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RNA Interference-Mediated Down-Regulation of 4-Coumarate: Coenzyme A Ligase in Populus Tremula Alters Lignification and Plant Growth

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ABSTRACT

The study of the lignin biosynthesis and ways of its modification by genetic engineering methods are important in both scientific and practical terms. The mechanisms of its regulation have not yet been well studied, and no efficient methods of lignification decrease have been developed, which would not cause any undesirable attributes in the phenotype. We have used the method of synthesis of small interfering RNAs to the fourth exon of the 4-Coumarate: Coenzyme A Ligase gene in transgenic aspen plants, which resulted in the decreasing expression of the 4CL1 gene in the plants of the PtXIII4CL2c, PtXIII4CL4a, and PtXVI4CL9b lines by 72%, 45%, and 75%, accordingly. The 4CL1 gene expression inhibition was accompanied by the growth (height) speed change in greenhouse conditions. The height of cloned plants PtXIII4CL2c and PtXVI4CL9b was validly lower than the control values by 20% and 12%, accordingly. At the same time, the PtXIII4CL4a clone was higher than the control plants by 23%. The total lignin content in the wood of 6-month old cloned plants PtXIII4CL2c, PtXIII4CL4a, and PtXVI4CL9b decreased by 23%, 13%, and 16%, accordingly. We detected inverse correlation between the decrease in the lignin content and the development of the red-brown color of the wood. The correlation ratio was -0.87. Thus, the growth reduction was observed when the 4CL1 gene expression was inhibited by more than 70%, while the lignin content decrease and color change took place in all lines under study.

KEYWORDS
Lignin biosynthesis, genetic engineering methods, Coenzyme A Ligase, cloned plants

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Introduction

Lignin is a complex natural polymer, which is a product of monolignols, derivatives of hydroxy-cinnamic alcohols (coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol) which is one of main components of the secondary cell wall of plants and determines mechanical properties of plant stems and upward

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transport. In the soft wood of coniferous plants in the temperate zone, the lignin content is between 24% and 33%, and in the hard wood of foliage plants it varies between 19% and 28% (Sarkanen & Hergert, 1971). The lignin content includes the "soft" acid-soluble lignin, the base of which are residues of the syringyl alcohol (S-monolignols), acid-tolerant "hard" lignin or Klason lignin, which consists of guaiacyl alcohol (G-monolignols) residues and a minor amount of p-hydroxyphenyl alcohol (H-monolignols) residues (Lundquist, 1992). It is more beneficial to produce paper, fuel, and fabric using wood containing less lignin, in which the "soft" lignin prevails, which is created by S-monolignols, as it facilitates the raw material delignification. Therefore, researchers worldwide seek efficient methods and targets to enable regulation of the content and composition of lignin in plants. The main object of scientific research are the enzymes of lignin biosynthesis in plants, the genes coding those enzymes, the transcription factors that regulate biosynthesis. Mutations of any genes in these enzymes lead to changes in the transcriptome and metabolome of the plant, as shown on the example of Arabidopsis. A frequent result of the lignin biosynthesis enzyme gene expression suppression with genetic engineering methods is a change in the chemical composition of wood and growth characteristics of the transformants (Anterola & Lewis, 2002; Blake et al., 2006). One of the most promising enzyme for the lignin content regulation in plants is the 4CL (4-Coumarate: Coenzyme A Ligase) enzyme, as it catalyzes the formation of precursors of all types of monolignols. At this point, a large number of isoforms of this enzyme has been detected in different plant species, but the most interesting is the group of 4CL1 enzymes as they catalyze the biosynthesis of lignin in developing xylem tissues (Hu et al., 1998). Researchers mainly use the antisense RNA synthesis technology to suppress the 4CL1 gene expression (Hu et al., 1999; Voelker et al, 2011; Li et al., 2003; Wagner et al., 2009; Kajita et al., 1997; Kajita, Katayama & Omori, 1996) in plants, which causes changes in enzyme activity and lignin content in woody and herbaceous plants. However, a more efficient method is the synthesis of interfering RNA for this gene. Therefore, this work targets to obtain transgenic aspen plants, in which the 4CL1 gene expression was suppressed using siRNA, and to analyze the lignin content in the transformants.

