

Characterization of Poplar Overexpressing Xylanase

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Abstract

We expressed *Hordeum vulgare* (barley) xylanase constitutively in *Populus tremula x alba* (poplar). The expression of xylanase reduced not only the amount of hemicellulose but also the amount of lignin in the secondary xylem. Stem and leaf growth rates were accelerated in transgenic plants. The stems also developed increased flexibility, especially in their younger parts, specifically, the regions above the 10th internode from the top. The secondary walls in the xylem would be slower to mature in xylanase-expressing poplar than in the wild type.

Key words: xylan, transgenic, xylanase, secondary wall, xylem differentiation.

Introduction

Xylan is the main hemicellulosic component of the secondary walls of higher plants (Shallom and Shoham 2003; Boussaid *et al.* 1999). Along with cellulose and lignin, it represents one of the major polymeric constituents of plant cell walls (Kulkarni *et al.* 1999; Bura *et al.* 2002). All three of these components interact via covalent and non-covalent linkages, in which xylan is found at the interface between lignin and cellulose. Thus, xylan is one of the most important of the components responsible for plant cell wall integrity (Beg *et al.* 2001). In addition, xylan is, after cellulose, the second most abundant organic compound on earth, accounting for approximately one-third of all renewable organic carbon (Timell 1967). Xylan always exists in large quantities in xylem: it constitutes 15~30% of xylem by weight in angiosperms, 7~10% in gymnosperms, and various high percentages (<30%) in annual plants (Singh *et al.* 2003). It occurs as a heteropolysaccharide with a homopolymeric backbone chain of 1,4-linked β -D-xylopyranose units substituted with acetyl, α -L-arabinofuranosyl, α -1,2-linked glucuronic and 4-methylglucuronic acid residues (Aspinall 1959).

Xylan is degraded by the widely distributed group of enzymes known as xylanases. Xylanases are produced by a plethora of organisms including bacteria, algae, fungi, protozoa, gastropods and arthropods (Prade *et al.* 1995); they are involved in the production of xylose, a primary carbon source for cell metabolism, as well as in plant cell infection by plant pathogens, which produce several kinds of xylanases to degrade cell walls and thereby gain easy access to plant cell interiors. Humans use xylanases in the pulp and paper industries: the hydrolysis of xylan facilitates the release of lignin from paper pulp, reducing the need for chlorine as a bleaching agent (Adler *et al.* 1987; Berlin 2005). Endoxylanases (1,4- β -D-xylan xylano-hydrolases, EC 3.2.1.8) hydrolyze xylan into xylo-oligosaccharides and xylose residues, while β -xylosidases (1,4- β -D-xylan xylo-hydrolases, EC 3.2.1.37) catalyze the

release of xylosyl residues from the non-reducing terminals of the oligosaccharides.

In this study, we overexpressed xylanase in poplar to examine whether the constitutive degradation of wall xylan has any effect on the process of cell wall formation or on the characteristics of cell walls. We also expected to clarify the function of xylan in the primary and secondary walls of higher plants.

Materials and Methods

Construction of the Plasmid Harboring Xylanase and Plant Transformation

Hordeum vulgare (barley) xylanase cDNA was amplified from first strand cDNA as a template by means of polymerase chain reaction (PCR) using a forward primer containing a *Bam*HI site (5P-GAGGATCCCAACGAGACCCTGGTG-3P) and a reverse primer containing an internal *Sac*I site (5P-GAAGAGCTCACAACCAATTA-TCCCTTGACG-3P) complementary to the DNA sequence (accession number U59312). The PCR fragment was cloned into a pGEM-easy vector and named pXyl100. The *Bam*HI-*Sac*I fragment was ligated into the *Bam*HI-*Sac*I site of the binary vector pBE2113-GUS, under control of the CaMV35S promoter and E12-6 enhancer sequences. The plasmid constructs were electroporated into *Agrobacterium tumefaciens* LBA4404, and leaves of aseptically flask-grown *Populus tremula x alba* were inoculated with the bacteria. Plantlets were grown in a growth chamber at 27°C under a photoperiod of 18 hours.

