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Lignin deficiency in transgenic flax resulted in plants with improved mechanical properties

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Abstract

Flax (*Linum usitatissimum* L.) is a very important source of natural fibres used by the textile industry. Flax fibres are called lignocellulosic, because they contain mainly cellulose (about 70%), with hemicellulose, pectin and lignin. Lignin is a three-dimensional polymer with a high molecular weight, and it gives rigidity and mechanical resistance to the fibre and plant. Its presence means the fibres have worse elastic properties than non-lignocellulosic fibres, e.g. cotton fibres, which contain no lignin.

The main aim of this study was to produce low-lignin flax plants with fibres with modified elastic properties. An improvement in the mechanical properties was expected. The used strategy for *CAD* down-regulation was based on gene silencing RNAi technology.

Manipulation of the *CAD* gene caused changes in enzyme activity, lignin content and in the composition of the cell wall in the transgenic plants. The detected reduction in the lignin level in the CAD-deficient plants resulted in improved mechanical properties. Young's modulus was up to 75% higher in the generated transgenic plants (CAD33) relative to the control plants.

A significant increase in the lignin precursor contents and a reduction in the pectin and hemicellulose constituents was also detected. A decrease in pectin and hemicellulose, as well as a lower lignin content, might lead to improved extractability of the fibres.

However, the resistance of the transgenic lines to *Fusarium oxysporum* was over two-fold lower than for the non-transformed plants. Since *Fusarium* species are used as retting organisms and had been isolated from retted flax, the increased sensitivity of the CAD-deficient plant to *F. oxysporum* infection might lead to improved flax retting.

Keywords: Cinnamyl alcohol dehydrogenase (CAD); Lignins; Gene silencing; Mechanical properties; Pathogen resistance; *Linum usitatissimum*

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1. Introduction

Flax (*Linum usitatissimum* L.) is an annual plant cultivated in temperate climate. It is considered a characteristic crop in central Europe. However, the low quality of fibres being produced on farms in Poland led to a rapid decrease in the demand for and area given over to flax growing in recent years. There has been a recent renewal of interest in natural fibres in Europe, and this seems an opportune moment to reestablish the flax fibre industry.

Flax fibres originate from the procambial cells of the protophloem and develop as bundles that encircle the vascular cylinder. In the process called retting, the degradation of polysaccharides, mainly pectin, hemicellulose and lignin, causes the release of the bundles of flax fibres from the other parts of the stem, in particular from the xylem. There are several recognized determinants that affect fibre quality. Flax cultivation is limited because of the low elastic properties of the fibres, which are worse than those of cotton fibres, and the low yield from the retting process, which also has an impact on the quality of the fibres.

The market value of fibres strongly depends on the mechanical properties. The partial degradation of the flax stems by fungal and bacterial enzymes during the retting process makes it possible to obtain clean fibres in a marketable form. The retting efficiency strongly depends on the method used. Several procedures have been investigated in the search for an efficient retting system. Water retting in a tank under controlled conditions, retting of standing flax following desiccation with glyphosate, and treatment with formic acid, propionic acid, formaldehyde, EDTA or sulphur dioxide were used, in the hopes of improving the fibre bundle separation from the rest of the plant stem. Although almost all were satisfactory on the laboratory scale, they failed to achieve commercial viability.

The traditional dew retting method is still the most widely performed. This is a relatively crude method in which the stalks are left in the field after the harvesting of the linseed, so microorganisms in the environment can digest the cell matrix polysaccharides. This biological process is weather dependent, and therefore uncontrollable. Also, the chemical composition of the flax stems strongly affects the degree of retting. More lignified fibres need longer exposure to fungal and bacterial enzymes. Long treatment causes the cellulose to decompose and the fibres to weaken. To overcome the problem of lignification, breeders harvest the plants before seed maturity and significantly before the onset of massive stem lignification. This is why flax breeders have taken an interest in a dual-purpose variety in which seed maturity is synchronized with the stage of fibre harvesting. Lignin synthesis reduction appeared to be a good way to optimize the yield of fibres and seeds.

Lignins are known to play a substantial role in plant adaptation to environmental conditions. They are large, complex polymers of three principal alcohols: coniferyl, sinapyl and *p*-coumaryl (Amthor, 2003). Cinnamyl alcohol dehydrogenase (CAD) is an enzyme that catalyzes the biosynthesis of lignin monomers (hydroxycinnamoyl-alcohols) from the corresponding aldehydes (Fig. 1), and remains a specific marker for lignification (Mitchell et al., 1994; Roth et al., 1997; Kim et al., 2002). Lignin monomers can also bind to polysaccharides (for example to hemicellulose) and proteins, yielding complex and sturdy structures (Amthor, 2003; Tobias and Chow, 2005). Flax fibres have a 3–5% lignin content providing them with their secondary cell wall mechanical resistance. These lignins are induced and deposited in response to pathogens, herbivores and other stresses (Carver et al., 1994; Cabane et al., 2004; Tobias and Chow, 2005). Cell wall deposition of lignins gives a physical barrier to pathogen infection. However, lignin accumulation decreases fibre plasticity and negatively affects the degree of stem retting and thus the fibre quality. It has been shown that the manipulation of lignin composition and content improves the efficiency of pulp- and paper-making processes (Blee et al., 2001). Down-regulation or silencing of the *CAD* gene has been performed for tobacco (Ralph et al., 1998), pine (Wagner et al., 2005) and pine callus cultures (Moller et al., 2005). In those studies, manipulation of the *CAD* gene was used to investigate the lignification process and to characterize the altered lignin structure isolated from mutants. Thus, the silencing of *CAD* was not directly applicable for the manipulation of the mechanical properties of plant fibres.

In this study, the gene silencing of a key enzyme of lignin synthesis (CAD) was used to produce low-lignin flax plants and thus improve their elastic properties. Preliminary data suggests that compensatory mech-

