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ORIGINAL PAPER

Physiological and transcriptional responses in the iron–sulphur cluster assembly pathway under abiotic stress in peach (*Prunus persica* L.) seedlings

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Abstract As one of the most indispensable element in mineral nutrition of plants, iron (Fe) is closely related to fruits quality and yield. However, molecular mechanisms towards Fe metabolism in fruit trees is largely unclear. In higher plants, iron–sulphur (Fe–S) cluster assembly occurs in chloroplasts, mitochondria and cytosol involving dozens of genes. In this study, we identified 44 putative Fe–S cluster assembly genes in peach (*Prunus persica* cv. 'Xiahui6'), and analyzed Fe–S cluster assembly gene expression profiles in response to abiotic stresses. Peach seedlings were more sensitive to iron deficiency, drought and salinity stress, evidenced in reduced photosynthetic performance and altered activity of nitrite reductase, succinate dehydrogenase and aconitase. In addition, Fe–S cluster assembly genes are differentially regulated by abiotic stresses. Iron depletion and drought stress are likely to affect Fe–S cluster assembly genes in leaves. Excess iron toxicity mainly induces Fe–S cluster assembly gene expression in roots, whereas salinity stress massively inhibits Fe–S cluster assembly gene expression in roots. Interestingly, we found that un-functional scaffolds are more prone to disappear during the long-term evolution in perennial woody plants. Our findings directly provide molecular basis for Fe metabolism in peach, and favorably reveal potential candidate genes for further functional determination.

Keywords Peach · Fe–S cluster assembly genes · Iron homeostasis · Abiotic stress

Introduction

Iron (Fe) is one of the most indispensable element in mineral nutrition of plants, especially in fruit trees (Tagliavini et al. 2000; Pestana et al. 2005). Iron deficiency is a major constraint for many fruit crops grown on calcareous soils, which is often assumed tacitly to negatively affect fruit yield, size and quality (Tagliavini et al. 2000; Tagliavini and Rombolà 2001; Pestana et al. 2005; Barton and Abadia 2006). However, molecular basis towards Fe metabolism in fruit trees is largely unclear.

Many metabolic pathways and cellular processes occurring in most sub-cellular compartments depend on the functioning of iron–sulfur (Fe–S) proteins (Balk and Pilon 2011; Couturier et al. 2013). For example, the Fe–S protein nitrite reductase (NiR) is crucial for chloroplastic nitrogen assimilation (Balk and Lobreaux 2005). Aconitase (ACO) and succinate dehydrogenase (SDH) are key enzymes involved in mitochondrial citric-acid cycle of glycometabolism. In particular, Fe–S cluster are cofactors of Fe–S proteins that play indispensible roles in photosynthesis, respiration, and DNA repair (Johnson et al. 2005; Lill and Muhlenhoff 2006; Rouault and Tong 2008; Lill 2009). A highly conserved Fe–S cluster assembly process includes Fe–S cluster formation on assembly scaffolds and transfer to target apo-proteins that calls for dozens of specific genes (Balk and Lobreaux 2005; Raulfs et al. 2008; Lill 2009). Approximately, forty more genes have been identified as Fe–S cluster assembly related genes in *Arabidopsis* (Balk and Pilon 2011), which are located in chloroplasts, mitochondria, cytosol, and nucleus, respectively (Table 1).

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Table 1 Complete list of Fe–S biosynthesis proteins in *Arabidopsis* and peach

	Protein names	Locus ID	
Plastid	NFS2	At1g08490	ppa005298 m
	SUFE1	At4g26500	ppa007330 m
	SUFE2	At1g67810	ppa017530 m
	SUFE3	At5g50210	ppa001921 m
	SUFA	At1g10500	ppa012351 m
	NFU1	At4g01940	ppa011214 m
	NFU2	At5g49940	ppa011050 m
	NFU3	At4g25910	ppa010743 m
	SUFB	At4g04770	ppa003788 m
	SUFC	At3g10670	–
	SUFD	At1g32500	ppa004982 m
	HCF101	At3g24430	ppa006650 m
	GRXS14	At3g54900	ppa012220 m
	GRXS16	At2g38270	ppa009373 m
Mitochondria	NFS1	At5g65720	ppa005512 m
	ISD11	At5g61220	ppa013993 m
	ISU1	At4g22220	ppa012356 m
	ISU2	At3g01020	–
	ISU3	At4g04080	–
	ISA1	At2g16710	ppa013203 m
	ISA2	At2g36260	ppa12351 m
	ISA3	At5g03905	ppa012679 m
	NFU4	At3g20970	ppa009781 m
	NFU5	At1g51390	–
	ADX1	At4g21090	ppa012568 m
	ADX2	At4g05450	ppa012238 m
	ADXR	At4g32360	ppa004960 m
	FH	At4g03240	ppa011940 m
	HSCA1	At4g37910	ppa002402 m
	HSCA2	At5g09590	ppa001973 m
	HSCA3	–	ppa002222 m
	HSCA4	–	ppa002489 m
	HSCA5	–	ppa002572 m
	HSCB	At5g06410	ppa016242 m
	INDL	At4g19540	ppa019981 m
	IBA57	At4g12130	ppa006632 m
	GRXS15	At3g15660	ppa012405 m
Cytosol	ATM3	At5g58270	ppa002114 m
	ERV1	At1g49880	ppa012227 m
	NAR1	At4g16440	ppa005089 m
	NBP35-1	At5g50960	ppa005998 m
	NBP35-2		ppa007759 m
	TAH18	At3g02280	ppa002941 m
	DRE2	At5g18400	ppa009994 m
	CIA1	At2g26060	ppa007909 m
	CIA2	At1g68310	ppa012624 m
	CIA3	–	ppa012624 m
	MMS19	At5g48120	ppa023072 m

All these ISC proteins in *Arabidopsis* were concluded in the previous studies of Balk and Pilon (2011). Their corresponding homologues in peach were identified by BLAST search in Peach Genome Database. These proteins are putatively located in plastids, mitochondria and cytosol respectively

In particular, the plastids harbor the SUF (sulphur mobilization) pathway and the mitochondria organelles use the ISC (iron–sulphur cluster) assembly pathway, which are working independently, whereas the cytosolic Fe–S cluster assembly depends on the emerging CIA (cytosolic iron–sulphur cluster assembly) pathway and mitochondria (Balk and Lobreaux 2005; Bernard et al. 2013). To date, nearly 60 more iron–sulfur proteins have been estimated or confirmed in *Arabidopsis* (Balk and Pilon 2011), which are physiologically important for plants growth and development. In plastids, Fe–S cluster proteins are reported to be involved in chlorophyll synthesis, nitrite reduction, sulfite reduction, and photosynthesis. In mitochondria, Fe–S cluster proteins are closely related to molybdenum cofactor biosynthesis, electron transfer in complex I and II, glutamate synthesis, and biotin synthesis. When it comes to cytosol and nucleus, Fe–S cluster proteins are involved in abscisic acid biosynthesis, ribosome assembly, DNA replication, and DNA repair (Balk and Lobreaux 2005; Balk and Pilon 2011; Stehling et al. 2012).

