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The acyl-activating enzyme PhAAE13 is an alternative enzymatic source of precursors for anthocyanin biosynthesis in petunia flowers

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Abstract

Anthocyanins, a class of flavonoids, are responsible for the orange to blue coloration of flowers and act as visual attractors to aid pollination and seed dispersal. Malonyl-CoA is the precursor for the formation of flavonoids and anthocyanins. Previous studies have suggested that malonyl-CoA is formed almost exclusively by acetyl-CoA carboxylase, which catalyzes the ATP-dependent formation of malonyl-CoA from acetyl-CoA and bicarbonate. In the present study, the full-length cDNA of Petunia hybrida acyl-activating enzyme 13 (PhAAE13), a member of clade VII of the AAE superfamily that encodes malonyl-CoA synthetase, was isolated. The expression of PhAAE13 was highest in corollas and was down-regulated by ethylene. Virus-induced gene silencing of petunia PhAAE13 significantly reduced anthocyanin accumulation, fatty acid content, and cuticular wax components content, and increased malonic acid content in flowers. The silencing of PhAAE3 and PhAAE14, the other two genes in clade VII of the AAE superfamily, did not change the anthocyanin content in petunia flowers. This study provides strong evidence indicating that PhAAE13, among clade VII of the AAE superfamily, is specifically involved in anthocyanin biosynthesis in petunia flowers.

Key words: AAE13, anthocyanin synthesis, malonic acid, malonyl-CoA, petunia.

Introduction

Flavonoids are secondary metabolites that are common to all higher plants. This category of compounds includes anthocyanins, flavanones, flavones, and flavonols (Koes et al., 1994). Anthocyanin pigments provide flowers with bright red and blue colors, and are induced in vegetative tissues by various signals (Mol et al., 1996). The anthocyanin biosynthetic pathway likely represents one of the best-studied examples of higher plant secondary metabolism (Fig. 1) (Koes et al., 2005; Rausher et al., 1999). The genes encoding flavonoid enzymes have been isolated from a variety of plant species. In plants, flavonoids are formed by adding three molecules of malonyl-CoA to a coumaroyl-CoA starter, which is catalyzed by chalcone synthase and forms naringenin chalcone (Halls and Yu, 2008).
Malonyl-CoA is the precursor for the formation of flavonoids and anthocyanins (Peer et al., 2001). It has been generally accepted that malonyl-CoA is formed almost exclusively by acetyl-CoA carboxylase (ACC; EC 6.4.1.2), which catalyzes the ATP-dependent formation of malonyl-CoA from acetyl-CoA and bicarbonate (Belkebir and Benhassaine-Kesri, 2013; Fatland et al., 2005).

In addition to ACCs, malonyl-CoA synthetase ligates malonic acid and CoA to generate malonyl-CoA directly in plants. Arabidopsis acyl-activating enzyme 13 (AtAAE13), a member of the clade VII AAE superfamily, was identified as a malonyl-CoA synthetase (Chen et al., 2011; Shockey and Fulda, 2003). Because aae13-null mutants grow poorly and accumulate malonic acid, AAE13 has been implicated in the detoxification of short-chain organic acids (Chen et al., 2011). The overexpression of AtAAE13 in Saccharomyces cerevisiae simultaneously increased lipid and resveratrol accumulation, and AAE13 partially complemented the temperature-sensitive acc1 mutant, replacing this key enzyme in central metabolism (Wang et al., 2014). Recently, Guan and Nikolau (2016) suggested that Arabidopsis AAE13 has two isoforms, translated from two types of transcripts—one that contains a mitochondrial-targeting pre-sequence, and one that does not—that are localized in both cytosol and mitochondria. In addition, bacterial malonyl-CoA synthetase has been used in fatty acid and flavonoid biosynthesis (Leonard et al., 2008; Park et al., 2011). However, it remains unknown whether the AAE13 pathway, which catalyzes the synthesis of malonyl-CoA from malonic acid, is involved in anthocyanin biosynthesis.

The petunia flower has served as a model for the study of flavonoid or anthocyanin synthesis (Gerats and Vandenbussche, 2005). More than 10 genes encoding enzymes and transcription regulators involved in anthocyanin synthesis, such as ANTHOCYANIN2 (AN2), AN1, and AN11, have been identified (Koes et al., 2005). The blue petal color of petunia phl mutants reflects a failure to hyperacidify vacuoles, and PH1 encodes a P3BATPase, hitherto known as a Mg^{2+} transporter in bacteria only, which resides in the vacuolar membrane (tonoplast) (Faraco et al., 2014).

