Research Article

Genetic modulation of RAP alters fruit coloration in both wild and cultivated strawberry

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Summary

Fruit colour affects consumer preference and is an important trait for breeding in strawberry. Previously, we isolated the Reduced Anthocyanins in Petioles (RAP) gene encoding a glutathione S-transferase (GST) that binds anthocyanins to facilitate their transport from cytosol to vacuole in the diploid strawberry Fragaria vesca. The parent of rap was the F. vesca variety ‘Yellow Wonder’ that develops white fruit due to a natural mutation in the FveMYB10 gene. Here, we investigated the application potential of RAP in modulating fruit colours by overexpression of RAP in F. vesca and knockout of RAP in the cultivated strawberry Fragaria × ananassa. Unexpectedly, the RAP overexpression in Yellow Wonder background caused formation of red fruit. In addition, the red coloration occurs precociously at floral stage 10 and continues in the receptacle during early fruit development. Transcriptome analysis revealed that the anthocyanin biosynthesis genes were not up-regulated in RAP-oct; rap myb10 flowers at anthesis and largely inhibited at the turning stage in fruit, suggesting a coloration mechanism independent of FveMYB10. Moreover, we used CRISPR/Cas9 to knockout RAP in cultivated strawberry which is octoploid. Six copies of RAP were simultaneously knocked out in the T0 generation leading to the green stem and white-fruited phenotype. Several T1 progeny have segregated away the CRISPR/Cas9 transgene but maintain the green stem trait. Our results indicate that enhancing the anthocyanin transport could redirect the metabolic flux from proanthocyanidin to anthocyanin production at early developmental stages of fruit and that RAP is one promising candidate gene in fruit colour breeding of strawberry.

Introduction

Cultivated strawberry (Fragaria × ananassa, octoploid) is one of the economically important fruit crops worldwide due to the attractive appearance, great taste and rich nutrition. Almost all strawberry cultivars bear red fruits. In recent years, a few white-fruited strawberry cultivars are developed such as the Chinese cultivars ‘Xiaobai’ and ‘Snow Princess’ (Zhao et al., 2018); these white strawberry fruits garner a much higher price than the red-fruited cultivars. Therefore, fruit colour is one of the breeding objectives of great interest in strawberry. However, development of new white-fruited cultivars is hampered by a shortage of required germplasms and the limited understanding of the underlying molecular mechanisms.

Anthocyanins are the principle pigments giving to the brilliant red colour of strawberry fruit (Kosar et al., 2004; Pillet and Folta, 2015; Silva et al., 2007). The anthocyanin biosynthetic genes have been identified in strawberry (Almeida et al., 2007; Pillet et al., 2015). In this pathway, phenylalanine is first metabolized into phenylpropanoids by phenylalanine ammonia-lyase (PAL), cinna-mate-4-hydroxylase (C4H) and 4-coumaroyl:CoA-ligase (4CL). Next, phenylpropanoids are synthesized into anthocyanidins sequentially by enzymes chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3 hydrolase (F3H), dihydroflavonol reductase (DFR) and anthocyanidin synthase (ANS). Subsequently, the glycosyltransferase GT catalyses glycosylation of anthocyanins. The flavonol synthase (FLS) as a branch pathway produces flavonols after F3H, whereas the leucoanthocyanidin reductase (LAR) and anthocyanidin reductase (ANR) catalyse the formation of proanthocyanidins after DFR. Genetic manipulation of these enzyme-coding genes could alter the level of these compounds. For instance, knock-down of CHS or FaGT1 reduced the anthocyanin pigments in fruit (Griesser et al., 2008; Hoffmann et al., 2006), while down-regulation of ANR led to ectopic anthocyanin accumulation in immature fruit (Fischer et al., 2014).

To facilitate genetic studies, the wild strawberry Fragaria vesca has been established as a model organism (Kang et al., 2013; Shulaev et al., 2011). Fragaria vesca offers the advantage of diploidy and existence of a wealth of natural variants. For example, ‘Yellow Wonder’ (YW) and ‘Hawaii 4’ (H4) are two F. vesca varieties bearing white fruit and red leaf petioles; by contrast, ‘Ruegen’ develops red fruit and red leaf petioles (Hartl et al., 2017; Hawkins et al., 2016; Slovin et al., 2009). This fruit colour difference is caused by a natural mutation in FveMYB10 that encodes a key transcription factor for fruit coloration.
(Hawkins et al., 2016; Lin-Wang et al., 2010). MYB transcription factors form the ‘MBW’ complex with one bHLH transcription factor and one WD40 protein; the MBW complex activates anthocyanin biosynthesis by promoting transcription of the enzyme-coding genes in the anthocyanin biosynthesis pathway (Xu et al., 2015). In this context, the red-fruited Rüegue variety, which is wild type for FveMYB10, is referred to as wild type, while the white-fruited ‘Yellow Wonder’ variety is referred to as rap myb10 mutant. Previously, we identified and isolated a mutant with green leaf petioles in a chemical mutagenesis screen of F. vesca strain ‘Yellow Wonder’. This mutant was designated as reduced anthocyanins in petioles (rap) (Luo et al., 2018). Since the rap mutant was generated in the ‘Yellow Wonder’ background, it was actually a rap myb10 double mutant. Bulk-segregant mapping by sequencing revealed that RAP codes for a glutathione S-transferase (GST) (Luo et al., 2018), which acts as a carrier protein for transferring anthocyanins from the cytosol into the vacuole (Kitamura et al., 2004; Li et al., 2011; Sun et al., 2012). Transient overexpression or knock-down of RAP in strawberry fruit at white-pink stages demonstrated that RAP activity is required for fruit coloration. These results prompted us to test the effect of this gene on fruit colour by stable transformation in F. vesca catalogue editing has been successfully performed in F. vesca (Feng et al., 2019; Xing et al., 2018; Zhou et al., 2018). This opens the door to new ways of engineering strawberry for improved traits and creation of allele diversity as is pioneered in this study.