Peach is among the most economically important fruits and the most genetically well-characterized species of the *Rosaceae* family (Jung et al. 2008). Regarding iron acquisition, peach is a Strategy I plant, which takes up iron as ferric chelates that mainly includes two steps: At first, ferric chelates were reduced at the roots surface. And then, the generated ferrous ions were absorbed across the roots plasma membrane (Kobayashi and Nishizawa 2012). Iron is translocated from roots to shoots via suitable chelating molecules and proper control of redox states between the ferrous and ferric forms, and imported into individual cells through transporters (Palmer and Guerinot 2009; Kobayashi and Nishizawa 2012). Subsequently, iron is imported into appropriate subcellular compartments for utilization in cellular function, including Fe–S cluster assemble, and to prevent it from accumulating in excess (Kobayashi and Nishizawa 2012).

Fe–S cluster assembly has been extensively studied in the model plant *Arabidopsis*, however, reports focusing on Fe metabolism, especially the Fe–S cluster assembly, in fruit trees is really rare. The knowledge on Fe–S cluster assembly favorably provides insights for the practice of fruit crops, i.e. breeding for higher output, better quality or more tolerant to stresses. In particular, drought (Crisosto et al. 1994; Ozturk et al. 2002), salinity (Mendlinger 1994; Colla et al. 2006), and iron deficiency (Pestana et al. 2005; Barton and Abadia 2006) in orchards are increasingly the major challenges to fruit productivity and quality. How the Fe–S cluster assembly genes respond to such abiotic stresses in peach seems to be interesting and significant to set about.

In this study, we identified 44 putative Fe–S cluster assembly genes in peach, and analyzed the physiological response of peach seedlings and expression profiles of each

Fe–S cluster assembly gene in response to abiotic stresses, including iron deficiency, iron toxicity, drought and salinity stresses. Particularly, our findings also indicated that un-functional scaffolds, especially of mitochondria ISC assembly pathway, were more likely to disappear in perennial higher plants during the long-term evolution.

Materials and methods

Plant material and growth condition

Peach (*Prunus persica* cv. 'Xiahui6') seedlings growing at the National Peach Germplasm Repository in Nanjing, China were used throughout this study. Seeds were washed with distilled water and germinated in soil in a green house. Germinated seedlings with similar growth status were transferred from soil to 1/2 MS solution (containing approximately 50 μM FeNa-EDTA, Murashige and Skoog 1962) for treatment, in a climate-controlled growth cabinet that maintained under 28/23 °C and 12/12 h light/dark (with 60 % relative humidity).

Stress treatment

For the iron excess treatments, germinated seedlings were grown in 1/2 MS nutrient solution containing 500 μM iron ($FeCl_3$, pH5.8). For the iron deficiency treatments, iron was omitted from the MS medium. In drought treatments, seedlings were grown in 1/2 MS nutrient medium supplied with 10 % (w/v) polyethylene glycol (PEG, pH5.8). In salinity treatments, seedlings were grown in 1/2 MS nutrient solution containing 100 mM NaCl (pH5.8). The seedlings were exposed to treatment for 72 h (for qRT-PCR determination) or 21 days (for physiological analysis) and photographed.

Physiological response analysis

Seedlings were rinsed in distilled water, separated into roots, stems, and leaves, and then weighed to obtain the fresh weight. The roots were scanned with an Epson (Long Beach, CA) Rhizo scanner (2004b), and data were acquired with Epson WinRHIZO software.

Photosynthetic analysis was carried out on a portable photosynthetic system LI-6400 (Li-COR, Lincoln, NE, USA) to determine net photosynthetic rate (P_N) and stomatal conductance (g_s) at the terminal leaflet of fully grown second leaf, as described by Kumar et al. (2006). Chlorophyll was extracted from fresh peach seedlings in 95 % ethanol, kept at 4 °C in darkness for 12 h, and then centrifuged at 1,000*g*, 4 °C for 10 min. The supernatant was used for determining absorbance at 665 and 649 nm to obtain chlorophyll *a* and chlorophyll *b*, respectively, and then calculate the total chlorophyll concentration.

Nitrite reductase (NiR) activity assay was carried out as described by Takahashi et al. (2001). ACO activity was checked according to the method (Kennedy et al. 1983). SDH activity was determined as described by Ackrell et al. (1978).

Iron determination was performed using the HNO_3–$HClO_4$ digestion method (Lu 2000). Collected seedlings were washed with distilled water, separated into roots, stems, and leaves, de-colored at 105 °C for 30 min, dried at 70 °C for 48 h, and weighted. Dried samples were ground into powders and were fully digested in 5 mL of concentrated HNO_3 overnight. After digestion for 4 h on Graphite Digestion Apparatus (Smart Block), iron was quantified on ICP-MS (IRIS Advantage, Thermo Electron, USA). Three biological replicates were used for each measurement.

Identification of Fe–S cluster assembly genes in peach

Protein sequences of 43 Fe–S cluster assembly genes of *Arabidopsis* (Balk and Pilon 2011; Stehling et al. 2012) were obtained at Phytozome Arabidopsis genome database (http://www.phytozome.net). These sequences were used as query to BLAST peach genome to identify peach homologues. Coding sequences of the identified putative Fe–S cluster assembly genes in peach were obtained at Genome Database for Rosaceae (Peach).

RNA extraction and quantitative real-time PCR

Total RNA was extracted from leaves, stems, or roots using Plant RNAKit (OMEGA). The extracted RNA was treated with gDNA Eraser to remove genomic DNA contamination, and was reverse transcribed into cDNA using PrimeScript™ RT reagent Kit (TaKaRa). Specific primers for ISC genes and control gene *Ubiquitin* in peach were designed using NCBI/Primer-BLAST on-line server. Primer sequences were listed in Online Resource 1. Quantitative real-time RT-PCR (qRT-PCR) was carried out on 7500 Real Time PCR System (Applied Biosystems, USA). The reaction was carried out in a 20 μL volume containing 1 μL of 1:20 diluted cDNA, 0.4 μM primers, and 10 μ SYBR Premix Ex Taq (TaKaRa). PCR condition for thermal cycling was as follows: 95 °C for 30 s, 40 cycles of 95 °C for 5 s and 60 °C for 34 s. The relative expression levels of the target genes were presented after normalization to the internal control from three independent biological repeats.

Statistical analysis

For all experiments, data were statistically analyzed using Student's *t* test in the SPSS 13.0 software (SPSS Chicago,

IL, USA). Details are described in figure legends. Graphs were produced using Origin 8.0 software.

