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ORIGINAL ARTICLE

Overexpression of *NbWRKY79* enhances salt stress tolerance in *Nicotiana benthamiana*

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Abstract

Main conclusion **A WRKY transcription factor encoding NbWRKY79, which was induced by salt and ABA was isolated from *Nicotiana benthamiana*. Overexpression of NbWRKY79 resulted in enhanced tolerance to salt stress.**

In plants, there are many families of transcriptional regulators, one of which is WRKY transcription factors, which have a significant effect on the adaptation to abiotic stress. Nevertheless, most of the mechanisms in plant to which WRKY genes are concerned to tolerate salinity are still undiscovered. In this study, a gene from *Nicotiana benthamiana*, NbWRKY79, was isolated and characterized. NbWRKY79 contains one WRKY domain and localizes in the nucleus. *NbWRKY79* was induced after the plant was exposed to salinity and abscisic acid (ABA). The overexpression of *NbWRKY79* remarkably enhanced the tolerance of tobacco plant to salinity, which was confirmed when the plant growth, root growth and chlorophyll content were studied through physiological analyses. The sensitivity to ABA-mediated seed germination and seedling root growth of *NbWRKY79* transgenic lines were increasing. In addition, the reduced accumulation of reactive oxygen species and malondialdehyde content as well as an increase in proline content and the activity of antioxidant enzymes such as superoxide dismutase, guaiacol peroxidase, catalase and ascorbate peroxidase during salt treatment were found, and these indicated that the transgenic plants enhanced tolerance to oxidative stress when comparing to the wild-type plants. Furthermore, it was found that ABA content and transcript levels of ABA-inducible genes, including *NbAREB*, *NbDREB* and *NbNCED*, were significantly increased in the salt stress conditions. These results recommend that *NbWRKY79* holds the key to salt stress response.

Keywords Abscisic acid · *NbWRKY79* · *Nicotiana benthamiana* · Overexpression · Reactive oxygen species · Salt stress

Abbreviations

ABA	Abscisic acid
APX	Ascorbate peroxidase
CAT	Catalase
DAB	3,3′-Diaminobenzidine
GUS	β-Glucuronidase
MDA	Malondialdehyde
NBT	Nitroblue tetrazolium
POD	Guaiacol peroxidase
qPCR	Quantitative-PCR
ROS	Reactive oxygen species
Semi qPCR	Semi-quantitative-PCR
SOD	Superoxide dismutase
YFP	Yellow fluorescent protein

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Introduction

Abiotic stress, one of which is salinity, is the principal cause of crop yield loss worldwide and adversely affects plant growth and productivity. Plants are sessile, so they must continually exhibit high-level tolerance against stresses from the environmental surroundings (Mittler 2006). To survive and grow under fluctuating and stressful environmental conditions, plants have evolved intricate mechanisms to recognize external signaling networks and to be evidence of adaptive responses at the physiological, biochemical, and molecular levels. In these responses, many genes involved in stress response are regulated to not only protect plant cells against environmental stress via the generation of important proteins and enzymes of metabolism but also control signaling pathways and gene expression. Transcription factors (TFs), key components of the signaling pathways, are present at the upstream region, which can regulate downstream genes that respond to stress and play an important role in supporting stress tolerances (Nakashima et al. 2009; Rushton et al. 2010). WRKY proteins are one of the largest families of plant transcription factors (Ülker and Somssich 2004). The WRKY transcription factors are named after the term WRKY domain, which has a highly conserved amino acid sequence, WRKYGQK, at the N terminus and an atypical zinc-finger structure at the C terminus (Eulgem et al. 2000; Eulgem and Somssich 2007; Rushton et al. 2010). According to the phylogenetic data of WRKY domains and the pattern of the zinc-finger-like motif, WRKY proteins are grouped into I, II, and III (Eulgem et al. 2000). Group II is subdivided into IIa, IIb, IIc, IId, and IIe (Rushton et al. 2010). There have been 104 and 74 *WRKY* genes identified in rice and *Arabidopsis thaliana*, respectively (Eulgem et al. 2000). All WRKY proteins that were characterized have one or two WRKY domains. The plant responses to biotic and abiotic stresses were controlled by WRKY proteins attaching to W-box elements (TTGACC/T) in the target gene promoters to up- or down-regulate them at the transcription level (Eulgem et al. 2000; Eulgem and Somssich 2007; Ren et al. 2010; Rushton et al. 1995). Many studies concentrated on defense-responsive WRKY proteins (Birkenbihl et al. 2012; Journot-Catalino et al. 2006; Li et al. 2006; Matsushita et al. 2013; Qiu and Yu 2009; Xu et al. 2006); however, a few studies have verified the roles of these proteins in abiotic stress responses (Yan et al. 2014). Recent evidence suggests that WRKY genes possibly participate in the positive and negative regulation of hormones and the abiotic stress response such as salinity, drought, osmotic, and heat stress (Chen et al. 2012; Li et al. 2013; Liu et al. 2014; Miller et al. 2008; Ren et al. 2010; Rushton et al. 2012). Nevertheless, which of the specific WRKY genes are specifically associated with abiotic stress tolerance are still unclear.

In this study, we isolated a novel group II *WRKY* gene, *NbWRKY79*, from *Nicotiana benthamiana* plant and characterized its function in response to salt stress. In comparison with wild-type plants, NbWRKY79 transgenic plants exhibited enhanced salt tolerance with remarkably increased levels of proline content, reduced levels of MDA, lower ROS content, higher activity of ROS-scavenging isoenzymes, and higher ABA content.

Materials and methods

Plant material, growth conditions, and treatments

Wild-type and transgenic *N. benthamiana* seeds were surface sterilized with a solution of 20% commercial bleach (2% sodium hypochlorite) for 10 min, and rinsed three times with sterile distilled water. For root growth measurements, 5-day-old seedlings cultured on one-half strength solid MS medium (Duchefa Biochemie, Haarlem, Netherlands) were transferred to one-half strength MS medium supplemented with different concentrations of NaCl (0, 200 and 400 mM) or ABA (0, 25 and 50 μM). The tobacco seedlings were cultured at 25 ± 2 °C with cool white fluorescent light (100 μE s^{-1} m^2 light intensity) under long-day condition (16 h light/8 h dark). Plates were oriented vertically with seedlings kept upside down. Salt stress was imposed on the soil-grown plants by treating with 100 mM NaCl daily. Three replicates were performed for each experiment.

Expression constructs

Homology search using an *N. benthamiana* Blast tool at the database Sol genomics network Boyce Thompson Institute for Plant Research (SGN, http://solgenomics.net/tools/blast/index.pl) was performed for isolation of the full-length cDNA sequence of *NbWRKY79*. The expressed sequence tags (ESTs) with accession number Niben.v0.4.2.Scf781 in *N. benthamiana* were obtained using *Nicotiana tabacum NtWRKY79* as the query nucleotide sequence. The design of the forward and reverse primers spanning the open reading frame of *NbWRKY79* was based on the ESTs. PCR amplifications were performed with *pfu* polymerase (Promega, Madison, WI, USA) in 1× reaction buffer, 0.1 mM dNTPs, 0.2 μM each of primers, and templates, under the following conditions: 2 min at 95 °C, followed by 30 cycles of 95 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min, and a final polymerization at 72 °C for 10 min. The resulting full-length *NbWRKY79* was cloned into the pENTR-D TOPO

(Invitrogen, Carlsbad, CA, USA) and subcloned into plant destination vector pH7WG2 (Plant Systems Biology, Ghent, Belgium) by LR recombination reaction (Invitrogen, Carlsbad, CA, USA). The integrity of the constructs was confirmed by sequence analysis.

For subcellular localization assay, *NbWRKY79* in pENTR-D TOPO (Invitrogen, Carlsbad, CA, USA) was subcloned into plant destination vector pSITEII-4C1 (Martin et al. 2009) by LR recombination reaction (Invitrogen, Carlsbad, CA, USA) to generate Venus fusion proteins.

