Transgenic aspens (Populus tremula) with the xyloglucanase gene from Penicillium canescens keep faster growth under semi-natural conditions

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Abstract

Background Recombinant carbohydrases genes are used to produce transgenic woody plants with improved phenotypic traits. However, cultivation of such plants in open field is challenged by a number of problems. Therefore, additional research is needed to alleviate them. Results Results of successful cultivation of the transgenic aspens (Populus tremula) carrying the recombinant xyloglucanase gene (sp-Xeg) from Penicillium canescens in semi-natural conditions are reported in this paper for the first time. Change of carbohydrate composition of wood was observed in transgenic aspens carrying the sp-Xeg gene. The transformed transgenic line PtXVXeg1b demonstrated accelerated growth and increased content of cellulose in wood of trees growing in both greenhouse and outside in comparison with the control untransformed line Pt. The accelerated growth was observed also in the transgenic line PtXIVXeg1c. Thicker cell-wall and longer xylem fiber were also observed in both these transgenic lines. Undescribed earlier considerable reduction in the wood decomposition rate of the transgenic aspen stems was also revealed for the transformed transgenic lines. The decomposition rate was approximately twice as lower for the transgenic line PtXVXeg3b in comparison with the control untransformedline Pt. Conclusion Our results showed that the plants carrying the recombinant sp-Xeg gene do not demonstrate a decrease in growth parameters in semi-natural conditions. However, in some transgenic lines, a change in the carbohydrate composition of the wood, an increase in the cell wall thickness and a decrease in the rate of decomposition of wood are observed.

Background

Forests play a huge role in the economy as a source of timber and many types of raw materials. The logging is growing every year worldwide [1]. Hardwood trees are of particular interest due to a lower content of resins and lignins, making them a convenient source of raw materials for various industries [2–4]. Aspen (*Populus tremula)* is currently one of the most promising hardwood species, fast-growing, with a wide distribution range. Aspen wood is increasingly used in the pulp, paper, and viscose industries, and for container, oriented strand board, and bioethanol production [5]. However, with a current high rate of logging of hardwood species, especially aspen, a shortage of raw materials is expected. Therefore, it is necessary not only to increase the efficiency and rationality of the use of wood, but also to establish forest plantations with increased productivity based on the new and improved tree forms and varieties. Their creation by traditional breeding is a long and not very efficient process [6], while the genetic engineering approach is considered as one of the promising methods for obtaining trees with desired properties [7].

Increase in plant cell size is possible by enzymatic softening the cell wall followed by its expansion due to increased intracellular pressure [8]. Xyloglucan is involved in formation of a strong cellular framework, a plant cell wall hemicellulose polysaccharide that cross-links nearby cellulose microfibrils [9]. Accordingly, softening of the cell wall occurs through separation of microfibrils by enzymes that cleave xyloglucans and weaken the bond between microfibrils [10]. Xyloglucanase is one of these enzymes

belonging to the carbohydrase group, which hydrolyses xyloglucans, weakening the cross-linking of cellulose microfibrils [8].

Currently, the overexpression of carbohydrases splitting xyloglucan is considered as a promising way of modifying the tree phenotype, increasing the productivity of woody plants and changing the composition and properties of wood. Shani et al. [11] produced transgenic aspen trees (*Populus tremula)* with an endoglucanase gene *cel1* from *Arabidopsis thaliana,* which had significant phenotypic changes, such as increased plant height, leaf size, stem diameter and a higher percentage of cellulose and hemicellulose in comparison with the untransformed control under greenhouse conditions. However, the results of field trials were not reported. Park et al. [12] showed that in white poplar (*Populus alba)* transformed with the xyloglucanase gene *AaXEG2* from *Aspergillus aculeatus,* increased length of stems and discoloration of leaves were observed in comparison with the control in the climatic chambers. There was also an increase in cellulose content and a reduction in hemicellulose in transgenic trees [12]. However, the growth rate of the transgenic trees in the field was lower than that of wild-type control trees [13].

The analysis of the world experience has shown that there are successful results in using of recombinant carbohydrases and xyloglucanases to increase the growth rate and improve the quality of aspen wood (genus *Populus).*. However, not all issues in this area are completely solved, especially for trees grown in field conditions. Therefore, the aim of this work is to analyze aspen with altered properties of wood carrying the recombinant xyloglucanase *sp-Xeg* gene from *Penicillium canescens* under semi-natural conditions as a preliminary stage before the field trials.

This article reports successful tests of transgenic aspen trees carrying a recombinant xyloglucanase gene *sp-Xeg* and growing under semi-natural conditions. Effects of xyloglucanase gene incorporation on growth parameters, chemical wood composition and rate of wood decomposition are also presented and discussed.

Results

Expression of xyloglucanase

Western blotting confirmed the presence of a recombinant *XegA* protein of the appropriate size (25 kDa) in the obtained 25 independent transgenic lines of aspen carrying the xyloglucanase gene *sp-Xeg* [14, 15]. Fig. 1 shows the results of Western blotting for 10 of the 25 lines,in which the recombinant protein was detected stably in all replicates of the assay.

Growth rate

Earlier, we noted that in a greenhouse environment, some transgenic linesshowed an increase in the growth rate [15]. Transgenic and control plants of aspen were transferred from the greenhouse conditions to an open air and grown under semi-natural conditions up to the age of 18 months. For the analysis, six

lines were chosen that demonstrated the presence of a recombinant protein. Four of them had an increased plants height in greenhouse conditions after two months in comparison with the *Pt* control line by 24.6% for the *PtXIVXeg1a* line, 15.7% for *PtXIVXeg1b,* 26.6% for *PtXVXeg1b* and 15.3% for *PtXVXeg3b.* The other two lines (*PtXIVXeg1c* and *PtXVXeg5a)* were not different from the control [15]. It should also be mentioned that although a tendency in increased tree height was observed for most of the transgenic trees in semi-natural conditions, statistically significant increase in tree height was observed only for the *PtXVXeg1b* line (Table 1). In the greenhouse, after two months of vegetation, this line was taller than non-transgenic *Pt* control by 26.6%, in the open air after 6 months of vegetation by 25.4%, and after 18 months by 14.6% (Fig. 2).

