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NOTE

The *Arabidopsis NST3/SND1* promoter is active in secondary woody tissue in poplar

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Abstract Wood biomass is one of the promising future materials for biofuels with no competing food uses. However, the higher cost to produce bioethanol from wood feedstocks is regarded as a priority issue. Genetic engineering techniques have been proposed to enhance the quality and quantity of wood materials to overcome the cost problem. Although many genetically engineered trees with applicable traits such as low lignin, a high syringyl to guaiacyl ratio and high cellulose content are generated, ectopic expression of an effector gene under a constitutive promoter can sometimes induce untoward side effects on plant growth and development. Our recent study demonstrated that *AtNST3/SND1* promoter of *Arabidopsis thaliana* is a candidate tool for driving a potent activator to enhance wood biomass production in poplar without any growth retardation. However, the tissue- and cell-dependent activity of the promoter remains to be elucidated. In the present study, we generated transgenic poplar expressing AtNST3/SND1promoter::GUS to examine in detail the activity of the *AtNST3/SND1* promoter. Histochemical analysis revealed that the promoter was predominantly active in secondary woody tissue. Our result indicates that the *AtNST3/SND1* promoter is an option for expressing an effector gene to modify secondary cell wall components and wood biomass.

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Introduction

Forest trees produce large amounts of lignocellulose, which is regarded as a renewable and cost-effective resource for bioenergy and industry. Wood biomass is expected to be one of the next-generation materials for biofuels since current bioethanol feedstocks such as corn and sugarcane compete with human food demands. Bioethanol produced from wood lignocellulose, however, would be more costly than that from food crops [1]. To reduce the cost of biofuel conversion, one goal is to modify and enhance the quality and quantity of wood materials using transgenic technology. Saccharification efficiency and ethanol production, for example, are gained in genetically engineered poplar, which shows lower lignin content [2, 3], an increased syringyl/guaiacyl (S/G) ratio in lignin [4], and modification of chemical linkages in lignin [5]. In addition, secondary cell walls, which represent a large part of wood biomass, are reinforced by introducing a transcriptional factor and sucrose catalytic enzyme into poplar [6–8]. In spite of many applicable successes, some studies report that ectopic expression of an effector gene under a constitutive promoter such as the cauliflower mosaic virus 35S promoter causes unexpected and negative effects on plant growth and development [7, 9, 10]. Use of a tissue-specific promoter to limit effector expression to appropriate cells and tissues is a solution designed to reduce the untoward side effects.

NAC SECONDARY WALL THICKENING-PROMOTING FACTOR3/SECONDARY WALL-ASSOCIATED NAC-DOMAIN PROTEIN 1 (*NST3/SND1*) and *NST1* are key

regulators of secondary cell wall formation in xylem fiber cells in *Arabidopsis thaliana* [11, 12]. The genes are members of the *VNS* (*VASCULAR-RELATED NAC-DOMAIN* (*VND*)-, *NST/SND*- and *SOMBRERO* (*SMB*)-related proteins) family and redundantly control secondary wall thickening in woody tissues. The double knockout mutant *nst1 nst3/snd1* is deficient in secondary wall of fiber cells, although the single mutants do not show a drastic phenotype affecting cell wall formation [12]. The *NST/SND* genes are highly expressed in xylem fibers of the inflorescence stem in *A. thaliana* [11, 12]. The xylem-dependent expression pattern is more specific to *NST3/SND1* than *NST1* since *NST1* is expressed to some extent in aboveground tissues such as flowers and leaves. The *AtNST3/SND1* promoter, showing woody tissue-specific activity, is a powerful tool for studying the biological process of wood-fiber development and the molecular function of genes that control cell wall formation in *A. thaliana* [13].

