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Insilico analysis of Arabidopsis ferric reductase oxidases (FRO) proteins associated with iron homeostasis

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Abstract
The ferric reduction oxidase (FRO) gene family is involved in various biological processes of plants and plays an essential role in metal homeostasis, tolerance, and signaling networks in response to several abiotic stresses. Our study describes the structural, functional characterization, and evolutionary relationships of eight Arabidopsis FRO proteins. The studies predicted the subcellular localization of FRO proteins to the plasma membrane, mitochondria, and chloroplast organelles. The structural analysis revealed localization of proteins onto the first and fifth chromosomes having 8-9exons and 8-10 transmembrane helices. The protein features of FRO proteins revealed 699-747 amino acids having 79600.02-84126.3 (Da) molecular weight. The six highly conserved protein motifs were predicted with 45-50 amino acids long representing ferric chelate reductase family domains. The phylogeny tree constructed using Clustal W divided the FRO proteins into two clusters and the interactome network revealed the co-expression of COPT1, NRAMP1, NRAMP3, NRAMP4, FRD3, OPT3, IRT1, IRT2, ZIF1, PYE proteins along with the seven FRO proteins.

Keywords: Insilico, Arabidopsis, reductase, associated, homeostasis

Introduction
Plant growth and development are greatly affected by the imbalance supply of mineral nutrition, affecting crop productivity (Victoria *et al*., 2012) [19]. The most needed transition micronutrient, iron (Fe), is found almost in all living organisms, contributing to the redox centers of proteins which are essential for respiration, biosynthesis of chlorophyll, and photosynthesis (Takagi *et al*., 1984) [18]. The rapid changes in the oxidative state of iron stimulate cellular function, regulation, electron transport, and various other metabolic functions (Grotz *et al*., 2006; Suzuki *et al*., 2012; Bashir *et al*., 2016) [7, 16, 2]. The gene regulation networks may alter the expression level in response to Fe toxicity (Quinet *et al*., 2012) [12], where the transporters and transcription factors are the key factors that are involved in iron translocation (Bashir *et al*., 2012) [16]. Therefore, the regulation of genes related to iron stress may help in plant adaptation against adverse conditions. The ferric reduction oxidases (FROs) gene encoding ferric reductase activity is executed by the ferric chelate reductase (FCR) enzyme which is mainly positioned in roots and shoots (Jeong *et al*., 2009) [10]. The plant FROs are expressed in different tissues depending on their locations within cell compartments and are responsible for iron homeostasis, transport, and stress response (Gama *et al*., 2017) [5] and Ferric-chelate reductase (FRE) was first identified in Arabidopsis (Robinson *et al*., 1999) [13].

More recently the subcellular localization of FRO family proteins was identified, where the authors reported that FRO2, FRO3, and FRO5 are expressed in roots having a role in iron uptake from the soil (Connolly *et al*., 2003) [4], and FRO6, FRO7, and FRO8 are positioned in shoots, while FRO1 and FRO4 gene expression occurs in both roots and leaves, but the expression is comparatively low (Wu *et al*., 2005; Mukherjee *et al*., 2006; Jeong *et al*., 2009; Jain *et al*., 2014) [23, 11, 10, 9]. In rice only two 'FRO-like' genes (OsFRO1 and OsFRO7) were identified, having unique functional characteristics in Fe uptake and abiotic stresses (Wang *et al*., 2013 [20]; Ishimaru *et al*., 2006 [8]). The expression of OsFRO1 was noticed in Zn, Mn, and Cu deficient rice leaves and later their role in iron homeostasis and bulky biomass under Fe toxic conditions was confirmed (Ruengphayak *et al*., 2015) [14]. However, till now, there has been no comprehensive study that can describe the role of FRO proteins, and their regulatory

mechanisms in plant growth, abiotic and metals stresses. This study aims to investigate the structural and Physico-chemical properties of Arabidopsis FRO proteins. Additionally, the conserved motifs prediction, systematic evolutionary and protein-protein interaction studies which reveals the functional protein domains and their interacting partners in the regulatory network.

Material and Methods

Retrieval of Eight FRO gene and protein sequences

Arabidopsis eight FRO gene sequences were retrieved from the NCBI database and the protein sequences were downloaded from the Uniprot database.

Analyses of FRO genes/ proteins

Physico-chemical features of FRO protein sequences were analyzed by the ProtParam tool (https://web.expasy.org/protparam) and transmembrane (TM) helix prediction was carried out by the TMHMM server (http://www.cbs.dtu.dk/services/TMHM). The cello server (http://cello.life.nctu.edu.tw) predicted the subcellular localization of proteins (Yu *et al*., 2006), and chromosomal locations, protein domain families, and functions were searched in ARAMEMNON (http://aramemnon.uni-koeln.de/).

Phylogenetic relationships and identification of conserved protein motifs: Multiple sequence alignment of FRO proteins was performed to identify conserved residues using Clustal Omega and the phylogenetic tree was constructed by the CLUSTALW tool using PhymL bootstrap and percent scoring method. Furthermore, the six conserved protein motifs of the proteins were characterized by MEME Suite 5.1.1 (http://meme-suite.org/tools/meme) with default parameters.

Structure analysis, Interactions, and co-expression of FRO proteins

The secondary structure of the protein was described by the SOPMA tool. Moreover, the transmembrane domains were constructed with Protter. The interactome network of eight FRO proteins was generated using the String server (http://string-db.org) visualized in Cytoscape (Szklarczyk *et al*. 2019) [17].

