Overexpressing *SgNCED1* in Tobacco Increases ABA Level, Antioxidant Enzyme Activities, and Stress Tolerance

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Abstract Abscisic acid (ABA) regulates plant adaptive responses to various environmental stresses. 9-*cis*-epoxycarotenoid dioxygenase (NCED) is the key enzyme of ABA biosynthesis in higher plants. A *NCED* gene, *SgNCED1*, was overexpressed in transgenic tobacco plants which resulted in 51–77% more accumulation of ABA in leaves. Transgenic tobacco plants decreased stomatal conductance, transpiration rate, and photosynthetic rate but induced activities of superoxide dismutase (SOD), catalase (CAT), and ascorbate-peroxidase (APX). Hydrogen peroxide (H_2O_2) and nitric oxide (NO) in leaves were also induced in the transgenic plants. Compared to the wild-type control, the transgenic plants improved growth under 0.1 M mannitol-induced drought stress and 0.1 M NaCl-induced salinity stress. It is suggested that the ABA-induced H_2O_2 and NO generation upregulates the stomatal closure and antioxidant enzymes, and therefore increases drought and salinity tolerance in the transgenic plants.

Keywords Abscisic acid (ABA) · Antioxidant enzymes · 9-*cis*-epoxycarotenoid dioxygenase (NCED) · Drought · Salinity

Introduction

The plant hormone abscisic acid (ABA) regulates the plant adaptive response to various environmental stresses and diverse physiologic and developmental processes. ABA induces stomata closure and reduces water loss via transpiration, which lead to an increased resistance to water stress-related environmental conditions (Zeevaart and Creelman 1988; Davies and Zhang 1991). ABA induces gene expression in response to various environmental stresses such as drought, salt, or chilling (Chandler and Robertson 1994). Signal molecules, hydrogen peroxide (H_2O_2) and nitric oxide (NO), are involved in the ABA-induced stomatal closure and gene expression and activities of antioxidant enzymes (Jiang and Zhang 2003; Desikan and others 2004; Zhou and others 2005b; Bright and others 2006; Zhang and others 2006, 2007). ABA-induced H_2O_2 production mediates NO generation, which in turn activates mitogen-activated protein kinase (MAPK) and results in upregulation of the expression and the activities of antioxidant enzymes (Zhang and others 2007). However, most data on ABA-induced gene expression and activities of antioxidant enzymes were based on investigations using exogenous ABA treatment. It has not been reported whether antioxidant enzyme activity is induced by upregulating endogenous ABA biosynthesis in transgenic plants.

ABA is synthesized from C_{40}-carotenoids, in which the oxidative cleavage of *cis*-epoxycarotenoids by 9-*cis*-epoxycartenoid dioxygenase (NCED) is the key regulatory step of ABA biosynthesis in higher plants (Liotenberg and others 1999; Seo and Koshiba 2002; Nambara and Marion-Poll 2005). *NCED* genes have been identified in several plant species such as bean (*Phaseolus vulgaris*) (Qin and Zeevaart 1999), cowpea (*Vigna unguiculata*) (Iuchi and others 2000), avocado (*Persea Americana*) (Chernys and Zeevaart 2000), *Arabidopsis* (Iuchi and others 2001), and peanut (*Arachis hypogaea*) (Wan and Li 2006). Plant *NCED* genes form a multigene family and show tissue-specific expression (Chernys and Zeevaart 2000; Tan and others 2003; Lefebvre and others 2006). *NCED* gene

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expression is induced by water deficit (Qin and Zeevaart 1999; Chernys and Zeevaart 2000; Tan and others 2003; Rodrigo and others 2006; Wan and Li 2006; Yang and Guo 2007) and salt stress (Iuchi and others 2000; Wan and Li 2006; Yang and Guo 2007). Overexpression of the *NCED* gene in transgenic plants resulted in ABA accumulation and increased drought tolerance (Thompson and others 2000; Iuchi and others 2001; Qin and Zeevaart 2002; Wan and Li 2006) and salt tolerance (Aswath and others 2005).

