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ORIGINAL PAPER

Functional Analysis of Two Orthologous *NAC* Genes, *CarNAC3*, and *CarNAC6* from *Cicer arietinum*, Involved in Abiotic Stresses in Poplar

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Abstract Abiotic stresses such as drought and high salinity inhibit plant growth and productivity. NAC transcription factors play a variety of important roles in plant development and abiotic stress responses. To date, the transformation of *NAC* genes into poplar plants has not been reported. In this study, we introduced the *CarNAC3* and *CarNAC6* salinity and drought tolerant genes from *Cicer arietinum* (chickpea) into *Populus deltoides* × *Populus euramericana* 'Nanlin895' (poplar) plants using *Agrobacterium tumefaciens*-mediated transformation. We verified the integration of the two genes into the poplar genome using polymerase chain reaction (PCR) and their stable expression was confirmed at the transcript level using real-time (RT)-PCR. The growth rates of roots and shoots and the clawed root rate increased in transformed plants grown in agar medium or in soil and increased in response to drought and salt stress conditions. Proline and photoprotectant pigment accumulation and antioxidant enzyme activities increased in response to abiotic stress, but the accumulation of photosynthetic pigments and malondialdehyde (MDA) decreased in transgenic lines relative to wild-type control plants. The *CarNAC3* transgene was expressed at higher levels than the *CarNAC6* transgene. Our results showed that the *CarNAC3* and *CarNAC6* genes enhanced the capacity for osmotic adjustment and increased antioxidant enzyme activity and suggest that these genes could play a significant role in improving drought and salt tolerance when expressed in poplar plants.

Keywords *Populus* · *CarNAC3* · *CarNAC6* · Transgenic · Drought tolerance · Salt tolerance · Functional analysis

Abbreviations

6-BA	N-6-benzyladenine
TDZ	Thidiazuron
AS	Acetosyringone
NPTII	Neomycin phosphotransferase
MS	Murashige and Skoog
ORF	Open reading frame
PCR	Polymerase chain reaction
RT-PCR	Reverse transcription polymerase chain reaction
MDA	Malondialdehyde
POD	Guaiacol peroxidase
SOD	Superoxide dismutase
ROS	Reactive oxygen species

Introduction

Plants must adjust to environmental conditions to coordinate their growth and development (Santner and Estelle 2009). Drought and high salinity are two important abiotic stresses that reduce plant growth and productivity (Nakashima et al. 2012). Many abiotic stress-responsive genes have been identified in plants using molecular techniques such as DNA microarrays (Fowler and Thomashow 2002; Rabbani et al. 2003; Yamaguchi-Shinozaki and Shinozaki 2006; Nakashima et al. 2009). NAC domain proteins are transcription factors (TFs) whose functions are related to plant development or biotic and abiotic stresses (Nakashima et al. 2012). Many NAC proteins have been reported to be involved in shoot apical meristem

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development (Souer et al. 1996), clawed ratio and lateral root (He et al. 2005), senescence (Guo and Gan 2006; Uauy et al. 2006), secondary cell wall formation (Mitsuda et al. 2007), and in response to fungal infection and to drought and high-salinity stress (Hu et al. 2006; Zheng et al. 2009). Previous research on NAC proteins focused on model plant species such as *Arabidopsis thaliana* (*Arabidopsis*) and rice (Olsen et al. 2005). The RESPONSIVE TO DEHYRATION 26 (*RD26*) gene from *Arabidopsis* was the first reported gene encoding a NAC protein (Yamaguchi-Shinozaki et al. 1992). The NAC domain was identified based on consensus sequences present in the petunia NAM and *Arabidopsis* AFAF1/2 and CUC2 protein sequences (Aida et al. 1997; Nakashima et al. 2012). Some *NAC* genes are upregulated by wounding and bacterial infection (Collinge and Boller 2001; Mysore et al. 2002; Hegedus et al. 2003) whereas others mediate viral resistance (Xie et al. 1999).

Hui et al. (2009) reported that the CarNAC3 protein from *Cicer arietinum* (chickpea) was 285 amino acids (aa) in length and included one conserved domain. The sequence responsible for the transcriptional activity of this protein was located in the C-terminal region. Under stress conditions, expression of the *CarNAC3* gene in poplar increased proline and photosynthetic pigment levels and antioxidant enzyme activities. Furthermore, its expression decreased the concentration of malondialdehyde (MDA) relative to that of wild-type (WT) control plants (Movahedi et al. 2014a).

The *CarNAC6* gene from chickpea encodes 308 amino acids and regulates transcription factors (Nakashima et al. 2012; Movahedi et al. 2014b). The *CarNAC6* gene belongs to the NAC domain protein family (*NAM*, *ATAF1, 2*, and *CUC2*), which enhances plant resistance to biotic and abiotic stresses and plays an important role in plant development (Movahedi et al. 2014b).

Sophisticated mechanisms have evolved in plants to protect against severe conditions through the synergistic actions of antioxidants and osmoprotectants (Al-Whaibi et al. 2011). Drought and high salinity stresses cause oxidative stress in plants (Ahmad et al. 2010; Barba-Espin et al. 2011). Plants respond to abiotic stress by increasing the formation of reactive oxygen species (ROS) including H_2O_2, $\bar{O}_2$, and hydroxyl radicals, which impair electron transport (Mishra et al. 2011; Nounjan et al. 2012). In response to increased ROS levels, plants produce an array of enzymatic and non-enzymatic antioxidant molecules to scavenge ROS (Krantev et al. 2008; Zhou et al. 2014). Superoxide dismutase (SOD), guaiacol peroxidase (POD), and catalase (CAT) activities and MDA levels are induced by cell membrane damage (Abdullahil et al. 2010; Dong et al. 2010; Mishra et al. 2011). Proline, a soluble osmolyte, plays an important role in adjusting plant osmylotic functions and protecting cell structures from abiotic stress (Abraham et al. 2010; Hou et al. 2013). Increased proline accumulation in transgenic poplar plants enhanced drought and salt tolerance (Ben et al. 2010; Su et al. 2011). Cho et al. (2012) reported that increasing the activity of antioxidant enzyme proline, influenced by abiotic stresses, cause to increase tolerance in plant. To date, the transformation of *NAC* genes into poplar plants has not been reported. In this study, we introduced the *CarNAC3* and *CarNAC6* genes into the poplar hybrid clone 'Nanlin895' (*Populus deltoides*× *Populus euramericana* 'Nanlin895') using *Agrobacterium tumefaciens*-mediated transformation. We compared the physiological and biochemical responses of transformed and wild-type poplar plants to abiotic stress. In addition, we investigated the effect of *CarNAC3* and *CarNAC6* gene expression in transgenic poplar plants on the antioxidant system and on osmotic protection against drought and high salinity stresses.

