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RP-HPLC-DAD FINGER PRINT ANALYSIS OF PHENOLIC EXTRACTS FROM TRANSGENIC FLAX

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Two flax lines overexpressing genes encoding three essential enzymes of the flavonoid synthesis pathway: chalcone synthase (CHS), chalcone isomerase (CHI), and dihydroflavonol reductase (DFR), were generated. Phenolic compounds were extracted from the flaxseed lines and the control sample (Linola) using 80% methanol. Then the complex of phenolic compounds present in the crude extract was hydrolysed at basic conditions. The crude and hydrolysed extracts were analysed using an RP-HPLC-DAD method. The chromatograms of the crude extracts of the control sample and transgenic lines were the same. The finger print analysis of the hydrolysed extracts shows the influence of the expression of genes encoding three above mentioned enzymes on the proportion between secoisolariciresinol diglucoside (SDG) and phenolic acids derivatives forming the phenolic complex in flaxseed.

INTRODUCTION

Successful research on elucidating biosynthesis pathways of components important for human health enabled the use of genetic modification techniques to develop crop varieties with increased amount of these compounds. The main aim of genetic engineering for food of plant origin over the past decade was to enhance its potential as nutraceuticals [Davies, 2007].

Flax (*Linum usitatissimum* L.) has traditionally been used as an important source of oil and fibre. Flaxseeds are rich in bioactive compounds important for human nutrition, such as α-linolenic acid and lignans [Tarpila *et al.*, 2005; Kamal-Eldin *et al.*, 2001]. Secoisolariciresinol diglucoside (SDG) is the main lignan of flax [Meagher *et al.*, 1999] and its content in defatted flaxseeds reaches 10-24 mg/g [Johnsson *et al.*, 2000]. Secoisolariciresinol diglucoside is known to have strong bioactive properties. SDG exerts a phytoestrogenic effect by acting as a precursor of "mammalian" lignans: enterodiol and enterolactone [Axelson *et al.*, 1984] and may also protect against prostate, breast and colon cancer [Jenab & Thompson, 1996; Adlercreutz, 2002; Lin *et al.,* 2002; Boccardo *et al*., 2004]. Antioxidant activity of flaxseed extracts and SDG was reported by Amarowicz *et al.* [1993, 1997], Niemeyer & Metzler [2003] and Hu *et al*. [2007]. A high content of lignans in flax made seeds of this plant an interesting raw material for food application within the concept of functional foods [Oomah & Mazza, 2001].

The present study reports the HPLC finger print analysis of phenolic constituents obtained from the transgenic flax with incorporated three genes of essential flavonoid biosynthesis pathway.

MATERIAL AND METHODS

Material

Flaxseeds (cv. Linola) were obtained from the Institute of Natural Fibers, Poznań, Poland.

Transgenic plants construction and transgenic plant selection

In this study, the transgenic plants overexpressing three genes encoding three essential enzymes of the flavonoid synthesis pathway from *Petunia hybrida*: chalcone synthase (CHS), chalcone isomerase (CHI), and dihydroflavonol reductase, were generated. Details of the transgenic plants construction and selection were described earlier by Lorenc-Kukuła *et al.* [2005].

Preparation of crude extract

Phenolic compounds were extracted from ground defatted seeds with hexane with 80% (v/v) aqueous methanol at 80°C for 15 min at a solid to solvent ratio of 1:10 (w/v) using a shaking water bath [Amarowicz *et al.,* 1995]. After extraction, the sample was centrifuged, supernatant was collected and pellet was re-extracted. The procedure was repeated twice more. Methanol from the combined extract was evaporated under vacuum at 40°C in a rotary evaporator and the remaining water was lyophilized.

Basic hydrolysis

Because lignans in flaxseeds are present in the form of a complex, the crude extract was resuspended and subjected to alkaline hydrolysis (0.3 mol/L NaOH) for 2 days at room

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temperature followed by neutralization using 6 mol/L HCl [Johnsson *et al.*, 2000]. Then the sample was evaporated and lyophilized.

