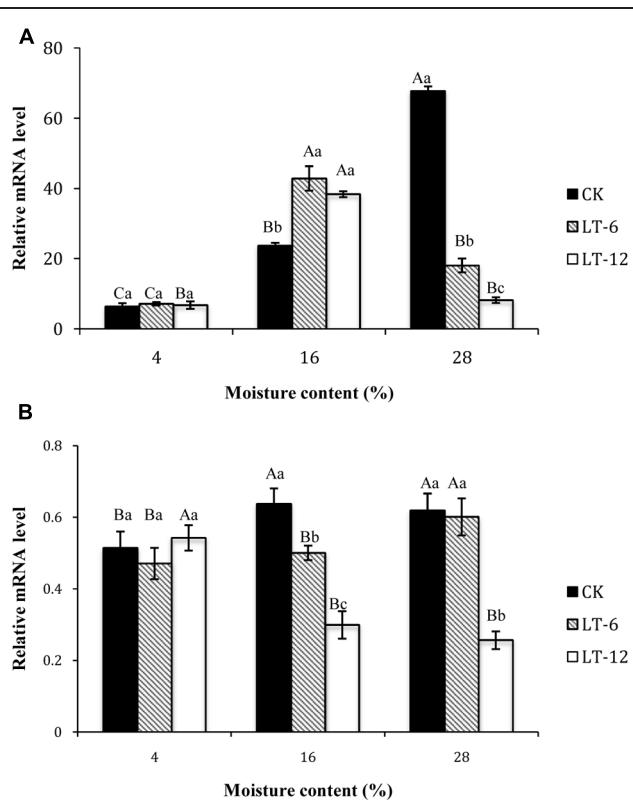
## Changes in Transcript Levels of Genes Related to Proline Biosynthesis and Catabolism during Storage

In the storage treatments of CK, the transcript level of *P5CS1* increased significantly ( $P < 0.05$ ) with seed MC increasing from 4 to 28% (Figure 6A). In LT-6 and LT-12, the highest transcript level of *P5CS1* occurred in seeds of 16% MC. There was no

## Discussion

Seed MC and storage duration greatly affect seed longevity during storage (Abba and Lovato, 1999; Mira et al., 2015). In this study, the germination percentage of oat seeds with 28% MC decreased significantly during storage (Figure 1). As the storing from 0 to 12 months, there was no significant difference for germination of oat seeds with 4% MC, but seeds with 28% MC did not germinate well, even in the CK. These findings suggested that low MC might be more important than the storage duration in terms maintaining the vigor of stored seeds. Similar results have been reported for seeds of many species under hermetic storage (Ellis et al., 1988; Zhang et al., 2010). The optimum MC for oat seeds may be 4%, but 16% MC was acceptable for seeds stored for less than 6 months.

The loss of seed germinability has been attributed to the accumulation of ROS (Wojtyla et al., 2006). Many reports have indicated that oxidative stress imposed by ROS is an important cause of seed deterioration during aging (McDonald, 1999; Rajjou



**FIGURE 6 | Effect of storage duration on *P5CS1* and *PDH1* gene expression of oat seeds with different MC at 4°C. (A) *P5CS1*; (B) *PDH1*.** The different letters indicate significant differences at 0.05 level among treatments as determined by the Duncan's multiple range test. Means with different capital letters indicate the significant differences of seed at different MC and same storage duration, and with different lowercase letters at the same MC and different storage duration. Data represent the mean  $\pm$  SD of three or more replicates.

and Debeaujon, 2008). In our study, the reduction of seed vigor was related to higher levels of ROS, such as  $O_2^-$  and  $H_2O_2$ , as seed MC increased from 4 to 28% (Figure 2). A loss of seed viability was shown to be positively correlated with increasing MC in seeds of sunflower, beech (*Fagus sylvatica*), holm oak (*Quercus ilex* L.) and cotton (*Gossypium hirsutum* L.; Bailly et al., 1996; Pukacka and Ratajczak, 2005; Kibinza et al., 2006). Higher MCs in aging seeds resulted in greater oxidative damage and ROS generation. In this study, as the storage duration extended from 0 to 12 months, the  $O_2^-$  production rate remained stable in seeds with 28% MC. The lowest  $O_2^-$  production rate in seeds with 4 and 16% MC was attained after 6 months of storage (Figure 2A). The  $H_2O_2$  content showed a similar trend; that is, the minimum level was in seeds with 4% MC after 6 months of storage (Figure 2B). These differences in  $H_2O_2$  contents might be related with the level of MC. However, antioxidant enzymes could be activated after 6 months storage or the contents of non-enzymatic antioxidants had increased in seeds with 4 and 16% MC. This result suggested that seeds with lower MCs were able to repair the deteriorated damages at 4°C and storage of 6 months.

Antioxidant enzymes and non-enzymatic antioxidants, including SOD, CAT, APX, and proline, contribute to reduce the concentration of ROS (Kishor et al., 1995; Mittal et al., 2012; Oliveira et al., 2012). SOD is one of the most important enzymes in cellular defense against ROS, as it could catalyze the  $O_2^-$  into  $H_2O_2$  (Kumar et al., 2010; Kumara et al., 2010). CAT and APX have been demonstrated to scavenge the  $H_2O_2$  produced by interacting under oxidative stress (Erdal et al., 2011; Yin et al., 2014). CAT reduces  $H_2O_2$  to water and dioxygen, and APX reduces  $H_2O_2$  to water and generates MDA (Noctor and Foyer, 1998). Activities of antioxidant enzymes have been observed to decrease in aged soybean, cotton, and sunflower (Bailly et al., 1996; Murthy et al., 2002; Goel et al., 2003). Increasing of MC promotes enzymatic oxidation and leads to rapid cellular damage, which decreases seed vigor (Gill and Tuteja, 2010). In this study, the changing of SOD activity was related with the level of MC and storage duration. There were no significant changes in CK with MC increasing, however, SOD activity decreased with MC increasing as stored 6 and 12 months (Figure 3A). There was different changing tendency between CAT and APX activity with MC increasing and storage duration prolonged. CAT and APX could respond rapidly to scavenge  $H_2O_2$  under stress. CAT activity was more responsive to the variation in higher MC compared to APX. The highest SOD activity in seeds with 4% MC occurred after 6 months of storage, but the  $O_2^-$  production rate and  $H_2O_2$  content reached the lowest level. Furthermore, the different changing for CAT and APX activity under moisture of 4% indicated that APX was more responsive than CAT to oxidative damage as the storage duration extended. These two enzymes may have complementary or interacting roles. The activities of SOD, CAT, and APX in seeds with 28% MC showed similar trends. The reductions in SOD, CAT, and APX activities may be because of ROS accumulated to toxic levels (Kong et al., 2014) or intolerant of the higher MC and/or long-term storage.

The accumulation of low-molecular weight metabolites acting as osmoprotectants, such as proline, is part of the adaptive response to stress in plants (Hoekstra et al., 2001). Kishor and Dange (1990) reported that proline protected cellular functions by scavenging ROS. In our study, proline content tended to increase with seed MC increasing. It's illustrated that proline accumulation in seeds could confer some adaptive advantages under stress. However, an increasing in proline content could also be considered as a stress-induced marker for oxidative damage during aging (Lei and Chang, 2012). As seeds storing 12 months, the proline contents decreased in seeds with 4 and 16% MC, but increased in seeds with 28% MC (Figure 5). Meanwhile, antioxidant enzyme activity also tended to increase in seeds with 4 and 16% MC, but decrease in those with 28% content MC during storage (Figure 3). This result suggested that proline played a more important role in adapting to oxidative stress in seeds with higher MC (28%), while antioxidant enzymes played a more important role in seeds with lower MCs (4 and 16%).

To elucidate the role of the ROS scavenging system during seed aging, we analyzed the transcript levels of genes encoding antioxidant enzymes by real-time PCR. The changes in transcript levels of *SOD1* which encodes a Cu/Zn SOD were not consistent with the SOD activity (Figure 4A). It indicated that *SOD1* was not

the main enzyme contributing to total SOD activity. Vaseva et al. (2012) proposed that Cu/Zn SOD was suppressed by Fe SOD in aging oat seeds. The transcript levels of *APX1* and *CAT1* in seeds with different MC were consistent with APX and CAT activities during storage, suggesting that *APX1* and *CAT1* were the major H<sub>2</sub>O<sub>2</sub>-scavenging enzymes for maintaining the balance of redox reaction in aged oat seeds. From these results, the expressions of *APX1* and *CAT1* in oat seeds were suppressed during aging and leading to H<sub>2</sub>O<sub>2</sub> accumulation. Similar results have been reported for rice seeds (Yin et al., 2014).

*P5CS1* catalyzes the first step of proline synthesis (Abraham et al., 2003). Overexpression of *P5CS1* improved stress tolerance in rice and wheatgrass (Choudhary et al., 2005). In this study, *P5CS1* transcript levels were significantly up-regulated with MC increasing from 4 to 28% in CK (**Figure 6A**). However, the expression of *P5CS1* presented the up-regulation at 16% MC and down-regulation at 28% during 6 and 12 months storage, while the proline content increased continually. This indicated that *P5CS1* transcript levels were affected significantly by the seed MC. Some studies have shown that suppression of *PDH1* increased the proline content and enhanced stress resistance (Nanjo et al., 1999; Armengaud et al., 2004). In our study, *PDH1* transcript levels showed the opposite trend in comparison with proline contents

in oat seeds stored 12 months, and reached lower levels at MCs of 16 and 28%. This implied that *PDH1* could improve stress resistance for seed aging and maintain seed vigor during long-term storage.

In summary, proline played the main role in adaptation to oxidative stress in seeds with higher MC (28%), while antioxidant enzymes (SOD, CAT, APX) played the main roles in seeds with lower MCs (4%, 16%) during storage at a low temperature (4°C). The transcript analyses showed that *SOD1* was not a main factor in total SOD activity, while *APX1* and *CAT1* were the main H<sub>2</sub>O<sub>2</sub>-scavenging enzymes in aging oat seeds. The transcript level of *P5CS1* was significantly affected by MC, and *PDH1* could improve stress resistance for seed aging and maintain seed vigor during long-term storage.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Transcriptomic profiling of linolenic acid-responsive genes in ROS signaling from RNA-seq data in *Arabidopsis*

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Linolenic acid (Ln) released from chloroplast membrane galactolipids is a precursor of the phytohormone jasmonic acid (JA). The involvement of this hormone in different plant biological processes, such as responses to biotic stress conditions, has been extensively studied. However, the role of Ln in the regulation of gene expression during abiotic stress situations mediated by cellular redox changes and/or by oxidative stress processes remains poorly understood. An RNA-seq approach has increased our knowledge of the interplay among Ln, oxidative stress and ROS signaling that mediates abiotic stress conditions. Transcriptome analysis with the aid of RNA-seq in the absence of oxidative stress revealed that the incubation of *Arabidopsis thaliana* cell suspension cultures (ACSC) with Ln resulted in the modulation of 7525 genes, of which 3034 genes had a 2-fold-change, being 533 up- and 2501 down-regulated genes, respectively. Thus, RNA-seq data analysis showed that an important set of these genes were associated with the jasmonic acid biosynthetic pathway including lipoxygenases (*LOXs*) and Allene oxide cyclases (*AOCs*). In addition, several transcription factor families involved in the response to biotic stress conditions (pathogen attacks or herbivore feeding), such as *WRKY*, *JAZ*, *MYC*, and *LRR* were also modified in response to Ln. However, this study also shows that Ln has the capacity to modulate the expression of genes involved in the response to abiotic stress conditions, particularly those mediated by ROS signaling. In this regard, we were able to identify new targets such as galactinol synthase 1 (*GOLS1*), methionine sulfoxide reductase (*MSR*) and alkenal reductase in ACSC. It is therefore possible to suggest that, in the absence of any oxidative stress, Ln is capable of modulating new sets of genes involved in the signaling mechanism mediated by additional abiotic stresses (salinity, UV and high light intensity) and especially in stresses mediated by ROS.