Materials and Methods

Production of transgenic aspen plants. Genetic transformation of aspen was performed using the plant material in vitro of the Pt (Populus tremula L.) genotype kindly provided by the Forest Institute of NASB (Padutov V.E., Gomel, Republic of Belarus). The agrobacterium-mediated transformation was performed with supervirulent strain CBE21 with plasmid vector pBI-4CL. The RNA-interfered structure 4CL is in the T-DNA region of this vector. It contains inverted repeats of the fourth exon of the 4-Coumarate:Coenzyme A Ligase gene of poplar (GenBank: AY043494.1), separated by a linker under the control of the 35S promoter (Figure 1). Being the selective gene, the binary vector T-DNA contains the nptII gene under the nopaline synthase promoter control. The interstices (8–10 mm) of microplants in vitro were used for inoculation. The regeneration and selection of transformants was performed in a MS medium (Murashige & Skoog, 1962) modified by the nitrate content (Shestibratov et al., 2008), supplemented with 3 mg/l of zeatin, 0.1 mg/l of IBA, 30 mg/l of kanamycin and 500 mg/l of claforan.

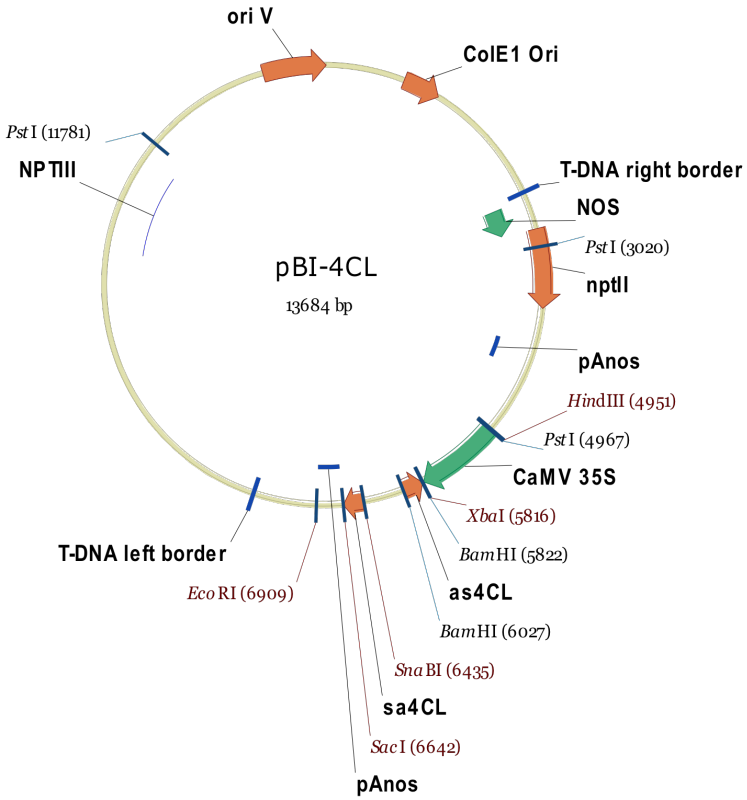

Figure 1. Plasmid vector pBI-4CL, containing inverted repeats of the fragment of the 4-Coumarate:Coenzyme A Ligase gene of poplar. NOS is the nopaline synthase promoter; nptII is the neomycinphosphotransferase gene; pAnos is the nopaline synthase terminator; CaMV35S is the 35S promoter of cauliflower mosaic virus; as4CL and sa4CL are the inverted repeats of the fragment of the 4-Coumarate:Coenzyme A Ligase gene of poplar (GenBank: AY043494.1)

