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RESEARCH PAPER

Iron homeostasis in tropical *indica* rice (*Oryza sativa* L.) cultivars having contrasting grain iron concentration

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Abstract Iron homeostasis was studied in two tropical *indica* rice cultivars viz. *Sharbati* (high Fe) and *Lalat* (low Fe) having contrasting grain Fe concentration. Plants were hydroponically grown with 5 concentrations of Fe (0.05, 2, 5, 15, 50 mg L^{-1}) till maturity. The effect of incremental Fe treatment on the plant was followed by analyzing accumulation of ferritin protein, activities of aconitase enzyme, enzymes of anti-oxidative defense and accumulation of hydrogen peroxide and ascorbic acid. Plant growth was adversely affected beyond 15 mg L^{-1} of Fe supplementation and effects of Fe stress (both deficiency and excess) were more apparent on the high Fe containing cultivar *Sharbati* than the low Fe containing *Lalat*. Level of ferritin protein and aconitase activity increased up to 5 mg L^{-1} of Fe concentration. *Lalat* continued to synthesize ferritin protein at much higher Fe level than *Sharbati* and the cultivar also had higher activities of peroxidase, superoxide dismutase and glutathione reductase. It was concluded that the tolerance of *Lalat* to Fe stress was because of its higher intrinsic ability to scavenge free radicals of oxidative stress for possessing higher activity of antioxidative enzymes. This, together with its capacity to sequester the excess Fe in ferritin protein over a wider range of Fe concentrations made it more tolerant to Fe stress.

Keywords Rice · Iron · Tolerance · Ferritin · Aconitase · Homeostasis

Abbreviations

MTT	Thiazolyl blue tetrazolium bromide
PMS	Phenazine methosulfate
CAT	Catalase
SOD	Superoxide dismutase
GR	Glutathione reductase

Introduction

Fe toxicity is a major problem for tropical rice cultivation in rainfed lowland and irrigated environments. Water-logged soil sustains ferrous form of Fe causing greater absorption of the element by plants (Santos and Oliveira 2007) and thus, leads to greater accumulation of the element in cells causing toxicity and subsequent loss of grain yield (Genon et al. 1994). Loss of 50 % grain weight was reported in rice plants with leaf Fe concentrations higher than 250 μg mL^{-1} on dry weight basis (Genon et al. 1994). Rice cultivars with lower physiological Fe concentration were found to have higher tolerance to Fe toxicity (Central Rice Research CRRI Annual Report 2007). Iron toxicity results from reaction of free Fe^{2+} ions with molecular oxygen leading to generation of reactive oxygen species (ROS) that injure cells. Ferritin is the only protein known to concentrate Fe to the level required by the cells and stores it in a soluble and biologically available form and releases it when required and thus, protect the cells against the toxic effects of excess Fe (Theil 1987). Iron chelation is another

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essential process that renders free Fe into a non-Fenton active form (Curie et al. 2009). Chelated forms of Fe exist in a variety of metalloproteins such as aconitase which contains Fe as (4Fe-4S) cluster. Thus, at a particular level of Fe nutrition, performance of any organism, for that matter rice plant, will depend on how Fe homeostasis is maintained.

Rice genotypes having contrasting grain Fe concentration had been found to have different levels of physiological Fe concentration in different plant parts (Panda 2010). Such genotypes may react differently when grown with different levels of Fe in growing media. In the present study, Fe homeostasis was studied in contrasting rice cultivars (with respect to grain Fe concentration) raised with different concentration of Fe. Plant responses were followed by analyzing Fe accumulation, activities of antioxidative enzymes namely peroxidase, catalase and superoxide dismutase, glutathione reductase and level of two other very important iron containing proteins namely ferritin which plays the universal central role in Fe homeosdtasis and the other one, aconitase, whose role in Fe homeostasis in plant is yet to be established..

Materials and methods

Plant culture and growth conditions

Two *indica* rice cultivars with contrasting grain Fe concentration viz. *Sharbati* (high, 26 ppm in brown rice) and *Lalat* (low, 7 ppm in brown rice) were originally identified by us from a stock of rice germplasm collected from the Rice Gene Bank of Central Rice Research Institute (CRRI), Cuttack, India (Panda et al. 2014). Seeds were surface sterilized by immersing in a 4 % solution of commercial sodium hypochlorite for 10 min, followed by rinsing three times with glass-distilled water. Seeds were germinated at 30 °C in darkness inside two layers of moistened filter disk placed on a petri dish. Seedlings were raised without Fe for 7 days in a hydroponic culture (in 100 mL plastic cup containing 50 mL nutrient solution) inside a glass house with ambient temperature and light. Each cup contained 35 rice seedlings which were supplied with fresh nutrient solution every day. The nutrient solution (Yoshida et al. 1976) contained (g L^{-1}) NH_4NO_3, 91.4; $NaH_2PO_4.2H_2O$, 40.3; K_2SO_4, 71.4; $CaCl_2$, 88.6; $MgSO_4.7H_2O$ 32.4; $MnCl_2.4H2O$, 1.5; $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.074; H_3BO_3, 0.934; $ZnSO_4.7H_2O$, 0.035; $CuSO_4.5H_2O$, 0.031;$C_6H_8O_7H_2O$, 11.9; $Na_2SiO_3.9H_2O$, 16.2. The seedlings were raised with half-strength nutrient solution for first 3 weeks and full-strength solution thereafter. Three- weeks-old seedlings were shifted to nutrient solutions with 5 levels of Fe (0.05, 2, 5, 15, 50 mg L^{-1}) supplied as Ferric-EDTA and grown to maturity. In all the cases, the nutrient solution was renewed every day and adjusted to pH 5.5 with 1 N NaOH or HCl, as required.