**Keywords:** linolenic acid, *Arabidopsis*, massively parallel sequencing, RNA-seq, methionine sulfoxide reductase, oxidative stress, ROS signaling

## INTRODUCTION

The poly-unsaturated fatty acid  $\alpha$ -Linolenic acid (Ln), with an 18-carbon chain and three *cis* double bonds, is an essential omega-3 fatty acid and organic compound found in seeds such as flaxseed, chia, soybean and various vegetable oils.

Ln, which can be released from several complex fatty acids mainly located in the membranes of organelles such as chloroplast is a precursor of the jasmonic acid (JA) phytohormone and, consequently, of the oxylipin pathway. It is generally accepted that phospholipase 1 (PLA<sub>1</sub>) is able to release Ln from the *sn1* position of galactolipids which is responsible for generating the JA substrate. The oxygenation of Ln is the initial step in JA biosynthesis which is carried out by plastid-located lipoxygenases (*LOXs*) at C-13 and is followed by the dehydration

of 13-hydroperoxy-octadecatrienoic acid caused by allene oxide synthase (AOS). The unstable epoxide generated is then cyclized stereospecifically and converted into 12-oxo-phytodienoic acid (OPDA) by allene oxide cyclase (AOC) followed by the reduction of OPDA to 3-oxo-2-(2'(*Z*)-pentenyl)-cyclopentane-1 octanoic acid by the *Arabidopsis* OPDA reductase (OPR3). Finally, the subsequent shortening of the carboxylic acid side chain is caused by the fatty acid  $\beta$ -oxidation machinery and is initiated by Acyl-CoA-oxydase1 (ACX1) (Wasternack and Hause, 2013; Wasternack, 2014b).

The regulation of JA biosynthesis is determined by a positive feedback loop, substrate availability and tissue specificity (Wasternack, 2007). This phytohormone mediates several processes during plant growth such as male and female organ as

well as embryo development, sex determination in maize, seed germination, seedling development, root growth, gravitropism, trichome and tuber formation, leaf movement and leaf senescence. Other processes mediated by JA include plant responses to desiccation, ozone, UV, osmotic, cold and light stresses as well as secondary metabolite formation and seasonal and circadian rhythm adaptations (Wasternack, 2014a).

Previously, the development of microarray technology enabled researchers to carry out large-scale studies and to analyze the response of thousands of genes in a single experiment. This technology has been used to analyze methyl-jasmonate-responsive genes in *Arabidopsis thaliana* cells (Pauwels et al., 2008) and to evaluate the role of different phytohormone-induced transcription factors in differential responses to damage caused by two different insect herbivores (Rehrig et al., 2014). These studies indicate that the application of jasmonate-derived molecules at an early stage prompted the expression of genes from the JA biosynthetic pathway and a later response in which a cellular metabolism is reprogrammed and cell cycle progression takes place, with the addition of a different response depending on the herbivore involved. Although most of these studies use jasmonate-derived molecules, to our knowledge, Ln, the principal component that engenders this pathway, has not been studied. Thus, jasmonate-responsive genes in *A. thaliana* have been identified with the aid of medium- and large-scale transcriptomic analyses using microarray technology (Pauwels et al., 2008). In recent years, new high-throughput sequencing methods, called massively parallel sequencing or RNA-seq, have emerged as a useful tool that could replace and improve upon existing methods given their advantages as compared to array-based methods (Wilhelm and Landry, 2009; Van Verk et al., 2013); (i) they do not depend on prior descriptions of the genomic sequence of the target species, making it easier to carry out gene expression studies of complex organisms; (ii) RNA-seq technology not only enables gene expression to be quantified but also facilitates the simultaneous identification of different isoforms, promoters, transcription start sites (TSS) and alternative splicing sites; (iii) RNA-seq is capable of detecting low-abundance transcripts; (iv) RNA-seq output is at the theoretical maximum of base pair resolution. As a result, RNA-seq data for higher plants have very recently started to be compiled (Lee et al., 2010; Li et al., 2012; De Cremer et al., 2013; Donà et al., 2013; Postnikova et al., 2013; Van Moerkercke et al., 2013).

The aim of the present study is to deepen our knowledge of how the linolenic acid precursor of jasmonic acid mediates new plant defense signaling pathways. The data indicate that Ln modulates the gene expression in the response to both biotic and abiotic stresses, which are especially mediated by reactive oxygen species (ROS). In this regard, a large-scale gene expression analysis has been carried out using paired-end RNA-seq technology developed by Illumina and is the first study of *Arabidopsis* to use this technique in order to increase our understanding of the Ln-signaling mechanism in the ROS metabolism. This technology has enabled us to gain an insight into transcriptional information and biological pathways that respond to Ln signaling which could not previously be identified by array-based methods applied to plants.

## MATERIALS AND METHODS

### PLANT MATERIAL, GROWTH CONDITIONS, AND TREATMENTS

*A. thaliana* cell suspension cultures (ACSC) were maintained in 200 ml liquid growth medium (Jouanneau and Péaud-Lenoë, 1967; Axelos et al., 1992) by gentle agitation at 120 rpm and 24°C under continuous 50  $\mu\text{E m}^{-2} \text{s}^{-1}$  PAR (photosynthetically active radiation) illumination in an incubator shaker (Multitron Standard model, Infors HT). Cells were subcultured with a 1/20th dilution every 7 days. To analyze the involvement of linolenic acid (Ln) in the mechanism of gene expression regulation, 9-day-old ACSC were incubated with 1 mM Ln (which is equivalent to 10  $\mu\text{mol Ln/g FW}$ ) and methanol (a fatty acid vehicle) and distilled water as controls. The treatments were applied under non-stress conditions. Due to ACSC growth in liquid medium, first step was the extraction by vacuum of liquid and pellets of cells were then harvested and used for RNA isolation. Samples were designated as control (C), vehicle (MeOH) and linolenic acid (Ln) ACSC.

### RNA SAMPLE PREPARATION AND HIGH-THROUGHPUT SEQUENCING

Total RNA from pooled ACSC was obtained using Trizol Reagent (Gibco-BRL), as described in the manufacturer's manual. RNA was then purified using a Spectrum Plant Total RNA kit (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's instructions. Any DNA contamination was removed by DNase I treatment on column (Roche, Basel, Switzerland). The RNA quality tests and concentrations were determined using a NanoVue™ Plus Spectrophotometer (GE Healthcare). RNA was pooled from each sample and complementary DNA (cDNA) libraries and sequencing in an Illumina HiSeq 1000 sequencer were generated by GeneSystems (Valencia, Spain). Two replicates of each sample were sequenced on different lanes in the flow cell.

### BIOINFORMATIC ANALYSIS

Quality control of sequencing was carried out using FastQC software (V0.10.1). Gene-expression was studied using DNASTar (ArrayStar 4) Qseq software for RNA-seq analysis ([www.dnastar.com](http://www.dnastar.com)) with the TAIR10 database as template. For mapping purposes, we used the  $k\text{-mer} = 63$  and 95% of matches parameters and the reads per kilobase per million mapped reads (RPKM) default normalization method. The gene ontology (GO) terms were loaded in the Blast2GO suite V.2.7.2 (Conesa et al., 2005; Conesa and Götz, 2008) in order to statistically analyze GO-term enrichment. Blast2GO integrated the Gossip package for statistical assessment of differences in GO-term abundance between two sets of sequences (Blüthgen et al., 2004). This package uses Fisher's exact test and corrects for multiple testing. A one-tailed Fisher's exact test was carried out using a false discovery rate (FDR) with a filter value of  $<0.01$ . Blast2GO returns GO terms over-represented at a specified significance value (Conesa and Götz, 2008). The results were saved in a Microsoft Excel datasheet, and charts were generated.

For functional annotation purposes, genes studied showing significant expression-level changes in response to linolenic treatment were analyzed using DAVID (Dennis et al., 2003; Da Wei Huang and Lempicki, 2008) and TAIR (<http://www.arabidopsis.org/tools/bulk/go/index.jsp>) databases.

## MEASUREMENT OF THIOBARBITURIC ACID REACTIVE SUBSTANCES (TBARS)

TBARS were measured using the method described above (Stewart and Bewley, 1980) involving the spectrophotometric measurement of the pink pigment produced by the reaction of thiobarbituric acid (TBA) with malondialdehyde (MDA) and other secondary lipid peroxidation products. The evaluation of absorbance at 532 nm gives a measure of the extent of lipid degradation. ACSC treated with 1 mM Ln were homogenized with liquid nitrogen to obtain a fine powder and were mixed with a solution containing 918 mM trichloroacetic acid, 25.6 mM thiobarbituric acid and 250 mM HCl (ratio 1/5; w/v). The mixture was heated at 85°C for 30 min, the reaction was stopped by abrupt placement in an ice-bath and the cooled mixture was centrifuged at 10000 g for 10 min. Absorbance at 532 and 600 nm was measured and, in order to correct for background absorption, absorbance values at 600 nm were subtracted from those at 532 nm, with the latter representing the absorption maximum of the TBA: MDA adduct. A molar extinction coefficient of 156,000 ( $1.56 \cdot 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ ) was used. All determinations were performed in triplicate and expressed in pmol MDA/g fresh weight.

## SPECTROPHOTOMETRIC DETERMINATION OF HYDROGEN PEROXIDE ( $\text{H}_2\text{O}_2$ )

Hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) content was measured as described by Jiang et al. (1990). Samples were incubated with a solution composed of 500  $\mu\text{M}$  ammonium ferrous sulfate, 50 mM sulphuric acid, 200  $\mu\text{M}$  xylenol orange and 200 mM sorbitol (ratio 1/2.5; w/v) in the dark for 45 min and then centrifuged at 2400 g for 10 min. Absorbance at 560 nm was measured and  $\text{H}_2\text{O}_2$  concentration was estimated as  $\text{H}_2\text{O}_2$  equivalent using a standard curve determined with commercial  $\text{H}_2\text{O}_2$ .

