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Subcellular iron transport genes in *Arabidopsis thaliana*: insights into iron homeostasis

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ABSTRACT

Subcellular iron homeostasis genes (SIHGs) (Vacuolar iron transporter 1 (VIT1), natural resistance-associated macrophage protein 3 and 4 (NRAMP3 and NRAMP4), ferric chelate reductase 7 and 8 (FRO7 and FRO8), and Permease in chloroplasts (PIC1) take part in sequestration, translocation and remobilization of iron between compartmentalized organs and cytoplasm. Therefore, to shed light on these genes' functions in different biological processes, in silico analyses of these proteins were conducted. Posttranslational modification (PTMs) analysis showed that all SIHGs can be dynamically regulated due to variations in their phosphorylation sites. The evolutionary tree of SIHGs revealed that VIT1, NRAMP3, and NRAMP4 may be derived from a common ancestral protein. In Protein-Protein Interaction (PPI) network analysis, the VIT1 gene was identified as an essential gene in subcellular iron homeostasis in *Arabidopsis*. Expression and co-expression analyses showed that these proteins may be components of various metabolic pathways, in a way explained as follows: NRAMP3 may be involved in starch metabolism, pathogen defense mechanism, and nutrient mobilization in senescence, and interact with aldehyde dehydrogenases (ADH), a protein superfamily regulating plant growth stages and regulated by abiotic stress mechanism. NRAMP4 is involved in iron uptake mechanism, acclimation, and stratification processes in dormant seeds with the stimulation of ethylene and CBFs. The VIT1 expression may be regulated by nicotianamide (NA). Salicylic acid (SA), as a plant hormone, may be the key activator of PIC1 expression. PIC1 appears to have taken part in DNA repair, pathogen response mechanisms, and sugar metabolism. Additionally, PIC1 may have vital roles in the cellular redox environment, in particular, chloroplast development. FRO8 may take part in SA-mediated systemic acquired resistance (SAR) network and DNA double-strand break repair mechanism. Lastly, similar to PIC1, FRO7 may be a component in DNA repair, SA, and pathogen defense mechanisms as well.

Key words: FRO7; FRO8, Iron homeostasis; NRAMP3; NRAMP4; VIT1

Introduction

Iron (Fe) homeostasis is a hot topic for plant mineral nutrition. Fe deficiency causes serious problems in human populations and approximately two billion people are reported to be influenced by Fe deficiency (Wu et al., 2002). Fe deficiency is also a problem in the plant kingdom. Its deficiency particularly affects respiration, chlorophyll biosynthesis, and photosynthetic electron transport in plants (Marschner, 1995). Fe is an important cofactor for fundamental biochemical activities in cells when it is taken up within a sufficient range (Vigani et al., 2013). However, excess Fe can be toxic in cells since it causes the generation of hydroxyl radicals damaging cellular components such as DNA and proteins (Halliwell et al., 1992).

Fe uptake in plants can be classified into the strategy I and strategy II, also known as reducing and chelating strategies, respectively. The main difference between the two strategies is based on the uptake form of Fe in strategy I

(Fe²⁺) and strategy II (Fe³⁺) (Connorton et al., 2017a; Kurt & Filiz, 2018). To regulate the proper storage and the use of iron, Fe must be safely translocated to some anatomical parts of the plant and compartmentalized into specific organelles in the cell (Jeong & Guerinot, 2009). In this respect, vacuoles are vital compartments and play important roles in iron storage and sequestration in plant cells. Several studies showed that the vacuole is an essential compartment for iron storage in seeds and vacuolar iron transporter 1 (*VIT1*) gene is identified as one of the key genes allowing this transport (Lanquar et al., 2005; Kim et al., 2006). In addition, *NRAMP3* and *NRAMP4* genes, the metal transporter proteins, belonging to the natural resistance-associated macrophage protein family, export the Fe from vacuoles (Lanquar et al., 2005).

In the chloroplast, Fe is used in chlorophyll biosynthesis, photosynthetic electron transport, Fe-S cluster assembly, and other crucial metabolic processes (Briat et al., 2007). Jeong & Connolly (2009) reported that chloroplast ferric chelate

reductase (*FRO7*) was essential for seedling survival under iron-limiting conditions. Proving this suggestion, the *fro7* mutants showed 75% less ferric chelate reductase activity and contained 33% less iron than wild-type chloroplasts. Another gene, permease in chloroplasts *1* (*PIC1*) or *TIC21*, also serves as a transmembrane protein in the inner envelope of *Arabidopsis* chloroplasts and contributes to Fe transport in chloroplast (Duy et al., 2007).

Mitochondria also demand larger amounts of Fe for the respiratory chain protein complexes and Fe-S clusters in plant cells (Briat et al., 2007). The Fe demand in mitochondria is relieved by expression of *FRO8*, a gene predicted to be localized in mitochondria (Heazlewood et al., 2004; Jeong & Connolly, 2009). Despite the functions of these genes in Fe metabolism, there are very limited studies about their involvement in other biological processes. Consequently, to explain these genes' functions in different biological processes in a more detailed way, *in silico* analyses of subcellular iron homeostasis genes/proteins were performed.