Fractionation of Cytoplasmic and Wall-bound Proteins

Stems were harvested, cut into several pieces, vacuum-infiltrated with demineralized water, and centrifuged for 15 min at 440 μ g to yield the apoplasmic solution. After the apoplasmic solution was extracted, 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 a buffer containing 1 M NaCl. Xylanase activity was measured through a reducing power assay conducted 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 2% (w/v) xylan in semimicroviscometers (Cannon Instrument Co., State College, PA, USA).

Preparation of An Antiserum and Western Blot Analysis

The xylanase gene pXyl100 was amplified for high expression by means of PCR using a forward primer containing a *Bam*HI site (5P-CATGGATCCCCGCGTCTACC-3P) and a reverse primer containing a *Xba*I site (5P-CCGTCTCCGTCAGGCGTCAGCC-3P). The PCR fragment was ligated into a pGEM-easy vector. Recombinant xylanase was expressed in *Escherichia coli* cells harboring the pET-21a expression vector fused with the full-length cDNA for its matured xylanase without a signal peptide region. 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 sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 10% sodium dodecyl sulfate, electrotransferred to Hybond-C Extra (Amersham, GE Healthcare, Chalfont St. Giles, UK), and probed with an antibody against the xylanase sequence followed by a second antibody, using an ABC high-horseradish peroxidase (HRP) immunostaining kit (Toyobo, Osaka, Japan).

Xylanase Assay

To detect xylanase activity in poplars, plants were grown on soil under cycles consisting of 16 hours of light and 8 hours of darkness at 28°C. After 4 weeks of culture, 100 mg of fresh weight plant tissues were macerated with liquid nitrogen and extracted with 2 mL of 50 mM sodium citrate buffer (pH 6.0). 3% birchwood xylan was used as a substrate (Sigma-Aldrich, St. Louis, MO, USA). After the plant tissues had been incubated for 2 hours at 30°C, an equal volume of Somogyi solution was added and boiled for 15 minutes at 100°C. After this mixture had cooled down, an equal volume of Nelson solution was added and mixed. This mixture was diluted with 3 times its volume of H₂O and its absorption spectrum was measured at 660 nm (Teather and Wood 1982).

Polysaccharide Content

The levels of certain polysaccharides in poplar xylem were determined through stepwise extraction (Park *et al.* 2004). The hemicellulose fraction was identified as

the 24% KOH-soluble fraction after EDTA extraction. The cellulose fraction was identified as the 24% KOH-insoluble fraction.

Lignin Content

The lignin content of the stem material was measured using the acetyl bromide method with approximately 15 mg extractive-free material (Fukushima and Hatfield 2004). 5 g of dry cell wall material was placed in a 250-mL round-bottom flask and 100 mL of acidic dioxane (90 mL of dioxane + 10 mL of 2 N HCl solution) was added. The suspension was refluxed under N₂ for 30 min, cooled to room temperature, and filtered through a glass fiber filter (GF/C, 47 mm, Whatman Inc., GE Healthcare). The insoluble cell wall residue that collected on the filter was washed with 20 mL of 96% dioxane, and the material that washed out was combined with the original dioxane extract. Sodium bicarbonate (4.0 g) was added, and the flask was placed on a rotary shaker for several minutes until the solution was neutralized. The solution was then filtered through a 0.45- μ m nylon membrane (Schleicher and Schuell, Keene, NH, USA) and diluted with water to a volume of 10~15 mL under reduced pressure on a rotary evaporator (water temperature of 40°C). It was then filtered through a 0.45- μ m nylon membrane once again. The suspension was centrifuged at 9000 g for 20 min and the supernatant was removed. The isolated lignin (after corrections for carbohydrate and protein contaminants) was dissolved in 5.0 mL of dioxane, and aliquots of 0.2, 0.3, 0.4, 0.5, and 0.6 mL were pipetted into culture tubes, frozen in liquid N₂, and placed on a freeze-dryer overnight. 0.5 mL of 25% acetyl bromide in glacial acetic acid (HAcBr) was added to each tube. A blank was included to correct for reagent background absorbance. Tubes were tightly capped with PTFE-lined caps and put into a 50°C water bath for 30 min. After their contents had cooled, all tubes received 2.5 mL of acetic acid (HAc), 1.5 mL of 0.3 M NaOH, and 0.5 mL of 0.5 M hydroxylamine hydrochloride solution. The tubes were shaken, and HAc was added to bring each tube to a final volume of 10.0 mL. Solutions were read in a spectrophotometer at 280 nm.