Stylosanthes guianensis is an important forage legume with drought and phosphate-deficiency tolerance in tropical and subtropical regions. Its chilling tolerance and antioxidant enzyme activities are increased by ABA treatment (Zhou and others 2005a), which is mediated by nitric oxide (NO) (Zhou and others 2005b). A *NCED* gene (*SgNCED1*) has been cloned from *S. guianensis* and showed an induced expression in response to drought, salinity, and chilling stress (Yang and Guo 2007). The objectives of this study were to analyze the function of *SgNCED1* in drought and salinity tolerance in transgenic tobacco and to investigate induction of an increased endogenous ABA on antioxidant enzyme activity.

Materials and Methods

Generation of Transgenic Tobacco

The full open reading frame (ORF) fragment of *SgNCED1* was amplified by polymerase chain reaction (PCR) from the plasmid pGEM-*SgNCED1* (Yang and Guo 2007), using two primers, NCED5 (CGAATCTAGATCAACCATGG CAGCA) and NCED6 (TTCACCCGGGATTCGAATCAA GCCT) containing *Xba*I and *Sma*I sites, respectively. The PCR product was digested with *Xba*I and *Sma*I and cloned into the pBI121 binary vector, which contains the 35SCaMV promoter. The new construct, pBI-*SgNCED1*, was transformed into *E. coli* and then delivered to strain EHA105 *Agrobacterium tumefaciens*. Tobacco (*Nicotiana tabacum* L. cv. Zhongyan 90) plants were transformed by coculturing leaf discs with *Agrobacterium tumefaciens*. The transformed calli were selected and regenerated on MS medium containing 100 mg L^{-1} kanamycin and 250 mg L^{-1} carbenicillin. The rooted plants were transferred to soil and grown in a greenhouse with natural illumination at 25–30°C.

Plant Growth

T$_1$ seeds of the transgenic plants were germinated on MS medium containing 100 mg L^{-1} kanamycin for selection of the segregated progeny of transgenic plants at room temperature under illumination of 80 μmol m^{-2} s^{-1}. All three tested lines (6, 8, and 16) showed approximately 3:1 segregation of kanamycin resistance. The surviving seedlings were then transplanted into soil after 8 weeks and cultivated in a growth chamber for 1 week at room temperature under illumination of 200 μmol m^{-2} s^{-1}. The seedlings were then moved to a greenhouse and stayed under natural light for another 4–5 weeks to grow strong enough for molecular analysis and physiologic measurements.

DNA Hybridization

Genomic DNA was extracted from 1 g of leaves by the hexadecyltrimethylammonium bromide method (Murray and Thompson 1980). To confirm integration of the transgene, genomic DNA (5 μg) was digested overnight at 37°C with *Eco*RI and separated by electrophoresis on 0.8% agarose gel. DNA was transferred to Hybond-N$^+$ nylon membranes (GE BioSciences Corp., Piscataway, NJ) and then was hybridized with the ^{32}P-labeled 0.8-kb fragment of *SgNCED1*. Hybridization was carried out as previously described (Guo and others 2003). The DNA filter was washed sequentially with $2 \times$ SSC, 0.1% SDS; $1 \times$ SSC, 0.1% SDS; and $0.5 \times$ SSC, 0.1% SDS for 15 min at 65°C.

Semiquantitative RT-PCR

Total RNA was extracted from mature leaves of the transgenic tobacco plants using TRIzol reagent (Invitrogen, Carlsbad, CA), treated with 1 unit μg^{-1} of RQ1 DNase (Promega, Madison, WI), and precipitated with 2 M LiCl to remove genomic DNA. Two micrograms of total RNA were used for reverse transcription with 80 units of M-MLV reverse transcriptase in the presence of oligo(dT)18 in a 10-μl reaction mixture. The PCR reaction contained 1 μl of the above reaction mixture as a template, dNTP, *Taq* DNA polymerase, and two primers, ZG33a (GCCGGT CACCACTTCTTCGACGG) and ZG34a (CTCTTCAAAC TCTCGTCGCATTC). The reaction was stopped after 22-28 PCR cycles. Another PCR reaction was made for 23 PCR cycles in the presence of two primers, ZG101 (TTGGATCTTGCTGGTCGT) and ZG102 (GATCCTC CAATCCAGACA), for amplifying *actin* as an internal control.

