J Wood Sci (2012) 58:281–289
DOI 10.1007/s10086-011-1247-1

ORIGINAL ARTICLE

Analyses of leaves from open field-grown transgenic poplars overexpressing xyloglucanase

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Received: 16 May 2011 / Accepted: 19 December 2011 / Published online: 23 February 2012

Abstract The transgenic expression of *Aspergillus* xyloglucanase cDNA (*AaXEG2*) with 35S promoter in the leaves of open field-grown poplars was studied. The level of xyloglucan in the transgenic poplars was decreased to 15–16% in the non-fertile soil (forest-field soil) and to 21–22% in the fertile soil (farming-field soil) compared with that of the wild-type poplars. The leaves exhibited a smaller surface area with more rounded teeth than those of the wild-type plants, similar to the sun leaf variety that was grown in the incubation room and subsequently greenhoused. The majority of total veins with water-conducting vascular bundles were shorter in the leaves of the transgenic poplars than those of the wild type. This decrease in vein length may result from a decrease in xyloglucan during leaf development, from which large numbers of proteins were markedly downregulated in the leaves of the transgenic plants via proteomic analysis. It seems likely that the leaves of the transgenic poplars came to relax the edges of their tooth rather than extend their veins as a result of the loosening of the xyloglucan cellulose networks in the leaves.

Keywords Xyloglucan · Field trial · Poplar · Leaf morphology · Sun leaf

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Introduction

Since both plant growth and morphology are controlled by the cell walls, a plant's development is determined by the nature of its walls during the growth phase. The skeletal component of the walls is formed by cellulose microfibrils, which are mostly tightened by the noncellulosic polysaccharide, xyloglucan, in the walls of dicotyledons [1, 2]. The degradation of xyloglucan in the cell wall promotes plant growth by increasing the plastic extensibility under turgor pressure [3]. Thus, the overexpression of xyloglucanase has been tested in open field-grown poplars in which *Aspergillus* xyloglucanase cDNA (*AaXEG2*) with 35S promoter has been used for the expression studies [4]. Two transgenic lines, trg300-1 and trg300-2, were employed along with wild-type plants. Genomic Southern hybridization revealed that the transgene may exist as one copy of trg300-1 and two copies of trg300-2 at different loci. The genes, which are integrated in genomes at a low copy number, as in these transgenic poplars, are expected to be expressed stably. Indeed, Western blot analysis revealed that *Aspergillus* xyloglucanase was expressed in the leaves of both transgenic poplar lines grown in the greenhouse, running at a position corresponding to the size (28 kDa) of the mature xyloglucanase. These results indicate that *AaXEG2* was integrated into the poplar genomes and expressed stably in individuals propagated by cuttings.

In a previous study on the transgenic expression of xyloglucanase in poplars grown in the incubation room, the transgenic plants exhibited an enhanced growth rate in terms of stem length and diameter, and produced wood with a higher specific gravity than the wild type [4]. In addition to these characteristics, the transgenic poplar produced sun leaf-like leaves as a result of the greater expansion of palisade parenchyma cells, even under the low-light conditions of the incubation room in which any plant would normally produce shade leaves. A sun leaf is usually produced under strong light conditions, and the leaf blade is, therefore, smaller and thicker than that of a shade leaf [5]. The leaves of the two transgenic lines were visibly greener, thicker, and smaller than those of the wild type. They resembled sun leaves in both the incubation room and the greenhouse, as if they had been grown under conditions of strong sunlight. This paper focuses on the leaf phenotype in open field-grown transgenic poplars overexpressing xyloglucanase.

The overexpression of xyloglucanase results in a decrease in xyloglucan content, which seems to be a kind of xyloglucan knockout in plant cell walls. The gene knockout mutant of xyloglucan xylosyltransferase should cause xyloglucan knockout. However, complete xyloglucan knockout has not yet been achieved by either overexpression or gene knockout. The former may be difficult due to the reduced accessibility of xyloglucan embedded in cellulose microfibrils for the overexpressed enzyme and the latter may be difficult because of the involvement of several isoenzyme genes for one transferase. An abnormal phenotype has been observed only in the roots and hairs of single and double knockout mutants for xyloglucan xylosyltransferases in Arabidopsis, in which a single mutant (*xxt5*) has short root hairs [6] and a double mutant (*xxt1* and *xxt2*) exhibited aberrant root hairs and slow growth [7] and altered cellulose organization [8], compared with wild-type plants.