Mechanical Properties of the Xylem Segment

The mechanical properties of the secondary xylem of transgenic and wild type plants were compared in stem segments taken from the tenth internode. For measurements of the modulus, the bark was removed to avoid complications from its potentially distinct mechanical properties, and the elasticity of the secondary xylem was measured by means of the tensile forced method using an automatic material testing machine as previously described (Park *et al.* 2004).

Results

Expression of Xylanase

Transgenic poplar was generated overexpressing a xylanase under control of a CaMV35S promoter (Figure 1). A gene product for xylanase was detected in the transgenic plants through western blotting using an antibody against recombinant xylanase; the molecular mass of the detected bands was equivalent to that of mature xylanase (Figure 2A). This gene product was found in both the cytosolic and the wall-bound fractions of transgenic poplar. Xylanase activity in the transgenic poplar was about 10 to 15 times higher than that in the wild type (Figure 2B). In terms of stem growth, transgenic poplar elongated somewhat faster than wild type did, whereas the diameters of the two genotypes increased at almost the same rate. The leaves had larger area than those of wild type (Figure 3). And the shape was wider on transgenic plants than on wild type plants, as shown in Figure 3, though its longitudinal length was similar in the two genotypes.



Figure 1. Transgenic poplar overexpressing xylanase.

Cell Wall Composition of the Stem

The greatest difference between transgenic and wild type plants was in the amount of hemicellulose found in the secondary xylem (Table 1). The amount of hemicellulose in the secondary xylem of transgenic plants was almost 15% less than that in wild type plants; in a primary tissue sample, in contrast, transgenic plants had 7% more hemicellulose than wild type plants (data not shown). Transgenic poplar therefore contained rather more cellulose than wild type poplar did.

