

RESEARCH PAPER

Transcriptional analysis of apple fruit proanthocyanidin biosynthesis

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Abstract

Proanthocyanidins (PAs) are products of the flavonoid pathway, which also leads to the production of anthocyanins and flavonols. Many flavonoids have antioxidant properties and may have beneficial effects for human health. PAs are found in the seeds and fruits of many plants. In apple fruit (*Malus × domestica* Borkh.), the flavonoid biosynthetic pathway is most active in the skin, with the flavan-3-ols, catechin, and epicatechin acting as the initiating units for the synthesis of PA polymers. This study examined the genes involved in the production of PAs in three apple cultivars: two heritage apple cultivars, Hetlina and Devonshire Quarrenden, and a commercial cultivar, Royal Gala. HPLC analysis shows that tree-ripe fruit from Hetlina and Devonshire Quarrenden had a higher phenolic content than Royal Gala. Epicatechin and catechin biosynthesis is under the control of the biosynthetic enzymes anthocyanidin reductase (ANR) and leucoanthocyanidin reductase (LAR1), respectively. Counter-intuitively, real-time quantitative PCR analysis showed that the expression levels of Royal Gala *LAR1* and *ANR* were significantly higher than those of both Devonshire Quarrenden and Hetlina. This suggests that a compensatory feedback mechanism may be active, whereby low concentrations of PAs may induce higher expression of gene transcripts. Further investigation is required into the regulation of these key enzymes in apple.

Key words: anthocyanidin reductase, apple, catechin, epicatechin, flavonoid, leucoanthocyanidin reductase, proanthocyanidin biosynthesis, regulation, tannin.

Introduction

Proanthocyanidins (PAs) are products of the flavonoid pathway, which also leads to the production of anthocyanins and flavonols. These three classes of secondary metabolites are called flavonoids and are synthesized from common precursor compounds. PAs (also known as condensed tannins) are produced from the condensation of flavan-3-ol units that accumulate in the vacuoles of plants (Dixon *et al.*, 2005; Lepiniec *et al.*, 2006). They are found in the bark of trees, leaves of tea and forage plants, and the seeds and fruits of many plants including grapes, apples, kiwifruit, cranberries, and persimmon. Anthocyanins, PAs, and

flavonols are all antioxidants and may have beneficial effects for human health when incorporated into the diet (Wolfe *et al.*, 2003; Tsao *et al.*, 2005; Stevenson and Hurst, 2007; Aron and Kennedy, 2008). PAs also impart astringency to fresh fruits, fruit juices, and wine, oxidize to form brown pigments in seeds and other tissues, and may act as feeding deterrents in reproductive tissues and developing fruits (Wrangham *et al.*, 1998; Forkner *et al.*, 2004). Apples provide a diet high in polyphenolics and flavonoids, and therefore represent a major source of dietary antioxidants (Vinson *et al.*, 2001; Lee *et al.*, 2003). Apples contain

Abbreviations: ANOVA, analysis of variance; ANR, anthocyanidin reductase; DAD, diode array detector; DAFB, days after full bloom; DFR, dihydroflavonol reductase; LAR, leucoanthocyanidin reductase; LC-MS, liquid chromatography/mass spectrometry; PA, proanthocyanidin; qPCR, real-time quantitative PCR.

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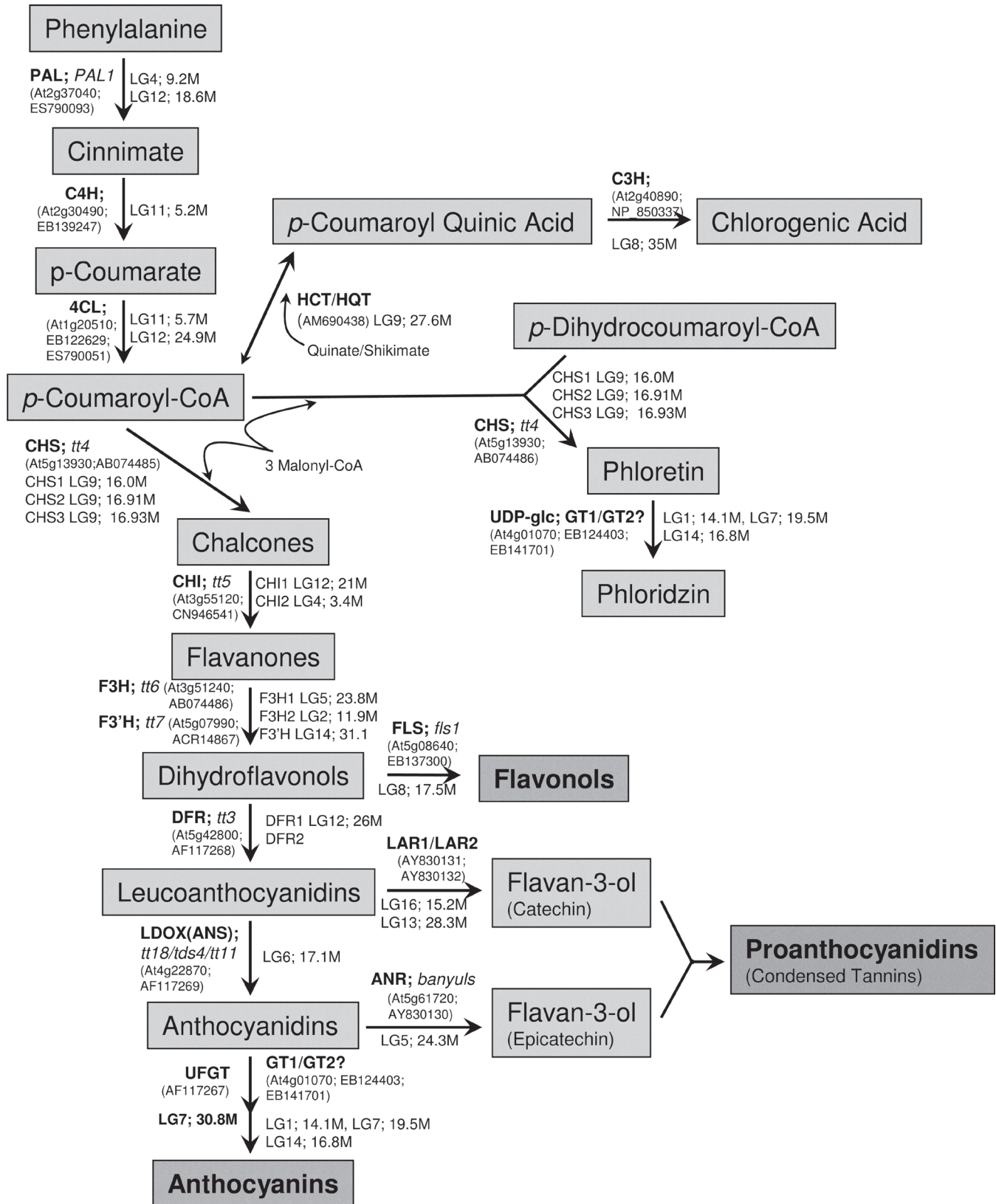


Fig. 1. Scheme of the apple polyphenolic pathway, showing structural genes involved in flavonoid biosynthesis in *Arabidopsis thaliana* seed and *Malus × domestica* fruit, annotated in bold with the TAIR locus and GenBank accession numbers, and their predicted linkage group in the apple reference genome, with approximate position (to the nearest 100kb). PAL, phenylalanine ammonia lyase; C4H, cinnamate-4-hydroxymate; 4CL, 4-coumarate:coenzyme A ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase, F3'H, flavanone 3'-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; UFGT, UDP-glucose flavonoid 3-O-glucosyl transferase; FLS, flavonol synthase; LAR1/2, leucoanthocyanidin reductase; ANR, anthocyanidin reductase; GT1/2, glycosyltransferases; HQT/HCT, quinate hydroxycinnamoyl/hydroxycinnamoyl CoA shikimate; C3H, *p*-coumarate 3-hydroxylase.

a large number of different types of polyphenolics, and the concentrations of these compounds within the apple vary among cultivars, growing conditions, and location within the tree (van der Sluis *et al.*, 2001; McGhie *et al.*, 2005; Tsao *et al.*, 2005).

