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# Comparison of transcriptional profiles of flavonoid genes and anthocyanin contents during fruit development of two botanical forms of *Fragaria chiloensis* ssp. *chiloensis*

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ABSTRACT

Difference in fruit pigmentation observed between two botanical forms of *Fragaria chiloensis* ssp. *chiloensis* (form chiloensis and form patagonica) was studied through transcriptional and chemical approaches. The proportion of different anthocyanins was demonstrated to be characteristic of each botanical form, with pelargonidin 3-glucoside being the most abundant in f. patagonica fruit and cyaniding 3-glucoside as the major one in f. chiloensis fruit. Partial gene sequences of the phenylpropanoid and flavonoid biosynthesis pathways were isolated from the native Chilean strawberry fruits, and used to design gene-specific primers in order to perform transcriptional analyses by qRT-PCR. These genes showed spatial, developmental, and genotypic associated patterns. The red fruit of f. patagonica exhibited higher transcript levels of anthocyanin-related genes and higher levels of anthocyanins compared to the barely pigmented fruit of f. chiloensis. The anthocyanin accumulation in *F. chiloensis* ssp. *chiloensis* fruits was concomitant with the particular progress of the transcriptional activity of genes involved in the biosynthesis of flavonoid pigments. The differences in anthocyanin contents, both in terms of type and quantity, between the two botanical forms of *F. chiloensis* ssp. *chiloensis* were coincident with the differential transcriptional patterns found in the anthocyanin-related genes.



## 1. Introduction

The native Chilean strawberry (*Fragaria chiloensis* (L.) Mill. ssp. *chiloensis* Staudt) is an octoploid species ($2n = 8x = 56$) of the Rosaceae family. This native strawberry has two botanical forms which are readily recognizable. *F. chiloensis* ssp. *chiloensis* f. patagonica is a wild plant with small fruits, red receptacle, and yellow or red achenes. On the other hand, *F. chiloensis* ssp. *chiloensis* f. chiloensis is a robust plant cultivated on a small scale that bears larger fruits, which are composed of a pinkish-white receptacle and red achenes when fully ripened. The latter is the maternal progenitor of the widely cultivated strawberry, *Fragaria* × *ananassa* Duch. (Hancock et al., 1999). *F. chiloensis* is characterized as having a high and particular aroma (González et al., 2009a), large fruit size (compared with all other wild species), and remarkable tolerance to infection by *Botrytis* (González et al., 2009b). These advantages, along with other characteristics, make it an important germplasm source both for its own development as a new exotic fruit crop, as well as for further development of new cultivars of the commercial strawberry (*F.* × *ananassa*).

These thus offer an interesting model to study fruit pigmentation by anthocyanins, considering that the color of the white and red botanical forms reflect the presence of such phytochemicals. Anthocyanins are natural colorants belonging to the flavonoid family of compounds, a secondary class of metabolites that are responsible for the red, violet and blue colors observed in flowers and fruits in a large number of plants. Due to the broad distribution of anthocyanins in the plant kingdom, their chemistry, distribution, biosynthesis and regulation have been extensively studied. This has resulted in the collection of a large amount of information concerning the production of plant pigments (Dooner et al., 1991; Holton and Cornish, 1995; Grotewold, 2006; Ferrer et al., 2008).

In the *Fragaria* genus, fruit color is determined by the accumulation of anthocyanins, the most abundant flavonoids in strawberry

*Abbreviations:* ANR, anthocyanidin reductase; ANS, anthocyanidin synthase; CTAB, cetyltrimethylammonium bromide; CHI, chalcone isomerase; CHS, chalcone synthase; C4H, cinnamate 4-hydroxylase; DAA, days after anthesis; DFR, dihydroflavonol reductase; FLS, flavonol synthase; F3H, flavanone 3-hydroxylase; GSP, gene specific primer; HPLC-DAD, high performance liquid chromatography-diode array detector; LAR, leucoanthocyanidin reductase; PA, proanthocyanidin; PAL, phenylalanine ammonia-lyase; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; TF, transcription factor; UFGT, UDP glucose:flavonoid 3-*O*-glucosyl transferase; 4CL, 4-coumarate:CoA ligase; f. chiloensis, *Fragaria chiloensis* ssp. *chiloensis* f. chiloensis; f. patagonica, *Fragaria chiloensis* ssp. *chiloensis* f. patagonica.

