Isolation and characterization of *MbWRKY2* gene involved in enhanced drought tolerance in transgenic tobacco

Deguo Han, Zhaoyuan Zhang, Haibin Ding, Lijing Chai, Wei Liu, Hongxia Li and Guohui Yang

Key Laboratory of Biology and Genetic Improvement of Horticultural Crops of Northeast Region, Ministry of Agriculture, College of Horticulture & Landscape Architecture, Northeast Agricultural University, Harbin, People’s Republic of China

**ABSTRACT**

Plant-specific WRKY transcription factors are widely involved in abiotic stress responses. In this study, a WRKY gene was isolated from *Malus baccata* (L.) Borkh and designated it as *MbWRKY2*. Subcellular localization showed that *MbWRKY2* was localized in the nucleus. The expression levels of *MbWRKY2* were up-regulated by abiotic stresses in *M. baccata*. When *MbWRKY2* was introduced into tobacco, it improved drought stress tolerance in transgenic plants. The transgenic tobaccos had the higher contents of chlorophyll, proline, relative water content, AsA, and GSH, increased activities of CAT, APX, SOD, and POD, and decreased levels of MDA, H$_2$O$_2$, and electrolyte leakage than wild-type, especially when dealt with dehydration treatment. Moreover, the *MbWRKY2-OE* plants enhanced the expression of oxidative stress response and stress-related genes involved in osmotic adjustment and membrane protection. These results suggest that *MbWRKY2* gene plays a positive regulatory role in drought stress response.

**Introduction**

As sessile organisms, plants are frequently exposed to variable environmental stresses, such as drought, salt, heat, chilling, pathogen attack, and nutrient deprivation, which adversely affect plant growth, development, and productivity (Gong & Liu 2013). Environmental stresses are perceived and transduced through a chain of signaling molecules that ultimately affect regulatory element of stress-inducible genes to initiate the synthesis of different classes of protein including transcription factors (TFs), enzymes, molecular chaperons, ion channels, and transporters or alter their activities (Mukhopadhyay et al. 2004). To avoid such deficiencies, plants have developed adaptable mechanisms to perceive external signaling networks and to manifest adaptive responses with appropriate physiological, cellular, and molecular changes (Liu et al. 2014). In these stresses, drought is the most severe threat to crop yield worldwide. Plants have evolved complex strategies to reduce potential damage of drought stress (Wang et al. 2009).

Among the numerous stress-induced genes, many TFs have been identified and studied, such as WRKY, NAC, and bZIP, which can interact with cis-elements present in the promoter regions of various abiotic stress-related genes and thus regulate the expression of many genes resulting in imparting tolerance to abiotic stresses. Among TFs, the WRKY genes received much attention in past decade. The WRKY proteins, which contain one or two conserved WRKY domains consisting of 60 amino acid region with a conserved WRKYGQKR motif in its N-terminus followed by a C$_2$H$_2$ or C$_2$HC zinc-finger motif, represent a large family of plant-specific TFs (Rushton et al. 2010). There are over 70 WRKY genes in *Arabidopsis* (Eulgem et al. 2000; Dong et al. 2003). Studies on WRKY genes predominantly point to an involvement in salicylic acid signaling and disease responses (Eulgem et al. 2000). Moreover, WRKY genes are involved in plant responses to drought, heat, cold, salinity (Shen et al. 2012; Liu et al. 2014; Wang et al. 2016), and oxidative stress (Rizhsky et al. 2004). Many WRKY TFs related to the drought stress responses have been isolated and studied in the recent years. For example, overexpressions of *OsWRKY30* (Shen et al. 2012), *TaWRKY1* (He et al. 2016), *CsWRKY2* (Wang et al. 2016), and *VvWRKY48* (Zhao et al. 2018) similarly enhanced the tolerance to drought stress in transgenic plants. But these studies mostly focused on model plants or crops, and the roles of the WRKY genes in *Malus* plant stress responses were less well known.

*Malus baccata* is widely used as an apple rootstock in northern China, but is also grown elsewhere as an ornamental tree and is highly resistant to low temperatures and drought (Xiao et al. 2008). To better understand the roles of WRKY genes involved in drought stress tolerance and to provide potentially genetic resources for the improvement of the drought resistance of *Malus* plant, a drought-responsive WRKY transcription factor was isolated from *M. baccata* and designated as *MbWRKY2*. Moreover, the overexpression of *MbWRKY2* in transgenic tobacco increased the tolerance to drought stress.