HPLC analysis

For the HPLC finger print analysis of phenolic compounds present in the extract before and after hydrolysis a Shimadzu system (Shimadzu Corp., Kyoto, Japan) consisting of two LC-10AD pumps, SCL-10A system controller, SPD-M-10A photo-diode array detector, and a prepacked LUNA C_{18} (4.6 × 250 mm, 5 μm, Phenomenex) was employed. A flow rate of 1 mL/min, and gradient elution of acetonitrile-water-acetic

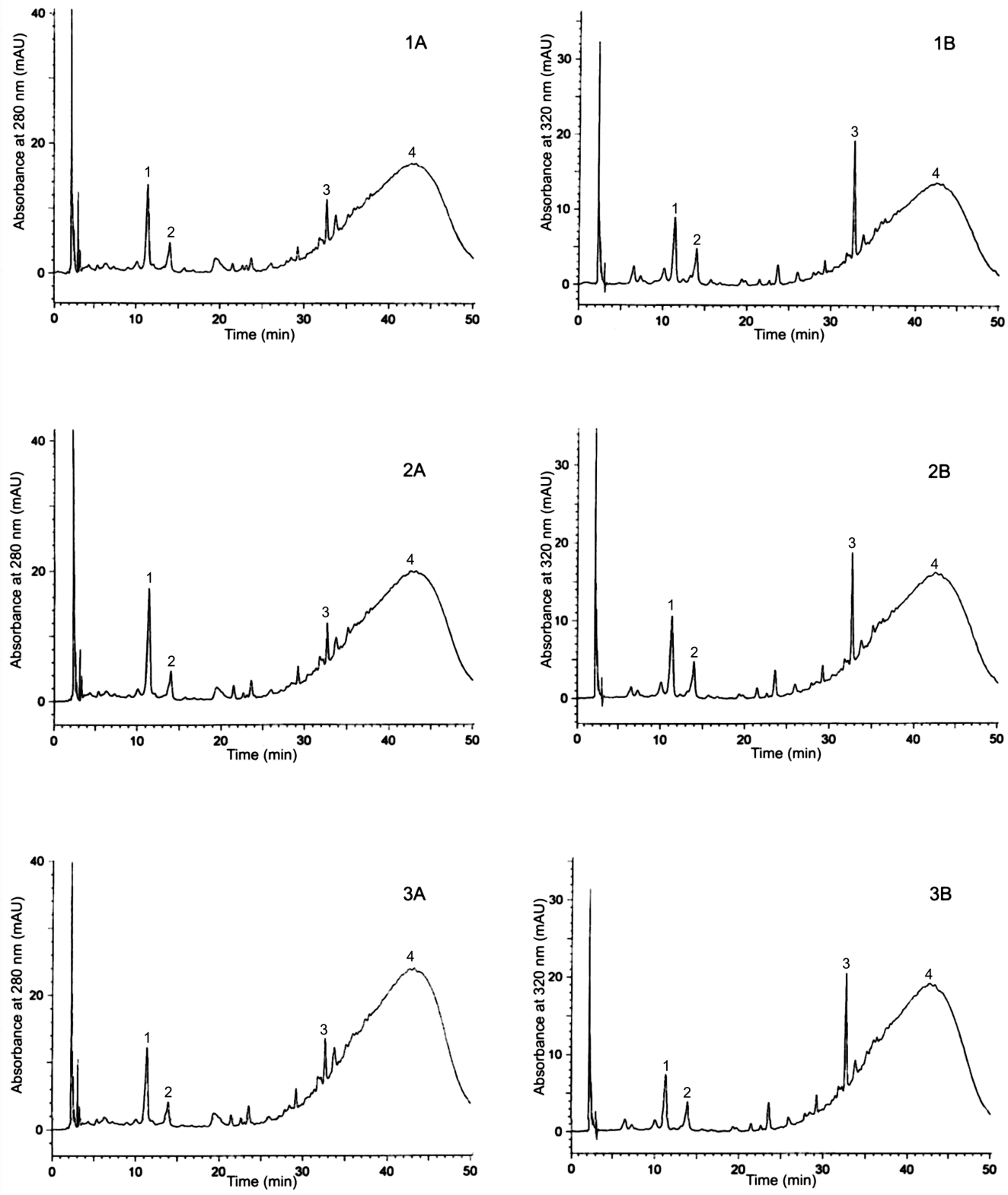

FIGURE 1. RP-HPLC chromatograms of the crude extracts of flaxseed phenolic compounds; 1 – Linola, 2 – line W92.40, 3 – line W92.72; A – chromatogram recorded at 280 nm, B – chromatogram recorded at 320 nm.