For promoter analysis, promoter region (2.0 kb) upstream the start codon of *NbWRKY79* gene was amplified using *N. benthamiana* genomic DNA as template and the following primers: forward, 5′-CCAACCACTG GAAGCTTTCA-3′ and reverse, 5′- TCCGAGGAAATAT ATATTGAAG-3′. The *NbWRKY79* promoter fragment was cloned into the pENTR-D TOPO (Invitrogen, Carlsbad, CA, USA) and subcloned into plant destination vector pHGWFS7 (Plant Systems Biology, Ghent, Belgium) to generate Pro*NbWRKY79*-β-glucuronidase (GUS) reporter construct. The transgenic lines were confirmed by PCR using promoter-specific primers.

Transient expression of fluorescent proteins in tobacco leaves

The *Agrobacterium* strain GV3011 harboring the YFP:*NbWRKY79*-derived construct was used for the transient experiment as described (Bhaskar et al. 2009). The *Agrobacterium* were grown at 28 °C in Luria–Bertani medium containing 50 mg l^{-1} of kanamycin. Overnight cultures were centrifuged and the cell pellets were reconstituted in infiltration medium (10 mM $MgCl_2$, 10 mM MES and 200 μM acetosyringone) to an optical density at 600 nm of 2, and then left at room temperature for 3–4 h. The cell suspensions of *Agrobacterium* strains harboring the YFP:*NbWRKY79* were infiltrated into leaves of 2–4-week-old *N. benthamiana* plants using a needleless syringe. Four days after the infiltration, the abaxial epidermis of infiltrated tobacco leaves was assayed for fluorescence through confocal laser-scanning microscopy (LSM780, Carl Zeiss, Germany). Nuclei were stained by adding one drop of 4′,6-diamidino-2-phenylindole (DAPI) staining solution (1 μg ml^{-1}) to epidermal cells. Epidermal cells were set in the dark condition for 15 min, and DAPI fluorescence was visualized by UV illumination.

Plant transformation

Pro*NbWRKY79*-β-glucuronidase (GUS) reporter and *NbWRKY79* constructs were introduced into *A. tumefaciens* strain GV 3101 by electroporation. *N. benthamiana* transformation was carried out using the leaf-dish method described by Horsch et al. (1985). The kanamycin-resistant plantlets regenerated from transformed callus were transferred to Vriezenveen 70L substrates (Potgrond Vriezenveen bv, Westerhaar, Netherlands) and grown in a growth chamber at 27 °C under a 16-h light and 8-h dark regime. Young plantlets in the pots were covered with clear plastic cups to retain moisture, and hardened off gradually by removing the cups. The seeds of 20 independent lines were harvested from these primary transformants. Ten T1 transgenic lines were obtained using kanamycin selection and PCR. The transgenic T2 lines were retained for further experiments.

Isolation and purification of total RNA

Total RNA was isolated from leaf tissues of 3-week-old *N. benthamiana* plants using a RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany), treated with DNase I (QIAGEN) to remove genomic DNA contamination, then purified and concentrated with a RNeasy MinElute Cleanup Kit (QIAGEN, Hilden, Germany). The yield and purity of total RNA was measured spectrophotometrically (NanodropND 2000, Nanodrop technologies, Wilmington, DE, USA). RNA samples of 2 μg μl^{-1} with high purity ($OD_{260/280}$ and $OD_{260/230}$ >2) were employed for further analysis.

Semi-quantitative PCR analysis

To assess the expression of *NbWRKY79* under salt stress or exposure to ABA, 3-week-old tobacco seedlings were treated with 200 mM NaCl or 50 μM ABA. The total RNA from stressed samples was isolated at five different time points and up to a maximum time of 48 h after imposition of salt or ABA treatment. Then cDNA was prepared. Semi-qPCR analysis was performed using *NbWRKY79* forward primer 5′-CTCCTAACGGTTCAGATGATGG-3′ and *NbWRKY79* reverse primer 5′-GATGCAGAGGATGTTCTGTCC-3′. The *N. benthamiana elongation factor 1-α* (*NbEF1-α*) gene was used as an internal loading control. Biological and technical replicates of each sample were applied for analysis.

The expression levels of combination of native endogenous *NbWRKY79* and overexpressing *NbWRKY79* in the wild-type plants or the transgenic lines were identified through semi-qPCR using the following primers: forward, 5′-CTCCTAACGGTTCAGATGATGG-3′ and reverse, 5′-GATGCAGAGGATGTTCTGTCC-3′. The forward primer 5′-CTCCTAACGGTTCAGATGATGG-3′ and the reverse primer 5′-CATGTACACACACA CAACGGG-3′, which were derived from the 3′-untranslated region of the *NbWRKY79* mRNA, were used to analyze the expression level of the native endogenous *NbWRKY79* mRNA in the transgenic lines.

Real-time PCR analysis

The cDNA was the result of the reverse transcription using 1 μg of total RNA in 20 μl of reaction according to the manufacturer's instructions (Promega, Madison, WI, USA). Quantitative real-time PCR (qRT-PCR) reactions were conducted to quantify the transcript levels of the selected genes using the StepOnePlus Real Time PCR system (Applied Biosystems, Foster City, CA, USA) and iQ™ SYBR® Green Supermix (Bio-RAD, Hercules, CA, USA). The *NbAREB1* (ABA-responsive element binding), *NbDREB* (dehydration-responsive element binding), and *NbNCED* (nine-cis-epoxycarotenoid dioxygenase) primers were used to determine *NbAREB*, *NbDREB*, and *NbNCED* expression levels under salt stress in this study by following the method of Yan et al. (2014) (Table S). The thermal cycles consisted of an initial 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Following PCR, a melting curve analysis was accomplished. Relative quantification of specific mRNA levels was analyzed using the cycle threshold (Ct) $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). All reactions were made in triplicates of three independent samples. To normalize the relative expression levels, *Nicotiana benthamiana β-actin* genes were used as the reference gene. All statistical analyses to identify the significant difference of relative expression of individual genes between control and NaCl-treated samples were made by one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range test, using the SPSS software (version 11.5 for Windows; SPSS, Inc., Chicago, IL, USA). The different letters were the means and comparisons with $p < 0.05$ were considered significantly different.

Histochemical detection of GUS activity

Expression using GUS assays in transgenic *N. benthamiana* plants containing reporter constructs was first examined in 20 independent T1 plants to determine lines with a consistent expression pattern. The T2 generation was analyzed in detail. For histochemical detection of GUS activity, seedlings or explants were treated as described by Jefferson et al. (1987). Plant tissues were cleaned up after GUS staining with ethanol and acetic acid (3:1) treatment to remove chlorophyll before visual examination using a Leica MZ12.5 stereomicroscope (Leica, Wetzlar, Germany).

Measurements of photosynthesis parameter, proline content and MDA content

The Portable Photosynthesis System CIRAS-2 was used to determine the photosynthetic rate of the wild-type plant and the transgenic lines as described by Li et al. (2014b). For the measurement of proline and MDA content, leaves of the wild-type plant and the transgenic lines were collected after 4 weeks of salt treatment. MDA content determination was done using the thiobarbituric acid (TBA) reaction as described by Ma et al. (2013). Proline content measurement was performed by the ninhydrin reaction method as described by Bates et al. (1973). All the assays were conducted at least three times per sample. The results were subjected to ANOVA using SPSS version 11.5 (SPSS, Inc., Chicago, IL, USA) and comparisons with $p < 0.05$ were considered significantly different.

In situ detection of reactive oxygen species

Superoxide anion and H_2O_2 levels in *N. benthamiana* leaves treated with 200 mM NaCl were visually detected by treating with nitroblue tetrazolium (NBT; Sigma, St. Louis, MI, USA) and 3,3′-diaminobenzidine (DAB; Sigma, St. Louis, MI, USA), respectively (Thordal-Christensen et al. 1997).

