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Research article

Temporal biosynthesis of flavone constituents in flax growth stages

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ABSTRACT

Previous studies showed that chalcone synthase (chs) silencing in flax *(Linum usitatisimum)* induces a signal transduction cascade that leads to extensive modification of plant metabolism. Result presented in the current study, performed on field grown flax plants – (across the whole vegetation period) demonstrates that, in addition to its role in tannin and lignin biosynthesis, the *chs* gene also participates in the regulation of flavone biosynthesis during plant growth. Apigenin and luteolin glycosides constitute the flavones, the major group of flavonoids in flax. Alterations in their levels correlate with plant growth, peaking at the flower initiation stage. Suppression of chs gene expression causes significant changes in the ratio of flavone constituents at the early stage of flax growth. A significant correlation between flavonoid 3′-hydroxylase (F3′H) gene expression and accumulation of luteolin glycosides has been found, indicating that flavone biosynthesis during flax growth and development is regulated by temporal expression of this gene. The lack of such a correlation between the flavone synthase (FNS) gene and flavone accumulation in the course of plant growth suggests that the main route of flavone biosynthesis is mediated by eriodictyol. This is the first report indicating the ratio of flavone constituents as a potent marker of flax growth stages and temporal expression of F3′H, the key gene of their biosynthesis.

1. Introduction

Flax development is a continuous process that begins when a seed germinates and is completed when the plant produces ripe seeds. Depending on overall environmental conditions, the life cycle of oilseed flax – from seeding to maturity – typically covers 13–17 weeks. The variety, date of sowing, geographical location, and weather conditions in individual growing seasons can all influence the duration of particular development stages. The degree of development of the flax field is determined by the average level of development of a representative sample of plants most often when more than 50% of the plants are on or off a particular stage of development.

The vegetative stage starts when the plant emerges from the soil and continues until the first flowers appear, and lasts about 6–8 weeks. The flax plant has one main stem with several branches. The main stem continues to extend and buds form at individual branches independently. Around this time, the first flower will begin to open and the reproductive stage starts. This stage is based on flowering, capsule development, seed development, and plant maturation. New flax flowers open early each morning and petals are usually shed by midday. The flowering period lasts typically from 2 to 3 weeks. After pollination, the ovary begins to mature and forms a capsule containing developing seeds. The next stage, ripening, starts 3–4 weeks after flowering and the capsule with seeds attains full maturity (dry, brownish yellow capsule). Flax plants can be considered as fully mature when 95% of the capsules reach their mature color and capsules contain seeds that rattle.

The color of flowers, mainly caused by flavonoids, may distinguish the flax varieties. The flavonoid biosynthetic pathway gives a wide range of phenolic compounds. However, flavone constituents are predominantly accumulated in the plant body.

Flavonoids are involved in many biological activities including the plant defense response to a broad spectrum of both abiotic and biotic stressors. For example, the relationship between flavonoid biosynthesis and the stress response in cereals was evidenced based on genetic, physiological and biochemical studies reviewed in (Khlestkina, 2013). In barley fruit pigmentation caused by phenolic compounds flavonoids play a protective role under stress conditions (Shoeva et al., 2016). Flavonoids are also involved in plant development. For example, in peach and nectarine these compounds vary during fruit growth and ripening. Expression of UDP-glucose-flavonoid-3-O-glucosyltransferase is high at the beginning and end of fruit growth, remaining low during

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Abbreviations: UPLC, Ultra Performance Liquid Chromatography; DW, dry weight; CHS, chalcone synthase; ROS, reactive oxygen species

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the other developmental stages. In addition, the expression of flavonol synthase and respective compound accumulation was temporal and tissue-specific (Ravaglia D 2013). Another example is temporal expression of genes such as flavonoid 3′-hydroxylase (F3′H) and flavone synthase and flavone accumulation during gentian flower development (Nakatsuka et al., 2005). Altogether the evidence suggests that the track of flavonoids biosynthesis pattern might serve as a tool for precise description of plant growth and development stages. Thus, in this study we analyzed flavonoid accumulation and expression of respective structural genes during flax growth. Control and CHS engineered plants were subjected to analysis. Beside their important role in plant biology, dietary flavonoids have diverse beneficial properties for animal cells, including activities as free radical scavengers and anticancer properties. Several studies have correlated the intake of these compounds with the lower incidence of certain chronic diseases such as cancers and cardiovascular and neurodegenerative diseases. Flavonoids are synthesized via a pathway whose genetics and biochemistry have been well characterized in several plant species (Schijlen et al., 2004). In flax the pathway consists of steps that are common to the synthesis of different classes of flavonoid mainly regulated at the level of transcription of genes such as chalcone synthase and chalcone isomerase. There are also branch steps, which are more specific for each type of flavonoid. Here we demonstrate the pattern of accumulation of flavone in the flax body during plant growth and development. The data suggest that the naringenin conversion step is controlled by flavone synthase (FNS) and flavanone 3-hydroxylase (F3H) genes, which leads to the synthesis of flavone or anthocyanin, respectively, which appears to be the branch step. The expression levels of the main flavone biosynthetic genes confirmed this hypothesis. The patterns of accumulation of flavone constituents reveals their conversion during plant growth and development. Apigenin, the predominant constituent of the flax plant body at the initial stage of plant growth, is converted to luteolin at the flowering stage and the ratio of both remains until full maturity although the compounds' content decreases. Since the two constituents of flavone significantly differ in antioxidant efficiency, it is suggested that in addition to their crucial role in the defense system as antioxidants, they influence plant growth and development by ROS-antioxidant interaction, producing signals modifying processes from cell proliferation and elongation to senescence and death.

To our knowledge such conversion within flavone constituents upon plant development have not been reported to date. In addition, we demonstrate that this effect is not affected by CHS gene suppression although the yield of flavone synthesis is reduced.

Although the regulatory mechanisms of phenolic compound flavone accumulation and respective gene expression are well known, the data from this study on temporal biosynthesis of flavone constituents in flax growth stages will provide a substantial basis for further elucidation of flavone biosynthesis in flax crop and compounds production for the market.

2. Materials and methods

2.1. Plant material

Flax seeds (*Linum usitatissimum* L., Linola 947) seventh-generation plants of the transgenic line W86 and control flax were field cultivated in Wroclaw on a semi-technical scale. The flax was harvested at four stages of flax plant development: seedling (4th week of growth), vegetative – stem extension stage (8th week), flowering period (11th week) and ripening (13th week). All samples were collected in the early afternoon to eliminate the day period impact and in the flowering stage to avoid collecting flowers (flower opening begins shortly after sunrise on clear, warm days and petals are shed in the early afternoon). Additionally, samples were taken at earlier stages (1 and 2 week) of plant development in order to determine the level of flavones and F3′H activity. All experiments (gene activity and metabolite content measurements) were performed in at least six independent biological replicates (plants from different areas of the field) and additionally for each biological sample three technical replicates.

2.2. Gene expression analysis with quantitative PCR

The mRNA level for the investigated genes was determined using real-time PCR. For each analysis at least three independent biological samples were used (in the case of 4-week-old plants and younger, whole plants were used, while for older plants the middle part of the stem, without seed capsules, flowers and roots were used). The total RNA isolation, cDNA synthesis and real-time PCR reactions were performed as previously described. The actin gene was used as a reference gene. The changes in transcript levels were presented as the relative quantification to the reference gene. Sequences of primers are presented in the Appendix.

2.3. Phenolic compound extraction

0.5 g of lyophilized flax stalk (in the case of 1–4-week-old plants, whole plants, while for older plants, the middle part of stem, without seed capsules, flowers and roots were used) was ground in a Retsch mill to a fine powder and extracted three times with methanol using an ultrasonic bath (15 min). After centrifugation (5 min, 5000 rpm, RT), the supernatant was collected. The remaining pellet was hydrolyzed in 2N NaOH at room temperature for 24 h. Extracts were adjusted to pH 3.0, and the samples were extracted three times with ethyl acetate and centrifuged (1 min, 5000 rpm, RT). The supernatants from booth steps of extraction were collected together, evaporated under a vacuum and resuspended in 1 ml of methanol.

