

# Maize *Lc* transcription factor enhances biosynthesis of anthocyanins, distinct proanthocyanidins and phenylpropanoids in apple (*Malus domestica* Borkh.)

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**Abstract** Flavonoids are a large family of polyphenolic compounds with manifold functions in plants. Present in a wide range of vegetables and fruits, flavonoids form an integral part of the human diet and confer multiple health benefits. Here, we report on metabolic engineering of the flavonoid biosynthetic pathways in apple (*Malus domestica* Borkh.) by overexpression of the maize (*Zea mays* L.) leaf colour (*Lc*) regulatory gene. The *Lc* gene was transferred into the *M. domestica* cultivar Holsteiner Cox via *Agrobacterium tumefaciens*-mediated transformation which resulted in enhanced anthocyanin accumulation in regenerated shoots. Five independent *Lc* lines were investigated for integration of *Lc* into the plant genome by Southern blot

and PCR analyses. The *Lc*-transgenic lines contained one or two *Lc* gene copies and showed increased mRNA levels for phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), flavanone 3 beta-hydroxylase (FHT), dihydroflavonol 4-reductase (DFR), leucoanthocyanidin reductases (LAR), anthocyanidin synthase (ANS) and anthocyanidin reductase (ANR). HPLC-DAD and LC-MS analyses revealed higher levels of the anthocyanin idaein (12-fold), the flavan 3-ol epicatechin (14-fold), and especially the isomeric catechin (41-fold), and some distinct dimeric proanthocyanidins (7 to 134-fold) in leaf tissues of *Lc*-transgenic lines. The levels of phenylpropanoids and their derivatives were only slightly increased. Thus, *Lc* overexpression in *Malus domestica* resulted in enhanced biosynthesis of specific flavonoid classes, which play important roles in both phyto-pathology and human health.

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## Abbreviations

ANS	Anthocyanidin synthase
ANR	Anthocyanidin reductase
BAP	6-Benzylaminopurine
CaMV	Cauliflower mosaic virus
CHI	Chalcone isomerase
CHS	Chalcone synthase
DAD	Diode array detection
DFR	Dihydroflavonol 4-reductase
FGT	UDP-Glucose:flavonoid 3- <i>O</i> -glucosyltransferase
FHT	Flavanon 3 beta-hydroxylase
FLS	Flavonol synthase
GA	Gibberellic acid
HPLC	High performance liquid chromatography
HC	Holsteiner Cox

IBA	Indole-3-butyric acid
LAR1+2	Leucoanthocyanidin reductase
Lc	Maize leaf colour
LC-MS	Liquid chromatography/mass spectroscopy
MS	Murashige and Skoog
PAL	Phenylalanin ammonia-lyase
TDZ	Thidiazuron
YEP	Yeast extract broth

## Introduction

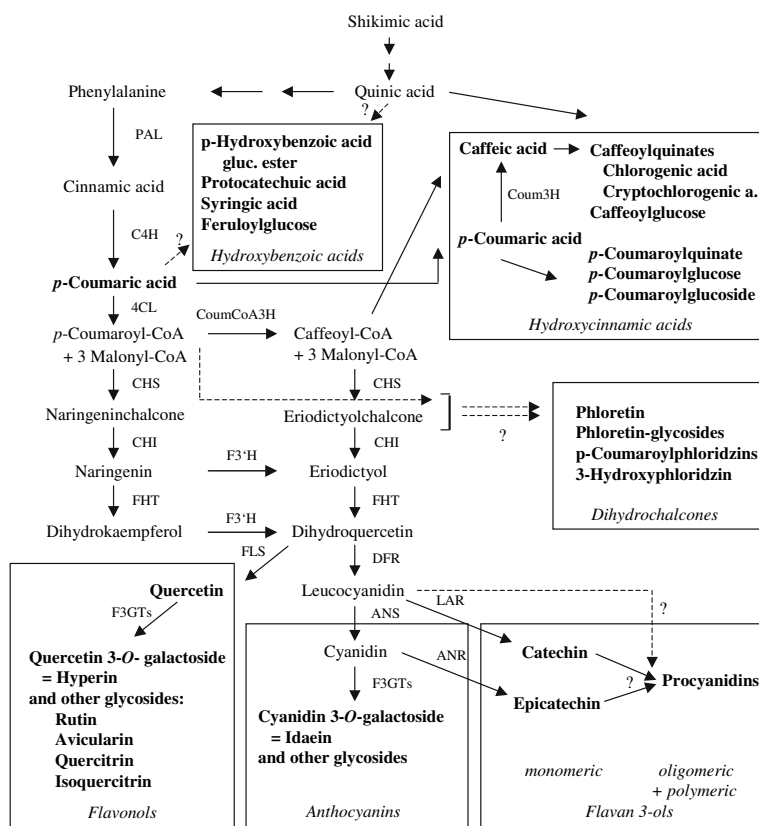
Flavonoids are plant polyphenolic compounds derived from the phenylpropanoid pathway. On the basis of their structure they can be classified into the classes flavanones, flavones, isoflavones, dihydroflavonols, flavonols, leucoanthocyanidins, flavan 3-ols (catechins and the polymeric proanthocyanidins) and anthocyanins. Representatives of most of these classes are also found in *Malus domestica* (Fig. 1). Chalcones are direct precursors of all flavonoids. The prominent dihydrochalcones present in *M. domestica* are chemical derivatives of them. Flavonoids have manifold functions in plants such as UV-protection, attraction of pollinators, warranty of pollen fertility, regulation of polar auxin transport (Taylor and Grotewold 2005), establishment of microbial symbioses and pathogen defence (Treutter 2005). In fruit crops such as *M. domestica*, flavonoids

are of utmost importance as they impart much of the colour and contribute to the flavour of the apple fruit by astringency of proanthocyanidins. Phytopathological work demonstrated the significance of flavonoids, especially of flavan 3-ols, in resistance reactions to apple scab, caused by the fungus *Venturia inaequalis* (Cooke) G. Wint., the most important apple disease in all cultivating regions (Römmelt et al. 1999; Bazzi et al. 2003; Leser and Treutter 2005).

