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Overexpression of *DnWRKY11* enhanced salt and drought stress tolerance of transgenic tobacco

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Abstract: *Dendrobium* seedlings showed low survival rate when they were transferred from *in vitro* conditions to greenhouse or field environment. One of the major reasons is their low tolerance to environmental changes. WRKY transcription factors are one of the largest families of transcriptional regulators in plants. They are involved in various biotic and abiotic stress responses. One *DnWRKY11* gene was isolated from *Dendrobium nobile*. To explore the function of *DnWRKY11* in *Dendrobium* defense responses to abiotic stress, it was overexpressed in tobacco. Under salt and drought stresses, the *DnWRKY11* transgenic tobacco showed higher germination rate, longer root length, higher fresh weight, higher activities of catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), and lower content of malonidialdehyde (MDA) than the wild type. These results proved the important roles of *DnWRKY11* in plant response to drought and salt stresses, and provided a potential gene for improving environmental stress tolerance of *Dendrobium* seedlings.

Key words: *Dendrobium nobile*; abiotic stress; functional identification; WRKY

Introduction

Plants are constantly challenged by various environmental changes, such as drought, salt, extreme temperatures and oxidative stress. To survive these challenges, plants have developed elaborate mechanisms to adapt themselves (Wu et al. 2009). During these processes, transcription factors play a central role in regulating the expression of specific stress related genes. WRKY transcription factors are one of the largest families of transcriptional regulators in plants. They are defined by the presence of one or two WRKY conserved domains of 60 amino acids characterized by the WRKYGQK motif at the N-terminus, and a Cys2His2 or Cys2HisCys zinc-binding motif at the C-terminus (Rushton et al. 2010). Based on the number of WRKY domains present and the type of zinc-binding motif, WRKY proteins was classified into groups 1, 2a to 2e, and 3 (Rushton et al. 2010). A large number of WRKY genes have been identified in plants (Mangelsen et al. 2008; Liu & Ekramoddoullah 2009; Pandey & Somssich 2009; Rushton et al. 2010; Song et al. 2010). They are involved in multiple biotic and abiotic stress responses, and developmental and physiological processes, such as anthocyanin biosynthesis, senescence, trichome development, starch biosynthesis, and hormone responses (Lagace & Matton 2004; Jiang & Yu 2009; Wu et al. 2009; Chen et al. 2010; Li et al. 2010; Miao & Zentgraf 2010; Ren et al. 2010; Liu et al. 2013).

Dendrobium is the second largest genus in the family Orchidaceae, which comprises over 1,100 species, and 74 species have been identified in China. Some species, such as *D. candidum*, *D. nobile*, and *D. chrysanthum*, containing various bioactive constituents, which makes them highly valued in Chinese traditional herbal medicine. Since their important roles in disease treatment and tonic effect, during the last few decades, wild *Dendrobium* have fallen into serious danger of extinction due to the excessive collection for growing medicinal demands and the limited natural resource in china. In recent years, *Dendrobium* seedlings were great obtained by the tissue culture technology. However, they showed low survival rate and slow growth when transferred from *in vitro* conditions to a greenhouse or field environment. The major reason is their low tolerance to environmental change. Hence, study the function of stress related gene of *Dendrobium* is important for improving their seedlings environmental stress tolerance. In the present study, one novel *DnWRKY11* was isolated from *D. nobile,* and its function in plant response to salt and drought stresses was analyzed by overexpressing it in tobacco.

Material and methods

Cloning of WRKY gene

Total RNA was extracted from *D. nobile*. First-strand cDNA was synthesized with first-strand cDNA synthesis kit (TransGen, Beijing, China) and used for PCR. Degenerate PCR primers were designed based on WRKY con-

served amino acid sequences. Primers sequences were: 5'-ATG GC(C/T) GT(C/T) GAT CT(A/C) ATC GG(A/C) TAC-3' (forward), 5'-TCA GAT C(A/T)C GCG (G/T)TG (A/G)G(G/T) (A/G)TC (A/G)A (A/G) GAC GA-3' (reverse). PCR conditions were: 94 ℃ for 3 min, 30 cycles of 94 ℃ for 30 s, 60 ℃ for 30 s, 72 ℃ for 90 s; and finally 72 ℃ for 10 min. The resulting PCR product was cloned and sequenced. The nucleotide sequence of the full-length cDNA was analyzed by using blast search of the GenBank Database at NCBI.

