Enhancement of growth and cellulose accumulation by overexpression of xyloglucanase in poplar

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Abstract Because the loosening of xyloglucan in the cell wall promotes plant growth (Takeda et al. (2002) Proc. Natl. Acad. Sci. USA 99, 9055–9060; Park et al. (2003) Plant J. 33, 1099–1106), we expressed *Aspergillus* xyloglucanase constitutively in *Populus alba*. The expression increased the length of stem even in the presence of sucrose. Increased stem growth was accompanied by a decrease in Young's elastic modulus in the growth zone but an increased elasticity in mature tissue. The increased internode length corresponded to an increase in cellulose content as well as specific gravity, showing that the removal of xyloglucan might cause an increase in cellulose density in the secondary xylem.

Key words: Cellulose deposition; Secondary wall; Transgenic poplar; Xyloglucan; Xyloglucanase

1. Introduction

Adjacent cellulose microfibrils are thought to be cross-linked by xyloglucans and the separation of microfibrils during elongation has been thought to require enzymes that cleave xyloglucan or loosen its binding to microfibrils [1]. Involvement of the cleavage of xyloglucan in cell elongation also agrees with a previous finding that the integration of xyloglucan oligosaccharides into pea stem segments solubilizes endogenous xyloglucan in the wall, weakens the cell wall and accelerates elongation [2], and that the trimming of cellulose microfibrils by overexpression of cellulase caused loosening of xyloglucan intercalation and the enlargement of cells with the increased amount of cellulose in *Arabidopsis thaliana* [3]. Here, we examined whether the constitutive degradation of wall xyloglucan contributes to the loosening of the cell wall. The aim is to assess whether a xyloglucan tether between cellulose microfibrils is a key parameter for growth rate and subsequent cellulose deposition in plants. By overexpressing xyloglucanase, it would be possible to study the function of xyloglucan in the primary and secondary walls of higher plants.

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2. Materials and methods

2.1. Plasmid construction and plant transformation

The *Aspergillus aculeatus* cDNA for xyloglucanase was amplified from the first strand cDNA as a template by polymerase chain reaction (PCR) using a forward primer containing a *Xba*I site (5′-GCTGCCAGTCTAGAGC GCCGCAGCGAC-3′) and a reverse primer containing an internal *Sac*I site (5′-CTCCCGTCAGCCG-CGGTCCACGCAAC-3′) complementary to the DNA sequence of *AaXEG2* (accession number AY160774). The *Xba*I–*Sac*I fragment for *AaXEG2* was subcloned into pBluescript II (SK$^-$). The xyloglucanase exhibits 98% identity at the DNA level and 99% identity at the amino acid level to *AaXEG1* (accession number AF043595) [4]. The signal peptide (Met^1 to Leu^{30}) of *Populus alba* cellulase was cloned in pGEM-T Easy vector from *PaPopCel1* cDNA (accession number D32166) by PCR using a forward primer containing a *Bam*HI site (5′-CTAGTGGATCCTTTGGAG-3′) and a reverse primer containing an *Xba*I site (5′-AGCATAGTCTAGAGAAGTGAAGGC-3′) [5]. The *Bam*HI–*Xba*I fragment was ligated into the pBluescript II (SK$^-$) harboring the *Xba*I–*Sac*I fragment for *AaXEG2*. The chimeric DNA for *PopCel1* signal peptide and *XEG2* mature protein was excised with *Bam*HI and *Sac*I, and inserted into the *Bam*HI–*Sac*I site of the binary vector pBE2113-GUS, under control of the CaMV35S promotor and E12-Ω enhancer sequences [6]. The plasmid constructs were electroporated into *Agrobacterium tumefaciens* LBA4404 [7], and leaves of aseptically flask-grown *P. alba* were inoculated with the bacteria [8]. Plantlets were grown in a growth chamber at 27°C under a photoperiod of 18 h.