We report that overexpression of RAP in stably transformed rap myb10 double mutant resulted in the development of red fruit, an unexpected phenotype since FveMYB10 has no function. Further, the red pigment of the fruit receptacle in RAP-ox; rap myb10 plants occurred precociously, at anthesis and early fruit developmental stages, when the anthocyanin biosynthesis pathway is not normally active. Hence, increased anthocyanin transport by RAP can directly affect fruit colour independent of ripening. Moreover, as a proof of concept, we knocked out six homeologs of RAP simultaneously in the cultivated strawberry to engineer fruit colour in the T0 generation by CRISPR/Cas9. Therefore, RAP is demonstrated here as a candidate target gene for breeding new colour varieties and the CRISPR/Cas9-generated white fruit F. ×ananassa can serve as a valuable germplasm for breeding.

**Results**

**Overexpression of RAP restored pigmentation in vegetative tissues**

YW5A73 is the 7th generation inbred line of the F. vesca variety YW, bearing red leaf petioles, white petals and light yellow carpels, called myb10 hereafter (Figure 1a). An ENU mutagenesis screen of myb10 led to the identification of the rap myb10 double mutant, which has green petioles due to reduced anthocyanin (Figure 1b) (Luo et al., 2018). RAP was shown to encode a glutathione S-transferase (GST) that facilitates anthocyanin transport from cytosol to vacuole. Transient overexpression of both RAP and FveMYB10 in the fruit tissue of rap myb10 resulted in pigmentation. To better understand the roles of RAP in fruit coloration, RAP driven by the 35S constitutive promoter was stably transformed into the rap myb10 double mutant. A total of 10 independent transgenic lines were obtained with similar phenotypes. Expression level of RAP was examined in two RAP-ox; rap myb10 lines (L1 and L2) by qRT-PCR, showing a great expression increase in leaf petioles of transgenic lines relative to rap myb10 (Figure S1). The RAP-ox; rap myb10 plants had red leaf petioles, indicating a rescue of the rap phenotype in leaf petioles (Figure 1c). In addition, some brown spots appeared on the leaf blades, and the stigma of each carpel became red, both of which are new phenotypes compared to myb10 and the wild-type Rüegue (Figure 1a,d). Closer observation showed that the coloration of epidermal cells was recapitulated in RAP-ox; rap myb10, in comparison with the pale-green colour of rap myb10 leaf petioles (Figure 1e). Cross sections of leaf petioles showed the same distribution of red cortex cells in RAP-ox; rap myb10 as that in wild type (Luo et al., 2018). Consistent with the observation, the total anthocyanin content was dramatically increased in the leaf petioles of RAP-ox; rap myb10 to a similar level to the wild type as well as myb10 single mutant (Figure 1f, P < 0.05, Tukey’s test). These results demonstrate that overexpression of RAP rescued the coloration in vegetative organs in rap myb10 and caused coloration in the stigma of carpels that is absent in wild type.

**Overexpression of RAP in myb10 background still resulted in fruit coloration**

FveMYB10 is a key positive transcription factor in strawberry fruit coloration (Lin-Wang et al., 2010); thus, myb10 is white-fruiting (Figure 2a) (Hawkins et al., 2016). The rap myb10 double mutant also makes white fruit (Figure 2b). RAP overexpression in rap myb10 was not expected to restore the fruit colour to red, as the anthocyanin pathway remains inactive due to the myb10 mutation. Unexpectedly, the RAP-ox; rap myb10 fruit turned red in the receptacle skin and flesh (Figure 2c), which contrasts with the WT fruit with red skin and white flesh (Figure 2d). The total anthocyanin contents were determined in both RAP-ox; rap myb10, myb10 single and WT. The anthocyanin content was dramatically increased in fruit of RAP-ox; rap myb10 compared to that of myb10 or rap myb10, but still significantly lower than that of WT (Figure 2e). These results suggest that RAP overexpression caused fruit coloration in an FveMYB10-independent manner.