PA biosynthesis results from a pathway controlled by genes encoding enzymes involved in the formation of the biochemical structure of the compound and by regulatory genes that control gene expression of these enzymes (Lepiniec *et al.*, 2006). The biochemical functions of these enzymes have been well established in some plant species (Holton and Cornish, 1995), and significant progress has been made in this area (Bogs *et al.*, 2005; Takos *et al.*, 2006c; Almeida *et al.*, 2007; Ikegami *et al.*, 2007). For many years, PA biosynthesis was assumed to branch from the anthocyanin pathway at the level of leucoanthocyanidin (Fig. 1). Most models of PA biosynthesis maintain that the extension units arise by condensation of molecular units derived from leucoanthocyanidin. However, this model fails in one important aspect: the stereochemistry of leucoanthocyanidin is 2,3-*trans* when formed by the proposed pathway, whereas in most cases the major extension unit is 2,3-*cis* (Xie and Dixon, 2005).

A possible solution to this stereochemical paradox was the discovery of the *BANYULS* (*BAN*) gene in *Arabidopsis thaliana*. Mutations in *BAN* result in a transparent testa associated with a lack of PAs in the seed coat (Devic *et al.*, 1999). Based on the phenotype and the protein sequence similarity of *BAN* to dihydroflavonol reductase (DFR; catalyses production of leucoanthocyanidins from dihydroflavanols), it was suggested that *BAN* encodes leucoanthocyanidin reductase (*LAR*) and therefore converts flavan-3,4-diols to the corresponding 2,3-*trans*-flavan-3-ols. However, expression of recombinant *BAN* proteins from *A. thaliana* and *Medicago truncatula* in *Escherichia coli* failed to produce *in vitro* *LAR* activity. Instead, *BAN* genes were shown to encode a new enzyme, anthocyanidin reductase (*ANR*), which converts anthocyanidins to 2,3-*cis*-flavan-3-ols (Xie *et al.*, 2003).

This discovery of the *BAN* gene highlighted two possible branch pathways for the formation of PAs in plant cells: (i) *LAR* synthesizing 2,3-*trans*-flavan-3-ol from leucoanthocyanidins (Tanner *et al.*, 2003); and (ii) *ANR*, encoded by the *BANYULS* gene (Devic *et al.*, 1999; Xie *et al.*, 2003), synthesizing 2,3-*cis*-flavan-3-ol from anthocyanidins (Fig. 1). PA biosynthesis in *Arabidopsis* is exclusively epicatechin (2,3-*cis*-flavan-3-ol)-based and limited to the seed coat, while many other plants produce both epicatechin and catechin (2,3-*trans*-flavan-3-ol)-based PAs of various amounts and compositions and in a range of different tissues (Dixon *et al.*, 2005). *Arabidopsis* lacks a functional *LAR* gene and it is not known what regulates *LAR* expression in other plants (Bogs *et al.*, 2007). In apple fruit (*Malus × domestica* Borkh.), the flavonoid biosynthetic pathway is most active in the skin (Lister *et al.*, 1994) with the *trans*- and *cis*-flavan-3-ols being catechin and epicatechin, respectively, acting as the initiating units for the synthesis of PA polymers. In apple (Takos *et al.*, 2006c), the *MdLAR1*, *MdLAR2*, and *MdANR* genes were cloned from the Cripps Red variety and real-time PCR carried out to correlate steady-state transcript levels with flavonoid accumulation (Takos *et al.*, 2006c). The results from this study indicated that transcription of these PA synthesis genes in apple skin was

differentially regulated from transcription of other flavonoid pathway genes.

Recent studies have elucidated the transcriptional regulation of anthocyanin accumulation in apple skin and flesh (Takos *et al.*, 2006a; Ban *et al.*, 2007; Espley *et al.*, 2007, 2009). Several of the flavonoid biosynthesis pathway genes involved in anthocyanin synthesis and accumulation are also required for the synthesis of PAs and flavonols (Fig. 1). However, the exact processes involved are not fully understood. The regulation of PA biosynthesis and accumulation has also been described in persimmon (Ikegami *et al.*, 2007; Akagi *et al.*, 2009a,b; Ikegami *et al.*, 2009) and grape (Czemmel *et al.*, 2009; Gagne *et al.*, 2009; Terrier *et al.*, 2009) fruit. Recently an apple orthologue of *Arabidopsis* TRANSPARENT TESTA GLABRA1 (*TTG1*), involved in production of seed coat PA-based colour, was shown to activate the *AtBAN* promoter (Brueggemann *et al.*, 2010). While these studies contribute to a global picture of flavonoid regulation, the specific transcriptional regulation of PAs in apple fruit is still not well understood.

In this study, we compared three apple cultivars: two heritage cultivars, Hetlina and Devonshire Quarrenden with high PA concentrations, and a commercial cultivar, Royal Gala, with a low PA concentration. To understand better the genes involved in the regulation of PAs in the flesh and skin of these cultivars, we isolated cDNA over a developmental period and compared the expression of flavonoid biosynthesis pathway structural genes by real-time quantitative PCR (qPCR). HPLC analysis provided an insight into the accumulation of PAs and other polyphenolics in these different apple cultivars. The results suggested that the regulation of gene expression of the apple PA pathway in fruit is well controlled, in a tissue- and developmentally specific fashion. However, comparisons of transcript levels between cultivars do not explain differences in final PA concentration or the magnitude of differences between tissues. Future study is required on the possible mechanism controlling final PA concentration in apple.

Material and methods

Tissue collection

Developing apple fruit from three cultivars were collected at five times throughout the 2007–2008 growing season. Because of differing flowering dates and maturity rates, each cultivar was sampled at five stages based on known developmental indices: 37, 65, 72, 100, and 128 days after full bloom (DAFB, tree ripe—as assessed by starch index and internal ethylene levels) for Hetlina; 30, 37, 65, 72, and 114 DAFB (tree ripe) for Devonshire Quarrenden; and 37, 65, 100, 128, and 135 DAFB (tree ripe) for Royal Gala. These fruit were sampled from trees at the Plant & Food Research orchard in Havelock North, Hawke's Bay, New Zealand. Twelve fruit were collected for each cultivar, from two different trees (Royal Gala and Hetlina) or one tree (Devonshire Quarrenden). For each apple, the skin, excised from the cortex, and the skin-free cortex were stored separately, frozen immediately in liquid nitrogen and stored at –80 °C. Confirmatory samples of stage 5 (tree ripe) apple fruit from the three cultivars were also taken during the 2009–2010 growing season.

Real-time qPCR expression analysis

RNA was isolated from the tissue samples collected (see above) using a method adapted from that previously described by Chang *et al.* (1993). The RNA was treated with DNase I using an Ambion DNA-free™ kit. After DNase I treatment, cDNA synthesis was carried out on 2 µg of each RNA sample using anchored-oligo(dT)₁₈ primers and random

hexamer primers following the protocol set out in the Roche Transcriptor First Strand cDNA synthesis kit.