Integration of the xyloglucanase gene had little effect on the leaf area of transgenic plants. Only two aspen lines showed a significant decrease in area - *PtXIVXeg1b* by 28% and *PtXVXeg3b* by 17% compared to the control plants (Table 2).

However, almost all transgenic lines changed the leaf shape - they significantly reduced the circularity. This was due to an increase in the length and decrease in the width of the leaf blade of transgenic plants. As a result, all the transgenic lines significantly changed the length-to-width ratio of the leaf by 14–23% compared to the control plants (Fig. 3). For all transgenic plants, there was a tendency to increase the number of leaves, but not statistically significant (Table 1).

Cellulose content in wood

The content of cellulose was measured in wood of all six studied transgenic lines. A significant increase in cellulose content was observed only in the *PtXIVXeg1c* and *PtXVXeg1b* lines (Table 1). The content of cellulose in other lines was on average at the control level. The reduction of cellulose content in transgeniclines was not detected. Thus, in terms of cellulose content, the *PtXIVXeg1c* line can be also considered as a prospective line in addition to the *PtXVXeg1b* line.

Carbohydrate composition of xylem

Xyloglucanase promotes cleavage of xyloglucan, thereby affecting the carbohydrate composition. A decrease in the content of pentosans (the main component of hemicellulose) was observed in all greenhouse plants [15]. In plants under semi-natural conditions, there was also a decrease in the content of pentosans in all transgenic lines in the youngest parts of the stem in comparison with control (Table 3). Less difference from control was observed in the older (18-month-old) parts of the stem, and four out of six lines demonstrated an increase in the content of pentosans. However, all changes were statistically insignificant.

The ratio of hemicellulose components was also affected in transgenic lines (Fig. 4). In the youngest parts of the plant, a significant decrease in the xylose content was observed in most of the lines,

accompanied by an increase in the content of glucose and sometimes arabinose (Fig. 4a). The content of galactose and mannose varied insignificantly. In the most mature wood, the proportions of xylose and glucose did not differ significantly from control, but the content of arabinose and mannose was slightly, but significantly increased (Fig. 4b). The relative content of rhamnose and fucose in aspen wood was relatively low: 1.5−2% of rhamnose, independently of the wood age, and 0.2−0.5% and 0.1−0.2% of fucose in the younger and older wood, respectively.

Libriform fiber analysis

The diameter and length of the libriform fiber measured in wood samples of six studied lines are presented in Table 4. A slight increase in the fiber diameter was observed in transgenic plants. The fiber length was significantly higher than in the control in two lines *PtXVXeg1b* and *PtXVXeg3b.* (Table 4).

Microscopy of xylem

Electron microscopy of xylem of 18-month-old aspen showed an increase of the cell wall thickness in prospective lines *PtXVXeg1b* (1.80 microns on average) and *PtXIVXeg1c* (1.47 microns on average) in the xylem cells of the first year of vegetation in comparison with non-transgenic control (1.16 microns) (Fig. 5).

Decomposition rate

Transgenic lines *PtXIVXeg1b* and *PtXVXeg3b* and control *Pt* were used in the decomposition experiment. These two transgenic lines were the most morphologically and biochemically similar to *Pt*. Analysis of the carbon dioxide emission during the decomposition of plant material showed that the stems of transgenic plants had a decomposition rate lower than in the control, especially for *PtXVXeg3b,* where it was about two times slower than in the control line *Pt* (Fig. 6). However, during root decomposition no significant difference in the carbon dioxide emission was detected between transgenic and control plants.

A significant decrease of nitrogen was observed in stems of transgenic plants in comparison with the control line, while no change for nitrogen was found in the roots. No change for carbon was found in both roots and stems of the transgenic lines in comparison with the control line (Table 5).

Discussion

The creation of highly productive woody plants with altered wood properties by increasing the cleavage of xyloglucan was considered in some studies as the most promising direction [12, 16]. However, successful results obtained in climatic chambers and greenhouse were not always confirmed in the field [13]. We tested our transgenic aspen lines with recombinant *sp-Xeg* gene also under semi-natural conditions: they had a closed root system and were grown in an open air during the entire vegetation

period. Measurements of their heights demonstrated that not all of the previously identified promising lines [15] have maintained a higher plant height. Convergence of phenotypic traits between transgenic and control lines was observed. *PtXVXeg1b* was the only line that maintained a high degree of productivity throughout the studies compared to the control. The increase in growth rate of the *PtXVXeg1b* line was relatively high in comparison with data published by other researchers [12]. Transgenic white poplars with xyloglucanase from *Aspergillus aculeatus* also tended to increase growth rates, but they were grown in climatic chambers [12]. When these plants were grown under greenhouse conditions, no difference in the growth rate was found [17]. Further field trials of these transgenic poplar plants showed that two transgenic white poplar lines trg300−1 and trg300−2 with overexpression of recombinant xyloglucanase from the fungus *Aspergillus aculeatus* showed a decrease in the growth rate and total biomass compared with non-transgenic control, while previously demonstrated an increase in productivity in climatic chambers. It was found that the deterioration in growth rates was due to a change in the transpiration process. In transgenic plants stomata dysfunction was observed [13]. The xyloglucanase *XegA,* which we used, is structurally identical to xyloglucanase from *Aspergillus aculeatus* (*AaXEG2)* at 70.46% based on the data from the NCBI database. Therefore, similar effects of the recombinant xyloglucanase *XegA* and xyloglucanase *AaXEG2* on plants were observed. The transgenic plants of the line *PtXVXeg1b* obtained by us mantained the tendency to increase the growth rate in all experimental conditions. It was observed that incorporation of hormonal control or transcription factor genes can affect the size of plant organs [18]. A similar effect was observed in aspens with xyloglucanase gene in our study. We observed the change in a leaf shape in all transgenic lines - the ratio of length to width increased, mainly due to a decrease in the leaf width (Table 3). The final size of plant organs, such as leaves, is controlled by two processes - cell division and cell expansion [19]. The size change can occur not in both, but only in the one dimension. For instance, the overexpression of the cytochrome P450 gene in *Arabidopsis* increased the length of the leaves without any change of their width [20]. Reducing leaf size is possibly associated with a decrease in the number and / or size of cells [21]. We found that a significant increase in the length of the wood fiber was observed in transgenic lines without any change in diameter (Table 4). Since xyloglucan is involved in changing the cell shape in growth and differentiation zones [22], the expression of the xyloglucanase gene could also alter the wall extensibility in the leaf cells, which changed the shape of the cells and subsequently the leaf shape. Moreover, the observed in our study tendency of increasing the number of leaves in transgenic plants may have a compensatory effect on the growth characteristics of these plants [15].

