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Full Length Article

Isolation and Preliminary Functional Analysis of *MbWRKY4* Gene Involved in Salt Tolerance in Transgenic Tobacco

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Abstract

WRKY genes are widely distributed in plants and closely linked to stress reactions, but the WRKY effects in signaling and responses of abiotic stresses have not been well understanded in *Malus* plants. In this research, a WRKY gene was cloned from *Malus baccata* and named it as *MbWRKY4*. Subcellular localization showed MbWRKY4 localized onto nucleus. The transcription level of *MbWRKY4* increased in *M. baccata* seedlings by treatments of salt, drought and abscisic acid. Over-expression of *MbWRKY4* in transgenic tobaccos contributed to higher salt tolerance. The transformed tobaccos had higher contents of chlorophyll and proline, increased activities of POD, APX and SOD, decreased levels of elecrolyte leakage, MDA, and H_2O_2 than WT, especially when dealt with salt treatment. Moreover, the *MbWRKY4* transgenic plants enhanced the expressions of oxidative stress response (*NtPOD*, *NtAPX* and *NtSOD*) when dealt with high salinity treatment. The results above indicated that *MbWRKY4* gene has an affirmative effect to high salinity response.

Keywords: Salt tolerance; *Malus baccata*; *MbWRKY4*; ROS-scavenging enzyme; Stress-related genes

Introduction

Plants are usually exposed to various abiotic stresses, such as salt, drought, heat, chilling, pathogen attack and nutrient deprivation, which adversely influence the development and productivity of plant (Gong and Liu, 2013). To avoid such deficiencies, plants have evolved adaptable mechanism to receive environmental signaling and to display emergency responses with appropriate physiological, cellulate, and molecular adjustments (Liu *et al*., 2014). Those external stresses frequently destroy the reactive oxygen species (ROS) homeostasis of plant, resulting in the oxidation stresses (Mittler, 2002). Among these stresses, high salt stress is considered as one of the biggest abiotic stresses for growth and yield of crop. Plant has developed different mechanisms to decrease the latent threat of salt stress (Liu *et al*., 2014).

Among the genes induced by various abiotic stresses, a lot of transcription factors (TFs) have been identified and studied, e.g., WRKY (possess 1–2 conservative WRKY regions consisting by 60 amino acid residues with a motif of WRKYGQK sequence, and named as WRKY), NAC (TFs similar to petunia NAM and *Arabidopsis* ATAF1/2, and CUC), and bZIP (basic region-leucine zipper), which interact with cis-elements current on promoter domains of different stress-related factors and therefore adjust the expression levels of functional genes leding to improved tolerances to these stresses (Agarwal *et al*., 2006).

The WRKY TFs family members in *Arabidopsis* and rice are more than 60 (Dong *et al*., 2003; Zhang *et al*., 2004; Rushton *et al*., 2010). Moreover, WRKY TFs were widely related to plant tolerances to salinity, heat, cold, drought, disease and oxidative stress (Pnueli *et al*., 2002; Rizhsky *et al*., 2004; Zhou *et al*., 2008; Niu *et al*., 2012; Shen *et al*., 2012; Liu *et al*., 2014). However, the effects of WRKY TFs in abiotic stress responses of *Malus* plant are still less well studied.

Malus baccata (L.) Borkh is widely used as an apple rootstock in northern China, but is also grown elsewhere as an ornamental tree (Xiao *et al*., 2008). To better comprehend the effects of WRKY TFs related to high salinity stress and to find potential gene resource for the enhancement to the high salinity resistivity of *Malus* plant, a salt-responsive WRKY transcription factor was cloned from *M. baccata* and named as *MbWRKY4*. Moreover, higher expression level of *MbWRKY4* in transformed tobacco increased the tolerance to high salinity stress.

Materials and Methods

Material and Treatments

In vitro grown seedlings of *M. baccata* were propagated and rooted on MS medium, and transferred to Hoagland's solution for domestication and growing for 50 d (Han *et al*., 2017). Then thirty seedlings were divided into three

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repetitions and dealt with drought treatment by laying on filter paper in clean bench. For high salinity stress and abscisic acid treatments, the roots of 30 seedlings (divided into three repetitions) were put into 300 m*M* NaCl or 0.1 m*M* ABA Hoagland's solution, respectively (Liu *et al*., 2014).

