J. Curr. Crop Sci. Technol., 2022; https://doi.org/10.29321/MAJ.10.000622

RESEARCH ARTICLE

Over-expression of *Echinochloa frumentacea Iron Regulated Transporter 2* Gene in Indica Rice var. ASD16

Chowdhury Rajen[1], Tushar Kashinath Manape[2], Ashokkumar Shanthinie[1], Eswaran Kokiladevi[1], Krish K. Kumar[1], Loganathan Arul[1], Shanmugam Varanavasiappan[1], Muthurajan Raveendran[1], Kizhaeral Sivathapandian Subramanian[3], Paramasivam Jeyaprakash[4] and Duraialagaraja Sudhakar[1*]

[1*]Department of Plant Biotechnology, Centre for Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University, Coimbatore - 641003, Tamil Nadu, India
[2]ICAR-Directorate of Onion & Garlic Research, Rajgurunagar, Pune, Maharashtra, India
[3]Directorate of Research, Tamil Nadu Agricultural University, Coimbatore-641003, Tamil Nadu, India
[4]Department of Plant Breeding and Genetics, Anbil Dharmalingam Agricultural College and Research Institute Tamil Nadu Agricultural University, Trichy – 620027, Tamil Nadu, India

Received : 14th June, 2022
Revised : 19th June, 2022
Accepted : 26th June, 2022

ABSTRACT

Iron (Fe) is one of the important minerals for all life forms, and its deficiency leads to serious health-related problems in humans. Rice, being a strategy II plant, uses the strategy I Fe-uptake system as well. Iron regulated transporters (IRT) primarily transport Fe from the soil to the plant. *IRT2* gene expresses in roots, and its expression is up-regulated under Fe deficient condition. In the present srudy, the *IRT2* gene isolated from barnyard millet, *Echinochloa frumentacea Iron regulated Transporter 2* (*EfIRT2*), driven by a constitutive promoter was introduced into rice to improve grain Fe content in an elite rice variety, ASD16. *Agrobacterium*-mediated transformation of ASD16 generated 95 events, and all of them were found to be transgenic based on PCR assays. Tissue localization studies using Scanning Electron Microscopy – Energy Dispersive X-Ray (SEM-EDX) analysis showed that the relative Fe content had increased in seed tissues. Fe estimation in unpolished transgenic rice using ICP-OES indicated a marginal increase of 15% in Fe content over wild-type ASD16.

Keywords: *Rice; IRT2; ICP-OES; SEM-EDAX; Iron; Biofortification*

INTRODUCTION

Iron (Fe) is an essential micronutrient for plants as it is involved in several physiological and biochemical functions, including photosynthesis, respiration, nitrogen assimilation, chlorophyll biosynthesis, DNA synthesis, and oxygen transport (Morrissey and Guerinot, 2009). Although Fe is abundant in soil, the availability of Fe is limited to plants due to its oxidized nature. It is present in the soil in the form of iron oxides and hydroxides. Fe deficiency symptoms in plants are common in calcareous soil, affecting yield and

quality. In humans, lack of Fe in the diet causes anemia and other health-related problems. Anemia affects 1.62 billion people, corresponding to 24.8% of the world population worldwide (WHO, 2008).

Plants have adapted different mechanisms for maintaining adequate Fe levels throughout their development. In non-graminaceous crops, protons are extruded by H+-ATPase, which lowers the rhizospheric pH, thereby increasing Fe solubility (Santi *et al*., 2005; Santi and Schmidt, 2009; Kobayashi and Nishizawa, 2012). Graminaceous plants employ a unique approach for Fe uptake from soil. They secrete phytosiderophores (PS) of the mugineic acid (MA) family (Mori and Nishizawa, 1987). As a result, Fe (III)-PS complexes form in the root zone and are taken up by the root system *via*. specialized transporter proteins known as yellow stripe (YS) or yellow stripe-like (YSL) (Curie *et al*., 2001; Inoue *et al*., 2009). Plants have sophisticated mechanisms to maintain cellular Fe homeostasis to cope with Fe deficiency. The vast information gathered over a few decades enabled a better understanding on the Fe's impact on plant homeostasis and Fe uptake systems by roots in the grass and non-grass plants. These systems are very efficient in Fe deficient conditions and may play a key role in Fe uptake in Fe sufficient conditions.

The ZIP ('ZRT, IRT-like Protein') family of membrane proteins (Eng *et al*., 1998; Guerinot, 2000), is divided into four sub-groups based on amino acid sequence similarity: Subfamily I, Subfamily II, LIV-1, and gufA (Gaither and Eide, 2001; Taylor *et al*., 2003). More than hundreds of ZIP family members have been found across prokaryotes to eukaryotes (Grass *et al*., 2002), insects (Mathews *et al*., 2005), and mammals (Mathews *et al*., 2006; Dufner-Beattie *et al*., 2003; Taylor and Nicholson, 2003). IRT1 is a primary Fe transporter belonging to the ZIP family that transport metal from soil to plant (Guerinot, 2000).