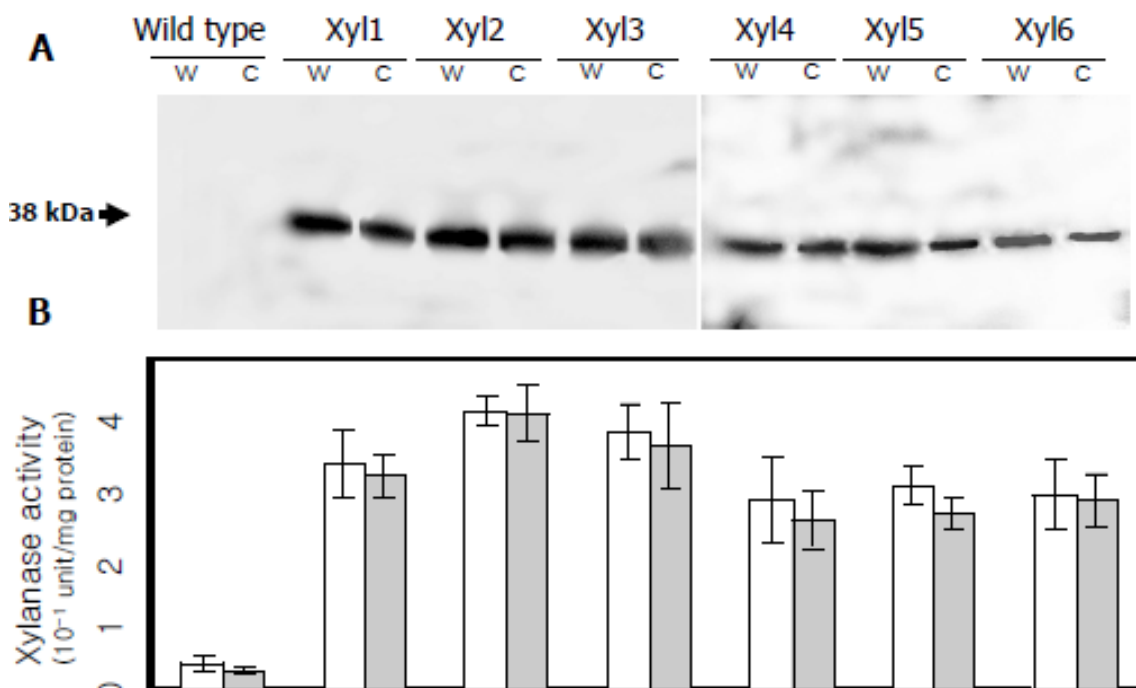


Figure 2. Identification of the gene product for xylanase and measurement of xylanase activity in poplar.

Western blot showing the gene product (upper panel). w, wall-bound fraction; c, cytoplasmic fraction. Arrow indicates 38-kDa gene product. Level of xylanase activity in stems (lower panel). White bar is for wall-bound, and gray bar is for cytoplasmic fraction. Each activity level represents the mean of three independently-tested plants, with individual values varying from the mean by up to 65%.

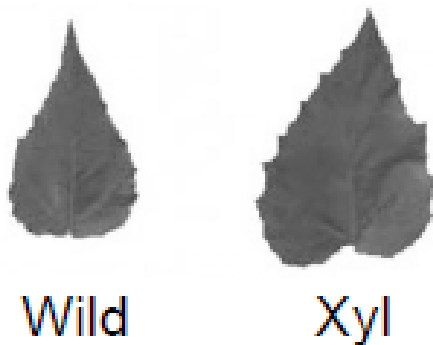


Figure 3. Effect of xylanase expression on leaf shape. Each of the leaves shown here was the eleventh from the top of its plant.

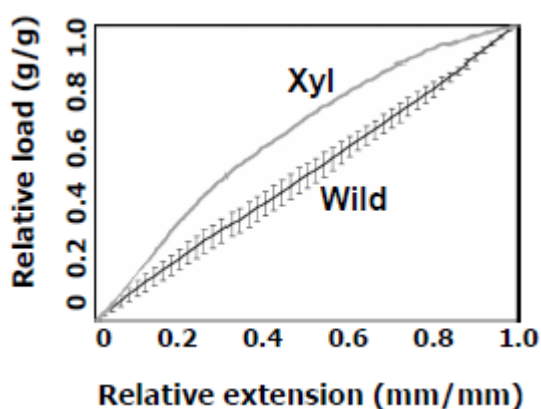


Figure 4. Effect of xylanase expression on relative load-extension curves. The load versus extension curves for each stem fragment are plotted as values relative to the point at which the fragment broke. The secondary xylem at the tenth internode was analyzed in this experiment; averaged curves are shown.

Table 1. Amounts of cell wall components in poplar stems (mg/g dry weight).

Genotype	Hemicellulose	Cellulose
Wild type	262 ± 16	533 ± 21
Transgenic	229 ± 18	530 ± 17

Sugar linkages were determined through methylation analysis, which revealed that 4-linked xylosyl residue was markedly decreased in transgenic plants at all internodes examined, namely, the first, sixth and tenth. The other linkages examined exhibited smaller differences between transgenic and wild type plants. The amounts of lignin in several internodes were fairly similar in transgenic and wild type plants, with low deposition in the young internodes, but lignin deposition was slower in transgenic internodes than in wild type, particularly from the fourth internode (Table 2).

