Enhanced Drought Tolerance of Tobacco Overexpressing *OjERF* Gene Is Associated with Alteration in Proline and Antioxidant Metabolism

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ABSTRACT. Drought stress is one of the major limiting factors for plant growth and development. The mechanism of drought tolerance has not been well understood. This study was designed to investigate proline and antioxidant metabolism associated with drought tolerance in transgenic tobacco (*Nicotiana tabacum*) plants overexpressing the *OjERF* gene relative to wild-type (WT) plants. The *OjERF* gene was isolated from mondo grass (*Ophiopogon japonicus*). The *OjERF* gene, driven by the CaMV35S promoter, was introduced into tobacco through agrobacterium (*Agrobacterium tumefaciens*)-mediated transformation. Five transgenic lines were regenerated, of which transgenic Line 5 (GT5) and Line 6 (GT6) were used to examine drought tolerance in comparison with WT plants in a growth chamber. Drought stress caused an increase in leaf malondialdehyde (MDA) and electrolyte leakage (EL), proline content, superoxide dismutase (SOD), and catalase (CAT) activity in both transgenic lines and WT plants. However, the transgenic lines had lower MDA content and EL and higher proline content, SOD and CAT activity relative to WT under drought stress. The activities of SOD and CAT were also greater in the transgenic lines relative to WT plants under well-watered conditions (Day 0). The *OjERF* activated the expression of stress-relative genes, including *NtERD10B*, *NtERD10C*, *NtERF5*, *NtSOD*, and *NtCAT1* in tobacco plants. The results of this study suggest that the *OjERF* gene may confer drought stress tolerance through upregulating proline and antioxidant metabolism.

Shortage of water limits plant growth and crop productivity in arid regions more than any other single environmental factors (Boyer, 1982). Plants undergo significant morphological and metabolic changes in response to drought (McCann and Huang, 2008; Zhang and Kirkham, 1996). Recent studies using genomics approaches have revealed that some transcription factors such as ethylene response factors (ERF) play vital regulatory roles in plant response to abiotic stresses (Agarwal et al., 2006; Jiang et al., 2009; Wang et al., 2010). Overexpression of ERF genes has been found to confer drought tolerance in tobacco plants (Trujillo et al., 2008).

Plants possess various mechanisms to cope with drought stress. Plants undergo osmotic adjustment in response to drought (Takahara and Akashi, 2006). Proline, one of the important compatible solutes, plays an important role in adjusting cell osmotic potential, maintaining plasma membrane integrity, and acting as a signaling molecule in response to drought stresses (Hare and Cress, 1997; Kishor et al., 2005). Man et al. (2010) noted that leaf proline content increased in response to drought in tall fescue (*Festuca arundinacea*) with a greater level of proline content in drought-tolerant cultivars relative to drought-sensitive cultivars. Goel et al. (2010) reported that transgenic tomato (*Solanum lycopersicum*) plants overexpressing the osmotin gene had higher proline content and leaf relative water content (RWC) under drought stress conditions. Proline also functions as free radical detoxification (Smirnoff and Cumbes, 1989).

Drought stress causes damage to plant cells through excess accumulation of reactive oxygen species [ROS (Zhang and Schmidt, 1999)]. Stomatal closure in response to drought results in declines in intracellular CO₂ level and rate of photosynthesis. Decreasing intracellular CO₂ levels result in the overreduction of components within the photosynthetic electron transport chain and electrons get transferred to O₂. This process generates ROS including singlet oxygen (¹O₂), superoxide radical (O₂^{•−}), hydrogen peroxide (H₂O₂), and hydroxyl radicals (OH[•]). These ROS may react with macromolecules and cause damage to proteins, lipids, and nucleic acids (McCann and Huang, 2008; Smirnoff, 2005).

Plants develop antioxidant systems to cope with drought stress (Zhang and Kirkham, 1994, 1996). Antioxidant enzymes and metabolites can scavenge ROS and protect cells under stress. Superoxide dismutases, which catalyze the dismutation of O₂^{•−} to H₂O₂, have been considered as the first line of defense against oxidative stress. Catalase can convert H₂O₂ into O₂ and H₂O. These antioxidant enzymes including those in the ascorbate–glutathione cycle [such as ascorbate peroxidase (APX) and glutathione peroxidase] and metabolites are important roles in plant tolerance to drought stress (Smirnoff, 2005; Zhang and Kirkham, 1996).

