Original Paper

Establishment of a novel system to elucidate the mechanisms underlying light-induced ripening of strawberry fruit with an *Agrobacterium*-mediated RNAi technique

Katsuyuki Miyawaki[1,*], Sachi Fukuoka[1], Yasuko Kadomura[2], Hirokazu Hamaoka[1], Taro Mito[1], Hideyo Ohuchi[1], Wilfried Schwab[3], Sumihare Noji[1]

[1] Department of Life Systems, Institute of Technology and Science, The University of Tokushima, Tokushima 770-8506, Japan;
[2] Department of Nutrition, Faculty of Medicine, The University of Tokushima, Tokushima 770-8503, Japan; [3] Biotechnology of Natural Products, Technical University München, Freising, Germany
* E-mail: miyawaki@bio.tokushima-u.ac.jp   Tel & Fax: +81-88-656-9310

Abstract   Traditional methods used to study strawberry ripening-related gene function are time-consuming, and require at least 15 months from initiating the transformation experiment until the first ripe fruits are available for analysis. To accelerate data acquisition during gene function studies, we explored a transient assay method that employs an *Agrobacterium*-mediated RNAi (AmRNAi) technique in post-harvest strawberry fruit, *Fragaria×ananassa* (*Fa*) cv. Sachinoka, a Japanese cultivar. Our results showed that artificial white light induced strong expression of *Fa′ chalcone synthase* (*Fa′ CHS*), *Fa′ chalcone isomerase* (*Fa′ CHI*), and *Fa′ flavonoid 3′-hydroxylase* orthologues (*Fa′ F3′ H*) in post-harvest fruit. *Fa′ CHS* and *Fa′ F3′ H* function was subsequently examined by performing AmRNAi with post-harvest fruit. Although reduction of light-induced *Fa′ F3′ H* expression by AmRNAi resulted in no significant change in anthocyanin content, reduction of *Fa′ CHS* significantly decreased anthocyanin levels, and up-regulated *Fa′ F3′ H* levels. Our results are consistent with previous data indicating that while *CHS* is required for anthocyanin accumulation during late stage strawberry fruit maturation, *Fa′ F3′ H* is not required. The novel system described here enabled gene function data to be available within 10 days of initiating the incubation period following infiltration. Therefore, we conclude our system is a valuable tool to elucidate the molecular mechanisms underlying light-induced ripening of strawberry fruit.

Key words: *Agrobacterium*-mediated RNAi, *chalcone synthase*, *flavonoid 3′-hydroxylase*, light-induced pigmentation, strawberry fruit.

Strawberry is a member of the genus *Fragaria* (Rosaceae), which is comprised of approximately 20 described species, including many hybrids and cultivars. The gene functions of several *Fragaria* members have been identified directly by appropriate assays, or inferred based on homology to genes of known function in other organisms (Manning 1998; Wein et al. 2002; Aharoni et al. 2001; Paolocci et al. 2011). While highly instructive, the traditional approaches applied to determine gene functions, particularly strawberry ripening-related genes, are time-consuming. It takes at least 15 months from the transformation experiment until the first ripe fruits are available for analysis (Hoffmann et al. 2006). Because of this extended experimental time frame, the molecular mechanisms underlying strawberry fruit ripening have

not been thoroughly investigated. However, a relatively new rapid assay system to assess gene functions related to strawberry fruit ripening has been proposed by Hoffman et al. (2006). A transient assay system where strawberry fruit genes were knocked down by RNAi with an intron-containing self-complementary hairpin RNA (ihpRNA) construct via *Agrobacterium* (*Agrobacterium*-mediated RNAi, AmRNAi) was successfully employed (Hoffmann et al. 2006). *Fragaria×ananassa chalcone synthase* (*Fa′ CHS*) was used as the reporter gene, and ihpRNA reduced *Fa′ CHS* function, which caused an immediate loss of fruit pigmentation; therefore easily detected (Hoffmann et al. 2006; Munoz et al. 2010). Hoffman et al. (2006) used the receptacles of growing fruits, which remained attached to the plant. We applied this method

to elucidate mechanisms underlying light-induced pigmentation of post-harvest fruit.