## DETERMINATION OF PROTEIN CARBONYL CONTENT

All procedures were performed at 0–4°C. Cell cultures were ground to a powder using a mortar with liquid nitrogen and were suspended in 100 mM Tris-HCl buffer, pH 7.5 (ratio 1/2; w/v) containing 5% (w/v) sucrose, 7% (w/v) PVPP, 0.05% Triton x-100, 0.1 mM EDTA, 1 mM PMSF and a commercial cocktail of protease inhibitors containing AEBSF, 1,10-phenanthroline, pepstatine A, leupeptine, bestatine and E-64 (Sigma, St. Louis, MO, USA). Homogenates were filtered through one layer of Miracloth (Calbiochem, San Diego, CA, USA) and centrifuged at 3000 g for 10 min. A 2% (w/v) streptomycin sulfate solution was added to each sample in order to precipitate nucleic acids, which may cause an erroneously high estimate of protein-bound carbonyl, agitated for 20 min and then centrifuged at 2000 g for 10 min. A 10 mM DNPH solution in 2 N HCl was then added to the protein pellet of each sample, with 2 N HCl only being added to corresponding sample aliquot reagent blanks. Samples were allowed to stand in the dark at room temperature for 1 h and were then precipitated with 20% (w/v) TCA for 15 min and centrifuged at 5000 g for 10 min. Protein pellets were washed three times with ethanol/ethyl acetate (1:1, v/v) to remove any free DNPH. Samples were then resuspended in 6 M guanidine hydrochloride, dissolved in 20 mM phosphate buffer, pH 2.3, at 37°C for

15 min with vortex mixing. Carbonyl content was determined from absorbance at 370 nm using a molar absorption coefficient of 22,000  $\text{M}^{-1} \text{ cm}^{-1}$  for DNPH (Levine et al., 1990) and also by measuring protein content at 280 nm. Simultaneously, a BSA standard curve, dissolved in 6 M guanidine hydrochloride and incubated at 37°C for 15 min, was constructed.

## FATTY ACID ANALYSIS OF *ARABIDOPSIS THALIANA*

The *Arabidopsis* lipid fraction of cell suspension cultures was analyzed using a gas chromatograph (GC) (Agilent 7890A). The Meth-Prep II (Alltech Chemicals Cat. No. 18007) GC reagent was used for the transesterification of the *Arabidopsis* lipid fraction for gas chromatographic analysis purposes, and a standard oil mixture (Supelco ref. 18919-1AMP) was used to calibrate the gas chromatograph. Each sample was placed in a microvial and evaporated under a stream of nitrogen, the lipids were dissolved in 48  $\mu\text{l}$  benzene and 50  $\mu\text{l}$  Meth Prep II reagent was added. Following the derivatization stage, a GC/MS analysis was carried out by injecting a 1  $\mu\text{l}$  solution. Analyses were carried out in a 7890A GC system (Agilent, USA) equipped with an SP-2560 capillary column (100 m  $\times$  0.25 mm  $\times$  0.25  $\mu\text{m}$ ) and a Quattro micro GC mass spectrometer (Waters, USA). The GC column procedure was as follows: initial temperature 140°C, maintained for 5 min, increased at 4°C  $\text{min}^{-1}$  to 250°C with a split ratio at injector port of 1:10.

## QUANTITATIVE REAL-TIME REVERSE TRANSCRIPTASE-PCR (qRT-PCR)

Total RNA from pooled of control, vehicle and linolenic acid ACSC was isolated as above, and first-strand cDNA was synthesized using the First Strand cDNA Synthesis kit (Roche) in a final volume of 20  $\mu\text{l}$  according to the manufacturer's instructions. Real-time PCR was performed in a CFX384 real-time PCR Detection System (Bio-Rad). Amplifications were carried out in 5  $\mu\text{l}$  of total volume containing 5 ng of cDNA, 2  $\mu\text{M}$  of specific primers (see **Supplemental Table 9**) and SsoFast EvaGreen Supermix (Bio-Rad). PCR conditions used consisted of an initial denaturation at 98°C for 30 s, followed by 39 cycles at 98°C, 5 s and 60°C, 30 s. After cycling, melting curves of the reaction were run from 72°C to 82°C. Results were normalized using Actin12 (AT3G46520), 18S rRNA (AT2G01010), and L2 (AT2G44065) as internal controls.

## DATA AVAILABILITY

The Illumina sequenced read data reported in this article have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive and are available under the Accession Numbers Bioproject ID: PRJNA273982 and SRP Study Accession: SRP052987.

## RESULTS

Fatty acid composition of *Arabidopsis* cell suspension cultures (ACSC) showed that linolenic acid (Ln) was the most abundant fatty acid, accounting for approximately 50% of total (0.134  $\mu\text{mol Ln/g FW}$ ), followed by linoleic acid, with 20% of total (**Supplemental Table 1**), showing that these data were consistent with those obtained by Bonaventure et al. (2003). As Ln is the jasmonic acid precursor and the major fatty acid in ACSC and

given that Ln-treatment did not cause oxidative damage in ACSC, it was therefore selected for this study.

In addition, ACSC treatment with 1 mM Ln for 1 h did not increase ROS content such as H<sub>2</sub>O<sub>2</sub> or caused oxidative damage as lipid and protein oxidation markers such as malondialdehyde (MDA) and protein carbonyl content, respectively, were not affected (**Supplemental Table 2**).

#### TRANSCRIPTOMIC ANALYSIS OF LN-RESPONSIVE GENES IN ACSC

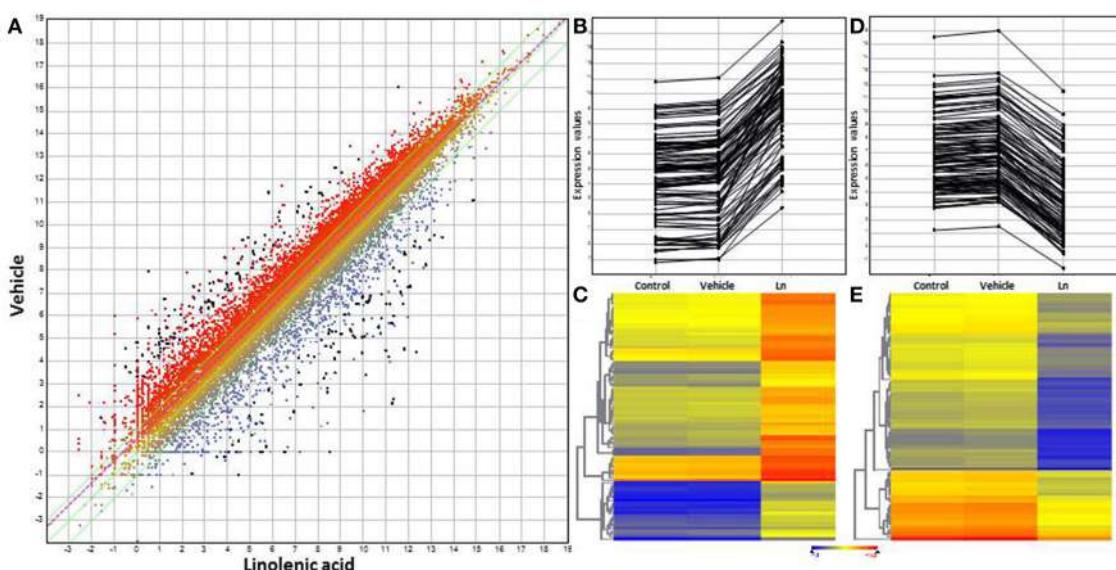
ACSC treated with 1 mM Ln (10  $\mu$ mol Ln/g FW) to observe a clear gene expression response due to Ln-treatment, methanol (vehicle) and distilled water (control) were harvested and used for total RNA isolation. Paired-end libraries were then prepared and sequenced as described in Material and Methods. Firstly, sequence quality was checked using the FastQC program and only reads with high quality values ( $\geq Q30$ ) were entered for mapping purposes (Phred values of 30 units or more which corresponds to a sequencing error rate of 0.1%). RNA-seq analysis using DNAsstar QSeq mapped the reads to the *Arabidopsis* transcriptome database (TAIR10) with highly stringent parameters of kmer = 63 and 95% of matches.

The gene-expression profile of two ACSC groups was firstly compared, one treated with distilled water and the other with 1 mM Ln (control vs. Ln). Importantly, to eliminate genes that respond to methanol, this comparison was filtered using vehicle-responsive genes, with control vs. vehicle comparison eliminating genes with a 1.5 FC due to methanol, henceforth referred to as control. RNA-seq analysis showed that Ln caused significant changes (95% of matches,  $p < 0.05$ ) in the gene-expression levels of 8947 ACSC genes, 7525 genes with annotations and 1422 genes coding for hypothetical proteins not considered for the rest of this study. From these 7525 genes, we selected 2FC up

and down Ln-responsive genes showing the modulation of 3034 genes, from which 533 were up- (**Supplemental Table 7**) and 2501 were down-regulated (**Supplemental Table 8**). Due to this large number of genes, we only show the trend for genes differentially expressed with 8FC up and down (**Figure 1**). In this sense, **Figure 1A** shows the scatter plot of total genes whose expression changes significantly in response to Ln. Panels **1B** and **1C** represent the line plot and heat map of 8FC over-expressed genes and panels **1D** and **1E** indicate the line plot and heat map of 8FC repressed genes.

