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Pleiotropic Effect of Phenolic Compounds Content Increases in Transgenic Flax Plant

KATARZYNA LORENC-KUKUŁA,[†] RYSZARD AMAROWICZ,[‡] JAN OSZMIAŃSKI,[§] PETER DOERMANN,[#] MICHAŁ STARZYCKI,[⊥] JACEK SKAŁA,[||] MAGDALENA ŻUK,[†] ANNA KULMA,[†] AND JAN SZOPA*,[†]

Institute of Biochemistry and Molecular Biology and Insitute of Genetics and Microbiology, Wrocław University, Przybyszewskiego 63/77, 51-148 Wrocław, Poland; Department of Food Science, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Tuwima 10, 10-747 Olsztyn, Poland; Department of Fruit, Vegetable and Cereals Technology, Agricultural University, Norwida 25, 50-375 Wrocław, Poland; Max Planck Institute of Molecular Plant Physiology, Am Muehlenberg 1, 14476 Golm, Germany; and Research Division, Plant Breeding and Acclimatization Institute IHAR, Strzeszyńska 36, 60-479 Poznań, Poland

The principal goal of this paper was to generate flax (*Linum usitatissimum* L.) plants with increased antioxidant properties. To accomplish this a vector containing a multigene construct was prepared, and transgenic plants overexpressing essential flavonoid biosynthesis pathway enzymes were generated and analyzed. The simultaneous expression of genes encoding chalcone synthase (CHS), chalcone isomerase (CHI), and dihydroflavonol reductase (DFR) resulted in a significant increase of flax antioxidant capacity. To investigate the determinants of higher antioxidant properties of transgenic plants, the phenolic acids and lignans compound contents were measured. In both green part and seed extracts from transgenic plants, the phenolic acids level was increased when compared to the control. The calculated correlation coefficient between phenolic acids content and antioxidant capacity (0.82 and 0.70 for green part and flaxseed, respectively) perfectly reflects their strong relationship. The increase in yield of transgenic plants and their higher resistance to *Fusarium culmorum* and *Fusarium oxysporum* when compared to the control plants was a characteristic feature. It was assessed a very high correlation (correlation coefficient = 0.9) between phenolic acids level in flaxseed extract and resistance to *F. culmorum*. The flowering date of transgenic plants was ∼3 weeks earlier than that of the control plants. Interestingly, a significant increase in monounsaturated fatty acids and a slight increase in lignans content accompanied the increase in antioxidant properties of flaxseeds.

KEYWORDS: Flax; antioxidant capacity; phenolic acids; lignans; fatty acids; *Linum usitatissimum* L.

INTRODUCTION

The flax plant is of great significance for industry. Seeds of the flax are used for extraction of oil and stems for fiber production. Linseed oil has commercial value as a component of adhesives, paints and varnishes, plasticizers, inks, and linoleum, and it is a precursor of nylon and composite materials. The flax fiber is commonly used in the textile industry. Because of linseed oil protection properties, it is recommended in gastrointestinal tract diseases (e.g., chronic peptic ulcer disease, chronic inflammations of mucous membranes) and respiratory tract diseases. Dietary properties of linseeds are also very important; they act positively on peristalsis and metabolism (*1*) and prevent skin diseases (*2*). The flaxseed lowers blood cholesterol and glucose levels (*3*−*5*). Linseed lignans inhibit cell proliferation and growth, making them potential anticancer agents. Studies in animals confirm the anticancer properties of flaxseed lignans (*6*). They showed a protective effect against colon and breast cancer (*7*), diabetes, hypercholesterolemic atherosclerosis (*8*), lupus nephritis (*9*), and immune and inflammatory reactions (*10*). Thus, linseed oil plays an important role in health care, pharmaceutics, and the food industry. Linseed oil from common flax cultivars is enriched in polyunsaturated fatty acids (PUFA) (*8*, *11*), which cause it to be very susceptible to oxidation. This is why the oil from those cultivars is suitable for industrial use rather than for human consumption. To improve it, new cultivars were created with a reduced content of 18:3 polyunsaturated fatty acid. In this study, we used a

* Corresponding author [e-mail szopa@ibmb.uni.wroc.pl; telephone (4871) 3756202; fax (4871) 3252930].
[†] Institute of Biochemistry and Molecular Biology, Wrocław University.
[‡] Polish Academy of Sciences.
[§] Agricultural University.
[#] Max Planck Institute of Molecular Plant Physiology.
[⊥] Plant Breeding and Acclimatizaion Institute IHAR.
[||] Institute of Genetics and Microbiology, Wrocław University.

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Linola cultivar that has a very low level (2.45%) of linolenic acid (*12*). The reduction in the unstable linolenic acid content resulted in the improvement of the oxidative status of this oil; however, even so it needs further protection against oxidation. Generally, lipids in flaxseeds are protected against peroxidation by the addition of natural antioxidants, that is, polyphenols (lignans). However, during extraction, lignans are not effectively extracted with oil. To avoid oxidation, flax oil is supplemented with vitamin E and stored in dark bottles in low temperature. None of these methods, however, is fully satisfactory, and an appearance of rancidity in flax oil is observed.