In addition to these implications in plant physiology and metabolism, flavonoids have attracted increasing interest as health promoting agents due to their antioxidant properties, their effects on mammalian enzymes (Geahlen et al. 1989; Cushman et al. 1991; Middleton et al. 2000), especially their actions at protein kinase and lipid kinase signalling pathways (reviewed by Williams et al. 2004). Results from several epidemiological studies revealed protective effects of flavonoids against cardiovascular diseases (Hertog et al. 1997), cancer (Knekt et al. 1997, 2000) and other age-related diseases such as dementia (Commenges et al. 2000) due to their anti-proliferative, anti-tumour and pro-apoptotic activities.

Apples are an important source of polyphenols in the human diet. These polyphenols include hydroxycinnamic acids (e.g. chlorogenic acid and *p*-coumaroyl glucoside), cyanidin-3-*O*-galactoside (idaein), flavan-3-ols/procyanidins (mainly catechin, epicatechin and procyanidins B2 and B5), flavonols (quercetin galactoside, glucoside, rhamnoside and arabinoside) and dihydrochalcones (mainly phloridzin

**Fig. 1** Flavonoid biosynthesis pathway in apple



and phloretin-2'-xyloglucoside; Treutter 2001; Tsao et al. 2005). Because of the high impact of flavonoids in plant defence and their great influence on quality and health of food and food products there is a growing interest in the development of agriculturally important food crops with optimized levels and composition of flavonoids.

Two classes of genes are determining the flavonoid pathway. Structural genes encode enzymes that directly participate in the formation of flavonoids and regulatory genes control the expression of the structural genes. Most known regulatory genes are homologous to *myb* and *myc* transcription factors, but some are also WD-repeat proteins or belong to other classes. One of the best characterized regulatory genes is the leaf colour (*Lc*) gene from maize (Ludwig et al. 1989). This gene, belonging to the *myc* type R gene family, encodes a protein with the basic-helix-loop-helix (bHLH) motif. *Lc* is one of the transcription factors which determines the amount, location and timing of anthocyanin accumulation in maize. The heterologous expression of *Lc* or combinations of *Lc* along with the *myb* type *Cl* (colourless) gene resulted in increased anthocyanin pigmentation in *Nicotiana tabacum* L., *Arabidopsis* (Lloyd et al. 1992), *Lycopersicon esculentum* Mill (Goldsbrough et al. 1996), a *Petunia* hybrid (Bradley et al. 1998), *Medicago sativa* L. (Ray et al. 2003) and *Caladium bicolor* (Li et al. 2005). Flavonoids of other classes have also been reported to accumulate after heterologous *Lc* expression. In the *Petunia* hybrid, an up-regulation of anthocyanin contents occurred accompanied by up-regulation of gene expression of most but not all enzymes of the flavonoid pathway (Bradley et al. 1998). Flavonol contents were positively and negatively influenced in a tissue-specific way. The coordinate expression of the maize regulatory genes *Lc* and *Cl* in transgenic *L. esculentum* resulted in an increased production of anthocyanins (in leaves) and of the flavonol kaempferol (in leaves and fruits; Bovy et al. 2002). In *M. sativa*, the transgenic *Lc* gene caused induction of anthocyanins and proanthocyanidins correlated with a reduction of flavone concentrations (Ray et al. 2003).

The study presented here reports on the introduction of the maize regulatory gene *Lc* into the *M. domestica* Borkh. cultivar Holsteiner Cox (HC) under control of the constitutive cauliflower mosaic virus (CaMV 35S) promoter to enhance flavonoid biosynthesis. Effects on the expression of phenylpropanoid and flavonoid genes and on the metabolite levels of anthocyanins, monomeric and polymeric flavan 3-ols (catechins, proanthocyanidins), flavonols, dihydrochalcones, hydroxycinnamic acids and hydroxybenzoic acids were considered in detail. This work was performed to comprehensively differentiate the various consequences of the heterologous *myc* transcription factor in a relevant fruit crop.

## Material and methods

### *Agrobacterium* strain and binary vector

The *Lc* gene of maize (Genbank M26227) was kindly provided by Ryan Peeler (Plant Biology Department, University of Georgia, Athens, GA, USA). The gene along with the CaMV35S promoter, was introduced into the binary vector pBI121 (Genbank accession no. AF485783) by replacing the GUS ( $\beta$ -glucuronidase) gene by *Hind*III and *Eco*RI sites. Additionally, pBI121 carries on its T-DNA the *nptII* gene coding for neomycin phosphotransferase II driven by the *nos* promoter. pBI-*Lc* thus confers kanamycin resistance in plants and was introduced into *Agrobacterium tumefaciens* EHA105 (Hood et al. 1993) through electroporation.

### Plant material

The plant material consisted of in vitro cultivated shoots of apple (*M. domestica* Borkh.) cv. HC. In vitro cultures were initiated from nodal segments. Plant material was obtained from the Chamber of Agriculture Lower Saxony, Germany. Shoot cultures were maintained as described elsewhere (Szankowski et al. 2003).

### Transformation and regeneration

The transformation of apple was done as described by Szankowski et al. (2003). In brief: explants obtained from the four youngest unfolded leaves from 4-week-old micro-propagated in vitro shoots were inoculated with agrobacteria (grown over night in YEP medium, centrifuged and resuspended in liquid MS medium to an OD of 0.8) and cocultivated in the dark on regeneration medium [MS salts and vitamins (Murashige and Skoog 1962), 3% sorbitol, 3  $\mu$ M TDZ, 1  $\mu$ M IBA and 0.3% gelrite; pH 5.7] for 3 days.