Leaf discs salt stress bioassay and estimation of chlorophyll

For the salt treatment, leaf discs (9 mm in diameter) were segregated from healthy, fully expanded leaves of 3-week-old wild-type plants and *NbWRKY79*-overexpressing lines. The discs were floated in liquid MS medium supplied with different concentrations of NaCl (0, 100, 200 and 400 mM) for 4 days in a growth chamber. Growth chamber conditions were maintained at 27 °C and 70% humidity under long-day regime (16 h white light/8 h dark). The effect of salt stress on leaf discs was estimated by quantifying the chlorophyll content from 10 leaf discs per wild-type plants and *NbWRKY79*-overexpressing lines. The leaf discs were submerged into liquid nitrogen and ground using the TissueLyser LT (QIAGEN, Hilden, Germany). The extract of chlorophyll was collected from plant materials by homogenizing leaf discs in 1-ml aliquots of 80% (v/v) chilled acetone, and the absorbance of the extract was measured. The significant difference between the samples at $p < 0.05$ was subjected to ANOVA using SPSS version 11.5 (SPSS, Inc., Chicago, IL, USA).

Protein extraction and antioxidant enzyme activity assays

After treatment with 200 mM NaCl, protein of tobacco leaves was extracted. Approximately 1 gam of tobacco leaves was ground in protein extract buffer (50 mM Tris–HCl, pH 7.4, 250 mM sucrose, 10 mM NaF, 10 mM Na_3VO_4, 1 mM sodium-tartrate, 10% v/v glycerol, 50 mM

$Na_2S_2O_5$, 1% SDS, 1 mM PMSF). Homogenized leaf tissue was centrifuged at 15,700*g* for 10 min at room temperature and the supernatant was collected. Protein content was identified by the BioRad Dc Protein Assay (Bio-Rad, Hercules, CA, USA) at OD_{750}.

Isoenzyme of SOD, CAT and POD were indicated as described by Wang and Yang (2005). APX in-gel activity was assayed using the method of Mittler and Zilinskas (1993). POD activity in the leaves was assayed according to Chen et al. (2002). The antioxidant enzyme activities of SOD, CAT and APX were determined as per the method of Kumar et al. (2008). Each assay was replicated at least three times per sample.

Quantification of endogenous ABA content

To determine the ABA accumulation under salt stress, 3-week-old tobacco plants were treated with or without 200 mM NaCl for one week. After treatment, the leaf samples were collected, weighed and ground in liquid nitrogen and homogenized in 80% (v/v) methanol containing 1 mM butylated hydroxytoluene overnight at 4 °C to extract the ABA. After centrifugation at 4000 g for 20 min at 4 °C, the supernatant was collected and then loaded onto a Sep-Pak C_{18} cartridge (Waters, Milford, MA, USA) and dried in N_2. The eluate containing ABA was dissolved in 0.01 M phosphate-buffered saline, pH 7.5. The endogenous ABA content was analyzed using an ABA ELISA Kit (Mybiosource, San Diego, CA, USA) according to the manufacturer's instructions.

Results

Isolation of a full-length cDNA encoding NbWRKY79 from *N. benthamiana*

A full-length cDNA clone of 744 nt was isolated and termed as *NbWRKY79*. The nucleotide sequence of the *NbWRKY79* cDNA contained an open reading frame, which encodes for a protein of 247 amino acids. The molecular mass of the predicted polypeptide was 27.68 kDa. The NbWRKY79 protein sequence showed significant homology to *Nicotiana tabacum* NtWRKY79, *Solanum tuberosum* StWRKY2-like, and *Nicotiana tomentosiformis* NtoWRKY33, with 88, 54, and 81% identity, respectively. Due to the high degree of homology with *Nicotiana tabacum* NtWRKY79, this new WRKY protein was designated as NbWRKY79. The gene sequence encoding NbWRKY79 was deposited in GenBank (accession no. KR559678). Sequence analysis indicated that the deduced protein contains a single WRKY DNA-binding domain (WRKYGQK) and a zinc-finger-like motif C2-H2 ($C\text{-}X_4\text{-}C\text{-}X_{23}\text{-}H\text{-}X\text{-}H$) (Fig. S1), which categorized this protein as a member of group II WRKY superfamily (Eulgem et al. 2000).

Expression of *NbWRKY79* is induced by salt stress and ABA

To analyze the expression profile of *NbWRKY79*, semi-quantitative RT-PCR was used. *NbWRKY79* was found to be up-regulated under salt and ABA treatments (Fig. 2). For salt stress, *NbWRKY79* transcription was induced within 6 h of NaCl treatment. The transcript level was significantly increased after 12 h of NaCl stress to seedlings and the level was continuously increased until 48 h (Fig. 1a). In the case of ABA treatment, the expression of *NbWRKY79* was increased and the highest level was reached at 6 h, we thus speculated that *NbWRKY79* is an ABA-sensitive gene (Fig. 1b). According to the result above, *NbWRKY79* may be concerned to salt stress and ABA adaptation.

Subcellular localization of NbWRKY79

To identify the subcellular localization of NbWRKY79 protein, we fused *YFP* with *NbWRKY79* under regulation of the *Cauliflower* mosaic virus 35S promoter (Fig. S2). In transient expression assay, *YFP-NbWRKY79* fusions and the control empty-vectors were introduced into leaf epidermal cells of *N. benthamiana* by *Agrobacterium*-mediated infiltration. The cells were dyed by DAPI to reveal nuclei and then investigated by a fluorescence microscopy. As a result, YFP-NbWRKY79 was shown to localize in the nucleus whereas the control YFP was distributed throughout epidermal cells (Fig. 3a). These findings suggest that NbWRKY79 is a nuclear protein.

Expression analysis using *NbWRKY79* promoter–GUS fusions in transgenic plants

To further elucidate whether the different roles of *NbWRKY79* in stress response correlate with differential expression patterns, GUS reporter gene fusions (Pro*NbWRKY79*:*GUS*) were constructed using approximately 2.0 kb of the upstream regulatory region for *NbWRKY79* gene (Fig. S3). We introduced the construct harboring the *NbWRKY79* promoter fused to the *GUS* reporter gene into the wild-type *N. benthamiana* plants. Non-transformed plants were also applied as a negative control. In consequence, no expression of GUS gene was observed in any tissues of non-transformed plants (Fig. S4) but the expression was detected in primary root and lateral root of plants transformed with Pro*NbWRKY79*:*GUS* (Fig. 3b). Next, we analyzed the inducible patterns of GUS

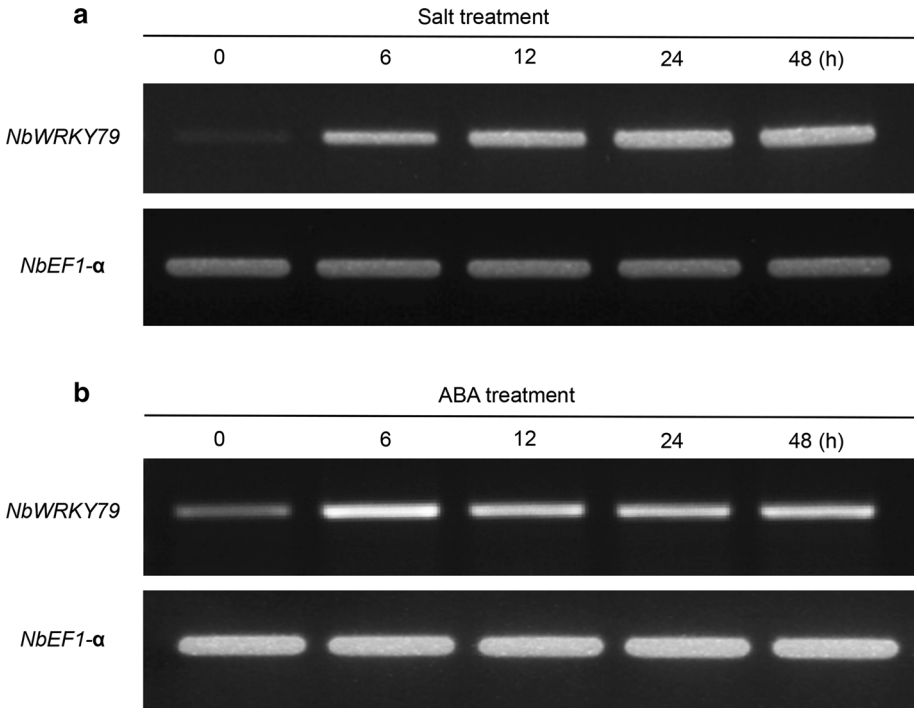


Fig. 1 Expression pattern of *NbWRKY79* after NaCl and ABA treatment to *N. benthamiana* seedlings. *NbEF1-α* was used as internal loading control. The experiments were repeated with three independent samples and similar results were obtained

