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PRODUCERS, BIOLOGY, SELECTION, GENETIC ENGINEERING

Expression of Xyloglucanase *sp-Xeg* Gene from *Penicillium canescens* Accelerates Growth and Rooting in Transgenic Aspen Plants

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Abstract—The properties of transgenic aspen (*Populus tremula*) clones carrying the recombinant gene of xyloglucanase *sp-Xeg* from *Penicillium canescens* have been analyzed. Complex modifications were revealed both in the composition of the wood and in the plant phenotype. Biometric analysis showed that shoot dimensions increased by 24.8%, 25% and 26% in the *PtXIV-Xeg1a*, *PtXVXeg1a* and *PtXVXeg1b* lines, respectively. The number of internodes in some transgenic clones also increased. Modifications in rhizogenesis have been shown for the first time in the plants with the recombinant gene of xyloglucanase: *in vitro* rooting efficiency exceeded the control value in 13 out of 25 lines. Maximum rooting efficiency was observed in the *PtXVXeg1a* line (3.2-fold higher than in the control). A reliable increase in the root system mass (by 20% to 52%) under greenhouse conditions was observed for 8 out of 25 clones. A lower pentosan content in the wood was shown for all lines. The data on xyloglucanase activity and pentosan content generally correlated with phenotypic modifications.

Keywords: xyloglucanase activity, rooting efficiency, pentosans, *Populus tremula*

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Plant cell growth is accompanied by cell wall expansion as a result of intracellular pressure. Xyloglucan is a hemicellulose polysaccharide in the plant cell wall structure. It is responsible for the crosslinks between adjacent cellulose microfibrils and, hence, the formation of a stable framework [1]. During plant cell growth, microfibrils are separated by the enzymes that cleave xyloglucans and weaken the crosslinks between the microfibrils [2]. One such enzyme is xyloglucanase from the carbohydrase group; it breaks the crosslink between cellulose microfibrils by hydrolyzing xyloglucans.

At present, overexpression of carbohydrases that cleave xyloglucan is considered as a promising method of phenotype modification and woody plant productivity enhancement. Park et al. [3] observed stem elongation and altered leaf color in the white poplar (*Populus alba*) transformed by the *AaXEG2* gene of xyloglucanase from *Aspergillus aculeatus* compared to the control. The aspen (*Populus tremula*) plants with the *cel1* gene of endoglucanase from *Arabidopsis thaliana* were characterized by significant phenotypic modifications, such as increases in plant height, leaf size, stem diameter, and cellulose and hemicellulose content as compared to the nontransgenic control [4]. Aspen plants with the recombinant *sp-Xeg* gene of xyloglucanase also showed changes in leaf morphology: a longer leaf stalk and shorter midrib [5]. *Paraserianthes falcataria* was genetically modified by the *pAaXEG* gene of xyloglucanase [6]. The resultant transgenic plants had longer internodes compared to the plants of the control line. Such results were also obtained for overexpression of xyloglucanase in poplar plants [3].

The cleavage of cell wall xyloglucans probably affects the whole spectrum of biochemical reactions and, in addition to the aforementioned increase in organ size, may influence other plant characteristics. This supposition is confirmed by studies with *Arabidopsis thaliana*, when an increase in xyloglucan hydrolysis intensity led to changes in the length of the leaf stalk [7], anther filaments, and midrib length, as well as in the trichome shape [8].

Abbreviations: OD—optical density, RT-PCR—PCR with reverse transcription, bp—base pair, PCR—polymerase chain reaction; dNTP—deoxynucleoside triphosphate(s); *Pt*—untransformed plants; WPM (woody plant medium)—rooting medium for woody plants.

It is known that the overexpression of recombinant genes is often accompanied by a pleiotropic effect; the influence of a single gene may lead to changes not in a single characteristic but in the whole phenotype and ontogenesis in general [3–5, 9, 10]. Thereupon, the correct assessment of the properties of new transgenic plants calls for a detailed analysis of complex parameters (growth rate, leaf morphology, rhizogenesis efficiency, root biomass, wood composition, etc.).

