JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

# Engineering Flax Plants To Increase Their Antioxidant Capacity and Improve Oil Composition and Stability

Magdalena Zuk,*[,†,‡] Anna Prescha,[§] Monika Stryczewska,[†,‡] and Jan Szopa[†,‡]

[†]Faculty of Biotechnology, University of Wrocław, Przybyszewskiego 63/77, 51-148 Wroclaw, Poland

[‡]Linum Foundation, Stablowicka 147/149, 54-066 Wroclaw, Poland

[§]Department of Food Science and Nutrition, Wroclaw Medical University, Nankiera 1, 50-140 Wroclaw, Poland

**ABSTRACT:** The composition of polyunsaturated fatty acids in the tissues is very important to human health and strongly depends on dietary intake. Since flax seeds are the richest source of polyunsaturated acids, their consumption might be beneficial for human health. Unfortunately, they are highly susceptible to auto-oxidation, which generates toxic derivatives. The main goal of this study was the generation of genetically modified flax plants with increased antioxidant potential and stable and healthy oil production. Since among phenylpropanoid compounds those belonging to the flavonoid route have the lowest antioxidant capacity, the approach was to inhibit this route of the pathway, which might result in accumulation of other compounds more effective in antioxidation. The suppression of the chalcone synthase gene resulted in hydrolyzable tannin accumulation and thus increased antioxidant status of seeds of the transgenic plant. This was due to the partial redirecting of substrates for flavonoid biosynthesis to the other routes of the phenylpropanoid pathway. Consequently, transgenic plants produced more (20−45%) polyunsaturated fatty acids than the control and mainly $\alpha$-linolenic acid. Thus, increasing the antioxidant potential of flax plants has benefits in terms of the yield of suitable oil for human dietary consumption.

**KEYWORDS:** *flax, Linum usitatissimum, flax oil, linolenic acid, seedcake, antioxidants*

## INTRODUCTION

Flax (*Linum usitatissimum L.*) seeds contain about 40% fats and are a moderate source of the fatty acid $\omega$-6 (linoleic acid) and the richest plant source of $\omega$-3 ($\alpha$-linolenic acid). Both $\alpha$-linolenic acid (ALA) and linoleic acid (LA) are essential fatty acids required in the diet because they cannot be synthesized by humans. In the body, they are desaturated and elongated to longer chain fatty acids. The main products of ALA and LA metabolism are eicosapentaenoic/docosahexaenoic acids and arachidonic acid, respectively. Since ALA and LA metabolism is controlled by the same enzymes ($\Delta$-desaturases), the presence of $\omega$-3 fatty acid can interfere with the activity of $\omega$-6 fatty acid. ALA also interferes with the biological activity of LA, blocking the production of arachidonic acid and also eicosanoids, which promote pathological processes (inflammation, platelet aggregation) such as atherosclerosis and general inflammatory reactions.[1] ALA's beneficial role for our health also lies in the prevention of chronic diseases and cardiovascular diseases, including cancer and rheumatoid arthritis.[2] Thus, in prophylaxis, it is important to decrease the ratio of $\omega$-6 to $\omega$-3 fatty acids in our diet. The high intake of $\omega$-6 in Western diets results in a $\omega$-6:$\omega$-3 ratio of about 20:1. The FAO/WHO-recommended ratio is significantly lower (5 to 10:1). In this situation flax seeds are now considered to be functional food.

The ratio of $\omega$-6 to $\omega$-3 in the oil from several flax cultivars is around 1:3, making it a suitable source of fatty acids for the human diet.[3]

On the other hand, there is an inverse correlation between polyunsaturated fatty acid content and oil stability and the shelf life. The high content of ALA in flax oil makes it highly susceptible to oxidation and rancidity. To prevent its rapid oxidation, flax oil is supplemented with antioxidants such as tocopherol and carotene and stored in dark glass jars. Even so, none of the protection methods are fully satisfactory and the oil cannot be used for frying. Manipulation of expression of genes coding for enzymes involved in antioxidant biosynthesis in flax might be a suitable approach for protecting polyunsaturated fatty acids against auto-oxidation and thus improving oil stability.

Plant phenolic compounds are metabolites with high levels of antioxidant activity. Almost all of them are synthesized from phenylpropanoid precursors. The main classes of plant phenolics are simple phenolics (e.g., coumaric acid, cinnamic acid, ferulic acid) and more complex phenolics such as flavonoids, lignans, and tannins. Besides their antioxidative activity, these compounds have a beneficial effect on human health, and when consumed together with essential unsaturated fatty acids, they can reduce the risk of various diseases.[2] Furthermore, plant phenolics form complexes and accumulate metal ions (e.g., ferric, zinc, magnesium), which are important for fatty acid metabolism. It was shown that a dietary deficiency in iron (a cofactor of desaturases) and zinc limits or increases, respectively, the $\beta$-oxidation of linolenic acid.[4] We have recently found that the overproduction of flavonoid compounds resulted in an increase in fatty acid accumulation in the oil from transgenic flax seeds. Changes in the fatty acid composition and an increase in their stability against oxidation were also detected.[5]

**Received:** February 2, 2012
**Revised:** April 20, 2012
**Accepted:** April 27, 2012
**Published:** April 27, 2012





dx.doi.org/10.1021/jf300421m | *J. Agric. Food Chem.* 2012, 60, 5003−5012

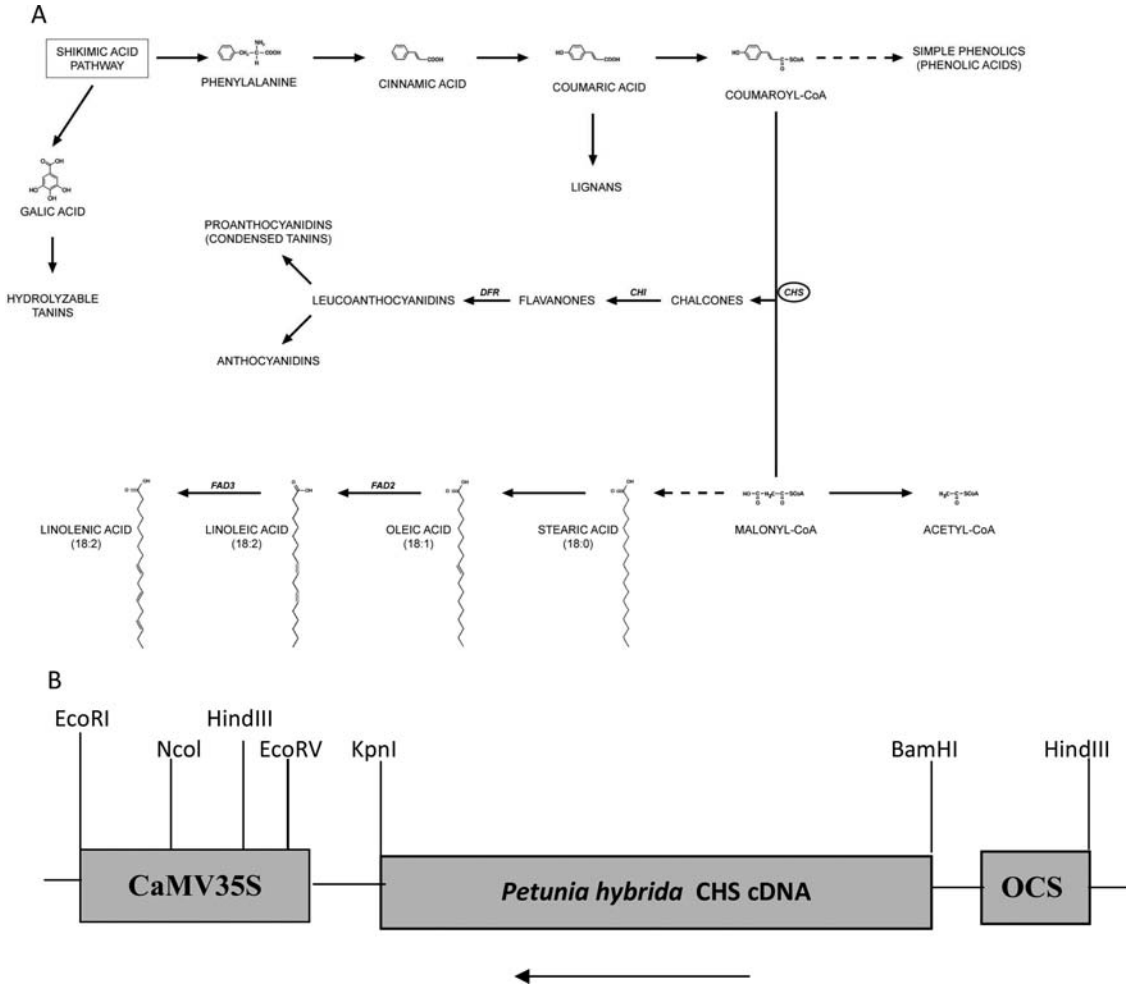


**Figure 1.** (A) Schematic diagram of phenylpropanoid pathway: CHS, chalcone synthase; CHI, chalcone isomerase; DFR, dihydroflavonol reductase; FAD, fatty acid desaturase. The targeted gene (CHS) was highlighted. (B) A diagram of gene construct used for transformation.