Materials and Methods

Plant Materials and Genetic Transformation

We transformed poplar using the LBA404 *A. tumefaciens* strain resistant to rifampicin (Hoekema et al. 1983) and kanamycin. Two binary vectors containing either the *CarNAC3* gene (GenBank accession number FJ356671.1) or the *CarNAC6* gene (GenBank accession number FJ477887.1) isolated from *C. arietinum* (chickpea) cultivar 209 (*PBI121/CarNAC3* and *PBI121/CarNAC6*, respectively) were prepared by the State Key Laboratory of Crop Genetics and Germplasm Enhancement of the National Center for Soybean Improvement at Nanjing Agricultural University, China (Hui et al. 2009). The *CarNAC3* gene was 1160 bp in length and contained a single open reading frame (ORF). The *CarNAC6* gene was 1154 bp in length and contained two opposing ORFs. The *PBI121/CarNAC3* plasmid was digested with *Xba*I to isolate an 858-nt fragment of *CarNAC3* and the *PBI121/CarNAC6* plasmid was digested with *Xba*I and *BamH*I to isolate a 924-nt fragment of *CarNAC6* for preparing transformant *Agrobacteria*. Expression of the *CarNAC3* and *CarNAC6* genes in these constructs was driven by the cauliflower mosaic virus (CaMV) 35S promoter, and both genes contained the nopaline synthase (NOS) gene terminator. *Agrobacterium* cultures were grown at 28 °C for 48 h in liquid Luria-Bertani medium supplemented with 50 mg/l rifampicin and 50 mg/l kanamycin (Horsch et al. 1988) with gentle shaking until an optical density (OD) of 0.7 was reached. After centrifugation of the cultures, the bacterial pellets were re-suspended in liquid Murashige and Skoog (MS) medium containing 5 % sucrose to prepare *Agrobacterium* infective suspensions which were cold-treated at 4 °C (Logemann et al. 2006). Leaves from *P. deltoides*× *P. euramericana* 'Nanlin895' (poplar) plants were pre-cultured for 2 days and then cut in 0.5×0.5 cm for inoculation by immersion into the *Agrobacterium* infective suspensions for 120 min at 28 °C

with constant agitation at 220 rpm. MS medium supplemented with 0.5 mg/l 6-benzylaminopurine (6-BA), 0.004 mg/l thidiazuron (TDZ), 4.0 g/l agar (semi-solid medium), 25.0 g/l sucrose, and 200 μM acetosyringone (AS) was used to co-cultivate the inoculated explants with *Agrobacterium* for 2 days at 28 °C without light.

Explants from 45 putative transformed lines for each of the two genes were transferred to MS selection medium containing 0.5 mg/l 6-BA, 0.004 mg/l TDZ, 6.0 g/l agar, and 25.0 g/l sucrose supplemented with 400 mg/l rifampicin and 50 mg/l kanamycin and grown at 23±1 °C and pH 5.8 under a 16/8-h photoperiod and 50–60 % relative humidity. The shoot tips (~5 cm) of regenerated shoots were excised from 42 *CarNAC3* to 43 *CarNAC6* lines and transferred to half-strength MS medium containing no added hormones for rooting under phytotron conditions. Forty well-developed poplar lines for each transgene were recovered to use in comparisons with untransformed WT poplar plants. Eight lines for each transgene were transferred to MS agar medium containing various salt concentrations. Thirty transgenic poplar lines for each gene and eight WT lines were transferred to the greenhouse for complementary experiments.

PCR and Real-Time-PCR

Total genomic DNA was extracted from young leaves of putative transformants using the CTAB method (Porebski et al. 1997) and DNA at concentrations of 900–1000 ng/μl determined using a BioDrop spectrophotometer (UK). Transformations were confirmed by the amplification of an 858-bp fragment from the *CarNAC3* gene by polymerase chain reaction (PCR) using the primers *CarNAC3* F (5′-ATGAATGGAAGA ACAAG-3′) and *CarNAC3* R (5′-ATATTCTCTATGGTCA TATAC-3′) and the amplification of a 924-bp fragment from the *CarNAC6* gene using the primers *CarNAC6* F (5′-ATAC ATGGCATCAATGGA-3′) and *CarNAC6* R (5′-ATAAAAAT TGAGGGTGTTGGAATTAG-3′). The PCR reactions were carried out under the following conditions: 94.5 °C for 5 min; 35 cycles of 94 °C for 35 s, 56 °C for 40 s, and 72 °C for 75 s, followed by 72 °C for 10 min. The PCR products were visualized on a 1 % agarose gel.

Total RNA was extracted from young leaves of transformed poplar plants using TRIzol (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions with RNA concentrations of 1100–1300 ng/μl determined using a BioDrop spectrophotometer. Real-time (RT)-PCR) reactions containing 4.0 μg of total RNA and an oligo-dT primer were carried out to synthesize first-strand complementary DNA (cDNA) using a PrimeScript One Step RT-PCR Ver. 2 kit (Takara Biotechnology, Dalian, China) according to the manufacturer's instructions. The RT-PCR products were re-amplified in 50-μl PCR reactions using the same PCR primers described above to confirm their identities. Absolute quantification of *CarNAC3* and *CarNAC6* transgene copy numbers was performed using RT-PCR and relative expression levels were determined by the $\Delta\Delta C_t$ method using an Applied Biosystems RT-PCR system (Applied Biosystems, USA) and Fast Start Universal SYBR Green Master (Rox; No. 04913914001: Roche, USA). Amplification was performed on 1000-, 500-, 250-, 125-, and 62.5-fold cDNA dilutions to create a standard curve using the primers *CarNAC3* messenger RNA (mRNA) F (5′-GTTTCAGATTTCACCCAACTGA TGA-3′) and *CarNAC3* mRNA R (5′-ATCAACTTCAGGGA TGATAGATGCA-3′) to amplify a 101-nt fragment from the *CarNAC3* cDNA and the primers *CarNAC6* mRNA F (5′-ATTTCACCCCACAGAAGAAGAACTA-3′) and *CarNAC6* mRNA R (5′-ATACCACTCTCTTTCTCCAACCTTT-3′) to amplify a 157-nt fragment from the *CarNAC6* cDNA. Expression of the housekeeping gene *β-actin* (NCBI Reference Sequence: XM-006370951.1) was used for normalization of expression. The primers *β-actin* F (5′-GACCTTCAATGTGC CTGCAA-3′) and *β-actin* R (5′-ACCATCACCAGAATCC AGCA-3′) were used to amplify a 100-nt fragment from the *β-actin* mRNA.

Plantlet Transplantation and Phenotypic Analysis

To analyze the phenotypic effects of *CarNAC3* and *CarNAC6* transgene expression, 40 putative transformed poplars lines were selected for each gene. Plants with three to four expanded leaves on the top bud were transplanted to fresh agar medium incorporating various NaCl concentrations for phenotypic analysis. The growth rates of roots and shoots of plantlets grown on NaCl concentrations of 0, 50, 100, 150, and 200 mM were measured over a period of 4 weeks. Each treatment was applied to eight putative transformed poplar lines for each gene.