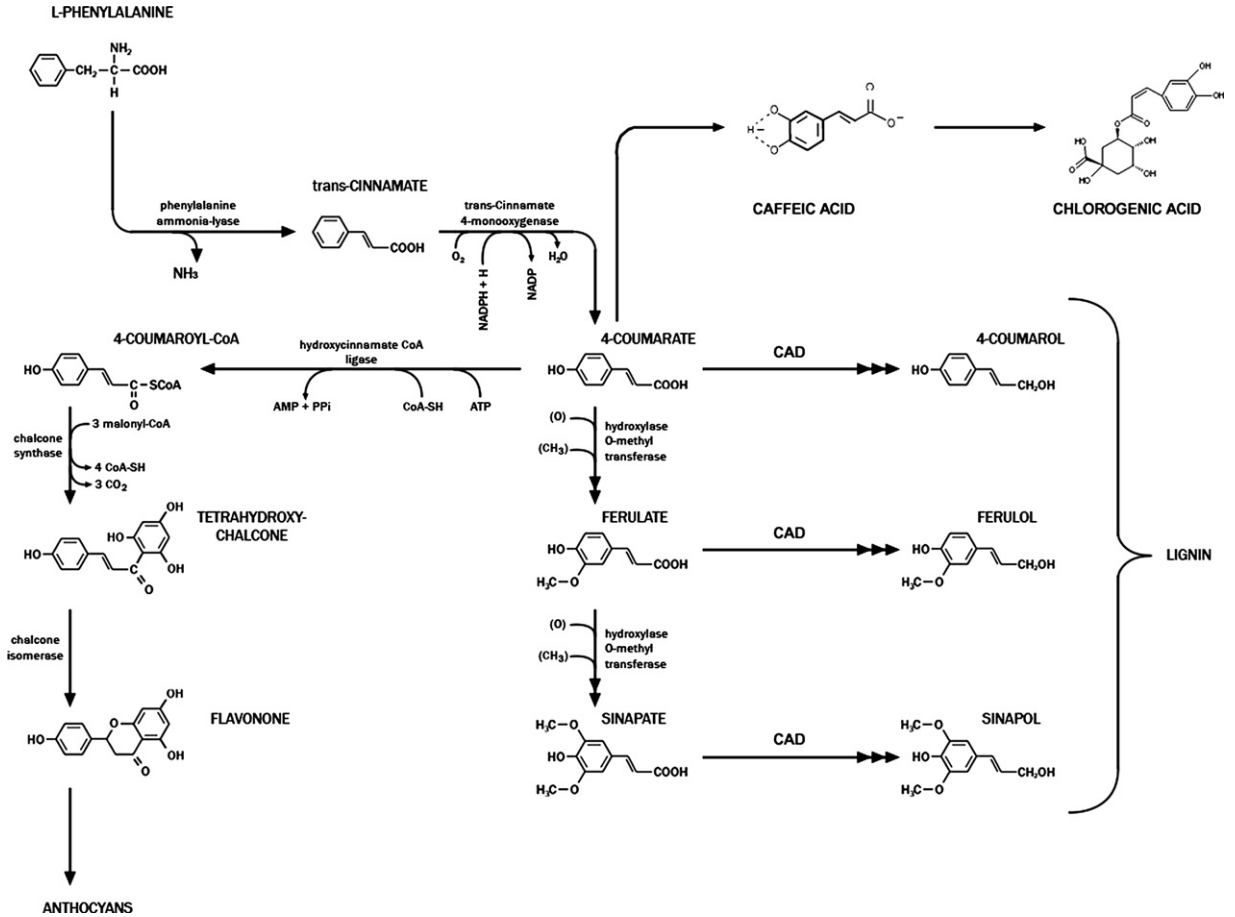

Fig. 1. Phenylpropanoid compounds in plants. Phenylpropanoids refers to a large number of compounds derived from phenylalanine. Phenylalnine-ammonia lyase (PAL) produces *trans*-cinnamate, which is hydroxylated to 4-coumarate. The reduction of CoA-activated carboxyl groups to alcohols by cinnamyl alcohol dehydrogenase (CAD) gives 4-coumarol, ferulol and sinapol, i.e. monolignols used for lignin synthesis.

anisms for a lack of lignin exist to modify the polysaccharides lignin would bind to. At present, the nature of these mechanisms and to what extent they could be exploited for industrial purposes is unknown. The contents of various polysaccharide and phenylpropanoid compounds were determined in CAD-reduced transgenic flax to begin to elaborate on the answers to these questions.

A decrease in lignin composites and an increase in phenolic acids was detected. This lignin content reduction did not affect the cellulose content. Although the transgenic plants showed a higher sensitivity to *Fusarium* infection, there was compensation in the form of improved retting.

2. Materials and methods

2.1. Plant material and growth conditions

Flax plants (*L. usitatissimum* L., fibrous cultivar Nike) were grown in tissue culture in a greenhouse under a 16 h light (21 °C), 8 h dark (16 °C) regime. The plants were grown on MS medium (Murashige and Skoog, 1962) containing 1% sucrose and 0.8% agar.

2.2. The plasmid construct

The binary plasmid pHellsgate2-CAD was used for transformation. pHellsgate2 (EMBL, acc. no.

AJ311874) contains cDNA encoding two 506-bp fragments of the *CAD* gene (EMBL, acc. no. DQ487210) from flax under a 35S CaMV promoter and an OCS terminator. The used sequence of *CAD* gene from *L. usitatissimum* exhibits 82% and 85% identity to the gene from *L. album* and *A. thaliana*, respectively. Two identical fragments of *CAD* cDNA were cloned in the sense and antisense orientation into the pHellsgate vector. Thus, this construct encodes self-complementary hairpin RNA (hpRNA), which ensures efficient gene silencing in plants. The presence of an intron in this construct enhances the effect of silencing (Wesley et al., 2001).

The vector pHellsgate2 was maintained in *E. coli* strain DB3.1 (Invitrogen, Carlsbad, CA, USA), because in this strain, the *ccdB* gene is not lethal and can be directly introduced into *Agrobacterium tumefaciens* for plant transformation.

2.3. Generation and screening of transgenic flax

Using a gene pulser (BioRad) at 2500 V, the plasmid pHellsgate-CAD was transformed by electroporation into the *Agrobacterium tumefaciens* strain C58C1, which contains the plasmid pGV2260.

The cotyledons and hypocotyls of fibrous flax were transformed with *Agrobacterium tumefaciens* by immersing the explants in the bacterial suspension for 2 days, as described elsewhere (Wróbel et al., 2004). The *Agrobacterium*-inoculated flax explants were transferred onto callus induction medium (MS medium containing 1 mg/l BAP, 0.05 mg/l NAA, 2.5% glucose, 2.5% sucrose and 0.8% agar). After 7 days, the explants were placed on shoot regeneration medium (MS medium containing 0.02 mg/l BAP, 0.001 mg/l NAA, 2.5% sucrose and 0.8% agar). Those shoots that reached 1–1.5 cm were cut off and transferred onto root induction medium (MS medium containing 1% sucrose and 0.8% agar). The callus and shoot induction media were supplemented with 25 μg/ml CAM (chloramphenicol) to ensure the selection of transformants with the introduced construct. The integration of the transgene into the created flax plants was analyzed using PCR and the northern blot method.

2.4. Pre-selection by PCR

Genomic DNA was isolated and used for PCR with primers specific for the 35S CaMV promoter (forward GAAAAGGAAGGTGGCTCCTA, reverse GGTCTTGCGAAGGATAGTGG). Those transgenic plants which showed the expected 220-bp fragment of the 35S CaMV promoter were analyzed further.

2.5. Northern blot analysis

The level of mRNA for the *CAD* gene was assayed using the northern blot method. The total RNA was isolated as described previously (Wilczynski et al., 1998; Wróbel et al., 2004) from tissue-cultured plants. Then, the RNA was blotted onto nitrocellulose and probed with cDNA encoding a 480-bp fragment of the *CAD* gene, labelled with ^{32}P.

2.6. Selection by PCR method

The incorporation of gene construct into genome was verified by amplification of its specific fragment. Genomic DNA was isolated and used for PCR reaction with forward primer specific for 35S CaMV promoter and reverse primer for *CAD* gene (35S CaMV forward GAAAAGGAAGGTGGCTCCTA, CAD reverse CAAGAAAGCTGGGTACTGACCA). The transgenic plants of two selected lines (CAD27 and CAD33) showed expected 880 bp fragment and this confirmed the presence of construct in flax genome.

2.7. Analysis of CAD activity

CAD was assayed spectrophotometrically as described by Hawkins and Boudet (1994). The assay was carried out at 30 °C in a final volume of 0.5 ml. The reaction mixture consisting of 100 mM Tris–HCl (pH 8.8), 2 mM coniferyl alcohol, 2 mM NADP and reaction was started by adding plant extract. CAD activity was monitored at 400 nm. The protein content was determined with the Bio-Rad Bradford protein reagent dye using bovine serum albumin as standard (Bradford, 1976).