Isolation and Expression Analysis

According to Han *et al*. (2015), the total RNA of different parts of 10 *M. baccata* seedlings (mature leaf, root, stem and new leaf) with different treatments above for 0, 4, 8, 12, 24 h, respectively were extracted with CTAB method (repeated three times). The cDNA (first strand) was generated according to the program of script II (Invitrogen, USA). PCR was carried out to acquire the *MbWRKY4* sequence wtith *M. baccata* cDNA as template. The primers of F1 and R1 (Table 1) were planned according to *M. domestica WRKY gene 4* (*MdWRKY4*: MDP0000507805) to identify the whole open reading frame (ORF) sequence. Then the primers of F1 & R1 were used to clone the ORF sequence of *MbWRKY4* from *M. baccata* by PCR method. The acquired DNA fragment was gel purified and connected to the pEASY-T1 vector (TransGen Biotech Co. Ltd, China) and sequenced (Invitrogen, Beijing).

The *MbWRKY4* gene expression level analysis was used real-time fluorescence quantitative PCR method (Han *et al*., 2017), with MF and MR (Table 1) as primers. The qPCR were incubated under the following procedure: 1 cycle of 94°C for 35 s; then 30 cycles at 94°C for 10 s, 58°C for 35 s. The reference gene was used with *Malus Actin* gene, using the primers of ApActF1, and ApActR1 (Table 1). Each sample was analysed in triplicate. According to Livak and Schmittgen (2001), the expression levels were analyzed with $2^{-\Delta\Delta C}{}_{T}$ method.

Subcellular Localization

The coding region of *MbWRKY4* was inserted into the pSAT6-GFP-N1 (a plant transient expression vector, provided by Prof. Kedong Xu, Zhoukou Normal University) by the restriction enzyme cutting sites of *Sac*I and *Sma*I. This transient expression vector contains a GFP at *Sac*I-*Sma*I sites. According to Xu *et al*. (2014), the plasmids of *MbWRKY4*-GFP were introduced into *Allium cepa* epidermal cells by injection. The fusion proteins of MbWRKY4-GFP (nucleus with DAPI: 4', 6-diamidino-2-phenylindole staining) were observed and photographed by confocal microscopy.

Vector Construction and Transformation

MbWRKY4 gene was added the sites of *Xba*I and *Bam*HI with PCR method. The pBI121-*MbWRKY4* plasmid was constructed by connection with the PCR products and pBI121, which were both digested by restriction enzymes of *Xba*I and *Bam*HI. The pBI121-*MbWRKY4* plasmids were transformed into tobaccos (*Nicotiana tabacum* cv. Xanthi) by method of An *et al*. (1986). The transgenic lines were choosed on MS medium with 40 mg dm^{-3} Kanamycin. The primers of AF and AR were used for RT-PCR detection of leaves of tobacco, with the *NtUbiquitin* (U66264.1) as reference gene (NtUbF1 and NtUbR1, Table 1). The PCR systems were executed for 30 and 25 circulations for *MbWRKY4* and *NtUbiquitin*, respectively.

Salt Tolerance Analysis

Thirty seedlings of each transformed tobacco lines (T_2 generation) and WT line were grown on the nutrient matrix (ratio of vermiculite: culture soil was 1:3) in flowerpots (diameter 10 cm) with regular management in a light incubator at 24±1°C under a 14 h light (120 μmol m^{-2} s^{-1})/18 ± 1°C under a 10 h dark regime for three weeks. For salt stress treatments, the transformed tobaccos (OE-3 and OE-7, randomly selected) and WT line were poured with 300 m*M* sodium chloride solution for 16 d and then re-watered to determine survival rate. The test was repeated for thee times for all treatments. During the treatments, the growing situations of all lines were recorded with camera by salt stress for 8 d (S8d) and 16 d (S16d). For survival experiments, the tobaccos were investigated with a re-watering normally as a recovery period for 6 d.