expression in *N. benthamiana* plants in response to salt stress. Salt treatments strongly induced GUS activity in primary roots, lateral roots, cotyledons, true leaves, and shoot apical meristem (Fig. 3c). In addition, GUS activity was detected in petals, sepals, anthers, stigma, and ovary in the transformed plants under salt stress (Fig. 3d). These results indicate that *NbWRKY79* has differential salt stress responses.

Overexpression of *NbWRKY79* exhibits enhanced tolerance to salt stress

Transgenic plants overexpressing *NbWRKY79* were produced to further examine the role of NbWRKY79. Two representative lines, *O.E1* and *O.E2,* were selected from ten T1 transgenic lines for further analyses by semi-quantitative PCR (Fig. S5a). Semi-quantitative RT-PCR results made manifest that all transgenic plants expressed a high level of *NbWRKY79* mRNA compared with the wild-type plant in normal condition (Fig. S5b). The expression level of native endogenous *NbWRKY79* gene remained unaltered in the wild-type plants and the transgenic lines. All transgenic lines were indistinguishable from the wild-type plants in both morphology and development throughout their life cycle (Fig. S4). Then their T2 homozygous line was selected for further analyses.

To ascertain the possible effects of *NbWRKY79* overexpression on salt tolerance, 5-day-old wild-type and *NbWRKY79*-overexpressing seedlings cultivated on MS medium were transferred to MS medium supplemented with 0 to 400 mM of NaCl. The growth of seedlings was monitored by measuring their root length after 5 days (Fig. 3a, b). Under control conditions, in the absence of both salt treatments, roots of wild-type seedlings and *NbWRKY79*-overexpressing lines grew at the same rate. At 200 mM NaCl, the root growth rate of wild-type plants and *NbWRKY79*-overexpressing lines was slower than the rate of those in the absence of NaCl. In comparison, seedlings of *NbWRKY79*-overexpressing lines grew faster than wild-type seedlings and had longer primary roots. The growth of roots in *NbWRKY79*-overexpressing lines was inhibited at 400 mM NaCl. The wild-type plants did not grow at this concentration.

To further examine the effect of *NbWRKY79* overexpression on vegetable growth stage, soil-grown wild-type plants and *NbWRKY79*-overexpressing lines were exposed to long-term high salinity. Three-week-old plants were irrigated with 200 mM NaCl solution for 4 weeks and physiological parameters were measured. The growth of the wild-type plants was more deferred than the growth of *NbWRKY79*-overexpressing lines (Fig. 3c). The wild-type lines had a severe signs of stress, particularly serious chlorosis and wilting, whereas *NbWRKY79*-overexpressing lines displayed mild symptoms. In addition, the transgenic lines had greater leaf area and photosynthesis rate than the wild-type plants (Fig. 3d, e). Compared with the wild-type plants, the transgenic lines exhibited remarkably higher levels of proline content (Fig. 3f) but lower levels of MDA content (Fig. 3g) under salt condition.

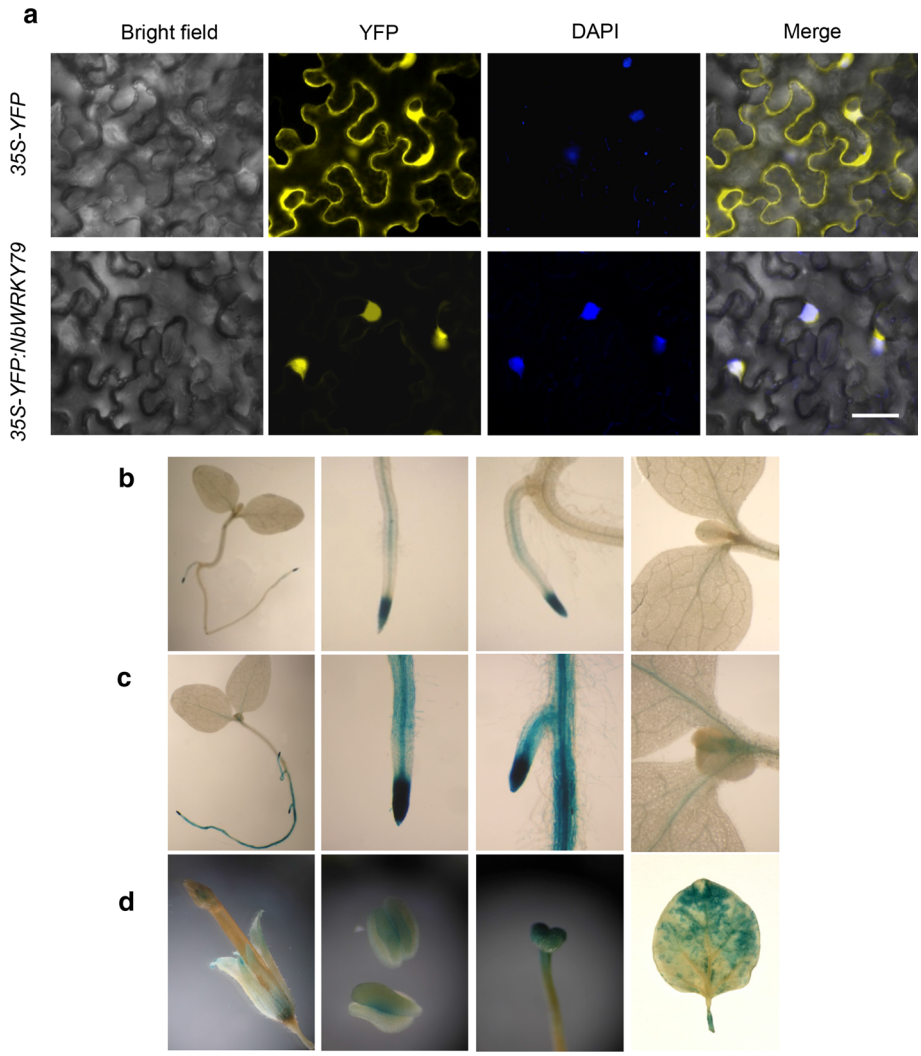
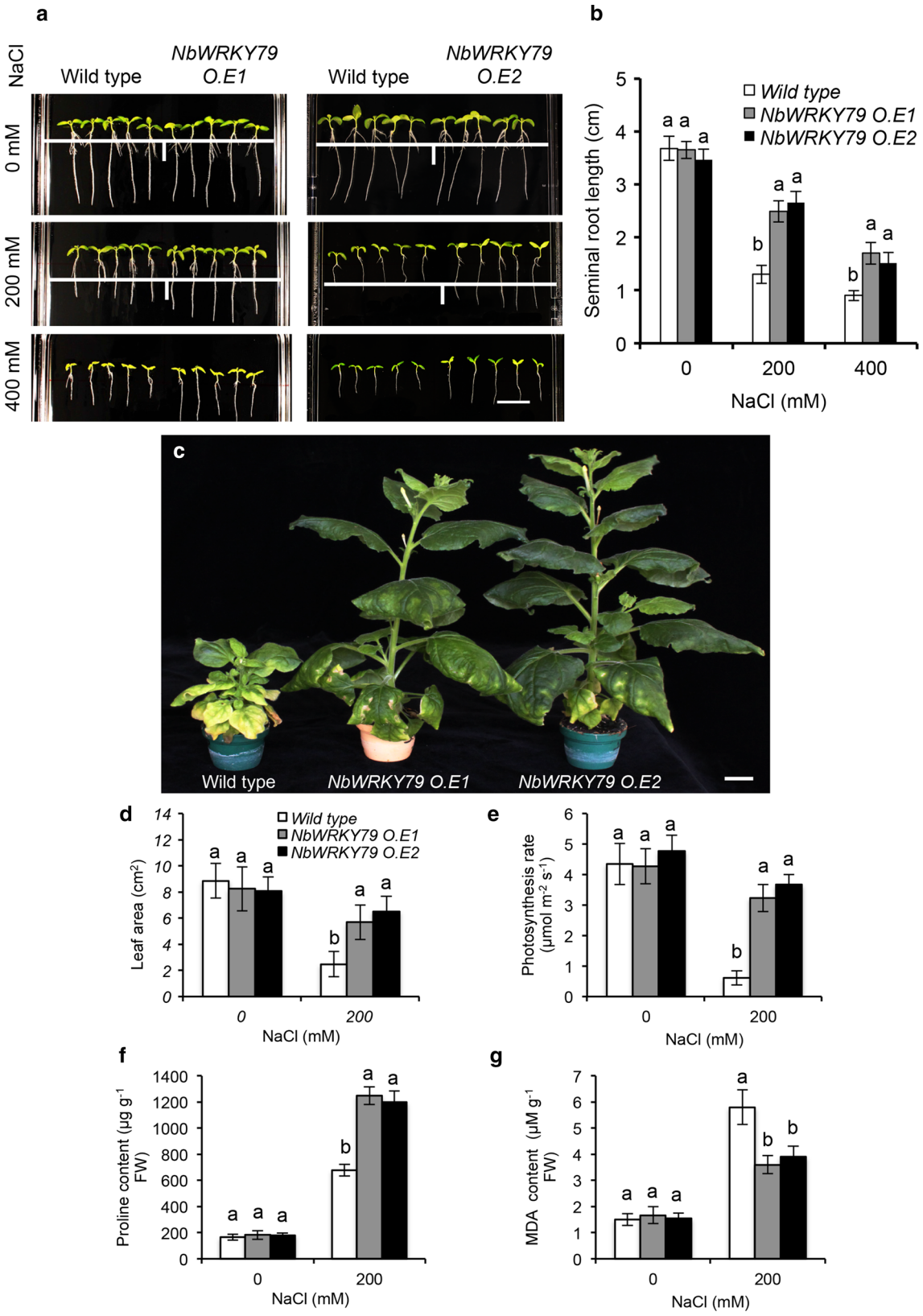