Determination of ABA Content

The fourth leaf (fully expanded) from the top was sampled from five individual plants as replicates. ABA content in *S. guianensis* or transgenic tobacco was determined using an ELISA (enzyme-linked immunosorbent assay) kit (made by China Agricultural University) according to the manufacturer's instructions and described previously (Yang and Guo 2007).

Measurement of Net Photosynthesis Rate (A), Stomatal Conductance (g_s), and Transpiration Rate (E)

A, g_s, and E were measured using a portable photosynthesis system (model LI-6400, LI-COR Biosciences, Lincoln, NE) according to the manufacturer's instructions. Measurements were conducted in the morning on intact leaves at PAR levels of 800 µmol m^{-2} s^{-1} after 10 min of equilibration to achieve steady-state conditions. Leaf temperature within the leaf chamber was controlled at 25°C and the CO_2 concentration was at 360 ppm at 80% relative humidity. The measurements were conducted from the fully expanded leaves of five individual plants as replicates.

Measurement of SOD, CAT, and APX Activity

For extraction of SOD and CAT, the fully expanded leaf samples (0.5 g) were ground in a mortar with pestle in 5 ml of 50 mM phosphate buffer (pH 7.8) at 4°C. For extraction of ascorbate-peroxidase (APX), leaf samples (0.5 g) were ground in a mortar with pestle in 5 ml of 50 mM cool phosphate buffer (pH 7.0, containing 1 mM AsA, 1 mM EDTA). The homogenates were centrifuged at 13,000g for 15 min at 4°C. The supernatants were recovered for determination of SOD, CAT, and APX, respectively, as described by Guo and others (2006). One unit of SOD activity was defined as the amount of enzyme required for inhibition of photochemical reduction of ρ-nitro blue tetrazolium chloride (NBT) by 50%. One unit of CAT and APX was defined as the amount of enzyme required for catalyzing the conversion of 1 µmol H_2O_2 (extinction coefficient = 0.0394 mM^{-1} cm^{-1}) or AsA (extinction coefficient = 2.8 mM^{-1} cm^{-1}) in 1 min, respectively. The protein content in the enzyme extracts was determined using Coomassie Brilliant Blue G-250 according to the method of Bradford (1976). Measurements were conducted from five individual plants as replicates.

Measurements of H_2O_2 and NO

The fully expanded leaf was sampled for H_2O_2 and NO assays. H_2O_2 and NO in leaf tissues were measured spectrophotomerically. For measurement of H_2O_2, fresh leaves (0.5 g) were frozen in liquid nitrogen and ground in a mortar and pestle in 4 ml of 5% (v/v) trichloroacetic acid. The homogenate was centrifuged at 10,000g for 20 min at 4°C. The supernatant was recovered and NO and H_2O_2 contents were determined as previously described (Zhou and others 2005b, 2006b). Fresh leaves (0.6 g) were frozen in liquid nitrogen and ground in a mortar and pestle in 3 ml of 50 mM cool acetic acid buffer (pH 3.6, containing 4% zinc diacetate). The homogenates were centrifuged at 10,000g for 15 min at 4°C. The supernatant was collected.

The pellet was washed with 1 ml of extraction buffer and centrifuged as before. The two supernatants were combined and 0.1 g of charcoal was added. After vortexing and filtration, the filtrate was collected. The mixture of 1 ml of filtrate and 1 ml of the Greiss reagent was incubated at room temperature for 30 min. Absorbance was determined at 540 nm. NO content was calculated by comparison to a standard curve of $NaNO_2$. Measurements were conducted from five individual plants as replicates.