Genes encoding apple flavonoid biosynthesis pathway enzymes and branch points were identified by best BLAST homology in the Plant & Food Research EST database (BioPipe and BioView – automated cDNA sequence annotation pipeline and viewer system © Dr Ross Crowhurst and The New Zealand Institute for Plant & Food Research Ltd) and in the apple genome (Velasco *et al.*, 2010). Where a location in the genome was apparent, with no contradiction between haplotypes, linkage group, and approximate position (to the nearest 100 kb), this is indicated on Fig. 1. Where putative gene family members existed, candidates were selected by abundance in fruit library tissues. Gene-specific primers corresponding to these genes were designed using Vector NTI version 9.0.0 (<http://www.invitrogen.com>) to a stringent set of criteria, enabling application of universal reaction conditions. The sequences of each primer pair and the relevant accession numbers are shown in Supplementary Table S1 at JXB online.

qPCR DNA amplification and analysis was carried out using a LightCycler[®] 480 Real-Time PCR System (Roche Diagnostics). All reactions were performed using the LightCycler[®] 480 SYBR Green I Master Mix (Roche Diagnostics) according to the procedure described by the manufacturer. Reactions were performed four times using 2.5 µl Master Mix, 0.25 µl each primer (10 µM), 1.25 µl diluted cDNA (1:50) and nuclease-free water (Roche Diagnostics) to a final volume of 5 µl. A negative water control was included in each run. Fluorescence was measured at the end of each annealing step. Amplification was followed by melting curve analysis with continual fluorescence data acquisition during the 65–95 °C melt. For each gene, a standard curve was generated using a cDNA serial dilution, and the resultant PCR efficiency calculations (ranging between 1.443 and 2.00) were imported into the relative expression data analysis. Relative expression levels were quantified using a developed quantification method (Andre *et al.*, 2009). Analysis of the raw data for this study used a single reference gene and gene-specific amplification efficiencies. *Malus × domestica Actin* (*MdActin*, GenBank accession number CN938023) was selected as the reference gene because of its consistent transcript level throughout fruit tissues and leaves. For each gene, a standard curve was generated using a cDNA serial dilution, and the resultant PCR efficiency (E) values (ranging between 1.443 and 2.00) were imported into the relative expression data analysis. For each biological sample, the relative quantity (RQ) of each target gene was determined by calculating the difference in quantification cycle value (Cq) between the average Cp value for each sample (from four technical replicates) and a calibrator value (i.e. a fixed Cp value was used for all samples; the minimum Cp value over the entire experiment).

$$RQ = E^{\Delta Cq}$$

$$\text{where } \Delta Cq = Cq_{\text{calibrator}} - Cq_{\text{target}}$$

The RQs of the target genes were normalized to the relative quantities of the reference gene *MdActin* to give normalized RQs (NRQs):

$$NRQ = RQ_{\text{target}}/RQ_{\text{actin}}$$

NRQ values were further rescaled to the sample with the lowest RQ over the entire experiment:

$$\text{Rescaled NRQ} = NRQ_{\text{target}}/NRQ_{\text{lowest}}$$

Error bars shown in the qPCR data are technical replicates, representing the standard deviation (SD) of four replicate qPCRs. These rescaled normalized relative quantities were used to compare the expression levels of the structural genes in the flavonoid biosynthetic pathway among the three apple cultivars over a developmental series.

Extraction and Identification of polypropanoids

Tissue samples from the 12 fruit collected for each cultivar, Hetlina, Devonshire Quarrenden, and Royal Gala, were used for extraction and identification of polypropanoids by HPLC.

For the total phenolics and flavan-3-ols developmental series analysis (Fig. 2), frozen samples of the skin and cortex from each cultivar at each developmental time from the 2007/2008 growing season were freeze dried, ground to powder under low light conditions, and the polyphenols extracted using absolute ethanol:water:formic acid (80:20:1, v/v/v) extraction buffer at a 5:1 buffer:sample ratio. The extraction mixture was homogenized using a vortex for 30 s and then incubated at 4 °C for 24 h. After centrifugation at 3000 g for 10 min, the supernatant was collected and stored at –20 °C. These extractions represented triplicate technical replicates of each tissue sample.

For mature fruit analysis (Fig. 3), frozen samples of the skin (0.5 g) and cortex (2 g) from each cultivar at the final tree-ripe stage 5 time point, from both the 2007/2008 and the 2009/2010 growing seasons, were ground under liquid nitrogen and the polyphenols extracted using 10 ml absolute ethanol:water (80:20, v/v) extraction buffer. The extraction mixture was homogenized using a vortex for 30 s and then shaken for 2 h at room temperature. After centrifugation at 6000 g for 15 min, the supernatant was collected and dried by evaporation using a Centriprep. The resulting pellet containing polyphenols was resuspended in 1 ml water:absolute ethanol (50:50, v/v), filtered through a 0.45 µm filter, and stored at –20 °C. These extractions represented triplicate technical replicates of the samples analysed.

HPLC analysis

For total phenolics and flavan-3-ols developmental series HPLC (Fig. 2), the HPLC system used to measure polyphenolics in sample extracts was a Waters Alliance 2690 with a Waters 996 photodiode array and Waters 474 fluorescence detectors (Waters, Milford, MA). The analytical column was a Zorbax SB-C18 (150 × 4.6 mm, 1.8 µm; Agilent, Melbourne, Australia) maintained at 40 °C. The injection volume was 5 µl. A gradient elution was performed with solvent A (5% formic acid in water) and solvent B (acetonitrile) at a flow rate of 0.8 ml min⁻¹. The solvent programme was as follows: 0–9 min, linear gradient from 0 to 20% solvent B; 10–18 min, linear gradient from 20 to 80% solvent B; 19–20 min, 80% solvent B isocratic; 21–22 min, linear gradient from 80 to 0% solvent B in order to return to the initial conditions before injecting another sample at 24 min. Spectral data were collected for the entire run, and the polyphenolic components were quantified by extracting chromatograms at 280, 370, and 530 nm. Quercetin, phloridzin, and phloridzin-xyloside were quantified at 280 nm, quercetin glycosides and chlorogenic acid at 370 nm, and cyanidin glycosides at 530 nm. Catechin, epicatechin, and procyanidins were quantified using fluorescence detection with excitation at 276 nm and emission at 316 nm. Chromatographic data were collected and manipulated using the Chromeleon[®] Chromatography Management System version 6.8. The polyphenolic standards quercetin 3-*O*-rutinoside, quercetin 3-*O*-galactoside, quercetin 3-*O*-glucoside, quercetin 3-*O*-rhamnoside, cyanidin 3-*O*-glucoside, and procyanidin B2 were purchased from Extrasynthese (Genay, France). Epicatechin, catechin, chlorogenic acid, phloridzin, and quercetin were purchased from Sigma (Sydney, Australia). Total phenolics values were calculated by totalling individual detection data for catechin, epicatechin, procyanidin B2, chlorogenic acid, anthocyanin, quercetin derivatives (flavonols), and phloridzin. Error bars shown are technical replicates, representing the standard error (SE) of three replicate HPLC extractions.

Mature fruit HPLC identification of the polyphenolic compounds (Fig. 3) was performed using a Ultimate 3000 system (Dionex, Sunnyvale, CA) equipped with a diode array detector (DAD). A 5 µl aliquot was injected onto a Dionex C18 Acclaim PolarAdvantage II column (150 × 2.1 mm internal diameter, 3 µm particle size). The mobile phases were water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). The flow rate was 0.35 ml min⁻¹, and the column temperature was 35 °C. The 42 min gradient was as follows: 0–5 min, 0–8% B; 6–10 min, 8–15% B; 11–20 min, 15–20% B; 21–27 min, 20% B linear; 28–34 min, 27–100% B; 35–36 min, 100% B linear; 37–42 min, 0% B, re-equilibration time. Simultaneous monitoring was set at 254, 280, and 320 nm, and at 520 nm for quantification. Polyphenol compounds were identified by their retention time and spectral data compared with standards and were

quantified using five-point calibration curves. High-UV-absorbing peaks were first putatively identified by liquid chromatography/mass spectrometry (LC-MS) from their mass spectra and from data in previous literature (Alonso-Salces *et al.*, 2004; Volz and McGhie, 2011). These assignments were confirmed by HPLC-DAD comparison of their UV spectra and retention times with those of authentic standards when available. Flavonols, hydroxycinnamic acids, and anthocyanins were quantified as rutin, chlorogenic acid, and cyanidin-3-galactoside equivalents, respectively. Oligomeric (up to hexamer) and polymeric procyanidins were determined according to their mass spectra and data from previous studies (Guyot *et al.*, 1998; Hamauzu *et al.*, 2005) and were quantified as catechin equivalents. Error bars shown in the HPLC data are technical replicates, representing the SE of three replicate HPLC extractions.

Mass spectrometry

Identification of the main phenolic compounds was performed by LC-MS using a LCQ Deca ion trap mass spectrometer fitted with an electrospray ionization interface (ThermoQuest, Finnigan, San Jose, CA) and coupled to a Surveyor™ HPLC instrument. Column and elution conditions were as described above for the HPLC-DAD analysis. Spectra were recorded in positive ion mode between 100 and 2000 atomic mass units.