**RAP overexpression changed the anthocyanin composition in fruit**

Prior studies identified most of the prominent anthocyanin compounds in leaf petioles and fruit of F. vesca using HPLC (Xu et al., 2014). To better understand the pigmentation caused by RAP overexpression that appears to exhibit fuchsia colour fruit, HPLC was used to determine anthocyanin compounds in leaf petioles and fruit of RAP-ox; rap myb10 and WT. Since the identity of each peak in the HPLC chromatograms is known, the three main peaks from leaf petioles of WT correlated with peonidin-3,5-diglucoside (peak 2), cyanidin-3-glucoside (peak 3) and peonidin-3-glucoside (peak 5) (Figure 2f). The leaf petioles of RAP-ox; rap myb10 also showed the same three peaks with similar proportions as WT, although the abundances of peak 3 and peak 5 were slightly increased. In fruit, however, the pigment
composition exhibited by WT and RAP-ox; rap myb10 was different. While the fruit of WT exhibited abundant cyanidin-3-glucoside (peak 3) and pelargonidin-3-glucoside (peak 4), it was low in peonidin-3-glucoside (peak 5) (Figure 2g). In contrast, the fruit of RAP-ox; rap myb10 has significantly higher peonidin-3-glucoside (peak 5) and much lower pelargonidin-3-glucoside (peak 4) than WT. In another word, fruit of RAP-ox; rap myb10 continued to produce peonidin-3-glucoside (peak 5), a pigment primarily present in leaf petioles (Figure 2f,g). These results indicate that RAP overexpression altered the anthocyanin composition in fruit.

Anthocyanin accumulation in fruit of RAP-ox; rap myb10 occurred at anthesis and early fruit developmental stages

Flower development in F. vesca was separated into 13 successive stages from flower meristem to anthesis (Hollender et al., 2012). For red-fruited strawberry, late fruit development is separated into green, white, turning, pink and red stages, respectively (Fait et al., 2008). The morphologies of flowers and fruits at different stages were shown in WT and RAP-ox; rap myb10 (Figure 3). The carpels and receptacles of WT are mostly greenish yellow, and their colour change only occurs much later in fruit development, at the turning stage (Figure 3a). In contrast, the carpel stigmata of RAP-ox; rap myb10 turn red at as early as floral stage 9, and fruit receptacles accumulate a large amount of anthocyanin from anthesis to six days after pollination (Figure 3b). The anthocyanin may not be produced any more at later fruit developmental stages, as the colour does not turn darker afterwards. To test this hypothesis, RAP driven by the 3SS promoter was transiently infiltrated into the myb10 fruit at white stage. More than ten fruits were examined, but none turned red when fully ripened (Figure S2), indicating that producing anthocyanins at the turning stage requires a functional FveMYB10. Therefore, we conclude that anthocyanin accumulation in fruit of RAP-ox; rap myb10 occurred at early developmental stages and stored in the vacuole until ripened.

Previously, down-regulation of ANR led to a redirection of the proanthocyanidin pathway to anthocyanin production in immature strawberry fruit (Fischer et al., 2014). Accordingly, we hypothesized that the proanthocyanidin content in the RAP-ox; rap myb10 fruit might be reduced owing to increased anthocyanin production. On the contrary, the proanthocyanidin levels were higher in the RAP-ox; rap myb10 fruit relative to WT at anthesis (fruit S1), 6 days after pollination, and the turning stage (Figure S3), indicating that both proanthocyanidin and anthocyanin biosynthesis were enhanced by RAP overexpression.

The anthocyanin biosynthetic pathway remained inactive in fruits of RAP-ox; rap myb10

To investigate the underlying molecular mechanism of fruit pigmentation in RAP-ox; rap myb10, fruit receptacles at anthesis (fruit S1) as well as the turning stage were collected from RAP-ox; rap myb10 and WT and subjected to RNA-seq. Three biological replicates for each sample led to a total of 12 libraries. Approximately 30 million reads per library were obtained and mapped against the F. vesca reference genome V4.0 (Edger et al., 2018). An average of 94.93% of raw reads could be uniquely aligned (Table S1). Differentially expressed genes between turning stage and anthesis in WT and RAP-ox; rap myb10 were identified, respectively (Data S1, fold change > 2, padj < 0.05). For WT, 4,831 genes were up-regulated and 6,534
genes were down-regulated at the turning stage when compared to S1. For RAP-ox; rap myb10, 4,470 were up and 6,177 were down in the same comparison. Moreover, 3787 genes were shared among the up-regulated genes, and 5442 genes were shared among the down-regulated genes (Figure 4a), suggesting a similar transcriptome profile during fruit development in these two genotypes. Next, we compared gene expression in fruit receptacles at anthesis (S1) between RAP-ox; rap myb10 and WT because of the striking difference in coloration. Consequently, only 453 genes were significantly up-regulated and 363 genes were down-regulated in RAP-ox; rap myb10 when compared with WT (Figure 4b). No GO term in the 'biological processes' category was enriched in these two gene lists.