To observe the effects of light on ripening receptacles, we examined whether it is possible to use post-harvest strawberry receptacles, instead of receptacles attached to the plant, which were utilized in the earlier investigation (Hoffmann et al. 2006). The use of post-harvest receptacles provides the capacity to control illumination conditions more easily and accurately. We also examined whether *Am*RNAi can be used in post-harvest receptacles. Ripening-related metabolites, and strawberry fruit genes have been previously identified by metabolite profiling, and cDNA microarray analyses in different developmental stages (Aharoni and O'Connell 2002). The three major anthocyanidins, i.e., pelargonidin (orange or red), cyanidin (pink or red), and delphinidin (purple or blue), which differ only in the hydroxylation pattern, are involved in strawberry plant pigmentation. The strawberry anthocyanin biosynthetic genes include *Fa′CHS*, *chalcone isomerase* (*Fa′CHI*), *flavonoid 3′-hydroxylase* (*Fa′F3′H*), and *dihydroflavonol 4-reductase* (*Fa′DFR*). CHS catalyzes naringenin formation; the precursor of several flavonoids derived from malonyl-CoA and *p*-coumaroyl-CoA, and is known as a key enzyme in flavonoid biosynthesis. F3′H is a member of the cytochrome P450-dependent monooxygenases (P450) superfamily, and an important gene/enzyme that adds an additional hydroxyl group to the flavan structure B-ring. It converts kaempferol to quercetin, and pelargonidin to cyanidin. Transcription factors *Fa′MYB1* and *Fa′MYB10* are known to respectively regulate anthocyanin biosynthesis in red ripe fruit tissue (Aharoni et al. 2001), and fruit ripening and color change (Lin-Wang et al. 2010). In this study, we investigated the expression profiles of six anthocyanin synthetic genes (*Fa′CHS*, *Fa′CHI*, *Fa′F3′H*, *Fa′DFR*, *Fa′MYB1*, and *Fa′MYB10*) during light-induced ripening of the post-harvest white-stage strawberry fruit. Furthermore, we analyzed *Fa′CHS* and *Fa′F3′H* functions by *Am*RNAi. Our results indicated *Fa′CHS* was required for pelargonidin 3-glucoside accumulation at late stage strawberry fruit ripening (Hoffmann et al. 2006), however *Fa′F3′H* was not necessary (Carbone et al. 2009). Our novel post-harvest RNAi strategy provided an examination of light-induced and ripening-related strawberry gene functions within 10 days of initiating the incubation period following infiltration.

Materials and methods

Strawberry fruit samples

The octaploid strawberry *Fragaria×ananassa* cv. Sachinoka was cultivated under greenhouse conditions from winter to spring. Strawberries were harvested either as full-sized green fruit (3–3.5 cm long), or white fruit with green achenes (W). Sepals and stamens were removed from the receptacle of each sample after harvest. The entire sample was sterilized with 70% ethanol, and sprayed with 0.2% AMISTAR 20 (Syngenta) to prevent mold. The cut ends of the stalks were wrapped with sterilized wet cotton, and the samples were placed in a plant box, and maintained at 23°C with a 16-h light : 8 h dark photoperiod under 14,000 lx irradiance provided by white fluorescent lights in a MLR-351 growth chamber (SANYO) (Figure 2B).

Plasmid construction and agro-infiltration

pBI-*GUS*-intron and pBI-*CHS*-*ihpRNA* (pBI-*CHSi*) constructs were used for control experiments (Hoffmann et al. 2006). The pBI-*GFP* binary vector (pBI-sense, antisense-GW) was purchased from Inplanta Innovations, Inc., Japan. cDNA fragments of *Fragaria×ananassa* cv. Sachinoka *F3′H* (*Fa′F3′H*) were isolated by performing homology-based RT-PCR, and 5′ or 3′-RACE PCR using degenerate and gene-specific primers (Table S1). The *Fa′F3′H* fragments were amplified using primers (forward: 5′-CTAGGAGCTCTCTAGATAAGGAGA CGTTCCGGCTTC-3′ and reverse: 5′-CTAGACTAGTGC TAGCGTCACATCCGCCAAGGTC-3′), and ligated into the pSK vector containing the *Xba*I/*Nhe*I or *Spe*I/*Sac*I restriction sites (sense or antisense direction, respectively), separated by an intron from the strawberry quinone oxidoreductase gene. The pSK-*Fa′F3′Hi* plasmid was cut with *Asc*I, containing the CaMV35S promoter and hairpin construct, and NOS terminator. The digested insert was cloned into the *Asc*I-cut pBI-*GFP* binary vector. The pBI-GFP-*Fa*F3′*Hi* construct was introduced into *Agrobacterium tumefaciens* strain GV2260.

The agro-infiltration method followed the Spolaore et al. (2001) protocol. For *Am*RNAi, the injection was repeated on days 2 and 4 following incubation. The samples injected with infiltration buffer, or the pBI-*GFP*-infiltrated fruit, were used as experimental controls.