The PCR analysis of transformants. From plants stably rooting on the selective medium, the total plant DNA was isolated by the S. Rogers & A. Bendich (1995) technique using 2×CTAB buffer, and analyzed for the absence of agrobacterium contamination, with primers VirB1 (5'-GGCTACATCGAAGATCGTATGAATG-3') and VirB2 (5'-GACTATAGCGATGGTTACGATGTTGAC-3') for the insertion of the selective gene nptII (primers Nos – 5'-CGCGGGTTTCTGGAGTTTAATGAGCTAAG-3'and NPTII-2 – 5'- GCATGCGCGCCTTGAGCCTGG-3') and insertion of the target structure (primers 35S 5'-CTGCCGACAGTGGTCCCAAAGATGGACCC-3' and 4CL1-low 5'-CCTGTTCTAGCAATGTGCTTGGCATT-3'). The amplification was performed under the following conditions: the reaction mixture contained 16 mM (NH4)2SO4, 200 μg/ml of bovine serum albumin, 200μM of each dNTP, 0.8μM of each oligonucleotide, 0.15 U/μl of Taq polymerase, 1–5 ng/μl of genomic DNA. To analyze the 4CL structure integration in the plant DNA, we added 5% of DMSO to the reaction mixture for PCR. Amplification mode: denaturation at 96°C (hot start) for 3 min; denaturation at 95°C for 45 sec; annealing at 62°C (nptII), 58°C (in the case of the VirB gene) or 59°C (the 4CL structure) for 45 sec; elongation at 72°C for 1 min; extension at 72°C for 5 min; 31 cycles. The reactions were

performed in thin-walled 200-µl tubes (QSP) in MJ MiniTM Gradient Thermal Cycler (BIO-RAD).

The RT-PCR analysis of the cDNA of transformants. The 4CL structure transcription was analyzed by the RT-PCR method. The total plant RNA was extracted using the TRIzol® reagent (Invitrogen, USA) according to the attached commercial method from vegetative material in vitro. The removal of genomic DNA from the samples was performed using turbo DNase (Ambion, USA) according to the protocol attached by the manufacturer. For cDNA synthesis, we took 160 ng of total RNA. RNA concentration was determined with the Thermo Scientific Nanodrop 2000 microspectrophotometer. cDNA synthesis was performed using M-MuLV reverse transcriptase, RNaseH- (SibEnzyme, Russia) using oligo-d(T)18 primer (Synthol, Russia) at a temperature of 37°C for 75 minutes. For the revertase inactivation, the mixture was heated to 70°C. We took 2 µl of the cDNA mixture as a template for PCR amplification using primers dirBam (5'- ATTAGGATCCGGATATGGAATGACCGAGGCAG-3') and revBam, (5'- GATTGGATCCTTTCATGATCTGATCACCCCG-3') under conditions similar to the PCR analysis of total DNA. To control RNA contamination with residues of the genomic DNA, PCR was performed with RNA preparations of each of the clones without revertase treatment. We used actin, primers Act-up (5'-TATGCCCTCCCACATGCCAT-3') and Act-low (5'-CATCTGCTGGAAGGTGCTGA-3') as the gene for labeling the signal level of the sought 4CL gene; the expected fragment size is 741 bp.

The 4CL gene expression analysis by reverse transcriptase PCR method in real time (RT-PCR). The level of gene expression was assessed by the PCR method in real time using the derived cDNA preparations. We used the SYBR Green intercalating dye as a fluorophore for the reaction products detection. The amplification was performed with the ANK 32 device (Synthol, Russia) using the reaction mixture for RT-PCR (M-427, Synthol, Russia). We used the following primers for PCR in real time: Actin1 – 5'-AAAGTGAAGATATTCAGCCTCTTGT-3'; Actin 2 – 5'-GCGACCCACAATGCTAGG-3'; 4CL1 – 5'-CTTTGTTAATAGCCCATCCAG-3'; 4CL2 – 5'-TGATTTCACAGCAAATGCAAC-3'. The amplification program algorithm is as follows: denaturation at 95°C for 5 min, 45 cycles (primer annealing and elongation at 60°C for 40 sec, denaturation at 95°C for 15 sec). The amplification specificity was assessed by melting curves. Actin was used as the reference gene. The target 4CL1 gene expression level in transgenic plants was normalized by the gene expression level of non-transgenic control plants. We calculated the gene expression level by the 2-ΔΔSt method (Livak & Schmittgen, 2001).