Plasmid construction

To construct vector, PCR was used to introduce *Sac*I and *Bam*HI restriction enzyme sites at 5' and 3' ends of the sequence of the *DnWRKY11*. The gene fragments were cloned into pEASY-T1 vector (TransGen, Beijing, China), sequenced and then moved into the *Sac*I/*Bam*HI sites of pSN1301 vector, resulting in plant expression vectors of pSN1301-*DnWRKY11*.

Tobacco transformation

The plant expression vectors were introduced into *Agrobacterium tumefaciens* EHA105 by electroporation. Consider the large size of *Dendrobium* seedlings, we did not choose *Arabidopsis* as receptor, and used *Nicotiana tabacum* cv. Huangmiaoyu as the gene transformation receptor to identify the function of *DnWRKY11* in response to abiotic stress. Tobacco leaf discs were transformed according to the methods of Wang et al. (2008). T1 seeds were collected, dried at 25 ℃, and sown on sterile media containing 80 μg mL^{-1} hygromycin to select the transformants. Surviving T1 plants were transferred to soil to set seeds (T2). T3 seeds were set the same way.

PCR analyses of transgenic plants

To confirm transgene insertion in transgenic tobacco, genomic DNA extraction from the transformants and PCR analysis were conducted. Two primers, GUS-5: 5'-GCA TGT TAC GTC CTG TAG AAA CCC-3' (forward), and GUS-3: 5'- CAA AGC CAG TAA AGT AGA ACG GT-3' (reverse), were designed to target the regions of the *GUS* report gene. Using tobacco genomic DNA as template, PCR was performed with a pre-denaturing condition of 94 ℃ for 3 min, 30 cycles of 94 ℃ for 30 s, 55 ℃ for 30 s, 72 ℃ for 80 s; and finally 72 ℃ for10 min.

Real-time PCR was carried out to analyze the expression of *DnWRKY11* in transgenic tobacco. Total plant RNA was extracted from T3 transgenic tobacco using Trizol reagent (TianGen, Beijing, China). PCR amplifications were performed three times for each RNA sample using primers of *DnWRKY11*P1 (5'-TCA ACG ACA ACG GGA ACG-3') and P2 (5'-ATG GAG GGC GGA GGA AGG A-3'). To verify the quality of cDNAs from different tissue samples, actin cDNA was amplified as an internal control using primer pairs P3 (5'-ATG GCG GAT GGG GAG GAC ATT-3') and P4 (5'-TTA GAA GCA TTT GCG GTG GAC-3') based on *N. tabacum* actin mRNA (GenBank accession No. AB158612.1). Real-time PCR amplification conditions consisted of an initial denaturizing step at 94 ℃ for 3 min, followed by 40 cycles of 20 s at 94 ℃, 20 s at 54 ℃, and 23 s at 72 ℃, with a fluorescence detection at 80 ℃ for 2 s.

Abiotic stress tolerance analysis

Seeds from wild type and T3 *DnWRKY11* transgenic tobacco were placed on 1/2 MS agar plates supplemented with distilled water or different concentrations of 200 mM NaCl and 200 mM mannitol, and cultured in a greenhouse (25 ℃, 16 h of light and 8 h of darkness). The germination rate of seeds was measured. A seed was scored as germination if the radicle completely penetrated the seed coat. The root lengths and fresh weights of transgenic plants were also measured after 20 d treatment with 200 mM NaCl and 200 mM mannitol.