The goal of this work was to investigate various phenotypic and biochemical properties of transgenic aspen plants with the recombinant *sp-Xeg* gene of xyloglucanase from the fungus *Penicillium canescens*. These include leaf morphology, the possibility of obtaining both highly productive and brachytic dwarf clones, pentosan content and its relation to plant growth.

EXPERIMENTAL CONDITIONS

Transgenic aspen plants. The transgenic aspen lines used in the work carried the recombinant *sp-Xeg* gene of xyloglucanase from the fungus *P. canescens* under the transcription control of the 35S promoter and the nopaline synthase (NOS) terminator that had been constructed previously at the Branch of the Institute of Bioorganic Chemistry, Russian Academy of Sciences [5]. The *sp-Xeg* gene codes for the chimeric xyloglucanase XegA with the signal peptide of white poplar cellulase [11].

RT-PCR analysis. Transcription of the chimeric *sp-Xeg* gene was analyzed by RT-PCR. Total plant RNA was extracted from plant material *in vitro* using TRIzol® reagent (Invitrogen, United Sates) by the commercial method (http://www.invitrogen.com). Genomic DNA was removed from the samples using DNAse I (Fermentas, United States) according to the manufacturer's protocol. cDNA was synthesized using M-MuLV reverse transcriptase (SibEnzyme, Russia) with the oligo-d$(T)_{15}$ primer (Sintol, Russia) at 37°C for 75 min. The mixture was heated to 70°C for inactivation of the reverse transcriptase. The reaction mixture was diluted fivefold with Milli-Q Water and introduced by 2 μL as a matrix during PCR with the primers Xeg-up (GAAATGGCTAATGCCACTACATT) and Xeg-low (GATTTAGGCAACATCGGCAG) (Evrogen, Russia). Amplification was carried out under the following conditions: the reaction mixture contained 16 mM of $(NH_4)_2SO_4$ (AppliChem, Germany), 200 μg/mL of bovine serum albumin (SibEnzyme), 200 μmole of each dNTP, 0.8 μmole of each oligonucleotide, 0.15 U/μL of *Taq* polymerase, and 1–5 ng/μL of genomic DNA. The amplification mode was as follows: denaturing at 96°C (hot start), 3 min; denaturing at 95°, 45 s; annealing at 62°, 45 s; elongation for 1 min at 72°; completion for 5 min at 72°; 30 cycles. The reactions were performed in thin-walled 200-μL Eppendorf test tubes in a MJ MiniTM Gradient Thermal Cycler (BIO-RAD, United States). RNA contamination with genomic DNA residues was controlled by PCR with RNA preparations of each clone without treatment with reverse transcriptase.

Analysis of xyloglucanase activity. Plant extracts were obtained from 4 individual aspen plants of each genotype (4 leaves of each plant, 1 to 1.5 g) cultivated for 1 month under greenhouse conditions. The leaves were ground in porcelain mortars in the presence of quartz sand in Tris-HCl buffer (pH 7.2, Panreac, Spain) at 4° till obtaining a homogenous suspension. The extracts were centrifuged at 4400 g. The supernatant was used for the Bradford protein assay [12] and for the determination of xyloglucanase activity. The endo-1,4-β-glucanase activity of transformed aspen plant extracts was determined by spectrophotometry with soluble tamarisk xyloglucan substrate stained with Remazolbrilliant Blue (Megazyme, Australia). The enzyme activity was assayed according to the manufacturer's recommendations (http://secure.Megazyme.com). Readings were taken at 590 nm; 0.25 mL of extraction buffer was added to the control sample instead of the plant extract. The enzyme activity was calculated using relative units; it was measured at OD_{590} in 1 mL of nontransformed plant (*Pt*) extract and recalculated per 1 mg of protein.