In this paper, the approach in which antioxidant compounds are synthesized in transgenic plants has been used. Thus, by the expression of cDNAs encoding key enzymes of polyphenols biosynthesis, the antioxidant capacity increases and thus protection of flax oil was expected. The plant polyphenols are a group of compounds that include flavonoids, phenolic acids, phenols, lignans, and tannins. The biological, pharmacological, and medicinal properties of the plant polyphenols have been extensively reviewed (*13*). It has been reported that they have multiple biological activities. It was found that they have antiallergenic, antiviral, and anti-inflammatory activities. It is supposed that consumed together with essential PUFA they lower the risk of various diseases. Their antioxidant activity has a great significance for the food's quality, because they inhibit enzymatic and nonenzymatic peroxidation (*14*). In the present study, we have generated flax plants overexpressed in three genes coding for key enzymes of the flavonoid biosynthesis pathway (chalcone synthase, CHS; chalcone isomerase, CHI; and dihydroflavonol reductase, DFR), which resulted in improved antioxidant properties. The measured antioxidant potential (IC_{50}) of transgenic plants was significantly increased, ranging from 1.5- to 6-fold in seeds and from 5- to 91-fold in leaves. The increased antioxidative properties of flax plants were accompanied by a significant increase in phenolic acids content in the transgenic plants.

MATERIALS AND METHODS

Plant Material. Flaxseeds (cv. Linola) were obtained from The Flax and Hemp Collection of the Institute of Natural Fibres, Poland. For preselection and selection, the transgenic and control plants were grown in tissue culture and were cultivated in a greenhouse under a 16 h light (21 °C)−8 h dark (16 °C) regimen. The plants in soil were grown in individual pots and were watered daily. For analysis, the control and selected transgenic plants were grown in a field, and seeds were harvested 3 months after transfer of the tissue-culture plants to the soil.

Transgenic Plants Construction. In this study, the transgenic plants overexpressing three essential enzymes of the flavonoid synthesis pathway from *Petunia hybrida*, CHS (EMBL/GenBank database accession no. X04080), CHI (EMBL/GenBank database accession no. X14589), and DFR (EMBL/GenBank database accession no. X15537) were generated. The cDNAs encoding enzymes were provided by Dr. I. Somssich. For cotyledons and hypocotyls explants transformation, the recently constructed binary vector containing three cDNAs in sense orientation under the control of 35S promoter and OCS terminator was used (*15*). The vector was introduced into *Agrobacterium tumefaciens* strain C58C1:pGV2260. Two-week-old cotyledons and hypocotyls (*16*) were transformed by submerging the explants in a suspension of *A. tumefaciens* containing binary vector. *A. tumefaciens* inoculated explants were subsequently transferred to callus induction and shoot regeneration medium (*17*).

Transgenic Plant Selection. The transgenic plants were preselected by Polymerase Chain Reaction (PCR) using primers specific for the kanamycin resistance gene and then selected by means of northern blot analysis. PCR was carried out with the use of specific primers for neomycine phosphotransferase (*npt II*) gene (forward, CCGACCTGTCCGGTGCCC; reverse, CGCCACACCCAGCCGGCC) on genomic DNA isolated from 3-week-old tissue-cultured plants as a template. Transgenic plants that contain the predicted *npt II* gene (475 bp fragment) product were further analyzed. The final selection was carried out by means of northern blot analysis. Total RNA was prepared from frozen young leaves using the guanidinium hydrochloride method (*18*). Total RNA was separated on 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 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. Five transgenic lines, W92.28, W92.40, W92.70, W92.72, and W92.86, that showed the highest mRNAs level were further analyzed.

Extraction of Leaves 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 flaxseeds 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. The combined extract was centrifuged at 14000*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.

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 from 1000 to 15 000 times with water and directly analyzed according to a published method (*19*). The 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 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 [micrograms of dry weight (DW), presented in percent total], which inhibits luminol chemiluminescence by 50%.

HPLC Analysis of Phenolic Acids. A 0.5 g sample of green parts of plants was subjected to hydrolysis (2 mL of 2 M HCl) for 60 min in a hot water bath. Then, 2 mL of 2 M NaOH and 6 mL of methanol were added, and aglycons were extracted using an ultrasonic bath for 15 min (*19*). Two hundred milligrams of defatted with hot hexane flaxseeds was mixed with 10 mL of 1.2 M HCl in 50% aqueous methanol for 2 h at 90 °C in glass screw-capped vials. The extract was allowed to cool and then sonicated for 5 min. Approximately 5 mL was centrifuged prior to injection onto a column (*20*). The extracts from green parts or seeds were centrifuged at 12000*g* for 10 min, and the supernatants were analyzed by HPLC. The HPLC system consisted of a pump (L-7100), a photodiode array detector (Merck-Hitachi L-7455), and a D-7000 HSM multisolvent delivery system. Separation of phenolic acids was done by a prepacked LiChroCART 125-3 Purospher RP-18 (5 μm) column (Merck). The mobile phase of 80% acetonitrile in 4.5% acetic acid (reagent A) and 4.5% acetic acid (reagent B) was delivered at a rate of 1 mL/min. Caffeic, chlorogenic, and ferulic acid detection was carried out by on-column measurement of absorption at 320 nm, and *p*-coumaric and protocatechuic acid detection was performed at 280 nm.