2.4. UPLC-PDA-MS analysis of phenolic components

Phenolic compounds were measured using UPLC combined with two detectors (PDA and MS). Simultaneous use of two techniques (UPLC-PDA-MS) allows us to obtain more detailed data. The extracts were analyzed on a Waters Acquity UPLC System with a 2996 PDA detector and Waters Xevo QTof MS System mass spectrometer, using an Acquity UPLC column BEH C18, 2.1 × 100 mm x 1.7 µm. The mobile phase was composed of solvent A (0.1% formic acid, pH 3) and solvent B (100% acetonitrile) in a gradient flow: 95% A/5% B at 1 min, 2–12 min gradient to 70% A/30% B; 13–20 min gradient to 0% A/100% B; and 21 min gradient at 95% A/5% B with a 0.4 ml/min flow rate. The MS spectra were recorded in ESI positive mode for 13 min in the 50–800 Da range. The parameters were: nitrogen flow 800 l/h, source temperature 70 °C, desolvation temperature cone 400 °C, capillary voltage 3.50 V, sampling cone 30 V, cone voltage ramp 40–60 V, scan time 0.2 s. The flow rate was 0.4 ml/min. The detection of coumaric, caffeic and ferulic acid, syringic acid and aldehyde, vanillin, petunidin, luteolin and apigenin derivatives was done at 320 nm and vanillic acid at 280 nm. The identity of the components was determined on the basis of retention time and UV and mass spectra of authentic standards (Sigma).

2.5. Isolation of the hydrolysable tannin fraction

2 g of lyophilized flax tissue (as described above) was extracted with 80% acetone (v/v) according to the published procedure (Amarowicz et al., 1995). The extract was centrifuged, evaporated to near dryness at 40 °C under a 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 hydrolysable tannins. The acetone was removed using a rotary evaporator, and the aqueous residue was condensed in a vacuum. The content of the phenolic groups was measured using the Prussian blue

method, and the hydrolysable tannin content was calculated using calibration curves prepared on standard substances and expressed as gallic acid equivalents.

2.6. Determination of cellulose content

The cellulose content was determined using the colorimetric method with the reagent anthrone, as described by Ververis (Ververis et al., 2004) and in our previous publication (Zuk et al., 2016).

2.7. Determination of lignin content

Determination of the total lignin content was performed using the acetyl bromide method, as described by Iiyama and Wallis (Iiyama, 1990).

2.8. Determination of pectin and hemicellulose content

Determination of the pectin and hemicellulose content was performed using the acetyl bromide method, as described previously (Iiyama, 1990, Zuk et al., 2016).

2.9. Antioxidant activity of plant tissue preparations

Free radical scavenging capacity of the methanolic extracts from plant stalk tissue was determined as described previously (Hasiewicz-Derkacz et al., 2015) using the stable free radical 2,2′-diphenylpicrylhydrazyl (DPPH) method.

2.10. Statistical analysis

All of the experiments were performed in at least six independent biological replicates (plants from different areas of the field) and additionally for each biological sample at least three technical replicates were done. The results are presented as the averages of independent replicates ± standard deviations. Statistical analyses were performed using Statistica 7 software (StatSoft, USA). The significance of the differences between the means was determined using Student's *t*-test. P-values are given separately for each experiment ($^{*}P < 0.05$, $^{**}P < 0.01$).

3. Results

3.1. Plant material characteristics

CHS-down-regulated flax (W86) has been obtained and characterized (Zuk et al., 2012). Briefly, in order to obtain genetically modified plants for agrotransformation, a cDNA construct was used for the CHS gene from *Petunia hybrida* in sense orientation under the control of the CaSV 35S promoter and the OCS terminator. Transgenic plants showed reduced expression of the endogenous CHS gene, which was accompanied by an increase in the level of hydrolysable and condensed tannins. Plants with low expression of the chs gene (W86) were also characterized by increased antioxidant properties, which resulted in increased resistance to *Fusarium* infection. An increase in the accumulation of polyunsaturated fatty acids was also found. In the analysis of the previous six generations, the total CHS gene expression level (measured in flax plant seedlings 3–4 weeks old) in transgenic plants was always lowered compared to the control plants (between 35% and 42% of the level observed for non-modified plants) and only slightly changed depending on the growing season. The overall conclusion that can be drawn from biochemical analysis of the CHS-down-regulated plants is that their biochemical characteristics are stable across several generations (Zuk et al., 2016). In this study transgenic plants of the 7th generation cultivated in the field were analyzed and compared to control plants.

The life cycle of the flax plant consists of a 45- to 60-day vegetative period (from the cotyledon up to the end of the stem extension stage – about 30 cm height), a 15- to 25-day flowering period (from the first visible buds up to late flowers), and a ripening period of 30–40 days (green capsule until ripe seed). Water stress, high temperature and disease can shorten any of these growth periods (Cullis, 2007). In this study, we revealed the expression patterns of phenolics biosynthesis-related genes and metabolite levels during four stages of flax plant development: seedling (4th week of growth – third pair of true leaves unfolded – start of leaf spiral); vegetative (8th week of growth – end of intensive stem extension); flowering (11th week – full flower, capsules start forming, continuation of branching); and ripening (13th week of growth – brown capsule, branches, stem and upper leaves green/yellow, middle leaves partially senescent, lower leaves dropped). Due to the interesting fluctuations in the level of flavones – apigenin and luteolin glycosides – observed during the experiments during the development of plants, we decided to collect samples from younger tissues (first and second weeks after germination of seeds) and determine the level of flavones and the activity of the key enzyme for the observed changes, i.e. F3′H.

3.2. Phenolic compounds and related gene activity during development of the plant

Of phenylpropanoids five groups of compounds have been detected during flax development. Flavones, anthocyanin, phenolic acids, polyphenols and benzoic derivatives were accumulated. The schematic representation of the general reaction leading to phenylpropanoid synthesis and examples of the major detected compounds in flax plant are presented in Fig. 1. Flavones were in the highest while anthocyanin was in the lowest quantity. They serve in flowers as co-pigments giving bluer colors to flowers. Twelve anthocyanins have been isolated from flax: the 3-glucosylrutinosides of pelargonidin, cyanidin and delphinidin; the 3-triglucosides of delphinidin and cyanidin, 3-diglucoside of delphinidin, 3-rutinosides of pelargonidin, cyanidin, and delphinidin, and 3-glucosides of pelargonidin, cyanidin and delphinidin (Dubois J.A, 1975). The main pigment was determined to be delphinidin 3-O-3-xylosylrutinoside. (Toki et al., 1995). In our study, we focused on the analysis of green parts of plants (stems) at successive stages of plant development, omitting developing flowers and maturing seed capsules. For this reason, the only anthocyanin compound identified in the analyzed tissues was the petunidin derivative. The content of this compound varies between 0.111 mg and 0.123 mg per gram of dry tissue at the initial stages of development (vegetative period), and in the flowering plants no significant differences were found between control plants and those characterized by decreased expression of the CHS gene. In mature plants the level of this compound drops distinctly – by 41% and 48% in control and modified plants respectively.

Apigenin and luteolin were detected as the main flavone compounds in flax (Table 1). In the early development stage (up to the 4th week) the dominant flavones are apigenin glucosides – the ratio of apigenin to luteolin in all studied plant types (control and transgene) varied from 7.2:1 to 5.5:1 (for control), or 6.3:1 to 5.2:1 (for transgene), the predominant flavone is apigenin-6.8-di-C-glycoside and only one luteolin derivative (luteolin-6-C-glucoside) was determined. In the flowering stage (7th to 11th week) the proportion of apigenin to luteolin changed to 1:3 (control) or 1:2.3 (transgenic plant) and these ratios were maintained until the yellow maturity stage (up to 13th week) and the prevalent flavone became luteolin-6-C-glucoside. At the flowering stage (here shown as week 9–11) the level of flavone was about 5 times higher than at the vegetative stage (weeks 2–8) and about 70% higher than at maturity. The fluctuation in apigenin and luteolin glucoside levels upon plant development correlated with activity of F3′H is presented in Fig. 2. The conversion of apigenin to luteolin might occur upon the hydroxylation reaction via eriodictyol conducted by flavonoid hydroxylase (F3′H) – see Fig. 3. Interestingly, the expression of the F3′H