Analysis of pentosan content. The specific pentosan content in the wood was assessed by the modified Tollens method [13] through the conversion of pentosans into furfural by means of distillation in the presence of HCl. A 0.1-g portion of air-dried sawdust was prepared from peeled plant stems under the age of 4 months. The OD value of the distillate was measured with a two-beam spectrophotometer at 277 nm. The wood dryness factor (K_{dry}) was determined by the formulas:

$$W = \frac{m_1 - m_2}{m_1 - m}, \quad K_{dry} = \frac{100 - W}{100},$$

where W is the relative wood moisture content; m is the mass of an empty weighing bottle, g; m_1 is the mass of a weighing bottle with a sample before drying, g; and m_2 is the mass of a weighing bottle with a sample after drying, g. The dry matter content of pentosans was calculated by the formula:

$$A = \frac{Dn}{m} K_{dry},$$

where A is the pentosan content in an air-dried sample, %; D is the mean OD value of furfural solution obtained by distillation; n is the coefficient of furfural percentage recalculation per pentosans (for hardwoods, $n = 2.434$); and m is the mass of a sawdust sample, g. For descriptive reasons, the percentage of pentosans was converted into absolute values: mg/g dry mass.

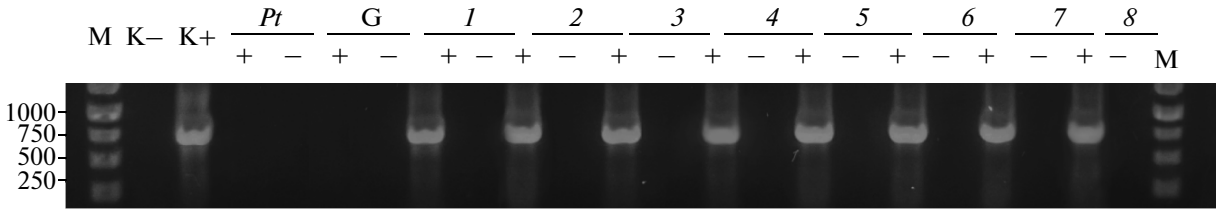

Fig. 1. RT-PCR analysis of the *sp-Xeg* gene expression in transgenic aspen plants: RNA preparations after treatment with reverse transcriptase (+); without treatment with reverse transcriptase (–); plasmid pBI-Xeg (K^+); water (K^-); nontransgenic control (*Pt*); transgenic control *PtIGUS5a* (G); transgenic lines (*1–8*): *PtXIVXegla* (*1*), *PtXVXegla* (*2*), *PtXVXeglb* (*3*), *PtXVXeg1c* (*4*), *PtXVXeg2b* (*5*), *PtXVXeg4c* (*6*), *PtXVXeg5c* (*7*), *PtXVIXeg1b* (*8*); molecular mass marker (SibEnzyme) (M).

