

The Strawberry Fruit Fra a Allergen Functions in Flavonoid Biosynthesis

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ABSTRACT The strawberry Fra a 1 allergen is a homolog of the major birch pollen allergen Bet v 1. It is synthesized by red ripe fruits of *Fragaria x ananassa* while white fruits of a mutant genotype, which is known to be tolerated by individuals affected by allergy, are devoid of it. Proteomic analyses have shown that Fra a 1 is down-regulated in the tolerated white-fruited genotype along with enzymes of the anthocyanin pigment pathway. In this study, we report the spatial and temporal expression of three *Fra a* genes that encode different isoforms, and the transient RNAi-mediated silencing of the *Fra a* genes in strawberry fruits of the red-fruited cultivar Elsanta with an ihpRNA construct. As a consequence of reduced levels of *Fra a* mRNAs, fruits were obtained that produced significantly decreased levels of anthocyanins and upstream metabolites. This effect is consistent with the parallel down-regulation of the phenylalanine ammonia lyase (*FaPAL*) and to a lesser extent of the chalcone synthase (*FaCHS*) transcript levels also found in these fruits. In naturally occurring white-fruited genotypes of *F. chiloensis* and *F. vesca*, *Fra a* transcript levels are higher than those of the red-fruited varieties, likely to compensate for the low expression levels of *FaPAL* and *FaCHS* in these mutant genotypes. The results demonstrate that *Fra a* expression is directly linked to flavonoid biosynthesis and show that the Fra a allergen has an essential biological function in pigment formation in strawberry fruit.

Key words: *Fragaria*; flavonoid; anthocyanin; phenylpropanoid; allergen; pathogenesis-related proteins.

INTRODUCTION

Plant food allergens belong to a rather limited number of protein families. The strawberry (*Fragaria x ananassa*) fruit allergen Fra a 1 is a member of the Bet v 1 superfamily named after the major birch pollen allergen and is ranked among a subfamily of cytoplasmic disease resistance-related proteins (Karlsson et al., 2004; Radauer and Breiteneder, 2007; Musidlowska-Persson et al., 2007). This family, termed pathogenesis-related proteins 10 (PR-10), is ubiquitously distributed among plants, including monocots and gymnosperms.

Although the allergenic properties of PR-10 proteins have been extensively studied, their biological function in plants is not as well understood. PR-10 proteins are regulated by hormones, biotic stress such as pathogen infection, and abiotic stress such as wounding, salt, and cold (Swoboda et al., 1995b; Wang et al., 1999; Liu et al., 2005; Jain et al., 2006; Yu et al., 2000; Mur et al., 2004). Identification of allergens that show cross-reactivity with anti-Bet v 1 antibodies yielded developmentally regulated isoforms that are highly expressed in pollen, seeds, fruits, and storage organs (Schenk et al., 2006). In birch, seven out of 13 PR-10 gene loci encode proteins that are specifically expressed in pollen (Schenk et al., 2006).

This suggests that the PR-10 genes constitute a multigene family in which some members are constitutively expressed in defined tissues while others are induced under certain environmental conditions (Wen et al., 1997).

A striking feature of the Bet v 1 protein structure is a large cavity that contains two tightly bound molecules of deoxycholate, which points to a possible biological function of PR-10-related proteins as plant steroid carriers (Gajhede et al., 1996; Marković-Housley et al., 2003). In light of the evolutionary relationship of the PR-10 proteins to other subfamilies of the Bet v 1 superfamily, it is tempting to propose that the Bet v 1 structure evolved as a versatile scaffold for binding a diverse array of polycyclic, mostly hydrophobic, low-molecular-weight

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compounds (Koistinen et al., 2005; Mogensen et al., 2002). Structural comparison shows that the Bet v 1 fold is also found in diverse protein families outside the plant kingdom whose functions are in most cases related to lipid binding (Neudecker et al., 2001). In addition, several members of the PR-10 family exhibit ribonuclease, antibacterial, and antifungal activities (Park et al., 2004; Bufe et al., 1996; Bantignies et al., 2000; Flores et al., 2002). Taken together, PR-10 isoforms can bear different functions that are not mutually exclusive.

In the search for strawberry genotypes with low allergen content, the proteomic variation within and between different varieties has been determined (Alm et al., 2007). Multiple *Fra a* protein spots were detected. The total allergen content varied due to growth conditions and was always lower in colorless (white) strawberry varieties than in red ones. The ripe colorless fruits that are tolerated by individuals affected by allergy were found to be virtually free from the strawberry allergen (Hjernø et al., 2006). Proteome analysis also showed that several catalytically active proteins like chalcone synthase (FaCHS), flavanone 3-hydroxylase (F3H), and dihydroflavonol reductase (DFR) were reduced. These enzymes belong to the anthocyanin biosynthesis pathway, which produces several compounds including pelargonidin 3-*O*-glucoside, the major red pigment of strawberry fruit (Griesser et al., 2008). The concomitant down-regulation of *Fra a* and some anthocyanin biosynthetic enzymes raised the question of whether these observations are related.

To determine whether there is a direct link between *Fra a* expression and pelargonidin 3-*O*-glucoside formation, we down-regulated *Fra a* gene expression in strawberry fruits. We did this using intron-containing constructs encoding self-complementary 'hairpin' RNA (ihpRNA) that transiently silence ripening-related genes in strawberry fruits (Hoffmann et al., 2006; Griesser et al., 2008). This method initiates an RNAi-induced degradation of specific mRNAs in strawberry fruit and provides a rapid assay for gene function analysis. Fruits were agro-infiltrated with the corresponding ihpRNAs and metabolite concentrations and flavonoid gene expression levels in the injected fruits were analyzed. Additional comparisons of the allergen expression levels in fruit of strawberry species that produce ripe, colorless fruit confirmed a close connection between *Fra a* expression and pigment formation.

RESULTS

Temporal and Spatial Expression of *Fra a* Genes

We identified three ESTs (M109B07, C201C06, and M117G04) in a private collection of strawberry fruit ESTs (FREST database) that showed substantial homology with the published *Fra a 1a* to *e* isoforms (Musidlowska-Persson et al., 2007). The full-length genes were obtained using cDNA prepared from ripe fruits. A comparative analysis of the amino acid sequences revealed that the sequence named M109B07 encodes a protein that is identical to *Fra a 1E*, while the other genes are new iso-

forms that were named *Fra a 2* and *3*. In the nomenclature proposed by Musidlowska-Persson and co-authors (2007), small letters denote isomeric nucleic acid sequences, while capital letters are used for isomeric amino acid sequences. The encoded proteins *Fra a 2* and *3* showed 58 and 59% amino acid identity to Bet v 1, and 79 and 76% to *Fra a 1E*, respectively (Figure 1). A particular feature of the *Fra a* protein sequences is a P-loop motif (GXGGXGXK; amino acid 47–55, Bet v 1, Figure 1) that is found in many DNA- and ATP/GTP-binding proteins (Saraste et al., 1990). The *Fra a 1e* gene is mainly expressed in roots and the transcript levels decrease from the open flower stage to the ripe fruit as demonstrated by gene expression analysis performed by quantitative PCR (Figure 2). In contrast, the *Fra a 2* gene is highly transcribed in the late stages of fruit ripening. *Fra a 3* displays a more uniform expression pattern with a three-fold higher transcript level in open flowers. Thus, the three *Fra a* genes clearly show distinct expression patterns and only *Fra a 2* is transcribed in a ripening-related manner.