Fig. 2 Localization of NbWRKY79 in *N. benthamiana* epidermal cells and tissue-specific expression patterns of *N. benthamiana* plants transformed with an *NbWRKY79* promoter driven GUS reporter gene (p*NbWRKY79*::GUS construct) in response to salt stress. **a** YFP:*NbWRKY79* constructs were transiently expressed in epidermal cells of *N. benthamiana* by agro-infiltration. Epidermal cells were observed by CLSM. Merged, merging of YFP and bright field imaged; YFP, representing of YFP signals; DAPI, staining with DAPI indicated the localization of cell nuclei. White bars, 10 μm. GUS expression patterns in uninduced transgenic seedlings (**b**) and in transgenic seedlings treated with NaCl (**c**). Five-day-old transgenic seedlings were treated without salt stress (Murashige and Skoog medium) or for 24 h with 200 mM NaCl. In untreated transgenic seedlings, GUS activity was found in root tip, lateral root and along vascular bundles. In transgenic seedlings treated with NaCl, GUS activity was presented in cotyledons, root (root tip, lateral root and along vascular bundles). Ten seedlings at least were examined and typical results are presented. **d** GUS activity in sepals, petals, anthers, stigma, ovary and leaf of transgenic plants treated with NaCl. Ten samples at least were examined and typical results are presented

The salt stress tolerance of *NbWRKY79*-overexpressing lines was further investigated using leaf-disc assay. Leaf discs from 3-week-old wild-type plants and *NbWRKY79*-overexpressing lines were soaked in the MS solutions including various NaCl concentrations, ranging from 0 to 400 mM, for 4 days. As shown in Fig. 4a, bleaching was perceived to a more severe extent in the wild-type plants than in the transgenic lines under salt treatment. This result was further confirmed through measurement of the chlorophyll content in the leaf discs after NaCl treatment (Fig. 4b).

NbWRKY79 overexpression decreases salt stress-induced ROS production

So as to further research the effect of salt stress on *NbWRKY79*-overexpressing lines, accumulation of ROS, $O_2^{\cdot-}$ and H_2O_2, leaves of the wild-type plants and *NbWRKY79*-overexpressing lines during salt treatment was tested by ROS staining using NBT and DAB, respectively. High amounts of ROS were observed in the wild-type


a
NaCl
Wild type
NbWRKY79 O.E1
Wild type
NbWRKY79 O.E2
0 mM
200 mM
400 mM
b
Wild type
NbWRKY79 O.E1
NbWRKY79 O.E2
Seminal root length (cm)
NaCl (mM)
c
Wild type
NbWRKY79 O.E1
NbWRKY79 O.E2
d
Leaf area (cm²)
NaCl (mM)
e
Photosynthesis rate (μmol m⁻² s⁻¹)
NaCl (mM)
f
Proline content (μg g⁻¹ FW)
NaCl (mM)
g
MDA content (μM g⁻¹ FW)
NaCl (mM)

◀**Fig. 3** Increased salt tolerance in transgenic lines overexpressing *NbWRKY79*. Five-day-old wild-type and *NbWRKY79*-overexpressing (O.E) lines geminated on one-half strength MS medium were transferred to one-half strength MS medium supplemented with 0 mM to 400 mM NaCl and solidified with agar. Seedlings were kept in a growth chamber at 25 °C for 5 days under 16 h of light (50 - 100 μmol m^{-2} s^{-1}) and 8 h of darkness daily. **a** Comparison of primary root length of the wild-type seedlings and *NbWRKY79*-overexpressing lines under salt stress. The plants grown on MS medium and MS medium supplemented with NaCl, and root lengths were after 5 days. *White bar*, 2 cm. **b** Comparison of root measurements. Data are the means of analyses of five individual seedlings from each line ± SD. **c** Phenotype of wild-type plant and *NbWRKY79*-overexpressing (O.E) *N. benthamiana* lines under salt stress. Soil-grown plants were irrigated with a 200 mM NaCl solution daily. Photos were taken 4 weeks after the plants were treated. **d** Leaf surface area. **e** Photosynthesis rate. **f** Proline content. **g** MDA content. The *graphs* represent the mean and SD over three replicates. The *different letters above the columns* indicate significant differences ($P < 0.05$) according to Duncan's multiple range test

leaves but not in the transgenic lines after 48-h exposure to 200 mM NaCl (Fig. 4c, d).