Adventitious shoot induction was performed on regeneration medium supplemented with 150 mg l<sup>-1</sup> ticarcillin (agrobacteria elimination) and 50 mg l<sup>-1</sup> kanamycin for selection of transgenic plant cells. Explants were cultured in the dark for 2 weeks at 24  $\pm$  1°C, then under 16/8 h photoperiod (70  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>) at the same temperature. The medium was changed every 2 weeks and after 6 weeks on regeneration medium they were cultured on elongation medium (MS salts and vitamins, 4.4  $\mu$ M BAP, 0.28  $\mu$ M GA<sub>3</sub>, 3% sucrose and 0.8% plant agar, pH 5.7).

### DNA analysis

For the detection of the transgenes in the genome of kanamycin selected, regenerated plants PCR and Southern blot analysis were performed. DNA was extracted from leaves

of *in vitro* plantlets (Doyle and Doyle 1990). The PCR reactions contained 0.2 U of *Taq* DNA polymerase (GeneCRAFT, Lüdinghausen, Germany), 16 mM  $(\text{NH}_4)_2\text{SO}_4$ , 67 mM Tris-HCl (pH 8.8), 1.5 mM  $\text{MgCl}_2$ , 200  $\mu\text{M}$  of each dNTP and 0.4  $\mu\text{M}$  of each primer in a volume of 25  $\mu\text{l}$ .

The primers 5'-ATG GCG CTT TCA GCT TCC CGA-3' and 5'-TGT ACC AAG CTC AAG CAC GCC-3' were used to amplify a specific *Lc*-fragment with the size of 583 bp under following conditions: 94°C for 1 min, followed by 30 cycles at 94°C for 45 s, 60°C for 45 s, 72°C for 45 s and with a final extension at 72°C for 5 min. The presence of the *nptII* gene was confirmed by using the primers 5'-GAT TGA ACA AGA TGG ATT-3' and 5'-CAT TTT CCA CCA TGA TAT TC-3', which amplify a 600 bp *nptII* specific fragment. The PCR reaction conditions were 95°C for 3 min, followed by 35 cycles at 94°C for 90 s, 63°C for 90 s, 72°C 150 s and with a final extension at 72°C for 10 min.

Southern blot analyses were performed to verify the integration of the transgenes. Twenty micrograms of genomic DNA were restricted with *HindIII* (single restriction site within the T-DNA) at 37°C overnight. DNA was separated in a 0.8% agarose gel and blotted on a positively charged nylon membrane (Boehringer, Mannheim, Germany). Membranes were hybridized with the digoxigenin-labelled PCR probes for *Lc*, incubated with alkaline phosphate conjugated anti-digoxigenin followed by the chemiluminescent substrate CDP-Star and finally exposed to X-ray film. All steps were performed following the supplier's instructions (Roche, Mannheim, Germany). Primers and PCR conditions for probe generation were the same as mentioned above.

#### Transcription profiling and real-time PCR

Three young *in vitro* leaves were randomly selected from each transgenic line and the untransformed control line and frozen in liquid nitrogen. The total RNA from these leaves was extracted using the Invisorb® Spin Plant-RNA Mini Kit (Invitex, Berlin, Germany). One  $\mu\text{g}$  of total RNA was treated with *Dnase* I (MBI Fermentas, St. Leon-Rot, Germany) to eliminate genomic DNA contaminations. Presence of DNA residues was tested using 1  $\mu\text{l}$  total RNA as template in a 25  $\mu\text{l}$  standard PCR reaction with primers specific for an apple gene encoding the elongation factor EF1 alpha. The PCR mixture contained 1  $\times$   $\text{NH}_4$ -buffer, 12 mM  $\text{MgCl}_2$ , 25 mM dNTPs, 12.5 mM of each primer (EF1forw 5'-TAC TGG AAC ATC ACA GGC TGA C-3' and EF1rev 5'-TGG ACC TCT CAA TCA TGT TGT C-3') and 0.625 U of *Taq* DNA polymerase (Invitex). The PCR reaction was performed by denaturation at 94°C for 5 min, followed by 30 cycles of 1 min denaturation at 94°C, 30 s annealing at 65°C and 1 min extension at 72°C. After a

final extension at 72°C for 5 min the amplified fragments were separated on a 0.8–1% agarose gel containing 0.5  $\mu\text{g ml}^{-1}$  ethidiumbromid in 1  $\times$  TAE buffer.

The remaining RNA was reverse transcribed using oligo(dT)<sub>18</sub> primers and the RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas) according to the manufacturer's instruction. The success of reverse transcription was verified by PCR of 1  $\mu\text{l}$  cDNA using primers EF1\_F1 and EF1\_R1 as described. Quantitative real-time PCR was performed using the iQ™ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) and first-strand cDNA as template on an iCycler iQ real-time PCR Detection System (Bio-Rad).

Gene specific amplification was evaluated by melt curve analysis and agarose gel electrophoresis. Single melt peaks were obtained for each gene. No primer dimers or other PCR artefacts were detected. To determine the amplification efficiencies and correlation efficiencies of each PCR reaction, 15, 30, 60 and 120 ng cDNA of the transgenic line T1 were analysed. The PCR efficiencies were used to transform the Ct-values into raw data for relative quantification. The determination of PCR efficiency and the calculation of the mRNA transcript levels were done using the Gene Expression Macro™ Version 1.1 (Bio-Rad). The methodology of this macro is based on the algorithms outlined by Vandersompele et al. (2002). The *Lc* gene transcription of each line was quantified relatively using the same primers and conditions as described. All samples were normalized using ribulose-1,5-bisphosphate carboxylase/oxygenase activase mRNA and mRNA of the *M. domestica* cDNA clone Mdfw2033f21.y1 (similar to the RNA polymerase subunit II) as internal control samples for each line. As no *Lc* gene transcription was expected in the non-transgenic HC the scaling of the *Lc* gene transcript levels was performed in relation to the mRNA expression level of the reference genes. The scaling of the genes involved in the flavonoid biosynthetic pathway of apple was performed relative to the mRNA expression level of the non-transgenic HC set to be one.