Secoisolariciresinol Diglucoside (SDG) Extraction and Measurement. A 0.5 g sample of flaxseeds was extracted four times with 5 mL of 80% methanol (v/v) using an Ultra-Turrax T25 (Janke & Kunkel IKR) homogenizer (60 s, 13500 rpm) at 70 °C. Prior to extraction, the seeds were defatted with hot hexane. The extract was centrifuged at 1750*g* for 10 min, and the supernatants were collected, combined, and evaporated to near dryness at 40 °C under vacuum.

The extract was then resuspended and subjected to alkaline hydrolysis (2 mL, 0.3 M aqueous sodium hydroxide) for 2 days at room temperature followed by acidification to pH 3 using 2 M hydrochloric acid (*21*). Before HPLC analysis, the mixture was desalted using a C_{18} reversed-SPE column (J. T. Baker; 3 mL; 500 mg gel per column). Prior to use, the column was washed with 5 mL of methanol followed by 5 mL of water. A portion of the hydrolyzed sample was applied onto the column and washed with water (2 × 5 mL) to remove the salt, and phenolic compounds were then eluted with 5 mL of methanol.

The SDG was separated on a prepacked LiChrospher 100 RP-18 column (4 × 250 mm, 5 μm; Merck, Darmstadt, Germany) and analyzed using a Shimadzu HPLC system (Shimadzu, Kyoto, Japan) consisting

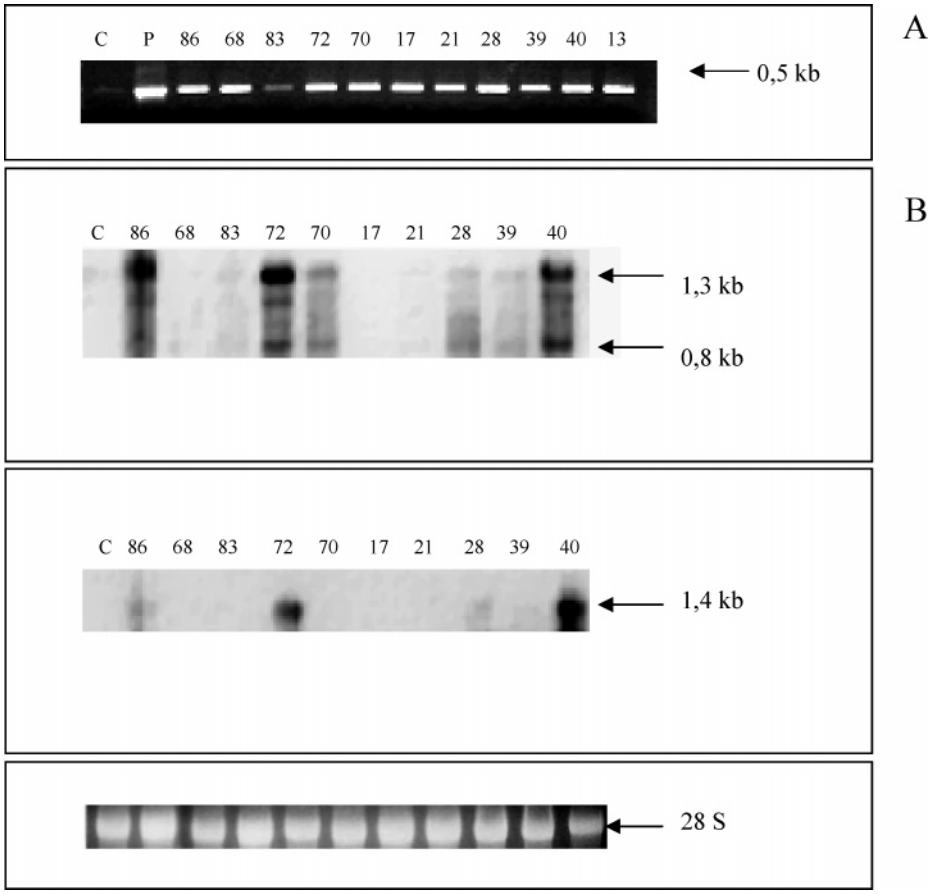

Figure 1. (**A**) Agarose gel electrophoresis of *npt II* gene PCR product (475 bp). Neomycine phosphotransferase gene (*npt II*) was amplified with the use of specific primers; a template was genomic DNA isolated from tissue-cultured flax plants. C, negative control (nontransformed plant); P, positive 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 at first hybridized with an equimolar mixture of ^{32}P-labeled CHI (0.8 kb) and CHS (1.3 kb) cDNAs (upper panel) and then after blotting extensively washed in hot (80 °C) water probed with labeled DFR (1.4 kb) cDNA (middle panel). A ribosomal RNA stained with ethidium bromide was used as a control of RNA applied onto the gel (bottom panel).

of a pump (LC-10ADVP), a system controller (SCL-10AVP), and a photodiode array detector (SPD-M10AVP). The mobile phase of water/acetonitrile/acetic acid (88:10:2; v/v/v) was delivered at a rate of 1 mL/min. The compound was detected at 280 nm (*22*).