2.8. Identification of lignin precursors and phenolic acids in flax by HPLC

A 500-mg sample of the green part of the plant was subjected to hydrolysis (2 ml 2 M HCl) for 60 min in a hot water bath. Then, 2 ml 2 M NaOH and 6 ml of methanol was added, and aglicons were extracted

using an ultrasonic bath (15 min). After centrifugation at 12,000 × *g* for 10 min, the supernatants were analyzed with HPLC. The HPLC system consisted of an L-7100 pump, a Merck Hitachi L-7455 photo-diode array detector and a D-7000 HSM Multisolvent Delivery System. Phenoloacid separation was conducted on a prepacked Merck LiChroCART® 125-3 Purospher® RP-18 (5 μm) column. The mobile phase contained 80% acetonitryle in 4.5% acetic acid (reagent A) or 4.5% acetic acid (reagent B) and was delivered at a rate of 1 ml/min. The detection of caffeic and ferulic acid was at 320 nm and *p*-coumaric acid at 280 nm.

2.9. Analysis of the lignin content in transgenic flax

Determination of the total lignin content was performed via the acetyl bromide method (Iiyama and Wallis, 1990). Lignins were isolated and analyzed from different transgenic plants grown in tissue culture, and the data was compared to that for the control plants. Ten milliliters of water was added to dried green parts of the plants (10–15 mg), and the samples were heated for 1 h at 65 °C and stirred every 10 min. Then, the samples were filtered through a GF/A glass fibre filter and rinsed sequentially in water, ethanol, acetone and finally diethyl ether. The filters were then placed in glass vials and heated overnight at 70 °C. Twenty-five percent of acetyl bromide (2.5 ml) was added to each vial and the vials were placed at 50 °C for 2 h. The cooled samples were mixed with 10 ml of 2N sodium hydroxide and 12 ml of acetic acid, and left overnight. The lignin content was analyzed with UV at 280 nm. Coniferyl alcohol was used to prepare a calibration curve, and the results are presented as equivalents of coniferyl alcohol.

2.10. Analysis of the cellulose content in flax

Cellulose content was determined using a colorimetric method with anthron reagent. For sequential release of lignin, hemicelluloses and xylosans, stem samples were incubated in a mixture of nitric and acetic acid (1:8 v/v, for 1 h at 100 °C), and then centrifuged. The pellet was washed twice with water (0.5 ml) and resuspended in 1 ml of 67% H_2SO_4 (v/v). The cellulose level was determined by spectrophotometry at 620 nm against cold anthrone reagent (Updegraff, 1969). The cellulose content was measured in control and transgenic plants from tissue culture.

2.11. Tensile testing

Tensile tests of fully hydrated stem specimens were conducted using the computer-driven Instron system (model 5542, High Wycombe, UK). Ten-millimeter long sections were cut from the basal part of the stems of plants grown *in vitro* and fixed between clamps covered with abrasive paper at an initial distance of 5.00 mm. Each specimen was stretched at a rate of 1 mm/min. The load–extension relationship recorded with the Instron system was the basis for the stress–strain diagram and stem material parameter calculations. Young's modulus, *E*, as determined automatically using Merlin (Instron, UK) software as the stress–strain ratio at the point of maximum slope of the stress–strain curve, corresponded to the initial linear range of the specimen deformation. The maximum stress was calculated as the ratio of the maximum tensile load, recorded by means of the Instron system, to the cross-sectional area of the stem. The cross-sectional area was evaluated at the mid-point of the specimen using a binocular microscope (Zeiss, Germany) and assuming an elliptical shape of the section. The tensile stiffness, *EA*, was calculated as the product of the Young's modulus, *E*, and the cross-section area, *A*.

2.12. The infection tests

Infection tests were performed by the mycelium method on 4-week-old CAD flax plants grown in tissue culture (Wróbel-Kwiatkowska et al., 2004). The fungi were grown for 7 days at 18 °C on potato–dextrose–agar (PDA) medium. The flax plants were transferred onto a solid culture of *Fusarium oxysporum*, and after 10–14 days, the numbers of infected flax plants (roots and hypocotyls) were determined and expressed as a percentage of the total flax plants used for the experiment.

2.13. Measurements of pectin and hemicellulose contents by GC–MS

Frozen plant tissue (100–150 mg) from tissue culture was powdered in liquid nitrogen, extracted with MeOH (14 ml g^{-1} FW), heated for 15 min at 70 °C

and centrifuged for 10 min at 14,000 rpm (Roesner et al., 2000). One thousand and five hundred microliters of water was added to the samples, which were then extracted with 750 μl of $CHCl_3$. A portion of the water phase was dried under a vacuum and used for derivatisation. Ribitol (120 $\mu g\ g^{-1}$ FW) was used as an internal standard added directly to the sample homogenate.

The dried extracts were mixed with methoxyamine hydrochloride (20 mg ml^{-1}) and incubated for 120 min at 37 °C. Then, the samples were derivatised with 70 μl *N*-methyl-*N*-trimethylsilyltrifluoroacetamide at 37 °C for 30 min and analyzed using GC–MS (Wróbel-Kwiatkowska et al., 2004). The GC–MS system consisted of a GC 8000 gas chromatograph, an AS 2000 autosampler, and a Voyager quadropole mass spectrometer (ThermoQuest, Manchester, UK). The chromatograms and mass spectra were evaluated using the MASSLAB program (ThermoQuest, Manchester, UK). The retention time and mass spectral library for peak quantification of metabolite derivatives was implemented within the MASSLAB method format.

2.14. Immunocytochemical detection of carbohydrate antigens in the cell walls

Sections of the flax stems were cut with a razor blade and fixed for 1 h in a mixture of 4% paraformaldehyde in 0.2 M phosphate buffer (PBS), pH 7.4. These sections were blocked in 5% BSA in 0.01 M PBS buffer, pH 7.3, to block the sites of non-specific antibody binding. After that, the sections were incubated for 12 h with the primary antibodies JIM7 and LM5 diluted in 0.01 M PBS containing 5% BSA. JIM7 binds to the pectin HGA (homogalacturonan), which shows 15–80% esterification and contains epitopes composed of methyl esterified residues of GalA (galacturonic acid) with adjacent or flanking unesterified residues (Willats et al., 2000; Clausen et al., 2003). LM5 detects four (1 → 4)-β-D-galactose residues (RG-I, rhamnogalacturonan I) (Jones et al., 1997).

The analyzed flax sections were washed in several changes of buffer (three times in 0.01 M PBS with 0.1% BSA and once in 0.01 M PBS containing 0.2% BSA). After that, the sections were incubated in darkness with secondary goat anti-rat antibodies conjugated to FITC (fluorescein isothiocyanate), diluted 1:40 (v/v) in a buffer of 0.01 M PBS with 0.2% BSA. The presence and abundance of carbohydrate epitopes was determined after covering the sections with antifade solution, i.e. 0.5% *p*-phenyldiamine in 0.01 M PBS, pH 11.7, mixed with glycerine 1:4 (v/v), using an IX70 Olympus fluorescence microscope equipped with a blue filter (excitation 490 nm, emission 525 nm). The analysis of picture was performed and registered with the Fluoview 500 programme.

3. Results and discussion

3.1. Generation and selection of transgenic flax plants

The construct pHellsgate2, designed for gene silencing in plants (Wesley et al., 2001) and containing two fragments of the *CAD* gene (Fig. 2a), was introduced into flax plants via the *Agrobacterium* transformation method. The presence of the cauliflower mosaic virus 35S promoter (35S CaMV) in this construct gives a strong and constitutive expression of the gene, and the insertion of sense and antisense fragments of the *CAD* gene guarantees the formation of hairpin RNA structures, which ensure efficient *CAD* gene silencing.

After transformation, cells were regenerated and the regenerants were pre-screened using the PCR method with specific primers for the 35S CaMV promoter (Fig. 2b). Unfortunately, the insertion of the T-DNA of the pHellsgate2-CAD construct into the flax genome revealed a negative regeneration and phenotype effect. Thirty percent of regenerants showed stunted growth and senesced after reaching a few milimeters in height (Fig. 3). Only plants which exhibited normal growth were taken for further analysis. From this group, 11 positive transgenic lines were obtained and used for selection based on the results of northern blot analysis.