To determine the levels of protective compounds and antioxidant enzyme activities, 15 each of *CarNAC3* and *CarNAC6* transgenic poplar lines were transferred to the greenhouse. After 3 days of acclimatization, the plantlets were transplanted to soil consisting of a 2:1 mixture of sterilized peat and perlite. During the first 2 weeks, humidity was maintained by spraying with water. After 2 weeks, the 15 lines for each transgene were irrigated with a series of increasing salt concentrations over a 4-week period. Eight untransformed WT lines served as controls. The plants were watered with 0 mM NaCl solution every 2 days during the first week. The NaCl concentration was then increased by 50 mM every week to a maximum of 150 mM. The amount of water supplied was kept constant and trays under the pots maintained the salt content of the soil. To impose drought stress on both the transgenic and WT lines, watering was then discontinued for 15 days. Shoot heights and the concentrations of photosynthetic pigments, proline, and malondialdehyde (MDA), and the activities of superoxide dismutase (SOD) and guaiacol

peroxidase (POD) were measured and compared between the transgenic and WT poplar lines.

In a complementary experiment, the clawed root rate was calculated using the formula: percent clawed roots=number of main roots/(main roots+lateral roots)× 100 to compare the effect of transgene expression on the clawed root phenotype in transgenic and WT lines grown for 4 weeks in medium containing 0 or 25 mM NaCl.

Determination of Chlorophyll, Proline, and MDA Concentrations and SOD and POD Activities

A 10-ml volume of 80 % acetone was added to 10 ml of young leaf tissue previously homogenized in liquid nitrogen and incubated for 24 h at 4 °C. The supernatant was collected by centrifugation at 10,000× *g* for 10 min at 4 °C. A microplate reader spectrophotometer (Bio-Rad) set to measure absorbance at 663.8, 646.8, and 470.0 nm was used to determine the absorbance of chlorophyll a (C_a), chlorophyll b (C_b), total chlorophyll (C_{a+b}), and total carotenoids (C_{x+c}) according to Lichtenthaler (1987). The SOD (EC1.15.1.1) activity and MDA concentration were determined according to the methods of Satoh (1978), Stewart and Bewley (1980), and Wang et al. (2012). The POD (E.C. 1.11.1.7) activity and proline content were measured using the methods of Pagariya et al. (2012) and Lei et al. (2007), respectively.

Statistical Analysis

All data analysis was carried out in triplicate using ANOVA with mean separations determined by Duncan's test calculated using SPSS version 16 (SPSS Inc., Chicago, IL, USA) and Excel 2013 software (Microsoft, Redmond, WA, USA). Differences were considered statistically significant when the confidence intervals showed no overlap of the mean values with an error value of 0.05.

Results

Analysis of *CarNAC3* and *CarNAC6* Nucleotide Sequences

Schematic diagrams of the *CarNAC3* and *CarNAC6* full-length cDNA sequences contained in the PBI121 binary vector constructs used for transformation of poplar are shown in Fig. 1. Based on analysis using the CLC Genomics Workbench 3 software (CLC, Taipei, Taiwan), the *CarNAC3* gene contained an 858-nt open reading frame (ORF), a 64-bp 5′-untranslated region (UTR), and a 238-bp 3′-UTR. The predicted CarNAC3 protein was 286 aa in length with an approximate molecular weight of 277 kDa, contained seven predicted α-helices, and the N-terminal region between amino acids 9 and 161 contained five subdomains according to Ooka et al. (2003). The *CarNAC6* nucleotide sequence contained two opposing ORFs that were 924 and 309 nt in length with 145-bp 5′- and 85-bp 3′-UTRs and 332-bp 5′- and 513-bp 3′-UTRs, respectively. The predicted positive-strand CarNAC6 protein was 307 aa in length with an approximate molecular weight of 299 kDa and contained seven predicted α-helices. The predicted complementary-strand CarNAC6 protein was 102 aa in length with an approximate molecular weight of 99 kDa and without predicted α-helix. Alignments of the CarNAC3 and CarNAC6 protein sequences were generated using the Clustal program in the CLC Genomics Workbench 3 software to detect conserved sequence motifs and to determine homology scores (Supplemental Data 1).

Confirmation of Transformation, Transgene Copy Number, and Relative Expression Levels

We confirmed the transformation of eight (each gene four lines) putative transgenic poplar lines that grew on MS selection medium supplemented with kanamycin using PCR followed by electrophoresis of the reaction products with

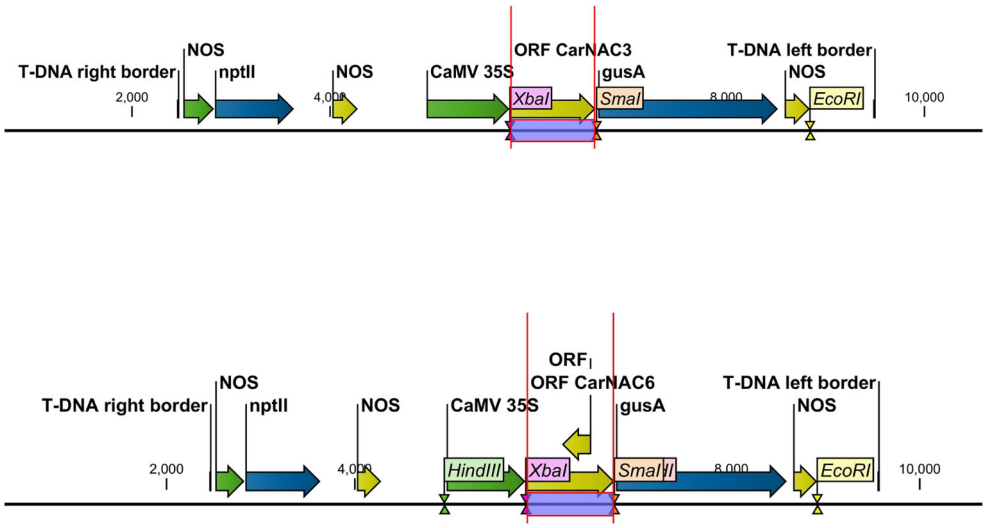


Fig. 1 Schematic diagram of the T-DNA regions of PBI121 binary vector constructs containing the *CarNAC3* and *CarNAC6* cDNA sequences with expression driven by the CaMV 35S promoter

DL2000 DNA Markers on a 1.2 % agarose gel. The production of bright gel bands from all of the transgenic lines corresponding to the relevant positive control bands produced from the *PBI121/CarNAC3* and *PBI121/CarNAC6* plasmid DNAs demonstrated that both transgenes were integrated into the poplar genome (Supplemental Data 2).