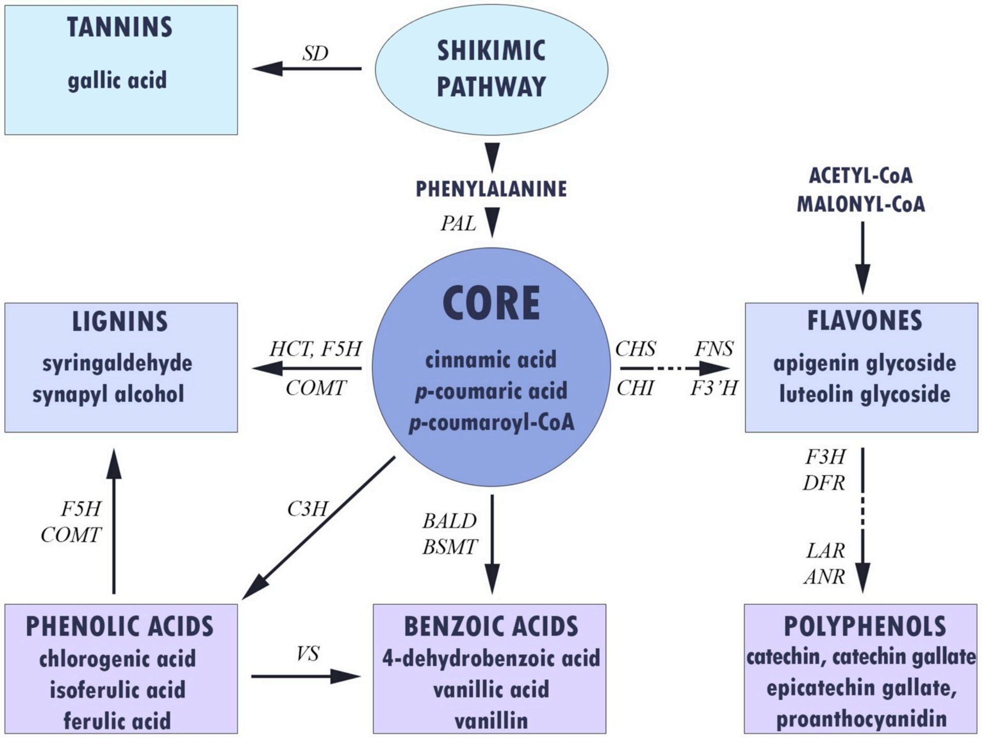

Fig. 1. The general reaction leading to phenylpropanoids and examples of the major detected compounds in flax plants. SD, shikimate dehydrogenase; PAL, phenylalanine ammonia lyase; CHS, chalcone synthase; CHI, chalcone isomerase; FNS, flavone synthase; F3′H, flavonoid-3′-hydroxylase; COMT, caffeic acid 3-O-methyltransferase; HCT, hydroxycinnamoyl-CoA:quinate/shikimate hydroxycinnamoyl transferase; F5H, ferulate 5-hydroxylase; C3H, 4-coumarate 3-hydroxylase; F3H, flavanone 3-dioxygenase; LAR, leucoanthocyanidin reductase; ANR, anthocyanidin reductase VS, vanillin synthase; BALD, benzaldehyde dehydrogenase; BSMT, benzoic acid methyltransferase.

encoding gene was firstly detected in 4-week-old plants at a low level and at the flowering stage (11 weeks) F3′H is significantly up-regulated. The indirect metabolite (eriodictyol) of the mentioned conversion of apigenin to luteolin was determined in mature tissue (13th week) at the level 0.034 mg/g DW for control and 0.061 mg/g DW for the CHS repressed plant. To our knowledge, this is the first report regarding the presence of this compound in flax and a possible way of bioconversion of apigenin to luteolin. The presence of hesperetin (4′-methoxy eriodictyol) – another flavanone compound which exhibits several pharmacological actions (mainly attributed to an antioxidant defense mechanism and suppression of pro-inflammatory cytokine production) (Li and Schluesener, 2017) was also found in the same tissue (0.034–0.036 mg/g DW). In the whole plant development, we can observe a clearly lower content of flavones in plants with chalcone synthase repression (from 45% in young plants – up to 8 weeks, through a decrease of about 19% in flowering plants, up to 23% in mature plants). The observed metabolic effect is mainly caused by reduced activity (generated during the genetic modification) of the *chs* gene (in young plants by about 60% and during flowering by almost 30%). In modified plants, we can also observe reduced activity of the gene encoding flavone synthase (FNS), the enzyme important for flavone biosynthesis (Fig. 4). Changes induced by modification of activity of the chalcone synthase gene, which is crucial for the phenylpropanoid pathway, are partially compensated by the increase of chalcone isomerase (CHI), flavanone 3-dioxygenase (F3H) and dihydroflavonol reductase (DFR) (in young plants) genes' activity. The action of F3H and DFR leads to production of leucoanthocyanidin, which can be, by activity of anthocyanidin synthase (ANS), converted to colored anthocyanidin (not detected in analyzed tissue). Leucoanthocyanidin and anthocyanidin are thought to be required for the synthesis of condensed tannins (proanthocyanidins). We have found that most of the catechins are present in the form of esters with gallic acid – catechin gallate and epicatechin gallate (Table 2). The compounds' content in control and transgenic flax is highly increased at flowering and especially at the maturity stage (when also proanthocyanidin B2 can be detected). In addition to CHS, anthocyanidin synthase (ANS), anthocyanidin reductase (ANR) and leucoanthocyanidin reductase (LAR) are downstream genes involved in the polyphenol biosynthesis pathway (see Fig. 5). The higher activity of leucoanthocyanidin reductase observed in transgenic plants resulted in slightly higher amounts of proanthocyanidins in these plants. The characteristic feature of W86 plants is high, in comparison to control plants, content of hydrolysable tannins starting at the flowering stage (0.49 ± 0.189 μg/gFW (control) and 1.02 ± 0.098 μg/gFW (W86) and particularly noticeable in mature plants (1.09 ± 0.01 and 1.87 ± 0.01 respectively). The increase in the content of both hydrolysable tannins and proanthocyanidins is particularly visible in the seeds produced by these plants – the seeds of transgenic plants W86 are dark brown while the unmodified plants produce yellow seeds (Zuk et al., 2012). We detected induction of the shikimate dehydrogenase (SDH) gene at the flowering stage of flax development. At this stage gallic acid was first detected in both groups of plants and its content was maintained until plant maturation. The induction of SDH gene expression was highly activated in CHS down-regulated flax, which corresponds to gallic acid accumulation (Table 2).

Trans-cinnamic acid, which is the product of phenylalanine conversion catalyzed by PAL (phenylalanine ammonia lyase) enzyme, is the branch point for p-coumaric acid (reaction catalyzed by cinnamate 4-hydroxylase) and benzoic acid derivatives. p-Coumaric acid might be converted to coumaroyl-CoA by 4CL (4-coumarate CoA ligase) and further by cinnamate 3-hydroxylase (C3H) and hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (HCT) to lignin or by chalcone synthase (CHS) to chalcones and then to proanthocyanidin and flavonoids. The balance between the synthesis of lignins (essential for the cell wall of the growing polymer plant) and the synthesis of flavonoids is an important parameter that should be analyzed during the development of the plant. The reduction in chalcone synthase gene expression in flax plants was accompanied by considerably (2-fold in fully developed stalks) higher expression of the phenylalanine ammonia lyase (PAL) gene, the first in the phenylpropanoid pathway, and subsequent changes in compound contents and expression of respective genes. The detected increase in coumaric acid, ferulic acid and isoferulic acid in the flowering stage (Table 3) may be caused by a greater demand for lignin precursors in the growing plant and might result from higher expression of p-coumarate-3-hydroxylase (C3H), caffeic

Table 1

Content and composition of flavones in stem tissue from control (Linola) and transgenic lines with CHS repression (W86). Flavone content is expressed in mg/g DW.