acid (5:93:2, v/v/v) [solvent A] and of acetonitrile-water-acetic acid (40:58:2, v/v/v) [solvent B], 0-50 min solvent B from 0 to 100% was used. The concentration of the sample dissolved in methanol was 8 mg/mL (crude extract) and 4 mg/mL (extract after hydrolysis); at injection volume 20 μL. The separation of compounds was monitored at 280 and 320 nm.

RESULTS AND DICUSSION

The chromatograms of the crude flaxseed extracts of two genetically-modified lines and control (Figure 1) were very similar and showed one main broad peak (4) with retention time of 42.5 min originating from the phenolic complex. The presence of one broad peak on the electrophoregram (capillary zone electrophoresis) of flaxseed crude extract, resulting from the presence of a phenolic complex, was reported before by Rybarczyk *et al.* [2008]. UV spectra of the phenolic constituents of the complex exhibited the maximum at 287 nm (Figures 2–4). Similar UV spectra of phenolic fractions separated from the crude flaxseed extract using Sephadex LH-20, RP-8 and silica gel column chromatography were described by Amarowicz *et al*. [1993, 1994, 1997]. On the chromatograms of the crude flaxseed extracts (Figure 1), additional peaks (1), (2) and (3) were also recorded with retention times of 11.39, 13.95, and 32.65 min, respectively. UV spectra of the separated compounds (1) and (3) showed maxima at 295 and 307 nm, respectively (Figure 2). UV spectra of compound (2) was characterised by maxima at 290 and 312 nm.

Figure 3 depicts the chromatograms of the extracts after basic hydrolysis. The absence of the broad peak is an evidence that the process of hydrolysis was complete. Secoisolariciresinol diglucoside (SDG) liberated from the complex was separated with retention time of 23.14 min. SDG UV spectrum displayed a maximum at 280 nm (Figure 4). This spectrum was similar to that of other lignan, nordihydroguiaretic acid (NDGA), which was reported before by Amarowicz *et al*. [1994]. The comparison of retention times of the peaks from samples with retention times of the original standards and the use of UV-DAD spectra (Figure 4) could tentatively identify the presence of two phenolic acids, namely *p*-coumaric and ferulic on the chromatograms of the hydrolysed samples (Figure 3).