#### LC-MS

A Bruker Daltonics esquire 3000<sup>plus</sup> ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) connected with an Agilent 1100 HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with a quaternary pump and a variable wavelength detector was used for LC-MS analysis. Components were separated on a Phenomenex, Aschaffenburg, Germany, Luna C-18 column (150 mm long  $\times$  2.0 mm inner diameter, particle size 5  $\mu\text{m}$ ) which was held at 25°C. The LC parameters went from 0% acetonitrile and 100% water (acidified with 0.05% formic acid) to 40% acetonitrile and 60% acidic water in 40 min, in

further 5 min to 100% acetonitrile and remained for 10 min at these conditions with a flow rate of  $0.2 \text{ ml min}^{-1}$ . The detection wavelength was 280 nm. The electrospray ionization voltage of the capillary was set to  $-4,000 \text{ V}$  and the end plate to  $-500 \text{ V}$ . Nitrogen was used as dry gas at a temperature of  $300^\circ\text{C}$  and a flow rate of  $10 \text{ l min}^{-1}$ . The full scan mass spectra were measured in a scan range from 50 to 800  $m/z$  with a scan resolution of 13,000  $m/z/s$  until the ICC target reached 20,000 or 200 ms, whatever was achieved first. Tandem mass spectrometry was carried out using helium as collision gas ( $3.56 \times 10^{-6} \text{ mbar}$ ) with the collision voltage set at 1 V. Spectra were acquired in the positive and negative ionization mode. Auto-tandem mass spectrometry was used to break down the most abundant  $[M+H]^+$ ,  $[M-H]^-$  or the  $[M+HCOO]^-$  ion of the different compounds of the extracts. Data analysis was performed using the DataAnalysis 3.1 software (Bruker Daltonics) and MZmine (<http://www.mzmine.sourceforge.net>; Katajamaa et al. 2006). Metabolites were identified by their retention times, mass spectra and product ion spectra in comparison with the data determined for authentic reference material. Signals of the compounds were integrated in their  $[M+H]^+$ ,  $[M-H]^-$  or  $[M+HCOO]^-$  ion traces. Induction factors were calculated from signal intensities.

#### Quantitative HPLC-DAD and identification of phenolic compounds

Analysis of leaves was done as follows: samples were lyophilized and ground in a ball mill. The extraction of phenolic compounds was done with methanol (100%) containing 6-methoxyflavone as an internal standard for 30 min in a cooled water bath during sonication. After centrifugation, the supernatant was evaporated and the residue redissolved in small quantities of methanol and injected for HPLC analysis. The phenolic compounds were separated on a column ( $250 \times 4 \text{ mm}^2$  i.d.) prepacked with Hypersil ODS, 3- $\mu\text{m}$  particle size, following a stepwise gradient using mixtures of solvent A (formic acid, 5% in water) and solvent B (methanol, gradient grade) from 95:5 (v/v) to 10:90 (v/v) with a flow rate of  $0.5 \text{ ml min}^{-1}$  (Treutter et al. 1994). The gradient profile (% B in A) used was as follows: 0–5 min, isocratic, 5% B; 5–15 min, 5–10% B; 15–30 min, isocratic, 10% B; 30–50 min, 10–15% B; 50–70 min, isocratic, 15% B; 70–85 min, 15–20% B; 85–95 min, isocratic, 20% B; 95–110 min, 20–25% B; 110–140 min, 25–30% B; 140–160 min, 30–40% B; 160–175 min, 40–50% B; 175–190 min, 50–90% B. For the HPLC determination of the dihydrochalcone glycoside phloridzin the extract was diluted by 200 with methanol and the phloridzin was analysed using a short column ( $12.5 \times 4 \text{ mm}^2$  i.d.) prepacked with LiChrospher 100 RP18, 5- $\mu\text{m}$  particle size, and a gradient range from 40 to 90% aqueous methanol.

Hydroxycinnamic acids (*p*-coumaric acids and caffeic acid derivatives), the dihydrochalcone glycosides and the flavonols (quercetin glycosides), were detected at 320, 280 and 350 nm, respectively, whereas the monomeric flavan-3-ols, the procyanidins and the dihydrochalcone phloretin were estimated at 640 nm after postcolumn derivatization with 4-dimethylaminocinnamic aldehyde (DMACA; Treutter 1989). Peak identification is published elsewhere (Mayr et al. 1995; Römmelt et al. 2003a).

## Results

### Generation of *Lc*-transgenic apple lines

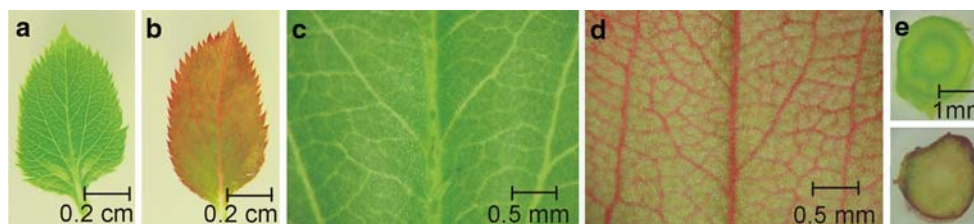
The maize *Lc* regulatory gene under the control of the CaMV 35S promoter was transferred into *M. domestica* cv. HC via *Agrobacterium tumefaciens*-mediated transformation. The shortened 2.2 kbp variant of the *Lc* gene lacking the inhibiting 200 bp of untranslated sequence was used (Lloyd et al. 1992). Explants inoculated with the agrobacteria and cultivated under kanamycin selection developed calli with red/purple groups of cells, later forming shoots. In regenerated shoots, the pigmentation was maintained (Fig. 2) which was neither observed in non-transformed control HC shoots nor in HC shoots transformed with other genes (Szankowski et al. 2003; Degenhardt et al. 2006; Rühmann et al. 2006). High levels of anthocyanins accumulated in leaves as well as in the stem (Fig. 2) of regenerated plantlets. Transgenic shoots developed much more callus on shoot propagation medium and this callus was dark red coloured. Contrarily, the callus of untransformed HC shoots was green. Furthermore, the growth of transgenic shoots was slower in comparison to non-transgenic shoots of the same cultivar.