Measurement the levels of electrolyte leakage, chlorophyll, proline, malondialdehyde (MDA) and hydrogen peroxide (H_2O_2): The thirty 3-week-old transformed tobaccos and WT line above were irrigated with 300 m*M* NaCl solution for 8 d, and leaves of all samples (before salt and after salt) were gathered for the determination. Elecrolyte leakage (EL) in leaves of all lines was determined according to the method described by Xing *et al*. (2011). Chlorophyll contents (Chl) in leaves were measured following the protocols of Aono *et al*. (1993). Proline contents (Pro) were assayed following the method of Irigoyen *et al*. (1992). The MDA contents in leaves were determined following the method of Dong *et al*. (2003). H_2O_2 contents were measured by the protocols of Alexieva *et al*. (2001). The measurements above were repeated for 3 times for all treatments.

Measurement of activities POD, APX and SOD: The activities of POD, APX and SOD in all the tobacco leaf samples exposed salt treatment for 8 d above were also measured. The POD activity was measured according to the method of Ranieri *et al.* (2000). APX activity was determined according the method of Zhang *et al*. (2011). The SOD activity was determined following the method of Beauchamp and Fridovich (1971). The relative quantifications related the activities of POD, APX and SOD in the transformed tobaccos to those of the WT line.

Reactive oxygen species (ROS) related genes' qPCR analysis: Three week-old tobacco plants were exposed to salt treatment for 8d, and then the leaves of ten strains from each line were sampled for ROS-related genes expression analysis by qRT-PCR method.

Table 1: The primers used in this study

Primer Name	Sequence(5′-3′)	Description
F1	ATGCTTCAAACGTATACTCTTCCA	full-length cDNA of *MbWRKY4*
R1	TCAGCACAGCAATGATTCAAAA	
MF	AACACCAATGAGATCCCAGAT	qRT-PCR for *MbWRKY4*
MR	TTGCATTAGGTTCATCTTCATC	
ApActF1	CTACAAAGTCATCGTCCAGACAT	qRT-PCR for *Actin*
ApActR1	TGGGATGACATGGAGAAGATT	
AF	CATCGTCAATGTCTCAATCCTCAGA	RT-PCR for *MbWRKY4*
AR	ATCTCTAGGCTAAAGGGAGCTTGC	
NtUbF1	TTAACACATGCAAGTCGGACG	RT-PCR for *NtUbiquitin*
NtUbR1	GAGACCTCAGTAGACAAAGCACATC	
NtPODF1	GCTGTTCGACGAGTTGTTAACAG	qRT-PCR for *NtPOD*
NtPODR1	CTCTGGCTGAGTTGTTGTTGG	
NtAPXF1	GTATTGACATTGCTATAAGACTCTT	qRT-PCR for *NtAPX*
NtAPXR1	CCCAAGGTATGGCCACCAGAGA	
NtSODF1	AGCTACATGACGCCATTTCC	qRT-PCR for *NtSOD*
NtSODR1	CCCTGTAAAGCAGCACCTTC	
NtUbF2	TCCAGGACAAGGAGGGTAT	qRT-PCR for *NtUbiquitin*
NtUbR2	CATCAACAACAGGCAACCTAG	

The primers of NtPODF1 and NtPODR1 were for *NtPOD* gene (AB178953.1); NtAPXF1 and NtAPXR1 for *NtAPX* gene (U15933.1); NtSODF1 and NtSODR1 for *NtSOD* gene (AB093097.1); NtUbF2 and NtUbR2 for *NtUbiquitin* gene (U66264.1). The qRT-PCR was performed as described above and all the primers were provided in Table 1. The expression levels of these genes were standardized relative to *NtUbiquitin.*

Statistical Analysis

Duncan multiple range tests were executed with SPSS 15.0. All the measurements and expression analyses above were repeated for 3 times for all treatments. Statistical differences were referred to as significant when $P<0.05$.

Results

Isolation and Phylogenetic Relationship of *MbWRKY4*

As shown in Fig. 1, the *MbWRKY4* sequence has a whole ORF of 1395 bp, the MbWRKY4 protein contains 464 amino acids having a theoretical molecular weight of 51.6 kDa and a predicted isoelectric point of 7.30. To investigate the homology with other plant WRKY TFs, 11 WRKY TFs from various species were analyzed with DNAMAN program (v.6.0). All the proteins of WRKY TFs include 2 WRKY domains (WRKYGQK) and 1 conserved C_2H_2 region (C-X_4-C-X_{22}-H-X-H) (Fig. 2A). The phylogenetic analysis showed that MbWRKY4 is more closely related to MdWRKY4.