Mechanical Properties of the Stem

Transgenic stems had lower moduli than wild type stems had. Load-extension curves were plotted as load against extension values relative to the breaking point for each value of max-extension. Load and elongation values were then expressed as values relative to the breaking point to allow for comparison between transgenic and wild-type plants. The results showed that transgenic plants had higher elongation values than wild-type plants. The load-extension curves of the stems of wild-type plants showed an arch pattern above the curves (Figure 4). Generally, the load-extension curves of brittle materials with many cross-links are more linear, meaning that brittle materials elongate less. In addition, brittle materials with many cross-links show load-extension curves with shallower slopes when the load and elongation values are expressed as values relative to the breaking point. The typical arch pattern and the large breaking-point values of elongation show that these transgenic plants have fewer cross-linking components than wild-type plants have.

Discussion

The transgenic expression of xylanase in poplar slightly increased the growth rates of stems and leaves and softened the stems in the early stages of xylem formation. The amount of hemicellulose in the transgenic poplar was 15% less than in wild type. Based on these observations, we have inferred that the expressed xylanase cleaves off xylans between transversely oriented cellulose microfibrils and lignin complexes, and promotes stem elongation by loosening the wall. The decreased relative elasticity of the secondary xylem of the transgenic poplar would be caused by a decrease in the number of xylan tethers between cellulose microfibrils and lignin complexes.

Despite the increased xylanase activity in their walls, these transgenic poplars always contained a certain quantity of wall-bound xylan. We suggest that this residual xylan represents xylan polymer that adheres tightly to the microfibrils and lignin, perhaps as a monolayer coating their surfaces. Portions of xylan molecules are known to bind microfibrils by means of extensive hydrogen bonds and may even intercalate within microfibrils (Berlin *et al.* 2006). If the xylanase expressed in our transgenic poplars attacks only intercalated xylan, it is reasonable to assume that any xylan coating microfibrils escapes degradation. In fact, the xylan that was degraded in our transgenic poplars appears to have existed as single molecules located between microfibrils and/or lignin, where xylan may exist as an entangled network giving rigidity to the wall. Consequently, it appears that degradation of the free form of xylan contributes to the loosening of the cell wall.

Table 2. Lignin content at each of several internodes in poplar stems (mg/g dry weight).

Genotype	Internode					
	Second	Third	Fourth	Fifth	Sixth	Tenth
Wild type	12 ± 0.9	20 ± 1.2	62 ± 1.6	163 ± 2.3	205 ± 2.1	228 ± 2.1
Transgenic	11 ± 0.8	13 ± 1.3	31 ± 2.1	95 ± 1.9	166 ± 1.8	170 ± 1.1

Certain forms of xylan molecules are known to induce lignin to bind to cellulose microfibrils with extensive hydrogen or covalent bonds; these xylan molecules may be even intercalated within the microfibrils (Bailey *et al.* 1992). Since our transgenic poplars exhibit low lignin content in the secondary xylem, xylanase would act by removing the side chains of the xylan polymer and thereby prevent it from binding to lignin. The relaxation that occurs as a result of side chain trimming would result in cell enlargement. If the deposition of lignin occurs in the side chains of xylan, the relaxation that occurs as a result of side chain trimming would decrease lignin deposition during the assembly of wall polysaccharides. We propose that both of the xylanase actions described above are probably involved in the enhancement of cell growth and the reduction of lignin accumulation in the transgenic poplar.

Lignin has a powerful effect on the mechanical properties of the cell wall, contributing to both its rigidity and its compressional strength (Niklass 1992). Low-lignin mutants of *Arabidopsis thaliana* develop irregularly-shaped xylem vessels with reduced mechanical strength (Jones *et al.* 2001). A similar type of deformation has been observed under conditions of extreme xylem tension, demonstrating the importance of the mechanical properties of conduit walls in water transport (Hacke *et al.* 2001). The decreased elasticity observed in these transgenic lines is consistent with a decreased number of xylan tethers between cellulose microfibrils and lignin.