Analysis of antioxidant enzyme activity in *NbWRKY79*-overexpressing plants

To look into the elemental mechanism of *NbWRKY79* that caused decreased ROS accumulation, and thus enhanced the stress tolerance, we surveyed the total activities of ROS-scavenging enzymes (SOD, POD, CAT and APX). Salt stress induced the ROS-scavenging enzyme activity in both the wild-type plants and the transgenic lines. Under normal growth conditions, the activities of SOD, POD, CAT and APX in the transgenic lines were 1.3-, 1.8-, 1.4- and 1.9- fold higher than wild-type plants, respectively (Fig. 5a–d). The total activities of antioxidant enzymes in the transgenic lines exposed to salt treatment were increased approximately 1.5-, 2.2-, 2.1- and 2.7-fold for SOD, POD, CAT, and APX, respectively (Fig. 5a–d). Activity in gel assay showed that the increased antioxidant enzyme activities might indicate the decreased oxidative damage in transgenic lines under salt stress. This observation suggests that *NbWRKY79* may be associated with ROS signaling (Fig. 5e–h).

Overexpression of *NbWRKY79* exhibits enhanced sensitivity to ABA

The increased transcription levels of *NbWWRKY79* in response to ABA treatment suggested that *NbWWRKY79* might play significant part in the ABA signaling pathway. In the absence of exogenously supplied ABA, germination patterns of the transgenic lines and the wild-type plants were similar (Fig. 6a). However, *NbWRKY79*-overexpressing lines showed lower germination than the wild-type plants in medium supplemented with different concentrations of ABA (25 and 50 μM) (Fig. 6a). In addition, during the post-germination growth stage, the root growth of *NbWRKY79*-overexpressing lines and wild-type seedlings was relatively similar in the absence of exogenously applied ABA (Fig. 6b). At 25 and 50 μM ABA, the root growth of transgenic lines was significantly inhibited and the inhibition level was more remarkable than that in the wild-type seedlings (Fig. 6b). The results indicated that overexpression of *NbWRKY79* increased the sensitivity of transgenic lines to ABA.

ABA accumulation and expression of ABA-inducible genes

The phytohormone ABA plays crucial parts in the response of plants to abiotic stresses such as high salinity (Cutler et al. 2010; Finkelstein et al. 2002). Salt stress-induced ABA accumulation was reported in many previous studies (Barrero et al. 2006; Jia et al. 2002; Ruiz-Sola et al. 2014). In this study, to determine whether salt stress tolerance in *NbWRKY79*-overexpressing lines is concerned with the ABA signal pathway, the endogenous ABA contents were assayed. All plants were initially cultivated under normal irrigating conditions and then treated with 200 mM NaCl for a week. Under normal conditions, the ABA level in *NbWRKY79*-overexpressing lines was close to that in the wild-type plants. Under salt stress, the ABA level was significantly increased in both the wild-type and the transgenic lines. The accumulated ABA in *NbWRKY79*-overexpressing O.E1 and O.E2 lines was 1.63- and 1.71-fold higher than in the wild-type plants, respectively (Fig. 7a). Furthermore, expressions of ABA-inducible genes, including *NbAREB1* (ABA-responsive element binding), *NbDREB* (dehydration-responsive element binding) and *NbNCED* (nine-cis-epoxycarotenoid dioxygenase) (Luo et al. 2012), were analyzed under salt stress. Figure 7b–d showed that the expression levels of ABA-inducible genes in the transgenic lines were higher than those in the wild-type plants under NaCl treatment. The results suggest that the endogenous ABA accumulation and the expression of some ABA-responsive genes might be altered by overexpression of *NbWRKY79*.

Discussion

WRKY proteins are one of the largest families of plant transcription factors and have approximately 100 members in *Arabidopsis* and rice (Eulgem et al. 2000). The *WRKY* gene family has been indicated to hold the key to regulation of the transcriptional reprogramming in plant stress

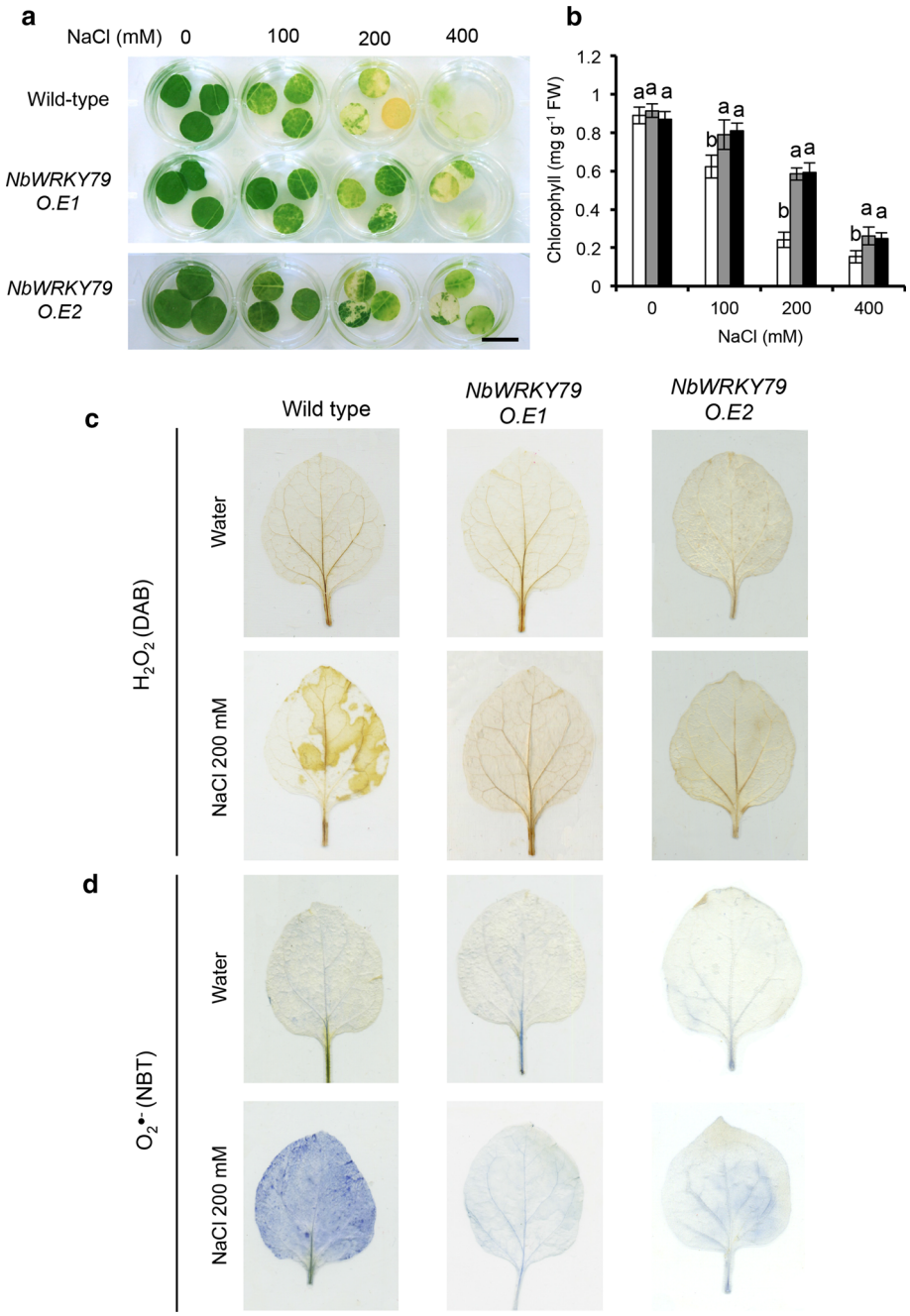


Fig. 4 Leaf disc assay and ROS generation in salt-stressed *N. benthamiana* leaves. **a** Leaf discs from the wild-type plants and *NbWRKY79*-overexpressing (O.E1 and O.E2) lines were treated with different concentrations of NaCl (0, 100, 200, and 400 mM). *Black bar*, 1 cm. **b** Quantification of chlorophyll content from the leaf disc assay. The graphs represent the mean and SD over three replicates. Wild-type and *NbWRKY79*-overexpressing (O.E1 and O.E2) leaves were taken from the plants, which were treated with 200 mM NaCl for two days. The tobacco leaves were stained with DAB (**c**) and NTB (**d**) to detect ROS accumulation in shoots and roots. A representative experiment from three with similar results is shown. The *different letters above the columns* indicate significant differences ($P < 0.05$) according to Duncan's multiple range test

