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Isolation and In Silico Analysis of Iron Regulated Transporter 2 Gene from Indian Barnyard Millet (*Echinochloa frumentacea* L.)

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A B S T R A C T

Iron is one of the essential mineral for all organisms and its deficiency leads to many serious problems in human being worldwide. Plants have evolved with specific ZIP transporters to uptake and transport the iron from the soil. IRT2 is one of the ZIPs family members, which has a role in uptake of Fe^{2+} from the soil. Indian barnyard millet (*E. frumentacea* L.) is one of the rich sources of iron among millets. *IRT2* gene of Indian barnyard millet is up regulated under iron deficient condition and hence we isolated *EfIRT2* gene. *In silico* analysis of *EfIRT2* gene showed that it codes for 386 amino acids, having molecular weight 40.54 kDa with pI 8.65. Its predicted structure showed that it is a plasma membrane protein with eight conserved domains and a signal peptide towards N-terminal end, with a Histidine signature of ZIPs family in between domain III and IV. Analysis based on deduced amino acid sequences of EfIRT2 showed highest similarity with SiIRT2 gene and clustered together with IRT2 proteins of Poaceae family members. Like other ZIPs family members, *EfIRT2* could be a potential candidate gene for biofortification of staple foods.

Introduction

Iron is an essential micronutrient and Fe deficiency is one of the most prevalent micronutrient deficiencies affecting around 2 billion people globally (Stoltzfus *et al*., 1998). About 30% of the global population is reported to be affected by anaemia due to Fe deficiency and it is also reported that 0.8 million deaths are caused annually due to Fe deficiency (WHO, 2002). Fe deficiency is ranked at sixth position among the risk factors of death and disability in developing countries with high mortality rates (WHO, 2002).

Fe deficiency is managed through food supplements and pills, which are not sustainable interventions in developing countries. Biofortification of staple food crops with enhanced iron in edible parts seems to be a viable strategy in bringing out sustainable solution to this problem. There are different approaches to enhance the iron content in crop plants. Application of Fe containing fertilizers, either by fertigation or foliar spray, can have significant effect on the accumulation of nutrients in edible plant

products (Grunes and Allaway, 1985). Secondly, genetic manipulation of iron content in food grains/vegetables through various breeding methods holds promising role in enriching iron in staple food crops (Goudia and Hash, 2015). Enhancement of iron through conventional breeding methods has resulted in only 2-3 fold increase in iron content, which is insufficient to reach the required target levels of 14 µg/g.

On the other hand, several attempts have been made through genetic engineering approaches to fortify staple food crops *viz*., rice, wheat and maize using candidate genes such as iron accumulation gene like *OsIRT1* (Lee and An, 2009), iron chelator gene *HvNAS* (Masuda *et al*., 2008), seed-specific expression of storage protein, ferritin (Vasconcelos *et al*., 2003) and enhancing iron bioavailability using phytase (*Afphytase*) which degrades phytic acid (Lucca *et al*., 2001). These studies have led to development of rice lines with enhanced iron accumulation in endosperm (15 µg/g Fe in polished rice) by over-expressing genes *viz*., *nicotianamine synthesis 2* (*OsNAS2*) and soybean ferritin (Trijatmiko *et al*., 2016). Based on results of several experiments, it is postulated that synergistic improvement of iron mobilization in soil, uptake of Fe by roots, transport of Fe from roots to shoots and finally accumulation in grains will help in developing crop varieties with enhanced iron in grains or edible parts.

Although iron is the fourth most metal element found in the earth's crust, its low solubility makes it unavailable to plant roots. Plants have evolved specialized system to take up iron from soil.

Fe transporter gene namely Iron Regulated Metal Transporter 1 (*IRT1*) has been established as the major iron uptake system from the soil, and candidate for iron biofortification (Tan *et al*., 2014, Boonyaves *et al*., 2016). IRTs are the members of the Zinc-Regulated Transporter/Iron-Regulated Transporters (ZRT/IRTs)-Related Protein (ZIP) transporter family. Among the *IRT* gene family members, *IRT2* is expressed in the outer layer of the root epidermal cells of *Arabidopsis thaliana* and upregulated under iron deficient condition, but to a lesser extent (Vert *et al*., 2001). Role of IRT2 in uptake of Fe and avoiding transport of manganese and cadmium has been reported (Vert *et al*., 2002; Shanmugam *et al*., 2011). This has made IRT 2 as one of the potential targets for genetic manipulation of iron accumulation in rice. In this context, identification of superior rice/cereal genotype exhibiting enhanced iron uptake/accumulation in grains and isolation of IRT from those genetic sources for genetic engineering applications are crucial in achieving the desired target.