**Materials and methods**

**Plant material and growth conditions**

*M. baccata* test-tube seedlings were rapidly propagated on MS + 0.5 mg L$^{-1}$ 6-BA + 0.5 mg L$^{-1}$ IBA for 30 d, and then returned to MS + 1.2 mg L$^{-1}$ IBA for 45 d for rooting. Finally, the seedlings were transferred to Hoagland solution for about 40 d for domestication and growth. When the seedlings had 8–9 mature leaves (fully expanded), they were exposed to air on filter paper with 20% relative humidity for dehydration
and drought treatment. For ABA and salt treatments, seedling roots were immersed in 0.1 mM ABA or 250 mM NaCl solution, respectively (Liu et al. 2014).

Isolation and qRT-PCR expression analysis of MbWRKY2

Total RNA was respectively extracted from root, new leaf (partly expanded), mature leaf (fully expanded), and stem, using the CTAB method (Han et al. 2015). The samples of all control and roots of treated plants were sealed after treatments of 0, 1, 3, 6, 12 h, respectively, frozen immediately in liquid nitrogen, and then stored at −80°C for RNA extraction. First strand cDNA was synthesized with 1 μg total RNA and 1 μL super script II enzyme (Invitrogen, USA) according to the manufacturer protocol. PCR was performed to obtain a whole sequence of MbWRKY2 by using the first strand cDNA of M. baccata as a template. A pair of primers (F1, 5'-ATGGGACCATTCAGCTGATATGAT-3' and R1, 5'-TGTTGGAAGTTTGTGAC-3') was designed according to the homologous regions of Malus domestica WRKY gene 2 (XP_008392469.1) to amplify the full-length cDNA sequence. The full-length cDNA of MbWRKY2 gene was isolated from M. baccata using PCR with F1 and R1 as primers. The obtained DNA fragments were gel purified and cloned into the pMD18-T vector (Takara) and sequenced (Invitrogen, Beijing).

The qRT-PCR expression analysis of MbWRKY2 to abiotic stress was analyzed by real-time PCR method in M. baccata and conducted according to Han et al. (2017). The primers for MbWRKY2 were designed for qRT-PCR from partial sequences isolated in this study, i.e. MF, 5’-TCTACAGAA-GAGAGTGGTCGTCTAT-3’ and MR, 5’-GAGAGATTTACGATCATGTTAA-3’. The relative gene expression data was analyzed using the 2^(-ΔΔCt) method (Livak & Schmittgen 2001).

Subcellular localization of the MbWRKY2 protein

The MbWRKY2 ORF was cloned into the SacI and SmaI sites of the pSAT6-GFP-N1 vector. This vector contains a modified red-shifted green fluorescent protein (GFP) at SacI-SmaI sites. The MbWRKY2-GFP construct was transformed into onion epidermal cells by particle bombardment as described earlier (Han et al. 2015). The transient expression of the MbWRKY2-GFP fusion protein was observed under confocal microscopy.

Vector construction and agrobacterium-mediated tobacco transformation

To construct an expression vector for transformation of tobacco, restriction enzyme cut sites of BamHI and EcoRI were added into MbWRKY2 cDNA at both 5’ and 3’ ends by PCR. To construct the pBI121-MbWRKY2 vector, the products of PCR and pBI121 were digested by BamHI and EcoRI, and linked together through the replacing of GUS gene. The MbWRKY2 gene driven under the CaMV 35S promoter was introduced into Nicotiana tabacum cv. Xanthi ecotype tobacco by Agrobacterium-mediated GV3101 transformation (An et al. 1986). Transformants were selected on MS medium containing 50 mg dm⁻³ Kanamycin. T₂ generation transgenic tobacco was used for further analysis. Expression levels of the MbWRKY2 in tobacco transgenic lines were analyzed by RT-PCR. The primers of MF and MR were used for RT-PCR detection of leaves of tobacco, with the NtUbiquitin gene (U66264.1) as reference gene (NTUbFl, 5’-TTAACACATGCAAGTCGGACG-3’ and NTUbR1, 5’-GAGACCTCAGTAACAAAGTCCA-3’). PCR was performed for 30 and 26 amplification cycles for MbWRKY2 and NtUbiquitin, respectively.