Statistical analysis of polyphenolic content

The statistical significance of variations in total polyphenolic compound content between cultivars was determined by one-way analysis of variance (ANOVA) of data (significant at $P = 0.00001$), followed by multiple comparisons using Tukey's test for least-squared-difference (LSD at $P = 0.05$).

Phylogeny and sequence alignment

Protein consensus sequences from the three apple cultivars (Hetlina, Devonshire Quarrenden and Royal Gala) were aligned with published protein sequences of reductases and a phylogenetic tree was generated using Geneious Pro 4.8.5 Tree Builder (Drummond *et al.*, 2010). The tree alignment options were: cost matrix = Blosum62, gap opening penalty = 10, gap extension penalty = 0.1, and alignment type = global alignment with free end gaps. The tree builder options were: genetic distance model = Jules-Cantor, tree build method = neighbour joining, and no outgroups.

Protein consensus sequences from the three apple cultivars were determined from the coding sequences of *ANR*, *LAR1*, and *LAR2* from each cultivar and aligned with translated apple genome reference sequences (Velasco *et al.*, 2010) and published protein sequences of *ANR*, *LAR1*, and *LAR2* (Takos *et al.*, 2006c) using Geneious Pro 4.8.5 Alignment (Drummond *et al.*, 2010). The alignment options were: cost matrix = Blosum62, gap opening penalty = 10, gap extension penalty = 0.1, and alignment type = global alignment with free end gaps.

Results

HPLC analysis reveals polyphenolic diversity between the three apple cultivars

Three apple cultivars were chosen for this study based on polyphenolic compound concentrations identified in heritage and commercially grown apples (Volz and McGhie, 2011). The two heritage apple cultivars, Hetlina and Devonshire Quarrenden, have high polyphenolic concentrations, while the commercial cultivar Royal Gala has low polyphenolic concentrations (Volz and McGhie, 2011). A full developmental series of fruit tissue was collected for the three cultivars over the 2007/2008 growing season (Fig. 2A). As flavonoid concentrations are affected by environmental cues

(Lin-Wang *et al.*, 2011), the results were validated in a second season (2009/2010). Stages 1–5 represent five time points within the apple growing season that allowed full coverage of the season. The full developmental series was used for extraction for HPLC analysis of phenolic compound composition. When total phenolic composition was examined per gram of fresh weight, it was apparent that apple skin consistently contained higher amounts of polyphenolic compounds than apple cortex across the whole growing season (Fig. 2B). When only catechin and epicatechin (total flavan-3-ols) were examined (Fig. 2C) a similar trend was observed as for total phenolics (Fig. 2B) with a decline in the total amount over the growing season. When the proportions of each compounds' contribution to the total phenolic profile were examined for both cortex and skin (Supplementary Fig. S1 in *JXB* online), the cortex was shown to contain predominantly chlorogenic acid with varying amounts of catechin, epicatechin, and procyanidin B2 across all three cultivars studied. Royal Gala showed the highest proportions of catechin, epicatechin, and procyanidin B2 across the growing season with Hetlina and Devonshire Quarrenden showing proportions similar to each other. The skin composition showed a significant decrease in the proportion of chlorogenic acid and an increase in flavonols and phloridzin. Royal Gala showed the highest catechin, epicatechin, and procyanidin B2 proportions of the three cultivars. Leaf tissue proportions were also calculated for Hetlina, Devonshire Quarrenden, and Royal Gala showing 85, 86, and 91% phloridzin content and 14, 13 and 8% flavonol content, respectively, with the other compounds studied making up the remainder (data not shown).

The phenolic composition of mature (stage 5) apples from the three cultivars was examined using an alternative HPLC protocol (Fig. 3 and Supplementary Table S2 at *JXB* online). For every compound, the phenolic amounts were higher in the apple skin than in the cortex, on a per gram of fresh weight basis. Apples make both catechin and epicatechin as primary subunits of procyanidin dimers and resulting oligomers and polymers. Catechin and procyanidin B1 (catechin dimer) were seen in all three cultivars, with the highest amounts in Devonshire Quarrenden. Amounts of epicatechin and procyanidin B2 (epicatechin dimer) were highest in Hetlina skin. The procyanidin B2 concentrations were approximately half that of epicatechin in Devonshire Quarrenden and Royal Gala but not in Hetlina, where they were similar in concentration. This could be an indication of a 'block' in the pathway leading to dimerization for the Devonshire Quarrenden and Royal Gala cultivars. Hetlina also had significantly higher total procyanidin oligomer and polymer concentrations than Devonshire Quarrenden and Royal Gala. Total quercetin derivatives (Fig. 1, flavonol branch) showed very high levels in the skin, with Hetlina the highest and Royal Gala the lowest, with no detectable amounts in the cortex in all three cultivars. Anthocyanins showed a similar pattern to flavonols, found in the skin of all three cultivars and not detected in the cortex, with Royal Gala the highest and Hetlina the lowest.

RNA expression profile of the phenylpropanoid pathway reveals tissue and cultivar differences

With the recent publication of the apple genome sequence (Velasco *et al.*, 2010), we were able to use the best BLAST