2.2. Preparation of an antiserum and Western blot analysis

Recombinant xyloglucanase (*AaXEG2*) was expressed in *Escherichia coli* cells harboring the pET-32 Xa/LIC expression vector fused with the full-length cDNA for its matured xyloglucanase. The recombinant protein was injected with Freund's adjuvant into a rabbit. The antiserum was precipitated with ammonium sulfate at 20–50% saturation. For Western blot analysis, proteins were subjected to electrophoresis with 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and electrotransferred to Hybond-C Extra (Amersham, USA) and probed with an antibody against AaXEG2 sequence followed by the second antibody with ABC high-horseradish peroxidase (HRP) immunostaining kit (Toyobo).

2.3. Fractionation of apoplastic, cytoplasmic and wall-bound fractions

Stems were harvested, cut into several pieces, vacuum infiltrated with demineralized water, and centrifuged for 15 min at 440×*g* to yield the apoplastic solution. After extraction of apoplastic solution, stems were homogenized in 20 mM sodium phosphate buffer (pH 6.2) in a mortar and the wall residue was washed three times. The extract obtained was designated as the cytoplasmic fraction. The wall-bound fraction was extracted from the wall residue with the buffer containing 1 M NaCl. Xyloglucanase activity was assayed viscometrically at 35°C for 2 h with 0.1 ml of enzyme preparation plus 0.9 ml of 10 mM sodium phosphate buffer (pH 6.2) containing 0.65% (w/v) tamarind xyloglucan in Cannon semimicroviscometers (Cannon Instrument). One unit of activity is defined as the amount of enzyme required to cause 0.1% loss in viscosity in 1 min under such conditions [9].

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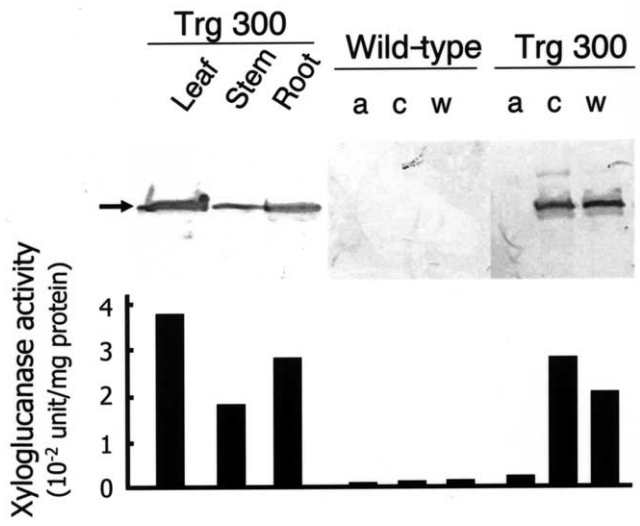

Fig. 1. AaXEG2 gene product and the level of xyloglucanase activity in poplar. Western blot of the gene product (upper part). a, apoplastic fraction; c, cytoplasmic fraction; w, wall-bound fraction. Arrow indicates 28-kDa gene product. Level of xyloglucanase activity in stem (lower part). The activities represent the mean of three independent plants for each line with individual values varying from the mean by $< 5\%$.

2.4. Growth measurements

Each stem (10 cm) was marked at 5-cm height, which was used as a reference point for measuring the height, diameter and number of internodes every fifth day. The length of stem was determined from top to the reference point. The first internode was defined as that below the uppermost leaf 3 mm in length. The fifth to ninth internodes were sampled for Western blot and enzymic activity. Dry weight was determined after freeze-drying the samples.

2.5. Fractionation and measurement of wall components

The primary wall was obtained from the first and second internodes, and the wall of the secondary xylem was obtained from the 10th internode after the bark was peeled off from the 10th internode of stem. Tissues were ground in liquid nitrogen and the resulting powder was freeze-dried. The sample was successively extracted three times with 10 mM sodium phosphate buffer (pH 7.0), three times with 0.1 M ethylenediamine tetraacetic acid (EDTA) (pH 7.0), and three times with 24% KOH containing 0.1% $NaBH_4$. The insoluble residue was solubilized with ice-cold 72% sulfuric acid. Total sugar in each fraction was determined by the phenol-sulfuric acid method [10]. Xyloglucan was determined by methylation analysis, by which the amount of xyloglucan was expressed as 4,6-linked glucose [11]. Lignin content was determined by the Klason method [12].

