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Engineering flax with increased flavonoid content and thus *Fusarium* resistance

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Abstract

Flavonoids are a group of secondary plant metabolites important for plant growth and development, and thus the regulation of their biosynthesis is of special interest. We used a transgenic approach for flavonoid content manipulation. The multigene construct contained the cDNAs for chalcone synthase (CHS), and chalcone isomerase (CHI) and dihydroflavonol reductase (DFR) were prepared. Following flax plants transformation, the levels of the products of the enzyme overproduction were assessed in leaves and seeds. The simultaneous expression of genes resulted in a significant increase in the levels of flavanones, flavones, flavonols and anthocyanins, suggesting those three overproducing enzymes efficiently control the flavonoid route of the phenylpropanoid pathway.

The increase in the flavonoid content in the transgenic flax plants might be the reason for observed, enhanced antioxidant capacity of those plants. The increased antioxidative properties of transgenic plants lead to improved resistance to *Fusarium*, the main pathogen of flax.

The changes in phenylpropanoids accumulation in transgenic plants affect cell wall carbohydrate content. Immunochemical studies revealed significant increase in carbohydrates, constituents of pectin and hemicellulose. Since pectins contribute to flax stem retting, the compounds increase might affect fibre production. An increase in pectin and hemicellulose content leads to enhanced disease resistance of those plants.

Keywords: Flax; Flavonoids; Antioxidant capacity; *Fusarium*; *Linum usitatissimum* L

1. Introduction

Plant phenylpropanoids are a broad and diverse group of low molecular weight secondary metabolites that include flavonoids, phenolic acids, phenols, lignans and tannins. To date, over 9000 flavonoids have been identified and this number is still increasing [1]. The pathway is initiated by the deamination of phenylalanine to cinnamic acid, and the following reactions lead to the formation of hydroxycinnamoyl CoA thioester, which is then the substrate for the branch pathways of flavonoid, lignin monomer, coumarin and simple ester (e.g. chlorogenic acid) synthesis [2]. Flavonoids constitute a class of phenylpropanoid; this metabolic route starts with the synthesis of chalcone in a reaction controlled by chalcone synthase (CHS), goes via flavanone and flavonol (the products of chalcone isomerase (CHI) action) and flavan (the product of dihydroflavonol reductase (DFR) activity) and ends with the synthesis of anthocyanidin and proanthocyanidin. The great diversity of these compounds results also from combinatorial

Abbreviations: CHS, chalcone synthase; CHI, chalcone isomerase; DFR, dihydroflavonol reductase; IC_{50}, the antioxidant potential; AAPH, 2, 2′-azobis (2-amidinopropane) dihydrochloride; HGA, homogalacturonan; RG-I, rhamnogalacturonan I

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modifications, such as glycosylation by glycosyltransferases, acylation by acyl transferases, hydroxylation by hydroxylases and methylation by methyl transferases [3]. The multitude of structures implies variable functions. Flavonoids are involved in many aspects of plant growth and development. They act as antioxidants, chelators of divalent cations [4], photoreceptors and visual attractors [5]. Flavonoids protect plants against pathogenic microorganisms [6], herbivores, UV radiation [7], and oxidative and temperature stresses. It has been reported that flavonoids, in particular flavonols, exhibit health-protecting activities, thanks to their strong antioxidant properties [5]. Their antioxidant activity has a great significance for food quality, because they may inhibit enzymatic and non-enzymatic peroxidation [8]. In addition, flavonoids have anti-allergenic, anti-viral, anti-inflammatory and vasodilatory activities [5,9].

All these features make them attractive targets for genetic engineering strategies. It is believed that an increased level of flavonoids strengthens a plant's defense against biotic and abiotic stresses. For example, it was noticed that transgenic tobacco with a suppressed level of phenolic compounds has shown increased disease susceptibility [10]. Knowing physiological functions of flavonoids, we have made an attempt to investigate the influence of increased content of these compounds on flax metabolism and plant resistance.

The goal of this study was to engineer the flavonoid biosynthesis route in flax plant via the simultaneous overexpression of three cDNAs, encoding CHS, CHI and DFR. Flax plant has a long history of traditional use both as a source of fibre and oil. Flax is grown for commercial use in over 30 countries of the world. In Poland, flax is the most important industrial oil and fibre crop. Flax diseases caused by fungal pathogen appeared the primary factor limiting plant production. Serious yield losses due to pathogen infection is reported. Thus, the other aim was to analyze the impact of flavonoids on flax resistance to pathogen infection. It was expected that the increase in antioxidant properties of transgenic plants might cause their resistance to *Fusarium* infection, the major fungal pathogen infecting flax plants, which caused the greatest yield decline.

2. Materials and methods

2.1. Plant material

Flax seeds (cv. *Linola*) were obtained from the Flax and Hemp Collection of the Institute of Natural Fibres, Poland. The transgenic and control plants were grown in tissue culture on MS medium [11] supplemented with 0.8% agar, 1% sucrose and 250 mg/L claforan. The control means the plants transformed with the empty vector. The transgenic and control plants were grown in tissue culture and for analysis the control and selected transgenic plants were transferred to the soil and cultivated in a greenhouse under a 16 h light (21 °C), 8 h darkness (16 °C) regime for acclimatization. The plants were grown in soil in individual pots and were watered daily for about 3 weeks. Then, plants were transferred and grown in a field, and seeds were harvested 3 months after the transfer of the tissue-cultured plants to the soil.

2.2. Transgenic plant construction and selection

Two-week-old cotyledon and hypocotyl explants were transformed. For transformation, we used the binary vector containing three cDNAs from *Petunia hybrida*, CHS (EMBL/GenBank database acc. no. X04080), CHI (EMBL/GenBank database acc. no. X14589) and DFR (EMBL/GenBank database acc. no. X15537) in the sense orientation under the control of the 35S promoter and OCS terminator [12].