Plants can increase tolerance to abiotic stress by altering stress-related gene expression (Jiang, et al., 2009). Wu et al. (2008) reported that the tobacco plants overexpressing the JERF3 gene improved tolerance to drought, salt, and freezing stress relative to wild-type plants. The transgenic tobacco plants had less accumulation of ROS under stress. It has been reported that the *NtLEA5* and *NtERD10C* genes encode hydrophilic late embryogenesis abundant (LEA) proteins that

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are identified to play critical roles in combating cellular dehydration (Hundertmark and Hincha, 2008). Huang et al. (2010) reported that overexpression of the *PtrABF* gene, a bZIP transcription factor isolated from *Poncirus trifoliata*, enhanced drought tolerance in tobacco. The results showed that the transgenic lines had higher activities of the three detoxifying enzymes (SOD, peroxidase, and CAT) and lower ROS content relative to WT plants.

Mondo grass is an evergreen perennial herb with a strong root system. It possesses good drought and heat tolerance and keeps green color even in winter (Zhang, 2003). It is widely distributed and cultivated in many areas of China (Qia et al., 2002). The *OjERF* gene, isolated from mondo grass, has a complete open reading frame of 1047 bp, encoding a 348 amino acid peptide with a predicted molecular mass of 39.08 kDa. Sequence analysis revealed that *OjERF* contains a conserved DNA-binding domain (AP2/ERF domain) of 58 amino acids, conserved Ala (A) and Asp (D) residues at the 14th and 19th, which shows that *OjERF* may be a member of the ERF subfamily.

The tobacco plant is an important cash crop in China and other regions and has also been widely used for genetic transformation. The objectives of this study were to introduce *OjERF* into tobacco plants and investigate drought tolerance of the transgenic tobacco overexpression of the *OjERF* gene in comparison with WT plants and examine if drought tolerance is associated with alteration in proline content and antioxidant enzyme activity.

Material and Methods

PLANT TRANSFORMATION VECTOR CONSTRUCTION AND GENE TRANSFORMATION. The ORF of *OjERF* cDNA, isolated from mondo grass using reverse transcription–polymerase chain reaction (RT-PCR), was introduced into PBI121 under the control of CaMV35S promoter with the restriction sites [XbaI and SmaI (Fig. 1A)]. This construct was introduced into tobacco plants through agrobacterium-mediated transformation (Hiei et al., 1994) during 22 Dec. 2009 through 26 Feb. 2010. When the kanamycin (Kan)-resistant shoots grew to ≈ 1.5 cm in length, they were transferred to the rooting medium [MS + 75 mg·L⁻¹ Kan + 200 mg·L⁻¹ carbenicillin (Carb)] for root growth. The transgenic plants were regenerated and grown for 30 d in

the growth medium (MS + 75 mg·L⁻¹ Kan + 200 mg·L⁻¹ Carb) from 27 Feb. through 29 Mar. 2010. The positive transformed plants were then identified by RT-PCR. The forward primer 5'-AGGAGCGATCATCTCCGACTTCATAC-3' and reverse primer 5'-CGATTTCATTGGCGTCATTACATTC-3' were used to amplify the *OjERF* gene using PCR. A total of five *OjERF* overexpressing transgenic lines (GT5, GT6, GT8, GT11, and GT15) were generated (Fig. 1B). Since no difference was found between the five transgenic lines in growth, thus the two lines (GT5 and GT6) were expected to represent the transgenic lines and used for drought stress study.