Contrasting the protein sequence of MbWRKY4 with other WRKY TFs, we discovered that MbWRKY4 has a higher homology with WRKY TFs. Moreover, an evolutionary tree was erected with amino acids (Fig. 2B) with DNAMAN (v.6.0). The phylogentic tree displayed that MbWRKY4, MdWRKY4 (MDP0000507805, from *Malus domestica*), PbWRKY26 (XP_009354509.1, from *Pyrus bretschneideri*), FvWRKY25 (XP_004294758.1, from *Fragaria vesca*) and PmWRKY33 (XP_008231402.2, *Prunus mume*) are grouped into the first cluster. TaWRKY19 (EU665430.1, from *Triticum aestivum*), VpWRKY2 (GU565706.1, from *Vitis pseudoreticulata*) and TaWRKY2 (EU665425.1, from *T. aestivum*) are grouped into the second cluster, then followed by GmWRKY25 (NP_001304523.1, from *Glycine max*), CoWRKY1 (OMP02348.1, from *Corchorus olitorius*), and JrWRKY24 (XP_018828546.1, *Juglans regia*) on their own cluster.

MbWRKY4 Expression Patterns

The expression levels of *MbWRKY4* in various tissues of *M. baccata* were measured with qPCR method. The *MbWRKY4* level was higher in root and leaf than in stem (Fig. 3A). The results showed that *MbWRKY4* increased in root under high salinity, drought, and ABA treatments (Fig. 3B, 3C and 3D). For high salinity stress, the *MbWRKY4* level increased at 4 h and ascended rapidly until 24 h (Fig. 3B). Under drought stress, the *MbWRKY4* level increased quickly and peaked to the max at 12 h, and kept high level at 24 h (Fig. 3C). For ABA treatment, the expression level of *MbWRKY4* increased quickly to the max at 8 h, and then decreased almost to the 4 h level (Fig. 3D).

MbWRKY4 Targeted onto Nucleus

As shown in Fig. 4, the MbWRKY4-GFP protein is preferentially only localized onto nucleus, while the pSAT6-GFP-N1 GFP (control) is distributed throughout the epidermal cell. The result indicated that MbWRKY4 is localized onto nucleus.

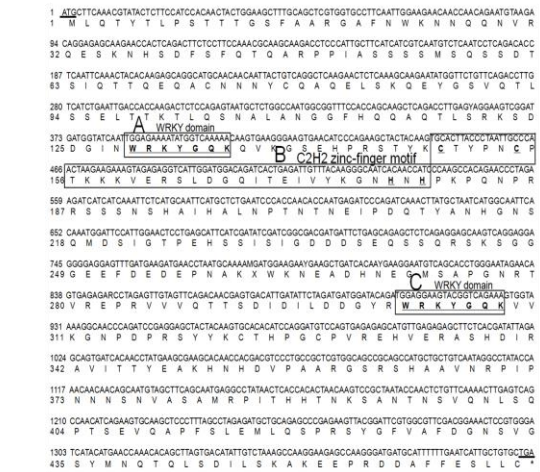

Fig. 1: Nucleotide and deduced amino acid sequences of *MbWRKY4*. (***A***) The WRKY domain. (***B***) The C_2H_2 zinc-finger motif. (***C***) The WRKY domain