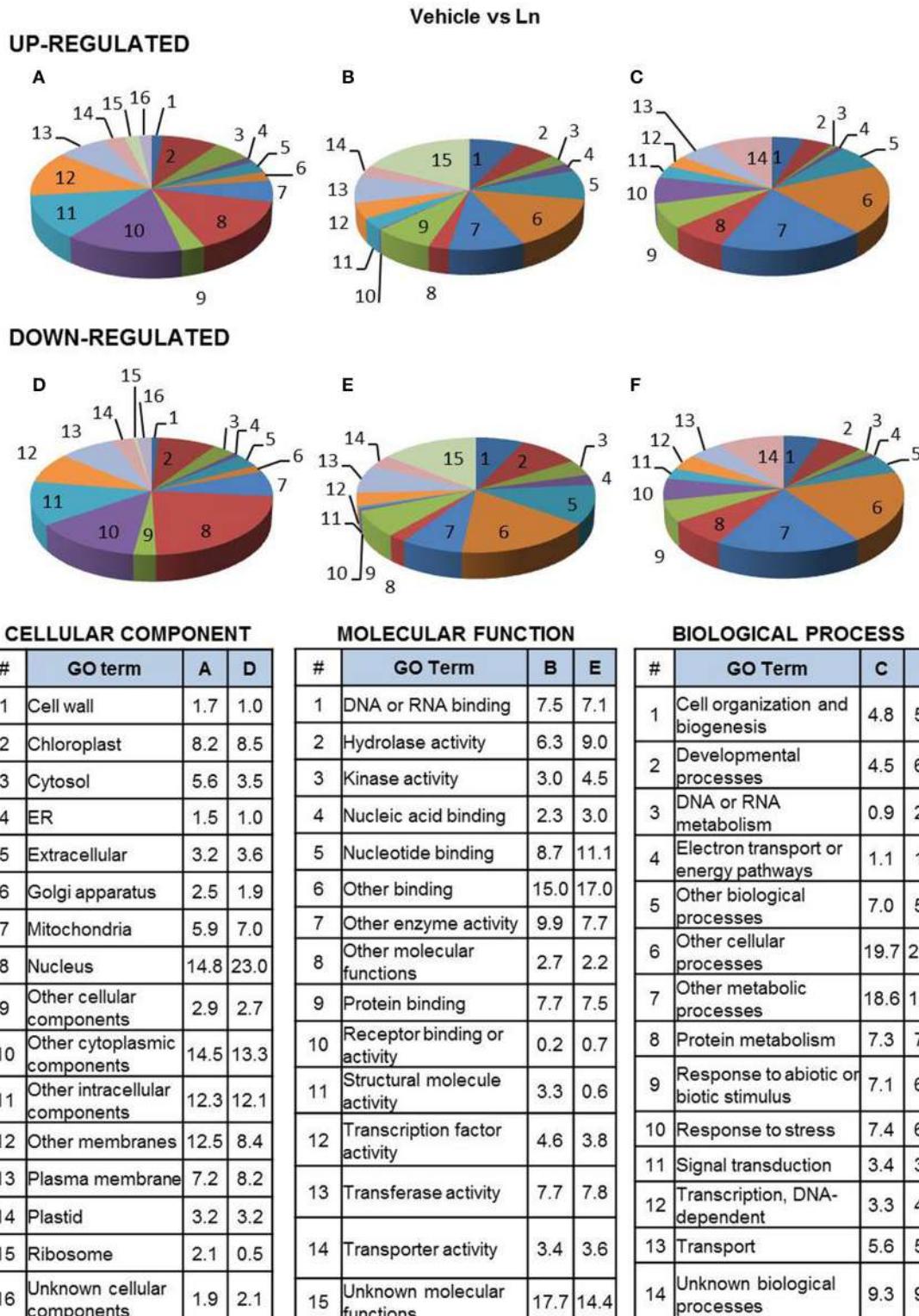
Functional classification of 2FC-Ln-induced genes shows that their products were mostly located in the nucleus, chloroplast and plasma membrane (**Figure 2A**). Furthermore, they were mostly characterized by nucleotide-, protein- and DNA/RNA-binding, transferase and hydrolase activity (**Figure 2B**). They were also predicted to be involved in the response to stress, biotic or abiotic stimuli and protein-metabolism processes (**Figure 2C**). On the other hand, the most abundant categories of 2FC-Ln-repressed genes in ACSC were also located in the nucleus, chloroplast, plasma membrane and mitochondria (**Figure 2D**). These genes showed hydrolase-, transferase-, nucleotide-, and protein-binding activity (**Figure 2E**) and were associated with the protein metabolism, developmental processes and stress responses (**Figure 2F**).

On the basis of these results, we decided to carry out a more detailed analysis of biological processes involved in Ln treatments. To do this, the GO terms obtained by the analysis were loaded in Blast2GO suite V.2.7.2 to statistically analyze GO-term enrichment. The unchecked two-tail box was used to analyze only positive enrichment. The test was carried out using a filter cut-off value of FDR  $< 1e^{-5}$  for up-regulated genes and an FDR  $< 0.001$  for down-regulated genes. The results are shown in **Figure 3**



**FIGURE 1 |** Scatter plot, line plot and heat map for 8-fold-change genes using DNAsstar software. **(A)** Scatter plot of total Ln-responsive genes. **(B)** Line plot of overexpressed genes. **(C)** Heat map for overexpressed genes. **(D)** Line plot of repressed genes. **(E)** Heat map for repressed genes. Distilled

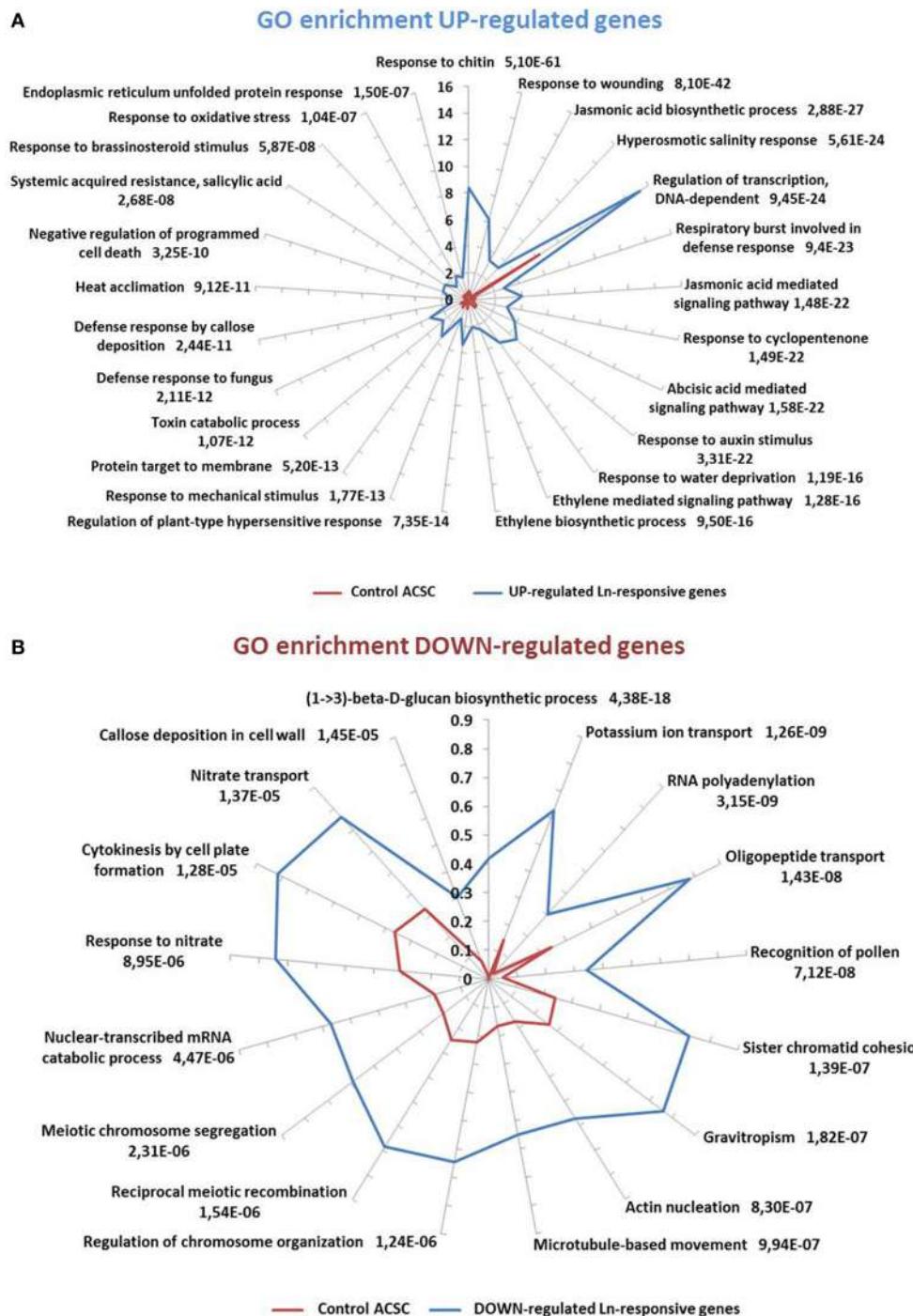
water and methanol-responsive genes were used to filter the results of Ln treatment. All graphs show 8-fold-change genes with 95% significant differential expression obtained by t-student test from whole Ln-responsive genes.

**FIGURE 2 | Functional classification of Ln-responsive genes in ACSC.**

Genes were classified by functional categories under the following gene ontology terms: cellular component (**A,D**), molecular function (**B,E**), biological

process (**C,F**), up-regulated genes (**A–C**) and down-regulated genes (**D–F**).

The number of genes assigned to each functional category is expressed as a percentage (%).



**FIGURE 3 | GO-term-enriched graph of biological processes of Ln-responsive genes. (A) Up-regulated genes. Node filter was set at FDR < 1e-5. (B) Down-regulated genes. Node filter was set at FDR**

< 0.001. Bars for up- and down-regulated genes are labeled with their corresponding P-values in Fisher's exact test against expressed control ACSC genes.

as the percentage of sequences annotated for each biological process GO term for both ACSC and Ln-responsive genes. Bars are labeled with their corresponding P-values in Fisher's exact test. Ln treatment of ACSC produced a significant response in the GO terms of up-regulated genes (Figure 3A). The GO terms of these genes were closely associated with biotic stress-related

processes such as responses to chitin and wounding and were closely connected with biosynthesis and the JA signaling pathway. GO enrichment analysis also highlighted the over-representation of processes associated with other important phytohormones such as abscisic acid, auxin, ethylene, salicylic acid and brassinosteroids. With regard to abiotic stress, Ln treatment affected

hyperosmotic salinity responses and heat acclimation, specifically in responses to oxidative stress through the response to hydrogen peroxide. With regard to the GO terms of down-regulated genes (**Figure 3B**), we found that the level of over-representation of biological processes was lower than in up-regulated genes. These processes were mostly associated with the synthesis of cell wall depicted by a (1-> 3)-beta-D-glucan biosynthetic process and callose deposition in cell wall together with the transport of potassium, oligopeptides and nitrate. Finally, mitotic- and meiotic-related processes such as sister chromatid cohesion, chromosome organization regulation, reciprocal meiotic recombination and meiotic chromosome segregation were observed.