IRT2, in contrast to *IRT1*, does not transport heavy metals such as cadmium in yeast. *IRT2* expression is observed exclusively in roots of *A. thaliana*, and the gene expression is enhanced by Fe deficiency. By fusing the IRT2 promoter to the GUS gene, Vert *et al*., (2001) showed that the IRT2 promoter was active in the external cell layers of the root sub-apical zone. These data supported the IRT2 transporter's role in Fe and zinc uptake from the soil in response to low Fe in the rhizosphere.

Strategy II Fe-uptake system in which Fe is absorbed by roots as an Fe^{3+}-phytosiderophore is exclusively used by members of Poaceae. Besides employing this strategy, rice possesses an additional Fe-uptake system which facilitates the plant to absorb Fe^{2+} directly. This strategy may be advantageous for better growth of rice in submerged conditions. Ishimaru *et al*., (2006) isolated *IRT2* gene from rice and showed that both *IRT1* and *IRT2* were expressed mainly in roots in response to low-Fe conditions.

Considering the potential of IRT2 in Fe transport within the plant system, Indian barnyard millet (*E. Frumentacea* L.), which has a relatively high iron content of up to 42.13 µg/g (Girish *et al.,* 2014), could be a good candidate as a source for IRT2 gene for biofortification study. Hence, *the IRT2* gene of barnyard millet was isolated at the our laboratory (Manape *et al*., 2017).

In this investigation, over-expression of *EfIRT2* in the rice model system demonstrated the functional significance of *IRT2*.

MATERIAL AND METHODS

Gene construct

A binary vector, pUH harboring *EfIRT2* (*Echinochloa frumentacea Iron regulated Transporter 2*) gene driven by maize ubiquitin1 promoter and nos terminator (Fig. 1) was used in the genetic transformation of rice. Besides *IRT2* expression cassette, the binary vector possessed the *hygromycin phosphotransferase II* gene for plant selection. The *Agrobacterium* strain, LBA4404 harbouring pUH-*EfIRT2* was used in the genetic transformation experiments.

Agrobacterium-mediated genetic transformation of rice *indica* cultivar ASD16

Immature seeds (14-16 days after flowering) of ASD16 were collected from Paddy Breeding Station, Tamil Nadu Agricultural University. Immature embryos were isolated from these seeds and used as explants for *Agrobacterium*-mediated transformation following the protocol of Hiei and Komari, (2008). Well proliferated and friable yellow calli were exposed to two rounds of stringent selection in 50 mgL^{-1} of hygromycin B. The calli that survived on hygromycin selection were subcultured onto pre-regeneration, regeneration, and rooting media. Regenerated plants with well-developed roots were hardened and maintained in the transgenic greenhouse (Plate 1).

PCR analysis of the putative transgenic rice

PCR assays were carried out to demonstrate the presence of *EfIRT2* and *hptII* genes in putative transgenic rice events by using the gene-specific primers (Table 1). These primers are expected to amplify 1254 bp,

and 686 bp DNA fragments, respectively. The amplified products were resolved on 0.8 per cent agarose gel, visualized on UV transilluminator upon ethidium bromide staining.

Histochemical analysis of *EfIRT2* transgenic rice grain by Prussian blue staining

The Perl's Prussian blue color staining was performed for a qualitative estimation of Fe concentration in the seeds (T_1) of transgenic rice events following the method described by Vasconcelos *et al.,* (2003) with slight modifications. The whole brown grains were stained with 3 % potassium ferrocyanide in acidic condition (3 % HCl) under vacuum for 8 h and washed with distilled water. The observation and documentation were done with a stereo microscope (Leica, Model S8 APO, USA).

Estimation of grain iron and zinc content in selected *EfIRT2* transgenic lines using ICP-OES

Inductively Coupled Plasma - Optical Emission Spectrometry (ICP-OES) was used to quantify the Fe and Zn contents in unpolished T_1 mature seeds harvested from *EfIRT2* transgenic rice lines maintained in the transgenic greenhouse. Two hundred and fifty milligrams of unpolished grains of wild type ASD16 and T_1 transgenic segregants for *EfIRT2 (EfIRT2-16, EfIRT2-20, EfIRT2-25, EfIRT2-28, EfIRT2-29, EfIRT2-30, EfIRT2-31, EfIRT2-32, EfIRT2-36, EfIRT2-42, EfIRT2-43, EfIRT2-44, EfIRT2-47, EfIRT2-49* and *EfIRT2-69)* were digested with concentrated nitric acid (Merck, USA) for 15 h in a vacuum hood and later digested on sand bath at 70 °C for 4-5 h till the solution became colorless. The digested sample volume was made up to 50 mL using 1 % nitric acid (Velu *et al.,* 2006) and used for estimation of Fe content in ICP-OES (Teledyne Leeman Labs, USA).

