INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
20–1287/2021/25–6–1222–1230
DOI: 10.17957/IJAB/15.1783
http://www.fspublishers.org

Full Length Article

FaTT10, a Laccasel-Like Polyphenol Oxidase Involved in the Accumulation of Proanthocyanins Monomer in Strawberry Fruit

Guoyan Hou†, Min Yang†, Yu Long, Binhua Li, Liangxin Wang, Xiaoyang Liu and Ya Luo*
College of Horticulture, Sichuan Agricultural University, Chengdu 611130, P. R. China
*For correspondence: luoya945@163.com
†Contributed equally to this work and are co-first authors
Received 09 September 2020; Accepted 08 February 2021; Published 10 May 2021

Abstract

Procyanidins (PAs) is the important flavonoids components in strawberry young fruits, which is synthesized through direct polymerization of flavanone-3-ol units. Numerous studies have been done on the synthesis of PAs, while the oxidation/polymerization processes have not been fully explored. *AtTT10*, a laccasel-like polyphenol oxidase, was recently reported to be participated in the oxidative polymerization of proanthocyanidins. Whether *FaTT10* has a positive effect on oxidative polymerization of strawberry fruit PAs is unclear. In this study, *FaTT10* in strawberries were isolated and analyzed. Transient overexpression of *FaTT10* decreased the accumulation of PAs monomer in strawberry fruit, whereas RNA interference increased the level of anthocyanin and PAs monomer. 4-Dimethylaminocinamaldehyde (DMACA) staining results also showed that lighter blue color in the *FaTT10*-RNAi fruit and concentrated blue color in the *FaTT10*-overexpression fruit. Meanwhile, PAs monomer showed a drop-and-rise tendency during strawberry fruit development and ripening. Furthermore, the expression of several flavonoids metabolism-related genes were affected by the altered *FaTT10* gene expression levels, such as L-phenylalanine ammonia-lyase *(PAL);* dihydroflavonol reductase *(DFR);* chalcone isomerase *(CHI);* flavanone 3-hydroxylase *(F3H);* 4-coumarate:CoA ligase *(4CL);* Cinnamate 4-hydroxylase *(C4H);* chalcone synthase *(CHS);* anthocyanin reductase *(ANR);* lecoanthocyanidins reductase *(LAR)* and anthocyanin synthase *(ANS).* In conclusion, these results revealed that *FaTT10* is specifically involved in oxidative polymerization of PAs by regulating the expression of flavonoids metabolism-related genes in strawberry fruits.

Keywords: *FaTT10*; Polymerization; Proanthcocyanins; Strawberry; Transient gene expression

Introduction

Proanthcocyanins (PAs) are a type of flavonoids formed by the polymerization of flavanone-3-ol units with C6-C3-C6 basic framework in plant, which are also known as condensed tannins. Based on the difference of C6-C3-C6 basic framework in polymerization degree, PAs is divided into monomers, oligomers (n = 2~5) and polymers (n ≥ 6) (Monagas *et al*. 2005). The degree of PA polymerization is closely related to fruit quality, such as the PA of tetramer begin to have bitter and astringent taste in fruit. With the increase of polymerization degree, astringency is also aggravated. PAs fulfils numerous functions, including protecting against microbial pathogens, insect pests, larger herbivores (Dixon *et al.* 2004) and affecting the flavor and storage quality of red wine (Cosme *et al*. 2009). PAs is also health beneficial in the human diet to lower the risk of chronic diseases.

The PAs biosynthesis pathways and its regulation have been basically clear through the study of model plants, such as *Arabidopsis* and Alfalfa. Early biosynthetic genes (EBGs) and late biosynthetic genes (LBGs) constitute PAs biosynthesis (Pelletier *et al*. 1999; Lepiniec *et al.* 2006). *Chalcone synthase* (*CHS*), *chalcone isomerase* (*CHI*), *flavonol 3-hydroxylase* (*F3H*) and *flavonol 3'-hydroxylase* (*F3'H*) belong to the EBGs, which participate in precursor biosynthesis for the whole flavonoid biosynthetic pathway. In the LBGs, *dihydroflavonol-4-reductase* (*DFR*), *leucoanthocyanidin reductase* (*LAR*), *anthocyanidin synthase* (*ANS*), *anthocyanidin reductase* (*ANR*), *TT19* (glutathione S-transferase) , *TT12* (MATE transporter) and *AHA10* (H^+-ATPase) are involved in PAs precursor synthesis as well as PAs compartmetalization, transport, oxidation and modification (Appelhagen *et al*. 2015). Among the LBGs, *ANR* and *LAR*, PAs meatabolic specific enzyme, could convert leuco anthocyanidins to flavane 3-alcohols, such as catechin (C), gallocatechin (GC), epicatechin (EC) and epigallocatechin (EGC). The next polymerization mechanism of PAs is still to be confirmed.

There are two general assumptions: (1) EC and EGC might be catalyzed by the action of flavane-3-alcohol gall acyltransferase to form flavan 3-O-gallate; (2) Under the

effect of plant laccase-like polyphenol oxidase, such as TT10, EC and EGC is condensed to form polyproanthcocyanins in the vacuoles (Zhao *et al*. 2010). At present, it is generally accepted that the synthesis of anthocyanin and proanthocyanidins is carried out on the cytoplasmic side of endoplasmic reticulum (ER). However, anthocyanin and PAs are widely distributed in cytosol, vacuole, ER, chloroplast, nucleus, small vesicles, apoplastic space, rhizosphere and pollen surfaces, but most of them are stored in vacuoles. How do these metabolites were transported to vacuoles and stored in vacuoles to polymerization, and even secreted to the surface of pollen grains through vacuole membranes and plasma membranes, and then polymerized has not been studied in depth.