In the present study, petunia PhAAE13 full-length cDNA was isolated. The expression of PhAAE13 was decreased by ethylene treatment and increased by ultraviolet B (UV-B) radiation. Virus-induced gene silencing (VIGS) of PhAAE13 significantly reduced anthocyanin accumulation and increased malonic acid accumulation in petunia. These results indicate that the new structural gene PhAAE13 plays an important role in anthocyanin biosynthesis.

**Fig. 1.** A simplified view of the anthocyanin biosynthesis pathway (Koes et al., 2005; Rausher et al., 1999). 3GT, UDP-glucose:flavonoid 3-O-glycosyl transferase; 4CL, 4-coumarate:CoA ligase; AAE13, acyl-activating enzyme 13 (malonyl-CoA synthetase); ACC, acetyl-CoA carboxylase; ANS, anthocyanin synthase; C4H, cinnamate-4-hydroxylase; CHI, chalcone flavanone isomerase; DFR, dihydroflavonol 4-reductase; F3H, flavanone 3′-hydroxylase; FAE, fatty acid elongase; PAL, phenylalanine ammonia-lyase.

**Materials and methods**

**Plant materials**

*Petunia hybrida* ‘Ultra’ plants were grown under greenhouse conditions (22–25 °C, 14 h light/10 h dark) as described by Yang et al. (2015). Eight to ten petunia flowers were harvested at anthesis (corollas 90° reflexed) and immediately placed in tap water. The stems, leaves, and roots were collected from plants at the vegetative stage when the plants were ~25 cm in height. All tissues were frozen in liquid nitrogen and stored at ~80 °C until used for RNA extraction. The fresh weights were measured immediately before freezing (Yang et al., 2015). All experiments were conducted at least three times with independently collected and extracted tissues unless otherwise noted.

**RNA extraction, RT-PCR, and cloning of the petunia PhAAE13, PhAAE3, PhAAE14, and PhACC2 genes**

Total RNA was isolated and reverse transcribed according to the methods of Liu et al. (2011). PhAAE13, PhAAE3, PhAAE14, and PhACC cDNAs were cloned according to previously described protocols (Yu et al., 2011). Degenerate primers were designed based on conserved sequences in AAE13 cDNA from Arabidopsis thaliana (AAM61199, NP_190468, NP_174340 and NP_174849), Solanum lycopersicum (XP_01341289, XP_00423495, XP_00433163 and XP_00425254), and Vitis vinifera (CBD6114, XP_02267459, XP_010655748 and XP_002288508). Degenerate primers (see Supplementary Table S1 at JXB online) were used to generate PCR products from Petunia cDNA. The remaining 5′ and 3′ cDNA sequences were isolated using rapid amplification of cDNA ends (RACE). Full-length cDNAs for PhAAE13, PhAAE3, PhACC1, and PhACC2, and the partial cDNA for PhAAE14, were isolated by RT-PCR using the specific primers (Supplementary Table S1).

**Sequence analysis**

Alignments were conducted using DNAMAN software, and a phylogenetic tree was generated using MEGA version 3.1 (Kumar et al., 2004). An identity search for nucleotides and translated amino acids was conducted using the National Center for Biotechnology Information (NCBI) BLAST network server (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

**Quantitative real-time PCR assays**

Quantitative real-time PCR (qPCR) assays were performed according to Liu et al. (2011). Analyses were conducted following the Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines (Bustin et al., 2009; Tan et al., 2014). Two genes, *Actin* (accession number: FN014209) and *Cyclophilin* (CYP) (accession number: EST883944), were used as internal reference genes to quantify the cDNA abundance (Mallona et al., 2010).
Similar results were obtained for both reference genes, and the data presented in this paper represent relative expression values calculated using Actin. The sequences of all primers used for qPCR analysis are described in Supplementary Table S2. Three biological replicates were analyzed for each treatment.

**Ethylene treatment**

Petunia flowers were treated with ethylene according to previously described protocols (Spitzer-Rimon et al., 2012; Tan et al., 2014). Flowers were harvested at anthesis and the stems were re-cut to 5 cm, placed in flasks with distilled water, sealed, and subsequently treated with 2 μl l⁻¹ ethylene for 0, 4, 8 and 16 h. The corollas from 8–10 flowers were collected at each time point, immediately frozen in liquid nitrogen, and stored at −80 °C for subsequent RNA extraction. Three biological replicates were analyzed for each treatment.

**UV-B treatment**

Petunia flowers were treated with UV-B according to previously described protocols (Ryan et al., 1998; Zhou et al., 2007). Flowers were harvested at anthesis and the stems were re-cut to 5 cm, placed in flasks with distilled water, and subsequently exposed to UV-B at 7.2 W m⁻² for 1, 3, and 5 h and then transferred to darkness. Control flowers were maintained for 1, 3, and 5 h in darkness. The corollas were collected at each time point, and total RNA was extracted from these samples for qPCR analysis. Three biological replicates were analyzed for each treatment.