Drought and Salt-tolerance Analysis

T_1 seeds of the transgenic plants were sterilized and germinated on the MS medium containing 100 mg L^{-1} kanamycin for selection of the segregated progeny of transgenic plants, whereas the wild-type seeds were germinated on the medium without kanamycin. The surviving seedlings were then transplanted onto a new MS medium containing 0.1M mannitol for drought stress treatment, or 0.1 M NaCl for salt stress. Three plants were transplanted on each medium in one bottle. Each treatment consisted of nine plants in three bottles. The plants growing on MS medium were used as controls. Fresh weight of shoots and roots were measured the eighth week after germination of seeds. The whole-plant weight (total weight) was calculated by shoot weight plus root weight. The relative growth was calculated by the percentage of fresh weight of a plant under stress to that under control conditions.

Statistical Analysis

All the measurements were repeated at least three times from different individual plants. Analysis of variance of all data was performed by the least significant difference (LSD) test at 0.05 probability level when the F test showed a significant ($P \leq 0.5$) effect using an SPSS program (SPSS Inc., Chicago, IL) .

Results

Increased ABA Content and Decreased Stomatal Conductance in Transgenic Tobacco

A total of 25 transgenic tobacco plants were identified by PCR amplification and DNA hybridization. The transgenic tobacco lines 6, 8, and 16 (T_0) exhibited growth similar to that of the wild-type control but had increased ABA content. They were therefore chosen for further studies. T_1 seedlings of the transgenic lines were investigated in this work. *Eco*RI was used for digestion of the DNA sample because there is no *Eco*RI site within the ORF of the *SgNCED1* sequence. DNA hybridization showed that the

SgNCED1 gene was integrated into the genomes of transgenic tobacco as one copy in lines 6 and 16 and two copies in line 8, whereas the wild-type control had no *SgNCED1* signal (Figure 1A). Because line 8 showed two copies of the transgene but had a kanamycin-resistant segregation ratio of 3:1, the *SgNCED1* gene might be integrated into a single locus on the same chromosome. Semiquantitative RT-PCR was further used to detect the *SgNCED1* mRNA. In comparison to the wild type, the three transgenic lines had PCR product bands, and two of them (lines 6 and 8) showed more abundant expression under nonstressed conditions (Figure 1B).

Abscisic acid (ABA) content in the leaves was measured. The tested transgenic tobacco lines had 51–77%
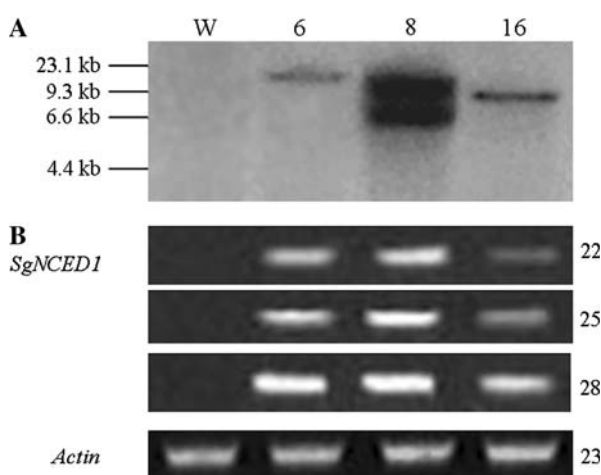