Drought tolerance analysis of transgenic tobacco

Seeds (T₂ generation) of transgenic plants (OE-8 and OE-25, randomly selected) were sown and germinated on the germination and culture matrix (nutrient soil: vermiculite ratio is 4:1) in flowerpots (diameter 10 cm) with normal management in a growth chamber at 25 ± 1°C under a light 8 h dark regime in parallel with wild-type (WT) seeds. Seedlings were grown for 3 weeks with regular irrigation prior to drought stress. Drought stress experiments were conducted by withholding water for 10 d. Plants were re-watered for 5 d to determine the survival rate. The experiments were performed twice with three independent leaves for each treatment at each time point. During the whole growth process, all tobacco seedlings were observed and recorded with a photograph on drought stress for 5 d (D5d), 10 d (D10d) and rewatered for 5 d (R5d).

Measurement the contents of chlorophyl, proline, MDA, H₂O₂, AsA, and GSH

Three-week-old transgenic tobacco (OE-8 and OE-25, randomly selected) and WT tobacco plants above were conducted by withholding water for 5 d, and the leaves of all samples (before drought and after drought) were collected for measurement. Chlorophyll contents (Chl) were measured according to the method of Aono et al. (1993). Proline contents (Pro) were assayed following the method of Toka et al. (2010). H₂O₂ contents in tobacco leaves were determined by the method described previously (Alexieva et al. 2001). MDA contents in tobacco leaves were measured according to Dong et al. (2003). AsA was assayed following Foyer et al. (1983) and GSH following the method of Zhang & Kirkham (1996).

Measurement the levels of relative water content and electrolyte leakage

The leaves (including samples of before drought and after drought) of 3-week-old transgenic and WT tobacco above were collected for measurement. Leaf relative water content (RWC) was estimated according to RWC (%) = (fresh weight – dry weight)/(turgid weight – dry weight)×100 (Wang et al. 2009). Electrolyte leakage (EL) in tobacco leaves was determined following the protocols described by Xing et al. (2011).
**Measurement of activities of CAT, APX, SOD, and POD**

The CAT, APX, SOD, and POD activities of all the tobacco leaf samples exposed drought treatment for 5 d above were also measured. CAT (EC 1.11.1.6) and APX (EC 1.11.1.1) activities were measured following the methods used previously (Zhang et al. 2011). SOD (EC 1.15.1.1) activities and POD (EC 1.11.1.7) activities were measured using the protocols described by Beauchamp and Fridovich (1971). Relative quantification relates the enzyme activity in the transgenic lines (OE-8 and OE-25) to that of the WT.

**Gene expression analyses of NtCAT, NtAPX, NtSOD, NtPOD, NtP5CS, and NtLEA5 in transgenic tobaccos and WT**

Three-week-old tobaccos were exposed to drought treatments for 5 d, and the leaf samples above were collected. Expressions of the ROS-related genes and stress-responsive genes in tobaccos (transgenic lines and WT) were analyzed by qRT-PCR. The qRT-PCR was performed as described above. The primers were: for NtCAT gene (U93244.1), 5′-AAGATGGCTCGCAAGTAAA-3′ and 5′-GCCTAGCATCTCCAGATGG-3′; for NtAPX gene (U15933.1), 5′-GTATGAGATCTCTAAGACCTCTT-3′ and 5′-CCCAGGTATGGCCACCAAGA-3′; for NtSOD gene (AB093097.1), 5′-AGCTACATGACGCCATTTCC-3′ and 5′-CCCTGTAAGACGACCTTC-3′; for NtPOD gene (AB178933.1), 5′-GCTTCTCAGAGTTGCTAA-3′ and 5′-CTCTGGGTGAGTTGTTG-3′; for NtP5CS gene (HM854026.1), 5′-TGGCCCTCCCCGTAACTCAATTCTC-3′ and 5′-GATA-3′; for NtLEA5 gene (AF053076.1), 5′-AAGATGGGGAAGAATCAAG-3′ and 5′-TGTTTAGATCCAGGTAGT-3′; for NtLEA5 gene (U66264.1), 5′-TCCAGACCAGGTGT-3′ and 5′-CATCAAACAGGCAACCAT-3′.