The vector was introduced into *Agrobacterium tumefaciens* strain C58C1:pGV2260 by using a gene pulser (BioRad) at 2500 V. *A. tumefaciens*-inoculated explants were subsequently transferred to a callus induction and shoot regeneration medium [13,35].

The transgenic plants were preselected via PCR using primers specific for the kanamycin resistance gene (*npt II*), and then selected by means of northern blot analysis. PCR was carried out using specific primers for the neomycine phosphotransferase gene (forward, CCGACCTGTCCGGTGCCC; reverse, CGCCACACCCAGCCGGCC) on genomic DNA isolated from 3-week-old tissue-cultured plants as a template. For PCR analysis, 1 μL of template (0.5 μg) was mixed with 1 μL of each primers (50 μM), 0.5 μL dNTP (40 mM), 2.5 μL of 10 × buffer (including 2 mM $MgCl_2$), 0.2 μL Taq polymerase (5 U/μL) and 18.8 μL of sterile water. Then, the amplification reaction was carried out in Biometra (UNO II) with preliminary denaturation at 94 °C for 3 min, followed by 25 cycles of 94 °C for 45 s, 67 °C for 45 s, and 72 °C for 1 min, with a final extension at 72 °C for 5 min.

Total RNA was prepared from frozen young leaves using the guanidinium hydrochloride method [14]. Total RNA was separated on an agarose gel [1.5% (w/v) agarose, 15% (v/v) formaldehyde] and blotted onto a Hybond N^+ (Amersham) filter. The membrane was hybridized overnight at 42 °C with ^{32}P-radiolabeled cDNAs (CHS, CHI and DFR) as probes and then washed three times with SSPE buffer containing 0.1% SDS for 30 min at 42 °C.

2.3. HPLC analysis of flavonoid content in transgenic flax plants

Grounded, dry green parts of flax (200 mg) were extracted with 7 mL of methanol/water (30/70, v/v) mixture contained 1 g/L L-ascorbic acid as antioxidant by sonication for 10 min at room temperature, shaking occasionally (BAS-10, Poland). A 1 g sample of flax seeds was extracted with 7 mL 35% aqueous methanol containing 1 g/L L-ascorbic acid, for 18 h at 20 °C in glass screw-capped

vials, and then sonificated at RT for 15 min. Next, the samples were centrifuged (5 min, 19,000*g*) and the clear supernatant was injected onto a HPLC column. The analysis of flavone and flavonol derivatives were carried out on a Merck-Hitachi L-7455 liquid chromatograph with a diode array detector (DAD) and quaternary pump L-7100 equipped with D-7000 HSM Multisolvent Delivery System (Merck-Hitachi, Tokyo, Japan) and an L-7200 autosampler. Separation was performed on a Synergi Fusion RP-80A 150 × 4.6 mm (4 μm) Phenomenex (Torrance, CA, USA) C-18 reverse-phase column. The oven temperature was set to 20 °C. The mobile phase was composed of solvent A (2.5% acetic acid) and solvent B (acetonitrile). The program began with a linear gradient from 0% B to 25% B at 36 min, followed by washing and reconditioning of the column. The flow rate was 1.0 mL/min, and the runs were monitored at the following wavelengths: flavone at 340 nm and flavonol derivatives at 360 nm. Photo-diode array (PAD) spectra were measured within wavelength range 200–600 nm at 2 nm interval. Retention times and spectra were compared with those of pure standards within 200–600 nm. Standards (apigenin, quercetin and kaempferol) were from Extrasynthese (France). Acetic acid, L-ascorbic acid, methanol and acetonitrile were purchased from Merck (Darmstadt, Germany).

2.4. Determination of flavanone content

A 35-mg sample of dried tissue-cultured plants or 35 mg of seeds were extracted with 400 μL of methanol in an ultrasonic bath for 30 min at RT. The extract was centrifuged at 14,000*g* for 10 min. The supernatant was mixed with 400 μL of 1% 2,4-dinitrophenylhydrazine (Aldrich), and heated for 50 min at 50 °C. Then, the solution was mixed with 1 mL of 1% KOH in 70% ethanol. After centrifugation at 1000*g* for 15 min, the supernatant was filtered through Whatman No. 1 filter paper. The absorbance at 495 nm was determined. Naringenin (Sigma) was used to prepare a calibration curve. Flavanone contents are reported as naringenin equivalents [15].

2.5. Determination of the total anthocyanin content via the pH-differential method

The total anthocyanin content was measured using a modified pH differential method [16]. A 15-mg sample of dried tissue-cultured plants or 15 mg of seeds was extracted with 1 mL of methanol/HCl (95:5, v/v) in an ultrasonic bath for 30 min at RT. The extract was centrifuged at 14,000*g* for 10 min. Two dilutions of the sample were performed: first, 100 μL of the supernatant was mixed with 900 μL of 0.025 M potassium chloride buffer, pH 1.0, and then, 100 μL of supernatant was mixed with 900 μL of 0.4 M sodium acetate buffer, pH 4.5. The solution was allowed to stand at RT for 15 min, and then the absorbance at 510 and 700 nm was measured, which allowed for haze correction. Absorbance readings were converted to total milligram of cyanidin 3-glucoside equivalents per 100 g DW using the molar extinction coefficient of 26,900 and an absorbance of $A = [(A510–A700)_{pH1.0}−(A510–A700)_{pH4.5}]$ [17].

2.6. Extraction of phenolic compounds from tissue-cultured plants and seeds for antioxidant level measurement

A 0.5-g sample of 3-week-old tissue-cultured plants or 0.5 g of seeds was dried at 70 °C. The flax seeds prior to drying were defatted using hot hexane. Then phenolic compounds were extracted three times with 1 mL of 80% methanol (v/v) in an ultrasonic bath for 15 min at RT. The combined extract was centrifuged at 14,000*g* for 10 min and the supernatant dried in a Speedvac. Polyphenols were resuspended in 500 μL of methanol and used for antioxidant properties measurements.