The total RNA isolated from different plants grown in tissue culture was blotted onto nitrocellulose and probed with cDNA encoding a 480-bp fragment of the CAD gene, labelled with ^{32}P.

Two transgenic lines (CAD27 and CAD33), with different levels of *CAD* silencing compared to wild-type plants, were chosen for further analysis (Fig. 2d). CAD27 had the highest level of *CAD* gene silencing. The regenerants which did not survive after transformation did not show even trace amounts of CAD mRNA

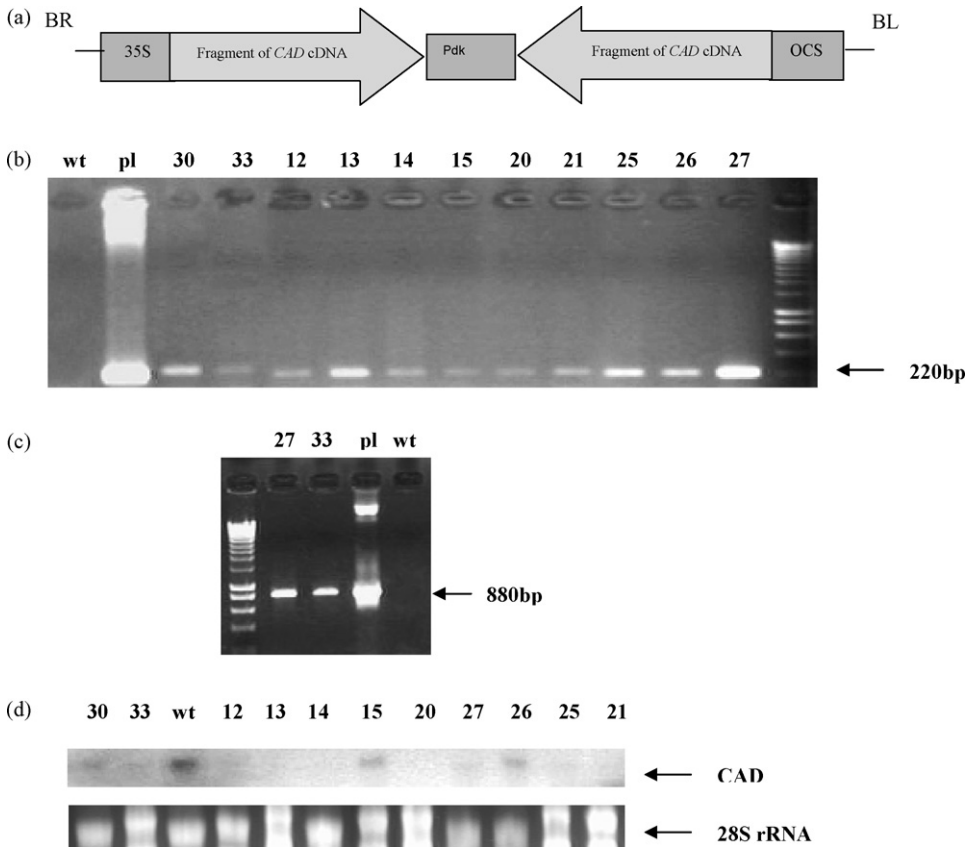

Fig. 2. (a) T-DNA of the binary vector pHellsgate2-CAD used for flax transformation. The construct, controlled by the 35S CaMV promoter and OCS terminator, was used to silence the CAD gene in flax. BR and BL, right and left T-DNA border sequence, respectively. 35S, promoter sequence from CaMV. OCS, terminator region of the octopin synthase gene. CAD, cinnamyl alcohol dehydrogenase from flax. CAM, the chloramphenicol resistance gene, localized in the intron, ensuring the selection of transformants with an intron. (b) Analysis of transgene integration by PCR. A fragment of the 35S CaMV promoter was investigated in the genomic DNA of transformed plants with specific primers. The expected length of the product was 220 bp. (c) Analysis of transgene integration by PCR method. A fragment of 35S CaMV promoter fused to *CAD* gene was amplified from genomic DNA as template. Two selected lines (CAD27 and CAD33) and wild-type plants were investigated. The expected length of the product (880 bp) was detected in transgenic lines. (d) An analysis of transgene expression by the Northern blot method. The total RNA was isolated from tissue-cultured flax plants and hybridised to a cDNA encoding fragment of the CAD gene as a probe. Fifty micrograms of total RNA was loaded into each lane, which was proved by gel staining with ethidium bromide. 28S ribosomal RNA is shown.

(data not shown). In CAD33, a transcript for *CAD* was found, but the level of produced mRNA was lower than in the control plants. The integration of the transgene in two selected lines (CAD27 and CAD33) was also confirmed by second PCR reaction with forward primer specific for 35S CaMV promoter and reverse primer specific to the fused *CAD* gene (Fig. 2c). Two selected lines (CAD27 and CAD33) showed expected product (880 bp), which was not found in untransformed, wild-type plants.

3.2. Analysis of CAD activity

CAD activity was analyzed in silenced transgenic lines to investigate whether silencing of *CAD* gene was followed by reduction in the enzyme activity. The activity of CAD was measured in two selected, transgenic lines (CAD27 and CAD33) and compared to wild-type, control plants as it was described in Section 2. Decrease in CAD activity was noticed for both transgenic lines in comparison to untransformed, control plants (Fig. 4).

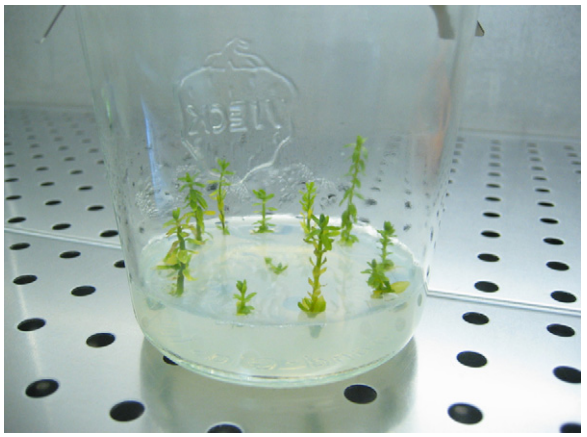

Fig. 3. The phenotype of regenerants obtained after transformation with the pHellsgate2-CAD vector. About 30% of flax plants after transformation and regeneration showed dramatically reduced growth and chlorosis (as shown in the picture), and did not survive. Only plants with normal growth were taken for all the further analyses.

The highest reduction in CAD activity was observed for line CAD27, which also showed the lowest *CAD* transcript level. Transgenic line CAD27 exhibited about 60% residual CAD activity, while CAD33 has approximately 80% residual CAD activity. These data revealed that silencing of *CAD* gene caused decrease in enzyme (CAD) activity.

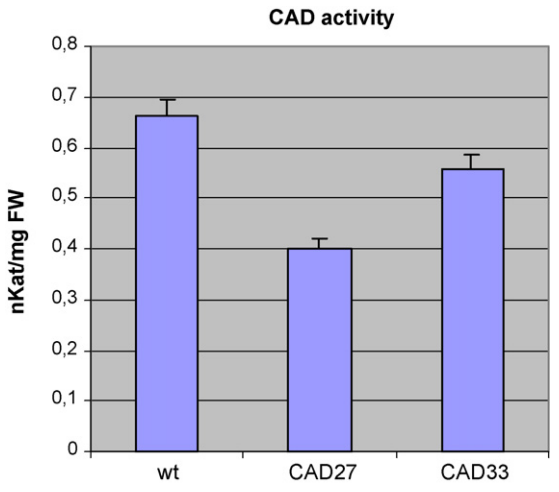

Fig. 4. CAD activity in the protein extract from two transgenic lines (CAD27 and CAD33) and wild-type plants. Enzyme assay was performed as it was described in Section 2. Data (±S.D.) was obtained from three to six samples per line.