We also employed proteomic approaches to establish the protein expression profiles of field-grown transgenic poplars. A mass tagging strategy using isobaric tags for relative and absolute quantification (iTRAQ) improves the identification of large sets of proteins and enables the analysis of relative protein expression levels among different samples [9, 10]. Using this method, we compared the levels of protein accumulation in transgenic poplars overexpressing xyloglucanase with those of wild-type plants. Prior to the proteomic analysis of leaves, we removed the ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) proteins using an immunoaffinity column, since RuBisCO constitutes approximately 35–40% of the total protein mass in green leaves [11, 12] and thus interferes with proteomics studies. Functional analysis of each of these identified proteins provided a great deal of information regarding the leaf phenotype, especially as it was related to the functional roles of xyloglucan in plants.

Materials and methods

Plant materials and size determination

One wild-type and two transgenic line poplars (trg300-1 and trg300-2) were used in this experiment. Their seedlings were produced by cuttings taken in 2006 and cultured in the greenhouse. Since all of the poplars (*Populus alba*) were female, they produced a large number of branches in their main stems so as to maximize pollen acquisition. The fourth leaves, counting from the uppermost leaf below 5 mm in length from the top of branch, were harvested from each line in July and August of 2010. Leaf surface size was calculated by converting the weight of each Xerox copy of the leaf into an area value. Leaf thickness was measured directly using a vernier caliper.

Field conditions

A field trial for the transgenic poplars was carried out in the field of the Forest Breeding Center in Hitachi, Japan (http://ftbc.job.affrc.go.jp/). Two kinds of soil were used for the trials. One was a forest-field soil, which contained average levels of soil nutrients found in Japanese forests. The other was a farming-field soil, which contained average levels of soil nutrients found in Japanese farming fields. The former was defined as a non-fertile soil and the latter as a fertile soil. Both types were layered onto the experimental field.

Six plots of poplar plantations were used for the field trial, of which three plots contained fertile soil and three contained non-fertile soil. Both of these three-plot sets consisted of one plot each of trg300-1, trg300-2, and wild-type plants, and each individual plot of 144 m^2 contained 25 trees arranged in a 5 × 5 grid with 2 m × 2 m spacing between the plants [13].

Chlorophyll content

Leaves were extracted with methanol; this extract was measured at 662 nm for chlorophyll *a* and at 646 for chlorophyll *b*. The amounts of these pigments were calculated according to the formulas of Lichtenthaler and Wellburn [14].

Xyloglucan degradation activity

Each leaf was homogenized with liquid nitrogen, and the resulting powder was ground with 20 mM sodium phosphate buffer (pH 6.2) containing 1 M NaCl in a mortar; the wall residue was washed three times. The combined supernatant was adjusted to 80% saturation with solid ammonium sulfate to generate a precipitate from which an aliquot protein was used. Xyloglucan degradation activity was determined by decolorization in the amount of the xyloglucan-iodine complex by use of the iodine-sodium sulfate method [15]. One unit of enzyme activity is defined as the amount of enzyme that causes 0.1% decrease in 640 nm of 0.9 ml of 0.5% (w/v) low molecular weight pea xyloglucan in 10 mM sodium phosphate buffer (pH 6.2) in 1 min at 35°C.

Microscopy

Leaves were fixed with 3.5% glutaraldehyde in a 20 mM sodium phosphate buffer (pH 7.2) overnight. The pieces were dehydrated in an ethanol series and embedded in Paraplast Plus (McCormick Scientific, St. Louis, MO, USA). Leaf sections of 5-μm thickness were obtained from the embedded samples. The sections were placed on glass slides with water and heated. After the Paraplast was removed, the sections were stained with 1% safranin, dehydrated with an ethanol series, and mounted with Biolite (Okenshoji, Tokyo, Japan). The sections were observed under a microscope and digital micrographs were taken. The thickness was measured on a computer at 40 points from 12 to 20 micrographs from each line and calculated using the scale bar.