Barnyard millet (*Echinochloa* sp.) is one of the oldest domesticated millets belonging to Poaceae family in the semi-arid tropics of Asia and Africa (Doggett, 1986). Indian barnyard millet (*E. Frumentacea* L.) is reported to possess fairly high level of digestible protein (12%), low carbohydrate content (58.56%), and rich amount of iron ranging from 19.56 to 42.13 µg/g in grains (Chandel *et al*., 2014; Patel *et al*., 2015).

Among cereals the presence of *IRT1* and *IRT2* genes have been predicted in *Oryza sativa, Oryza brachyantha, Brachypodiun distachyon, Setaria italica, Sorghum bicolor, Zea mays, Hordeum vulgare,* etc., but no efforts have been made to isolate and characterize *IRT* genes in an iron rich small millet *E. frumentacea*. In this study, we isolated a full-length gene encoding *EfIRT2* and predicted its physical and chemical properties using bioinformatics tools. This is the first report on isolation and characterization of an iron regulated transporter 2 gene from Indian barnyard millet.

Materials and Methods

Amplification of partial fragment of *EfIRT2* gene

Genetically pure seeds of Indian barnyard millet (*E. frumentacea* L. cv. CO 1) were sown on the soil and genomic DNA was extracted from 0.5 g of leaf samples collected from 7 day-old seedlings by CTAB method (Doyle and Doyle, 1987) with slight modifications for amplification of full-length *EfIRT2* gene from genomic DNA.

IRT2 genomic DNA sequences of 7 species of Poaceae family; *viz. B. distachyon, H. vulgare, O. sativa, O. brachyantha, S. italica*, *S. bicolor* and *Z. mays*, were retrieved from Phytozyme *v9.1* and aligned using ClustalX *v2.0* online tool. The degenerate primer set (dIRT2-F1 and dIRT2-R2, Table 1) was designed in highly conserved region. Partial fragment of *IRT2* gene sequence was PCR amplified by using 50 ng genomic DNA as template using PCR conditions of initial denaturation for 5 min at 94 ºC followed by 35 cycles of 1 min at 94 ºC, 1 min at 58 ºC 1.30 min at 72 ºC and final extension for 10 min at 72 ºC. The amplified fragment was purified using PCR product purification kit (BioBasic Inc. Canada) and sequenced (Scigenom Pvt. Ltd., Cochin).

Amplification and cloning of full-length genomic DNA encoding *EfIRT2*

The 5' and 3' ends of *EfIRT2* were amplified by thermal asymmetric interlaced PCR (TAIL-PCR) as described by Liu *et al*., (1995). Following gene specific primers were used to amplify 5' end (5P, 5S, 5T; Table 1) and 3' end (3P, 3S, 3T; Table 1) along with 6 degenerate primers (AD1, AD2, AD3, AD4, AD5 and AD6; Table 1). The tertiary products of TAIL-PCR were purified and sequenced. The overlapping sequences of 5' and 3' ends of IRT2 gene were aligned with partially amplified fragment to obtain full-length nucleotide sequence of *IRT2* gene. The ORF and intron region of *EfIRT2* gene were predicted by FGENESH online tool (http://www.softberry.com/berry.phtml?topic=fgenesh&group=programs&subgroup=gfind) using *S. italica* IRT2 gene as a reference gene. The full-length *IRT2* gene was amplified from genomic DNA with ORF specific primer set (ORF_F and ORF_R, Table 1) using proof reading XT-5 polymerase (Genei, Bangalore). PCR conditions used were initial denaturation for 5 min at 94 ºC followed by 35 cycles of 45 sec at 94 ºC, 45 sec at 62 ºC, 90 sec at 72 ºC and final extension for 10 min at 72 ºC. The PCR amplified product was resolved on 0.8% agarose gel. Amplified *IRT2* gene products were purified and cloned into pJET1.2 vector (ThermoScientific Inc. USA) and five independent positive clones were sequenced in both the directions.