2.7. The antioxidant capacity

The chemiluminescence method was used to determine the antioxidant activity of the extracts. A methanol extract of flax was diluted in the range of 1000–15,000 times with water, and directly analyzed according to the published method of Lukaszewicz et al. (2002) [18]. This experiment was performed in a final volume of 250 μL on white microplates in a solution containing 0.1 M Tris–HCl buffer, pH 9.0, and 4 mM 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH), freshly prepared. The luminol solution (100 μM) and diluted extract were automatically injected. The photons produced in the reaction were counted on an EG&G Berthold LB96P microplate luminometer at 30 °C. The antioxidant potential (IC_{50}) was defined as the amount of flax extract (μg DW) that which inhibits luminol chemiluminescence by 50%.

2.8. Evaluation of flax resistance to Fusarium culmorum and Fusarium oxysporum infections via the mycelium method

Flax seeds were immersed in 96% ethanol for 1 min and then washed three times with sterile water and placed on a plate. After 7 days of growth on MS medium, the seedlings were inoculated by placing them on medium with *F. culmorum* or *F. oxysporum*. The fungi were cultured for 7 days at 18 °C on potato-dextrose-agar (PDA) medium [19]; 10–14 days after transfer, the number of infected flax seedlings (roots and hypocotyls) was counted and expressed as a percentage of the total seedlings used for the experiment [20].

2.9. Growth inhibition test of F. oxysporum

Fungi was cultured for 3 days at 18 °C on solid PDA medium. The flavonoid extracts from non-transformants, transgenic lines and water as control were spotted where indicated. Extracts from tissue-cultured plants (100 mg) were prepared according to the procedure describing

extraction of phenolic compounds with the exception that the compounds were finally resolved in 500 μL of water.

2.10. Immunochemical detection of carbohydrate antigens in the cell wall

The 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. Then, the 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, sections were incubated for 12 h with the primary antibodies JIM 7 and LM 5 (PlantProbes, UK) diluted in 0.01 M PBS containing 5% BSA. The dilution of the antibodies was 1:20 (v/v) for JIM 7 and 1:5 (v/v) for LM 5. JIM 7 binds to the pectin HGA (homogalacturonan), showing 15–80% esterification. The recognized epitopes are composed of methyl esterified residues of galacturonic acid with adjacent or flanking unesterified residues [21,22]. LM 5 detects four (1→4)-β-D-galactose residues [23]. The analyzed flax sections were then washed in several changes of buffer (0.01 M PBS with 0.1% BSA three times and 0.01 M PBS containing 0.2% BSA once). After that, sections were incubated in darkness with secondary goat anti-rat antibodies conjugated to FITC (fluorescein isothiocyanate), antibodies were diluted 1:40 (v/v) in 0.01 M PBS buffer with 0.2% BSA. The levels of carbohydrate epitopes were determined after the sections had been covered with antifade solution, i.e. 0.5% *p*-phenyldiamine in 0.01 M PBS, pH 11.7, mixed with glycerin 1:4 (v/v), using an IX70 Olympus fluorescence microscope equipped with a blue filter (excitation 490 nm, emission 525 nm). An analysis of picture was performed and registered with the Fluorview 500 program.

2.11. Statistical analysis

To determine whether there was any difference between a control and transgenic plants the statistical post-hoc Tukey test (Statistica 6.0) was used. Values of $P<0.05$ were considered as significantly different.

3. Results

3.1. Transgenic plant generation and selection

The hypocotyl and cotyledon explants of flax plants were transformed with a multigene vector containing three cDNAs encoding key enzymes of flavonoid biosynthesis, according to the *Agrobacterium* method [13]. The construct, consisting of CHS, CHI and DFR cDNAs from *P. hybrida* under the control of CaMV 35S promoter and OCS terminator, was inserted into the genome of the flax plants [12]. *Petunia* DFR has been used for the reason that it preferentially converts dihydromyricetin to leuco-delphinidin [24]. Delphinidin 3-*O*-3-xylosylrutinoside was determined as the main pigment in *Linum grandiflorum* [25].

The obtained regenerants were prescreened using the PCR method with specific primers for the neomycin phosphotransferase gene. Plants that exhibited a 475-bp (*npt II*) DNA fragment were used for further selection by means of northern blot analysis (Fig. 1). The two transgenic lines (40 and 72) that showed the highest level of mRNA for the three introduced cDNAs were used for further analysis.

3.2. Phenotype analysis

There were no visible differences in leaf shape and size or petal and seed color when compared to the non-transformed plants. However, the obtained transgenic plants showed a higher yield of seeds per plant compared to the control plants. Transgenic plants produced more seeds per plant than non-transformed plants. The fresh weight of a single seed and the number of seeds per bag were not changed statistically, however, in case of line 72 both these parameters were higher than in the control. The fresh weight of transgenic seed per bag increased by about 23% in line 72 (statistically significant) and 19% in line 40. The data are presented in Fig. 2.

3.3. Total flavonoid content in transgenic plants

All the transgenic lines produced the respective mRNAs, suggesting full functionality of the gene construction used for flax transformation. This was then verified at the metabolic level. The final step in transgenic plant selection was the analysis of total flavonoid level (calculated as the sum of all measured flavonoid compounds) in the generated transgenic