The main structural motif of xyloglucan in genus *Populus* is a repeating block of the XXXG type consisting of three glucose residues containing the xylose substituent (X) and one unsubstituted glucose residue (G) [23]. In the two-year-old wood of the control aspens, the content was quite similar to this indicator, both in absolute values and in the ratio of xylose, 3.6. However, in young areas the ratio was sharply reduced to 1.8, because xylose decreased and cellulose increased simultaneously at the same time. The incorporation of the xyloglucanase gene has altered the composition of the polysaccharide matrix, which primarily resulted in an increase in glucose and a decrease in xylose that caused defects in the formation of xylan. It was most significant in young plants, but was also observed in more mature

wood, although to a lesser extent (most significantly in the *PtXIVXeg1a* and *PtXIVXeg1b* lines). This suggests that the incorporation of the xyloglucanase gene promoted the formation of hemicellulose, which is characteristic of the earlier stage of plant development. In the more mature wood, the effect of the xyloglucanase gene was minimal - the content of the main components did not differ much from the control, although the share of the minor components - mannose and arabinose - increased slightly. The third component, galactose, practically did not change. However, we noted significant changes in the relative content of other hemicellulose polysaccharides, which may be due to an indirect effect on the biosynthesis of the secondary cell wall. Our data partially agree with the results of Baba et al. [24], according to which 10% decrease in polysaccharide matrix was noted in the poplar with xyloglucanase gene, as well as a slight decrease in xylose due to galactose and arabinose. Unfortunately, in this work the age of plants was not specified. A significant decrease in xylose was observed in all *Populus deltoides* lines with suppression of the glycosyltransferase gene by interfering RNA [25], where it occurred due to galactose and mannose, but the glucose did not change. The reverse effect was in *Populus euramericana* with interfering RNA on endoglucanase gene, where xylose increased substantially, and glucose was reduced, while other hemicellulose sugars - mannose, galactose, arabinose, rhamnose and fucose remained unchanged [26]. We found that the incorporation of the xyloglucanase gene in our transgenic aspens led to a decrease in the content of hemicellulose in young wood. In addition, the gene xyloglucanase has changed the composition of xyloglucan, making it more characteristic for an early age. We also showed that this effect is weakened with the age of the plant, and it is practically absent in the second year wood. It is possible that compensatory mechanisms for the formation of hemicellulose are amplified with age, and, in addition, it could be promoted by our plants being grown under semi-natural conditions.

It was demonstrated earlier that the cellulose formation can indirectly affect the binding of microfibrils to each other by xyloglucan filaments, and, therefore, the cleavage of which can probably have a stimulating effect on the cellulose biosynthesis [27]. Our plants had an increase in the cellulose content by 7.4% and 11.3% in the lines *PtXVXeg1b* and *PtXIVXeg1c,* respectively. Our cellulose content data are in consensus with the data on electron microscopy of xylem of transgenic plants, as well as with the data on libriform measurement. The length of the fibers has increased in some transgenic lines. This is due to the specific interaction of xyloglucanases with the cell walls resulted to the separation of the cellulose microfibrils that led to the cell size increase due to the increase of intracellular pressure [8]. An increase in the cellulose content may be associated with an increase in the thickness of the cell walls, especially if the gelatinous layer increases [28]. We noted that the thickness of the cell wall of xylem in transgenic plants exceeded the control values and averaged 1.63 microns, whereas in control plants it was 1.16 microns. This is comparable to the increase in cellulose content in these plants.

Changes in the wood composition can affect also biogeochemical processes in ecosystems. In our earlier studies, we measured the decomposition rate of aspen wood in lines with the xyloglucanase gene using the method of mass loss [29]. Significant differences in the decomposition rate between transgenic and control plants were found at the early stages in the transgenic roots, but not in stems. However, this method is not accurate enough, since it does not take into account the coefficient of microbial

conversion, which includes the increase in the biomass of microorganisms during decomposition [30]. For a more accurate determination of the decomposition rate in plants, it is recommended to measure the intensity of the CO_2 emission [31]. In our study we used this method in stems and roots, which are rarely used both in decomposition experiments. According to Zhang et al. [32], the overwhelming number of decomposition experiments were conducted with leaves or needles, and branches and roots are only occasionally used. Meanwhile, leaves and roots constitute a significant proportion of annual litter, and the built-in gene has an effect on the composition of the wood. Although our main experiment was conducted for two years, we used 6-month-old samples to study decomposition because they better represent the structure of litter in the natural conditions. According to Freschet et al. [33], 41% of the annual forest litter consists of leaves, 11% of the branches with up to 5 *mm* in diameter and 48% of the roots with up to 2 *mm* in diameter.

Measurement of the intensity of the CO_2 emission showed that for wood of stems of transgenic plants, a significant decrease in the rate of decomposition is characteristic. Such a change may be due to a decrease in the nitrogen content of these plants by 15% on average. A number of studies reported that the nitrogen content was directly related to the decomposition rate of plant residues [32, 34, 35]. The higher nitrogen content increased the rate of decomposition, and the lower content reduced it. Perhaps, for the same reason, differences in the emission of carbon dioxide during the decomposition of the roots of our transgenic plants were not detected. It is likely that an increase in the thickness of the cell wall can also affect the rate of wood decomposition [36]. Thus, based on the results of the aspen tests in semi-natural conditions, it can be concluded that the recombinant *sp-Xeg* gene has a complex effect on the plant organism. Recombinant gene influenced not only the growth parameters of transgenic plants, but also the content of cellulose, plant fiber, and decomposition rate of wood. Among all transgenic aspen lines with the *sp-Xeg* gene, *PtXVXeg1b* was identified as the most promising line, which under all test conditions maintained an increased growth rate, had a higher cellulose content and a thicker cell wall of xylem fibers.