Determination of Lipid Content in Flaxseeds by GC Method. Methyl esters of fatty acids (FAMEs) were prepared from seeds by extraction with 1 mL of 1 N HCl in methanol and incubation at 80 °C for 90 min (*23*). Then 1 mL of 0.9% NaCl and 1 mL of hexane were added to the extract, mixed, and centrifuged at low speed (3 min at 100 rpm). The hexane phase was collected, and the lipids were concentrated in a N_2 stream and stored at −20 °C. The methyl esters were quantitated by gas chromatography, using pentadecanoic acid as an internal standard (*24*).

Evaluation of Flax Resistance to *Fusarium culmorum* and *Fusarium oxysporum* Infection by Mycelium Method. Flaxseeds 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*. Fungi were cultured for 7 days at 18 °C on potato−dextrose−agar (PDA) medium; 10−14 days after transfer, the number of infected flax seedlings (roots and hypocotyls) was counted and expressed as percentage of total seedlings used for the experiment (*25*).

RESULTS

Cloning Vector. For the plant transformation, the new pBinW88 multigene vector was recently prepared (*15*). Briefly, the plasmid pUC 19 was equipped with sequence recognized and cut by three rare (*Asc*I, *Fse*I, and *Sex*AI) restriction enzymes and thus able to clone up to five genes into it. Three cassettes containing CHS, CHI, and DFR cDNAs sense oriented, each under the control of a 35S promoter and OCS terminator, were introduced into *Asc*I, *Fse*I, and *Sex*AI restriction sites, respectively.

Transgenic Plant Generation and Selection. The hypocotyl and cotyledon explants of flax plants were transformed according to the *Agrobacterium* method (*17*), and several transgenic lines overexpressing key enzymes of the flavonoid synthesis pathway were generated. The transgenic plants were preselected by means of PCR with primers specific for *npt II* selection gene and then selected by northern blot analysis. **Figure 1A** shows the result, and plants exhibiting the expected 475-bp fragment in PCR were taken for further selection. The selection was based on mRNA analysis with the use of respective radiolabeled cDNAs. Northern blot of flax transgenic plants with overexpression of CHS, CHI, and DFR genes probed with radioactively labeled respective cDNAs is shown in **Figure 1B**. The transgenic lines W92.40, W92.72, and W92.86 showed all three bands representing respective mRNAs; in other cases, two bands (W92.70, W92.39) or no signal (W92.17, W92.21) at all was detected.

Because it is well-known that the positive correlation between mRNA and protein level is often lacking for unknown reasons,

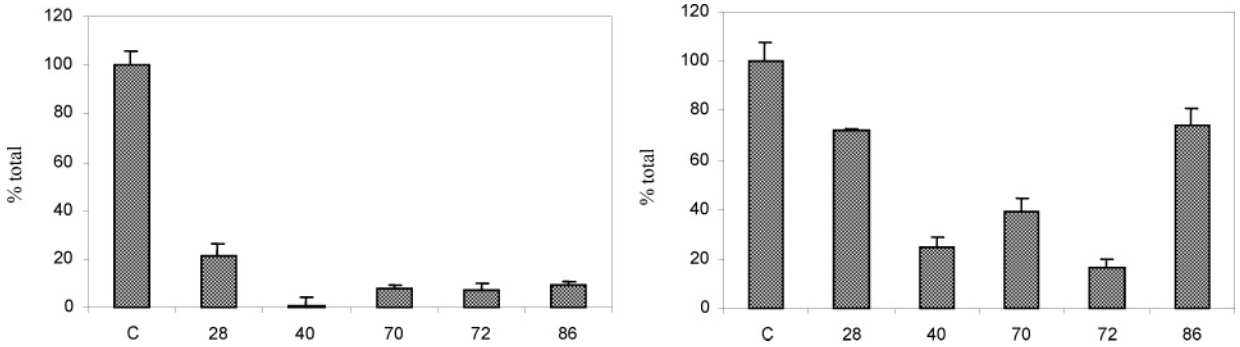

Figure 2. Antioxidant potential (IC_{50}) of green parts (left) and seeds (right) extract from the control (C) and transgenic plants (numbered) overexpressed flavonoid biosynthesis enzymes. The analysis of flax extracts was performed as specified under Materials and Methods. The mean value ($n = 4$) ± SD is presented, $P < 0.01$.

Table 1. Determination of Phenolic Acid Content in Green Parts and Flaxseeds[a]

A. Phenolic Acid Content in Green Parts (mg/100 g)

line	protocatechuic acid	caffeic acid	ferulic acid	*p*-coumaric acid	chlorogenic acid
control	660.20 ± 38.13	25.32 ± 2.93	25.50 ± 2.87	172.74 ± 9.17	52.18± 5.59
W92.28	816.82 ± 48.55	33.07 ± 3.19	33.91 ± 3.09	157.32 ± 6.32	56.77 ± 6.99
W92.40	760.66 ± 25.12	30.42 ± 2.28	28.75 ± 2.27	204.64 ± 8.73	51.07 ± 4.76
W92.70	821.18 ± 58.82	32.58 ± 3.21	33.79 ± 3.00	171.18 ± 5.11	54.60 ± 4.51
W92.72	759.83 ± 56.99	32.64 ± 3.33	29.92 ± 3.58	177.36 ± 7.77	57.33 ± 3.58
W92.86	832.27 ± 49.00	34.50 ± 2.18	35.95 ± 3.29	188.12 ± 7.30	53.83 ± 2.65