responses (Chen et al. 2012; Rushton et al. 2010). Preceding studies have indicated that a huge number of genes encoding WRKY transcription factors in plants are induced under biotic stresses. In *B. cinerea*, some *WRKY* genes including *WRKY70* and *WRKY33*, which are similar to genes in *Arabidopsis*, are responsive to necrotrophic fungal pathogens (AbuQamar et al. 2006; Zheng et al. 2006). However, it is still unknown regarding the function of WRKY proteins in abiotic-stress conditions. Changes in the expression patterns of *WRKY* genes impact various signaling pathways and regulatory networks. WRKY proteins have numerous potential transcriptional activation and repression domains in their structure; therefore, they can activate or repress gene transcription (Rushton et al. 2010). In addition, preceding studies have reflected that WRKY proteins are concerned with plant ABA signaling. Hence, WRKY proteins may function as activators or repressors in ABA signaling (Chen et al. 2012; Miller et al. 2008; Wang et al. 2009; Xie et al. 2005; Zou et al. 2004). A novel *WRKY* gene termed *NbWWKY79* from *Nicotiana benthamiana* was isolated and characterized in this study. Overexpression of *NbWRKY79* exhibited a significant

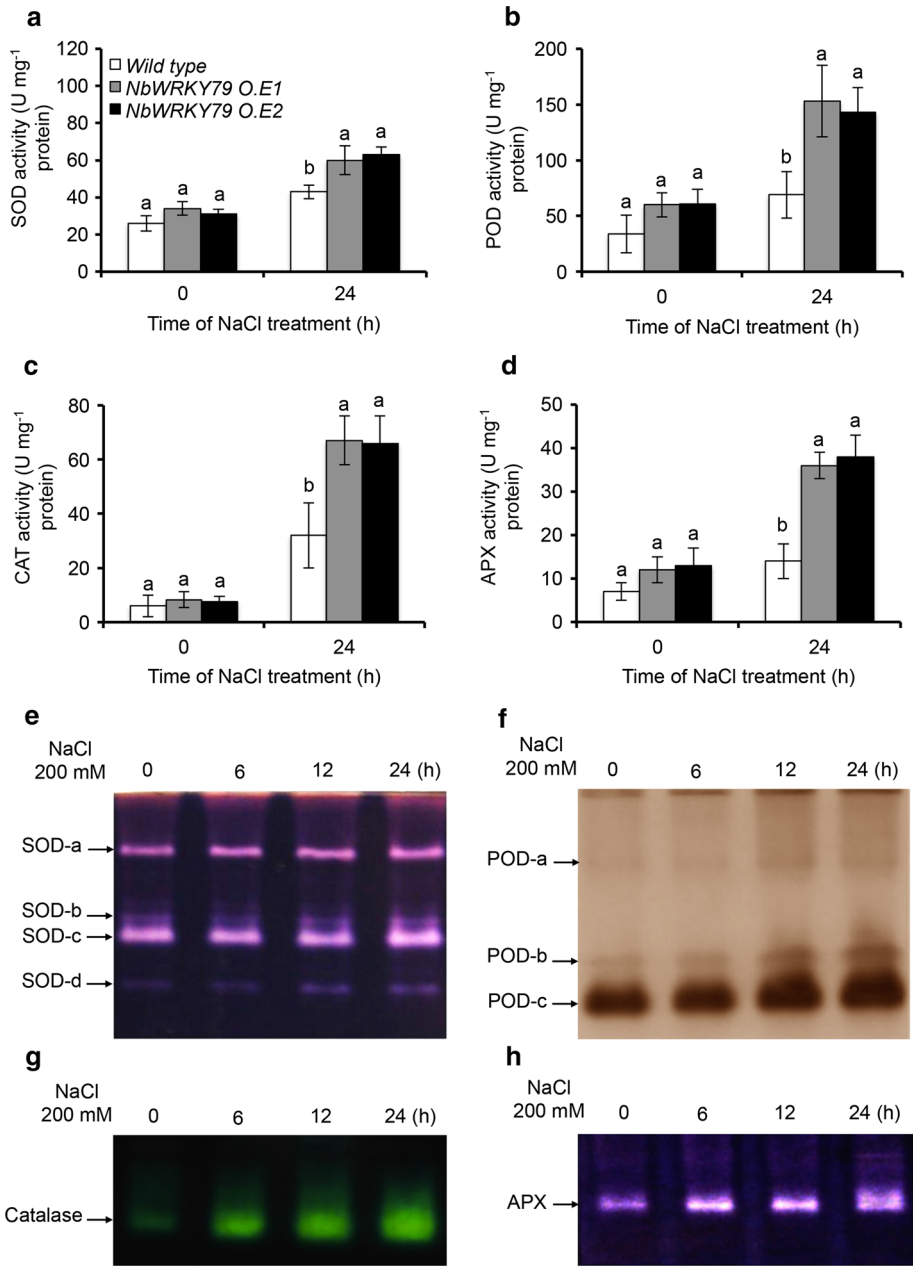
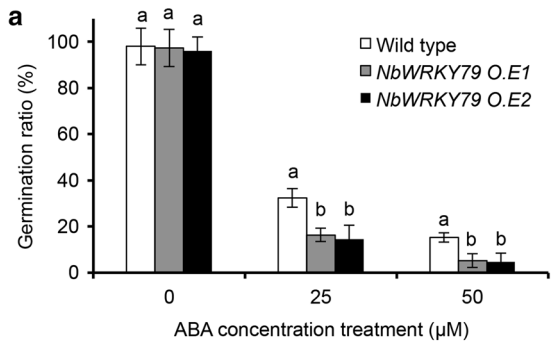


Fig. 5 Relative activity of antioxidant enzymes, SOD—superoxide dismutase (**a**), POD—peroxidase (**b**), CAT—catalase (**c**), APX—ascorbate peroxidase (**d**) and on gel-activity of antioxidant enzymes SOD—superoxide dismutase (**e**), POD—peroxidase (**f**), CAT—catalase (**g**) and APX—ascorbate peroxidase (**h**) in leaves of *NbWRKY79* overexpressing (O.E1 and O.E2) lines to these in leaves of wild-type plants. Protein was extracted from the leaves, which were treated with 200 mM NaCl for 2 weeks. The activity of the antioxidant enzymes was quantified. The graphs represent the mean and SD over three replicates. The *different letters above the columns* indicate significant differences ($P < 0.05$) according to Duncan's multiple range test. For the on gel-activity of antioxidant enzymes, protein was extracted from leaves of *NbWRKY79*-overexpressing lines, which were treated with 200 mM NaCl for 0-24 h. Native PAGE of leaf extracts containing 200 mg protein. The experiments were repeated with three independent samples and similar results were obtained

increase in plant tolerance to salt stress (Figs. 3, 4). The transcription of *GhWRKY17* and *GhWRKY25* (overexpressing in *N. benthamiana*) belonging to group I and II *WRKY* genes was also strongly induced during the early development period in cotton (*Gossypium hirsutum*) after exposure to salt and ABA treatment (Liu et al. 2015; Yan et al. 2014). Further analysis showed that *NbWRKY79* associated with plant salt stress response through ABA signaling pathway and the regulation of ROS accumulation. These results transpired the participation of *NbWRKY79* genes in plant tolerate to salinity.

