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Differential expression of iron-sulfur cluster biosynthesis genes during peach flowering

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Abstract

Iron is required for the Fe-S cluster assembly which occurs in chloroplasts, mitochondria, and cytosol and here we characterized 44 Fe–S cluster biosynthesis genes and investigated their expression profiles during different peach flowering stages. Quantitative real-time PCR analysis shows that the highest expression of most peach Fe-S cluster biosynthesis genes appeared in the full bloom stage. Also, the highest Fe accumulation occurred in the full bloom stage followed by beginning bloom, petal fall, and bud swell stages. Activities of nitrite reductase (NiR) and succinate dehydrogenase (SDH) were closely correlated to the flower Fe content, whereas the aconitase (ACO) activity kept steady during the whole flowering process. Moreover, shading treatment significantly reduced Fe accumulation and NiR, SDH, and ACO activities of the full blooming flowers. Seventeen Fe-S cluster biosynthesis genes were down-regulated in response to a shading treatment. In particular, plastid sulfur mobilization genes were sensitive to the shading treatment.

Additional key words: aconitase, Fe homeostasis, nitrite reductase, *Prunus persica*, shading, succinate dehydrogenase.

Introduction

Many metabolic pathways and cellular processes depend on iron-sulfur (Fe-S) proteins which are found in all major sub-cellular compartments such as plastids, mitochondria, cytosol, and nucleus (Lill 2009, Balk and Pilon 2011). The Fe-S protein nitrite reductase (NiR) is famous for chloroplastic nitrogen assimilation, aconitase (ACO) and succinate dehydrogenase (SDH) are crucial enzymes involved in the mitochondrial citric-acid cycle. In particular, a Fe-S cluster is present in proteins that play indispensable roles in photosynthesis, respiration, DNA repair, S and N assimilation, plant hormones, and protein translation (Johnson *et al*. 2005, Lill and Muhlenhoff 2006, Rouault and Tong 2008, Lill 2009, Balk and Pilon 2011).

A highly conserved Fe-S cluster biosynthesis process includes Fe-S cluster formation on scaffolds and transfer to target apo-proteins which calls for dozens of specific genes (Balk and Lobreaux 2005, Raulfs *et al*. 2008, Lill 2009, Couturier *et al*. 2013). Notably, more than 40 genes have been identified as Fe-S cluster biosynthesis genes in *Arabidopsis* (Balk and Pilon 2011, Couturier *et al*. 2013), rice (Liang *et al*. 2014), and soybean (Qin *et al*. 2015). They are located in plastids, mitochondria, and cytosol. In particular, plastids harbor the sulfur mobilization (SUF)-like pathway and mitochondria use the iron-sulfur cluster (ISC) assembly pathway independently, whereas the cytosolic Fe-S cluster assembly depends on the emerging cytosolic iron-sulfur cluster assembly (CIA) pathway (Balk and Lobreaux 2005, Bernard *et al*. 2013). The knowledge of Fe-S cluster biosynthesis in model plants favorably provides insights into investigation of woody plants.

In orchards, Fe is needed for mineral nutrition in fruit trees (Tagliavini *et al*. 2000, Pestana *et al*. 2005). Fe deficiency negatively affects fruit yield, fruit size, and quality (Tagliavini *et al*. 2000, Tagliavini and Rombolà

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Abbreviations: ACO - aconitase; CIA - cytosolic iron-sulfur cluster assembly; FS - flowering stage; ICP-AES - inductively coupled plasma atomatic emission spectrometry; ISC - iron-sulfur cluster; NiR - nitrite reductase; RT-qPCR - real-time quantitative PCR; SDH - succinate dehydrogenase; SUF - sulfur mobilization.
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2001, Pestana *et al*. 2005, Barton and Abadia 2006). However, a molecular basis towards Fe metabolism in woody angiosperms is not completely understood. How Fe-S cluster biosynthesis genes behave during flowering process of fruit trees remains essentially unknown.

In plants, sunlight not only provides energy for photosynthesis but also acts as environmental signal that regulates floral induction (Van Doorn and Kamdee 2014). Direct sunlight shading is often limiting factor which negatively affects bud formation and flower development (Stutte and Martin 1986, Menzel and Simpson1988, Jacobs and Minnaar 1998, Wu *et al*. 2007). However, peach is typical sun-loving fruit crop, and shading significantly reduces peach pollen germination and flower development (Wu *et al*. 2007), which adversely affects further cross pollination or natural self-pollination. How Fe-S cluster biosynthesis genes response to shading also seems interesting to set about.