#### LINOLENIC ACID-RESPONSIVE GENES INVOLVED IN JASMONATE-AND BIOTIC STRESS-RELATED PROCESSES

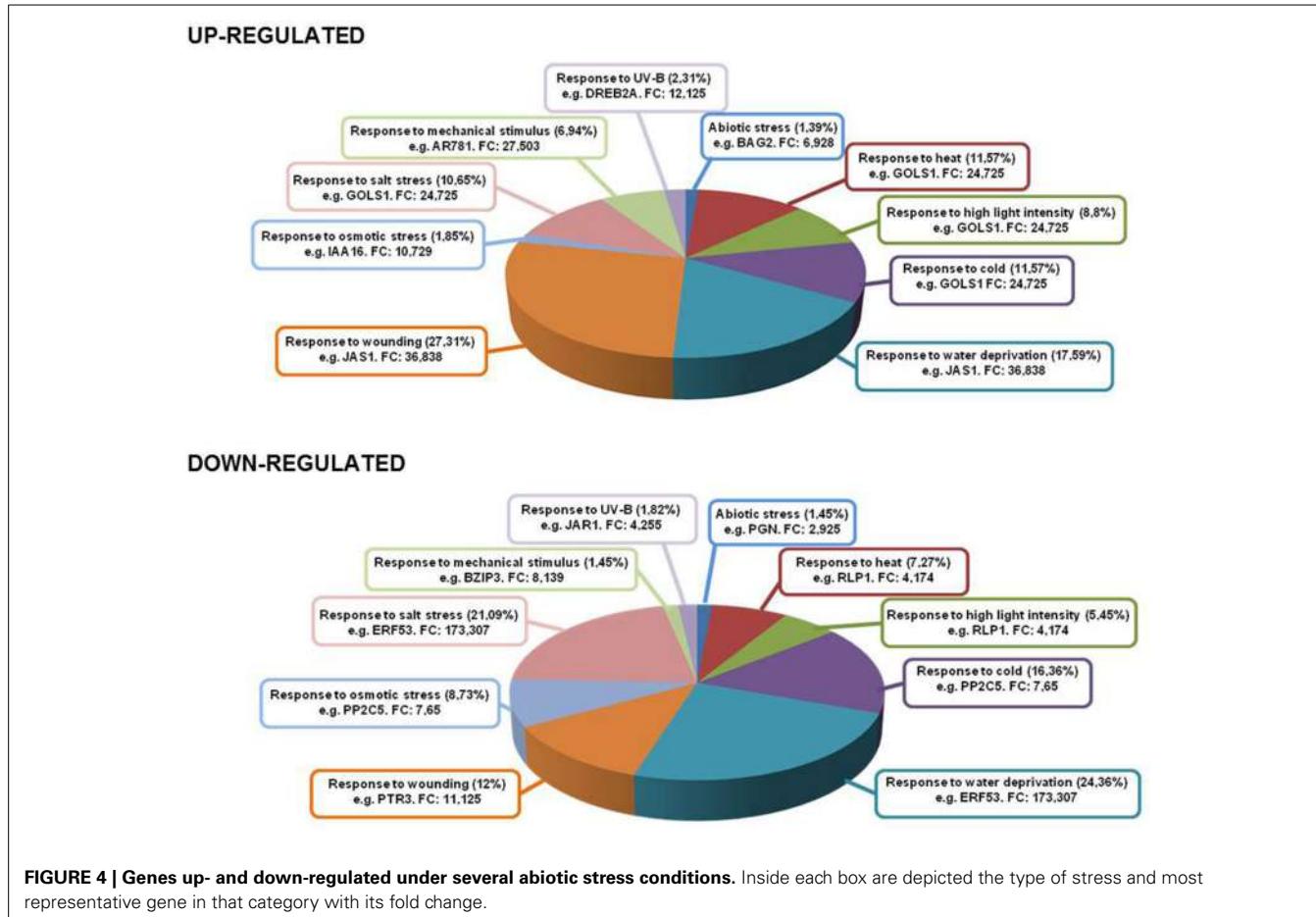
Ln can be released from the plasma membrane by certain lipase enzymes in response to stress (Yang et al., 2012) and, for this reason, the regulation of JA-related genes in response to Ln treatment was predicted. Several genes involved in the JA biosynthetic pathway were induced: *DGL* (*AT1G05800*), *LOX4* (*AT1G72520*), *LOX3* (*AT1G17420*), *AOS* (*AT5G42650*), *AOC3* (*AT3G25780*), *OPR3* (*AT2G06050*), and *OPCL1* (*AT1G20510*) (see **Supplemental Table 3**). Furthermore, several *JAZ* genes encoding proteins that repress JA signaling and are targeted by E3-ubiquitin ligase *SCF<sup>COI1</sup>* for proteasome degradation in response to JA (Chico et al., 2008), were up-regulated in response to Ln treatment. Among these, RNA-seq analysis showed an increase in the transcript levels of *JAZ10* (*AT5G13220*), *JAZ6* (*AT1G72450*), *JAZ5* (*AT1G17380*), *JAZ9* (*AT1G70700*), and *JAZ2* (*AT1G74950*). Two groups of induced transcriptional regulators of jasmonate biosynthesis and the JA-mediated signaling pathway were also detected. On the one hand, a set of activators were encoded mainly by *MYC2* (*AT1G32640*), a (bHLHzip)-type transcription factor (TF) that directly interacts with *JAZ* proteins, and *ORA47* (*AT1G74930*), the AP2/ERF protein postulated to be a positive regulator of JA biosynthesis (Pauwels et al., 2008). On the other hand, the repressors were encoded by *ZAT10* (*AT1G27730*) and *AZF2* (*AT3G19580*) that contain an ERF-associated amphiphilic repression domain and may act as both positive and negative regulators of plant defenses (Sakamoto et al., 2004; Mittler, 2006). In addition to the genes mentioned above, treatment of ACSC with Ln led to up-regulation of transcription factors from different families including ERF (*AT4G34410*, *AT1G28370*, *AT3G15210*, *AT5G47220*, and *AT3G23240*). *WRKY* (*AT1G80840*, *AT5G22570*), *bHLH* (*AT2G46510*) and *MYB* (Myeloblast) (*AT3G23250*, *AT3G06490*, *AT1G74430*, *AT1G57560*, *AT4G37260*, *AT2G16720*, *AT5G67300*, *AT3G28910*, *AT4G34990*, *AT4G38620*, and *AT5G37260*), mainly involved in the response to JA stimuli, and *NAC* (*AT5G08790*, *AT1G01720*, *AT1G52890*, *AT3G15500*, and *AT4G27410*) transcription factors were also found, as previously described (Pauwels et al., 2008). On the other hand, only a few genes playing a role in JA biosynthetic processes were down-regulated. *LOX5* (*AT3G22400*) activity in roots has been shown to facilitate green peach aphid colonization of *Arabidopsis* foliage (Nalam et al., 2012), indicating that the repression of this enzyme could act as an ACSC defense mechanism. Finally, in relation to the JA-mediated signaling pathway,

Ln provoked gene down-regulation of carbonic anhydrase (CA) enzymes *AT5G14740* and *AT3G01500* and different *MYB* transcription factor families (*AT5G59780*, *AT2G36980*, *AT2G31180*, and *AT5G61420*) in response to JA stimuli.

The RNA-seq study shows that Ln provoked over-expression of *WRKY40* (*AT1G80840*) (see **Supplemental Table 4**) which was the most induced gene involved in defense responses, biotic stimulus and bacterium detection, negative defense response regulation and immune response regulation. *WRKY40* is a pathogen-induced transcription factor that plays a positive role in JA-mediated defense (Xu et al., 2006), showing that Ln treatment launches a set of defense mechanisms against pathogen attacks. In this regard, *RRTF1* (*AT4G34410*), involved in responding to chitin and fungus and in respiratory burst as part of a defense response, was the most up-regulated gene in the RNA-seq analysis, with an FC of 496.97. With regard to pathogen attacks, Ln treatment led to the up-regulation of *BAG2* (*AT5G62100*) which belongs to the BAG (Bcl-2-associated athanogene) protein family and also with *JAS1/JAZ10* (*AT5G13220*), playing a role in systemic acquired resistance (SAR), being another highly up-regulated gene. With respect to Ln-repressed genes, a broad diversity of genes was associated with a variety of cellular processes such as *LRR* proteins *AT1G74360*, *AT1G51790*, *AT1G34420*, *AT1G35710*, and *AT4G08850*, which mainly respond to chitin and respiratory burst associated with defense responses. A large set of disease-resistant proteins were also down-regulated in response to Ln treatment, specifically in relation to defense responses and defense response signaling pathways (see **Supplemental Table 4**). This family of genes, of which Ln is a repressor, may be involved in responses to pathogens and abiotic stress (Rushton et al., 2010).

#### LINOLENIC ACID-RESPONSIVE GENES INVOLVED IN ABIOTIC STRESS SITUATIONS

The treatment of ACSC with 1 mM Ln provoked the over-expression of genes involved in different abiotic stress situations (**Figure 4**). The highest induction levels (FC = 24.725 up) were found in galactinol synthase (*GOLS1*, *AT2G47180*) which responds to heat, high light intensity, cold, salt stress and water deprivation (see **Supplemental Table 5**). Furthermore, different genes associated with heat responses also responded to Ln treatment such as heat stress transcription factors (*AT1G52560*, *AT2G26150*, *AT4G25200*, *AT2G20560*, *AT5G56030*, *AT2G32120*, *AT1G74310*, *AT5G37670*, *AT4G21320*, and *AT1G16030*) and to water deprivation as transcription factor *DREB2A* (*AT5G05410*) was highly induced. This gene has been shown to respond to dehydration and high salt stress (Liu et al., 1998). As is well known, Ln induces the jasmonate pathway involved in the response to biotic stress situations and wounding among other processes. An important set of over-expressed genes, accounting for 27.31% of the total, was involved in the response to wounding, with, as described previously, the most up-regulated gene being *JAS1/JAZ10* among other *JAZ* proteins. *JAZ* genes are rapidly induced by JA, suggesting the presence of a negative feedback loop to replenish the *JAZ* protein pool and to dampen the response to JA (Chini et al., 2007; Thines et al., 2007). A high percentage of the Ln-repressed genes were involved in the response to water deprivation and salt stress, accounting