Biometric studies of 6-month greenhouse plants. Transgenic aspen plants transformed with RNA-interference-mediated structure with inverted repeats of the 4CL gene of poplar were planted in a greenhouse to adapt to the protected ground conditions (Biotron artificial climate station). At the end of the first growing season, these plants were subjected to a biometric research. We measured the plants' height from the root collar to the apical bud. We determine the height of 50 sample plants. The measurement results were expressed in cm. The statistical processing was provided by a system of Excel spreadsheets (Microsoft Office 2003). The coefficient of variation of the standard error of the mean was calculated (SEM). The comparative assessment of the mean values, we used the Student's t-test (Takahashi, 1976).

Lignin content analysis in the wood of 6-month greenhouse plants. For the analysis, we used the wood of transgenic plants grown in protected soil for 6 months (one vegetative period). We removed bark from stems, dried, ground, and used to determine the dryness coefficient and for the analysis. 1 g of the sample was dried to constant weight at 103+2°C, cooled in a closed weighing bottle with weighed sample in a desiccator for 2 hours, and then weighed. The wood dryness coefficient was found by the following formulas:

$$Kdry = \frac{100 - W}{100}\ ; W = \frac{m\,1 - m\,2}{m\,1 - m};$$

where W is relative humidity of the wood m is the mass of empty weighing bottle, g; m1 is the mass of the weighing bottle with the weighed sample before drying, g; m2 is the weight of the weighing bottle with the weighed sample after drying, g.

All subsequent chemical analyzes involved the calculation of the absolutely dry wood sample by multiplying the value of the taken weighed air-dried sample by Kdry. We determined acid-soluble and acid-insoluble lignin by the Klason method (Chiang & Funaoka, 1990).

The weight proportion of Klason lignin in wood was determined as a percentage by the following formula:

$$L_1 = \frac{m}{g} \times 100$$

where L1 is the Klason lignin fraction, m is the lignin mass, g is the mass of oven-dry sample wood (Obolenskaya, Elnitskaya & Leonovich, 1991).

The collected after boiling filtrate was used to find acid-soluble lignin. For this, we used a spectrophotometer to determine the absorbance in the ultraviolet region (the wavelength of 205 nm) in 1-cm cuvettes. The content of acid-soluble lignin was calculated by the following formula:

$$L_2 = \frac{(A_s - A_b) \times V}{110 \times W} \times 100$$

where L2 is the proportion of acid-soluble lignin, As is the sample absorption, Ab is the solvent absorption, W is weighed wood, V is the solution volume (Lakin, 1990).

The total lignin content was calculated as the amount of acid-insoluble Klason lignin and acid-soluble lignin. The obtained results were expressed as a percentage of lignin in dry wood.

Analysis of the cellulose content in the wood of 6-month greenhouse plants. The cellulose content in the wood of transgenic plants was determined by Kyurshner's nitrogen and alcohol method (Obolenskaya, Elnitskaya & Leonovich, 1991).For the analysis, 50 ml of nitrogen and alcohol mixture, consisting of one volume of concentrated nitric acid and four volumes of 95% ethanol, was added to 1 g of air-dry sawdust, then a reflux condenser was attached, and the mixture was refluxed for 1 hour. After refluxing, the sawdust was allowed to settle, the liquid was carefully poured through a dried to

constant weight fritted glass filter. The remaining sawdust reflux procedure was carried out 4 times. The absence of lignins was monitored with phloroglucinol saline solution. After the last treatment the cellulose was filtered off with the same dried to constant weight fritted filter, washed with 300 ml of hot distilled water (90°C) using a pump. The filter with cellulose was then dried at 103±2°C to constant weight and weighed.

Mass fraction of "raw" cellulose in absolutely dry wood was calculated by the following formula:

$$C = \frac{m1 - m}{g} \times 100;$$

where m1 is the mass of the filter and cellulose, g; m is the mass of the empty filter, g; g is the mass of absolutely dry sample wood, g.

To calculate the mass fraction of cellulose in dry matter, we multiplied the obtained value of the "raw" cellulose mass fraction by dryness ratio Kdry. The dryness ratio was found as described above. The obtained results were expressed as a percentage of lignin in dry wood.