For immunofluorescence labeling, leaves were delignified in 5 ml of 8% sodium chlorite solution containing 1.5% acetic acid by shaking at 50 rpm at 40°C for 40 h. The leaves were then washed with water, extracted with 0.1 M KOH containing 0.1% $NaBH_4$ by shaking at 50 rpm at 40°C for 24 h, and again washed completely with water. After blocking for 1 h with 3% (w/v) non-fat dry skim milk in sodium phosphate-buffered saline (pH 7.0), the leaves were incubated with a tenfold dilution of monoclonal antibody CCRC-M1 (CarboSource, Athens, GA, USA) for 2 h, washed with sodium phosphate-buffered saline (pH 7.0) containing 0.05% Triton X-100 (PBST), and then incubated with a 50-fold dilution of anti-mouse IgG conjugated with alkaline phosphatase for 1 h in darkness. The samples were then washed with sodium phosphate-buffered saline (pH 7.0) containing 0.05% Triton X-100 (PBST) and stained with a reagent [16].

Wall analysis

Each leaf was ground in liquid nitrogen and the resulting powder was homogenized six times with 20 mM sodium phosphate buffer (pH 7.0) and three times with water. The residue was then freeze-dried as a wall preparation. The wall preparation was then extracted with 24% KOH containing 0.1% $NaBH_4$. The insoluble wall residue (cellulose fraction) was washed twice with water and extracted with acetic/nitric reagent (80% acetic acid/concentrated nitric acid, 10:1) in a boiling water bath for 30 min [17]. The insoluble material was weighed after the material was washed with water and freeze-dried. The alkali-soluble fraction was neutralized, dialyzed, and freeze-dried for use

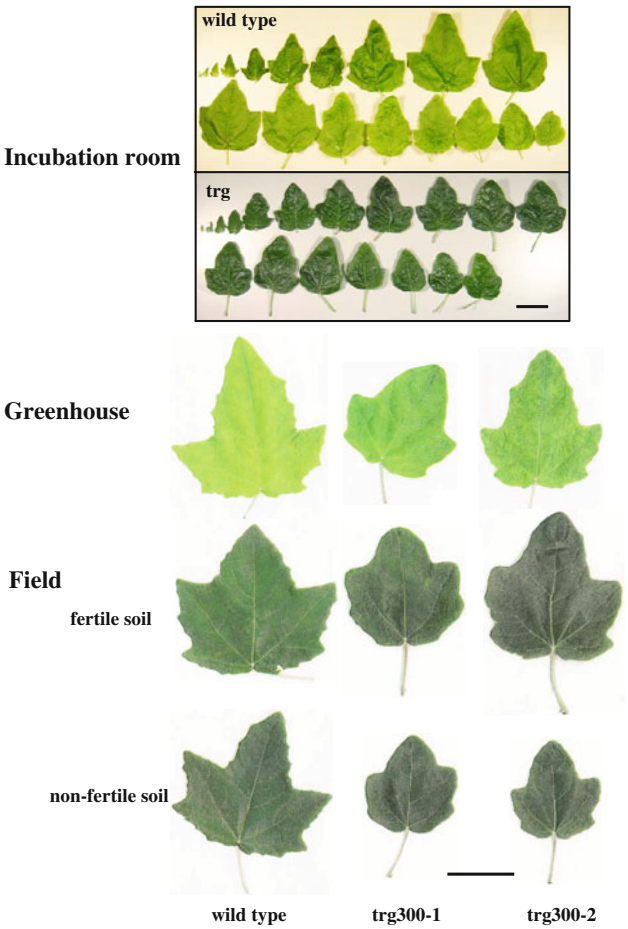

Fig. 1 Phenotypes of leaves in the transgenic poplars grown in the incubation room, greenhouse, and open field. Incubation room: whole leaves taken from the top to the bottom of one wild-type poplar and one transgenic poplar grown in pots in the incubation room. *Bar* 3 cm. Greenhouse and field: the fourth leaf from the top shoot (5 mm) of wild type, trg300-1, and trg300-2 poplars grown under greenhouse and field (fertile and non-fertile soils) conditions. *Bar* 3 cm