Modification of plant properties through genetic transformation should always be assessed for its environmental impact. In particular, how transgenic aspen can affect forest ecosystems and soil. A computer simulation of virtual forest plantations consisted of the *PtXVXeg1b* clone and non-transgenic plants was carried out earlier using the EFIMOD simulation model that can predict carbon and nitrogen flows in forest ecosystems with strong feedback mechanism between soil and stand [37]. This model experiment showed that effect of growing transgenic aspens on carbon and nitrogen flows was not different from effect of non-transgenic plants in controls.

Conclusions

The representative panel of transgenic aspen lines with the constitutive expression of recombinant xyloglucanase gene *sp-Xeg* from *Penicillium canescens* was analyzed. It was proved that the expression of *sp-Xeg* recombinant xyloglucanase leads to a change of thickness of wood fiber and the plant growth. Electron microscopy showed an increase in cell wall thickness in transgenic lines. Libriform analysis also

showed an increase in the length and width of the vascular fiber in transgenic plants. An increase in wood fiber parameters is likely to affect growth. For the first time, it was shown that transgenic aspen plants with the gene of recombinant xyloglucanase of fungal origin under test conditions close to the field (semi-natural conditions) do not demonstrate growth reduction. In transgenic plants, *sp-Xeg* recombinant xyloglucanase alters the composition of carbohydrate-containing substances in wood. The change in the content of cellulose and hemicellulose is confirmed by the data obtained in the analysis of the xylem monomeric sugars composition. For the first time, an analysis of carbon dioxide emissions during the decomposition of plant material was carried out. It showed that the stems of transgenic plants had a decomposition rate lower than the control ones.

Methods

Transgenic aspens

Transgenic aspen lines with the introduced recombinant *sp-Xeg* xyloglucanase gene from the fungus *Penicillium canescens* under the transcriptional control of the 35S promoter and nopaline synthase terminator were analyzed. The *sp-Xeg* gene encodes a chimeric xyloglucanase *XegA* with a white poplar cellulase signal peptide [14]. The *in vitro*-derived trees were adapted to *in vivo* conditions in the climatic chambers for a month and were grown in greenhouses for a further month. In total, 25 transgenic lines, two control lines - non-transgenic wild-type line (*Pt*) and a transgenic line with the inserted gene *β*-glucuronidase (*PtlGus5a)* were studied, respectively. Each line (genotype) was represented by 50 plants (ramets). The plants were grown in individual plastic containers with a volume of 1 liter (with peat to perlite ratio of 3:1) and after two months of growth in the greenhouse moved to semi-natural conditions in an open air with additional watering and feeding. Semi-natural conditions are the cultivation of potted plants outside of the greenhouse [38]. Such growing conditions are close to the field and allow us to estimate the resistance of plants to various biotic and abiotic factors. Nontransgenic control aspens (natural variant) were randomized with aspens of transgenic lines and grown together alongside each other under the same conditions. Under these conditions the plants were grown up to the age of one and a half years with wintering in natural conditions.

After two months of growth in the greenhouse and before transfer to semi-natural conditions, the height and number of leaves were measured. After another 4 months of vegetation, growth was measured, samples were taken for carbohydrate composition measurement, and some of the plants were used to analyze the decomposition rate of the stem wood. The second part wintered and continued to grow for another year, and then, at the age of 18 months, the growth, content of cellulose and pentosans were measured, and samples were taken for carbohydrate composition, microscopy and libriform, and the annual results of the decomposition experiment were evaluated.

The presence of the recombinant gene and protein expression were confirmed in all 25 transgenic lines. Analyses of growth parameters, specific content of cellulose, pentosans, carbohydrate composition of xylem, measurement of libriform were performed on six transgenic lines *PtXIVXeg1a, PtXIVXeg1b,*

PtXIVXeg1c, PtXVXeg1b, PtXVXeg3b and *PtXVXeg5a* and the control line *Pt.* Electron microscopy was carried out on the plants of the *PtXIVXeg1c, PtXVXeg1b,* and *Pt* lines. The decomposition rate was measured in the plants of the *PtXIVXeg1b, PtXVXeg3b* and *Pt* lines.

Western blot analysis

Total protein extracts were obtained from the leaves of 6-month-old transgenic and control plants *ex vitro* by the addition of an extraction buffer [0.175 M Tris / HCl (pH 8.8), 5% SDS (w/v), 15% glycerol (v/v), 0.3 M mercaptoethanol] [39]. Electrophoretic separation of proteins was performed according to Laemmli [40] in a 12% polyacrylamide gel. Electrotransfer of the separated polypeptides was carried out on a BioTrace nitrocellulose membrane (Pall, USA) by semi-dry transfer on a TE70PWR transblotter (Amersham, USA). Polyclonal rabbit anti-xyloglucanase antibodies from *P. canescens* fungus were used as the primary antibodies. Monoclonal goat anti-rabbit antibodies conjugated with alkaline phosphatase (Sigma, USA) were used as the secondary antibodies. Immunocomplexes were detected using a stabilized Western Blue substrate (Promega, USA).

Growth indicators

To study each line, 40 plants were used. The length of the stem was measured from the root neck to the apical bud. The number of leaves was calculated from the apical bud and to the root neck. The parameters of the leaf blade were measured using the LAMINA software [41] in the second year of vegetation. Height was measured at the age of 2, 6 and 18 months and analyzed using the Statistica 7.0 software (https://www.tibco.com/products/tibco-statistica).

Analysis of the specific cellulose content

Median internodes of 20 plants per each 18-month-old line were used to determine the content of cellulose by the Kurschner-Hanak nitrogen-alcohol method [42]. The content was recalculated taking into account the weight of an absolutely dry sample.