3.3. Identification of lignin precursors and phenolic compounds by HPLC and GC–MS in CAD-deficient plants

The phenylpropanoid pathway in plants yields precursors of phenolic compounds, including lignins, and the flux of compounds between different routes of the phenylpropanoid pathway was reported on (Matsuda et al., 2005). Therefore, their levels in transgenic flax plants were measured. The amount of phenolic acids increased in all the analyzed transgenic lines, with the exception of chlorogenic acid in CAD27 (Fig. 5). An increase in the *p*-coumaric and ferulic acid contents (lignin precursors) was observed (Fig. 5). An almost two-fold increase in the ferulic acid content and a 30% increase in *p*-coumaric acid were detected in CAD27 plants. CAD33 showed the highest level of chlorogenic acid: almost two-fold higher than for non-transformants. Thus, the lack of the CAD enzyme in transgenic plants led to an increased accumulation of lignin precursors.

3.4. Analysis of lignin and cellulose contents in transgenic flax

The total lignin content was determined using the acetyl bromide method as described in Section 2. We measured a 40% decrease in the total lignin content in CAD27 relative to the wild-type plants and a 16% reduction in CAD33 plants (Fig. 6).

Analysis of lignin content is in agreement with data of enzyme (CAD) activity. The highest reduction in enzyme activity (observed for CAD27 line) was accompanied by the highest decrease in its product, i.e. lignin content. Thus silencing of *CAD* was effective in lignin engineering in flax.

At 60–70% of the fibre weight, cellulose is a major component of mature flax fibres (Gorshkova et al., 1996). The level of this metabolite was assessed in transgenic flax plants and compared to the level for wild-type plants. The β-1,4 links between the sugar units in cellulose resulted in strong and stable fibres (Vincent, 1999). The theoretical modulus of the cellulose molecule is 250 GPa, but the experimental data showed approximately 130 GPa. The chemical and physical properties of cellulose make it comparable with other engineering materials (Vincent, 1999). No statistically important changes in cellulose level were

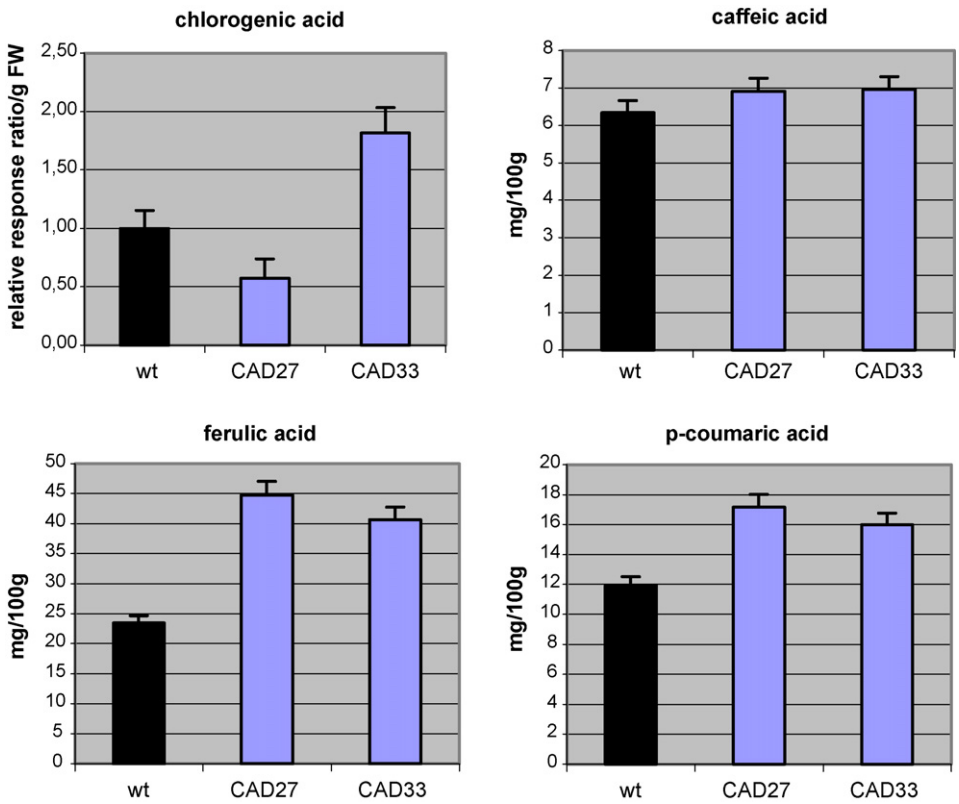

Fig. 5. Determination of lignin precursor and phenolic acid levels by HPLC and GC–MS. The total amount of *p*-coumaric, ferulic and caffeic acids was determined by HPLC. The level of chlorogenic acid was measured by GC–MS. Data (±S.D.) was obtained from three samples per line. Sinapic acid was not detectable.

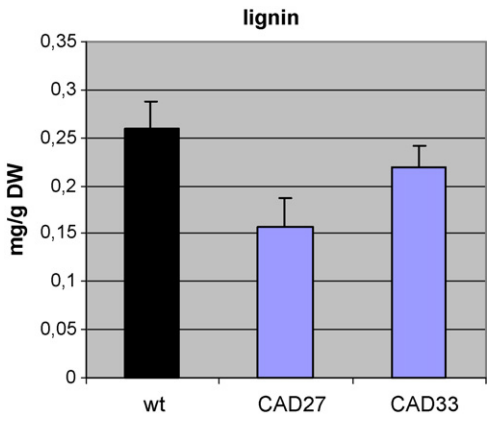

Fig. 6. Determination of the lignin level in control (wild-type) and transgenic plants (numbered). The lignin content was measured using the acetyl bromide method. Data (±S.D.) was obtained from three to six samples per line.

found in the analyzed plants from the two transgenic lines in comparison to the untransformed control plants (Fig. 7). Thus, lignin manipulation through *CAD* silencing did not have any effect on cellulose content. This is very important from an industrial point of view, because the flax stem is a source of fibres used by the textile industry. It was recently reported that the manipulation of another enzyme in the phenylpropanoid pathway affects cellulose synthesis. The antisense inhibition of the 4-coumarate:CoA ligase gene in transgenic quaking aspen led to a reduction in the lignin content by about 45% concomitant with an increase in the cellulose content by about 15% (Hu et al., 1999). The different response of flax and trees to lignin content reduction might result from species specificity with respect to the secondary metabolism.

The analysis of the cellulose and lignin contents in transgenic and control flax plants revealed that lignin

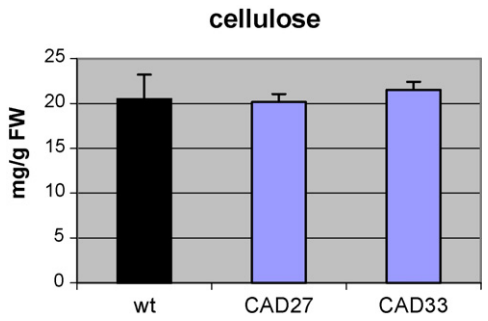

Fig. 7. The cellulose content in transgenic plants (numbered) in comparison to control plants (wild-type). The measurements were conducted as specified in Section 2. Data (±S.D.) was obtained from three to six samples per line.