At the time of panicle emergence, flag leaf of the main tiller was excised from the leaf base and kept into an ice bag for further analysis. Biochemical analyses (estimation of total Fe, assay and electrophoresis of peroxidase, catalase, superoxide dismutase, glutathione reductase, aconitase, extraction and visualization of ferritin protein, determination of hydrogen peroxide, ascorbate and net photosynthesis) of the plant samples were carried out as per the procedures given in the following subsections.

Estimation of total Fe

Completely dried plant parts (100–500 mg for green tissues such as leaf and 2 g for grain) were digested in a di-acid mixture (HNO_3 and $HCLO_4$ in 15:2 ratio). Samples were immersed overnight in 15 mL conc. HNO_3 in a conical flask. On the next say, 2 mL $HClO_4$ was added and the sample was digested on a hot plate at 60 °C for 2 h followed by further digestion at 90 °C until white fumes of $HCLO_4$ started effervescing out. The left over liquid (about 2 mL) was transferred to a 50 mL volumetric flask with several washings with double distilled water and the volume was made up to the mark. The solution was filtered with Whatman No. 41 filter paper and used for estimation of Fe in an atomic absorption spectrophotometer (GBC, Avanta).

Chlorophyll estimation

Samples of fresh leaves (0.5 g) were homogenized in cold mortar and pestle with 80 % pre-chilled aqueous acetone. Homogenates were centrifuged at 5000 g and the supernatants were collected in new tubes. The process was repeated three times by mixing the residue with additional acetone. Volume of the pooled supernatants was measured. Total chlorophyll content of the sample was estimated according to method of Arnon (1949).

Determination of photosynthetic attributes

The assimilatory characters such as net photosynthesis (*Pn*) and stomatal conductance (*gs*) were determined using a portable photosynthesis system (LI-6200, LICOR, Nebraska, USA). Data were recorded in the second fully expanded leaf from the top of the plant. All the measurements were taken between 11.00 and 12.00 h under natural sun light.

Extraction and assay of aconitase (EC 4.2.1.3)

Aconitase activity was assayed following the procedure of Bacon et al. (1961). Fresh leaves (0.5 g) were chopped into small pieces and homogenized in a pre-chilled mortar and pestle with 2.5 mL of cold extraction buffer (0.2 M

Tris-HC1, pH 8.5, containing 0.5 M sucrose and 5 mM EDTA). The homogenate was centrifuged at 13,000 g for 20 min at 4^0C. The supernatant was used for enzyme assay. The assay mixture (3.0 ml) contained 0.05 M sucrose, 0.5 M sodium (±) isocitrate buffered with 0.1 M Tris. HCl (pH 8.0 and 25 to 100 µl leaf extract The mixture was thoroughly mixed and absorbance was measured at 240 nm at 5 min interval over a period of 30 min. A control (without sodium (±) isocitrate) was also run. Micromoles of aconitate formed were calculated from an extinction value of 0.71 for 0.2 mM cis-aconitate (Racker 1950). One unit of aconitase enzyme activity was described as the amount of enzyme that formed one µmole of cis-aconitate h^{-1}.

Nondenaturing polyacrylamide gel electrophoresis and staining for aconitase activity

Aliquots of crude protein extract (containing 100 µg protein) were run on non-denaturing polyacrylamide gel (7.5 %) and enzyme activity was visualized on the gel by a specific stain (Harris and Hopkinson 1976). The staining solution contained 0.08 M Tris. HCl, 0.1 % *cis*-aconitic acid, 0.02 M $MgCl_2$, 0.01 % NADP, 0.01 % MTT, 0.002 % PMS and 2-3 units of isocitrate dehydrogenase. The gel was incubated in the staining solution for 1.5 h at 30 °C for development of enzyme activity band.

Extraction, electrophoresis and staining of ferritin protein

Ferritin protein was extracted from 1.0 g fully opened fresh plant leaves following the second procedure of Laulhere et al. (1988). Electrophoresis of ferritin protein was carried out on a 6 % native polyacrylamide gel, and ferritin protein was selectively stained with Feren S (Chung 1985). The staining solution consisted of freshly prepared mixture of 0.75 mM Feren S and 15 mM thioglycollic acid (in 2 % acetic acid). The gel was immersed in the staining solution till ferritin bands were visible as greenish yellow band.

Determination of ascorbate (AsA) content

Fresh flag leaves (0.5 g) were ground to fine powder in liquid nitrogen. The powdered tissues were homogenized with 2 mL pre-chilled trichloro acetic acid (6 %, w/v) and kept on ice for 15 min. The homogenate was centrifuged at 4^0C for 5 min at 15,000 g and the supernatant was immediately assayed for AsA content following the procedure of Kampfenkel et al. (1995).

Determination of hydrogen peroxide (H_2O_2) content

Fresh flag leaves (0.5 g) were ground to fine powder in liquid nitrogen. The powdered leaf tissues were homogenized with 5 mL pre-chilled trichloro acetic acid (0.1 %, w/v). The homogenate was centrifuged at 12,000 g for 15 min and the supernatant was assayed for H_2O_2 content following the method described by Velikova et al. (2000).

Antioxidative enzyme assays

Leaf segments (0.5 g) were ground to fine powder in a mortar and pestle using liquid nitrogen. Soluble proteins were extracted by homogenizing the powder in 10 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1 % polyvinylpyrrolidone (PVP) and 1 mM ascorbic acid (in case of peroxidase). The homogenate was centrifuged at 15,000 g for 20 min at 4 °C and the supernatant was used for enzyme assay.