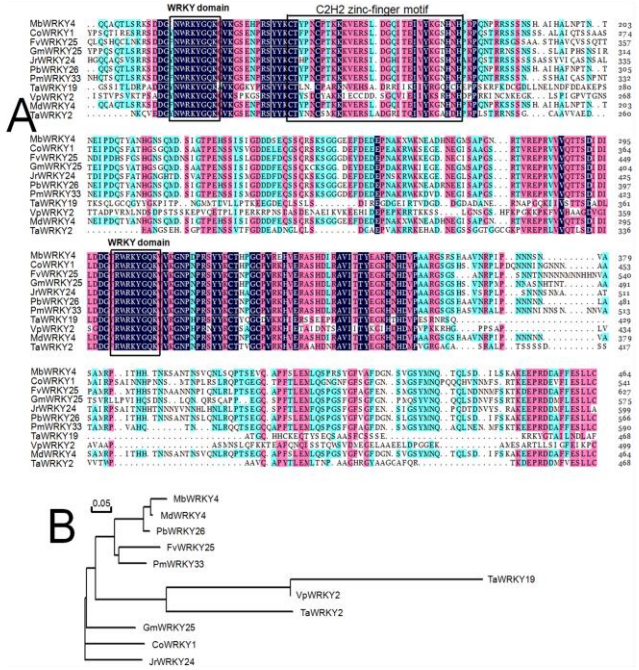

Fig. 2: Comparison and phylogenetic relationship of MbWRKY4 with other reported WRKY transcription factors

(A) Complete alignment comparison of MbWRKY4 with other plant WRKY transcription factors. Positions containing identical residues are shaded in navy blue, while conservative residues are shown in green. All the WRKY proteins contain two WRKY domains and one C_2H_2 zinc-finger motif.

(B) Phylogenetic tree analysis of MbWRKY4 and other plant WRKY transcription factors. The tree was constructed by the neighbour-joining method with DNAMAN (v.6.0). The gene accession numbers are listed as follows: MdWRKY4 (MDP0000507805), PbWRKY26 (XP_009354509.1), FvWRKY25 (XP_004294758.1), PmWRKY33 (XP_008231402.2), TaWRKY19 (EU665430.1), VpWRKY2 (GU565706.1), TaWRKY2 (EU665425.1), GmWRKY25 (NP_001304523.1), CoWRKY1 (OMP02348.1), and JrWRKY24 (XP_018828546.1)

Over-expression of *MbWRKY4* Contributed to Higher Salinity Tolerance

To study the effect of *MbWRKY4* in high salinity treatment in tobacco, we collected transformed tobacco lines over-expression *MbWRKY4*. Among the 24 transgenic strains, 6 lines (OE-1, OE-3, OE-5, OE-7, OE-8 and OE-9) were affirmed by RT-PCR method with WT as control (Fig. 5A).

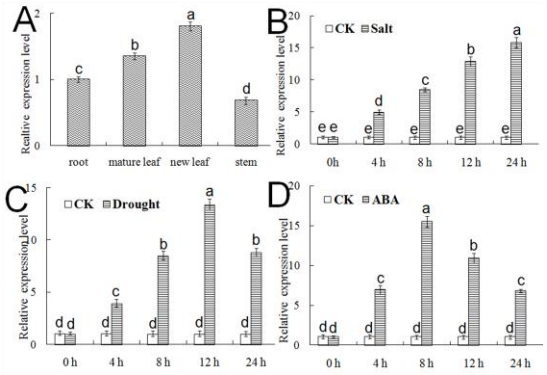

Fig. 3: Quantitative real-time PCR analysis of expression patterns of *MbWRKY4* in different organs and in response to various treatments. The expression of *MbWRKY4* in untreated root was defined as CK

(A) Expression patterns of *MbWRKY4* in root, new leaf, mature leaf, and stem under normal conditions. (B) Relative expression levels of *MbWRKY4* in root under salt treatment. (C) Relative expression levels of *MbWRKY4* in root under drought treatment. (D) Relative expression levels of *MbWRKY4* in root under ABA treatment. Data represent means and standard errors of three replicates. Different letters above columns indicate ($P<0.05$) significance using Duncan's multiple range test differences between treatments

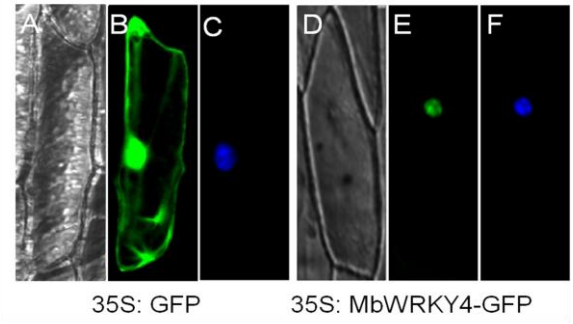

Fig. 4: Subcellular localization of MbWRKY4. Transient expressions in onion epidermal cells of 35S-GFP and 35S-MbWRKY4-GFP translational product were visualized by fluorescence microscopy. The transient vector harboring 35S-GFP and 35S-MbWRKY4-GFP cassettes were transformed into onion epidermal cells 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 24 h

