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Expression profiling of iron deficiency responsive microRNAs and gene targets in rice seedlings of Madhukar x Swarna recombinant inbred lines with contrasting levels of iron in seeds

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Abstract

Aims To test if microRNAs are involved in iron (Fe) homeostasis in *Oryza sativa*.

Methods Recombinant inbred lines (RILs) of rice with contrasting levels of iron in seeds (high iron line HL, low iron line LL) and parent Swarna were grown in Fe sufficient (+Fe) and deficient (−Fe) environment. miRNAs whose target genes underlie the QTLs mapped for iron concentration (mapped in our previous study) were identified using bioinformatics. The expression analysis of these miRNAs and their targets along with few other miRNAs involved in nutrient homeostasis was done in root and shoot tissue. Real time PCR was used to study the relative expression of miRNAs and their target genes.

Results Out of nine miRNAs used in this study, 7 miRNAs-miR156, 168, 172, 162, 167, 171, and 398 showed down-regulation under Fe deficiency in root and shoot of high iron line when compared with Fe sufficient condition. Further, most of the miRNAs showed down-regulation while their target genes showed up-regulation under Fe deficiency in roots of all three genotypes (HL, LL and Swarna) suggesting

roots are more responsive to Fe deficiency. Important role of miRNAs in iron homeostasis was analyzed by comparing the expression of these miRNAs in HL, LL and Swarna under +Fe and –Fe.

Conclusion MicroRNAs showed differential expression in +Fe and –Fe environment. Further, their expression is more effectively regulated in root under Fe deficiency. This indicates that miRNAs might be playing regulatory roles in iron homeostasis in rice. This study suggests that Fe deficiency responsive miRNAs are involved in cross talk between other nutrients stress.

Introduction

Rice is the primary or secondary staple food crop for more than half of the world. However, it is a poor source of essential micronutrients such as iron (Fe) and zinc (Zn) in the diet of rice consumers. Fe is an important micronutrient for plants as well and is involved in biological oxidation-reduction reactions. It plays a key role in chlorophyll biosynthesis, photosynthetic and respiratory electron transport, nitrogen assimilation, and several other physiological and biochemical processes and thus a key determinant of biomass production (Briat et al. 2015; Buckhout et al. 2009). Though iron is abundant in arable land, it is often unavailable to plants due to low solubility of its oxidized form Fe^{+3}. Thus Fe can easily become a major constraint for plant growth.

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Higher plants have developed strategies to regulate its homeostasis (Hindt and Guerinot 2012). One of the prime targets in this respect is the control of iron acquisition in the root. Fe deficiency causes a metabolic imbalance which is lethal to plant growth e.g., impairing chlorophyll biosynthesis, chloroplast development, and photosynthesis. On the other hand, anaerobic conditions in acidic soils may lead to cellular Fe overload, triggering toxicity in plants. Hence, Fe levels within plant cells must be tightly regulated (Kong and Yang 2010). Iron metabolism in plants is regulated by protein encoding genes and non-coding microRNAs (Waters et al. 2012).

MicroRNAs (miRNAs) are 21–24 nt small RNAs regulating gene expression at the post-transcription levels by mRNA degradation or translation repression (Bartel 2004). The majority of plant miRNAs target transcription factors and are therefore thought to regulate the key steps of biological pathways (Bari et al. 2006; Chiou et al. 2006; Bartel 2004). These small RNAs regulate expression of their target genes involved in diverse biological processes including plant development and response to abiotic and biotic stresses (Sailaja et al. 2014; Jones and Dangl 2006; Sunkar and Zhu 2004). MicroRNAs are also involved in the regulation/signalling of metal toxicity response and nutrient stress (Mendoza-Soto et al. 2012; Ben Amor et al. 2009; Hobert 2008; Jones and Dangl 2006). An earlier study in *Arabidopsis* reported the involvement of miRNAs in Fe homeostasis (Kong and Yang 2010), however, miRNAs association in Fe homeostasis has not been reported in *Oryza sativa* yet. Expression of miR169b, miR169c, miR172c, miR172d, miR173 and miR394b in roots and miR169c, miR172c, miR172d, miR173, miR394a and miR394b in shoots was affected by Fe deficiency in *Arabidopsis* (Kong and Yang 2010). Since there are similarities in the response pathway to Fe deficiency in *Arabidopsis* and rice e.g., IDE1-like sequences and involvement of bHLH transcription factors (Buckhout et al. 2009; Kobayashi et al. 2003, 2007; Ogo et al. 2007; Ling et al. 2002), it is reasonable to hypothesize a similar complexity in the pathway of iron regulation in rice.