Results

Retrieval of Eight FRO gene and protein sequences

Arabidopsis eight FRO gene sequences as FRO1 (AT1G01590), FRO2 (AT1G01580), FRO3 (AT1G23020), FRO4 (AT5G23980), FRO5 (AT5G23990), FRO6 (AT5G49730), FRO7 (AT5G49740), and FRO8 (AT5G50160) were retrieved from NCBI database and the protein sequences of the above eight FRO proteins as FRO1 (Q9LMM2), FRO2 (P92949), FRO3 (F4I4K7), FRO4 (Q8W110), FRO5 (Q9FLW2), FRO6 (Q8RWS6), FRO7 (Q3KTM0) and FRO8 (Q8VY13) were downloaded from the Uniprot database (Table 1).

Table 1: Information on structural protein properties of Arabidopsis eight FRO proteins

Uniprot Id	Entry name	Size	Subcellular localisation	Molecular Weight	pI	Instability Index	TMD
Q9LMM2	FRO1_ARATH	704	Plasma membrane and other locations	79600.02	9.56	41.78	9
P92949	FRO2_ARATH	725	Plasma membrane	81501	9.37	39.61	9
F4I4K7	FRO3_ARATH	717	Mitochondrial membrane	80933.76	9.79	38.06	8
Q8W110	FRO4_ARATH	699	Plasma membrane and other locations	80250.88	9.44	39.86	10
Q9FLW2	FRO5_ARATH	707	Plasma membrane	81166.07	9.37	40.58	10
Q8RWS6	FRO6_ARATH	738	Plasma membrane	83457.97	7.94	37.76	10
Q3KTM0	FRO7_ARATH	747	Chloroplast membrane	84126.3	6.82	35.17	10
Q8VY13	FRO8_ARATH	728	Mitochondrial membrane	83230.13	9.58	47.28	9

Analyses of FRO genes/ proteins

The eight FRO proteins were encoded with residues of 699-747 amino acids having 79600.02-84126.3 (Da) molecular weight. The pI and instability index values ranged from 6.82-9.56 and 35.17-47.28 respectively. Notably, all of these FRO proteins showed 8-10 transmembrane helices. The subcellular localization prediction revealed five (FRO1, FRO2, FRO4, FRO5, FRO6) proteins to the plasma membrane, two (FRO3, FRO8) to the mitochondria, and one (FRO7) to the chloroplast (Table 1). Among the eight, three were localized on the first chromosome, and the remaining five on to the fifth chromosome (Figure 1). The exon number among the eight proteins ranged between 8-9 (Figure 2). Using Aramemnon, the protein domain families were predicted, wherein the FAD-binding domain, Ferric reductase like trans membrane component, Ferric reductase NAD-binding domain were identified in all the eight FRO proteins and Oxidoreductase NAD-binding domain was found in FRO2 and FRO8 proteins (Figure 3).