Results

Identification of Fe–S cluster assembly genes in peach

Taking 43 *Arabidopsis* Fe–S cluster assembly sequences as references, we identified 44 putative ISC biogenesis genes from peach genome by carrying out sequence BLAST (Table 1), and verified protein domains by using InterProScan 4.8 (http://www.ebi.ac.uk/Tools/pfa/iprscan/) and CD search (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) web servers. For each pair of orthologs, there is a 40–89 % identity in protein sequences between peach and *Arabidopsis*, indicating that Fe–S cluster assembly genes are highly conserved across higher plants. Name and locus ID of the 44 putative peach Fe–S cluster assembly genes and their proposed subcellular and functional localization of these proteins were shown in Table 1 and Fig. 1. Notably, although ATM3 and ERV1 are actually mitochondrial proteins, they were grouped as part of cytosolic CIA machinery in that their functions are required for Fe–S cluster assembly in cytosol (Lill and Muhlenhoff 2008; Balk and Pilon 2011). In particular, SUFC as a scaffold is missing from peach genome. In addition, scaffolds of ISU2, ISU3 and NFU5 are missing from peach mitochondrial ISC machinery. Chaperones are reported to function in the Fe–S cluster delivery from mitochondrial ISU scaffolds to target proteins (Vickery and Cupp-Vickery 2007; Ciesielski et al. 2012). Notably, peach genome encodes additional chaperones of HSCA3, HSCA4, and HSCA5, suggesting that peach seedlings probably depends on more chaperones that used for Fe–S cluster delivery to target proteins.

Peach plants utilize less scaffolds for mitochondrial ISC machinery

It is worthy mentioning that two alternative scaffolds, ISU3 and NFU5 for the mitochondrial ISC machinery, are also missing from the rice genome (Liang et al. 2013). The orthologs of ISU3 and NFU5 in *Arabidopsis* was expressed so lowly that these two genes are likely to be non-functional or pseudogenes (Leon et al. 2003). Probably, we speculate that nol-functional scaffolds are likely to be lost during mitochondrial ISC machinery evolution, which needs further verification. In order to support this proposition, we identified the ISU and NFU family orthologs via blasting the genome of nine higher plants species, including two *Brassicaceae* plant (*Arabidopsis* and *Thellungiella*), two *Gramineous* plant (*Brachypodium* and rice), one *Solanaceae* plant (tomato), one *Salicaceae* plant (Polar), one *Rutaceae* plant (orange), and two *Rosaceae* plant (peach and apple) (Table 2). Our findings showed that three herbaceous plant including *Arabidopsis*, *Thellungiella*, and *Brachypodium* possessed all ISU and NFU members, while rice, tomato and polar missed ISU3 in their genome. Interestingly, three representative fruit trees of orange, apple and peach missed both ISU2 and ISU3 in their genome (Table 2). Different from three annual herbaceous plant, i.e. *Arabidopsis*, *Thellungiella* and *Brachypodium*, two annual angiosperm plant (rice and tomato) and four perennial woody plants (polar, orange, apple and peach) missed NFU5 gene in their genome (Table 2). Probably, such findings indicate that perennial woody plants utilize less scaffolds for Fe metabolism, especially in mitochondrial ISC machinery. And non-functional scaffolds are more prone to disappear during long-term evolution.

Expression profiles of Fe–S cluster assembly genes in peach seedlings

To verify the expression profiles of putative Fe–S cluster assembly genes in peach, we performed qRT-PCR determination. Results showed that all 44 genes except SUFE2 were expressed in tested organs, including leaves, stems and roots (Fig. 2). For chloroplast/plastid SUF machinery genes, the expression level was higher in leaves than in stems or roots, which is consistent with their potential function in photosynthesis (Ye et al. 2006a). The most highest-expressed genes of plastid SUF machinery were NFU1 and GRXS14 in leaves. While SUFE2 seemed to be an distinctive chloroplastic protein as its transcript level was not detected in the tested organes. In contrast, half of the 20 mitochondrial Fe–S cluster assembly genes were evenly expressed throughout entire peach plants, whereas six genes was expressed higher in leaves and 4 genes higher in roots (Fig. 2). Except for TAH18 that mainly expressed in roots, most cytosolic Fe–S cluster assembly genes were expressed evenly in leaves, stems, and roots, or the difference in their expression between tissues was not significant (Fig. 2). In particular, the most remarkable genes ISU1 (a scaffold, Gerber et al. 2003; Tone et al. 2004) was highly expressed in leaves, indicating that this gene is probably driven by a stronger promoter in peach. Possibly, ISU1 may be a dominant scaffold in mitochondria of peach. Dramatically, HSCA1 as a chaperone was the least expressed that could hardly be detected in all tested tissues. However, expression level of HSCA2, HSCA3, HSCA4 and HSCA5 were several times of HSCA1, implying that HSCA1 may not be an essential chaperone for mitochondrial ISC pathway in peach seedlings. Notably, ADXR and ERV1 as electron transfers were

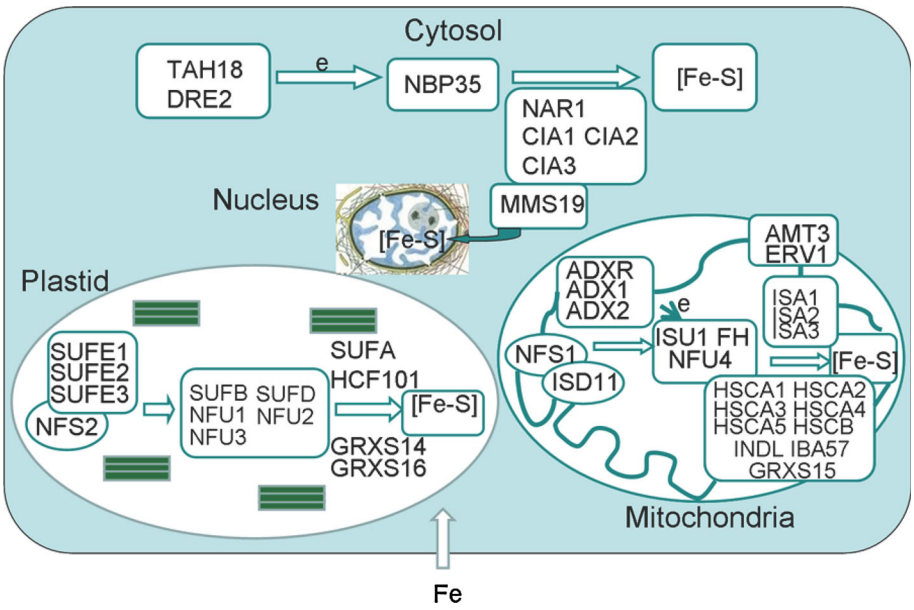

Fig. 1 The proposed function model of Fe–S cluster assembly in peach cells. The obtained 44 proteins involved in ISC biosynthesis were putatively localized in plastids, mitochondria, and cytosol, respectively. The proposed function model were simply schematized, taking *Arabidopsis* as a reference (reviewed in Balk and Pilon 2011)

the second least expressed genes, especially in leaves. While another two electron transfers of TAH18 and DRE2 were expressed at higher level throughout whole plant, nearly eight times of ADXR expression, indicating that peach seedlings are prone to preferentially take TAH18 and DRE2 as electron transfers.