Stage of development	1 week		2 weeks		4–8 weeks		9–11 weeks		12–14 weeks	
mg/g DW	control	W86	control	W86	control	W86	control	W86	control	W86
Apigenin-6.8-di-C-glycoside	1.2705 ± 0.006	0.6621 ± 0.006	2.1456 ± 0.177	2.7613 ± 0.170	2.431 ± 0.245	1.346 ± 0.306	2.641 ± 0.305	2.360 ± 0.294	1.677 ± 0.225	1.632 ± 0.069
Apigenin glucosyl-arabinoside	0.2269 ± 0.002	0.1520 ± 0.0084	0.512 ± 0.005	0.2043 ± 0.005	0.743 ± 0.077	0.519 ± 0.115	1.100 ± 0.125	0.898 ± 0.117	0.562 ± 0.078	0.528 ± 0.032
Apigenin galactosyl-arabinoside	0.3036 ± 0.008	0.1983 ± 0.018	0.5833 ± 0.037	0.8658 ± 0.057	0.965 ± 0.103	0.570 ± 0.110	1.396 ± 0.170	1.170 ± 0.149	0.821 ± 0.103	0.821 ± 0.047
Apigenin-8-C-glucoside	0.0785 ± 0.004	0.0932 ± 0.001	0.764 ± 0.005	0.843 ± 0.011	0.133 ± 0.020	0.125 ± 0.031	0.633 ± 0.072	0.552 ± 0.098	0.506 ± 0.069	0.354 ± 0.019
Apigenin-6-C-glucoside	ud	ud	ud	ud	0.180 ± 0.026	0.161 ± 0.040	1.462 ± 0.189	1.301 ± 0.205	0.949 ± 0.133	0.795 ± 0.039
Luteolin diglucoside	ud	ud	ud	ud	0.209 ± 0.007	0.034 ± 0.007	0.891 ± 0.105	0.786 ± 0.099	0.577 ± 0.082	0.682 ± 0.041
Luteolin-6-C-glucoside	0.3057 ± 0.003	0.2301 ± 0.018	0.5256 ± 0.033	0.7408 ± 0.048	1.030 ± 0.103	0.357 ± 0.097	12.59 ± 1.175	10.00 ± 1.163	9.567 ± 1.300	7.122 ± 0.423
Luteolin-8-C-glucoside	ud	ud	ud	ud	0.199 ± 0.021	0.129 ± 0.017	4.823 ± 0.600	3.835 ± 0.601	3.118 ± 0.450	2.289 ± 0.136
Flavones	**2.1852 ± 0.023**	**1.3357 ± 0.051**	**4.5305 ± 0.287**	**5.4149 ± 0.298**	**5.894 ± 0.59**	**3.245 ± 0.72**	**25.55 ± 2.73**	**20.91 ± 0.72**	**17.78 ± 2.45**	**13.70 ± 0.80**
Apigenin: Luteolin	**7.2:1**	**6.3:1**	**5.5:1**	**5.2:1**	**3:1**	**5.2:1**	**1:3**	**1:2.3**	**1:2.9**	**1:2.4**
Petunidin derivate	ud	ud	ud	ud	0.116 ± 0.032	0.111 ± 0.031	0.124 ± 0.075	0.090 ± 0.015	0.073 ± 0.016	0.059 ± 0.003

The results are the mean values ± SD (n = 6 × 3); ud – undetermined. The significance of the differences between the means was determined using Student's *t*-test (P < 0.05).

acid O-methyltransferase (COMT), ferulate 5-hydroxylase (F5H) and 4-hydroxycinnamoyl-CoA lyase (4CL) genes (Fig. 6). All these changes strongly suggest the increase in unit level for lignin biosynthesis, and the lignin content is notably higher (6-fold) at the flowering stage of plant development in both group of plants in comparison to young plants. The content of lignin in transgenic plants is higher than in control plants. During the maturation of plants, the situation is dramatically changed, and in the transgenic plants the level of lignin not only does not grow but decreases markedly (by about 26%). The only genes from the lignin biosynthesis route that are significantly reduced in transgenic plants are 4CL, F5H and p-hydroxycinnamoyl-coenzyme A: quinate shikimate p-hydroxycinnamoyltransferase (HCT). Thus, F5H and HCT genes are somehow co-regulated with the CHS gene in lignin but not in flavonoid biosynthesis. This might suggest that biosynthesis of both groups, lignin and flavonoid compounds, in flax plants is regulated independently. The data on lignin content (Table 4), its precursors (Table 3) as well as selected and potential genes involved in biosynthesis of these compounds (Fig. 7) are presented.

Changes in the content of cell wall polymers are an important and necessary phenomenon for the proper development of the growing plant. The main polymers that build the cellular walls of flax are cellulose, pectin, hemicellulose and the mentioned lignin. It seems that the proportions of individual polymers are under strict control of cell wall sensing genes, and in the case of changes in the content of one of the polymers, a specific compensation mechanism works (Zuk et al., 2016). Changes in the content of cell wall polymers in plants during development are shown in Table 4. During the development of the plant we can observe progressive lignification – more pronounced in the case of control plants. In plants with reduced expression of chalcone synthase, the slower growth of lignin content is compensated by the greater content of cellulose.

Ferulic acid is the precursor for vanillin derivatives (vanillin and vanillic acid), and these compounds have been identified at each stage of plant development (see Table 5), but their content was not changed significantly during development. The level of the compounds is significantly increased at the flowering stage, but they are in the lowest quantity when compared to other phenylpropanoids. The key genes of their synthesis, such as benzaldehyde dehydrogenase (BALD) and benzoic acid methyltransferase (BSMT), are highly expressed in flax (Fig. 7) but they do not reflect the compounds' content and do not seem to be affected by CHS expression.

3.3. Antioxidant activity changes during plant development

Phenolic compounds such as flavones, vanillin or phenolic acids are known for their activity as antioxidants. It is therefore reasonable to assess how changes in the content of these compounds resulting from the development of the plant will affect the antioxidant potential of the plant. The ability to capture free radicals is important for the plant under stress conditions (e.g. pathogenic infection, drought, cold). Passing through the successive stages of development is also associated with the synthesis of free radicals and, consequently, stress (Sanz et al., 2015, Zimmermann and Zentgraf, 2005). As the plants grew, a significant increase in antioxidant capacity was observed (measured by the DPPH test). In young tissues the percentage of quenching reached 21–23% in the older tissue (at flowering stage – 42%) to fall back to the level of 25% in the mature tissue. There were no significant differences in the antioxidant rate between the examined plant types. This proves that the observed increase in antioxidative capacity is most likely due to the higher content of phenolic compounds but is subject to regulation in development and not through the activity of the *chs* gene. It is also possible that physiological regulation is superior to genetic regulation (resulting from gene expression).

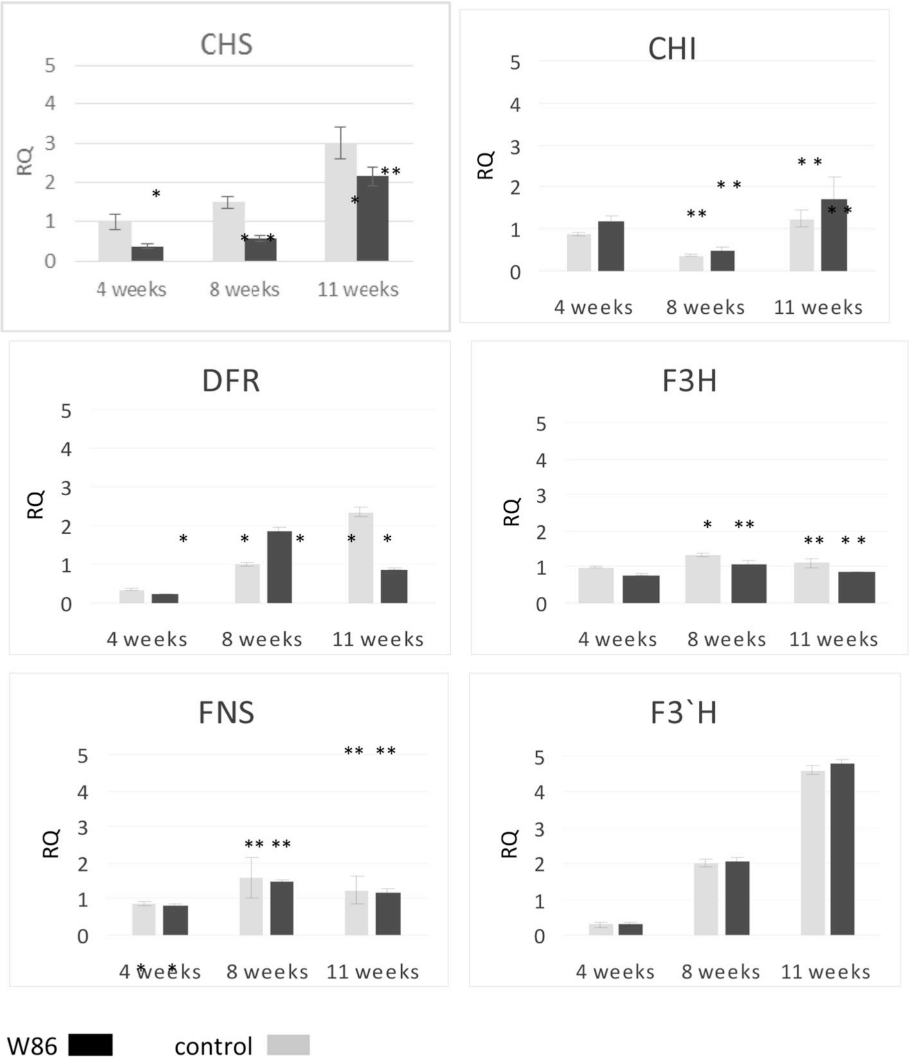

Fig. 2. Schematic representation of fluctuation in flavone levels (apigenin glucosides and luteolin glucosides) and activity of F3′H (flavonoid 3′-hydroxylase) during plant growth. The area highlighted in gray shows the flowering time.

