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Expression Analysis of Two NAC Transcription Factors *PtNAC068* and *PtNAC154* from Poplar

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Abstract *NAM/ATAF/CUC* (*NAC*) family genes comprise one of the largest families of transcription factors in plant genomes and are widely expressed in developing woody tissues. In the present study, we constructed plant transformation vectors using the β-glucuronidase (*GUS*) reporter gene system and detected the promoter expression patterns derived from the *PtNAC068* and *PtNAC154* genes of Chinese white poplar (*Populus tomentosa* Carr.) in transgenic poplars (*Populus alba* × *Populus glandulosa*). The results showed that the *GUS* expression driven by *PtNAC068* and *PtNAC154* promoters may be more complex in poplar than they are in *Arabidopsis*. Histochemical GUS assays showed that *GUS* activity driven by *PtNAC068* promoter was mainly in vascular tissues of stems, leaves, petioles, and roots, while that driven by *PtNAC154* promoter was confined to the developing secondary xylem of stems and veins of leaves. The transcript level of both *PtNAC068* and *PtNAC154* in successive internodes below the apex was found to be much higher in IN5-10 compared to that in IN2-4 as measured by real-time RT-PCR, suggesting that *PtNAC068* and *PtNAC154* upregulation is related to secondary growth in poplar. *GUS* expression in internodes 3–8 of *ProNAC068::GUS* transgenic plants was 30-fold higher than that in *ProNAC154::GUS* transgenic plants. The differences in the expression pattern and transcript level of mRNA accumulation indicate that *PtNAC068* and *PtNAC154* may be involved in two distinct aspects of vascular tissue development.

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Abbreviations

aa	Amino acid
CTAB	Cetyl trimethylammonium bromide
GUS	β-Glucuronidase
IN	Internode
NAC	NAM/ATAF/CUC
NPTII	Neomycin phosphotransferase II
RT-qPCR	Real-time quantitative PCR
X-gluc	5-Bromo-4-chloro-3-indoxyl-beta-D-glucuronic acid

Introduction

The formation of wood (secondary xylem) involves sequential developmental events encompassing the differentiation of xylem mother cells from the vascular cambium, cell division, cell expansion, cell wall thickening, programmed cell death, and formation of heart wood (Plomion et al. 2001). Several classes of transcription factors in tree species have been shown to be associated with secondary growth and wood formation such as AUXIN RESPONSE FACTOR (ARF), CLASS III HOMEODOMAIN–LEUCINE ZIPPER (HD-ZIPIII), KANADI (KAN), MYB, and NAM/ATAF/CUC (NAC) (Demura and Fukuda 2007; Du and Groover 2010). NAC domain proteins comprise a large protein family represented by 135 genes in *Arabidopsis* (http://planttfdb.cbi.pku.edu.cn/family.php?fam=NAC), 140 genes in rice (Fang et al. 2008), 152 genes in tobacco (Rushton et al. 2008), 101 genes in soybean genome (Pinheiro et al. 2009), and 163 genes in poplar (Hu et al. 2010). The NAC transcription

factors contain a highly conserved N-terminal domain involved in DNA binding and a C-terminal region highly divergent in sequence and length (Xie et al. 2000; Duval et al. 2002; Tran et al. 2004). N-terminal NAC domain contains a unique transcription factor with a fold consisting of a twisted sheet surrounded by a few helical elements (Ernst et al. 2004). The NAC domain has been implicated in nuclear localization, DNA binding, and the formation of homodimers or heterodimers with NAC domain proteins (Ernst et al. 2004; Olsen et al. 2005; Jensen et al. 2010). The C-terminal regions of NAC proteins confer the regulation diversities of transcriptional activation activity (Duval et al. 2002; Taoka et al. 2004; Yamaguchi et al. 2008; Shen et al. 2009).

