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NOTE

Growth and root sucker ability of field-grown transgenic poplars overexpressing xyloglucanase

Toru Taniguchi · Ken-ichi Konagaya · Manabu Kurita · Naoki Takata · Katsuaki Ishii · Teiji Kondo · Fumiaki Funahashi · Seiichi Ohta · Tomomi Kaku · Kei'ichi Baba · Rumi Kaida · Takahisa Hayashi

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Abstract Xyloglucan is thought to be a key hemicellulose cross-linking adjacent cellulose microfibrils in plant cell walls. The growth traits of transgenic poplars (*Populus alba*) with decreased xyloglucan from overexpression of *Aspergillus aculeatus* xyloglucanase were characterized during a 4-year field trial. The field-trial site consisted of two blocks, a fertile soil block and a non-fertile soil block, determined by soil analysis. In the fertile block, the growth of aboveground biomass of the transgenic poplars was reduced to 24–44 % compared to that of wild-type poplars, in contrast to the growth seen in chamber and greenhouse conditions. In the non-fertile block, the aboveground biomass of transgenic poplars was also smaller than that of the wild-type poplars. Because poplars reproduce asexually by root suckers, we also compared the formation of root suckers from transgenic and wild-type poplars. Root suckers formed less frequently from transgenic poplars than from wild-type poplars. The growth rates of root suckers from transgenic poplars were also slower than those from wild-type poplars. The results showed that constitutive degradation of xyloglucan impairs poplar growth and vegetative reproduction ability.

Keywords Transgenic poplar · Xyloglucanase · Field trial · Growth · Root sucker

T. Taniguchi (✉) · K. Konagaya · M. Kurita · N. Takata · K. Ishii
Forest Bio-Research Center, Forestry and Forest Products Research Institute, Ibaraki 319-1301, Japan
e-mail: toru.t@affrc.go.jp

T. Kondo
Forest Tree Breeding Center, Forestry and Forest Products Research Institute, Ibaraki 319-1301, Japan

F. Funahashi · S. Ohta
Gradute School of Agriculture, Kyoto University, Kyoto 606-8502, Japan

T. Kaku · K. Baba
Research Institute for Sustainable Humanosphere, Kyoto University, Uji, Kyoto 611-0011, Japan

R. Kaida · T. Hayashi
Department of Bioscience, Tokyo University of Agriculture, Setagaya-ku, Tokyo 156-8502, Japan

Introduction

Cellulose microfibrils form the skeletal component of plant cell walls. Xyloglucan is thought to be a key hemicellulose that cross-links adjacent cellulose microfibrils. A decrease in the cross-linkage in cell elongation, and an increase in the growth rate and internode length of transgenic poplars in growth chamber conditions are attributable to the degradation of xyloglucan by overexpression of *Aspergillus* xyloglucanase cDNA (*AaXEG2*) controlled by the cauliflower mosaic virus (CaMV) 35S promoter [1]. Cellulose deposition is likely affected by xyloglucan cross-links, since the deposition of cellulose is increased in transgenic poplars [1]. Xyloglucan tightens cellulose microfibrils in gelatinous layers in tension wood and provides tensile stress in the wall structure [2].

As xyloglucan tethers adjacent cellulose microfibrils together, degradation of the tether alters cell growth and cell-wall characteristics, and could modify the wood and fiber properties. Indeed, saccharification and ethanol production accompanying enzymatic hydrolysis of xylem was accelerated in transgenic poplars overexpressing *AaXEG2* [3]. Degradation of xyloglucan enlarges the cellulose microfibril width in the fibers of transgenic poplars [4].

Research is currently being conducted on trees genetically modified to resist fungal [5] or insect attacks [6], to accelerate growth [7], and to alter lignin metabolism [8]. Field trials in the natural environment are very important for clarifying the true traits of transgenic trees. According to domestic regulations, however, biosafety assessments are needed prior to any field trial. Therefore, we performed biosafety assessments of transgenic poplars overexpressing *AaXEG2* [9]. There was no distinguishable difference in growth between the transgenic and wild-type poplars when they were grown in a greenhouse, though the young transgenic poplars grew faster than the wild-type poplars in a growth chamber. Allelopathic tests showed that the transgenic poplars did not produce harmful substances. Based on these experimental data and the scientific literature on poplar species, it was concluded that the transgenic poplars do not disturb the biological diversity of the surrounding environment, even when they are submitted to field trials.