In this report, we present a new approach for improving fatty acid composition and oil stability from seeds of transgenic plants transformed with the gene that controls the phenylpropanoid pathway. The approach is based on the partial redirection of substrates from flavonoid biosynthesis to other routes of the phenylpropanoid pathway, which might enhance the antioxidative status of seeds. The way by which this approach might be accomplished is cosuppression of the flax endogenous chalcone synthase gene. In this study, was demonstrated that cosuppression of chalcone synthase resulted in tannin accumulation in flax seeds. The overproduction of tannin in flax resulted in a beneficial ratio of $\omega$-6 to $\omega$-3 fatty acids (1−2:1) in the oil, and yielded a higher stability than found in the control plants. The tannin accumulation caused changes in seed color from light yellow to dark brown.

To the best of our knowledge, this is the first report on transgenic flax plants producing highly stable oil with roughly equal amounts of $\omega$-6 and $\omega$-3 fatty acids suitable for human dietary consumption.

## MATERIALS AND METHODS

**Plant Material.** The control flax seeds (cv. *Linola*) were obtained from the Flax and Hemp Collection of the Institute of Natural Fibres, Poland. Transgenic plants were derived from our previous study,[5] transgenic plants W92 used for comparison, and our own cultivation, transgenic plants W86. For this study, the control and selected homozygous transgenic plants were grown in field, and seeds were harvested after 3 months and analyzed.

**Transgenic Plant Construction and Selection.** For flax plant transformation, we used a binary vector containing cDNA from *Petunia hybrida*, encoding chalcone synthase (CHS, EMBL/GenBank database acc. no. X04080) in the sense orientation under the control of the 35S promoter and OCS terminator (see Figure 1B). The transgenic plants were preselected via PCR using primers specific for the neomycin phosphotransferase (*Npt*II) gene on genomic DNA isolated from tissue-cultured plants as a template. Plants were then selected via the RT-PCR method on total cDNAs as a template.

**Isolation of Homozygous Plants.** The primary regenerants were maintained in small containers and cultivated in the greenhouse until seeds had been produced. To facilitate the identification of the homozygous transgenic lines, the seeds were placed on kanamycin-containing medium (100 mg/L) to test for the segregation of the T-DNA loci. The resistant plants were then relocated to the greenhouse, and the resulting F2 seeds were tested in the seed germination assay to identify homozygous lines. The seeds from homozygous lines were used for field cultivation (F3 generation).

Young leaves obtained from the field plants were used to detect the transgene in the flax genome. Genomic DNA was isolated as a template for polymerase chain reaction (PCR) with primers specific for petunia chalcone synthase (forward, AGCGCATGTGT-GAAAAATCAA; reverse, AATGTCGTCACCAACAAGGCTA). Concomitantly, the endogenous flax chalcone synthase gene amplified in PCR (forward CAAGATCACCCACGTAATATTCTGC; reverse AATGTCGTCACCAA CAAGGCTA) was also analyzed. Seeds were

harvested only from plants that showed the presence of the introduced gene and further used for CHS gene expression analysis.

**Expression Analysis of the CHS Gene in the Transgenic Plants.** Total RNA was isolated from plant material using the Total RNA Kit (A&A Biotechnology, Poland). cDNAs were synthesized from these RNAs using a SuperScriptII Reverse Transcriptase system (Invitrogen). One microliter of a cDNA sample from the RT reaction was used for semiquantitative PCR using Phusion Polymerase (Finzymes). For the PCR reaction, the same primers as for the analysis of genomic DNA were used. As a control housekeeping gene, actin (forward, ACACAGATCATGTTCGAGAC; reverse AGAGCATACCCTTCGTAGA) was used. Seeds were harvested only from plants that showed suppression of the endogenous CHS gene, and those were used throughout this study.

**Biochemical Analysis of Seeds.** *Determination of the Total Anthocyanin Content via the pH-Differential Method.* Total anthocyanin from 15 mg of seeds was extracted using 1 mL of methanol/HCl (95:5, v/v) in an ultrasonic bath for 30 min. The extract was centrifuged at 14000*g* for 10 min. Two dilutions of the sample were performed: first, 100 $\mu$L of the supernatant was mixed with 900 $\mu$L of 0.025 M potassium chloride buffer, pH 1.0, and then 100 $\mu$L of the supernatant was mixed with 900 $\mu$L of 0.4 M sodium acetate buffer, pH 4.5. Both solutions were allowed to stand at room temperature for 15 min, and then the absorbances at 510 and 700 nm were measured, which allowed for haze correction. The control sample containing only buffer solutions was also performed to avoid the influence of such solution on obtained results. The results were calculated using calibration curves prepared on standard substances and reported as cyanidin-3-*O*-glucoside equivalents.[6]

*HPLC Analysis of Flavonol Content in the Transgenic Flax Plants.* One gram of flax seeds was extracted with 7 mL of 35% aqueous methanol containing 1 g/L L-ascorbic acid as an antioxidant, for 18 h at 20 °C in glass screw-capped vials, and then sonicated for 15 min. Next, the samples were centrifuged (5 min, 19000*g*) and the clear supernatant was injected onto a HPLC column. The analysis of flavonol derivatives was carried out on a Merck-Hitachi L-7455 liquid chromatography 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 $\mu$m) Phenomenex (Torrance, CA, USA) 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 for 36 min, followed by washing and reconditioning of the column. The flow rate was 1.0 mL/min, and the runs were monitored at 360 nm.

The retention times were compared, and the amounts of flavonols were calculated using calibration curves prepared on standard substances (Sigma).

*Secoisolariciresinol Diglucoside (SDG) and Phenolic Acid Extraction and Measurement.* A 0.25 g sample of flax seeds defatted with hot hexane was extracted three times with 1.5 mL of 80% methanol (v/v) for 10 min at 70 °C. The extract was centrifuged and evaporated to near dryness at 40 °C under a vacuum. The extract was then resuspended and subjected to alkaline hydrolysis (1 mL, 0.3 M aqueous NaOH) for 2 days at room temperature, followed by neutralization using 2 M HCl.[7] The extract was analyzed on a Waters Acquity UPLC System with a 2996 PDA detector, using an Acquity UPLC column BEH C18, 2.1 × 100 mm, 1.7 $\mu$m. The mobile phase was A = acetonitrile/B = 20 mM ammonium formate, pH 3, in a gradient flow with a protocol of 1 min 10%/90% A/B; a 2–6 min gradient from 10%/90% A/B to 30%/70% A/B; and a 7 min gradient from 30% to 100% A with a 0.4 mL/min flow rate. The compound was detected at 280 nm (SDG) and 320 nm (phenolic acids) and compared to standard substances' retention times (RT): coumaric acid glucoside, RT 1.7; caffeic acid glucoside, RT 1.85; ferulic acid glucoside, RT 2.32; caffeic acid, RT 3.95; *p*-coumaric acid, RT 5.22; ferulic acid, RT 5.96; SDG, RT 5.48 min.The amounts of SDG and phenolic acids were calculated using calibration curves prepared on standard substances (Sigma).