As shown in Fig. 5B, these were no obvious differences between the WT tobacco and *MbWRKY4*-OE (OE-3 and OE-7, randomly selected) lines after 3 weeks of growth on culture matrix with normal conditions (Unt). However, the improved salt tolerances in transformed lines were easily found (S8d, S16d) under salt stress (irrigated with 300 m*M* NaCl solution). Moreover, a higher survival rate of transformed tobaccos after recovery was discovered, 76.9% for OE-3; 82.7% for OE-7, compared to WT, only 22.3% (Fig. 5C).

Over-expression of *MbWRKY4* Confers to Higher Chlorophyll and Proline Levels

In order to study the reasons why the transgenic tobaccos had the better appearences under the salt stress, the chlorophyll and proline levels of all lines (both WT and transgenic tobaccos OE-3, OE-7) under normal irrigation and salt stress were measured. The transgenic tobaccos had the higher chlorophyll and proline contents than WT when dealt with salt treatment (Fig. 6A and 6B).

MbWRKY4-OE Tobaccos Accumulated Less EL, MDA and H_2O_2

In order to study the reasons why the transformed tobaccos had the higher levels of chlorophyll and proline, with the better appearances under the salt stress, the EL, MDA and H_2O_2 levels of all lines (both transgenic tobaccos OE-3, OE-7 and WT line) under normal irrigation and salt stress were measured. The EL, MDA and H_2O_2 levels in leaves of transgenic tobaccos were significantly lower relative to WT,

especially when dealt with salt stress for 8 d (Fig. 6C, 6D and 6E), revealing the WT line had higher severity membrane damage with salt stress.

Over-expression of *MbWRKY4* 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-related enzymes e.g., POD, APX and SOD in both transgenic tobaccos (OE-3 and OE-7) and WT line before and after salt stress were also measured. Under normal water management, the POD, APX and SOD activities in transformed tobaccos were 1.2, 1.1- and 1.1-fold higher than those in wild-type, respectively. When dealt with salt stress, the POD, APX and SOD activities enhanced in transgenic plants about 1.8, 1.6- and 1.7-fold than in WT line, respectively (Fig. 6F, 6G and 6H). Under salt stress, the activities of ROS-scavenging enzymes in transformed tobaccos and WT line had the similar change tendency. 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 membrane.

Over-expression of *MbWRKY4* Confers to Enhanced Expression Levels of ROS-related Genes

In order to research what caused the higher antioxidant enzyme activities (POD, APX and SOD) in transgenic tobaccos (OE-3 and OE-7) than in WT line, the expression levels of *NtPOD*, *NtAPX* and *NtSOD* in all lines were also determined by real-time PCR. The results indicated that the expression levels of 3 genes in transformend tobaccos were 2.3-, 1.7- and 2.1-fold than those in WT with regular water management. When dealt with salt stress, the expression levels of *NtPOD*, *NtAPX* and *NtSOD* enhanced in each line, which in the transgenic tobaccos were about 4.9, 4.5- and 3.9-fold higher than WT line, respectively (Fig. 7).

Discussion

The WRKY TFs had significant functions in regulation of transcriptional levels of many genes related to abiotic and biotic stress responses in plants (Chen *et al*., 2012). In this research, a WRKY gene was isolated from *M. baccata* and named it as *MbWRKY4*. Sequence analysis showed that MbWRKY4 transcription factor contains 2 WRKY regions and 1 C_2H_2 motif (C-X_4-C-X_{22}-H-X-H). Evolutionary tree indicated that MbWRKY4 is a new member of WRKY family. There are about 95.8, 93.2, 83.1, 82.1, 75.3, 73.4, 72.9, 66.8, 61.2 and 46.2% amino acid homologies between MbWRKY4 and MdWRKY4, PbWRKY26, FvWRKY25, PmWRKY33, CoWRKY1, GmWRKY25, JrWRKY24, TaWRKY2, VpWRKY2, TaWRKY19, respectively (Fig. 2B). All the WRKY TFs include 1–2 WRKY regins and one conserved C_2H_2 or C_2HC motif (Liu *et al*., 2014).