We planted two transgenic poplar lines and wild-type poplars at a field trial site in March 2007. All of the poplars were cut down 4 years later, in December 2010. During the field trial, we investigated above- and belowground growth traits of the transgenic poplars. Poplars reproduce asexually by root suckers. Therefore, to compare the vegetative dispersal capacity of transgenic and wild-type poplars, root sucker formation was monitored for 4 years, and the growth rate of root suckers was compared.

Materials and methods

Field trial establishment

Two transgenic poplar lines, trg300-1 and trg300-2, overexpressing *A. aculeatus* xyloglucanase *AaXEG2* (accession number AY160774) with *PopCel1* signal peptide [10] under the control of the CaMV35S promoter and E12Ω enhancer sequences, were produced by *Agrobacterium* infection [1]. A field trial with 1-year-old rooted cuttings of these two transgenic and control wild-type poplars was established in March 2007 in Hitachi, Ibaraki, Japan. The field trial site consisted of two blocks, a fertile soil block and a non-fertile soil block, determined by soil analysis. Both blocks consisted of three plots, one plot each of trg300-1, trg300-2 and wild-type poplars. Twenty-five trees were planted in each plot, arranged in a 5×5 grid with 2×2 m spacing. Spacing between each plot was 7.5 m. The field-trial site was surrounded by an 8-m tall fence with a 1-m deep underground concrete wall. Distance from the fence and wall to the poplars was 7.5 m on the north, east and south sides, and 29 m on the west side.

Soil analysis

In July 2007, 100 cc of soil was taken at depths of 0–5 and 5–10 cm from six randomly selected points in each plot. The soil samples were air-dried and passed through a 2-mm sieve, followed by measurement of bulk density and pH (2.5:1 in water). Exchangeable base cations (K, Ca and Mg) were extracted with 1 M ammonium acetate buffered at pH 7, and their concentrations were determined by atomic absorption spectrophotometry (iCE 3300; Thermo Fisher Scientific, Tokyo, Japan). Cation exchange capacity (CEC) was measured using the ammonium acetate (pH 7.0) method [11]. Available phosphorus (P) was determined using extraction with dilute acid fluoride [12]. Total carbon (C) and total nitrogen (N) were determined by an NC analyzer (JM1000CN; J-Science, Tokyo, Japan) after pulverizing samples. Percent base saturation (BS) was calculated as exchangeable base cations divided by CEC. Data for soil at depths of 0–5 and 5–10 cm were averaged in each replicate. For available nitrate analysis, a soil sample was taken at a depth of 5–10 cm from four points in each plot in September 2007, air-dried and passed through a 2-mm sieve. Available nitrate was extracted from the soil sample with hot water (80 °C) for 12 h, followed by 0.1 M potassium chloride extraction for 30 min at room temperature. Nitrate concentration was determined by a colorimetric procedure [13, 14].

Aboveground growth

Tree height and diameter of stems at approximately 10 cm above the ground were measured once a month from April to October in 2007 and 2008, and from April to September in 2009 and 2010 for all of the poplars. Mean heights and diameters of all the poplars in each plot except for those used for bending or nutrition content experiments (data not shown) were used for growth curve formation. Volume index (D^2H) was calculated from the height (H) and diameter (D) of individual trees. At the end of December 2010, all trees were cut down near the ground surface and the fresh weight of the aboveground parts was measured. All branches were subsequently detached form the stem and the fresh weight of branches of individual trees was determined. Aboveground biomass was determined after drying portions of stems and branches to a constant weight at 105 °C.

Belowground growth

For the analysis of belowground growth, the belowground parts of sample trees from each line were excavated at the end of December every year. More specifically, after measuring tree height and diameter, approximately 4–5

thicker horizontal roots in their diameter of a sample tree were selected, and then, these roots were traced carefully to a diameter larger than 1–2 mm. After excavation, the longest horizontal root length was measured. In 2007 and 2008, one sample tree from each line was excavated, and in 2009 and 2010, two sample trees from each line were excavated.