**Agroinoculation of tobacco rattle virus vectors**

To generate tobacco rattle virus (TRV) plasmids (pTRV2) containing the 3’ untranslated region of PhAAE13, PhAAE3, and PhAAE14 (TRV-PhAAE13, TRV-PhAAE3, and TRV-PhAAE14), sequences of approximately 250 bp of each gene were amplified by PCR using forward and reverse primers (Supplementary Table S3), and the PCR products were inserted into the pTRV2 vector. Agrobacterium tumefaciens (strain GV3101) transformed with pTRV1 and pTRV2 derivatives was prepared as previously described (Spitzer-Rimon et al., 2012; Tan et al., 2014). The Agrobacterium culture was grown overnight at 28 °C in Luria-Bertani medium with 50 mg l⁻¹ kanamycin and 200 mM acetosyringone. The cells were harvested and resuspended in inoculation buffer containing 10 mM MgCl₂, 2 mM acetosyringone, and 10 mM N-(trimethylsilyl)trifluoroacetamide/1% trimethylchlorosilane (Pierce Biotechnology) for 30 min. The metabolites were derivatized with 100 μl N-methyl-N-(trimethylsilyl)trimethylsilyl trifluoroacetamide (Supplementary Table S2) for 30 min. The sample was subsequently transferred to a 200 μl glass insert and analyzed by GC-MS.

Next, 1.0 μl of the solution was injected at a 1:1 split ratio on to a HP 6890 GC equipped with a 30 m Rtx-5 Sil MS column (Restek; 0.25 mm internal diameter and 0.25 mm film thickness) coupled to a HP 5973 MS. The injection port and transfer arm were maintained at 280 °C. Separation was achieved with a temperature program of 80 °C for 3 min, ramped at 5 °C/min to 315 °C and maintained for 6 min. The MS source was maintained at 230 °C, and the quadrupole was maintained at 150 °C and scanned using a mass-to-charge ratio of 50–800. Three biological replicates were analyzed for each treatment.

**Fatty acid analysis**

The fatty acid composition and content analysis was performed as previously described by Park et al. (2015) with minor modification. Approximately 10 mg of corollas were transmethylated at 95 °C for 90 min in 1 ml methanol containing 5% (v/v) H₂SO₄. Glyceryl triheptadecanoate was added to each sample as an internal standard. The mixture was cooled to room temperature for approximately 30 min. After transmethylation, 1.5 ml aqueous 0.9% NaCl was added, and the fatty acid methyl esters (FAMEs) were recovered by three sequential extractions with 1 ml hexane. Total FAMEs were concentrated under nitrogen gas and analyzed via gas chromatography (GC-2010, Shimadzu, Japan) on a 30 m × 0.32 mm DB-23 column (Agilent, USA). The oven temperature was held at 190 °C for 10 min and then varied from 160–230 °C at 5 °C min⁻¹. The final oven temperature was maintained at 230 °C for 10 min. The fatty acids were identified based on comparison of retention times and mass spectra against standards. Three biological replicates were analyzed for each treatment.

**Cuticular wax components analysis**

The cuticular wax components analysis was performed as previously described (King et al., 2007). Cuticular waxes were extracted from 8–12 g of petal tissue by washing once with 100 ml and once with 50 ml hexane for 30 s. The hexane was removed from the combined extracts by rotary evaporation. The wax fraction (< 30 mg) was then dissolved in hexane and applied to a 1 g silica gel column, which had been equilibrated with hexane. Lipids were then eluted with 10 ml of (i) hexane, (ii) pentene-stabilized chloroform, (iii) acetone, and (iv) methanol, and wax-esters were eluted in the chloroform. Fractions were then analyzed by GC-MS. A Shimadzu GCMS-QP2010 Ultra was used to perform all GC-MS analyses. Sample injections volumes were 1 μl. Plant waxes were analyzed using a Rtx-5MS column (30 m, 0.25 mm internal diameter, 0.25 μm film; Restek, Bellefonte, PA, USA) using a temperature program of 100 °C for 1 min, increasing to 300 °C at 20 °C min⁻¹, held at 300 °C for 10 min, increasing to 350 °C at 20 °C min⁻¹, then held at 350 °C for 10 min. Three biological replicates were analyzed for each treatment.

**Statistical analyses**

Statistical analysis was performed using one-way ANOVA followed by Duncan’s multiple range test with at least three replicates. Values of P≤0.05 were considered significant.
Results

Cloning the petunia PhAAE13 cDNA sequence

Using the homologous cloning and RACE-PCR method, the full-length cDNA of PhAAE13, containing a 1794 bp open reading frame, was obtained. The predicted PhAAE13 protein comprises 597 amino acids and has a calculated molecular mass of 66.3 kDa.