As a crucial phytohormone, plant development and various stress responses were related to ABA, activating the gene expression in response to stresses (Chinnusamy et al. 2004). That the changes of ABA at the cellular and whole-plant levels increase ABA content is helpful to plants after exposure to stress conditions (Xiong and Zhu 2003). Salt stress can induce ABA accumulation when ABA synthesis is activated and ABA degradation is inhibited. The accumulation of ABA was set off by salt stress, which in turn induces the expression of stress-related genes through ABA-dependent and -independent regulatory systems (Zhu 2002). In this study, expression of *NbWRKY79* was up-regulated by exogenous ABA. The *NbWRKY79*-overexpressing lines were significantly more sensitive to exogenous ABA than those in wild-type plants



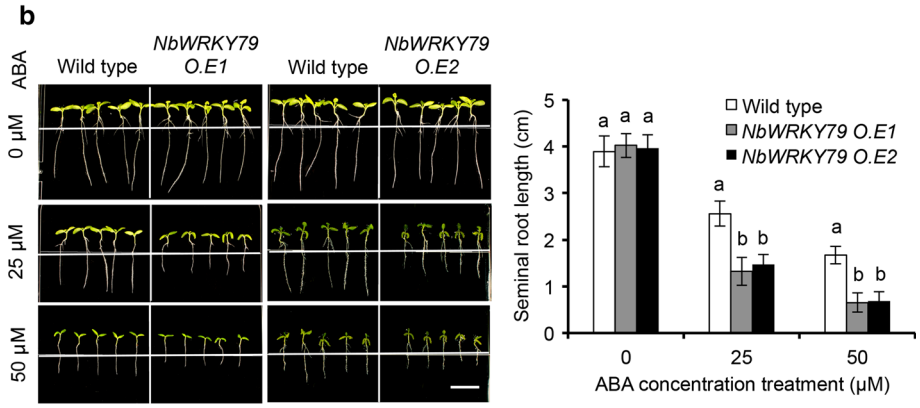


Fig. 6 Response of *NbWRKY79*-overexpressing lines seed germination (**a**) and seedling root growth (**b**) to the application of exogenous ABA. The *different letters above the columns* indicate significant differences ($P < 0.05$) according to Duncan's multiple range test. *White bar*, 2 cm

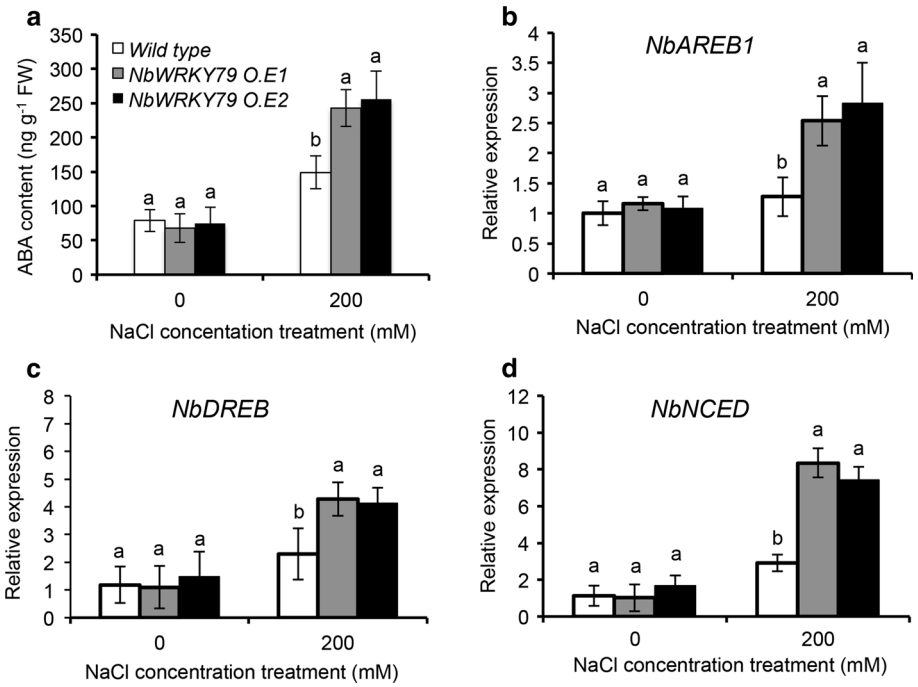


Fig. 7 ABA accumulation and ABA-inducible gene expressions in the wild-type and *NbWRKY79* (O.E)-overexpressing lines. **a** Endogenous ABA levels in the leaves of the wild-type plants and *NbWRKY79* transgenic lines were determined under normal condition and salt treatment with 200 mM NaCl. The expression levels of ABA-inducible genes, *NbAREB* (**b**), *NbDERB* (**c**) and *NbNCED* (**d**) in the wild-type plants and transgenic lines during the salt stress responses as determined using qPCR. The values represent the mean ± SE of three independent experiments. The *different letters above the columns* indicate significant differences ($P < 0.05$) according to Duncan's multiple range test

(Fig. 6). The *NbWRKY79*-overexpressed plants exhibited more accumulation of ABA under salt stress compared with wild-type plants (Fig. 7). In addition, the expression of *NbAREB*, *NbDREB* and *NbNCED*, reported to have function in the ABA-dependent and -independent pathways, was up-regulated under salt stress in the transgenic lines (Fig. 7). These findings suggest that *NbWRKY79* is concerned with the ABA-dependent signaling pathways under salt stress conditions.

Salt stress can induce the accumulation of ROS, which causes damage to cellular membrane, mediates lipid peroxidation, and leads to oxidative stress (Banu et al. 2009; Bowler et al. 1992; Leshem et al. 2007; Mazel et al. 2004). It is critical to regulate ROS accumulation under salt stress. Prior studies have shown that ROS accumulation is modulated through the ABA-triggered regulatory genes involved in ROS production and ROS scavenging (Mittler et al. 2004). However, the regulatory mechanisms of ABA-dependent ROS signaling under salt stress are still unclear (Yan et al. 2014). In the present study, accumulation of ROS in the wild-type plants and the transgenic lines in response to high-salt stress was determined by histochemical staining of DAB and NBT. Our study provided the evidence that *NbWRKY79*-overexpressed plants exhibited a lower ROS accumulation and MDA content in the transgenic lines compared to wild-type plants under salt stress (Fig. 3). Furthermore, this study revealed that the activity of ROS-scavenging enzymes including SOD, POD, CAT, and APX in the transgenic lines was higher than that in the wild-type plants in both normal and salt stress conditions (Fig. 5). Another study has reported that overexpression of a gene from cotton plants (*Gossypium hirsutum*), named *GhWKRY44*, increases ability to scavenge reactive oxygen species (ROS) (Li et al. 2014a). Once the activities of ROS-scavenging enzymes are up-regulated, they support plants from oxidative damage (Apel and Hirt 2004; Suzuki et al. 2012). These results indicate that *NbWRKY79* is concerned with the regulation of SOD, POD, CAT, and APX activities, which resulted in the suppression of ROS accumulation so as to endure less from oxidative damage under salt conditions.

In this study, *NbWRKY79* gene encoding for a WKRY transcription factor whose transcription was induced by salt and ABA was extracted from *N. benthamiana*. *NbWRKY79* exhibits important physiological functions in salt stress response. Overexpression of *NbWRKY79* caused enhanced tolerance to salt stress, which reflected a partial correlation between the activation of ROS-related antioxidant enzymes and reduced accumulation of ROS under salt stress. Our study also indicated that salt stress tolerance in the transgenic plants might involve ABA signaling pathway. Importantly, overexpression of *NbWRKY79* was achieved without having an effect on their phenotypes under normal conditions (Fig. S6). Overall, our findings identified *NbWRKY79* as a potential genetic resource for ameliorating salt tolerance in plants.

Author contribution statement TNN and LHT conceived and designed the research. TNN and DSM conducted experiments. TNN and NVT analyzed data. TNN and LHT wrote the manuscript. All authors read and approved the manuscript.

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