2.6. Determination of mechanical properties of stem segments

The native stem was clamped in 10 mM MES/KOH buffer (pH 6.2) at 23°C. The experimental conditions were 0.5 g/min for load at 5 mm span. The tensile force was loaded in the longitudinal direction of stems and the Young's modulus was measured by using an automatic material testing machine (Toyo).

2.7. Determination of specific gravity

After measuring the length of the 10th internode, the bark was peeled off. The secondary xylem was dried in an oven at 70°C and dry weight measured. Then, the xylem was completely filled up with water under the reduced pressure and the tissue hung on a wire was put into the water bath. The weight increase by increased water volume was determined by a scale. The specific gravity was calculated as the ratio of xylem dry weight to increase in water weight at 25°C. Then, the tissue was ground into powder in a mini-mixer (Zojirushi) and subjected to the determination of cellulose [13].

3. Results

3.1. Expression of xyloglucanase

We generated 54 independent transgenic poplar lines that expressed a xyloglucanase (AaXEG2) from *A. aculeatus* [14] under the control of a constitutive promoter and the *PopCel1* signal sequence [5]. By a Western blot, the xyloglucanase was present in the leaf, stem, and root of transgenic no. 300 (Trg 300), running at a position corresponding to the size of the mature xyloglucanase (Fig. 1, upper part). In the stem of the transgenic plant, the protein was abundant in the cytosol and wall-bound fractions, and a faint signal was detected in the soluble apoplastic fraction. The xyloglucanase activity in the wall-bound fraction was about 20-fold higher in the stem of Trg 300 than in that of the wild type (Fig. 1, lower part).

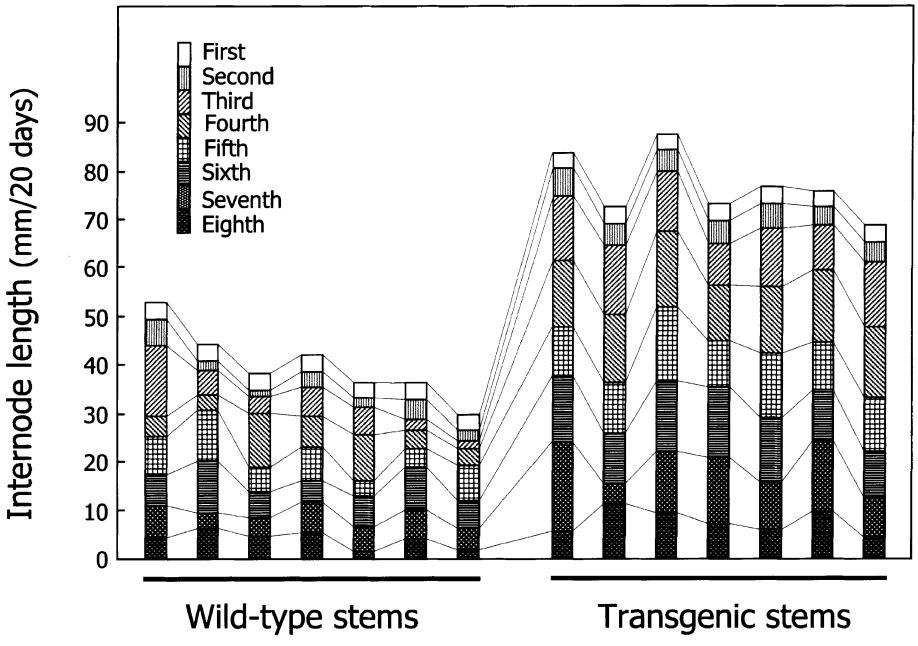

Fig. 2. Effect of AaXEG2 transgenes on the elongation of sterile stem in the presence of sucrose.