Fe and Zn localization study in rice grain using SEM-EDX

Unpolished seeds of transgenic rice lines and wild-type ASD16 were selected for the Fe and Zn content estimation and localization through Scanning Electron Microscopy – Energy Dispersive X-Ray (SEM-EDX) analysis. Seeds were immersed in 0.5 mL of standard fixative consisted of 0.1 mL of glutaraldehyde (2.5%), 0.1 mLof paraformaldehyde (2.0%) and 0.3 mL of 0.1M sodium phosphate buffer (pH 7.2) and kept at room temperature for 2 hrs and @ 4 °C for 48 hrs. Longitudinal and cross sections of seeds were taken to estimate the percentage of Fe and Zn content in the endosperm, embryo, and aleurone layer. The sections were washed with 0.1 M sodium phosphate buffer twice with 10-min intervals. The sectioned seeds were lyophilized (10-20 min) for dehydration and subjected to sputter coating, and processed for SEM and EDX analysis at different magnifications (Ahmed *et al.,* 2019).

RESULTS AND DISCUSSION

The Fe and Zn contents of endosperm in polished rice of popular varieties fall in the ranges 2 - 5 µg Fe g^{-1} and 15 - 20 µg Zn g^{-1} dry weight (Sperotto *et al.,* 2012). Lower quantities of Fe and Zn in grains, particularly in the endosperm of rice, a food crop for about 50% of the global population, could cause deficiency diseases in resource-poor who consume rice as a principal source of calories (Banakar *et al.,* 2017). Over-expression of Fe homeostasis genes in rice could significantly enhance the grain Fe content as reported by several groups (Johnson *et al.,* 2011; Lee *et al.,* 2012; Masuda *et al.,* 2009; Boonyaves *et al.,* 2017; Banakar *et al.,* 2017). Constitutive over-expression of the *OsNAS* gene family revealed single-gene strategies for effective Fe- and Zn-biofortification of rice endosperm (Johnson *et al.,* 2011). Constitutive over-expression of *OsNAS2* and endosperm specific ferritin gene expression resulted in Fe accumulation beyond the targeted level (15 µg g^{-1}) in rice under field conditions (Trijatmiko *et al.,* 2016). Previous studies reported that the constitutive over-expression of *IRT1* from different sources had increased the Fe content in rice grains (Lee and An, 2009; Tan *et al*., 2015). In the present study, we transformed rice with an *IRT2* gene cloned from barnyard millet and established its Fe uptake role in rice.

Generation of transgenic events with *EfIRT2*

A total of 95 transgenic events were generated through *Agrobacterium*-mediated transformation of rice immature embryo. The putative transgenic plants were then confirmed by Polymerase Chain reaction (PCR) for the presence of selectable marker gene *hptII* and *EfIRT2* transgene and resultant PCR products had expected amplicon sizes of 686 bp and 1254 bp, respectively (Plate 2 and 3). Out of 2425 embryos used for the co-cultivation, 95 transgenic events were generated with a transformation efficiency of 3.92 per cent (Table 2). All the 95 events were found to contain transgene sequences, *hpt,* and *EfIRT2*.

Histochemical analysis of *EfIRT2* transgenic rice grain by Prussian blue staining

Twenty-five transgenic events were selected for qualitative assessment of Fe content in brown rice (based on the availability of seeds). Four independent readings were taken for Prussian blue staining intensity on a scale of 1 to 5. The scores ranged from 1 (wild type) to 4.25. (Table 3). Fifteen *EfIRT2* transgenic lines with higher scores in Prussian blue straining were selected for Fe and Zn estimation using the ICP-OES method. Some of the lines with higher scores could not be included due to limited availability of seeds.

Estimation of Fe and Zn content in selected *EfIRT2* transgenic lines

Fifteen *EfIRT2* transgenic lines along with wild type ASD16 were selected for Fe and Zn estimation of T_1 brown rice using ICP-OES. Transgenic events *EfIRT2*-29 (45 mg /kg^{-1} DW), *EfIRT2*-30 (41 mg /kg^{-1} DW), *EfIRT2*-43 (41 mg /kg^{-1} DW) and *EfIRT2*-49 (40 mg /kg^{-1} DW) had marginal (15%) increase in Fe content as compared to the wild type ASD16 (39 mg /kg^{-1} DW) (Table 4, Fig. 2).

Fe and Zn localisation study in rice grain using SEM-EDX

Fe and Zn elemental composition analysis on percentage basis from embryo, aleurone layer, and endosperm of wild type ASD16 and transgenic lines *viz*., *EfIRT2*-30 and *EfIRT2*-49 was carried out using SEM-EDX. No structural or texture change was observed in the transgenic lines compared to the wild-type ASD16. Embryo, aleurone layer and endosperm have shown a differential response to Fe-uptake between the non-transgenic ASD16 and transgenic ASD16 lines (Table 5). The relative Fe content in the embryo has increased from 0.91 % (% mol wt. as provided by SEM-EDAX) in wild-type ASD16 to 1.57 per cent and 1.73 per cent for the transgenic lines *EfIRT2*-30 and *EfIRT2*-49, respectively. In the aleurone layer, relative Fe content has increased from 0.60 per cent in wild type ASD16 to 0.78 and 1.52 per cent for *EfIRT2*-30 and *EfIRT2*-49, respectively. Similarly, in the endosperm, relative Fe content has increased from 0.52 per cent in wild type ASD16 to 1.07 and 1.05 per cent for *EfIRT2*-30 and *EfIRT2*-49, respectively.