Of all the proteins found that may be involved in the transport/polymerization process, *Arabidopsis Transparent Testa 10* (*TT10*) has attracted the considerable interest from researchers. *TT10* encodes a laccasel-like polyphenol oxidase which is identified from *Arabidopsis* seed coat color mutant (transparent seed coat). This protein was secreted to the extraplastid side under the guidance of the front end signal peptide, and may convert colorless, soluble PAs or PAs monomer derivatives into polyproanthcocyanins. Next, the polyproanthcocyanins combines with cell wall proteins to form insoluble substances deposited in seed coat, thus showing the special color of seeds at maturity (Pourcel *et al*. 2005). There is very limited research about *TT10* in non-model plant. Recent research showed that silencing of *BnTT10* delayed the pigment accumulation of seed coat at the mature stage, and also increased soluble PAs levels in the seed coat by more than three times as much as that in the control. Interestingly, the insoluble PAs content increased by 1.2–1.9 times and the lignin content decreased significantly (Zhang *et al*. 2013). It is speculated that *BnTT10* is involved in the synthesis of proanthocyanidins and lignin. In addition, transforming *DkLAC1* gene into the *TT10* mutant of *Arabidopsis thaliana* could partially or completely restore its phenotype (Hu *et al*. 2013), which indicated that *DkLAC1* also participated in the oxidative polymerization of proanthocyanidins.

Strawberry (*Fragaria* x *ananassa* Duch.) is a good source of flavonoids, such as anthocyanins, flavan-3-ols, flavonols and simple phenols (Pérez *et al*. 1997; Lin-Wang *et al*. 2014). An integrated study at biochemical and molecular level have been conducted on different developmental stages (Kosar *et al*. 2004; Almeida *et al.* 2007) and various strawberry genotypes (Wang and Lewers 2007; Carbone *et al.* 2009) However, little is known about the transportation, polymerization and storage process of these compounds in strawberry fruits. Considering the important role of flavonoids in the nutritional quality of strawberry, we aimed at functionally characterizing the actual role of *FaTT10* in PAs oxidation/polymerization, in hoping to provide a promising gene candidate for manipulating strawberry flavonoids biosynthesis and PAs polymerization.

Materials and Methods

Plant material

Strawberry (*Fragaria* × *ananassa* cv. Benihoppe) plants were grown in plastic greenhouse in Chengdu, China. According to the method of Jia *et al*. (2013), strawberry fruit development and ripening stages were classified in small green (SG), large green (LG), de-greening stage (DG), white (WT), initial red (IR), partial red (PR) and full red (FR), seven developmental stages. At each stage, ten fruits were collected and snap-frozen in liquid nitrogen, and then were quickly stored at -80°C until processed. Three independent biological replicates were in this experiment.

Phylogenetic analysis

GDR (https://www.rosaceae.org/) and TAIR (https://www.arabidopsis.org/) were used to download protein sequences. Homologues of *TT10* genes were identified by using BioEdit. Phylogenetic relationships of the FaTT10 related proteins were constructed with the neighbor-joining statistical methods and the bootstrap method (1000 replications) using the MEGA 7 program.

RNA extraction and cDNA synthesis

An RNA extraction kit (TaKaRa) was used for the total RNA extraction. RNA sample integrity and concentration were checked by using an Agilent 2100 bioanalyzer and a NanoDrop ND-1000 spectrophotometer. Before generating the first cDNA, the genomic DNA was eliminated, and then cDNA was synthesized using PrimeScript RT Reagent Kit (Takara, Dalian, China) following the manufacturer's protocol. PCR was performed at 37°C for 15 min, followed by 85°C for 3 sec, and then 12°C for 15 min.

Plasmid construction

A 650 bp cDNAs fragment specifically targeting *FaTT10 gene* was amplified using appropriate primers (sense: 5′-CGGGATCCGTCACCATTCATTGGCACGGAGT-3′, antisense 5′-CGGAATTCCAGCGTAAGCAGAGGAAGCCAT-3′) cloned into the *BAMH* I and *EcoR* I sites of pTRV2. Then, pTRV1, pTRV2, and pTRV2 derivatives pTRV2-*TT10* were separately transformed into *Agrobacterium* strain GV3101 for RNAi. For overexpression of *FaTT10* gene, a 1719 bp cDNAs fragment of *FaTT10* was amplified using specific primers (*FaTT10*: sense 5′-TGCTCTAGAATGGCTGGGTTTAACAAAATGGGAG-3′, and antisense 5′-CGGGATCCCTAAGACTTGGAGCAAGGAGGCAT-3') cloned into the *BAMH* I and *Xba* I sites of pCAMBIA1301. Then pCAMBIA1301, or pCAMBIA1301 derivatives pCAMBIA1301- *TT10* was transformed into

Agrobacterium strain GV3101 for overexpression. *Agrobacterium*-mediated virus infiltration into strawberry fruits was performed based on the method of Jia *et al*. (2011). The *Agrobacterium* suspension was infiltrated into every DG fruit, and then the injected fruit was placed in a growth chamber at 23°C under 16 h light/8 h dark and 80~90% relative humidity for 6 days. During this period, the above injection was repeated twice at the next day and the third day after the first infiltration. Each treatment had ten uniformly sized fruit, and with three replicates.