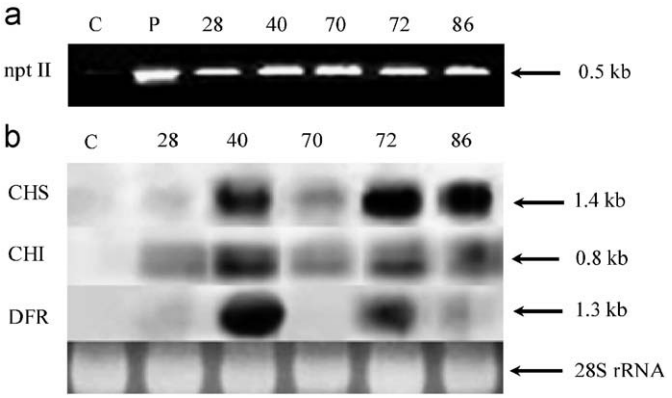

Fig. 1. (a) Agarose gel electrophoresis of *npt II* gene PCR product (475 bp). Neomycine phosphotransferase gene (*npt II*) was amplified with the use of specific primers; the template was genomic DNA isolated from tissue-cultured flax plants. C, negative control (non-transformed plant); P, positive control (plasmid containing an *npt II* gene); the different transgenic lines are numbered. (b) Northern analysis of RNA isolated from tissue-cultured plants of the control (C) and independent transgenic lines (numbered). Thirty micrograms of total RNA from each sample was loaded in each lane. The blot was hybridized with ^{32}P-labeled CHS (1.3 kb), CHI (0.8 kb) and DFR (1.4 kb) cDNAs. A ribosomal RNA stained with ethidium bromide was used as a control of RNA applied onto the gel.

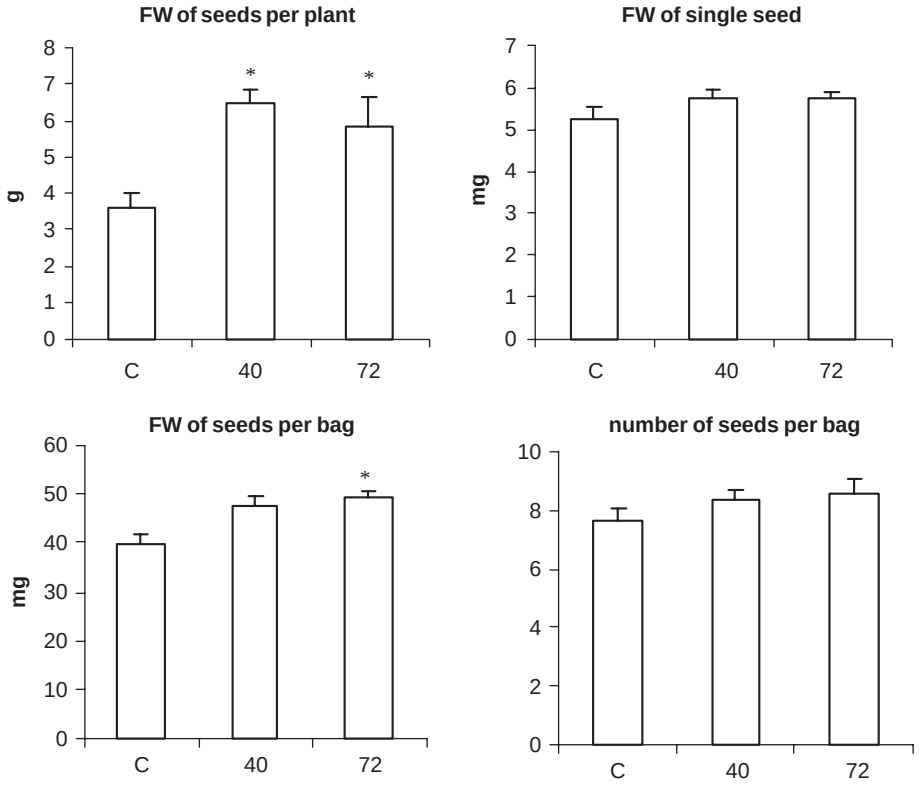

Fig. 2. The yield of flax seeds from the control (C) and transgenic lines (numbered). The mean value ($n = 30$) ± S.D. is presented. *Statistically significant ($P < 0.05$).

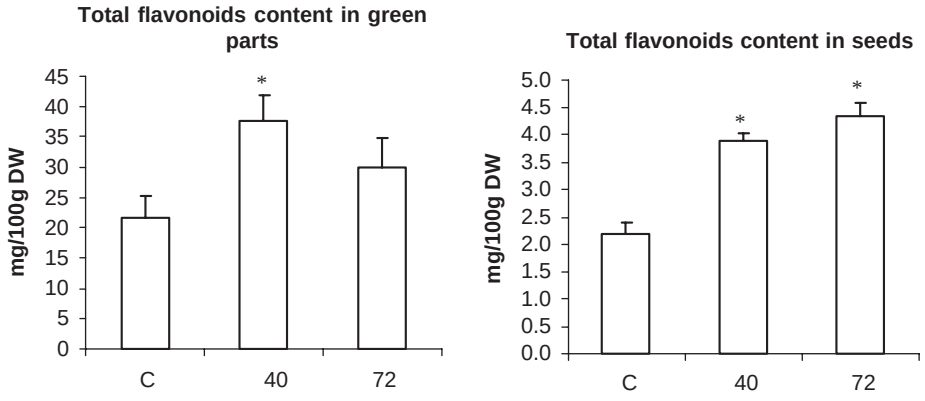

Fig. 3. Determination of total flavonoid content in the control (C) and transgenic flax plants (numbered). The mean value ($n = 4$–6) ± S.D. is presented. *Statistically significant ($P < 0.05$).

flax plants, in both the leaves and seeds. The extracts from transgenic lines overexpressing essential enzymes of the flavonoid synthesis pathway exhibited increased total flavonoids production in both green parts and seeds; the data are presented in Fig. 3. A significant relationship between the total flavonoid content and the mRNA level from the introduced cDNAs was found in the transgenic plants. The respective calculated correlation coefficients for the level of mRNAs encoding CHS, CHI, and DFR (calculated from northern blot densitometry) and total flavonoid content was 0.86 for the green parts, and 0.99 for the seeds. Thus, the increase in flavonoid level resulted from expression of the introduced cDNAs. Both transgenic lines exhibited an increased content of metabolites along the flavonoid route. Thus, the action of the three enzymes in concert increased the flux of substrates starting from flavanones and ending with anthocyanins.