Transient Down-Regulation of *Fra a* Genes in Strawberry Fruits

In some naturally occurring strawberry varieties bearing ripe colorless fruits, the *Fra a* isoforms have been shown to be

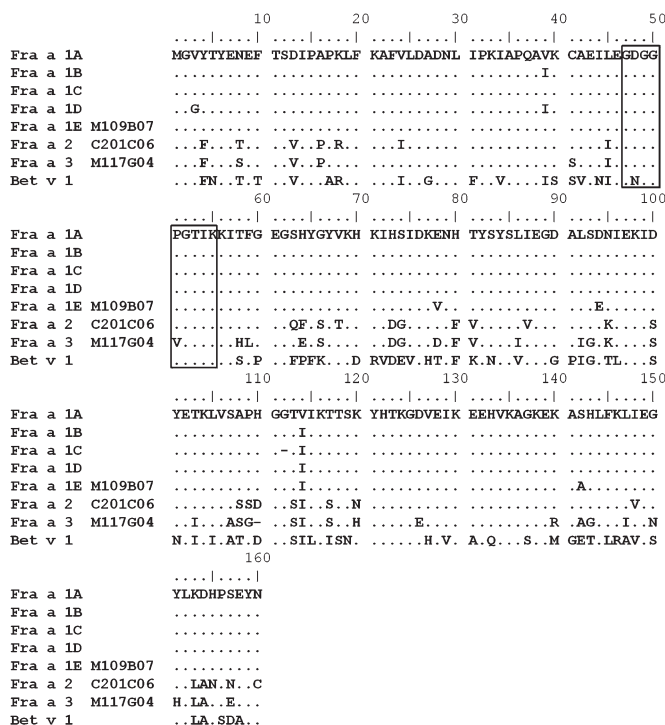


Figure 1. Amino Acid Comparison of Bet v 1 and Related Isoforms from *Fragaria x ananassa*.

Fra a 1A to Fra a 1E show the encoded amino acid sequences of the genomic clones isolated by Musidlowska-Persson et al. (2007). M109B07, C201C06, and M117G04 sequences are deduced from ESTs from a private collection. The P-loop motif GXGGXGXK is boxed.

reduced along with some structural enzymes of the flavonoid pathway (Hjernø et al., 2006). However, a direct regulatory function for *Fra a* has not been previously considered. To investigate a possible regulatory function of *Fra a* in the flavonoid pathway, we assembled ihp-constructs of the *Fra a 1e* and *Fra a 3* gene for RNAi-mediated down-regulation of allergen gene expression during fruit ripening. Vectors were generated by inserting a fragment of each gene (*Fra a 1e* and *Fra a 3*) in the sense and antisense orientation interrupted by an intron in the pBI-Intron construct to replace GUS (Hoffmann et al., 2006). We expected co-silencing of the *Fra a 2* gene by the

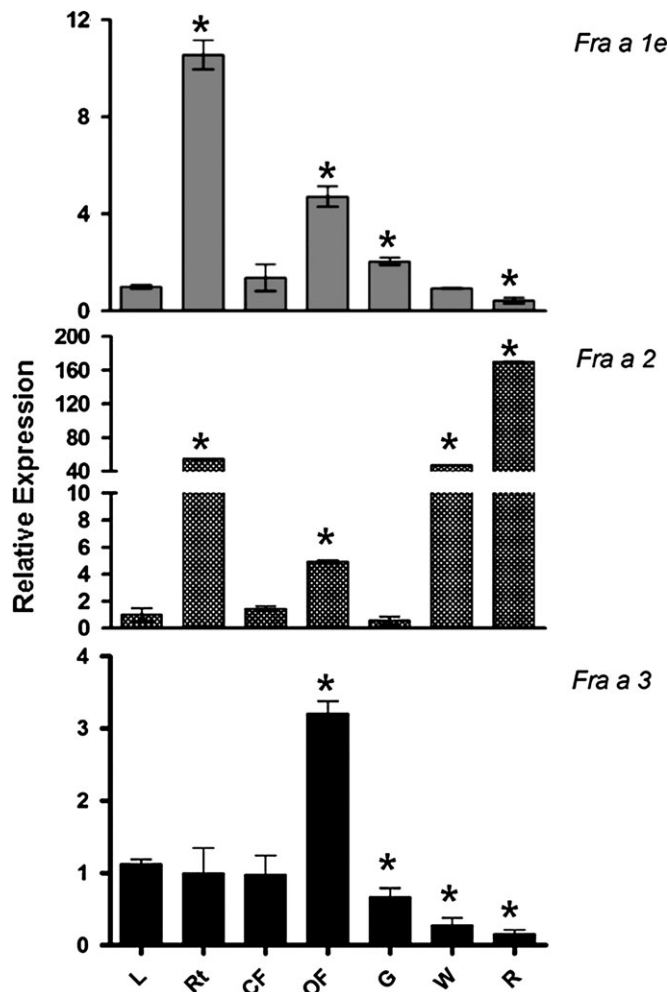


Figure 2. QPCR Analysis of *Fra a 1e*, *Fra a 2*, and *Fra a 3* Gene Expression in Various Tissues of *F. x ananassa* cv. Camarosa.

Analysis was performed with primers specific for the three genes. Values are normalized to the interspacer gene, which was used as control and set to one. RNA samples were taken from *F. x ananassa* vegetative tissues leaves (L), roots (Rt), and closed (CF) or fully mature flowers (OF), as well as from green (G), white (W), or red (R) fruits. Error bars show the standard error (SE) of three independent pools. Statistically significant values are marked with an asterisk ($p < 0.05$). The statistical analysis was performed by paired the t -test function implemented by SigmaStat software (Supplemental Table 1).

ihp-constructs targeted to *Fra a 1e* and *Fra a 3* because the three *Fra a* genes show nucleotide identities to each other of more than 78%. Recent investigations have shown that RNAi-mediated silencing may be less specific than was originally thought, since off-target transcripts with only partial complementarity were also down-regulated (Jackson and Linsley, 2004). An *Agrobacterium* suspension containing either the pBI-*Fraa1ei* or the pBI-*Fraa3i* construct was injected into attached fruits 14 d after pollination. Fruit infiltrated with *Agrobacterium* containing the pBI-Intron (empty vector) or pBI-*FaCHSi* vector (carrying a fragment of the *FaCHS* gene in the sense and antisense orientation interrupted by an intron) were used as negative and positive controls of the silencing effect, respectively.