The results of the multiple sequence alignment showed that PhAAE13 had 68.1% identity to AtAAE13 (accession number: AAM61199) and 87.4% identity to SIAAE13 (accession number: XP_010314289). Similar to other clade VII AAE proteins, the primary structure of predicted PhAAE13 contains conserved AMP-binding and ACS (acyl-CoA synthetase) domains (Fig. 2; Black and DiRusso, 2007; Black et al., 1997; Shockey et al., 2003; Weimar et al., 2002). A detailed sequence alignment of the clade VII AAE homologs showed a highly conserved AMP-binding motif (PS00455) and ACS domains (Fig. 2).

Phylogenetic analysis of AAE13 proteins

Because AAE13 belongs to clade VII of the AAE superfamily (Shockey et al., 2003), we constructed a phylogenetic tree based on the AAE13 protein sequences of five plants and Arabidopsis AAE3 and AAE14, members of clade VII of the AAE superfamily. The phylogenetic tree showed that the PhAAE13 protein is most similar to AtAAE13 (Fig. 3).

To further elucidate the evolutionary relationship among AAE13-like proteins in plants, we examined the AAE13-like amino acid sequences of all known plant proteins. Using amino acid sequences derived from GenBank, a phylogenetic tree was generated with MEGA software (Supplementary Fig. S1). In all plant species examined to date, a small gene family encodes the homomeric AAE13. In most plants, one or two genes encode AAE13, except in Brassica napus, which has three AAE13s. The PhAAE13-coding amino acid sequence showed 57.6% and 60.9% identity to Selaginella moellendorffii SmAAE13a and SmAAE13b, respectively. In addition, the monocotyledon rice OsAAE13 showed 54.6% and 57.4% identity to the pteridophyte S moellendorffii SmAAE13a and SmAAE13b, respectively, suggesting that AAE13 protein sequences are highly conserved.

Expression analysis of PhAAE13

The expression pattern of PhAAE13 was examined in different plant organs, during bud development and in response to ethylene treatment using qPCR.

As shown in Fig. 4A, qPCR analysis demonstrated that the mRNA levels of PhAAE13 were highest in the corollas and lower in the roots, stems and leaves, similar to the expression of Petunia flavonone 3-hydroxylase (PhF3H) (accession number: AF022142), an anthocyanin synthesis gene (Fig. 4B).

Ethylene affects light-induced anthocyanin synthesis (Craker and Wetherbee, 1973). In the present study, the expression of PhAAE13 and PhF3H in corollas significantly decreased after ethylene treatment for 4-16 h (Fig. 4C, D).

Because flavonols often accumulate to high levels in flowers, providing protection from UV-B light (Albert et al., 2009; Li et al., 1993; Ryan et al., 2002), the effects of UV-B on PhAAE13 expression were examined using qPCR. The transcriptional level of PhAAE13 in corolla significantly increased from 1 to 3 h and remained stable from 3 to 5 h after UV-B treatment; similar results were seen for PhF3H (Fig. 4E, F).

To examine the expression of PhAAE13 during bud development, this process was divided into six stages: S1 (0.5 cm), S2 (1.0 cm), S3 (2.0 cm), S4 (3.0 cm), S5 (4.0 cm) and S6 (anthesis) (Fig. 4I). The qPCR analysis revealed that the expression of PhAAE13 is high during floral bud development, increases from S1 to S3, then decreases until S5, and slightly increases again at anthesis (S6) (Fig. 4G). The pigment of corollas gradually deepened, and the anthocyanin accumulation increased from stage S1 to S5 (Fig. 4G, H).

Silencing of PhAAE13 results in a significant reduction of anthocyanin content in petunia

To characterize PhAAE13 functionally, a loss-of-function approach was implemented using a TRV-mediated VIGS strategy optimized for Petunia ‘Ultra’ (Violet line) (Tan et al., 2014).

Following the known requirements for efficient gene silencing (Burch-Smith et al., 2006), the construct for silencing PhAAE13 was designed. To ensure that the dedicated VIGS construct targeted PhAAE13, approximately 250 bp fragments of 3′ untranslated sequences of the Petunia PhAAE13 and chalcone synthase J (PhCHSJ) cDNAs were cloned from petunia cDNA into a pTRV2 vector to examine the silencing of PhAAE13 and PhCHS (positive control), respectively.