Analysis of the specific pentosan content

The specific content of pentosans in wood was estimated using the modified Tollens method [43, 44] by converting them to furfural during distillation in the presence of HCl. For this analysis, two 10 *cm* long cuts of stem per plant, representing the 1st and the 2nd year growing wood, respectively, were taken from 18 months plants. The cuts were stripped off their bark, and the air-dried sawdust samples weighing 0.1 *g* were prepared. The optical absorption of the distillate was measured by a two-beam spectrophotometer at a wavelength of 277 nm. The dryness coefficient of wood (C_{dry}) was calculated from the following formulas: [Due to technical limitations, this equation is only available as a download in the supplemental

files section.] and [Due to technical limitations, this equation is only available as a download in the supplemental files section.], where W—the relative humidity of the wood, m—the mass of the empty bag (*g*)„ m1 and m2—the weights (*g*) of the bag with the sample before and after drying, respectively. Then, the content of pentosans in dry matter was calculated according to the formula: [Due to technical limitations, this equation is only available as a download in the supplemental files section.], where A—the percentage of pentosans in the air-dry sample, D—the average optical absorption of the furfural solution obtained from the distillation, n—the conversion factor for the percentage of furfural to pentosans (2.434 for hardwoods), m—the mass of the sawdust sample (*g*)„ C_{dry}—coefficient of dryness. For clarity, the percentage of pentosans was converted to absolute values as *mg* / *g* dry weight.

Analysis of hemicelluloses monosaccharide

Monomeric sugars of hemicelluloses (arabinose, fucose, galactose, glucose, mannose, rhamnose, and xylose) were measured by standard alditol acetate method [45, 46]. For the analysis of the composition of monosaccharides, two 10 *cm* long cuts of stem per plant without bark, representing the 1st (6 months) and the 2nd (18 months) year growing wood, respectively, were taken from 18-month-old plants. Samples of 5 *mg* of wood sawdust were hydrolyzed with 2M trifluoroacetic acid (TFA) at 100° C for 5 hours. The mixture of neutral monosaccharides was converted to alditol acetates and identified by gas chromatography–mass spectrometry (GC-MS) analysis using the GCMSQP 2010 Plus chromatograph (Shimadzu Corporation, Japan) with the HP–5MS column (60 *m* × 0.32 *mm* × 0.25 *μm*).. Myo-inositol was used as an internal standard. Helium was used as the carrier gas. The temperature of the injector was 150° C. The column temperature was increased from 60° C to 250° C at a rate of 2° C/min, and, then, held for 10 minutes [47].

Microscopy

For the microscopy analysis, samples of 18-month-old plants were taken from the lower part of the stem (wood of the second year of cultivation) in three replicates. A total cut of all tissues of the stem was made. To prepare the cross sections, the samples were embedded in the epoxy-resin mixture containing DER–332, DER–732, DDSA, and DMP–30 [48]. The transverse sections were obtained using the ultra microtome Reichert Om U2 (Austria) with glass knives, stained with methylene blue, azur-II, and basic fuchsin [49] and photographed with the AxioImager M1 light microscope (CarlZeiss, Germany).The slices were scanned, and the thickness of the cell walls was estimated using the AxioVision 4.8.1 software package (CarlZeiss, Germany).

Measurement of the libriform fibers

For the measurement of the libriform fibers, samples of the 18-month-old plants were taken from the bottom of the stem without bark (wood of the second year of cultivation) in three replicates. Samples

were macerated using acetic acid and sodium chlorite, and the length and diameter of their libriform fibers were measured [50].

Measurement of the aspen decomposition rate

The decomposition rate was estimated by analyzing the emission of carbon dioxide during plant material decomposition [51]. In the experiment, sifted through a 0.5 mm fine sieve, washed and sterilized sand was used as a substrate. The plant tissue (stems and roots) from the 6-month-old plants was ground in a porcelain mortar, and, then, dried at 65° C for three days, and 100 mg of this dried and ground tissue were placed in a glass tube with 2 g of sand and sealed with rubber stoppers. To ensure the decomposition in the test tubes, an aqueous extract of the forest plant litter was added. Distilled water was also added to the tubes in an amount of 50% of the total moisture capacity of the sand (taking also into account the water needed to restore the initial mass of plant tissue). Then, the tubes were placed in a thermostat at 22° C for 48 weeks. Samples of air were sampled from the tubes every 8 weeks, and their carbon dioxide gas was analyzed using a gas chromatograph Crystallux 4000M (Research and Production Company «Meta-chrom», Yoshkar-Ola, Russia). All samples were analyzed also for C and N content by gas chromatography using the Euro EA-CHNSO Elemental Analyser (HEKAtech GmbH, Wegberg, Germany).

Declarations

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author upon request.

Author contributions

Conceptualization: E. O. V., V. G. L., K. A. S., data curation: E. O. V., N. M. S., V. A. B., V. G. L., formal analysis: E. O. V., V. G. L., funding acquisition: K. A. S., K. V. K., investigation: E. O. V., N. M. S., V. A. B., V. G. L., project administration: K. A. S., writing—original draft: E. O. V., writing—review & editing: E. O. V., V. G. L., K. V. K., K. A. S.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1 Height, number of leaves and cellulose content (± SD) in the 18-month-old aspen lines in semi-natural conditions

Line	Height, cm	Number of leaves	Cellulose content, mg/g
Pt (control)	59.4 ± 1.97	24.2 ± 0.84	383.2 ± 10.97
PtXIVXeg1a	61.1 ± 2.14	26.2 ± 1.05	381.2 ± 12.33
PtXIVXeg1b	53.3 ± 1.91	25.3 ± 0.88	397.6 ± 11.52
PtXIVXeg1c	59.3 ± 2.34	25.1 ± 0.90	426.4 ± 12.39*
PtXVXeg1b	69.6 ± 2.67*	29.4 ± 0.99*	411.3 ± 11.57*
PtXVXeg3b	51.2 ± 1.85	25.2 ± 0.82	388.7 ± 11.44
PtXVXeg5a	60.5 ± 2.02	25.0 ± 1.07	379.6 ± 10.86