Assays of antioxidant enzyme activities and malondialdehyde content

To test the response of *DnWRKY11* transgenic plants to abiotic stresses, three-week wild type and T3 transgenic seedlings were placed in Hogland supplemented with distilled water or different concentrations of NaCl (200 mM) and mannitol (200 mM) for 24 h. The activities of catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), and the content of malonidialdehyde (MDA) in seedlings were measured according to the method of song et al. (2009).

Results

DnWRKY11 gene

In the present study, one novel *WRKY* gene was isolated from *D. nobile*, and designated as *DnWRKY11* (GenBank accession number: KF767689). The full-length *DnWRKY11* cDNA was 921 bp and encoded 306 amino. Compared with other plant WRKY proteins, the deduced *DnWRKY11* protein shared 97, 95 and 95% similarity with *BnWRKY11, AtWRKY11* and *VvWRKY11* from *Brassica napus, Arabidopsis thaliana* and *Vitis vinifera*, respectively. The deduced DnWRKY11 possesses one WRKY domains that contain the highly conserved amino acid sequence WRKYGQK and one Cys2His2 zinc finger motif at the position of 259–291 bp, and belongs to group 2 WRKY transcription factors.

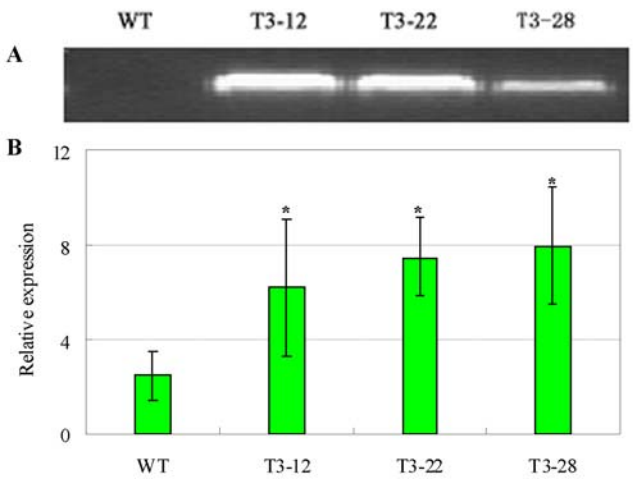

Fig. 1. PCR analyses of transgenic plants. A – PCR application of *GUS* gene confirmed the transgenic lines of 12, 22 and 28; B – Real-time PCR analyzed the expression of the *DnWRKY11* gene in transgenic tobacco lines of 12, 22 and 28.