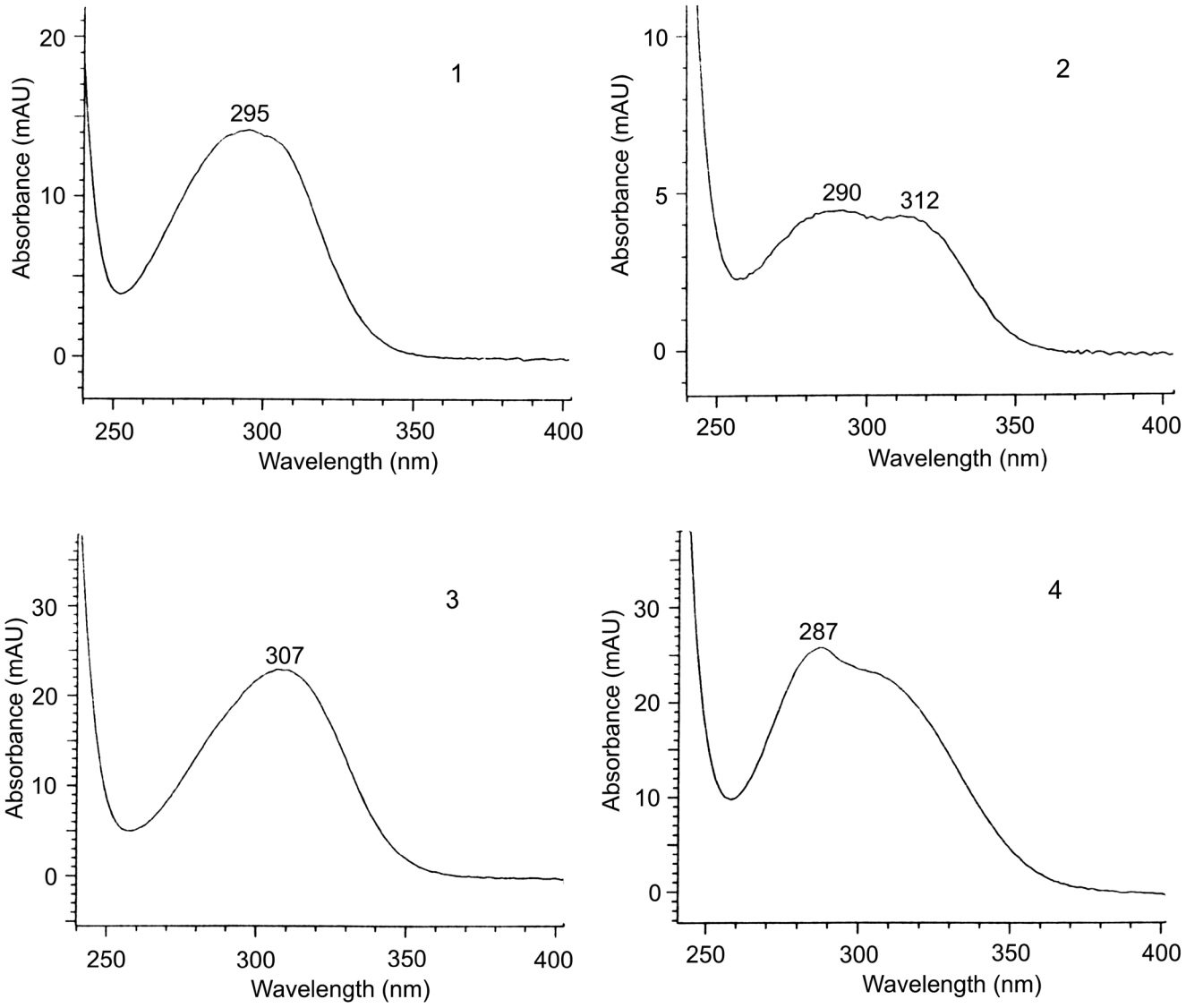

FIGURE 2. UV spectra of phenolic compounds detected in crude extracts; 1, 2, 3, 4 – numbers of peaks as reported in Figure 1.