A recent study by Li *et al.,* (2022) it was observed that the reversal of the growth defects involving Zn and Fe uptake in a mutant yeast when ZmIRT2 was overexpressed. They suggested that *ZmIRT1*, *ZmIRT2* and *ZmYS1* may function in a cooperative manner to maintain Zn and Fe homeostasis in ZmIRT2 overexpressing plants. Other studies have suggested that IRT2 was unable to restore the wild-type phenotype of *IRT1* defective mutant plants (irt1-1) in *Arabidopsis* when over-expressed (Varotto *et al.,* 2002, Vert *et al.,* 2009).

However, in the present study it was observed that the marginal improvement of Fe and Zn level in rice grain when grown under normal condition (Table 4). Vert *et al.* (2009) have demonstrated that *IRT1* is upregulated in 35S::*IRT2* plants and suggested its role in the depletion of cytosolic iron by *IRT2*, thereby making the plant Fe store deplete.

Table 1. List of primers used for characterizing the transgenic plants

S. No.	Primer Code	Primer sequence	Amplicon Size	PCR conditions
1	EfIRT2-F	AGGATCCATGTCTTCACAAACAGTGCCAAG	1254 bp	94 °C for 5 min : 1 cycle 94 °C for 45 sec 62 °C for 45 sec : 30 cycles 72 °C for 1.30 min 72 °C for 10 min : 1 cycle
2	EfIRT2-R	AGGTACCTCACGCCCACTTTGCCATG		
3	hptII-F	GACGTCTGTCGAGAAGTTT	686 bp	94 °C for 5 min : 1 cycle 94 °C for 45 sec 58 °C for 45 sec : 30 cycles 72 °C for 45 sec 72 °C for 7 min : 1 cycle
4	hptII-R	GCCTCCAGAAGAAGATGTTG		

Table 2. Details of genetic transformation of ASD16 with pUH-*EfIRT2* construct

No. of batches of co-cultivation done	No. of embryos co-cultivated	No. of transgenic events generated	Transformation efficiency (%)
38	2425	95	3.92

Table 3. Screening of transgenic events expressing *EfIRT2* based on Prussian blue staining

S.No.	Event name	Average
1.	Event-1	2.5
2.	Event-2	2
3.	Event-6	3.5

4.	Event-7	2.75
5.	Event-8	3.25
6.	Event-14	3
7.	Event-16	4.25
8.	Event-18	1.75
9.	Event-20	2.5
10.	Event-25	3.75
11.	Event-26	2
12.	Event-27	2.25
13.	Event-28	2.5
14.	Event-29	3
15.	Event-29	3
16.	Event-30	2.5
17.	Event-31	2.75
18.	Event-32	2.5
19.	Event-36	2.75
20.	Event-42	2.75
21.	Event-43	2.5
22.	Event-44	2.5
23.	Event-47	2.75
24.	Event-49	3.25
25.	Event-69	2.5
26.	ASD16 Wild type	1

Table 4. Fe and Zn content in unpolished rice, ASD16 transformed with *EfIRT2*

Event Name	Fe (mg/Kg DW)	Zn (mg/Kg DW)
ASD16-1	39	31
EfIRT2-16	23	41
EfIRT2-20	29	27
EfIRT2-25	37	50
EfIRT2-28	19	79
EfIRT2-29	45	59
EfIRT2-30	41	51
EfIRT2-31	32	68
EfIRT2-32	34	35
EfIRT2-36	20	34
EfIRT2-42	36	55
EfIRT2-43	41	69
EfIRT2-44	27	68
EfIRT2-47	35	24
EfIRT2-49	40	46
EfIRT2-69	35	62

Table 5. Iron concentration (per cent of total elements) in different tissues of seeds of transgenic ASD16

Event Name	Embryo (Fe)	Aleurone (Fe)	Endosperm (Fe)
ASD16 (WT)	0.91	0.60	0.52
EfIRT2 Event 30	1.57	0.78	1.07
EfIRT2 Event 49	1.73	1.52	1.05

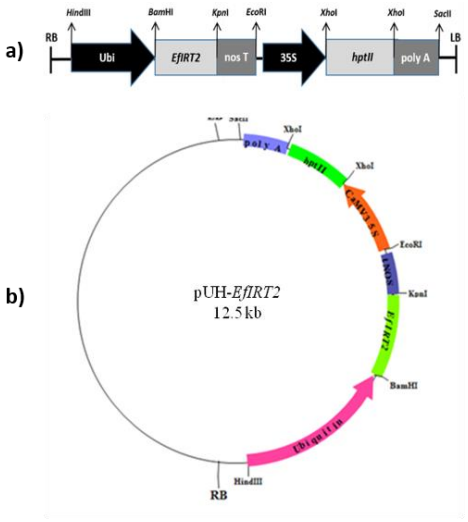


Figure 1. a) The T-DNA of pUH-*EfIRT2* in the backbone of pUH binary vector.