B. Phenolic Acid Content in Seeds (mg/100 g)

line	caffeic acid	ferulic acid	*p*-coumaric acid
control	12.34 ± 0.14	29.78 ± 0.67	32.27 ± 0.57
W92.28	12.91 ± 0.36	27.39 ± 0.84	32.75 ± 1.03
W92.40	17.01 ± 0.54	32.90 ± 0.96	42.94 ± 1.16
W92.70	13.10 ± 0.29	31.50 ± 0.80	37.32 ± 0.88
W92.72	13.78 ± 0.22	30.78 ± 0.85	35.12 ± 0.79
W92.86	10.26 ± 0.12	25.47 ± 0.81	29.34 ± 0.66

[a] HPLC determination of phenolic acid content in green parts (**A**) and seeds (**B**) from the control (C) and the transgenic flax plants (numbered). Phenolic acids were separated on a prepacked LiChroCART 125-3 Purospher RP-18 (5 μm) column (Merck). The mean value ($n = 3$–6) ± SD is presented, $P < 0.01$.

for further experiments we have chosen all transgenic lines showing positive results in PCR and northern blot analysis.

The level of transgene expression usually depends on its copy number, but it might also derive from different nucleotide context of their location in the genome; however, this is pure speculation, because we do not have evidence to support this view.

Antioxidant Activity of Extracts from Flax Plants. The final step of transgenic plant selection was antioxidant capacity analysis in both leaves and seeds. To evaluate the antioxidant properties of transgenic flax extracts, a chemiluminescence method was used. It is based on the chemiluminescent response of luminol to oxidation. Antioxidants in the model system scavenge the reactive oxygen species, which prevents them from oxidizing the luminol and quenching the chemiluminescent light response.

In this study, five independent transgenic lines, W92.28, W92.40, W92.70, W92.72, and W92.86, selected by means of PCR and northern blot, were assayed for antioxidant capacity. Results were expressed as the IC_{50} parameter, which is the amount of extract inhibiting luminescence by 50%, and are shown in **Figure 2**. The lower the IC_{50}, the higher the antioxidant potential. The extracts from all transgenic lines overexpressing essential enzymes of the flavonoid synthesis pathway showed significant decrease in IC_{50} compared with control plants. Thus, the antioxidant potential of flax plants increased from 1.5- to 6-fold in seeds and from 5- to 91-fold in leaves. The highest antioxidant capacity of leaves and seeds was obtained for transgenic lines W92.40, W92.70, W92.72, and W92.86. The calculated correlation coefficients for the level of mRNA encoding CHS, CHI, and DFR (measured by northern densitometry) and IC_{50} are −0.79, −0.93, and −0.55, respectively. Thus, the principal goal of this study was successfully accomplished, and transgenic plants with significant increases in antioxidant capacity were obtained. It should be pointed out that on the basis of the coefficient value the antioxidant status of transgenic plant almost entirely resulted from expression of introduced cDNAs.

Analysis of Phenolic Acids and Lignans Contents in Transgenic Plants. Due to the significant increase in antioxidant capacity of transgenic plants, the increase in phenolic compounds content was expected. Thus, we have measured the antioxidant compound content both in the green part (leaves and stems) of the plant and in seeds. In leaves and stems extract, the phenolic acids level was increased from 13 to 26%, depending on the transgenic line, compared to the control plant (**Table 1A**). Similarly, in flaxseeds extract, the phenolic acids level was increased in almost the same range (from 7 to 25%), and respective data are presented in **Table 1B**. In one case (W92.86), however, a decrease in phenolic acids level (12.5%) was observed. It was observed that there is a very high negative correlation (correlation coefficient of −0.82) between phenolic acids level and the antioxidant parameter IC_{50} of leaves and stems extracts. In addition, a high correlation coefficient value

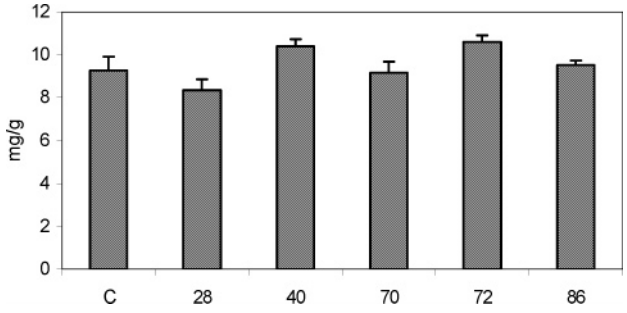

Figure 3. HPLC determination of secoisolariciresinol diglucoside content in seeds from the control (C) and transgenic flax plants (numbered). SDG separation was done on a prepacked LiChrospher 100 RP-18 column (4 × 250 mm, 5 μm; Merck, Darmstadt, Germany). The mean value (n = 3) ± SD is presented, $P < 0.05$.

(−0.7) for phenolic acids level and antioxidant capacity of flaxseed was calculated. Thus, it is suggested that the phenolic acids content determines the antioxidant status of transgenic green organs and flaxseeds.