3.4. Determination of flavanone content

The initial step in the flavonoid route of the phenylpropanoid pathway is the synthesis of naringenin chalcone

through condensation of three malonyl-CoA units with *p*-coumaroyl-CoA, which are derived from carbohydrate metabolism and phenylpropanoid pathway, respectively. The reaction is catalyzed by the action of CHS. The naringenin chalcone is a branch point for all flavonoid biosynthesis [26]. Chalcones are very labile compounds and could be accumulated as dihydrochalcones or rapidly converted to the colorless flavanone naringenin by the CHI.

Since our gene construction contained cDNA for CHS and CHI enzyme, the flavanone compound content was measured. The data obtained showed an increase in flavanone level in the green parts of plants and in flax seeds from plants of line 40 and line 72; the data are presented in Fig. 4.

3.5. Determination of flavone and flavonol contents

Flavanone is another branch point in the biosynthesis of flavonoids. Flavanones act as a precursor for the synthesis of flavones and 3-OH–flavanones (dihydroflavonols). Dihydroflavonols can then be converted into flavonols by FLS (flavonol synthase) or into anthocyanins by DFR (dihydroflavonol 4-reductase).

HPLC analysis of tissue cultured plants from transgenic lines (40 and 72) reveals increase in apigenin derivative (flavone) accumulation compared to the control. In line 40 and line 72 transgenic flax, the production of quercetin derivative (flavonol) increased in the green parts and in seeds. Moreover, analysis reveals increase in kaempferol derivative (flavonol) accumulation in green parts and also in seeds; the data are presented in Fig. 4.

3.6. Determination of anthocyanin content

Since the third gene in our transgenic construction was DFR, the product of its activity was measured. We have chosen the *Petunia* DFR. It is known that petunia cannot accept dihydrokaempferol as a substrate, thus limiting the type of anthocyanins produced to only di- and tri-hydroxylated. It is also well known that compounds with increased number of hydroxyl groups (di- and tri-hydroxylated) are stronger antioxidants than mono-hydroxylated and play important role in response to stress [27,28]. The direct products of DFR activity are leucoanthocyanidins. These compounds can be converted to the anthocyanidins and/or to the proanthocyanidins.

Thus, we measured the anthocyanins content in the transgenic lines and found an increase in the green parts and in the flax seeds; the data are presented in Fig. 4.

3.7. Antioxidant activity of extracts from transgenic plants

Flavonoids were shown to exhibit antioxidant properties [4]. Since there was an increase in the total flavonoid compounds detected in transgenic plants, an increase in antioxidant capacity was expected. The antioxidant properties of both the green parts (leaves and stems) and flax seeds were assessed via the chemiluminescence method, as described elsewhere [29]. The antioxidant potential is expressed as the IC_{50} value, which denotes the amount of plant extract inhibiting the oxidation of luminol by 50%. The antioxidant properties of the green parts and seeds of transgenic plants were higher than those of the non-transformed plant. The antioxidant potential of transgenic flax plants increased 106-fold in the green parts of line 40 and 14-fold in line 72. Moreover, six-fold increase of antioxidant capacity in line 40 and four-fold in line 72 in the seeds was observed. Fig. 5 shows the antioxidant level of the transgenic and control plants for both the leaves and seeds. The calculated correlation coefficients for the expression of CHS/CHI/DFR (based on northern blot densitometry) and IC_{50} were -0.89 for the green parts and -0.83 for the seeds. This data indicate that the antioxidative status of the transgenic plants mainly resulted from the activation of the flavonoid biosynthesis pathway, through the overexpression of the three key flavonoid genes.

3.8. Infection of transgenic flax plants to F. culmorum and F. oxysporum

Another goal of this study was to investigate the physiological significance of increased phenylpropanoid compound contents for transgenic plants; of greatest interest was their role in protecting against pathogen infection. It was expected that the increase in the antioxidant properties of transgenic plants might cause their higher resistance to pathogen infection. Indeed, resistance to *F. culmorum* and *F. oxysporum* was higher in the transgenic plants than in the control plants; the data are presented in Fig. 6. Moreover, the significant relationship between the flavonoid contents and resistance to *Fusarium* in the transgenic plants was assessed. In the case of *F. culmorum*, the calculated correlation coefficient was -1.00, and for *F. oxysporum* it was -0.96. These results correlate well with the antioxidative properties in the transgenic flax plants. The correlation coefficient for antioxidative capacity and pathogen infection was 0.85 for *F. oxysporum* and 0.81 for *F. culmorum*. Thus, an increased level of flavonoids yielded a better antioxidative status and resulted in efficient plant protection against pathogenicity.

3.9. Growth inhibition test of F. oxysporum

To verify the direct effect of flavonoids on fungi growth, we have grown *F. oxysporum* on PDA medium supplemented with the control and transgenic plant flavonoid extracts. It was expected that the extract from transgenic plants would be able to inhibit the growth of *Fusarium* mycelium. This was exactly the case (Fig. 7); the flavonoid extracts from transgenic plants inhibited the mycelium growth. The extract from the line 40 revealed the highest inhibition and a lower, but still significant percentage was