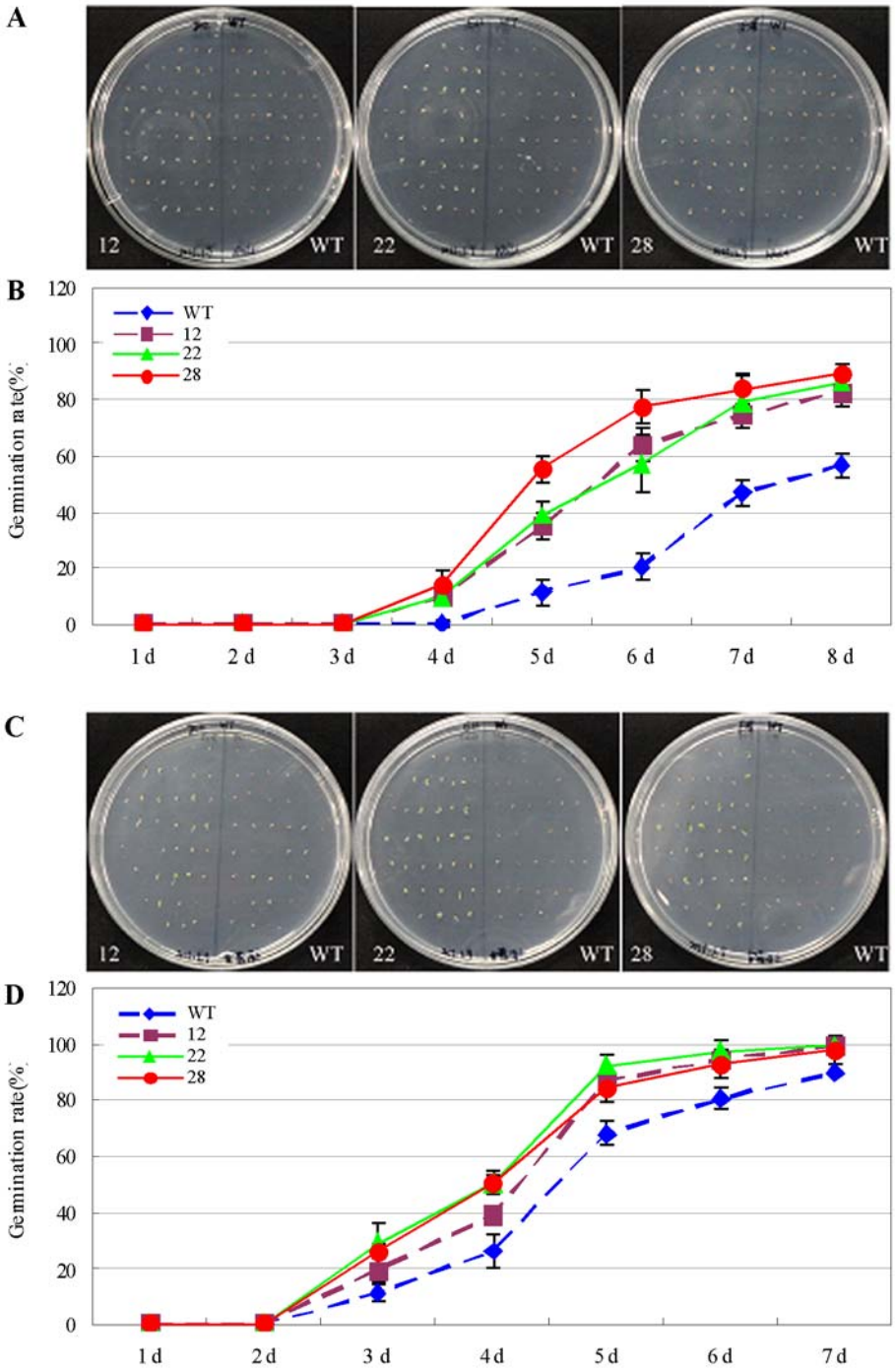

Fig. 2. Overexpression of *DnWRKY11* in tobacco enhanced transgenic plants seed generation under both salt and drought stress. Seeds germination phenotype of three transgenic lines under salt stress (A) and drought stress (C). Seeds germination rates of three transgenic lines under salt stress (B) and drought stress (D).

Expressing DnWRKY11 in tobacco

To investigate the potential function of *DnWRKY11* in response to abiotic stress, it was introduced into tobacco via *Agrobacterium*-mediated transformation. 46 independent *DnWRKY11* transgenic plants were obtained by kanamycin-resistant screening. Three independent transgenic lines of DnWRKY11-12, DnWRKY 11-22 and DnWRKY11-28, were confirmed as transgenic plants after GUS PCR application (Fig. 1A). And the *DnWRKY11* was highly expressed in DnWRKY11-12, DnWRKY11-22 and DnWRKY11-28 transgenic tobacco lines (Fig. 1B).

Overexpression of DnWRKY11 in tobacco enhanced plants salt and drought tolerance

Under non-stress conditions, no significant difference was observed in phenotype between wild type and the transgenic plants (data not shown). On 1/2 MS medium, the germination rates of wild type and *DnWRKY11* overexpression lines also showed no ob-