*Evaluation of Proanthocyanidin Content.* For these measurements, 15 mg of seeds was used. Proanthocyanins were hydrolyzed with 1 mL of *n*-butanol–HCl (95:5, v/v) and 33 $\mu$L of 2% (w/v) $NH_4Fe(SO_4)_2 \cdot 12H_2O$ in 2 M HCl for 40 min at 95 °C. The extract was centrifuged at 14000*g* for 10 min, and the supernatant was used for proanthocyanin content evaluation. Proanthocyanin detection was carried out by measuring absorption at 540 nm, and the proanthocyanin content was calculated using calibration curves prepared on standard substances and expressed as catechin equivalents.[8]

*Isolation of the Tannin Fraction.* Defatted flax seeds (10 g) were ground and extracted with 80% acetone (v/v) according to the published procedure.[9] The extract was centrifuged, evaporated to near dryness at 40 °C under vacuum, and then dissolved in 5 mL of ethanol and applied onto a column (1 × 10 cm) packed with Sephadex LH-20 gel. At first, the low molecular weight phenolic compounds were eluted with ethanol (25 mL). Then 30 mL of acetone:water (1:1, v/v) was used to elute the hydrolyzable tannins. The acetone was removed using a rotary evaporator, and the aqueous residue was condensed in vacuum. The content of the phenolic groups was measured using the Prussian blue method,[10] and the hydrolyzable tannin content was calculated using calibration curves prepared on standard substances and expressed as gallic acid equivalents.

*Antioxidant Capacity Measurements.* The chemiluminescence method was used to determine the antioxidant activity of the seed extracts. A methanol (1 mL) extract of 150 mg of seeds was diluted in the range of 1000 to 15,000 times with water, and directly analyzed according to the published method. This experiment was performed in a final volume of 250 $\mu$L 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 mM) and the 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 (IC50) was defined as the amount of flax extract (mg/g DW) that inhibits luminol chemiluminescence by 50%.[3]

**Oil Preparation and Biochemical Analysis.** *Oil Preparation.* Five kilograms of flax seeds was used for oil pressing using a Table OilPress Type20 device (Skeppsta Maskin AB). Flax seeds were ground and transferred to the oil press for the cold pressing of the oil.

*Determination of Fatty Acid Content.* Fatty acid methyl esters (FAMEs) were extracted from the oil using 0.5 M KOH in methanol, after the sample had been neutralized using 1.25 M HCl in methanol. Then methyl esters of the fatty acids were extracted with hexane. The hexane phase was collected, and the lipids were concentrated in a $N_2$ stream and stored at −20 °C. The methyl esters were quantified by gas chromatography (Agilent Technology 6890N with FID detector) using pentadecanoic acid as an internal standard.[11]

*Determination of Phenolic Compound Content.* Total phenolic compounds in methanol extracts (90% v/v) of the oil were measured using the Folin–Ciocalteu method.[12] The phenolic compound content was calculated as equivalents of caffeic acid.

*Determination of Tocopherol, Plastochromanol-8 and $\beta$-Carotene Contents.* The oil samples were dissolved in hexane and filtered using a syringe membrane filter (0.2 $\mu$m) (Pall Gelman Laboratory). The tocopherol, plastochromanol-8, and $\beta$-carotene contents were determined via high-performance liquid chromatography (HPLC, Waters Milford 600 with a fluorimetric detector, excitation 290 nm, emission 330 nm) with $\beta$-tocopherol as an internal standard. For the analysis of $\beta$-carotene, a UV–vis detector (450 nm) was used.[13]

**Oil Stability Analysis.** *Peroxide Value Measurement.* The peroxide value of the oil was determined by measuring the amount of iodine that was formed by the reaction of peroxides (formed in the oil) with the iodine ion. The peroxide value was measured as the mol/dm$^3$ content of sodium thiosulfate ($Na_2S_2O_3$).

*Determination of the Conjugated Diene Concentration.* The conjugated diene concentration was determined spectrophotometrically (234 nm) according to the published method.[14] Linoleic acid was used as a standard.

*Evaluation of Flax Resistance to Fusarium Infection 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, and 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.[15]

*Statistical Analysis.* Data on the parameters measured were statistically analyzed using ANOVA, followed by Kruskal−Wallis test. All of the calculations were carried out using the STATISTICA 7.1 software package (StatSoft Polska, Poland). Values of $p < 0.05$ were considered significant.

## RESULTS

**Transgenic Plant Generation and Selection.** The hypocotyl and cotyledon explants of flax plants were transformed with a vector containing cDNA encoding the key enzyme of flavonoid biosynthesis, chalcone synthase from *Petunia hybrida* (see Figure 1), using *Agrobacterium* mediated transformation. The petunia gene was used due to its high homology to the flax gene, which might provide a cosuppressive effect on expression of both genes, and also facilitate its detection in the flax genome. The gene construct was inserted into the genome of the flax plants.[5] The obtained regenerants were preselected using the PCR method with specific primers for the neomycin phosphotransferase gene. Plants for which a 495-bp (*Npt*II) DNA fragment was indicated were used for further selection via RT-PCR analysis (Figure 2). The three transgenic lines (W86.7, W86.10, and W86.11) that showed the lowest level of endogenous mRNA encoding CHS were used for the field trial and homozygous line isolation, and then for biochemical analysis. Although detailed analysis of the W86 transgenic plants was the main goal of this study, the plants of the transgenic lines overproducing flavonoid compounds (W92 type) previously described[5] are now being used for comparison.

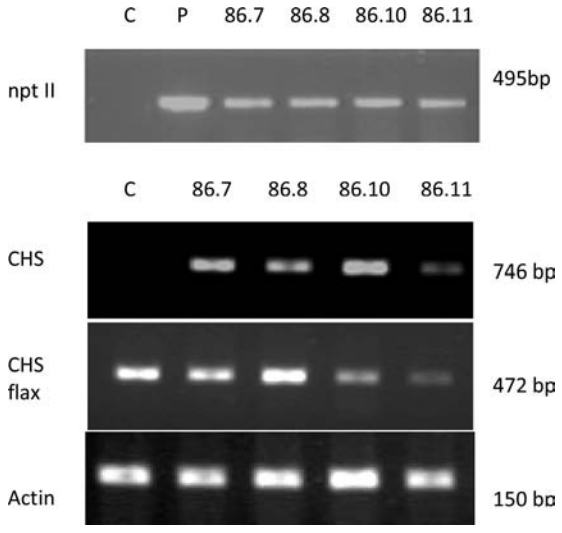


**Figure 2.** Preselection (A, top) and selection (B, bottom) of transgenic flax. (A) Agarose gel electrophoresis of PCR products (495 bp) of the *Npt*II gene; C, nontransformed plants (negative control); P, plasmid used for transformation (positive control); transgenic plants are numbered. (B) RT-PCR analysis of transgenic (CHS) and endogenous (CHS flax) chalcone synthase gene expression in control (C) and transgenic plants (numbered). Total RNA was subjected to RT-PCR semiquantitative analysis using the actin (Actin) gene as the internal control; the lengths of the PCR products are marked on the right.

**Isolation of Homozygous Plant.** The seeds obtained from the primary regenerants (F1) were grown on MS medium containing kanamycin for the segregation of the T-DNA loci. Plants resistant for kanamycin were then transferred to the greenhouse; after two cycles of cultivation we identified homozygous lines. Field-grown homozygous plants from F3 seeds were analyzed throughout this study.

The integration of the petunia cDNA coding for CHS into the flax genome was confirmed via PCR. Genomic flax DNA, which served as a template, was isolated from plants of the F3 generation and probed with primers specific for the introduced gene. The results of the PCR reaction are presented in Figure 3.

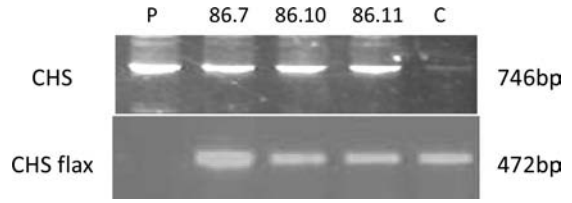


**Figure 3.** Detection of the introduced petunia gene in homozygous transgenic W86 flax lines. Agarose gel electrophoresis of PCR products on genomic flax DNA as a template revealed the presence of the introduced petunia gene in homozygous flax lines (numbered). CHS, petunia chalcone synthase gene product (746 bp); CHS flax, product of endogenous chalcone synthase gene (472 bp); P, positive control (plasmid DNA); C, nontransformed plants.

