Heterologous Expression of AtWRKY57 Confers Drought Tolerance in Oryza sativa

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Drought stress is a severe environmental factor that greatly restricts plant distribution and crop production. Recently, we have found that overexpressing AtWRKY57 enhanced drought tolerance in Arabidopsis thaliana. In this study, we further reported that the Arabidopsis WRKY57 transcription factor was able to confer drought tolerance to transgenic rice (Oryza sativa) plants. The enhanced drought tolerance of transgenic rice was resulted from the lower water loss rates, cell death, malondialdehyde contents and relative electrolyte leakage while a higher proline content and reactive oxygen species-scavenging enzyme activities was observed during stress conditions. Moreover, further investigation revealed that the expression levels of several stress-responsive genes were up-regulated in drought-tolerant transgenic rice plants, compared with those in wild-type plants. In addition to the drought tolerance, the AtWRKY57 overexpressing plants also had enhanced salt and PEG stress tolerances. Taken together, our study indicates that over-expressing AtWRKY57 in rice improved not only drought tolerance but also salt and PEG tolerance, demonstrating its potential role in crop improvement.

Keywords: AtWRKY57, drought tolerance, Oryza sativa, Stress, ROS

INTRODUCTION

Drought is a critical abiotic stress that severely restricts crop production (Zhu, 2002). With the process of evolution, plants have gained a variety of strategies with the purpose of avoiding drought stress by reducing water loss or increasing water uptake. Nevertheless, other strategies need to protect plant cells from damage when water is exhausted and tissue dehydration unavoidable (Verslues et al., 2006). Additionally, the molecular, cellular, and whole-plant levels strategies should be coordinated to adapt to drought stress (Yu et al., 2008).

Under drought- or salt-stress conditions, plants accumulate reactive oxygen species (ROS) (Verslues et al., 2006). In living cells, ROS such as superoxide, hydrogen peroxide (H2O2), and hydroxyl radicals are generated as harmful substances via aerobic metabolism. Through partially reduced or activated derivatives of oxygen, ROS can destroy DNA, proteins and carbohydrates, resulting in cell death (Mittler et al., 2004). A master level of ROS gives rise to the oxidation of biomolecules, such as lipids, nucleic acids and proteins, which caused cellular damage. When CO2 fixation is restricted under environmental stress conditions, the photosynthetic electron transport system generates ROS (Asada, 1999). To defend oxidative stress, organisms have
evolved an effective system to protect themselves. For example, numerous stress-related genes were induced by ROS in response to oxidative stress in these defensive systems (Demple and Amabile-Cuevas, 1991; Gasch et al., 2000; Desikan et al., 2002). As higher plants have the ability to coordinately regulate multiple antioxidant genes, they are much tolerant to oxidative stresses.

Normally, the maintenance of routine homeostasis is achieved through the ROS-scavenging system in plant cells, which is mainly mediated by enzymatic defenses, including superoxide dismutase (SOD), catalase (CAT), and peroxidases (POX) (Mittler et al., 2004). Generally, SODs, which catalyze the dismutation of superoxide into oxygen and H$_2$O$_2$, provide the first line of defense against ROS in various subcellular compartments, such as chloroplast, mitochondria and cytosol (Raychaudhuri and Deng, 2000). The physiological role of CAT is to break down H$_2$O$_2$ in the cell (Scandalios, 2002). Therefore, increased CAT activity would result in H$_2$O$_2$ degradation. PODs are a group of enzymes that catalyze the oxidation of many substrates (e.g., phenolic compounds) at the expense of H$_2$O$_2$ (Asada, 1987). The increased activity of these enzymes would decrease ROS levels. Recent reports have demonstrated that transgenic rice plants with enhanced ROS-scavenging abilities had improved drought tolerance (Ouyang et al., 2010; Zhang et al., 2011). For example, OsSIK1 functions in stress signaling through scavenging and detoxification of ROS. In OsSIK1-overexpressing plants, the high levels of POD and CAT enzymes resulted to low levels of H$_2$O$_2$ (Ouyang et al., 2010). Similarly, the improved drought tolerance of HRF1-overexpressing transgenic rice plants was partially resulted from the increased ROS-scavenging activities (Zhang et al., 2011).