Catalase (EC 1.11.1.6) activity was determined as per the procedure of Aebi (1984). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 10 mM H_2O_2 and 100 µl of enzyme extract in a 3 ml volume and the consumption of H_2O_2 was measured at 240 nm for 3 min.

Peroxidase (EC 1.11.1.7) was assayed following method of Hammerschmidt et al. (1982). Enzyme activity was determined by following decrease in A_{420} for 1.5 ml of a reaction mixture containing 0.45 mL of 0.1 M phosphate buffer (pH7.0), 0.75 mL of guaiacol (8 mM), 0.25 mL solution H_2O_2 (18 mM) and 50 µl crude enzyme extract. Enzyme activity was calculated using extinction coefficient of 25 mM^{-1} cm^{-1}.

Total SOD (EC 1.15.1.1) activity was assayed by monitoring inhibition of photochemical reduction of nitro blue tetrazolium (Walker et al. 1987). An aliquot of 0.1 mL enzyme extract was placed in a transparent glass test tube and 4.9 mL of 50 mM phosphate buffer (pH7.8) containing riboflavin, TEMED, nitroblue tetrazolium and EDTA to final concentrations of 2.3 µM, 4.3 mM, 0.112 mM, and 0.1 mM, respectively. The reaction mixture was illuminated for exactly 15 min at a light intensity of 5000 lx. One unit of SOD activity was defined as the amount of enzyme that caused 50 % inhibition of the reduction of nitroblue tetrazolium as monitored at 560 nm.

Glutathione reductase (EC 1.6.4.2) activity was determined by following the oxidation of NADPH at 340 nm (extinction coefficient 6.2 $mM^{-1}cm^{-1}$) for 3 min in an assay mixture (1 ml) containing 50 mM potassium phosphate buffer (pH 7.8), 2 mM Na_2EDTA, 0.15 mM NADPH, 0.5 mM GSSG, and 100 µl of enzyme extract. The reaction was initiated by adding NADPH. Corrections were made for the background absorbance at 340 nm, without NADPH (Schaedle and Bassham 1977).

Protein content was determined according to Bradford (1976) using BSA as standard.

RT-PCR expression analysis

Total RNA samples were extracted from plant tissues using the RNeasy Plant Mini Kit (Qiagen, Germany) as per manufacturer's instructions. First-strand cDNAs were synthesized from total RNA using "Revert Aid First Strand cDNA synthesis kit" (Fermentas, Canada). RT-PCR was performed using PCR Master mix kit (Fermentas, Canada) on an Eppendorf Master Cycler with running conditions of 94 °C for 2 min followed by 40 cycles of 94 °C for 15 s, 65 °C for 30 s, 72 °C for 1 min. The housekeeping gene, ß-tubulin, was used as an internal control. All the primers used in RT- PCR reactions are given in Table 1.

Statistical analysis

The experiments were conducted in a randomized complete block design and the results were presented as the means ± sd of 3 independent replicates. Means were tested by *t*-test at 5 % level of significance.

Results & discussion

In this study, effect of Fe level in the growing medium was tested on growth, Fe acquisition, levels of ferritin protein, H_2O_2, ascorbate and the activities of aconitase, peroxidase, catalase and superoxide dismutase in two tropical rice cultivars having contrasting grain Fe concentration.

Effect of iron level on plant growth and iron concentration in grains and flag leaves

Plant growth increased significantly from 0.05 to 15 mg L^{-1} level of Fe supplementation and decreased thereafter in both the rice cultivars (*Sharbati* and *Lalat*). *Sharbati* appeared to be more adversely affected by lower as well as higher levels of Fe in the growing medium. Iron concentration in the growing medium seemed to differentially affect flowering of the two the rice cultivars. While *Sharbati* showed flowering with 15 mg L^{-1} level only, *Lalat* showed flowering at both 5 and 15 mg L^{-1} Fe in the nutrient solution (Supplementary Fig. 1).

Iron concentration of the flag leaf of both the rice cultivars increased with increase in Fe level in the culture medium (from 0.05 mg to 15 mg L^{-1}) and declined thereafter and the concentration was higher in *Sharbati* as compared to *Lalat* at different levels of Fe supplementation (Fig. 1a). Grains of *Sharbati* also possessed higher Fe concentration than *Lalat* at maturity (Fig. 1b).

Thus, these contrasting cultivars responded to a varying degree in terms of plant growth and accumulation of Fe in flag leaf (Fig. 1a and Supplementary Fig. 1). Plant growth was drastically affected in both the cultivars beyond 15 mg L^{-1} of Fe in the culture medium. Similar to plant growth, Fe accumulation in the flag leaf also declined beyond 15 mg L^{-1}. The high Fe containing cultivar, *Sharbati* also had higher Fe concentration in the flag leaf; however, the cultivar appeared to be more sensitive to both low as well as high level of Fe in the growing medium than the low Fe containing cultivar, *Lalat*. Emergence of panicle clearly showed the difference in response of the two cultivars. Thus, there might be a negative relationship between the severity of Fe stress symptoms and Fe concentration of flag leaf and grain. The cultivar *Lalat,* in spite of having

Table 1 Primer sequences of the genes of interest and the house keeping gene used in the study