Four weeks after infection, the flowers of TRV-PhAAE13-infected and TRV-PhCHS-infected plants showed a loss of anthocyanin pigmentation phenotype to various degrees (Fig. 5B, C), but the flowers of control plants and TRV-infected plants remained purple (Fig. 5A).

In total, the corollas of some flowers in one plant infected with TRV containing the PhAAE13 or PhCHS fragment were uniformly white to different degrees, and some flowers showed reduced pigmentation in the tube and at the base of the limb. However, infection with TRV containing the PhCHS fragment decreased anthocyanin production in the corollas to much lower levels compared with infection with TRV containing the PhAAE13 fragment (Fig. 5B, C, I); infection with TRV containing the PhCHS fragment decreased anthocyanin production in the corollas by up to 95% relative to controls, while infection with TRV containing the PhAAE13 fragment decreased anthocyanin production by up to 65% relative to controls. Moreover, anthocyanin production in the filaments and styles of the white flowers in PhAAE13- and PhCHS-silenced plants was reduced (Fig. 5H). In addition, the content of anthocyanins in mature leaves and stems was significantly reduced in PhAAE13-silenced plants (Fig. 5J, K), although this reduction was not visible to the human eye.

Transcript accumulation was examined using primers that anneal outside the gene region of PhAAE13 targeted for
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Compared with transcript accumulation in control plants, the mRNA accumulation of PhAAE13 in white flowers of PhAAE13-silenced plants was significantly reduced (Fig. 6A). The expression levels of PhAAE13 in the purple flowers of the infected plants were similar to those in the flowers of control plants.

Previous studies have shown decreased fertility in Arabidopsis aae13 mutants (Chen et al., 2011). However, in the present study, fertility was not significantly affected, and fruits and seeds developed normally in PhAAE13-silenced plants. Other growth behaviors of PhAAE13-silenced plants were indistinguishable from those of control plants (Supplementary Fig. S2).

Fig. 2. Alignment of PhAAE13 with A. thaliana AtAAE13 (BAB02683, AAM61199), S. lycopersicum SLAAE13 (XP_010314289), V. vinifera VvAAE13 (XP_002279139), and O. sativa OsAAE13 (ECC71525). White text on a black background indicates identical residues across all five sequences; dark gray shading indicates identical residues in four out of five sequences; light gray shading indicates similar residues in three out of five sequences and/or conserved substitutions. The arrow represents the start site of the sequence of another protein (ctAAE13) that is localized to the cytosol in Arabidopsis. The sequence underlined with a solid line is the conserved 12-amino acid AMP binding motif and that underlined with a dotted line is the ACS (acyl-CoA synthetase) conserved domain. The alignments were generated using DNAMAN software.
PhAAE13 silencing slightly increases the expression of PhACC1 and PhACC2

It has been suggested that ACC catalyzes the biosynthesis of malonyl-CoA (Belkebir and Benhassaine-Kesri, 2013; Fatland et al., 2005). Thus, we examined the effects of PhAAE13 silencing on the expression of PhACC. First, we isolated two full-length PhACC cDNAs, named PhACC1 and PhACC2, using the homology cloning method. Alignment and phylogenetic analysis confirmed that PhACC1 and PhACC2 are the homologs of Arabidopsis ACC1 and ACC2 (Supplementary Figs S3 and S4). qPCR analysis showed that the mRNA levels of both PhACC1 and PhACC2 were slightly but significantly increased in the corollas of PhAAE13-silenced plants compared with wild-type plants (Fig. 7A).

PhAAE13 silencing increases malonic acid accumulation in corollas

To further understand the function of PhAAE13, the malonic acid content in white corollas of PhAAE13-silenced plants at anthesis was examined. GC-MS was used to measure the tissue concentrations of malonic acid in extracts from white corollas at anthesis. In the flowers of PhAAE13-silenced plants, the malonic acid levels were 60 µg g⁻¹ fresh weight, comparable to that in wild-type flowers, which contained 25 µg g⁻¹ fresh weight (Fig. 8). This result showed that PhAAE13 silencing induced the accumulation of malonic acid in corollas.

Effects of silencing of PhAAE13 on fatty acid contents in corollas

Because malonyl-CoA is the precursor of fatty acid biosynthesis, we examined the fatty acid content in corollas of PhAAE13-silenced and control plants. The content of 5

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**Fig. 3.** Phylogenetic tree of clade VII of AAEs. Petunia PhAAE13 was aligned with A. thaliana AIAAE13 (AAM61199), AIAAE3 (NP_190468); AIAAE14 (NP_174340), S. lycopersicum SIAAE13 (XP_010314289), V. vinifera VvAAE13 (CBI36114), and O. sativa OsAAE13 (EEC71525) using DNAMAN.