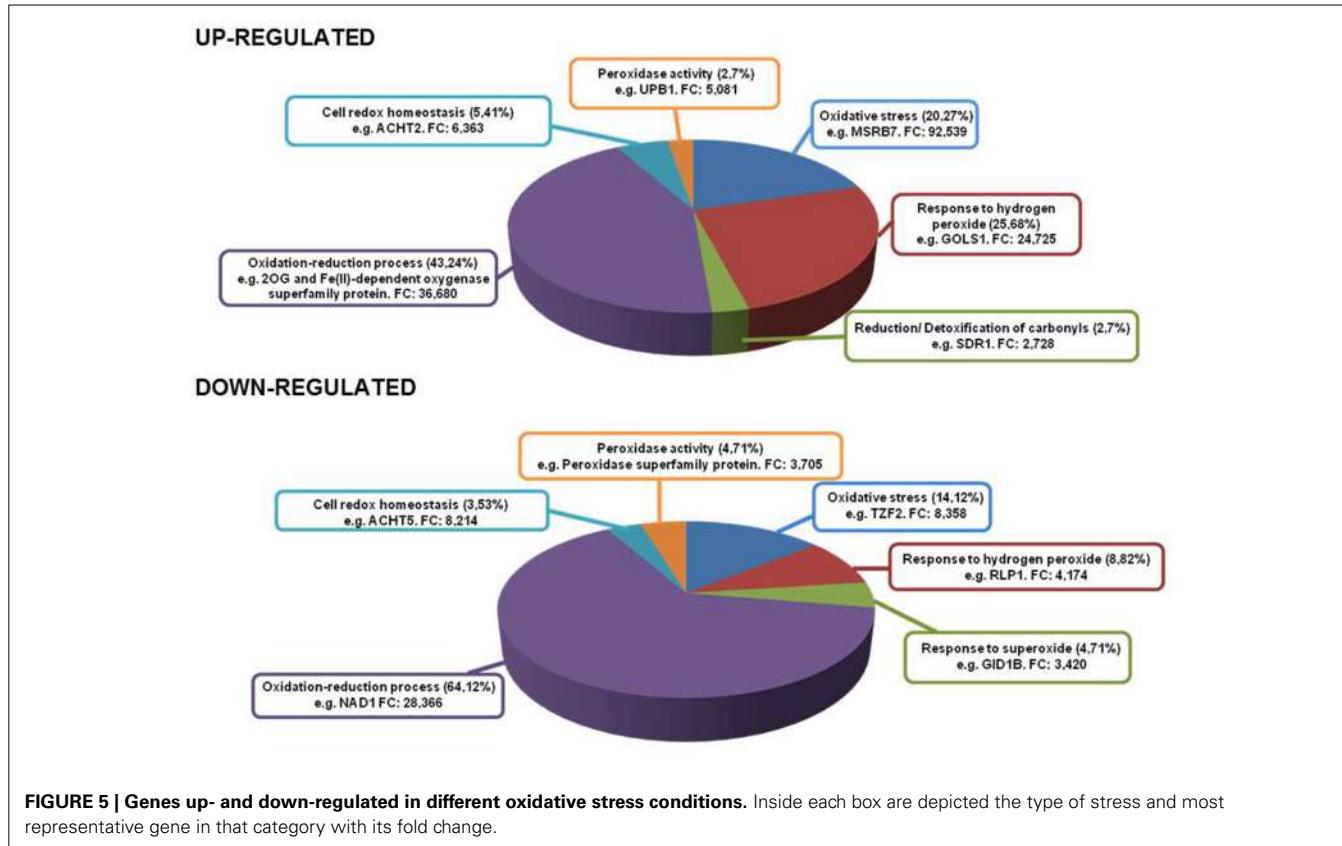


for 24.36 and 21.09% of down-regulated Ln-responsive genes, respectively. The highly repressed *AtERF53* (*AT2G20880*) is associated with drought stress responses (Cheng et al., 2012), while peptide transporter 3 (*AT5G46050*), involved in the response to wounding, was also repressed. Finally, another important group of down-regulated genes was activated in the response to cold and osmotic stress (16.36 and 8.73%, respectively), with the inhibition of PP2C5 (*AT2G40180*) acting as an MAPK phosphatase that controls MAPK levels and thus modulates innate immunity, JA and ethylene levels in *Arabidopsis* (Schweighofer et al., 2007).

#### LINOLENIC ACID-RESPONSIVE GENES IN OXIDATIVE STRESS SITUATIONS

With the aid of this (see **Supplemental Table 6**) transcriptomic analysis, we found a large amount of heat shock proteins (HSPs) and heat shock transcription factors (HSFs) associated with the responses to hydrogen peroxide ( $H_2O_2$ ), accounting for 25.68% of total genes up-regulated (*AT1G52560*, *AT2G26150*, *AT4G25200*, *AT2G20560*, *AT2G32120*, *AT1G74310*, *AT5G37670*, *AT4G21320*, and *AT1G16030*) (**Figure 5**). Recently, *HsfA2* expression has been shown to be induced under different types of oxidative stress conditions such as  $H_2O_2$  treatment (Miller and Mittler, 2006), with its overexpression producing a higher level of tolerance to several environmental stresses (Li et al., 2005). Furthermore, Ln treatment induced the expression of a set of

glutathione S-transferases (*AT2G29480*, *AT2G29470*, *AT2G29420*, *AT2G29490*, *AT2G29450*, *AT2G29460*, *AT2G47730*, *AT1G17170*, and *AT2G29440*) and of methionine sulfoxide reductase B7 (MSRB7, *AT4G21830*, FC 92.539 up). In this respect, another up-regulated enzyme associated with the detoxification of oxidized proteins was the alkenal reductase (*AT5G16970*). The largest percentage of up-regulated genes was associated with oxidation-reduction processes which accounted for 43.24% of total genes. Among them, we found members of 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily, as the most over-expressed genes, and a significant proportion of members of the cytochrome P450 superfamily (CYP450) (*AT5G06900*, *AT4G19230*, *AT1G64950*, *AT2G27690*, *AT5G63450*, *AT5G47990*, *AT4G15393*, *AT5G06905*, *AT4G31500*, and *AT5G25180*) encoding for fatty acid hydroxylases among others. These enzymes are capable of producing different compounds of cutin, a part of the cuticle that protects plants against various stresses (Kolattukudy, 1980). We also detected the induction of alternative oxidase 1D (*AT1G32350*) which plays an important role in metabolic and signaling homeostasis during abiotic and biotic stress in plants (Vanlerberghe, 2013). Finally, we also observed gene up-regulation of the monodehydroascorbate reductase 3 (MDAR3) enzyme (*AT3G09940*) which encodes an enzyme involved in the oxidation-reduction process. This enzyme is present in the ascorbate-glutathione cycle and is responsible for



the regeneration of reduced ascorbate, a major antioxidant in plant cells. In this regard, an increase in the transcript levels of the peroxisomal MDAR1 gene has been observed in *Pisum sativum* leaves subjected to mechanical wounding (Leterrier et al., 2005). This indicates that MDAR3 gene induction in *Arabidopsis* caused by Ln treatment may initiate the functioning of the ascorbate-glutathione cycle and thus control ROS production.

On the other hand, Ln treatment generated the down-regulation of several genes involved in oxidative stress (14.12%) such as OXS2 (AT2G41900) and OXS3 (AT5G56550). These genes have been shown to play a role in stress tolerance as they may act as chromatin remodeling factors in relation to the stress response to protect DNA or alter its transcriptional selectivity (Blanvillain et al., 2009). Finally, the most abundant down-regulated functional category was the oxidation-reduction process, accounting for 64.12% of total suppressed genes. Among these genes, the electron transport chain was inhibited, principally the components of NADH dehydrogenase complex I and cytochrome oxidase complex III (AT2G07785, AT2G07689, ATMG00650, ATMG00285, AT4G16790, ATMG01360, ATMG00513, ATCG00890, ATCG01250, ATMG00580, ATMG00990, ATCG01090, ATMG00510, ATCG01080, ATCG01070, ATCG01100, ATCG01050, AT2G07751, and ATCG01010). It has been reported that most photosynthesis-related genes are down-regulated after herbivore attack and may allow attacked plants to reinvest resources in other processes such as defense (Halitschke et al., 2001; Hui et al., 2003). This indicates that Ln was capable of reducing photosynthesis-related genes, possibly to counterbalance the induction of defense traits.

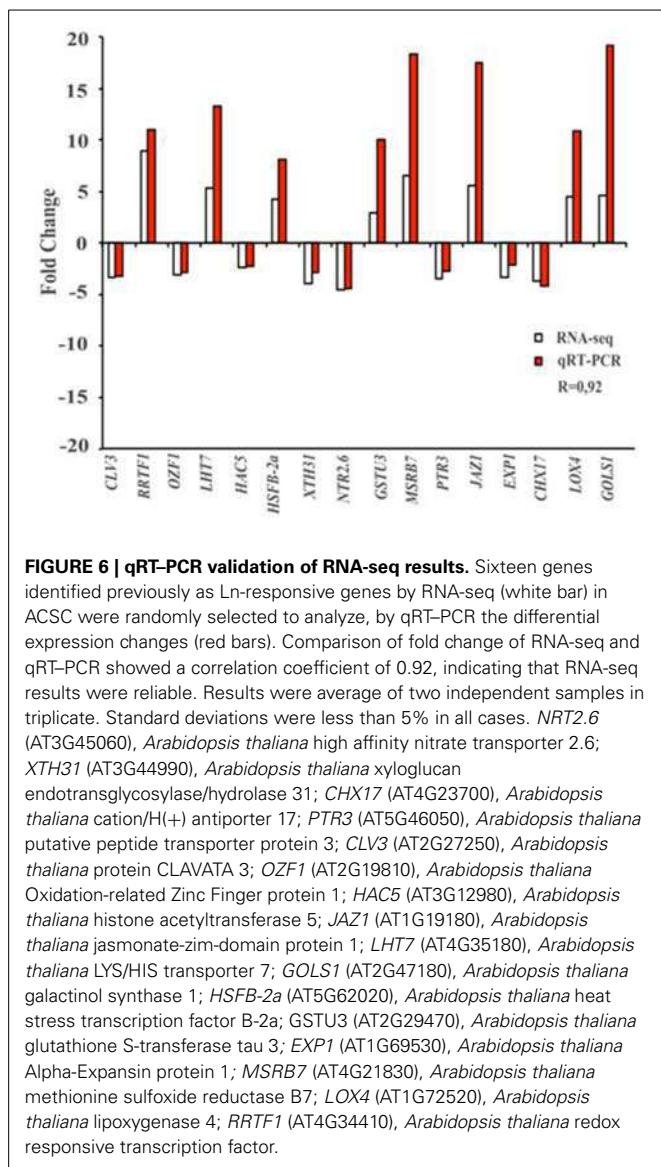
## VALIDATION OF LN-RESPONSIVE GENES BY QUANTITATIVE REAL-TIME REVERSE TRANSCRIPTION-PCR (qRT-PCR)

To validate RNA-seq results, we randomly assigned several Ln-responsive genes to conduct the expression analysis by qRT-PCR. Figure 6 shows the comparison between the qRT-PCR and RNA-seq analysis, showing that all the Ln-responsive genes tested and previously identified by RNA-seq were confirmed by qRT-PCR. The results showed a positive correlation between the two approaches (with a correlation coefficient of 0.92), indicating that the RNA-seq expression analysis performed is highly reliable.

## DISCUSSION

LN is an important molecule in plant physiology due to is the precursor of jasmonate pathway, a key component of plant defense (Wasternack and Hause, 2013). In this respect, Ln has been shown to be involved in the plant's gene responses to pathogen attacks and mechanical wounding caused by insect feeding (Wasternack, 2014a). However, little is known about the role played by Ln in gene responses to other abiotic stress situations and specifically those mediated by genes which regulate the cellular redox state and/or mediated by an oxidative stress. With the aid of RNA-seq technology, we have analyzed the transcriptional effect of Ln on ACSC by using a Ln treatment which does not provoke oxidative stress, thus enabling new gene sets involved in ROS cross-talk signaling to be identified.