Sl. no	Gene	Primer sequence	Forward / Reverse	Annealing temp, °C	Amplicon size, bp
1	*Ferritin*	5′-TCACTCTTCACCCGCCGCG-3′ 5′-TCGACGAACTTTTGCCTAGC-3′	Forward Reverse	62.5	338
2	*Aconitase*	5′-ACCTTATAGATGTGGAGCCTTG C-3′ 5′-AACTGCAAAACCCTTGAAACCAA-3′	Forward Reverse	61.0	128
3	*Fe-SOD*	5′TGCACTTGGTGATATTCCACTC-3′ 5′-CGAATCTCAGCATCAGGTATCA-3′	Forward Reverse	59.0	297
4	*Cat A*	5′-GAAGATTGCGAATAGGCTCAAC -3′ 5′-GTGGCATTAATACGCCAGTACA -3′	Forward Reverse	59.5	305
5	Cytosolic *PDX*	5′-GACAAGAAACCCTCTGCAGTTT-3′ 5′-GTAGTCTGCTGGTTCACACTGG-3′	Forward Reverse	61.0	305
6	ß-tubulin	5′-TCTTCCACCCTGAGCAGCTC -3′ 5′-AACCTTGGAGACCAGTGCAG -3	Forward Reverse	62.0	147

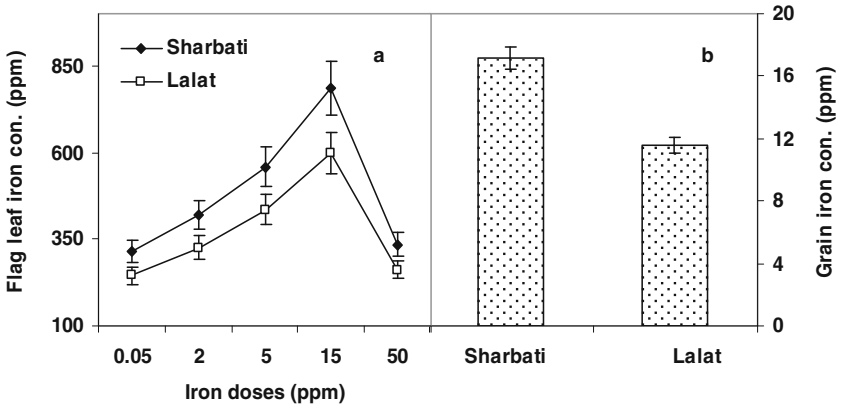

Fig. 1 Effect of Fe concentration in nutrient solution on Fe accumulation in the flag leaf (**a**) and dehusked grains (**b**) of two *indica* rice cultivars (*Sharbati* and *Lalat*). Seedlings received half-strength nutrient solution for the first 3 weeks. Three-weeks-old seedlings were shifted to nutrient solutions with five levels of Fe (0.05, 2, 5, 15, 50 mg L^{-1}) supplied as Ferric-EDTA. Dehusked grains were collected from plants raised in nutrient solution with 2 mg L^{-1} of Fe. *Vertical bars* represent mean ± sd of 3 replicates. Means were tested by *t*-test at 5 % level of significance

lower grain Fe concentration, is more tolerant to Fe stress (Fig. 1 and Supplementary Fig. 1).

Hydrogen peroxide (H_2O_2) and ascorbate level in flag leaves

In both the cultivars, accumulation of H_2O_2 increased with increase in the level of Fe in culture medium and the level was higher in *Sharbati* than *Lalat* (Fig. 3a). Both the cultivars showed decrease in ascorbate concentration in the flag leaf with increase in Fe supplementation from 0.05 to 50 mg L^{-1} in the growing medium (Fig. 3b) and overall content of ascorbate was higher in the flag leaf of *Lalat* than that of *Sharbati*.

Increased accumulation of Fe in the plant led to onset of oxidative stress which resulted in an increase in H_2O_2 and decrease in ascorbate content (Supplementary Fig 2a and 2b). Thus, adverse effect of Fe on plant growth might have resulted from the oxidative stress caused by greater absorption and accumulation of Fe by the rice plant. It was reported that Fe deficiency enhances accumulation of ascorbate in the cells (Zaharieva and Abadía 2003) and its requirement is low in the presence of high level of Fe. The same is true for the high Fe containing cultivar, *Sharbati* in which internal Fe level was found to be higher (Fig. 1a and b).

Expression of ferritin gene and accumulation ferritin protein in flag leaves

Ferritin protein concentration of the flag leaf increased with level of Fe-treatment in the culture medium from 0.05 to 5 mg L^{-1} in both the rice cultivars and declined at 50 mg L^{-1} (Fig. 2a). Expression of ferritin gene as observed with RT-PCR coincided with the fluctuation of ferritin protein level (Fig. 2a and b). However, contrary to grain Fe concentration, accumulation of ferritin protein and expression of ferritin gene was higher in *Lalat* as compared to *Sharbati* and like plant growth, both the processes seemed to be more severely affected at lower as well as higher Fe levels (Fig. 4a and b). However, between the two cultivars decline in ferritin protein level was more gradual in *Lalat* as compared to an abrupt decline in *Sharbati*.