In previous studies, we identified 44 putative Fe-S cluster biosynthesis genes in peach genome (Song *et al*. 2014). In this study, we characterized and determined expression profiles of peach Fe-S cluster biosynthesis genes during different flowering stages, especially under shading treatment. Our findings would provide a direct molecular basis for Fe metabolism in flowering process of perennial woody trees.

Materials and methods

Plants and treatments: Five-year-old peach (*Prunus persica* L. cv. Hongfenjiaren) trees growing at the National Peach Germplasm Repository (Nanjing, China) were used throughout this study. Hongfenjiaren is new cultivar of ornamental peaches with pink double petal and dense flowers. The full bloom date is in late March in the Nanjing area (Ma *et al*. 2011). The peach orchard was divided into three plots in which three trees per plot were selected for sample collection. One hundred flowers were randomly picked up with three biological replicates at different flowering stages (FS), including bud swell (FS1, 24th March), beginning bloom (FS2, 26th March), full bloom (FS3, 27th March), and petal fall (FS4, 28th March 2014).

For shading treatment, the flowering shoots of one tree in each group were covered with double layer (yellow outside and black inside) paper bags (*Yunong Co.*, Qingdao, China) on 4th March (the flower bud formation period), and flower samples were collected on 23rd March (the full bloom date of control flowers).

Physiological analysis: One hundred flowers were collected and fresh mass was determined. For Fe content analysis, samples were dried, digested using concentrated HNO_3-$HClO_4$ overnight, and then subjected to inductively coupled plasma atomatic emission spectrometry (ICP-AES; *Thermo Electron*, Waltham, USA). Activity of ACO was carried out as described by Liang *et al*. (2014). One unit of ACO activity was defined as the amount of the enzyme that isomerized 1.0 µmol of citrate to isocitrate per min at pH 7.4. Activity assays of NiR and SDH were determined using relevant detection kits (*Nanjing Jiancheng Bioengineering Institute*, Nanjing, China), according to the manufacturer's descriptions. One unit of NiR activity was defined as the amount of the enzyme required to catalyze reduction of 1 µmol NO_2^-, and one unit of SDH activity was defined as the amount of the enzyme that decreased the rate of FAD reduction by an absorbance change of 0.01 per min in absorbance recorded at 600 nm. Three biological replicates were used for each measurement.

Extraction of RNA and RT-qPCR: Total RNA was extracted from flower samples using a universal plant RNA extraction kit (*BioTeKe*, Beijing, China) and then was reverse transcribed into cDNA using a *PrimeScript*™ RT reagent kit (*TaKaRa*, Kyoto, Japan). Real time quantitative PCR (RT-qPCR) was carried out with a *7500* real time PCR system (*Applied Biosystems*, New York, USA), using a *SYBR Premix Ex Taq* (*TaKaRa*) reaction kit. The *ubiquitin* gene (Genebank No. KJ598788) was used as internal control (Tong *et al*. 2009, Song *et al*. 2014, 2015). Specific primers of Fe-S cluster biosynthesis genes and the control gene are listed in Table 1 Suppl. An RT-qPCR reaction procedure was as follows: 95 °C for 30 s, 40 cycles of 95 °C for 5 s and 60 °C for 34 s, and then 72 °C for 60 s. After amplification, fluorescent data were converted to threshold cycle values. The concentration of sample was determined by relating a threshold cycle value to a standard curve recorded in the *7500 System SDS* software (*Applied Biosystems*). To calculate the starting template concentration and RT-qPCR efficiency for each sample, the linear regression of the log (fluorescence) per cycle number data was used according to the description of Ramarkers *et al*. (2003) by taking the logarithm on both sides of an equation as follows: $\log (Nc) = \log (No) + \log (Eff) \times C$, where Nc is fluorescence, No is the initial concentration of a transcript, Eff is efficiency, and C is the cycle number. The relative expression of target genes were presented after normalization to the internal control (*ubiquitin*) from three independent biological replicates of cDNA templates.