About 10–14 d after injection, the pBI-*FaCHSi*-infiltrated fruits developed white sections—a clear sign of impaired anthocyanin accumulation due to the silencing of chalcone synthase, a key enzyme in the anthocyanin biosynthesis pathway (Hoffmann et al., 2006; Figure 3A). Fruits injected with *Agrobacterium* harboring the pBI-Intron control vector developed a uniform red color, confirming that the normal accumulation of anthocyanins during fruit ripening is not affected by infiltration with *Agrobacterium* cells (Figure 3B). Fruits agro-infiltrated with pBI-*Fraa1ei* and pBI-*Fraa3i* consistently showed white regions similar to the chimeric pBI-*FaCHSi* phenotype, which can be explained by inhomogeneous silencing

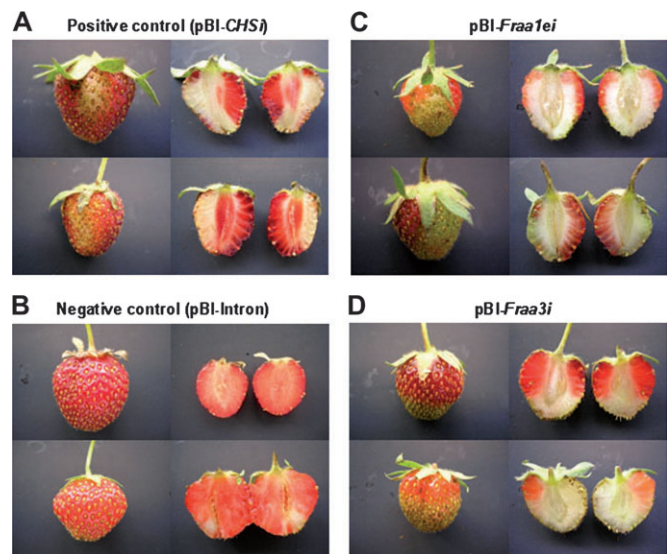


Figure 3. Strawberry Fruit Phenotypes.

(A) Fruits of *F. x ananassa* cv. Elsanta after 14 d of infiltration with *A. tumefaciens* harboring pBI-*CHSi* for RNAi-mediated down-regulation of chalcone synthase (*FaCHS*). Loss of pigmentation confirms the silencing of the target gene. (B) Fruits infiltrated with *A. tumefaciens* containing the control vector pBI-Intron. Healthy red fleshy fruits show that strawberry withstand the injection of *A. tumefaciens*. (C, D) Fruits after 14 d of infiltration with *A. tumefaciens* harboring pBI-*Fraa1ei* and pBI-*Fraa3i*, respectively. Lack of pigmentation demonstrates the effect on anthocyanin formation.

effects (Figure 3C and 3D). These results demonstrate that RNAi-mediated transcript degradation of *Fra a* genes directly affects anthocyanin accumulation. However, overexpression of *Fra a* allergens yielded phenotypes that were identical to those of control fruits agro-infiltrated with the pBI-Intron (Supplemental Figure 1). Thus, the fruit color cannot be further intensified by increasing *Fra a 1e* and *Fra a 3* transcript levels.

Metabolite Analyses

Pelargonidin 3-*O*-glucoside is the predominant anthocyanin pigment in strawberry fruit, and is usually accompanied by cyanidin 3-*O*-glucoside and pelargonidin 3-*O*-glucoside-6'-*O*-malonate. Analysis of anthocyanin levels and related compounds in fruits agro-infiltrated with pBI-Intron, pBI-*Fraa1ei*, and pBI-*Fraa3i* revealed significantly lower levels of phenolic acid derivatives, kaempferol 3-*O*-glucoside, and pelargonidin derivatives, except for the 3-*O*-rutenoside only in those fruits injected with the *Fra a* silencing constructs (Table 1). The concentrations of other metabolites were either not affected or slightly higher after the injections. Some of these anthocyanin-related compounds, like the proanthocyanidins and quercetin 3-*O*-glucoside, are already formed prior to the agro-infiltration and are not further produced in the later rip-

ening stages (Griesser et al., 2008). Thus, their levels are not affected by the ihp constructs and remain almost unchanged in comparison with their concentrations in the pBI-Intron infiltrated fruits. Although fruits infiltrated with pBI-*Fraa1ei* and pBI-*Fraa3i* displayed a phenotype similar to fruits injected with pBI-*FaCHSi*, there are important differences in the metabolic shifts caused by the different ihp vectors harboring different genes (Hoffmann et al., 2006). In pBI-*FaCHSi*-silenced fruits, the levels of FaCHS downstream metabolites and cinnamoyl-glucose-ester were decreased in comparison with their levels in control fruits, while upstream metabolites like the phenylpropanoid derivatives accumulated as a consequence of the reduced FaCHS activity (Hoffmann et al., 2006; Figure 4). However, in fruits infiltrated with the pBI-*Fraa1ei* and pBI-*Fraa3i* constructs, all metabolites of the anthocyanin biosynthesis pathway showed lower levels than the controls, indicating a control point early in this pathway that is likely affected by the *Fra a* gene expression level, *Fra a* protein concentration, or levels of the pathway intermediates.

Transcript Levels

To confirm the suppression of *Fra a* isoforms at the molecular level, we performed quantitative PCR. Primers were designed to anneal to the *Fra a* genes outside of the region used in the

Table 1. Changes in Metabolite Levels Detected by LC-MS in Ripe Fruits Injected with pBI-Intron, pBI-*Fraa1ei*, or pBI-*Fraa3i* Constructs.

Compounds	pBI-Intron	pBI- <i>Fraa1ei</i>	pBI- <i>Fraa3i</i>
<i>Phenolic acid derivatives</i>			
<i>p</i> -Hydroxybenzyl-glucose-ester	0.32 ± 0.02	0.12 ± 0.01	0.16 ± 0.02
Caffeoyl-glucose-ester	0.48 ± 0.05	0.10 ± 0.01	0.25 ± 0.05
Cinnamoyl-glucose-ester	1.86 ± 0.20	0.56 ± 0.10	0.64 ± 0.09
<i>p</i> -Coumaroyl-glucose-ester	0.93 ± 0.10	0.33 ± 0.04	0.61 ± 0.23
<i>p</i> -Coumaroyl-glucoside	0.27 ± 0.02	0.13 ± 0.01	0.25 ± 0.04
Feruloyl-glucose-ester	0.28 ± 0.02	0.08 ± 0.01	0.15 ± 0.02
<i>Flavonols</i>			
Quercetin 3- <i>O</i> -glucoside	0.13 ± 0.01	0.11 ± 0.01	0.16 ± 0.02
Kaempferol 3- <i>O</i> -glucoside	1.01 ± 0.07	0.36 ± 0.02	0.47 ± 0.04
Catechin	0.41 ± 0.06	0.47 ± 0.07	0.94 ± 0.15
<i>Proanthocyanidins</i>			
(Epi)catechine (Isomer 1)	1.04 ± 0.11	1.07 ± 0.09	1.66 ± 0.10
(Epi)catechine (Isomer 2)	1.03 ± 0.15	1.57 ± 0.16	2.60 ± 0.23
(Epi)afzelechine (Isomer 1)	0.10 ± 0.01	0.08 ± 0.01	0.11 ± 0.01
(Epi)afzelechine (Isomer 2)	0.14 ± 0.01	0.13 ± 0.02	0.15 ± 0.01
<i>Anthocyanins</i>			
Cyanidin 3- <i>O</i> -glucoside	1.11 ± 0.11	1.03 ± 0.20	0.83 ± 0.18
Pelargonidin 3- <i>O</i> -glucoside	56.91 ± 3.46	32.00 ± 2.39	27.97 ± 8.30
Pelargonidin 3- <i>O</i> -glucoside-6'- <i>O</i> -malonate	34.97 ± 2.27	14.53 ± 1.44	12.41 ± 2.47
Pelargonidin 3- <i>O</i> -rutenoside	1.52 ± 0.16	1.52 ± 0.14	2.19 ± 0.10

The values shown are the mean of independent measurements ± SE (mg kg⁻¹-equivalent 4-methyl-umbelliferyl-glucoside) of 20 fruits for each construct. Increased or decreased level was confirmed by statistical analysis using the *t*-test function implemented by SigmaStat software. Values that show significant differences between pBI-*Fraa1ei* or pBI-*Fraa3i* and pBI-Intron (control) fruits are highlighted by gray boxes (Dunne's Method, *P* < 0.05).