vious difference. About 50% seeds started to germinate at the second day, and nearly all the seeds germinated at the fourth day (data not shown). However, under salt stress condition, *DnWRKY11* transgenic seeds showed more stress tolerance than wild type. About 10, 10 and 14% seeds of DnWRKY11-12, DnWRKY11-22 and DnWRKY11-28 transgenic tobacco germinated at four day, but no seeds started to germinate in wild type. After eight day, about 86, 83 and 89% seeds of DnWRKY11-12, DnWRKY11-22 and DnWRKY11-28 transgenic tobacco germinated, only 56% seeds of wild type germinated (Fig. 2A, B). Similarly, under drought stress condition, *DnWRKY11* also enhanced transgenic seeds stress tolerance. At the three day, about 19, 29 and 26% seeds of DnWRKY11-12, DnWRKY11-22 and DnWRKY11-28 transgenic tobacco germinated, only 11% seeds of wild type germinated (Fig. 2C, D). After seven day, about 99, 100 and 98% seeds of DnWRKY11-12, DnWRKY11-22 and DnWRKY11-28 transgenic tobacco germinated, but the seeds germination rate of wild type was only 90% (Fig. 2C, D).

After three weeks under salt and drought stresses, the root length and fresh weight of wild type and transgenic tobacco seedlings were assayed, respectively. Under salt stress condition, the root length of DnWRKY11-12, DnWRKY11-22 and DnWRKY11-28 was 1.21, 1.52 and 1.49 cm, respectively, and the wild type was 0.93 cm. The fresh weight of DnWRKY11-12, DnWRKY11-22 and DnWRKY11-28 was 2.84, 3.11 and 3.14 mg, respectively, and the wild type only was 2.15 mg (Fig. 3A). Under drought stress, the root length of DnWRKY11-12, DnWRKY11-22 and DnWRKY11-28 was 2.22, 2.89 and 2.69 cm, respectively, but the wild type was only 1.43 cm. The fresh weight of DnWRKY11-12, DnWRKY11-22 and DnWRKY11-28 was 6.74, 9.01 and 9.54 mg, respectively, and the wild type was only 3.84 mg (Fig. 3B). These results suggested that overexpression of *DnWRKY11* in tobacco enhanced plant salt and drought stresses tolerance.

Overexpression of DnWRKY11 enhanced the antioxidant enzyme activities and reduced the content of MDA in tobacco

After 24 h under salt and drought stresses, *DnWRKY11* transgenic seedlings of DnWRKY11-12, DnWRKY11-22 and DnWRKY11-28 showed higher activities of CAT, POD and SOD than that in the wild type seedlings (Fig. 4). Moreover, lower content of MDA was observed in DnWRKY11-12, DnWRKY11-22 and DnWRKY11-28 transgenic lines than that in wild type seedlings (Fig. 5), indicated that overexpressing *DnWRKY11* in tobacco enhanced plant oxidation resistance against salt and drought stresses.