We determined *CarNAC3* and *CarNAC6* transgene copy numbers using RT-PCR. Based on the formula, $X=Y-$intercept/slope degree where X=copy number and $Y=C_t$, the value

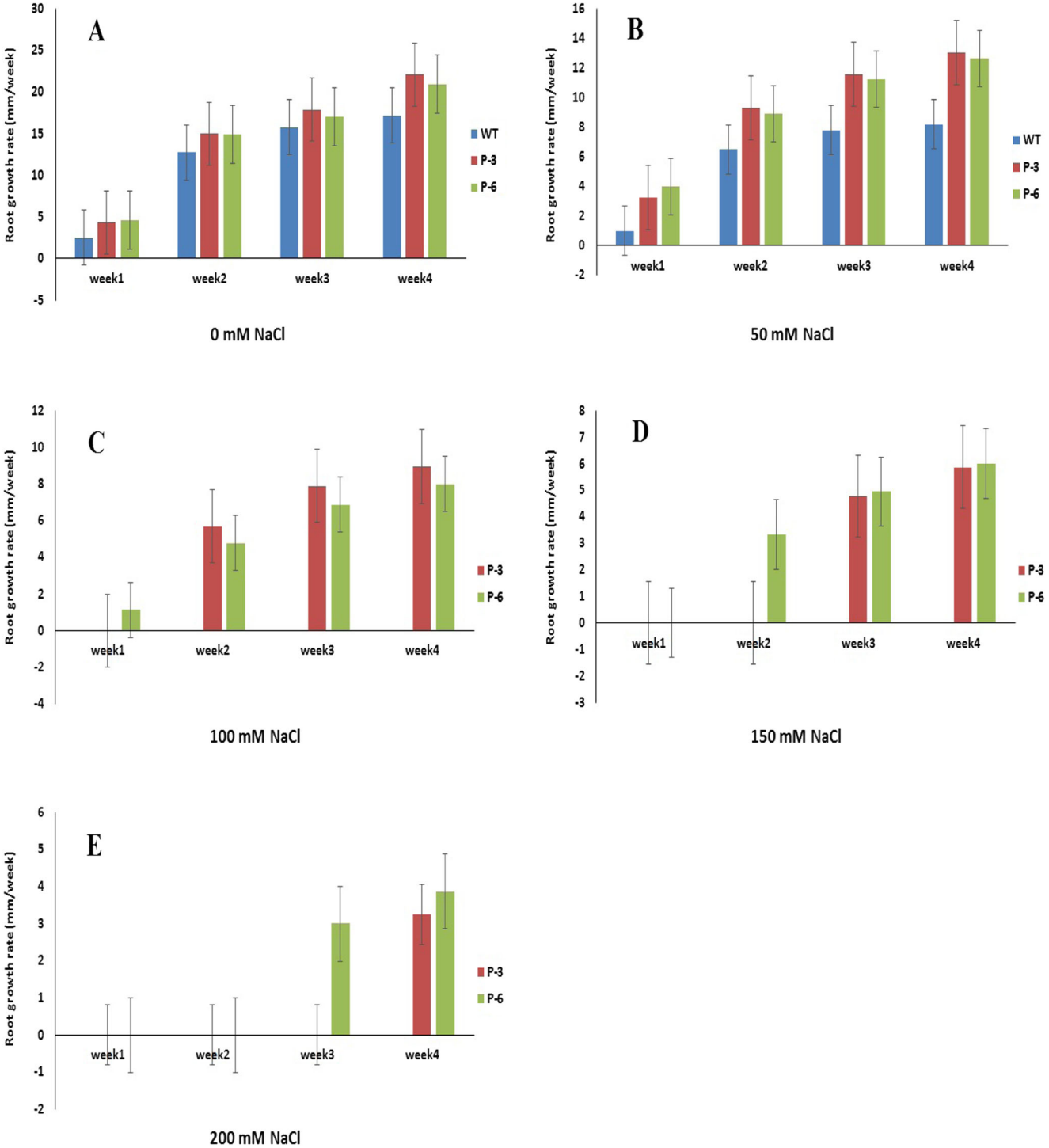


Fig. 2 Comparison of root growth rates (mm/week) in transformed poplar plants and wild-type (WT) plants grown for 4 weeks in agar medium containing various NaCl concentrations. **a**–**e** Root growth rates in 0, 50, 100, 150, and 200 mM NaCl concentrations. *P-3 CarNAC3* plants, *P-6 CarNAC6* plants

average of four lines of *CarNAC3* gene copy number was 6.668 with a slope of −3.35 and an R^2 value of 1.0 (Supplemental Data 3c). The value average of four lines of *CarNAC6* gene copy number was 5.933 with a slope of −3.557 and an R^2 value of 0.999 (Supplemental Data 3f).

The C_t values for the *CarNAC3* and *CarNAC6* genes during the exponential phase of amplification were 1.6 and 1.5, respectively (Supplemental Data 3a, d). Comparative quantitative analysis using the $\Delta\Delta C_t$ method with expression of the *β-actin* housekeeping gene serving as an internal reference showed that *CarNAC3* expression was approximately fivefold higher than that of *CarNAC6*, taking into account the similar gene copy numbers (Supplemental Data 4c).