RNA isolation and Quantitative real-time RT-PCR analysis

Prior to RNA isolation, achenes were removed from the receptacles. Total RNA was isolated using the RNAqueous kit with Plant RNA Isolation Aid (Ambion, Carlsbad, CA, USA). First-strand cDNA was synthesized from 250 ng of total RNA using the Superscript III First-Strand cDNA Synthesis System (Invitrogen), and oligo d(T) as primers. Quantitative RT-PCR was performed on an ABI 7900HT system (Applied Biosystems, Tokyo, Japan). *Fa′CHS* and *Fa′F3′H* forward primers were designed at the upstream RNAi target region. Gene specific primers were used to investigate expression levels (Table S1). PCR reactions were performed in a mixture containing 1–4.6 μl of diluted cDNA, 100 nM of each primer, and 5 μl of 2× Power SYBR® Green PCR Master Mix (Applied Biosystems). The following amplification parameters were applied: 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. The target quantity in each sample was normalized to the reference control (*GAPDH* or *actin*) using the comparative ($2^{-\Delta\Delta Ct}$) method following the manufacturer's

instructions.

Anthocyanin extraction

For anthocyanin extraction, minced receptacles were weighed and placed in 1% HCl in methanol, and incubated for 4–8 h in the dark at 4°C. Following extraction and centrifugation at 12,000 rpm for 10 min, the supernatant absorbance was measured at 530 and 657 nm. The anthocyanin concentration was calculated using $A_{530}-A_{657}$ per gram fresh weight (Santiago-Domenech et al. 2008). For cyanidin 3-*O*-glucoside or pelargonidin 3-*O*-glucoside quantitative analysis in *Am*RNAi fruit, LC-MS analysis was performed as described previously (Hoffmann et al. 2006; Munoz et al. 2010).

Statistical analysis

Data were expressed as mean±standard deviation (SD). Statistical analyses were performed using a two-tailed Student's *t*-test.

Results

Expression analysis of pigmentation-related genes during light-induced pigmentation in strawberry fruit

We performed homology-based RT-PCR with degenerate primers (Table S1), and 5′ or 3′-RACE PCR using anchor- and gene-specific primers to isolate cDNA fragments of *Fragaria×ananassa* cv. Sachinoka *F3′H* (*Fa′F3′H*). The deduced amino acid sequence of the *Fa′F3′H* clone (GenBank accession no. AB665441) exhibited 70% amino acid identity to *Arabidopsis thaliana F3′H* (NP_196416), 79% amino acid identity to grape *Vitis vinifera F3′H* (CAI54278), and 88% amino acid identity to apple *Malus×domestica F3′H* (ACR14867) (Figure S1). Alignment was performed with ClustalW. Domains conserved among many cytochrome P450s, including the heme-binding site (HBS) and oxygen-binding site (OBS), were detected in the *Fa′F3′H* sequence. In addition, the characteristic hydrophobic membrane anchor and substrate recognition site 1 (SRS1) were present at the N-terminal site. Therefore, a comparative analysis revealed the *Fa′F3′H* clone is a gene encoding the F3′H enzyme.

When W stage fruit was re-exposed to white fluorescent light at 4,000 lx for 16 h per day in the growth chamber, red pigmentation was observed in the skin following 5 days of light exposure. The fruit skin and flesh were completely red after 10–14 days (data not shown). However, fruit color or softening in early green stages (G1–G2) was not affected by light treatment (data not shown). In contrast, when exposed to 14,000 lx, red pigmentation was observed after 2.5 days of irradiation, and the entire fruit (skin and flesh) was red after 5 days of light treatment (Figure 1A). Anthocyanin concentration was elevated following approximately 7.5 days exposure to white fluorescent light (Figure 1B). The onset of anthocyanin synthesis occurred at about 2.5 days, and was complete in approximately one week under 14,000 lx.

Fa′CHS, *Fa′CHI*, *Fa′DFR*, *Fa′F3′H*, and *Fa′MYBs* expression was examined using quantitative RT-PCR analysis during post-harvest receptacle pigmentation in the growth chamber. A marked increase in *Fa′CHS* and *Fa′CHI* transcripts were detected after 5 days of incubation, and a subsequent decrease after 7.5 days of incubation (Figures 1C, E). *Fa′F3′H* relative expression level at 5 days following incubation was higher than at 2.5 days, but relatively lower than prior to incubation (Figure 1D). *Fa′DFR*, *Fa′MYB10*, and *Fa′MYB1* were also up-regulated during incubation (Figures 1F–H).