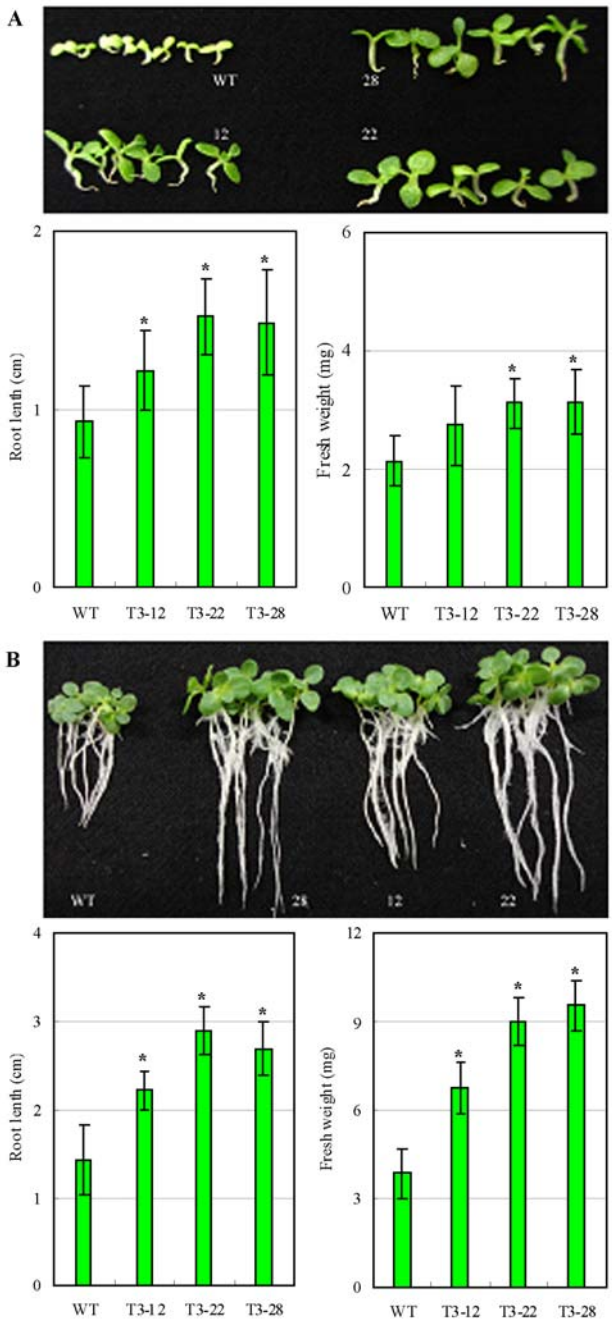

Fig. 3. Overexpression of *DnWRKY11* increased plants tolerance to salt and drought stress. A – Root length and fresh weight of transgenic plants under salt stress; B – Root length and fresh weight of transgenic plants under drought stress.

Discussion

WRKY protein is one of the major transcription factors regulating plant development and response to various biotic and abiotic stresses. Its functions were extensively explored in various plants, especially in rice and *Arabidopsis*. In rice, overexpression of *OsWRKY11* enhanced drought tolerance of transgenic seedlings, and increased survival rate of green plant parts (Wu et al. 2009). In *Arabidopsis*, overexpression of *OsWRK45*, *OsWRK72* and *GmWRKY54* enhanced salt and drought tolerance, overexpression of *AtWRKY25* and *AtWRKY33* increased salt tolerance, while overexpression of GmWRKY13 led to increased sensitivity to salt and drought stresses (Zhou et al. 2008; Qiu & Yu 2009; Song et al. 2010; Li et al. 2011). However, no data existed about the role of WRKY genes in mediating