Analysis of the pentosan content in the wood of 6-month greenhouse plants. The specific content of pentosans in wood was determined by modified Tollens method (Obolenskaya, Elnitskaya & Leonovich, 1991), through converting them into furfural by distillation in the presence of HCl. The distillate optical absorption value was measured by two-beam spectrophotometer at a wavelength of 277 nm. The subsequent calculation of the pentosan content in dry matter was performed by the following formula:

$$A = \frac{D \times n}{m} \times Kdry;$$

where A is the percentage of pentosans in the air-dry weighed sample; D is the average value of the optical absorption of the furfural solution obtained by distillation; n is the 'furfural to pentosans' percentage recalculation coefficient (for hard wood, it is 2.434); m is the mass of sawdust sample, g; Kdry is the dryness ratio. The obtained results were expressed as a percentage of pentosans in dry wood.

Determination of the correlation between the change in the lignin quantity in wood and the transformants' wood color. The comparison of the transgenic aspen clones' wood color changes was provided by taking photographs of transversal section of 3 transgenic clones and the control genotype. We used the Corel PHOTO-PAINT X4 software to determine the R/G/B indicator and the Excel software to calculate the individual value of R/G/B. Using the Spearmen correlation method (Statistica 10 software), we determined the correlation between the wood color and the specific quantity amount of lignin.

Results

We obtained 14 kanamycin resistant lines in the course of aspen interstices' agrobacterium-mediated transformation with the structure containing inverted repeats of the fourth exon of the 4-Coumarate: Coenzyme A Ligase gene of poplar. The efficiency of transformation equaled to 17.5%. For subsequent analyses, we selected three lines: PtXIII4CL2c, PtXIII4CL4a, and PtXVI4CL9b.

These demonstrate good rooting on nutrient medium containing selective antibiotic kanamycin in the concentration of 30 mg/l. The PCR analysis of the total DNA of transformants showed no agrobacterium contamination, insertion of selective gene nptII and target structure 4CL1 lines in all three transformant lines (data not provided). The analysis of the 4CL1 gene expression involved the evaluation of the native 4CL1 gene transcripts' presence in preparations of the total RNA of the transformants by RT-PCR. Maximum inhibition of the 4CL1 gene expression compared to the source genotype Pt was found in clones PtXIII4CL2c (72%) and PtXVI4CL9b (75%) (Figure 2). The suppression of the 4CL1 gene expression in clone PtXIII4CL4a was equal to 45%. The visualization of the RT-PCR reaction products with electrophoregram also allows detecting different levels of gene 4CL1 expression in the clones under study, compared to control samples (Figure 3, A,B). Plants of these lines were propagated by micropropagation and acclimatized in greenhouse conditions. Plants were grown for 6 months in 1.5 l pots in a greenhouse at standard farming conditions for woody plants. When plants ceased growing, we measured the height of the control plants and transgenic clones. According to our data, clones PtXIII4CL2c and PtXVI4CL9b and were validly lower than the control values by 20% and 12%, accordingly. Clone PtXIII4CL4a was higher than the control plants by 23% (Figure 4). The findings were statistically valid, $p < 0.05$.

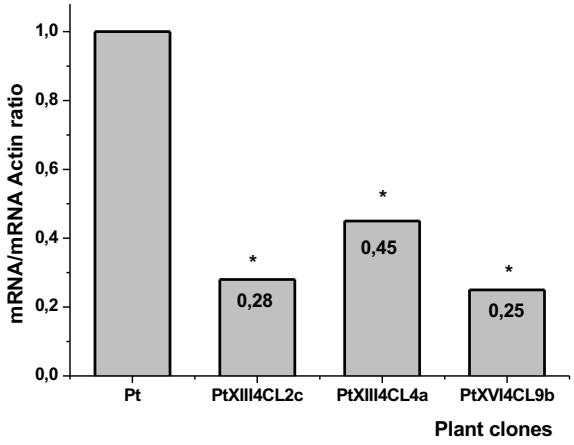

Figure 2. RT-PCR analysis of the expression of the native 4-coumarate-CoA ligase gene in transgenic aspen plants