To evaluate the effects of light exposure on transcriptional gene regulation, we compared gene expression levels in post-harvest fruit that were either exposed to full light, or shaded from light in the growth chamber (Figures 1I–K). *Fa′CHS*, *Fa′CHI*, and *Fa′MYB10* expression levels increased at 2.5 days in the light-exposed group, compared to the shaded group. *Fa′F3′H* expression levels increased continuously at 2.5, 5, and 7.5 days after incubation. In contrast, *Fa′DFR* and *Fa′MYB1* expression levels were not significantly different between the two groups.

Transient gene expression in post-harvest receptacles by agro-infiltration with pBI-GFP and pBI-GUS-intron

Agro-infiltration was used to achieve transient gene expression in the W stages of strawberry fruit following detachment from the plant (Figure 2A). Post-harvest receptacles were injected with an *Agrobacterium* suspension containing the pBI-*GFP* or pBI-*GUS*-intron binary vector. GFP expression was detected 2 days after infiltration; at which time it was partially detected, and complete expression was observed until 5 days after infiltration (Figures 2C, D); GFP expression was not observed in achenes. A reporter *GUS* intron gene (pBI-*GUS*-intron) was used to verify that the observed GUS activity was not due to its expression in *Agrobacterium* cells (Hoffmann et al. 2006). Compared with control fruit injected with buffer, GUS activity was widely visualized by histochemical staining using X-Gluc, a substrate for beta-glucuronidase, 1 wk post infiltration (Figures 2E, F). Achenes did not exhibit GUS activity. Five days following injection, fruit did not exhibit red coloration, however all samples were red after 10 days. Side effects of *Agrobacterium* suspension infiltration (up to a final OD_{600} of ≤2.4) were not observed in injected immature fruit (e.g., premature ripening and decomposition), therefore nearly full-sized (3–3.5 cm long) white fruit was used for *Agrobacterium*-mediated RNAi (*Am*RNAi).
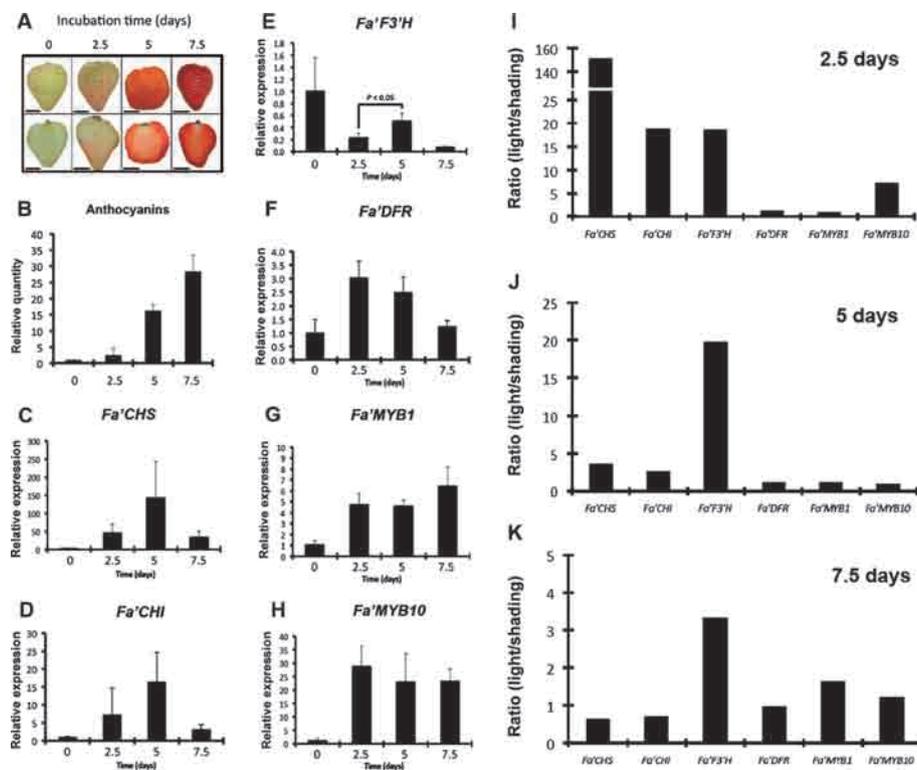
Figure 1.   Expression profiling of strawberry anthocyanin genes during light-induced ripening of post-harvest fruit. (A) Pigmentation induction of white stage fruit following irradiation by white fluorescent light. Representative phenotypes are shown after several days of treatment in growth chambers. (B) Relative total anthocyanin levels at different time points. (C–H) QPCR analysis of *Fa'CHS* (C), *Fa'CHI* (D), *Fa'F3'H* (E), *Fa'DFR* (F), *Fa'MYB1* (G), and *Fa'MYB10* (H). Data are normalized with a reference control (*Fa'GAPDH*). (I–K) Effects of light on the expression of anthocyanin synthetic genes. The ratio of the gene expression level in the light and shade was calculated at 2.5 (I), 5 (J), and 7.5 days (K) after incubation, respectively. Data are expressed as mean±S.D. (n=4–6). Scale bars indicate 1 cm.
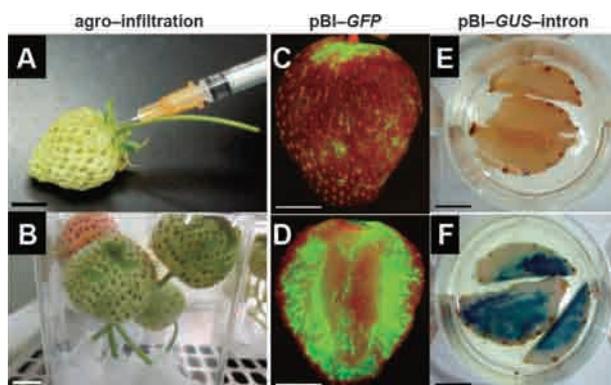