Both the cultivars accumulated maximum amount of ferritin protein in the flag leaf at 5 mg L^{-1} of Fe beyond which it declined (Fig. 2a and b); however, response of the two cultivars to higher level of Fe was different. While the degree of decline was gradual in *Lalat*, it was a steep decline in *Sharbati* (Fig. 2a and b). Inhibition of ferritin synthesis by higher level of Fe has not been

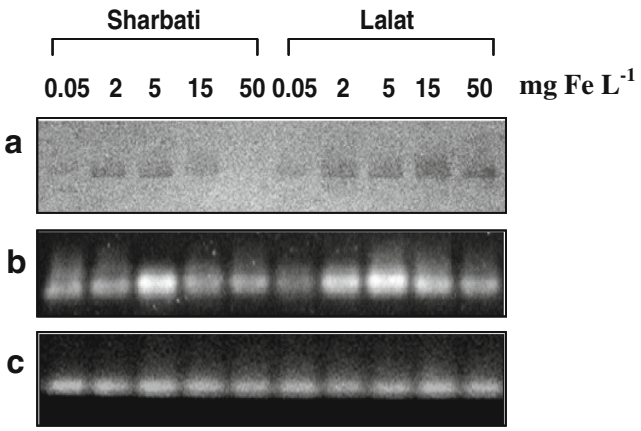

Fig. 2 Effect of Fe level on ferritin protein accumulation (**a**) and ferritin gene expression (**b**) in the flag leaves at panicle emergence stage of two *indica* rice cultivars (Sharbati and Lalat) grown in hydroponic culture. β-tubulin (**c**) was used as loading control in RT-PCR. Seedlings received half-strength nutrient solution for the first 3 weeks. Three-weeks-old seedlings were shifted to nutrient solutions with five levels of Fe (0.05, 2, 5, 15, 50 mg L^{-1}) supplied as Ferric-EDTA. Hundred microgram of protein extract was loaded in each lane of a 7.5 % native polyacrylamide gel. The gel was stained with Fe specific stain, Feren S. RT-PCR products were electrophoresed on a 1.5 % agarose gel

reported earlier and the influence seemed to be affected at the transcriptional level of ferritin gene (Fig. 2b). Differential response of the two cultivars to higher level of Fe might be due to the fact that the low Fe containing cultivar, *Lalat*, might be inefficient in absorption and translocation of the element within the plant (Fig. 1 and unpublished data from this lab). At similar level of Fe nutrition, internal Fe pool in *Lalat* might be smaller than *Sharbati* and hence ferritin synthesis went on until internal Fe level was high enough to cause decline in ferritin synthesis. *Lalat,* thus sequestered more Fe in ferritin protein, rendering the element unavailable for Fenton reaction and this might have given its higher tolerance to Fe excess. Relationship between Fe level and ferritin accumulation had been well documented. Excess of Fe was reported to cause increase in transcription ferritin gene and accumulation of ferritin protein (Lescure et al. 1991; Lobreaux et al. 1992; Majerus et al. 2007; da Silveira et al. 2009).

Expression of aconitase gene and accumulation aconitase activity in flag leaves

In both the rice cultivars, aconitase activity increased with increase in the level of Fe in culture medium from 0.05 mg L^{-1} to 5 mg L^{-1} and declined beyond that (Fig. 3a). In comparison with *Lalat*, the enzyme activity was higher in *Sharbati*. Aconitase enzyme activity on native PAGE (Fig. 3b) and expression of the aconitase gene (Fig. 3c) followed a pattern similar to that of the enzyme activity at different levels of Fe-treatment.

Aconitase is not only an Fe containing enzyme but also plays an important role in Fe homeostasis in animals (a similar role has not yet been established in plants). The relationship between Fe nutrition and aconitase activity was reported by Bacon et al. (1961) in mustard plant. Active aconitase is an Fe-sulfur cluster (4Fe-4S) containing enzyme that catalyses reversible isomerization of citrate to isocitrate in TCA cycle. Loss of one Fe from the Fe-sulfur cluster i.e. (3Fe-4S) inactivates its activity (Kennedy et al. 1983) and decreases cellular energy metabolism. Deficiency of the element has been shown to decrease activity of the enzyme significantly and replenishment restored its normal activity (Balk and Lobreaux 2005). In our experiment, aconitase activity was found to be low under both high and low level of Fe in the growing medium. Lower activity of the enzyme arose from suppression of transcription of aconitase gene rather than loss of activity of the enzyme under low Fe nutrition (Fig. 3a, b and c) and how this control is imposed needs to be studied in future. However, aconitase inactivation has a protective role for plants. The inactivation reduces electron flow through the mitochondrial transport chain, and thereby down regulates production of