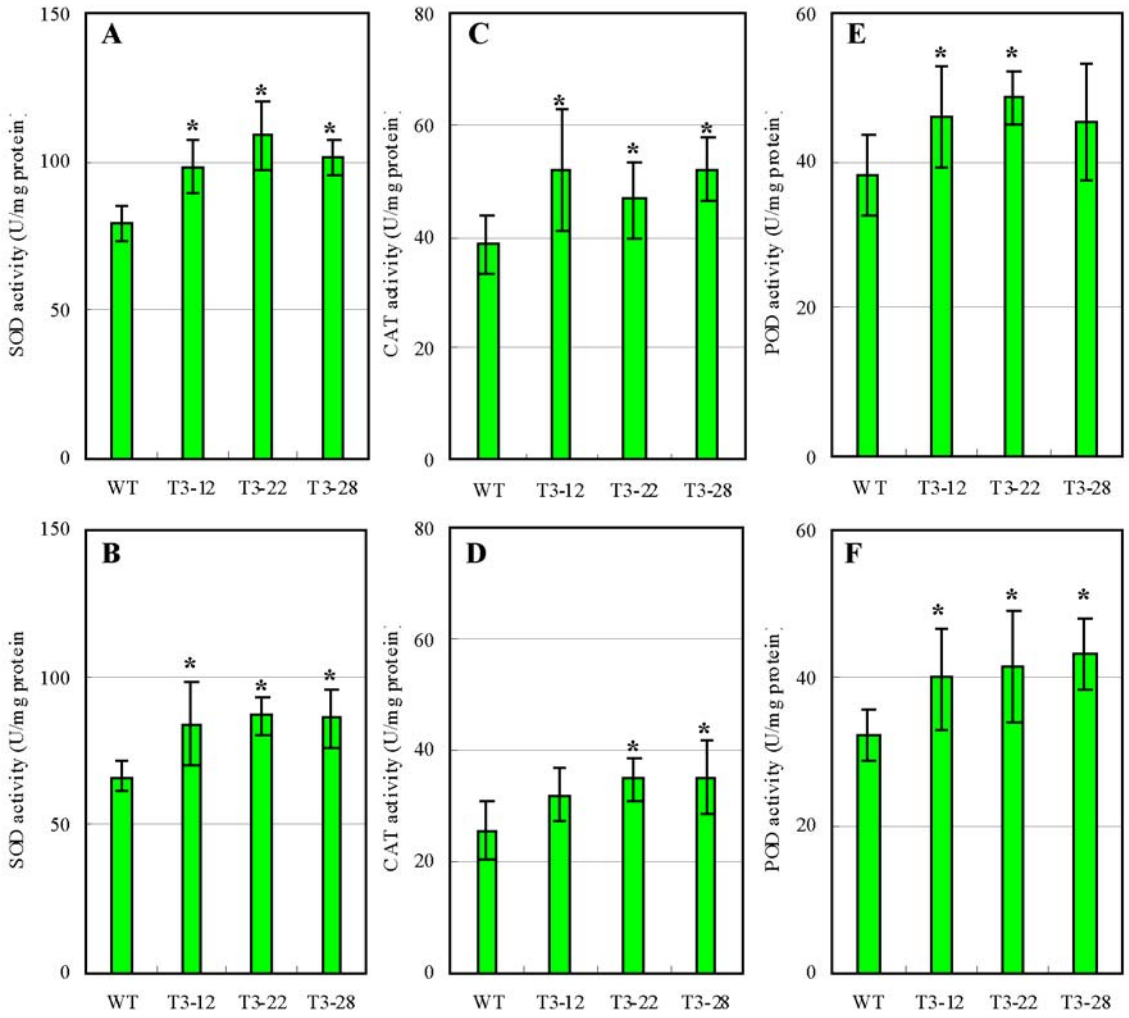

Fig. 4. Overexpression of *DnWRKY11* enhanced antioxidant enzyme activities and reduced the content of MDA of three-week transgenic tobacco seedlings after stress treated for 24 h. SOD activities in transgenic lines 12, 22 and 28 under salt stress (A) and drought stress (B); CAT activities in transgenic lines 12, 22 and 28 under salt stress (C) and drought stress (D); POD activities in transgenic lines 12, 22 and 28 under salt stress (E) and drought stress (F).

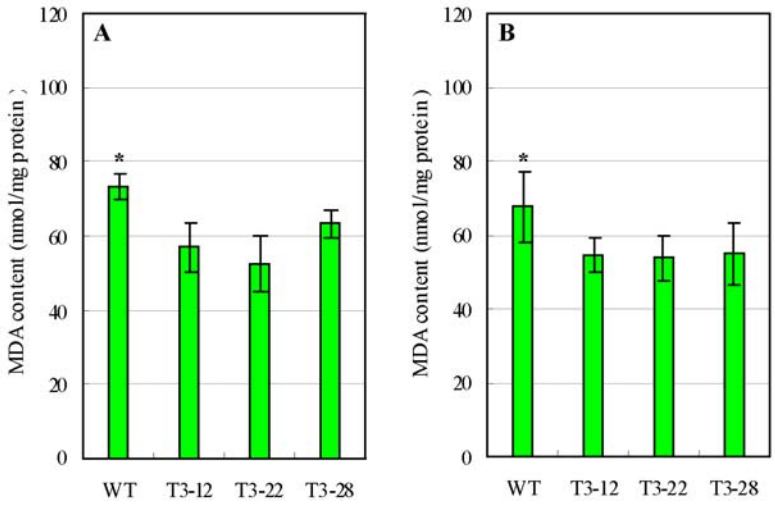

Fig. 5. Overexpression of *DnWRKY11* reduced the content of MDA of three-week transgenic tobacco seedlings after stress treated for 24 h. MDA content in transgenic lines 12, 22 and 28 under salt stress (A) and drought stress (B).