Figure 2.   Transient gene expression in the receptacle by agro-infiltration with pBI-*GFP* and pBI-*GUS*-intron. (A) The *Agrobacterium* suspension was evenly injected throughout the fruit using a sterile 1 ml syringe with 27-G needle. (B) The infiltrated fruits were maintained in a plant box, and transferred to a growth chamber. (C, D) GFP expression of pBI-*GFP*-infiltrated fruit in frontal view (C) and sectional view (D) at 5 days after infiltration. (E, F) GUS staining in controls (E) and pBI-*GUS*-intron-infiltrated fruit (F) at 7 days after infiltration. Scale bars indicate 1 cm.
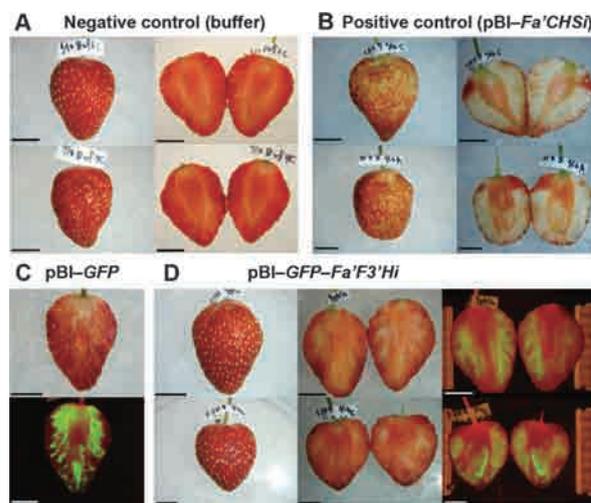

Figure 3.   Phenotypes by *Agrobacterium*-mediated RNAi (*A*mRNAi). (A) Fruits injected with buffer as a negative control. Seven days after injection, fruits exhibited entirely red flesh. (B) Fruits 7 days following infiltration with pBI-*CHSi* for *A*mRNAi of *chalcone synthase* (*Fa'CHS^AmRNAi*). Loss of pigmentation confirmed the silencing of the target gene. (C) Fruit infiltrated with *Agrobacterium* containing the control vector pBI-*GFP*. After 7 days of incubation, the infiltrated fruit demonstrated that strawberry withstands *Agrobacterium* and exogenous gene expression. (D) Fruit after 7 days of pBI-*GFP-Fa'F3'Hi* infiltration for *A*mRNAi of *flavonoid 3'-hydroxylase* (*Fa'F3'H^AmRNAi*). The reduction of pigmentation was observed in the GFP-co-expressing region. Scale bars indicate 1 cm.

Fa′CHS or *Fa′F3′H* transient down-regulation in post-harvest W-stage strawberry fruit

We targeted the *Fa′CHS* gene as a control for RNAi experiments to examine whether AmRNAi can be used to silence a gene expressed during light-induced post-harvest strawberry fruit maturation. An *Agrobacterium* suspension containing the pBI-*CHSi* or pBI-*GFP-FaF3′Hi* construct was injected into post-harvest white fruit. If the entire fruit turned red by day 5, or exhibited partial color change but remained hard due to incomplete pollination, the samples were excluded before the third infiltration (days 4 or 5). The *Fa′CHS* shRNA construct was based on the sequence obtained from *Fragaria×ananassa* cv. Elsanta. The target sequence region of two *Fa′CHS* genes between Elsanta and Sachinoka showed 97% nucleotide sequence identity (294/303 bp). One week following incubation, 100% of the control fruit turned completely red ($n=9/9$) (Figure 3A), however, the pBI-*CHSi*-infiltrated fruit (*Fa′CHS^{AmRNAi}* fruit) showed a white-red chimeric phenotype ($n=17/23$, 74%) (Figure 3B), a sign of impaired anthocyanin accumulation (Hoffmann et al. 2006; Munoz et al. 2010).