PLANT CULTURE AND DROUGHT STRESS TREATMENT. This study was conducted in a growth chamber of the Turfgrass Research Institute at Beijing Forestry University, Beijing, China. On 3 Apr. 2010, the WT and *OjERF*-overexpressing transgenic tobacco plants were transplanted into pots (25 cm diameter, 20 cm deep) containing soil:peat:vermiculite (3:1:1) and grown in the growth chamber with temperatures at 25/23 °C (day/night), photosynthetic active radiation at 400 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, 16-h photoperiod, and 60% to 65% relative humidity. The plants were irrigated by hand daily. On 14 June, the plants were subjected to drought stress by withholding irrigation. The soil water content was monitored using time domain reflectometry system (Soilmoisture Equipment, Santa Barbara, CA). The soil moisture content was 31.5% at 0 d, 21.0% at 5 d, 15.5% at 10 d, 10.5% at 15 d, and reached $\approx 6\%$ at 20 d (3 July 2010). Leaf tissues were sampled at 0, 5, 10, 15, and 20 d of drought stress and used for analysis of RWC and EL. Additional leaf samples were collected, frozen with liquid nitrogen, and stored at -80 °C for proline content and antioxidant activity determination.

LEAF RELATIVE WATER CONTENT MEASUREMENT. Leaf RWC was determined according to Barrs and Weatherley (1962) with some modifications. Briefly, fresh leaves (100 mg) were weighed [fresh weight (FW)] and then saturated in distilled water for 4 h and their turgid weights (TW) were determined. The samples were then dried in an oven at 80 °C for 24 h and weighed [dry weight (DW)]. The RWC was determined as follows: $\text{RWC} = (\text{FW} - \text{DW})/(\text{TW} - \text{DW}) \times 100$.

LEAF MALONDIALDEHYDE AND ELECTROLYTE LEAKAGE MEASUREMENT. Leaf tissues (50 mg) of WT and transgenic tobacco plants were homogenized in 1 mL of 80% ethanol and centrifuged at 16,000 g_n for 20 min. The supernatant was transferred to a new tube, and 1.6 mL of reaction solution containing 20% w/v trichloroacetic acid (TCA) and 0.5% w/v thiobarbituric acid was added. The mixture was heated in a water bath at 95 °C for 30 min, cooled immediately, and centrifuged at 10,000 g_n for 10 min. The absorbance of supernatant was determined at 450, 532, and 600 nm. The formula for the calculation of MDA content was: $\text{MDA content (micromoles per gram FW)} = [6.45(A_{532} - A_{600}) - 0.56A_{450}] \times V/W$, where V is the volume of cuvette and W is the weight of leaf tissues in each sample.

The EL was measured according to the method of Zhang and Ervin (2009) with some modifications. Fresh leaf discs (100 mg) were washed and then placed in Falcon tubes with 20 mL distilled water. The tubes were shaken for 24 h and an initial electrical conductance (C1) was determined using a conductivity meter (Model DDSJ_308A; Shanghai Precision and Scientific, Shanghai, China). The tubes were boiled for 20 min, cooled down to 25 °C, and the final electrical conductivity (C2) was measured. The EL was calculated according to the formula: $\text{EL (\%)} = (\text{C1}/\text{C2}) \times 100$.

Fig. 1. Construction of vector (A) and reverse transcription–polymerase chain reaction (RT-PCR) identification (B) of *OjERF* transgenic tobacco plants. (A) The construction map of 35S:*OjERF* in a binary vector. (B) Analysis of *OjERF* expression in wild-type (WT) and transgenic tobacco lines (GT5, GT6, GT8, GT11, and GT15) by RT-PCR amplifications. NtActin was used to equalize cDNA concentrations of different plant samples.

ASSAY OF LEAF PROLINE CONTENT. Frozen leaf tissues (100 mg) were crushed with liquid nitrogen and extracted with a pestle in an ice-cold mortar with 4 mL of 3% 5-sulfosalicylic acid solution. The homogenate was filtered with a filter paper (#2), and the filtrate was used for the analysis. The proline content was determined spectrophotometrically at 520 nm according to Bates et al. (1973).

ASSAY OF ANTIOXIDANT ENZYME ACTIVITY. Frozen leaf samples (0.25 g) were crushed in liquid N₂ and extracted with a pestle in an ice-cold mortar with 4 mL of 50 mM sodium phosphate buffer [pH 7.0 (Sambrook et al., 1989)]. The extract was centrifuged at 15,000 *g*_n for 20 min. The supernatant was collected for analysis of SOD and CAT activity.