In silico analysis

The ORF and intron region of *EfIRT2* gene were predicted by FGENESH online tool. The nucleotide sequences of *EfIRT2* gene from genomic DNA and predicted ORF (cDNA) were compared using ClustalW online tool to confirm the intron region predicted by FGENESH tool. Deduced amino acid sequence form predicted *EfIRT2* ORF was used as a query to identify homologous genes in different plant species using BLASTp search tool against protein sequences in the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Multiple sequence alignment of other known IRT sequences from related crop species *viz. S. italica* IRT2 (XP_004986332.1), *O. sativa* IRT2 (XP_015629246.1), *O. brachyantha* IRT2 (XP_006651668.1), *S. bicolor* hypothetical protein (XP_002464109.1), *Z.*

mays hypothetical protein (XP_008665793.1), *S. italica* IRT1 (XP_004982221.1), *O. sativa* IRT1 (XP_015632375.1), *B. distachyon* IRT1 (XP_014752016.1), and *H. vulgare* IRT (ACD71460.1), was carried out using CLUSTALW tool in BioEdit software in order to find conserved domains. This amino acids alignment was used for phylogenetic analysis by MEGA6 software using the maximum likelihood method with 1,000 bootstrap replications by Poisson model.

Various other properties viz., secondary structure (PSIPHRED; http://bioinf.cs.ucl.ac.uk/psipred/); pI/Mw (Compute pI/Mw; http://web.expasy.org/compute_pi/); sub-cellular localization (ProtCompv9.0; http://www.softberry.com/berry.phtml?topic=protcomppl) and trans-membrane protein prediction (DAS-TMfilter server; http://mendel.imp.ac.at/sat/DAS/DAS.html) were also analyzed.

Results and Discussion

Isolation of *EfIRT2* gene

Partial fragment of approximately 950 bp was PCR amplified from barnyard millet genomic DNA using a pair of primers designed in conserved domain (Figure 1a) and the amplified DNA was sequenced. TAIL-PCR resulted in the amplification of 250 bp fragment at the 5'end and 150 bp fragment at the 3'end. These fragments were purified and sequenced. Using these 5'and 3'end sequences, full-length sequence of EfIRT2 was deduced and new primers were designed to amplify the full-length coding sequence of *EfIRT2* from genomic DNA (Figure 1b).

Amplified full-length coding sequence was confirmed by sequencing. Genomic DNA sequences of *EfIRT2* were deposited in NCBI-GenBank database (Accession no. KX832298).

In silico analysis of *EfIRT2* gene

ORF prediction of deduced nucleotide sequence by FGENESH tool and comparison of genomic DNA and predicted cDNA nucleotide sequences of *EfIRT2* gene revealed that genomic DNA sequence of *EfIRT2* gene had 1254 bp ORF, comprising two exons with an intron of size 93 bp (+706 to +798 bp) (Fig. 2). BLASTn results showed that *EfIRT2* gene sequence shared significant homology with *IRT2* genes of other related crop species *viz.* 95% with *S. italica* (XM_004986275.2), 91% with *O. sativa*, 90% with *O. brachyantha* and 90% with *Z. mays*. It also shared significant homology with *IRT1* gene of other grass family members *viz.* 90% with *O. brachyantha*, 89% with *O. sativa*, 89% with *S. italica*, 88% with *B. distachyon* and 86% with *Z. mays* (results not shown).

Conserved domain analysis by multiple sequence alignment of amino acids of EfIRT2 and other IRTs of related species revealed that it had 3 conserved domains, among them C-terminal domain is highly conserved but N-terminal domain exhibited greater degree of variation across different members (Figure 2). The phylogenetic tree was constructed based on the amino acid alignments of 10 IRT proteins. Phylogenetic analysis of EfIRT2 with other IRT2 family members of related species showed that EfIRT2 formed a close/tight lineage with predicted SiIRT2 whereas separate/distant lineage from IRT1 of other grass family species (Figure 3).

Prediction of putative secondary structure using PSIPHRED tool revealed that EfIRT2 protein contains 15 coils, 13 alpha helices and 6 beta-strands (Figure 4). It was predicted that it encoded a protein of 386 amino acids with a molecular mass of 40.54 kDa and pI of 8.65. Sub-cellular localization by ProtComp *v9.0* online tool predicted that EfIRT2 protein was a typical plasma membrane transmembrane

protein with a certainty score of 0.86 to/far 0.9. The transmembrane prediction tool of EfIRT2 protein revealed that EfIRT2 protein has 8 potential transmembrane α helices (amino acids: 69-88, 104-117, 146-163, 236-251, 265-269, 296-314, 324-350, 364-378). A highly conserved region containing the ZIP signature sequence of histidine motif (amino acid sequence HCHGHGHM, from 195-202, shown by * in figure 2) was located in between III and IV transmembrane domain. A potential signal peptide (amino acid 8-24) was also predicted in the N-terminal domain.