We recently enhanced wood biomass production in poplar without any growth retardation using a gene construct containing the *AtNST3/SND1* promoter to drive an *AtNST3/SND1* homologue in *Oryza sativa* [14]. However, the tissue- and cell-dependent activity of *AtNST3/SND1* promoter remains unclear in poplar. In this study, we generated transgenic hybrid poplar carrying *β-glucuronidase* (*GUS*) driven by the *AtNST3/SND1* promoter to investigate in detail the tissue- and cell-dependent activity of the promoter in woody perennial poplar. Histochemical GUS assay clarified that the promoter showed a secondary woody tissue-dependent expression pattern. This result indicates that the *AtNST3/SND1* promoter is a promising tool to modify secondary cell wall components and biomass of wood materials through transgenic technology.

Materials and methods

Plant material and generation of transgenic plants

Sterile rooted cuttings of *Populus tremula* × *Populus tremuloides* (wild-type clone T89) were cultured in 0.5 × Murashige and Skoog medium (pH 5.7) containing 0.8% (w/v) agar at 25 °C under a cycle of 16-h light (50 µmol m^{-2} s^{-1})/8-h dark. Transgenic poplar was generated with the expression binary vector pBCKK-AtNST3/SND1pro::GUS, by *Agrobacterium*-mediated transformation [12, 15]. The binary vector harbors a 3028-bp region upstream of the start codon of *AtNST3/SND1* (Gene ID At1g32770). Genomic DNA was extracted by the simple method of the KAPA3G Plant PCR Kit (Kapa Biosystems, Wilmington, MA USA) from leaves of transgenic poplar. PCR was performed by a KAPA3G plant DNA polymerase according to the manufacturer's instructions using the primer pair 5′-CTATGTCTCGTCGAGTCCTACCACC-3′ and 5′-TCCTGATTATTGACCCACACTTTG-3′. Transformants were potted to soil mix (3:1 fertilized peat moss:vermiculite, v/v) and grown at 20 °C under long-day conditions (18-h light at 200 µmol m^{-2} s^{-1}/6-h dark).

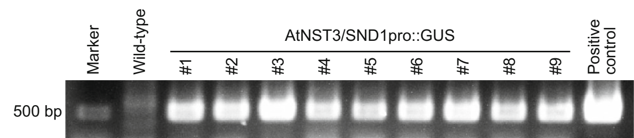

Fig. 1 Generation of transgenic poplar events expressing AtNST3/SND1pro::GUS. The expression gene cassette was amplified as a 554-bp PCR product from genomic DNA of transgenic poplar

Histochemical assays

Histochemical GUS assays were performed as described by Takata and Taniguchi [15]. Plant tissues such as leaves, stems and roots were harvested from transformants growing for 32 days in soil mix and fixed in cold 90% acetone for 30 min. Tissues were washed with 50 mM sodium phosphate (pH 7.0) and then incubated in GUS staining solution [50 mM sodium phosphate (pH 7.0), 0.5 mg ml^{-1} 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 0.1% Triton X-100] at 37 °C for 16 h. Samples postfixed in 2.5% (v/v) glutaraldehyde in 50 mM sodium phosphate (pH 7.0) were bleached in ethanol:acetic acid (6:1, v/v) for leaf blades and 70% ethanol for leaf veins, petioles, stems and roots. Leaf blades were cleared with chloral hydrate/glycerol solution. Other tissues were infiltrated through a series of 25, 33, 50, 66, 75, and 100% 2.3 M sucrose in 100 mM sodium phosphate (pH 7.0). Leaves and stems were embedded in SCEM-L1 mounting medium (Leica, Solms, Germany) and frozen in liquid nitrogen-cooled hexane. Cryosections of 50 µm were cut using a Leica CM3050 S cryomicrotome (Leica). Roots were sectioned at a thickness of 80 µm using a vibratome (Dosaka EM, Kyoto, Japan). Samples were imaged by a Leica MZ FLIII stereomicroscope and a Leica DMR microscope (Leica).