Recently, NACs are known to be regulated in diverse processes of plant development including plant organ senescence (Guo and Gan 2006), stress-induced peel pitting (Fan et al. 2007), fruit ripening (Liu et al. 2009), xylem cells differentiation (Kubo et al. 2005; Zhao et al. 2005), and secondary cell wall biosynthesis (Mitsuda et al. 2007; Zhong et al. 2007; Mitsuda and Ohme-Takagi 2008). For the model tree species, *Populus*, a total of 163 full-length NAC genes were identified by a comprehensive analysis of NAC gene family, and they were phylogenetically clustered into 18 distinct subfamilies (Hu et al. 2010). Grant et al. (2010) studied NACs which may have a putative involvement in secondary cell wall synthesis as negative or positive cell wall regulators. For example, *XYLEM NAC DOMAIN 1* (*XND1*) overexpression in poplar resulted in severe stunting and reduction in cell size and number, vessel number, and frequency of rays in the xylem (Grant et al. 2010). Overexpression of *PopNAC154* in poplar reduced height growth and increased the relative proportion of bark versus xylem (Grant et al. 2010). Yamaguchi and Demura (2010) summarized the function and regulation of the NAC domain proteins belonging to the VND/NST/SND1 (VASCULAR-RELATED NAC-DOMAIN PROTEIN/NAC-SECONDARY WALL THICKENING PROMOTING FACTOR/SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN) subfamily controlling secondary wall formation. *PtrWND2B* and *PtrWND6B* were able to activate the promoters of poplar wood biosynthetic genes and a number of other poplar wood-associated transcription factors (Zhong et al. 2009). PtrWNDs are proposed to be master transcriptional switches regulating a cascade of downstream transcription factors and thereby mediating the coordinated activation of wood biosynthetic genes (Zhong and Ye 2010). Shen et al. (2009) performed a genomewide bioinformatic analysis of NAC genes for plant cell wall development and identified a total of 1,232 NAC proteins from 11 different organisms, including 148 *PtNAC* genes from *Populus*, by sequence phylogeny based on protein DNA-binding domains. The NAC proteins were classified into eight subfamilies (NAC-a to NAC-h).

In *Arabidopsis*, *ANAC012* and *ANAC073* are NAC family transcription factors which were highly expressed in the secondary xylem tissue (Ko et al. 2006). In our previous study, both proteomic and microarray analysis were used to study the gene expression profiles during the different stages of the secondary vascular system regeneration in Chinese white poplar (*Populus tomentosa* Carr.) (Du et al. 2006; Wang et al. 2009). A number of tissue-specific genes including NAC transcription factors showed transcript-level differences at different regeneration stages (Wang et al. 2009). According to the cDNA microarray data, the transcription of *PtNAC068* and *PtNAC154* was abundant at later regeneration stages when xylem mother cells were in differentiation (Wang et al. 2009). Therefore, we selected these two NAC transcription factors, which are highly homologous with *NST3/ANAC012* and *SND2/ANAC073* from *Arabidopsis*, respectively. To further elucidate their roles in wood formation, we isolated their promoters in order to drive GUS as a reporter to study their expression patterns in vascular tissues in poplar. The results showed that these two NAC promoters were expressed in vascular tissues with obvious differences in pattern and abundance, which provides clues for further dissection of the roles of NAC transcription factors in the development of vascular tissues.

Materials and Methods

Plant Material, Bacterial Strains, and Vectors

Chinese white poplar plants (*P. tomentosa* Carr.) were grown in a greenhouse. The top three expanded leaves were used as the source material for DNA extraction. Hybrid poplar (*P. alba* × *P. glandulosa*) clone 84 K was cultured and used for genetic transformation. *Escherichia coli* DH5α and *Agrobacterium tumefaciens* GV3101 were applied in DNA manipulation and plant transformation, respectively. pGEM-T Easy (Promega, Madison, WI, USA) and pBI121 vectors were kept in the laboratory.

Bioinformatic Analysis of *PtNAC068* and *PtNAC154*

The amino acid sequences of the *Arabidopsis NST3/ANAC012* (At1g32770) and *SND2/ANAC073* (At4g28500) were retrieved from The Arabidopsis Information Resource (http://www.arabidopsis.org/index.jsp). The sequences were BLASTed against the database of poplar protein sequences at the Joint Genome Institute (JGI; http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html). The alignment of deduced amino acid (aa) sequences and phylogenetic tree were

computed using DNAMAN program (version 5.2.2.0; Lynnon Corp., Pointe-Claire, Quebec, Canada). The occurrence of putative *cis*-acting regulatory elements was studied using PLACE program (http://www.dna.affrc.go.jp/PLACE/index.html) (Higo et al. 1999).

Construction of *ProNAC068::GUS* and *ProNAC154::GUS* Constructs

Genomic DNA was isolated from young leaves of poplar plants using cetyl trimethylammonium bromide method (Sambrook and Russell 2001). Based on phylogenetic analysis, gw1.XI.947.1 (http://genome.jgi-psf.org/cgi-bin/dispGeneModel?db=Poptr1_1&id=232967) and estExt_fgenesh4_pg.C_640203 (http://genome.jgi-psf.org/cgi-bin/dispGeneModel?db=Poptr1_1&id=827160) were used as templates to amplify the poplar *NAC068* and *NAC154* promoters, respectively, from *P. tomentosa* genomic DNA using promoter-amplified primers (Table 1), and the fragments of 901 bp spanning −733 to +168 for *NAC068* promoter and 668 bp spanning −667 to +1 for *NAC154* promoter were amplified, respectively. The two fragments were cloned into the pGEM-T Easy vector and sequenced using T7 and SP6 primers from outside of the promoter sequence. DNA sequencing was used to verify the veracity of sequences.