Iron is directly related to plant growth and development as it has a crucial role in biosynthesis and maintenance of chlorophyll structure and function (Terry and Abadia, 1986). Iron uptake and transport in plants influence iron accumulation in plant organs such as leaves, grains, and fruits, which are important food sources for humans and animals. ZIPs are the major Fe^{2+} transporter, both in graminaceous as well as in non-graminaceous plants. Under iron deficient conditions, significant induction of *IRT2* was observed in root epidermal cells of *Arabidopsis* (Vert *et al*., 2001), green gram (Muneer *et al*., 2014) and tomato (Eckhardt *et al*., 2001). It has generally been reported that Fe transport genes are highly up-regulated during mineral starvation (Kerkeb *et al*., 2008; Shanmugam *et al*., 2011; Vert *et al*., 2002).

Since Indian barnyard millet belongs to Poaceae, EfIRT2 amino acid sequences showed maximum homology and formed a cluster in dendrogram with IRT2 of others members of Poaceae crops. EfIRT2 amino acid sequences showed maximum similarity (95%) and tight lineage with *S. italica* IRT2, as both *S. italica* and *E. Frumentacea* are belonging to millet group and may have more genome similarity and hence *IRT2* might be having similar function. Our results predicted that EfIRT2 is having 386 amino acids with a molecular mass of 40.54 kDa and pI of 8.65. Similarly, Vatansever *et al*., (2015) have analyzed IRT2 from 8 different plant species and reported IRT2 having amino acid sequences ranging from 324 to 408 residues with molecular weights ranging from 33.86 to 41.38 kDa and pI ranging from 6.11 to 6.85.

In general, most of the eukaryotic transporters are located at membrane of chloroplast, vacuole, mitochondria, plasma membrane. ZIP family members have eight predicted transmembrane domains (Guerinot, 2000). Eide *et al*., (1996) reported that AtIRT1 is a transmembrane protein, with 8 transmembrane domains, a histidine motif in between transmembrane domain III and IV and a potential signal peptide (sensing domain). A highly conserved region containing histidine motif is the ZIP signature that proposed to be involved in the formation of a cytoplasmic metal binding site (Eide *et al*., 1996). Signal peptides are the N-terminal amino acid sequences that direct proteins to organelles like chloroplast, mitochondria and are required for their transport across membranes from their site of synthesis in the cytoplasm for post-translational modifications of proteins (Patron and Walker, 2007). Sensing domain of AtIRT1 might mediate posttranslational regulation (Eide *et al*., 1996; Guerinot, 2000). Our results also predicted that EfIRT2 is a typical plasma membrane transmembrane protein with 8 potential transmembrane α helices, histidine motif in between transmembrane domain III and IV and a potential signal peptide. Therefore, it is a member of ZIP family and a potential signal peptide may have a role in transport of EfIRT2 to the specified organelle for post-translational modifications. In general, the protein has peculiar secondary structure, but their conserved domains in secondary structure helps in folding the proteins and determines the function (Fig. 5).

Table.1 List of primers used in this study

S. No	Primer name	Sequences (5'…..3')
1	dIRT2-F1	CGCCTGAAGCTVATCG
2	dIRT2-R2	ACSAGCGCCATGTAGTG
3	5P	TGTGGAAGGTGAGCATGAG
4	5S	GTGAGGTCGTTGAAGGAG
5	5T	GACGACGACGAAGAGGTTGC
6	3P	AGATGGGCATCGTGGTGCACT
7	3S	GATGAAGTCGGTGCTGGTCT
8	3T	ATCGTCGTCGGGCTGCTGAA
9	AD1	NGTCGASWGANAWGAA
10	AD2	TGWGNAGSANCASAGA
11	AD3	AGWGNAGWANCAWAGG
12	AD4	STTGNTASTNCTNTGC
13	AD5	NTCGASTWTSGWGTT
14	AD6	WCAGNTGWTNGTNCTG
15	sqF	AGATGGGVATCGTGGTGCA
16	sqR	CACTTGGCCATGACGGACAT
17	ORF_ F	ATGTCTTCACAAACAGTGCCAAG
18	ORF_R	TCACGCCCACTTTGCCATG
19	Act1F	TGTGATGTTGATATCAGGAAGGA
20	Act1R	GGGACCGGTTTCGTCATACT