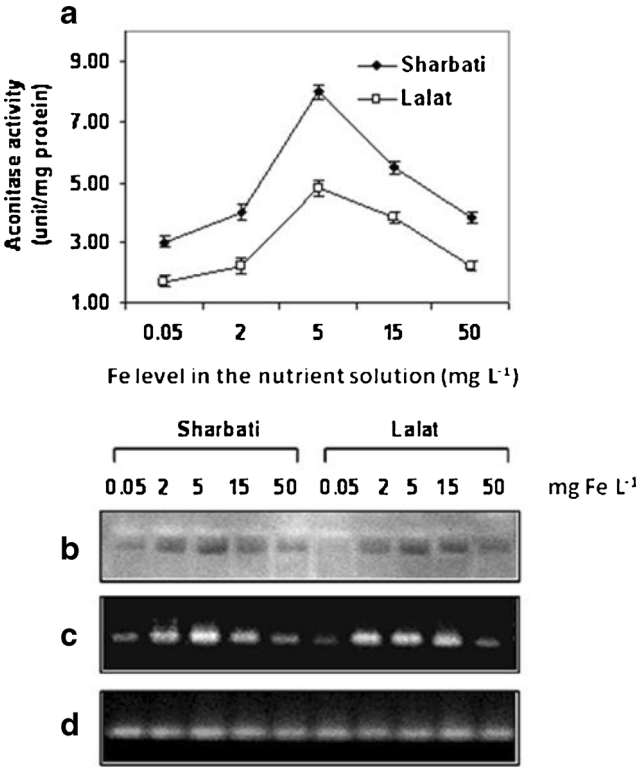

Fig. 3 Effect of Fe level on aconitase enzyme activity (**a**), activity staining of aconitase enzyme on native PAGE (**b**), aconitase gene expression by RT-PCR (**c**) in the flag leaves at panicle emergence stage of two *indica* rice cultivars (Sharbati and Lalat) grown in hydroponic culture. β-tubulin (**d**) was used as loading control in RT-PCR. Seedlings received half-strength nutrient solution for the first 3 weeks. Three-weeks-old seedlings were shifted to nutrient solutions with five levels of Fe (0.05, 2, 5, 15, 50 mg L^{-1}) supplied as Ferric-EDTA. Hundred microgram of protein extract was loaded in each lane of a 7.5 % native polyacrylamide gel. The gel was stained with specific stain for the enzyme. RT-PCR products were electrophoresed on a 1.5 % agarose gel. *Vertical bars* represent mean ± sd of 3 replicates. Means were tested by *t*-test at 5 % level of significance

reactive oxygen species as byproduct of respiration (Gardner and Fridovich 1991).

Activity of anti-oxidative enzymes and expression of their genes in flag leaves

Activity of peroxidase increased from 0.05 to 2 mg L^{-1} level of Fe and declined thereafter (Fig. 4a). While in *Sharbati*, the increase and decline in enzyme activity in response to varying levels of Fe were gradual, in *Lalat* the enzyme activity rapidly increased from 0.05 to 2 mg L^{-1} level of Fe and declined abruptly when the level of Fe was increased further. The pattern of peroxidase enzyme activity on native PAGE (Fig. 6b) and expression of gene following RT- PCR (Fig. 4c) were similar to that of the assay results of the enzyme (Fig. 6a) at different levels of Fe in the growing medium.

Catalase activity in the flag leaf increased from 0.05 to 5 mg L^{-1} level of Fe in the culture medium in both the

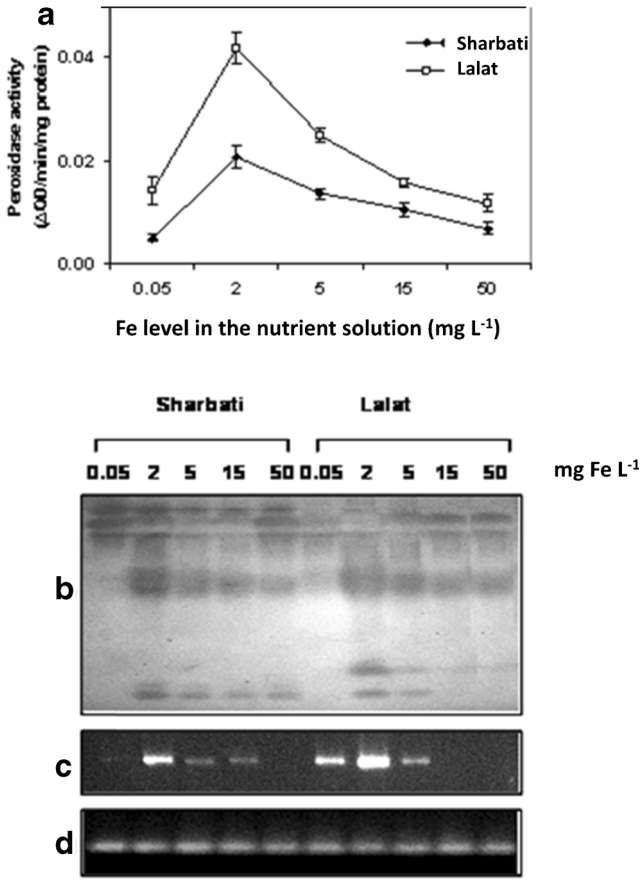

Fig. 4 Effect of Fe level on the activity of peroxidase (**a**), activity staining of peroxidase on native PAGE (**b**), peroxidase gene expression by RT-PCR (**c**) in the flag leaves at panicle emergence stage of two *indica* rice cultivars (Sharbati and Lalat) grown in hydroponic culture. β-tubulin (d) was used as loading control in RT-PCR. Seedlings received half-strength nutrient solution for the first 3 weeks. Three-weeks-old seedlings were shifted to nutrient solutions with five levels of Fe (0.05, 2, 5, 15, 50 mg L^{-1}) supplied as Ferric-EDTA. Hundred microgram of protein extract was loaded in each lane of a 7.5 % native polyacrylamide gel. The gel was stained with specific stain for the enzyme. RT-PCR products were electrophoresed on a 1.5 % agarose gel. *Vertical bars* represent mean ± sd of 3 replicates. Means were tested by *t*-test at 5 % level of significance

