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ISOLATION AND CHARACTERIZATION OF IRON ACQUISITION RELATED GENES INVOLVED IN EARLY STAGE IRON DEFICIENCY RESPONSE IN FOXTAIL MILLET (*SETARIA ITALICA*)

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ABSTRACT: Foxtail millet (*Setaria italica*) is minor millet known to have high grain nutritive value especially iron compare to that of major staple cereal rice. Any increase in quality of millets might have significant role in combating micronutrient malnutrition for human health over the world. For the development of nutria rich rice, isolation of the genes involves in the metal homeostasis is the proven and successful approach for beneficial malnourished population. In our study 13 genes involved in the iron acquisition and transport were used for gene expression analysis by using semi quantitative RT-PCR. Leaf and root tissue were collected from Fe sufficient and Fe deficient grown Foxtail millet CO (Te)-7 plant at early stage. The expression level of *VIT1*, *NAAT*, *FER1*, *FRO2*, *OsIRO2*, *YSL2 SiNAS2*, and *OsIDFE1* genes showed up regulation in Fe deficient leaves. *VIT1* and *Si022298* genes showed up regulation in Fe deficient roots. *NAAT*, *FER1*, *OsIRO2*, *YSL2*, *SiNAS2* and *Si036196* genes showed down regulation in Fe deficient roots. On the basis of above expression analysis we have selected *SiNAS2* gene for further cloning and characterization using PCR based amplification. Amplification of full length *SiNAS2* gene was performed on tissue collected from CO (Te)-7 genotype using *SiNAS2* specifically designer primer. These result in generating an amplicon of 2.8 kb full length for genomic DNA. These amplified fragments were cloned in pCAMBIA1301 followed by transformation of agrobacterium cell. The positive clone was screen and conforms for *SiNAS2* insert by colony PCR. Plant transformation and over expression studies using this gene construct will be useful for enhancing grain iron content in staple crops like rice and genetically simple solution to Fe deficiency disorders affecting billions of people throughout the world.
Key words: Foxtail millet, genes amplification, expression analysis, cloning and characterization.

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INTRODUCTION

Iron deficiency is the most common nutritional disorder worldwide 2 billion people – over 30% of the world's population is anemic, mainly due to iron deficiency [1]. Poor grain micronutrient contents (iron and zinc) in cereals is the primary cause of prevalence of such nutritional deficiency related disorders amongst population having cereals based diet, especially those dwelling in developing world [2]. One of the strategic approaches to address this is the use of alternative healthy food choices like minor millets that form a good dietary source of nutritional elements. Foxtail millet (*Setaria italica*) is the second most largely cultivated millet. Foxtail millet (*Setaria italica*), a member of the family Poaceae, is one of the oldest cereal crops.

Foxtail millet is a minor yet important crop in some areas of the world. It has strong adaptability to abiotic stresses, especially drought, and poor soil. It also has high nutritional value. Foxtail millet is twice richer in protein, four times richer in mineral and fat and thrice richer in calcium as compared to rice [3]. Foxtail millet has reportedly more genetic characterization among the other small millets [4] giving it a great potential for gene identification, mining, cloning and transformation. Among the several genes governing these nutritive traits (Fe in particular),*SiNAS2* genes have been recognized as a rich source prominent in the early stages of growth to manipulate the Fe levels in the consumable portions of the crop to combat malnutrition. Employment of such effective strategies and discovering even better sources of effective genes from micronutrient rich non- model crop genomes could provide a sustainable and genetically simple solution to Fe and Zn deficiency disorders affecting billions of people throughout the world.

MATERIALS AND METHODS

Plant material & Growth Conditions:

The plant material used for the study includes foxtail millets one genotype CO (Te)-7. Seeds were surface sterilized with 0.1% Bavistin followed by rinsing with distilled water. The treated seeds were germinated on petri dishes and 7 day old seedling were transferred to coco peat culture pots supplies with Yoshida solution [5] and maintained under greenhouse condition (Fig1). Nutrient solution was supplies within a four days interval.