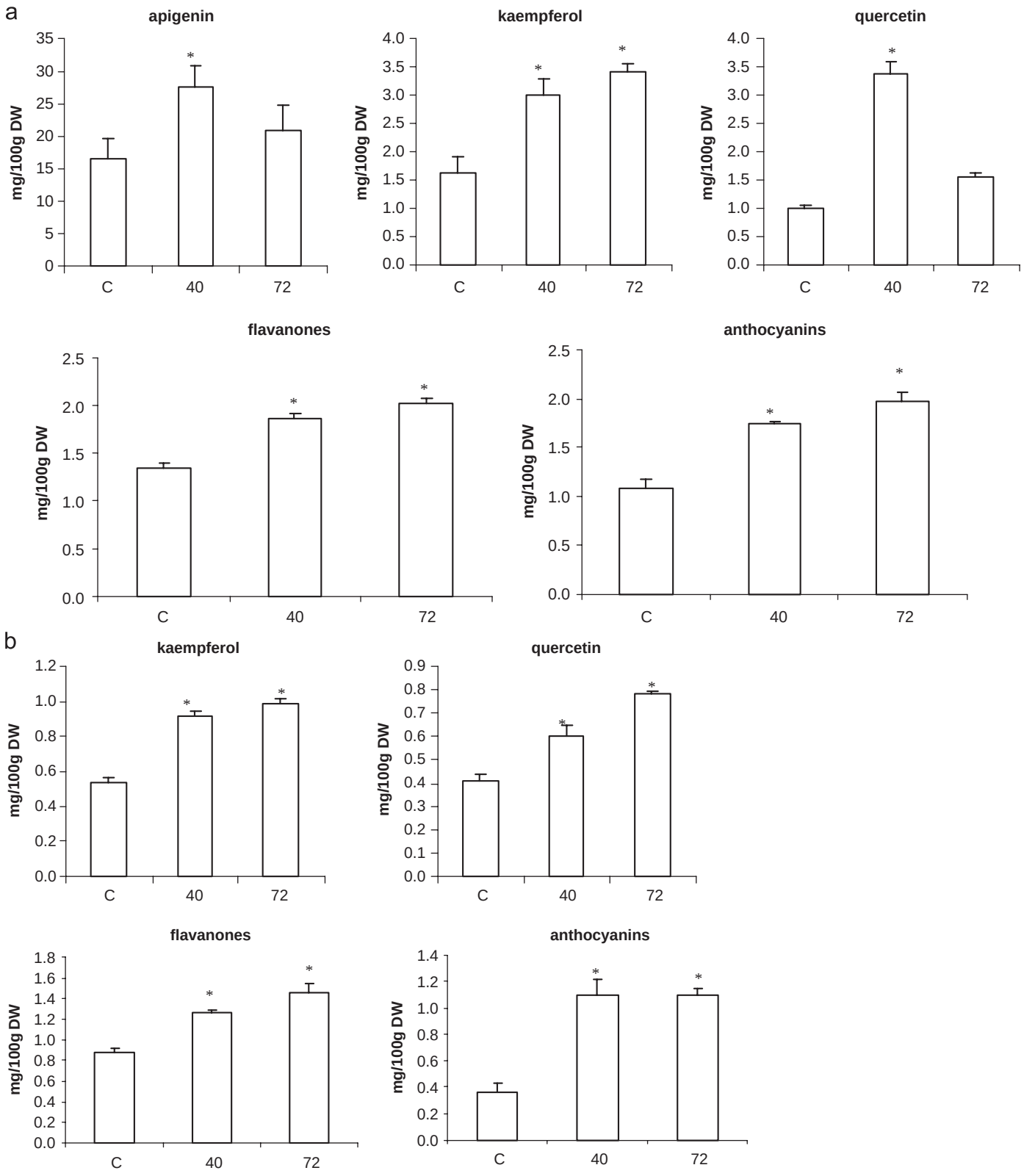

Fig. 4. Determination of flavonoid content in the transgenic flax plants. (a) Flavonoid (flavanone, apigenin, kaempferol, quercetin and anthocyanins) content from green parts of the control (C) and the transgenic flax plants (numbered). The mean value ($n = 4$–6) $\pm$ S.D. is presented. *Statistically significant ($P < 0.05$), (b) Flavonoid (flavanone, kaempferol, quercetin and anthocyanins) content from seeds of the control (C) and the transgenic flax plants (numbered). The mean value ($n = 4$–6) $\pm$ S.D. is presented. *Statistically significant ($P < 0.05$).

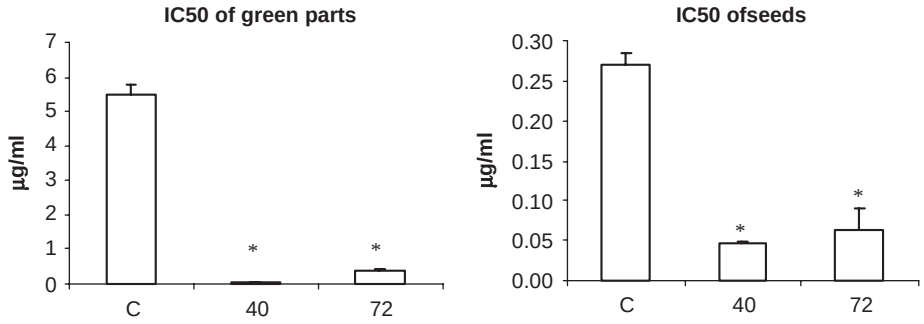

Fig. 5. The antioxidant potential (IC_{50}) of extracts from green parts and seeds from the control (C) and transgenic plants (numbered) overexpressing flavonoid biosynthesis enzymes. The analysis of flax extracts was performed as specified in Materials and Methods. The mean value ($n = 10$) plus and minus S.D. is presented. *Statistically significant ($P < 0.05$).

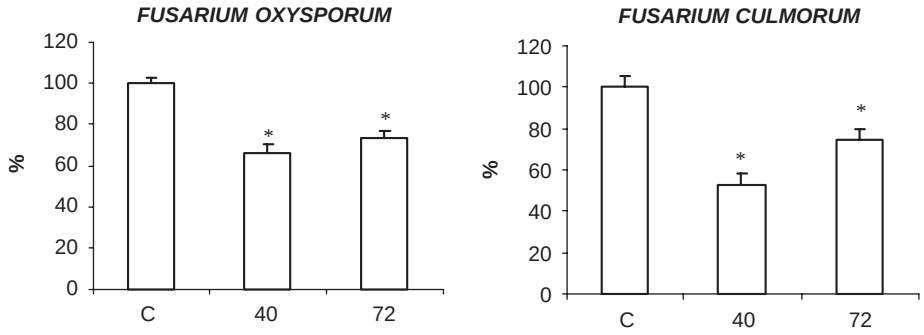

Fig. 6. The resistance of the control (C) and the transgenic lines to *F. oxysporum* and *F.culmorum* (mycelium method). 7-day-old flax seedlings were transferred onto a fungal culture grown for 7 days at 18 °C on potato-dextrose-agar (PDA) medium. After 10–14 days, the number of infected flax seedlings (roots and hypocotyls) was counted and the result is expressed as a percentage of the total seedlings used for the experiment. The mean value ($n = 9$) ± S.D. is presented. *Statistically significant ($P < 0.05$).