Quantitative real-time PCR (qRT-PCR) analysis

The qRT-PCR was used to analyse the expression level of *FaTT10* gene and structural genes of PA biosynthesis pathway in the *FaTT10*-overexpression, *FaTT10*-RNAi and control fruit. The specific qRT-PCR primers designed for structural genes were following: L-phenylalanine ammonia-lyase *(PAL, sense, 5'*- CTCGGTCCACTAATTGAAG-3'; antisense, *5'*- AATGCCTTGTTCCTTGAA-3' *);* Cinnamate 4-hydroxylase *(C4H, sense, 5'*-TGCCCTTGGCTTCATGACT-3'; antisense, *5'*-GCTTGACACTACGGAGAAAGGT-3'*);* 4-coumarate:CoA ligase *(4CL, sense, 5'*-ACAAGACAATCACGAGTTCA-3'; antisense, *5'*-AGCAGTAGGTGTGGAGAG-3'*);* chalcone synthase *(CHS sense, 5'*- CAAGCCTGAGAAGTTAGAAG-3'; antisense, *5'*- AAACAACACACAAGCACTA -3'*);* chalcone isomerase *(CHI, sense, 5'*-CAAGCCTGAGAAGTTAGAAG-3'; antisense, *5'*-AAACAACACACAAGCACTA-3'*);* flavanone 3-hydroxylase *(F3H, sense, 5'*-ATCACCGTTCAACCTGTGGAAG-3'; antisense, *5'*-TCTGGAATGTGGCTATGGACAAC-3'*);* dihydroflavonol reductase *(DFR, sense, 5'*- GCTACATCTGTTCATCAC-3'; antisense, *5'*-GTCAAGTTCTCCTCAATG-3'*);* anthocyanin reductase *(ANR, sense, 5'*-CCTGAATACAAAGTCCCGACTGAG-3'; antisense, *5'*-GTACTTGAAAGTGAACCCCTCCTTC-3'*);* anthocyanin synthase *(ANS, sense, 5'*-GCTGCTCTCATCTCAACTAA-3'; antisense, *5'*- CCTGCTCAACATCGTCTT -3*);* lecoanthocyanidins reductase *(LAR, sense, 5'*-GGTGATGGCACGGTTAAAGC-3'; antisense, *5'*-CTCCCACAGTGAAGCAAGTCC -3'*);* and *Actin (sense, 5'*-TTCACGAGACCACCTATAACTC-3'; antisense, *5'*-GCTCATCCTATCAGCGATT -3'*).* qRT-PCR procedures was: holding for 3 min at 95°C, followed by 40 cycles for 10 s at 95°C, annealing for 30 s at 55°C and 72°C for 15 s. Relative fold changes in gene expression were analyzed using the $2^{-\Delta\Delta CT}$ method.

4-dimethylaminocinamaldehyde (DMACA) staining assay

DMACA staining method was used for the observation of PAs accumulation patterns in *FaTT10* RNAi and overexpression fruit (Li *et al.* 1996). Longitudinal sections of fruits about 0.2 cm thick were soaked and decolorized in ethanol solution (3:1, v/v) and glacial acetic acid for 12–20 h, and rinsed with 75% ethanol for12 h, then rinsed with distilled water. After dyeing with 0.6% (w/v) DMACA solution in a cold mixture of methanol and 6 M HCL (1:1, v/v) for 2 min, photographic records were taken.

Determination of PAs monomer and anthocyanin content

PAs monomer content was determined by using the PAs determination kit (Shagnhai MLBIO Biotechnology Co., Ltd. and Suzhou Keming Biotechnology Co., Ltd.). The quantification of total anthocyanin was measured by the pH differential spectrophotometry method (Cheng and Breen 1991). Briefly, the extraction was conducted from 0.5 g mixed strawberry fruit homogenized with 1.8 mL of cold 1% HCl-ethanol. Then, the homogenate was centrifuged at 8, 000 × *g* for 25 min at 4°C. Next, the supernatants were collected for determination of the total anthocyanin content. Anthocyanin concentration was expressed as mg pelargonidin 3-glucoside equivalents 100 g^{-1} of fresh weight.

Confocal microscopy

Full coding sequence of the *FaTT10* was amplified with primer subc-TT10-F and subc-TT10-R. The open reading frame was then fused to the N-Terminal of the GFP marker in a modified pC1302-35S-GFP vector after Nco I and Spe I digestion. The correct construct was subsequently transformed into the *Agrobacterium tumefaciens* strain GV3101 and transiently expressed in *Nicotiana benthamiana* leaves epidermal cell. Stains with the marker (ABRC stock CD3-1003) were used as positive and reference control. GFP signals were captured by Nikon A1 Confocal Laser Microscope.

Results

Phylogenetic tree of TT10-like homologues for strawberry

To further identify the characteristics of *FaTT10*, a neighbor-joining phylogenetic tree was constructed with 1000 bootstrap replicates. Scale bars indicate 0.05 estimated amino acid substitutions per site. Numbers above branches indicate the reliability of the associated taxa clustering. The phylogenetic tree showed FaTT10 is more closely related to *Fragaria vesca* than *Pyrus bretschneideri*, but clearly distant from that *Arabidopsis* (Fig. 1).

Changes of PAs monomer content during strawberry fruit development

PAs monomer content was measured during strawberry fruit development. The results showed that the PAs monomer

content was mainly accumulating at early and late stage during fruit development. The lowest PA monomer was observed at WT stage, which was 6.56 mg 100 g^{-1} FW, and then followed by a gradual increase and reached a highest value at FR stage, which was 71 mg 100 g^{-1} FW and increased by 10.83 fold compared with that of the fruit at WT stage (Fig. 2).

Silencing of the *FaTT10* promoting the accumulation of proanthocyanidins monomer

To test the role of *FaTT10* in PAs polymerization, *FaTT10* was knocked down by RNAi. Thirty fruits with pedicel were injected with the *FaTT10*-RNAi vector *in vitro*. Six days after infiltration, the phenotypic changes of the control and RNAi fruits were observed. The fruit phenotype showed that strawberry fruits grew normally, and there were no adverse symptoms such as fruit shrinkage, mold growth and virus infection *in vitro*, which indicated that the control and *FaTT10*-RNAi fruits could be inoculated with *Agrobacterium in vitro* and did not affect the observation of strawberry fruits phenotype after gene silencing. The results showed that there were more colored fruits in *FaTT10*-RNAi group than that in control (Fig. 3A, B). Meanwhile, qRT-PCR results showed that *FaTT10* expression level was significantly down-regulated by 0.54fold in the *FaTT10*-RNAi fruit compared with the control (Fig. 3C), and the content of anthocaynin and PAs monomer increased by 3.0fold and 2.81fold, respectively (Fig. 3D, E).