Under environment stress conditions, the stress-related proteins not only function in protecting cells from damage but also regulate the expression of downstream genes for signal sensing, perception and transduction (Kreps et al., 2002; Seki et al., 2002). These proteins can be classified into two groups. The first group protein plays a crucial role to avoid cellular injury, such as detoxification enzymes, Late Embryogenesis Abundant (LEA) proteins, and the key enzymes for osmolyte biosynthesis (Kreps et al., 2002; Seki et al., 2002). The second group includes numerous transcription factors involved in further regulation of transcriptional control and signal transduction. The CBF/DREB factor, Basic Leucine Zipper families, CUC transcription factor, NAM, plant nuclear factor Y (NF-Y) B subunits, zinc finger and ATAF, belong to this group (Umezawa et al., 2006; Nelson et al., 2007; Takasaki et al., 2010). Studies on these transcription factors will contribute to uncover the respect for commercially improving drought tolerance in crops through genetic engineering.

The WRKY family consists of 74 and 102 members in Arabidopsis thaliana and Oryza sativa, respectively (Eulgem et al., 2000; Wu et al., 2005); and majority of them play critical roles in biotic and abiotic stress responses (Eulgem and Sommer, 2007; Miller et al., 2008). Recently, increasing evidences confirmed that numerous of WRKY genes are involved in drought stress. For example, ABO3/WRKY63 plays a key role in plant responses to ABA and drought stress (Ren et al., 2010). Overexpression of a stress-induced OsWRKY45 significantly confer drought tolerance in Arabidopsis and rice (Qiu and Yu, 2008; Tao et al., 2011). Especially, our previous study demonstrated that overexpression of AtWRKY57 improved drought tolerance by directly targeting the promoter sequences of NCED3 to increase the content of ABA in Arabidopsis (Jiang et al., 2012). These evidences give us a hypothesis that the improvement of plant drought tolerance might be realized through gene manipulation approaches. To test this hypothesis, we further over-expressed AtWRKY57 in rice and demonstrated that the stress tolerance of the transgenic rice under drought conditions was significantly improved. 3,3′-Diaminobenzidine (DAB) and nitro blue tetrazolium (NBT) staining analyses showed that the ROS levels in transgenic lines were lower than in control plants after drought-stress treatment. Consistent with the low ROS levels, the antioxidative enzyme activities were also enhanced in the transgenic lines. Moreover, high expression levels of stress-responsive genes also supported the drought tolerance in transgenic lines. Overall, our results indicated that the over-expression of AtWRKY57 in rice conferred the adaptation of rice to drought tolerance by reducing ROS damage and up-regulating the expression of stress-responsive genes.

**MATERIALS AND METHODS**

**Construction and Transformation of AtWRKY57 in Rice**

The full-length cDNA sequence of AtWRKY57 was obtained from Arabidopsis using the same method as described in our previous study (Jiang et al., 2012). The full coding sequence of AtWRKY57 was cloned into pUN1301 in the sense orientation behind the Ubiquitin promoter. Then the T-DNA was transformed into ZH11 (Oryza sativa L. ssp. japonica cv. Zhonghua11) via the Agrobacterium tumefaciens-mediated method (Hiei et al., 1994). After transformation, the calli were selected from half-strength (MS) medium containing 100 μg/ml hygromycin. Seedlings with hygromycin-resistant were transplanted to soil in a growth chamber.

**Plant Growth Conditions**

The sterilized Oryza seeds sowed on medium and kept in a growth chamber at 22°C under long-day conditions [16 h light/8 h dark cycles]. One week generation, seedlings were then transplanted in soil and half-strength MS medium supplemented with 1.5% (W/V) sucrose for drought stress, NaCl and PEG treatments. The soils are commonly used loam, mixed 50% humus soil, 30% coconut tree branny, 20% red clay.