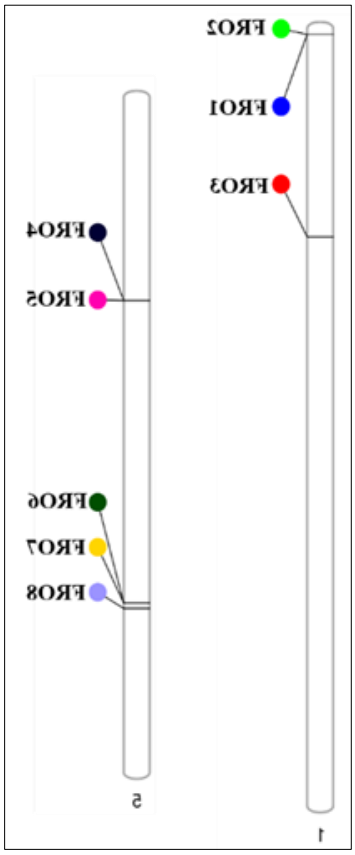

Fig 1: Representation of eight FRO genes on the Arabidopsis chromosomes

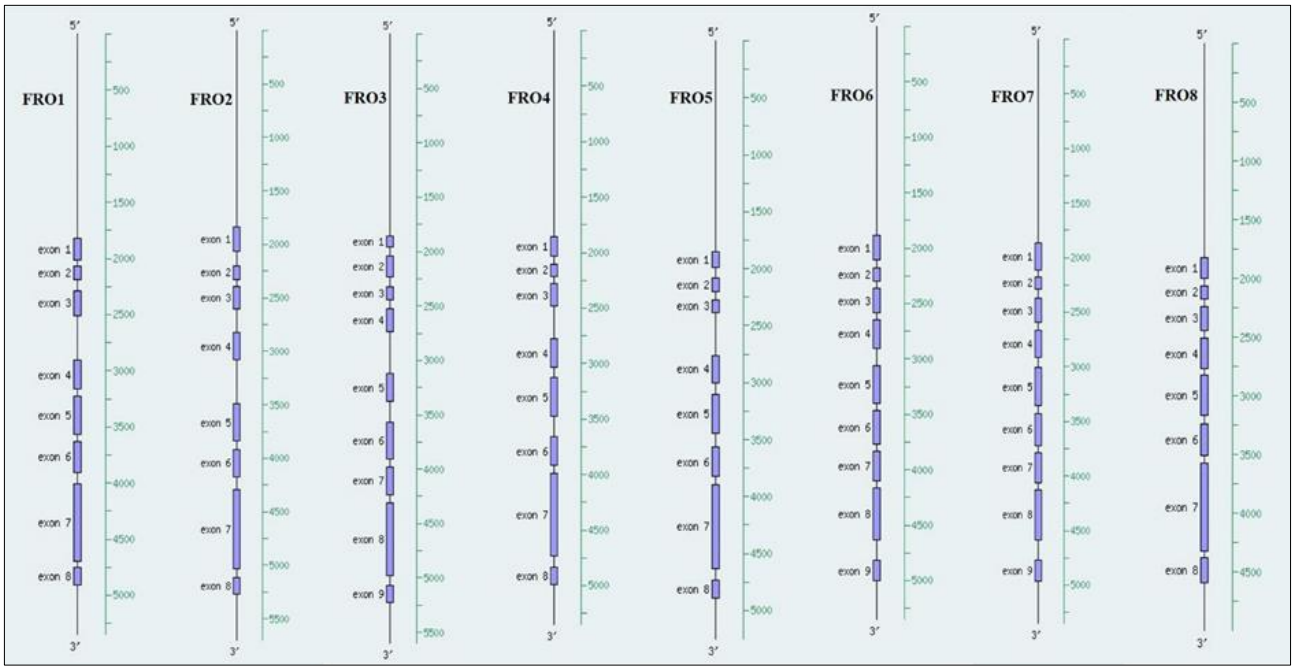

Fig 2: Representation of several exons, intron regions, and their positions on the gene