Statistical analysis: For all experiments, data were statistically analysed using the independent sample *t*-test in the *SPSS 13.0* software (*SPSS*, Chicago, USA). Data were compared between the control and shading treatments, and details are described in the figure legends. The graphs were produced using the *Origin 8.0* software.

Results

Flowering details of peach cv. Hongfenjiaren trees were obtained during the years 2012, 2013, and 2014 by measuring flower fresh mass, size, and shape. A typical flowering process, observed in 2014, is shown in Fig. 1 Suppl. together with representative flowers from the bud to bloom. The flowering process can be divided into four distinct stages, FS1 to FS4. During FS1 (24th March), leaf buds stopped expanding and flower buds began to swell. Flower buds began to bloom in FS2 (26th March) with an increasing mass gain and volume. Flowering stage 3 (27th March) was characterized by a fast flower development and blooming flowers reached its final full size. Finally, FS4 (28th March) was the period when flowers drooped and petals began to fall. The fresh mass of 100 peach flowers gradually increased from FS1 to FS3 but began to decrease in FS4 (Fig. 1*A*).

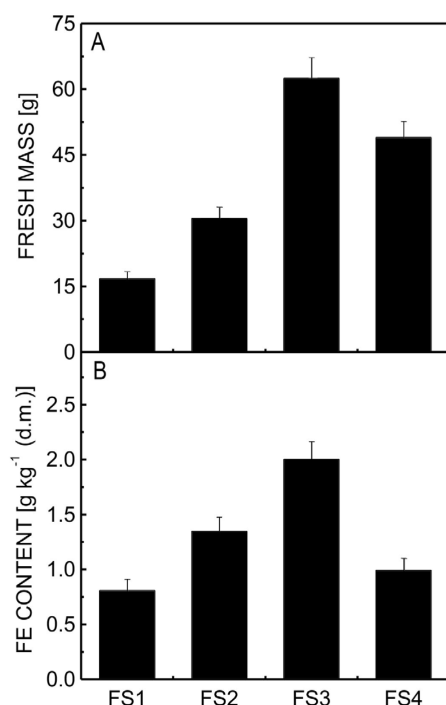

Fig. 1 Fresh mass (*A*) and Fe content (*B*) at different flowering stages including bud swell (FS1, 24th March), beginning bloom (FS2, 26th March), full bloom (FS3, 27th March), and petal fall (FS4, 28th March) in the year 2014. Data are means from three independent replicates ± SEs.

Similarly to the fresh mass gain, Fe content in flowers increased from bud to bloom, and then decreased in FS4 (Fig. 1*B*). The activities of NiR and SDH were positively correlated to the flower Fe content, and their highest activities were in FS3, whereas the ACO activity kept steady, especially from FS1 to FS3, and slightly decreased in FS4 (Fig. 2).

In previous studies, 44 putative Fe-S cluster biosynthesis genes were isolated and identified from the peach genome (Song *et al*. 2014). Names, locus ID, proposed subcellular and functional localization of these Fe-S cluster biosynthesis genes are given in Table 2 Suppl. In particular, 44 Fe-S cluster biosynthesis genes were unevenly distributed on 8 scaffolds, and scaffold 6 had 9 genes, whereas scaffold 2 had none. Gene structure analysis shows that all the peach Fe-S cluster biosynthesis genes possessed at least one intron, with the exception of *SUFE1* and *NFS1* that had no introns. Notably, *ATM3* as ATP-binding cassette transporter possessed 17 introns, varied distinctly in length implying that this gene is more prone to be post-transcriptionally modified.

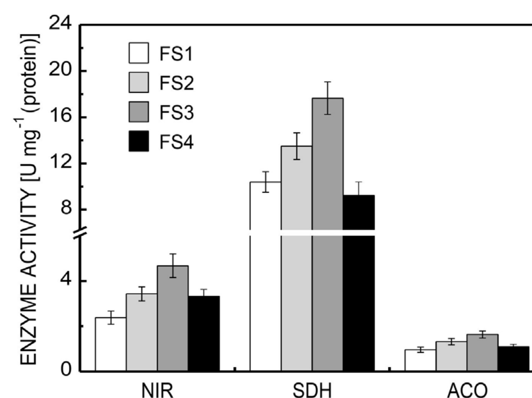

Fig. 2 Activities of NIR, SDH, and ACO during different peach flowering stages (FS1 - FS4; see Fig. 1). Means from three independent replicates ± SEs.