**Drought-Tolerance Assays**

Drought-tolerance assays were performed using 4-week-old plants. The transgenic rice and control seedlings were
transplanted in the same pot and treated with drought stress by withholding water for 20 days. Three independent pots repeated at the same time and a representative result displayed. Three independent experimental replications were conducted.

To evaluate the water loss rates, flag leaves were detached from the plants and weighed at designated time intervals at room temperature. The proportion of fresh weight lost was calculated based on the initial plant weight. At least three biological replicates for each sample were used for the calculation.

**Trypan Blue, DAB and NBT Staining**

For DAB staining, leaf sections of approximately 5 cm in length were cut and soaked in a 1% solution of DAB in 50 mM Tris-HCl buffer (pH 6.5). After 30 min vacuum infiltrating, the immersed leaves were incubated in the dark for 20 h at room temperature. And then the leaves were bleached by bath in boiling ethanol until the brown spots appeared clearly. The area of brown spots are represented the DAB reaction degree to H$_2$O$_2$.

Leaf sections of approximately 5 cm in length were excised to detect superoxide accumulation by a 0.1% solution of NBT in 10 mM potassium phosphate buffer (pH 7.8) as described previously (Fitzgerald et al., 2004). After 15 min vacuum infiltrating, the immersed leaves were incubated overnight at room temperature. After incubation, the leaves were fixed and cleared in alcoholic lacto-phenol (2:1:1, 95% ethanol: lactic acid: phenol) at 65°C for 30 min, rinsed with 50% ethanol, and then rinsed with water. When NBT interacts with superoxide, a blue precipitate forms is visible in leaves.

**Proline (Pro) Content, Malondialdehyde (MDA) Content, and Electrolyte Leakage Measurements**

The proline concentration was determined as described (Bates, 1973). Approximately 0.5 g of transgenic and control leaf segments were homogenized in 10 ml 3% aqueous sulfosalicylic acid and centrifuged at 3,000 × g for 20 min. 2 ml of supernatant was reacted with 2 ml acid ninhydrin and 2 ml glacial acetic acid in a test tube at 100°C for 1 h, cooled on ice, and the absorbance at 520 was measured. L-Pro was used as a standard to calculate the proline concentration.

The MDA content was determined as described (Heath and Packer, 1968) with slight modifications. Approximately 1 g of transgenic and control leaf segments were homogenized in 10 ml of 10% trichloroacetic (v/v) and centrifuged at 5,000 × g for 10 min. 2 ml of supernatant was reacted with 2 ml thiobarbituric acid in a test tube at 100°C for 15 min, quickly cooled on ice, and the absorbance at 532 was measured. The MDA content was confirmed using the extinction coefficient of 155 nM$^{-1}$ cm$^{-1}$, and expressed as nmol g$^{-1}$ FW.

The relative ion leakage was checked following the method of Clarke et al. (2004). For the above assays, each data point is the average of three replicates. At least three experiments were performed, and the results are consistent. The result from one set of experiments is presented here.

**Oxidative Enzyme Activity Measurements**

The leaves of 4-week-old rice seedlings were dehydrated for 2 h, and then homogenized in a solution of 50 mM sodium phosphate buffer (pH 7.8) containing 1% polyvinylpyrrolidone and 10 mM β-mercaptoethanol in an ice-cold mortar. After centrifugation (13,000 × g, 15 min) at 4°C, the supernatant was used to identify SOD, POD and CAT activity levels. U min$^{-1}$ mg$^{-1}$ protein was represented the enzyme activity of SOD, POD, and CAT.

The ability to inhibit the photochemical reduction of NBT chloride was used for the determination of the total SOD activity as described by Beauchamp and Fridovich (1971). The reduction of NBT by 50% of the quantity of enzyme required was defined as one unit of SOD activity.

The activity of POD was determined as described by Maehly and Chance (1954). Three milliliter of reaction mixture contained 30 μl enzyme extract, 5.4 mM guaiacol, 50 mM sodium acetate buffer (pH 5.6), and 15 mM H$_2$O$_2$. The oxidation of guaiacol to tetrahydrochloroal was contributed to the increase in absorbance monitored at 470 nm. A 0.01 absorbance increase per min at 470 nm was defined as one unit of POD activity.