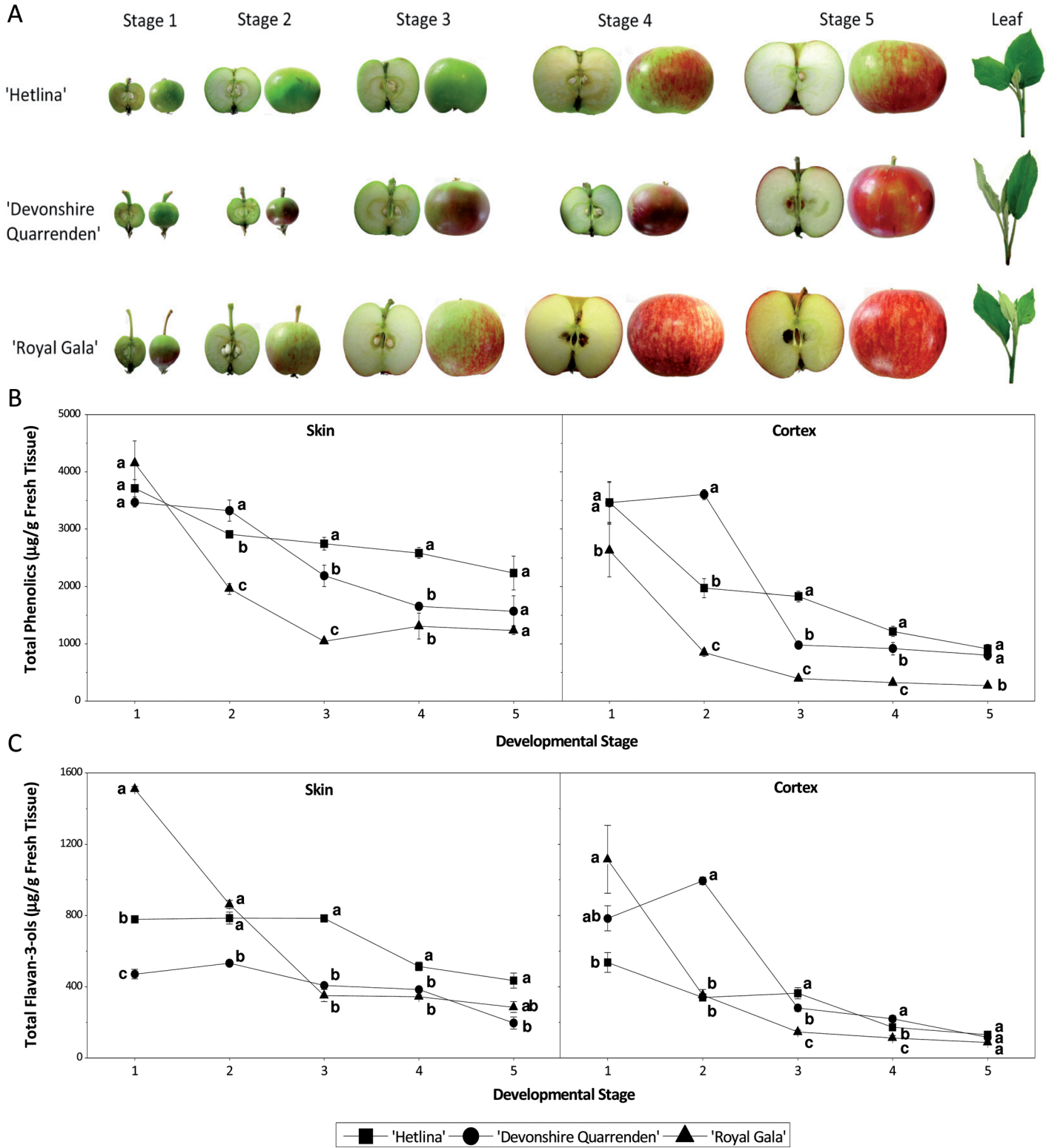


Fig. 2. (A) Apple fruit developmental series. Developing Hetlina, Devonshire Quarrenden, and Royal Gala apple fruit were collected at five time points throughout the 2007/2008 growing season. (B) HPLC data of apple skin and cortex for total polyphenolic composition for Hetlina, Devonshire Quarrenden, and Royal Gala over a developmental series. (C) HPLC data of apple skin and cortex for total flavan-3-ols (catechin and epicatechin) for Hetlina, Devonshire Quarrenden, and Royal Gala over a developmental series. Error bars show SE. Data points with the same letter are not significantly different at $P = 0.05$, using one-way ANOVA analysis, followed by a multiple-comparison t -test.

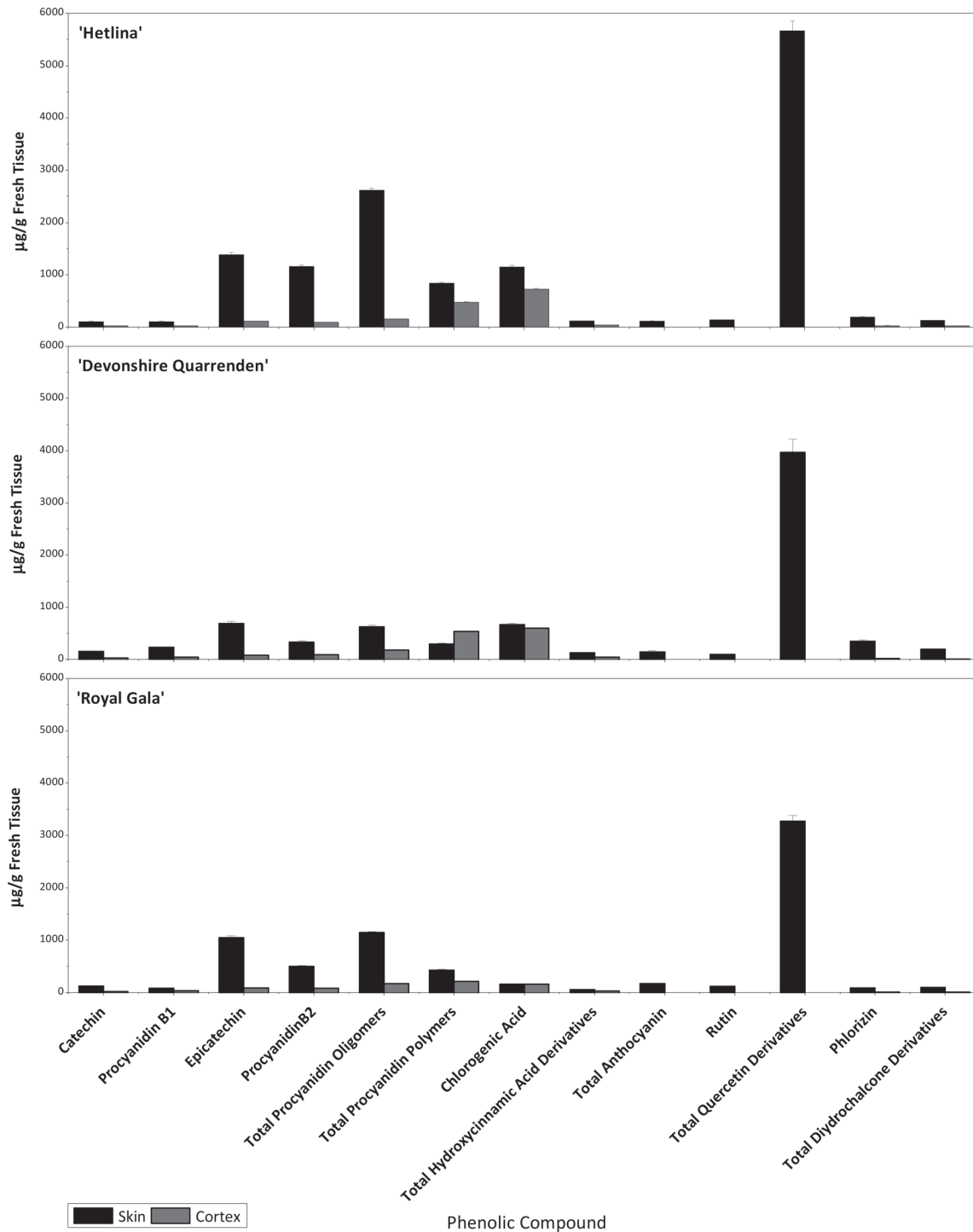


Fig. 3. HPLC analysis for identification of the polyphenolic compounds in tree-ripe (stage 5) apple skin and cortex from Hetlina, Devonshire Quarrenden, and Royal Gala cultivars. Error bars show SE.

match to known *Arabidopsis* genes to add several potential new steps to the phenylpropanoid pathway (Fig. 1). For example, good matches to gene family members for *CHS* and *CHI* were identified. In addition, linkage group information is shown in Fig. 1, which agrees with recent mapping and reverse genetics of apple phenylpropanoid quantitative trait loci (Chagne *et al.*, 2012).

Gene expression analysis of apple skin (Fig. 4) revealed some surprising observations. Royal Gala showed the highest

expression of the core phenylpropanoid biosynthetic genes *PAL*, *C4H*, *CHS*, *CHI*, *F3H*, *DFR*, and *ANS*. Genes encoding the synthesis of chlorogenic acid and phloretin/phloridzin have not yet fully been characterized. However, expression levels of the published *LARI* and *ANR* genes (Takos *et al.*, 2006c) were also highest in Royal Gala skin. *FLS* expression was highest in Devonshire Quarrenden tree-ripe fruit but was still not as high as in leaf. A lower expression level of *FLS* in Hetlina could direct more substrate down the phenylpropanoid pathway towards