Fig.1 Amplification of EfIRT2 gene

(a)Genomic DNA of *E. Frumentacea* was used as a template for amplification of partial sequence of EfIRT2 (~950 bp); (b)Amplification of full length sequence of EfIRT2 gene from genomic DNA (1254 bp)
M- 1 kb ladder.

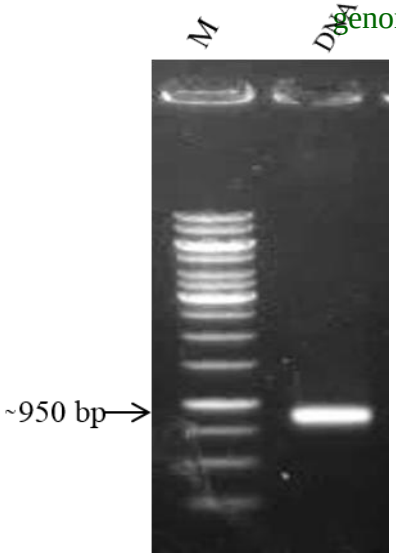


Figure 1a. Partial *EfIRT2*

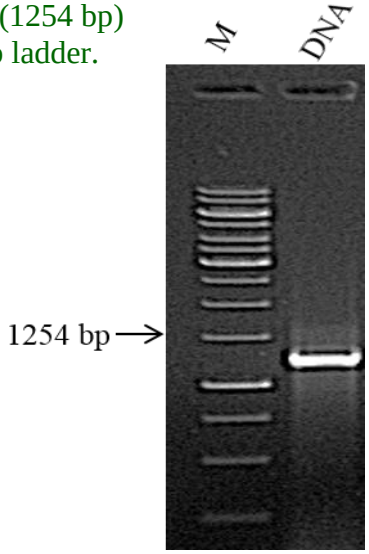


Figure 1b. Full length *EfIRT2*

Fig.2 EfIRT2 gene nucleotide sequence comparison

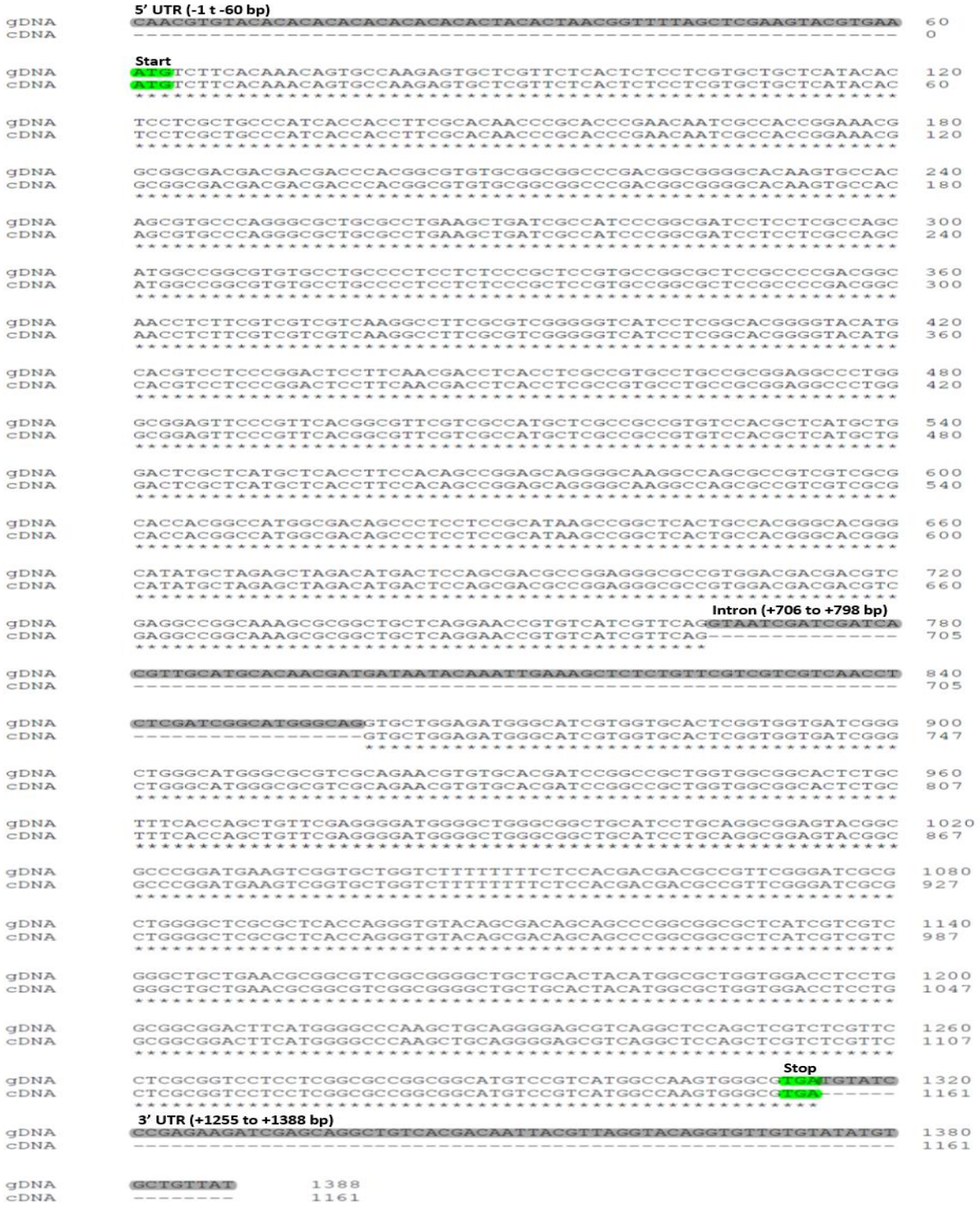