The activity of CAT was measured following the method of Cakmak and Marschner (1992) by determining the rate of H$_2$O$_2$ disappearance at 240 nm. Three milliliter of reaction mixture contained 30 μl enzyme extract, 10 mM H$_2$O$_2$ and 50 mM phosphate buffer (pH 7.0). A 0.01 absorbance decrease per min at 240 nm was defined as one unit of CAT activity.

For each enzyme's activity, the data points are the average of three replicates. Three experiments were performed, and the results are consistent. The result from one set of experiments is presented here.

**Salt and Osmotic Tolerance Assays**

For salt tolerance assays, 2-week-old seedlings grown on half-strength MS agar medium were transferred into half-strength MS liquid medium for 2 weeks growth and then transferred into half-strength MS liquid medium supplemented with 175 mM NaCl and incubated at 22°C under long-day conditions for 2 days. After 2 days of NaCl treatment, the seedlings were transferred into half-strength MS liquid medium for 7 days recovery.

For PEG tolerance assays, 2-week-old seedlings grown on half-strength MS agar medium were transferred into half-strength MS liquid medium for 2 weeks of growth and then transferred into half-strength MS liquid medium supplemented with 25% PEG6000 (v/v) and incubated at 22°C under long-day conditions for 4 days. After 4 days of PEG treatment, the seedlings were transferred into half-strength MS liquid medium for 7 days recovery.

The transgenic rice and control seedlings were transplanted in the same pot for NaCl and PEG treatments. Three independent pots repeated at the same time and a representative result displayed in the manuscript. Three independent experimental replications were conducted.
Real-Time RT-PCR Analysis
For the real-time RT-PCR analysis, the same method was used as described in our previous studies (Jiang et al., 2012, 2014). We conducted three independent experiments (three biological replications and three technological replications in every independent experiment) and one representative result was displayed. All of the primer sequences used in real-time RT-PCR analysis were listed in Supplementary Table 1.

Statistical Analysis
Statistically significant differences (P < 0.05) based on the Student’s t-test computed by the SigmaPlot10.0. Data are the means ± SE of three independent experiments (3 biological replications and three technological replications in every independent experiment).

RESULTS
Constitutive Expression of AtWRKY57 in Transgenic Rice Lines
In our previous study, we confirmed that overexpression of AtWRKY57 significantly enhanced drought tolerance in Arabidopsis (Jiang et al., 2012). To explore whether AtWRKY57 plays an important role in improving the agronomic traits through gene manipulation approaches, we introduced this gene to rice. More than 20 transgenic lines were generated and five lines were randomly selected to check AtWRKY57 expression by northern blotting (Supplementary Figure 1). And then two lines, Line 3 and Line 5, were chosen for further analysis (Supplementary Figure 1). There were no significant differences in morphology between the control and transgenic plants (Figure 1A).

Improved Drought Tolerance in AtWRKY57 Transgenic Plants
The transgenic lines and control seeds were germinated simultaneously on half-strength MS agar medium containing 2% sucrose with or without hygromycin at 100 μg/ml and then planted in soil after 1 week. Four-week-old plants were treated with natural drought stress (not supplied with water). The control plants showed wilting symptoms 6 days before the transgenic lines. After 14 days treatment, the transgenic plants did not display any drought-stress symptoms, while the wild-type plants exhibited severe drought symptoms (Figure 1B). Up to 20 days of treatment, the control showed obvious drought-stress symptoms (Figure 1C). When plants were re-watered, only 12.3% of control plants were survived and most of them never recovered; however, all of the transgenic rice plants survived (Figures 1D,E). These results suggested that these transgenic rice plants acquired significantly improved drought tolerance. Soil moisture contents and their dynamics showed in Supplementary Figure 2.