The SOD activity was determined using the procedure of Chowdhury and Choudhuri (1985) and Zhang and Kirkham (1994) with slight modifications. The 3 mL reaction mixture contained 6.3 mM nitrobluetetrazolium chloride (NBT), 0.13 mM riboflavin, 13 mM methionine, 10 mM ethylenediaminetetraacetic, 50 mM phosphate buffer (pH 7.8), and 60 µL of enzyme extract. Riboflavin was added last. The reaction solution was irradiated at 70 µmol·m⁻²·s⁻¹ photons for 10 min. A non-irradiated reaction mixture that did not develop color served as the control and its absorbance was subtracted from the absorbance at 560 nm. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the rate of NBT reduction.

The CAT activity was determined by monitoring the decomposition of H₂O₂ according to the method of Beers and Sizer (1952). The 3 mL reaction mixture contained 50 mM phosphate buffer (pH 7.0), 15 mM H₂O₂, and 0.1 mL enzyme extract. The reaction started once the enzyme extract was added. The decrease in absorbance at 240 nm was recorded in 1 min. One unit of CAT activity was defined as a decrease of absorbance by 0.1 per minute.

GENE EXPRESSION ANALYSIS. RT-PCR was used to monitor transcriptional levels of seven stress-responsive genes in WT and transgenic plants. Total RNAs for RT-PCR were isolated from leaf tissues in 5-week-old plants using the TRIzol reagent according to the manufacturer's instructions (TRIzol Reagent; Tiangen Biotech, Beijing, China). The first-strand cDNA was synthesized from 1.5 µg of total RNA using M-MLV reverse transcriptase (Promega, Madison, WI) as described by the manufacturer's instructions. There were three replicates for RT-PCR. The tobacco actin gene was used to equalize the concentrations of the cDNA samples. Synthesized cDNA was used to carry out PCR amplifications using gene-specific primers (Table 1).

EXPERIMENTAL DESIGN AND STATISTICAL ANALYSIS. A randomized complete block design was used with three replications. The data from the three treatments (WT, G5, and G6) at each

sampling date were subjected to an analysis of variance (ANOVA) using SPSS (SPSS Version 17.0 for Windows; IBM Corp., Armonk, NY). In addition, the data for each treatment at different sampling dates were analyzed using ANOVA. Means separation was performed using Duncan's multiple-range test at 5% probability level.

Results

GENERATION OF TRANSGENIC TOBACCO PLANTS. Sequencing and bioinformatics analysis showed the *OjERF* gene, isolated from mondo grass, has a complete open reading frame of 1047-bp, encoding a 348 amino acid peptide with predicted molecular mass of 39.08 kDa and pI of 5.11. Sequence analysis revealed that *OjERF* contains a conserved DNA-binding domain (AP2/ERF domain) of 58 amino acids, conserved Ala (A) and Asp (D) residues at the 14th and 19th, which shows that *OjERF* may be a member of the ERF subfamily. This gene has been submitted to the National Center for Biotechnology Information with the accession number of JN055434.

Five transgenic lines (GT5, GT6, GT6, GT8, GT11, and GT15) were obtained. After analyzing the expression of the *OjERF* gene in five independent transgenic lines, two lines (GT5 and GT6) were selected for drought stress experiment. The transgenic tobacco plants showed a 764-bp amplification fragment when amplified with *OjERF* gene-specific primers; whereas no amplification was observed in the WT plants (Fig. 1B).

LEAF RELATIVE WATER CONTENT. No difference in RWC was found between the transgenic lines and WT under well-watered conditions (0 d). The RWC declined gradually during drought stress treatment (Fig. 2). The GT5 and GT6 had consistently higher RWC than the WT plants as measured at Day 5 through Day 20 of drought stress. At 20 d, leaf wilting was observed in WT plants, but not in GT5 and GT6 plants (Fig. 3). The LWC was reduced by 50.5% in WT plants, 21.3% in GT5, and 21.5% in GT6 as measured at 20 d relative to 0 d (Fig. 2).