In our previous report, recombinant inbred lines (RILs) were developed from the cross Madhukar x Swarna and QTLs were mapped for grain iron and zinc concentrations. Several RILs with high Fe and Zn concentrations were identified during this study (Anuradha et al. 2012). Further, expression analysis of candidate genes underlie or close to mapped QTLs was performed in selected RILs with contrasting level of iron in seeds

(Agarwal et al. 2014). In this study, we identified 5 miRNAs whose target genes underlie QTLs mapped in our previous study. In addition, miRs169, 395 and 171 were selected for expression analysis based on earlier reports on their involvement in Fe and other nutrients homeostasis (Khraiwesh et al. 2012; Waters et al. 2012; Kong and Yang 2010). Expression of miR162 was included as it regulates DCL1 (dicer like protein) expression and miRNA biogenesis (Vazquez et al. 2004). To investigate the involvement of miRNA mediated pathways in Fe homeostasis in rice, the expression pattern of these 9 miRNAs and their putative targets was analyzed in seedlings of two RILs with contrasting levels of Fe/Zn in seeds. These seedlings were grown under Fe sufficient and deficient conditions. Expression analysis of miRNAs shows distinct differential expression in response to Fe deficiency suggesting the role of miRNAs in Fe homeostasis in rice seedlings.

Materials and methods

Plant material and growth conditions

Seeds of rice (*Oryza sativa*) cultivar Swarna and two RILs (Madhukar x Swarna) 185 M (high Fe line) designated as HL and 270 M (low Fe line) designated as LL with contrasting level of iron concentration in seeds were germinated on wet filter paper for 1 week. One week old rice seedlings were transferred to plastic boxes containing nutrient solution for 10 days based on our previous study (Agarwal et al. 2014). The solution contained KNO_3 (3 mM), $MgSO_4$ (0.5 mM), $CaCl_2$ (1.5 mM), K_2SO_4 (1.5 mM), NaH_2PO_4 (1.5 mM), H_3BO_3 (25 μM), $MnSO_4$ (1 μM), $ZnSO_4$ (0.5 μM), $(NH_4)6Mo7O_{24}$ (0.05 μM) $CuSO_4$ (0.3 μM), Fe-EDTA (40 μM) with pH adjusted to 6.0 with KOH. For+Fe treatment, Fe-EDTA was present in the nutrient solution but for −Fe treatment, Fe-EDTA was not added. Seedlings were grown in hydroponics in a growth chamber at 30 °C/22 °C (day/night) temperatures with a 12-h-light/12-h-dark regime. Both control (+Fe) and stress (−Fe) treatments consisted of three independent replications, with eight seedlings per box. LL and Swarna showed severe chlorosis in 17 days old seedlings while HL was able to withstand in Fe deficient medium (Agarwal et al. 2014). Shoots and roots were immediately frozen in liquid N_2 after harvesting and used for RNA isolation.

Identification of miRNAs

QTLs for Fe concentration in grain were mapped in our previous study (Anuradha et al. 2012). Here, we identified the putative target genes and corresponding miRNAs present within the QTLs (Table 1) mapped on chromosome 1, 5, 7 and 12. Most of the targets were identified on chromosome 7. The corresponding miRNAs of the identified target genes were retrieved from miRbase (http://www.mirbase.org/). In addition to this, 3 miRNAs (miRs169, 395 and 171) reported to be responsive to Fe and other nutrient deficiency were also analysed.