Transpiration water loss is an important factor related to drought tolerance. Flag leaves were detached and the changes of fresh weight were determined over a 200-min period to assess the water loss rate of transgenic and control plants. A slower water loss rate was displayed in the transgenic lines’ leaves than the control’ (Figure 1F). The reduced water loss rate is favorable for an increased drought tolerance in the transgenic lines. In response to drought stress, stomata often close to limit water loss by transpiration. Given that water loss rate were lower in two transgenic lines than in control plants, we further investigated whether stomata density and/or stomata aperture affects this progress. White nail polish blotting was used to count the stomata density and measure stomata aperture. The ratio of stomatal width to length indicated the degree of stomatal closure. The results showed that the stomata density didn’t displayed significant difference between control and two transgenic plants leaf adaxial surface (Supplementary Figures 3A,B). However, two transgenic lines’ stomata showed more quick closure than control’ under dehydration treatments (Supplementary Figure 3C). These results suggest that more quick closure of stomata in two transgenic lines result in the lower water loss rate, which may be critical for transgenic plants to adapt to drought stress.

The accumulation of proline in plant is associated with adaptation to environmental stress through metabolic adjustments (Ábrahám et al., 2003). We also checked the proline contents of transgenic and control plants under normal growth and drought-stress conditions to characterize the physiological basis for the improved stress tolerance. No differences in the proline contents were observed in the leaves of transgenic and control plants under normal conditions (Figure 1G). However, under drought conditions, transgenic plants began to accumulate proline after 14 days and further accumulated an up to fourfold higher proline content compared with the levels prior to drought stress, whereas control plants showed a low increase in proline. This result demonstrates that the proline accumulation corresponded to the increased drought tolerance of transgenic plants.

Decreasing the ROS Damage in AtWRKY57 Transgenic Plants
Leaves of control plants began to produce brownish lesions after 14 days of drought stress (Figure 2A). In contrast, none of the transgenic plants exhibited lesion formation grown under the same conditions. Lesion formation was accompanied by significant trypan blue staining that indicates cell death in the control leaves (Figure 2B).

Stress usually causes damage via oxidative damage in plants including the generation of ROS, represented as H2O2 and superoxide (Zhu, 2001; Mittler, 2002; Xiong and Zhu, 2002). As activation of AtWRKY57 enhanced the drought tolerance of transgenic rice plants, we further determined whether AtWRKY57 is involved in drought tolerance via ROS detoxification. Transgenic rice and control seedlings were subjected to DAB staining and NBT staining to detect H2O2 and superoxides in their leaves. After 14 days of drought stress, the transgenic plants had very few brown H2O2 and superoxide
FIGURE 1 | Improved drought tolerance in AtWRKY57 transgenic rice. (A) Before drought treatment. (B) Drought for 14 days. (C) Drought for 20 days. (D) Recovery for 7 days after 20 days drought treatment. Drought stress was imposed on 4-week-old T3 transgenic seedlings in greenhouse. Drought experiments were repeated three times and at least 40 plants for each individual lines were used in each repeated experiment and one representative picture was shown. (E) Survival rate after 20 days drought stress. Values are mean ± SE (n = 40 plants, *P < 0.05). (F) Rate of water loss by detached leaves from control and transgenic plants. Values are the mean ± SE (n = 6 plants, **P < 0.01). (G) Proline content in the leaves of 4-week-old transgenic and control plants with or without drought treatments. Values are the mean ± SE of three independent experiments (*P < 0.05). FW, Fresh weight.

Overall, these results indicated that the over-expressing AtWRKY57 gene in rice increased the tolerance to drought stress by decreasing ROS damage.

The Enhanced ROS-Scavenging Ability and High-Level Expression of Oxidative Enzyme Genes in AtWRKY57 Transgenic Plants

A decreased cell viability and even cell death was resulted from the over-accumulation of ROS; therefore, scavenging ROS avoids or alleviates the harmful effects on plant under stress conditions. In the ROS-scavenging mechanisms of plants, POD, SOD, and CAT are key enzymes (Mittler, 2002; Xiong and Zhu, 2002; Apel and Hirt, 2004), and are involved in the H2O2 elimination. Following drought stress, the enzyme activities of seedlings were subjected to measurement. Under normal growth conditions, POD, SOD, and CAT activity levels were not different; however, after 14 days of drought stress, the activities of the antioxidative enzymes were all significantly enhanced in the AtWRKY57-overexpressing plants compared with those in the control plants (Figures 3A–C). These results suggested that over-expression of