Since the CHS gene sequence from flax is already known, the presence of the endogenous chalcone synthase gene was also probed. All three lines of transgenic plants (W86.7, W86.10, and W86.11) of the third generation exhibited the introduced genes, and the endogenous CHS gene was also detected. Thus, the isolated homozygous transgenic lines were stably transformed.

**Phenotype Analysis.** There were no visible differences in the leaf shape and size or the petal between the transformed and nontransformed plants in field-grown plants. However, the plants of W86 transgenic lines displayed changes in seed color. The color of the seeds changed from the light yellow typical for the control to dark brown for transgenes, and this feature was stably inherited through the F2 and F3 generations (Figure 4).

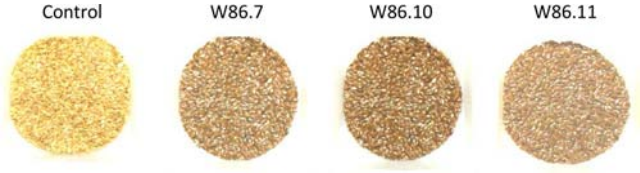


**Figure 4.** Phenotype of flax seeds from the control and transgenic lines overproducing tannins (numbered).

**Analysis of the Metabolites in Seeds.** The phenylpropanoid pathway is central to the formation of phenolic compounds. The condensation of *p*-coumaroyl-CoA and malonyl-CoA resulted in chalcones that served as a direct substrate for flavonoid biosynthesis (Figure 1). This reaction is catalyzed by chalcone synthase (CHS). Chalcones are converted to flavanones via isomerization catalyzed by chalcone

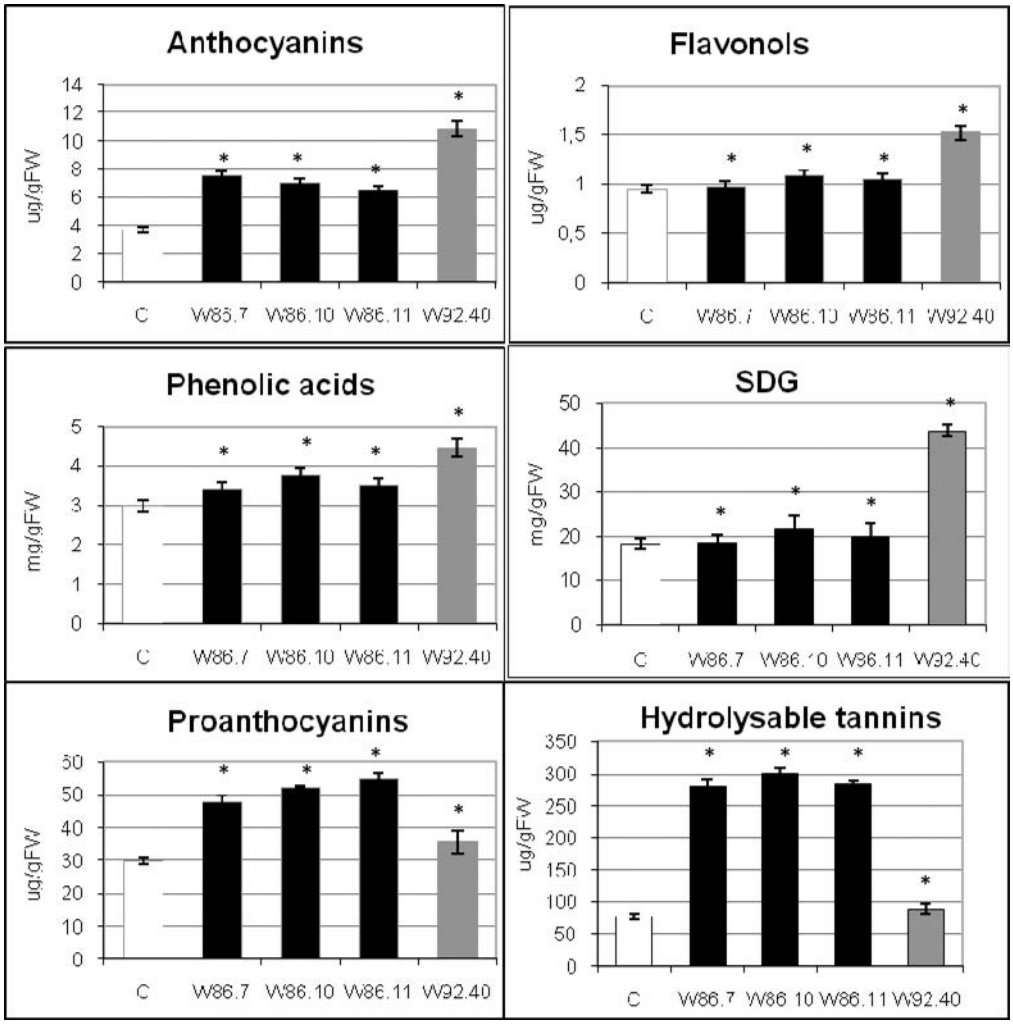


**Figure 5.** Content of metabolites from the phenylopropanoid pathway determined in seeds from control (C) and transgenic flax plants (numbered). Seeds were extracted with methanol (anthocyanin, flavonol, secoisolariciresinol diglucoside, phenolic acid), *n*-butanol−HCL (proanthocyanidin), or acetone (hydrolyzable tannin). The content of anthocyanidin glucoside, flavonols, proanthocyanidins, and hydrolyzable tannins was determined spectrophotometricaly and expressed in cyanidin-3-*O*-glucoside, naringenin, catechin, and gallic acid equivalents, respectively. The secoisolariciresinol diglucoside (SDG) and phenolic acids were identified via UPLC analysis based on standard retention time, and the compound contents were calculated using prepared calibration curves. The mean value ($n$ = 5−6) ± SD is presented; *statistically significant ($p$ < 0.05).

isomerase (CHI). Hydroxylation reactions convert flavanones to dihydroflavonols, which are converted to flavonols in a desaturation reaction. The dihydroflavonols are reduced by dihydroflavonol reductase (DFR) to form colorless leucoanthocyanidins, which are then converted to anthocyanidins. Flavonoids can polymerize to form proanthocyanidins, which are frequently referred to as condensed tannins.

It was previously reported that transgenic flax plants show significant changes in the level of flavonols (kaempferol, quercetin), phenolic acids, and anthocyanin upon the overexpression of CHS, CHI, and DFR cDNAs (W92 plants).[5] Those transgenic lines showed a significant (nearly 3-fold) increase in the total anthocyanin content (Figure 5, W92.40 is shown for comparison). Consistently with this, the suppression of CHS (transgene W86) resulted in a lower level of anthocyanins but still significantly higher (about 1.7-fold) than for the control plants (Figure 5).

The total flavonol content was also higher in the plants from transgenic lines that overexpressed three petunia genes (W92 type) compared to nontransgenic plants. Again for W86 plants a lower content was measured, but relative to the control plant, a 10% increase was detected.

Flavonoids can be converted to proanthocyanidins, so the compound content was analyzed in transgenic plants of the third generation and compared to the control. The level of proanthocyanins in the seeds from W86 transformants increased by about 56−80%, while only 18% increase for plants overexpressing three flavonoid genes (W92 type) was detected (Figure 5).

The transgenic lines W86 showed a small increase of simple phenolics (phenolic acids) content in comparison to the control plants (14−26%), but a distinct increase (on average 49%) in the content of these compounds in W92 plants was detected (Figure 5). As indicated on the UPLC chromatograms (Figure 6), in all of the analyzed plants the dominant phenolic acids were ferulic acid (aglycon and glucoside), *p*-coumaric acid (aglycon and glucoside), and caffeic acid glucoside.