*significantly different from *Pt* at $P \leq 0.05$ based on ANOVA

Table 2 Leaf trait parameters (± SD) of the 18-month-old aspen lines

Line	Area, mm^2	Circularity	Length, mm	Width, mm	Length to width
Pt (control)	5776.3 ± 203.9	91.6 ± 1.79	96.3 ± 2.85	81.0 ± 2.51	1.20 ± 0.04
PtXIVXeg1a	4921.6 ± 172.2	85.5 ± 1.91*	100.1 ± 3.09	69.1 ± 2.03*	1.47 ± 0.05*
PtXIVXeg1b	4176.3 ± 156.7*	86.0 ± 1.85*	89.9 ± 2.77	65.3 ± 2.59*	1.39 ± 0.04*
PtXIVXeg1c	5526.3 ± 197.8	87.1 ± 1.09*	102.4 ± 2.35	75.0 ± 2.77	1.38 ± 0.04*
PtXVXeg1b	6020.8 ± 210.7	90.1 ± 2.14	105.1 ± 2.71	77.7 ± 2.56	1.37 ± 0.04*
PtXVXeg3b	4784.6 ± 167.4*	88.2 ± 1.15*	95.5 ± 1.98	68.9 ± 2.09*	1.39 ± 0.05*
PtXVXeg5a	5901.5 ± 212.4	86.6 ± 1.55*	108.8 ± 2.79*	76.2 ± 2.45	1.45 ± 0.05*

*significantly different from *Pt* at $P \leq 0.05$ based on ANOVA

Table 3 The percentage of pentosans (± SD) in different parts of the 18-month-old aspen lines

Line	Wood of the 6 month of cultivation	Wood of the 18 month of cultivation
Pt	23.1 ± 0.57	18.8 ± 0.56
PtXIVXeg1a	17.5 ± 0.53*	17.8 ± 0.48
PtXIVXeg1b	20.8 ± 0.46	20.7 ± 0.60
PtXIVXeg1c	21.8 ± 0.94	20.3 ± 0.58
PtXVXeg1b	21.5 ± 0.53	17.9 ± 0.50
PtXVXeg3b	21.3 ± 0.76	19.8 ± 0.55
PtXVXeg5a	23.0 ± 0.61	22.2 ± 0.61

* significantly different from *Pt* at $P \le 0.05$ based on ANOVA

Table 4 Mean diameter and length (± SD) of wood fiber in aspen lines

Line	Mean diameter, *mkm*	Mean length, *mkm*
Pt (control)	19.58 ± 1.84	488.9 ± 12.7
PtXIVXeg1a	20.14 ± 1.80	506.7 ± 14.3*
PtXIVXeg1b	19.76 ± 1.61	487.6 ± 11.6
PtXIVXeg1c	20.16 ± 1.27	461.8 ± 17.4
PtXVXeg1b	20.01 ± 1.93	512.0 ± 10.8*
PtXVXeg3b	19.57 ± 1.54	524.3 ± 13.0*
PtXVXeg5a	21.98 ± 1.93	487.8 ± 15.2

* significantly different from *Pt* at $P \le 0.05$ based on ANOVA

Table 5 Percentage of nitrogen and carbon (± SD) in the wood of the aspen lines

Line	Stems		Roots	
	nitrogen	carbon	nitrogen	carbon
Pt	1.23 ± 0.12	45.8 ± 1.5	1.7 ± 0.3	45.5 ± 1.5
PtXIVXeg1b	1.04 ± 0.19*	46.1 ± 1.5	1.9 ± 0.4	45.5 ± 1.5
PtXVXeg3b	1.05 ± 0.18*	45.9 ± 1.5	1.8 ± 0.3	44.9 ± 1.4

* significantly different from *Pt* at $P \le 0.05$ based on ANOVA

Figures
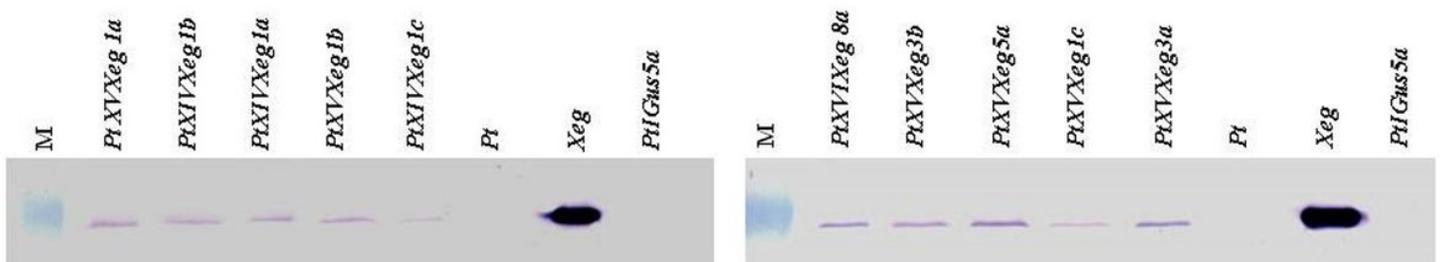
Figure 1

Western blot analysis of protein extracts of transgenic aspens carrying the recombinant gene sp-Xeg. M - standard protein molecular marker 26 kDa, Xeg - fungal extract, Pt - non-transgenic control, PtlGus5a -