Dendrobium seedlings defense responses to environmental changes or abiotic stresses. In the present study, a novel *DnWRKY11* gene was isolated from *D. nobile*. Its deduced protein contains one typical WRKY domains

WRKYGQK and one Cys2His2 zinc finger motif, and belongs to group 2 WRKY transcription factors. To explore the function of *DnWRKY11* in *Dendrobium* defense responses to abiotic stress, it was overexpressed in tobacco.

In cereals, a-amylase enzymes are gibberellin (GA)-inducible and abscisic acid (ABA)-repressible proteins, which involve in seed germinating and post-germinating growth by hydrolyzing starch. In wild oat, two WRKY transcription factors, ABF1 and ABF2, have been found to bind to W boxes in the promoter of the a-amylase gene*a-Amy2,* and were related to the seed germination (Rushton et al. 1995). In rice, OsWRKY51 and OsWRKY71 were key regulators mediating the crosstalk of GA and ABA in aleurone cells and embryos, and were found to encode repressors of the rice *RAmy1A* a-amylase and the barley *Amy32b* a-amylase genes (Zhang et al. 2004; Xie et al. 2005, 2006). OsWRKY24, a novel type of transcriptional repressor, were found to inhibit both GA and ABA signaling in aleurone cells by repressing the expression of the β-glucuronidase gene driven by the GA-inducible Amy32b α-amylase promoter (Amy32b-GUS) and the ABA-inducible HVA22 promoter (HVA22-GUS) (Zhang et al. 2009). In addition, in *Arabidopsis*, T-DNA insertion mutants showed the possible role of AtWRKY2 as a negative feedback regulator of ABA-mediated arrest of seed germination and post-germination growth (Jiang & Deyholos 2009). In the present study, as shown in Fig. 2, under drought and salt stresses, the seeds of *DnWRKY11* transgenic tobacco showed higher germination rate than the wild type, indicated that the *DnWRKY11* gene played positive role in enhancing seeds tolerance to drought and salt stresses, and did not involve in ABA- or GA-mediated arrest of seed germination.

An appropriate intracellular balance between ROS generation and scavenging exists in all cells. This redox homeostasis requires the efficient coordination of reactions in different cell compartments and is governed by complex signal transduction pathways. Plants possess an array of antioxidant enzymes that can protect cells from oxidative damage. These enzymes include SOD, CAT and POD, which work together with other enzymes to promote the scavenging of ROS (Hernandez et al. 2001). SOD catalyzes the dismutation of O_2^- to H_2O_2 and O_2. CAT is present in the peroxisomes of nearly all aerobic cells (Dionisio-Sese & Tobita 1998). It can protect the cell from H_2O_2 by catalyzing its decomposition into O_2 and H_2O (Foyer & Noctor 2000). POD is widely distributed in all higher plants and protects cells against the destructive influence of H_2O_2 by catalyzing its decomposition through the oxidation of phenolic and enodiolic cosubstrates (Asada 1992; Borsani et al. 2001). In the present study, compared with the wild type, higher activities of SOD, CAT, POD (Fig. 4) and lower content of MDA (Fig. 5) were detected in the transgenic tobacco seedlings, suggesting that overexpression of *DnWRKY11* enhanced the activities of antioxidant enzymes and by which promoted growth of *DnWRKY11* transgenic seedlings under drought and salt stresses (Fig. 3), indicated the positive role of *DnWRKY11* in activating plant resistance to abiotic stresses.

In conclusion, overexpression of *DnWRKY11* in tobacco enhanced seeds and seedlings tolerance to salt and drought stresses, demonstrated its positive role in regulating plant abiotic stress response. This study provided a potential gene for improving environmental stress tolerance of *Dendrobium* seedlings.

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