Materials and Methods

Sequence analysis of SIHG

The SIHG sequences were retrieved from Uniprot (<http://www.uniprot.org>). The exon structures and amino acid lengths of the proteins were identified in Phytozome v12.1 (<http://phytozome.jgi.doe.gov/pz/portal.html>; Goodstein et al., 2012). Protein domains were determined by Pfam 31.0 (<https://pfam.xfam.org>; Finn et al., 2016). The subcellular localizations of proteins were checked with CELLO server (<http://cello.life.nctu.edu.tw/>; Yu et al., 2006). Transmembrane helices of proteins were found on TMHMM Server v. 2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>; Krogh et al., 2001). Posttranslational modifications of SIHG were established by NetPhos 3.1 Server (<http://www.cbs.dtu.dk/services/NetPhos/>; Blom et al., 1999).

Phylogenetic and protein structure analyses

The Phylogenetic tree of SIHG was constructed with Mega 7 software (Kumar et al., 2016) using the maximum likelihood (ML) method with 1000 replicates. The gene sequences, other than *Arabidopsis*, in the phylogenetic tree, were collected from NCBI Reference Sequence Database (RefSeq) and NCBI protein database. The gene sequences of species used for phylogenetic analysis were as follows: *Morus notabilis*, *Zea mays*, *Nicotiana attenuata*, *Nicotiana tabacum*, *Glycine max*, *Populus trichocarpa*, *Solanum lycopersicum*, *Cicer arietinum*, *Oryza sativa japonica*, *Brachypodium distachyon*, *Brassica Napus*, *Malus domestica*, *Arachis hypogaea*, and *Vigna unguiculata*. Tertiary structures were predicted by Phyre² server (<http://sbj.bio.ic.ac.uk/phyre2/>; Kelley & Sternberg, 2009) and, the secondary structures of proteins were found by Sopma Server

(https://npsa-prabi.ibcp.fr/cgi-bin/secpred_sopma.pl; Combet et al., 2000). The 3D structures were validated through Ramachandran plots on Rampage server (<http://mordred.bioc.cam.ac.uk/~rapper/rampage.php>; Lovell et al., 2002). The proteins were superimposed on I-TASSER (<https://zhanglab.ccmb.med.umich.edu/TM-score/>; Zhang & Skolnick, 2004) and Click server (<http://cospi.iiserpune.ac.in/click/>; Nguyen et al., 2011) to have TM-score and overlap values, respectively.

Protein interaction, expression and co-expression analyses

Putative protein-protein interaction network of SIHG proteins was constructed using STRING v10 server (<https://string-db.org/cgi/help.pl?sessionId=UuGcudKqG854>; Szklarczyk et al., 2015). Expression analyses were conducted on Atted-II (<http://atted.jp>; Obayashi et al., 2018) and Genevestigator (Hruz et al., 2008) platforms. For expression analyses on the Genevestigator, the genes of interest were queried on the database and expression profiles were constructed by perturbations tool. The studies providing high, medium or low expression of the interested genes by developmental stages were chosen from the dataset and interpreted. Regarding co-expression analyses on the Genevestigator, the co-expression tool was employed to reveal the identification of the most correlated genes with each SIHG. Twofold change as the expression ratio and a significance level of 0.05 were applied as filtering criteria for the co-expressed genes with each SIHG. The default correlation threshold was used for the analysis. The genes most correlated with our investigated targets were interpreted based on the literature.

Results and discussion

Sequence analyses of gene/protein

For sequence analyses, the six SIHG (*VIT1*, *NRAMP3*, *NRAMP4*, *FRO7*, *FRO8*, and *PIC1*) were analyzed (Table 1). While the exon numbers ranged from three to nine, the protein lengths varied between 250 and 747 amino acid residues. The PF01988 (VIT1 domain) in *VIT1* gene, PF01566 (NRAMP domain) in *NRAMP3* and 4 genes, PF01794, PF08022, and PF08030 (NAD binding domains) in *FRO7* gene, PF12263 (Protein of unknown function: DUF3611) in *PIC1* gene, PF01794 (Ferric reductase like a transmembrane component), PF08022 and PF08030 (NAD binding domain) in *FRO8* gene were identified as domain structures. The number of transmembrane helices (TMH) was found between three and 12 and the highest number of TMHs was identified as 12 in NRAMP4 protein. The phosphorylation sites of serine and threonine or tyrosine were found between 21 and 118, indicating the dynamic regulations of SIHG in *Arabidopsis*.

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Table 1. Sequence properties of six subcellular iron homeostasis genes (SIHG) s

Transcript ID (Phytozome)	Uniprot ID	Gene name	Exon number	Subcellular location	Protein length (aa)	Domain family	TMH	PTM
At2g01770	Q9ZUA5	VIT1	4	Vacuole	250	PF01988	5	21
At2g23150	Q9SNV9	NRAMP3	4	Vacuole	509	PF01566	11	42
At5g67330	Q9FN18	NRAMP4	3	Vacuole	512	PF01566	12	61
At5g49740	Q3KTM0	FRO7	9	Chloroplast	747	PF01794 PF08022 PF08030	10	118
AT2g15290	Q9SHU7	PIC1(TIC21)	4	Chloroplast	296	PF12263	3	77
At5g50160	Q8VY13	FRO8	8	Mitochondria	728	PF01794 PF08022 PF08030	10	92

Legend: PF01988: VIT1, PF12263: Protein of unknown function (DUF3611), PF01566: Nramp, PF01794: Ferric reductase like transmembrane component, PF08022: FAD-binding domain, PF01794, PF08022, PF08030: NAD binding domain, TMH: transmembrane helices (TMHMM Server v. 2.0) PTM: Post-translational modification.