Fig.3 Multiple sequence alignment of EfIRT2 protein

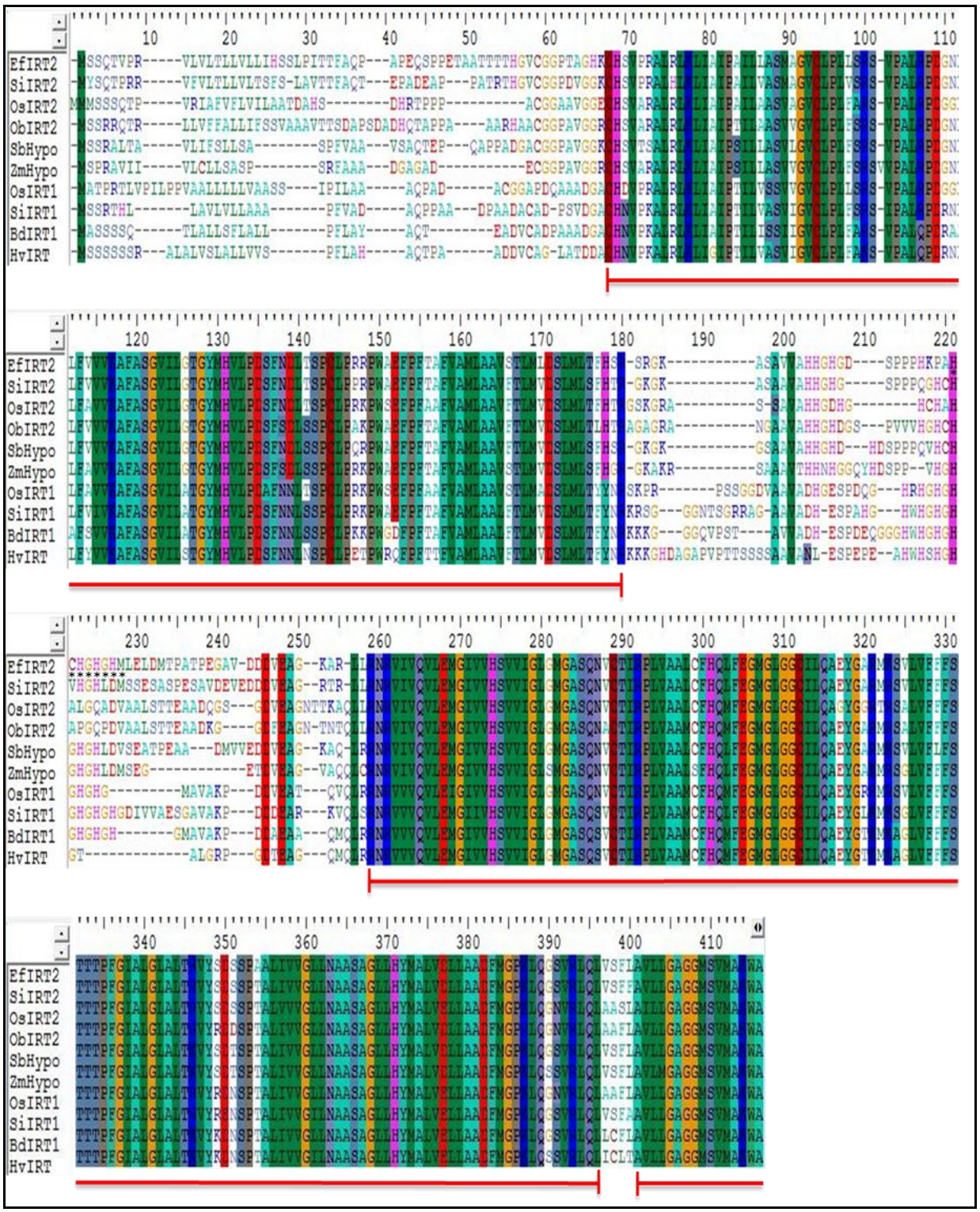


Fig.4 Phylogenetic analysis of EfIRT2 protein

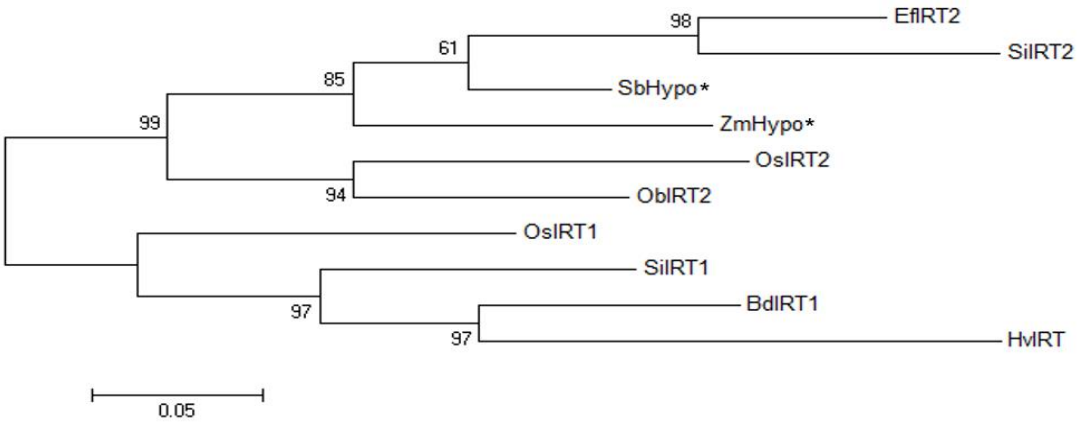
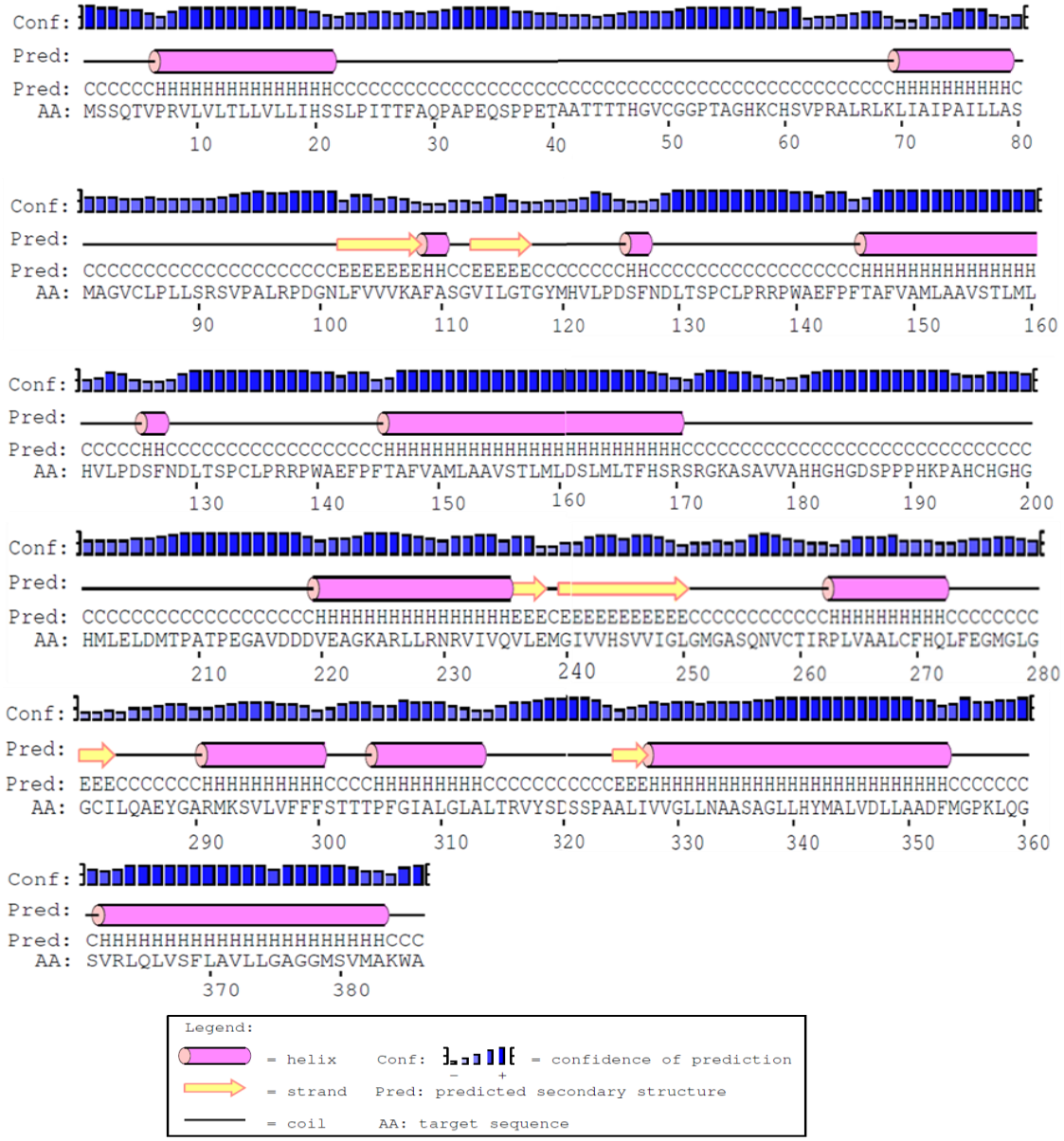