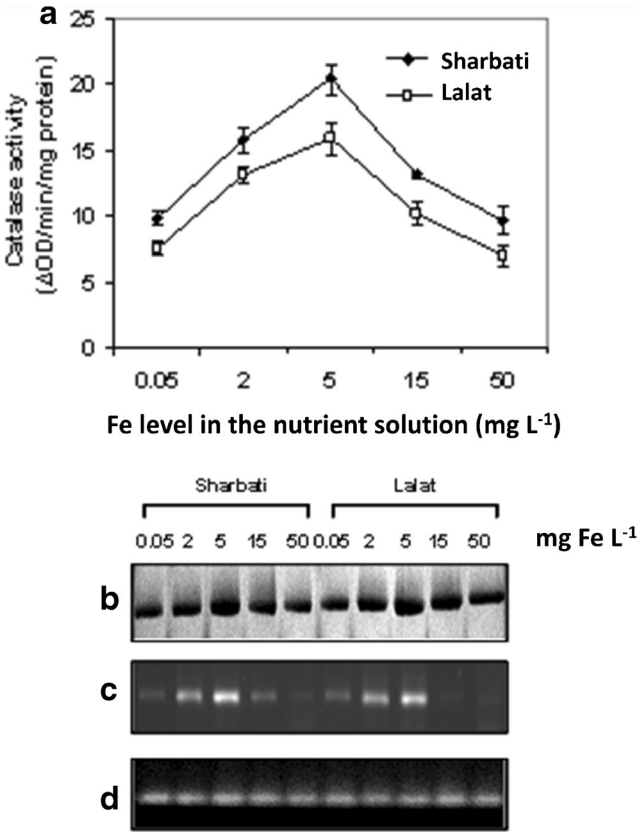

Fig. 5 Effect of Fe level on the activity of catalase (**a**), activity staining of catalase on native PAGE (**b**), catalase (*catA*) gene expression by RT-PCR (**c**) in the flag leaves at panicle emergence stage of two *indica* rice cultivars (Sharbati and Lalat) grown in hydroponic culture. β-tubulin (**d**) was used as loading control in RT-PCR. Seedlings received half-strength nutrient solution for the first 3 weeks. Three-weeks-old seedlings were shifted to nutrient solutions with five levels of Fe (0.05, 2, 5, 15, 50 mg L^{-1}) supplied as Ferric-EDTA. Hundred microgram of protein extract was loaded in each lane of a 7.5 % native polyacrylamide gel. The gel was stained with specific stain for the enzyme. RT-PCR products were electrophoresed on a 1.5 % agarose gel. *Vertical bars* represent mean ± sd of 3 replicates. Means were tested by *t*-test at 5 % level of significance

rice cultivars (Fig. 5a). However, activity of the enzyme was higher in *Sharbati* than *Lalat* at different levels of Fe. The fluctuation in the activity of the enzyme in native PAGE (Fig. 5b) matched with that of the gene expression of the enzyme (Fig. 5c) at different levels of Fe in the growing medium.

Superoxide dismutase showed increased activity with increase in Fe level in the culture medium from 0.05 to 2 mg L^{-1} and the enzyme activity declined thereafter in both the rice cultivars (Fig. 6a). Compared to *Sharbati*, activity of the enzyme was higher in *Lalat*. In both the rice cultivars expression of superoxide dismutase gene (Fig. 6c) also increased from 0.05 to 2 mg L^{-1} and declined at higher levels Fe. The pattern of superoxide dismutase activity on native PAGE (Fig. 6b) and its gene expression (Fig. 6c) was similar to that of enzyme activity (Fig. 8a) at different levels of Fe in the growing medium. However, while in *Lalat* expression of the gene was vigorous at high levels of Fe, there was significant fall in gene expression in *Sharbati* at similar levels Fe.

Activity of glutathione reductase enzyme in the flag leaf decreased initially with increase in the Fe level in the growing medium from 0.05 to 15 mg L^{-1} and then increased rapidly in both the rice cultivars (Supplementary Fig. 3a). As compared to *Sharbati*, activity of the enzyme was higher in *Lalat* at all levels of Fe nutrition. The assay results were further substantiated by activity of the enzyme on native PAGE (Suuplentary Fig. 3b).

Differences in the activity of anti-oxidative enzymes between the two cultivars clearly showed that the cultivars

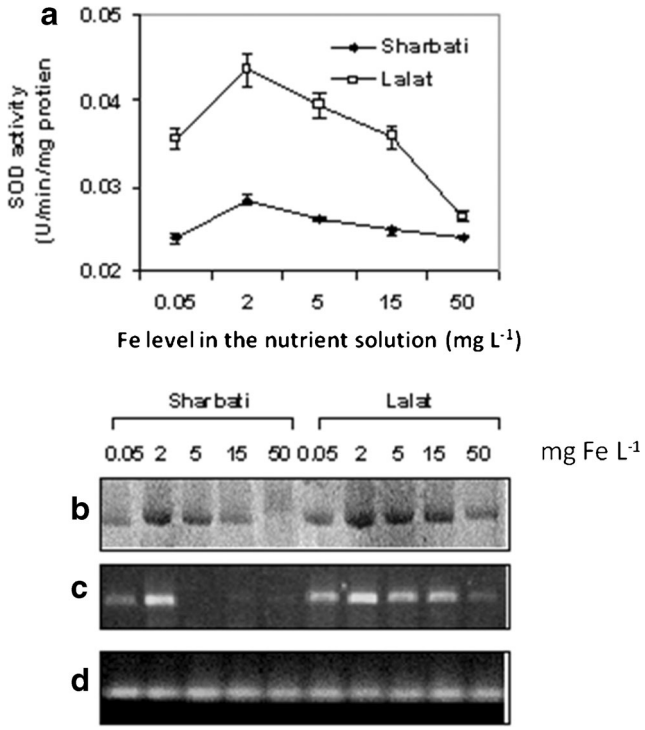

Fig. 6 Effect of Fe level on the activity of superoxide dismutase (SOD) (**a**), activity staining of SOD on native PAGE (**b**), sod gene expression by RT-PCR (**c**) in the flag leaves at panicle emergence stage of two *indica* rice cultivars (Sharbati and Lalat) grown in hydroponic culture. β-tubulin (**d**) was used as loading control in RT-PCR. Seedlings received half-strength nutrient solution for the first 3 weeks. Three-weeks-old seedlings were shifted to nutrient solutions with five levels of Fe (0.05, 2, 5, 15, 50 mg L^{-1}) supplied as Ferric-EDTA. Hundred microgram of protein extract was loaded in each lane of a 7.5 % native polyacrylamide gel. The gel was stained with specific stain for the enzyme. RT-PCR products were electrophoresed on a 1.5 % agarose gel. *Vertical bars* represent mean ± sd of 3 replicates. Means were tested by *t*-test at 5 % level of significance