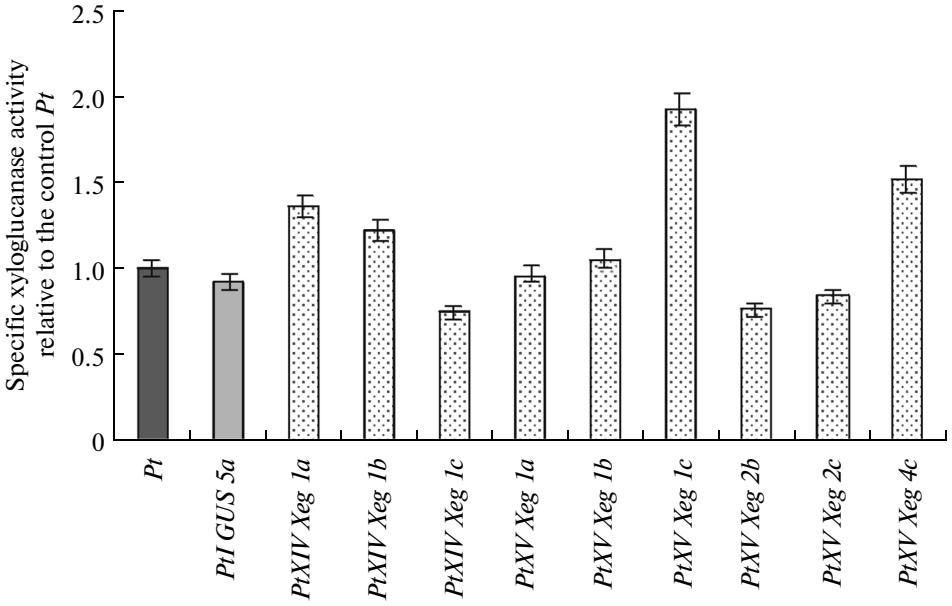

Fig. 2. Xyloglucanase activity in transgenic lines and control plants (*Pt*, *PtIGUS5a*) of aspen grown under greenhouse conditions.

Analysis of plant biometrics parameters. Microclonal reproduction of 40–50 plants of each of the 25 clones, the nontransgenic control *Pt*, and the transgenic control *PtIGUS5a* (the plant carrying the *GUS* reporter gene) was performed to investigate the influence of recombinant *sp-Xeg* gene expression on the phenotype of transgenic aspen plants. Rooting efficiency was measured under *in vitro* conditions on the sixth day after replanting to the woody plant medium (WPM) with the addition of sucrose (30 g/L) and agar-agar (9 g/L). The length of each root was measured in all plants. Rooting efficiency (Y) was calculated by the formula [14]:

$$Y = NL/100,$$

where N is the average number of roots per plant and L is the average root length of a plant, mm.

Four-month-old plants with a closed root system were obtained under greenhouse conditions (Artificial Climate Station Biotron, the Branch of the Institute of Bioorganic Chemistry). The phenotypic changes in transgenic plants were assessed by measuring the following five parameters: plant height, the number of internodes, root system mass, and the length of leaf stalk and midrib of mid-shoot leaves. The biometric parameters were analyzed in 40 plants of each genotype. All data were obtained from three independent experiments.

The data were processed by the mathematical methods of statistics [15] using the Dunkan's Multiple Range Test by ANOVA-1 (the method of single-factor analysis of variance) and Statistica 7.0.

RESULTS AND DISCUSSION

RT-PCR analysis of transcription of the recombinant *sp-Xeg* gene in transgenic aspen plants. Twenty-five transgenic lines [5] carrying the *sp-Xeg* gene of recombinant xyloglucanase from *Penicilium canescens* were obtained as a result of agrobacterial transformation of aspen of the *Pt* genotype. The expression of *sp-Xeg* in the tissues of greenhouse plants was confirmed by RT-PCR of total RNA preparations of these