This result is particularly crucial in terms of these proteins' functions since the posttranslational modifications (PTMs) expand proteome diversity, resulting in functional divergence in gene expression, signaling, protein stability and interactions, and enzyme kinetics in plants (Friso & van Wijk, 2015). The protein sequence identity values ranged from 0.032 to 0.748 and showed lower sequence identities. The lowest and highest identity values were found between FRO7&VIT1 and NRAMP3&NRAMP4, respectively. FRO7 and FRO8 proteins belonged to the same gene family, showed lower identity as 0.264 than NRAMPs, proving the high level of genetic variations in NRAMP3 and 4 genes.

Phylogenetic analyses

The phylogenetics of SIHG s was constructed according to ML method with 1000 bootstraps and the results showed that SIHG s were grouped into five main groups (NRAMP, VIT1, PIC1, FRO8, and FRO7 groups) (Figure 1).

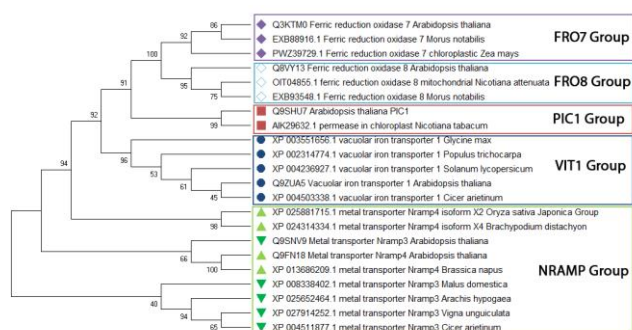


Figure 1. The phylogeny of subcellular iron homeostasis genes (SIHG) s in various species. The tree was built by ML method with 1000 bootstraps in MEGA 7.0 software.

FRO7 and FRO8 were sister groups (100% bootstrap value) and outgroup to PIC1 with 91% bootstrap value. In a

similar manner, VIT1 has shared the same ancestor with FRO7, FRO8, and PIC1 with 92% bootstrap value. However, NRAMP genes showed a clear divergence among monocots and dicots. Nonetheless, a divergence was not observed among NRAMP3 and NRAMP4 genes. AtNRAMP3 was split from other NRAMP3s. The identification of the function of a protein requires a combination of versatile schemes such as sequence similarity, fold identification, surface cavities and binding pockets, residues binding to DNA, and etc. In addition, interactions of the proteins in a metabolic pathway are also a distinctive tool to help understand their functions (Watson et al., 2005). At this juncture, it was reported that VIT1, NRAMP3-4 have complementary functions and interactions in seedling process in which VIT1 forms Fe pools; and NRAMP3 and NRAMP4 remobilize these pooled Fe for strong seedling germination in endodermal cells under iron limiting conditions (Nouet et al., 2011; Eroglu et al., 2017). In sum, the phylogeny of SIHG s showed that similar gene families distributed homogeneously.

Tertiary structure analyses of SIHG proteins

Phyre² server was employed for predictions of 3D structures of SIHG s. Model validations of investigated proteins were checked with Ramachandran plot analysis on the Rampage server. Residues in models were varied in a range of 85.9 -97.6% in the favored and allowed region. Therefore, the model qualities were good. The secondary structure analyses on Sopma Server showed variations between 35.34 -60.4% for α -helices, 7.20 -21.82% for extended strands, 3.04-5.20% for beta turns and 25.78-39.76% for random coils. To make inference about protein structure similarities, we superimposed proteins as pairwise using the Click server and also had structural similarities' TM-scores in the I-TASSER server (Supplemental File, Table 1). Although percentages of structural overlaps of

proteins and their RMSD scores were provided by the Click server, these two parameters are not sufficient to come to a conclusion about the degree of similarities of the compared proteins. Therefore, we employed TM-score metrics which are known for their robustness against traditional problems like root-mean-square-deviation (RMSD) (Zhang & Skolnick, 2004). The results showed that the highest structural similarities were between NRAMP3 and NRAMP4 with a 0.54 TM score and 91.55% structural overlap. This result is in line with the phylogenetic analysis. Moreover, although the highest TM scores were obtained from FRO8-PIC1 (0.21), PIC1-VIT1 (0.21), NRAMP3-VIT1 (0.17), FRO8-VIT1 (0.16) and NRAMP4-VIT1 (0.16) superimpositions, these results were not found to be important since their TM scores were not between 0.5-1. Lastly, pairwise superimposition of NRAMP3-PIC1 and of NRAMP4-PIC1 were generated a considerable overlap value; however, TM scores of these superimpositions, which were 0.15 and 0.13 respectively, showed random structural similarity.