### Integration analysis

Five transgenic lines were regenerated on kanamycin selective medium. Results of Southern blot analyses (Fig. 3) revealed the integration of at least one copy of the *Lc* gene in lines T1, T2, T3 and T4 and two gene copies in line T5. No hybridization signal occurred in the DNA sample of non-transformed control plants.

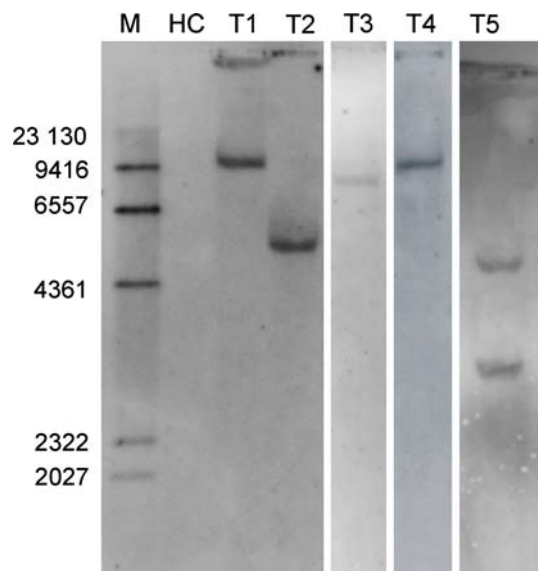
### Transcription profiling

Real-time RT-PCR using gene specific primers (Table 1) was performed to obtain transcription profiles for the transgenes *nptII* and *Lc* as well as for the genes of the flavonoid biosynthetic pathway encoding PAL, CHS, CHI, FHT, FLS, DFR, LAR1+2, ANS, ANR and FGT. The transcripts of *nptII*, *Lc* (Fig. 4), and structural flavonoid genes were



**Fig. 2** a–e In vitro plants of Holsteiner Cox transformed with pBI-*Lc* (line T1) and regenerated under selective pressure with 50 mg l<sup>-1</sup> kanamycin exhibit strong anthocyanin pigmentation in leaves (b, d)

and stems (e, lower part, cross-section of a stem) compared to non-transformed leaves (a, c) and stems (e, upper part)



**Fig. 3** Southern hybridization of *Hind*III digested DNA isolated from *Lc*-transgenic apple plants and respective control plants. Genomic DNA was hybridized to a 583 bp *Lc*-probe. Lanes: **M** molecular-weight markers II, DIG labelled (Roche), **HC** negative control (DNA from non-transformed Holsteiner Cox plants), **T1–T5** DNA isolated from transgenic lines

detected in tissues of the transgenic lines T1–T5. No *nptII* or *Lc* expression was observed in non-transgenic control samples, while *rubisco* expression was detected in all cDNA samples (Fig. 4). The *Lc* gene transcription levels of the transgenic lines ranged between 137 (line T4) and 291 (line T2; Figs. 5, 6).

The relative expression levels of the structural genes revealed that almost all *Lc* transgenic lines showed significantly increased transcription levels for *pal*, *chs*, *fht*, *dfr*, *lar*, *ans* and *anr* in comparison to the non-transgenic wild type (Fig. 6). Especially *ans* transcription was strongly induced in all lines. In contrast, *fgt* was clearly induced in only some lines. Neither *chi* nor *fls* were induced by the *Lc* transgene.

#### Metabolic profiling

Metabolic profiling of polyphenolic secondary metabolites was performed by quantitative HPLC-DAD and LC-MS

analysis to obtain absolute compound concentrations as well as mass spectral data. A number of anthocyanins, monomeric and polymeric flavan 3-ols (catechins, proanthocyanidins), flavonols, dihydrochalcones, hydroxycinnamic acids and hydroxybenzoic acids were identified and quantified from leaves of untransformed in vitro plants and the five in vitro lines transgenic for 35S-*Lc* (Table 2, Fig. 7). Generally, members of almost all analysed polyphenol classes were induced by *Lc*. Strongest rises were observed for the anthocyanin idaein (12-fold), epicatechin (14-fold), catechin (41-fold) and proanthocyanidins (Fig. 7). Remarkably, only two distinct proanthocyanidins, namely B2 and a yet unidentified proanthocyanidin were selectively induced by mean factors of 58 and 134, respectively (Fig. 7). The concentration of hyperin (quercetin-3-O-galactoside) as the prominent *M. domestica* flavonol was only slightly increased while levels of other flavonol glycosides remained unchanged or were even reduced (Table 2). Also within the large pool of dihydrochalcones (phloretin, phloridzin and phloretin 2'-xyloglucoside) as the dominant *M. domestica* polyphenol class, the levels in the transgenic lines increased only slightly (up to threefold). Some hydroxycinnamic acids, hydroxybenzoic acids and their derivatives showed rises up to a mean factor of about 2.

## Discussion

### Phenotype

The recombinant expression of the maize *Lc* regulatory gene enhances flavonoid biosynthesis in transgenic *M. domestica*. *Lc* transgenic HC shoots were phenotypically distinguishable from non-transgenic plantlets by strong anthocyanin accumulation in leaves and stems. *Lc*-transgenic shoots also grew slower in comparison to non-transformed shoots. Similar growth inhibiting effects due to *Lc* expression were observed in a *Petunia* hybrid (Bradley et al. 1998), in *L. esculentum* and in *Arabidopsis* transgenic lines, where calli failed to give rise to shoots or the shoots failed to root (Quattrocchio et al. 1993). Since flavonoids

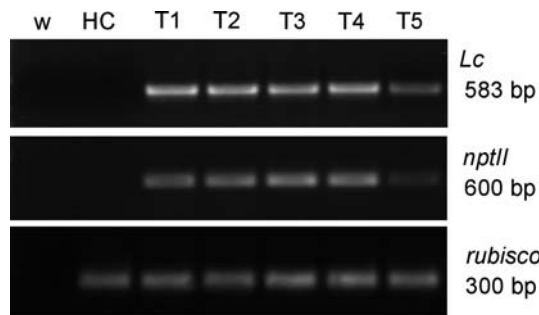
**Table 1** Primers used for RT- and quantitative real-time PCR