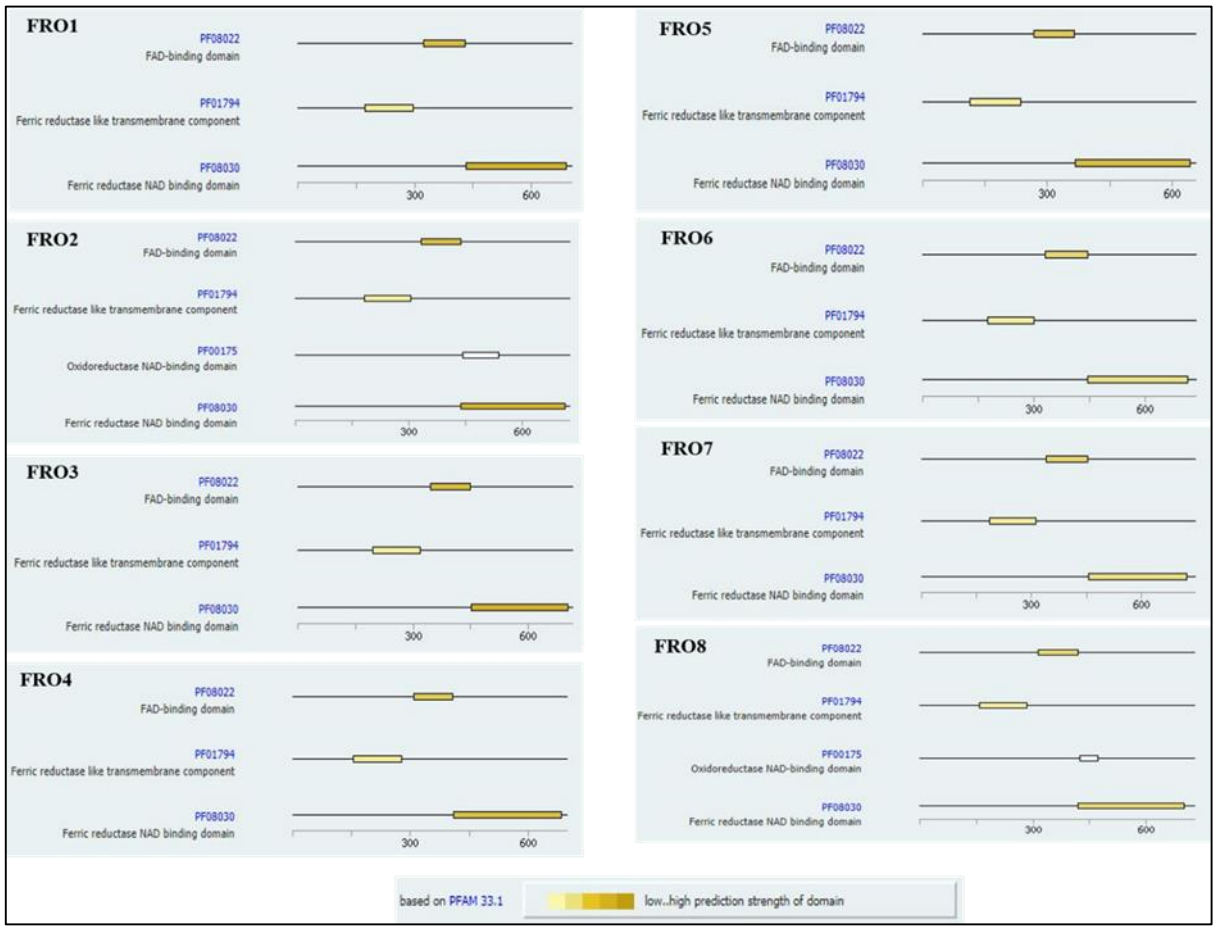

Fig 3: Characterization of functional domains of FRO proteins in Arabidopsis

Phylogenetic relationships and identification of conserved protein motifs

We have used the MEME tool to search for the six most conserved motifs in eight FRO proteins. Motifs 1, 2, 3, 4 were 50 long residues of amino acids, while motif 5 was 21 and motif 6 was 45 residues long (Figure 4). The analysis showed that motif 2 (SARILPCDTLELTFSKNPMLHYSPTSIL FLNIPSISKLQWHPFTITSSSK), 3 (SIDKJAVSVEGPY GPASPDFLRHESLVLVAGGSGITPFISIIRDLJYRSR), 4 (GNICLAFLFFPVARGSSLLPLVGJTSESSIKYHIWLGHIV MFFFTVHGLC), 5 (DVGVLVCGPKKMREEVAKICS), 6 (FKPQPSDQPISPILGPNSFLWLGVILLSSFIIFJIIIGIITRY YI) belongs to the ferric-chelate reductase (PLN02292) superfamily and motif 1 (LAGEIALVAGLLMWVTSLPSIR RKYFEVFFYTHHLYIVFIVFFVLHVGDS) belong to oxidoreductase/ferric-chelate reductase (PLN02844) super family. The phylogenetic tree constructed using the CLUSTALW tool divided the eight proteins into two clusters wherein FRO6 and FRO7 formed one cluster and the remaining six formed the second cluster (Figure 5).

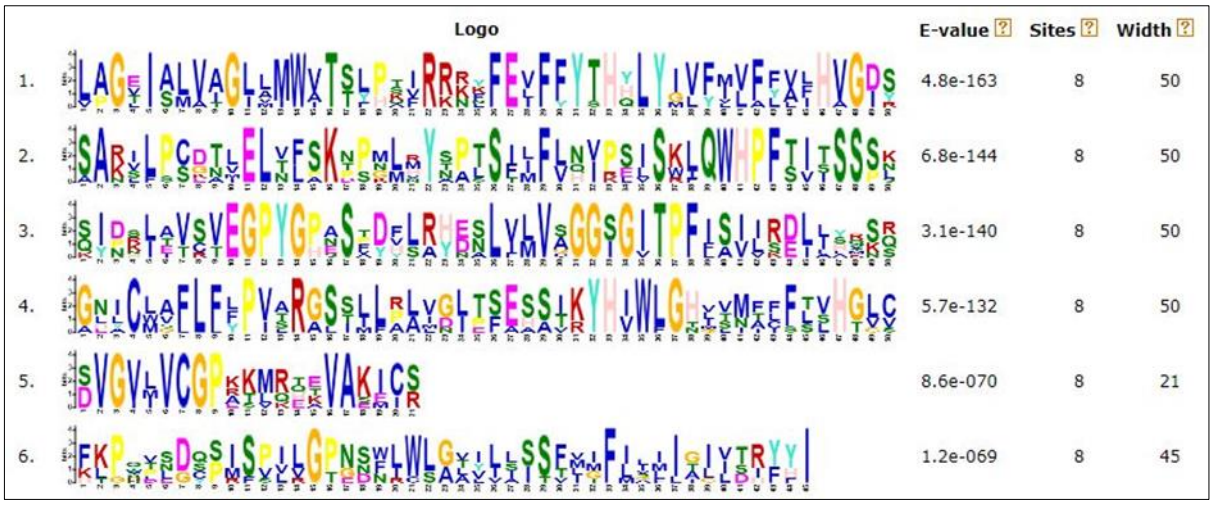

Fig 4: Identified conserved motifs in FRO proteins using MEME software

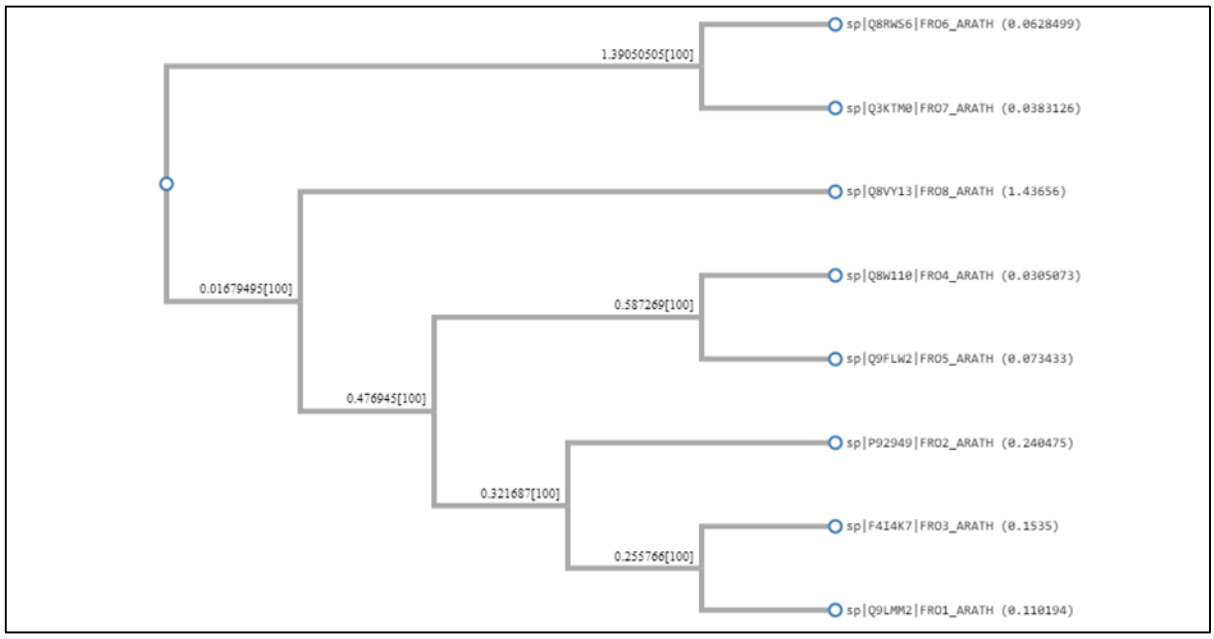

Fig 5: Phylogenetic tree representing the evolutionary relationships among the FRO proteins