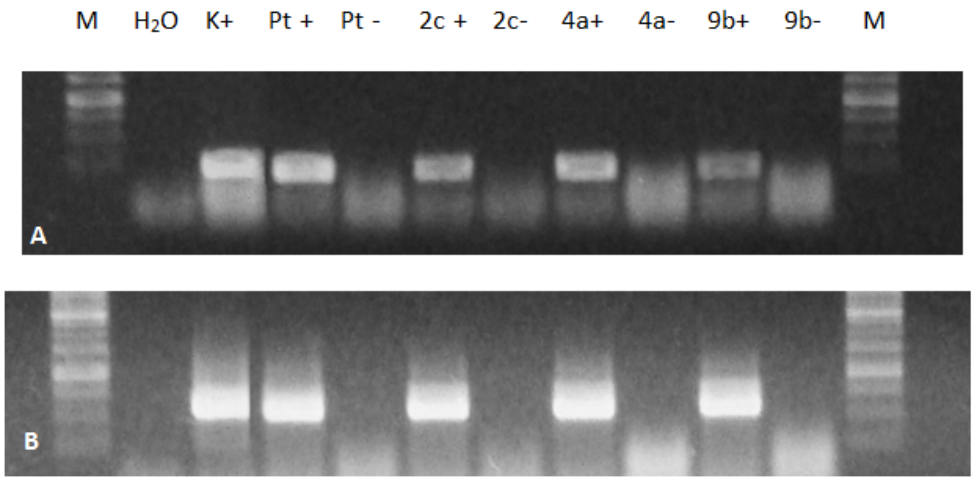

Figure 3. RT-PCR analysis of the 4CL1 gene expression (A) in transgenic aspen plants relative to the reference actin gene (B). K + is the total DNA from the CBE 21 agrobacterial strain, transformed with the pBI-4CL structure (A), or total plant DNA of non-transgenic plant Pt (B); Pt are non-transgenic plants; 2c, 9b, 4a are transgenic plants: PtXIII4CL2c, PtXVI4CL9b, and PtXIII4CL4a, accordingly; "+" are RNA preparations after treatment with reverse transcriptase; "-" are RNA preparations without reverse transcriptase treatment; M is the molecular marker

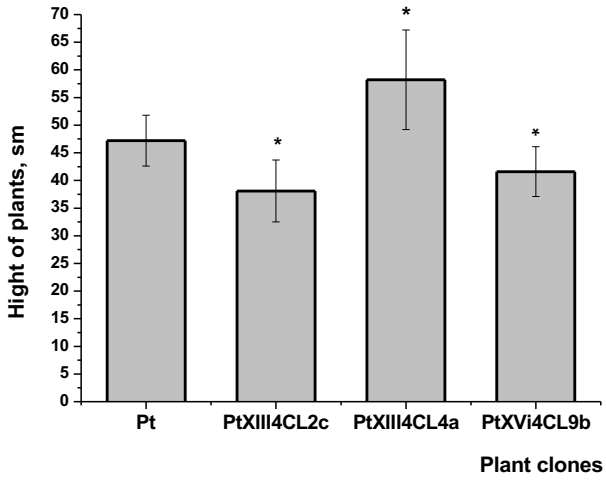

Figure 4. The height of greenhouse plants at the age of 6 months. The results of two biological replications are presented; the sample includes 50 plants for each clone. Differences are valid relative to control samples (Pt), $p<0.05$.

After biometric studies plant stems were collected and used for analyzing the main wood components. The results of the experiments are provided in Table 1.

Total lignin content reduced by 23%, 16%, and 13% in the wood of the PtXIII4CL2c, PtXVI4CL9b, and PtXIII4CL4a cloned plants, accordingly. Maximum inhibition of the 4CL1 gene expression was observed in clones

PtXIII4CL2c and PtXVI4CL9b; these clones demonstrated the greatest lignin content reduction. It should be noted that in all the analyzed clones, the Klason lignin quantity significantly reduces in comparison to the control plants. The minimum Klason lignin content was also noted in the PtXIII4CL2c (16.4%) and PtXVI4CL9b (18.1%) clones compared to non-transgenic control plants (21.6%). These clones demonstrated the greatest decrease of acid-soluble lignin, as well.

Table 1. Specific content of main wood components in the stems of 6-month transgenic plants.