Primer		Sequence 5'→3'	Accession number <sup>a</sup>	T <sub>m</sub> °C	PCR efficiency %	Correlation coefficient
PAL	F	AAGGGAAGTGCTGATTGAACAT	X68126	56.5	104.0	0.967
	R	CTTGGAACACCTTGTCCACTC		60.3		
CHS	F	CTAGTGACACCCACCTTGAYAG	DQ026297, AB074485, AF494402, AF494401	61.2	77.5	0.990
	R	AGAAGAGYGAGTTCCAGTCYGA		60.3		
CHI	F	GGTCCGTTTGAGAAAATTCATTC	AF494398, AF494400, AF494399	56.5	126.6	0.999
	R	KGTTTTCTATCACCRYGTTTGC		57.5		
FHT	F	TCAACCAGTGGAAGGAGCTT	AY965340, AY965339	57.3	95.6	0.999
	R	GTCCTGCAGTTGCTGTTCTT		59.4		
FLS	F	GAAAGCAATGAGGGTACAATCC	AF119095, AY965343	58.4	109.0	1.000
	R	AACAGATGGAGGCCAAATCTTA		56.5		
DFR	F	AGTCCGAATCCGTTTGTGTC	AY227729, AY227728, AB074488, AF117268	57.3	99.4	0.996
	R	TTGTGGGCTTGATCACTTCA		55.3		
LAR	F	TCTTGCCCTACTTTGACAAC	AY830131, AY830132, DQ139836, DQ139837	57.9	69.2	0.998
	R	AGAGTTCCCACTTCCACATCA		57.9		
ANS	F	CACCTTCATCTCCACAACAT	DQ156905, DQ381771, AB074487, AF117269	57.9	113.1	0.995
	R	ATGTGCTCAGCAAAAGTTCGT		55.9		
ANR	F	CCACCTCACAGCACTACAAGAG	DQ139835, DQ099803, AY830130	62.1	139.7	0.985
	R	GCAAATTTCCAAGCTGTCTTCT		56.5		
FGT	F	CCCTTCTGTGGTCTATCAAGG	DQ156906, AF1172667	60.3	100.5	0.986
	R	TGGTAAAAACTCCATCTCCAC		58.4		
RNAPOL II <sup>b</sup>	F	ATATGCCACCCGTTCTCTACT	CN579456	60.3	95.8	0.997
	R	CACGTTCCATTTGTCCAAACTT		56.5		
RUBISCO <sup>b</sup>	F	GCTTGTCCAAGAGCAAGAGAAT	Z21794	58.4	73.7	0.999
	R	CTCCCTCCCCTCAATTATAACC		60.3		

ANR anthocyanidin reductase, ANS anthocyanidin synthase, CHI chalcone isomerase, CHS chalcone synthase, DFR dihydroflavonol 4-reductase, FHT flavanon 3 beta-hydroxylase, FGT UDP-glucose:flavonoid 3-O-glucosyltransferase, FLS flavonol synthase, LAR leucoanthocyanidin reductase, PAL phenylalanine ammonia-lyase, RNAPOL II similar to RNA polymerase subunit II, RUBISCO ribulose-1,5-bisphosphate carboxylase/oxygenase activase

<sup>a</sup> Database information used for primer design

<sup>b</sup> Internal standard, used for normalization

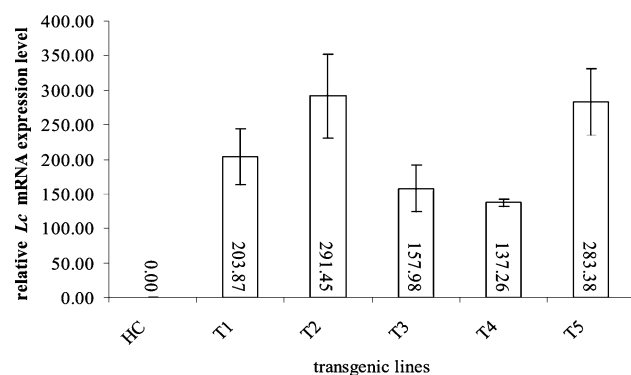


**Fig. 4** Gel electrophoresis of PCR products of *Lc* transgenic apple lines and the untransformed HC control after real-time PCR using *Lc*, *nptII* and *rubisco* specific primers. *w* blank; *HC* untransformed Holsteiner Cox; (T1–T5) *Lc* transgenic lines

Induction of structural gene expression

Phenotypic colour change is in accordance with transcription analyses which confirmed an accompanied increase in transcription levels of most anthocyanin structural genes except *chi* (Fig. 6). Especially expression of anthocyanidin synthase (ANS), the enzyme converting leucoanthocyanidins into anthocyanidins, dramatically increased in *Lc*-transgenic lines. Similar expression pattern changes, especially the unaffected CHI expression, were also found in *Lc/CI* transgenic *L. esculentum* (Bovy et al. 2002). Gene expression of the diverging pathways to epicatechin (ANR) or catechin (LAR) was induced in *Lc* overexpressing *M. domestica* plants, too, but not the FLS responsible for the divergent pathway to the flavonols. The coordinate transcriptional regulation of ANR and LAR1 by a bHLH transcription factor was also found in transgenic *Lotus corniculatus* expressing the maize bHLH *Sn* gene (Paolucci et al. 2007).

can negatively regulate polar auxin transport in vivo (Taylor and Grotewold 2005), disturbances in transport of endogenous auxins might be the reason.