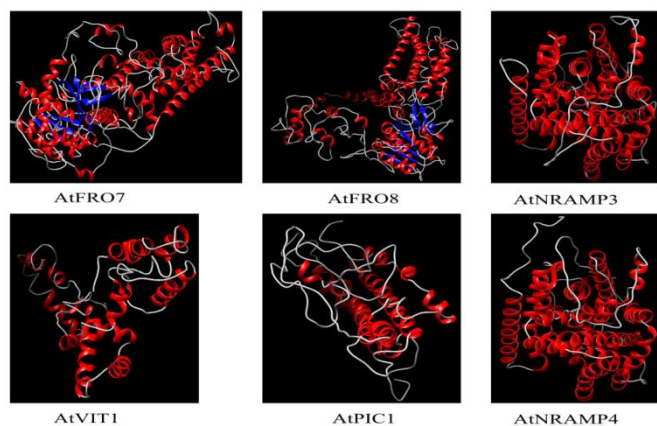


Figure 2. The predicted tertiary structures of SIHG proteins by Phyre² server. The red, blue, and grey colors indicate helix, sheet, and random coil structures, respectively.

The network of protein-protein interactions

The protein-protein interactions (PPIs) studies are versatile resources to reveal unknown functions of proteins at the molecular level and to provide a glimpse into complex cellular networks (Fukao, 2012). In this study, all SIHG were used to construct the PPI network and a total of six nodes and nine edges were identified (Figure 3). Slavic et al., (2016) reported that vacuolar sequestration of Fe is vital for detoxification of excess iron and vacuolar iron transporters (VITs) play significant roles in this process. The PPI topology revealed that VIT1 was a central protein in the network and had direct connections to all genes except for PIC1, confirming the essential role of VIT1 in subcellular iron homeostasis in *Arabidopsis*. Duy et al. (2007) stated that PIC1 comes from the cyanobacterial origin and, most likely, it functions in iron permease in the chloroplast envelope. In the PPI network, the divergence of PIC1 (TIC21) from others

may be related to the cyanobacterial origin and essential functions in plants.

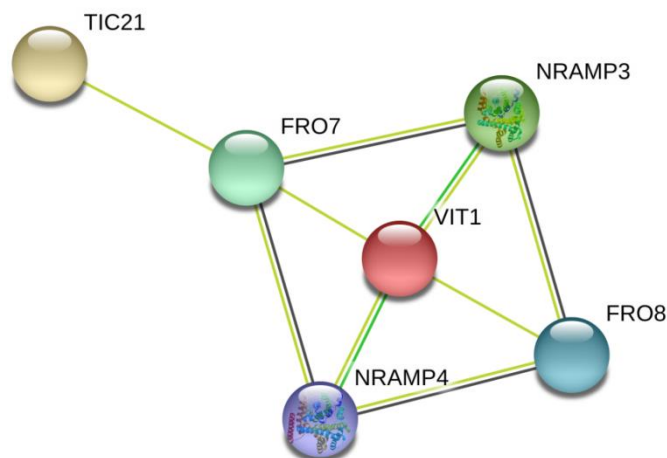


Figure 3. The PPI network of six SIHG genes in STRING server. Number of nodes and edges were identified as six and nine, respectively. Average local clustering coefficient was 0.667.

Co-expression networks

The increasing amount of genomic information obtained from plant species may support obtaining deep insight into molecular mechanisms (Li et al., 2015). The co-expression networks are efficient tools to annotate unknown genes in plant genomes (Serin et al., 2016). In this study, co-expression networks were constructed using four genes, including *PIC1*, *NRAMP3*, *NRAMP4*, and *FRO8* by ATTED-II (Figure 4) and Genevestigator co-expression databases (Supplemental File, Table 2-5).

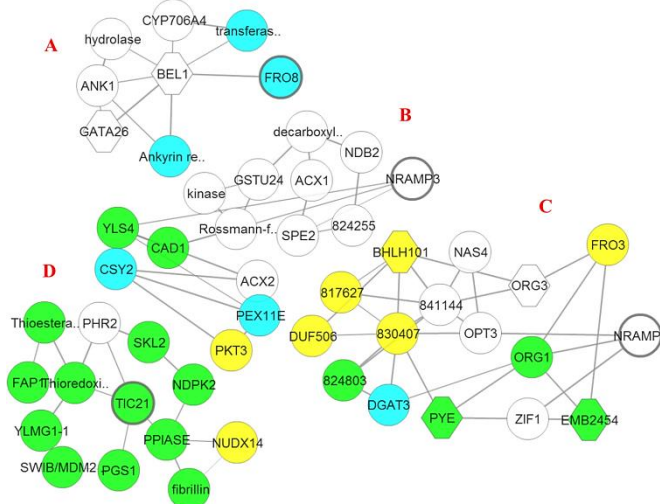


Figure 4. Co-expression network of SIHG. A, B, C, and D capitals represent the network of *FRO8*, *NRAMP3*, *NRAMP4*, and *PIC1*(TIC21) available on ATTED-II server, respectively.

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The *FRO8* gene had one direct connection with the homeobox (*BEL1*) (*AT5G41410*) gene (Figure 4A). The *BEL1*-like family (*BELL*) of transcription factors are ubiquitous among plant species and they regulate a range of developmental processes by interacting with *KNOTTED1*-like proteins (Sharma et al., 2014), suggesting that *FRO8* gene may play roles in plant development. Also, supporting this evidence, the Genevestigator co-expression network analysis showed that *BEL1* and *KNAT4* (Homeobox protein knotted-1-like 4 and a.k.a, *AT5G11060*) genes had the highest correlation coefficient values with *FRO8*; i.e. 0.49 and 0.48, respectively (Supplemental file, Table 2).