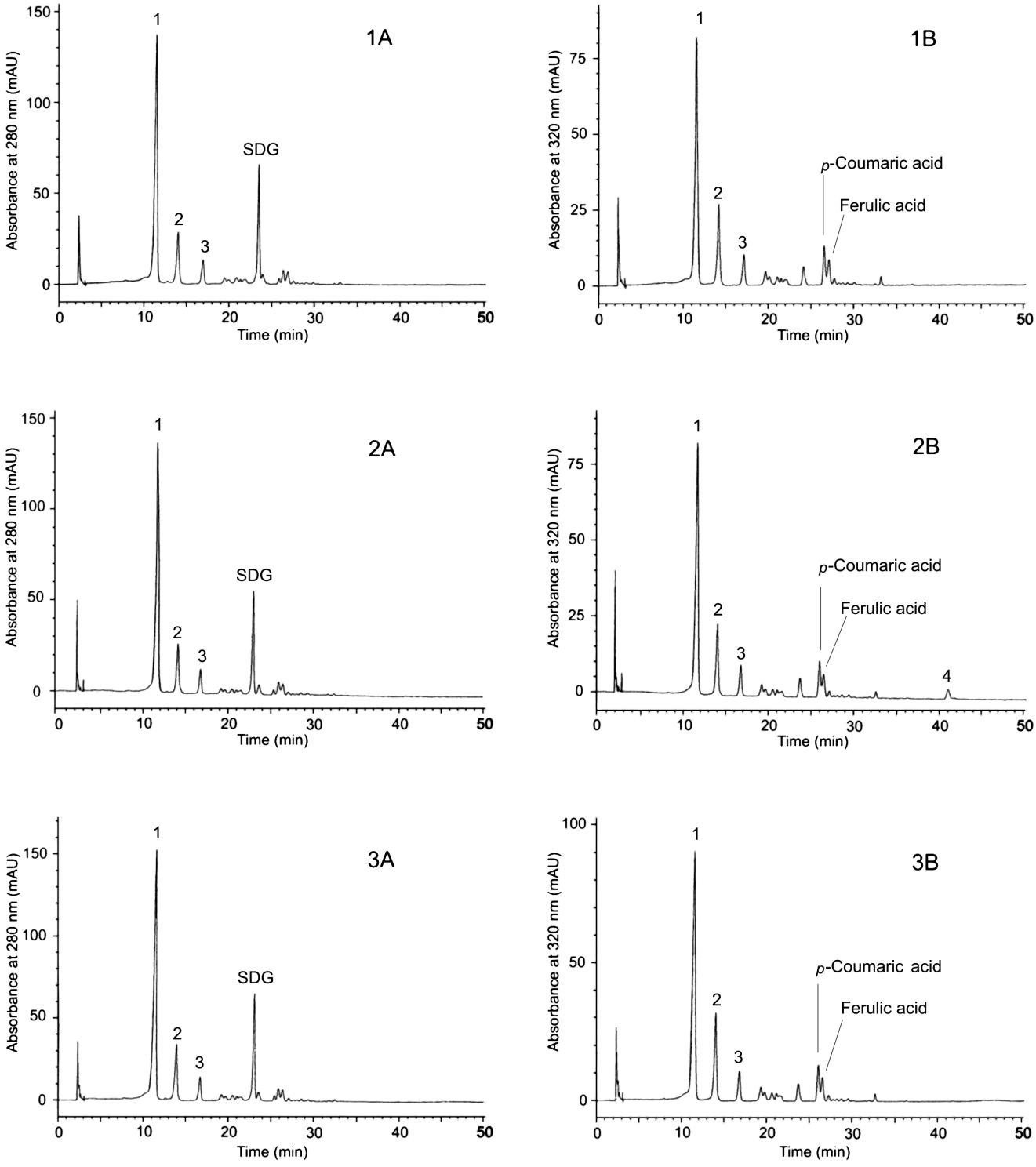

FIGURE 3. RP-HPLC chromatograms of the extracts of flaxseed phenolic compounds after alkaline hydrolysis; 1 – Linola, 2 – line W92.40, 3 – line W92.72; A – chromatogram recorded at 280 nm, B – chromatogram recorded at 320 nm.

After basic hydrolysis three peaks (1), (2), (3) originating from strong polar phenolic compounds were recorded on the chromatograms (Figure 3). Maxima of their UV spectra were noted at 295 (1) and 290 nm (2, 3), (Figure 5). UV spectrum of compound (2) possessed a shoulder at 320 nm. The chromatogram of flaxseed phenolic compounds liberated after basic hydrolysis obtained in this work is very similar to that reported by Johnsson *et al.* [2002]. In the cited paper, the authors reported that peaks (1) and (2) originated from 4-O-β-glucopyranosyl-*p*-coumaric acid and 4-O-β--glucopyranosyl-ferulic acid, respectively. The similarity of retention times of peaks (1) and (2) on chromatograms of the samples before and after hydrolysis (Figures 1 and 3) and their UV spectra confirms, for the first time ever, the presence of small amounts of *p*-coumaric and ferulic glucosides in the crude extract.

TABLE 1. Ratio of the area of selected peaks on HPLC chromatograms to the area of SDG peak.

Material	S_1/S_{SDG}	S_2/S_{SDG}	S_3/S_{SDG}	$S_{p\text{-Coumaric}}/S_{SDG}$	$S_{Ferulic}/S_{SDG}$
Linola	1.92	0.64	0.27	0.15	0.24
W92.40	2.18	0.56	0.23	0.14	0.25
W92.72	1.83	0.67	0.22	0.13	0.23

S – peak area; 1, 2, 3 number peaks in Figure 3; peaks were recorded at 320 (1, 2, 3 and those from *p*-coaumaric and ferulic acids) and 280 nm (from SDG).