The structural and regulatory genes in the anthocyanin biosynthesis pathway have been identified in F. vesca (Pillet et al., 2015). Expression levels of all these genes indicated by TPM (Transcripts Per Million) were determined according to the RNA-seq data. First, we checked the expression level of RAP. RAP was greatly induced in fruit receptacles at turning compared to anthesis in WT (Figure 4c), which is consistent with our previous study (Luo et al., 2018). Moreover, RAP was expressed at a much higher level in RAP-ox; rap myb10, irrespective of the developmental stages (Figure 4c), owing to the 35S constitutive promoter. Subsequently, expression levels of 15 structural genes, from the upstream FvePAL1 to the downstream glycosyltransferase gene FveGT1, were analysed. None of these genes was expressed at a greater than twofold level in fruit of RAP-ox; rap myb10 at anthesis (S1) compared with WT (Table S2), suggesting an inactive status of anthocyanin biosynthesis. However, seven genes were dramatically reduced in fruit of RAP-ox; rap myb10 compared with WT at the turning stage (Figure 4c), owing to the lack of a functional FveMYB10. Moreover, expression of the regulatory genes FveMYB10 and FvebHLH3 was not significantly changed in these pairwise comparisons (Table S2). The anthocyanin and proanthocyanidin pathways make use of mostly the same enzymes. Of note, the proanthocyanidin pathway is active.
in the immature fruit (Figure 4c, Table S2). Altogether, these results indicate that the anthocyanin biosynthesis pathway was not transcriptionally enhanced in the RAP-ox; rap myb10 fruit at both anthesis and the turning stages. This strongly suggests that the precocious and ectopic fuchsia pigment of the flowers and fruit at different developmental stages in Ruegen. Image showing the longitudinal section of the flowers and fruit at different developmental stages in Ruegen. (b) Images showing the longitudinal section of the flowers and fruit at different developmental stages in RAP-ox; rap myb10. Stages 9 (S9), 10 and 12 were defined according to Hollender et al., 2012. Fruit at 6 days (6d), 10 days and 13 days after pollination and the turning stage were shown. Scale bars: 250 µm (S9, S10, S12, anthesis); 1 mm (6d, 10d, 13d); 1 cm (turning).

Generation of the rap mutants in cultivated strawberry created by CRISPR/Cas9

White-fruited strawberry cultivars are rare and popular in the market. Since transient knock-down of RAP in fruits of the wild and cultivated strawberries resulted in reduced pigments (Luo et al., 2018), we applied CRISPR/Cas9 to edit the RAP gene in a Chinese red-fruited strawberry cultivar ‘Ningyu’. According to the sequenced genome of cultivated strawberry (Edger et al., 2019), there are three homeologous RAP loci in chromosome 1, including FxaC_1-2g38550, FxaC_1-4g34540 and FxaC_1-1g01220 (Figure S4). The gene model of FxaC_1-1g01220 is truncated at the 5' end compared to the other two homeologs. Their coding sequences share a high level of similarity; only a few SNPs exist. The sgRNA1 target site is the 27-45th nucleotides in the coding sequences of FxaC_1-2g38550 and FxaC_1-1g01220 (Figure S5). The two sgRNAs are driven by the Arabidopsis U6-26 and U6-29 promoters, respectively, and the construct also contains a 35S::GFP cassette for visual screen of positive transgenic calli and plants (Tang et al., 2018).

Nine independent transgenic lines (L1-9) in the T0 generation were validated by the presence of GFP fluorescence and positive PCR amplification of GFP (Figure S6). Most of these transgenic plants had green petioles such as L1 and L2 (Figure 5a), but some of them showed normal coloration in leaf petioles such as L9 (Figure 5a). For short, these transgenic lines are called rapCR. CRISPR/Cas9-induced mutations were further examined at the target sites in seven independent T0 lines by PCR, cloning PCR fragments in a vector and Sanger sequencing of individual colonies. In rapCR-L1 with green leaf petioles, the sgRNA1 target site was heterozygous: one allele was wild type; the other allele had a 1-bp insertion; and three different alleles were detected at the sgRNA2 target site, including 15-bp deletion, a combination of short insertion and deletion and long insertion (Figure 5b). In rapCR-L2 with green leaf petioles, 1-bp insertion and 2-bp deletion were found at the sgRNA2 target site. In rapCR-L9 with red leaf petioles, there was no mutation at both target sites. The other four lines examined also harboured short insertions or deletions at both target sites (Figure S7). In this case, the mutation frequency of sgRNA2 was higher than sgRNA1. These results indicate that the RAP homologs were successfully mutated in cultivated strawberry.

Characterization of the rapCR plants

Fruit coloration was carefully observed in the rapCR plants. Mature fruit of the wild-type ‘Ningyu’ had red receptacle skin and red flesh (Figure 6a). In contrast, rapCR (L1 and L2) produced white receptacle fruit skin and white flesh, resembling other white-fruited strawberry cultivars (Zhao et al., 2018). Consistent with the observation, total anthocyanin content was dramatically reduced in the rapCR fruit in comparison with the wild-type fruit (Figure 6b). These results indicate that knockout of the RAP gene is sufficient to abolish anthocyanin accumulation in the fruit of cultivated strawberry.