transgenic negative control, PtXIVXeg1a, PtXIVXeg1b, PtXIVXeg1c, PtXVXeg1a, PtXVXeg1b, PtXVXeg1c, PtXVXeg3a, PtXVXeg3b, PtXVXeg5a, and PtXVIXeg8a are transgenic lines
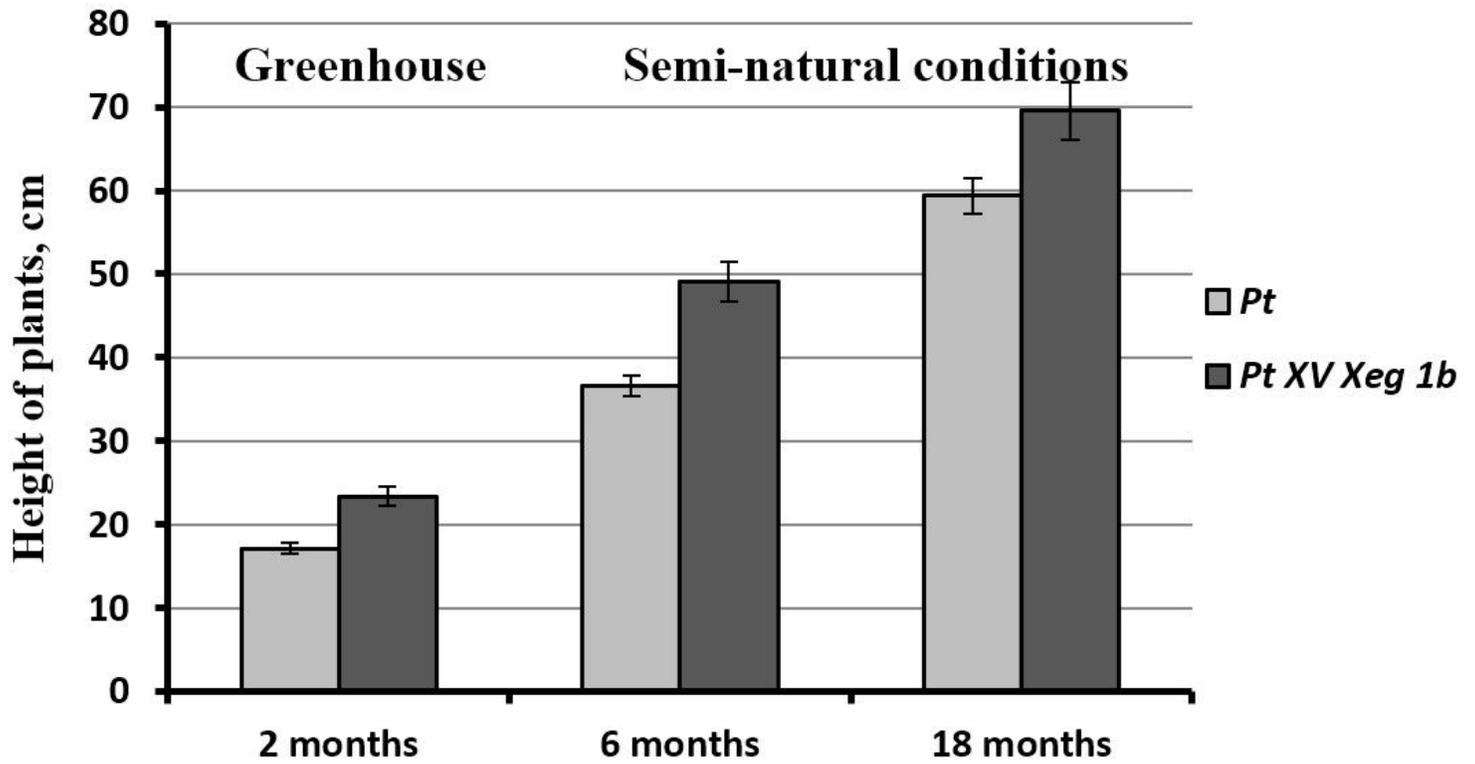
Figure 2

Growth rate of the transgenic (PtXVXeg1b) and non-transgenic (Pt control) aspen lines

Figure 3

Aspen leaves under semi-natural conditions. Left - Pt control, right – line PtXVXeg1b
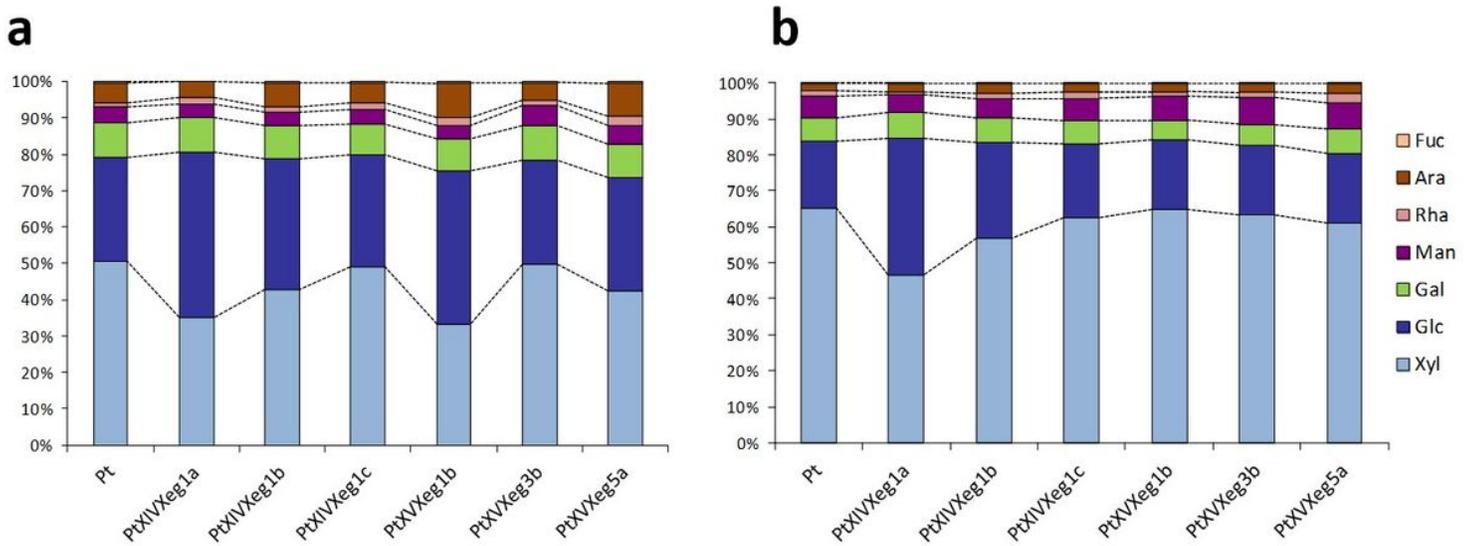
Figure 4