Fe–S cluster assembly genes are differentially regulated by abiotic stresses

To investigate biological response and Fe–S cluster assembly gene expression profiles in peach, we applied various abiotic stresses to peach seedlings, including iron depletion, excess iron toxicity, PEG-induced drought stress and NaCl-induced salinity. Results showed that iron depletion, drought and salinity treatments caused severe phenotype to peach seedlings (Online Resource 2). Notably, most of the Fe–S cluster assembly genes responded to at least one treatment (Online Resource 3). The most dramatical gene was GRXS16, an 2Fe-2S transfer (carrier protein) in plastid SUF machinery, whose expression could be directly regulated by all abiotic stresses. In contrast, 11 genes of NFU1, GRXS14, ISA2, FH, HSCA3, ERV1, NAR1, NBP35-2, DRE2, CIA2, and MMS19 exhibited no response to any treatment (Online Resource 3). Interestingly, Fe–S cluster assembly genes are differentially regulated by abiotic stresses, i.e. of iron deficiency and PEG treatments more specifically affect Fe–S cluster assembly genes in peach shoots, whereas excess iron toxicity and NaCl treatments mainly affect Fe–S cluster assembly genes in roots (Figs. 3e, f, 4e, f).

In particular, genes of the mitochondrial ISC machinery were more sensitive to abiotic stresses (Figs 3, 4), subsequently with plastid (SUF) and cytosolic (CIA) machinery genes. Seventeen out of 20 genes (85 %) in mitochondria, 10 out of 13 genes (77 %) in plastid, and 5 out of 11 genes (45 %) in cytosol, respectively, were responsive to abiotic stresses (Online Resource 3). Furthermore, eight out of the 10 (80 %) responsive genes in plastid and 10 out of the 17 (59 %) responsive genes in mitochondria were intricately regulated by multiple abiotic stresses. However, only ATM3 and CIA2 in cytosolic machinery responded to two kinds of abiotic stresses (Online Resource 3).

Iron depletion likely affects Fe–S cluster assembly genes in shoots while excess iron toxicity induces massive Fe–S cluster assembly gene expression in roots

Iron is an indispensible element in mineral nutrition of plants, and iron deficiency inhibits growth status (Tagliavini and Rombolà 2001; Liang et al. 2013). In this present studies, iron depletion severely hindered growth of peach seedling (Online Resource 2). Dramatically, iron deficiency caused more severe retardation in shoots, evidenced in withered and chlorotic leaves, than in roots. Fresh weight of leaves, stems and roots was reduced approximately 55, 39 and 20 %, respectively (Fig. 3a). Correspondingly, photosynthetic performance was obviously destroyed, embodied in significantly reduced net photosynthetic rate P_N, stomatal conductance g_s and total leaf chlorophyll concentration (Table 3). In particular, iron was mainly accumulated in roots, and iron depletion significantly decreased the tissue iron concentration, especially in roots (Fig. 3c). Notably, iron depletion obviously increased the enzyme activity of NiR in roots and ACO in all tested

Table 2 Orthologs of ISU and NFU members in nine species of higher plants

Species	Arabidopsis	Thellungiella	Brachypodium	Rice	Tomato	Polar	Orange	Apple	Peach
ISU1	At4g22220	Thhalv10026418 m	Bra020855	Os01g47340	Solyc03g112900	Potri.015G077500	orange1.1g030644 m	MDP0000778166	ppa012356 m
ISU2	At3g01020	Thhalv10028164 m	Bra013601	Os05g49300	Solyc07g007450	Potri.012G081700	–	–	–
ISU3	At4g04080	Thhalv10029418 m	Bra029483	–	–	–	–	–	–
NFU1	At4g01940	Thhalv10028839 m	Bra000905	Os03g20010	Solyc01g079220	Potri.002G192200	orange1.1g027469 m	MDP0000245391	ppa011214 m
NFU2	At5g49940	Thhalv10014572 m	Bra037947	Os11g07916	Solyc01g103710	Potri.004G222600	orange1.1g026830 m	MDP0000285539	ppa011050 m
NFU3	At4g25910	Thhalv10026125 m	Bra013933	Os06g47940	Solyc05g044630	Potri.006G165600	orange1.1g038446 m	MDP0000952041	ppa010743 m
NFU4	At3g20970	Thhalv10021272 m	Bra031245	Os05g06330	Solyc11g007120	Potri.010G237400	orange1.1g023823 m	MDP0000150995	ppa009781 m
NFU5	At1g51390	Thhalv10011711 m	Bra018906	–	–	–	–	–	–

organs, which are representative Fe–S proteins for plant metabolism (Balk and Lobreaux 2005), whereas there was no change in SDH activity (Fig. 3d). In addition, iron deficiency mainly affected three genes for plastid SUF machinery in roots and GRXS16 in shoots, whereas largely affected mitochondrial and cytosolic Fe–S cluster assembly genes in shoots, except for HSCA5 in roots (Fig. 3e). Dramatically, IBA57 was greatly up-regulated sixfold in leaves. As a chaperone, HSCA1 was the most remarkable gene that was increased throughout whole plant under iron deficiency (Fig. 3e).

Excess iron toxicity obviously inhibited plant trunk development and roots elongation, but with normal and green leaves (Online Resource 2). Fresh weight of stems and roots, total root length and total root surface area was obviously decreased (Figs. 3a, b). However, leaf fresh weight and total leaf chlorophyll concentration was not changed under iron toxicity (Figs. 3a, Table 3), implying that peach seedlings might possess better tolerance to iron toxicity. Correspondingly, the net photosynthetic rate P_N and stomatal conductance g_s was similar to that of control conditions (Table 3), which may practically explain the normal green leaves and mildly impaired plant growth. In particular, iron toxicity significantly increased the enzyme activity of SDH in roots and NiR throughout entire plant, whereas there was no change in ACO activity (Fig. 3d). Dramatically, iron toxicity largely enhanced expression of ten Fe–S cluster assembly genes in roots (Fig. 3f). Notably, IBA57 was most sensitive to excess iron, whose expression was obviously enhanced in all tested organs (Fig. 3f). Presumably, these findings indicating that these genes are closely related to iron metabolism that maintaining 'luxury utilization' of external-iron or depositing excess iron to where it does not bother plant metabolism, which possibly further secures the roots iron uptake and transportation systems to avoid iron toxicity of plant growth. Simultaneously, the tissue iron accumulation was indeed significantly increased (Fig. 3c). Together, these findings definitely contribute to the better tolerance of peach seedlings to iron toxicity.