Various components of flaxseeds could reduce the free radicals level; thus, we have speculated that their antioxidative properties might derive partially from other antioxidative compounds content. Thus, we have also measured the content of the diphenolic compound, SDG, the major component of lignans, which might contribute to the antioxidative status of transgenic seeds (*26*). Only slight increases in two transgenic lines (12% in W92.40 and 14% in W92.72) were detected, and the data are presented in **Figure 3**. It should be pointed out, however, that those plants showed the highest antioxidant properties. A high positive correlation (correlation coefficient of 0.7) between SDG level and antioxidant capacity of flaxseeds was calculated. Thus, the level of lignans may partially affect the antioxidant capacity of the seeds from two transgenic lines, but certainly does not reflect the entire antioxidant status of all investigated lines.

Lipid Composition of Transgenic Flaxseed. Because of the significant increase in antioxidant capacity of seeds from transgenic plants, the protection of unsaturated fatty acids might occur. Thus, it was of interest to measure the fatty acids composition from seeds. The flaxseed oil was examined by gas chromatography equipped with flame ionization detection. Surprisingly, a significant increase in monounsaturated fatty acids in most transgenic lines was detected. The highest increase (54%) was observed in line W92.72 (**Figure 4**). Simultaneously, a decrease in polyunsaturated acids (PUFA) content (from 1 to 12%) in those seeds was noticed (**Figure 5**).

Interestingly, a very high positive correlation coefficient (0.91) was calculated for monounsaturated fatty acids content and the antioxidant capacity of flaxseeds. It should be pointed out that the calculated correlation coefficient for PUFA showed also a high, but negative, value. The highest increases were observed for stearic acid (∼53%) and oleic acid (∼38%) levels, and the data are presented in **Table 2**. The linoleic acid (18:2) level is decreased by 9% in W92.40 transgenic line and by 13% in W92.72; simultaneously, SDG levels in both lines increased by 12 and 14%, respectively. Thus, the increase in the antioxidant potential of seeds resulted in significant changes in fatty acids composition.

Phenotype Analysis. The obtained transgenic plants were visually indistinguishable from the nontransformed control plants. Transgenic plants, however, showed ∼3 weeks earlier flowering compared to the control plants. Additionally, the yield of seeds [fresh weight (FW) of seeds per plant] and fresh weight of seeds per bag were higher than for the control plants. Transgenic plants produced >36% on average (15−62%, depending on the transgenic line) more seeds per plant compared with nontransformant (**Table 3**). In only one case (W92.28) was

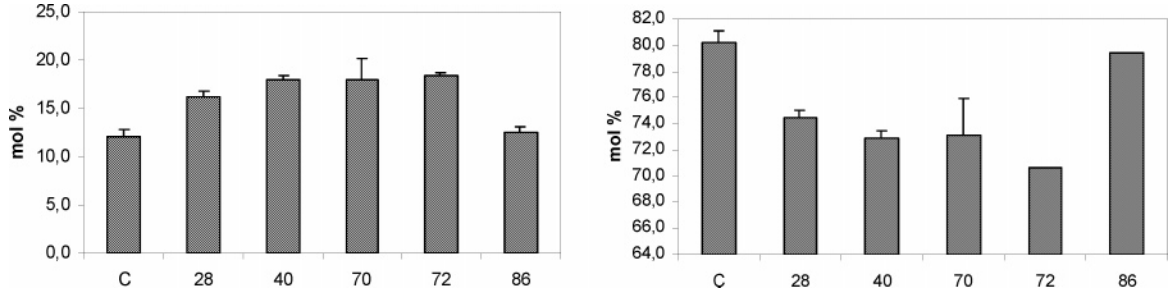

Figure 4. Determination of MUFA (left) and PUFA (right) content in control (C) and transgenic seeds (numbered). The extraction and analysis were performed as specified under Materials and Methods. The methyl esters were quantitated by gas chromatography, using pentadecanoic acid as an internal standard. The mean value (n = 4) ± SD is presented, $P < 0.05$.

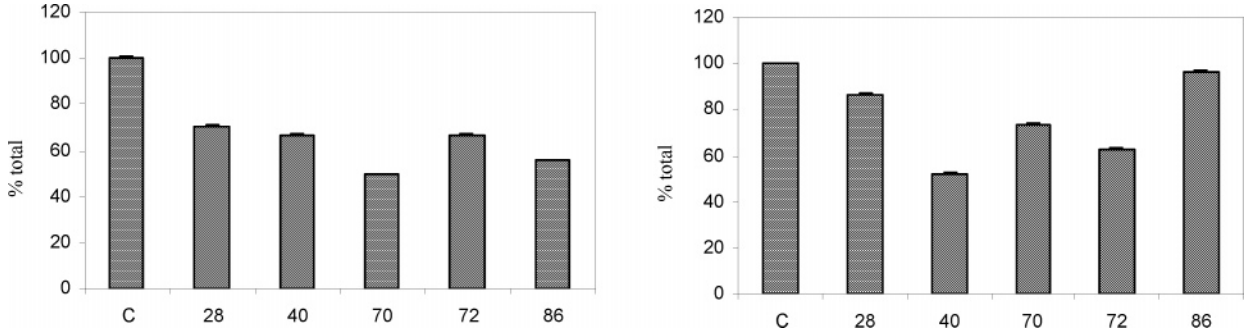

Figure 5. Evaluation of resistance of the control (C) and the transgenic lines to *F. culmorum* (right) and *F. oxysporum* (left) by mycelium method. Seven-day-old flax seedlings were transferred onto fungi culture grown for 7 days at 18 °C on potato–dextrose–agar (PDA) medium; 10–14 days later the number of infected flax seedlings (roots and hypocotyls) was counted and the result expressed as percentage of total seedlings used for the experiment. The mean value (n = 9) ± SD is presented, $P < 0.01$.