**Fig. 5** *Lc* gene mRNA expression levels of six *Lc* transgenic apple lines (T1–T5) determined by real-time PCR. The values are expressed relative to the level of mRNA transcript levels of the reference genes. Values are the mean of three replicates  $\pm$ SE

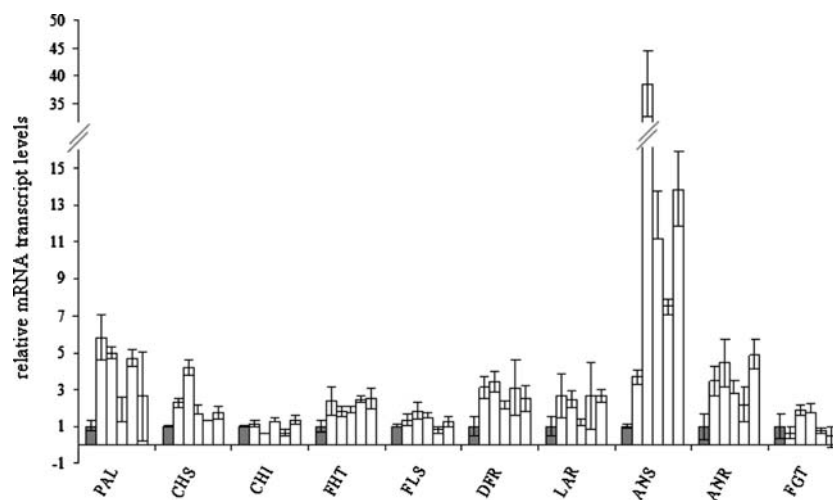
### Metabolites

The *Lc* protein caused pronounced enhancement of accumulation of anthocyanins, of monomeric flavan 3-ols (catechin, epicatechin) and of some polymeric flavan 3-ols (proanthocyanidins). Generally, this corresponds well with the observed changes in transcription levels of structural genes responsible for anthocyanin and flavan 3-ol biosynthesis (Fig. 1, 6). However, the reason for the strongly increased levels of distinct proanthocyanidins (B2 and another unidentified dimer), and catechin as one of their supposed precursors remains unknown. While the level of procyanidin B2 (epicatechin-(4 $\beta$ -8)-epicatechin) was 58-fold increased in the *Lc* transgenic lines the concentration of B5 (epicatechin-(4 $\beta$ -6)-epicatechin) was only sevenfold

higher. From the current data, no obvious correlation of transcript levels with either the stereochemistry of the respective monomers or the types of inter-flavonoid linkage can be seen. The most likely explanation seems the existence of specific condensing enzymes that are differentially regulated and, thus, differentially influenced by the transgenic *Lc* factor. However, only recently, the ANS catalysed dimerization of catechin monomers has been described (Wellmann et al. 2006). Thus, it is also conceivable that ANS, which is highly expressed in the *Lc* transgenic lines, plays an important role in the formation of selected proanthocyanidins.

The concentration of hyperin (quercetin 3-*O*-galactoside) is increased in the transgenic lines, but other quercetin glycosides are reduced and therefore the total amount of flavonols is unchanged. UDP-glycosyl:flavonoid 3-*O*-glycosyltransferases often show broad substrate specificity for anthocyanidins as well as for flavonols (Ford et al. 1998). Therefore, the induced UDP-galactosyl:flavonoid 3-*O*-galactosyltransferase from anthocyanin (idaein) biosynthesis may cause this shift towards galactosylated quercetin at the cost of the other quercetin glycosides. Furthermore, the levels of some hydroxycinnamic acids and some hydroxybenzoic acids are slightly increased by *Lc*.

The enhanced PAL expression, but also the rise in some hydroxycinnamic acids, argues for a rise in *p*-coumaroyl-CoA substrate supply for flavonoid biosynthesis. The alternative explanation, a drain of substrates from the major pool of dihydrochalcones can hardly be tested for, as the levels of dihydrochalcones in the *Lc* lines are almost unaffected. With respect to the enormous concentrations of the dihydrochalcones, their biological variation would hide



**Fig. 6** mRNA expression levels of genes involved in flavonoid biosynthetic pathway in *Lc* transgenic apple lines (T1–T5, white bars from left to right) determined by real-time PCR. The values are expressed relative to the level of transcripts in the untransformed Holsteiner Cox (dark grey) set to be one. Values are the mean of three replicates  $\pm$ SE.

*PAL* phenylalanine ammonia lyase, *CHS* chalcone synthase, *CHI* chalcone isomerase, *FHT* flavanon 3- $\beta$ -hydroxylase, *FLS* flavonol synthase, *DFR* dihydroflavonol 4-reductase, *LAR* leucoanthocyanidin reductase, *ANS* anthocyanidin synthase, *ANR* anthocyanidin reductase, *FGT*, UDP-glucose:flavonoid 3-*O*-glucosyltransferase



**Table 2** Concentrations of phenolic compounds in leaves of untransformed (HC) and transgenic (T1–T5) in vitro apple lines

Compound	HPLC (mg g <sup>-1</sup> of dry weight)					
	HC	T1	T2	T3	T4	T6
Anthocyanins						
Idaein	Below detection limit	0.99	0.52	0.20	0.35	0.46
Monomeric flavan 3-ols						
Total	0.10	4.23	4.61	4.51	2.89	3.87
Procyanidins						
Derivatives of catechin, total	0.05	0.63	0.39	0.47	0.41	0.46
Derivatives of epicatechin, total	1.28	2.23	2.30	2.40	2.25	2.35
Flavonols						
Total	6.26	8.24	9.86	6.94	8.35	7.41
Dihydrochalcones						
Total	95.07	106.99	78.42	88.26	104.10	121.32
Cinnamic acid deriv.						
Total	0.43	1.72	0.84	1.41	1.05	1.54
Other phenolic compounds						
Total	0.54	0.67	1.27	1.19	0.51	0.70

Values represent the mean of two biological replicates

what can be expected for a substrate drain explaining the observed rises in anthocyanins and flavan 3-ols. Nevertheless, this substrate drain may also take place. It appears that dihydrochalcones synthesizing enzymes are not induced by *Lc*, this could indicate a generally different regulation for them in comparison to the regulation of flavonoid structural genes. The closely related pear (*Pyrus communis* L.), which is also a member of the *Maloidae*, does not contain dihydrochalcones (Chalice and Williams 1968; Andreotti et al. 2006). This hints to a phylogenetically young pathway in *M. domestica*.