Root sucker

Root sucker formation was monitored and quantified inside the field every year. The formation of root suckers was also monitored in a range of 10 m outside the field from April to October every year. For evaluation of the root sucker growth rate, the heights of root sucker plants were measured for approximately 40 days from the end of July 2010 in the fertile block after weeding, when root suckers had been trimmed to approximately 5 cm above the ground.

RNA extraction and real-time PCR

AaXEG2 expression was assessed by real-time PCR in the fourth year of the field trial. Three trees of varying height were selected from each plot of transgenic poplars. One tree from the plots of wild-type poplars was used for sampling. Five samples of young leaves at shoot tips were collected from each of the trees in the middle of June 2010, and frozen in liquid nitrogen. Three and one root suckers from each plot of transgenic and wild type poplars, respectively, were also used for *AaXEG2* expression assessment. Total RNA was extracted using the RNeasy Plant Mini kit (Qiagen, Valencia, CA, USA), and treated with DNase I (Qiagen) to remove residual genomic DNA, according to the manufacturer's instructions. For the synthesis of first-strand cDNA, 1 μg of total RNA was reverse-transcribed using oligo(dT) and random primers with PrimeScript II 1st Strand cDNA synthesis kit (Takara, Shiga, Japan) in a volume of 20 μl.

Real-time PCR was performed with *Power* SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and StepOnePlus Real-Time PCR System (Applied Biosystems). The PCR thermal-cycling conditions were performed according to the manufacturer's instructions. The eukaryotic initiation factor 4A-8 (*eIF4A-8*) was used as an internal standard, based on their constitutive expression in poplar [15]. Primers used for amplification of the targeted *AaXEG2* gene were 5′-TCCTACAGCGGC GATACCA-3′ and 5′-CTCTTGACGCTGCTGCTACC T-3′ with a production size of 71 bp. The *elf4A-8* gene was amplified with primers 5′-CGAAGTGGACGTTTTGGA AGA-3′ and 5′-TTCTGTCATCATCCCTTGTCACA-3′, giving a production size of 64 bp.

Results and discussion

Soil nutrition at field trial site

The field trial site was divided into two blocks according to soil nutritional content (Table 1). We named one block "fertile block", in which available N, available P, exchangeable base cation (K Ca, and Mg) contents, pH, and BS were significantly higher than those in another block, "non-fertile block". Total C and the C/N ratio in the non-fertile block were higher than in the fertile block, indicating a higher content of less-decomposed organic matter in the non-fertile block and, in turn, resulting in a higher CEC. Within individual soil blocks, there were some significant differences among plant lines in soil chemical composition and properties. However, these differences were not so large as compared to the differences between fertile and non-fertile blocks. After 4 years,

Table 1 Means of soil chemical properties in field trial site

Plant line	pH		Total C (g/kg)		C/N		Avail. N (mg/kg)		Avail. P (mg/kg)		K (g/kg)		Ca (g/kg)		Mg (g/kg)		CEC (cmol/kg)		BS (%)	
Fertile block																				
wt	6.03	a	35.55	e	12.25	c	18.73	a	17.36	b	0.40	a	1.25	b	0.16	a	15.43	c	75.36	a
trg300-1	6.01	ab	36.65	d	12.25	c	14.20	b	21.02	ab	0.36	b	1.32	a	0.16	a	15.49	c	76.94	a
trg300-2	5.97	b	37.89	c	12.34	c	13.28	b	21.11	a	0.38	ab	1.30	ab	0.16	a	14.44	d	81.96	a
Non-fertile block																				
wt	5.15	cd	86.77	ab	16.35	a	2.60	c	7.04	e	0.11	d	0.32	c	0.04	bc	21.50	a	15.01	b
trg300-1	5.08	d	103.99	a	17.46	a	2.80	c	9.51	d	0.12	d	0.28	d	0.03	c	21.61	a	13.39	b
trg300-2	5.27	c	71.34	b	15.36	b	3.05	c	12.07	c	0.19	c	0.45	c	0.05	b	19.01	b	22.85	b

C/N ratio of total C to total N, *CEC* cation exchange capacity, *BS* base saturation

Values with different letters are significantly different by *t* test ($p < 0.05$)