LEAF MALONDIALDEHYDE CONTENT AND ELECTROLYTE LEAKAGE. There was no difference in MDA between the transgenic line and WT under well-watered conditions (0 d). The MDA content was increased in the WT plants and the transgenic lines during drought stress (Fig. 4). The GT5 and GT6 plants had lower MDA content relative to the WT plants as measured at Day 10 through Day 20 of drought stress. The MDA content in WT plants was 2.4- and 2.8-fold greater than that in GT5 and GT6 lines, respectively, when measured at 20 d.

No difference in EL was found between WT plants and the transgenic lines. The EL gradually increased as drought stress progressed (Fig. 5). The transgenic lines had less EL relative to WT plants as measured at Day 5 through Day 20. The EL level

Table 1. Gene-specific primers of the corresponding genes for reverse transcription–polymerase chain reaction amplifications in tobacco plants.

Gene name	Accession no.	Forward primer	Reverse primer
NtERD10A	AB049335	5' TCTGCTGCGAAACAATA 3'	5' TCCACGGCACATCACTA 3'
NtERD10B	AB049336	5' GGCAATCCCATTCGTCA 3'	5' TCTCCTTCATCCCTTCTT 3'
NtERD10C	AB049337	5' CGTGGTTTGTGTTGATTT 3'	5' CCTCTGTTTCCTCGTATT 3'
NtSOD	AB093097	5' AGCAACTCCACGGCTTCC 3'	5' CAATTTGGCGACGGTAGG 3'
NtCAT1	U93244	5' GGAGGAGCAAATCACAGC 3'	5' TGGAGCATTAGCAGGAAG 3'
NtAPX2	D85912	5' GAGCCCATCAAGGAGCAG 3'	5' AAGCAGGATCAGAAAGGAGA 3'
NtERF5	AY655738	5' AAGCCCACTAGAAATAG 3'	5' GTATCAAATGTCCCTAAC 3'
NtActin	AB158612	5' ATAGTAAGCAACTGGGACG 3'	5' CTCTGAGCCAATGGTAAT 3'

Fig. 2. Leaf relative water content (RWC) responses of tobacco wild-type (WT) and transgenic lines (GT5 and GT6) to drought stress treatment. The error bars indicate $\pm$ SD. The bars marked with different letters for a given treatment indicate the difference between the sampling dates for the given treatment was significant at $P \leq 0.05$. The bars marked with an asterisk (*) represent that the difference between the transgenic line and control was significant ($P \leq 0.05$) at the given sampling date (n = 3).

Fig. 3. Tobacco wild-type (WT) and transgenic lines overexpression of *OjERF* gene (GT5 and GT6) grown under well-watered conditions [A (0 d)] and subjected to drought stress for 20 d [B (20 d)].

was 70.4% in the WT plants, 42.5% in GT5, and 34.2% in GT6 when measured at 20 d.

LEAF PROLINE CONTENT. There was no difference in leaf proline content between WT plants and the transgenic lines at 0 d (well-watered conditions). The proline content in GT5 and GT6 increased more quickly than WT plants in response to drought stress (Fig. 6). Higher proline content was found in the transgenic lines relative to WT plants as measured at Day 5 through Day 20 of drought stress. The proline content was 1.6-fold higher in GT5 and 1.7-fold higher in GT6 relative to WT plants at 20 d of stress.

Fig. 4. Leaf malondialdehyde (MDA) content response of tobacco wild-type (WT) and transgenic lines overexpressing the *OjERF* gene (GT5 and GT6) to drought stress treatment. The error bars indicate $\pm$ SD. The bars marked with different letters for a given treatment indicate the difference between the sampling dates for the given treatment was significant at $P \leq 0.05$. The bars marked with an asterisk (*) represent that the difference between the transgenic line and control was significant ($P \leq 0.05$) at the given sampling date (n = 3).

Fig. 5. Leaf electrolyte leakage responses of tobacco wild-type (WT) and transgenic lines (GT5 and GT6) to drought stress treatment. The error bars indicate $\pm$ SD. The bars marked with different letters for a given treatment indicate the difference between the sampling dates for the given treatment was significant at $P \leq 0.05$. The bars marked with an asterisk (*) represent that the difference between the transgenic line and control was significant ($P \leq 0.05$) at the given sampling date (n = 3).