b) Vector map of binary vector pUH-*EfIRT2*

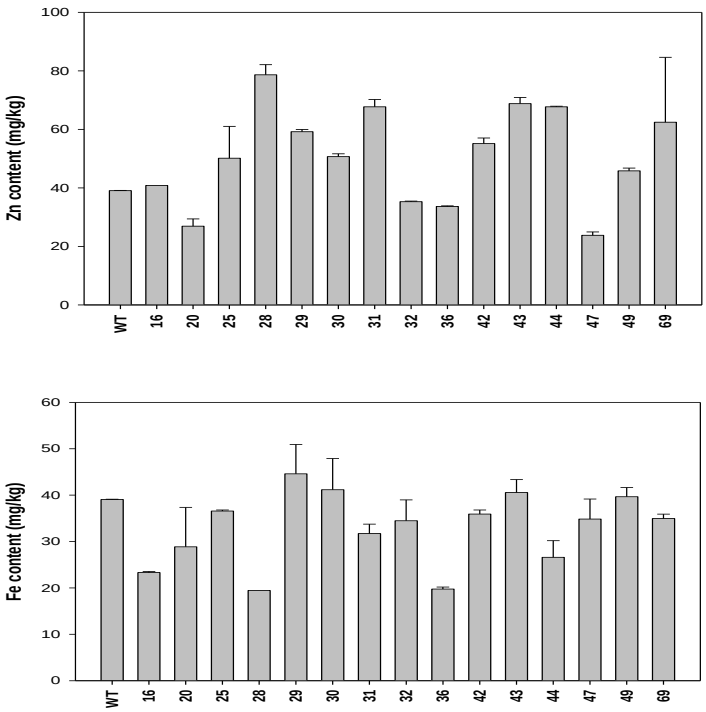
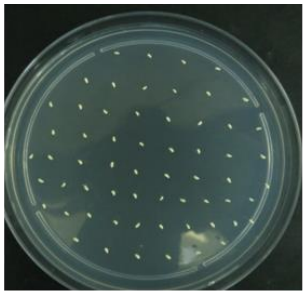


Figure 2. Fe and Zn content in T_1 transgenic lines of *EfIRT2*

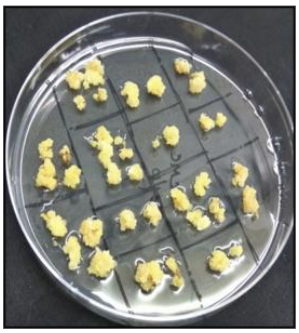
X-axis is the respective rice lines and Y-axis is Fe or Zn content (mg kg^{-1}). WT is the wild-type ASD16, 16, 20, 26, 28, 29, 30, 31, 32, 36, 42, 43, 44, 47, 49, 69 are the *EfIRT2* transgenic lines.