PAs are colorless before oxidation, and blue products are produced when they react with DMACA reagents. Therefore, DMACA reagents are commonly used to stain fresh plant tissues. The distribution and content of PAs can be determined by the localizaiton and color of blue products. In this experiment, PAs were located in the control and *FaTT10*-RNAi fruit tissue and seemed to be more concentrated in the stele of fruit, followed by the pith, the vascular bundle, and the cortex (Fig. 3F). Furthermore, the blue color of the stele, the pith and the vascular bundle in control was more concentrated compared with that of *FaTT10*-RNAi fruit, which suggested that silencing *FaTT10* gene could inhibit the accumulation of PAs in strawberry fruit. Taken together, silencing of *FaTT10* gene up-regulated the content of anthocyanin and PAs monomer, which led to the inhibition of PAs content in strawberry fruit.

Overexpression of *FaTT10* inhibiting the accumulation of proanthocyanidins monomer

To further explore *FaTT10* gene function in PAs polymerization of strawberry fruit, overexpression *FaTT10* via agro-infiltration was also applied in this study. The control and the *TT10*-overexpression fruit showed the similar fruit phenotype on the sixth day after *Agrobacterium* injection, which fruit surface was white or partly red (Fig. 4A, B). Meanwhile, qRT-PCR results demonstrated that the

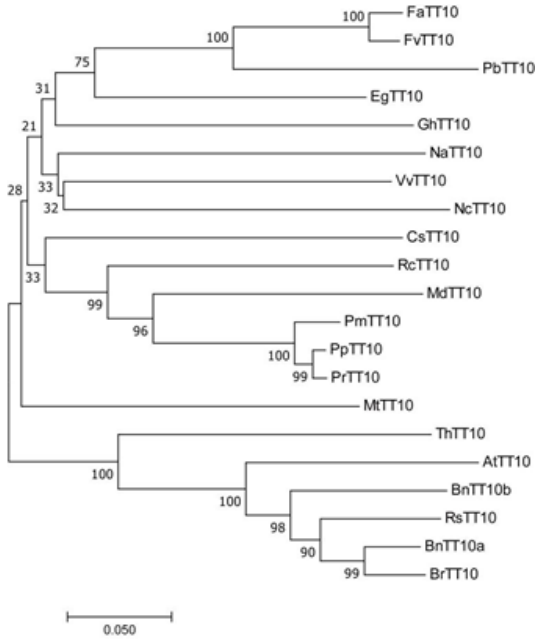

Fig. 1: Phylogenetic analyses of *FaTT10* and its closest relatives. Phylogenetic analysis was performed by a neighbor-joining algorithm using MEGA 7 program. Gene IDs were listed as: *Prunus persica* PpTT10 XP_007200191, *Prunus dulcis* PrTT10 VVA36043, *Prunus mume* PmTT10 XP_008237408, *Malus domestica* MdTT10 XP_028965267, *Pyrus bretschneideri* PbTT10 XP_009351840, *Fragaria vesca* FvTT10 XP_011464921, *Citrus sinensis* CsTT10 XP_006473165, *Vitis vinifera* VvTT10 XP_002272689, *Gossypium hirsutum* GhTT10 XP_016748740, *Arabidopsis thaliana* AtTT10 NC_003076, *Brassica napus* BnTT10a ADV03954, *Brassica napus* BnTT10b ADV03952, *Brassica rapa* BrTT10 XP_009129702, *Raphanus sativus* RsTT10 XP_018435646.1, *Tarenaya hassleriana* ThTT10 XP_010521293, *Medicago truncatula* MtTT10 XP_003603035, *Rosa chinensis* RcTT10 XP_024189647, *Eucalyptus grandis* EgTT10 XP_010031937, *Nicotiana attenuata* NaTT10 XP_019264267, *Nymphaea colorata* NcTT10 XP_031502939

transcriptional level of *FaTT10* was significantly up-regulated by 11fold in the *FaTT10*-overexpression fruit compared with that of the control fruit (Fig. 4C), which meant that the *FaTT10* gene was successfully overexpressed in the strawberry fruits. Furthermore, almost no anthocaynin was detected (data not listed), but PAs monomer decreased by 0.23fold (Fig. 4D). DMACA dyeing showed that the spatial accumulation of PAs was also in the stele, pith, vascular bundle and cortex of *FaTT10*-overexpression and control fruit, and concentrated blue color was mainly observed in the stele, and pith of *FaTT10*-overexpression fruit compared with that of control fruit (Fig. 4E). Above all, overexpression *FaTT10* gene inhibited the PAs monomer content, while promoted the accumulation of PAs.

Alteration of *FaTT10* genes expression affects the transcripts of PAs biosynthesis-related genes

In order to explore the mechanism of *FaTT10* gene in the

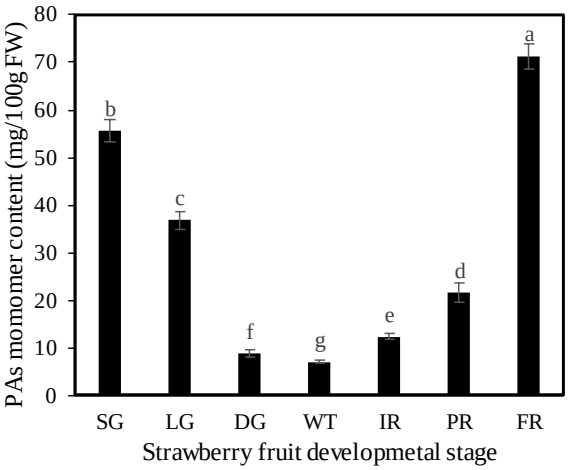

Fig. 2: PAs monomer content during strawberry fruit development. Small green (SG, 7 d after anthesis), large green (LG, 14 d after anthesis), de-greening (DG, 18 d after anthesis), white (WT, 20 d after anthesis), initial red (IR, 23 d after anthesis) and full red (FR, 28 d after anthesis). Values are means ± SD of three biological replicates. An overall significant difference ($P \leq 0.5$) is represented by different lower-case letters as determined by Duncan's multiple range test