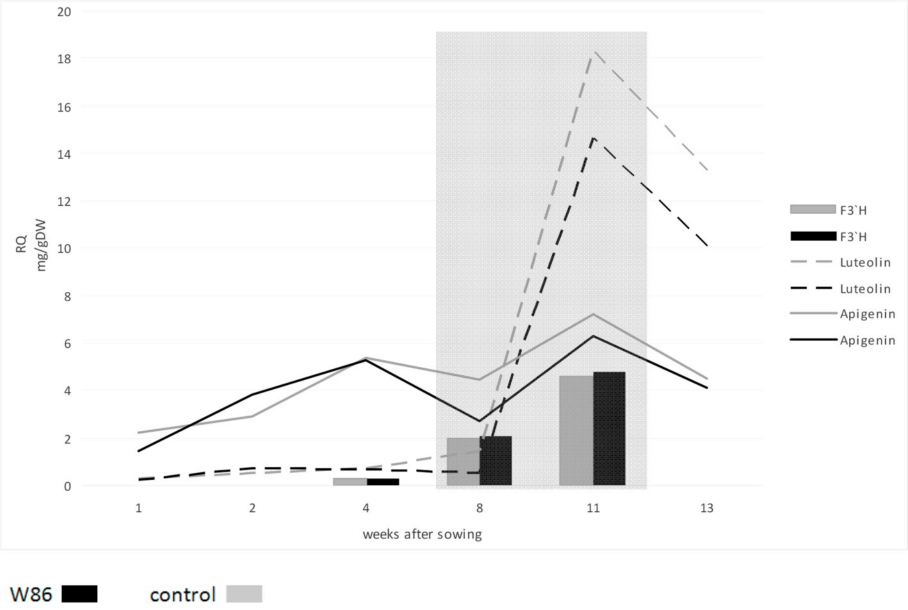

Fig. 3. Expression levels of flavone synthesis genes. The mRNA level of flavone synthesis genes (CHS, chalcone synthase; CHI, chalcone isomerase; DFR, dihydroflavonol reductase; F3H, flavanone 3-dioxygenase; FNS, flavone synthase; F3′H, flavonoid-3′-hydroxylase) of W86 flax in comparison with control flax obtained from the real-time RT-PCR analysis. Data represent the mean ± standard deviations from three independent experiments. The significance of the differences between the means was determined using Student's *t*-test (*P < 0.05,**P < 0.01). RQ – relative quantity.

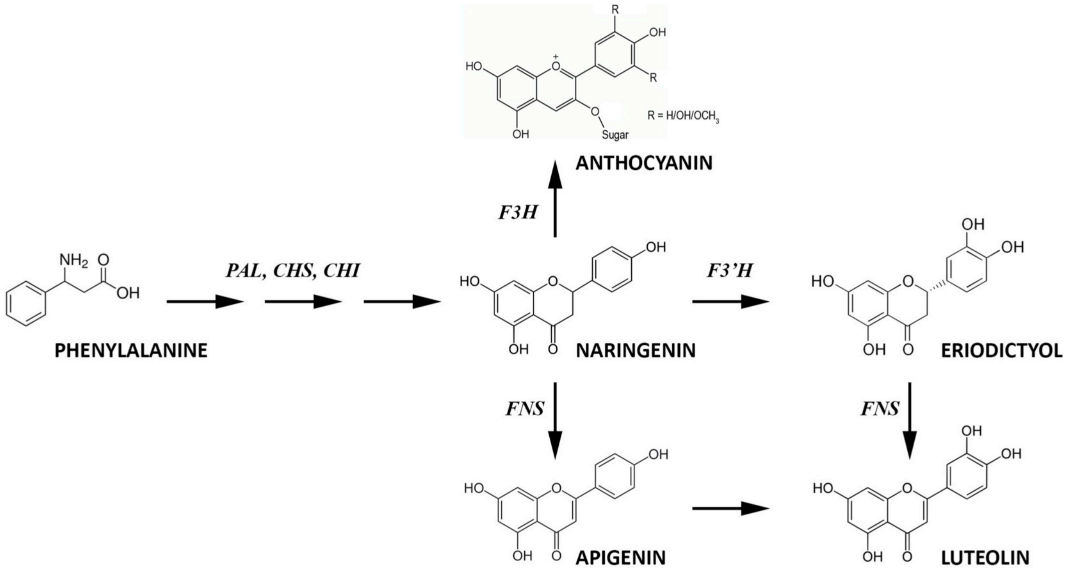

Fig. 4. Schematic diagram of branch-points leading to flavone formation. PAL, phenylalanine ammonia lyase; CHS, chalcone synthase; CHI, chalcone isomerase; FNS, flavone synthase; F3′H, flavonoid-3′-hydroxylase; F3H, flavanone 3-dioxygenase.

4. Discussion

Plants produce a number of phenolic compounds for which the shikimic acid route and phenylalanine provide the structural basis. The main groups of phenolic compounds are flavonoids, benzoic acid derivatives (e.g. vanillin), phenolic acids (e.g. coumaric, ferulic, caffeic), lignin and tannin (Dixon and Pasinetti, 2010). The first committed reaction of the flavonoid biosynthesis pathway is the condensation of one p-coumaroyl-CoA molecule with three molecules of malonyl- CoA catalyzed by chalcone synthase (CHS) that forms chalcones. Chalcones are precursors for the biosynthesis of a range of flavonoids including flavanones, flavones, isoflavones, flavonols, anthocyanins and proanthocyanins. CHS gene expression is rapidly induced upon environmental factors and exerts control over the flux through the flavonoid route. Indeed, several reports suggest the key regulatory role of CHS gene expression in flavonoid biosynthesis (Muir et al., 2001; Sun et al., 2015). CHS gene silencing in flax leads to inhibition of lignin biosynthesis, but increase of proanthocyanidin and hydrolysable tannin contents. Rerouting the substrates from lignin biosynthesis to other classes of the phenolic biosynthesis pathway was suggested. Probably, in *Arabidopsis* suppression of key lignin biosynthesis genes (HCT, C3H) results in lignin synthesis reduction, which leads to redirection of the metabolic flux into flavonoids through CHS activity. This, however, is not the case in eucalyptus plants (*Eucalyptus globulus*), where a positive correlation between CHS transcript content and lignin and flavonoid levels was detected (ShinyaT et al., 2014). This suggests that biosynthesis of both groups, lignin and flavonoid compounds, might be independently regulated.

The chalcones are unstable compounds and thus do not accumulate in plants. The reaction catalyzed by chalcone isomerase converts chalcone into flavanone. Flavanones can also be formed spontaneously at physiological pH and therefore CHI is not thought to be important for control over route flux. However, there are examples indicating that CHI does exert some control over flux into flavonoid branches of the phenylpropanoid pathway. For example, in barley and nectarine, the genes of CHS, CHI, F3H, and DFR are coordinately co-expressed in flavonoid and proanthocyanidins synthesis (Shoeva et al., 2016). Hydroxylation of flavanone at the 3-position by the action of flavanone 3-hydroxylase (F3H) leads to the formation of dihydroflavonol, which is catalyzed by dihydroflavonol 4-reductase (DFR) to leucoanthocyanidin. Leucoanthocyanidin dioxygenase (also called anthocyanidin synthase, ANS) catalyzes the conversion from the colorless leucoanthocyanidin to the colored anthocyanidin. Leucoanthocyanidin and anthocyanidin are thought to be required for the synthesis of condensed tannins (proanthocyanidins). On the other side of the reaction, flavone synthase (FNS) is a key enzyme for flavone biosynthesis; it catalyzes the dehydration reaction of flavanone naringenin to form apigenin and/or luteolin. Eriodictyol, the hydroxylation product of naringenin catalyzed by F3′H, can also undergo dehydration by FNS, producing apigenin and/or luteolin. In flax flavones were detected as the main compounds.