Monomeric sugars composition in wood of the 6- (a) and 18- (b) month-old aspen lines. Fuc – fucose, Ara – arabinos, Rha – rhamnose, Man – mannose, Gal – galactose, Glc – glucose, Xyl – xylose
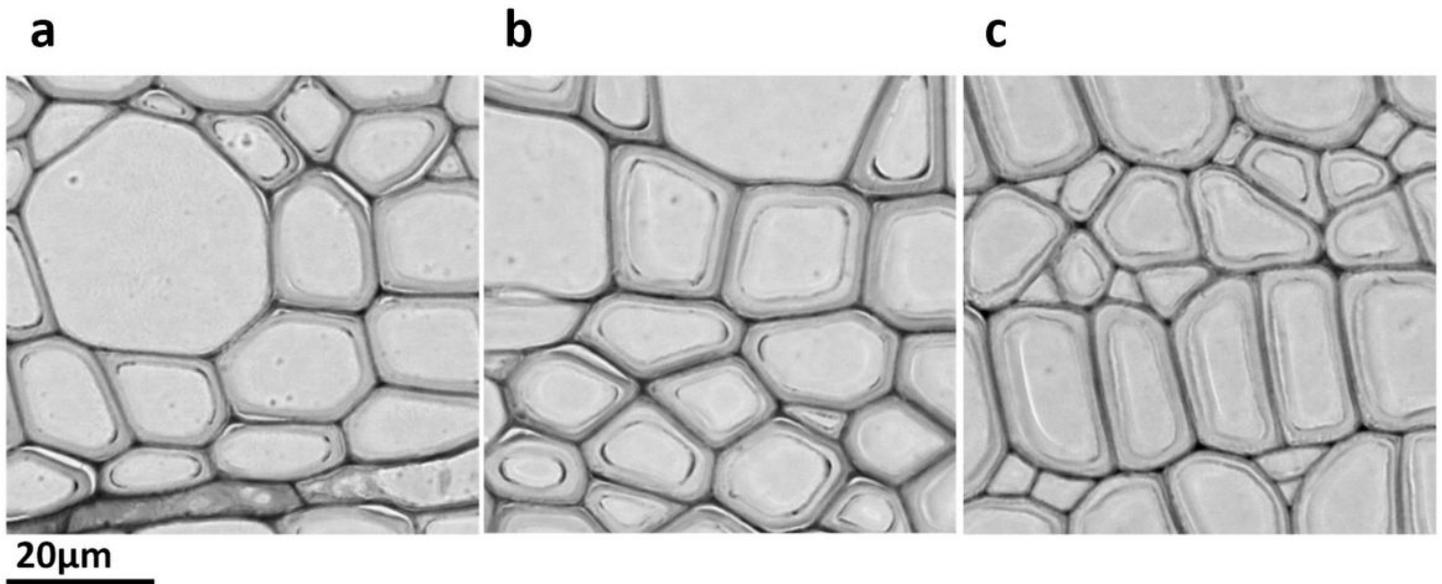
Figure 5

Micrograph of cell slices of plant xylem with different cell wall thickness of 1.16 ± 0.044 microns in control line Pt (a) and 1.47 ± 0.045 and 1.80 ± 0.056 in transgenic lines PtXIVXeg1c (b) and PtXVXeg1b (c), respectively
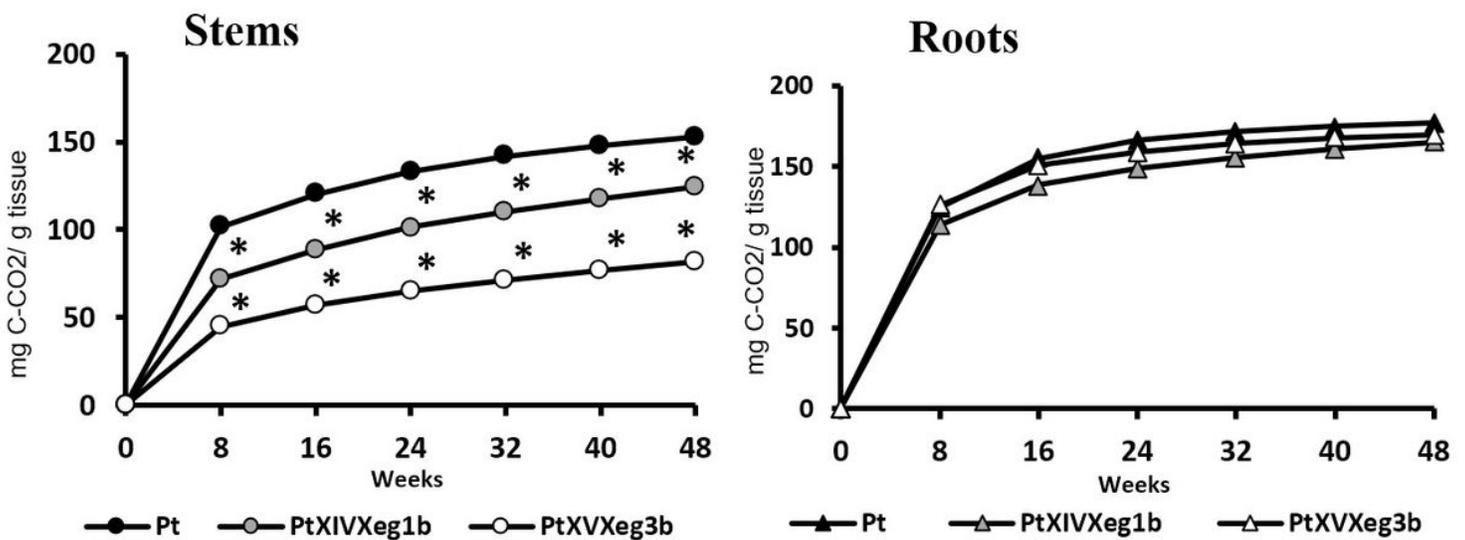
Figure 6

Cumulative CO_2 emissions during decomposition of stems and roots of transgenic (PtXIVXeg1b and PtXVXeg3b) and control (Pt) aspens during the year; *statistically significantly different from Pt at $P \leq$ 0.05 based on ANOVA