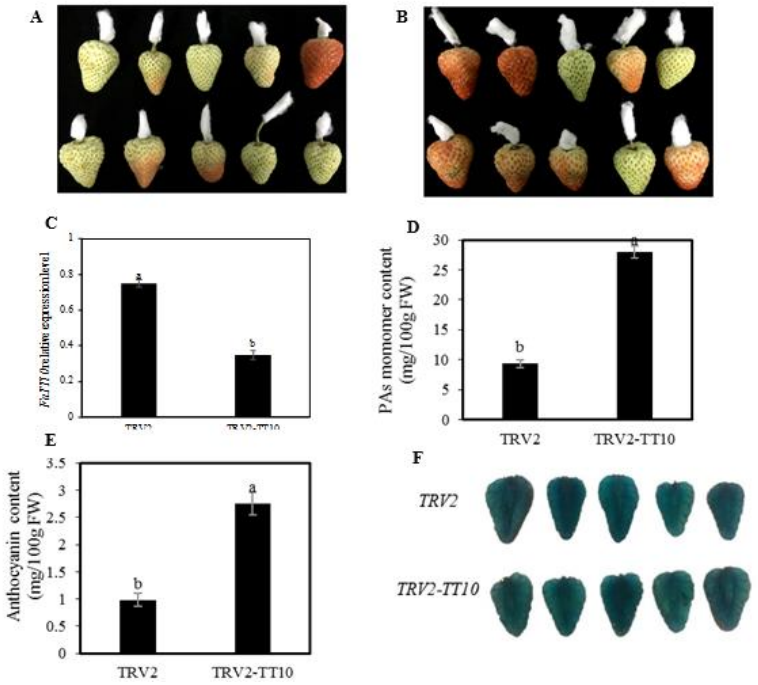

Fig. 3: VIGS for the *FaTT10* gene in strawberry fruit. (**A**) The phenotype of control fruit inoculated with *Agrobacterium* containing TRV2 *in vitro*. (**B**) The phenotype of virus-induced *FaTT10* silencing in strawberry fruits. (**C**) *FaTT10* expression level in the control and RNAi fruits by qRT-PCR. (**D**) PAs monomer content in the control and *FaTT10*-RNAi fruits. (**E**) Anthocaynin content in the control and *FaTT10*-RNAi fruits. (**F**) DMACA staining of longitudinal sections of the control and *FaTT10*-RNAi fruits. Values are means ± SD of three biological replicates. An overall significant difference ($P \leq 0.5$) is represented by different lower-case letters as determined by Duncan's multiple range test

regulation of strawberry PAs biosynthesis, a set of genes related to flavonoids metabolism, such as L-phenylalanine ammonia-lyase *(PAL);* cinnamate 4-hydroxylase *(C4H);* 4-coumarate CoA ligase *(4CL);* chalcone synthase *(CHS);* chalcone isomerase *(CHI);* flavanone 3-hydroxylase *(F3H);* dihydroflavonol reductase *(DFR);* anthocyanin reductase *(ANR);* anthocyanin synthase *(ANS)* and lecoanthocyanidins reductase *(LAR)*, were measured using both overexpression and RNAi fruit. The results showed that there were significant differences in the expression levels of the above genes between the RNAi fruit and the control fruit, except *ANS* gene (Fig. 5A). According to the characteristics of

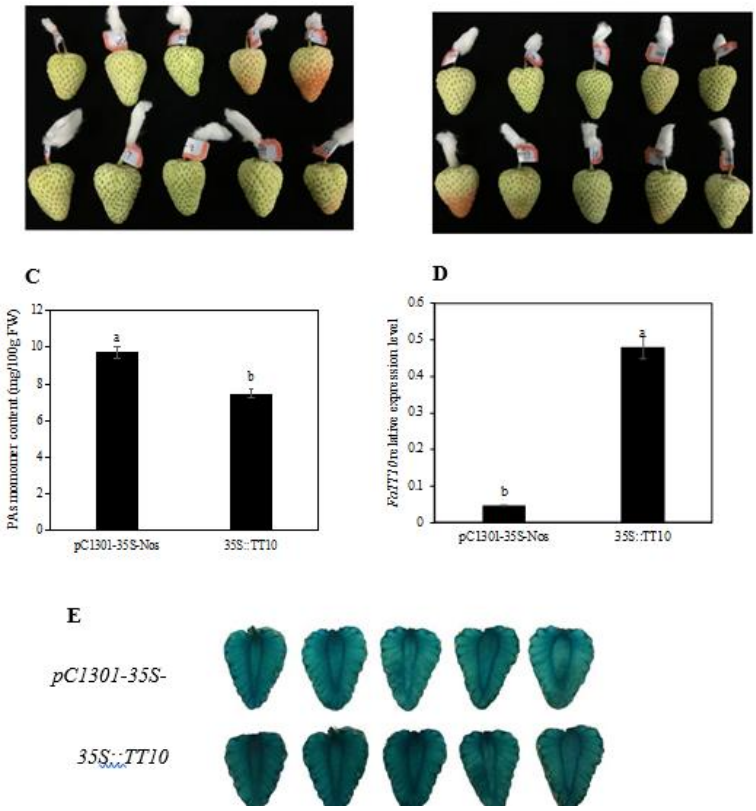

Fig. 4: Overexpression for the *TT10* gene in strawberry fruit. (**A**) The control fruit phenotype on the 6th day *in vitro*. (**B**) The *FaTT10* overexpression fruit phenotype on the 6th day *in vitro*. (**C**) *FaTT10* expression level in the control and overexpression fruits. (**D**) PAs monomer content in the control and overexpression fruits. (**E**) DMACA staining of longitudinal sections of the control and overexpression fruits. Values are means ± SD of three biological replicates. An overall significant difference (P≤0.5) is represented by different lower-case letters as determined by Duncan's multiple range test