To test whether the CRISPR/Cas9-induced mutations could be stably inherited into the next generation, the T1 seedlings of rapCR (L1) were characterized. A total of 30 seedlings were germinated, all of which had green hypocotyls and leaves. Among them, twenty-five seedlings were GFP fluorescence positive, and five seedlings lost the GFP fluorescence (Figure 6c),...
indicating that the transgene might have been segregated out in these T1 progeny of rapCR plants. To confirm the loss of the transgene, PCR was performed to test for the presence of GFP or Cas9 in the genomic DNA extracted from these plants. As expected, there were bright bands for the nine GFP-positive plants, while no bands for the three GFP-negative plants (Figure 6d). These results suggest that the CRISPR/Cas9 transgene could induce genetically stable traits while being erased from the genome through sexual reproduction. The resulting transgene-free and white-fruited strawberry ‘Ningyu’ could serve as a germplasm for future breeding of new strawberry varieties.

**Discussion**

Previously, we identified RAP acting as a principal GST anthocyanin transporter in leaf petioles and fruit in F. vesca (Luo et al., 2018). In other fruit crops, GSTs also play important roles in fruit or flower pigmentation (Cardoso et al., 2012; Cheng et al., 2015; El-Sharkawy et al., 2015; Hu et al., 2016; Jiang et al., 2019; Liu et al., 2019; Zhao et al. 2019). However, none of them has been stably overexpressed by germ-line transformation to modulate flower or fruit colours in these crops, especially in the background of impaired ripening-associated fruit coloration. Here, we reveal
that overexpressing RAP resulted in fruit coloration independent of fruit ripening with altered anthocyanin compounds in *F. vesca*, which suggests a completely different mechanism to enhance anthocyanin biosynthesis. Most excitingly, CRISPR-based knockout of RAP in cultivated strawberry could cause white fruit colour due to reduced anthocyanin accumulation in fruit. Therefore, RAP is a promising candidate gene for breeding fruit colour variations in strawberry. In fact, creation of the transgene-free and white-fruited ‘Ningyu’ by CRISPR demonstrates an immediate application of knowledge learned in a diploid ‘model’ towards modulating traits in the cultivated strawberry (Gaston *et al.*, 2019).

**RAP** overexpression promotes coloration in the immature fruit receptacles

It is known that proanthocyanidins are abundantly produced in the immature receptacle, while anthocyanins accumulate only when the fruit colors. It is known that proanthocyanidins are abundantly produced in the immature receptacle, while anthocyanins accumulate only when the fruit colors.

![Figure 5](image_url)

*Figure 5* Generation of the rap<sup>CR</sup> mutants in cultivated strawberry. (a) Images showing three rap<sup>CR</sup> transgenic plants (L1, L2 and L9) in *F. × ananassa* cultivar ‘Ningyu’. (b) Mutations induced at the sgRNA1 and sgRNA2 target sites in rap<sup>CR</sup> (L1, L2 and L9) in the T0 generation. The sgRNA is indicated with red. The mutated nucleotides are indicated with blue, and deletions are indicated with dashes. For n/m, m indicates the number of clones examined, and n indicates the number of bacterial colonies showing the indicated sequence.

![Figure 6](image_url)

*Figure 6* rap<sup>CR</sup> mutants develop white fruits and some segregate away the transgene in the T1 generation. (a) Images showing the mature fruit of the rap<sup>CR</sup> mutants. (b) Total anthocyanin contents in the mature fruit. Asterisks indicate significant difference to the wild-type ‘Ningyu’ (Student’s *t*-test, ** *P* < 0.01, mean ± SD, *n* = 3). (c) The T1 seedlings of rap<sup>CR</sup>-L1 (line 1) with or without GFP fluorescence. For each seedling, there are two adjacent images: the left is a regular image, and the right shows GFP fluorescence. One seedling is GFP positive, and the other is GFP negative. (d) Cropped gel image showing the PCR products of GFP and Cas9 in different T1 progeny of rap<sup>CR</sup>-L1. RAP was used as a positive control of PCR. For GFP and Cas9, +: positive control using the transformation vector; -: negative control using genomic DNA of nontransgenic plant. For RAP, +: positive control using genomic DNA of nontransgenic plant; -: negative control using ddH<sub>2</sub>O. Scale bars: 1 cm (a); 0.5 cm (c).
the fruit start to ripen in strawberry as well as other fruit crops (Bianco et al., 2009; Carbone et al., 2009; Fait et al., 2008; Gniesser et al., 2008; Hartl et al., 2017; Schaart et al., 2013). The reason is that the anthocyanin biosynthetic genes are developmentally regulated by transcription factor complexes consisting of one MYB transcription factor, one bHLH transcription factor and one WD-40 protein (Xu et al., 2015). MYB10 is the key activator of anthocyanin biosynthesis in strawberry, which is not expressed at early developmental stages of fruit, but greatly induced during fruit ripening (Lin-Wang et al., 2010; Lin-Wang et al., 2014; Luo et al., 2018; Medina-Puche et al., 2014). Consistent with this knowledge, a number of anthocyanin biosynthetic genes are also developmentally up-regulated in red-fruited strawberry variety Ruegen according to our RNA-seq data, such as CHS, F3H, DFR and GT1 (Figure 4). As an anthocyanin transporter, ectopic expression of RAP/GST in fruit is not expected to cause pigmentation in fruit when the anthocyanin biosynthetic pathway is not activated. However, our results showed that RAP overexpression greatly increased the anthocyanin contents in fruit receptacles at anthesis (Figure 2), indicating that this pigmentation bypassed the developmental control. At the turning stage, expression of anthocyanin biosynthetic genes was not up-regulated in RAP-ox; rap myb10, in a sharp contrast to that in WT Ruegen (Figure 4). When RAP was transiently overexpressed in the myb10 (YYV5AF7) fruit via agro-infiltration, which is carried out at the white stage (a few days before turning), no pigmentation occurred (Figure S2), indicating that anthocyanins in the mature fruit of RAP-ox; rap myb10 are not synthesized at the turning stage.