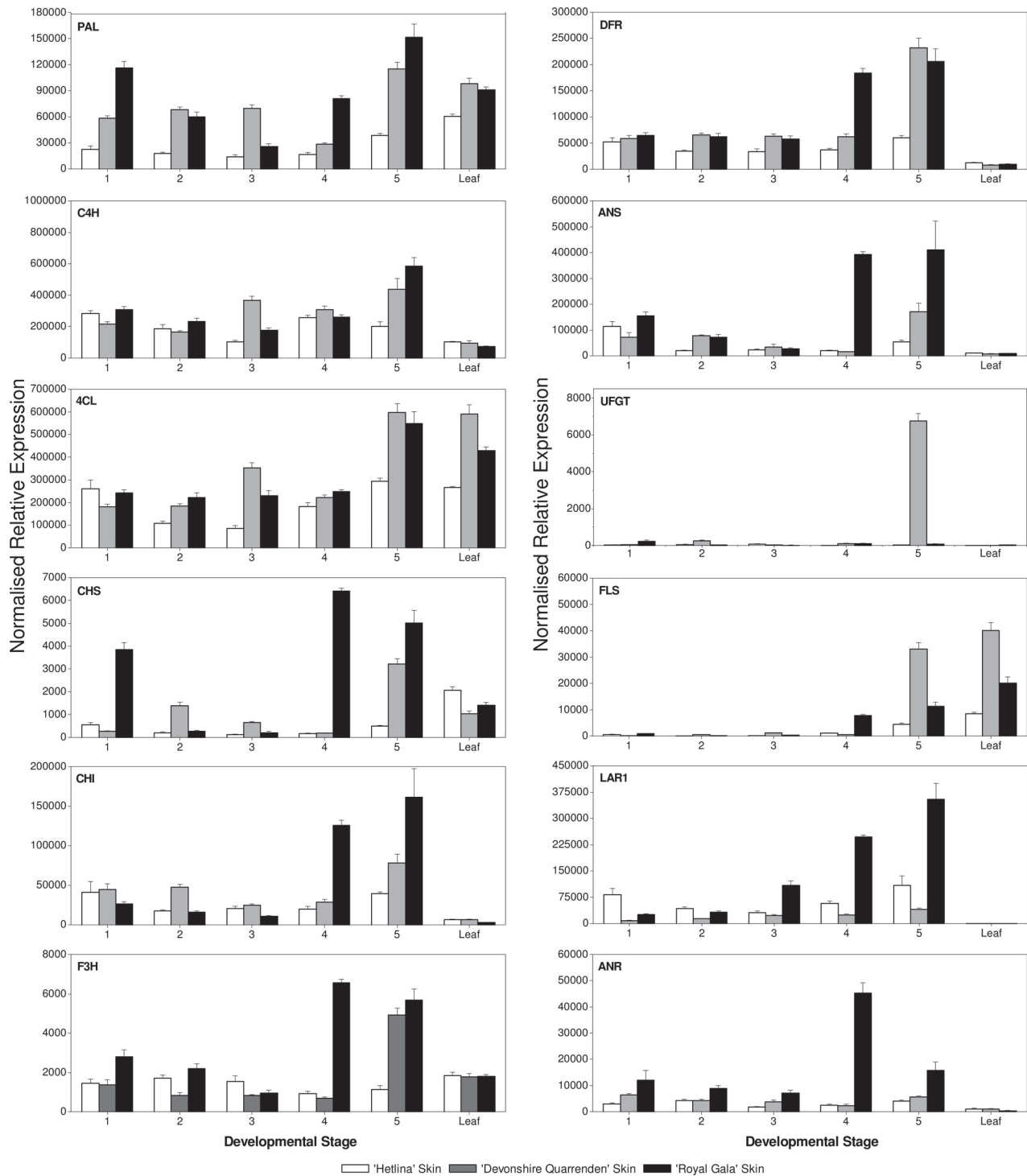


Fig. 4. Real-time qPCR expression analysis for all major steps of the polyphenolic biosynthetic pathway in apple skin from Hetlina, Devonshire Quarrenden, and Royal Gala cultivars. *MdActin* used as the reference gene. Error bars show SD.

anthocyanin and PAs. However, Hetlina also had the highest concentration of quercetin derivatives (Fig. 3). Expression analysis revealed that certain genes were expressed in a highly fruit-specific manner. All the assayed genes showed high expression values (normalized to *MdActin*) with good fold induction during fruit development. In general, transcript levels increased in the skin with fruit development, even though total skin phenolics declined on a fresh weight basis (Fig. 2B).

As apple flesh is by far the bulk of the tissue eaten by the consumer, the importance of phenolics in this tissue and the genes that control compound synthesis were examined separately (Fig. 5). In the cortex, there were generally much lower expression levels for *PAL*, *CHS*, *CHI*, *F3H*, *DFR*, and *ANS* compared with those in the skin, for all cultivars (Fig. 5). In contrast, cortical expression of *C4H* and *4CL* was similar to skin expression levels (Figs 4 and 5). In the apple cortex, expression of the reductases *LAR1* and

ANR was significant, even though the flesh had lower catechin, epicatechin, procyanidin B1, and procyanidin B2 concentrations in the cortex (Fig. 3). *LAR1* expression was similar in the cortex and skin (Figs 4 and 5). *ANR* expression levels were significantly lower in Royal Gala flesh than in skin. However, in Hetlina and Devonshire Quarrenden, *ANR* expression levels were similar in skin and flesh.

Another striking example of mRNA expression not corresponding to compound concentrations occurred with flavonol synthesis. Quercetin derivatives were very high in the skin but not detected in the cortex using HPLC (Supplementary Table S1). However, in Royal Gala, *FLS* showed cortical expression that was higher than skin expression at maturity (relative to reference genes). Expression of *FLS* in Hetlina was lower but still

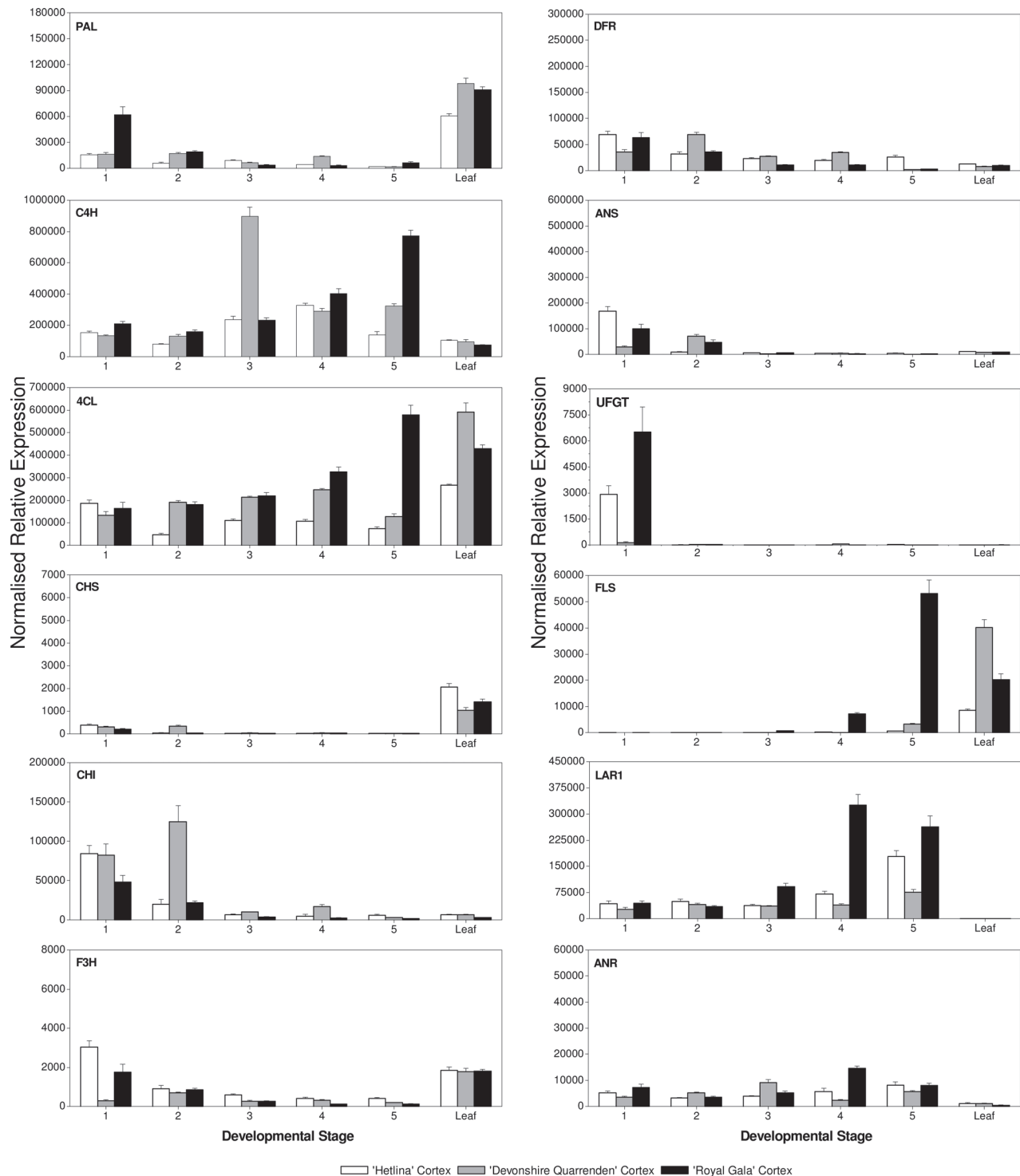


Fig. 5. Real-time qPCR expression analysis for all major steps of the polyphenolic biosynthetic pathway in apple cortex from three apple cultivars, Hetlina, Devonshire Quarrenden, and Royal Gala. *MaActin* used as the reference gene. Error bars show SD.