For both promoters, forward and reverse primers were added to a linker, which could be recognized by *Hind*III (AAGCTT) and *Xma*I (CCCGGG). After digestion with *Hind*III and *Xma*I, the two fragments were directionally inserted into pBI121, which was cut with the same enzymes to remove the 35S promoter. The constructs *ProNAC068::GUS* and *ProNAC154::GUS* (Fig. 1) were then isolated from transformed *E. coli* DH5α cells and transformed into *A. tumefaciens* GV3101. All of the above procedures were performed as described by Sambrook and Russell (2001).

Transformation and Characterization of Hybrid Poplar Clone 84 K

Leaves from cultured hybrid poplar 84 K plantlets were used for the transformation via *A. tumefaciens* GV3101 harboring *ProNAC068::GUS*, *ProNAC154::GUS* and pBI121, respectively, as described by Zhou et al. (2009). Infected leaf discs were co-cultured in differentiation Murashige and Skoog (MS) basal medium supplemented with 0.5 mg/L 6-benzylaminopurine (6-BA) and 0.05 mg/L α-naphthaleneacetic acid (NAA) in the dark for 3 days at 25°C, and then transferred on the same medium with 30 mg/L kanamycin and 200 mg/L timentin under a 16/8-h (day/night) photoperiod with a light intensity of 50 μmol m^{-2} s^{-1} provided by cool white fluorescent tubes. Individual regenerated shoots about 1~2 cm high were removed and induced for rooting on 1/2 MS medium containing 0.1 mg/L IBA, 0.05 mg/L NAA, 30 mg/L kanamycin, and 200 mg/L timentin. The putative transgenic plants were screened by genomic PCR amplification of the *NPTII* (neomycin phosphotransferase II) gene (494 bp) using detection primers (Table 1). Another set of primers for *ProNAC068::GUS* was used to confirm the correct promoter–transgene fusions (630 bp of the amplified DNA fragment) with forward primer at the promoter region and reverse primer at the *GUS* region (Table 1). Similarly, a pair of primers for *ProNAC154::GUS* was used to amplify a 401-bp fragment (Table 1). These plants were farther confirmed using GUS staining as described below. Positive transgenic plants (15–20 lines of each construct) together with controls (WT, wild plants) were transferred to a greenhouse. After 3 months, these plants were transplanted in the field in May 2010. A total of six to eight cuttings per line were potted for propagation in a greenhouse in January 2011. Two-month-old plants were used for histochemical GUS staining assay and real-time quantitative PCR.

Table 1 Primers used in this study

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
Promoter-amplified primers		
PtNAC068	CTGTAGTCTCTCGTTTGAGC	ATCTTCAGGCATTTTTGCTA
PtNAC154	AGCTCTCACGCATCAGATTGC	TTCCTTTGGTTTCCTCTTATG
Detection primers		
NPTII	ATCTCCTGTCATCTCACCTTGCTCCT	TCAGAAGAACTCGTCAAGAAG
ProNAC068::GUS	TCTTCTCCAAGGCTTAGACCCC	ACTTCCTGATTATTATTGACCCACA
ProNAC154::GUS	TTCTTTCAAGCCCTTCGTG	CTGCCCAACCTTTCGGTAT
Real-time PCR primers		
PtNAC068	CAATGGTCAGTCTCAGGTTCC	TCCCATGGCTCAAGCTTATT
PtNAC154	CTGCATG ATATGGTGGCCTA	CCCTCAAGAATCTGGCTCCT
GUS	AGAGCTGATAGCGCGTGACA	GTGTGAG CGTCGCAGAACAT
β-tubulin	GATTTGTCCCTCGCGCTGT	TCGGTATAATGACCCTTGGCC

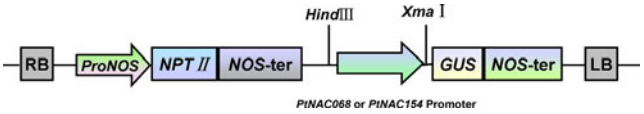

Fig. 1 Schematic representations of *ProNAC068::GUS* and *ProNAC154::GUS* constructs. *LB* and *RB*, left and right T-DNA borders, respectively; *NOS-ter*, nopaline synthase terminator; *NPTII*, neomycin phosphotransferase II; *GUS*, β-glucuronidase. The diagram is not drawn to scale