Table 1 Leaf phenotype, chlorophyll content and xyloglucanase activity of the transgenic poplars

	Fertile field			Non-fertile field			Green house		
	Wild type	trg300-1	trg300-2	Wild type	trg300-1	trg300-2	Wild type	trg300-1	trg300-2
Area (cm^2)	22.3 ± 1.2^{a}	17.3 ± 1.0^{b}	21.5 ± 0.7^{a}	18.3 ± 0.8^{b}	13.5 ± 0.8^{c}	13.2 ± 0.8^{c}	19.1 ± 1.0^{ab}	15.5 ± 1.1^{bc}	17.3 ± 1.5^{abc}
Thickness (mm)	0.122 ± 0.004^{a}	0.160 ± 0.007^{b}	0.156 ± 0.004^{b}	0.166 ± 0.004^{b}	0.162 ± 0.003^{b}	0.162 ± 0.004^{b}	0.110 ± 0.006^{a}	0.117 ± 0.007^{a}	0.127 ± 0.004^{a}
Fresh weight (g)	0.314 ± 0.022^{ac}	0.355 ± 0.019^{a}	0.455 ± 0.027^{b}	0.328 ± 0.017^{a}	0.304 ± 0.024^{ac}	0.303 ± 0.019^{ac}	0.248 ± 0.019^{c}	0.243 ± 0.009^{c}	0.268 ± 0.023^{ac}
Primary vein length (mm)	55.4 ± 2.0^{a}	46.2 ± 1.5^{bc}	51.7 ± 1.4^{ab}	49.2 ± 1.1^{ab}	41.7 ± 1.2^{c}	42.3 ± 1.3^{c}	57.8 ± 2.5^{a}	49.1 ± 1.8^{abc}	51.9 ± 2.7^{ab}
Numbers of secondary veins	7.1 ± 0.4^{ab}	6.7 ± 0.2^{a}	7.3 ± 0.2^{ab}	8.1 ± 0.2^{b}	6.9 ± 0.2^{a}	6.6 ± 0.2^{a}	8.8 ± 0.5^{b}	6.9 ± 0.3^{a}	9.1 ± 0.3^{b}
Total lengths of secondary veins (mm)	156 ± 6^{ab}	125 ± 5^{cd}	158 ± 7^{ab}	177 ± 6^{a}	127 ± 5^{cd}	114 ± 5^{d}	142 ± 7^{bce}	120 ± 7^{de}	138 ± 5^{ce}
Petiole length (mm)	24.8 ± 1.3^{ab}	22.9 ± 0.8^{b}	26.6 ± 0.7^{a}	20.0 ± 0.9^{bcd}	22.1 ± 1.1^{bc}	20.5 ± 1.0^{bcd}	10.8 ± 0.9^{e}	18.3 ± 1.4^{c}	16.3 ± 1.2^{cd}
Chlorophyll *a* (μg)	9.6	14.5	10.4	9.0	8.6	7.5	7.1	9.61	4.8
Chlorophyll *b* (μg)	2.0	1.6	2.1	1.6	1.9	1.5	1.1	2.5	1.0
Xyloglucanase activity (10^2 U/mg protein)	1.2	37.5	27.9	0.9	87.0	93.5	1.0	60.4	79.7

The first leaf was defined as the uppermost leaf below 5 mm in length from top, and the fourth leaves from the internode were sampled. Twenty to twenty-five leaves for each poplar were used in these experiments. Data represent the mean ± SE of independent plants of a single line. Values with different letters indicate statistically significant differences according to Student's *t* test ($P < 0.05$)

Fig. 2 Leaf cross sections of fertile field-grown poplars. *Bar* 100 μm

in methylation analysis. Partially methylated alditol acetates were analyzed using an Agilent gas chromatography-mass spectrometer with a glass capillary column (0.25 mm × 15 m) of DB-225 [18].