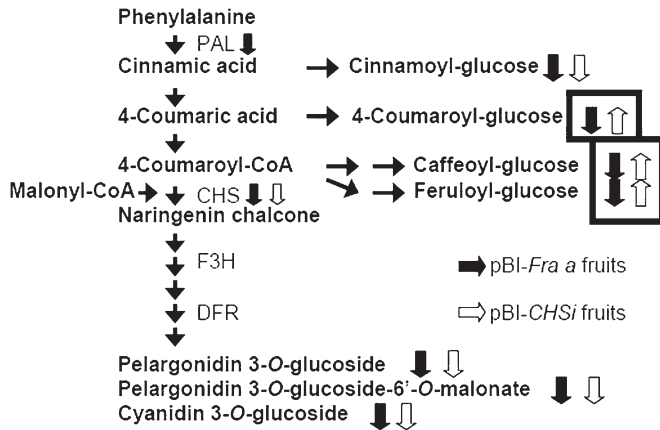


Figure 4. Scheme of the Anthocyanin and Phenylpropanoid Pathway.

Black and white arrows visualize the effects on the metabolite and selected gene expression levels caused by the down-regulation of the *Fra a* and *FaCHS* genes, respectively (Hoffmann et al., 2006). Arrows point downward when the levels decrease in comparison with the levels in the pBI-Intron fruits (control) and vice versa. PAL phenylalanine ammonia lyase, CHS chalcone synthase, F3H flavanone 3-hydroxylase, DFR dihydroflavonol reductase.

silencing constructs. One of the primers annealed in the 5'-UTR region to make the product gene-specific. Expression levels of the three *Fra a* genes, *FaCHS*, and *FaPAL* were determined relative to an interspacer 26S-18S RNA housekeeping gene (Figure 5). The *FaCHS* and *FaPAL* genes are highly expressed in red ripe fruit and encode key enzymes of the anthocyanin pathway, thought to be important regulatory points (Weisshaar and Jenkins, 1998; Supplemental Figures 2 and 3). The *Fra a* messages in the pBI-*Fraa1ei*- and pBI-*Fraa3i*-infiltrated fruits were strongly reduced to less than 40% of the levels when compared with fruits agro-infiltrated with *Agrobacterium* carrying the empty vector pBI-Intron. The reduced transcript levels of *Fra a 2* confirmed off-target silencing that is probably due to the high nucleotide sequence similarities of the *Fra a* genes (Jackson and Linsley, 2004). Interestingly, *Fra a* seems to also have a -regulatory function because the *FaCHS* and, in particular, *FaPAL* expression levels were significantly reduced in pBI-*Fraa1ei* and pBI-*Fraa3i*-injected fruits. Furthermore, agro-infiltration of the pBI-*FaCHSi* construct not only caused the expected reduction of *FaCHS* transcript levels, but also clearly reduced the expression levels of *Fra a 1e* and *Fra a 3*, while *FaPAL* expression was slightly increased and *Fra a 2* messages were not affected. These results demonstrate a clear link between *Fra a* expression and flavonoid formation.

Fra a Transcript Levels in Naturally Occurring White-Fruited Varieties

To further substantiate the connection of *Fra a* gene transcription and color formation in strawberry fruit, we also quantified the expression levels of *Fra a 1e*, *Fra a 2*, and *Fra a 3* as well as *FaPAL* and *FaCHS* genes in ripe fruits of a red-fruited *F. vesca* (cv. Reina de los Valles, red variety) and a white-fruited

F. vesca variety (cv. White Vesca, white variety), and in red- and white-fruited *F. chiloensis* varieties (cv. Red chiloensis and cv. White chiloensis). The transcript levels of the *Fra a* genes are substantially higher (two- to five-fold) in ripe fruits of the white-fruited *F. chiloensis* cultivar than in the red variety (Figure 6A). Similarly, in *F. vesca*, the levels of *Fra a 2* and *Fra a 3* mRNAs are higher in white than in red fruits, but *Fra a 1e* shows lower expression levels in white fruits (Figure 6B). *FaPAL* and *FaCHS* transcript levels are lower in the white *F. chiloensis* and almost absent in the white *F. vesca* variety (Figure 6C and 6D). It seems that in these white mutants, *Fra a* expression is up-regulated to compensate for the loss of pigments caused by the low expression levels of *FaPAL* and *FaCHS*. But still the fruits have no color, so a higher amount of *Fra a* mRNA does not imply red color. Lower *Fra a* protein levels have recently been determined in other white strawberry genotypes (Hjernø et al., 2006). Since gene transcript levels were not provided by the authors, comparisons of gene expression levels and protein concentrations between the different white genotypes cannot be made.

DISCUSSION

Although the biosynthetic pathway of anthocyanins is one of the most extensively studied pathways of plant secondary products and most enzymes and genes have been characterized, the regulation, vascular deposition, and sequestration are comparatively little understood (Springob et al., 2003). Our study shows that the *Fra a* allergen, which is homologous to the major birch pollen allergen Bet v 1, has a functional role in this pathway and even though the detailed mechanism remains to be clarified, our data suggest that *Fra a* functions as a carrier of pathway intermediates or as a (co-)transporter of anthocyanins into the vacuole, in accordance with recent results of Bet v 1 superfamily members.

Eight DNA sequences, all containing one intron, were recently obtained with PCR primers designed for cloning of *Fra a* genomic DNA from three different strawberry varieties (Musidlowska-Persson et al., 2007). Five deduced amino acid sequences (*Fra a* 1A-E) showed sequence similarity of greater than 97% and only seven positions of amino acid variability. Thus, the authors concluded that the sequence variability in the strawberry allergen *Fra a* 1 is small, both within and between strawberry varieties, and that the large proteomic variation previously detected in 2-D electrophoreses must be due to differences in post-transcriptional modifications (Alm et al., 2007, 2006; Musidlowska-Persson et al., 2007; Hjernø et al., 2006). In order to find expressed allergen sequences in the strawberry fruit, we searched an EST collection and found three EST sequences showing high homology to the published *Fra a* sequences. Three full-length cDNAs were then cloned from these sequences. Although one of the sequences was identical to *Fra a 1e*, the two others are new isoforms whose deduced protein sequences show only 79 and 76% amino acid identity to *Fra a* 1E, and appear to belong to new subfamilies.