Our findings indicate that Ln is associated with the plant's response to different abiotic stress conditions including hyperosmotic salinity and heat acclimation. Finally, one of the



**FIGURE 6 | qRT-PCR validation of RNA-seq results.** Sixteen genes identified previously as Ln-responsive genes by RNA-seq (white bar) in ACSC were randomly selected to analyze, by qRT-PCR the differential expression changes (red bars). Comparison of fold change of RNA-seq and qRT-PCR showed a correlation coefficient of 0.92, indicating that RNA-seq results were reliable. Results were average of two independent samples in triplicate. Standard deviations were less than 5% in all cases. *NRT2.6* (AT3G45060), *Arabidopsis thaliana* high affinity nitrate transporter 2.6; *XTH31* (AT3G44990), *Arabidopsis thaliana* xyloglucan endotransglycosylase/hydrolase 31; *CHX17* (AT4G23700), *Arabidopsis thaliana* cation/H(+) antiporter 17; *PTR3* (AT5G46050), *Arabidopsis thaliana* putative peptide transporter protein 3; *CLV3* (AT2G27250), *Arabidopsis thaliana* protein CLAVATA 3; *OZF1* (AT2G19810), *Arabidopsis thaliana* Oxidation-related Zinc Finger protein 1; *HAC5* (AT3G12980), *Arabidopsis thaliana* histone acetyltransferase 5; *JAZ1* (AT1G19180), *Arabidopsis thaliana* jasmonate-zim-domain protein 1; *LHT7* (AT4G35180), *Arabidopsis thaliana* LYS/HIS transporter 7; *GOLS1* (AT2G47180), *Arabidopsis thaliana* galactinol synthase 1; *HSFB-2a* (AT5G62020), *Arabidopsis thaliana* heat stress transcription factor B-2a; *GSTU3* (AT2G29470), *Arabidopsis thaliana* glutathione S-transferase tau 3; *EXP1* (AT1G69530), *Arabidopsis thaliana* Alpha-Expansin protein 1; *MSRB7* (AT4G21830), *Arabidopsis thaliana* methionine sulfoxide reductase B7; *LOX4* (AT1G72520), *Arabidopsis thaliana* lipoxygenase 4; *RRTF1* (AT4G34410), *Arabidopsis thaliana* redox responsive transcription factor.

most interesting processes we observed was the response to oxidative stress which led us to analyze the interaction between Ln and signaling mediated by oxidative stress-related processes, about which very little is known.

#### LINOLENIC ACID REGULATES BIOSYNTHESIS AND SIGNALING OF JASMONATES IN ACSC. INVOLVEMENT OF LINOLENIC ACID IN BIOTIC STRESS SITUATIONS

Ln treatment provoked the over-expression of several genes associated with jasmonate-biosynthetic processes, including lipoxygenases which constitute the first step in the formation of these molecules (Wasternack, 2014b). This phenomenon is probably due to an increase in JA levels after Ln treatment followed by a self-activation stage in JA biosynthesis that has been reported extensively in the literature (Wasternack, 2007). In addition, the application of Ln to ACSC resulted in the up-regulation of *ERF1* (AT3G23240) which acts downstream of the intersection between

ethylene and jasmonate pathways. It has also been suggested that this gene is a key element in the integration of both signals for regulating defense response genes (Lorenzo et al., 2003). We also observed an increase in the number of transcripts of several transcription factor families such as *WRKY*, *bHLH*, *MYB*, and *NAC*. The over-expression of all these genes shows that polyunsaturated fatty acid Ln induces the oxylipin pathway in order to produce JA-related phytohormones and consequently all components involved in its generation and functioning. In addition, several down-regulated Ln genes active in jasmonate-related processes were detected. Carbonic anhydrase (CA) was found to be a down-regulated enzyme. In this regard, in some stress situations such as wounding and herbivore attacks, CA gene expression in wounded *Capsicum annuum* leaves was observed to be lower than that in control leaves. This suggests that proteins playing a role in photosynthesis are down-regulated by metabolic reconfiguration in order to maintain a balance between defense and tolerance (Mahajan et al., 2014). Furthermore, several *MYB* transcription factors were down-regulated. In plants, *MYB* genes are a large family functionally active in regulating several defense processes (Kirik et al., 1998; Stracke et al., 2001), indicating that Ln launches a mechanism capable of improving the plant's defenses in different stress situations.

The release of Ln from membranes in response to intracellular signaling events is a key stage in the activation of defensive genes, with phospholipases being the enzymes responsible for both basal and stimulus-induced production of JA. Phospholipase A has actually been active in wound- and systemin-induced JA formation in tomato (Narváez-Vásquez et al., 1999), while phospholipase D  $\alpha$ 1 also promotes wound-induced accumulation of free Ln and JA in *Arabidopsis* (Zien et al., 2001; He et al., 2002). Elicitation of wound responses in plants appearing upon mechanical wounding (abiotic stress) or herbivore attacks (biotic stress) is one of the most prominent examples and extensively studied areas where JA/JA-Ile is involved as a signal. In this regard, RNA-seq analysis enabled us to identify Ln-regulated genes which play a role in the biotic, abiotic and oxidative stress responses of ACSC. With respect to genes participating in biotic stress responses, our analysis revealed the over-expression of *WRKY40* and *RRTF1* transcription factors. Although the role played by *RRTF1* in plant defense still needs to be rigorously tested, recent reports indicate its involvement in regulating redox homeostasis during stress, with *RRTF1* expression being dependent on COI1, a key regulator of JA signaling (Khandelwal et al., 2008; Wang et al., 2008). A direct physical *in vivo* interaction has also been shown to exist between *WRKY40* and *RRTF1*, indicating that this WRKY transcription factor acts as a direct transcriptional repressor of *RRTF1* (Pandey et al., 2010). We also detected the up-regulation of the *BAG2* gene which has been identified as containing a regulator of plant programmed cell death (PCD). Given that AtBAG family members have been shown to inhibit plant PCD pathways in response to stress (Doukhanina et al., 2006), Ln may act as a promoter of cell survival and resistance to biotic stress situations. In this respect, we detected over-expression of the *JAS1/JAZ10* TF which is a negative regulator of JA signaling in *Arabidopsis* seedlings and of disease susceptibility to *Pseudomonas syringae* strain DC3000 (Demianski et al., 2012). This activity is mediated

by the JAZ10.4 alternative splice variant that lacks the Jas motif in the C-terminal and mediates interaction with COI1 and MYC2 (Moreno et al., 2013). Up-regulation of this gene may be related to the JA self-regulation mechanism which controls jasmonate over-production.

#### **LINOLENIC ACID INDUCES KEY GENES OF ENZYMES INVOLVED IN THE PLANT'S DEFENSE AGAINST ABIOTIC STRESS**

An important finding produced by RNA-seq data analysis was the regulation by Ln treatment of abiotic stress response genes. Most previous studies have focused on biotic stress responses and the oxylipin pathway following the application of JA-related molecules. Nevertheless, the role of these molecules in processes participating in abiotic stress situations, particularly in relation to oxidative stress, is not very well known. In this regard, we detected an up-regulation of the galactinol synthase enzyme (GOLS1, AT2G47180) with a FC of 24.725. GolS catalyzes the first stage in the biosynthesis of raffinose family oligosaccharides (RFOs) from UDP-galactose and also RFO-derived molecules like raffinose and stachyose which intervene in the accumulation of osmoprotectants during seed development. In this respect, galactinol synthase has been shown to play a key role in the accumulation of galactinol and raffinose under abiotic stress conditions such as drought, high salinity and cold (Taji et al., 2002). In addition to these findings, high intracellular levels of galactinol and raffinose have recently been demonstrated to correlate with increased tolerance to methylviologen (MV) treatment as well as salinity and chilling stress conditions. This suggests that these molecules may scavenge hydroxyl radicals protecting plant cells from oxidative damage caused by these stresses (Nishizawa et al., 2008). These findings indicate that Ln could mediate plant responses to abiotic stresses by inducing an important defense mechanism mediated by this galactinol synthase. It is important to note that a large percentage of up-regulated genes in response to Ln treatment encoded heat shock proteins (HSPs) or chaperones. As several different abiotic stress conditions can cause protein dysfunction, maintaining these proteins in their functional conformations and preventing the aggregation of non-native proteins are particularly important for cell survival under stress conditions. These HSPs can play a crucial role in protecting plants against stress by re-establishing the normal protein conformation and thus cellular homeostasis (Wang et al., 2004). In this regard, Ln could initiate a defense mechanism mediated by the induction of key components like these protein stabilizers involved in defending the plant against different abiotic stresses. In conclusion, Ln is capable of modulating the expression levels of different genes participating in a wide variety of abiotic processes such as drought, salinity and wounding. The Ln poly-unsaturated fatty acid is involved in other pathways not directly associated with biotic responses and also with several abiotic stress situations, indicating that it plays a very important role as a signaling mediator.

#### **LINOLENIC ACID RESPONSE AGAINST OXIDATIVE STRESS: INDUCTION OF METHIONINE SULFOXIDE REDUCTASE (MSRB7) AND ALKENAL REDUCTASE**

Until now, most studies have focused on the exogenous administration of JA-related molecules such as methyl-jasmonate, which

act as signaling molecules in order to induce responses to biotic stress situations like pathogen attacks and wounding induced by herbivores. For this reason, an important aim of this study has been to identify the genes and potential metabolic pathways involved in the regulation of the cellular redox state and/or mediated by an oxidative stress in response to Ln treatment under non-oxidative stress conditions. Remarkably, in this regard, we detected the over-expression of methionine sulfoxide reductase B7 (MSRB7, FC 92.539). Methionine oxidation by ROS (John et al., 2001) leads to the formation of MetSO (Boschi-Muller et al., 2008) which could alter both the activity and conformation of many proteins (Dos Santos et al., 2005; Rouhier et al., 2006). This enzyme catalyzes the reduction of methionine sulfoxides back to methionine and can also repair oxidized proteins and protect against oxidative damage (Moskovitz, 2005). Begara-Morales et al. (2014) showed that MSRB7 was highly induced by S-nitrosoglutathione (GSNO), suggesting that this enzyme plays a very important role in the oxidative metabolism and specifically in nitric oxide metabolism. It is therefore important to determine whether a poly-unsaturated fatty acid such as Ln is able to induce a key gene in an enzyme involved in protection against methionine oxidation, indicating crosstalk between redox status and the nitric oxide metabolism. Another important enzyme detected by this RNA-seq analysis relating to oxidized protein detoxification was the alkenal reductase enzyme. Oxidative stress produced in some biotic/ abiotic stress situations can lead to the production of ROS which damage biomolecules such as proteins and lipids. Because linoleic and linolenic acids are sources of many short-chain carbonyls due to peroxidation, biomolecules are threatened by the toxicity of reactive compounds including  $\alpha,\beta$ -unsaturated carbonyls, which are involved in the pathophysiological effects associated with oxidative stress in cells and tissues (Yamauchi et al., 2011). This enzyme catalyzes the reduction of the  $\alpha,\beta$ -unsaturated bond of reactive carbonyls which are active in anti-oxidative plant defenses (Mano et al., 2005). Finally, we also detected the induction of various glutathione S-transferase genes and a large percentage of several members of the CYP450 superfamily. In this regard, oxidative burst, involving the rapid production of enormous amounts of ROS, is one of the first mechanisms of defense against certain biotic and abiotic stresses such as wounding, cells subjected to mechanical stress or pathogen attacks. Faced with these oxidative attacks, damaged plants initiate a series of defense mechanisms by, for example, releasing Ln from cell membranes throughout several lipases. In this sense, we observed that Ln was able to enhance the expression of MSRB7 and alkenal reductase which could repair oxidative modifications in proteins caused by the high levels of ROS generated in these stress situations. Moreover, the glutathione metabolism appears to be of crucial importance due to the large number of glutathione S-transferase genes induced. These enzymes play a crucial role in detoxification of peroxidised lipids, thus contributing to the defense response potentiated by Ln. Furthermore, the induction of genes from members of the CYP450 family generates compounds as protectors and potent elicitors of defense mechanisms (Kolattukudy, 1980; Schweizer et al., 1998). In this regard, CYP94C1 has been reported to be induced at the transcriptional level by the methyl-jasmonate

stress hormone (Kandel et al., 2007) which corroborates the findings obtained by this RNA-seq analysis.