Preparation of proteins and iTRAQ labeling

Leaves (ca. 2 g) were homogenized with 7 M urea containing 2 M thiourea, 4% (w/v) CHAPS, and 10 mM dithiothreitol. The homogenates were centrifuged at 10,000*g* for 20 min at 4°C, and the supernatants were obtained. The protein concentration was determined using a 2-D Quant Kit (GE Healthcare, Buckinghamshire, UK). One hundred micrograms of protein from each sample was

precipitated by the addition of eight volumes of cold acetone and kept overnight at −20°C. The pellets were dried briefly. Then, proteins were digested with trypsin followed by labeling with the iTRAQ reagent (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Samples from wild-type and transgenic plants were labeled with iTRAQ reagent 114 and 115, respectively.

Mass spectrometric analysis and iTRAQ data analysis

LC–MS/MS analyses were performed on an LTQ-OrbitrapXL (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a nano-ESI source and coupled to a Paradigm MG2 pump (Michrom Bioresources, Auburn, CA, USA) and an autosampler (HTC PAL, CTC Analytics, Zwingen, Switzerland). Peptide mixtures were separated on a MagicC18AQ column (100 μm × 150 mm, 3.0 μm particle size, 300 Å, Michrom Bioresources) with a linear gradient from 100% A buffer (0.1% formic acid in 2% acetonitrile) to 5–30% B buffer (0.1% formic acid in 90% acetonitrile) for 80 min, 30–95% B buffer for 10 min, and 95% B buffer for 4 min and finally decreased to 5% B buffer, at a flow rate of 500 nl/min. Up to three high-energy C-trap dissociation (HCD) spectra were acquired in a data-dependent acquisition mode following each full scan (*m*/*z*, 350–1,500).

Protein identification and quantification for iTRAQ analysis were carried out using Proteome Discoverer (Thermo Fisher Scientific) against the NCBI non-redundant database with green plant taxonomy. Search parameters included iTRAQ labeling at N-terminus and lysine residues, cysteine modification by methyl methanethiosulfonate. The false discovery rate was confirmed to be less than 5%. Relative protein abundances were calculated using the ratio of the iTRAQ reporter ion in the MS/MS scan. Their mass spectrum patterns were later queried in a National Center for Biotechnology Information (NCBI) database search.

Results

The open field-grown transgenic poplars experienced a high temperature of above 36°C in August 2009 but typically experienced a high of around 30–35°C in July and August. They experienced a low of below −7°C in February 2009 but typically experienced a low of −6 to −7°C with approximately 5 cm snowfall between December and February. The dry season took place from November to January and the rainy season from June to July. The wind speed was rather high, with an average of 15 m/s from January to April, with a maximum wind gust of 30.3 m/s in February 2008. A 965 hPa typhoon struck the poplars with a maximum wind gust of 50 m/s on September 7, 2007. Earthquakes occurred numerous times in the field, twice with a magnitude of 4, 18 times with a magnitude of 3, 59 times with a magnitude of 2, and 155

Table 2 Composition of wall components in the leaves of the transgenic poplars

Composition	Content (w/w%)[a]					
	Fertile soil			Non-fertile soil		
	Wild type	trg300-1	trg300-2	Wild type	trg300-1	trg300-2
Cellulose	43.9	47.4	46.8	42.4	46.3	47.8
Hemicellulose	20.7	17.8	18.8	20.4	18.4	18.2
Fucosyl terminal	0.51	0.23	0.24	0.25	0.25	0.35
Arabinosyl terminal	1.48	1.29	1.39	1.00	1.31	1.42
Arabinosyl 5-linked	1.56	1.38	1.85	1.45	1.88	1.71
Xylosyl terminal	0.98	0.43	0.56	1.05	0.50	0.62
Xylosyl 2- or 4-linked	7.39	8.65	8.72	7.32	8.30	8.40
Glucosyl 4-linked	2.59	1.72	2.00	3.20	1.97	1.00
Glucosyl 4,6-linked	3.02	0.63	0.66	03.10	0.48	0.50
Galactosyl terminal	0.44	0.60	0.67	0.40	0.68	0.85
Galactosyl 2-linked	0.40	0.10	0.10	0.32	0.11	0.11
Galactosyl 4-linked	1.00	1.51	1.53	1.40	1.53	1.83
Galactosyl 3,6-linked	0.15	0.24	0.29	0.25	0.34	0.36
Mannosyl 4-linked	0.18	0.44	0.46	0.30	0.65	0.48