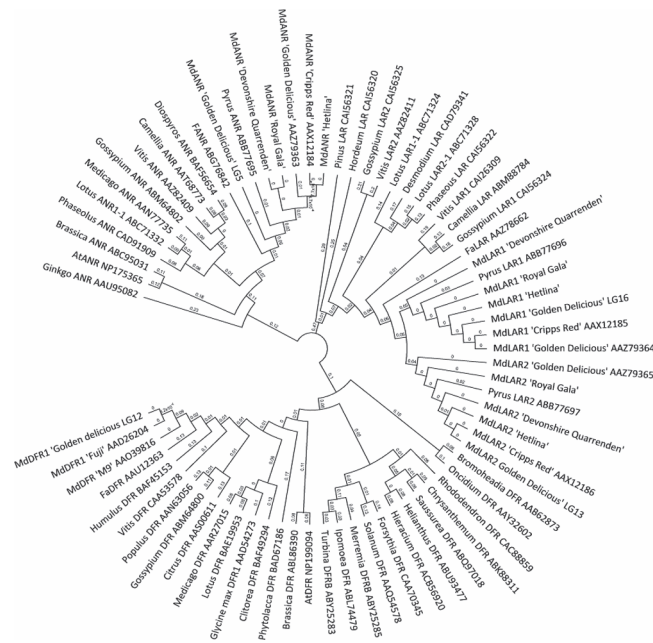


Fig. 6. Phylogenetic relationships for the deduced amino acid sequences of the *ANR*, *LAR1*, *LAR2*, and *DFR* biosynthetic genes from *Malus × domestica* and other species. All *ANR* protein sequences clustered together, as did *LAR1*, *LAR2*, and *DFR*.

significant. As a proportion, flavonols made up almost 50% of skin total polyphenolics in all three cultivars, and nothing in the flesh (Supplementary Fig. S1). Therefore, other processes must be occurring to explain the absence of flavonols in the flesh.

Phylogenetic analysis of apple phenolic reductases

Because of the lack of correlation between gene expression and polyphenolic concentrations, we sequenced the full-length genes of the reductase family members (*LAR1*, *LAR2*, and *ANR*) from all three cultivars in our study and compared these with sequences from the recently published Golden Delicious (Velasco *et al.*, 2010) and Cripps Red (Tako *et al.*, 2006c) reductases. The deduced protein sequences showed high sequence homology among the three cultivars studied here and the additional cultivars. All *Malus × domestica* *ANR* protein sequences clustered together, with the addition of the *Pyrus* *ANR* sequence, as did *LAR1*, *LAR2*, and *DFR1* (Fig. 6).

The *LAR1* protein sequences were almost identical among the five cultivars (Fig. 7B). Only a single amino acid residue appeared to differ in Devonshire Quarrenden at position 59. As this alteration did not occur in Hetlina, it is unlikely to be responsible for a difference in compound concentrations among cultivars. *LAR1* and *LAR2* also shared high sequence homology among cultivars and with one another, and were on homologous linkage groups (LG16 and LG13, respectively). They are therefore likely to represent a genome-wide duplication that occurred in apple evolution (Velasco *et al.*, 2010). All *LAR1* sequences contained the motif LELLQEEEDQK located between residues 335 and 345, while *LAR2* sequences contained PVHEEN in this region at the C-terminal end (without the protein sequence marked I in Fig. 7B). *LAR1* and *LAR2* also both contained sequence motifs common to the reductase–epimerase–dehydrogenase family of

proteins. These included the Rossmann dinucleotide-binding domain (sequence motif GXXGXXG) starting at Gly¹⁹ and three residues, Ser¹²⁹, Tyr¹⁴⁸, and Lys¹⁵¹, critical for the catalytic site (Tako *et al.*, 2006c).

Alignment of the *ANR* protein sequences also showed high sequence homology with very little variation among cultivars or in comparison with the grape *ANR* protein sequence. The *ANR* protein sequences for all the cultivars studied contained the Rossmann dinucleotide-binding domain (motif GXGXXA) starting at Gly¹⁷ (Tako *et al.*, 2006c). There were 11 amino acid differences between the Golden Delicious sequence and the other cultivars (Fig. 7A). However, none of these changes correlated with differences between high and low polyphenolic apples. Most changes were within the Golden Delicious reference, so may represent sequencing errors from the high-throughput approach used to generate the genome sequence (Velasco *et al.*, 2010). At position 65, there was an amino acid change in Hetlina and Cripps Red, while at position 228 Royal Gala had a unique amino acid change. These may be relevant for enzymatic activity.

Discussion

Apples are a major source of dietary polyphenolics (Sampson *et al.*, 2002). These compounds have beneficial effects for human health, as well as implications for apple taste and appearance. We sought to characterize the accumulation of PAs, anthocyanins, and flavonols in diverse genotypes of apple. Apples contain high concentrations of these compounds, and there are a diversity of different types, including flavonols (quercetin glycosides), cinnamic acids (chlorogenic and caffeic acids), flavanols (catechin, epicatechin, and polymeric PAs), dihydrochalcones (phloridzin), and anthocyanins (cyanidin glycosides) (McGhie *et al.*, 2005).