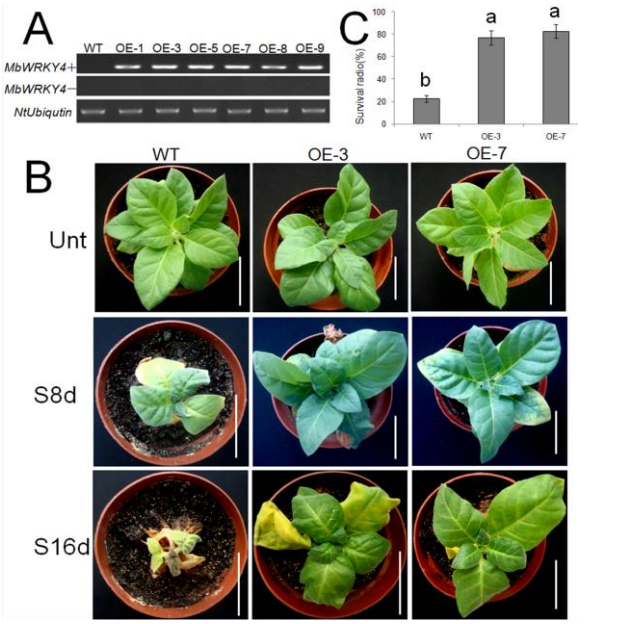

Fig. 5: Expression of *MbWRKY4* in transgenic tobacco and over-expression of *MbWRKY4* in tobacco improved salt stress tolerance

(A) Expression levels of *MbWRKY4* in wild-type (WT) and *MbWRKY4*-OE transgenic T_2 lines revealed by semi-quantitative RT-PCR. PCR products using *MbWRKY4*-specific primers with (top) and without (middle) prior reverse transcription were stained by ethidium bromide, the RT-PCR products with *NtUbiquitin* gene (U66264.1) primers (bottom) was used as control

(B) Over-expression of *MbWRKY4* in tobacco improved salt stress tolerance in *MbWRKY4* transgenic lines. WT, OE-3 and OE-7 were respectively shown the wild-type and transgenic tobacco seedlings grown on culture matrix. T_2 seeds were grown for 3 weeks in growth chamber with normal water management (***Unt***: untreatment), then irrigated with 300 mM NaCl solution for 8 d (***S8d***) and 16 d (***S16d***). Scale bars 5 cm

(C) Survival rates of seedlings in WT and transgenic T_2 lines after recovery

Previous studies had showed that WRKY TFs were diffusely existed in rice, *Arabidopsis*, soybean, maize, cotton, orange, peach, etc., which were known to be related to abiotic (Rushton *et al*., 2010; Chen *et al*., 2012) and biotic stress responses (Pandey and Somssich, 2009).

The expression level of *MbWRKY4* was higher in root and leaf than in stem (Fig. 3A), which showed that *MbWRKY4* may have an important functions in active parts. The transcription levels of *MbWRKY4* in roots were markedly increased by drought and salt treatments (Fig. 3B and C). The expression of *MbWRKY4* was markedly affected by ABA treatment, which rised in the first stage, and then decreased (Fig. 3D). ABA has been proved to mediate numerous stress reactions by regulating the transcription levels of the stress-related genes (Chinnusamy *et al*., 2004). In the present research, the expression of *MbWRKY4* increased by ABA treatment, which suggested that *MbWRKY4* possibly involved in the ABA-related signal pathway. These results above suggested that *MbWRKY4* gene had participated in stress responses in *M. baccata.*