Histochemical GUS Staining

Histochemical staining for GUS enzyme activity was performed by incubating leaves, stems, petioles, and roots of transgenic and wild-type plants as described previously (Hawkins et al. 1997). In brief, stems and petioles were hand-cut into 2- to 4-mm sections, fixed for 20 min in 90% cold acetone, and then washed twice in rinsing solution (50 mM $NaPO_4$ pH 7.2, 0.5 mM $K_3Fe[CN]_6$, 0.5 mM $K_4Fe[CN]_6$). Plant materials were vacuum-infiltrated for 30 min at room temperature and then stained with 5-bromo-4-chloro-3-indoxyl-beta-D-glucuronic acid (X-gluc) staining solution (50 mM $NaPO_4$, pH 7.5, 0.5 mM $K_3Fe[CN]_6$, 0.5 mM $K_4Fe[CN]_6$, and 2 mM X-gluc), followed by overnight incubation at 37°C in X-gluc staining solution. Removal of chlorophyll was carried out via an ethanol series as follows: 15%, 30%, 50%, 70%, 85%, 95%, and 100%, each for 1 h. Thereafter, stems and petioles were embedded in paraffin (Paraplast Embedding Media- Paraplast Plus, Sigma, St. Louis, MO, USA) and cut into 12-μm-thick transverse sections with a microtome (LEICA RM 2135, Germany). Sections of stems and petioles were mounted on glass slides and viewed and photographed using a Carl Zeiss Axioimager A1 universal transmitted-light microscope. Images of leaves and roots were acquired using a scanner (EPSON PERFECTION V700 PHOTO, USA).

Total RNA Extraction and Real-Time Quantitative PCR (RT-qPCR)

Total RNA were extracted from the poplar samples using the RNeasy Plant Kit (Qiagen, Valencia, CA, USA) with DNase treatment. Purified RNA was quantified spectrophotometrically (NANODROP 8000, Thermo, Wilmington, NC, USA) and analyzed in denaturing 1.0% (*p*/*v*) agarose gel. cDNA synthesis was performed with 5 μg of total RNA, oligo-dT (18), and superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) according to the protocol of the manufacturer in a total volume of 20 μl.

Total RNA derived from the nine stem internodes in development series and stem apex of wild type plants were used to analyze the transcript levels of *PtNAC068* and *PtNAC154*. In addition, internodes 3–8 from three transgenic plants and wild-type plants were selected to analyze the transcript levels of *GUS* gene. Real-time quantitative PCR was performed using a 7500 Real Time PCR System (Applied Biosystems, CA, USA) and a SYBR® Premix Ex Taq™ Kit (TaKaRa, Tokyo, Japan) as described by Phillips et al. (2009). Primer pairs were designed using Primer3 Input 0.4.0 (http://frodo.wi.mit.edu/primer3/) to amplify fragments between 150 and 200 bp. To quantify the expression level of *PtNAC068*, *PtNAC154*, and *GUS*, the poplar *β-tubulin* gene was used as an endogenous control gene. Primers in real-time quantitative PCR were listed in Table 1. PCR reactions were prepared in 20-μl volumes containing 2 μl of 15-fold diluted cDNA synthesized above, 10 μl 2× SYBR® Premix Ex Taq™, 0.4 μl 10 μM forward primer, 0.4 μl 10 μM reverse primer, 0.4 μl 50 × RO × reference dye, and 6.8 μl sterile distilled water. Six replications per sample were carried out in parallel, and data analysis was performed as described by Pfaffl (2001).

Results

Bioinformatic Analysis of *PtNAC068* and *PtNAC154*

NST and SND are classified into different phylogenetic subgroups and motif clades (Shen et al. 2009). NST3/ANAC012 and SND2/ANAC073 were grouped into subgroups c-3 of the NAC-c subfamily and g-9 of the NAC-g subfamily, respectively. *PtNAC068* and *PtNAC154* have been identified as *Populus* homologs of *Arabidopsis NST3/ANAC012* (At1g32770) and *SND2/ANAC073* (At4g28500), respectively, by searching the JGI *Populus* (*Populus trichocarpa* v1.1) genome database. Two poplar genes, gw1.XI.947.1 and gw1.I.5485.1, were most closely related to *Arabidopsis NST3/ANAC012*. The 367 aa encoded by the *PtNAC068* gene consists of five conserved subdomains (A–E) located in NAC domain (Fig. 2a). These two genes are highly similar, sharing 91% identity in amino acid sequences (Fig. 2c). Therefore, only gw1.XI.947.1 (*PtNAC068*) was selected for promoter analysis.

As shown in Fig. 2b, estExt_fgenesh4_pg.C_640203 (*PtNAC154*) and eugene3.00070120 are highly similar by alignment of aa sequences. They are in the same clade with *Arabidopsis SND2/ANAC073*, sharing 97% identity in aa sequences (Fig. 2c). Therefore, estExt_fgenesh4_pg.C_640203 was used for promoter analysis.