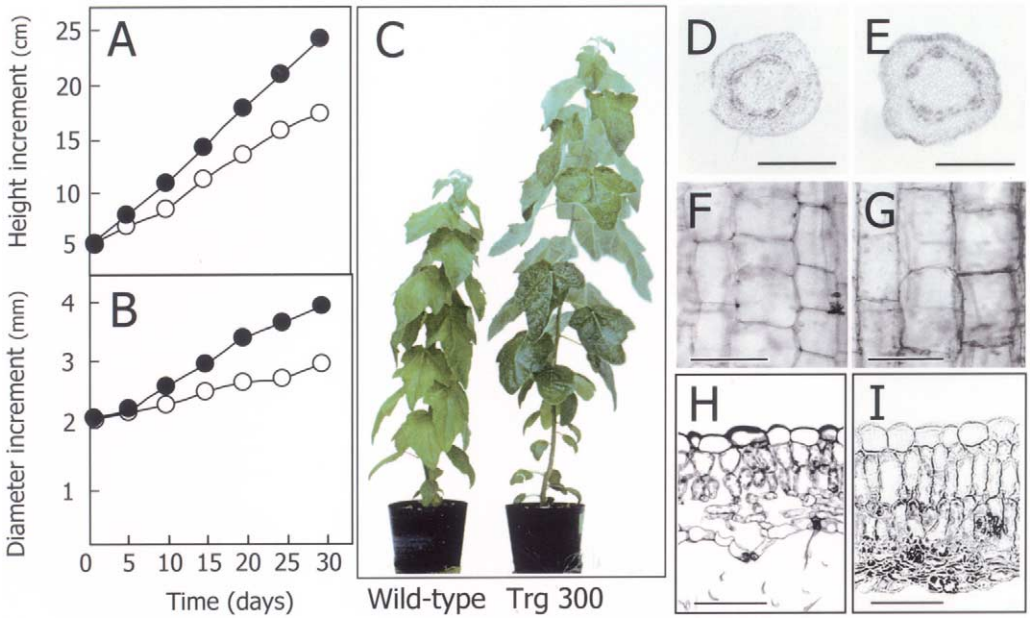

Fig. 3. Effect of AaXEG2 transgenes on stem and leaf cell growth. Stem elongation (A) and diameter growth (B) represent the mean of five independent plants for each line with individual values varying from the mean by $<6\%$. Open circles: wild type; closed circles: Trg 300. 30-day-old wild type and Trg 300 plants (C). Transverse section of the second internode in the wild type (D) and in Trg 300 (E). Bar = 1 mm. Longitudinal section of the second internode in the wild type (F) in Trg 300 (G). Bar = 10 μm. Cross-section of the 10th leaf in the wild type (H) and in Trg 300 (I). Bar = 50 μm.

When the total proteins extracted from sterile tissues were subjected to gel filtration, immunodetected xyloglucanase peaked at 30 kDa and did not form a complex with xyloglucanase inhibitor protein (data not shown) [15].

3.2. Growth of transgenic plants

When sterile shoots (1 cm length) were cultured in Murashige and Skoog agar medium containing 3% sucrose and 4 μM indole butylic acid, each internode elongated longer in the transgenic than in the wild type (Fig. 2). The non-sterile stem elongated faster in Trg 300 than in the wild type (Fig. 3A and C), and the diameter increased (Fig. 3B). Although transverse sections of elongating stems (the first internode) showed no difference in cell dimensions (radial or tangential) among the genotypes (Fig. 3D and E), longitudinal sections showed that the parenchymal cells in the elongating region were longer in the transgenic than in the wild type (Fig. 3F and G). Leaf epidermal cells were expanded in the transgenic line, whereas they were thin and flat in the wild type (Fig. 3H and I), and the palisade parenchymal cells in the transgenic were larger and surrounded by less air space. In addition to the change in shape, the transgenic leaf was about 1.3-fold greater in dry weight compared to the wild type and was visibly greener (Fig. 3C).