## AUTHOR CONTRIBUTIONS

The experiments were conceived and designed by: JBB, FC, FL, BS, CM, and JCB. The experiments were performed by: CM, BS, JCB, MP, RV, FL, AF, JJ, JE, FC, and JBB. The data were analyzed by: CM, JBB, JCB, and FL. The paper was written by: CM, JBB, FC, FL, and JCB.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://www.frontiersin.org/journal/10.3389/fpls.2015.00122/abstract>

**Supplemental Table 1 | Fatty acid composition of *Arabidopsis thaliana* cell suspension cultures (ACSC).**

**Supplemental Table 2 | Effect of Ln on oxidative stress markers.**

**Supplemental Table 3 | Ln-responsive genes involved in jasmonate-related processes.**

**Supplemental Table 4 | Ln-responsive genes involved in biotic stress situations.**

**Supplemental Table 5 | Ln-responsive genes involved in abiotic stress situations.**

**Supplemental Table 6 | Ln-responsive genes involved in oxidative stress conditions.**

**Supplemental Table 7 | 2FC-up-regulated genes by Linolenic acid-treatment.**

**Supplemental Table 8 | 2FC-down-regulated genes by Linolenic acid-treatment.**

**Supplemental Table 9 | Oligonucleotides used for qRT-PCR.**

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# Reactive sulfur species (RSS): possible new players in the oxidative metabolism of plant peroxisomes

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Peroxisomes are ubiquitous organelles with a notable oxidative metabolism. In plants, these subcellular compartments have been shown to be involved in the metabolism of reactive oxygen and nitrogen species (ROS and RNS), whose components, hydrogen peroxide and nitric oxide (NO), are important molecules involved in signaling processes. The presence of new elements in plant peroxisomes such as glutathione reductase (GR), sulfite oxidase (SO), glutathione (GSH), and S-nitrosoglutathione (GSNO) indicates the involvement of these organelles in the sulfur metabolism. This could suggest the participation of a new family of molecules designated as reactive sulfur species (RSS) which will possibly provide new functions for peroxisomes.

## CRITICAL VIEW

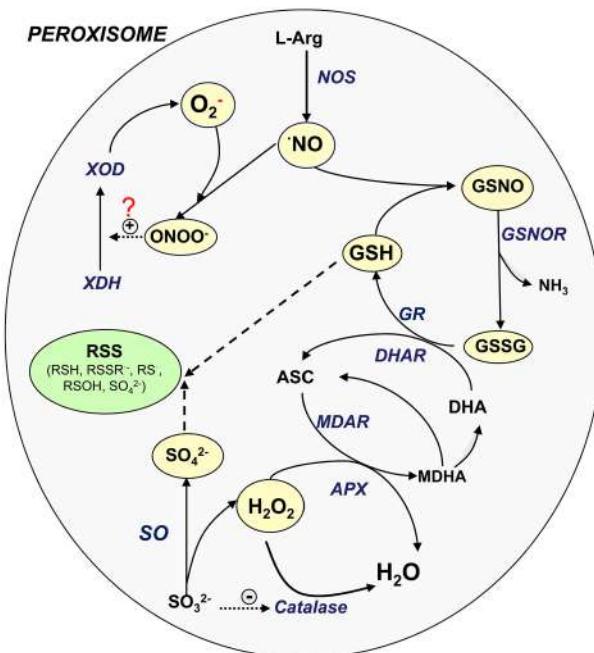
Peroxisomes are remarkable subcellular compartments given their simple morphology (granular/fibrillar matrix bounded by a single membrane) which does not reflect the complexity of their enzymatic composition (Hayashi et al., 2000; Baker and Graham, 2002; del Río et al., 2006). In plant cells, peroxisomes are involved in the photorespiration cycle, fatty acid  $\beta$ -oxidation, the glyoxylate cycle, and the metabolism of ureides (Corpas et al., 1997; Baker and Graham, 2002; del Río et al., 2006; Hu et al., 2012), thus indicating that these organelles play a role in key physiological processes such as seed germination, plant development, fruit ripening, and senescence. Plant peroxisomes have been shown to be a

source of ROS including molecules such as superoxide radicals ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ), and RNS (del Río et al., 2006; del Río, 2011; Corpas et al., 2013; Corpas and Barroso, 2014b). RNS include NO and related molecules such as peroxynitrite ( $ONOO^-$ ) and GSNO which are characterized by a broad spectrum of physiological/pathological activities. Both these molecular families (ROS and RNS) include radical molecules containing an unpaired electron as well as non-radical molecules and can also have dual effects depending on their cellular concentration. Thus,  $H_2O_2$  and NO at low concentrations can function as signal molecules in the cell or may cause damage to cell components when overproduced as a consequence of adverse conditions (Valderrama et al., 2007; Chaki et al., 2011; Signorelli et al., 2013).

Sulfur (S) is an essential mineral for plant growth and development (Leustek and Saito, 1999; Rausch and Wachter, 2005; Hawkesford and De Kok, 2006). It is present in thiamin (B1) and pantothenic acid (B5) vitamins, amino acids (cysteine and methionine), biotin and molybdenum cofactors, and prosthetic groups (Leustek and Saito, 1999) and also in secondary sulfur compounds (polysulfides, glucosinolates, and phytochelatins). In addition, other organic sulfur compounds, such as thiols, GSH, GSNO, and sulfolipids, play an important role in physiological processes and plant stress conditions (Brychkova et al., 2007; Münchberg et al., 2007). In animal cells, the gas hydrogen sulfide ( $H_2S$ ) has been

shown to be generated from L-cysteine by the pyridoxal-5'-phosphate-dependent enzyme. Thus, endogenous  $H_2S$  can act as a neuromodulator in rat brain (Abe and Kimura, 1996). In higher plants, recent evidence indicates that  $H_2S$  is actively involved in the regulation of ethylene-induced stomatal closure and also interacts with  $H_2O_2$  to regulate the plasma membrane  $Na^+/H^+$  antiporter system under salinity stress (Hou et al., 2013; Li et al., 2014). The term RSS has been proposed in order to designate a group of sulfur-related molecules that are formed *in vivo* under oxidative stress conditions in animal systems (Giles et al., 2001, 2002; Jacob et al., 2004). These molecules include thiyl radicals (RS $\cdot$ ), disulfide-S-oxides [RS(O) $_2$ SR] and sulfenic acids (RSOH). Thus, high cellular GSH concentrations in an oxidative environment and the decomposition of S-nitrosothiols generate disulfide-S-oxides (Tao and English, 2004). These mechanisms can modulate the function of sulfur proteins throughout the redox status of biological thiols (Jacob and Anwar, 2008). Accordingly, disulfide formation is an important cysteine redox reaction in many proteins that affects its function, with thioredoxins and peroxiredoxins being good examples.

In plant peroxisomes, the presence of important sulfur compounds such as GSH (non-enzymatic antioxidants) (Jiménez et al., 1997; Müller et al., 2004) and GSNO (transport and storage vehicle for NO) has been demonstrated (Barroso et al., 2013). Furthermore, the presence of enzymes such as GR



**FIGURE 1 | Signaling cross-talk between NO, ROS, and RSS.** Model of the interaction amongst the different ROS, RNS, and sulfur metabolism into plant peroxisomes. ASC, ascorbate, reduced form; DHA, ascorbate, oxidized form (dehydroascorbate); GSH, glutathione, reduced form; GSNOR, nitrosoglutathione reductase; GSSG, glutathione, oxidized form; NO, nitric oxide; NOS, L-arginine-dependent nitric oxide synthase; MDAR, monodehydroascorbate reductase;  $ONOO^-$ , peroxynitrite; SO, sulfite oxidase; XDH, xanthine dehydrogenase; XOD, xanthine oxidase; RSS, reactive sulfur species; RSH, thiol;  $RSSR^-$ , disulfide radical; RS, thyl radical; ROSH, sulfenic acid;  $SO_4^{2-}$ , sulfate.

(Jiménez et al., 1997; Romero-Puertas et al., 2006), *S*-nitrosoglutathione reductase (GSNOR) (Reumann et al., 2007; Barroso et al., 2013) and SO (Eilers et al., 2001; Nakamura et al., 2002; Nowak et al., 2004; Hänsch and Mendel, 2005) involved in the sulfur metabolism has also been reported. These new insights lead us to suggest that peroxisomes may play a role in the RSS metabolism, as has been demonstrated for ROS and RNS. **Figure 1** shows the potential interactions among the different ROS, RNS, and sulfur-containing compounds in peroxisomes. NO is generated by L-arginine-dependent nitric oxide synthase (NOS) activity (Corpas and Barroso, 2014a) which can react with superoxide radicals generated by xanthine oxidase to form peroxynitrite ( $ONOO^-$ ). This RNS is a highly oxidant compound capable of catalyzing the conversion of xanthine dehydrogenase to xanthine oxidase (Corpas et al., 2008) or inducing protein nitration (Radi, 2013). NO can also react with GSH to form GSNO which

can be decomposed by GSNOR activity through the generation of GSSG (oxidized form) and  $NH_3$ . GSSG is reduced by GR as a component of the ascorbate-glutathione cycle.  $H_2O_2$ , which is mainly generated by flavin-oxidases, is decomposed either by catalase or ascorbate peroxidase (APX). SO catalyzes the conversion of sulfite to sulfate with the concomitant generation of  $H_2O_2$  (Hänsch et al., 2006). It has been reported that low concentrations of sulfite inhibit catalase activity (Veljović-Jovanović et al., 1998), which could therefore be a means of regulating both enzymes.

In this context, the interactions of ROS, RNS and possibly RSS components in plant peroxisomes open up new challenges and a new area of research to determine the biochemical interactions and potential functions of these reactive species of oxygen, nitrogen and sulfur in peroxisomes, some of which play a very important role as signaling molecules in physiological and phyto-pathological processes (Yamasaki, 2005).

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