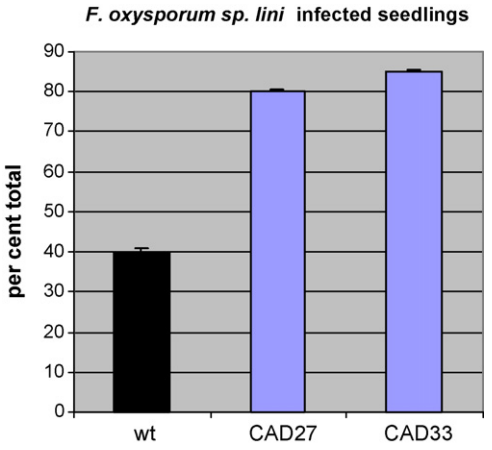

Fig. 9. The infection tests of the CAD plants to *F. oxysporum* in comparison to the non-transformants (wild-type). The results of the infection were estimated 10–14 days after inoculation. The numbers of infected flax plants were determined and expressed as a percentage of the total flax plants used for the experiments. The data is the means ± S.D. and was obtained from 10 plants per line.

makes up 9.6% of the cellulose content in control plants and 5.96% and 7.78% in CAD27 and CAD33, respectively. Thus, *CAD* silencing caused changes in the lignin/cellulose ratio and resulted in about a 40% reduction of the lignin content in CAD27.

3.5. Stem tensile properties

One of the aims of this study was to investigate if the lignin decrease in CAD-deficient plants influenced their mechanical properties. The biomechanical parameters of whole plants derived from tissue culture were measured using standard tensile Instron tests. The performed tests showed improved stem mechanical properties in terms of the tension of CAD-transgenic plants grown *in vitro* relative to untransformed, wild-type plants. Two fundamental parameters were evaluated from the experimental stress–strain curve, the strength and Young's modulus. The maximum stress (load per unit area) recorded during the test, a measure of the material strength, was respectively equal to 1.66 and 1.87 MPa for CAD27 and CAD33, while its value for the untransformed control plants was only 1.39 (Fig. 8). The gene manipulation had a more prominent impact on Young's modulus, *E*. The CAD27 and CAD33 plants respectively displayed average stem material stiffness, expressed as the *E*, of 25.7

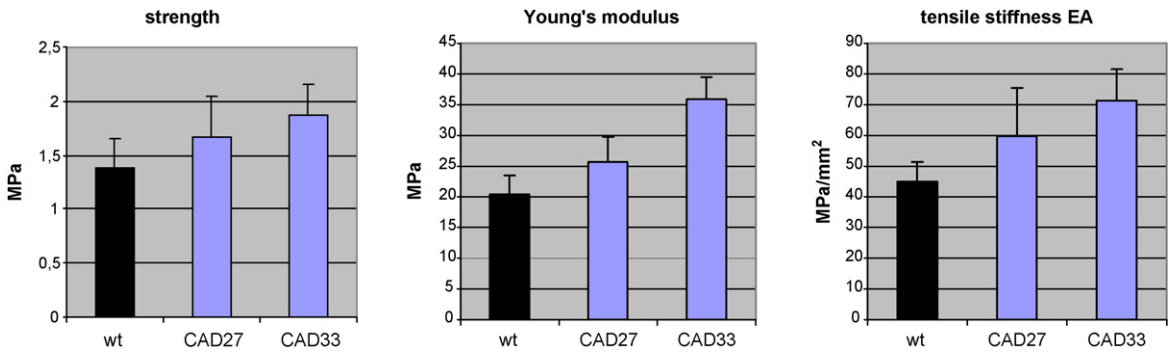

Fig. 8. The mechanical parameters measured in different transgenic CAD lines compared to the control plants (wild-type). The mechanical parameters of the transgenic plants were measured as indicated in Section 2. The data is the means ± S.D. and was obtained from five plants per line.

and 35.9 MPa, i.e. values 26% and 76% higher than the wild-type ($E = 20.4$ MPa) (Fig. 8). Similar effects were confirmed in another experiment (data not shown). The transgenic and control, wild-type plants were also characterized with respect to the stem stiffness, EA, defined as the product of Young's modulus, E, and the cross-section area, A, which is a measure of the resistance of the stem as a structure against tension (Fig. 8).

Theoretically, beneficial mechanical modifications may result from modifications of the following stem properties: (i) an increased relative amount of reinforcing tissues and (ii) improved mechanical properties of fibres. The results of this study do not allow a conclusion to be drawn as to the extent to which the changes in tissue arrangement or cell wall composition are responsible for the modification. However, the alteration in stem strength and stiffness corresponded to an increased ratio of cellulose to lignin and cellulose to pectins in the stems of the transgenic plants relative to the non-transformed ones. Cellulose is the fibrous component of the cell wall considered a composite, while hemicellulose, pectins and lignins are matrix components. The fibrous element contributes the most efficiently to the tensile resistance and the capability to carry tensile loads. An increased proportion of the fibrous element to the matrix components