3.3. Cell wall analysis of stems

The transgenic primary cell wall had a similar amount of pectin (EDTA-soluble) as did the wild type but had less hemicellulose (24% KOH-soluble) and more cellulose (insoluble) as shown in Table 1. The decreased other polysaccharide was almost quantitatively accounted for by decreased wall-bound xyloglucan, the level of which in the transgenic was only about 20% of the level in the wild type. In the secondary xylem, a trace of wall-bound xyloglucan was found, and the amount was lower for the transgenic in the same proportion as the decrease in the primary cell wall (Table 1). The transgenic secondary cell wall had a relatively low amount of the other polysaccharides (pectin and hemicellulose) and lignin but had significantly more cellulose, the level of which was more than 10% greater than that in the wild type.

3.4. Mechanical properties of stems

To compare the mechanical properties of stems between elongating and mature regions, we used the second, fourth,

Table 1
Composition of wall components in the stems

Sample	0.1 M EDTA (mg/g dry weight)	24% KOH (xyloglucan) (mg/g dry weight)	Insoluble (mg/g dry weight)	Lignin (mg/g dry weight)
First and second internodes (whole stem)				
Wild type	208	243 (60.0)	345	0
Trg 300	207	185 (11.7)	355	0
10th internode (secondary xylem)				
Wild type	55.0	223 (0.080)	420	223
Trg 300	50.8	200 (0.0053)	467	210

The amounts represent the mean of three independent plants for each line with individual values of fractions varying from the mean by $<2\%$.

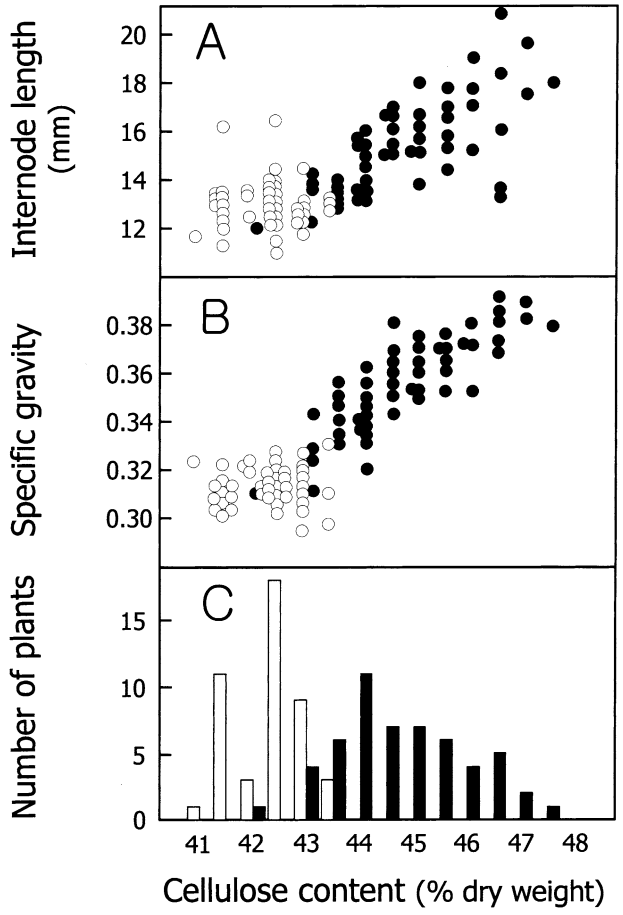

Fig. 4. Effects of increased cellulose contents on the specific gravities of the secondary xylems and internode lengths. Open circles and bars: wild type; closed circles and bars: transgenic.

fifth and 10th internodes (Table 2). We could not use the first internode for the determination of Young's modulus because it was too short. The second internode was growing over most of its length (at least more than 3.5 mm) at the time of sampling and represents elongating tissue. For measurements of the modulus, the bark was removed to avoid complications from its potentially distinct mechanical properties, and the Young's modulus of the secondary xylem measured by the tensile forced method using an automatic material testing machine [16]. Bark could not be separated from the second or third internodes, so the modulus reported for the second internode reflects the whole organ. The transgenic stem had a lower modulus in the second internode compared to the wild type but in the remaining internodes the modulus was increased gradually from fourth to 10th internodes with the increased length of internode (Table 2).