Sample collection and DNA preparation:

Leaf tissue samples were harvested at early stage for DNA. DNA was extracted by CTAB method with little modification.

Extraction of RNA and semi-quantitative RT-PCR

Leaves and roots samples were harvested at early stage for RNA extraction. Tissues were frozen in liquid nitrogen immediately to be used for RNA extraction using Trizol reagent / Himedia RNA miniprep purification kit and quantified with NanoDrop Spectrophotometer ND-1000® (NanoDrop Technologies, USA). RNA isolated and quantified was used for cDNA was synthesized using BIORAD iScript^{TM} cDNA Synthesis Kit. Semi-quantitative RT-PCR reactions were carried out with 10 µl of the reaction solutions using gene specific primers. Additional reaction components were: 10 mM polymerase buffer, 1mM dNTPs, 0.1 units Taq polymerase and 1 µM specific primers. The amplified PCR product was run on 1.5 percent agarose gel prepared in Tris acetate ethyldimethyl tetra acetic acid (TAE) buffer and visualized by UV illuminator after staining with ethidium bromide. The presence of amplicons and their respective intensity were recorded under gel documentation system. The relative intensity of amplicons provided the basis for quantification of level of expression of gene as high, moderate, low and negligible.

PCR primers for generating inserts for cloning

Phire tag polymerase was used for DNA amplification with PCR for the Si*NAS2*. PCR of Si*NAS2* was performed of pre amplification denaturation at 98^{o}C for 1minute followed by thirty five cycles each of 98^{o}C for 0.10 seconds, 69^{o}C for 0.15 seconds and 72^{o}C for 0.30 sec, and final extension at 72^{o}C for 1 minute. The amplified Si*NAS2* was run on 1.5 percent agarose gel prepared in Tris acetate ethyldimethyl tetra acetic acid (TAE) buffer and visualized by UV illuminator after staining with ethidium bromide. DNA bands were compared with 1kb ladder as marker to identify the positions of required band. PCR products were cut out of the gel and purified with the GenElute Gel extraction kit (Sigma) as recommended by producer.

Restriction double digestion and cloning ligation reactions

PCR was performed on genomic DNA were purified to be used as inserts. Inserts as well as vector were prepared by restriction double digestion through two enzymes (*Nco I* and *Pml I)* (Fig 4B*).*The digested vector and insert were gel purified again and ligated (Fig 4C) to ligation mixture (20 µl) ligation buffer (4 µl), containing vector DNA(5 µl), insert DNA (8 µl), ligase enzyme (2.0 µl) and Mili Q water (1.0 µl) was incubated at 24 °C for 60 minutes for sticky end ligation °C for 5 min.

Preparation of chemically competent cells

To prepare chemically competent cells, culture a strain of *Agrobacterium* strain *LBA4404* was initiated by inoculated in 50 ml LauriaBartini (LB) medium at 28 °C with 150 rpm shaking two days. The starter culture was poured into 250 ml LB medium, and incubated at 28 °C with 150 rpm until the O.D600 reached at 0.4-0.5. Bacterial cells of *Agrobacterium* strain *LBA4404* were made chemically competent by using competent cell preparation kit from Merck Bioscience (CAT# 611660100011730, KT26).

Transformation of competent *Agrobacterium* cells via heat shock method

Transformation was performed on *Agrobacterium cells* by heat shock treatment (42^{o} C for 45 sec) by mixing 5 µl of ligation mixture and 100 µl of competent cells in an eppendorf on ice. LB media (250 ml) was poured and kept on constant shaking (250 rpm, 1 hour) at 37 °C. Transformation mixture (100 µl) was spread on LB kanamycin plates and incubated at 28 °C overnight. Plates were observed for transformed colonies and screened.