The first leaf was defined as the uppermost leaf below 5 mm in length from top, and the fourth leaves from the internode were sampled

[a] Content (w/w%) was calculated from the wall preparation. Each data point represents the mean of three independent plants of a single line, with individual values of fractions varying from the mean by <3%

times with a magnitude of 1 around Hitachi in Ibaraki. Every year, insects would attack the leaves of the poplars, and several plants would lose more than 25% of their leaves as a result. It should be noted that the transgenic poplars were grown under natural and not artificial conditions.

Figure 1 (upper box) shows whole leaves from a 28-cm tall seedling of one transgenic poplar and a 22-cm tall seedling of one wild-type poplar, grown in pots in an incubation room under low-light conditions (3000 lux). The leaves taken from the transgenic line poplar appear a darker green and have smaller leaf sizes but longer petioles compared to those of the wild-type plant. In the case of the field-grown transgenic poplars (Fig. 1), these leaves remained smaller and had more rounded teeth than those of the wild type, although the intensity and shade of green for both the transgenic and wild-type leaves was much more similar. Based on the quantitative analysis of the leaf phenotype from each plot (Table 1), the transgenic expression was confirmed to result in smaller leaf sizes even in the field, in which the smallest leaves were found in the transgenic poplars grown in the non-fertile field (forest-field soil). Although there was no increase in the leaf thickness between the different field-grown transgenic plants, the spongy parenchymal cells were more greatly expanded in the transgenic poplars grown in the field (Fig. 2). Leaf sizes of the transgenic and the wild-type poplars in the fertile soil (farming-field soil) were nearly identical, although the leaf thickness and chlorophyll content were higher in the transgenic plants. Nevertheless, the transgenic poplars exhibited shorter primary and secondary vein lengths than the wild-type poplars, particularly in the secondary veins at the base of the leaf. This is in agreement with the finding [19] that the greatest percent change in the relative spatial growth rate occurred at the base of the leaf and close to the outer margin in poplars. It should be noted that the level of transgenic expression was slightly decreased in the poplars grown in the fertile soil. A high level of xyloglucanase activity corresponded to a decrease in the leaf size and chlorophyll content in the non-fertile soil, whereas low-level activity corresponded to an increase in leaf thickness in the fertile soil. In the case of the wild-type poplars, their leaves in the fertile soil were larger in size, but thinner than those in the non-fertile soil. In the greenhouse (Fig. 1; Table 1), the leaves of the transgenic poplars remained smaller and thicker with longer petioles and they contained a slightly higher level of chlorophyll than those of the wild type.

Xyloglucanase overexpression resulted in a decrease in the amount of xyloglucan in the leaves of the transgenic poplars. This was confirmed by methylation analysis of the hemicellulose fraction of the leaves, in which the proportion of 4,6-linked glucose on the xyloglucan backbone for the transgenic leaves was approximately 15.5–16.1% from the non-fertile soil and 20.9–21.8% from the fertile soil of the corresponding wild-type values (Table 2). A decrease in xyloglucan was also observed by immunostaining (Fig. 3) using a mouse monoclonal antibody as CCRC-M1 against an α-L-Fuc (1 $\rightarrow$ 2)-β-Gal epitope. The staining intensity in the wild type was uniform in the young leaves from the top to the bottom of each leaf but revealed a high density in the veins of the old leaves, exhibiting a high level of xyloglucan content because of the high density of cells which are composed of vascular bundles, xylem, and parenchymal cells. A decrease in xyloglucan could occur on the entire area of the leaf but not in a specific tissue type in the transgenic plants. This is because xyloglucan had been constitutively degraded in all tissues and organs of the poplars via a 35S promoter for a period of 4 years.