We found that the RAP-ox; rap myb10 fruit accumulates red pigments from anthesis to a few days after fertilization (Figure 3). In addition, most of the anthocyanin/proanthocyanidin biosynthetic genes are expressed in flowers at anthesis, albeit at a lower level than the turning stage in WT (Figure 4). These genes in young fruit might be transcriptionally regulated by other MYB transcription factors instead of MYB10 required for ripening. Previous study found that knock-down of ANR, coding for an enzyme directing to the proanthocyanidin branch, resulted in prominently visible anthocyanin accumulation in the immature receptacles and in the stigmata of carpels (Fischer et al., 2014). Our RAP-ox; rap myb10 plants exhibited similar phenotypes to the ANRI lines. Accordingly, one hypothesis would be that enhancing anthocyanin transport into vacuoles by increased activity of RAP/GST could effectively deplete certain intermediate or precursors and hence channel the metabolic flux from the proanthocyanidin pathway to the anthocyanin pathway.

Editing of RAP by CRISPR/Cas9 could be used for colour breeding in strawberry

CRISPR/Cas9 is an extremely useful tool to translate new findings into plant breeding (Gao, 2018). In this study, we used an optimized CRISPR/Cas9 vector called pKSE401G (Tang et al., 2018), which has a 35S::GFP cassette allowing visual screen of positive calli and regenerated shoots during transformation. In this vector, zCas9 was under the control of two tandem constitutive 35S promoters, and the two sgRNA scaffolds were driven by the Arabidopsis U6-26 and U6-29 promoter, respectively (Xing et al., 2014). This vector has been proven highly effective in knocking out multiple RAP homologs in the cultivated strawberry and yields white fruit colour. Application of CRISPR to cultivated strawberry has not been widely reported; a recent study knocked out FaTM6 by CRISPR/Cas9 in cultivated strawberry but the plants were sterile preventing analysis of T1 progeny (Martin-Pizarro et al., 2018). CRISPR/Cas9 has also been successfully applied in diploid F. vesca (Zhou et al., 2018). Another report edited genes in both diploid and octoploid strawberry (Xing et al., 2018). Thus far, different CRISPR/Cas9 vectors and promoters used for driving sgRNAs and Cas9 are able to work well in both wild and cultivated strawberry (Feng et al., 2019; Wilson et al., 2019). In this study, we also show that the CRISPR/Cas9-induced phenotypes could be stably inherited, and the transgene could be segregated away into the next generation. Together, CRISPR/Cas9 technique appears to work well in strawberry and holds great promise in the engineering and modulating useful traits as demonstrated here.

In strawberry, the diploid species F. vesca is frequently used as a model system to study basic biology questions and identify key genes controlling important horticultural traits. To facilitate the translation of new findings in the basic research, it is particularly valuable to establish the genome editing technology in cultivated strawberry. As an octoploid species, it is highly possible that there are multiple homeoalleles for one gene in cultivated strawberry. For instance, there are six copies of RAP in three of the four homoeologous chromosome 1 (Figure S4). CRISPR/Cas9 could modify multiple alleles simultaneously using one or limited number of sgRNAs in polyploid species (Wang et al., 2014; Yang et al., 2017), hence a great option for genetic modification of target genes in cultivated strawberry. CRISPR/Cas9 would elicit new editing events at the target sites during sexual propagation, thereby acquiring a greater proportion of mutants in the descendants than following the classical Mendelian model (Yang et al., 2017), which may greatly increase the selection efficiency of target traits, especially for the polyploid crops.

Working model for the mechanism of RAP-mediated fruit coloration in strawberry

A working model was proposed to demonstrate the mechanism of RAP-mediated fruit coloration in strawberry (Figure 7). For wild type, proanthocyanidins are mainly produced in fruit at early developmental stages. At the turning stage, expression of FveMYB10 is greatly increased to promote the expression levels of anthocyanin biosynthesis and transport genes, which leads to fruit coloration because of the accumulation of anthocyanins in vacuole. For RAP-ox; rap myb10, enhancing anthocyanin transport into vacuoles by increased abundance of RAP/GST could somehow make the proanthocyanidin biosynthetic enzymes synthesize anthocyanins at early developmental stages, in addition to synthesize proanthocyanidins, thus leading to fruit coloration precociously.

Experimental procedures

Plant materials and growth conditions

Two F. vesca varieties, Yellow Wonder 5AF7/YW5AF7 and Ruegen, and one F. × ananassa cultivar ‘NingYu’ were used in this study. Plants were grown in a growth room under a light intensity of 100 μmol/m²/s with a 16/8 h light/dark photoperiod at 25°C.