To verify the expression profiles of Fe-S cluster biosynthesis genes during flowering, we carried out RT-qPCR determination. The results show that all 44 genes were differentially expressed throughout the whole flowering process, with the exception of *HSCA1* and *ERV1* that were not detected in peach flowers (Fig. 3). Notably, the highest expression of most genes appeared in FS3, whereas *ADX1* and *DRE2* were evenly expressed throughout FS1 - FS4 (Fig. 3). Although weakly expressed in peach flowers, the expressions of *HSCA5*, *ATM3*, and *MMS19* were enhanced over 3 fold during FS3 compared to FS2 (Fig. 3). In particular, the most remarkable gene was *ISU1*, whose expression was the highest throughout whole flowering (Fig. 3). Possibly, *ISU1* may be a dominant scaffold used for mitochondrial ISC machinery in peach flowers. Although *ERV1* is essential for plant respiration and viability specifically in cytosol (Levitan *et al*. 2004), it could hardly be detected in peach flowers. Moreover, HSCA1 as chaperone could hardly be detected during the whole flowering process, whereas *HSCA2*, *HSCA3*, and *HSCA4* were highly expressed, especially during FS4 (Fig. 3), implying that *HSCA2*, *HSCA3*, and *HSCA4*, instead of *HSCA1*, may be essential chaperones for Fe-S cluster biosynthesis during peach flowering.

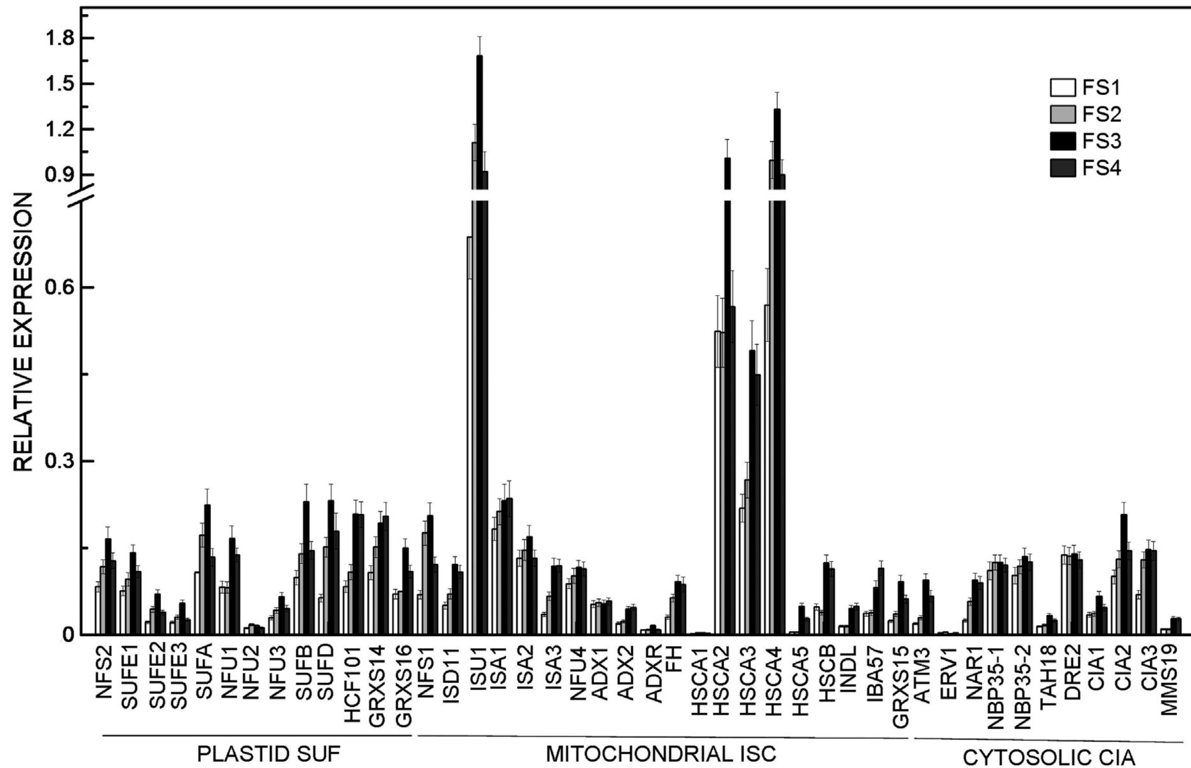

Fig. 3. Differential expression of Fe-S cluster biosynthesis genes during peach flowering. The relative expressions are presented after normalization to the internal control *ubiquitin*. Means from three independent replicates ± SEs.