Since simple phenolics are the precursors for lignan biosynthesis, the quantity of this compound was also measured. The level of secoisolariciresinol diglucoside (SDG), the main flax lignan, was only very slightly higher (about 10%) in plants

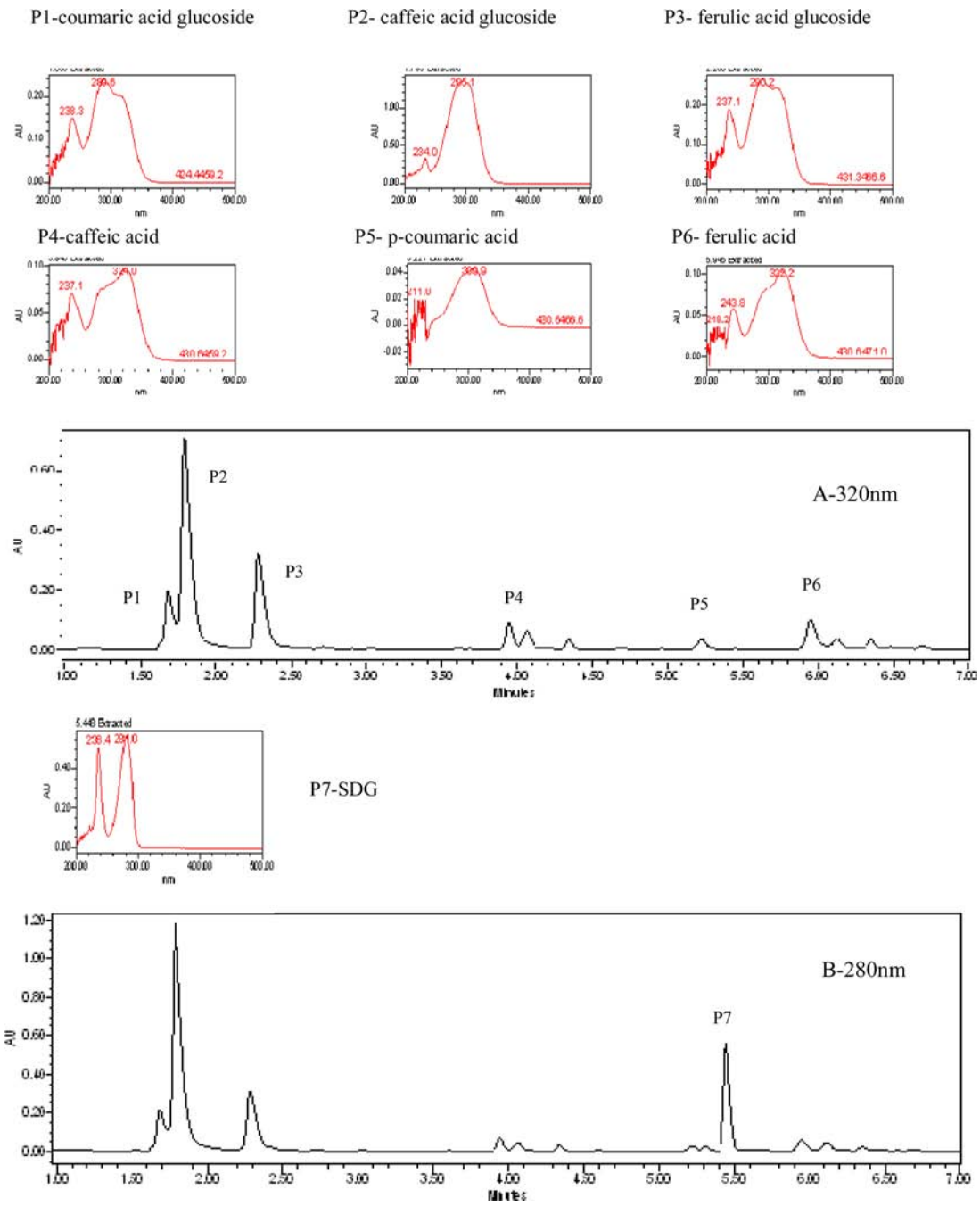


**Figure 6.** Identification of phenolic acids and secoisolariciresinol diglucoside (SDG) in transgenic seeds by UPLC analysis of flax seed crude extracts. (A) Chromatogram taken at 320 nm for phenolic acid identification. (B) Chromatogram taken at 280 nm for SDG identification. The UV spectra of the identified compounds are inserted.

with CHS suppression (W86 type) when compared to the control. However, the compound content in the plants that overproduced CHS (W92 plants) was significantly higher (over 2-fold) than for control plants.

The compounds of the phenylpropanoid pathway that might be responsible for seed color changes (lines W86) are tannins. The ring cells of the parenchyma and the pigment cells of the testa may contain dark tannin substances.[4] Thus, we performed an analysis of hydrolyzable tannins. For seeds of W86 transgenic plants, about 4-fold increase in the level of hydrolyzable tannin was detected when compared to the control (Figure 5). The content of these compounds in the seeds from W92 type plants was the same as for the control.

In summary, the data strongly suggest that suppression of the flax CHS gene only partially affects flavonoid biosynthesis and strongly influences the tannin biosynthesis route of the phenylpropanoid pathway. It is speculated that suppression of this gene resulted in redirection of the flavonoid route's substrate to hydrolyzable tannin synthesis, and thus led to the changes in seed color.

**Antioxidant Capacity of Extracts from Seeds.** It is known that phenolic compounds synthesized from phenylpropanoid precursors exhibit strong antioxidant properties. Since genetic modification performed on flax plants resulted in an increase in their content in seed extracts, the respective enhancement in antioxidant properties was expected. The


dx.doi.org/10.1021/jf300421m | J. Agric. Food Chem. 2012, 60, 5003−5012

antioxidant potential is expressed as the IC-50 value, which denotes the amount of plant extract inhibiting the oxidation of luminol by 50%. As expected, the extracts from the seeds of W86 transgenic lines showed a significant increase (by about 40−50%) in antioxidant capacity (Figure 7). However, a much higher increase (5-fold on average) in antioxidant potential was detected for W92 plants.

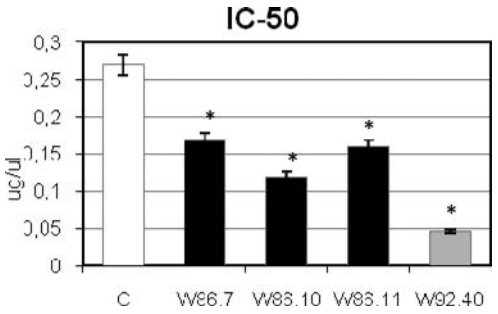


**Figure 7.** Antioxidant potential (IC-50) of extracts from seeds of the control (C) and transgenic plants overproducing tannins (W86 type), and for comparison a transgenic line accumulating flavonoids (W92.40) is included. The mean value ($n$ = 5−6) ± SD is presented; *statistically significant ($p$ < 0.05).

**Analysis of Fatty Acid Composition in Seeds.** Flax seeds have long been used in the human and animal diet and in industry as a source of oil. Since the antioxidant capacity of seeds from transgenic lines was significantly increased, protection against oxidation and thus improvement of the fatty acid content and composition in seeds was expected. This was the case: an increase in total as well as in unsaturated acid content in all transgenic plants was detected; the data are shown in Table 1. A 50% increase in the content of total fatty acids (mainly linolenic acid, 30−34-fold in independent transgenic lines) was found in the seeds from W86 transgenic lines, compared to the control. Roughly the same increase (20% on average) was measured for fatty acids in the seeds from the W92 plants. In the case of W92 transgenic lines, the changes in fatty acid composition mainly concerned linoleic acid (35% on average).

*Preparation of Oil from Seeds.* Seeds from field-grown transgenic line W86.11 (2010 season) were used as a source of oil. The oil was produced by the typical industrial method including seed grinding and cold pressing. There was only a slight difference in the yield of oil from control (25%) and W86 seeds (22%) and no difference compared to W92 (25%) transgenic seeds.

*Analysis of Fatty Acid Composition and Stability of Oil.* As expected, the composition of fatty acids in cold pressed oil from W86.11 plants was similar to that indicated for seeds (Table 2).