spots within the leaf segments, whereas more than half of the leaf area of the control plants became brown (Figures 2C,D). The leaf segments of control plants displayed more brown areas than compared transgenic plants. These results confirmed that over-expressing AtWRKY57 in rice could efficiently remove the H2O2 and superoxide produced during drought stress. Malondialdehyde, acting as a biomarker for lipid peroxidation, is an effect of oxidative damage deriving from decomposition product of polyunsaturated fatty acid hydroperoxides. The MDA contents in transgenic lines and controls were similar under normal growth conditions, but there was a significant difference after drought stress. Then, the MDA contents of two AtWRKY57 over-expressing lines were significantly lower than those of control plants (Figure 2E).

Electrolyte leakage, an indicator of membrane damage, was also measured following drought stress. The results showed that the leaves of two AtWRKY57 over-expressing lines exhibited significantly lower electrolyte leakage levels, compared to those of control leaves (Figure 2F). After 14 days of drought stress, more than 60% of the ions leaked from cells in control plants, whereas the ion leakage of AtWRKY57 over-expressing lines was less than 50%.
AtWRKY57 gene may enhance the ROS-scavenging ability, which decreases ROS damage.

To test whether drought stress modifies transcript levels, the expression levels of several antioxidant genes were measured. Consistent with the increase of antioxidative enzymes activities, control and two transgenic plants up-regulated the transcript levels of *OsCAT B*, *OsCu/Zn-SOD1*, *OsCu/Zn-SOD2* and *OsPOD* in response to drought stress, with a greater increase in the transgenic plants (Figures 3D–G). This was enhancing the capacity to decompose H$_2$O$_2$ and superoxide in the leaves.

**High-Level Expression of Stress-Response Genes in AtWRKY57 Transgenic Plants**

To better understand the mechanisms of drought tolerance conferred by over-expressing *AtWRKY57*, the expressions of several stress-related genes were investigated. As shown in Figure 4A, the expression levels of a pyrroline-5-carboxylate synthesis gene (*OsP5CS*; Igarashi et al., 1997) were strongly induced in transgenic lines under drought stress compared with those in control plants. This higher expression level of *OsP5CS* was consistent with the higher proline content in two *AtWRKY57* overexpressing lines (Figure 1G). However, the expression level of an ABA synthesis gene (*OsNCED5*) was not significantly different in transgenic lines and control plants after drought stress (Figure 4B). Dehydration-responsive element-binding (DREB) transcription factors play key important roles in plant-stress responses. DREB proteins encoding by *OsDREB1A* and *OsDREB2A* were strongly up-regulated in drought stress, with a greater increase in the transgenic plants compared to the control plants (Figures 4C,D). We also checked another two well-characterized drought resistance-related genes (*OsRab21* and *OsRab16D*), and found that they were significantly affected by water stress. Their expression levels were obviously higher in transgenic plants than in control after drought treatment (Figures 4E,F).

These results indicated that over-expressing *AtWRKY57* gene in rice may enhance the expression of some stress-response genes and finally increase the tolerance to drought stress.

**Improved NaCl and PEG Tolerance in AtWRKY57 Transgenic Plants**

Our results revealed that the constitutive expression of *AtWRKY57* enhanced the drought tolerance in rice (Figure 1). Given the function of WRKY-type regulators in abiotic stress, we further explored the functions of *AtWRKY57* in NaCl and PEG stress conditions. We tested the survival rates of transgenic and control plants on MS medium additionally added with 175 mM NaCl. The control plants displayed more severe phenotype, including leaf curves and dehydration, than the transgenic lines after 2 days of NaCl treatment (Figures 5A,B). When plants were recovered in fresh MS medium, none of the control plants survived but most of the transgenic lines reversed (Figure 5C;
Supplementary Figure 4). We also tested the survival rate of transgenic lines and control plants on MS medium supplemented with 25% PEG6000. The control plants showed more severe phenotype than transgenic lines after 4 days PEG treatments (Figures 5D,E). When plants were recovered in fresh MS medium, none of the control plants survived, but all of the transgenic lines lived (Figures 5F,G; Supplementary Figure 5).