**Fig. 4.** Expression of PhAAE13 determined using qPCR. (A, B) Expression of (A) PhAAE13 and (B) PhF3H in different organs. (C, D) Expression of (C) PhAAE13 and (D) PhF3H in corollas in response to exogenous ethylene. (E, F) Expression of (E) PhAAE13 and (F) PhF3H in corollas in response to UV-B. (G) Expression of PhAAE13 and (H) anthocyanin accumulation in corollas during flower development. Relative expression levels are shown as fold-change values. Data are presented as means±SD (n=3). Three repetitions are included in the data presented. Data were generated from different flowers from different plants grown in parallel. (I) Images showing the six developmental stages of petunia buds. (This figure is available in colour at JXB online.)
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of the 25 fatty acids examined was significantly reduced in PhAAE13-silenced plants compared with that of control plants; among these, a reduction of 54% of the content of C22:2 (cis-13-docosenoic acid) and 37% of C23:0 (tricosanoic acid), relative to controls, was detected (Supplementary Table S5). The content of the other 20 fatty acids detected was not significantly changed in PhAAE13-silenced plants.

Effects of PhAAE13 silencing on the cuticular wax components content of corollas

Because cytosolic malonyl-CoA is also the precursor for the biosynthesis of cuticular wax components, we examined the cuticular wax components content in PhAAE13-silenced and control plants. The content of 6 of the 10 cuticular wax components examined was significantly reduced in PhAAE13-silenced plants compared with control plants; among these components, a reduction of 46.4% of the content of C25 esters and 41.5% of C23 esters was detected (Supplementary Fig. S5).

Silencing of both PhACC1 and PhACC2 results in a significant reduction of anthocyanin content in petunia

We further examined whether PhACC1 and PhACC2 are involved in anthocyanin accumulation. TRV-PhACC1 and TRV-PhACC2 vectors were constructed and used to silence PhACC1 and PhACC2 in petunia. The flowers of PhACC1-
PhACC2- and PhAAE13-silenced plants all showed similar anthocyanin pigmentation phenotypes and anthocyanin accumulation in flowers (Fig. 5C, D, E, H, I). However, in leaves and stems, PhACC1- and PhACC2-silenced plants did not show significant reductions in anthocyanin pigmentation compared with control plants (Fig. 5J, K).

qPCR showed that the level of PhACC1 mRNA was significantly reduced in the pink flowers of TRV-PhACC1 treated plants, while levels of PhAAE13 and PhACC2 were not significantly changed (Fig. 7B, C). Similarly, the level of PhACC2 mRNA was significantly reduced in the flowers of TRV-PhACC2 treated plants, while levels of PhAAE13 and PhACC1 were not significantly changed.

Silencing of the other two genes in clade VII of PhAAEs does not change the anthocyanin content of flowers

To further examine the specificity of PhAAE13 involvement in anthocyanin accumulation, the cDNAs encoding PhAAE3 and PhAAE14, which belong to clade VII of the PhAAE superfamily, were isolated. The multiple sequence alignment and phylogenetic analysis of clade VII of PhAAEs and their homologs in Arabidopsis are shown in Supplementary Figs S6 and S7.

When TRV-PhAAE3 and TRV-PhAAE14 vectors were used to silence PhAAE3 and PhAAE14 in petunia, the flowers of PhAAE3- and PhAAE14-silenced plants and the TRV control all showed the same anthocyanin pigmentation phenotype and anthocyanin accumulation in the flowers, leaves and stems (Fig 5A, F, G, H, I). In addition, the colors of the filaments and styles of the flowers of PhAAE3- and PhAAE14-silenced plants were not significantly changed compared with the TRV control (Fig. 5H). The qPCR analysis showed that the expression of PhAAE3 and PhAAE14 was significantly reduced in plants infected with the corresponding (TRV-PhAAE3 and TRV-PhAAE14) vectors, while the expression of PhAAE13, PhAAE3, and PhAAE14 was not significantly changed in plants infected with vectors carrying the homologous genes (Fig. 6A).

Discussion

Anthocyanins are the major pigments in the flowers of higher plants. Many structural genes of anthocyanin biosynthesis, including CHI, CHS, F3H, and DFR, have been cloned and identified (Weiss, 2000). Malonyl-CoA is an important intermediate in anthocyanin synthesis (Mol et al., 1996). Previously, ACC was identified as the key enzyme of malonyl-CoA synthesis in anthocyanin biosynthesis (Sasaki and Nagano, 2004). Recently, AAE13 was identified as malonyl-CoA synthetase in Arabidopsis (Chen et al., 2011). The results obtained in the present study show that PhAAE13, the key synthase of malonyl-CoA, plays an important role in anthocyanin biosynthesis in petunia flowers.