RNA/Small RNA isolation and cDNA synthesis

RNA and small RNAs were isolated from frozen samples (~200 mg) of leaves and roots of 17 days old seedlings, using the mirVana miRNA isolation kit (Ambion) according to the manufacturer's instructions. cDNA synthesis of small RNAs was performed using the miScript reverse transcription kit (Qiagen) as per the manufacturers' instructions. cDNA of mRNA was synthesized from 2 μg of total RNA in a 25-μl reaction mixture with M-MLV reverse transcriptase (Promega, Madison, WI, USA).

Table 1 List of identified miRNAs and their targets which underlie the QTLs mapped for Fe concentration (Anuradha et al. 2012)

S.No	QTL	Regulator/Target	miRNA
1	qFe1.1	Squamosa promoter binding protein-like (SPL TF)	miR156
2	qFe1.1	Squamosa promoter binding protein-like (SPL TF)	miR156
		Auxin responsive factor (ARF)	miR167
3	qFe7.1	Copper/zinc-superoxide dismutase (Cu/ZnSOD)	miR398
		AP2 domain transcription factor (AP2 TF)	miR172
4	qFe7.2	ARGONAUTE9 protein (AGO)	miR168
		Leaf development protein Argonaute (AGO)	miR168
		Squamosa-promoter binding protein (SPL TF)	miR156
		Copper/zinc-superoxide dismutase (Cu/ZnSOD)	miR398
		AP2 domain transcription factor (AP2 TF)	miR172
5	qFe12.1	AP2 domain transcription factor (AP2 TF)	miR172
		Auxin responsive factor (ARF)	miR167
6	qFe12.2	Auxin responsive factor (ARF)	miR167

Quantitative real-time PCR (RT-qPCR)

The first strand cDNA was used as a template for RT-qPCR to analyze abundance of mature miRNAs. Expression analysis of each miRNA was performed using RT-qPCR-based assay. Primers used for RT-qPCR of miRNAs and their putative targets are listed in Table 2 and Table 3, respectively. U6 was used as internal control for small RNA (Ding et al. 2011) and *OsActin1* (Lee et al. 2011) for miRNA targets. RT-qPCR was performed using double-stranded DNA binding dye Syber Green. miScript SYBR Green PCR Kit (Qiagen) and SYBR Premix Ex-Taq (Takara) were used to quantify miRNA and targets, respectively, in an ABI GeneAmp 7500 Sequence Detection System. Real-time PCR analysis of miRNAs was done using miRNA-specific forward primers (Table 2) and the miScript universal primer (reverse primer) provided in miScript SYBR Green PCR Kit. Each reaction was run in duplicate and the melting curves were constructed using Dissociation Curves Software (Applied Biosystems), to ensure that only a single specific product is amplified. Validation experiments were performed to test the efficiency of the target amplification and the efficiency of the reference amplification. Relative transcript levels for each sample were obtained using the 'comparative Ct method' (Applied Biosystems). The mean threshold cycle (Ct) value obtained after each reaction was normalized to the Ct value of reference gene whose expression was consistent across the conditions. Further $\Delta\Delta$CT values were calculated using the formulae $\Delta\Delta CT = \Delta CT$ of test sample $-\Delta CT$ control sample, and then fold difference was calculated from $2^{-\Delta\Delta Ct}$. Similarly, ΔCT standard deviation was calculated as given at www3.appliedbiosystems.com/…/generaldocuments/cms_042380.pdf. In case of downregulation of miRs, $\Delta\Delta CT$ calculation was positive. Here, if the test sample has value of 0.25, it suggests 1/4 the amount of target RNA as the calibrator.

Results

The identified RILs developed from the cross Madhukar x Swarna containing contrasting level of Fe concentration in seeds were utilized to study the differential expression of miRNAs. One RIL 185(M) with high grain Fe and Zn (123 ppm Fe and 102 ppm Zn) designated as HL, the other RIL 270(M) with low grain Fe