Fig. 1 DNA hybridization and semiquantitative RT-PCR analysis of the transgenic tobacco plants (lines 6, 8, and 16) overexpressing *SgNCED1* in comparison to the wild-type control (W). (**A**) indicates the genomic DNA (10 μg) digested with *Eco*R1. The blot was probed with the ^{32}P-labeled *SgNCED1* fragment (0.8 kb). (**B**) indicates the RT-PCR product of *SgNCED1* at 22, 25, and 28 PCR cycles and *actin* (an internal control), respectively
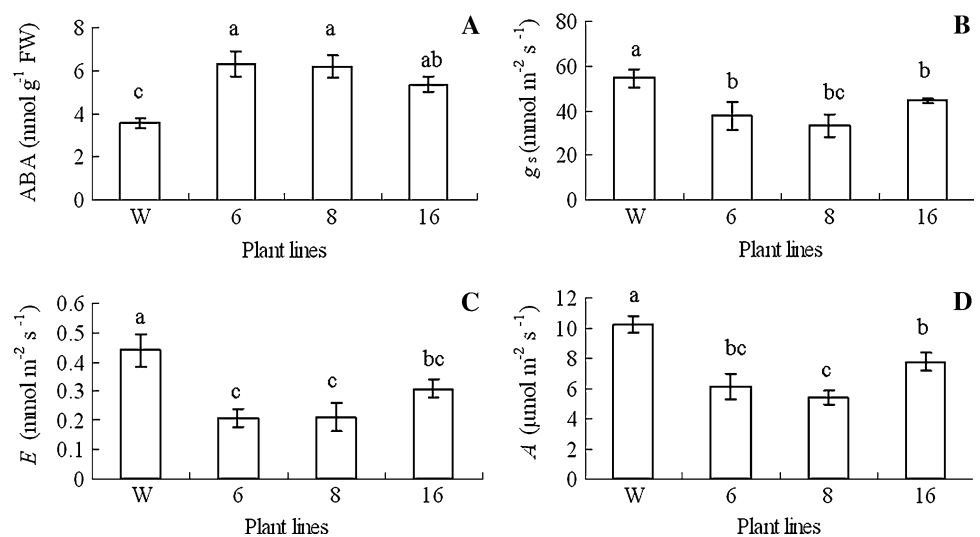
higher levels of ABA than the wild-type control (Figure 2A), however, it did not show a positive correlation with *SgNCED1* mRNA levels in the transgenic lines. Line 8 had the highest level of *SgNCED1* mRNA, but its endogenous ABA level was similar to the two other transgenic plants (Figure 2A).

One of the effects of ABA is to induce stomatal closure, which reduces transpiration and photosynthesis. Stomatal conductance (g_s) was decreased by 30, 39, and 19%, respectively, in the transgenic lines 6, 8, and 16 (Figure 2B), indicating that the expression of *SgNCED1* resulted in stomatal closure. Transpiration rate (E) and net photosynthetic rate (A) were also decreased in the transgenic tobacco plants compared to those in the wild-type control. E was decreased by 53, 52, and 30%, respectively, in the transgenic lines 6, 8, and 16 (Figure 2C), whereas A was decreased by 40, 47, and 24%, respectively, in the three transgenic lines (Figure 2D).

Antioxidant Enzyme Activities and H$_2$O$_2$ and NO Contents in Transgenic Tobacco

Antioxidant enzymes play an important role in the abiotic stress tolerance of plants and are induced by treatment with ABA (Zhou and others 2005a, b). SOD, CAT, and APX activities were measured in the transgenic plants in comparison to the wild-type control. SOD activity was 22–27% higher in the transgenic lines 6 and 16 than in the wild-type control (Figure 3A). CAT and APX activities were elevated in all the tested transgenic lines compared to those in the wild-type control. CAT activity was induced by 71, 29, and 40% in the transgenic lines 6, 8, and 16, respectively (Figure 3B), and APX activity was increased by 50, 40, and 54%, respectively (Figure 3C).

Hydrogen peroxide and NO in leaves were also measured spectrophotometerically. More H$_2$O$_2$ and NO were

Fig. 2 ABA content (**A**), stomatal conductance (g_s, **B**), transpiration rate (E, **C**), and net photosynthesis rate (A, **D**) in leaves of the transgenic tobacco lines in comparison to the wild-type control under normal growth conditions. The data are means of five independent measurements from five individual plants. The same letter above the columns indicates that the difference is not significant at $P = 0.05$

accumulated in the tested transgenic lines than in the wild-type control (Figure 4). H_2O_2 level was increased by 54, 32, and 43% in the transgenic lines 6, 8, and 16, respectively (Figure 4A); NO level was increased by 40, 44, and 53%, respectively (Figure 4B).