Drought stress mainly enhances Fe–S cluster assembly gene expression in shoots

Drought suppresses plant growth with concomitant cellular dehydration and generally prompts stomata to close which restricts the net photosynthetic rate (Ozturk et al. 2002; Pal et al. 2013). In this present study, PEG-induced drought stress caused the worst phenotype to peach seedling, evidenced in dramatically withered and chlorotic leaves and retarded roots (Online Resource 2). Fresh weight of leaves, stems and roots was reduced approximately 76, 48 and 44 %, respectively (Fig. 4a). And total root length and

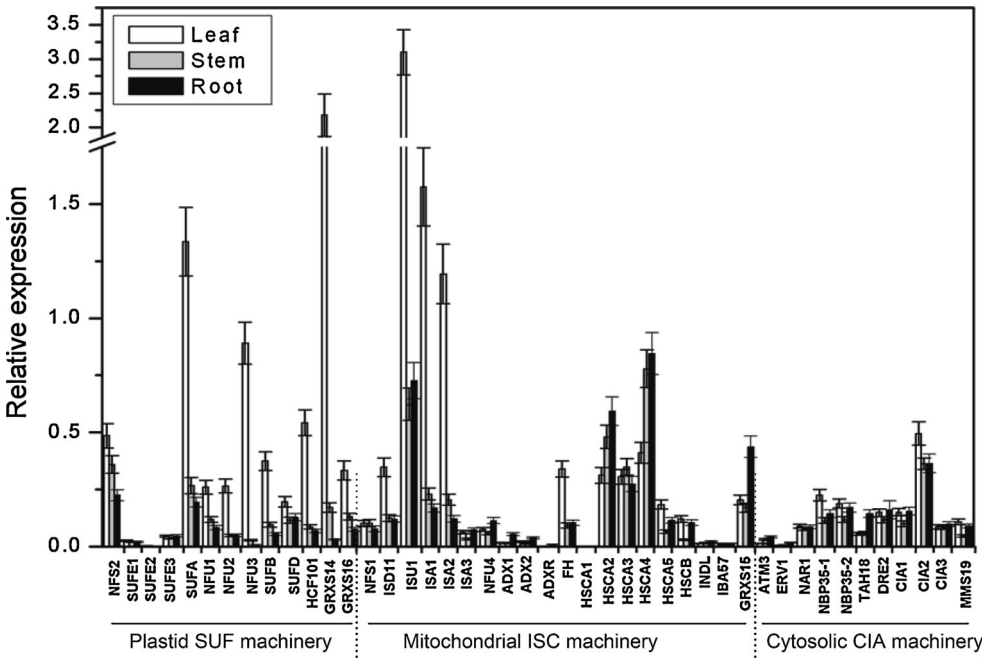

Fig. 2 Expression of 44 Fe–S cluster assembly genes. Seedlings were grown in 1/2 MS solution (50 μM $FeCl_3$, control conditions) for 3 days before qRT-PCR examination. Expression values are given as a ratio relative to the values of actin. Data are the means of values obtained from three independent replicates ± SD. The *dashed lines* was used to separate the genes into the plastid SUF machinery, mitochondria ISC machinery and cytosolic CIA machinery

surface area was obviously decreased (Fig. 4b). Correspondingly, photosynthetic performance was greatly destroyed by drought stress, i.e. significantly reduced net photosynthetic rate P_N, stomatal conductance g_s and total leaf chlorophyll concentration (Table 3). In particular, drought stress significantly enhanced the tissue iron concentration in all tested organs (Fig. 4c). Notably, PEG treatment obviously increased the activity of NiR in roots and ACO activity in all tested organs, whereas decreased SDH activity in roots (Fig. 4d).

Totally, nineteen Fe–S cluster assembly genes were responsive to drought stress, which mainly enhanced these genes in shoots (Fig. 4e). Being a putative delivery protein that weakly expressed under control conditions, INDL was the most remarkable gene, which was up-regulated almost 11 fold in leaves and 8.5 fold in stems (Fig. 4e). Notably, five genes of SUFE1, SUFE3, ADXR, HSCA1 and TAH18 were more sensitive to excess iron, whose expression was obviously enhanced in all tested organs (Fig. 4e). In particular, cysteine desulfurases (NFS) provide sulphur for Fe–S cluster assembly (Pilon-Smits et al. 2002), and SUFE1&3 were reported to be activators of NFS (Xu and Møller 2006; Murthy et al. 2007). Favorably, we speculate that the increased expression level of SUFE1&3 may contribute to enhance the NSF abundance. Indeed, the expression of NSF2 and NSF1 in leaves were simultaneously elevated (Fig. 4e). Hardly expressed under normal conditions, HSCA1 (chaperone) and ADXR (electron transfer) were largely activated under drought stress, indicating that their functions may be required for plant tolerance to drought stress.

Salinity stress mainly affects Fe–S cluster assembly gene expression in roots

Salinity stress is one of the major environmental factor limiting fruit growth and productivity (Mendlinger 1994; Colla et al. 2006). In this study, NaCl-induced salinity stress caused severe phenotype to peach seedling (Online Resource 2), evidenced in obviously reduced tissue fresh weight (Fig. 5a), decreased total root length and surface area (Fig. 5b), impaired photosynthetic performance and total leaf chlorophyll concentration (Table 3). Simultaneously, roots iron concentration was significantly reduced, whereas there was no change in the aboveground parts (Fig. 5c). Notably, NaCl treatment obviously reduced the activity of NiR in leaves, and SDH and ACO activity in all tested organs (Fig. 5d).

In addition, fifteen Fe–S cluster assembly genes were responsive to NaCl treatment. Unexpectly, expression of most genes was decreased in roots (Fig. 5e). In particular, fourteen of these genes belong to the plastid and mitocondria (Fig. 5e). Half of the 14 responsive members, i.e. NFU2, NFU3, SUFB, SUFD, ISU1, NFU4, and NBP35-1, are typical scaffolds (reviewed in Balk and Pilon 2011). Therefore, we speculate that salinity stress probably impairs the chloroplastic SUF and mitochondrial ISC assembly pathway via deactivating alternative scaffolds, especially in roots, which further destroyed the internal iron homeostasis and plant metabolism. Indeed, roots iron concentration and the activity of representative Fe–S proteins, including NiR, SDH, and ACO, was decreased by NaCl treatment (Fig. 5c, d). Notably, HSCA2 as an