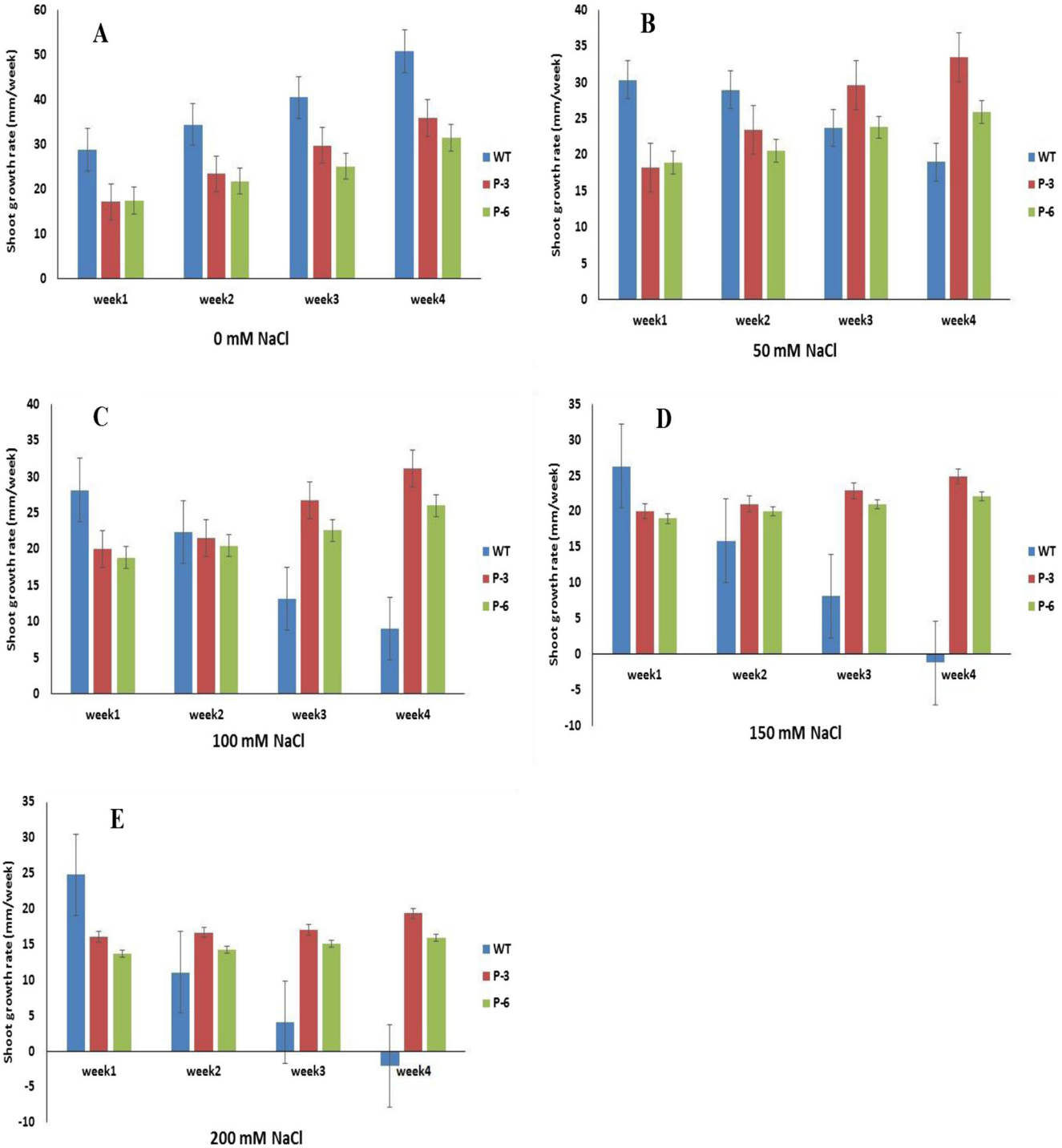


Fig. 3 Comparison of shoot growth rates (mm/week) in transformed poplar and WT plants grown for 4 weeks in agar medium containing various NaCl concentrations. **a**–**e** Shoot growth rates in 0, 50, 100, 150, and 200 mM NaCl. *P-3 CarNAC3* plants, *P-6 CarNAC6* plants

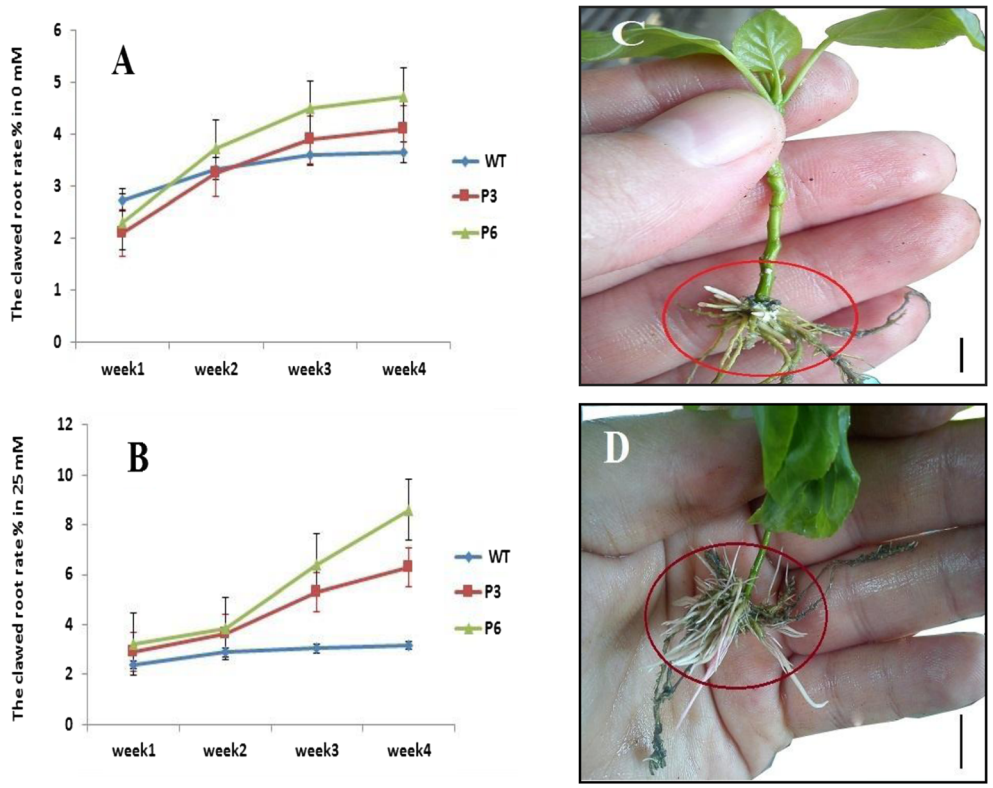


Fig. 4 Comparison of clawed root rates (%) in WT and transgenic poplar plants after 4 weeks of growth. **a** Clawed root rate (%) in the absence of stress (0 mM NaCl). **b** Clawed root rate under mild salt stress (25 mM NaCl). **c** A representative *CarNAC3* transgenic poplar plant after the third week of treatment with 25 mM NaCl. **d** A representative *CarNAC6* transgenic poplar plant after the third week of treatment with 25 mM NaCl. *P-3 CarNAC3* plants, *P-6 CarNAC6* plants. *Scale bar* 1 cm

Effects of Transgenic *CarNAC3* and *CarNAC6* Expression on Growth Phenotypes of Poplar Plants

We examined the effects of *CarNAC3* and *CarNAC6* gene expression on salt tolerance in transgenic poplar plants as indicated by root and shoot growth rates and the clawed root rate in plants grown on MS agar medium containing various concentrations of NaCl. Both transgenic and WT poplar plants exhibited normal root growth in medium lacking NaCl over a 4-week period, but the transgenic poplar plants exhibited a higher rate of root growth than the WT plants (Fig. 2a). Under mild salt stress (50 mM NaCl), the leaves of WT plants turned yellow and their root growth rates were lower than those of the transgenic poplar lines (Fig. 2b). These results showed that, under mild salt stress, the transgenic lines maintained normal root growth rates while the WT lines did not.

Under moderate salt stress (100 mM NaCl), most leaves of the WT plants appeared withered and yellow and root growth ceased, whereas the transgenic plants exhibited normal root growth. Under this condition, the *CarNAC3* transgenic plants started to root during the second week (Fig. 2c). Under high salt stress (150 mM NaCl), the WT plants did not survive, but all of the transgenic lines remained unaffected until the fourth week. Root growth ceased in some of the transgenic lines during the first and second weeks. Under severe salt stress (200 mM NaCl), root growth continued in all of the transgenic lines but with different rates and the *CarNAC6* transgenic plants exhibited greater root growth than the *CarNAC3* transgenic plants, consistent with the higher expression of *CarNAC6* than *CarNAC3* in roots (Fig. 2d, e).

In contrast, the transgenic and WT plants maintained normal shoot growth rates during the 4-week period in the absence of salt stress (0 mM NaCl), with the WT plants exhibiting higher shoot growth rates than the transgenic lines (Fig. 3a). Increasing the NaCl concentration to mild or moderate levels of salt stress (50 and 100 mM NaCl) decreased the shoot growth rates in WT plants but had no effect on the transgenic plants, which maintained approximately constant growth rates with a higher rate in the *CarNAC3* plants than in the *CarNAC6* plants (Fig. 3b, c).