Plasmid construction

Construction of the RAP-ox vector was as described previously (Luo et al., 2018). To construct the single guide RNA (sgRNA)-Cas9 vector for the RAP homologs, two sgRNAs, respectively, targeting RAP at 27 bp (sgRNA1: TTAGGGCAGCTGCCCCCAG) and 771 bp (sgRNA2: TGGTCGCCCTTCTTGTAAGAA) downstream of the translation initiation codon were designed using the web
server CRISPR-P (http://cbi.hzau.edu.cn/cgi-bin/CRISPR). Two ATU6 promoter-sgRNA-AtU6 terminator cassettes were amplified by PCR using pCBC-DT112 as the template, and then, the PCR fragments were inserted into pHSEG401 (Tang et al., 2018) by Golden Gate Assembly and confirmed by Sanger sequencing. These two constructs were transformed into Agrobacterium tumefaciens strain GV3101 for plant transformation. The primers used for making these constructs are listed in Table S3.

Stable transformation of wild and cultivated strawberry

Agrobacterium-mediated transformation of the wild strawberry F. vesca was performed as described previously (Feng et al., 2019). Positive transgenic calli and regenerated plants were selected by using both antibiotics and visual screen of GFP fluorescence during transformation. The GFP fluorescence was detected using a fluorescence dissecting microscope (Micro-shot Technology Limited, Guangzhou, China, MZX81). Transformation of the cultivated strawberry ‘Ningyu’ was similar to the wild strawberry with minor modification. The 5’++ sequence of the cultivated strawberry ‘Ningyu’ was similar to the wild strawberry with minor modification. The 5’++ medium contains 1 × Murashige and Skoog (MS), 2% sucrose, 4 mg/L thidiazuron, 0.2 mg/L IBA and 0.7% phytoagar, pH 5.8. Additionally, 250 mg/L carbenicillin and 2 mg/L hygromycin were used to inhibit the growth of Agrobacterium and negative calli.

Measurement of total anthocyanins

Approximately 0.5 g fresh strawberry fruit or petioles was ground to powder using liquid nitrogen, added to 5 mL extraction solution (methanol: H2O: formic acid: trifluoroacetic acid, 70:27:2:1) and placed in a refrigerator at 4 °C for 2 h in the dark. The supernatant was transferred to a new tube by filtration. The absorbance was measured at 530 and 657 nm by an ultraviolet spectrophotometer (Hoefer Vision, SP-2001). The total anthocyanin content was calculated using the following formula: QAnthocyanins = (A530−(0.25 × A657))/M, where QAnthocyanins is the amount of anthocyanins, A530 and A657 are the absorbance at the indicated wavelengths, and M is the fresh weight of the plant material used for extraction (Zhang et al., 2009). All samples were measured as triplicates in three independent biological replicates.

Measurement of proanthocyanidins

Approximately 0.1-0.2 g fresh samples were ground to powder in liquid nitrogen, added to 1 ml extraction solution (acetone: H3O: glacial acetic acid, 70:29.5:0.5) and placed in a refrigerator at 4 °C for 24 h in the dark. The solution was centrifuged at 10,000 g for 10 min, and the supernatant was transferred to a new tube and mixed with equal volume of chloroform. After centrifugation, the supernatant was transferred to a new tube and mixed with the same volume of n-hexane. After centrifugation, the subnatant was isolated and stained with 0.1% p-dimethylaminocinnamaldehyde (DMACa) for about 15 min at room temperature. Finally, the absorbance at 640 nm was measured. The total proanthocyanin levels were calculated as procyanidin equivalents using procyanidin B2 (Sigma-Aldrich, MO) as standards.

HPLC analysis of anthocyanins in strawberry petioles and fruit

Approximately 0.5 g fresh strawberry fruit or petioles was ground to powder using liquid nitrogen, added to 2.5 mL extraction solution (methanol: H2O: hydrochloric acid, 80:20:0.1) and placed in a refrigerator at 4 °C for 12 h in the dark. The mixture was centrifuged at 9,000 g for 20 min. The supernatant was filtered through a microporous membrane (0.45 μm) into a brown sample vial. HPLC analysis was performed using a Daqing LC-20AT system. Separation was performed using a Develosil-ODS C18 column (5 μm, 4.6 × 250 mm). The mobile phase was 0.1% formic acid in water (solvent A) and methanol (solvent B) at a flow rate of 0.6 mL/min. The linear gradient of phase B was as follows: 0-10 min, 10-25%; 10-15 min, 25-30%; 15-50 min, 30-50%; 50-60 min, 50-60%; 60-68 min, 60-10%; and 68-70 min, 10%. The UV-visible light detector wavelength was set at 510 nm for detecting anthocyanins. Cyanidin (Cy) 3-gluc (Aladdin, Cat# 27661-36-5) was used as the authentic standard.

RNA-seq and data analysis

Total RNA was extracted from the fruit samples of RAP-ox; rap myb10 and WT (Ruegen), including receptacle and carpels, at anthesis and turning stage using the Plant Total RNA Isolation Kit (Sangon Biotech, Shanghai, China, Cat# B518661), respectively. Each sample had three biological replicates, each of which contained 3-6 fruits. RNA-seq libraries were constructed using the PE150 method and sequenced on Illumina HiSeq X Ten (Novogene, Beijing, China). Raw reads in each library (approximately 8G) were mapped against the F. vesca genome Fvb with the v2.0.a2 annotation (Li et al., 2017) using the program STAR.