Expression Patterns of *PtNAC068* and *PtNAC154* Conferred by Their Promoters

Histochemical GUS staining of all transgenic plants was performed in order to study tissue-specific expression. Transgenic poplar plants for both promoters showed a

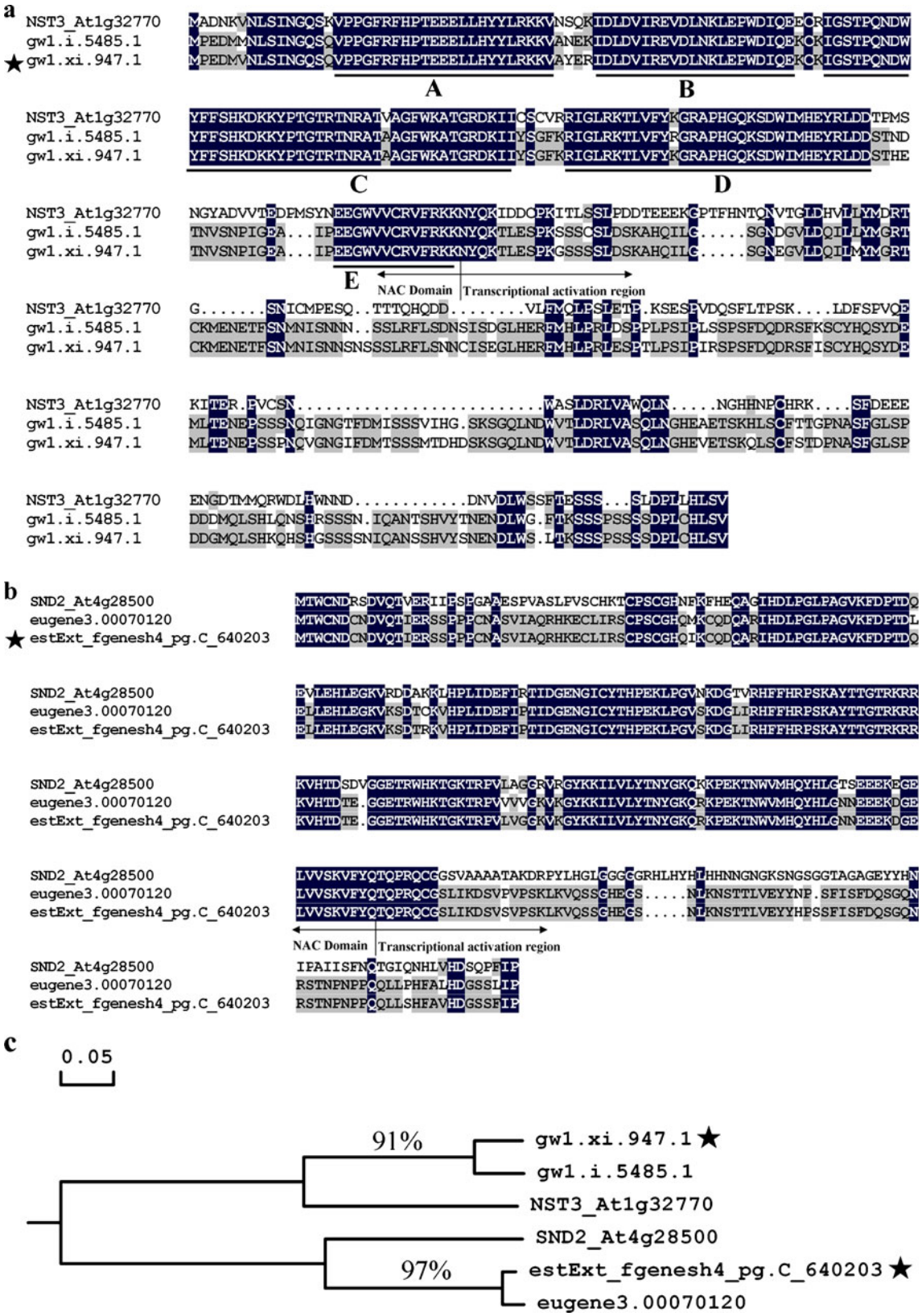

Fig. 2 Analysis of the aa sequences of the putative *PtNAC068* and *PtNAC154* encoding genes and their relationship to *Arabidopsis* NST3/ANAC012 and SND2/ANAC073. **a** *Arabidopsis* NST3/ANAC012 and its *Populus* homologs. **b** *Arabidopsis* SND2/ANAC073 and its *Populus* homologs. **c** Phylogenetic analysis of *Arabidopsis* NST3/ANAC012 and SND2/ANAC073 and their *Populus* homologs; *figures on branches* are bootstrap values. The *black areas* indicate the conserved amino acids. *Asterisks* indicate the genes used for promoter analysis