Fig. 4. The effect of shading on flower development. Flowering shoots were covered with paper bags on 4th March (the flower bud formation period) and flower samples collected on 27th March (the full bloom of control flowers).

In this study, sunlight shading treatment obviously hindered flower development evidenced by more aborted buds and dwarfed and decolorant flowers (Fig. 4). In particular, the fresh mass of 100 FS3 flowers was greatly reduced to approximately 60 % compared to the control flowers (Table 1). Further, the Fe content significantly decreased, and simultaneously the activities of NiR and SDH weres significantly reduced, whereas no change was observed in the ACO activity (Table 1). Totally, 17 peach Fe-S cluster biosynthesis genes were responsive to the shading treatment, and their expressions were mainly reduced with the exception of *ISA1* and *TAH18* that were enhanced (Fig. 5). Notably, the plastid SUF machinery genes were more sensitive to the shading treatment, and 9 out of 13 genes were massively down-regulated, whereas the other 4 genes (*SUFE3*, *SUFD*, *NFU1*, and

Table 1. The physiological analysis of full blooming flowers under a shading treatment. Values are means ± SEs from three sets of biological replicates. *Asterisks* indicate significant differences between the control and shading treatment at $P < 0.01$ (*t*-test).

Full bloom stage	Control	Shading
Fresh mass [mg flower^{-1}]	625 ± 53	248 ± 3.1**
Iron concentration [g kg^{-1} (d.m.)]	2.0 ± 0.3	1.3 ± 0.2**
NiR activity [U mg^{-1} protein)]	4.7 ± 0.4	3.3 ± 0.3**
SDH activity [U mg^{-1} protein)]	17.6 ± 1.4	11.3 ± 1.2**
ACO activity [U mg^{-1} protein)]	1.6 ± 0.2	1.4 ± 0.2

GRXS16) were not affected (Fig. 5). The mitochondrial ISC machinery genes were less sensitive to the shading treatment, and expression of 4 (*NSF1*, *ISU1*, *FH*, and *HSCA4*) out of 20 genes decreased, but expession of the *ISA1* gene significantly increased. As concerns the cytosolic CIA assembly machinery, 3 (*ATM3*, *DRE2*, and *MMS19*) out of 11 genes were down-regulated under the shading treatment (Fig. 5). Although weakly expressed under the control conditions, the *TAH18* gene was nearly two fold up-regulated after being suffered from the shading treatment (Fig. 5).