**Table 2. Content of Principal Fatty Acid (mg/mL) Fraction and Antioxidant Levels in Cold Pressed Oil from Control and Transgenic Seeds Overproducing Tannins (W86.11)[a]**

| | control | W86.11 | W92.40 |
|---|---|---|---|
| 16:0 | 51.25 ± 0.64 | 37.35 ± 0.34 | 50.76 ± 0.77 |
| 18:0 | 30.15 ± 0.35 | 29.63 ± 0.74 | 27.01 ± 0.68 |
| 18:1 | 154.82 ± 2.32 | 125.27 ± 2.05 | 111.81 ± 1.94 |
| 18:2 | 589.01 ± 5.78 | 321.78 ± 4.04 | 708.26 ± 8.35 |
| 18:3 | 17.21 ± 0.67 | 414.95 ± 6.21 | 21.49 ± 0.84 |
| phenolic compounds | 1.19 ± 0.04 | 1.32 ± 0.02 | 1.27 ± 0.01 |
| tocopherols | 8.34 ± 0.61 | 8.41 ± 0.22 | 8.36 ± 0.72 |
| plastochromanol-8 | 1.15 ± 0.07 | 1.22 ± 0.20 | 1.32 ± 0.40 |
| $\beta$-carotene | 0.01 ± 0.00 | 0.02 ± 0.00 | 0.02 ± 0.00 |

[a]For comparison a transgenic line accumulating flavonoids (W92.40) is included.

The high level of linolenic acid in the oil from W86 plants, although beneficial because of its biological activity, might however significantly reduce the oil stability and thus its shelf life. Therefore, the parameters that describe oil stability were measured. The oil stability was determined by measuring the peroxide value (prime products of lipid peroxidation) and conjugated diene content (secondary products of lipid peroxidation). The data are presented in Table 3. The peroxide value parameter shows decrease by about 72% for oil from W86 seeds when compared to the control. For W92 this parameter

**Table 1. Content and Composition of Fatty Acid in Seeds from Control (Linola) and Transgenic Lines Overproducing Tannins (W86.7, W86.10, W86.11)[a]**

| cultivation/fatty acid | control | W86.7 | W86.10 | W86.11 | W92.40 |
|---|---|---|---|---|---|
| 16:0 | 12.62 ± 0.44 | 10.09 ± 0.35 | 12.53 ± 0.94 | 12.42 ± 1.01 | 13.89 ± 1.02 |
| 16:1 | 0.16 ± 0.00 | 0.20 ± 0.05 | 0,21 ± 0.01 | 0.22 ± 0.04 | 0.27 ± 0.01 |
| 16:2 | 0.12 ± 0.64 | 0.14 ± 0.01 | 0.16 ± 0.00 | 0.17 ± 0.01 | 0.18 ± 0.00 |
| 16:3 | 0.10 ± 0.01 | 0.13 ± 0.04 | 0.12 ± 0.01 | 0.10 ± 0.03 | 0.15 ± 0.02 |
| 18:0 | 7.52 ± 0.34 | 8.50 ± 0.05 | 7.38 ± 0.98 | 10.18 ± 0.64 | 7.03 ± 0.53 |
| 18:1 | 41.75 ± 4.64 | 41.49 ± 3.87 | 54.22 ± 4.08 | 42.11 ± 3.56 | 32.11 ± 3.47 |
| 18:2 | 147.00 ± 3.34 | 91.72 ± 3.24 | 102.88 ± 2.94 | 110.25 ± 3.94 | 197.84 ± 4.44 |
| 18:3 | 4.43 ± 0.64 | 151.51 ± 7.98 | 132.83 ± 6.91 | 139.99 ± 6.54 | 6.06 ± 0.88 |
| 20:0 | 0.26 ± 0.00 | 0.23 ± 0.00 | 0.24 ± 0.01 | 0.26 ± 0.00 | 0.16 ± 0.00 |
| 20:1 | 0.19 ± 0.03 | 0.23 ± 0.04 | 0.27 ± 0.00 | 0.19 ± 0.00 | 0.30 ± 0.02 |
| 22:0 | 0.12 ± 0.02 | 0.14 ± 0.00 | 0.15 ± 0.00 | 0.14 ± 0.02 | 0.15 ± 0.00 |
| 22:1 | 0.10 ± 0.00 | 0.19 ± 0.04 | 0.18 ± 0.00 | 0.18 ± 0.01 | 0.20 ± 0.00 |
| 24:0 | 0.03 ± 0.00 | 0.04 ± 0.00 | 0.05 ± 0.00 | 0.06 ± 0.00 | 0.05 ± 0.00 |
| satd fatty acids | 20.55 ± 2.44 | 19.00 ± 2.34 | 20.35 ± 1.99 | 23.06 ± 2.64 | 21.28 ± 2.15 |
| polyunsatd fatty acids | 151.65 ± 3.64 | 243.5 ± 4.85 | 235.99 ± 5.04 | 250.51 ± 4.97 | 204.23 ± 5.24 |
| total fatty acids | 214.4 ± 5.87 | 304.61 ± 5.64 | 311.22 ± 6.02 | 316.27 ± 5.99 | 258.39 ± 4.98 |

[a]For comparison a transgenic line accumulating flavonoids (W92.40) is included. The fatty acid content in seeds is expressed in mg/gFW.

**Table 3. Peroxide Value and Conjugated Diene Content in Heated Oil from Control and Transgenic Seeds Overproducing Tannins (W86.11)[a]**

| | peroxide value [mequiv of $O_2$/kg of oil] | conjugated diene [mmol/kg of oil] |
|---|---|---|
| control | 5.76 ± 0.089 | 11.86 ± 0.27 |
| W86.11 | 1.64 ± 0.1 | 7.14 ± 0.16 |
| W92.40 | 0.32 ± 0.03 | 5.23 ± 0.09 |

[a]For comparison a transgenic line accumulating flavonoids (W92.40) is included.

decreases by about 90%. The conjugated diene content, another parameter that describes the antioxidant potential of oil, was also significantly lower (ranging from 40 to 55% of the control value) in the transgenic plants.

*Analysis of Metabolites with Antioxidant Potential in Oil.* Since it was found that the oil from transgenic plants was more stable than the control, the concentrations of metabolites with antioxidant activity were measured. The data are presented in Table 2. There was a slight but statistically significant increase in the total phenolics compared to the control. Also, a slight increase was detected in the content of fat-soluble antioxidants (tocopherols, plastochromanol-8, $\beta$-carotene) in the oil from transgenic seeds compared to the control. Whether these slight increases in antioxidant contents resulted in oil stability changes is as yet an open question. However, as reported from an in vitro study, the addition of either carotene or quercetin at micromolar concentration reasonably protected flax lipids against oxidation, while a higher concentration of these compounds increased lipid susceptibility to peroxidation.[16]

*Fusarium oxysporum and F. culmorum Resistance.* Flax infection by pathogenic fungi can lead to about a 20% loss in yield. It is thus important from an agronomic point of view that any genetic modification of flax desirable for increased metabolite production does not affect the plant's sensitivity to pathogenic microorganisms.

Thus, we measured the resistance of young cotyledons obtained from the seeds of control and transgenic plants against *F. culmorum* and *F. oxysporum* infection.

There were significant changes in the resistance of transgenic plants against *F. culmorum* and *F. oxysporum* infections. The level of this resistance reflects the antioxidant capacity of the flax (Figure 8). The improved resistance to *F. oxysporum* and *F. culmorum* infection shown by the plants of the W86 transgenic lines suggests their high suitability for farming.

In summary the suppression of chalcone synthase gene expression resulted in the accumulation of hydrolyzable tannin in flax plant. Since the first stage in plant pathogen infection is free radical production[17] and tannins reveal antioxidative function, the increased plant protection against pathogen infection was expected. This was the case, transgenic plant showed higher resistance against fusarium infection than control plants. The other advantage of tannin accumulation was higher oil stability, which is probably also the result of tannin antioxidative function.