ANTIOXIDANT ENZYME ACTIVITY. The SOD activity was higher in the transgenic plants relative to WT plants under non-stress conditions (0 d). The SOD activity in WT plants increased from 0 d through 15 d and declined thereafter, whereas SOD activity in transgenic lines increased from Day 0 through Day 20. The SOD activity was higher in GT5 and GT6 plants relative to WT plants at all sampling dates. At 15 d, SOD activity in GT5 and GT6 was 1.2-fold and 1.4-fold higher, respectively, when compared with that in the WT plants. The SOD activity was increased by 33.1% in GT5 and 33.7% in GT6, whereas it declined by 36.8% in WT plants as measured at 20 d relative to 15 d of drought stress (Fig. 7).

The CAT activity was higher in the transgenic lines relative to WT plants under well-watered conditions (0 d). The CAT activity in WT plants increased from Day 0 through Day 15 and declined thereafter, whereas CAT activity in transgenic lines increased from Day 0 through Day 20. The CAT activity was higher in GT5 and GT6 plants relative to WT plants at all

Fig. 6. Leaf proline content responses of tobacco wild-type (WT) and transgenic lines (GT5 and GT6) to drought stress treatment. The error bars indicate $\pm$ sd. The bars marked with different letters for a given treatment indicate the difference between the sampling dates for the given treatment was significant at $P \leq 0.05$. The bars marked with an asterisk (*) represent that the difference between the transgenic line and control was significant ($P \leq 0.05$) at the given sampling date ($n = 3$).

sampling dates. The CAT activity in GT5 and GT6 was 2.9- and 3.0-fold greater than that in WT plants, respectively, as measured at 20 d (Fig. 8).

ACTIVATION OF STRESS-RELATED GENES BY OVEREXPRESSION OF *OjERF*. The expression of the seven genes (*NtERD10A*, *NtERD10B*, *NtERD10C*, *NtERF5*, *NtSOD*, *NtCAT1*, and *NtAPX2*) were enhanced in the transgenic GT6 when compared with those in WT plants. However, *NtERD10A* and *NtAPX2* expression level in the transgenic line GT5 were similar to those in the WT plants (Fig. 9).

Discussion

The results of this study indicated that the transgenic tobacco plants overexpressing the *OjERF* gene had lower MDA content and EL relative to WT plants under drought stress conditions. This is supported by previous studies (Jung et al., 2007; Tang et al., 2005; Trujillo et al., 2008) who reported that overexpression of ERF genes can effectively enhance stress tolerance in several plant species. In our study, the transgenic lines (GT5 and GT6) had higher RWC and less lipid peroxidation (less MDA) and greater cell membrane integrity (less EL) relative to WT plants. The results of this study suggest that overexpression of the *OjERF* gene could be an effective approach to improve drought tolerance in tobacco plants.

The results of the study also showed that the transgenic lines had higher leaf proline content relative to WT plants under drought stress. This is consistent with a previous study by Hsieh et al. (2002) who found that proline content was higher in the plants overexpressing ERF genes relative to WT tomato plants under drought stress. It has been documented that leaf proline content is correlated with plant tolerance to abiotic stresses (Karaba et al., 2007; Quan et al., 2010). The results of our study suggest that improvement of drought tolerance in the transgenic lines may be associated with upregulation of proline metabolism.

The results of this study indicated that the transgenic tobacco plants overexpressing the *OjERF* gene had higher antioxidant (SOD and CAT) activity relative to WT plants under non-stress

Fig. 7. Leaf superoxide dismutase (SOD) activity responses of tobacco wild-type (WT) and transgenic lines (GT5 and GT6) to drought stress treatment. The error bars indicate $\pm$ sd. The bars marked with different letters for a given treatment indicate the difference between the sampling dates for the given treatment was significant at $P \leq 0.05$. The bars marked with an asterisk (*) represent that the difference between the transgenic line and control was significant ($P \leq 0.05$) at the given sampling date ($n = 3$).