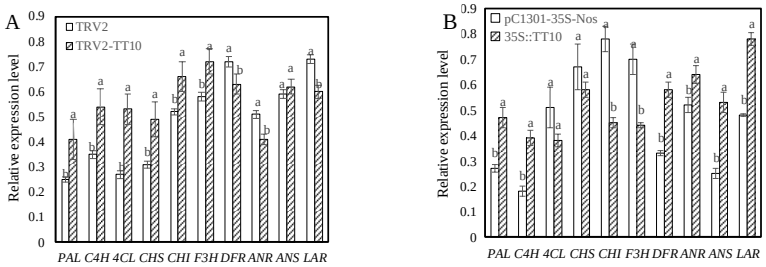

Fig. 5: Alteration of *FaTT10* expression affects transcripts of a set of PAs biosynthesis related genes in silencing and overexpressing fruit. Values are means ± SD of three biological replicates. (**A**) mRNA expression level of PAs biosynthesis relevant genes in RNAi fruit. (**B**) mRNA expression level of PAs biosynthesis relevant genes in overexpression fruit

genes expression level, it can be roughly divided into two categories: one is that the expression level in RNAi-*FaTT10* fruit was significant up-regulated compared with that in control fruit, including *PAL*, *C4H*, *4CL*, *CHS*, *CHI* and *F3H* (Fig. 5A). The expression levels of *4CL* were up-regulated by more than twice, *PAL* and *C4H* were up-regulated by about 60%, *CHS*, *CHI* and *F3H* were up-regulated by 48, 29 and 24%, respectively. Another kind of genes, including *DFR*, *ANR* and *LAR*, which expression levels were down-regulated by 14, 20 and 19% in RNAi-*FaTT10* fruit compared with that in control fruit, respectively (Fig. 5A).

In addition, the expression level of PAs biosynthesis related genes was also quantified in *FaTT10* overexpression fruit and control fruit. Among these structural gene of PAs biosynthesis, the expression level of *PAL*, *C4H*, *DFR*, *ANR*, *ANS* and *LAR* genes was significant up-regulated in *FaTT10* overexpression fruit compared with that in control fruit, while the expression level of *CHI* and *F3H* genes was significant down-regulated (Fig. 5B). Taken together, these changes in the expression of genes associated with the

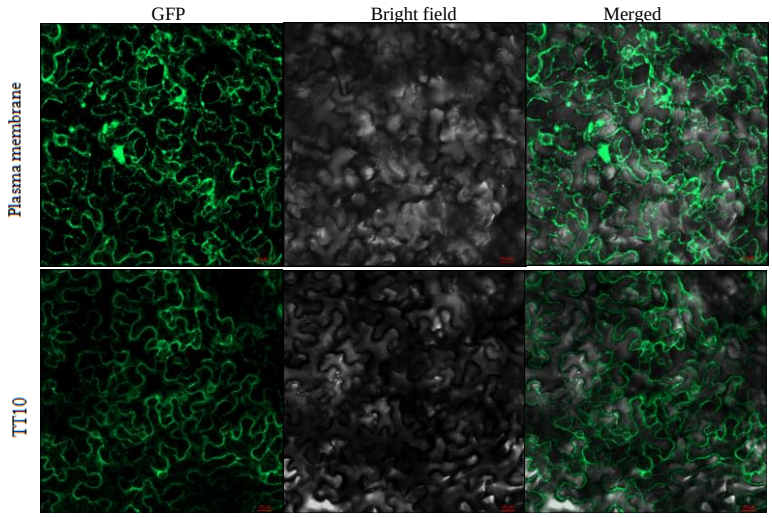

Fig. 6: Sub-celluar localization of strawberry TT10 protein in the epidemis of *Nicotiana benthamiana* leaves. Left, UV-excited GFP green fluorescence signals. Middle, bright field. Right, merged image. Scale bars = 20 μm

biosynthesis of PAs in *FaTT10* RNAi and overexpression strawberry fruit suggested that *FaTT10* were involved in the regulation of PAs biosynthesis by regulating the expression levels of PAs biosynthesis-related genes.

Sub-celluar localization of FaTT10

After 36 h infection with *Agrobacterium* containing pC1302-35S-GFP::TT10 vector and pC1302-35S-GFP empty vector respectively, the epidemis of *Nicotiana benthamiana* leaves were observed by fluorescence confocal microscope. The results showed that the GFP signal was mainly detected at the plasma membrane in cells transfected with the empty vector and in cells expressing the TT10 fusion proteins (Fig. 6), suggesting that TT10 might be membrane proteins.

Discussion

Laccase (EC 1.10.3.2) is ubiquitous in bacteria, fungi, insects and higher plants. The research on fungi laccase are the most reported, which function is associated with enhancing virulence of pathogenic bacteria (Salas *et al.* 1996), lignin degradation (Eggert *et al.* 1997) and pigment synthesis (Fang *et al.* 2010). The research on plant laccase is mainly focused on lignin synthesis (Sterjiades *et al*. 1992; Bao *et al*. 1993; Ranocha *et al.* 2002) stress response (Li and Steffens 2002) and pigment synthesis (Cai *et al.* 2006). Through our previous gene cloning, sequence analysis and subcellular localization prediction, it was showed that the protein encoded by *FaTT10* gene belongs to the laccase family and anchors at plasma membrane (Mo *et al*. 2019). In this study, sub-celluar localization experiment confirmed *TT10* located in the plasma membrane in present study. Phylogenetic analysis strengthened TT10 share common genetic ancestry in *Rosacea* species because of close evolutionary relationships (Fig. 1). In future work, it would be interesting to explore the overall evolutionary history of the whole TT10 family from a wider species range.

Strawberry was identified as potential sources of B-type proanthocyanidins. (Epi) catechin and (epi) afzelechin was always the terminal unit of proanthocyanidin oligomers (Gu *et al.* 2003). Through proanthocyanidin determination of 15 strawberry cultivars in Spain, it was found that the concentration of proanthocyanidin ranged from 53.9 mg 100 g^{-1} to 168.1 mg 100 g^{-1}, which was genotype-dependent and (epi) catechin was the main proanthocyanidin oligomers in strawberry fruit (Buendía *et al.* 2010). In the previous study, it has been reported that decreased PAs coupled with the decreasing *FaTT10* expression level during strawberry fruit development and ripening (Mo *et al.* 2019), while the content of PAs monomers decreased gradually in fruit from SG to WT stage and then followed by a gradual increased from WT to FR stage (Fig. 2). The simplest hypothesis for this is that *FaTT10* specifically regulates the content of PAs monomers could be an integrate part of regulation mechanism for oxidative polymerization of PAs.