Since most structural genes involved in phenylpropanoid biosynthesis and the main metabolites are now available, as a result of previous work as well as the results of this study, we attempted to determine their expression profiles to elucidate how they are expressed during

Table 2
The content and composition of tannins and their precursors in stem tissue from control (Linola) and transgenic lines with CHS repression (W86). Tannin content is expressed in μg/g DW.

μg/g DW	4–8 weeks		11 weeks		13 weeks	
	control	W86	control	W86	control	W86
Proanthocyanidin B2	ud	ud	ud	ud	1.56 ± 0.31	1.74 ± 0.28
Catechin	0.083 ± 0.012	0.126 ± 0.009	0.447 ± 0.039	0.481 ± 0.085	1.36 ± 0.06	1.39 ± 0.06
Catechin gallate	0.373 ± 0.028	0.915 ± 0.156	2.005 ± 0.208	2.681 ± 0.575	1.13 ± 0.32	1.23 ± 0.08
Epicatechin gallate	ud	ud	ud	ud	1.12 ± 0.09	1.43 ± 0.20
Polyphenols	**0.456 ± 0.040**	**1.041 ± 0.165**	**2.452 ± 0.247**	**3.162 ± 0.660**	**5.17 ± 0.78**	**5.79 ± 0.62**
Gallic acid	ud	0.003 ± 0.001	0.050 ± 0.005	0.057 ± 0.006	0.046 ± 0.003	0.089 ± 0.004

The results are the mean values ± SD (n = 6). Significance of the differences between the means was determined using Student's *t*-test ($P < 0.05$).

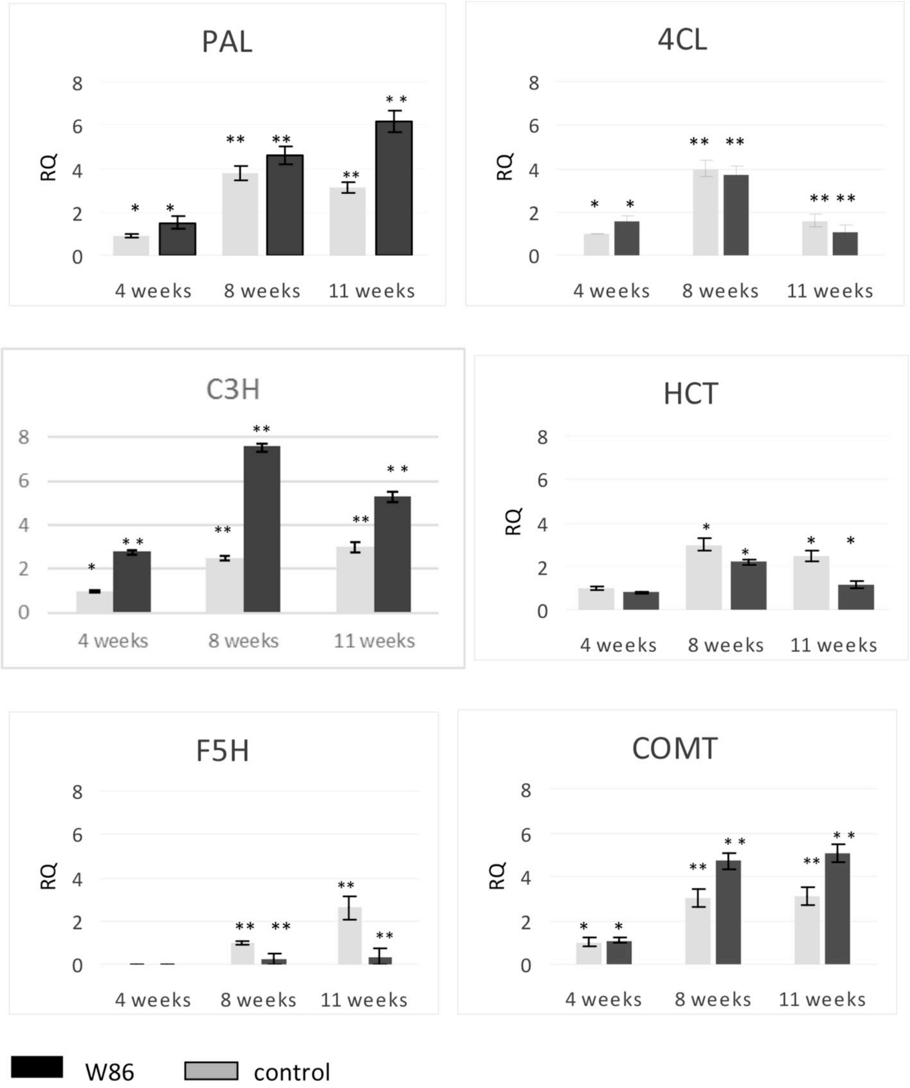

Fig. 5. Expression levels of tannin synthesis genes. The mRNA level of tannin synthesis genes (LAR, leucoanthocyanidin reductase; ANR, anthocyanidin reductase; SDH, shikimate dehydrogenase) of W86 flax in comparison with control flax obtained from the real-time RT-PCR analysis. Data represent the mean ± standard deviations from three independent experiments. The significance of the differences between the means was determined using Student's *t*-test (*$P < 0.05$,**$P < 0.01$). RQ – relative quantity.

plant development and how they regulate biosynthesis of different groups of phenolics in CHS-modified flax plants.

In this study, we revealed the expression patterns of phenolics biosynthesis-related genes during four stages of flax plant development, i.e. seedling, vegetative (stem extension stage), flowering and ripening. In addition, accumulation levels of the compounds representing the main routes of the phenylpropanoid pathway such as flavonoids, phenolic acids and benzenoid derivatives were determined by high performance liquid chromatography analysis.

This is the first systematic research that aims to determine temporal biosynthesis of phenolics and expression patterns of their related genes in CHS gene engineered flax and during plant development and thus generate useful information. Of the phenylpropanoids, five groups of compounds have been detected in different stages of flax development.

Table 3
Content and composition of lignin precursors (phenolic acids) in stem tissue from control (Linola) and transgenic lines with CHS repression (W86). The phenolic acid content is expressed in mg/g DW.

mg/g DW	4–8 weeks		11 weeks		13 weeks	
	control	W86	control	W86	control	W86
Chlorogenic acid	2.405 ± 0.147	2.544 ± 1.064	5.156 ± 0.706	5.846 ± 0.482	1.793 ± 0.276	1.481 ± 0.112
Coumaric acid	0.079 ± 0.001	0.075 ± 0.024	0.105 ± 0.014	0.096 ± 0.011	0.050 ± 0.007	0.030 ± 0.004
Ferulic acid	0.820 ± 0.139	0.555 ± 0.142	0.427 ± 0.096	0.377 ± 0.038	0.079 ± 0.024	0.082 ± 0.004
Isoferulic acid	0.036 ± 0.015	0.017 ± 0.005	0.039 ± 0.004	0.040 ± 0.008	0.023 ± 0.004	0.020 ± 0.004
Phenolic acids	**3.341 ± 0.02**	**3.192 ± 1.23**	**5.728 ± 0.82**	**6.360 ± 0.54**	**1.946 ± 0.31**	**1.615 ± 0.11**
Syringaldehyde	0.024 ± 0.006	0.011 ± 0.002	0.073 ± 0.004	0.076 ± 0.004	0.016 ± 0.008	0.008 ± 0.002

The results are the mean values ± SD ($n = 6$). Significance of the differences between the means was determined using Student's *t*-test ($P < 0.05$).