Agro-infiltration with pBI-*GFP* showed the absence of GFP expression in achenes. However, one week following incubation, pBI-*GFP*-infiltrated fruit exhibited nearly complete red-colored flesh, including the GFP expressing regions (Figure 3C). Compared with buffer only injected control fruit, total anthocyanin levels in pBI-*GFP*-infiltrated fruit showed no significant change at 10 days post-incubation (1.00 ± 0.07, $n=4$, and 0.97 ± 0.30, $n=3$; data not shown). The W stage strawberry fruit exhibited the absence of pigmentation changes following agro-infiltration with the *Agrobacterium* GV2260 strain carrying the vector pBI-*GFP*, as a marker to visualize the *Agrobacterium*-infected region. *Fa′F3′H^{AmRNAi}* fruit showed a weaker phenotype than *Fa′CHS^{AmRNAi}* fruit, therefore we used the pBI-*GFP-Fa′F3′Hi* vector to select samples expressing *Fa′F3′H* shRNA efficiently, based on GFP fluorescence. The pBI-*GFP-Fa′F3′Hi*-infiltrated fruit exhibited a GFP negative efficiency of 31% ($n=12/39$), and GFP positive efficiency of 69% ($n=27/39$). In GFP positive pBI-*GFP-Fa′F3′Hi*-infiltrated fruit (*Fa′F3′H^{AmRNAi}* fruit) samples, a mild reduction in color intensity was observed in GFP-expressing regions, with 60% efficiency ($n=16/27$) (Figure 3D).

Analyses of total anthocyanin and gene expression levels in post-harvest fruit

Achenes were completely removed from receptacles prior to anthocyanin or RNA extraction for anthocyanin concentration and gene expression analyses. A comparative gene expression level analysis one week after incubation revealed marked reductions in *Fa′CHS* in the *Fa′CHS^{AmRNAi}* fruit expression levels, relative to
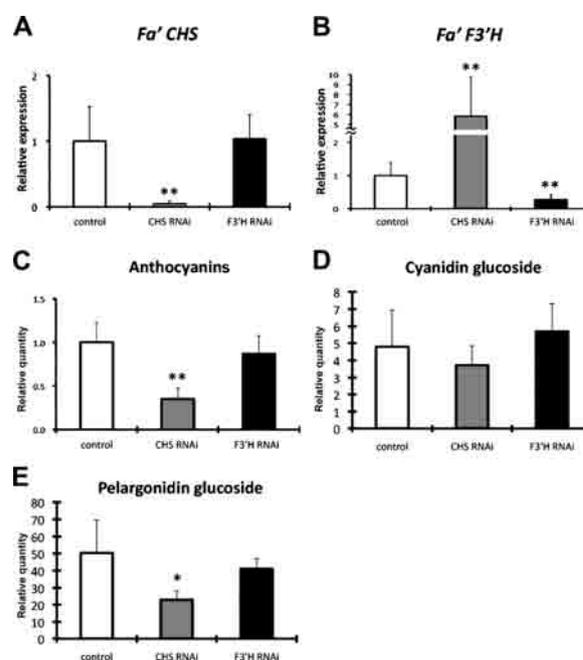

Figure 4. Analyses of gene expression and anthocyanin levels. (A, B) Relative expression levels of *Fa′CHS* (A), and *Fa′F3′H* (B) in control fruit ($n=6$), *Fa′CHS^{AmRNAi}* fruit ($n=9$), and *Fa′F3′H^{AmRNAi}* fruit ($n=14$), respectively. (C–E) Relative concentration of total anthocyanins (C), cyanidin glucoside (D), and pelargonidin glucoside (E) in control fruit ($n=3–6$), *Fa′CHS^{AmRNAi}* fruit ($n=4–9$), and *Fa′F3′H^{AmRNAi}* fruit ($n=6–12$). Significant differences, relative to the control, are indicated by * $p<0.05$ and ** $p<0.01$.

controls (Figure 4A). It was clear that suppression of the *Fa′CHS* gene by RNAi resulted in strong inhibition of anthocyanin production. In the *Fa′CHS^{AmRNAi}* fruit, *Fa′F3′H* expression was notably increased (Figure 4B), likely as compensation for the low anthocyanin concentration. RNAi-mediated *Fa′F3′H* silencing caused an expected down-regulation of *Fa′F3′H* transcript levels in GFP-positive and color-reduced fruit, while *Fa′CHS* levels remained unchanged, indicating that an apparent reduction in pigmentation was due to decreased *Fa′F3′H* expression levels by RNAi.