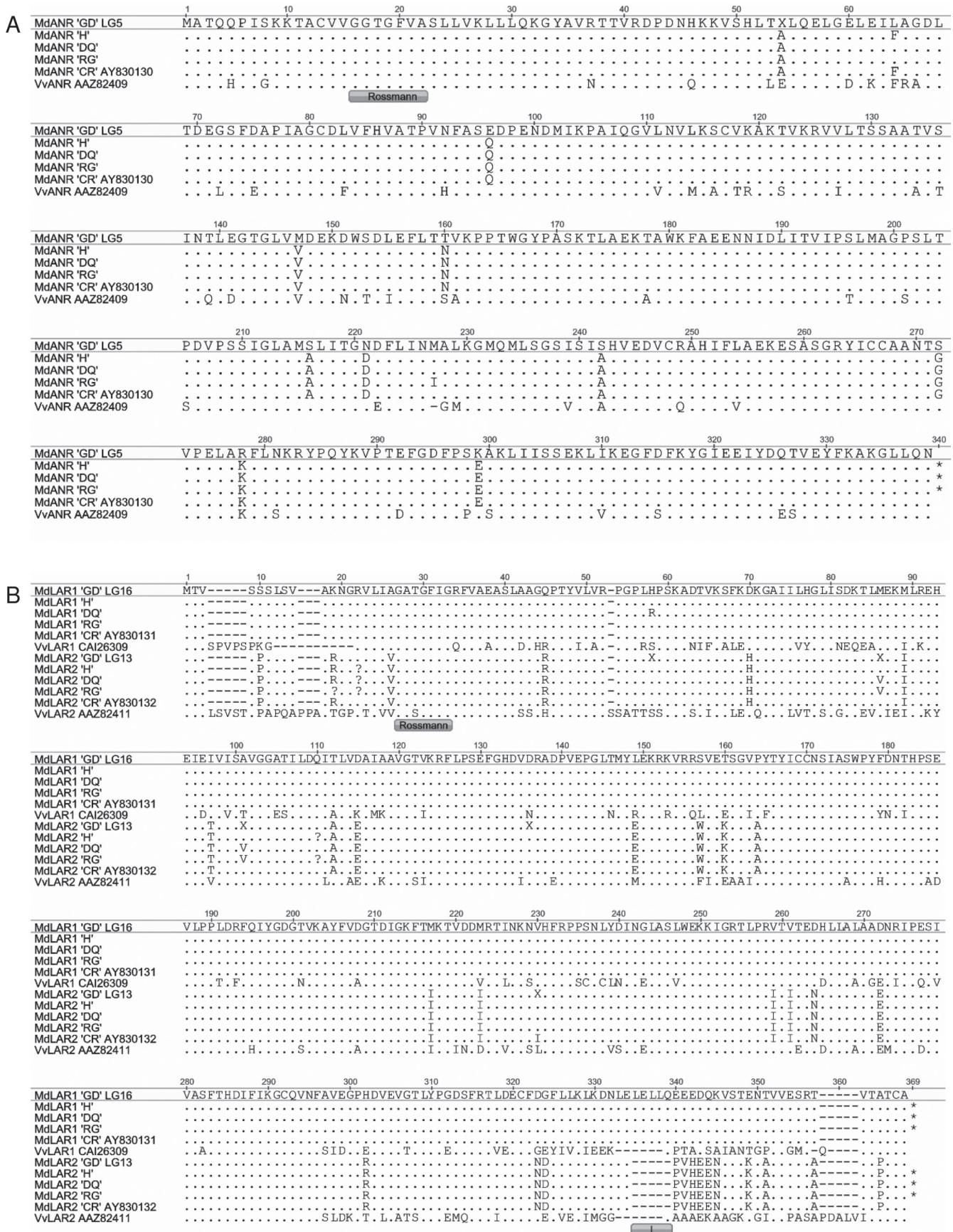


Fig. 7. Protein sequence alignment of various apple cultivars for (A) ANR protein and (B) LAR1 and LAR2 protein. The conserved reductase Rossmann dinucleotide-binding domain (Bottoms *et al.*, 2002) is shown for ANR, LAR1, and LAR2. An additional motif at the C-terminal end differing between LAR1 and LAR2 is marked I (Takos *et al.*, 2006c).

The profile of these types is affected by cultivar (McGhie *et al.*, 2005; Wojdylo *et al.*, 2008), environment (McGhie *et al.*, 2005; Lin-Wang *et al.*, 2011), and growth conditions (Takos *et al.*, 2006b). Our results confirmed previous reports of restriction of some polyphenolic components to the skin, for example, anthocyanins and flavonols (quercetin glycosides) (Takos *et al.*, 2006a,b,c; Ban *et al.*, 2007; Espley *et al.*, 2007). Other compounds such as chlorogenic acid, phloridzin, epicatechin, and condensed tannins are present in both skin and flesh (McGhie *et al.*, 2005; Volz and McGhie, 2011).

Our HPLC analysis showed that tree-ripe fruit from the heritage cultivar Hetlina contained higher amounts of phenolic compounds than the commercial cultivar Royal Gala or the heritage cultivar Devonshire Quarrenden. PAs and condensed tannins are the predominant apple phenolic compounds, making up 80% of the total (Wojdylo *et al.*, 2008). We have compared the genes involved in the production of PAs in three apple cultivars: two heritage apple cultivars, Hetlina and Devonshire Quarrenden, with high polyphenolic amounts, and a commercial cultivar, Royal Gala, with low polyphenolic amounts. Epicatechin and catechin biosynthesis is under the control of biosynthetic enzymes ANR and LAR1, respectively. Counter-intuitively, real-time qPCR analysis showed that expression levels of Royal Gala *LAR1* and *ANR* were significantly higher than those of both Devonshire Quarrenden and Hetlina. However, although Royal Gala was lowest in total polyphenolics and flavan-3-ols, the proportion of flesh and skin polyphenolics that were in the PA fraction was highest in Royal Gala.

The expression of apple flavonoid pathway genes and PA concentration has been measured previously (Takos *et al.*, 2006b; Szankowski *et al.*, 2009). In the study carried out by Takos *et al.* (2006b), fruit bagging of red skinned Cripps' Red resulted in downregulation of the entire apple anthocyanin and flavonol pathway. Transcripts of *ANR* and *LAR1* were less affected by bagging. For example, there was a 40- and 70-fold decrease in *UFGT* and *FLS* transcript levels, respectively, without light, while *ANR* and *LAR1* declined only two- to four-fold (Takos *et al.*, 2006b). Apple skin PAs were little affected by this treatment (100 d in bags on the tree), while anthocyanins disappeared completely and flavonols declined by 40% (Takos *et al.*, 2006b). Further investigation is required into the regulation of these key enzymes in apple.

A study by Szankowski *et al.* (2009) investigated the consequences of blocking anthocyanin biosynthesis in apple by silencing *ANS* in transgenic plants of a red-leaved apple cultivar. As expected, anthocyanin biosynthesis was strongly reduced. What was not expected was an increase in epicatechin biosynthesis in the *ANS*-silenced plants, as well as a strong decrease in the epicatechin derivatives procyanidin B2, B5, and E-B5. There was a decrease in *ANR* transcript levels, which is counter-intuitive to the increase in epicatechin biosynthesis. Szankowski *et al.* (2009) suggested a second biosynthetic pathway to epicatechin as an alternative explanation for the increase in epicatechin amounts in the *ANS*-silenced apples. They suggested several alternative hypotheses for the increase in epicatechin, such as epimerization of catechin to epicatechin, depolymerization in a non-stereospecific manner from oligomeric epicatechin derivatives, or redundant secondary ANS enzyme activity of another

dioxygenase of the flavonoid pathway. Royal Gala, a commercial variety not known for its astringency, displayed significantly higher gene expression levels for key procyanidin biosynthesis enzymes such as *LAR1* and *ANR* than the heritage cultivars Hetlina and Devonshire Quarrenden. There appear to be other events occurring in each cultivar that contribute to the final polyphenolic profile.

A study into apple flesh polyphenolic content suggested that polyphenols accumulated in the first 30–50 d of fruit development (Renard *et al.*, 2007), followed by a marked decrease in concentration between 30 and 60 d for both cider and table apple cultivars. After that point, polyphenol concentration would evolve mostly by dilution, which they postulated could also explain the dramatic decrease in total phenolics towards maturity of the fruit. Renard *et al.* (2007) suggest a possible link between fruit growth phases and polyphenol accumulation via modifications of enzyme activities, as well as a correlation between the end of active biosynthesis and the beginning of cell enlargement. They concluded that the possible mechanisms are still unknown but that the metabolic shift between cell proliferation and cell expansion clearly appears to include the polyphenol biosynthesis pathway. Our observation that the transcripts of *ANR* and *LAR1* increased towards maturity further suggests that enzymatic activities are vital to PA and condensed tannin concentrations as fruit mature.

Our study showed that procyanidin concentration in apple is only partially regulated at the transcriptional level. As can be seen in Fig. 2, total phenolics decreased according to the developmental stage, probably associated with a decrease in astringency, whereas the relative gene expression data followed the opposite trend. For example, apples at stages 4 and 5 generally had higher levels of gene expression for most enzymes and in all cultivars. This suggests the presence of degradation processes or polymerization to an extent that the compounds are no longer extracted, which increases with maturity. Alternatively, polyphenolic content per gram of dry weight may be relatively constant, suggesting a dilution effect as fruit grows towards maturity.

The discrepancies between metabolite concentrations and gene expression could be explained by the relative importance of some of the following factors: (i) spontaneous chemical degradation of the compounds due to variations in the conditions *in planta* (such as pH); (ii) active degradation of the compounds by enzymes such as oxidases and peroxidases; (iii) variation of biosynthetic enzymatic activities by post-translational modification; (iv) an absence of substrate supply; and (v) the presence of an additional reductase or redundant secondary ANS enzyme activity of another dioxygenase of the flavonoid pathway.

Conclusions

In this study, we have shown strong evidence that the procyanidin concentration observed in both heritage and commercial apple cultivars is only partially regulated at the transcriptional level, with a combination of events occurring in each cultivar to contribute to a final polyphenolic profile. In order to determine the regulatory events that are occurring in apple fruit, both transcriptional and post-translational modifications of the biosynthetic steps need investigation. The ratio of catechin:epicatechin

in the PA polymers determined via thiolysis of the condensed tannins will also highlight a point at which epicatechin and catechin amounts are determined.

Supplementary data

Fig. S1. Pie charts showing proportions of individual polyphenolic compounds that constitute the total polyphenolic amounts displayed in Fig. 1B for (a) skin and (b) cortex for each of the cultivars Hetlina, Devonshire Quarrenden and Royal Gala.

Table S1. Forward and reverse primers for the apple genes used in qPCR analysis.

Table S2. HPLC analysis for identification of the polyphenolic compounds in tree-ripe (stage 5) apple skin and cortex from Hetlina, Devonshire Quarrenden and Royal Gala cultivars.

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