Structural analysis of FRO proteins

On average, the alpha helix of all these FRO proteins ranged from 37.26% to 41.48% of the protein structure. In addition, the extended strand contains 21.02% to 23.99% of the structural organization of FRO proteins. As expected, random coil contains stands roughly around 35-40% of locations of all FRO proteins (Figure 6). None of these FRO proteins displayed the presence of 3_{10} helix, Pi helix, beta bridge, beta-turn, bend region, and ambiguous state in structure. The arrangement of transmembrane domains of Arabidopsis FRO proteins in the cell membrane was visualized using the Protter server which helps to identify the signal peptides, disulfide bonds, variants, extracellular and intracellular cytoplasmic strands (Figure 7).

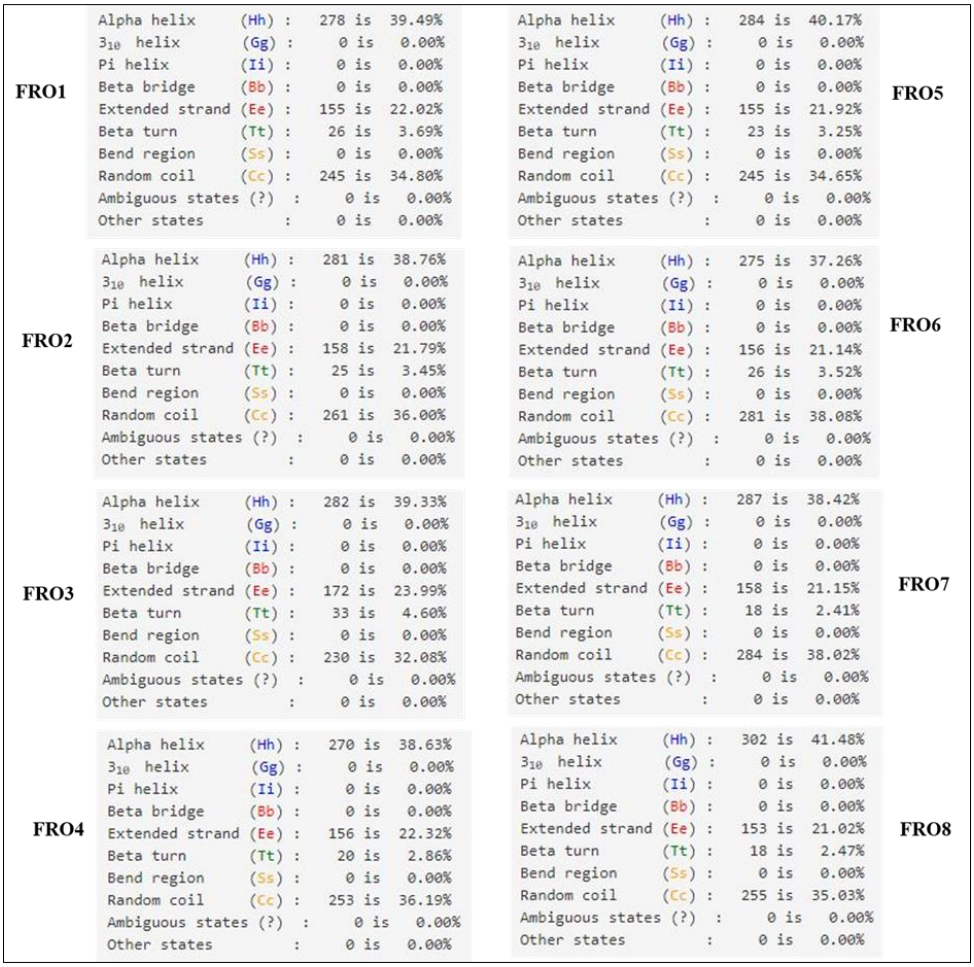

Fig 6: Representation of Arabidopsis eight FRO secondary protein structural properties