In present study, our results showed that inhibition of *Fa*TT10 genes results in more colored fruit (Fig. 3B) and an increase in the level of anthocyanin and PAs monomer (Fig. 3D) in strawberry fruit. In contrast, overexpression of *FaTT10* genes can decrease the accumulation of PAs monomer (Fig. 3D). Similar results were obtained in *Brassica napus* and *Arabidopsis*, showing that the content of soluble PAs in *AtTT10* mutant seeds (Pourcel *et al.* 2005) and *BnTT10*-RNAi seeds (Zhang *et al*. 2013) was higher than that in wild type. In addition, DMACA staining results also showed that lighter blue color in the *FaTT10*-RNAi fruit (Fig. 3F) and concentrated blue color in the *FaTT10*-overexpression fruit (Fig. 3E) were observed. In Chinese PCNA persimmon, the concentrated blue color was observed in overexpression *DkLAC1* leaves by DMACA staining, as

well as the increased soluble and insoluble PA (Mo 2015).

The end products of the flavonoid biosynthetic pathway are PAs. Various MYB–bHLH–WDR (MBW) protein complexes and flavonoid synthesis structure gene (*ANR, LAR* and *ANS)* (Feng *et al.* 2013; Xiao *et al.* 2014) are involved in the regulation of PAs synthesis at the transcriptional level (Xu *et al.* 2014a; Xu *et al.* 2015). In present study, alternation of *FaTT10* expression level affected the flavonoid biosynthetic pathway, which includes up-regulation of *PAL*, *C4H*, *DFR*, *ANR*, *ANS* and *LAR* expression level (Fig. 5B) in *FaTT10*-overexpression fruit, and down-regulation of LBGs (*DFR*, *ANR* and *LAR*) transcriptional level (Fig. 5A) in *FaTT10*-RNAi fruit compared with respective control. In note, down-regulation of *DFR*, *ANR* and *LAR* was also observed during strawberry fruit development (Xu *et al*. 2014b).

Conclusion

In this study, PAs monomer contents first decreased and then increased gradually during strawberry fruit development and ripening. Transient knock-down of *FaTT10* or overexpression increased or decreased the level of PAs monomer, accompanied by the changes in PAs contents and gene expression on flavonoids synthesis pathway. The above results indicate that *FaTT10* contributes to proanthocyanins polymerization.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (3180817); the State Education Ministry, Key projects of Sichuan Provincial Education Department (172A0319) and Key projects of Sichuan Provincial Science and Technology Department (2018NZ0126) and Service Station Projects of New Rural Development Research Institute, Sichuan Agricultural University (2018, 2020).

Author Contributions

Ya Luo was responsible for the design of the experiments and the main writing of the manuscript. Most of the experiments and data analysis was completed by Gouyan Hou and Min Yang. All authors participated in the discussion of the results and comments on the manuscript.

Conflict of Interest

All the authors declare no conflict of interest.

Data Availability

All data included in this study are available upon request by contact with the corresponding author.

Ethics Approval

This article does not contain any studies with human participants or animals performed by any of the authors.

References

Almeida JRM, ED'Amico, A Preuss, F Carbone. CHR Vos, B Deiml, F Mourgues, G Perrotta, TC Fischer, AG Bovy, S Martens, C Rosati (2007). Characterization of major enzymes and genes involved in flavonoid and proanthocyanidin biosynthesis during fruit development in strawberry (*Fragaria* × *ananassa*). *Arch Biochem Biophys* 465:6171–6181

Appelhagen I, N Nordholt, T Seide, K Spelt, R Koes, F Quattrochio, M Sagasser, B Weisshaar (2015). TRANSPARENT TESTA 13 is a tonoplast P_{3A}-ATPase required for vacuolar deposition of proanthocyanidins in *Arabidopsis thaliana* seeds. *Plant J* 82: 840–849

Bao W, DM O'malley, R Whetten, RR Sederoff (1993). A Laccase associated with lignification in *Loblolly Pine* xylem. *Science* 260:672–674

Buendía B, MI Gil, JA Tudela, AL Gady, JJ Medina, C Soria, JM López, FA Tomás-Barberán (2010). HPLC-MS Analysis of Proanthocyanidin Oligomers and Other Phenolics in 15 Strawberry Cultivars. *J Agric Food Chem* 58:3916–3926

Cai XN, EJ Davis, J Ballif, MX Liang, E Bushman, V Haroldsen, J Torabinejad, YJ Wu (2006). Mutant identification and characterization of the laccase gene family in *Arabidopsis*. *J Exp Bot* 57:2563–2569

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

Cheng GW, PJ Breen (1991). Activity of phenylalanine ammonia-lyase (PAL) and concentrations of anthocyanins and phenolics in developing strawberry fruit. *J Amer Soc Hortic Sci* 116:865–869

Cosme F, JM Ricardo-Da-Silva, O Laureano (2009). Effect of various proteins on different molecular weight proanthocyanidin fractions of red wine during wine fining. *Amer J Enol Viticult* 60:74–82

Dixon RA, DY Xie, SB Sharma (2004). Proanthocyanidins – a final frontier in flavonoid research? *New Phytol* 165:9–28

Eggert C, U Temp, KEL Eriksson (1997). Laccase is essential for lignin degradation by the white-rot fungus *Pycnoporus cinnabarinu*. *FEBS Lett* 407:89–92

Fang WG, EKK Fernandes, DW Roberts, MJ Bidochka, RJS Leger (2010). A laccase exclusively expressed by *Metarhizium anisopliae* during isotropic growth is involved in pigmentation, tolerance to abiotic stresses and virulence. *Fung Genet Biol* 47:602–607