Transgenic Tobacco Exhibits Increased Drought and Salt Tolerance

The wild-type and transgenic plants were similar in size when they were grown on MS medium. The growth of wild-type plants was significantly inhibited on the medium containing mannitol or NaCl, whereas that of the transgenic lines was less reduced than the wild-type plants (Figure 5). The shoot weight was similar in all plants under
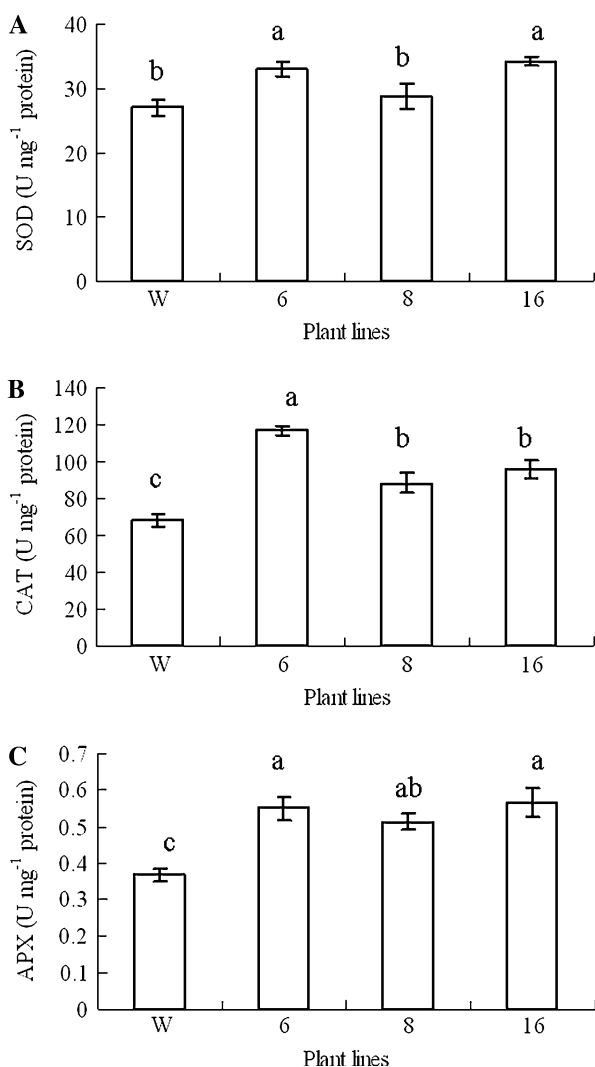
Fig. 3 SOD (**A**), CAT (**B**), and APX (**C**) activities in leaves of the transgenic plants in comparison to the wild-type control under normal growth conditions. The data are means of five independent measurements from five individual plants. The same letter above the columns indicates that the difference is not significant at $P = 0.05$
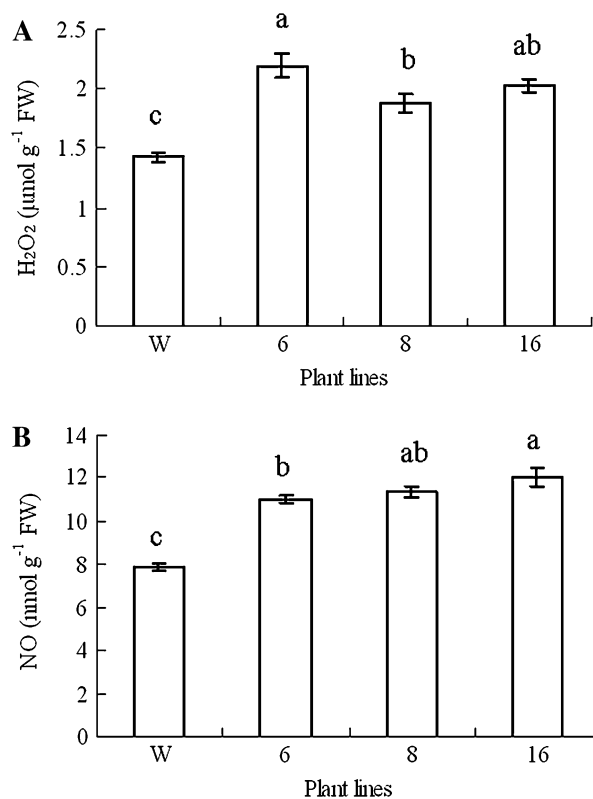
Fig. 4 Hydrogen peroxide (**A**) and nitric oxide (**B**) contents in leaves of the transgenic plants in comparison to the wild-type control under normal growth conditions. The data are means of five independent measurements from five individual plants. The same letter above the columns indicates that the difference is not significant at $P = 0.05$