Total anthocyanin concentration in *Fa′CHS^{AmRNAi}* fruit was significantly reduced to less than 35% of control fruit levels (0.35 ± 0.12, $n=9$, and 1.00 ± 0.24, $n=12$, respectively; $p<0.01$) (Figure 4C). However, a significant difference in total anthocyanin concentration between the control and *Fa′F3′H^{AmRNAi}* fruit was not detected (1.00 ± 0.24, $n=12$, and 0.87 ± 0.20, $n=12$, respectively). Two major anthocyanins, cyanidin 3-glucoside and pelargonidin 3-glucoside, were quantified by LC-MS analysis. However, relative quantity of cyanidin 3-glucoside or pelargonidin 3-glucoside in *Fa′F3′H^{AmRNAi}* fruit was indistinguishable from control fruit (Figures 4C, D). While results showed the relative quantity of cyanidin 3-glucoside in *Fa′CHS^{AmRNAi}* fruit was unchanged, the level of pelargonidin 3-glucoside was significantly reduced in RNAi fruit (Figures 4C, D).

Discussion

In this study, we investigated a light-induced ripening system with post-harvest strawberry fruit, and developed a protocol to effectively elucidate fruit ripening mechanisms using an *Am*RNAi technique in a small, artificial environment. In post-harvest W stage fruit, the onset of anthocyanin synthesis occurred at approximately 2.5 days after incubation initiation, and was completed in approximately one week, when the fruit was exposed to white light irradiation at 14,000 lx. *Fa′CHS*, *Fa′CHI*, *Fa′DFR*, *Fa′MYB1*, and *Fa′MYB10* expression levels increased during incubation at 2.5–5 days (Figure 1). This period may correspond to the natural turning stage. Transcript levels of *Fa′CHS* and *Fa′CHI* genes have previously been shown to be relatively high at the turning stage, when the strawberry receptacle begins a gradual pigmentation change during developmental maturation (Carbone et al. 2009; Salvatierra et al. 2010). *Fa′F3′H* expression, however, was intense during the early stages but decreased as maturation progressed (Carbone et al. 2009). *Fa′F3′H* expression was markedly up-regulated in light-exposed fruit in contrast to shaded fruit (Figure 1). These results indicated that major anthocyanin synthetic genes were expressed in post-harvest fruit, and were dependent on irradiance level.

When an *Agrobacterium* suspension containing the pBI-*GUS*-intron or pBI-*CHSi* was infiltrated into the post-harvest fruit, the resulting fruits expressed *GUS* mRNA, or were white-red chimeric phenotypes (Figures 2F, 3B). These data are very consistent with previous results obtained when growing fruit receptacles that remained attached to plants (Hoffmann et al. 2006). Post-harvest fruit, therefore, can be effectively applied to study the influence of light exposure on anthocyanin synthetic gene expression.

The *Fragaria* genome may contain several *CHS* genes, and many of these genes are expressed during fruit development and maturation (Almeida et al. 2007). Our data indicated we successfully cloned and analyzed one *Fa′CHS* gene, which was expressed primarily during late stage fruit development. Most flavonol glucosides and proanthocyanidins in *Fragaria* are primarily synthesized in unripe green fruit, with anthocyanins accumulated in appreciable amounts during latter fruit ripening stages (Halbwirth et al. 2006). In several Japanese strawberry cultivars, including Sachinoka, the relative percentage of cyanidin 3-glucoside was high during the early color development stage, although pelargonidin 3-glucoside was the predominant pigment (66–94% of total anthocyanins) in mature receptacles (Yoshida et al. 2002). In post-harvest strawberry fruit, relative *Fa′F3′H* induction levels were much lower than preincubation levels, whereas the opposite was true for *Fa′CHS*. Although reduction of light-induced *Fa′F3′H* expression

by *Am*RNAi techniques resulted in no significant change in the relative amount of cyanidin 3-glucoside, decreased *Fa′CHS* expression resulted in a substantial reduction of pelargonidin 3-glucoside, and up-regulation of *Fa′F3′H*. The up-regulation of *Fa′F3′H* may be compensation for low anthocyanin concentrations. It is interesting to note that although CHS is an upstream enzyme of F3′H in the anthocyanin pathway, reduction of light-induced *Fa′CHS* expression by AmRNAi techniques resulted in the absence of a significant change in cyanidin 3-glucoside levels compared to controls. These results suggested that light-induced pigmentation predominantly enhanced pelargonidin synthesis, as observed in natural fruit ripening (Halbwirth et al. 2006).