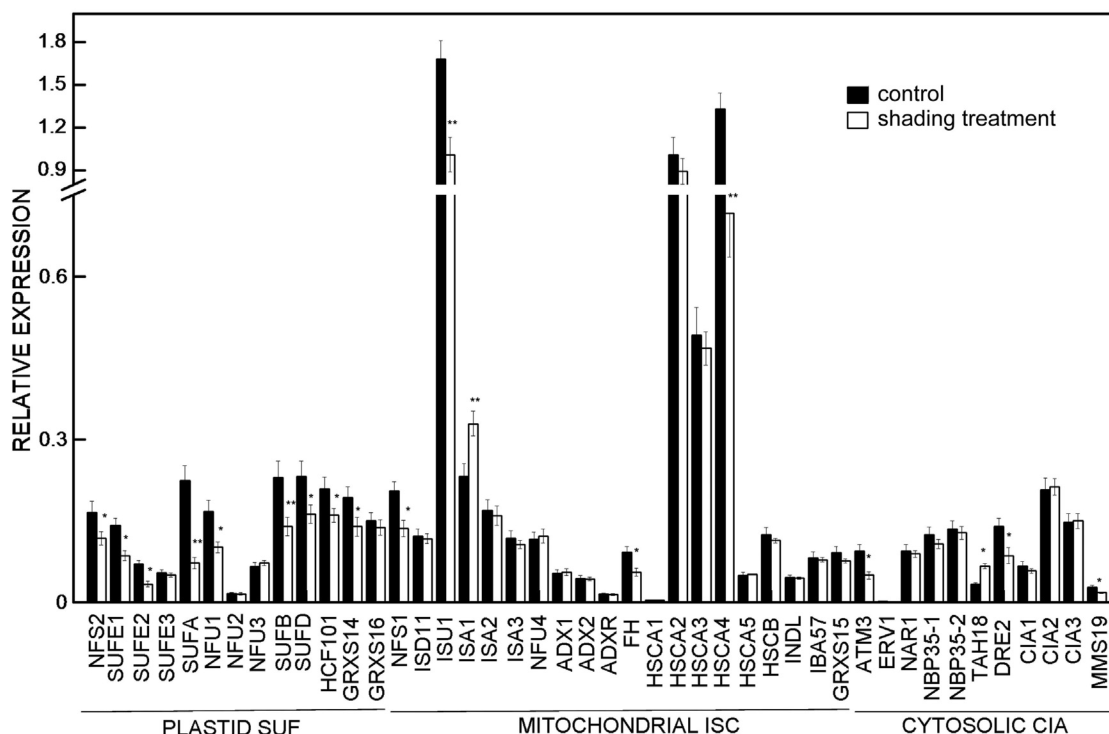

Fig. 5. The response of Fe-S cluster biosynthesis genes to a shading treatment. The relative expressions are presented after normalization to the internal control *ubiquitin*. Means from three independent replicates ± SEs. *Asterisks* indicate ignificant differences between the shading treatment and control plants (* - $P < 0.05$, **- $P < 0.01$, *t*-test).

Discussion

In plants, the Fe–S cluster biosynthesis machinery mainly includes Fe-S cluster formation on biosynthesis scaffolds and transfer to target proteins that requires dozens of genes (Balk and Lobreaux 2005, Raulfs *et al*. 2008, Balk and Pilon 2011, Couturier *et al*. 2013). In this study, we characterized 44 Fe-S cluster biosynthesis genes in peach and determined expression patterns during peach flowering in relation to flower Fe homeostasis and Fe metabolism. The quantitative analysis shows that the Fe-S cluster biosynthesis genes were differentially expressed during the flowering process, and the shading treatment mainly affected expression of the plastid SUF machinery genes. This study helps to elucidate flower Fe metabolism, and reveals potential genes for further functional verification.

The highest expression of most Fe-S cluster biosynthesis genes appeared in FS3 (Fig. 3), which may directly explain the maximum Fe content and activities of NiR, ACO, and SHD during this stage (Figs. 1 and 2). Together, functioning Fe-S cluster biosynthesis genes is crucial for flower Fe metabolism and so maintaining the internal Fe homeostasis, which further facilitates Fe-dependent metabolic pathways or other cellular processes (reviewed in Couturier *et al*. 2013).

Interestingly, the expression profiles of the Fe-S cluster biosynthesis genes in the peach flowers were slightly distinct from that in 1-month-old peach seedling leaves (Song *et al*. 2014). The most abundant genes in leaves of the 1-month peach seedlings are *ISU1*, *GRXS14*, and *ISA1*. The expression patterns of flower Fe-S cluster biosynthesis genes were similar with roots or stems of 1-month-old peach seedlings, and the highly expressed genes were *ISU1*, *HSCA4*, and *HSCA2*, which were all belonging to the mitochondrial ISC machinery pathway. Encoding a scaffold protein (Gerber 2003, Tone 2004), *ISU1* is the most remarkable gene which is highly expressed not only in flowers but also throughout the whole plant of 1-month peach seedlings (Song *et al.*

2014). We guess that ISU1 may be a dominant scaffold of mitochondria Fe-S cluster biosynthesis in peach. It is worth to mention that HSCA1 as chaperone could hardly be detected in flowers, whereas *HSCA2*, *HSCA3*, *HSCA4*, and *HSCA5* genes were highly expressed (Fig. 3), which is consistent with previous studies in 1-month-old peach