Clone	Total lignin, %	Klason lignin, %	Acid-soluble lignin, %	Cellulose, %	Pentosans, %
Pt	24.2±1.5	21.6±1.01	2.6±0.56	41.3±1.84	19.3±2.26
PtXIII4CL2c	18.5±1.03*	16.4±1.03*	2.1±0.6	41.1±0.5	18.9±0.5
PtXIII4CL4a	21.1±1*	18.7±0.49*	2.4±0.63	41.4±0.56	17.6±0.17
PtXIV4CL9b	20.3±0.45*	18.1±0.42*	2.2±0.03	42.6±0.46	17.3±0.16

The results of 2-3 biological replicates ± standard deviation are provided
*Valid difference with respect to the control samples, Mann-Whitney test

No significant changes in the cellulose and pentosan content in the wood of transgenic clones compared to control plants were found (Table 1).

During the preparation of plant stems for the chemical composition analysis of the wood, we noticed that the transgenic plants' stems were painted in red-brown color. To establish a correlation between the change in the total lignin content and the wood color change (the R/G/B indicator), we calculated the correlation ratio by Spearmen's statistical method. The coefficient had a value of -0.87, indicating that there is an inverse relation between the two attributes (Figure 5). This value shows that the lignin content decrease in the wood of transgenic plants correlates with the development of red-brown coloration of the stem.

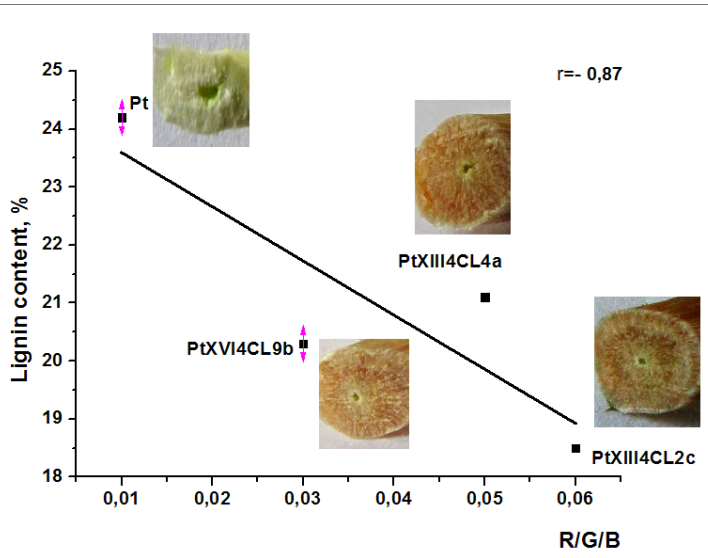

Figure 5. Correlation between the wood color depth indicator (R/G/B) and the specific content of total lignin

Discussion

Lignin biosynthesis in plants is a complex multi-step process, which is governed by many factors at different synthesis stages. Any influence, whether stressful stimuli or change in the gene expression, transcription factors, enzyme activity, naturally or artificially, leads to change in the content and composition of the final product, lignin. The possibility of artificial control of the lignin content and composition has been attracting many scientists around the world, since the use of raw materials with low lignin content is more cost-effective. Researchers obtain transgenic herbaceous and woody plants with reduced expression of the PAL, CCOMT, CYP84, CAD, COMT, CCR and 4CL genes (Anterola and Lewis, 2002). Affecting the level of expression of these genes, we can reduce the lignin content and change the ratio of S- and G-monolignols in its composition: the PAL activity decrease slightly reduces the Klason lignin content in tobacco plants; while the decrease in C4H activity leads, conversely, to an increase in the Klason lignin content and increased share of G-monolignols (Anterola and Lewis, 2002). The most common method for inhibiting the gene expression, used in genetic engineering, is the synthesis of antisense RNA in the cell to inhibit the translation of mRNA complementary to the desired gene. For example, in Pilate's work (Pilate et al., 2002) the synthesis of antisense RNA to the CAD and COMT genes led to a 10% decrease in the lignin content in poplar. The use of antisense RNA to suppress the 4CL gene expression also leads to a lignin content reduction from 21.6% to 17% in Arabidopsis, i.e. a 21% decrease (Kajita, Katayama & Omori, 1996), 35% decrease in pine (Wagner et al., 2009), and 10% (Voelker et al., 2011) and 28% (Tian et al., 2013) in poplar. The results of our research demonstrate up to 23% decrease in the lignin content (Table 1). This is an important economic indicator, because delignification of raw wood and removal of acid-insoluble lignin is the most expensive and environmentally unfriendly stage of the process.