**Statistical analysis**

Duncan multiple range tests were performed by using SPSS 13.0 program. Statistical differences were referred to as significant when p < .05.

**Results**

**Isolation of MbWRKY2 and phylogenetic relationship with other WRKY TFs**

Sequence analysis showed that the MbWRKY2 cDNA has a complete open reading frame of 966 bp, the predicted MbWRKY2 comprises 321 amino acids with a theoretical isoelectric point of 5.29 and a predicted molecular weight of 35.6 kDa. To investigate the evolutionary relationship among plant WRKY TFs, 9 proteins of WRKY TFs from different species were analyzed by DNAMAN (v.6.0). As shown in Figure 1, all the deduced amino acid sequence of WRKY TFs include a WRKY domain (WRKYGQ) and one conserved C2H2 zinc-finger motif (C-X3-C-X23-H-X-H). Comparing the amino acid sequences of MbWRKY2 with other WRKY TFs, we found that MbWRKY2 has a high identity to the WRKY TFs.

Additionally, a phylogenetic tree (neighbor-joining) was constructed with the amino acid residues (Figure 2) by MEGA program (v.4.1). Phylogenetic analysis demonstrated that the majority of 26 isolated proteins belong to 7 different subgroups of I, II-a, II-b, II-c, II-d, II-e, and III. MbWRKY2 was clustered into II-a subgroup, and more closely related to the BhWRKY1.

**Expression patterns of MbWRKY2 under abiotic stresses in M. baccata**

The spatial-specific expression of MbWRKY2 in different tissues of M. baccata was determined by qRT-PCR. The result shows that MbWRKY2 mRNA is more abundant in new leaf and root than in mature leaf and stem (Figure 3(a)). The results showed that MbWRKY2 increased in root under drought, high salinity, and ABA treatments (Figure 3(b)) by the qRT-PCR method. Under drought stress, the expression levels of MbWRKY2 began to increase after 1 h dehydration treatment and increased gradually until the experiment was concluded at 12 h. For high salinity stress, the expression level of MbWRKY2 increased rapidly and reached the stable high level for 12 h. The expression level of MbWRKY2 increased rapidly and peaked at 6 h, and then decreased almost to the beginning level in response to ABA treatment.

**MbWRKY2 was localized in the nucleus**

As shown in Figure 4, the MbWRKY2-GFP fusion protein is targeted into the nucleus with 4′,6-diamidino-2-phenylindole (DAPI) staining, whereas the control GFP alone is distributed throughout the cytoplasm. These results showed that the MbWRKY2 is a nucleus localization protein.

**Overexpression of MbWRKY2 confers enhanced tolerance to drought stress**

In order to investigate the role of MbWRKY2 in response to drought stress in plants, we generated transgenic tobacco with overexpression of MbWRKY2 under the control of the CaMV 35S promoter. Among 27 transformed lines, six of them (OE-1, OE-2, OE-8, OE-16, OE-21, and OE-25) were confirmed by using RT-PCR analysis with WT line as control (Figure 5(a)).

As shown in Figure 5(b), no significant difference existed in appearance between WT and MbWRKY2-OE (OE-8 and OE-25) lines after 3 weeks of growth on culture matrix with normal conditions, both types of tobaccos grew well (Unt: withholding water). However, when dealt with drought stress (withholding water), improved drought tolerance in transgenic plants was observed (D5d, D10d). A higher survival rate of transgenic plants after rewatering for 5 d (R5d), 76.9% for OE-8, 82.6% for OE-25, compared to WT, only 22.2% (Figure 5(c)).
**Overexpression of MbWRKY2 in transgenic tobaccos conferred to higher levels of chlorophyl, Pro, RWC, AsA and GSH under drought stress**

In order to study the reasons why the transgenic tobaccos had the better appearances under the drought stress, the Chl, Pro, RWC, AsA and GSH levels of all lines (both transgenic tobaccos OE-8, OE-25, and WT line) under normal irrigation and drought stress were measured. The transgenic tobaccos had the higher contents of chlorophyl, proline, and RWC than WT when dealt with drought treatment (Figure 6(a–c)). When dealt with dehydration stress, there were marked increases in the concentrations of AsA and GSH in both WT and the transgenic lines. However, the accumulations in the concentrations of AsA and GSH were significantly higher in the two transgenic lines than WT in response to drought stress (Figure 6(g,h)).