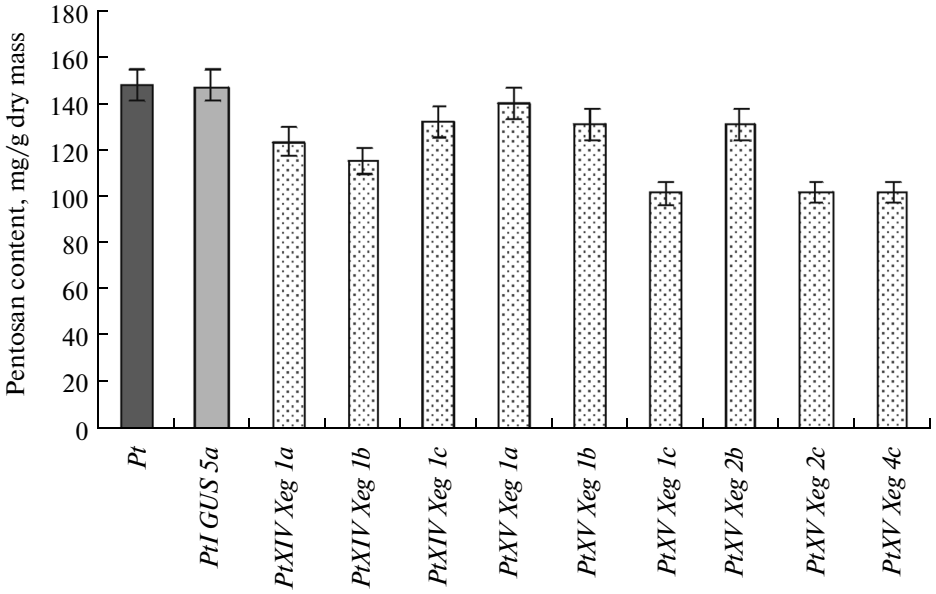

Fig. 3. Specific pentosan content in the wood of transgenic lines and control plants (*Pt*, *PtIGUS5a*) of aspen.

transgenic plants. The preparations of all 25 clones were analyzed. Nontransgenic aspen plants of genotype *Pt* and transgenic line *PtIGUS5a* carrying the *GUS* reporter gene were used as a control. The amplification product of expected size (762 bp) was found in all lines with the integrated xyloglucanase gene taken for the analysis, which confirms the presence of transcripts of the recombinant *sp-Xeg* gene (Fig. 1).

Analysis of xyloglucanase activity in transgenic aspen plants. Xyloglucanase activity was analyzed in plant extracts from 25 transformed and 2 control lines grown under greenhouse conditions. Figure 2 shows the results of analysis of xyloglucanase activity in the plant extracts from 11 clones grown in a greenhouse. An increase in xyloglucnase activity relative to the *Pt* control was observed in the four genotypes: *PtXVXeglc* (by 92%); *PtXVXeg4c* (by 51%); *PtXIVXegla* (by 35%); and *PtXIVXeglb* (by 21%). The enzyme activity in the other two transgenic lines not presented in Fig. 2 was comparable with that in the control.

Analysis of specific pentosan content. Pentosans are the main sugars forming hemicellulose. The expected effect of overexpression of recombinant xyloglucanase is a reduction of the specific pentosan content in the cell walls of transgenic plants. The pecific pentosan content was 148 mg/g dry mass in the control lines; it varied from 100 to 140 mg/g dry mass in all of the transgenic clones under study. Figure 3 shows the data on pentosan content in 11 lines of aspen plants. The maximum decrease in pentosan content was recorded in the lines *PtXVXeg1c, PtXVXeg2c*, and *PtXVXeg4c*: up to 100.1, 102.1, and 102.3 mg/g dry mass, respectively.

Analysis of biometric parameters. The biometric analysis of plants under greenhouse conditions showed a change in the height of transgenic plants compared to the nontransgenic control genotype (*Pt*). A considerable increase in shoot height (by 23–26%) was noted for the lines *PtXIVXeg1a, PtXVXeg1a*, and *PtXVXeg1b*; however, the height of one of the isolated lines, *PtXVXeg1c*, was below the control by approximately 14% (Fig. 4). An increased number of internodes was noted in three transgenic lines (Fig. 5). The maximum (18%) difference from the control (*Pt*) was observed in plants of the clone *PtXIVXeg1a*; the minimum difference was observed in plants of the clone *PtXVXeg1c* (by 24% below the control).

The previous comparative analysis of leaf morphology in transgenic aspen clones showed an increase in the length of leaf stalk and a decrease in the length of midrib [5]. Figure 6 presents the typical laminas of the transgenic plant *PtXIVXeg1a* and the control genotype *Pt*. In 20 out of 25 lines, the leaf stalk to midrib length ratio was reliably higher than in the control (data not shown).