In the present study, we cloned the full-length cDNA of PhAAE13. The phylogenetic tree (Fig. 3) showed that PhAAE13 protein was most similar to AtAAE13 in Arabidopsis, suggesting that PhAAE13 is a homolog of AtAAE13. The phylogenetic tree of the AAE13s in all known plants in NCBI and the high similarity between the AAE13 members demonstrated that AAE13 is a small, conserved gene subfamily (see Supplementary Fig. S1).

Flavonoid biosynthesis genes are typically coordinately regulated by developmental and environmental cues, such as growth stage (Lepiniec et al., 2006). Furthermore, metabolic channeling has been proposed for flavonoid biosynthesis enzymes (Jorgensen et al., 2005; Winkel, 2004), suggesting the need for tight co-regulation of protein amounts. In the petunia ‘Ultra’, the anthocyanins primarily accumulated in the flower organs. The expression of PhAAE13, similar to that of PhF3H, was highest in corollas. Flavonoid accumulation in seedlings is developmentally regulated and parallels the expression of the early genes of flavonoid biosynthesis, CHS, CHI, and F3H in Arabidopsis (Pelletier et al., 1999). Here, we observed that the accumulation of anthocyanins in buds is developmentally regulated, and, although there is no close correlation between the anthocyanin content and PhAAE13.
expression during development of the corollas, PhAAE13 expression is not low (Fig. 4G, H). A previous study has identified two types of AAE13 transcripts, with the resulting proteins being localized to the cytosol and mitochondria, respectively (Guan and Nikolau, 2016); this observation could partially explain the lack of close correlation between the anthocyanin content and PhAAE13 expression during development of the corollas.

Ethylene markedly suppresses anthocyanin accumulation (Craker and Wetherbee, 1973), while the CoA-mediated inhibition of ethylene biosynthesis and the prevention of ethylene activity by silver increases the anthocyanin content of maize (Zea mays) seedlings (Rengel and Kordan, 1987). Similarly, the petals of transgenic tobacco (Nicotiana tabacum) plants expressing the mutant melon (Cucumis melo) ethylene receptor gene ethylene response1 (ETR1H69A) accumulate higher levels of anthocyanins than control plants (Takada et al., 2005). In the present study, ethylene treatment decreased the expression of F3H, an anthocyanin biosynthesis gene, suggesting the negative regulation of ethylene on anthocyanin biosynthesis in petunia corollas; the level of PhAAE13 mRNA was also down-regulated by ethylene (Fig. 4C, D).

Flavonoids are strong UV-absorbing metabolites that primarily accumulate in epidermal cells after UV induction, suggesting that these molecules function as a protective shield (Schmelzer et al., 1988). One of the most general responses of plants to UV light is the transcriptional activation of flavonoid biosynthesis genes (Abbruzzese et al., 1986; Favory et al., 2009; Koes et al., 1994; Ryan et al., 2002). Consistently, in the present study, the mRNA levels of both PhAAE13 and PhF3H were up-regulated after UV-B treatment (Fig. 4E, F).

Chen et al. (2011) identified AtAAE13 as malonyl-CoA synthase, which catalyzes the formation of malonyl-CoA from malonic acid. Malonyl-CoA is the precursor for the formation of flavonoids and anthocyanins (Peer et al., 2001). In the present study, PhAAE13 silencing reduced anthocyanin biosynthesis and malonic acid accumulation. These results further suggested that the formation of malonyl-CoA is catalyzed through AAE13, with malonic acid as the substrate. The canonical view of flavonoid and anthocyanin biosynthesis suggests that malonyl-CoA is almost exclusively formed via acetyl-CoA carboxylase, which catalyzes the ATP-dependent formation of malonyl-CoA from acetyl-CoA and bicarbonate. In the present study, PhAAE13 silencing increased malonic acid accumulation and significantly reduced the anthocyanin content of corollas (Figs 5C and 8), suggesting that PhAAE13 is an alternative enzymatic source of precursors for anthocyanin biosynthesis in petunia flowers. In addition, the amounts of 5 of the 25 fatty acids and 6 of the 25 cuticular wax components detected were significantly reduced in PhAAE13-silenced plants compared with controls, suggesting a role for PhAAE13 in the biosynthesis of fatty acids and cuticular wax components in petunia.

CHS is a unique enzyme that catalyzes the synthesis of chalcone by coumaroyl-CoA. In the present study, VIGS-mediated silencing of PhCHS showed a stronger reduction of anthocyanin biosynthesis than that associated with PhAAE13 silencing. In addition, there was the patchy appearance typical of VIGS. Infection with TRV-PhCHS led to white patches, while infection with TRV-PhAAE13, TRV-PhACC1, and TRV-PhACC2 led to pink patches (Fig. 5C), which was repeatedly observed. These results indicate that in addition to PhAAE13, malonyl-CoA synthesis is catalyzed by another enzyme, PhACC1 and PhACC2 in petunia flower.