The three first neighbors were detected for the *NRAMP3* gene; *aspartate aminotransferase 3* (*Asp 3* or *At5g11520*), *arginine decarboxylase 2* (*At4g34710*), and *NAD(P)-binding Rossmann-fold superfamily protein* (*At5g19440*) (Figure 4B). However, the co-expression network constructed in Genevestigator showed different results (Supplemental file, Table 3). Pearson correlation scores of these results in Genevestigator showed that *CAD8* (*Cinnamyl alcohol dehydrogenase 8* or *AT4G37990*), *SRG1* (*Protein SRG1* or *AT1G17020*), *Vacuole membrane protein KMS1* (*KMS1* or *AT4G14950*) and *Asp 3* were the most correlated genes with *NRAMP3*. The scores were 0.521 for *CAD8*, 0.489 for *SRG1*, 0.481 for *KMS1* and 0.476 for *ASP3*. The co-expression of *NRAMP3* reveals that *NRAMP3* may also partake in various metabolisms.

CAD genes are one of ten genes involving in the biosynthesis of three different monolignols, a major component of lignin and a crucial part of pathogen defense. This enzyme family catalyzes the reduction reaction of three cinnamyl aldehydes to alcohols at the final stage of the coupling of different monolignols. Manipulation of *CAD* genes are of more importance to obtain less recalcitrant lignocellulosic material for biofuel production, compared to repression of genes involving in an early stage of lignin biosynthesis, since this manipulation changes lignin biosynthesis and thereby causes less impaired plant growth, increase of the digestibility, and saccharification efficiency (Bukh et al., 2012).

The conversion of aldehydes to alcohols, or vice versa, takes place in the presence of alcohol or aldehyde dehydrogenases (*ADH*), reducing nicotinamide adenine dinucleotide (i.e., NAD^+ to NADH). This enzyme family particularly targets aldehydes, yielding cinnamyl alcohol (Zucca et al., 2009). *ADH* is found in chloroplast and the mono iron enzyme is upregulated under dark anoxic conditions as a key regulator involving in ethanol production (Van Lis et al., 2016). The upregulation of *ADH* is associated with improved starch accumulation. Other than that, *ADH* is reported to be expressed highly under various environmental conditions such as dehydration and low temperature and in

response to abscisic acid (*ABA*). Fruit ripening, seedling and pollen development processes in plants are also reported to be regulated in the presence of *ADH* (Thompson et al., 2010). In closing, *NRAMP3* is co-expressed with genes involving in starch metabolism and pathogen defense mechanism and may interact with aldehyde dehydrogenases (*ADH*), a protein superfamily regulating plant growth stages which are regulated by abiotic stress mechanism.

At5g53450 (*OBP3-responsive gene1* or *ORG1*), *At5g13740* (*Zinc induced facilitator 1* or *ZIF1*) and *At4g16370* (*Oligopeptide transporter* or *OPT3*) were found as three putative neighbors for *NRAMP4* gene (Figure 4C). Nonetheless, the co-expression network for *NRAMP4* in Genevestigator (Supplemental file, Table 4) showed that *PAP14* (*Probable plastid-lipid-associated protein 14, chloroplastic* or *AT5G53450*), *OPT3*, *Transcription factor bHLH47* (*bHLH47*, *Popeye-PYE-* or *AT3G47640*), *ORG3* (*Transcription factor ORG3* or *ORG3*) and *ZIF1* were the most co-expressed genes with 0.553, 0.487, 0.474, 0.447 and 0.446 coefficient scores, respectively.

Seed viability is particularly dependent on iron transport, regulated by *OPT3* (Gayomba et al., 2015). On the other hand, *PYE* (*bHLH47*) negatively regulates *FRO3*, *NAS4*, and *ZIF1*. (Gollhofer et al., 2014; Li et al., 2016). Transporter families like *NRAMP* were reported to act in either transport of the metals from the plasma membrane to cytoplasm or redistribution of them from intracellular compartments into the cytoplasm (Hu et al., 2012). However, it appears that *PYE* network may negatively affect *NRAMP4*, by interacting with *ZIF1*, and may inhibit transport and redistribution of metals, particularly iron. To summarize, *NRAMP4* generally seemed to be co-expressed with genes involved in iron uptake mechanism and may be inhibited by *PYE* network.

The phosphatidylglycerolphosphate synthase 1 (*At2g39290*), thioredoxin superfamily protein (*At3g52960*), photolyase/blue-light receptor 2 (*PHR2* or *At2g47590*), and rhodanese-like domain-containing protein (*At5g19370*) were identified as neighbors of *PIC1* gene (Figure 4D). Nonetheless, the co-expression network in Genevestigator for *PIC1* led to different gene interactions than the network obtained from Atted-II (Supplemental file, Table 5). The highest correlation coefficients for *PIC1* gene were as follows: 0.6672 for *PRXIII* (*Peroxiredoxin-2E, chloroplastic* or *AT3G52960*), 0.666 for *CJD1* (*Chloroplast J-like domain 1* or *AT1G08640*), 0.659 for *UPP* (*Uracil phosphoribosyltransferase, chloroplastic* or *AT3G53900*) and 0.655 for *PHR2* (*Blue-light photoreceptor PHR2* or *AT2G47590*). *PIC1* functions as a transmembrane protein and involve in Fe transport into the chloroplast. *PHR2* is also suggested to be part of a network for chloroplast development. Therefore, a cross-talk may be suggested for

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PIC1 and PHR2 under iron deficiency due to their roles in chloroplast development (Joshi et al., 2013).