Shoot growth stopped during the fourth week in the WT plants subjected to 150 mM NaCl, but the transgenic lines were unaffected (Fig. 3d). Under severe salt stress (200 mM NaCl), all of the transgenic lines maintained normal shoot growth rates, but some of the WT plants withered and none maintained normal shoot growth (Fig. 3e).

The effects of the transgenes on the clawed root rate were high in both types of transgenic lines and increased significantly under mild salt-stress conditions (Fig. 4a, b). The *CarNAC6* transgenic plants exhibited a higher clawed root rate than the *CarNAC3* transgenic plants (Fig. 4c, d).

Evaluation of Drought and Salt Tolerance of Transgenic *CarNAC3* and *CarNAC6* Poplar Plants Under Greenhouse Conditions

When grown in soil in the greenhouse without salt stress, the WT plants exhibited higher growth rates after 3 weeks than the transgenic plants and the transgenic *CarNAC6* plants grew at higher rates than the transgenic *CarNAC3* plants (Fig. 5a). To compare the growth of the transgenic *CarNAC3* and *CarNAC6* plants with that of the WT plants under drought and salt stress conditions, growth continued to be monitored after commencing irrigation with NaCl concentrations of 0, 50, 100, and 150 mM at the start of the fifth week. For all treatments, the plants were irrigated at intervals of 3 days for 2 weeks. Each treatment included three clones of the transformed poplar lines with three replications and the averages of the data from all of the clones were used for statistical analysis.

The growth of the WT plants in soil watered with 50 mM NaCl continued, but at a much lower rate than when watered without NaCl and was suppressed by 100 mM NaCl, while the transgenic lines maintained normal growth rates at both NaCl concentrations (Fig. 5b). These results indicated that the growth of the transgenic *CarNAC3* and *CarNAC6* plants was enhanced under salt stress conditions relative to that of the WT plants. The growth rates of the *CarNAC3* plants in 100 and 150 mM NaCl were significantly higher than those of the *CarNAC6* plants, possibly due to the much higher expression of the *CarNAC3* transgene (Fig. 5c). These results indicated that the growth of the transgenic *CarNAC3* and *CarNAC6* poplar plants was superior to that of the WT plants under

Fig. 6 Antioxidant activities in WT and transgenic poplar plants under drought- and salt-stress conditions. **a**, **b** Proline accumulation in transgenic *CarNAC3* and *CarNAC6* transgenic plants and WT control plants under drought- and salt-stress conditions. **c**, **d** Superoxide dismutase (*SOD*) enzymatic activity in *CarNAC3* and *CarNAC6* transgenic and WT control plants under drought- and salt-stress conditions. **e**, **f** Guaiacol peroxidase (*POD*) enzymatic activity in *CarNAC3* and *CarNAC6* transgenic plants and WT control plants under drought- and salt-stress conditions. **g**, **h** Malondialdehyde (*MDH*) accumulation in response to drought and salt stresses in *CarNAC3* and *CarNAC6* transgenic plants and WT plants. *P-3 CarNAC3* plants, *P-6 CarNAC6* plants