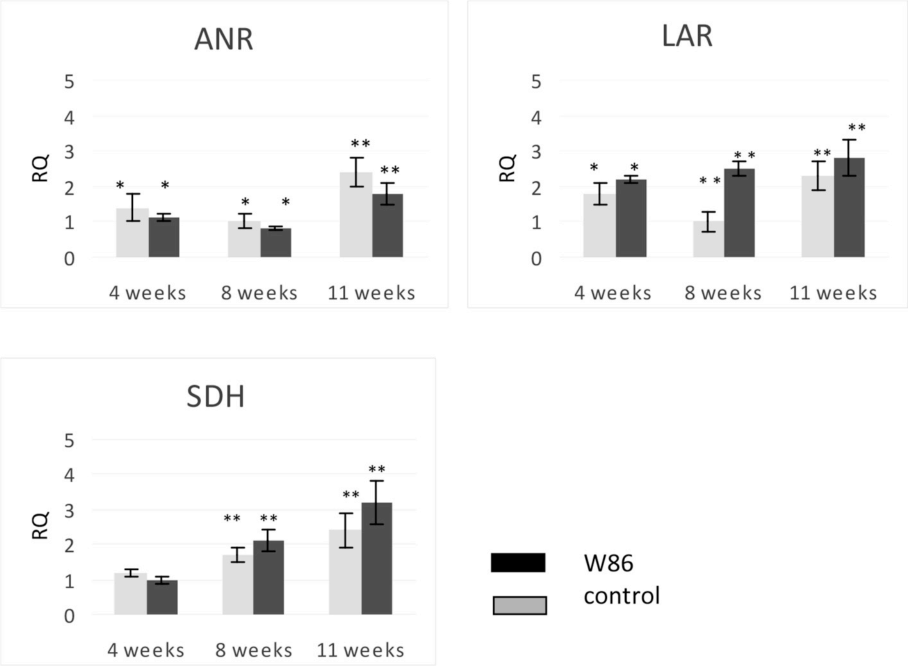

Fig. 6. Expression levels of lignin synthesis genes. The mRNA level of lignin synthesis genes (PAL, phenylalanine ammonia lyase; 4CL, 4-coumarate CoA ligase; COMT, caffeic acid 3-O-methyltransferase; HCT, hydroxycinnamoyl-CoA:quinate/shikimate hydroxycinnamoyl transferase; F5H, ferulate 5-hydroxylase; C3H, 4-coumarate 3-hydroxylase) of W86 flax in comparison with control flax obtained from the real-time RT-PCR analysis. Data represent the mean ± standard deviations from three independent experiments. The significance of the differences between the means was determined using Student's *t*-test (*P < 0.05,**P < 0.01). RQ – relative quantity.

Table 4
Content and composition of cell wall polymers in stem tissue from control (Linola) and transgenic lines with CHS repression (W86). The cell wall polymer content is expressed in mg/g DW.

mg/g DW	4–8 weeks		11 weeks		13 weeks	
	control	W86	control	W86	control	W86
Lignin	2.95 ± 0.05	4.2 ± 0.06	17.86 ± 0.05	23.85 ± 0.02	21.3 ± 0.04	15.6 ± 0.013
Cellulose	318 ± 24.6	228 ± 18.3	478 ± 36.5	508 ± 43.2	578.87 ± 48.8	654.78 ± 25.3
Pectin	44.0 ± 0.01	35.2 ± 0.02	33.8 ± 0.02	28.3 ± 0.02	35.8 ± 0.02	35.2 ± 0.01
Hemicellulose	18.28 ± 0.02	18.08 ± 0.01	18.33 ± 0.04	23.30 ± 0.03	25.45 ± 0.02	28.89 ± 0.05

The results are the mean values ± SD (n = 6). Significance of the differences between the means was determined using Student's *t*-test (P < 0.05).

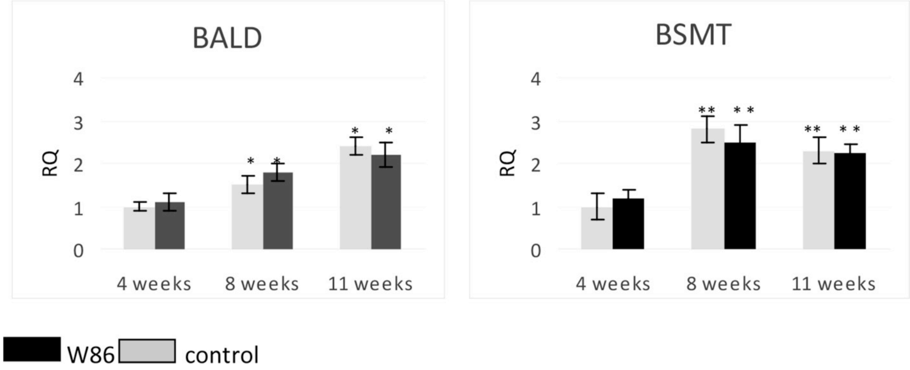

Fig. 7. Expression levels of benzoic derivate synthesis genes. The mRNA level of flavone synthesis genes (abbreviation explained in text) of W86 flax in comparison with control flax obtained from the real-time RT-PCR analysis. Data represent the mean ± standard deviations from three independent experiments. The significance of the differences between the means was determined using Student's *t*-test (*P < 0.05,**P < 0.01). RQ – relative quantity.

Table 5
Content and composition of benzoic derivates in stem tissue from control (Linola) and transgenic lines with CHS repression (W86). The cell wall polymer content is expressed in mg/g DW.

mg/g DW	4–8 weeks		11 weeks		13 weeks	
	control	W86	control	W86	control	W86
4-hydroxybenzoic acid	0.024 ± 0.004	0.013 ± 0.002	0.006 ± 0.001	0.004 ± 0.001	0.021 ± 0.006	0.016 ± 0.004
Vanillic acid	0.291 ± 0.028	0.326 ± 0.052	0.298 ± 0.020	0.317 ± 0.060	0.134 ± 0.014	0.115 ± 0.024
Vanillin	0.049 ± 0.008	0.062 ± 0.005	0.232 ± 0.018	0.254 ± 0.030	0.039 ± 0.020	0.018 ± 0.005
Benzoic derivates	**0.364 ± 0.046**	**0.412 ± 0.062**	**0.555 ± 0.044**	**0.595 ± 0.098**	**0.197 ± 0.046**	**0.157 ± 0.034**

The results are the mean values ± SD (n = 6). Significance of the differences between the means was determined using Student's *t*-test (P < 0.05).

Flavones, phenolic acids, anthocyanin, proanthocyanidins and benzoic derivatives were accumulated, and flavones were present in the highest while anthocyanin was present in the lowest quantity. Flavones are widely distributed in the plant kingdom. They serve in flowers as co-pigments with anthocyanins, giving bluer colors to flowers such as gentian.

Apigenin and luteolin were detected as the main flavone compounds in flax. In the early development stage (up to the 8th week) the ratio of apigenin to luteolin in all studied plant types (control and transgene) was from 3 (for control) to 5 (for transgene) while in the flowering stage it changed to 0.3 (control) or 0.4 (transgenic plant), and these ratios were maintained until the yellow maturity stage. At the flowering stage the level of flavone was about two times higher than at the initial stage and about 70% higher than at maturity. The conversion of apigenin to luteolin might occur upon hydroxylation via eriodictyol conducted by flavonoid hydroxylase (F3′H). Interestingly, at the flowering stage F3′H is significantly up-regulated.

Most flowering plants mainly accumulate either flavone or flavonol pigments; for example, snapdragon and torenia plants accumulate flavone rather than flavonol, while petunia and tobacco plants accumulate flavonol. Both flavone compounds (apigenin, luteolin) were also accumulated in developing gentians. Their accumulation peaked at the flowering stage and in flowers, apigenin accumulation was about three times higher than that of luteolin, whereas an almost equal amount was detected in the leaves. Contrary to flax plant, the ratio between these compounds was however unchanged during development (Nakatsuka et al., 2005).

Since the enzymes of F3H (flavanone 3-hydroxylase) and FNS (flavone synthase) are at the branching point for flavone and flavonoid biosynthesis, it is suggested that the switching of gene expression from FNS to F3H is considered critical for changing the biosynthesis pathway from flavone to anthocyanin. The suggestion is supported by the finding that in flax the FNS/F3H gene expression ratio is slightly but systematically higher than 1.0 and is maintained during plant development stages. It is interesting that at the flowering stage of development the accumulation of flavone is affected by CHS gene expression level. Lower CHS activity resulted in a slight decrease of F3H and FNS gene expression. Interestingly, F3′H gene expression is not affected by CHS down-regulation. In petunia, the expression of CHS and DFR was increased during flower development corresponding to anthocyanin pigmentation, whereas that of F3′H and FNS was maximal at the early stage of flower development (BruglieraF. et al., 1999). In snapdragons, of these two gene groups, one involves both CHS and CHI and another involves F3H, DFR and ANS. The first two enzymes (CHS and CHI) of the pathway are precursors for flavone as well as anthocyanin biosynthesis (Martin et al., 1991). It has been reported that flavonoid biosynthesis-related genes coordinately regulate several classes of compounds within the phenylpropanoid pathway. It should be pointed out that such analysis is valuable for understanding the molecular background of plant development. Based on metabolite analysis, the phenylpropanoid biosynthesis pathway studied here was classified into three groups, which could be roughly defined as early, middle and late stages of plant growth. The earlier developmental stage might be characterized by a relatively high level of proanthocyanidins, a low level of hydrolysable tannins, and a medium level of phenolic acids and benzoic derivatives. The middle stage might be characterized by the highest quantity of all analyzed compounds and the late stage might be described as containing the lowest level of all compounds except the flavones, which are of medium quantity.