Fig. 8. Leaf catalase (CAT) activity responses of tobacco wild-type (WT) and transgenic lines (GT5 and GT6) to drought stress treatment. The error bars indicate $\pm$ sd. The bars marked with different letters for a given treatment indicate the difference between the sampling dates for the given treatment was significant at $P \leq 0.05$. The bars marked with an asterisk (*) represent that the difference between the transgenic line and control was significant ($P \leq 0.05$) at the given sampling date ($n = 3$).

(Day 0) and drought stress conditions. Overexpression of the *OjERF* gene may alter antioxidant enzyme (SOD and CAT) metabolism, leading to increased enzyme activity regardless of growing conditions. Under drought stress conditions, the two transgenic lines had higher activities of SOD and CAT relative to WT plants, indicating improvement of drought tolerance is associated with the antioxidant enzyme activity in the transgenic plants. This is supported by the previous study by Wu et al. (2008) who noted that expression of *JERF3* in tobacco plants increased SOD activity and suppressed ROS under cold stress. Cellular antioxidants, including enzymatic and non-enzymatic antioxidants, are important plant defense mechanisms to protect the cells against damage resulting from ROS. Among enzymatic antioxidants, SOD plays a pivotal role in removing ROS by converting O_2^- into H_2O_2 (Apel and Hirt, 2004). Hydrogen peroxide can be eliminated by CAT and APX (Scandalios et al., 1997). The results of our study showed that

Fig. 9. The expression of stress-related genes as influenced by overexpression of the *OjERF* gene in tobacco transgenic lines (GT5 and GT6) in comparison with wild-type (WT) tobacco plants under normal growth conditions. The transcript levels of seven stress-responsive genes were determined by reverse transcription–polymerase chain reaction using specific primers. Tobacco *NtActin* was used as an internal control.

the activities of SOD and CAT continued to increase in GT5 and GT6, whereas they declined in WT plants during the late stage of the drought stress period from Day 15 through Day 20. This is consistent with a previous study by Hertwig et al. (1992) who found that leaf CAT activity declined in response to stresses. Higher antioxidant activity in the transgenic line may scavenge ROS more effectively relative to WT plants under severe drought stress conditions. Deployment of better ROS scavenging systems might be an integral part of defense against drought in the transgenic plants overexpressing *OjERF*.

It has been documented that ERF family members can regulate the expression of numerous stress-related genes (Yamaguchi-Shinozaki and Shinozaki, 2006). *NtERD10A*, *NtERD10B*, and *NtERD10C* encode hydrophilic LEA proteins that play important roles in combating cellular dehydration (Hundertmark and Hinch, 2008). Kasuga et al. (2004) reported that overexpressing *AtDREB1A* in tobacco increased expression of *NtERD10B* under cold and drought stress. *NtERD10C* is induced by drought and cold and can act as functional genes to anticipate in abiotic stress response. Some ERF proteins can regulate the expression of detoxification enzyme genes to enhance abiotic stress (Wu et al., 2008). The *NtSOD*, which encodes SOD, was reported to enhance oxidative stress tolerance in tobacco plants (Slooten et al., 1995). *NtCAT1* and *NtAPX2* encode CAT and chloroplastic APX, respectively. They are considered to use H_2O_2 as an electron acceptor to catalyze a number of oxidative reactions. The results of this study provided the evidence showing that the expression of *NtSOD* and *NtCAT1* was enhanced in the transgenic tobacco plants overexpressing *OjERF* under normal conditions. The results also showed that the *NtERF5* gene was upregulated in the tobacco transgenic plants overexpressing the *OjERF* gene. Wu et al. (2007) reported that ERF protein JERF1 that modulates abscisic acid biosynthesis-related gene enhanced tobacco plant tolerance to salinity and cold stress. The results of this study indicated that overexpression of the *OjERF* gene can activate other stress-related genes, conferring stress tolerance in tobacco plants.

In summary, the transgenic tobacco plant overexpression of the *OjERF* gene had greater tolerance to drought stress relative to WT plants. The enhanced stress tolerance of the transgenic plants may be associated with upregulation of proline content, antioxidant enzyme activity, and activation of stress-related genes. The overexpression of *OjERF* gene could be an effective approach to improve drought tolerance of tobacco plants.

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