**MbWRKY2-overexpression tobaccos accumulated less MDA, H$_2$O$_2$ and EL under drought stress**

We also measured the MDA, H$_2$O$_2$, and EL levels of all lines (OE-8, OE-25 and WT) under normal irrigation and drought stress. The MDA, H$_2$O$_2$, and EL levels in leaves of transgenic tobaccos were significantly lower relative to WT, especially when dealt with drought stress for 5 d (Figure 6(d–f)). When dealt with drought stress, the levels of MDA, H$_2$O$_2$, and EL in WT tobaccos were about respectively 1.5-, 1.9-, and 1.6-fold than transgenic lines, which indicated that the WT line had more severe membrane damage. These results indicate that the overexpression of MbWRKY2 in transgenic tobaccos confers greater tolerance to the oxidative stress associated with drought stress.

**Overexpression of MbWRKY2 confers to enhanced antioxidant enzyme activities**

Aim to research the reason why WT tobaccos had more severe membrane damage relative to transgenic lines, the activities of ROS-scavenging enzymes such as CAT, APX, SOD, and POD in both transgenic tobaccos (OE-8 and OE-25) and WT line before and after drought stress were also measured. Under normal water management, the activities of CAT, APX, SOD, and POD in the transgenic lines were 1.2-, 1.1-, 1.3-, and 1.2-fold higher than WT line, respectively. When dealt with drought stress, the activities of POD, SOD, and CAT increased in all lines approximately 1.6-, 1.5-, 1.8-, and 1.7-fold than WT, respectively (Figure 6(i–l)). The change tendency of activities of ROS-scavenging enzymes between the WT and transgenic lines remained more or less the same. Compared to WT line, the transgenic tobaccos had the higher ROS-scavenging enzymes activities so that they can remove more reactive oxygen radicals and protect integrality of the membrane.

**Overexpression of MbWRKY2 confers to enhanced expression levels of ROS-related genes and stress-related genes**

In order to research what caused the higher antioxidant enzyme activities (CAT, APX, SOD, and POD) in transgenic
tobaccos (OE-8 and OE-25) than in WT line, the expression levels of NtCAT, NtAPX, NtSOD, and NtPOD in all lines were also measured by the qRT-PCR method. The result showed that the expression levels of three genes were 1.7-, 1.6-, 1.9-, and 2.5-fold higher than the WT under normal water management. Under drought stress, the expression levels of NtCAT, NtAPX, NtSOD, and NtPOD increased in all lines, which in the transgenic tobaccos were about 3.6-, 4.7-, 3.1-, and 4.8-fold than WT line, respectively (Figure 7(a–d)).

Moreover, the expression levels of genes that function in osmotic adjustment and membrane protection including NtP5CS and NtLEA5 were significantly up-regulated in the two transgenic lines than WT in response to drought stress (Figure 7(e,f)). It is suggested that MbWRKY2 enhanced drought tolerance in the transgenic lines via adjusting osmotic potential, maintaining membrane integrity, and removing ROS.

Discussion

The WRKY TFs have been proved to play important functions in the regulation of transcriptional reprogramming related to plant biotic and abiotic stress responses. All the WRKY TFs include a WRKY domain (WRKYGQK) and one conserved C2H2 or C2HC zinc-finger motif (Liu et al. 2014). These results showed that the WRKY TFs family was highly conserved during evolution. Previous reports have indicated that WRKY TFs genes are widely distributed in Arabidopsis, rice, soybean, strawberry, grape, and peach, which are known to be involved in abiotic (Ramamoorthy et al. 2008) and biotic stress responses (Pandey & Somsiss 2009).