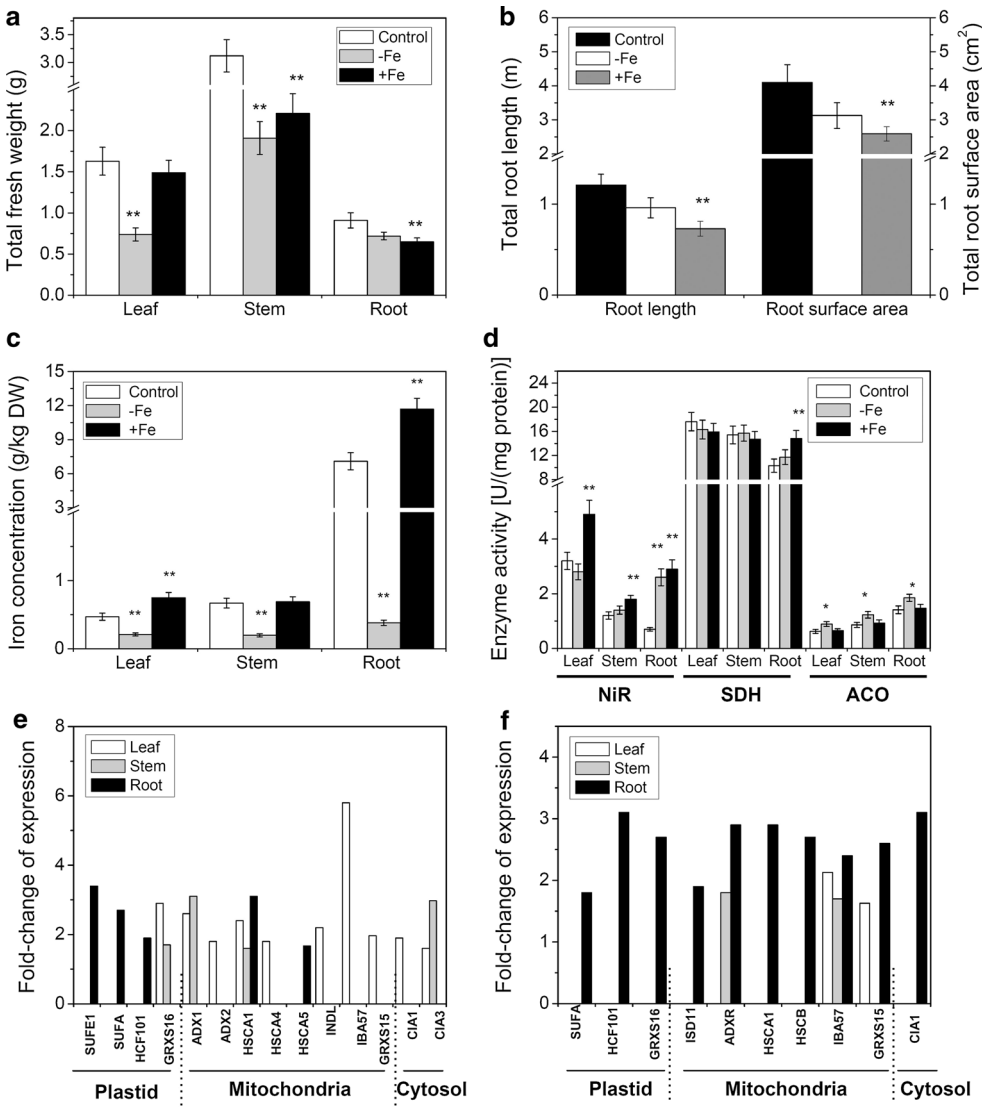

Fig. 3 Physiological response and expression changes in Fe–S cluster assembly genes in response to iron supply. Seedlings were grown in 1/2 MS solution supplied with 50 (control conditions), 0 (-Fe, iron depletion), and 500 μM (+Fe, excess iron) $FeCl_3$ for 72 h (for qRT-PCR determination) or 21 days (for physiological analysis). **a** Total fresh weight. **b** Total root length and surface area. **c** Tissue iron concentration. **d** Enzyme activity of concentration of NiR, ACO and SDH. **e** Fold-change of expression under iron depletion. **f** Fold-change of expression under excess iron stress. Data are the means of values obtained from three independent replicates ±SD. *Asterisks* indicate statistical differences between plants under control and stress treatment. ($0.01 < {*}P < 0.05$, ${**}P < 0.01$, independent-samples *t* test)

chaperone was greately down-regulated in all tested organs under NaCl treatment (Fig. 5e), indicating that this gene might not be essential for plant tolerance to salinity stress.

Discussion

Although the Fe–S cluster assembly in life is highly complex, mechanisms of Fe–S cluster assembly are generally considered to be highly conserved from prokaryotes to eukaryotes (Balk and Lobreaux 2005; Lill 2009). Fe–S cluster assembly machinery mainly includes Fe–S cluster formation on assembly scaffolds and transfer to target proteins and requires dozens of genes (Balk and Lobreaux 2005; Raulfs et al. 2008). In this present study, we have identified 44 putative Fe–S cluster assembly genes in peach. Investigation of gene expression profiles indicate that Fe–S cluster assembly genes are differentially regulated by abiotic stresses. In particular, iron deficiency and PEG treatments more specifically affect Fe–S cluster assembly genes in peach shoots, whereas excess iron toxicity and NaCl treatments mainly affect Fe–S cluster assembly genes in roots (Figs. 3, 4, 5, Online Resource 3). Mighty, this study not only provides direct molecular evidence in Fe metabolism in peach, but also reveals potential genes for further functional verification and molecular breeding of new peach varieties with enhanced tolerance to abiotic stress.

Encoding a chloroplastic SufE-like protein, SUFE2 was not detected in all tested organs in this study. By contrast, SUFE1 and SUFE3 were ubiquitously expressed in peach seedlings, though their expression level were relative low (Fig. 2). Remarkably, AtSUFE2 expression was flower-specific and high in pollen of *Arabidopsis* (Murthy et al. 2007). Assumedly, SUFE2 may have a special function in peach flower formation, especially pollen development, that