We analyzed the efficiency of rhizogenesis *in vitro* in transgenic and control aspen lines (Table 1 shows the data on 14 experimental and 2 control lines). The rooting efficiency *in vitro* in 13 out of 25 transgenic lines (not all of them are presented in Table 2) was higher than in the control. In the clone *PtXVXeg1a*, it exceeded the control value 3.2-fold (see Table 1).

Root biomass was reliably higher under greenhouse conditions compared to the control in 8 out of 25 clones. However, the root biomass of some plants (e.g., the clone *PtXVXeg1c*) proved to be lower by 40% than in the control. Simultaneous enhancement of the rooting efficiency *in vitro* and the root system biomass compared to nontransgenic plants was noted for the

Fig. 4. Plants of the control and transgenic aspen clones (from left to right): control *Pt*; clone with enhanced growth rate (by 25%) *PtXVXeg1a*; the clone with reduced growth rate (by 14%) *PtXVXeg1c*. Figure shows the typical representatives of each group of plants.

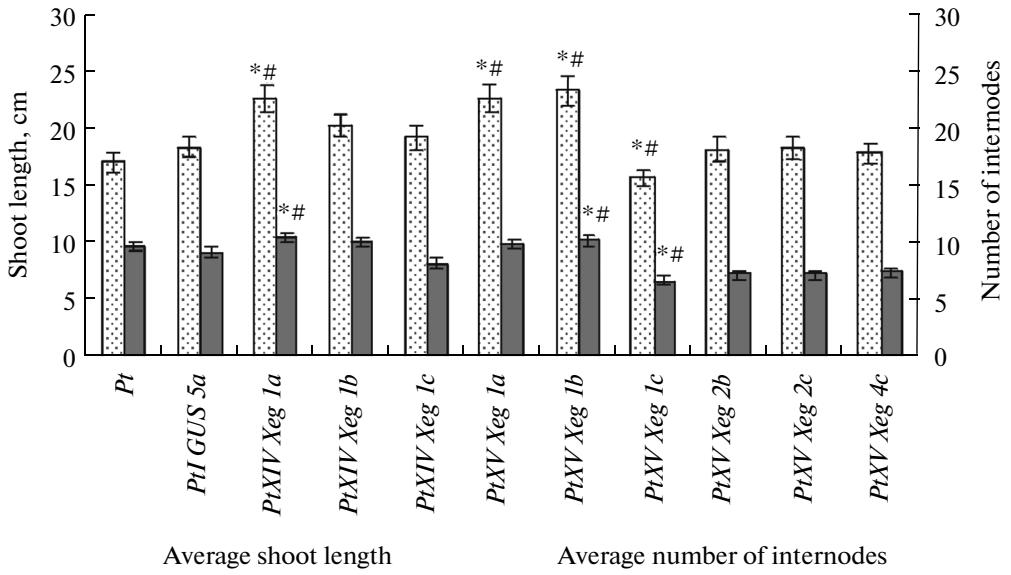

Fig. 5. Comparative analysis of the height and number of internodes in transgenic and control plant clones (*, statistically reliable difference from *Pt*, $p < 0.05$; #, statistically reliable difference from *PtIGUS5a*, $p < 0.05$).

clones *PtXVXegla*, *PtXVXeglb, PtXVXeg2c, PtXVXeg3b, PtXVXeg4b, PtXVXeg5b*, and *PtXVXeg5c*.

Based on the results of biometric analysis, the lines *PtXIVXeg1a, PtXVXeg1a* and *PtXVXeg1b* are of interest for further experiments in the open ground: their biometric parameters generally exceed the control values (Table 2).