References

- Adler, E.; G. Brunow; K. Lundquist. 1987. Investigation of the Acid-catalyzed Alkylation of Lignin by Means of NMR Spectroscopic Methods. *Holzforschung* 41: 199-207.
- Aspinall, G.O. 1959. Structural Chemistry of the Hemicelluloses. *Adv Carbohydr Chem* 14: 429-468.
- Bailey, M.J.; P. Biely; K. Poutanen. 1992. Interlaboratory testing of methods for assay of xylanase activity. *J Biotechnol* 23: 257-270.
- Beg, Q.K.; M. Kapoor; L. Mahajan; G.S. Hoondal. 2001. Microbial Xylanases and their Industrial Applications: A review. *Appl Microbiol Biotechnol* 56: 326-338.
- Berlin, A.; N. Gilkes; A. Kurabi; R. Bura; M. Tu; D. Kilburn; J. Saddler. 2005. Weak Lignin-binding Enzymes: A Novel Approach to Improve the Activity of Cellulases for Hydrolysis of Lignocellulosics. *Appl Biochem Biotechnol* 121: 163-170.
- Berlin, A.; M. Balakshin; N. Gikes; J. Kadla; V. Maximenko; S. Kudo; J. Saddler. 2006. Inhibition of Cellulase, Xylanase and β -glucosidase Activities by Softwood Lignin Preparations. *J Biotech* 125: 198-209.
- Boussaid, A.; J. Robinson; Y. Cai; D.J. Gregg; J. Saddler. 1999. Fermentability of the Hemicellulose-derived Sugars from Steam-exploded Softwood (Douglas-fir). *Biotechnol Bioeng* 64: 284-289.
- Bura, R.; S. Mansfield; R. Bothast; J. Saddler. 2002. SO₂-catalyzed Steam Explosion of Corn Fiber for Ethanol Production. *Appl Biochem Biotechnol* 98: 59-72.
- Fukushima, R.S.; R.D. Hatfield. 2004. Comparison of the Acetyl Bromide Spectrophotometric Method with Other Analytical Lignin Methods for Determining Lignin Concentration in Forage Samples. *J Agric Food Chem* 52: 3713-3720.
- Hacke, U.G.; J.S. Sperry; W.T. Pockman; S.D. Davis; K.A. McCulloch. 2001. Trends in Wood Density and Structure are Linked to Prevention of Xylem Implosion by Negative Pressure. *Oecologia* 126: 457-461.
- Jones, L.; A.R. Ennos; S.R. Turner. 2001. Cloning and Characterization of *irregular xylem4 (irx4)*: A Severely Lignin-deficient Mutant of *Arabidopsis*. *Plant J* 26: 205-216.
- Kulkarni, N.; A. Shendye; M. Rao. 1999. Molecular and Biotechnological Aspects of Xylanases. *FEMS Microbiol Rev* 23: 411-456.
- Niklas, K. 1992. *Plant Biomechanics: An Engineering Approach to Plant Form and Function*. Chicago: Univ. of Chicago Press.
- Park, Y.W.; K. Baba; Y. Furuta; I. Iida; K. Sameshima; M. Arai; T. Hayashi. 2004. Enhancement of Growth and Cellulose Accumulation by Overexpression of Xyloglucanase in Poplar. *FEBS Lett* 564: 183-187.
- Prade, R.A. 1995. Xylanases: From Biology to Biotechnology. *Biotech Genet Eng Rev* 13: 100-131.
- Shallom, D.; Y. Shoham. 2003. Microbial Hemicellulases. *Curr Opin Microbiol* 6: 219-228.
- Singh, S.; A.M. Madlala; B.A. Prior. 2003. Thermomyces Lanuginosus: Properties of Strains and their Hemicellulases. *FEMS Microbiol Rev* 27: 316.
- Teather R.M.; P.J. Wood. 1982. Use of Congo-red Polysaccharide Interactions in Enumeration and Characterization of Cellulolytic Bacteria from the

- Bovine Rumen. *Appl Environm Microbial* 43: 777-480.
- Timell, T.E. 1967. Recent Progress in the Chemistry of Wood Hemicelluloses. *Wood Sci Technol* 1: 45-70.
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