differed in their response to Fe stress. Enzyme activity fluctuated significantly in response to variation in the levels of Fe in the growing medium: activities of the Fe containing enzymes viz. peroxidase, catalase and SOD were low under both Fe-deficient and - excess situations (Figs. 4a, 5a and 6a) whereas, activity of glutathione reductase, a non-Fe enzyme was higher under both high and low Fe level in the growing medium. Among the four anti-oxidative enzymes, activities of peroxidase, SOD and glutathione reductase were higher in the low Fe containing cultivar, *Lalat*. Higher catalase activity in *Sharbati*, although remained very high up to 5 mg L^{-1}, could not provide sufficient protection against Fe stress (Supplementary Fig. 1). This means that apparent tolerance of *Lalat* against oxidative stress might not be due to higher activity of the enzymes at higher Fe level rather due to higher inherent activity of these enzymes in this cultivar (which was seen at all Fe levels). It is possible that the higher level of Fe in the tissues of *Sharbati* (Fig. 1) produced more reactive oxygen species and the prevailing antioxidative enzymes could not detoxify them to the physiologically safe level rendering the plant susceptible to oxidative stress. Data on enzyme assay and activity staining on native PAGE were corroborated by the RT-PCR results. Contrary to this work, Dasgan et al. (2003) suggested that activity of Fe containing antioxidative enzymes such as peroxidase, catalase and superoxide dismutase was related to the internal Fe status of the plant. On the other hand, glutathione reductase, a non-Fe containing antioxidative enzyme had very similar activities in tomato cultivars differing in physiological Fe status (Dasgan et al. 2003).

Photosynthetic attributes of flag leaves

Net photosynthetic rate (Fig. 7a) and stomatal conductance (Fig. 7b) of the flag leaf increased with increase in Fe-level in the growing medium from 0.05 to 2 mg L^{-1} and declined marginally thereafter in both the rice cultivars. However, in *Lalat*, stomatal conductance recorded a significant reduction at higher levels of Fe in the growing medium.

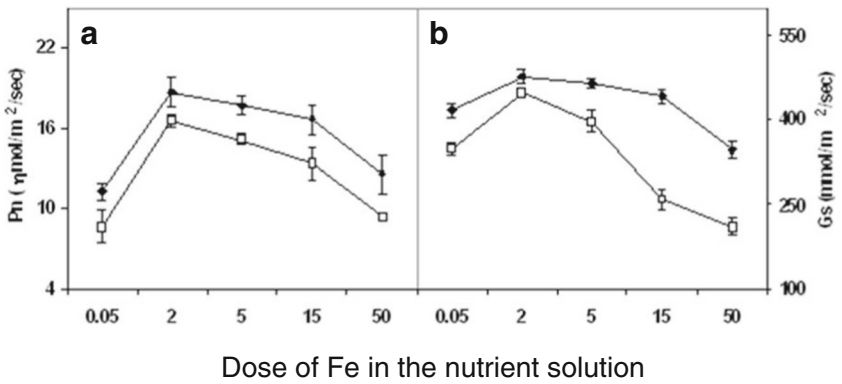

Fig. 7 Effect of Fe level on net photosynthesis (Pn) (**a**) and stomatal conductance (Gs) (**b**) of the flag leaves of two *indica* rice cultivars (Sharbati and Lalat) grown in hydroponics. Seedlings received half-strength nutrient solution for the first 3 weeks. Three-weeks-old seedlings were shifted to nutrient solutions with five levels of Fe (0.05, 2, 5, 15, 50 mg L^{-1}) supplied as Ferric-EDTA. *Vertical bars* represent ± sd of 3 replicates. Means were tested by *t*-test at 5 % level of significance

Iron is essential for chlorophyll synthesis without having any direct involvement in the process. The link between photosynthetic apparatus activity and protective role of ferritin in oxidative stress has already been reported (Busch et al. 2008). Ferritin protein protects the photosynthetic apparatus and minimizes the incidence of photo-oxidative stress and this action of ferritin is dependent on Fe availability (Briat et al. 2010). In our study, net photosynthesis and stomatal conductance remained higher in the high Fe containing cultivar, *Sharbati* (Fig. 7). Increase in Fe level in the growing medium also resulted in increased photosynthetic activity. However, excess of Fe in the culture medium became detrimental for photosynthesis as it resulted in generation of reactive oxygen species which might have caused damage to membranes of chloroplast thereby disrupting the photosynthetic electron transport thereby reducing net photosynthesis (Nikolic and Kastori 2000; Li et al. 2002).

It is, thus, concluded that the low Fe containing cultivar *Lalat* maintained Fe homeostasis by sequestering the excess Fe in ferritin protein over a wider range of Fe concentration and this might be due to the fact that at the same level of Fe in the growing medium, accumulation of the element in the plant was much lower compared to the high Fe containing cultivar, *Sharbati*. *Lalat* also showed high intrinsic capacity for scavenging free radicals of oxidative stress due to higher level of antioxidative enzymes such as peroxidase, superoxide dismutase and glutathione reductase. This, together with its ability to sequester Fe in ferritin over a wider Fe level imparts tolerance of *Lalat* to Fe stress.

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Compliance with ethical standards

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