It has already been mentioned that the overexpression of carbohydrases in woody plants is considered in some works as a promising technique for the creation

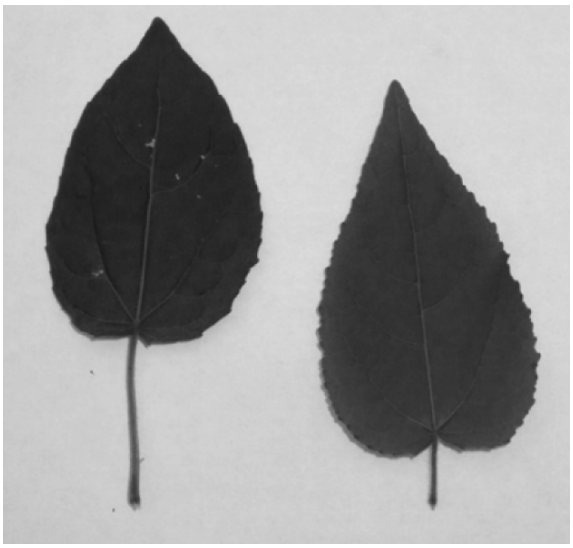

Fig. 6. Leaves of transgenic line *PtXVXeg1a* (on the left) and of the control (on the right).

of highly productive plants. A higher rate of plant growth and an increased internodal length and stem diameter were observed in the respective transgenic plants. The alteration of morphological characters was accompanied by the changes in the chemical composition of wood in the plants with overexperssion of xyloglucanase genes. White poplar also showed a 24% reduction of hemicellulose content and a multifold enhancement of xyloglucanase activity in stems [3].

Table 1. Rhizogenesis of transgenic aspen plants and plants of the control group

Clone	Rooting efficiency in vitro	Root mass, g dry mass
Pt	1.74 ± 0.03^{a}	3.89 ± 0.09^{a}
Pt I GUS 5a	1.45 ± 0.06^{a}	2.99 ± 0.11^{a}
Mean values of the control	1.6 ± 0.05^{a}	3.44 ± 0.10^{a}
Pt XIV Xeg 1a	2.38 ± 0.06^{b}	3.00 ± 0.09^{a}
Pt XIV Xeg 1b	3.03 ± 0.07^{b}	3.32 ± 0.10^{a}
Pt XIV Xeg 1c	3.09 ± 0.08^{b}	3.50 ± 0.11^{a}
Pt XV Xeg 1a	5.16 ± 0.1^{c}	4.40 ± 0.14^{a}
Pt XV Xeg 1b	2.57 ± 0.04^{b}	6.00 ± 0.18^{b}
Pt XV Xeg 1c	1.79 ± 0.08^{a}	1.44 ± 0.10^{c}
Pt XV Xeg 2a	1.38 ± 0.04^{a}	2.80 ± 0.08^{a}
Pt XV Xeg 2c	4.47 ± 0.05^{c}	4.03 ± 0.12^{a}
Pt XV Xeg 3a	3.83 ± 0.03^{b}	3.60 ± 0.11^{a}
Pt XV Xeg 3b	2.66 ± 0.06^{b}	3.93 ± 0.12^{a}
Pt XV Xeg 4b	1.86 ± 0.06^{a}	7.26 ± 0.22^{b}
Pt XV Xeg 4c	1.14 ± 0.05^{a}	3.90 ± 0.12^{a}
Pt XV Xeg 5b	2.87 ± 0.04^{b}	6.36 ± 0.19^{b}
Pt XV Xeg 5c	2.83 ± 0.08^{b}	6.14 ± 0.18^{b}

Different letters indicate groups with values reliably different from the control, according to the Dunkan's Multiple Range Test by ANOVA-1 at $p \le 0.05$ (^{a}, higher than the control; ^{b}, at level of the control; ^{c}, lower than the control).

The results of analysis of the specific pentosan content (which naturally drops with the increasing xyloglucanase activity) in the wood of our transgenic clones are in agreement with the literature data [3]. Many of the analyzed lines were characterized by lower pentosan content. The analysis of protein extracts from greenhouse plants showed that xyloglucanase activity in the *PtXVXeg1c* line was 1.9-fold higher than in the control plant; at the same time, the pentosan content in the wood of this clone was minimal: 102 mg/g, i.e., 13% lower than in the control (148 mg/g of dry mass). Such a dependence was also noted for other clones: e.g., in the clone *PtXIVXeg1a*, xyloglucanase activity was 1.5-fold higher and the pentosan content was 124 mg/g dry mass, i.e., 16% lower than in the control.