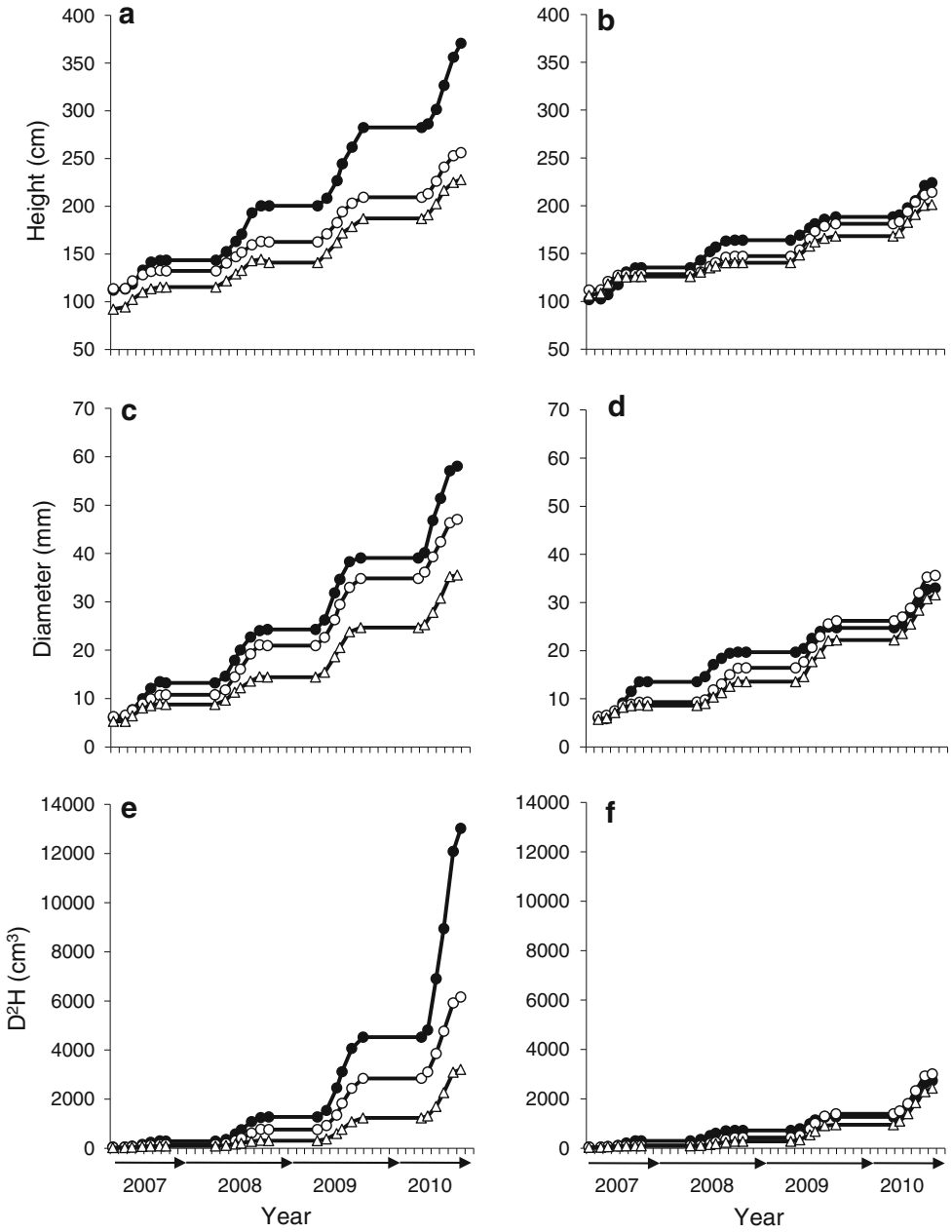

Fig. 1 Growth curves for height (**a**, **b**), diameter (**c**, **d**) and volume index (**e**, **f**) of transgenic and wild-type poplars grown in fertile (**a**, **c**, **e**) and non-fertile (**b**, **d**, **f**) blocks during a 4-year field trial. *Filled circles*, wild type; *open circles*, trg300-1; *open triangles*, trg300-2

although exchangeable base cations and P were slightly reduced in the fertile block (data not shown), they were still higher than those in the non-fertile block. This indicated that a better nutrient condition in the fertile block compared to the non-fertile block was maintained during the field trial.