nonstressed conditions. It decreased significantly in the wild-type plants under drought stress and salinity stress, but it was not significantly altered in all transgenic lines under salinity stress. Although shoot weight decreased significantly in all transgenic lines under drought stress, it was still much higher than that in the wild-type plants (Figure 5A). Drought stress had no significant influence on root weight in the wild-type control and transgenic line 6, but it increased root weight in the transgenic lines 8 and 16. Salinity stress had no significant influence on root weights in all tested lines (Figure 5B). The wild-type plants had total plant weights similar to those of the transgenic lines under nonstressed conditions. The plant weight and the relative growth in the wild-type control was decreased significantly under drought and salt stresses, whereas those in the transgenic lines were little affected or were maintained at unstressed levels (Figure 5C, D).

Discussion

Oxidative cleavage of *cis*-epoxycarotenoids catalyzed by NCED is the main regulatory step in the biosynthesis of

Fig. 5 Fresh weights of shoots (**A**), roots (**B**), whole plant (**C**), and relative growth (**D**) of the transgenic tobacco plants in comparison to the wild-type controls. The transgenic lines and wild-type controls were growing in the MS medium containing 0.1 M mannitol as drought stress, 0.1 M NaCl as salinity stress, or MS medium alone as control. The data are means of three independent measurements. The same letter above the columns indicates that the difference is not significant at $P = 0.05$ using the Tukey test
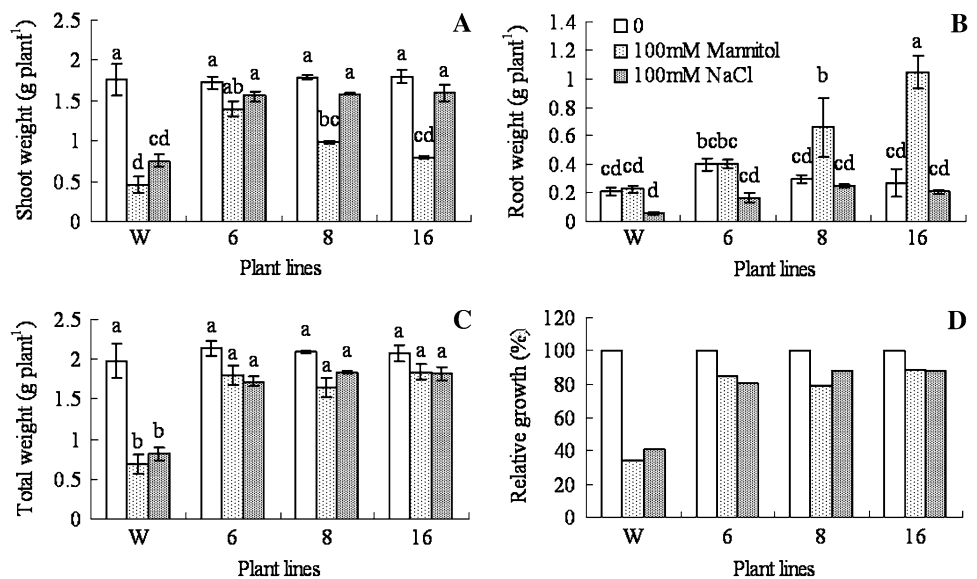
abscisic acid (ABA) in higher plants (Xiong and Zhu 2003; Nambara and Marion-Poll 2005) and occurs in the plastids (Seo and Koshiba 2002). DNA hybridization and semi-quantitative RT-PCR revealed that the *SgNCED1* gene was integrated into the genomes and transcribed in transgenic tobacco plants. Its overexpression in the transgenic plants resulted in 51–77% more accumulation of ABA in leaves. A putative chloroplast transit peptide locates at the N-terminus of *SgNCED1* (Yang and Guo 2007), which targets the NCED protein to the plastids after it is translated (Qin and Zeevaart 1999; Iuchi and others 2000). Overexpression of *NCED*s from other plant species also increased ABA content in the transgenic plants (Thompson and others 2000, 2007; Iuchi and others 2001; Qin and Zeevaart, 2002; Lefebvre and others 2006; Wan and Li 2006).