(Plate 2a) Rice Seeds for isolation of immature embryos

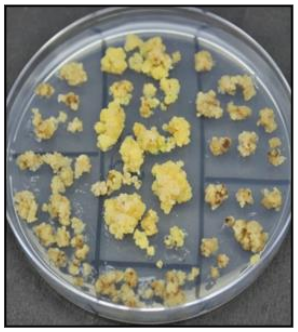


(Plate 1b) Co-cultivation

(Plate 1c). Co-cultivated embryo germination

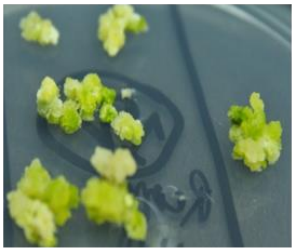


(Plate 1d) Sub culturing of calli



(Plate 1e) Selection-I

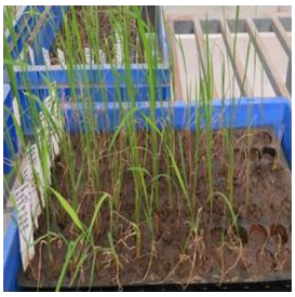
(Plate 1f) Selection-II



(Plate 1g) Regeneration

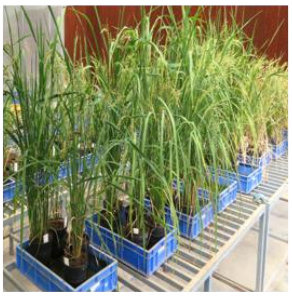
(Plate 1h) Rooting plate

(Plate 1i) Rooting bottle



(Plate 1j) Plants hardening in pro-trays

(Plate 1k) Plants in Pots



(Plate 1l) Different Transgenic Events

Plate 1. Stages of rice transformation

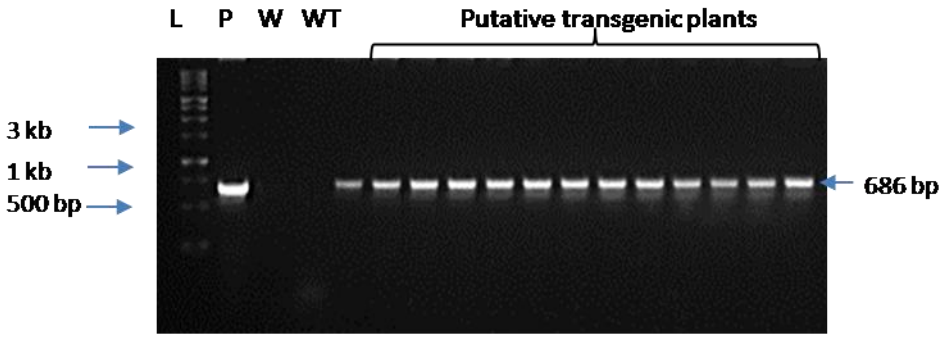


Plate 2. PCR analysis of putative transgenic *EfIRT2* events for the presence of *hpt* gene

L is 1 kb DNA ladder, P is pUH-*EfIRT2* plasmid positive control, W is water control, WT is wild type ASD16, Transgenic events with 686 bp *hptII* PCR amplicon

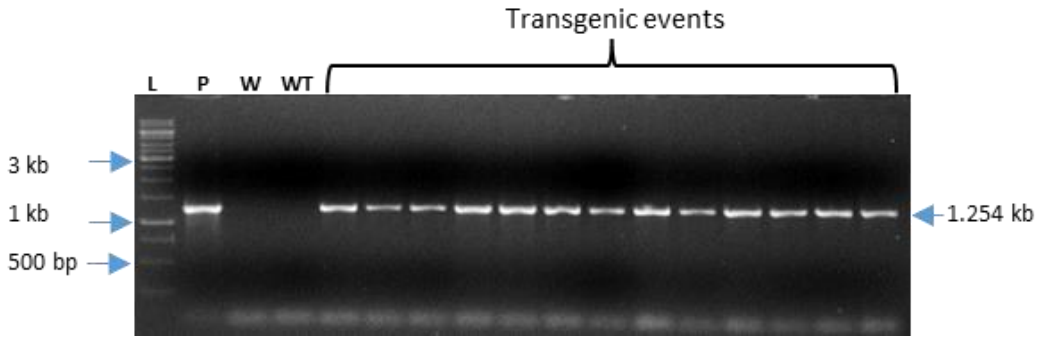


Plate 3. PCR analysis of putative transgenic *EfIRT2* events for the presence of *EfIRT2* gene

L is 1 kb DNA ladder, P is pUH-*EfIRT2* plasmid positive control, W is water control, WT is wild type ASD16, Transgenic events with 1.254 kb *EfIRT2* PCR amplicon

CONCLUSION

Biofortification is a viable and long-term strategy for addressing micronutrient deficiencies. It is possible to achieve desirable micronutrients in target tissues by engineering staple food crops with iron homeostasis genes. Though *IRT2* is suggested as a potential candidate for enhancing iron content, we observed a marginal improvement of 15 % of Fe in rice grain of the best event. Further studies on the expression of the transgenes are required to understand the efficiency of *EfIRT2* gene in rice.

Funding and Acknowledgment

The authors acknowledge the financial support provided to Rajen Chowdhury by ICMR-SRF (Ref. no. 3/1/JRF-2015/HRD-LS/132/92173/77) and funding by ICAR-.CRP on biofortification.

Ethics statement

No specific permits were required for the described field studies because no human or animal subjects were involved in this research.

Originality and plagiarism

Authors should ensure that they have written and submit only entirely original works, and if they have used the work and/or words of others, that this has been appropriately cited. Plagiarism in all its forms constitutes unethical publishing behavior and is unacceptable.

Consent for publication

All the authors agreed to publish the content.

Competing interests

There were no conflict of interest in the publication of this content

Data availability

All the data of this manuscript are included in the MS. No separate external data source is required. If anything is required from the MS, certainly, this will be extended by communicating with the corresponding author through corresponding official mail; dsudhakar@hotmail.com

Author contributions

Research grant-ICMR & ICAR, conceptualization-DS, Experiments- CR, AS, TKM, Guidance -DS, EK, KKK, LA, SV, MR, KSS, PJ , Writing original draft – DS, CR, Writing- reviewing &editing - DS, EK, KKK, LA, SV, MR, KSS, PJ.

REFERENCES

Ahmed, B., Rizvi, A., Zaidi, A., Khan, M.S and J. Musarrat. 2019. Understanding the phyto-interaction of heavy metal oxide bulk and nanoparticles: evaluation of seed germination, growth, bioaccumulation, and metallothionein production. *RSC Adv.,* **9 (8)**.