The other neighbor of PIC1, Phosphatidylglycerol (PG), is a ubiquitous membrane phospholipid and it is synthesized by a pathway shared in prokaryotes and eukaryotes (Dowhan 1997). Tanoue et al., (2014) stated that PG biosynthesis is essential for the embryo development and normal membrane structures of the chloroplast, and mitochondria in *Arabidopsis*.

As for thioredoxins, they are small proteins catalyzing the thiol-disulfide interchange and playing important roles in the regulating redox environment of the cell (Gelhaye et al., 2005). These co-expression data about *PIC1* gene showed that PIC1 have vital roles in cellular redox environment, in particular, chloroplast development.

Digital expression analyses

To find out at which developmental stages SIHG are activated, the expression analysis was conducted in the Genevestigator (Table 2).

Table 2. The expression levels of SIHG genes in terms of developmental stages

Gene ID	Gene Nam	G S	S d	Y R	D S	B	Y F	D F	F S	M S	S
At2g01770	VIT1	M	L	L	L	L	L	L	M	M	M
At2g23150	NRA MP3	M	M	M	M	M	M	M	M	M	M
At5g67330	NRA MP4	H	H	H	H	H	H	H	H	H	H
At5g49740	FRO7	M	M	H	H	H	H	H	H	H	M
AT2g15290	PIC1	H	H	H	H	H	H	H	H	H	H
At5g50160	FRO8	M	M	M	M	M	M	M	M	M	L

Legend: GS: germinated seed, Sd: seedling, YR: young rosette, DS: developed rosette, B: bolting, YF: young flower, DF: developed flower, FS: flower and siliques, MS: mature siliques, S: senescence. Also, L, M, and H show the low, medium, and high levels of expression.

The results showed that *NRAMP4* and *PIC1* were expressed in all growth stages at high levels. *FRO7* expression was found higher at all stages except germinated seed, seedling, and senescence. Interestingly, *NRAMP3* was expressed at medium level in all stages; as for *VIT1*, it was generally expressed at low levels.

NRAMP3 and *NRAMP4* are functionally redundant genes and take part in the mobilization of stored iron from vacuoles during germination along with *VIT1* (Lanquar et al., 2005). Nonetheless, although *NRAMP3* and *NRAMP4* are suggested to be expressed in a similar pattern in *Arabidopsis* under iron deficiency (Lanquar et al., 2005) and in *Thlaspi*

caerulescens under Fe starvation, and Zn excess and Cd toxicity (Oomen et al., 2009), the expressions of *NRAMP3* and *NRAMP4* have different expression patterns in developmental stages. The difference in our analysis may be explained by collecting data from various perturbations such as light, chemicals and etc. under different developmental stages.

NRAMP3 was reported to be transcribed at a medium level in a mutant which is a lack of starch synthase III. In that experiment, the leaf starch content was stated to increase (Li et al., 2009). This result shows that *NRAMP3* may involve in starch metabolism, explained in the co-expression analysis in this study. Also, Balazadeh & Mueller-Roeber (2008) identified *ANAC059* and *ANAC092* genes as targets of senescence-affiliated transcription factors. In this experiment, the *NRAMP3* was induced at a moderate level at the MS stage. The involvement of *NRAMP3* in this process is explained by nutrient mobilization (particularly nitrogen and iron) from senescent organs to seeds under stress and nutrient-scarce conditions. Consequently, *NRAMP3* may play a role in nutrient mobilization in senescence and starch metabolism (López-Millán et al., 2016).

As regards *NRAMP4*, it was upregulated highly in all stages of plant life but its expression under dormancy and germination inducing conditions was somehow different. Narsai et al. (2011) suggested that upregulated subset genes during stratification, germination and post-germination may be induced at specific stages. The regulation of these subsets is dependent on the stratification of seeds having dormancy. Ethylene signaling begins during stratification and mitochondrial function occurs at the germination stage. If hormonal stimulation (ethylene) takes place at the germination, the germination fails or if plants are not stratified then, hormonal stimulation never occurs and seeds never germinate. Consequently, the upregulation of *NRAMP4* may be suggested to be started earlier than germination for dormant seeds. In addition, Kendall et al., (2011) stated that seed maturation is a temperature-dependent process in which *DELAY OF GERMINATION (DOG1)*, a dormancy-controlling gene, and hormones of gibberellin and abscisic acid were stimulated by low temperatures. *DOG1* and dormancy regulation is regulated by C-REPEAT BINDING FACTORS (CBFs), acting in low temperatures. Since *NRAMP4* was highly induced in seed maturation, *NRAMP4* may also be upregulated by CBFs. Collectively, *NRAMP4* may take part in acclimation and stratification processes in dormant seeds by stimulation of ethylene and CBFs.