Identification of positive bacterial transformants:

Positive bacterial transformants initially colony PCR was performed (phire tag polymerase) by picking single colonies which appeared on LB kanamycin plates after overnight incubation at 28 °C. Si*NAS2* gene fragment was amplified by primers and detected on agarose gel electrophoresis. Plasmid pCAMBIA/Si*NAS2* was isolated using Invitrogen's PureLink™ Quick Plasmid Miniprep Kit (Cat# K210010).Single colonies which appeared on LB kanamycin plates after overnight incubation at 28°C.

RESULTS AND DISCUSSION

This study showed varied level of expression (low to high) in leaves and roots tissues at early stage among selected Foxtail millet genotypes CO (Te)-7 and was found to be expression high in Fe deficient leaves and low Fe deficient roots. The expression pattern of *FER1* gene showed uniform level of expression in Fe sufficient leaves, roots and Fe deficient leaves but negligible expression of Fe deficient roots (Fig 3). Expression of *OsFER1* gene in flag leaf with variation in level of expression was found among four rice cultivars [6]. *OsYSL*2 gene expressed in Fe sufficient and deficient leaves, whereas, low expression was observed in Fe sufficient roots(Fig 3). This gene showed negligible expression in Fe deficient roots. The observed expression pattern signifies deficiency induced up-regulation of *OsYSL2* in leaves but not in root tissues. In rice crop, promoter *b*-glucuronidase (GUS) analysis revealed that *OsYSL2* was expressed in companion cells in iron sufficient roots [7]. *OsNAAT* gene show moderate level of expression in Fe sufficient leaves, roots and deficient leaves. But low expression was observed in Fe deficient roots indicating deficiency induced down- regulation in root tissues. Two barley *NAAT* genes, *HvNAAT-A* and *HvNAAT-B*, were also cloned through enzyme purification from Fe-deficient barley roots [8]. *OsVIT1* gene shows constitutively high level of expression under both the conditions (Fe sufficient and deficient) and in both the tissue types. *OsIRO2* gene shows high level of expression in Fe sufficient leaves and roots (Fig 3). The expression was maintained to similar levels in leaf tissues under deficiency while a strong down-regulation was observed in case of roots. *OsIDEF1* gene show moderate level of expression in Fe sufficient and deficient leaves, low level of expression in Fe sufficient roots but no expression in Fe deficient roots (Fig 3). *FRO2* gene show high level of expression in Fe sufficient leaves, roots and Fe deficient leaves moderate level of expression in Fe deficient roots (Fig 3). Transcript of *NAS*1 gene show moderate level of expression in Fe sufficient leaves, roots and deficient leaves but no expression in Fe deficient roots was observed. Similarly, *OsNAS*2 transcript shown high level of expression in deficient leaves and moderate to low level of expression in sufficient leaves and roots.No expression was observed in deficient root (Fig 3). *SiNAS2* gene show moderate level of expression in Fe sufficient leaves and deficient leave. No expression in Fe sufficient and deficient roots (Fig 3). *SiNAS3* transcript shown high level of expression in Fe sufficient leaves and roots but low level of expression Fe deficient leaves and no expression in Fe deficient roots (Fig 3).*Si0222198* gene shown moderate level of expression in Fe sufficient and deficient leaves and high level of expression in Fe sufficient and Fe deficient roots (Fig 3).Si036196 gene shown moderate level of expression in Fe sufficient leaves, roots and Fe deficient leaves but low expression in Fe deficient roots (Fig 3). Transcripts of *OsNAS1* and *OsNAS2*, the expression of which is up-regulated by Fe deficiency, were detected not only in rice Fe-deficient roots, but also in chlorotic leaves [8]. In maize Isolated and characterized three *NAS* genes in maize (*ZmNAS*), and demonstrated that there are two types of *NAS* genes with different expression patterns; *ZmNAS1* and *ZmNAS2* are expressed only in roots under Fe-deficient conditions, and *ZmNAS3* is expressed under Fe-sufficient conditions. Discuss the role of NA in Fe-deficient roots and in Fe-sufficient leaves [9].The expression level of *VIT1*, *NAAT*, *FER1*, *FRO2*, *OsIRO2*, *YSL2 SiNAS2*, and *OsIDFE1*genes show up regulation in Fe deficient leaves (Table 1). *VIT1* and *Si022298* genes show up regulation in Fe deficient roots (Table 1). *NAAT*, *FER1*, *OsIRO2*, *YSL2*, *SiNAS2* and *Si036196* genes show down regulation in Fe deficient roots (Table 1). Amplification of full length *SiNAS2* gene using tissue collected from CO (Te)-7genotype using *SiNAS2* specifically designer primer which generated 2.8 kb full length for genomic DNA. The amplified fragments were clone in pCAMBIA1301 followed by transformation of agrobacterium cell. The positive clone was screen and conforms for *SiNAS2* insert by colony PCR (Fig 5b). Plant transformation and over expression studies using this gene construct will be useful for enhancing grain iron content in staple crops like rice and genetically simple solution to Fe deficiency disorders affecting billions of people throughout the world.