Banakar, R., Fernandez, A. A., Díaz-Benito, P., Abadia, J., Capell, T., and P. Christou. 2017. Phytosiderophores determine thresholds for iron and zinc accumulation in biofortified rice endosperm while inhibiting the accumulation of cadmium. *J.Exp.Bot.*, **68**: 4983–4995.

Boonyaves, K., Wu, T. Y., Gruissem, W., and Bhullar, N. K. 2017. Enhanced grain iron levels in rice expressing an iron-regulated metal transporter, nicotianamine synthase, and ferritin gene cassette. *Front. Plant Sci.*, **8**, 130.

Girish, C., Meena, R. K., Mahima, D., and K. Mamta. 2014. Nutritional properties of minor millets: neglected cereals with potentials to combat malnutrition. *Curr. Sci.*, **107(7),** 1109-1111.

Curie, C., Panaviene, Z., Loulergue, C., Dellaporta, S. L., Briat, J. F. and E. L. Walker. 2001. Maize yellow stripe1 encodes a membrane protein directly involved in Fe (III) uptake. *Nature.*, **409(6818)**: 346-349.

Dufner-Beattie, J., Wang, F., Kuo, Y. M., Gitschier, J., Eide, D. and G. K. Andrews. 2003. The acrodermatitisenteropathica gene ZIP4 encodes a tissue-specific, zinc-regulated zinc transporter in mice. *J.Biol.Chem.,* **278(35)**: 33474-33481.

Eng, B. H., Guerinot, M. L., Eide, D. and M. H. Saier. 1998. Sequence analyses and phylogenetic characterization of the ZIP family of metal ion transport proteins. *J.Membr.Biol.*, **166(1):** 1-7.

WHO, 2008. Worldwide prevalence of anaemia 1993–2005 : WHO global database on anaemia. Edited by de Benoist B, McLean E, Egli I and Cogswell M. World Health Organization, Switzerland.

Gaither, L. A., and D. J. Eide, 2001. The human ZIP1 transporter mediates zinc uptake in human K562 erythroleukemia cells. *J.Biol.Chem.,***276(25):** 22258-22264

Grass, G., Wong, M. D., Rosen, B. P., Smith, R.L. and Rensing, C., 2002. ZupT is a Zn (II) uptake system in Escherichia coli. *J.Bacteriol.*, **184(3):** 864-866.

Guerinot, M. L. 2000.The ZIP family of metal transporters.*BiochimicaetBiophysicaActa (BBA)-Biomembranes.,***1465(1-2):** 190-198.

Hiei, Y. and T. Komari, 2008. *Agrobacterium*-mediated transformation of rice using immature embryos or calli induced from mature seed. *Nat.Protoc.*, **3(5):** 824-834.

Inoue, H., Kobayashi, T., Nozoye, T., Takahashi, M., Kakei, Y., Suzuki, K., Nakazono, M., Nakanishi, H., Mori, S. and N. K. Nishizawa. 2009. Rice OsYSL15 is an iron-regulated iron (III)-deoxymugineic acid transporter expressed in the roots and is essential for iron uptake in early growth of the seedlings. *J.Biol.Chem.,***284(6):** 3470-3479.

Ishimaru, Y., Suzuki, M.,Tsukamoto, T., Suzuki, K., Nakazono, M., Kobayashi, T., Wada, Y., Watanabe, S., Matsuhashi, S., Takahashi, M., Nakanishi, H., Mori, S. and N. K. Nishizawa. 2006. Rice plants take up iron as an Fe^{3+}-phytosiderophore and as Fe^{2+}. *Plant J.*, **45:** 335–346.

Johnson, A. A. T., Kyriacou, B., Callahan, D. L., Carruthers, L., Stangoulis, J., Lombi, E. and M. Tester. 2011. Constitutive overexpression of the *OsNAS*gene family reveals single-gene strategies for effective iron-and zinc-biofortification of rice endosperm. *PLoS One*, **6:** e244769 (1-11).

Kobayashi, T. and N.K. Nishizawa. 2012. Iron uptake, translocation, and regulation in higher plants. *Annu. Rev. Plant Biol.,***63:** 131-152.

Lee, S. and G. An. 2009. Over-expression of *OsIRT1* leads to increased iron and zinc accumulations in rice. *Plant Cell Environ.,* **32:** 408-416.

Lee, S., Kim, Y. S., Jeon, U. S., Kim, Y. K., Schjoerring, J. K., and G. An. 2012. Activation of rice nicotianamine synthase 2 (OsNAS2) enhances iron availability for biofortification. *Mol. Cells*, **33(3),** 269-275.

Li, S., Song, Z., Liu, X., Zhou, X., Yang, W., Chen, J. and R. Chen. 2022. Mediation of Zinc and Iron Accumulation in Maize by ZmIRT2, a Novel Iron-Regulated Transporter. *Plant Cell Physiol.,* **63(4):** 521-534.