The expression of the *VIT1* pattern was reported to be different from *NRAMPs* (Bashir et al., 2016). *VIT1* was suggested to be expressed moderately during pollen tube growth and development (Pina et al., 2005). Pollen

development and pollen tube passage take place according to the nicotianamine (NA) function. The NA is of importance for the distribution of Fe from phloem to sink organs (Schuler et al., 2012). Connorton et al. (2017b) suggested that the success of biofortification is related to iron flux into vacuoles, emanating from NA stimulation. Consequently, VIT1 expression may be regulated by NA.

PIC1 is a key regulator of chloroplast development and required for all plant developmental stages (Bashir et al., 2016). The highest expressions of PIC1 were found at the DS stage. Interestingly PIC1 was highly transcribed at the DS stage together with new novel genes and pathways involving in the DNA double-strand break mechanism. Based on the expression pattern of PIC1, we may suggest that *PIC1* may be one of the genes on one of the pathways of DNA repair mechanism (West, 2007). As a proof that may strengthen this suggestion, Zhang (2014) reported that iron-requiring proteins in eukaryotes, regulated by iron homeostasis genes, are key agents of DNA replication, DNA repair, metabolic catalysis, iron regulation, and cell cycle progression. Iron is used as a cofactor by DNA polymerases/primases, DNA helicases, and ribonucleotide reductases, the enzymes involving in DNA repair and replication mechanism. Additionally, at the DS stage, Suzuki et al. (2005) found that constitutively and overexpressed multiprotein bridging factor 1c (*MBF1c* or *At3g24500*) increased biotic (bacterial infection) and abiotic stress tolerance against salinity, heat, and osmotic stressors. Transcription of the *MBF1c* gene increases sugar and defense transcripts in response to heat stress. The increased sugar (sucrose) concentration in roots triggers ferric chelate reductase activity and iron uptake genes such as *FRO2*, *IRT1*, and *FIT* in roots (Lin et al., 2016). Thus, more iron is taken up by root epidermis and complexed to the NA, produced by nicotianamine synthase 2 (*NAS2*). At this stage, the excess iron is either transported to the vacuole or moved to the pericycle using symplastic route for NA chelation again. The second chelation is taken place by *NAS4* and after chelation; iron is loaded into the xylem (Palmer et al., 2013) and distributed to the leaves. At this point, probable upregulation *PIC1* in sugar metabolism seems to be stimulating the transportation of iron into chloroplasts.

PIC1 upregulation in response to *MBF1c* stimulus can also be explained by plant hormones. Salicylic acid (SA) (Khan et al., 2015), jasmonate (JA) (Li et al., 2010), and ABA (Lee & Luan, 2012) function as key regulators in both biotic and abiotic stress conditions. *MBF1c* plays a key role in heat tolerance in response to heat shock in *A. thaliana* and acts in upstream of SA together with SA-regulated pathogenesis-related 1 (*PR1*). Consequently, *PIC1* may be suggested as a component of abiotic and biotic stress pathway induced by *MBF1c*. Similarly, *PIC1* is highly upregulated in parallel with the upregulation of *ERD15* (*Early Responsive to*

Dehydration 15), a drought-responsive gene that is induced during a pathogen attack (Helenius et al., 2006). Apart from that, a negative relationship is present between the overexpression of *ERD15* and ABA responsiveness, a drought-responsive hormone. Moreover, *ERD15* induces SA-dependent pathogen defense expression (Shao et al., 2014). Corroborating this suggestion, Chandran et al. (2009) found that under virulent powdery mildew infection *chloroplastic isochorismate synthase 1* (*ICS1*), a gene involving in the synthesis of SA, influences processes like redox, vacuolar transport/secretion, and signaling. Thus, *ICS1* mutants show susceptibility for powdery mildew infection. *ICS1* is also known for its involvement in iron and calcium homeostasis. In parallel to this, under Silverleaf whitefly (SLWF) infection, a phloem-feeding insect, *PIC1* was identified as one of 700 transcripts upregulated (Kempema et al., 2007). As a result, the upregulation of *PIC1* may be related to SA mediated systemic acquired resistance (SAR) mechanism. Thus, we can conclude that the SA as a plant hormone may be the key activator of *PIC1* expression. Moreover, *PIC1* involves sugar, DNA repair and pathogen response mechanisms.

As for *FRO7* and *FRO8*, they are shoot-specific genes (Kim & Guerinot, 2007); however, *FRO8* was restrictively shoot-specific in senescence and both genes reduced ferric iron to ferrous iron in chloroplast and mitochondria in different developmental stages, respectively (Jain & Connolly, 2013). West (2007) also stated that, in parallel with *PIC1*'s involvement in the DNA repair mechanism, *FRO8* was upregulated at medium level in the DNA double-strand break repair mechanism. As mentioned above, this may be stemming from iron-requiring proteins' involvement in DNA repair and the replication mechanism (Zhang, 2014). At this juncture, *FRO8* also seems to be one of the downstream genes of immune responses. In *Arabidopsis*, the *RAD51* gene (a gene involving in DNA double-strand repair mechanism) binds to promoters of defense genes under SAR. The binding is mediated by SA and *BREAST CANCER 2* (*BRCA2*) gene (a main defense-related gene regulator). *BRCA2*- *RAD51* involve in plant immune responses (Wang et al., 2010). In this network, two newly identified DNA damage proteins, *SSN2* and *RAD51D*, interacts with *SNI1*, a negative regulator of *NPR1* (a key regulator of the salicylic acid-mediated SAR), to regulate pathogen-related gene expression. These pieces of evidence show that *FRO8* may be downstream genes of SA-mediated SAR (Song et al., 2011).