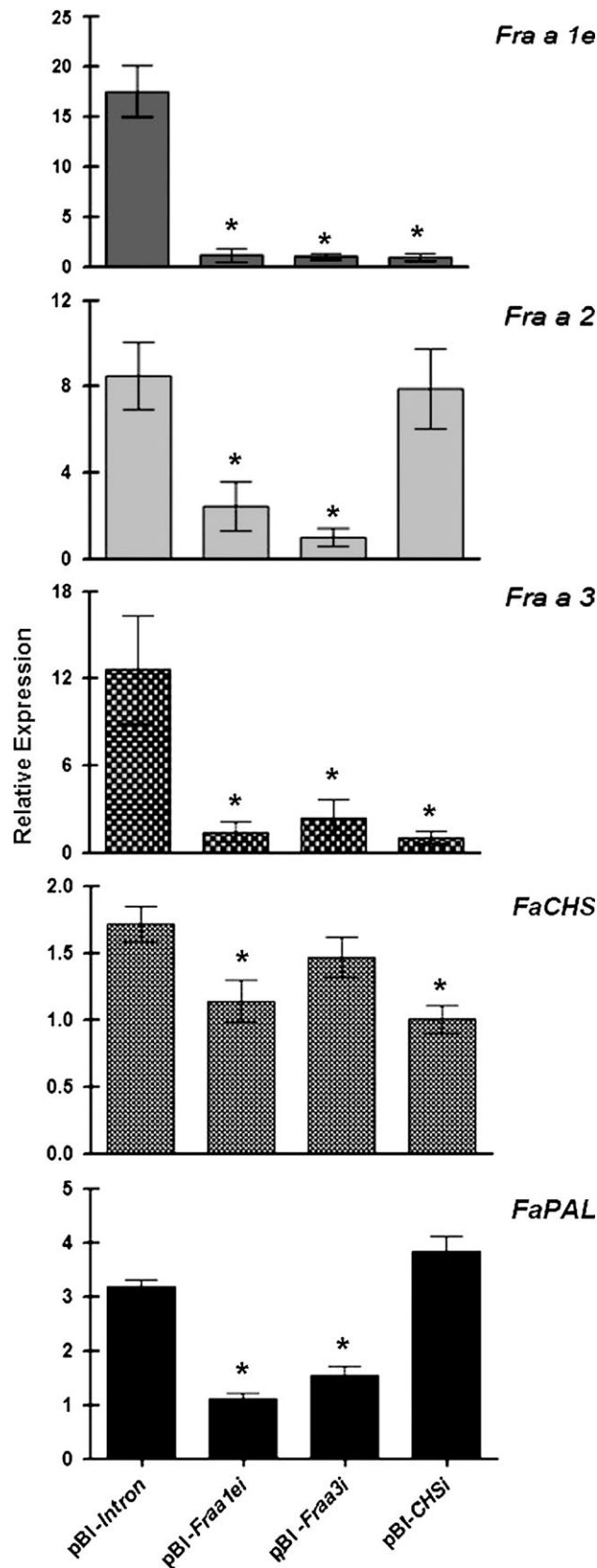


Figure 5. Relative Gene Expression Levels in Differently Treated Fruits as Determined by qPCR.

Both *Bet v 1* and the homologous gene from apple (*Malus x domestica* Borkh.) *Mal d 1* are members of gene families consisting of at least 20 (Swoboda et al., 1995a) and 15 (Atkinson et al., 1996) isoforms, respectively, that differ in amino acid sequence and immunological impact (Ferreira et al., 1996). Sequencing of cDNA libraries of one cultivar yielded 12 members of the *Mal d 1* family (Beuning et al., 2004). If we assume that strawberry, like apple, contains a relatively large family of *Fra a*-related genes, this would mean that the approach using degenerate primers probably leads to an underestimation of the total number of isoforms. The previously used primers were degenerate in only one nucleotide. Thus, it is not surprising that strawberry cultivars, which are also octoploid, contain several *Fra a* isoforms that might have different roles.

Genes of the *Mal d 1* family are not only expressed in different tissues, but a large number of them are also transcribed in the same tissue, namely in ripe fruit skin (Beuning et al., 2004). Similarly, we observed that although the *Fra a* genes studied in this work are expressed in fruit, only *Fra a 2* showed a specific ripening-related expression pattern consistent with the development of an allergic reaction by sensitive individuals against strawberry fruit. It is known from *Bet v 1* that very small changes in amino acid sequence can change orthologs from being hyperallergenic to hypoallergenic, so it is reasonable to suggest that *Fra a 2* might be a hyperallergenic isoform (Ferreira et al., 1998).

Fra a 1, like *Bet v 1*, is homologous to the group of PR-10 proteins that were originally characterized at the transcriptional level, showing increased gene expression in stress situations, such as during microorganism infection (Radauer and Breiteneder, 2007). Although the allergenic properties of PR-10 proteins in terms of structure, epitopes, and epidemiology are well documented, limited functional studies have been performed on the corresponding proteins (Viehts et al., 2002). However, it is conceivable that the unusual tertiary structure of the *Bet v 1* superfamily members, the internal cavity, plays a central role in the biological function of the molecule and probably does so through specific interaction with unknown ligands (Gajhede et al., 1996). This hypothesis is supported by the observation of the high 3-D structural similarity of *Bet v 1*-homologous proteins to the START domain of MLN64, a protein involved in the mobilization of cholesterol in human placenta and brain, although there is no sequence homology (Tsujishita and Hurley, 2000; Watari et al., 1997). Moreover, the PR-10 member from mung bean binds

Relative transcription levels of *Fra a 1e*, *Fra a 2*, *Fra a 3*, *FaCHS*, and *FaPAL* normalized to the interspacer gene were determined in fruits agro-infiltrated with pBI-Intron, pBI-Fraa1ei, pBI-Fraa3i, and pBI-CHSi. Values represent means $\pm$ SE of three independent measurements (fruits). Statistically significant values in comparison with the values for pBI-Intron are marked with an asterisk ($p < 0.05$). The statistical analysis was performed by paired *t*-test function implemented by SigmaStat software (Supplemental Table 2).

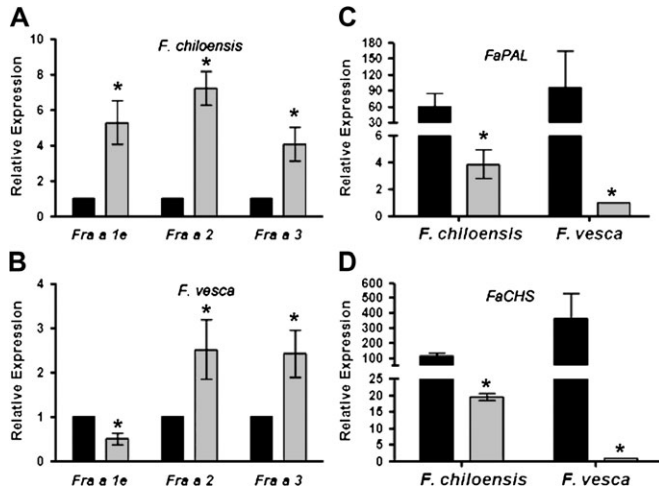


Figure 6. Expression Levels of *Fra a 1e*, *Fra a 2*, and *Fra a 3* allergens (A, B) as well as *FaPAL* (C), and *FaCHS* (D) in Fruits of the Red (Black Bars) and White (Gray Bars) Fruited *F. chiloensis* Cultivar (A, C, D) and *F. vesca* cultivar (B, C, D).