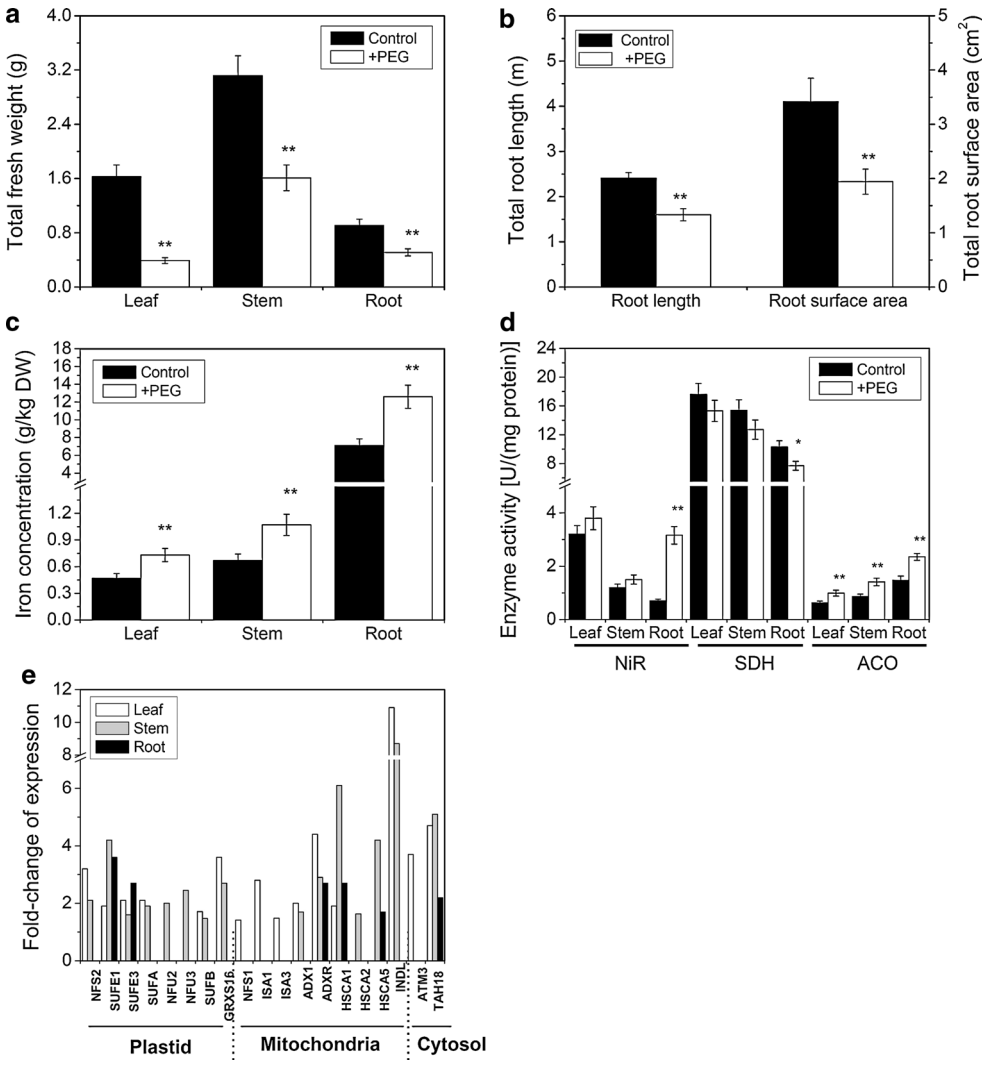

Fig. 4 Physiological response and expression changes in Fe–S cluster assembly genes under drought stress. Seedlings were grown in 1/2 MS solution supplied with 10 % (w/v) PEG6000 (+PEG) for 72 h (for qRT-PCR determination) or 21 days (for physiological analysis). **a** Total fresh weight. **b** Total root length and surface area. **c** Tissue iron concentration. **d** Enzyme activity of concentration of nitrite NiR, ACO and SDH. (e) Fold-change of expression under drought stress. Data are the means of values obtained from three independent replicates ±SD. *Asterisks* indicate statistical differences between plants under control and stress treatment. ($0.01 < {*}P < 0.05$, ${**}P < 0.01$, independent-samples *t* test)

Table 3 Photosynthetical performance analysis of peach seedlings under abiotic stress

Treatment	Chlorophyll concentration (g/kg FW)	P_N [(mg CO_2/(10 cm^2 h)]	g_s [g/(m^2 h)]
Control	1.17 ± 0.12	9.45 ± 0.57	0.24 ± 0.02
−Fe	0.43 ± 0.05**	4.35 ± 0.31**	0.16 ± 0.01**
+Fe	1.12 ± 0.11	8.95 ± 0.61	0.22 ± 0.03
+PEG	0.36 ± 0.04**	2.41 ± 0.22**	0.06 ± 0.01**
+NaCl	0.47 ± 0.06**	4.75 ± 0.53**	0.13 ± 0.02**

Seedlings were exposed to treatment of iron depletion (−Fe), excess iron toxicity (+Fe), drought stress (+PEG) and salinity stress (+NaCl) for 21 days before examination. Data are given as the mean ±SE from three independent experiments. *Asterisks* indicate statistical differences between treatments (** $P < 0.01$, independent samples *t* test)

still needs further verification. In *Arabidopsis*, purified SUFE1 and SufE2 were reported to activate the cysteine desulfurase activity of NFS2, and SUFE3 might be required for the interaction with NFS2 and for synthesis/repair of its Fe–S cluster (Xu and Møller 2006; Ye et al. 2006b; Murthy et al. 2007). The significance of these findings in *Arabidopsis* are highly necessary and pressing to be further verified in the typical woody angiosperm plant peach, especially of flower formation and fruit development.

Sufficient functional scaffolds are required for Fe–S cluster assembly in plants. However, typical scaffolds i.e. ISU2, ISU3 and NFU5 were shown to be lost in several perennial plants, especially in woody fruit trees (Table 2). Definitely, such scaffolds are not essential for Fe–S cluster assembly pathway in these chosen plants. Mighty, higher plants has undergone an intricate and long-term evolution in the Fe–S cluster assembly pathway, especially in mitochondrial ISC machinery, and perennial plants are more likely to evolve a strategy of limiting un-functional scaffolds. Interestingly, other ten scaffolds, including SUFB, SUFC, SUFD, NFU1-4, ISU1, and NBP35-1 and NBP35-2, are constitutively expressed at a moderate to high level. Notably, these genes were not responsive to external iron