ABA induces stomata closure and decreases transpiration rate and therefore increases drought tolerance in plants (Zeevaart and Creelman 1988; Davies and Zhang 1991). Drought tolerance is increased in transgenic plants overexpressing *NCED* genes (Iuchi and others 2001; Qin and Zeevaart 2002; Wan and Li 2006). The higher level of ABA in the transgenic tobacco lines overexpressing *SgNCED1* was accompanied by a decreased stomatal conductance and transpiration rate. The transpiration rate is also decreased in transgenic *Arabidopsis* overexpressing *AtNCED3* (Iuchi and others 2001). Gas exchange through the stomata is important in the control of photosynthesis. The transgenic lines had a 24–47% lower photosynthetic rate, which resulted from lower stomatal conductance.

The role of antioxidant enzymes in abiotic stress tolerance has been well documented (Noctor and Foyer 1998). Higher antioxidant enzyme activities are associated with tolerance in the drought- or salinity-tolerant cultivars of some crops (Dionisio-Sese and Tobita 1998; Sreenivasulu

and others 2000; Srivalli and others 2003; Guo and others 2006). ABA induces the expression and activities of SOD, CAT, and APX in plants (Zhu and others 1994; Guan and others 2000; Zhou and others 2005a). This work demonstrated first that transgenic tobacco plants overexpressing *SgNCED1* had significantly higher levels of SOD, CAT, and APX than the wild-type plants.

Hydrogen peroxide and NO are important signals involved in plant growth and development as well as in abiotic stress tolerance (Neill and others 2002; Uchida and others 2002). Exogenous ABA treatment induces production of H_2O_2 and NO, which in turn induce stomatal closure and increased expression and activities of antioxidant enzymes in plants (Jiang and Zhang 2003; Desikan and others 2004; Zhou and others 2005b; Zhang and others 2007). H_2O_2 and NO levels in leaves were higher in the transgenic tobacco lines overexpressing *SgNCED1* in comparison to the wild-type plants, indicating that the increased endogenous ABA biosynthesis induced generation of H_2O_2 and NO, which may upregulate stomatal closure and induction of antioxidant enzyme activities.

The transgenic lines were similar in size to the wild-type plants. The fresh weights of shoots and whole plants were similar among the transgenic lines and the wild-type control under normal growth conditions, indicating that the decreased photosynthesis in the transgenic lines did not affect plant growth. It is possible that the respiratory CO_2 loss via stomata was also limited. Plant growth was significantly inhibited in the wild-type plants under drought stress or salinity stress, whereas the transgenic lines were inhibited less than the wild-type control, indicating that overexpressing *SgNCED1* in the transgenic lines increased drought and salinity tolerance. It is interesting that among the transgenic lines, line 6 showed a slightly reduced

growth of shoots and an unaltered growth of roots under drought stress, whereas lines 8 and 16 exhibited significantly reduced growth of shoots but significantly increased growth of roots. The mechanism(s) underlying root growth promotion in lines 8 and 16 require further study. Nevertheless, the present work demonstrates that *SgNCED1* is a good candidate gene for use in crop improvement for abiotic stress tolerance.

In summary, overexpression of *SgNCED1* in transgenic tobacco plants resulted in improved ABA biosynthesis, which led to the lower levels of stomatal conductance, transpiration, and photosynthesis. The transgenic plants also exhibited increased activities of SOD, CAT, and APX and production of H_2O_2 and NO, as well as tolerance to drought and salinity stresses. It is suggested that ABA-induced H_2O_2 and NO upregulate the stomatal closure and antioxidant enzymes and therefore increase drought and salinity tolerance in the transgenic plants.

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