The p-coumaroyl-CoA synthesized by coumarate-CoA ligase (4CL) is the branching point for both the most representative phenylpropanoid compound, chlorogenic acid, and flavone. Its expression is highly increased at the flowering stage, which suggests its regulatory role in flax development. The genes coding for 4-coumarate 3-hydroxylase enzyme (C3H), caffeic acid 3-O-methyltransferase (COMT) and ferulate 5-hydroxylase (F5H) situated along the phenolic acid biosynthesis route increased at the flowering stage and their expression was higher in the case of CHS modified transgenic plants compared to controls. But their expression level does not reflect CHS expression. Ferulic acid is the precursor for vanillin derivatives and the compounds have been identified at each stage of plant development, but their content did not change significantly during development.

Benzenoid derivatives are widespread mediators of the plant response to environmental stress in plants. For example, vanillin is a strong antioxidant of antibacterial function, salicylic acid is a key signal compound in plant responses to different pathogens, and volatile benzenoid derivatives are present in flower scent in over a hundred plant species. This was the reason for investigation of benzenoid during flax development. The compound level is significantly increased at the flowering stage, but they are present in the lowest quantity compared to other phenylpropanoids. The key genes of their synthesis such as benzaldehyde dehydrogenase (BALD) and benzoic acid methyltransferase (BSMT) are highly expressed in flax, but it is not reflected in the compounds' content and also it does not seem to be CHS expression affected.

Polyphenolic compounds are widely distributed in plants. They contribute to color and flavor, plant disease defense and damage caused by abiotic stress and are involved in pigment biosynthesis (Kurauchi et al., 2011). Notably, polyphenols are responsible for the protective effects of plants against a variety of human diseases including cancer (Thomasset et al., 2007). Among the polyphenols, catechins are the major components. They are biosynthesized by different branches of the phenylpropanoid biosynthetic pathway and the CHS gene encodes the key enzyme for the first step of their biosynthesis (Wang et al., 2012). In addition to CHS, anthocyanidin synthase (ANS), anthocyanidin reductase (ANR) and leucoanthocyanidin reductase (LAR) are downstream genes involved in the polyphenol biosynthesis pathway. However, to what extent these genes affect catechin biosynthesis and compositions in plant development remains elusive. We have found that most of the catechins are present in the form of esters with gallic acid. The compounds' content in control and transgenic flax is highly increased at the flowering stage. In nectarine it was found that the ANR and LAR genes are responsive to both developmental and environmental stimuli of plant organs (Ravaglia et al., 2013). In CHS-modified flax the LAR gene expression was up-regulated while ANR was down-regulated during all investigated flax developmental stages. This might suggest that in flax polyphenols are synthesized under the control of the LAR gene independently of CHS activity or gallic acid biosynthesis regulates the level of its esters. Three pathways have been proposed for the biosynthesis of gallic acid i. p-oxidation of the side-chain trihydroxycinnamic acid; ii. dehydrogenation of shikimic acid, presumably with 3-dehydroshikimic acid as an intermediate; and iii. hydroxylation of protocatechuic acid. In tea the pathway through shikimic acid and phenylpropanoid was found to be most effective (Saijo, 1983). We detected induction of shikimate dehydrogenase (SDH) gene expression at the flowering stage of flax development and at this stage gallic acid was detected and its content was maintained until plant maturation. The induction of SDH gene expression was highly activated in CHS down-regulated flax, which corresponds to gallic acid accumulation. Temporal accumulation of all catechins was also detected in tea leaves (Zhang et al., 2016). Since catechin accumulation in flax differed at different developmental stages, we suggest that this route of the phenylpropanoid pathway appears to be responsive to both developmental and environmental stimuli.

In summary, dynamic changes in the quantity of flavone constituents during flax growth and development have been demonstrated. Moreover, the conversion of apigenin to luteolin is the most intriguing. Both compounds showed antioxidant properties but differed in antioxidant efficiency and other parameters such as absorption and emission properties (Amat A et al., 2009, Leopoldini et al., 2004). The bond dissociation enthalpy (BDE) of the O–H bonds in –OH groups which substitute the phenyl ring (B ring) differed significantly between compounds. This important parameter determined antioxidant efficiency. A lower value meant a weaker O–H bond and easier free radical inactivation. BDE for luteolin is very low (74.54 kcal/mol) compared to apigenin (82.20 kcal/mol), which suggests its high functionality as an antioxidant, the regulator of free radical status. This functionality is strongly reduced for apigenin. Free radicals act at sensitive subcellular sites, playing a critical role in the deleterious and beneficial effects of maturation and senescence of various plant organs (Leshem, 1988). Since the production of reactive oxygen species (ROS) is genetically programmed, induced environmentally and during development and affects primary and secondary metabolism (Shao et al., 2008), the accumulation of flavone constituents with different antioxidant efficiency is required and developmentally regulated. Thus in addition to the crucial role in the defense system as antioxidants, flavones might influence plant growth and development by ROS-antioxidant interaction, producing different types of signals modifying processes from cell proliferation and elongation to full maturity (senescence). To our best knowledge, this is the first report on a broad analysis of different classes of phenylpropanoids involved in flax development and indicating the potential of flavone constituents in this process. Such analysis is valuable for understanding the molecular background of plant development.

Apart from the crucial function for plants, flavones possess beneficial properties for human health. They are known antioxidant, anti-inflammatory, antibacterial and antifungal factors. Dietary flavones serve as free radical scavengers and have anticancer properties (Sak, 2014, Zhang et al., 2006, Zhao et al., 2016). For example, baicalin and wogonin, the major bioactive flavones produced in large amounts by the roots of *S. baicalensis*, provide a variety of specific health benefits, such as antifibrotic activity in the liver, and antiviral and anticancer properties (Fox J. T et al., 2012, HimejiOhtsuki et al., 2007, Nayak et al., 2014). They specifically promote apoptosis in tumor cells but have low or no toxicity in healthy cells (Baumann et al., 2008, Parajuli et al., 2009). Thus, apart from flavones' significance for flax development, we are interested in elucidating the peak of their biosynthetic pathway for applications involving increased production of these bioactive compounds.

5. Conclusions

Flax (*Linum usitatissimum*) is an annual plant whose life cycle from sowing to maturity is usually 12–14 weeks, but it depends on general environmental conditions that may delay its development and productivity. Secondary metabolites, such as flavonoids, in addition to their usefulness for therapeutic, aromatic and culinary purposes, are also an important part of the plant defense system against environmental stress. In flax flavonoids, the flavone subclass, represented by apigenin and luteolin, was detected as the major components. We have shown that the ratio of apigenin to luteolin significantly decreases in the plant at flowering stage. Conversion of apigenin to luteolin occurs after eriodictyol hydroxylation led by flavonoid hydroxylase (F3′H). At the flowering stage, the expression of the F3′H gene was significantly increased. Both flavone constituents showed antioxidant properties but differed in the effectiveness of antioxidants. Because the production of reactive oxygen species (ROS) is genetically programmed, environmentally induced and during development and affects primary and secondary metabolism, the accumulation of flavone components with different antioxidant efficiency and developmental regulation is required. Thus, in addition to the key role in the defense system as antioxidants, flavones can influence the growth and development of plants through the interaction of ROS-antioxidant.

Conflicts of interest

The authors declare that they have no competing interests.

Author contributions

Magdalena Zuk and Jan Szopa designed the research; Jakub Szperlik perform all metabolite content analysis, Magdalena Zuk and Agata Hnitecka perform genes expression analysis, Magdalena Zuk and Jan Szopa wrote the article. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.07.009.

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