The height of the obtained transgenic plant clones *PtXIVXeg1a, PtXVXeg1a* and *PtXVXeg1b* is comparable with the data of Shani [4] and Park [3]. The correlation between the xyloglucanase activity of greenhouse plants and the biometric parameters of their height are of particular interest, because xyloglucanases are involved in plant growth [6]. Clones with high enzyme activities (*PtXVXeg1c* and *PtXVXeg4c*) (see Fig. 3) are not higher or even lower than the control plants (see Fig. 5). Indeed, the constitutive overexpression of foreign genes is sometimes accompanied by abnormalities in plant growth and the phenomenon of dwarfism [16]. At the same time, the *PtXVXeg1a* and *PtXVXeg1b* genotypes, where the recombinant enzyme activity is slightly higher than the control values (see Fig. 3), are reliably higher than in nontransgenic plants (see Fig. 5). Thus, the findings do not suggest any direct dependence between the level of recombinant enzyme activity and the degree of phenotypic changes in transgenic plants. Such a phenomenon was observed in some other studies; e.g., one work [17] showed the absence of a direct relationship between the level of *4CL* gene expression and a change in the biomass content in poplar plants. We noted a significant increase in plant height that is associated with a change in the number of internodes (see Fig. 5). This peculiarity is typical of most of the obtained transgenic clones; such observations have not been reported previously.

One of the unexpected effects of the *sp-Xeg* gene was the modification of root formation. An enhanced efficiency of rhizogenesis *in vitro* for some genotypes was expressed by an increase in root system biomass under greenhouse conditions. The positive effect of xyloglucanase gene overexpression on formation of the root system has not been noted previously for

Table 2. Basic characteristics of the most promising clones

Parameter \ Line	Control, *Pt*	*PtXIV Xeg1a*	*PtXVXeg1a*	*PtX VXeg1b*
Height, mm	171.20	227.60	228.00	233.30
Rooting efficiency	2.14	2.38	5.16	2.57
Xyloglucanase activity relative to the control	1.00	1.35	1.02	1.06
Pentosan content	148.32	124.52	140.45	131.88

plants of the genus *Populus*; it was shown only to influence the formation of root suckers [10]. The observed effect is probably associated with the influence of xyloglucanase on the properties of cell walls and the capacity of root cells for elongation and/or signal transduction via auxin receptors [18]. Growth rate enhancement must be accompanied by an increase in the amount of substances entering the plants [19]. Clones with an enhanced growth rate (*PtXIVXeg1a, PtXVXeg1a* and *PtXVXeg1b*) demonstrated higher rooting efficiency; at the same time, a reduction of rhizogenesis was observed in the clone *PtXVXeg1c* with reduced growth (see Table 1). An enhancement of rhizogenesis efficiency and root system biomass may be accompanied by improvement of the characteristics of above-ground organs during further growth.

Morphological changes also affected the leafstalk and midrib length: a reliable increase in the leafstalk to midrib length ratio was recorded in 20 lines out of 25 transgenic plants, which confirms the data obtained previously [5]. Similar results on the increase in leafstalk length [7] and decrease in midrib length [8] were obtained in the case of intensified xyloglucan cleavage in *Arabidopsis thaliana* plants, but for woody plants they have been shown for the first time in this work.

Thus, the three highly productive transgenic aspen lines *PtXIVXeg1a, PtXVXeg1a* and *PtXVXeg1b* were selected during greenhouse cultivation (see Table 2). The selected clones have a number of advantages over wild-type plants and other clones: an enhanced growth rate, higher rooting efficiency, and reduced content of hemicellulose. Further studies will provide new data on the biology of these clones during open ground cultivation.

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