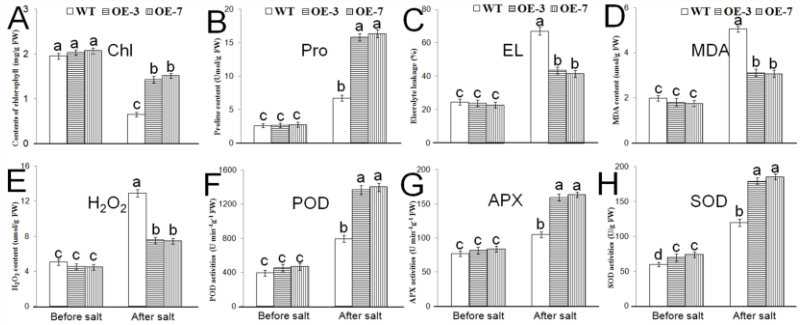

Fig. 6: Analysis of chlorophyll (Chl), proline (Pro), elecrolyte leakage (EL), oxidants, and antioxidant enzymes between WT and transgenic T_2 tobaccos (OE-3 and OE-7) under normal water management or irrigated with 300 mM NaCl solution for 8 d, respectively. Data represent means and standard errors of three replicates. Different letters above columns indicate ($P<0.05$) significance using Duncan's multiple range test differences between treatments

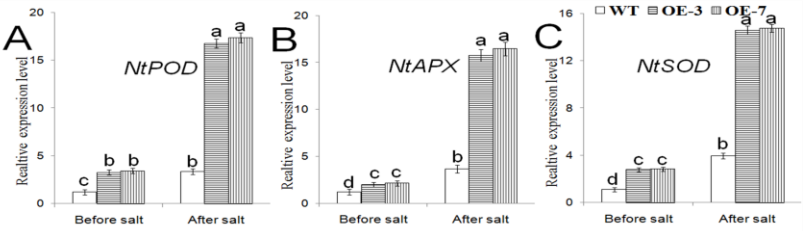

Fig. 7: Expression of ROS-related genes in WT and transgenic T_2 tobaccos (OE-3 and OE-7). A–C The relative transcript levels of *NtPOD*, *NtAPX*, and *NtSOD* under normal water management or irrigated with 300 mM NaCl solution for 8 d in WT and transgenic tobaccos, respectively. The ROS-related genes expression levels were normalized against the *NtUbiquitin* gene (U66264.1) expression level. Data represent means and standard errors of three replicates. Different letters above columns indicate ($P<0.05$) significance using Duncan's multiple range test differences between treatments

Subcellular localization study indicated that MbWRKY4 was localized onto the nucleus (Fig. 4), which is consistent with previous research. Some other WRKY TRs were reported to localize also in nucleus (Pandey and Somssich, 2009; Liu *et al*., 2014).

Salt stress can cause the accumulation of ROS and induce lipid peroxidation, which may destroy the cytomembrane structure and result in oxidative stress (Huda *et al*., 2013). MDA levels were the organic compound with the formula $CH_2(CHO)_2$ and usually defined as markers for lipid peroxidation (Caradonna and Mauro, 2016). Electrolyte leakage reflected membrane damage severity due to abiotic stress (Xing *et al*., 2011). Under salt stress, the *MbWRKY4*-overexpression transgenic tobaccos generated smaller amounts of MDA and H_2O_2, and had the lower EL levels than in WT (Fig. 6). The chlorophyll contents were usually used as markers for salt stress degree (Liu *et al*., 2014). Proline, which has been associated with the general stress response, may also be cryoprotective, since proline overproducers display an enhanced salt tolerance (Toka *et al*., 2010).

Moreover, the *MbWRKY4*-OE transgenic tobaccos had higher levels of ROS-related enzyme activities (POD, APX and SOD) than WT line under regular water management and salt stress (Fig. 6G and H). These results suggest that over-expression of *MbWRKY4* in transgenic tobaccos stimulates the enhanced expressions of 3 ROS-scavenging genes (*NtPOD*, *NtAPX* and *NtSOD*), and results in the more high levels of ROS-related enzyme activities. Consequently, higher protective enzyme activities lead to the inhibition of ROS cumulation for suffering less oxidative injures under high salinity stress. This is the reason why over-expression of *MbWRKY4* in transgenic tobaccos confers higher levels of chlorophyll and proline (Fig. 6A and B), enhanced tolerance (Fig. 5B) and the higher survival rate to salt stress (Fig. 5C).

Conclusion

The expression level of *MbWRKY4* was affected by salt and drought stresses, and ABA treatments. Over-expression of *MbWRKY4* in transformed tobaccos contributed to improved

tolerance to high salinity stress. It was possibly related to the activations of ROS-scavenging antioxidant genes & enzymes, resulting in less accumulation of ROS under high salinity stress. Hence, *MbWRKY4* provided a superior genetic resource in plants for increasing the salt tolerance.

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