3.5. Specific gravity

The 54 independent transgenic poplars produced longer internodes and a larger amount of cellulose in the secondary xylem than the wild type (Fig. 4A and C). The cellulose content in the 10th internode was also assessed by measuring specific gravity, which showed relatively larger values in the transgenic than in the wild type (Fig. 4B). The specific gravity was increased in the transgenic probably by increasing the density of cellulose, which could result in an increase in cellulose content. In addition, the increased cellulose content might correspond to an increase in internode length, after which elongation growth was accelerated.

4. Discussion

The transgenic expression of xyloglucanase in poplar increased the length of stems in the presence of sucrose, independent from source tissue-dependent growth. Then, we have inferred that the expressed xyloglucanase cleaves off xyloglucans cross-linked with transversely oriented cellulose microfibrils and promotes stem elongation by loosening the wall [1]. The decreased Young's modulus in the elongating regions of the transgenic lines is consistent with a decreased amount of xyloglucan tethers between cellulose microfibrils.

Despite the high levels of xyloglucanase, the transgenic poplars always contained a residual quantity of cell wall-bound xyloglucan. We suggest that this residual represents xyloglucan polymer that is tightly adhering to the microfibrils, perhaps as a monolayer coating the surface [17]. Portions of xyloglucan molecules are known to bind microfibrils by extensive hydrogen bonds and may even intercalate within microfibrils [1]. Because the xyloglucanase probably does not attack xyloglucan coating microfibrils [18], it is reasonable that this portion of xyloglucan would escape degradation. Instead, the degraded xyloglucan appears to be derived from the portions of the xyloglucan polymer that are present between microfibrils, where the polymer may exist as an entangled network to give rigidity to the wall. Consequently, it appears that degradation of the free form of xyloglucan contributes to the loosening of the cell wall.

Since the deposition of cellulose was increased in the secondary xylem of the transgenic lines as well as in the primary wall, it is possible that cellulose deposition is affected by xyloglucan cross-links. If cellulose formation is restricted by the entanglement with xyloglucan, the relaxation resulting from cleavage of cross-linking xyloglucans may accelerate cellulose

Table 2
Length and Young's modulus of each internode

Internode	Length (mm)		Young's modulus ($\times 10^6$ Pa)	
	Wild type	Trg 300	Wild type	Trg 300
First	3.00	3.00	ND	ND
Second	4.22 ± 1.39	4.31 ± 1.43	3.65 ± 0.50	1.52 ± 0.45
Third	7.22 ± 1.75	9.01 ± 1.76	ND	ND
Fourth	10.74 ± 1.93	15.21 ± 1.66	8.70 ± 1.40	14.40 ± 0.41
Fifth	12.58 ± 2.53	17.58 ± 2.10	155 ± 16	171 ± 14
10th	13.12 ± 3.38	18.10 ± 1.40	370 ± 23	452 ± 36

ND, not determined.
Five independent plants for each line were used for determination.

biosynthesis and deposition. We propose that such a relation is involved in the enhancement of cellulose deposition in the transgenic poplars. Another possibility is that high turnover of xyloglucan occurs in the secondary wall, where xyloglucan might cross-link microfibrils. Although xyloglucan accumulated in the primary layer of developing xylem fibers [19], the majority of xyloglucan could be deposited during secondary wall formation. Therefore, overexpression of xyloglucanase might further promote xyloglucan turnover, controlling the secondary wall from mechanical stresses due to xyloglucan cross-linkings.

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