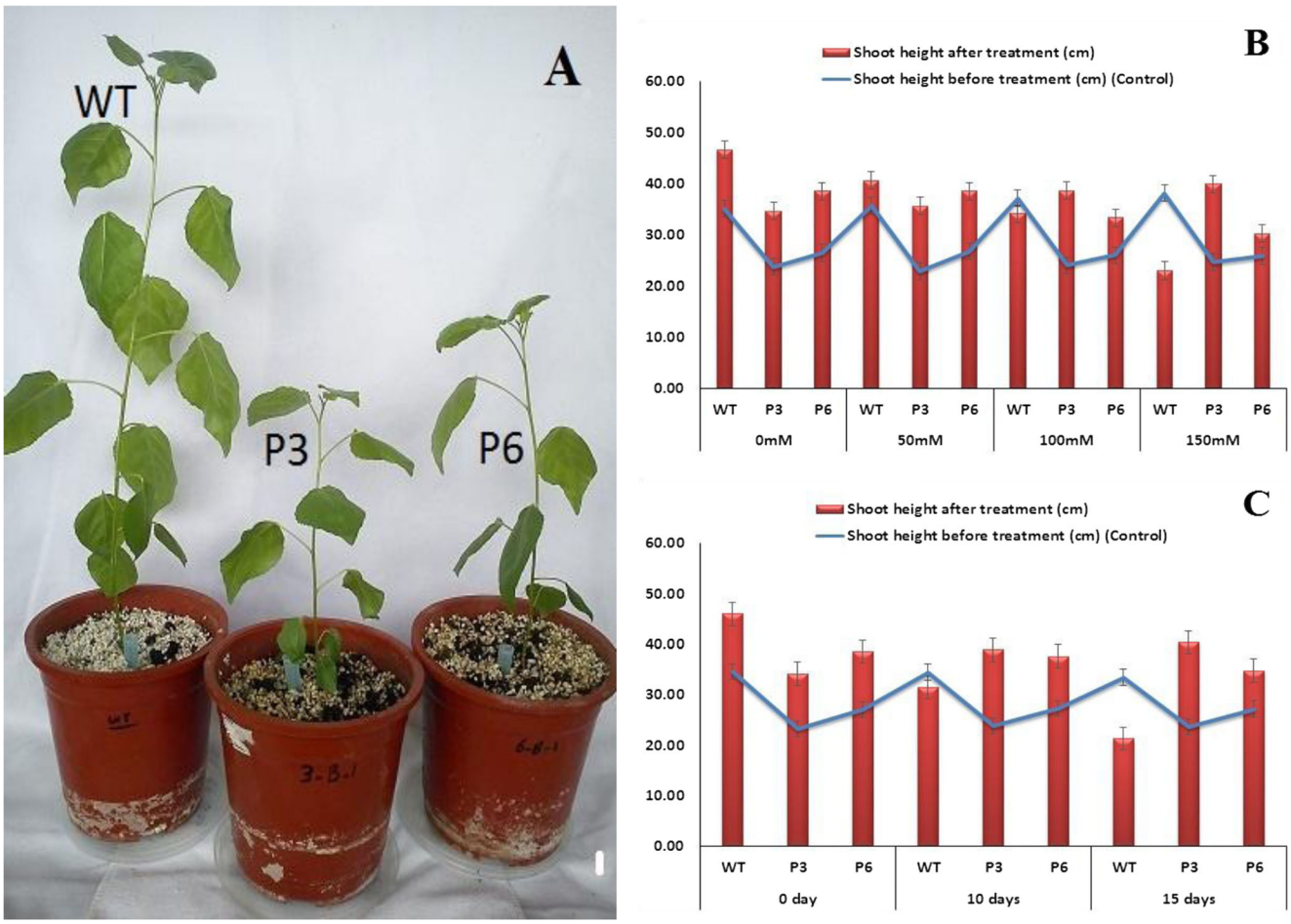
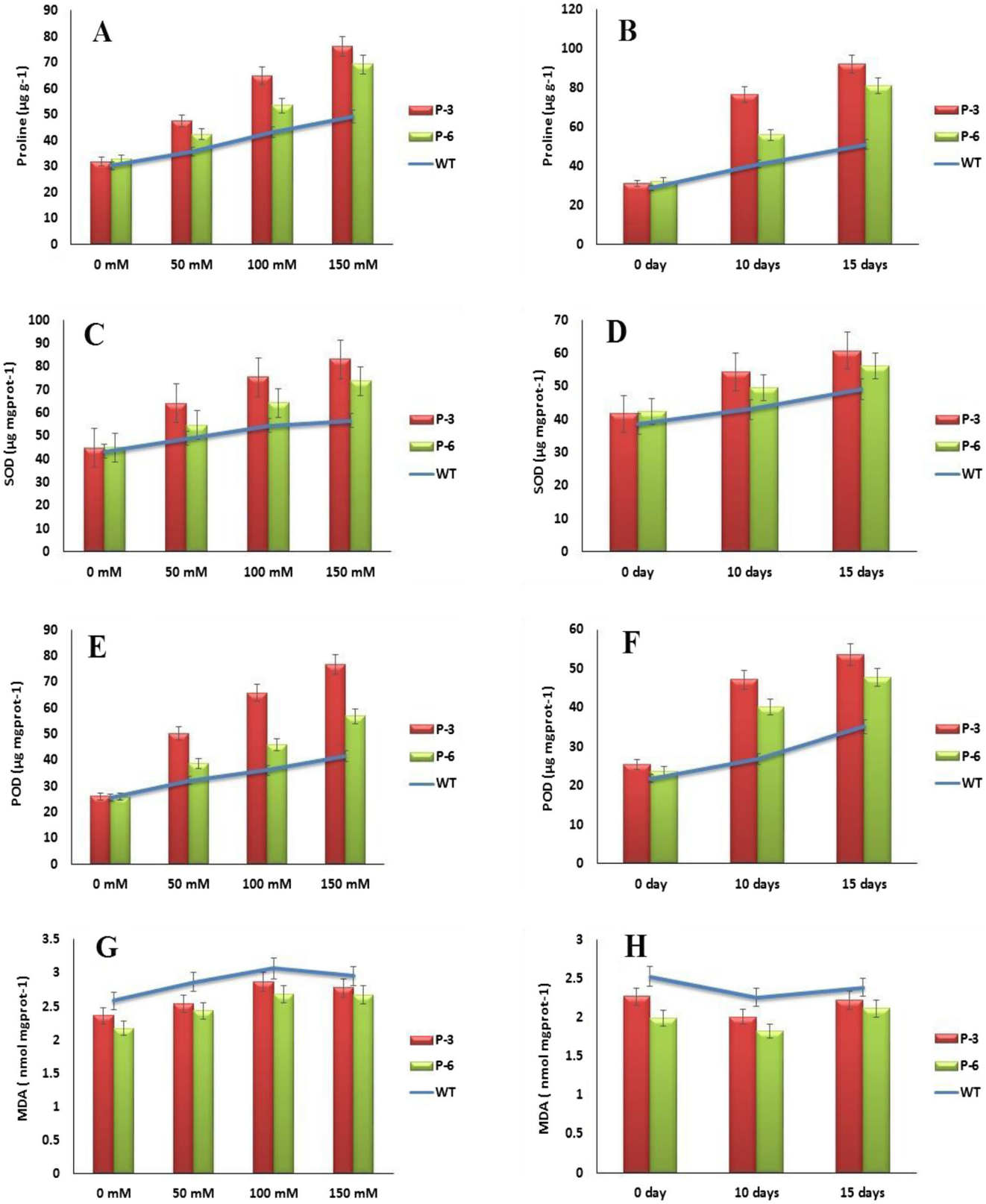


Fig. 5 Comparison of shoot heights between WT and transgenic poplar plants with standard *error bars*. **a** Representative WT and transgenic *CarNAC3* and *CarNAC6* poplar plants after 3 weeks. **b** Shoot height before and after salt stress with various NaCl concentrations. **c** Shoot height before and after drought stress for various lengths of time. *P-3 CarNAC3* plants, *P-6 CarNAC6* plants. *White scale bar*, 1 cm



salt-stress conditions. These results were also consistent with the much higher expression level of the *CarNAC3* transgene under conditions of severe drought and salt stress, as determined by RT-PCR.

Proline, MDA, Chlorophyll, and Carotenoid Amounts and SOD and POD Activities

Proline, one of the most important osmoprotectants in plants, protects cells from damage caused by drought and salt stresses. The amounts of proline were significantly higher in the *CarNAC3* and *CarNAC6* plants than in the WT control plants under drought- and salt-stress conditions (Fig. 6a, b). SOD activity did not increase significantly in WT plants in response to drought or salt stress, but did increase significantly in all of the transgenic lines (Fig. 6c, d). In addition, POD activity was significantly higher in the transgenic lines than in the WT plants under all treatment conditions (Fig. 6e, f).

In the absence of salt stress, the MDA concentrations in the expressing *CarNAC3* and *CarNAC6* plants were 1.1- and 1.2-fold lower respectively than WT plants mathematically (Fig. 6g, h). After salt treatment for 2 weeks, the MDA concentrations increase significantly in the *CarNAC3* and *CarNAC6* transgenic lines.

In the absence of salt stress, the chlorophyll and carotenoid concentrations in the transgenic lines were similar to those in the WT plants (Fig. 7a, b). Under drought- and salt-stress conditions, the chlorophyll concentrations decreased significantly in both the transgenic and WT plants in contrast to the carotenoid levels, which increased. Unlike the transgenic plants, the decrease in the chlorophyll content was greater in response to salt stress than to drought stress in the WT plants. The increases in the carotenoid levels in the WT and transgenic plants were similar but were greater the transgenic plants. These results demonstrated that the effects of transgenic expression of the *CarNAC3* and *CarNAC6* genes were greater under salt stress than under drought stress. The *CarNAC3* lines were more tolerant of both stress conditions than the *CarNAC6* lines in all experiments.