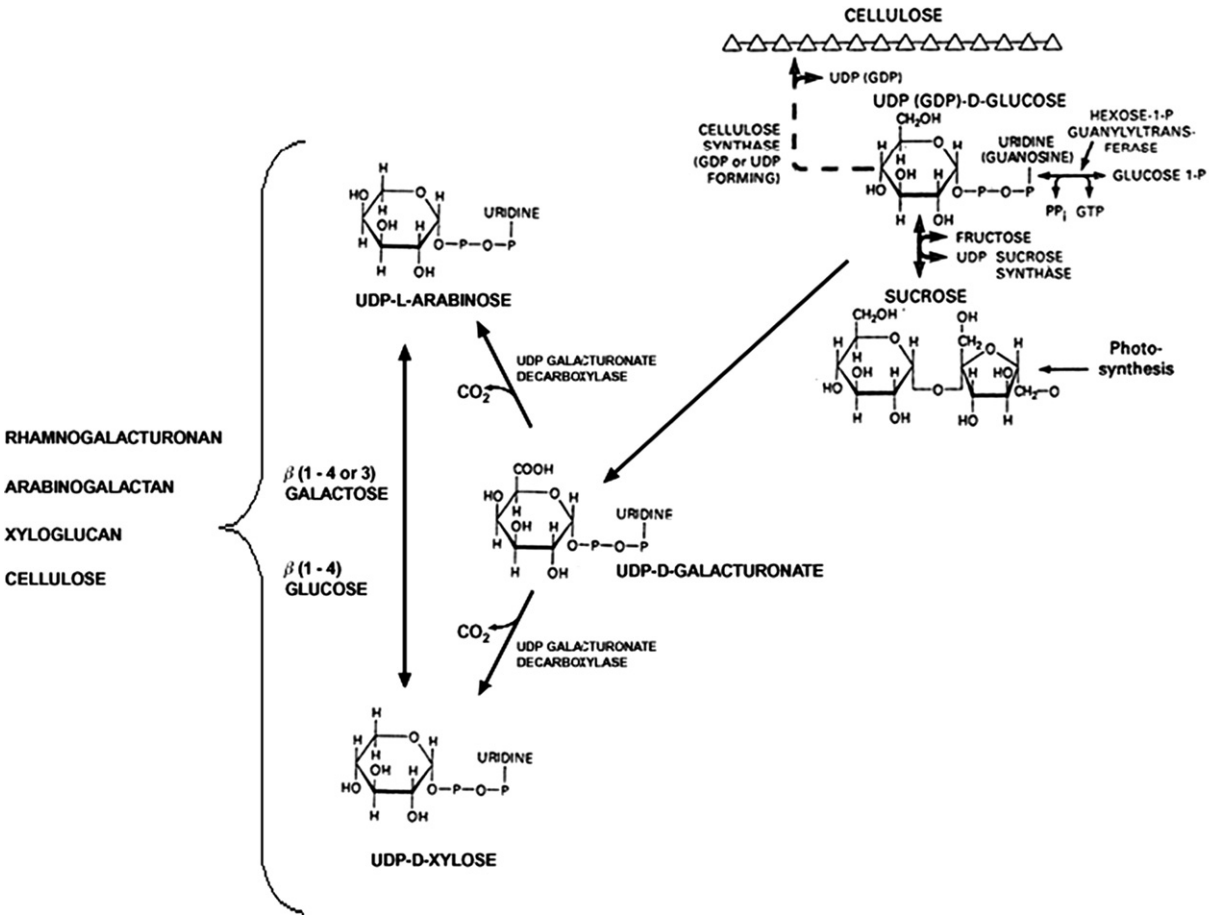

Fig. 10. Formation of compounds used for the synthesis of cell walls in plants. Cell walls consist of cellulose fibres, hemicelluloses (xylans, xyloglucans, derivatives of mannose, galactose, and fucose) and arabinogalactans which bind to cellulose. Pectins contain acidic sugars (galacturonic acid) and neutral sugars (rhamnose, galactose, arabinose), glue cells together, support negative charges, which bind Ca^{2+} and Mg^{2+}, and are highly hydrated. Lignins, phenolic polymers of lignols, are distributed in secondary cell walls.

(lignins, hemicelluloses, pectins), as found in the CAD-transgenic plants, reasonably explains the observed improvement in the stem mechanical properties. The strength and stiffness of the stem measured in the flax plants grown in culture are comparable in magnitude to the properties of highly watered soft tissues, but not to the mature flax fibres used in the industry. Therefore, this data may be considered only as an indication that the used genetic technology may potentially improve the mechanical properties of flax produced by mature plants grown in the field. Our further studies aimed to prove this thesis.

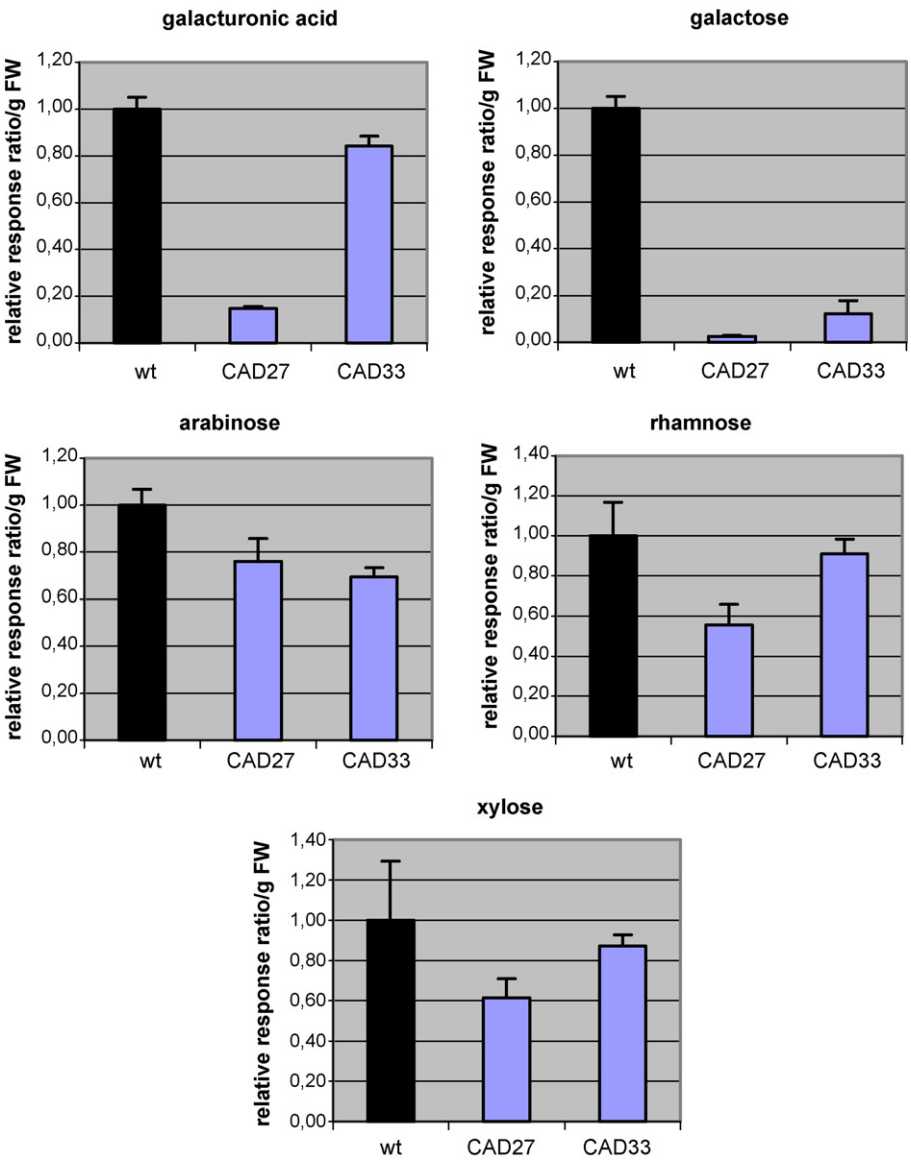

Fig. 11. An analysis of pectin and hemicellulose constituents in control (wild-type) and transgenic plants (numbered) measured by GC–MS. The level of hemicellulose (galactose, xylose) and the content of pectin constituents (galacturonic acid, rhamnose, galactose, arabinose) were determined as described in Section 2. Data (±S.D.) was obtained from three samples per line and expressed as the relative response ratio per g FW.

3.6. Transgenic flax resistance to F. oxysporum

Transgenic flax plants were analyzed using the mycelium method (Wróbel-Kwiatkowska et al., 2004), and their resistance to *F. oxysporum* was measured. The percentage of infected transgenic flax plants was higher than for the control plants (Fig. 9). The two tested transgenic lines (CAD27, 33) showed almost the same level of resistance to *F. oxysporum* infection, and they were about two-fold less resistant than the control plants.

Since *Fusarium* species are known to be retting organisms producing pectinases, xylanases, mannanases and cellulases (Henriksson et al., 1997), the noticed decrease in the resistance of the CAD-deficient plants may lead to an improvement in flax retting. However, this needs to be verified using field-grown plants.