These results showed that consecutively expressing AtWRKY57 enhanced not only drought tolerance but also the NaCl and PEG stress tolerance in rice.

**DISCUSSION**

Combination of abiotic and biotic stresses used to limit the production of crop. Drought severely restricts crop production as the most important abiotic stress (Boyer, 1982; Rockstrom and Falkenmark, 2000). In a previous study, we confirmed that over-expressing AtWRKY57 significantly conferred drought tolerance in Arabidopsis (Jiang et al., 2012). These results suggested that AtWRKY57 may improve crops’ drought adaptability using gene manipulation. In this study, we evaluated the role of AtWRKY57 in transgenic rice after drought stress.

The drought-tolerance phenotype of AtWRKY57 transgenic rice plants were the result of a collection of physiological indexes observed in the over-expressing plants. AtWRKY57 overexpressing plants displayed higher survival rates most likely because the water loss was reduced in these plants compared to control plants under drought conditions (Figure 1F). P5CS, catalyzing proline biosynthesis, is critical for the increasing of osmotolerance. Drought, salt, and abscisic acid induce the expression of OsP5CS and the conferred osmotolerance is resulting from an up-regulated expression of OsP5CS which increases proline content in transgenic plants (Xiang et al., 2007). The significantly higher transcript levels of OsP5CS were consistent with the high proline content in transgenic plants after drought stress (Figures 1G and 4A). These results confirmed that the transgenic plants’ adaptation to drought stress was associated with mechanisms of dehydration avoidance through proline metabolic adjustments. Programmed

**FIGURE 3** | Enhanced the ROS-scavenging ability and the expression of oxidative enzymes genes in transgenic rice. (A–C) POX, SOD, and CAT activities in the leaves of 4-week-old transgenic and control plants before and after drought stress. Values are the mean ± SE of three independent experiments (*P < 0.05). FW, Fresh weight. Relative expression of oxidative enzymes genes Cu/Zn-SOD1 (D), Cu/Zn-SOD2 (E), POX8.1 (F), and CatB (G) in the leaves of 4-week-old transgenic and control plants before and after drought stress. Values are the mean ± SE of three independent experiments (*P < 0.05).
cell death (PCD) and lesion formation in some lesion mimic mutants, such as lsd1, was mainly caused from the elevated levels of extracellular superoxide (Jabs et al., 1996). We observed high levels of superoxide, H$_2$O$_2$ and cell death in control plants than in transgenic lines. We believe that the lesion formation in control plants results from their reduced capability to detoxify ROS compared with transgenic plants (Figure 2A). These results are similar to those of Yang et al. (2004), who reported that salicylic acid-deficient transgenic rice contains elevated levels of superoxide and H$_2$O$_2$ and exhibits spontaneous lesion formation in an age- and light-dependent manner.

Drought or salt-stress conditions promoted the accumulation of ROS in plants. MDA is often considered as a reflection of cellular membrane degradation or dysfunction and is also an important intermediary agent in ROS scavenging. Thus, high level of MDA causes PCD and induces toxicity to plant cells (Apel and Hirt, 2004; Hou et al., 2009). High ability of ROS-scavenging enzymes decreased over-accumulated ROS levels which induces PCD in plants (Mittler, 2002; Apel and Hirt, 2004; Chaves and Oliveira, 2004; Farooq et al., 2009; Hou et al., 2009). In AtWRKY57 transgenic plants, lower levels of PCD, DAB and NBT staining, MDA content and relative electrolyte leakage were detected (Figures 2B–F), but increased SOD, POD, and CAT activity levels (Figures 3A–C) and elevated oxidative enzyme genes’ transcript levels (Figures 3D–G) were detected after drought stress, demonstrating that they were better protected from oxidative damages through the enhanced capability to scavenge ROS.