Results and discussion

Nine transgenic events were generated by *Agrobacterium*-mediated transformation (Fig. 1), and plants from two independent events, #1 and #3, were assayed histochemically. Figure 2 shows the GUS staining pattern of these transgenic poplar plants in the leaf blade (Fig. 2d, l), leaf main vein (Fig. 2e, m), petiole (Fig. 2f, n), young stem (Fig. 2c, k), mature stem (Fig. 2a, b, i, j) and root (Fig. 2g,

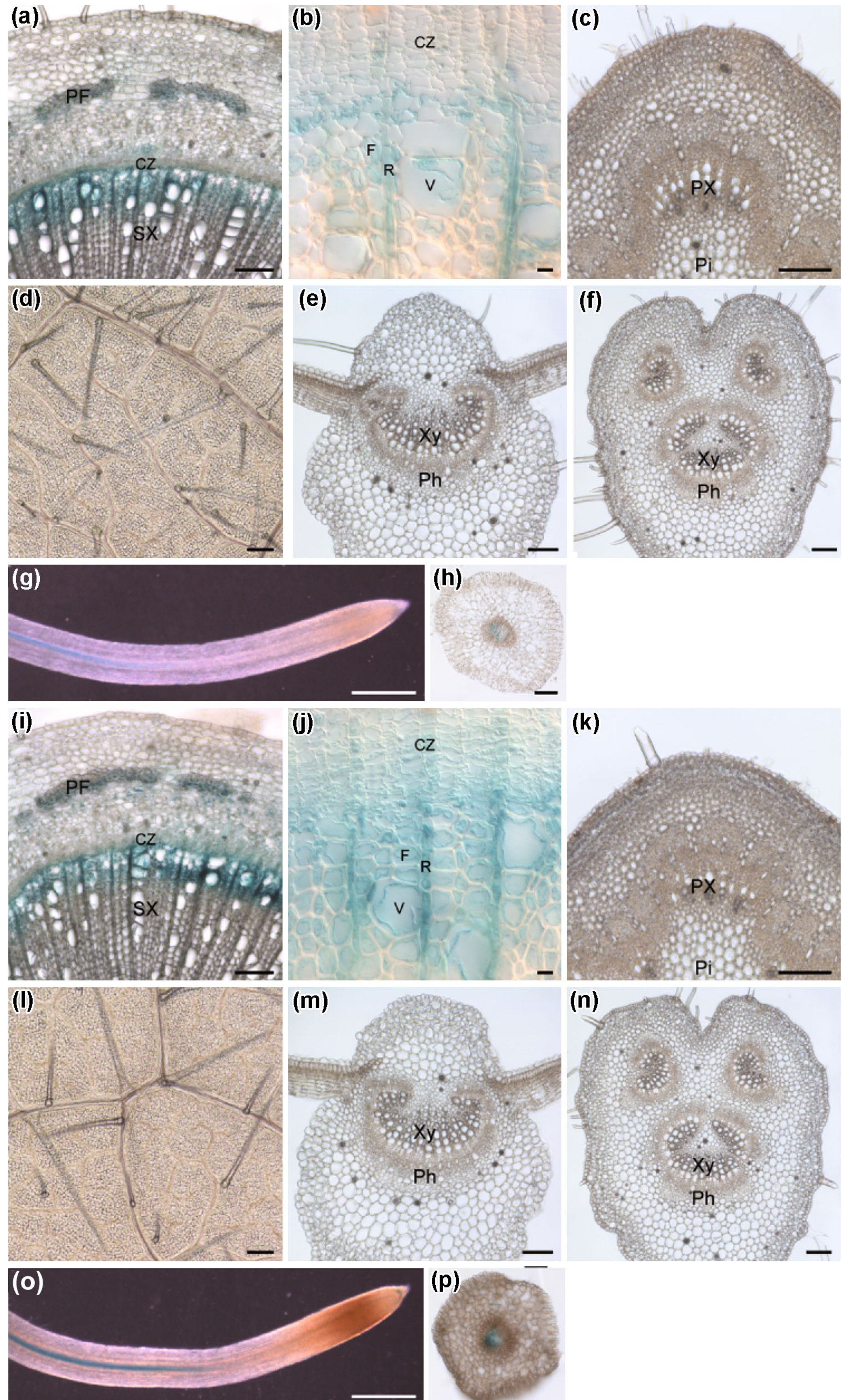

Fig. 2 Histochemical GUS analysis in two independent transgenic events (#1, **a**–**h**; #3, **i**–**p**). The images are from mature stem (**a**, **b**, **i**, **j**), young stem (**c**, **k**), leaf blade (**d**, **l**), a cross section of the main vein (**e**, **m**), a cross section of the petiole (**f**, **n**), root (**g**, **o**) and a cross section of the root (**h**, **p**). *CZ* cambial zone, *F* xylem fiber, *PF* phloem fiber, *Ph* phloem, *Pi* pith, *PX* primary xylem, *R* ray parenchyma cell, *SX* secondary xylem, *Xy* xylem, *V* vessel element. *Bars* indicate 1 cm (**g**, **o**), 100 µm (**a**, **c**–**f**, **h**, **i**, **k**–**n**, **p**) and 10 µm (**b**, **j**)

h, o, p). The *AtNST3/SND1* promoter was active in secondary xylem, phloem fibers and root xylem (Fig. 2a, b, g–j, o, p). However, no GUS staining was detected in other woody tissues such as primary xylem of young stems or the vascular system of leaves and petioles (Fig. 2c–f, k–n). The independent transgenic events, #1 and #3, showed similar GUS staining pattern in all tissues examined (Fig. 2). Taken together, our data indicate that the *AtNST3/SND1* promoter has secondary woody tissue-dependent activity in hybrid poplar.

In developing secondary xylem, the *AtNST3/SND1* promoter was active in wood fibers, vessel elements and ray parenchyma cells (Fig. 2b, j). In *A. thaliana*, *AtNST3/SND1* is predominantly expressed in secondary xylem fibers and differentiating vessel elements of the hypocotyl, and in interfascicular fibers of inflorescence stems [11, 12]. Thus, the *AtNST3/SND1* promoter shows a similar cell-specific expression pattern in poplar. In the *Populus* genome, four *NST/SND* orthologues (*VNS09/WND2A/SND1-B1*, *VNS10/WND2B/SND1-B2*, *VNS11/WND1B/SND1-A2* and *VNS12/WND1A/SND1-A1*) have been conserved and coordinately control secondary cell wall formation in xylem cells and phloem fibers [16–18]. Phylogenetically, *VNS09/WND2A/SND1-B1* and *VNS10/WND2B/SND1-B2* are orthologous to *A. thaliana NST1* and *NST2*, and *VNS11/WND1B/SND1-A2* and *VNS12/WND1A/SND1-A1* are orthologous to *NST3/SND1* [16, 17]. The *Populus NST/SND* orthologues are mainly expressed in wood fibers, developing vessels, xylem ray parenchyma cells and phloem fibers [16]. Interestingly, *VNS11/WND1B/SND1-A2*, an *AtNST3/SND1* orthologue, was not detected by in situ hybridization in primary xylem of young stems and petioles [17]. The tissue-dependent expression of *VNS11/WND1B/SND1-A2* appears to be consistent with the GUS staining pattern in AtNST3/SND1pro::GUS-expressing poplar (Fig. 2). These results suggest that the transcriptional regulation of *NST3/SND1* orthologues could be evolutionarily conserved between herbaceous *Arabidopsis* and perennial poplar.

A tissue- and cell-specific promoter is an important and effective tool for precisely controlling the expression pattern of an effector gene for establishing a target trait in transgenic plants. Dozens of xylem tissue-dependent promoters have been identified in poplar and some are used for genetic modification of wood properties [5, 6, 15, 19–24]. Although woody tissue-specific promoters are highly expressed in mature xylem tissue, many show expression not only in secondary xylem but also in primary xylem and the vascular system of petioles and leaves [15, 19, 21, 23, 24]. The present study demonstrates that the *AtNST3/SND1* promoter activity is dominant in secondary woody tissue of poplar trees. Using the *AtNST3/SND1* promoter conjugated with a potent activator for secondary cell wall formation, we previously reported that transgenic hybrid poplar had a thicker secondary cell wall, higher xylem intensity and higher stem Young's modulus without any growth defects [14]. Together, the *AtNST3/SND1* promoter is an option for expressing an effector gene only in secondary woody tissue to enhance the industrial value of wood.

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