## DISCUSSION

Flax seeds have long been used in the human and animal diet, and in industry as a source of oil. Recently, there has been growing interest in flax plants as a source of nutraceuticals. It is suggested that flax fat can be significant in maintaining human well-being, promoting health and even treating diseases such as

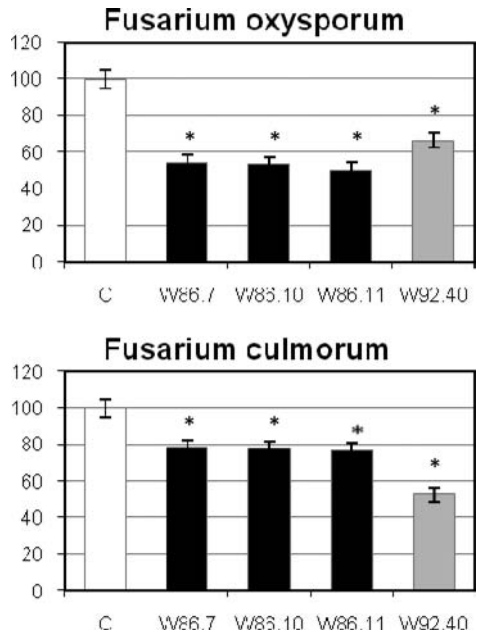


**Figure 8.** Resistance of control (C) and transgenic plants overproducing tannins (W86 type), and for comparison a transgenic line accumulating flavonoids (W92.40) to *Fusarium oxysporum* and *Fusarium culmorum* infection (percentage of infected flax seedlings). The mean value ($n$ = 5−6) ± SD is presented; *statistically significant ($p < 0.05$).

coronary heart disease, some kinds of cancer, and neurological and hormonal disorders.[1,2] The beneficial effects are mostly due to the flax lipids. Flax oil is a moderate source of linoleic acid (about 16% of the total fatty acids), an essential fatty acid of the $\omega$-6 family, and the richest plant source of $\alpha$-linolenic acid (about 57% of the total fatty acids), which is the parent fatty acid of the $\omega$-3 family, the polyunsaturated fatty acids (PUFAs).

In light of these findings, flax oil appeared to be a suitable source of essential fatty acids for the human diet. Most of the flax cultivars produce oil with a $\omega$-3 to $\omega$-6 ratio ranging around 0.3:1.[16]

Unfortunately, essential fatty acids are highly susceptible to oxidation. Therefore, flax oil has a very short shelf life. Most importantly, the oxidation of fatty acids yields several toxic compounds (e.g., conjugated diene). Only certain cultivars such as Linola, obtained via mutation breeding, are suitable for the commercial preparation of edible oil.[18,19] However, the oil from Linola exhibits an extremely high $\omega$-6 to $\omega$-3 ratio (30:1), so its health beneficial effect is rather limited.

To prevent rapid oxidation, flax oil is often cold-pressed, supplemented with vitamin A and E, and/or stored in dark glass jars and under nitrogen atmosphere. Since none of these protection methods are fully satisfactory, further improvements are being sought. The genetic engineering approach could involve the overproduction of various natural antioxidants within the flax grains. In addition to preventing fat rancidity, antioxidants such as polyphenols might also have a beneficial effect on human health.[2] Flavonoids protect plants against pathogenic microorganisms,[20] herbivores, UV radiation,[21] and oxidative and temperature stresses. It has been reported that flavonoids exhibit health-protecting activities due to their strong antioxidant properties.[22] Their antioxidant activity has a great significance for food quality, because they may inhibit enzymatic and nonenzymatic peroxidation. In addition,

flavonoids have antiallergenic, antiviral, anti-inflammatory, and vasodilatory activities.[22]

Similar health promoting activities are postulated for condensed and hydrolyzable tannins.[23] It was also indicated that gallic acid inhibits mast cell-derived inflammatory allergic reactions by blocking histamine release and proinflammatory cytokine expression. Furthermore, in vivo and in vitro antiallergic effect of gallic acid suggests a possible therapeutic application of this agent in inflammatory allergic diseases.[24] Tannins, as common components of food and beverages of plant origin, influenced their taste by providing a more or less appreciated astringency. As versatile medicinal agents they have been widely used in traditional folk medicine to cure a variety of physical disorders.[25]Tannins will be also very important in animal (especially cattle) feeding. Tannins bind to rumen cell wall degrading microorganisms and inhibit microbial growth or enzyme activity. They also interact with feed proteins and decrease their availability to the rumen microorganisms. The retardation of the wasteful ruminal protein degradation is a major task for ruminant nutritionists and is achieved by the selective inhibition of Gram-positive bacteria by feed antibiotics. Feed antibiotics will be banned in the European community soon, so tannins could be one natural alternative to manipulate ruminal protein degradation.[26]

All these features make phenylopropanoid compounds attractive targets for genetic engineering strategies aimed at producing transgenic plants with increased antioxidant properties. Thus, the goal of our previous study was to increase the antioxidant potential of flax to give greater stability of PUFAs against oxidation. Indeed, simultaneous and maximal overexpression of petunia chalcone synthase (CHS), chalcone isomerase (CHI), and dihydroflavonol reductase (DFR) in selected transgenic lines (W92 type) was found to result in significant accumulation of flavonoids in seeds from transgenic plants, and thus an increase in the stability of unsaturated fatty acids against oxidation was detected.[5] However, the increase in antioxidant status of the seeds does not improve the ratio of $\omega$-6 to $\omega$-3 fatty acids in oil from these seeds. The oil from W92 plants exhibits an extremely high $\omega$-6 to $\omega$-3 ratio (20:1), and therefore its health beneficial effect is rather low.[5]

In this study, a different approach for changing plant antioxidant capacity was taken. It is based on redirecting the substrates for flavonoid biosynthesis to other branches of the phenylpropanoid pathway upon suppression of chalcone synthesis. Introducing the chalcone synthase gene into flax explants resulted in suppression of both exogenous and endogenous CHS gene expression.

The molecular background for the suppression of endogenous CHS gene expression is as yet unknown, but it was noticed that overexpression of CHS in petunia plants resulted in its cosuppression, which is now identified as an siRNA effect.[27] Thus, we speculate that highly expressed exogenous CHS leads to siRNA generation and/or subsequent gene modification. Whatever the reason, the inhibition of endogenous CHS expression in flax might result in the redirection of upstream substrates (from the shikimic acid pathway, Figure 1) for flavonoid biosynthesis to the tannins. Indeed, the plants of the W86 transgenic lines produced a higher level of hydrolyzable tannins than the control, which resulted in the change of seed color from light yellow to dark brown. In the course of this study, homozygous plants of the third generation were studied and showed stable inheritance of this phenotypic feature.

An interesting result was obtained for the PUFA level in oil from seeds of the W86 transgenic lines. Their quantity significantly increases (about 50%). During plant development, the fatty acid composition changes due to their turnover within lipids and the formation of oxidized polyunsaturated fatty acids. PUFA oxidation plays a regulatory role in plant adaptation to diverse and variable environmental conditions. It might thus suggest that accumulated antioxidants in transgenic seeds protect PUFAs against auto-oxidation during plant development and maturation. This is perhaps the reason for the higher accumulation of PUFAs in the oil from transgenic seeds compared to the control.

However, the most intriguing results that have been obtained concern PUFA composition in oil from W86 transgenic plants. The level of $\alpha$-linolenic acid in oil from these plants increases about 25-fold compared to the control. Thus the ratio of $\omega$-6 to $\omega$-3 in oil from W86 seeds is about 1.5:1, which is quite close to that recommended by the FAO/WHO (5 to 10:1) for the human diet. Additionally, the oil stability is significantly improved. The conjugated diene formation in the oil from transgenic plants oxidized at 140 °C decreases by about 40% compared to the control oil. Also, the peroxide value, which indicates products of primary lipid oxidation, significantly decreases (a 3.5-fold decrease) upon oil oxidation compared to the control.

It is as yet difficult to explain the high accumulation of $\alpha$-linolenic acid in oil from transgenic W86 plants instead of linoleic acid, which accumulates in the oil from W92 transgenic lines.[28] The simplest explanation would be the interconversion of linoleic to $\alpha$-linolenic acid via activation of the desaturation reaction in W86. It is known that the desaturating enzyme contains iron and the desaturation of fatty acids depends on electron transfer from NADH via iron-containing cytochromes. Therefore, the iron deficiency might limit the desaturation of fatty acids. Since W86 plants contain a high quantity of tannins that bind metal ions, it is quite possible that this is the mode of desaturase activation. The other possibility is simply based on the protection of unsaturated fatty acids against oxidation by accumulated antioxidants in transgenic seeds. PUFAs are oxidized either via auto-oxidation or by enzymes such as lipoxygenases or $\alpha$-dioxygenases, and the products of the latter play a role in distinct stress and developmentally regulated pathways. Since the tannins and proanthocyanins (although structurally distinct) are known to bind proteins, it is quite possible that binding protein enzymes limits their oxidation activity. Plants of the W86 lines have a high tannin content and showed this process most effectively.