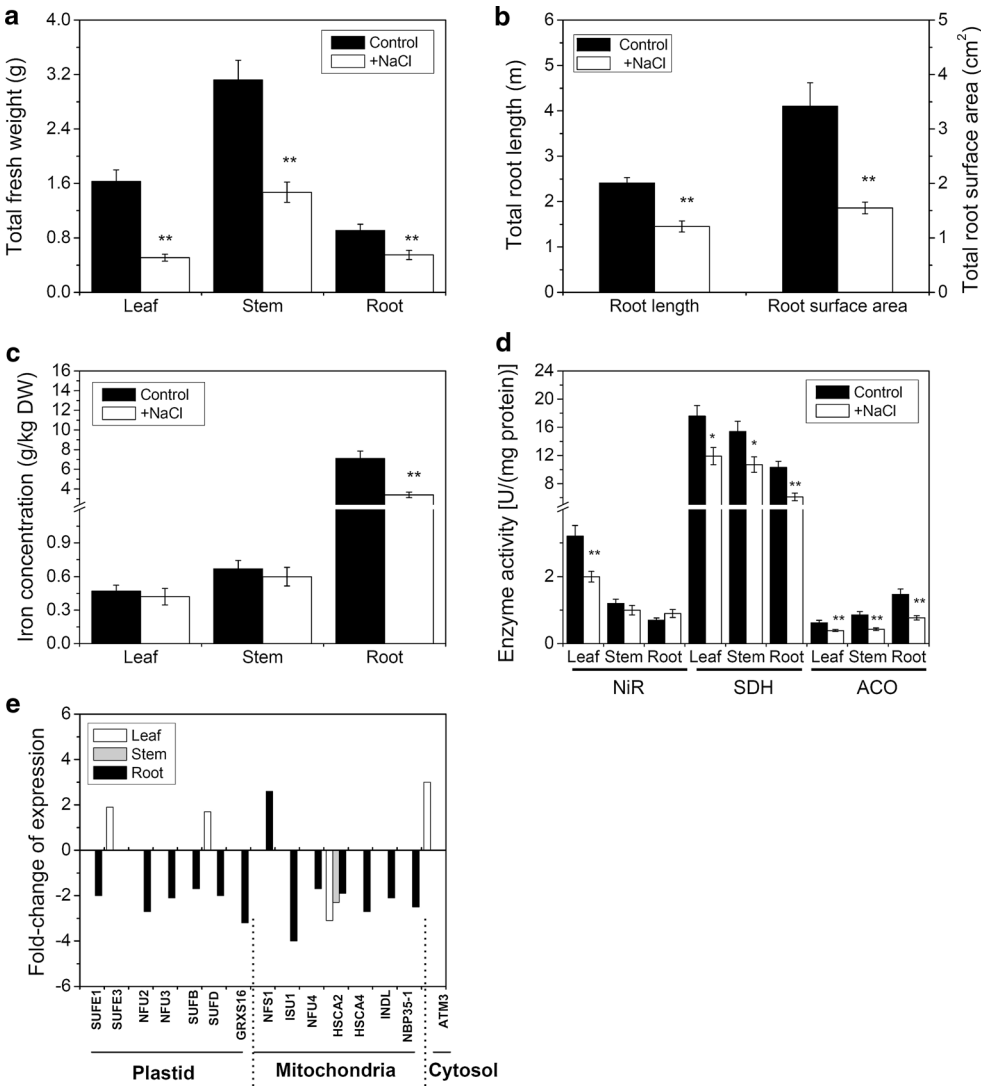

Fig. 5 Physiological response and expression changes in Fe–S cluster assembly genes under NaCl stress. Seedlings were grown in 1/2 MS solution supplied with 100 mM NaCl (+NaCl) for 72 h (for qRT-PCR determination) or 21 days (for physiological analysis). **a** Total fresh weight. **b** Total root length and surface area. **c** Tissue iron concentration. **d** Enzyme activity of concentration of NiR, ACO and SDH. **e** Fold-change of expression under NaCl stress. Data are the means of values obtained from three independent replicates ±SD. *Asterisks* indicate statistical differences between plants under control and stress treatment. ($0.01 < *P < 0.05$, $**P < 0.01$, independent-samples t test)

supplies, either iron depletion or excess iron toxicity. Nonetheless, we speculate that these ten genes are functionally sufficient for Fe metabolism in peach. Contrast to the above mentioned scaffolds, HCF101 expression in peach roots was highly dependent on iron levels, and either iron depletion or iron toxicity could significantly enhance its transcript levels. While HCF101 expression was more constitutive in peach shoots that was not affected by external iron status. In *Arabidopsis*, HCF101 belongs to the P-loop NTPases that specifically assembles and transfer [4Fe–4S] clusters to photosystem I in chloroplasts (Lezhneva et al. 2004; Schwenkert et al. 2010; Stockel and Oelmuller 2004). Supposedly, HCF101 may be more likely to be involved in chloroplast iron homeostasis and photosynthesis, especially under iron depletion.

As carrier proteins, GRXS14 and GRXS16 are 2Fe-2S transfers in cytosolic SUF assembly pathway, which are were shown to be able to bind one 2Fe–2S cluster per dimer with the aid of glutathione (Bandyopadhyay et al. 2008; Cheng et al. 2006; Yadav et al. 2012). However, the expression profiles of them are dramatically different in peach. In particular, the transcript level of GRXS14 was 5 times of GRXS16 in leaves, whereas GRXS16 expression was 3 times of GRXS14 in roots (Fig. 2). Notably, GRXS16 is the most inert gene, which had response to any treatment in this present study. Iron depletion especially enhanced the expression of GRXS16 in shoots, while iron toxicity enhanced the expression of GRXS16 in roots. On the contrary, GRXS14 expression is more constitutive in peach, with no response at all to any treatments (Online Resource 3). The distinct expression and response to abiotic stresses indicated that GRXS14 may be the major carrier protein in chloroplasts of leaves, while GRXS16 may be required for plant tolerance to abiotic stresses, which possibly made it be a candidate gene in peach breeding for better traits of stress tolerance.

Previously studies showed that FH (frataxin) may be the iron source for Fe–S cluster assembly in *Arabidopsis*, and

knockout of this gene caused embryo lethality (Busi et al. 2006; Vazzola et al. 2007). Recently, FH was reported to play a role in regulating NFS1 activity in *Arabidopsis* mitochondria (Turowski et al. 2012). Considering its steady expression throughout the entire plant that responsed to no abiotic stress (Fig. 2 and Online Resource 3), we speculate that FH is absolutely indispensible for mitochondrial ISC assembly pathway in peach, which may not only be the iron source, but also presumably be involved in regulating NFS1 activity.

Being the reductase for ADX (Adrenodoxin), ADXR is thought to have a function of electron transfer in mitochondrial ISC assembly pathway (Picciocchi et al. 2003; Takubo et al. 2003). Compared with ADX1 and ADX2, ADXR expressed at an extremely low level and was highly activated under high external iron supply and drought stress. Simultaneously, both of iron supply and drought stress caused an increase of internal iron accumulation, especially in roots. Mighty, ADXR is prone to play the role of reductase especially when the tissue iron concentration was sufficiently high.

Being as delivery proteins for Fe–S proteins, i.e. INDL for respiratory complex I (Bych et al. 2008; Sheftel et al. 2009) and IBA57 for radical SAM (S-adenosyl methionine) enzyme (Gelling et al. 2008; Sheftel et al. 2012). Although both INDL and IBA57 were expressed at a dramatically low level (Fig. 2), they were easier responsive to different abiotic stresses. Taking the most remarkable HSCA1 for example, HSCA chaperone family members were also highly responsive to various abiotic stresses. These findings favorably revealing important roles for such genes in tolerance to adverse environmental stresses and in iron homeostasis of peach seedlings.

As an famous ATP-binding cassette transporter, ATM3 plays key role in the biogenesis of cytosolic Fe–S proteins in *Arabidopsis* (Chen et al. 2007; Bernard et al. 2009; Luo et al. 2012). Although had no response to external iron levels, ATM3 expression was up-regulated, especially in leaves, by drought and salinity stresses (Fig. 4f). Mighty, ATM3 is more likely to take part in the tolerance to natural stresses.

In conclusion, we identified 44 putative Fe–S cluster assembly genes in peach, which were differentially regulated by abiotic stresses. These genes may differently contribute to iron homeostasis and stress tolerance in peach seedlings. Notably, we found that un-functional scaffolds are more prone to disappear during the long-term evolution. Our findings directly provide molecular basis for Fe–S luster assembly in peach, and favorably reveal potential candidate genes for further functional determination.

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