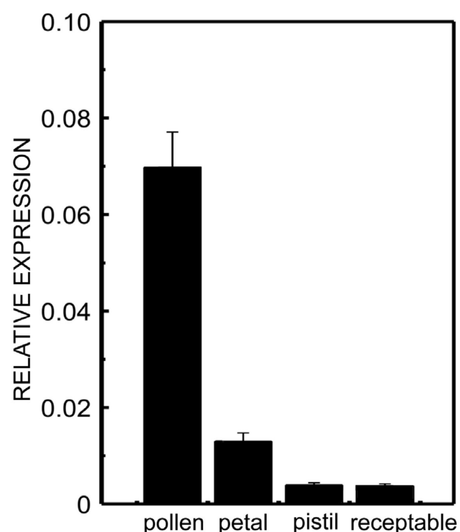

Fig. 6 The tissue-specific expression of *SUFE2*. Full bloom flowers were collected and then separated into parts of petal, pollen, pistil, and receptacle before real time quantitative PCR examination. The relative expression level of the gene is presented after normalization to the internal control *ubiquitin*. Data are means of values obtained from three independent replicates ± SEs.

seedlings (Song *et al*. 2014). These findings demonstrate that HSCA1 may not be essential chaperone for the mitochondrial Fe-S cluster biosynthesis pathway in peach. Notably, ERV1 as the first identified mito-chondrial sulfhydryl oxidase is essential for respiration and vegetative growth (Levitan *et al*. 2004). However, the *ERV1* gene was not detected in either peach flowers (Fig. 3) or 1-month seedlings (Song *et al*. 2014) implying that this gene may not be directly involved in peach Fe metabolism.

Encoding a chloroplastic SufE-like protein, *AtSUFE2* is specifically expressed in *Arabidopsis* flowers (Murthy *et al*. 2007). Similarly, the *SUFE2* gene was obviously expressed in the peach flowers (Fig. 3) but cannot be detected in 1-month-old peach seedlings (Song *et al*. 2014). Further verification shows that *SUFE2* was highly expressed in pollen but weekly in petal, pistil, and receptacle (Fig. 6). These findings again indicate that *SUFE2* may have a special function in peach pollen formation or development. Notably, SUFE1 and SUFE2 were reported to activate cysteine desulfurase activity of NFS2 in *Arabidopsis*, and SUFE3 might be required for interaction with NFS2 and for synthesis/repair of its Fe-S cluster (Xu and Møller 2006, Ye *et al*. 2006, Murthy *et al*. 2007). Such significant findings in *Arabidopsis* are necessary to be verified in typical woody angiosperm fruit trees, including peach, especially during flower formation and fruit development.

Sunlight is essential for floral induction and development (Van Doorn and Kamdee, 2014) and sunlight shading inhibits flower development (Stutte and Martin 1986, Menzel and Simpson1988, Jacobs and Minnaar 1998, Wu *et al*. 2007). In this study, the shading treatment hindered peach flower development and blooming (Fig. 4), which is consistent with previous findings of Wu *et al*. (2007). Notably, nearly 40 % of the peach Fe-S cluster biosynthesis genes were responsive to shading. They were mainly down-regulated, especially those in the chloroplastic SUF machinery pathway (Fig. 5). We speculate that shading possibly obstructed Fe-S cluster biosynthesis in flowers, which further ruined flower Fe metabolism. Indeed, both the Fe accumulation and the activities of the crucial enzymes NiR and SDH significantly decreased under the shading treatment (Table 2).

Sufficient functional scaffolds are required for Fe-S cluster biosynthesis in plants (Balk and Lobreaux 2005, Couturier *et al*. 2013). There are at least nine scaffold encoding genes in peach, and five genes belong to the chloroplastic SUF machinery pathway. Although *ISU1* was down-regulated under shading, its expression was still higher than the expressions of the *NFU2-4* and *NBP35* genes which were not changed under the shading treatment (Fig. 5). Probably, these genes are prone to function as scaffolds in the flower Fe-S cluster biosynthesis pathway, especially under shading treatment. Moreover, ADX and ADXR are crucial to transfer an electron in the respiratory complexes of mitochondria (Picciocchi *et al*. 2003, Takubo *et al*. 2003). In this study, the *ADX1*, *ADX2*, and *ADXR* genes were steadily expressed, even under the shading treatment (Fig. 5) indicating that these genes were essential for the mitochondrial ISC assembly pathway in peach flowers. Interestingly, *TAH18* and *DRE2* as cytosolic CIA assembly genes were more responsive to the shading treatment, but expression of *DRE2* decreased, whereas that of *TAH18* was enhanced (Fig. 5). Likely, peach flowers prefer to utilize more TAH18 to transfer electrons during the cytosolic CIA assembly pathway under shading treatment.

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