#### Mode of transcription factor action

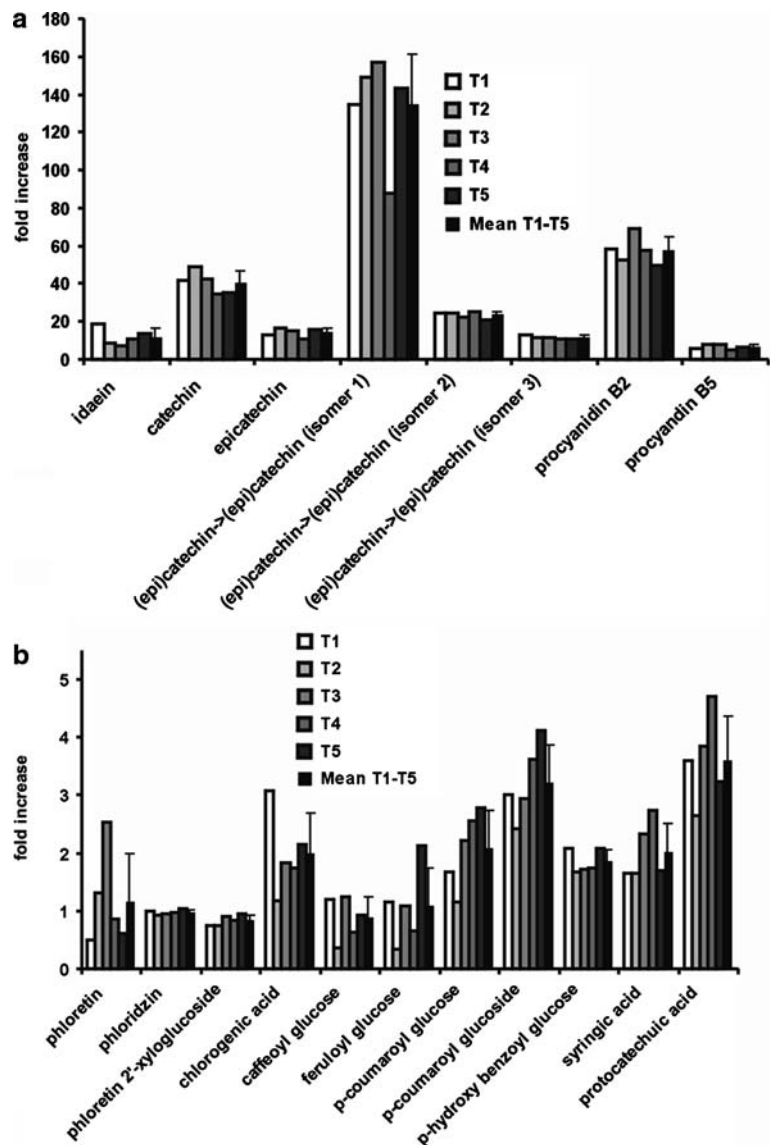
In maize and many other plants, the presence of both *myb* and bHLH (*myc*) type transcription factors is required for anthocyanin expression (e.g. Grotewold 2005; Heim et al. 2003). In HC apple, constitutive *Lc* expression was sufficient to strongly enhance anthocyanin, catechins and specific proanthocyanidin accumulation, making it likely that interacting *myb*-type transcription factors are expressed in the cultivar under the described experimental conditions. Recently, the light induced transcription factors *MdMYB1* and *MdMYB10*, determining apple fruit colour, were described (Espley et al. 2006; Takos et al. 2006). In *MdMYB10* a signature motif for the interaction with bHLH proteins was identified (Espley et al. 2006). It may be expected that the *Lc* protein interacts with or even induces *myb*-type transcription factors, which leads to the observed induction of anthocyanins and other phenylpropanoids. Further work is required to confirm this.

#### Perspective

In this study we have shown that heterologous expression of maize *Lc* enhanced flavonoid biosynthesis in leaf tissues of apple. Whether the effect will also be true for the fruit appears to be strongly influenced by and depending on expression patterns of other transcription factors and can only be determined when plants have fruited.

Flavonoids have become targets for genetic plant modification to modify flower colour and to improve forage traits, crop resistance and healthiness of the human diet. Especially flavan-3-ols and their derivatives have significant health promoting effects and show even pharmacological activities. The role of flavonoids in pathogen resistance is well documented (Jambunathan et al. 1990; Byrne et al. 1996; Römmelt et al. 1999, 2003b; Halbwirth et al. 2003). Studies on apple provided evidence for the significance of flavonoids in resistance to apple scab (*V. inaequalis*). Flavan 3-ols accumulate at the infection site (Treutter and Feucht 1990) and their concentration is associated with field resistance (Mayr et al. 1997). Inhibition of PAL, catalysing the first step of the phenylpropanoid pathway and supplying the precursors for flavonoid biosynthesis, led to severe apple scab symptoms on leaves of a resistant cultivar (Mayr et al. 1997), whilst vice versa enhanced biosynthesis improved resistance (Römmelt et al. 1999; Bazzi et al. 2003; Leser and Treutter 2005). Flavonoids also play a major role in post-harvest resistance of fruits and vegetables (Lattanzio et al. 1994; Lattanzio 2003). Based on these studies it can be assumed, that *Lc*-transgenic apple plants may exhibit increased pathogen resistance.

**Fig. 7 a, b** *Lc*-dependent increase in the levels of flavonoids (**a**) and other phenolic secondary metabolites (**b**) determined by LC-MS. Values are expressed as fold induction of metabolite levels in transgenic lines relative to metabolite levels in the untransformed controls. The *black bar* represents the mean  $\pm$  SD of values obtained from all transgenic lines (T1–T5)



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