The MbWRKY2 was structurally similar to BhWRKY1, which was isolated from Boea hygrometrica under high-drought stress. Identifying BhWRKY1 belongs to group II-a WRKY factors because of it shared homology with group II-a WRKY proteins (Wang et al. 2009). Phylogenetic analysis demonstrated that MbWRKY2 was clustered into II-a subgroup and more closely related to the BhWRKY1 (Figure 2). The expression of MbWRKY2 was much enriched in new leaf and root than that in mature leaf and stem (Figure 3(a)), which indicated that MbWRKY2 may play an important role in active organs. When treated with drought and salt stresses, the transcript levels of MbWRKY2 in roots were markedly increased (Figure 3(b)). The expression of MbWRKY2 was markedly affected by ABA treatment, which rised in the first stage, and then decreased. ABA has been proved to
mediate many stress responses through regulating the expression levels of the stress defense genes (Chinnusamy et al. 2004). Moreover, TaWRKY1 played an important role in drought stress, which was obviously up-regulated by ABA treatment (He et al. 2016). In this study, the expression analysis showed that MbWRKY2 substantially induced by the treatment of drought, salt, and ABA, may be involved in the abiotic stress response via the ABA-dependent signaling pathway. These results above suggested that MbWRKY2 gene had participated in stress responses in *M. baccata*.

Subcellular localization experiment has revealed that MbWRKY2 is preferentially localized in the nucleus (Figure 4), which is consistent with previous research. Some other WRKY TFs were reported to localize also in the nucleus (Ramamoorthy et al. 2008; Liu et al. 2014; Wang et al. 2016). The chlorophyl content and RWC were usually used

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**Figure 3.** Expression patterns of MbWRKY2 in different organs and in response to various treatments. The expression of MbWRKY2 in untreated roots was defined as CK. (a) Expression patterns of MbWRKY2 in root, new leaf (partly expanded), mature leaf (fully expanded), and stem under normal conditions. (b) Relative expression levels of MbWRKY2 in root under drought treatment. (c) Relative expression levels of MbWRKY2 in root under salt treatment. (d) Relative expression levels of MbWRKY2 in root under ABA treatment. Data represent means and standard errors of three replicates. Different letters above columns indicate ($p < .05$) significance using Duncan’s multiple range test differences between treatments.

**Figure 4.** Subcellular localization of MbWRKY2. Transient expressions in onion epidermal cells of 35S-GFP and 35S-MbWRKY2-GFP translational product were visualized by fluorescence microscopy. The transient vector harboring 35S-GFP and 35S-MbWRKY2-GFP cassettes were transformed into onion epidermal cells by particle bombardment. The photos were taken in bright light (a, d), or in the dark for GFP images (b, e) and DAPI-stained images (c, f) after incubation for 22 h.
as the markers for drought stress degree, which in BdWRKY36-overexpressing tobaccos were more higher than those in WT line under drought stress (Sun et al. 2015). Proline, which has been associated with the general stress response, may also be cryoprotective, since proline overproducers display an enhanced drought tolerance (Toka et al. 2010).

The 35S:MbWRKY2 transgenic tobacco exhibited a markedly increased tolerance to drought (Figure 5(b,c)). Overexpression of BhWRKY1 also enhanced the tolerance to drought stress in transgenic tobacco through regulating the expression level of BhGolS1, which leads to the accumulation of raffinose family oligosaccharides in drought-tolerant Boea hygrometrica leaves (Wang et al. 2009). The overexpression of stress-responsive WRKY genes TaWRKY2 and TaWRKY19 in Arabidopsis has been reported to enhance the tolerance to drought and salt (Niu et al. 2012). Drought stress can cause the accumulation of ROS and induce lipid peroxidation, which can damage cytomembrane structure and lead to oxidative stress (Huda et al. 2013). MDA is the organic compound with the formula CH₂(CHO)₂ and usually used as a marker for lipid peroxidation (Dong et al. 2003). EL reflects membrane injury severity after abiotic stresses (Xing et al. 2011). Under drought stress, the MbWRKY2-overexpression transgenic plants generated smaller amounts of MDA and H₂O₂, and had the lower EL levels than in WT (Figure 6). Similarly, overexpressing of FcWRKY40 in transformed tobaccos increased the tolerant to drought stress by alleviating H₂O₂ accumulation via regulation of the antioxidant genes (Gong et al. 2014).