The transcript levels for several stress-tolerant genes were more elevated in AtWRKY57 transgenic rice than in control plants under drought-stress conditions (Figure 4). It is interesting that the relative transcript levels of OsNCED5 and the ABA content were not significantly changed (Figure 4B; Supplementary Figure 6) in AtWRKY57 transgenic plants under drought-stress conditions, which may demonstrate that there were different regulatory mechanisms in transgenic Arabidopsis and transgenic rice. Our previous study revealed that the activated expression of AtWRKY57 conferred Arabidopsis transgenic plants drought tolerant by elevating the ABA contents through directly binding the promoter sequence of AtNCED3 (Jiang et al., 2012). In this study, we found that the enhanced capability to scavenge ROS was important for AtWRKY57 overexpressing transgenic rice plants to tolerate drought stress (Figures 2 and 3). Interestingly, there are increasing studies demonstrated that the same gene may have different regulatory functions and/or mechanisms when overexpressed in different plants species, such as in rice, cotton and Arabidopsis. For example, OsWRKY45 overexpressing transgenic rice showed sensitivity to drought stress (Tao et al., 2011); however, heterologous overexpression of OsWRKY45 in Arabidopsis conferred plants drought tolerant mainly resulting from the reduction of transpiration rate (Qiu and Yu, 2008). Overexpression of OsSNAC1 enhanced drought tolerance of transgenic rice plants by targeting genes that control ROS homeostasis and stomatal closure (Yu et al., 2013), whereas overexpressing OsSNAC1 rendered
transgenic cotton plants more drought tolerance by reducing transpiration rate and enhancing root development (Liu et al., 2014). Heterologous expression of the \textit{AtDREB1A} gene in peanut conferred transgenic plants drought and NaCl tolerance by upregulating proline synthesis to better osmotic adjustments (Sarkar et al., 2014), while the \textit{AtDREB1A} transgenic \textit{Arabidopsis} enhanced drought by activating some stress-related genes expression (Liu et al., 1998). Thus, it’s possible that there may be diversified regulatory functions and/or mechanisms for one protein to regulate different physiological processes in different species under stress conditions.

WRKY transcription factors belong to a large family that functions under a variety of abiotic stresses. Our results provided evidences that overexpressing \textit{AtWRKY57} also increased the tolerance to salt and PEG stresses (Figures 1 and 5), demonstrating that this is a potential candidate gene for crop improvement. Recently, several studies confirmed that overexpression of some stress-related genes may enhance drought tolerance in rice (Dubouzet et al., 2003; Park et al., 2005; Chen et al., 2008; Hou et al., 2009; Huang et al., 2009; Cui et al., 2011; Gao et al., 2011; Yang et al., 2012; Zou et al., 2012). However, a persistent problem is that the constitutive over-expression of stress-related genes often result in abnormal development and thus reduces crop productivity (Kasuga et al., 1999; Hsieh et al., 2002; Dubouzet et al., 2003; Nakashima et al., 2007; Priyanka et al., 2010). The improvement in drought tolerance should be perfectible without limitation in plant growth and production (Cattivelli et al., 2008). Yu’s study confirmed that the heterologous expression of \textit{AtEDT1/HDG11} in rice significantly improved its drought tolerance and also simultaneously increased the grain yield under both normal and drought-stress conditions (Yu et al., 2013). In our study, \textit{AtWRKY57} transgenic plants underwent normal development compared with controls.

\textbf{FIGURE 5} | Improved NaCl and PEG tolerance in transgenic rice. (A) Before NaCl treatment. (B) NaCl treatment for 2 days. (C) Recovery for 7 days after 2 days NaCl treatment. (D) Before PEG treatment. (E) PEG treatment for 4 days. (F,G) Recovery for 7 days after 4 days PEG treatment. NaCl and PEG stress was imposed on 4-week-old T3 transgenic seedlings under water culture conditions in greenhouse. Experiments were repeated three times and at least 30 plants for each individual lines were used in each repeated experiment and one representative picture was shown.
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SUPPLEMENTARY MATERIAL

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AUTHOR CONTRIBUTIONS

YJ designed and performed experiments, interpreted data, and wrote the article. DY designed experiments, and edited the article. YQ and YH interpreted data and edited the article. Both authors read and approved the final article.
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