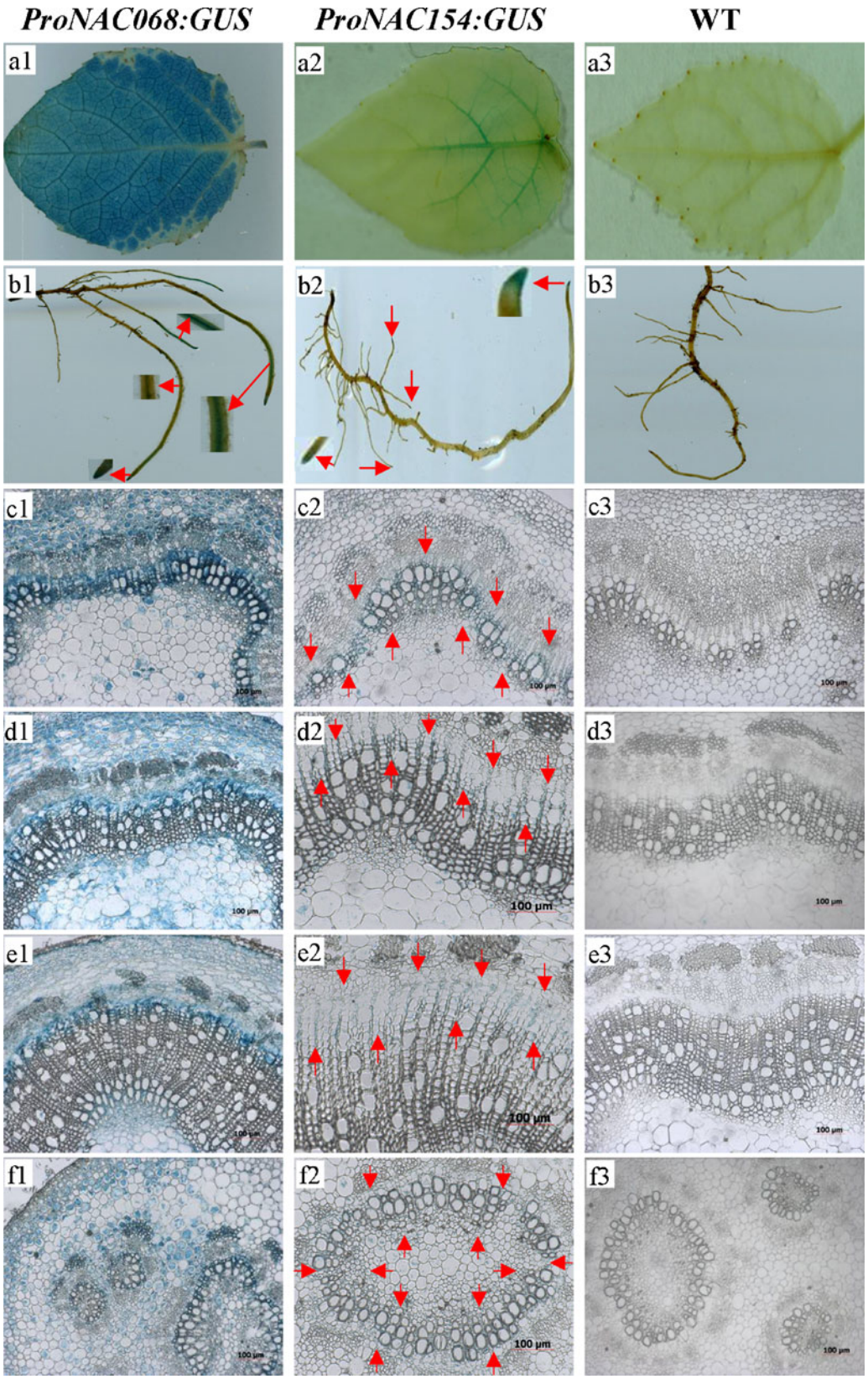

Fig. 3 Histochemical localization of GUS activity in transgenic poplars grown in greenhouse. **a1**–**f1** The *ProNAC068::GUS* transformants, **a2**–**f2** the *ProNAC154::GUS* transformants, **a3**–**f3** The wild-type (*WT*) poplar plants serving as negative controls. **a** The first expanding leaf, **b** adventitious root, **c**–**e** Transverse sections of IN 3, 5, 9; **f** Transverse sections of petiole from the fifth expanded leaf. The *stained* (or *enlarged*) areas were indicated with *red arrows* if necessary. *Scale bar* = 100 μm

great diversity of expression patterns. GUS activity driven by *PtNAC068* promoter was detected in whole leaf with vein in predominance (Fig. 3a1), vascular strands and root tips of roots (Fig. 3b1), and phloem and xylem of internode 3 (IN3) below the apex (Fig. 3c1). GUS expression was also observed in immature xylem of IN5 (Fig. 3d1) and IN9 (Fig. 3e1). With the increased degree of lignification, no GUS activity was detected in mature xylem cells (Fig. 3e1). In petiole cross-sections, blue staining was also mainly observed in vascular tissues (Fig. 3f1). In WT plants, no blue staining was detected in all the above-mentioned tissues (Fig. 3a3–f3). Thus, the activity of *PtNAC068* promoter was almost equally distributed to the vascular tissue of leaves, stems, petioles, and roots.