Table 2. Fatty Acids Composition of Lipid from Control and Transgenic Seeds[a]

line	fatty acid composition (mol %)					
	16:0	16:1	18:0	18:1	18:2	18:3
control	5.53 ± 0.12	0.04 ± 0.01	2.30 ± 0.20	11.95 ± 1.42	77.71 ± 1.57	2.45 ± 0.08
W92.28	5.73 ± 0.21	0.07 ± 0.01	3.59 ± 0.26	16.05 ± 1.00	72.17 ± 0.92	2.32 ± 0.04
W92.40	5.72 ± 0.07	0.03 ± 0.00	3.26 ± 0.12	17.81 ± 1.18	70.62 ± 0.83	2.28 ± 0.14
W92.70	5.65 ± 0.34	0.03 ± 0.00	3.27 ± 0.43	17.85 ± 4.06	70.70 ± 4.68	2.44 ± 0.15
W92.72	6.18 ± 0.57	nd[b]	4.53 ± 0.22	18.43 ± 0.16	67.74 ± 1.09	2.88 ± 0.41
W92.86	5.14 ± 0.03	nd	2.95 ± 0.08	12.48 ± 0.58	76.78 ± 0.46	2.62 ± 0.13

[a] Results are expressed in mole percent (mol %). The mean value ($n = 4$) ± SD is presented, $P < 0.05$. [b] nd, not detectable.

Table 3. Yield of Flaxseeds from the Control and Transgenic Lines[a]

line	FW of seeds per plant (g)	FW of seeds per bag (mg)	no. of seeds per bag	FW of single seed (mg)
control	4.28 ± 0.22	42.95 ± 1.17	8.34 ± 1.0	5.15 ± 0.44
W92.28	3.25 ± 0.18	52.51 ± 1.29	8.66 ± 1.1	6.06 ± 0.23
W92.40	5.03 ± 0.29	49.23 ± 1.03	8.71 ± 0.6	5.65 ± 0.31
W92.70	6.43 ± 0.11	47.78 ± 1.11	8.59 ± 1.2	5.56 ± 0.20
W92.72	4.91 ± 0.32	51.28 ± 1.00	8.95 ± 0.4	5.73 ± 0.37
W92.86	6.94 ± 0.19	47.33 ± 1.43	8.88 ± 0.7	5.33 ± 0.25

[a] The mean value ($n = 30$) ± SD is presented, $P < 0.05$.

a decrease in yield of seeds (24%) observed. The second generation (F2) plants produced 30% more seeds than the control (data not shown). The fresh weight of a single seed was slightly higher (10%) than for the control plants.

Resistance to *F. culmorum* and *F. oxysporum* Infection. The major fungal pathogen infecting flax plants is *Fusarium*, which caused the greatest yield decline. It was expected that the increase in antioxidant properties of transgenic plants might cause their resistance to pathogen infection. This was the case; resistance to *F. culmorum* and *F. oxysporum* in the case of transgenic plants was higher compared to the control plants (**Figure 5**). The high positive correlation (correlation coefficient of 0.9) was established between resistance to *Fusarium* and phenolic acids content. There is also a high positive correlation (correlation coefficient of 0.7) between resistance to *F. culmorum* and SDG contents in transgenic lines where increased quantities of this compound were detected. Surprisingly, the high positive correlation (correlation coefficient of 0.9) was also established between resistance to *Fusarium* and MUFA content. This finding pointed out that MUFA greatly contribute to plant immune response to pathogen infection.

DISCUSSION

Our effort has been directed toward genetically improving the market properties of flax oil. Flaxseed is a rich source of PUFA and lignans with beneficial health effects. However, the market value of flaxseed oil is limited by the perishability of PUFA, which are highly susceptible to peroxidation, and only certain cultivars of the flax with low contents of 18:3 fatty acids are suitable for commercial preparation of edible oil. The reaction of reactive oxygen species (ROS) with PUFA has been extensively studied because of their involvement in rancidity and the development of undesirable flavors and odor in food. Lipid peroxidation in plant membranes would degrade unsaturated fatty acids and accumulate dangerous products. ROS are associated with carcinogenesis, atherosclerosis, aging, inflammation, and other pathophysiological conditions (*27*). Therefore, inactivation or elimination of ROS may be beneficial in terms of reducing the risk of cancer and other diseases. Thus, dietary or medicinal intake of antioxidants could be an important regimen for fortifying the body's defense system against oxygen free radical-induced carcinogenesis (*28*). In both industrial and edible uses, an increase in antioxidative properties of flaxseeds is desirable and has been a major aim of the genetic manipulation. For this reason, we intended to increase the number of antioxidants in flax, and we accomplished this aim by the introduction of genes coding for key enzymes of the flavonoid biosynthesis pathway, that is, chalcone synthase, chalcone isomerase, and dihydroflavonol reductase, into the flax genome. The plants selected by means of PCR and northern blot showed correlation between antioxidant capacity content and mRNA level (correlation coefficient of −0.77). The selected transgenic lines with the highest expression of introdced cDNAs revealed significant increases in antioxidant capacity when compared to control. The antioxidant potential of flax plants increased from 1.5- to 6-fold in seeds and from 5- to 91-fold in leaves. The highest antioxidant capacities were found for transgenic lines W92.40, W92.70, W92.72, and W92.86, and those lines were further investigated.