As regards *FRO7*, Jeong & Connolly (2009) reported that no matter what form of iron is transported into the chloroplast through *PIC1*, active involvement of *FRO7* and *PIC1* has still not been identified. Nevertheless, as does happen in Strategy I, *FRO7* is highly expected to be affiliated with, *IRT1*, a transporter protein, which imports ferrous iron into the

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chloroplast. In our analysis, we observed a considerable similar expression pattern at the development stage between PIC1 and FRO7. In the analysis, the highest FRO7 expression was observed in the DS stage like PIC1. And similar to PIC1, FRO7 was highly transcribed in DNA repair mechanism (West 2007) under conditions upregulating ERD15 (Helenius et al., 2006) and MBF1c (Suzuki et al., 2005). FRO7 may also be regulated by SA mechanism and be involved in pathogen defense mechanism (Chandran et al., 2009; Kempema et al., 2007).

The expression of SIHG from *Arabidopsis* under different stressors was shown in Figure 5.

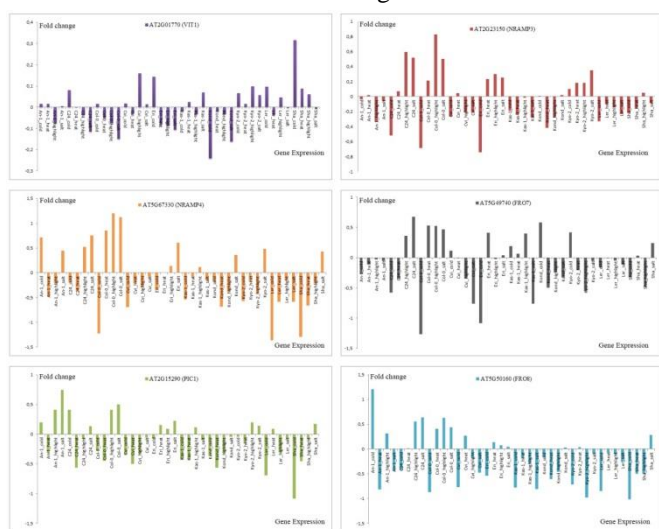


Figure 5. *VIT1*, *NRAMP3*, *NRAMP4*, *FRO7*, *PIC1*, and *FRO8* expressions in ten *Arabidopsis* ecotypes exposed to cold (10°C), heat (38°C), highlight (800 μm photons m⁻²s⁻¹), and salt (100 mM NaCl) perturbations for 3 hours. The ecotypes were Antwerp (An-1, Belgium), Cape Verdian Islands (Cvi), Columbia (Col-0, United States), Coimbra (C24, Portugal), Erigsboda (Eri, Sweden), Kashmir (Kas1, India), Kondara (Kond, Tajikistan), Kyoto (Kyo-2, Japan), Landsberg (Ler, Poland) and Shadara (Sha, Tajikistan).

The lowest expression was observed in *NRAMP4* in the Landsberg ecotype under cold stress (-1.361 fold). On the other hand, the highest expression among SIHG was found in *FRO8* under An-1_Cold treatment as 1.21 fold. The *NRAMP4* was the most downregulated gene by 65 % in terms of all treatments and ecotypes whereas *VIT1* and *FRO8* were least downregulated genes by 50 %. Moreover, *VIT1*, *NRAMP3*, *NRAMP4*, *FRO7*, *PIC1*, and *FRO8* were highly transcribed under cold (20%), heat (15%), salt (17.50%), cold-heat (10% for both), salt (15%), and heat-salt (12.50% for both) perturbations, respectively.

Rasheed et al. (2016) suggested that *FRO8* and *VIT1* upregulated in roots under drought stress. Specifically, *FRO8* was transcribed between the fifth and ninth days of drought treatment whereas the expression of *VIT1* was the highest in the seventh day of it. The activation of these genes in a

specific stage of drought may be related to the detoxification strategies of *Arabidopsis*. Free iron in cells (Fe²⁺) induces the formation of one of the highly reactive oxygen species (ROS) and reactive hydroxyl (-OH) through Fenton reaction (Becana et al., 1998). The high concentrations of intracellular metal ions are compartmentalized in vacuoles by means of several transporters (Manara, 2012) like *VIT1* (Slavic et al., 2016). In case that sequestration of excess iron is not effective to prevent iron toxicity, plants employ oxidative stress defense mechanism; i.e., signaling molecules such as hormones, and stress proteins like heat shock proteins (Manara, 2012) to prevent ROS (like reactive hydroxyl) formation, damage of DNA, lipids and proteins (Connolly & Guerinot, 2002). As a result, the involvement of *VIT1* and *FRO8* in drought stress may be stemmed from detoxification of excess iron and the prevention of DNA repair, respectively.

Conclusion

In this study, we tried to shed light on the probable functions of SIHG in various metabolic pathways. The results are particularly important to understand and manipulate, plant stress mechanisms in response to biotic and abiotic stresses as well as to develop new crops for biofortification studies. Furthermore, they could contribute to the understanding of Fe homeostasis in plants.

Author contribution statement

EF and FK conceived the study. FK and EF wrote the manuscript; and all authors read, edited, and approved the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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