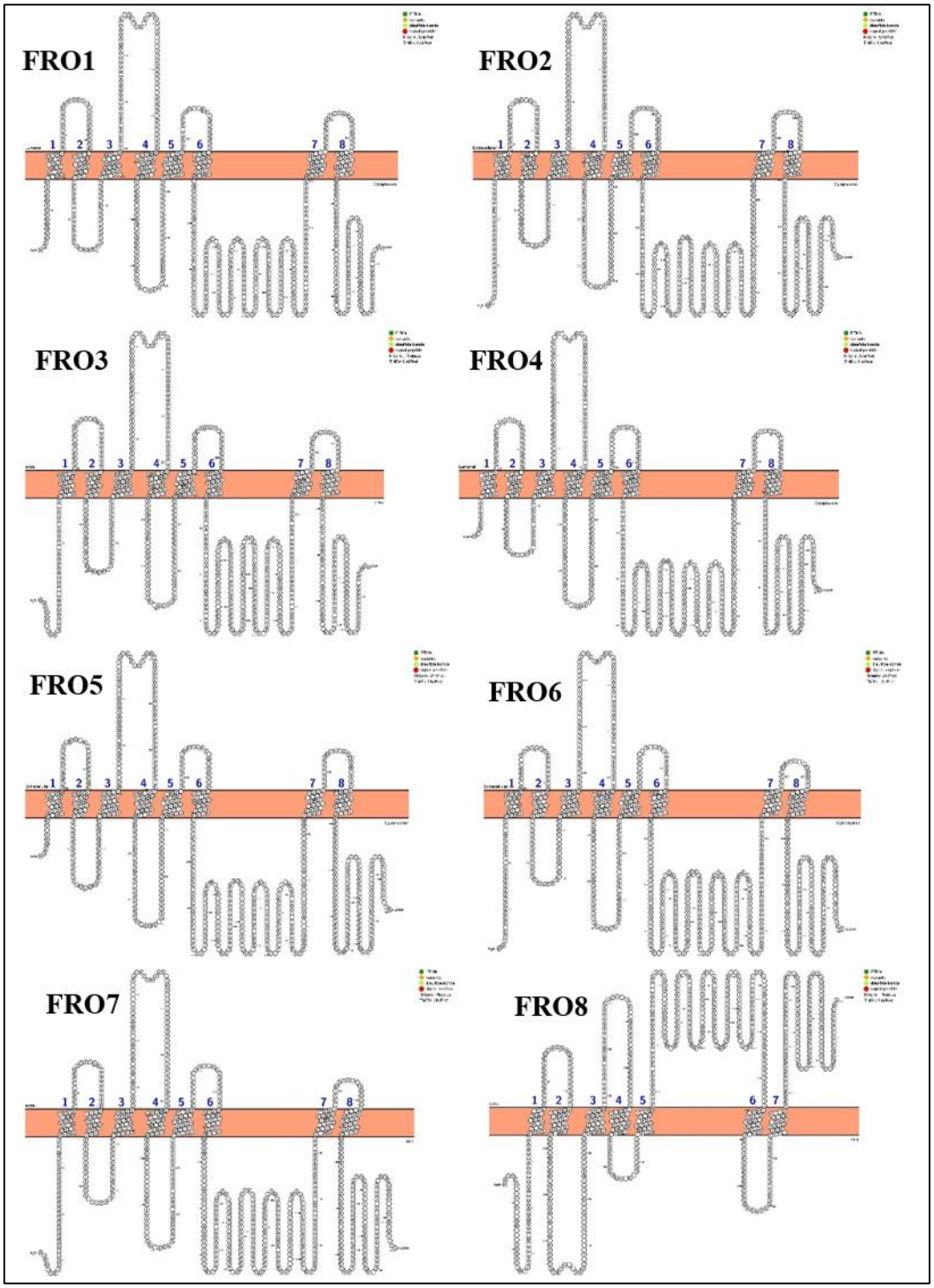

Fig 7: Representation of Transmembrane domains in Arabidopsis FRO proteins

Interactions and co-expression of FRO proteins

The interacting partners of Arabidopsis FRO proteins were predicted using UniProt protein ids as an input to the String database. The interactome network was created using seven FRO proteins out of eight and all the seven FRO proteins shown direct and indirect interactions among themselves. The proteins related to metal ion uptake, IRT1, IRT2, NRAMP1, NRAMP3, NRAMP4, NAS1, and the transcription factors involved in the iron homeostasis pathway, bHLH 100, bHLH101, bHLH38, and bHLH39 were co-expressed with the FRO2, FRO3 protein, whereas COPT1, FRD3 and OPT3 transporter proteins shown co-expression with the FRO4 protein (Figure 8). The FRO6 was predicted to be co-expressed with the FRO7. However many other proteins related to uptake, transport, and storage were reported in the Interactome network.

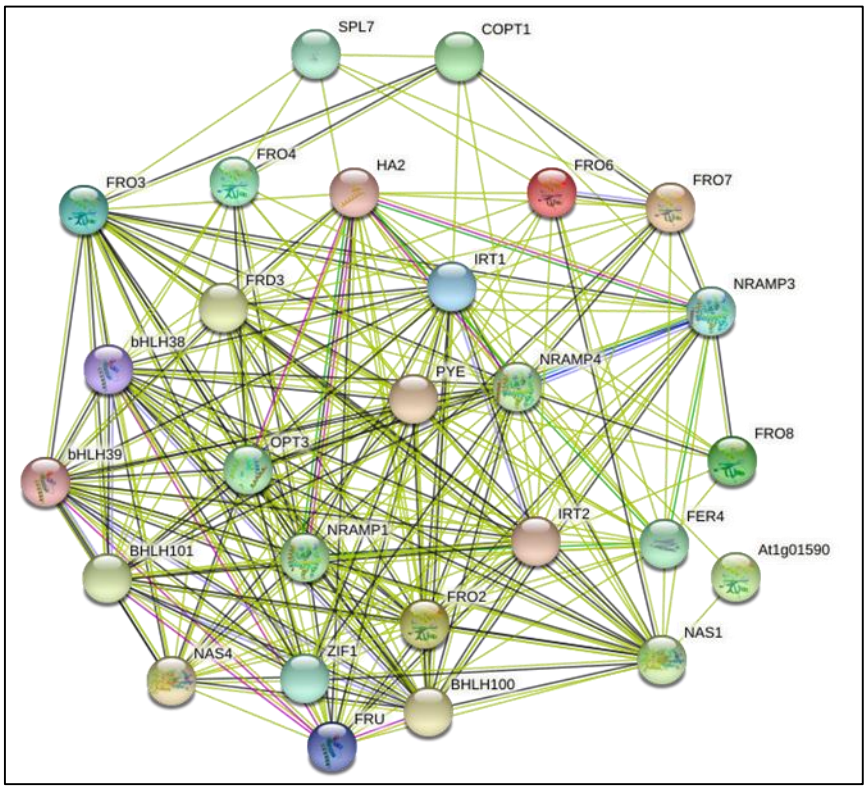

Fig 8: Representation of eight FRO Protein interactome network and their interacting partners

Discussion

The FRO gene encoding ferric reductase activity is executed by the FCR enzyme which is mainly positioned in roots and shoots. Iron chelate reductase is required by the non-graminaceous plants for Fe uptake and eight genes related to FRO were isolated in Arabidopsis plants. Among the eight FRO genes, FRO2 was considered as a major gene in iron chelate reductase activity wherein its expression is specific to roots and induced expression was noted in roots at Fe deficiency conditions.