Figure 7  Diagram showing the RAP functions in fruit coloration in strawberry. (a) Proanthocyanidin production in fruit of wild type at early developmental stages. (b) Anthocyanin production in fruit of wild type at the turning stage. (c) Anthocyanin and proanthocyanidin production in fruit of RAP-ox; rap myb10 at early developmental stages. Of note, MYB10 regulates expression of the enzyme-coding genes at the turning stage; some other MYBs play this role at early developmental stages. Regular fonts of protein names indicate low abundance; bold fonts indicate high abundance. Thick arrows indicate strong activities; thin arrows indicate weak activities. [Colour figure can be viewed at wileyonlinelibrary.com]
(Dobin et al., 2013) in 2-pass mode. FeatureCounts (Liao et al., 2014) was used to count the number of reads for each gene. Differentially expressed genes were identified by using the R package DESeq2 (Love et al., 2014).

qRT-PCR
Total RNA was extracted from young leaves of RAP-ox; rap myb10 and rap myb10 using a Plant Total RNA Isolation Kit (Sangon Biotech, Shanghai, China, Cat# SK8631). Approximately 1 μg of total RNA was used for cDNA synthesis using a PrimeScript™ RT reagent kit (TaKaRa, Shiga, Japan, Cat# RR047A). For qRT-PCR, a total volume of 10 μL reaction mixture was used containing 5 μL of 2 × SYBR Green PCR master mix (Cat# 172-5124, BioRad), 0.25 μL of each primer (Table S3), 3.5 μL ddH2O and 1 μL of 4 × diluted cDNA. Amplification was performed using a QuantStudio 7 Flex system (Applied Biosystems, Waltham, MA, USA). The reaction programme consisted of one cycle of 50 °C for 2 min and 95 °C for 10 min, followed by 50 cycles of 95 °C for 15 s, 60 °C for 20 s and 72 °C for 20 s (fluorescence signal collection) and followed by 1 cycle of 95°C for 15 s, 60 °C 1 min and 95 °C 15 s. The expression level of each gene was calculated using the 2^ΔΔCt method (Livak and Schmittgen, 2001). FvHd1g05910/Gene11892 was used as the internal control. All analyses were repeated three times using biological replicates.

Transient gene expression in strawberry fruit
Agrobacterium-mediated transient gene expression in strawberry fruit was performed as described (Pi et al., 2019). The Agrobacterium GV3101 culture harbouring the RAP-ox construct was suspended into the injection buffer (MS salt, 2% sucrose, pH 5.8) to reach an OD 600 of 0.8-1.0. The mixture was injected into the myb10 (YW5AF7) fruit at the white stage with a 1-ml syringe. The injection buffer was used as the negative control. More than 10 fruits were injected for each. The GFP signal and fruit coloration were observed about one week after the injection.

Detection of genome editing events
Genomic DNA was extracted from the rap28 transgenic plants and the wild-type ‘Ningyu’ by a Plant Genomic DNA Isolation Kit (TSINGKE Biological Technology, Beijing, China, Cat# TSP101-200). Full length sequences of the RAP homologs were amplified by PCR and inserted into the T vector by using the pEASY-T1 Cloning Kit (TransGen Biotech, Beijing, China, Cat# CT101). A total of 10-20 bacterial colonies for each transgenic plant were selected for Sanger sequencing to examine the induced mutations.

Statistical analyses
Statistical analyses were performed with SPSS v22.0 (IBM Corp., Armonk, NY, USA). Pairwise comparisons were determined using Student’s t-test (*, P < 0.05; **, P < 0.01). The comparison between multiple samples was determined using Tukey’s test, and significant differences at the P < 0.05 level are indicated by different letters.

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Conflict of interest
The authors declare that there is no conflict of interest.

Authors’ contributions
CK and ZL designed the experiments. QG and HL performed the experiments. YL analysed the RNA-seq data. CK, QG and ZL wrote the manuscript.

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**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Figure S1** Expression level of *RAP* in the *RAP-ox; rap myb10* transgenic lines.

**Figure S2** Transient overexpression of *RAP* in fruit starting from white stage failed to change the fruit color.

**Figure S4** Genomic sequences of the *RAP* homeologs in cultivated strawberry.

**Figure S5** Sequence alignment of *RAP* from *F. vesca* (gene 31672) and its homeologs in the cultivated strawberry *F. × ananassa*.

**Figure S6** Genotyping of the *rapCR* transgenic lines in ‘Ningyu’ in T0.

**Figure S7** Mutations induced in the other four *rapCR* mutants (L3-6) with green petioles in the T0 generation.

**Table S1** Summary of RNA-seq read statistics.

**Table S2** Expression levels of the anthocyanin pathway genes according to RNA-seq.

**Table S3** The list of primers used in this study.

**Data S1** Differentially expressed genes identified by pairwise comparison of the RNA-sequencing samples.