In conclusion, the assay system reported here is suitable for gene regulatory function analyses under varied light quality and quantity conditions during strawberry fruit ripening and pigmentation maturation. These data provide new insights into the relationship between light signaling and ripening in fruit.

References

Aharoni A, O'Connell AP (2002) Gene expression analysis of strawberry achene and receptacle maturation using DNA microarrays. *J Exp Bot* 53: 2073–2087

Aharoni A, De Vos CH, Wein M, Sun Z, Greco R, Kroon A, Mol JNM, O'Connell AP (2001) The strawberry *FaMYB1* transcription factor suppresses anthocyanin and flavonol accumulation in transgenic tobacco. *Plant J* 28: 319–332

Almeida JR, D'Amico E, Preuss A, Carbone F, de Vos CH, Deiml B, Mourgues F, Perrotta G, Fischer TC, Bovy AG, Martens S, Rosati C (2007) Characterization of major enzymes and genes involved in flavonoid and proanthocyanidin biosynthesis during fruit development in strawberry (*Fragaria*×*ananassa*). *Arch Biochem Biophys* 465: 61–71

Carbone F, Preuss A, De Vos RCH, D'Amico E, Perrotta G, Bovy AG, Martens S, Rosati C (2009) Developmental, genetic and environmental factors affect the expression of flavonoid genes, enzymes and metabolites in strawberry fruits. *Plant Cell Environ* 32: 1117–1131

Halbwirth H, Puhl I, Haas U, Jezik K, Treutter D, Stich K (2006) Two-phase flavonoid formation in developing strawberry (*Fragaria*×*ananassa*) fruit. *J Agric Food Chem* 54: 1479–1485

Hoffmann T, Kalinowski G, Schwab W (2006) RNAi-induced silencing of gene expression in strawberry fruit (*Fragaria*×*ananassa*) by agroinfiltration: a rapid assay for gene

function analysis. *Plant J* 48: 818–826

Lin-Wang K, Bolitho K, Grafton K, Kortstee A, Karunairetnam S, McGhie TK, Espley RV, Hellens RP, Allan AC (2010) An R2R3 MYB transcription factor associated with regulation of the anthocyanin biosynthetic pathway in Rosaceae. *BMC Plant Biol* 10: 50–66

Manning K (1998) Isolation of a set of ripening-related genes from strawberry: their identification and possible relationship to fruit quality traits. *Planta* 205: 622–631

Muñoz C, Hoffmann T, Escobar NM, Ludemann F, Botella MA, Valpuesta V, Schwab W (2010) The strawberry fruit Fra a allergen functions in flavonoid biosynthesis. *Mol Plant* 3: 113–124

Paolocci F, Robbins MP, Passeri V, Hauck B, Morris P, Rubini A, Arcioni S, Damiani F (2011) The strawberry transcription factor *FaMYB1* inhibits the biosynthesis of proanthocyanidins in *Lotus corniculatus* leaves. *J Exp Bot* 62: 1189–1200

Salvatierra A, Pimentel P, Moya-Leon MA, Caligari PDS, Herrera R (2010) Comparison of transcriptional profiles of flavonoid genes and anthocyanin contents during fruit development of two botanical forms of *Fragaria chiloensis* ssp. *chiloensis*. *Phytochemistry* 71: 1839–1847

Santiago-Doménech N, Jiménez-Bemúdez S, Matas AJ, Rose JKC, Muñoz-Blanco J, Mercado JA, Quesada MA (2008) Antisense inhibition of a pectate lyase gene supports a role for pectin depolymerization in strawberry fruit softening. *J Exp Bot* 59: 2769–2779

Spolaore S, Trainotti L, Casadoro G (2001) A simple protocol for transient gene expression in ripe fleshy fruit mediated by *Agrobacterium*. *J Exp Bot* 52: 845–850

Wein M, Lavid N, Lunkenbein S, Lewinsohn E, Schwab W, Kaldenhoff R (2002) Isolation, cloning and expression of a multifunctional *O*-methyltransferase capable of forming 2,5-dimethyl-4-methoxy-3(2*H*)-furanone, one of the key aroma compounds in strawberry fruits. *Plant J* 31: 755–765

Yoshida Y, Koyama N, Tamura H (2002) Color and anthocyanin composition of strawberry fruit: changes during fruit development and differences among cultivars, with special reference to the occurrence of pelargonidin 3-malnoylglucoside. *J Japan Soc Hort Sci* 71: 355–361