Moreover, it was found that MbWRKY2-overexpression transgenic plants possess the higher levels of antioxidants such as AsA and GSH as well as the higher activities of ROS-scavenging enzymes (CAT, APX, SOD, and POD) in comparison with WT line under normal water management and drought stress (Figure 6(g–l)). The higher expression
Figure 6. Analysis of chlorophyl (Chl), RWC, proline (Pro), oxidants, electrolyte leakage (EL), AsA, GSH, and antioxidant enzymes between WT and transgenic T2 tobaccos (OE-8 and OE-25) under normal water management (Before drought) or withheld irrigation for 5 d (After drought), respectively. (a) Chl, (b) RWC, (c) Pro, (d) MDA, (e) H$_2$O$_2$, (f) EL, (g) AsA, (h) GSH, (i) CAT, (j) APX, (k) SOD, (l) POD. Data represent means and standard errors of three replicates. Different letters above columns indicate ($p < .05$) significance using Duncan’s multiple range test differences between treatments.

Figure 7. Expression levels of stress-responsive genes in WT and transgenic T2 tobaccos (OE-8 and OE-25). (a–f) The relative transcript levels of NtCAT, NtAPX, NtSOD, NtPOD, NtP5CS, and NtLEA5 under normal water management or withheld irrigation for 5 d in WT and transgenic tobaccos, respectively. Data represent means and standard errors of three replicates. Different letters above columns indicate ($p < .05$) significance using Duncan’s multiple range test differences between treatments.
levels of the ROS-scavenging genes such as \textit{NiCAT}, \textit{NiAPX}, \textit{NiSOD}, and \textit{NiPOD} in \textit{MbWRKY2}-overexpression plants would produce an increase in the activities of these enzymes, thus conferring more tolerance to drought stress. These results above suggest that overexpression of \textit{MbWRKY2} in transgenic tobaccos results in the higher levels of ROS-scavenging genes/enzymes. The results above were similar to the overexpression of \textit{BdWRKY36} in tobaccos (Sun et al. 2015). Furthermore, the expression levels of \textit{AtRD29A} and \textit{AtNCED3} were regarded as indicators for drought stress, which were more higher in \textit{VfWRKY48}-overexpressing \textit{Arabidopsis} than WT line when dealt with drought stress (Zhao et al. 2018). Consequently, higher protective enzyme activities lead to the suppression of ROS accumulation in order to suffer less from oxidative damage under drought stress. It is the possible reason why overexpression of \textit{MbWRKY2} in transgenic tobaccos conferred enhanced tolerance and had the higher survival rate to drought stress.

Under stress environment, the late embryogenesis abundant proteins are regarded as the protector for membranes integrity, and enhance the tolerance to osmotic stress (Chinnusamy et al. 2004; Niu et al. 2012). The overexpression of \textit{MbWRKY2} in transgenic tobaccos led to a greater up-regulation of \textit{NtLEA5} and enhanced the tolerance to drought stress. It is possible that \textit{MbWRKY2} plays a crucial role in helping plants to survive from drought stress by regulating the expression level of \textit{NtLEA5}. Higher expression level of \textit{NtLEA5} in \textit{MbWRKY2}-OE plants contributed to better membrane protection and facilitating osmotic adjustment in response to drought stress. In addition, the \textit{Ntp5CS} (a proline synthase gene) had significantly higher expression levels in \textit{MbWRKY2}-overexpression tobaccos than WT under drought stress. These results suggested that the higher proline contents in \textit{MbWRKY2}-overexpression plants are likely to result from the increased expression levels of \textit{Ntp5CS} under drought stress.

In conclusion, a WRKY transcription factor encoding \textit{MbWRKY2}, which was induced by drought and salt stresses, and ABA treatment, was isolated from \textit{M. baccata}. Overexpression of \textit{MbWRKY2} in tobacco resulted in enhanced tolerance to drought stress. This was partially correlated with the activation of ROS-related antioxidant genes/enzymes and stress-related genes involved in osmotic adjustment and membrane protection, leading to less accumulation of ROS under drought stress. The results suggest that \textit{MbWRKY2} may work with other factors to promote expression of stress-responsive genes specifically under stress conditions. More importantly, overexpression of \textit{MbWRKY2} in tobacco was achieved without affecting their phenotypes under normal conditions. Therefore, \textit{MbWRKY2} provides a potentially excellent genetic resource for improving drought tolerance in plants.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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