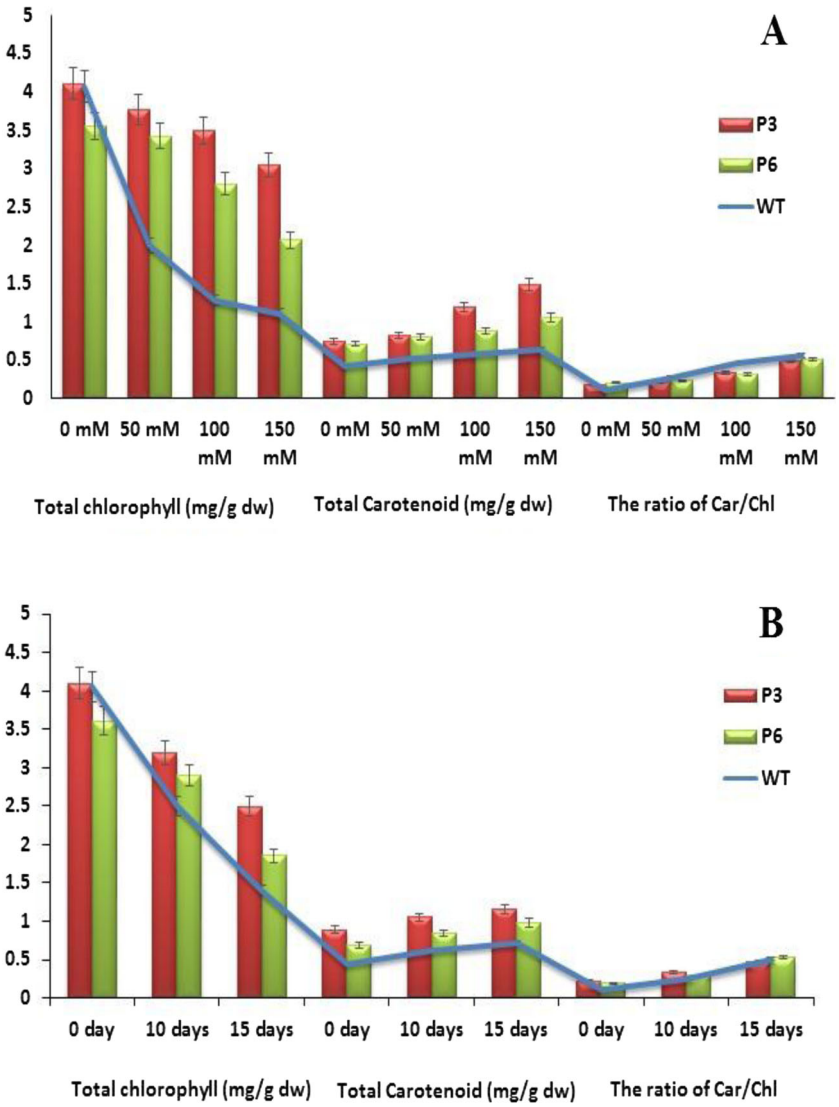


Fig. 7 Comparison of photosynthetic pigment levels in *CarNAC3* and *CarNAC6* transgenic plants and WT plants under drought- and salt-stress conditions. **a** Effects of various levels of salt stress on chlorophyll and carotenoid contents. **b** Effects of drought stress on chlorophyll and carotenoid contents. *P-3 CarNAC3* plants, *P-6 CarNAC6* plants

Discussion

The presence of related domains in different proteins may indicate that the proteins have similar biological functions. A phylogenetic analysis of the CarNAC3 protein sequence placed it in the NAC-LIKE ACTIVATED BY AP3/PI (NAP) subgroup containing the NAP, AtNAM, AtNAC2, ANAC047, NAM-B1, GmNAC1, and GhNAC5 proteins (Hui et al. 2009). NAC-type TFs play important roles in the control of responses that confer tolerance to environmental stresses such as drought and salt stress. Overexpression of stress-responsive Arabidopsis *SNAC-A* genes such as *RD26* and *ATAF1* and rice *SNAC-A* genes such as *SNAC1*, *OsNAC6/SNAC2*, and *OsNAC5* enhanced drought and salt stress tolerance (Hu et al. 2006; Nakashima et al. 2007; Takasaki et al. 2010). Transgenic plants overexpressing the *OsNAC6* gene exhibited less growth than transgenic plants overexpressing the *SNAC1* or *OsNAC5* genes, which exhibited growth similar to that of control plants (Hu et al. 2006; Nakashima et al. 2007; Takasaki et al. 2010). The *CarNAC3* gene was expressed primarily in blooming flowers, similar to *GmNAC1* and *NAP* (Sablowski and Meyerowitz 1998; Meng et al. 2007). Moreover, a close homolog of the CarNAC3 and AtNAC2 proteins was shown to be a transcription factor that acts downstream of the auxin signaling pathway to promote lateral root formation (He et al. 2005). Based on these prior results, we investigated the role of *CarNAC3* in lateral root development. In this study, we transformed the *CarNAC3* and *CarANC6* genes into poplar to investigate the effects of their expression in comparison with WT poplar plants. We confirmed the integration of these genes into the poplar genome and their expression using PCR and RT-PCR.

Proline accumulation can affect various physiological processes of plants, such as osmotic adjustment, which is an important mechanism that confers tolerance to drought and salt stress conditions. Proline plays a critical role as an osmoprotectant in the regulation of gene expression in plants subjected to salt stress (Bursy et al. 2007). Proline accumulation could increase the capacity for osmotic adjustment and increase the level of abiotic stress tolerance. In our study, the proline levels in WT and transgenic lines were approximately similar in the absence of stress, but drought and salt treatments caused increased *CarNAC3* and *CarNAC6* expression in transformed plants and an associated increase in proline accumulation (Fig. 6a, b). Therefore, the osmotic adjustment capacity of the transgenic lines was enhanced relative to the control plants. The improved osmotic adjustment capacity resulting from *CarNAC3* and *CarNAC6* expression may enable the maintenance of chlorophyll and carotenoid levels to preserve plant growth while subjected to drought and salt stress conditions (Fig. 7a, b). SOD and POD activities and MDA concentration are commonly used indicators of the magnitude of a plant response to abiotic stress (Mittova et al. 2004). Transgenic expression of the *CarNAC3* and *CarNAC6* genes was shown to increase SOD and POD activities significantly under abiotic stress conditions and to decrease MDA concentrations markedly (Song et al. 2006; Bhagat et al. 2011). In this study, the SOD and POD activities in transgenic lines increased markedly in response to drought and salt stress conditions (Fig. 6c–f) and the MDA concentrations decreased significantly in response to drought stress and severe salt stress relative to the control plants (Fig. 6g, h).

These results suggest that *CarNAC3* and *CarNAC6* gene expression in poplar reduces oxidative damage to membranes and increases salinity and drought tolerance against stresses. Furthermore, the *CarNAC3* gene may play a central role in this resistance due to its higher level of expression than that of the *CarNAC6* gene. In the absence of stress, all of the *CarNAC3* and *CarNAC6* transgenic lines exhibited SOD and POD activity levels similar to those of the WT plants, but under abiotic stress conditions, the *CarNAC3* transgenic lines exhibited higher activity levels than the *CarNAC6* transgenic lines. These results suggest that *CarNAC3* and *CarNAC6* perform different functions in oxidative stress responses.

In summary, we introduced the *CarNAC3* and *CarNAC6* genes into poplar and confirmed by molecular analyses integration of both into the poplar genome and their stable expression at the transcript level. *CarNAC3* gene expression mediated osmotic protection and induced antioxidant enzyme systems to higher levels than did *CarNAC6* gene expression resulting in improved drought and salt tolerance.

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Conflict of Interest The authors do not have conflicts of interest to declare.

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