The insilico analysis showed the existence of 8-10 transmembrane helices in all eight FRO proteins and are localized in chromosome 1 and chromosome 5. The position and organization of the coding sequence of a gene are considered to be critical factors in predicting evolutionary relations and functional genomics potentialities. In this study all the eight FRO genes showed 8-9 exons, suggesting that these FRO genes are phylogenetically closer to each other. Conserved motifs are identical sequences that are maintained by natural selection and in plants, a highly conserved sequence plays a functional role and can be useful for further studies (Wong *et al.* 2015) [22]. Among the eight FRO proteins, we searched for six motifs using the MEME tool. Out of which five motifs matched with the ferric chelate reductase family and observed the oxidoreductase family domain in the motif 1.

The FROs cover the superfamily of flavocytochrome located in the cellular membrane that transfers electrons from intracellular donors to extracellular acceptors such as iron or molecular oxygen (Robinson *et al.*, 1999) [13]. The major functional domains of FRO genes consist of six membrane-spanning regions, two heme, or ferric reductases-like, transmembrane components, which are a highly conserved core protein throughout the flavocytochrome family and crucial for cell surface ferric reductase activity (Schagerlof *et al.*, 2006; Wang *et al.*, 2013 [21]). The flavin adenine dinucleotide (FAD-binding-8) and nicotinamide adenine dinucleotide (NAD-binding-6) domains likely coordinate two intramembranous heme groups leading to superoxide formation and are instrumental for electron transfer.

Interactome map and co-expression analysis were performed using the seven FRO proteins in the STRING platform. In the interactome map, the FRO proteins seemed to be associated with several interaction partners involved in iron uptake, transport, and storage. The proteins related to metal ion uptake, IRT1, IRT2, NRAMP1, NRAMP3, NRAMP4, NAS1, and the transcription factors involved in the iron homeostasis pathway, bHLH 100, bHLH101, bHLH38, and bHLH39 were co-expressed with the FRO2. AtIRT1 and AtIRT2 are the metal transporters studied in Arabidopsis, belong to ZIP family metal transporters, function along with the FRO activity aiding in the transport of Fe^{2+} and Zn^{2+} ions across the root plasma membrane from the soil. NRAMP (natural resistance-associated macrophage protein) family of transporters are also involved in the transport of divalent metals which is present on either intracellular vesicles or the plasma membrane. Two membranes of NRAMP family metal transporters (AtNRAMP3 and AtNRAMP4) in Arabidopsis, play a role in the mobilization of Fe from the vacuole during early seedling development. The FER was the first transcription factor, encoding the bHLH transcription factor involved in regulating Fe responsive genes in tomato plants. In Arabidopsis, a functional ortholog of FER was identified as FIT (FER-like iron deficiency-induced transcription factor) (Colangelo *et al.*, 2004) [3]. FIT in Arabidopsis functions in regulating the expression of Fe uptake genes FRO2 and IRT1 in Fe deficiency conditions (Yuan *et al.*, 2005) [23]. FIT is known to interact with other bHLH transcription factors which are seen to be upregulated in leaves and roots in Fe deficiency conditions. The bHLH subgroup 1b (bHLH 38, bHLH39, bHLH100, bHLH101) transcription factors interact with the FIT in regulating the Fe uptake genes (Yuan *et al.*,

2008) [24]. FRD3 is a multidrug and toxin efflux transporter localized to the plasma membrane of the root cell (Green *et al*., 2004) [6]. It is specifically expressed in roots and involved in the efflux of NA into the xylem.

Conclusion

In conclusion, the analysis showed similar physicochemical properties, gene organization, and conserved motifs of FRO proteins related to the Fe ion transport. Sequence homology and phylogenetic tree of FRO proteins showed the closest evolutionary relationship. In addition, the interactome map displayed the co-expression of FRO proteins to Fe uptake transport and storage proteins.