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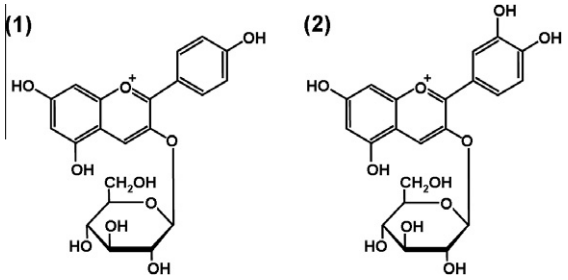


**Fig. 1.** Chemical structures of anthocyanins measured in *Fragaria chiloensis* ssp. *chiloensis* f. chiloensis and f. patagonica fruits. (**1**), Pelargonidin 3-glucoside; (**2**), cyanidin 3-glucoside.

fruits (Hannum, 2004). Pelargonidin 3-glucoside (**1**) is the predominant anthocyanin in several varieties of red strawberries, usually followed by pelargonidin 3-rutinoside and cyanidin 3-glucoside (**2**) (Gil et al., 1997; Kosar et al., 2004; Tulipani et al., 2008). These three compounds represent more than 95% of the total anthocyanins in the cultivated strawberry (Lopes da Silva et al., 2007). *F. chiloensis* f. patagonica fruit also showed similar levels of pelargonidin 3-glucoside (**1**) (Simirgiotis et al., 2009) to *F.* × *ananassa* (Kosar et al., 2004), when fully ripe. However, at the same developmental stage, f. chiloensis had lower levels of anthocyanins, with cyanidin 3-glucoside (**2**) being the major anthocyanin followed by pelargonidin 3-glucoside (**1**) (Simirgiotis et al., 2009). This indicates differences in anthocyanin compositions between the two botanical forms of the native Chilean species of *F. chiloensis* ssp. *chiloensis*.

Flavonoid genes involved in anthocyanin biosynthesis exhibit up-regulation during ripening leading to fruit pigmentation in *F.* × *ananassa* thereby establishing a positive correlation between transcript levels of flavonoid genes and anthocyanin accumulation (Manning, 1998; Almeida et al., 2007; Carbone et al., 2009).

In the present work, cDNA fragments of genes from phenylpropanoid (*PAL*, *C4H* and *4CL*) and flavonoid biosynthetic pathway (*CHS*, *CHI*, *F3H*, *DFR*, *ANS*, *UFGT*, *LAR* and *ANR*) were isolated from the native Chilean strawberry. The differential transcriptional profiles of these genes were analyzed in f. chiloensis and f. patagonica through four different fruit developmental stages and tissues by qRT-PCR. In parallel, the accumulation of the two major anthocyanins, pelargonidin 3-glucoside (**1**) and cyaniding 3-glucoside (**2**; Fig. 1), in these native strawberries was quantified by HPLC-DAD. The relationship between transcriptional profiles and anthocyanin contents present in both botanical forms is discussed in terms of the modulation of flavonoid gene expression in the determination of the different pigmentation patterns observed in the white- and red-fruited Chilean native strawberries.

## 2. Results and discussion

### *2.1. Transcriptional profiles of genes involved in biosynthesis of phenolic compounds in fruits at different developmental stages*

The phenylpropanoid biosynthesis pathway is part of the secondary metabolism of plants, and its branches transform the amino acid, phenylalanine, into a variety of important phytochemicals, including lignins, stilbenes, coumarins, salicylates, sinapate esters and flavonoids. The structural diversity of compounds derived from phenylalanine is due to the action of enzymes and enzyme complexes that bring about regio-specific condensation, cyclization, aromatization, hydroxylation, glycosylation, acylation, prenylation, sulfation and methylation reactions (Noel et al., 2005).