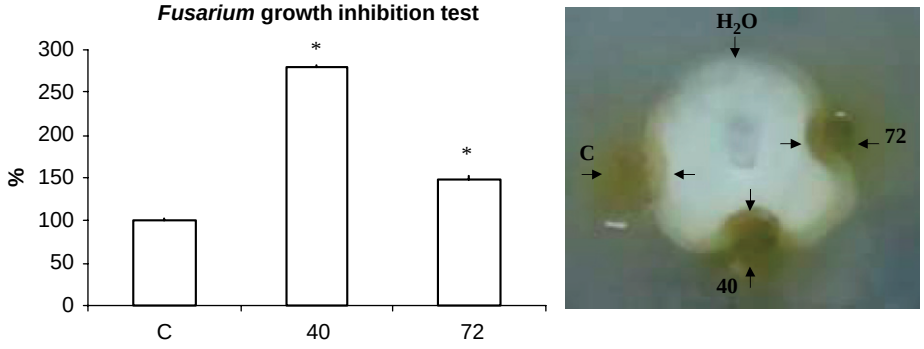

Fig. 7. The inhibition of mycelium growth of *F. oxysporum*. Fungi was cultured for 3 day at 18 °C on solid PDE medium, where indicated (arrows) and the extracts from the control (C) and transgenic plants (numbered) were applied. The inhibition of growth of fungi was measured as a diameter (mm) of area with fungi growth retardation (dark spots) and expressed as a percentage of the control. Measurement of fungi growth inhibition was marked by arrows. The mean value ($n = 15$) ± S.D. is presented. *Statistically significant ($P < 0.05$).

noted for the plants from the line 72. The crucial role of the flavonoids overproduction in transgenic flax resistance to *Fusarium* infection is strongly supported by the data obtained.

3.10. Immunochemical detection of pectin and hemicellulose epitopes

Cell wall provides a physical barrier for pathogen invasion. Therefore, studies of its constituents are meaningful. For flax, pectins are of special interest for two reasons. Firstly, pectin polysaccharide (non-acidic) promotes pathogen adhesive effect [30] and, secondly, they affect stem-retting process and thus fibre quality [31]. Hence, we investigated flax shoots for pectin and hemicellulose by means of immunostaining (Fig. 8). The JIM 7 antibody performed the detection of different oligosaccharide epitopes, recognizing the HGA epitope with a high degree of methyl esterified residues of GalA (galacturonic acid) with adjacent or flanking unesterified residues, and

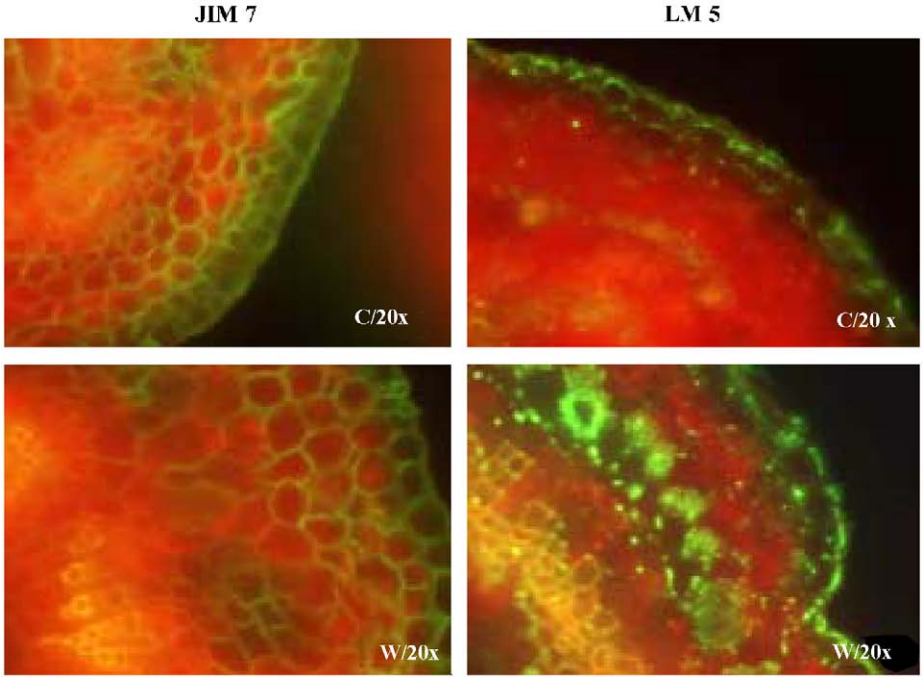

Fig. 8. An immunocytochemical comparison of oligosaccharyde epitopes in flax shoots from transgenic (designated as W) and control plants (C). The plants were from a 4-week in vitro culture: (a) stem cells probed with the JIM 7 antibody, magnification 20 × and (b) stem cells probed with the LM 5 antibody, magnification 20 × . The immunodetection was performed as described in Section 2.10.

LM 5 recognizes four (1→4)-β-D-galactose residues. The hand-cut flax stem sections from the 4-week-old tissue-cultured plants were probed with this specific monoclonal antibody. The immunofluorescence labeling showed the differences in the contents of oligosaccharide epitopes in the transgenic plants when compared to the control plants. The increase of the highly esterified homogalacturonan regions recognized by JIM 7 antibody was detected (Fig. 8). Moreover, the increased of relative content of (1→4)-β-D-galactose epitopes probed with antibody LM 5 was also observed.