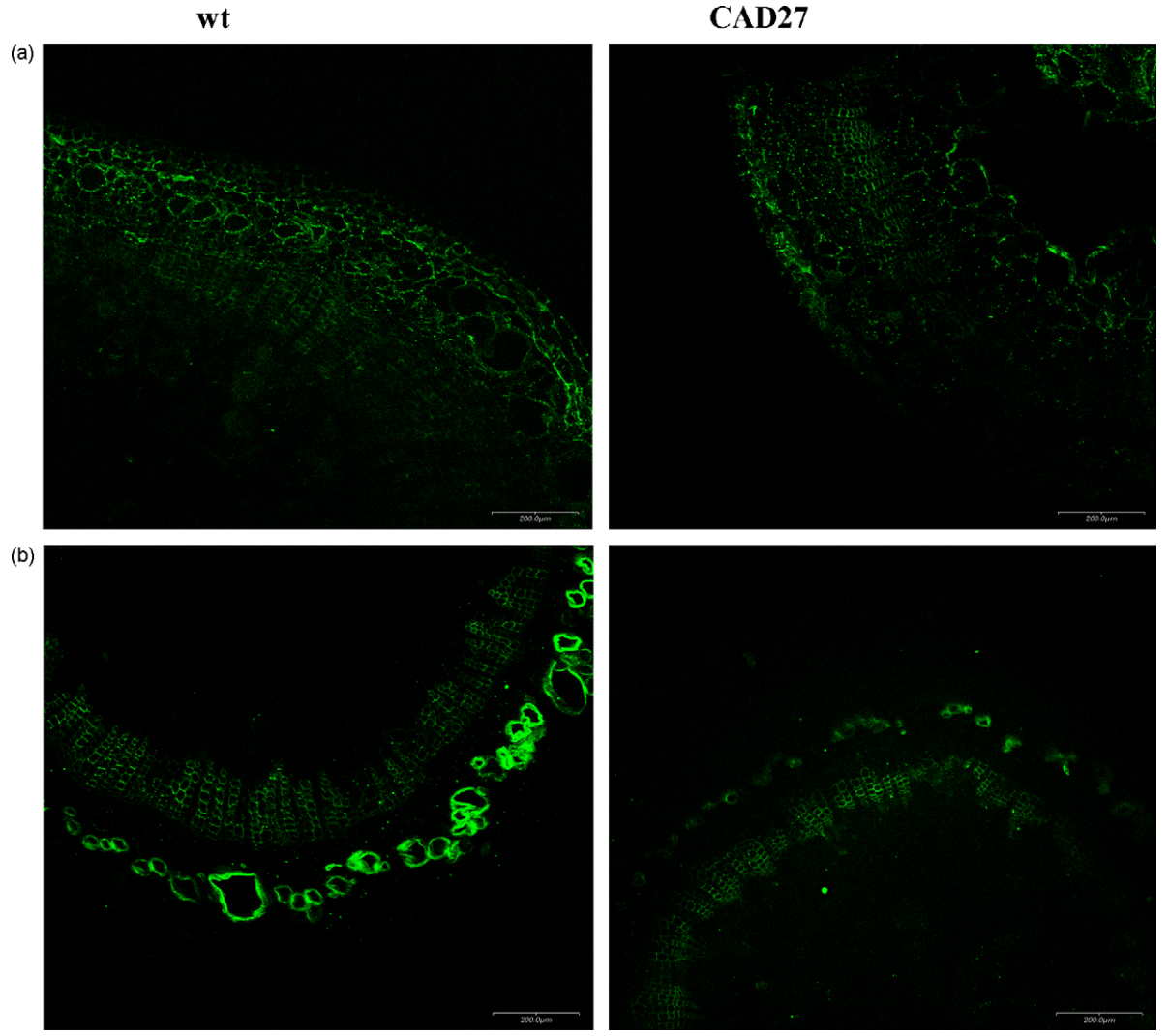

Fig. 12. Immunolabelling of flax stem cells with the JIM7 (a) and LM5 (b) antibodies revealed the respective distributions of galacturonic acid (a) and galactose (b) epitopes as indicated by green fluorescence. The examined cells derived from the CAD27 transgenic line showed relatively lower pectin and hemicellulose contents when compared to the wild-type plants. The plants were derived from a 30-day *in vitro* culture, grown on media containing MS with 0.8% agar and 1% sucrose in 16 h day/8 h night conditions, at 21 °C/16 °C. Resolution 100×, exposure time: 2.0 s. The immunodetection was performed as described in Section 2.

The observed changes in flax resistance concurred with the known role of lignin in providing resistance and a physical barrier to pathogen infection (Carver et al., 1994; Cabane et al., 2004; Tobias and Chow, 2005). The reduced quantity of lignin in the CAD plants resulted in an increased susceptibility of those plants to pathogen infection.

3.7. The content of pectin and hemicellulose constituents in transgenic flax

The manipulation of lignin synthesis through *CAD* silencing has broad consequences for the content of non-cellulosic carbohydrates, i.e. pectins and hemicellulose (Figs. 10 and 11).

There was an effect on the quantity of carbohydrates, constituents of hemicelluloses. The level of xylose and galactose was reduced in the transgenic lines, primarily in CAD27 plants (Fig. 11).

The constituents of pectins were also affected in the generated transgenic lines. The content of rhamnose, arabinose, galacturonic acid and galactose was decreased in transgenic flax plants. The highest reduction in the pectin content was observed for CAD27, which exhibited the lowest measured level of galactose, galacturonic acid, and rhamnose (Fig. 11). Galacturonic acid was reduced more than 5-fold, while a 10-fold reduction in the amount of galactose was detected. As hemicellulose and especially pectins are degraded during retting by microorganisms, the observed reduction in their components might have a positive effect on the release of flax fibres from the stems.

It should be pointed out that the analyses of all the metabolites were performed on sterile plants derived from tissue cultures, in order to eliminate the influence of any biotic or abiotic stresses on the metabolic pattern.

3.8. Immunodetection of carbohydrate antigens in the cell walls of CAD-deficient plants

Since the data from the metabolic profiling showed a decrease in the level of carbohydrates, which are constituents of pectin and hemicellulose in CAD flax plants, we investigated the occurrence of two carbohydrate epitopes: galacturonic acid (GalA) and (1 → 4)-β-D-galactose, typical constituents of pectin and hemicellulose.

Tissue-cultured transgenic flax plants from line CAD27 and wild-type plants were used as the donor material for the immunocytochemical detection of selected antigens. Immunolabelling with the JIM7 antibody revealed widespread distribution of the analyzed epitopes in flax stem cross-sections, as indicated by green fluorescence (Fig. 12a). Sections of the stems in wild-type plants showed a higher GalA content than CAD27 plants. Strong labelling of cells was observed in the xylem, phloem and parenchyma.

Immunolabelling of flax stem cells with the LM5 antibody also gave a strong signal, but only in the phloem of flax fibres and xylem cells (Fig. 12b). The difference in the relative content of (1 → 4)-β-D-galactose residues in CAD27 and control plants was observed.

Our observations provide evidence that the transgenic line CAD27 exhibits lower galactose and galacturonic acid contents. The immunolabelling pattern of flax stems with both antibodies (JIM7, LM5) is in agreement with the chemical data, which showed a more than 5-fold reduction in galacturonic acid and a 10-fold reduction in galactose content in the transgenic lines.

4. Conclusion

Silencing of the cinnamyl alcohol dehydrogenase (*CAD*) gene was used for lignin manipulation in flax plants. The flax *CAD* gene was cloned into an RNAi construct (pHellsgate2) and introduced to the genome of a fibrous cultivar of flax plants (*L. usitatissimum* L., cv. Nike).

Silencing of *CAD* resulted in decrease of CAD activity (by about 20–40%) and lignin content (transgenic line CAD27 showed reduced total lignin amount by about 40%) and caused changes in the composition of the cell walls. A lower content of the constituents of pectin and hemicellulose was also observed. However, lignin engineering does not affect the cellulose level.

The reduction in the lignin content in CAD-deficient plants resulted in an improvement in the elastic properties of flax plants; transgenic plants showed increased mechanical properties in comparison to non-transformants. The increased proportion of the fibrous element (cellulose) to the matrix components (lignins, hemicelluloses, pectins) in the cell wall found in CAD-

transgenic plants may explain the noticed improvement in the mechanical properties of the stem.

Unfortunately, the decreased level of lignin led to about a two-fold lower resistance to fungi. The tested pathogen *F. oxysporum* is involved in the retting process. The increased susceptibility to this pathogen might however be the reason for the easier release of flax fibres during retting.

The genetic manipulations done in this study might have potential commercial significance for the textile industry, i.e. in the improvement of the elastic properties of flax fibres and in the retting process.

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