Comparing the results reported in Figure 1, no effects of the expression of genes on the phenolic compounds forming the complex were found. Some effects occurred on the chromatograms of the samples after hydrolysis. The chromatogram of phenolics from the line W92.40 recorded at 320 nm (Figure 3 2B) showed an additional peak (4) with retention time of 41.08 min. UV spectra of the compound giving peak (4) were characterised by the maximum at 322 nm (Figure 5). Additional interesting information is provided in Table 1. The proportions of the peak areas on chromatograms of the hydrolysed extracts of Linola and two lines were different. The sample of the line W92.40 showed the S_1/S_{SDG} ratio to be higher, and the S_2/S_{SDG} ratio to be lower as compared to Linola. In the case of the line W92.72, these relations appeared adverse. In both transgenic lines, the S_3/S_{SDG} ratio was lower than that of Linola. The relation between the area of two phenolic acids peaks and SDG peak in Linola and transgenic lines was almost the same.

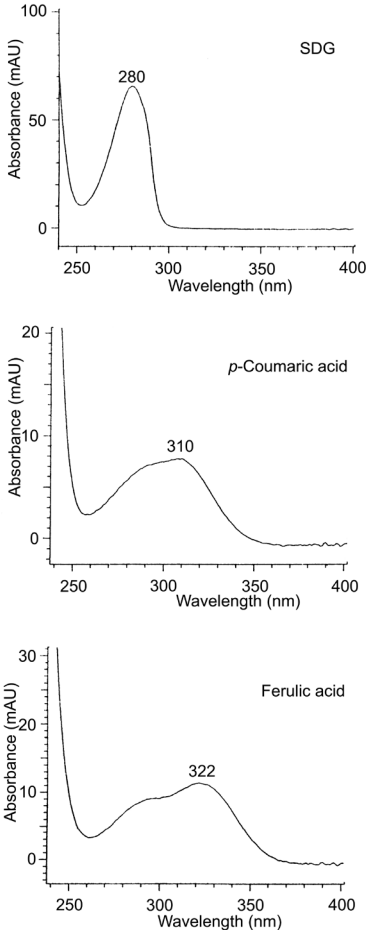

FIGURE 4. UV spectra of SDG, *p*-coumaric acid and ferulic acid detected in hydrolysed extracts of flaxseed (see Figure 3).

CONCLUSIONS

The results of RP-HPLC-DAD finger print analysis show the influence of expression of genes encoding chalcone synthase, chalcone isomerase, and dihydroflavonol reductase on the quantitative proportion of secoisolariciresinol diglucoside (SDG) and phenolic acids derivatives forming the phenolic complex in flaxseed. The different proportions of phenolic constituents in the complex may modify the antioxidant activity of the extract, which was previously reported by Lorenc-Kukuła *et al.* [2005]. Small amounts of *p*-coumaric and ferulic glucosides are present in flaxseed also in the free form.

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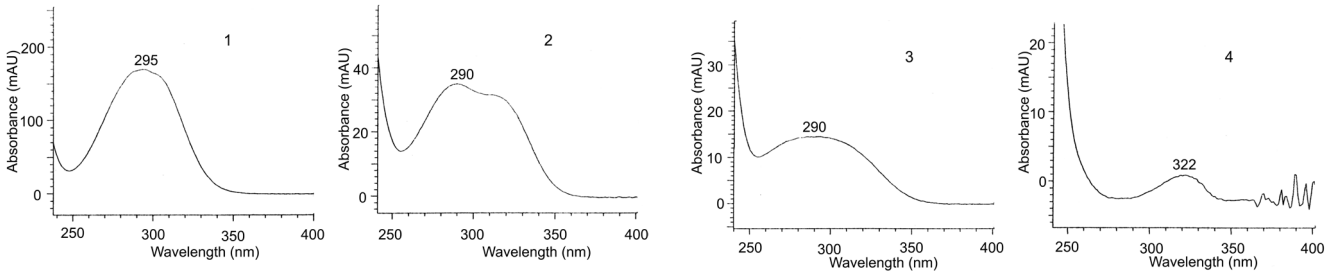

FIGURE 5. UV spectra of phenolic compounds detected in extracts after basic hydrolysis; 1, 2, 3, 4 – numbers of peaks as reported in Figure 3.

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