AaXEG2 expression in transgenic poplars

Since all the transgenic poplars sampled in the field trial were expressing *AaXEG2* as assessed by real-time PCR, the transgene was expressed during the 4-year field trial without gene silencing that might occur due to genome rearrangement, DNA methylation, or insertion site interaction [15–19]. *AaXEG2* expression level in wild-type poplars was essentially 0. Mean relative expression levels of *AaXEG2* in trg300-2 were 2.1 times higher than those in trg300-1. This difference might be attributable to the copy number of transgenes, which was one and two in trg300-1 and trg300-2, respectively.

Aboveground growth

The *Populus* species is nutrient demanding [20]. Therefore, as expected, all the poplars (wild type and two transgenic lines) grew faster in the fertile block than in the non-fertile

block (Fig. 1a–f). The growth of transgenic poplars, however, was slower than that of wild-type poplars in the fertile block. The mean height, diameter, volume index (D^2H), and aboveground biomass of the transgenic line, trg300-1, after 4 years were 69, 81, 47 and 44 %, respectively, of those of wild-type poplars in the fertile block (Table 2; Fig. 1a, c, e). The growth of trg300-2 was more inhibited than that of trg300-1, and both the mean volume index and aboveground biomass of trg300-2 was only approximately 25 % of those of wild-type poplars (Table 2; Fig. 1e). In the non-fertile block, the growth of both transgenic poplar lines was inferior to that of wild-type poplars for the first 2 years (Fig. 1b, d), though there was no significant difference in aboveground biomass between transgenic and wild-type poplars in the fourth year (Table 2), which was likely due to a slowing in the growth rate of wild-type poplars from soil nutrient deficiency.

An *Arabidopsis* mutant lacking detectable xyloglucan exhibited aberrant root hairs and produced smaller plants [21]. Transgenic poplars constitutively overexpressing *AaXEG2* grown in a field also had reduced xyloglucan content and had fewer root hairs than wild-type poplars (Kaida et al., unpublished data). Root hairs are important for plant to uptake nutrients and water from soil. Therefore, reduction of the growth rate of the transgenic poplars in the field trial is thought to be associated with decreased root hairs. The ratio of branch mass to aboveground mass of the transgenic lines was significantly lower than that of wild-type trees in the present study (Table 2). These lower ratios of branch mass might be also associated with the low growth capacity of transgenic poplars.

Compared to the wild-type poplars, the less biomass of the transgenic poplars in field condition was in contrast to artificial environment conditions (growth chamber and greenhouse) [1, 9]. The disparity in growth performance between field and artificial condition like the transgenic poplars overexpressing *AaXEG2* has been reported, for example, for transgenic poplars down-regulating lignin biosynthesis genes [8, 22]. The multiple stresses to which poplars were exposed in the field likely caused these disparities in growth performance.

Belowground growth

Adult trees of *Populus alba* are well known to spread their horizontal roots vigorously [23], although growth of their vertical roots is less vigorous than that of their horizontal roots. Since root suckers (see below) grow from the horizontal root, length of the root shows the range or area in which root suckers may arise. Volk et al. [24] reported that 5-year-old hybrid poplars had root lengths ranging from 8.3 to 10.2 m. Every year from 2007 to 2010, the longest horizontal root length of one or two trees of each line was measured at the field trial site. In 2007, the longest horizontal root lengths of wild-type, trg300-1 and trg300-2 poplars were 2, 1.3 and 1.7 m, respectively. In 2008 those were 3.4, 3.4, 2.4 m, respectively; in 2009, those were 5.3, 5.4, 2.8 m, respectively, and in 2010, those were 12.9, 5.5, 5.8 m, respectively.

The root length of poplars increased, as expected, with tree height, and there was no definite difference in relationships between root length and tree height among wild-type and transgenic lines (Fig. 2).