In order to study the transcriptional profiles of flavonoid genes in the Chilean native strawberry of red and white fruit, fragments

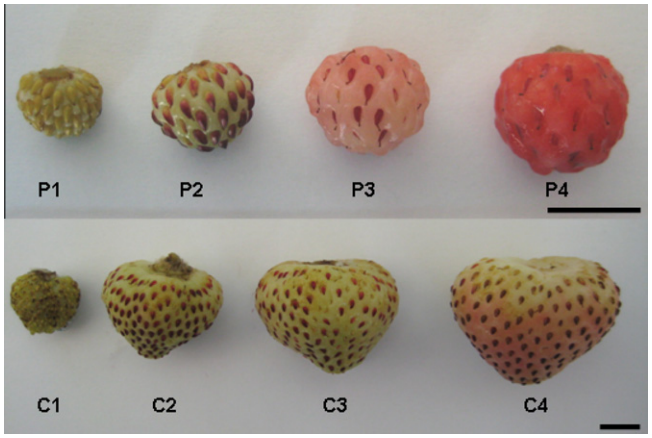


**Fig. 2.** Developmental and ripening stages of native Chilean strawberry fruit. Four different developmental stages for *F. chiloensis* ssp. *chiloensis* fruits: (A) red fruited botanical form patagonica; (B) white–pinkish fruited botanical form chiloensis. Bars are equivalent to 1 cm.

of genes involved in this biosynthetic pathway were isolated. These gene fragments showed a high nucleotide homology with phenylpropanoid and flavonoid genes from other plant species (Supplementary Table 2). Suitable primers for transcriptional analysis by

**Table 1**
Primer sequences of the phenylpropanoid and flavonoid genes and housekeeping gene (*GAPDH*) used for qRT-PCR. All primers were designed from partial sequences isolated in this work, except for *FLS* primer, which was designed directly from a *F.* × *ananassa* sequence found in public database (GenBank number accession DQ087252).

| Target gene | Primers (forward/reverse) | Amplicon size (bp) | Efficiency (%) |
|---|---|---|---|
| PAL | 5′-CAAGGGCGGCGATGCTAGTAAG-3′<br>5′-CCAAGTCACCCGACGACGAGAT-3′ | 153 | 96.5 |
| C4H | 5′-CTGTAAGGAGGTGAAGGAGAAGAGG-3′<br>5′-CTGTTGAGCGTCCAGGATGTG-3′ | 139 | 97.1 |
| 4CL | 5′-ACTTGGTCAGGGATATGGGATG-3′<br>5′-GCACCAGTTTCAGGGTCTACG-3′ | 150 | 95.4 |
| CHS | 5′-CCGACTACTACTTTCGTATCACCA-3′<br>5′-ACTACCACCATGTCTTGTCTTGC-3′ | 190 | 94.3 |
| CHI | 5′-TTTTCAATGGCTTTCGCTTCTG-3′<br>5′-GTGACAATGATACTACCGCTGACG-3′ | 119 | 94.7 |
| F3H | 5′-GTGCGCCACCGTGACTACTC-3′<br>5′-ATGCCTTTGTCAATGCCTCC-3′ | 157 | 95.4 |
| DFR | 5′-GGGTGGTGTTTACATCTTCGG-3′<br>5′-CTGCTTGCTCGGCTAGAGTTT-3′ | 156 | 96.5 |
| ANS | 5′-ATCGTCATGCACATAGGCGACACC-3′<br>5′-CCTTGGGCGGCTCACAGAAAA-3′ | 130 | 97.1 |
| UFGT | 5′-ATCGTGGCTTGACAAACAGAA-3′<br>5′-TGACCACAAGAATGGAACCCTA-3′ | 133 | 94.2 |
| ANR | 5′-CATCCAAGGCGAAGACCAT-3′<br>5′-TCATACTTAAACAACTGAGACCACC-3′ | 167 | 96.5 |
| LAR | 5′-GGTGATGGCACGGTTAAAGC-3′<br>5′-CTCCCACAGTGAAGCAAGTCC-3′ | 156 | 100.4 |
| FLS | 5′-TTATCTTTGGGGTTAGGGCTTGAA-3′<br>5′-GAGAATGGTGAGGGCGGACA-3′ | 162 | 98.1 |
| GAPDH | 5′-TCCATCACTGCCACCCAGAAGACTG-3′<br>5′-AGCAGGCAGAACCTTTCCGACAG-3′ | 132 | 93.0 |