However, at a lower 4CL gene expression level led not only to a reduction in the lignin content, but also a disorder in cell wall formation in conductive paths and a slowdown of the plants' growth rate. An analysis of the known experience of the 4CL gene expression suppression in woody plants (poplar (Hu et al., 1998; 1999; Voelker et al., 2011), pine (Wagner et al., 2009)) showed the following modifications of the plant phenotype: a decrease in plant survival, dwarfing, tillering, and other disorders of plant growth and development, reduced lignin content, and increased cellulose content. In contrast to the anomalies demonstrated by the majority of researchers, there is the work by W.J. Hu et al. (1999), who managed to suppress the Pt4CL1 enzyme activity, but the resulting transgenic plant lines, although characterized by low activity of the 4CL enzyme and a decrease in the lignin content by 5–40%, at the same time showed an increased growth rate, increased number of interstices, and increasing rhizogenesis. W.J. Hu et al. (1999) carried out their research on greenhouse plants and our results were also obtained from greenhouse plants, but the clones with maximum suppression of the 4CL1 gene expression demonstrated a decrease in the plant height by 12–20% (Figure 3), similarly to most researchers. In the work by L. Li et al. (2003), the authors used the xylem-specific Pt4CL1P promoter of poplar. It ensured the 4CL gene's antisense sequence expression only in the tissues of conducting vessels, causing local inhibition of the gene expression; therefore, no growth abnormalities were found in the transgenes.

An interesting work is the work of Chinese researchers who carried out partial suppression of the 4CL gene expression in poplar Populus tomentosa Carr. using the RNA-interference method (Lakin, 1990). The plants were planted in open ground, they were characterized by normal height and stem diameter, comparable to the control plants during the six months of vegetation; the enzyme expression level was reduced by about 10–15% compared to the native 4CL gene, while the lignin content decreased by 11.67% and 4.38%; in one line, the cellulose quantity reduced by 7.23%, and in the other increased by 1.93%. In addition, the content of S-monolignols increased and G-monolignols decreased. Thus, the use of interfering RNA in the work of Chinese scientists allowed reducing the lignin content by 11.67%. In the same work, the use of the antisense RNA technology allowed reducing the lignin content by 28% (Tian et al., 2013).

The obtained results of reduction the lignin content by 24%, 16%, and 13% are better than the data of the group of scientists headed by X.M. Tian (2013), and our results' values are closer to those obtained with the antisense RNA technology. Furthermore, the height of the clones with maximum lignin content reduction was also lower than the control values in our studies, as had been noted by researchers who used the antisense RNA technology. The plants, which we grew in greenhouses, typically had denser leaves, that had a darker color and were smaller in size, compared to the control plants' leaves. S.L. Voelker et al. (2011) noted a decrease in the biomass of transgenic plants with reduced expression of the 4CL gene, however, mainly due to the reduced wood amount. In his research, X. Gengshou (2013) obtained transgenic rice plants with a slight decrease in the 4CL gene expression by the RNA interference method. The plant typically had reduced height, reduced number of shoots, a 10.17% decrease in the lignin content, and darker leaves. Thus, among known works, we managed to achieve the maximum reduction in the lignin content by means of the RNA-interference technology in our experiments.

A number of researchers pointed out the appearance of red-brown color in transgenic plants with reduced 4CL gene expression in pine (Wagner et al., 2009), in poplar to 24-60% (Voelker et al., 2011), and in poplar with reduced CCR gene expression (Leple et al., 2007). We also observed color change in the stems of transgenic plants. The depth and area ofcolor propagation along the stem depended on the lignin content reduction in the wood of plants (Figure 4). Our studies are the first to demonstrate this dependence.

Conclusion

Thus, we have shown that lowering the 4CL1 gene expression by the RNA interference technology leads to a significant reduction in the lignin content, lower plant height, and development of the red-brown color in proportion to the lignin content reduction. Both the content of Klason lignin and acid-soluble lignin noticeably reduced. The obtained results contribute to the study of the lignification processes and lignin biosynthesis process regulation by genetic engineering methods.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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