Feng HJ, XH Tian, YC Liu, YJ Li, XY Zhang, BJ Jones, YQ Sun, J Sun (2013). Analysis of flavonoids and the flavonoid structural genes in brown fiber of upland cotton. *PLoS One* 8; Article e58820

Gu LW, MA Kelm, JF Hammerstone, G Beecher, J Holden, D Haytowitz, RL Prior (2003). Screening of foods containing proanthocyanidins and their structural characterization using LC-MS/MS and thiolytic degradation. *J Agric Food Chem* 51:7513–7521

Hu QN, C Luo, QL Zhang, ZR Luo (2013). Isolation and characterization of a *Laccase* gene potentially involved in proanthocyanidin polymerization in oriental persimmon (*Diospyros kaki* Thunb.) fruit. *Mol Biol Rep* 40:2809–2820

Jia HF, YH Wang, MZ Sun, BB Li, Y Han, YX Zhao, XL Li, N Ding, C Li, WL Ji, WS Jia (2013). Sucrose functions as a signal involved in the regulation of strawberry fruit development and ripening. *New Phytol* 198:453–465

Jia HF, YM Chai, CL Li, D Lu, JJ Luo, L Qin, YY Shen (2011). Abscisic acid plays an important role in the regulation of strawberry fruit ripening. *Plant Physiol* 157:188–199

Kosar M, E Kafkas, S Paydas, KHC Baser (2004). Phenolic composition of strawberry genotypes at different maturation stages. *J Agric Food Chem* 52:1586–1589

Lepiniec L, I Debeaujon, JM Routaboul, A Baudry, L Pourcel, N Nesi, M Caboche (2006). Genetics and biochemistry of seed flavonoids. *Annu Rev Plant Biol* 57:405–430

Li L, JC Steffens (2002). Overexpression of polyphenol oxidase in transgenic tomato plants results in enhanced bacterial disease resistance. *Planta* 215:239–247

Li YG, G Tanner, P Larkin (1996). The DMACA-HCl protocol and the threshold proanthocyanidin content for bloat safety in forage legumes. *J Sci Food Agric* 70:89–101

Lin-Wang K, TK McGhie, M Wang, Y Liu, B Warren, R Storey, RV Espley, AC Allan (2014). Engineering the anthocyanin regulatory complex of strawberry (*Fragaria vesca*). *Front Plant Sci* 5; Article 651

Mo LR (2015). *Isolation and Characterization of ADH and PDC Genes Involved in Natural Loss of Astringency in Chinese PCNA Persimmon*. Huazhong Agricultural University, Wuhan, Hubei, China

Mo Q, YJ Ling, F Mo, C Ge, S Luo, Y Luo (2019). Cloning and Expression Analysis of *FaTT10* in *Fragaria ananassa*. *Genomics Appl Biol* 38:699–706

Monagas M, B Hernández-Ledesma, I Garrido, PJ Martín-lvarez, C Gómez-Cordovés, B Bartolomé (2005). Quality assessment of commercial dietary antioxidant products from *Vitis vinifera* L. grape seeds. *Nutr Cancer* 53:244–254

Pelletier MK, IE Burbulis, B Winkel-Shirley (1999). Disruption of specific flavonoid genes enhances the accumulation of flavonoid enzymes and end-products in *Arabidopsis* seedlings. *Plant Mol Biol* 40:45–54

Pérez AG, R Olías, J Espada, JM Olías, C Sanz (1997). Rapid determination of sugars, nonvolatile acids, and ascorbic acid in strawberry and other fruits. *J Agric Food Chem* 45:3545–3549

Pourcel L, JM Routaboul, L Kerhoas, M Caboche, L Lepiniec, I Debeaujon (2005). *TRANSPARENT TESTA 10* encodes a laccase-like enzyme involved in oxidative polymerization of flavonoids in *Arabidopsis* seed coat. *Plant Cell* 17:2966–2980

Ranocha P, M Chabannes, S Chamayou, S Danoun, A Jauneau, AM Boudet, D Goffner (2002). Laccase down-regulation causes alterations in phenolic metabolism and cell wall structure in poplar. *Plant Physiol* 129:145–155

Salas SD, JE Bennett, KJ Kwon-Chung, JR Perfect, PR Williamson (1996). Effect of the laccase gene, *CnLAC1*, on virulence of *Cryptococcus neoforman*. *J Exp Med* 184:377–386

Sterjiades R, JFD Dean, KEL Eriksson (1992). Laccase from sycamore maple (*Acer pseudoplatanus*) polymerizes monolignols. *Plant Physiol* 99:1162–1168

Wang SY, KS Lewers (2007). Antioxidant capacity and flavonoid content in wild strawberries. *J Amer Soc Hortic Sci* 132:629–637

Xiao YH, Q Yan, H Ding, M Luo, L Hou, M Zhang, D Yao, HS Liu, X Li, J Zhao, Y Pei (2014). Transcriptome and biochemical analyses revealed a detailed proanthocyanidin biosynthesis pathway in brown cotton fiber. *PLoS One* 9; Article e86344

Xu WJ, C Dubos, L Lepiniec (2015). Transcriptional control of flavonoid biosynthesis by MYB–bHLH–WDR complexes. *Trends Plant Sci* 20:176–185

Xu WJ, L Loïc, D Christian (2014a). New insights toward the transcriptional engineering of proanthocyanidin biosynthesis. *Plant Signal Behav* 9; Article e28736

Xu WP, H Peng, TB Yang, B Whitaker, LH Huang, JH Sun, P Chen (2014b). Effect of calcium on strawberry fruit flavonoid pathway gene expression and anthocyanin accumulation. *Plant Physiol Biochem* 82:289–298

Zhang K, K Lu, C Qu, Y Liang, R Wang, Y Chai, JN Li (2013). Gene silencing of *BnTT10* Family genes causes retarded pigmentation and lignin reduction in the seed coat of *Brassica napus*. *PLoS One* 8; Article e61247

Zhao J, YZ Pang, RA Dixon (2010). The mysteries of proanthocyanidin transport and polymerization. *Plant Physiol* 153:437–443