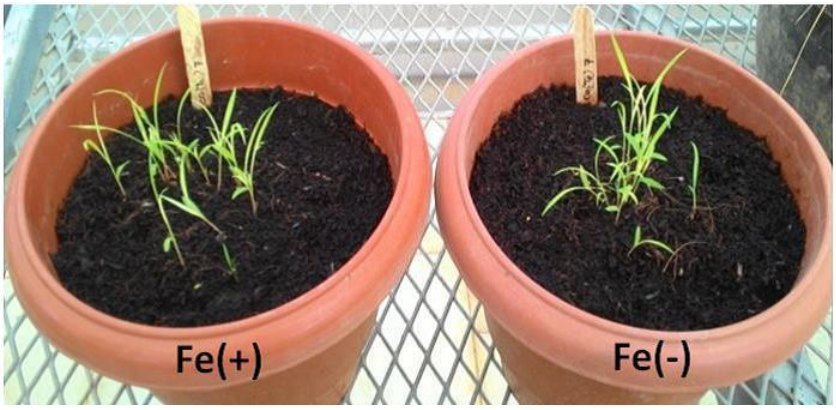

Figure 1: plant of Foxtail millet (variety Co (Te)-7) raised under Fe sufficient and deficient condition

Fe Sufficient Fe deficient

Figure 2: Co (Te)-7 variety of Foxtail millet under Fe sufficient and deficient conditions