Fig.5 Secondary structure prediction of EfIRT2 protein



The amino acid sequence alignment results showed that there were 3 conserved domains in secondary structure of EfIRT2 which are highly conserved among other IRT2 of other species of Poaceae. It is hypothesized that EfIRT2 has function of Fe uptake similar to other ZIP family members.

In conclusion, this study shows that *EfIRT2* gene possesses high similarity and tight lineage with other ZIPs members of Poaceae family, the encoded protein has presence of eight conserved transmembrane domains along with histidine signature of ZIPs family and its plasma membrane location. All these above characteristics of the gene are sufficient to consider the *EfIRT2* as a potential candidate gene for transport of Fe and may provide an alternative gene for iron biofortification of crops.

The nucleotide sequences of *EfIRT2* gene from genomic DNA (gDNA) and predicted ORF using FGENESH (cDNA) were aligned using ClustalW tool and compared. UTR regions, start codon, stop codon and intron region are highlighted.

Multiple sequence alignment of amino acid sequences of EfIRT2 with IRT family members of selected plant species. Gaps (dashed lines) were introduced for optimal alignment. The region underlined with red bar indicates the conserved domain. Alignments were performed using ClustalW program.

Phylogenetic relationship between EfIRT2 and IRT members of other grass species (Si- *S. italica*L, Sb- *S. bicolor*, Zm- *Z. mays* L, Os- *O. sativa* L, Bd- *B. distachyon*, Hv- *H. vulgare*. Hypo* indicates hypothetical protein. The phylogenetic tree was constructed with the MEGA 6.0 software using Maximum Likelihood method, and the bootstrap values are in per cent, secondary structure of deduced EfIRT2 amino acid sequence predicted by PSIPRED protein structure prediction server.

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