Manape, T.K., Varanavasiappan, S., Raveendran, M., Robin, S., Rajeswari, S. and D. Sudhakar. 2017. Isolation and In Silico Analysis of Iron Regulated Transporter 2 Gene from Indian Barnyard Millet (Echinochloafrumentacea L.). *Int. J. Curr.Microbiol. Appl. Sci.,***6(4):** 1576-1587.

Masuda, H., Usuda, K., Kobayashi, T., Ishimaru, Y., Kakei, Y., Takahashi, M., Higuchi, K., Nakanishi, H., Mori, S. and N. K. Nishizawa. 2009. Overexpression of the barley nicotianamine synthase gene HvNAS1 increases iron and zinc concentrations in rice grains. *Rice*, **2(4),** 155-166.

Mathews, W. R., Ong, D., Milutinovich, A. B., and M. Van Doren. 2006. Zinc transport activity of Fear of Intimacy is essential for proper gonad morphogenesis and DE-cadherin expression. *Development.,* **133 (6):**1143–1153.

Mathews, W.R., Wang, F., Eide, D.J. and M.Van Doren. 2005. Drosophila fear of intimacy encodes a Zrt/IRT-like protein (ZIP) family zinc transporter functionally related to mammalian ZIP proteins. *J. Biol. Chem.,* **280(1):** 787-795.

Mori, S. and N. Nishizawa. 1987. Methionine as a dominant precursor of phytosiderophores in Graminaceae plants. *Plant cell physiol.,* **28(6):** 1081-1092.

Morrissey, J. and M.L.Guerinot. 2009. Iron uptake and transport in plants: the good, the bad, and the ionome. *Chem. Rev.*, **109(10):** 4553-4567.

Santi, S. and W. Schmidt. 2009. Dissecting iron deficiency-induced proton extrusion in Arabidopsis roots. *New Phytol.,* **183(4):** 1072-1084.

Santi, S., Cesco, S., Varanini, Z. and R. Pinton. 2005. Two plasma membrane H+-ATPase genes are differentially expressed in iron-deficient cucumber plants. *Plant Physiol. Biochem.,* **43(3):** 287-292.

Sperotto, R. A., Ricachenevsky, F. K., de Abreu Waldow, V., and J. P. Fett. 2012. Iron biofortification in rice: it's a long way to the top. *Plant Sci.,* **190**, 24-39.

Tan, S., Han, R., Li, P.,Yang, G., Li, S., Zhang, P., Wang, W. B., Zhao, W. Z. and L. P. Yin. 2015. Over-expression of the *MxIRT1* gene increases iron and zinc content in rice seeds. *Transgenic Res.,***2 4:** 109-122.

Taylor, K. M. and R. I. Nicholson. 2003. The LZT proteins; the LIV-1 subfamily of zinc transporters. Biochimicaet Biophysica Acta (BBA)-*Biomembranes.,***1611(1-2):** 16-30.

Taylor, K. M., Morgan, H. E., Johnson, A., Hadley, L. J. and R. I. Nicholson. 2003. Structure–function analysis of LIV-1, the breast cancer-associated protein that belongs to a new subfamily of zinc transporters. *Biochem. J.*, **375(1):** 51-59.

Trijatmiko, K. R., Dueñas, C.,Tsakirpaloglou, N., Torrizo, L., Arines, F. M., Adeva, C.,Balindong, J.,Oliva, N., Sapasap, M. V. and J. Borrero. 2016. Biofortified indica rice attains iron and zinc nutrition dietary targets in the field. *Sci. Rep.,* **6:** 19792.

Varotto, C., Maiwald, D., Pesaresi, P., Jahns, P., Salamini, F. and D. Leister. 2002. The metal ion transporter IRT1 is necessary for iron homeostasis and efficient photosynthesis in Arabidopsis thaliana. *Plant. J.,* **31(5):** 589-599.

Vasconcelos, M., Datta, K., Oliva, N., Khalekuzzaman, M., Torrizo, L., Krishnan, S., Oliveira, M., Goto, F. and S. K. Datta. 2003. Enhanced iron and zinc accumulation in transgenic rice with the ferritin gene. *Plant Sci.,***164(3):** 371-378.

Velu, G., Kulkarni, V. N., Muralidharan, V., Rai, K.N., Longvah, T., Sahrawat, K. L. and T. S. Raveendran. 2006. A rapid screening method for grain iron content in pearl millet. *Int. Sorghum and Millets Newsletter.*, **47:** 158-161.

Vert, G., Barberon, M., Zelazny, E., Séguéla, M., Briat, J. F. and C. Curie. 2009. Arabidopsis IRT2 cooperates with the high-affinity iron uptake system to maintain iron homeostasis in root epidermal cells. *Planta.,* **229(6):** 1171-1179.

Vert, G., Briat, J. F. and C. Curie. 2001. Arabidopsis IRT2 gene encodes a root-periphery iron transporter. *The Plant J.,* **26(2):** 181-189.