References

1. Bashir K, Ishimaru Y, Nishizawa NK. Molecular mechanisms of zinc uptake and translocation in rice. Plant Soil 2012;361:189–201. doi: 10.1007/s11104-012-1240-5.
2. Bashir K, Rasheed S, Kobayashi T, Seki M, Nishizawa NK. Regulating subcellular metal homeostasis: The key to crop improvement. Front. Plant Sci 2016;7:1192. doi: 10.3389/fpls.2016.01192.
3. Colangelo EP, Guerinot ML. The Essential Basic Helix-Loop-Helix Protein FIT1 Is Required for the Iron Deficiency Response. The Plant Cell Online 2004;16(12):3400–3412. doi:10.1105/tpc.104.024315
4. Connolly EL, Campbell NH, Grotz N, Prichard CL, Guerinot ML. Overexpression of the FRO2 ferric chelate reductase confers tolerance to growth on low iron and uncovers posttranscriptional control. Plant Physiol 2003;133:1102–1110. doi: 10.1104/pp.103.025122.
5. Gama F, Saavedra T, Dandlen S, de Varennes A, Correia PJ, Pestana M *et al*. Silencing of the FRO1 gene and its effects on iron partition in Nicotiana benthamiana. Plant Physiol. Biochem 2017;114:111–118. Doi: 10.1016/j.plaphy.2017.03.004.
6. Green LS, Rogers EE. FRD3 Controls Iron Localization in Arabidopsis. Plant Physiology 2004;136(1):2523–2531. doi:10.1104/pp.104.045633
7. Grotz N, Guerinot ML. Molecular aspects of Cu, Fe and Zn homeostasis in plants. Biochim. Biophys. Acta (BBA) Mol. Cell Res 2006;1763:595–608. doi: 10.1016/j.bbamcr.2006.05.014.
8. Ishimaru Y, Suzuki M, Tsukamoto T, Suzuki K, Nakazono M, Kobayashi T *et al*. Rice plants take up iron as an Fe^{3+}-phytosiderophore and as Fe^{2+} Plant J 2006;45:335–346. doi: 10.1111/j.1365-313X.2005.02624.x.
9. Jain A, Wilson GT, Connolly EL. The diverse roles of FRO family metalloreductases in iron and copper homeostasis. Front. Plant Sci 2014;5:100. doi: 10.3389/fpls.2014.00100
10. Jeong J, Connolly EL. Iron uptake mechanisms in plants: Functions of the FRO family of ferric reductases. Plant Sci 2009;176:709–714. doi: 10.1016/j.plantsci.2009.02.011
11. Mukherjee I, Campbell NH, Ash JS, Connolly EL. Expression profiling of the Arabidopsis ferric chelate reductase (FRO) gene family reveals differential regulation by iron and copper. Planta 2006;223:1178–1190. doi: 10.1007/s00425-005-0165-0.
12. Quinet M, Vromman D, Clippe A, Bertin P, Lequeux H, Dufey I *et al*. Combined transcriptomic and physiological approaches reveal strong differences between short-and long-term response of rice (*Oryza sativa*) to iron toxicity. Plant Cell Environ 2012,1837-1859. doi: 10.1111/j.1365-3040.2012.02521.x
13. Robinson NJ, Procter CM, Connolly EL, Guerinot ML. A ferric-chelate reductase for iron uptake from soils. Nature 1999;397:694–697. doi: 10.1038/17800.
14. Ruengphayak S, Ruanjaichon V, Saensuk C, Phromphan S, Tragoonrung S, Kongkachuichai R *et al*. Forward screening for seedling tolerance to Fe toxicity reveals a polymorphic mutation in ferric chelate reductase in rice. Rice 2015;8:36. doi: 10.1186/s12284-014-0036-z.
15. Schagerlöf U, Wilson G, Hebert H, Al-Karadaghi S, Hägerhäll C. Transmembrane topology of FRO2, a ferric chelate reductase from Arabidopsis thaliana. Plant Mol. Biol 2006;62:215-221. doi: 10.1007/s11103-006-9015-0.
16. Suzuki M, Bashir K, Inoue H, Takahashi M, Nakanishi H, Nishizawa NK. Accumulation of starch in Zn-deficient rice. Rice 2012;5:9. doi: 10.1186/1939-8433-5-9.
17. Szklarczyk D, Gable AL, Lyon D, Junge A, Wyder S, Huerta-Cepas J *et al*. STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. Nucleic Acids Res 2019;47:607-613.
18. Takagi SI, Nomoto K, Takemoto T. Physiological aspect of mugineic acid, a possible phytosiderophore of graminaceous plants. J. Plant Nutr 1984;7:469–477. doi: 10.1080/01904168409363213.
19. Victoria FDC, Bervald CMP, da Maia LC, de Sousa RO, Panaud O, de Oliveira AC. Phylogenetic relationships and selective pressure on gene families related to iron homeostasis in land plants. Genome 2012;55:883–900. Doi: 10.1139/gen-2012-0064.
20. Wang GF, Li WQ, Li WY, Wu GL, Zhou CY, Chen KM. Characterization of rice NADPH oxidase genes and their expression under various environmental conditions. Int. J. Mol. Sci 2013;14:9440–9458. doi: 10.3390/ijms14059440
21. Wang GF, Li WQ, Li WY, Wu GL, Zhou CY, Chen KM. Characterization of rice NADPH oxidase genes and their expression under various environmental conditions. Int. J. Mol. Sci 2013;14:9440–9458. doi: 10.3390/ijms14059440
22. Wong A, Gehring C, Irving HR. Conserved Functional Motifs and Homology Modeling to Predict Hidden Moonlighting Functional Sites. Frontiers Bioeng. Biotech 2015;3:82.
23. Wu H, Li L, Du J, Yuan Y, Cheng X, Ling HQ. Molecular and biochemical characterization of the Fe (III) chelate reductase gene family in Arabidopsis thaliana. Plant Cell Physiol 2005;46:1505–1514. doi: 10.1093/pcp/pci163
24. Yuan Y, Wu H, Wang N, Li J, Zhao W, Du J *et al*. FIT interacts with AtbHLH38 and AtbHLH39 in regulating iron uptake gene expression for iron homeostasis in Arabidopsis. Cell Research 2008;18(3):385–397. doi:10.1038/cr.2008.26
25. Yuan YX, Zhang J, Wang DW, Ling HQ. AtbHLH29 of Arabidopsis thaliana is a functional ortholog of tomato FER involved in controlling iron acquisition in strategy I plants. Cell Research 2005;15(8):613-621. doi:10.1038/sj.cr.7290331