In *ProNAC154::GUS* transgenic plants, GUS activity on the transverse sections of stems and petioles revealed a strong association with secondary wall deposition. *PtNAC154* promoter was active only in veins close to the basal section in leaf (Fig. 3a2) and in the tips of roots (red arrows) (Fig. 3b2). GUS activity driven by *PtNAC154* promoter was observed throughout the xylem of IN3 (Fig. 4c2) but restricted to the developing secondary xylem tissues in lignifying stems at IN5 and IN9 as indicated between two rows of red arrows (Fig. 4d2, e2). Similarly, GUS activity in petioles was observed in xylem areas as shown in Fig. 4f2. Altogether the data suggested that *PtNAC154* promoter is active only in developing secondary xylem where the secondary wall thickening occurs.

Expression Level Analysis of *PtNAC068*, *PtNAC154*, and *GUS* by RT-qPCR

In order to verify the transcriptional changes during primary and secondary growth phases of the stem development, we further analyzed the expression profiles of *PtNAC068* and *PtNAC154* using RT-qPCR. IN1 was too short to sample; thus, stem apex including IN1 was selected for analysis with IN2-10. The two genes exhibited similar expression profiles in successive internodes below the apex (Fig. 4a). We detected a 1.2–2.0-fold higher level of expression of *PtNAC068* in IN5-10 compared with that in IN2-4. The transcript level increased from IN5 and kept stable in IN5-10; thus, the high expression of *PtNAC068* may be associated to the secondary growth of stems (Fig. 4a). On the other hand, the expression of *PtNAC154* exhibited a lower level in stem apex and IN2-4 but increased two- to three-fold in the following INs (Fig. 4b), coincident also with the development of secondary tissues (Fig. 3d2, e2).

As illustrated in Fig. 4c, *GUS* activity was 3-fold and 94-fold lower in *ProNAC068::GUS* and *ProNAC154::GUS* transgenic plants as compared to that in *35S::GUS*

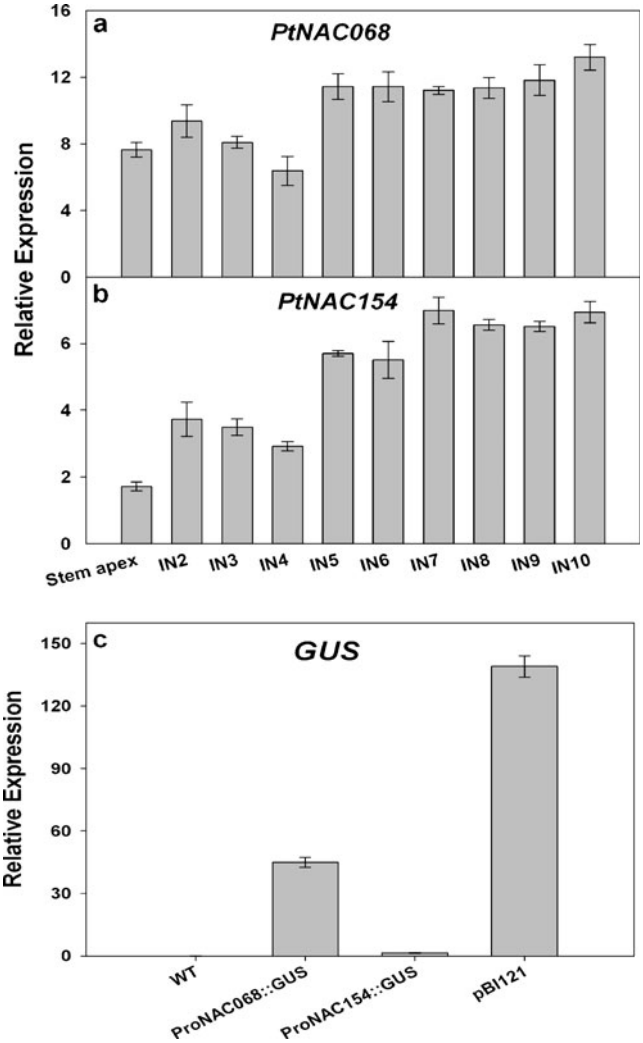

Fig. 4 Expression analysis using RT-qPCR. **a** Relative transcript abundance of *PtNAC068* in different internodes from WT, **b** relative transcript abundance of *PtNAC154* in different internodes from WT, **c** relative transcript abundance of *GUS* in transgenic plants as compared to wild type. Relative expression in tissue samples collected from greenhouse-grown hybrid poplar clone 84 K. Relative transcript quantities were determined by normalizing with data of *β-tubulin* as a control. *IN*, internode; *error bars* are standard deviations over three biological replicates

(pBI121), respectively. Notably, *GUS* expression in *ProNAC068::GUS* transgenic plants was 30-fold higher than that in *ProNAC154::GUS* transgenic plants.