To characterize quantitatively the compounds responsible for the antioxidant potential of transgenic plants, the level of phenolic acids and lignans was measured. In both green parts and seeds extracts, the phenolic acids level was increased compared to the control plant. A high negative correlation (correlation coefficient of −0.82) between phenolic acids level and the IC_{50} parameter of leaves and stems extract was found. Also, a high correlation (correlation coefficient of −0.7) for phenolic acids level and IC_{50} parameter of seed extract was calculated. The high negative correlation for measured parameters suggests the significance of phenolic acids in the antioxidative status of transgenic flax.

We have detected that in certain lines the increase in antioxidant properties of flaxseeds is accompanied by an increase in lignans level. Lignans are widespread phenolic compounds, derived from the phenylpropanoid metabolic pathway (*29*). Defatted flaxseed flour is one of the richest sources of lignans (*30*). The major components of the lignan's family are secoisolariciresinol diglucoside (SDG), matairesinol, isolariciresinol, and pinoresinol (*11*, *31*). It is known that SDG is the precursor of the major mammalian lignans enterodiol and enterolactone that exert anticarcinogenic properties (*11*, *29*, *31*). Some of these compounds have phytoestrogen properties and may protect humans against hormone-dependent cancers such as breast cancer (*29*, *32*, *33*). We have noted that there is a high positive correlation (correlation coefficient of 0.7) between the major component of lignans, the SDG level, and the antioxidant capacity of flaxseeds. Thus, the level of lignans could affect the antioxidant capacity of the seeds; it should be, however, pointed out that this suggestion is valid for only two (W92.40, W92.72) of the analyzed transgenic lines and does not reflect the entire antioxidant status of all investigated lines. For the other lines, for which SDG levels were similar to that

of the control plants, the antioxidant potential entirely depends on the content of phenolic acids.

Because phenolic acids together with lignans could protect lipids against peroxidation and have hydroxy radical scavenging activity (*32*, *34*), we have analyzed the fatty acids composition in seeds from the control and transgenic plants. Transgenic seeds showed a significant increase (32% in average) in MUFA content and a decrease (7.5% in average) in PUFA level. A very high negative correlation (correlation coefficient of −0.91) was found between monounsaturated fatty acids and the IC_{50} antioxidant parameter for extract from flaxseeds, thus suggesting a highly positive effect of MUFA level in protection against free radicals. It was reported that purified PUFA from flaxseeds could enhance in vivo and in vitro lipid peroxidation in tissue (*32*). Also reported is the significant role of other components (lignans, fiber, and proteins) in oil protection against lipid peroxidation (*32*). Thus, we speculate that increases in phenolic acid, MUFA, and lignan levels concomitant with a decrease in PUFA content resulted in higher antioxidative properties of generated plants.

The increase in stearic and oleic acids levels in transgenic seeds was noted. It is well-known that oil enriched with oleic or stearic acid is more stable during cooking and solid at room temperature (*35*). Interestingly, oil enriched with oleic acid lowers the risk of coronary heart disease (*36*).

It is suggested that the increase in antioxidant capacity may result in increasing plant growth (*38*), resistance to UV-B radiation (*37*), and protection against infectious agents (*39*). Transgenic flax plants were visually indistinguishable from the control plants but were characterized by a higher yield of seeds (fresh weight of seeds per plant) and higher numbers of seeds per bag when compared to the control. Resistance to fungi was also the characteristic feature of transgenic plants. The highest resistance was observed in transgenic lines W92.40 and W92.72, for which the highest content of polyphenols and antioxidant potential were also observed. It was assessed that there is a very high correlation (correlation coefficient of 0.9) between phenolic acids level in flaxseed extract and resistance to *F. culmorum*. There is also a high positive correlation (correlation coefficient of 0.7) between resistance to *F. culmorum* and SDG contents. It is interesting that the high positive correlation (correlation coefficient of 0.9) was calculated for resistance to *Fusarium* and MUFA content.

In conclusion, we found that transgenic flax overexpressing three cDNAs encoding the key enzymes of the phenolic compounds biosynthesis pathway showed increased phenolic acids content. The consequence is the modification of the fatty acids composition in seeds and a significant increase in antioxidant capacity. Subsequently, flax plant yield increase and resistance to pathogen were detected.

ABBREVIATIONS USED

CHS, chalcone synthase; CHI, chalcone isomerase; DFR, dihydroflavonol reductase; IC_{50}, antioxidant potential; AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SDG, secoisolariciresinol diglucoside; ROS, reactive oxygen species.

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