Values represent means $\pm$ SE of three independent pools. Statistically significant values are marked with an asterisk ($p < 0.05$). The statistical analysis was performed by the *t*-test function implemented by SigmaStat software (Supplemental Table 3).

cytokinins and their analogs with nanomolar affinity, while the major allergens from cherry, Pru av 1, Bet v 1, and Bet v 1l, were all reported to bind phytosteroids (Fujimoto et al., 1998; Neudecker et al., 2001; Mogensen et al., 2002; Marković-Housley et al., 2003). But Bet v 1 also interacts with fatty acids and flavonoids, whereas the Hyp-1 protein from *Hypericum perforatum*, which shows high sequence similarity with PR-10 proteins, is able to convert the phenolic compound emodin to hypericin *in vitro* and norcoclaurine synthases couple dopamine with 4-hydroxyphenylacetaldehyde (Mogensen et al., 2002; Bais et al., 2003; Minami et al., 2007). NMR studies revealed that proteins of the PR-10c subfamily bind to cytokinin, flavonoid glycosides, sterols, and emodin (Koistinen et al., 2005). From these results, it was concluded that PR-10 proteins have a role in the storage and transport of biologically important molecules or provide a scaffold that promotes enzymatic reactions.

The carrier function was recently substantiated by an investigation that showed that Bet v 1 binds and permeabilizes membranes (Mogensen et al., 2002; Marković-Housley et al., 2003; Koistinen et al., 2005; Mogensen et al., 2007). Two major bound stages (A and B) with distinct secondary structure compositions were identified, which predominate in the pH ranges ~ 9.5 – 6.5 and ~ 5 – 2.5 , respectively. In addition, one state was able to induce complete leakage of a fluorescent dye from vesicles, indicating that this state is partially inserted into and significantly perturbs bilayer structures (Mogensen et al., 2007). The Bet v 1 homolog Mal d 1, which shares 59% sequence similarity to Bet v 1, showed the same ability

as Bet v 1 to undergo structural changes and to permeabilize vesicles in the B-state. *Fra a 1e*, *Fra a 2*, and *Fra a 3* have sequence similarities of 70, 71, and 74% with Bet v 1.

Because our study revealed that the down-regulation of *Fra a* transcript levels results in reduced pigment formation in strawberry fruit, we suggest that the encoded proteins might be involved in processes leading to the formation and/or the accumulation of anthocyanins. Since the general biosynthetic enzymes of the pelargonidin 3-*O*-glucoside pathway are known and the protein sequences of the Bet v 1 homologs do not share significant similarity with any known anthocyanin biosynthesis enzyme but Bet v 1 isoforms are able to bind phenolic compounds and permeabilize membranes, we assume that *Fra a* is involved in the storage and/or transport of intermediates of the flavonoid pathway. It is known that Bet v 1 alone binds reversibly to membranes without penetration to the other side, but ligand binding could facilitate translocation. Therefore, we propose that *Fra a* proteins might mediate transport of the intermediates of the flavonoid pathway from one biosynthetic enzyme to the other or facilitate the transport of anthocyanins through the tonoplast into the vacuole. The A state of the Bet v 1-homolog that predominates in the pH-neutral medium of the cytosol could bind, for example, pelargonidin 3-*O*-glucoside, which may trigger translocation. Within the vacuole at a pH of 5, the B-state is formed, which eventually releases the anthocyanin. This is in agreement with the release data for the fluorescent dye where only the B-state released the dye (Mogensen et al., 2007). Thus, *Fra a* isoforms might bind and transport flavonoid pathway intermediates within the cytosol or through membranes.

Vacuolar transport of flavonoids in petunia is mediated by glutathione-S-transferase (GST), but instead of forming glutathione conjugates with flavonoids, GST binds flavonoids and is suggested to serve as a cytoplasmic flavonoid carrier protein (Müller et al., 2000). A similar role was proposed for PR-10c proteins in the storage of flavonoids and their transport to the site where they are needed most (Koistinen et al., 2005). Recently, it has been shown that two proteins with high sequence similarity to the multidrug and toxic extrusion family act *in vitro* as vacuolar H⁺-dependent acylated anthocyanin transporters (Gomez et al., 2009). The acyl conjugation was essential for the uptake. However, a passive, carrier-mediated transport, similar to the one suggest for *Fra a*, of oleanolic acid monoglucuronides into the vacuole was reported for *Calendula officinalis* (Szakiel and Janiszowska, 2002).

Our data also indicate that the function of *Fra a* is monitored by an unknown mechanism because silencing of the allergen gene expression affects *FaPAL* and *FaCHS* transcript levels leading to a shut-down of the anthocyanin biosynthetic pathway. One isoform of the *Fra a* transcripts or proteins could function as a direct regulator of *FaPAL* and *FaCHS* gene expression. This result is in agreement with the experimental data of the proteomic screening of a white-fruited cultivar where the strawberry Bet v 1 homologous allergen was down-regulated in concert with enzymes (F3H, DFR, *FaCHS*) of the flavonoid

pathway (Hjerno et al., 2006). However, gene expression levels were not determined in the latter publication. In fruits of naturally occurring white-fruited *F. chiloensis* and *F. vesca* varieties, used in this study, *Fra a* mRNA levels were up-regulated probably to compensate for the low anthocyanin concentration. To allow comparison of both studies, future work should focus on the determination of the expression levels of the *Fra a* genes in the strawberry variety used for the proteome analysis and on the quantification of *Fra a* isoenzyme levels in the white- and red-fruited varieties used in this work.

RNAi-mediated silencing of *FaCHS* caused the expected down-regulation of the chalcone synthase transcript level and surprisingly also a reduction in *Fra a 1e* and *Fra a 3* messages while the level of *Fra a 2* remained unchanged. It seems that *Fra a 1e*, *Fra a 3*, and *FaCHS* gene expression is closely related and that the mutual regulation is highly specific, as the ripening-induced *Fra a 2* expression was not affected. This observation points to different functions of the individual isoforms.

As a result of the plant's interaction with changes in its environment, specific flavonoids and other phenolic and polyphenolic metabolites are produced and accumulated (Dixon and Paiva, 1995). The biosynthesis of flavonoids is constitutive in plants, being internally controlled during normal growth and development, such as new vegetative leaf growth and reproductive organ development. But flavonoid biosynthesis may also be induced by exogenous stimuli, such as changes in light and temperature, or elicited by damage to the plant caused by physical agents, herbivore attack, and microbial invasion. These damages also induce the expression of PR-10 (Bowles, 1990). Hence, both flavonoids and PR-10 expression respond to similar stimuli, which is consistent with PR-10s having a biological function in that pathway. Furthermore, the expression pattern of a PR1 promoter–GUS gene correlated well, temporally and spatially, with the developmental expression observed for GUS fusions, with the promoters derived from genes coding for enzymes in the phenylpropanoid pathway (Warner et al., 1994). The PR1 promoter contains sequence motifs similar to those implicated as important in the regulation of phenylpropanoid pathway genes and another PR gene.