Discussion

In this study, we examined the expression patterns for the poplar orthologs predicted for the *Arabidopsis* NAC domain transcription factors *NST3/ANAC012* and *SND2/ANAC073*. The results from histochemical GUS staining indicate that *PtNAC068* and *PtNAC154* are involved in two distinct aspects of xylem development. In *ProNAC068::GUS* trans-

genic plants, *GUS* expression was mainly detected at vascular tissues of stem and petiole, especially in their xylem tissues (Fig. 3c1–3f1). Intensive staining in stem was observed during the early stages of xylem differentiation (IN3; Fig. 3c1) but predominantly in xylem undergone secondary wall thickening (IN5 and IN9; Fig. 3d1, e1). This suggests that the expression of *PtNAC068* may be more complex in poplar than it is in *Arabidopsis*, where GUS activity driven by *ANAC012/NST3* promoter was preferentially localized in the (pro)cambium region of inflorescence stem and root and also detected in the midrib of leaf veins and the basal part of siliques (Ko et al. 2007). Thus, it seems that the expression of *PtNAC068* in poplar may be widely distributed in vascular tissues at the early developmental stage but becomes more specific in differentiating xylem leading to the maturation of xylem.

In *Arabidopsis*, GUS activity driven by *ANAC073* promoter was observed throughout the vascular system in young seedlings and located to xylem cells of the stem and root within the vascular tissue in adult plants (Ko et al. 2006). In poplar, *PtNAC154* promoter was active in leaf vein at the basal sections, root tips, and developing xylem cells. In dicots, leaf vein arises and subsequently differentiates in a basipetal order to form a continuous, branching system with smaller veins diverging from larger ones and the smallest veins forming a closed reticulum (Dengler and Kang 2001). Therefore, lignification of leaf veins proceeds basipetally from the apex to the petiole during leaf development (Candela et al. 1999). The *GUS* gene expression driven by *PtNAC154* promoter in leaves was detected specifically in immature veins (Fig. 3a2). In addition, histochemical staining also revealed *GUS* expression in the meristematic zone of developing roots in the transgenic poplar plants where vascular tissues initiated (Fig. 3b2). Furthermore, GUS activity was evident in developing secondary xylem in the stems (Fig. 3c2–e2). Collectively, GUS activity driven by *PtNAC154* promoter was confined to the developing vascular tissues in leaves, stems, and roots. This indicates that the expressions of *PtNAC068* and *PtNAC154* in poplar are different in pattern and level in the developing stages of vascular tissues; thus, they may be involved in different aspects of vascular tissue development.

Wood formation starts at the shoot apical meristem of the young plant. The shoot apical meristems develop vascular cambium that is able to generate all of the cell types necessary for wood formation. In poplar, internodes 2–3 only have primary xylem and phloem and a full ring of cambium initials enabling radial secondary stem growth. Internodes 4–7 are transitional regions where secondary growth has initiated the lignification of xylem and followed by phloem fiber development. Below internode 5, secondary phloem and secondary xylem are well developed (Dharmawardhana et al. 2010; Grant et al. 2010). In the present experiment, successive internodes below the apex were sampled to analyze the expression levels of *PtNAC068* and *PtNAC154* by quantitative real-time RT-PCR. The results showed that the transcript levels of *PtNAC068* and *PtNAC154* were higher in IN5-10 compared with IN2-4. The expression profiles of *PtNAC068* and *PtNAC154* were consistent with the previous results (Dharmawardhana et al. 2010), which profiled their expression in stem segments that spanned primary to secondary growth using a genomic microarray. The expression data suggest that *PtNAC068* is highly expressed in vascular tissues, while *PtNAC154* is relatively low but xylem specific. Therefore, *PtNAC154* is possibly involved in the regulation of secondary xylem differentiation in an elaborate manner. The expression of both *PtNAC068* and *PtNAC154* increases following the progress of the development of secondary growth in stem but is activated in different strength and space during this process, suggesting that the intricate process of wood formation in poplar requires a complex regulatory network; thus, their roles in this process deserve further investigation.

In summary, we have investigated the expression level and pattern of *PtNAC068* and *PtNAC154* in poplar and suggested that these two NAC genes may be involved in different aspects of vascular tissue development. Their possible roles in the regulation of secondary xylem development are worth to be farther explored. In addition, the tissue-specific and quantitatively different expression of *GUS* was achieved using *PtNAC068* and *PtNAC154* promoters. These promoters could be used in driving target genes specifically expressed in wood-forming tissues to study their functions as well as to improve wood property through genetic engineering.

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