Table 2 Growth properties of wild type and transgenic poplars (trg300-1 and trg300-2) grown in field trial for 4 years

Plant line	n[a]	Aboveground biomass (kg)			Ratio of branch mass[b]		
Fertile block							
wt	16	4.34	±0.33	a	0.63	±0.01	a
trg300-1	14	1.90	±0.27	b	0.55	±0.02	b
trg300-2	16	1.05	±0.10	c	0.54	±0.00	b
Non-fertile block							
wt	17	1.09	±0.15	c	0.62	±0.00	a
trg300-1	18	0.92	±0.11	c	0.52	±0.02	b
trg300-2	13	0.76	±0.13	c	0.55	±0.00	b

Data are given as the mean ± SE

[a] Numbers of individual trees used for calculation of growth properties are shown

[b] Ratio of branch biomass to aboveground biomass

Values with different letters are significantly different by t test ($p < 0.05$)

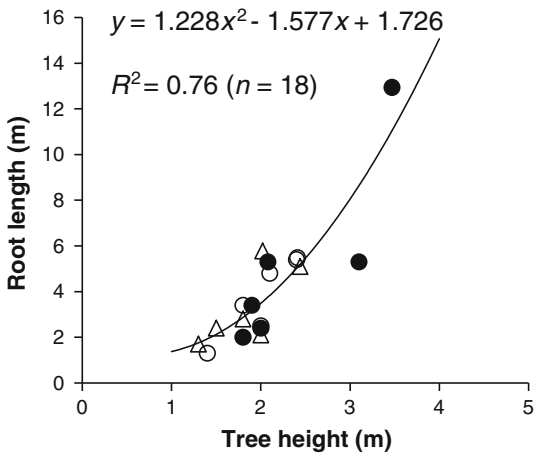

Fig. 2 Relationship between root length and tree height of poplars grown during a 4-year field trial. *Filled circles*, wild type; *open circles*, trg300-1; *open triangles*, trg300-2

Table 3 Frequency of root sucker formation in field trial

Years	Fertile block			Non-fertile block		
	wt	trg300-1	trg300-2	wt	trg300-1	trg300-2
2007	0	0	0	0	0	0
2008	2	1	0	1	0	0
2009	67	16	4	10	2	4
2010	311	35	33	56	8	9

Root suckers

Populus alba reproduce asexually by root suckers, adventitious shoots originating from the horizontal root [20]. In our field trial, formation of four root suckers was first detected in 2008 (Table 3), three of them appeared within 50 cm from wild-type poplars, and another one appeared near transgenic poplar trg300-1. In 2009, root suckers appeared in all plots. In 2010, the total number of root suckers had increased to 452. Leaves of root suckers of both the transgenic lines had more rounded teeth than those of wild-type poplars, resembling those of transgenic poplars grown in the greenhouse and field. The real-time PCR analysis revealed that the root suckers of both transgenic lines stably expressed *AaXEG2* for 4 years and that the expression level in root suckers of wild-type poplars was essentially 0 (data not shown). Root suckers more frequently formed in the fertile block than in the non-fertile block, and there were fewer root suckers from transgenic lines than from wild-type poplars (Table 3). These differences in root sucker formation were probably related to the aboveground growth (Table 2). No root suckers appeared outside the field trial site during the 4-year period.

Root sucker growth rate was compared between wild-type and transgenic poplars in the fertile block. New shoot growth from trimmed root suckers of both wild-type and transgenic poplars was observed 11 days after weeding. After 39 days, the mean height of root suckers from wild-type, trg300-1 and trg300-2 poplars were 32, 23 and 24 cm, respectively. The height was significantly lower in both transgenic lines than that in wild-type poplars (t test; $p < 0.05$). This lower growth rate of root suckers from transgenic lines might be associated with the lower growth rate of transgenic poplars.

Conclusion

In a 4-year field trial, we evaluated growth characteristics of transgenic poplars with decreased xyloglucan from overexpression of *AaXEG2*. The field trial showed that the aboveground biomass of transgenic poplars was reduced by constitutive overexpression of *AaXEG2*, in contrast to the growth chamber and greenhouse conditions in which smaller poplar plants were grown in pots with sufficient moisture and nutrients. In addition to biomass reduction, root sucker formation and growth was reduced in transgenic poplar lines. It is known that a knockout *Arabidopsis* mutant with altered xyloglucan biosynthesis disrupts root hair growth [21]. Leaves of the transgenic poplars are smaller and greener than those of wild-type poplars [9]. Disturbed root hairs and abnormal leaves might be associated with reduced growth of transgenic poplars in the field. Distinct expression of xyloglucanase in stems or xylem by a specific promoter will not disrupt root hair growth and not affect leaf morphology. Consequently, there will be no interruption of growth traits, and improved xylem suitable for pulping and saccharification can be expected.

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