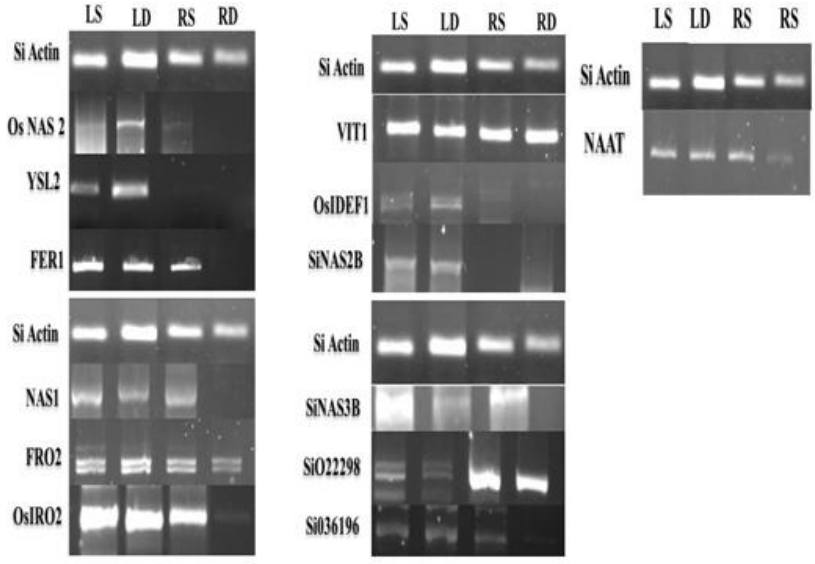

Figure 3: Expression analysis

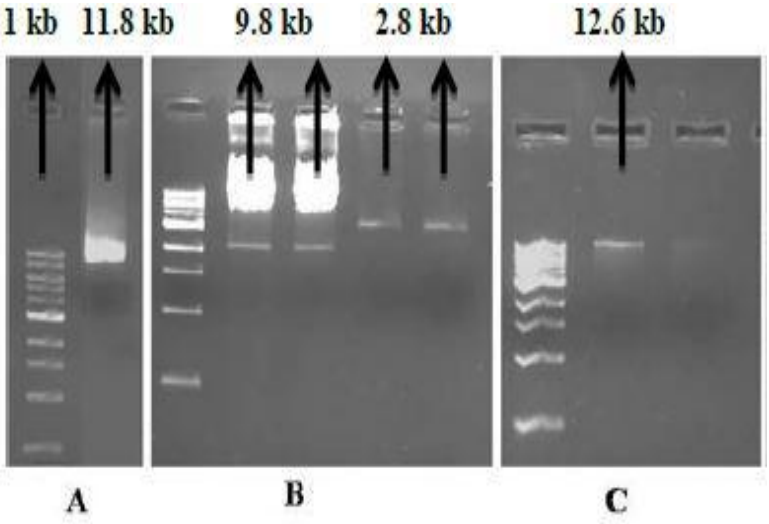

Figure 4: A- Undigested plasmid B- Digested plasmid and insert C-Ligation (plasmid+ insert)

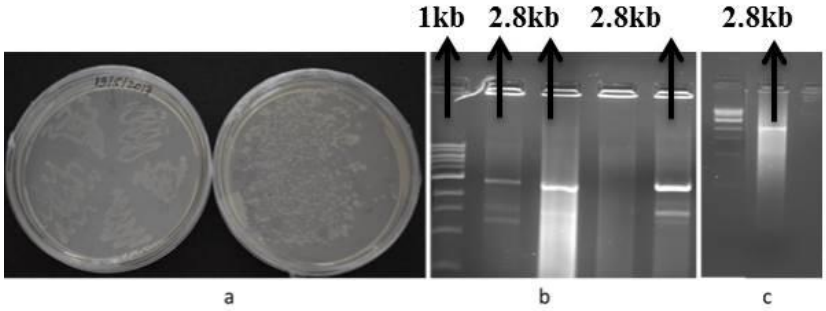

Figure 5: a- cloning plate b-Colony PCR c-plasmid PCR

Table1. Expression of metal homeostasis related genes in tissues

Sr no	Genes	Leaf sufficient	Leaf deficient	Root sufficient	Root deficient
1	OsNAS2				
2	YSL2				
3	FER1				
4	OsIRO2				
5	NAAT				
6	VIT1				
7	OsIDEF1				
8	SiNAS2				
9	SiNAS3				
10	Si036196				
11	Si022298				
12	NAS1				
13	FRO2				

High
Moderate
Low
Negligible

CONCLUSION

Majority of genes under study showed varied level of expression (low to high) in leaves and roots tissues at early stage among selected foxtail millet genotypes and was found to be expression high in Fe deficient leaves and low Fe deficient roots. The amplified fragments were clone in pCAMBIA1301 followed by transformation of agrobacterium cell. The positive clone was screen and conforms for *SiNAS2* insert by colony PCR. Plant transformation and over expression studies using this gene construct will be useful for enhancing grain iron content in staple crops like rice and genetically simple solution to Fe deficiency disorders affecting billions of people throughout the world.

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