In a study in Arabidopsis, all of the homozygous aae1 mutant plants exhibited strong defects in growth and development, and after 39 d growth, many of the mutant plants died and the others remained small and chlorotic (Chen et al., 2011). However, in the present study, except for the reduction of anthocyanin biosynthesis in the flowers, the growth behavior of PhAAE13-silenced plants was indistinguishable from that of control plants. It is likely that infection with TRV-PhAAE13 led to partial PhAAE13 silencing in petunia (Fig. 6A), while homozygous aae1 mutant plants show loss-of-function mutations. Moreover, in Arabidopsis, all wild-type and hemizygous aae13 plants showed normal growth and development (Chen et al., 2011). In addition, the height of the seedlings after infection was 10–15 cm, and the effects of PhAAE13 silencing on young seedlings were not observed.

PhAAE13 belongs to clade VII of the AAE superfamily, which in Arabidopsis contains three genes, AtAAE13, AtAAE3, and AtAAE14. Kim et al. (2008) and Foster et al. (2012) identified Arabidopsis AtAAE14 (At1g30520) and AtAAE3 (At3g48990) as o-succinylbenzoyl-coenzyme A ligases acting in phylloquinone and oxalyl-CoA synthetase, respectively; whether AtAAE14 and AtAAE3 are involved in anthocyanin biosynthesis remains unknown. In the present study, petunia PhAAE3 and PhAAE14 were isolated, and VIGS-mediated silencing of both PhAAE3 and PhAAE14 did not change anthocyanin biosynthesis in petunia. The genes from clade VII almost defy categorization into a single clade because all three sequences are quite divergent relative to the other members of the superfamily (Shockey et al., 2003). These results suggested that among the members of clade VII of the AAE superfamily, PhAAE13 is specifically involved in anthocyanin biosynthesis. In addition, the increase in the level of PhACC mRNA could reflect a compensation mechanism of malonyl-CoA biosynthesis in PhAAE13-silenced corollas. These findings may partially explain that PhAAE13 could be redundant or predominant over cytosolic ACC isoforms.

Recently, Guan and Nikolau (2016) showed that the cytosolic AA13 protein in Arabidopsis is not essential because there is a redundant malonyl-CoA generating system provided by a cytosolic acetyl-CoA carboxylase, while the mitochondrial AA13 protein is essential for plant growth. The aae13-1 mutant showed typical metabolic phenotypes associated with a deficiency in the mitochondrial fatty acid synthase system, including the depletion of lipoylation of the H subunit of the photosynthetic enzyme glycine decarboxylase, increased accumulation of glycine and glycolate, and reduced sucrose levels (Guan and Nikolau, 2016). However, in the present study, VIGS-mediated PhAAE13 silencing reduced the production of anthocyanin, which is synthesized in the cytoplasm, suggesting that the cytosolic PhAAE13 protein is essential for anthocyanin biosynthesis in petunia corollas. In addition, the transcriptional level of the two possible types
of PhAEE13 transcripts should be reduced in plants infected with TRV-PhAEE13, which contains the common 3’ UTR sequence of PhAEE13 transcripts, although the plants did not show a visible change in plant growth; the cause of this finding could be that the VIGS-mediated approach achieved only partial silencing of PhAEE13.

Supplementary data

Supplementary data are available at JXB online.

Table S1. Primer sequences used for cloning genes.
Table S2. Primer sequences used in quantitative real-time PCR.
Table S3. Primer sequences used in VIGS.
Table S4. Comparative analysis of the amino acid sequence of AAE13s with their closest homologs in Arabidopsis, tomato, Vitis vinifera and Oryza sativa.

Table S5. Effects of PhAEE13 silencing on the content of fatty acids in corollas in Petunia.

Fig. S1. Phylogenetic tree of AAE13s.
Fig. S2. Phenotype of wild-type and PhAEE13-silenced plants.
Fig. S3. Alignment of PhACCs with AtACCs.
Fig. S4. Phylogenetic analysis of ACCs.
Fig. S5. Effects of PhAEE13 silencing on the content of cuticular wax components.
Fig. S6. Alignment of clade VII of AAEs.
Fig. S7. Phylogenetic analysis of clade VII of AAEs.

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Conflict of interest

The authors declare that there are no conflicts of interest.

Author contributions

YY and LJ designed the research; CG, LH, ZH, and WQ performed the research; ZH and WQ analyzed the data; and YY and LJ wrote the paper.

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