Based on the crystal structures of Bet v 1 and Pru av 1 isoforms and protein–ligand interaction studies, it can be stated that PR-10 proteins are capable of binding several different types of ligands (phytohormones, fatty acids, and flavonoids) using different binding sites for different compounds (Gajhede et al., 1996; Marković-Housley et al., 2003; Neudecker et al., 2001; Koistinen et al., 2005; Mogensen et al., 2002). Thus, the presence of multiple Bet v 1, Mal d 1, and *Fra a* 1 isoforms could reflect the necessity of numerous carriers for the transport of different metabolites into different target compartments.

In addition to the cavity, the 3-D structure of Bet v 1 and Pru av 1 shows a P-loop motif (GXGGXGXK; amino acid 47–55, Bet v 1, Figure 1) that is found in many DNA- and ATP/GTP-binding proteins (Saraste et al., 1990). It is conserved in the hypoallergenic ortholog from periwinkle, the cytokinin-binding homolog in mung bean, and the *Fra a* isoforms (Laffer et al., 2003;

Fujimoto et al., 1998; Figure 1). These data indicate a potential role of the allergens in signaling, which is also supported by the down-regulation of *FaPAL* and *FaCHS* transcript levels in pBI–*Fraa1ei* and pBI–*Fraa3i* fruit. The recent isolation of a small protein that interacts with Mal d 1 provides additional support for the role of allergens in signaling (Pühringer et al., 2003). It was suggested that this protein could act to stabilize a transport complex within the plant, while binding to divergent isoforms, and the presence of only one gene in apple would be consistent with a signaling role. Only recently, regulators of type 2C protein phosphatases (PP2Cs), key players in abscisic acid (ABA) signal transduction, were identified in *Arabidopsis* and share structural similarity with class 10 pathogen-related proteins, although they have only 12% sequence similarity with Bet v 1 (Ma et al., 2009). The regulators were shown to bind ABA, to mediate ABA-dependent inactivation of PP2Cs *in vitro*, and to antagonize PP2C action *in planta*. Similarly, our new data suggest the existence of a signaling pathway for the *Fra a* function but the detailed analysis of the signaling cascade remains to be performed.

The present study was motivated by the observation that the strawberry Bet v 1-homologous allergen is down-regulated together with enzymes of the flavonoid biosynthesis pathway in colorless strawberry mutants (Hjerno et al., 2006). Among the proteins that were co-down-regulated were chalcone synthase, dihydroflavonol reductase, and flavanone 3-hydroxylase. Our functional analysis indicates that the *Fra a* genes are essential for color formation in strawberry fruits. The results demonstrate that the reduced *Fra a* gene expression achieved in the transiently transformed fruits accounts for the reduced expression of flavonoid pathway genes and subsequently for the white phenotype observed in the transformed fruits. However, other white-fruited varieties bearing different mutations in the anthocyanin pathway like those used in this study show higher *Fra a* expression levels when compared with their red-fruited counterparts. Altogether, the findings provide strong evidence that *Fra a* expression is directly linked to anthocyanin formation and confirms that the *Fra a* allergen has an essential biological function in pigment formation in strawberry fruit.

METHODS

Plant Material

The octoploids *Fragaria x ananassa* (cv. Camarosa) and *Fragaria chiloensis* (spp. *chiloensis* forma *chiloensis*, white fruits; spp. *chiloensis* forma *patagonica*, red fruits), as well as diploid *Fragaria vesca* L. (Reina de los Valles, red fruits; spp. *vesca* forma *alba* (Her.) Staudt, white fruits), were used for real-time quantitative PCR. Tissues harvested for RNA extraction were immediately frozen in liquid nitrogen and stored at –80°C until RNA isolation. The octoploid strawberry *Fragaria x ananassa* cv. Elsanta was used for transfections. Standard growing conditions were maintained at 25°C and a 16-h photoperiod under

120 $\mu\text{mol m}^{-2} \text{sec}^{-1}$ irradiance provided by Osram Fluora lamps (Munich, Germany). For genetic and molecular analyses, fruits were injected 14 d after pollination and harvested 10–14 d after injection.

Cloning of *Fra a* Genes

The full-length sequences of *Fra a 1e*, *Fra a 2*, and *Fra a 3* genes were isolated from cDNA of red fruit of strawberry (*F. x ananassa* cv. Camarosa) using primers *Fra a 1e* (forward: 5'-AAG CGG CCG CCA TGG GTG TTT ACA CTT ATG-3' and reverse: 5'-AAG GCG CGC CCG TTG TAT TCG CTG GGG TG-3'), *Fra a 2* (forward: 5'-AAG CGG CCG CCA TGG GTG TGT TCA CTT ATG-3' and reverse: 5'-AAG GCG CGC CCA CAG TAT TCA TTA GGA TTG-3'), and *Fra a 3* (forward: 5'-AAG CGG CCG CCA TGG GTG TGT TCA CAT ACG-3' and reverse: 5'-AAG GCG CGC CCG TTG TAT TCC TCA GGA TG-3'). The forward and reverse primers contained an *NotI* and an *Ascl* restriction site, respectively. PCR-product gels were purified, digested with *NotI/Ascl*, and then cloned into *NotI/Ascl*-cut pEntry-D/Topo vector. The resulting plasmids were named pEntry-*Fra a 1e*, pEntry-*Fra a 2*, and pEntry-*Fra a 3*.

Isolation of Nucleic Acids and qPCR Analysis

Total RNA from three independent pools (six to seven strawberry fruits per pool) at different ripening stages, roots, leaves (fully expanded), green (about 7 d after blooming), white (about 14 d after blooming), red (about 30 d after blooming) fruits and flowers were extracted according to Manning (1991). Agro-infiltrated fruits were analyzed individually. Extracted RNA was treated with Amplification Grade DNase 1 (Sigma-Aldrich, Steinheim, Germany) to remove any DNA contamination prior to cDNA synthesis. Reverse transcription (RT) was carried out using 1 μg of total RNA and the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA), while qPCR analysis was performed using the iCycler system (Bio-Rad) as described previously (Benítez-Burraco et al., 2003). Specific primers for the coding region of each gene were designed to investigate the expression of *Fra a 1e* (forward: 5'-CAT GGA GGA ACC ATC ATC AA-3' and reverse: 5'-CAC CCC AGC GAA TAC AAC TAA-3'), *Fra a 2* (forward: 5'-CGT GGA GAT CAA GGA AGA GC-3' and reverse: 5'-GGA ACA TCA GCG GAA CAA AT-3'), *Fra a 3* (forward: 5'-GTT AAG GCC GGA AAG GAA AG-3' and reverse: 5'-GAT CGA GCA GAT TGA CAC CA-3'), *FaPAL* (forward: 5'-GAT GCA AAG GCT AAG GCA AG-3' and reverse: 5'-AGC CCT AAC GCT CTC AAC CT-3'), and *FaCHS* (forward: 5'-GCC TTT GTT TGA GCT GGT CT-3' and reverse: 5'-CCC AGG AAC ATC TTT GAG GA-3'). The results were normalized with an inter-spacer 265–185 RNA housekeeping gene (forward: 5'-ACC GTT GAT TCG CAC AAT TGG TCA TCG-3 and reverse: 5'-TAC TGC GGG TCG GCA ATC GGA CG-3').