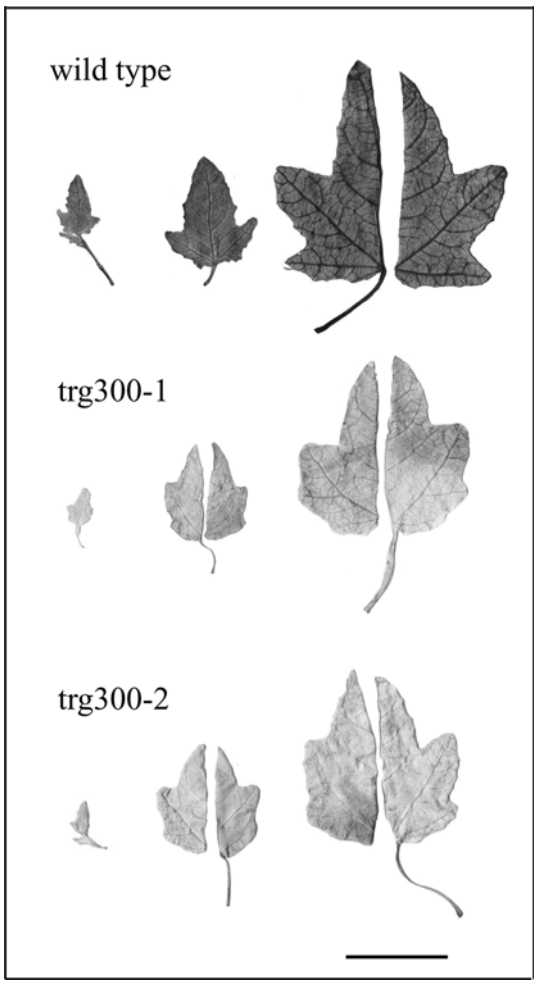

Fig. 3 Immunostaining of xyloglucan by CCRC-M1 in the leaves of fertile field-grown poplars. *Bar* 3 cm

Discussion

Under the strong sunlight in the field, smaller leaves, but not sun leaves, were produced by the transgenic poplars, whereas they produced smaller and thicker sun leaf-like leaves under the low-intensity artificial light in the incubation room. Although a longer petiole may be formed by an increase in xyloglucan-degrading activity in both poplar [4] and Arabidopsis [20], such a phenotype has apparently been cancelled in the field-grown poplar (Fig. 1 and Table 1). Leaf teeth and area extensibility could be still controlled in the field by a decrease in xyloglucan in the walls of the leaves. There are many genes that have been reported as having leaf length and width [21] and leaf tooth formation [22] qualities, most of which have been found in Arabidopsis. To date, neither of the transgenic model plants has been tested in the open field and a decrease in xyloglucan did not correspond to its leaf phenotypes. Although the transgenic plants have always been studied under artificial rather than natural conditions, we must make an effort to learn more about their response to an open field environment.

Table 3 List of differentially expressed proteins in the leaves of transgenic poplars