4. Discussion

Overexpression of principal genes is thought to be useful tool in understanding the route of flavonoid biosynthesis in flax. Route modification may lead to an increased level of health-promoting compounds, and also may enhance plant resistance against pathogen infection, thereby may increase plant yields. To accomplish these goals, we introduced cDNAs coding for the essential enzymes of the flavonoid biosynthesis route (CHS, CHI, and DFR) into the flax genome. All the transgenic lines produced the respective mRNAs, showing full functionality of the introduced gene construction. Moreover, the transgenic lines exhibited an increased content of flavonoids. Recently, it was demonstrated that in tomato the coordinating expression of a four-gene construct containing CHS, CHI, flavanone 3-hydroxylase (F3H) and flavonol synthase (FLS) cDNAs from *Petunia* resulted in an increased level of flavonols both in the peel and flesh of the tomato fruit [26].

Here, the transgenic flax plants with overexpression of CHS, CHI and DFR cDNAs from *Petunia* revealed full functionality of the ectopically introduced genes. We observed increased content of flavonols (kaempferol and quercetin derivatives), flavones (apigenin derivatives), flavanones and anthocyanins content. We also recently reported on an increase in anthocyanin synthesis due to the expression of a single cDNA for DFR in potato [12]. To investigate the physiological significance of an increase in the level of flavonoids, the antioxidant properties of an extract from transgenic flax were measured. All the extracts from the transgenic plants with the ectopic expression of the introduced genes showed a significant decrease in IC_{50} relative to the control. It is suggested that an increase in antioxidant capacity may result in protection against infectious agents [5] and cause an increase in plant growth [32]. The most aggressive pathogen infecting flax is *F. oxysporum*. Its invasion involves an oxidative burst (oxidative radicals) that is a part of the hypersensitive resistance, inducing lignin synthesis that provides a physical barrier to the pathogen on the one hand and results in plant tissue lesion on the other hand. It is suggested that the transgenic plant with elevated antioxidant capacity could show higher tolerance to oxidative burst caused by biotic and abiotic stresses. The other possibility is that transgenic plants with improved antioxidant capacity would be able to diminish or counteract the damages caused by reactive oxygen species. Data published so far supports the thesis that antioxidants could be part of a constitutive pool of compounds responsible for plant resistance to stresses.

Therefore, the promising approach to generate more resistant transgenic plants is to engineer secondary metabolites (e.g. flavonoids) that confer resistance to different kinds of pathogens. For instance, phenylpropanoids inhibit the activity of fungal hydrolytic enzymes and, in onion, resistance to *Colletotrichum cercinous* is related to catechol and protocatechuic acid [33]. In potato tubers, resistance to *Streptomyces scabies* is related to chlorogenic acid [34]. Moreover, in order to obtain a potato plant with an increased pool of antioxidants, we overexpressed the key genes controlling flavonoid biosynthesis in potato. Although effective in protecting the potato plants against pathogen infection (*Erwinia carotovora* ssp. *carotovora*), the yield of tubers was significantly reduced [12].

Here, we demonstrated that a higher resistance to fungal infection was characteristic for transgenic flax with overexpression of the CHS, CHI and DFR genes. Thus, the engineering of total flavonoids in the flax by coordinating the expression of the three genes coding for CHS, CHI and DFR was reached in the flax plant. We assessed the very high correlation coefficient between flavonoid content and resistance to *Fusarium*, which relates to an important function of the flavonoids in pathogenesis. Another important conclusion is that this concert action of three enzymes increased flax antioxidant capacity, which strengthened the plant's response to pathogen infection. The data obtained from analysis of transgenic plants were strengthened by in vitro experiment in which the growth of *F. oxysporum* was strongly inhibited by addition of flavonoid extracts from transgenic plants.

The interesting to note is the analysis of pectin constituents in transgenic flax. The immunochemical analysis of the control and transgenic plants treated with antibody against pectin and hemicellulose epitopes (LM 5 and JIM 7) revealed increase of the esterified homogalacturonan regions and also increase of relative content of (1-4)-β-D-galactose epitopes in the transgenic plants. Since malonyl-CoA, the basic substrate for flavonoid pathway derives from carbohydrate metabolism, we could speculate that the higher flavonoid pathway enzyme amounts might compete with pectin biosynthetic enzymes for malonyl CoA and thereby with decreasing amounts of pectic polysaccharides. But on the contrary, we observed increased content of pectin constituents in the case of transgenic flax plants. The reason is, however, yet unknown.

The increase in pectin constituents might have affected resistance to pathogen attack. In another study it was observed that changes in pectin and hemicellulose content was accompanied by changes in resistance to *F. oxysporum* infection [35]. A decrease in pectin and hemicellulose constituents (galactose, galacturonic acid) was accompanied by about two-fold lower resistance of transgenic flax to fungi. In agreement with this is the finding that W92 plants, for which we have noticed the increased level of pectin and hemicellulose, showed improved torerance to *F. oxysporum* and *F. culmorum*. However, for another transgenic flax where polygalacturonase and rhamnogalacturonase were overexpressed, which resulted in decrease of pectin content, improved resistance to *Fusarium* was noticed [31]. Thus, we speculate that pectin constituents are not the only components of flax defense against *Fusarium* infection and that response to pathogen attack is such a complex mechanism that one component of its cannot be evaluated as the direct reason for changes in pathogen resistance. It was also shown that methylesterification of pectin is crucial in the infection process [36]. The high level of the resistance of potato tubers was noticed when pectin was highly methylated, because such compound was difficult to degrade by pectinases of the pathogen (*Erwinia chrysanthemi*) [36]. It is also known that pectin methylesterase (PMEs), which de-esterified methylesterified forms of pectin, are regulated by specific proteinaceous inhibitors (PMEIs), and that transgenic *Arabidopsis* plants with overexpression of PMEI were more resistant to the fungi [37].

In agreement with this is the finding that W92 plants showed higher than control level of esterified GalA epitopes recognized by JIM 7 antibody. However, we did not analyze the total level of methylesterified pectin in these plants.

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