To the best of our knowledge, this the first report on the effect of phenylpropanoid pathway manipulation on fatty acid metabolism in plants. The flax generated through this manipulation appeared to be a remarkable source of health-promoting oil.

## AUTHOR INFORMATION

### Corresponding Author

*E-mail: mzuk@ibmb.uni.wroc.pl. Tel, fax: 48 71 3252930.

### Funding

This study was supported by Grants NN310079038, NR12009-06, and NR 12017110 from the Ministry of Science and Higher Education.

### Notes

The authors declare no competing financial interest.

## ABBREVIATIONS USED

ALA, α-linolenic acid; LA, linoleic acid; PUFAs, polyunsaturated fatty acids; SDG, secoisolariciresinol diglucoside; UPLC, ultraperformance liquid chromatography; RTPCR, reverse transcriptase PCR; DW, dry weight; FW, fresh weight

## REFERENCES


(1) Lu, D. Y.; Tsao., Y. Y.; Leung, Y. M.; Su, K. P. Docosahexaenoic acid suppresses neuroinflammatory responses and induces heme oxygenase-1 expression in BV-2 microglia: implications of antidepressant effects for omega-3 fatty acids. *Neuropsychopharmacology* **2010**, *35* (11), 2238−2248.

(2) Massaro, M.; Scoditti., E.; Carluccio, M. A.; De Caterina, R. Nutraceuticals and prevention of atherosclerosis: focus on omega-3 polyunsaturated fatty acids and Mediterranean diet polyphenols. *Cardiovasc. Ther.* **2010**, *28*, 13−19.

(3) Lukaszewicz, M.; Matysiak.-Kata, I.; Skała, J.; Fecka, I.; Cisowski, W.; Szopa, J. Antioxidant capacity manipulation in transgenic potato tuber by changes in phenolic compounds content. *J. Agric. Food Chem.* **2004**, *52*, 1526−1533.

(4) Muir, A. D., Westcott, N. D., Eds. *Flax: The genus Linum*; Taylor & Francis: Canada, 2003.

(5) Lorenc-Kukuła, K.; Amarowicz., R.; Oszmiański, J.; Doermann, P.; Starzyki, M.; Skała, J.; Żuk, M.; Kulma, A.; Szopa, J. Pleiotropic Effect of Phenolic Compounds Content Increases in Transgenic Flax Plant. *J. Agric. Food Chem.* **2005**, *53*, 3685−3692.

(6) Francis, F. J. Food colorants: anthocyanins. *Crit. Rev. Food Sci. Nutr.* **1989**, *28*, 273−314.

(7) Johnsson, P.; Kamal-Eldin, A.; Lundgren, L. N.; Aman, P. HPLC method for analysis of secoisolariciresinol diglucoside in flaxseeds. *J. Agric. Food Chem.* **2000**, *48*, 5216−5219.

(8) Porter, L. J.; Hrstich, L. N.; Chan, B. G. The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. *Phytochemistry* **1986**, *25*, 223−230.

(9) Amarowicz, R.; Piskuła, M.; Honke, J.; Rudnicka, B.; Troszyńska, A.; Kozłowska, H. Extraction of phenolic compounds from lentil seeds (Lens culinaris) with various solvents. *Pol. J. Food Nutr. Sci.* **1995**, *45*, 53−62.

(10) Graham, H. D. Stabilization of the Prussian Blue Color in the Determination of Polyphenols. *J. Agric. Food Chem.* **1992**, *40*, 801−805.

(11) Prescha, A.; Świędrych., A.; Biernat, J.; Szopa, J. Increase in lipid content in potato tubers modified by 14−3-3 gene overexpression. *J. Agric. Food Chem.* **2001**, *49*, 3638−3643.

(12) Naczk, M.; Shahidi, F. Extraction and analysis of phenolics in food. *J. Chromatogr., A* **2004**, *1054*, 95−111.

(13) Velasco, L.; Goffman., D. Tocopherol,plastochromanol and fatty acid patterns in the genus Linum. *Plant Syst. Evol.* **2000**, *221*, 77−88.

(14) Recknagel, R. O.; Glende, E. A., Jr. Spectrophotometric detection of lipid conjugated dienes. *Methods Enzymol.* **1984**, *105*, 331−337.

(15) Starzycki, M. Badania nad odpornością rzepaku ozimego (*Brassica napus* L.), na patogena *Leptosphaeria maculans* (Desm.) Ces. et de Not./*Phoma lingam* (Tode ex Fr. Desm.), powodującego suchą zgniliznę roślin kapustnych. *Monografie i rozprawy naukowe IHAR* **1998**, *1*.

(16) Lukaszewicz, M.; Szopa, J.; Krasowska, A. Susceptibility of lipids from different flax cultivars to peroxidation and its lowering by added antioxidants. *Food Chem.* **2004**, *88*, 225−231.

(17) Kostyn, K.; Czemplik., M.; Kulma, A.; Bortniczuk, M.; Skała, J.; Szopa, J. Genes of phenylpropanoid pathway are activated in early response to Fusarium attack in flax plants. *Plant Sci.* **2012**, DOI: 10.1016/j.plantsci.2012.03.011.

(18) Dribnenki, J.; Green, A. G. Linola '947' low linolenic flax. *Can. J. Plant Sci.* **1995**, *75*, 201−202.

(19) Green, A. G. A mutant genotype of flax (Linum usitatissimum L.) containing very low levels of linolenic acid in its seed oil. *Can. J. Plant Sci.* **1986**, *66*, 499−503.

(20) Cushni, T.; Lamb, A. Antimicrobial activity of flavonoids. *Int. J. Antimicrob. Agents* **2005**, *26*, 343−56.

(21) Ryan, K. G.; Swinny, E.; Markham, K. R.; Winefield, C. Flavonoid gene expression and UV photoprotection in transgenic and mutant Petunia leaves. *Phytochemistry* **2002**, *59*, 23−32.

(22) Harborne, J. B.; Williams, Ch. A. Advances in flavonoids research since 1992. *Phytochemistry* **2000**, *55*, 481−504.

(23) Fernandes, A.; Fernandes, I.; Cruz, L.; Mateus, N.; Cebral, M.; De Freitas, V. Antioxidant and biological properties of bioactive phenolic compounds from Quercus suber L. *J. Agric. Food Chem.* **2009**, *57*, 11154−11160.

(24) Sang-Hyun, K.; Chang-Duk, J.; Kyongho, S.; Byung-Ju, C.; Hyunjeung, L.; Seunja, P.; Seung, Ho L; Hye-Young, S.; Dae-Keun, K.; Tae-Yong, S. Gallic Acid Inhibits Histamine Release and Pro-inflammatory Cytokine Production in Mast Cells. *Toxicol. Sci.* **2005**, *91* (1), 123−131.

(25) Haslam, E. Natural polyphenols (vegetable tannins) as drugs:possible modes of action. *J. Nat. Prod.* **1996**, *59*, 205−215.

(26) Muetzel, S.; Becker., K. Extractability and biological activity of tannins from various tree leaves determined by chemical and biological assays as affected by drying procedure. *Anim. Feed Sci. Technol.* **2006**, *125*, 139−149.

(27) De Paoli, E.; Dorantes-Acosta, A.; Zhai, J.; Accerbi, M.; Jeong, D. H.; Park, S.; Meyers, B. C.; Jorgensen, R. A.; Green, P. J. Distinct extremely abundant siRNAs associated with cosuppression in petunia. *RNA* **2009**, *15* (11), 1965−1970.

(28) Zuk, M.; Kulma, A.; Dymińska, L.; Szołtysek, K.; Prescha, A.; Hanuza, J.; Szopa, J. Flavonoid engineering of flax potentiate its biotechnological application. *BMC Biotechnol.* **2011**, DOI: 10.1186/1472-6750-11-10.