Ten to fourteen days after injection, agro-infiltrated fruits were individually freeze-dried in a lyophilizer and ground to a fine powder. Fifty milligrams of the powder from each fruit was used for RNA extraction, followed by DNase 1 treatment and RT, as described before. cDNA was used for qPCR experiments in the injected fruits using specific primers

designed to amplify the 5'-UTR for *Fra a 1e* (forward: 5'-TCC TTA TTC CCT GCT TGC TT-3' and reverse: 5'-AGG TGA TCT TCT TGA TGG TTC C-3'), and the 3'-UTR for *Fra a 2* (forward: 5'-CGT GGA GAT CAA GGA AGA GC-3' and reverse: 5'-GGA ACA TCA GCG GAA CAA AT-3'), and *Fra a 3* (forward: 5'-GTT AAG GCC GGA AAG GAA AG-3' and reverse: 5'-GAT CGA GCA GAT TGA CAC CA-3'). Primers for *FaPAL* and *FaCHS* were as indicated above.

Preparation of ihp Constructs and Overexpression Constructs

The *pBI-Intron* and *pBI-CHSi* constructs were generated as described previously (Hoffmann et al., 2006). For the construction of *pBI-Fraa1ei* and *pBI-Fraa3i*, a partial sequence of both genes was PCR-amplified from strawberry cv. Camarosa cDNA using gene-specific primers (*Fra a 1e* forward: 5'-GCT AGC CAG CCA CTA CGG GTA CG-3' and reverse: 5'-TCT AGA GTT GTA TTC GCT GGG GTG-3'; *Fra a 3*, forward: 5'-GCT AGC CTT ACC TTC CAT ATA TAT AGC TG-3' and reverse: 5'-TCT AGA GTG CTC TTG ATG ATG GA-3'). The forward and reverse primers contained an *NheI* and an *XbaI* restriction site, respectively. The amplified fragments were cloned into a pGemT vector and then digested with *NheI/XbaI*, obtaining fragments of approximately 300 pb (*Fra a 1e*) and 400 pb (*Fra a 3*), which were used for ligation into the binary vector pSK that contained *XbaI/NheI* and *SpeI/SacI* (*Ec/136II*) restriction sites separated by an intron from strawberry (Hoffmann et al., 2006). To do this, the vector was cut with *XbaI* and *NheI*, and the fragment was ligated in the sense direction. Second, the vector was digested with *SpeI* and *Ec/136II* and the fragment was inserted in the antisense direction, obtaining the intron-hairpin construct pSK-*Fra a 1ei* and pSK-*Fra a 3i*. These plasmids were cut with *Ascl*; the digested fragment was cloned into the *Ascl*-cut pBI121 obtaining the constructs pBI-*Fra a 1ei* and pBI-*Fra a 3i*. For the *Fra a 1e-pXCSG-StrepII* and *Fra a 3-pXCSG-StrepII* construction, the full-length sequences of the *Fra a 1e* and *Fra a 3* genes were cloned into the pEntry-D/TOPO vector (as described above) and then they were cloned into the pXCSG-StrepII vector using the LR reaction of GATEWAY (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. The resulting plasmids *Fra a 1e-pXCSG-StrepII* and *Fra a 3-pXCSG-StrepII* were introduced into *Agrobacterium tumefaciens* strain GV3101.

Transfection of Strawberry by Agro-Infiltration

The *Agrobacterium tumefaciens* strains AGL0 containing the pBI-*FaCHSi*, or pBI-Intron, or pBI-*Fraa1ei*, or pBI-*Fraa3i* were grown at 28°C in LB medium with appropriate antibiotics according to Hoffmann et al. (2006). When the culture reached an OD₆₀₀ of about 0.8, *Agrobacterium* cells were harvested and re-suspended in a modified MMA medium (MS salts, 10 mM MES pH 5.6, 20 g l⁻¹ sucrose). The *Agrobacterium* suspension was evenly injected throughout the entire attached fruit about 14 d after pollination by using a sterile 1-ml hypodermic syringe.

Metabolite Analysis

Metabolites in individual strawberries were analyzed about 14 d after injection. Harvested strawberry fruit were ground in liquid nitrogen. Approximately 200 mg of frozen material was thawed, centrifuged (16 000 g, 10 min) and the supernatant was used directly for LC–UV–ESI–MSⁿ analyses. Relative quantification and statistical evaluation were performed according to Hoffmann et al. (2006).

Liquid Chromatography–UV–Electrospray Ionization–Tandem Mass Spectrometry (LC–MS)

The system used for metabolite analysis was a Bruker esquire 3000 plus mass spectrometer, equipped with an Agilent 1100 HPLC system composed of an Agilent 1100 quaternary pump and an Agilent 1100 variable wavelength detector. The column was a Eurospher C18 column, particle size 5 µm, 10 × 2 mm (Grom Analytik & HPLC GmbH, Rottenburg, Germany). The ionization parameters were as follows: the voltage of the capillary was 3074 V and the end plate was set to –500 V. The capillary exit was –109.8 V and the Octopole RF amplitude 120 Vpp. The temperature of the dry gas (N₂) was 300°C at a flow of 10 l min^{–1}. The full scan mass spectra of the metabolites were measured from m/z 50–800 until the ICC target reached either 20 000 or 200 ms. Tandem mass spectrometry was performed using helium as the collision gas, and the collision energy was set at 1.00 V. Mass spectra were acquired in negative and positive ionization modes. Auto-tandem mass spectrometry was used to break down the most abundant [M+H]⁺, [M–H][–], or [M+HCOO][–] ions of the different compounds of the strawberry extracts. The LC parameters went from 0% acetonitrile and 100% water (acidified with 0.05% formic acid) to 50% acetonitrile and 50% acidic water in 50 min, then in 20 min to 100% acetonitrile, held for 10 min at these conditions, then returned to 100% water and 0% acetonitrile in 5 min at a flow rate of 0.2 ml min^{–1}. The detection wavelength was 280 nm.

Reference Compounds

Phenylpropanoyl glucose esters were enzymatically synthesized with FaGT2 (Lunkenbein et al., 2006). Pelargonidin 3-O-glucoside, quercetin 3-O-glucuronide, quercetin 3-O-glucoside, kaempferol 3-O-glucuronide, kaempferol 3-O-glucoside catechin, and epicatechin were obtained from Roth, Karlsruhe, Germany. Proanthocyanidins and pelargonidin 3-O-glucoside-6'-O-malonate were isolated from strawberry and identified according to Fossen et al. (2004) and Gu et al. (2003).

Sequence data from this article can be found in the EMBL/GenBank data libraries under accession numbers GQ148818 and GQ148819.

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online*.

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