Annotation	gi number	Matched peptide no.	Sequence coverage (%)	Functional classification	Average ratio Transgenic/ Wild type
(Ratio > 1.3)					
Small rab2-related GTPase [*Populus trichocarpa*]	222854715	3	7.63	Membrane transporter	2.21
Heat shock cognate 70 kDa protein 2 (Hsc70.2) [*Arabidopsis thaliana*]	12644165	1	9.29	Stress related	1.41
Vitamin-b12 independent methionine synthase, 5-methyltetrahydropteroyltriglutamate-homocysteine [*Populus trichocarpa*]	224104961	2	6.23	Methionine related	1.37
Beta 1 tubulin [*Zea mays*]	295851	10	43.12	Cytoskeleton	1.32
(Ratio < 0.7)					
Caffeoyl-CoA-*O*-methyltransferase [*Acacia auriculiformis* × *Acacia mangium*]	161701632	1	7.69	Lignin synthesis	0.69
Putative methionine synthase [*Arabidopsis thaliana*]	164520572	2	5.19	Methionine related	0.68
Superoxide dismutase [Cu–Zn] (EC 1.15.1.1) [*Populus trichocarpa*]	118484653	7	23.09	Stress related	0.67
Putative cysteine-rich repeat secretory protein 20 precursor [*Arabidopsis thaliana*]	158564065	1	7.94	Stress related	0.67
Caffeoyl-CoA-*O*-methyltransferase [*Bambusa oldhamii*]	126723796	1	6.69	Lignin synthesis	0.66
Phenylalanine ammonia lyase [*Nicotiana tabacum*]	164454787	1	13.43	Lignin synthesis	0.65
Malate dehydrogenase 2, mitochondrial precursor (mNAD-MDH 2) [*Arabidopsis thaliana*]	75311246	5	8.76	Mitochondria related	0.65
UDP-glucuronate decarboxylase [*Populus trichocarpa*]	118485318	2	9.79	Polysaccharide synthesis	0.65
Ran binding protein [*Nicotiana benthamiana*]	118488201	2	5.37	Stress related	0.65
Annexin [*Populus trichocarpa*]	118487795	1	9.22	Stress related	0.61
40S ribosomal protein S21 [*Populus trichocarpa*]	118484720	5	15.71	Others	0.61
Tubulin [*Rosa hybrid cultivar*]	164457733	2	8.90	Cytoskeleton	0.6
Adenine phosphoribosyltransferase [*Physcomitrella patens* subsp. *patens*]	162679359	4	8.89	Nucleoside synthesis	0.57
Cytochrome *b*5 [*Oryza sativa*]	162684231	3	12.65	Sugar transport related	0.54
Membrane-associated progesterone binding protein 3 [*Arabidopsis lyrata* subsp. *lyrata*]	162680944	2	9.41	Others	0.52
Copper/zinc-superoxide dismutase [*Litchi chinensis*]	164654158	3	8.32	Stress related	0.44

Comparative proteomic data revealed 20 proteins in the leaves that were differentially expressed as either upregulated or downregulated proteins in the transgenic poplars with a greater than ± 30% change, as listed in Table 3. There was no significant difference between the proteins of the two transgenic poplar lines grown in either the fertile or non-fertile fields. The transgenic poplars expressed markedly high levels of Rab2-related GTPase, which could be membrane transporter related but may be involved in the removal of apoptotic cells [23]. However, the amounts of lignin synthesis-related and stress-related proteins were greatly decreased. This is in agreement with the finding that the transgenic poplars have shorter veins that are composed of the water-conducting vascular bundles required for lignin [24]. Therefore, the more rounded tooth of the transgenic poplars leaves (Fig. 1) may be caused by the prevention of vein extension. It is likely that xyloglucan is related to lignin deposition in the veins during leaf development. The walls of leaf cells could then alter the leaf morphology as a result of the decreased levels of cross-linked cellulose microfibrils caused by the absence of xyloglucan. In the case of mangium [25], overexpression of xyloglucanase accelerates the heteroblastic development and, in the case of sengon [26], a decrease in xyloglucan disturbs the biological clock of the plant by altering the closing movements of the leaves.

The leaves were obtained in July 2010, when the poplars had received a sufficient amount of rain but had experienced strong insect attacks at around 30°C as well as moderate levels of earthquakes, although stress-related proteins were downregulated in the transgenic plants (Table 3). Stress-related proteins, such as superoxide dismutase and copper/zinc-superoxide dismutase, were upregulated in the wild-type plants but not in the transgenic plants. Nevertheless, the leaves of the transgenic plants had been triggered by light stress to form sun leaf-like leaves [27]. It seems likely that the leaves of the transgenic poplars came to relax the edges of their tooth rather than extend their veins as a result of the loosening of the xyloglucan cellulose networks in the leaves.

Acknowledgments We thank Ms. Hikari Ishikawa and Ms. Ruttikan Prayong for their assistance with leaf size determination. This work was supported by JSPS KAKENHI (Grant-in-Aid for Scientific Research [A]19208016). This paper is also a part of the outcome of the JSPS Global COE Program (E-04): In Search of Sustainable Humanosphere in Asia and Africa.

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