Table 2 List of primers used for microRNA expression using qRT-PCR

S.No	miRNA	Primer Sequence	Target known	References
1	miR156	TGACAGAAGAGAGTGAGC	SPL transcription factors	Xie et al. 2007; Ding et al. 2011
2	miR162	TCGATAAACCTCTGCATC	DCL1	Xie et al. 2003
3	miR167	TGAAGCTGCCAGCATGAT	Auxin responsive factors (ARFs)	Huang et al. 2009; Valdés-López et al. 2010; Zhou et al. 2012
4	miR168	TCGCTTGGTGCAGATCGG	ARGONAUTE protein (AGO)	Liu et al. 2008
5	miR169	CAGCCAAGGATGACTTGC	NFY/MtHAP2-1	Liu et al. 2008; Zhou et al. 2007; Li et al. 2008; Zhao et al. 2009
6	miR171	TGATTGAGCCGCGCCAAT	SCL transcription factors	Xie et al. 2007; Zhou et al. 2008; 2012
7	miR172	AGAATCTTGATGATGCTG	AP2 transcription factors	Valdés-López et al. 2010; Zhou et al. 2012
8	miR395	GTGAAGTGCTTGGGGGAA	APS/AST	Jones-Rhoades and Bartel 2004; Sunkar and Zhu 2004; Liu et al. 2008
9	miR398	TGTGTTCTCAGGTCACCC	Cu/ZnSOD, COX5b.1, CCS	Sunkar et al. 2006; Zhou et al. 2008; Valdés-López et al. 2010
10	U6	CGATAAAATTGGAACGATACAGA	**Internal control**	Ding et al. 2011

and Zn (2 ppm Fe and 22 ppm Zn) concentration designated as LL and Swarna (22.5 ppm Fe and 27.2 ppm Zn) were used. The root and shoot tissue of these genotypes were taken to study the expression of miRNAs in +Fe and –Fe growth medium.

Differential expression of miRNAs in rice genotypes in response to Fe deficiency

Out of the 9 miRNAs analysed in this study, 7 miRNAs showed down regulation in root and shoot of HL, the high iron line 185(M) under –Fe compared to +Fe (Fig. 1). These miRNAs were miR156, 162, 167, 168,

171, 172, and 398. In general, these miRNAs showed similar response in root and shoot except miR162 and 167 where root showed more down-regulation than shoot. The expression of miR169 and 395 was not observed in root while both were up-regulated in shoot. In particular, miR395 showed 5.19 fold up-regulation in shoot in response to Fe deficiency in HL.

The low iron line 270(M) showed considerable variation in expression of miRNAs in root and shoot under -Fe when compared with +Fe. Here, all the 9 miRNAs showed down-regulation in root tissue. The shoot showed up-regulation of miR162, 171, 172, 398 and down-regulation of miR167 and 168. Major

Table 3 List of primers of gene targets of microRNAs used for expression analysis using qRT-PCR

| S.No.	Target	Locus ID	Forward Primer	Reverse Primer
1	SPL TF (SPL4)	LOC_Os02g07780.1	TTGATGGAGCACCGGATCTTC	TGTGAATTGGATGACGCGAGA
2	DCL1	LOC_Os03g02970.1	GCGAGGAATGCACTTGTGGTC	TGCCTGGTGAACATCTGAGCA
3	*OsARF12*	LOC_Os04g57610	AGGAGTTGCTGGTGAGGGAGGT	TGGATGGAGCCTCTGTTGTCCC
4	AGO1	Wu et al. 2009	CTGTTATCTAAGACGGAACGACCT	TGATTTGAGTAGCCACACTGAAAG
5	NF-YA (NF-YA3)	LOC_Os12g42400.3	TTCGTTCTATGGTGGTGCTGT	TTCGGTGGCTGGTTCTATTGG
6	SCL TF	LOC_Os02g44360.1	TGTCCTCCTCCTCTTGCCATC	GCTGCTGCGGTTGTAGAGCA
7	AP2 TF	LOC_Os05g03040.2	TTCTCCAAAGCCGCCACGACA	TCAATGGTGGTGGTGATGGCGG
8	APS	LOC_Os03g53230.1	ATTCATCCGCAACCACTGC	TCCTCCACCACCACCACTACCT
9	Cu/ZnSOD	LOC_Os07g46990.1	CCTTCTGGAGTCTTCCTCATCA	GCAAGCACAGCAACAGCCTTC
10	*OsACT1*	Lee et al. 2011	TGGAAGCTGCGGGTATCCAT	TACTCAGCCTTGGCAATCCACA
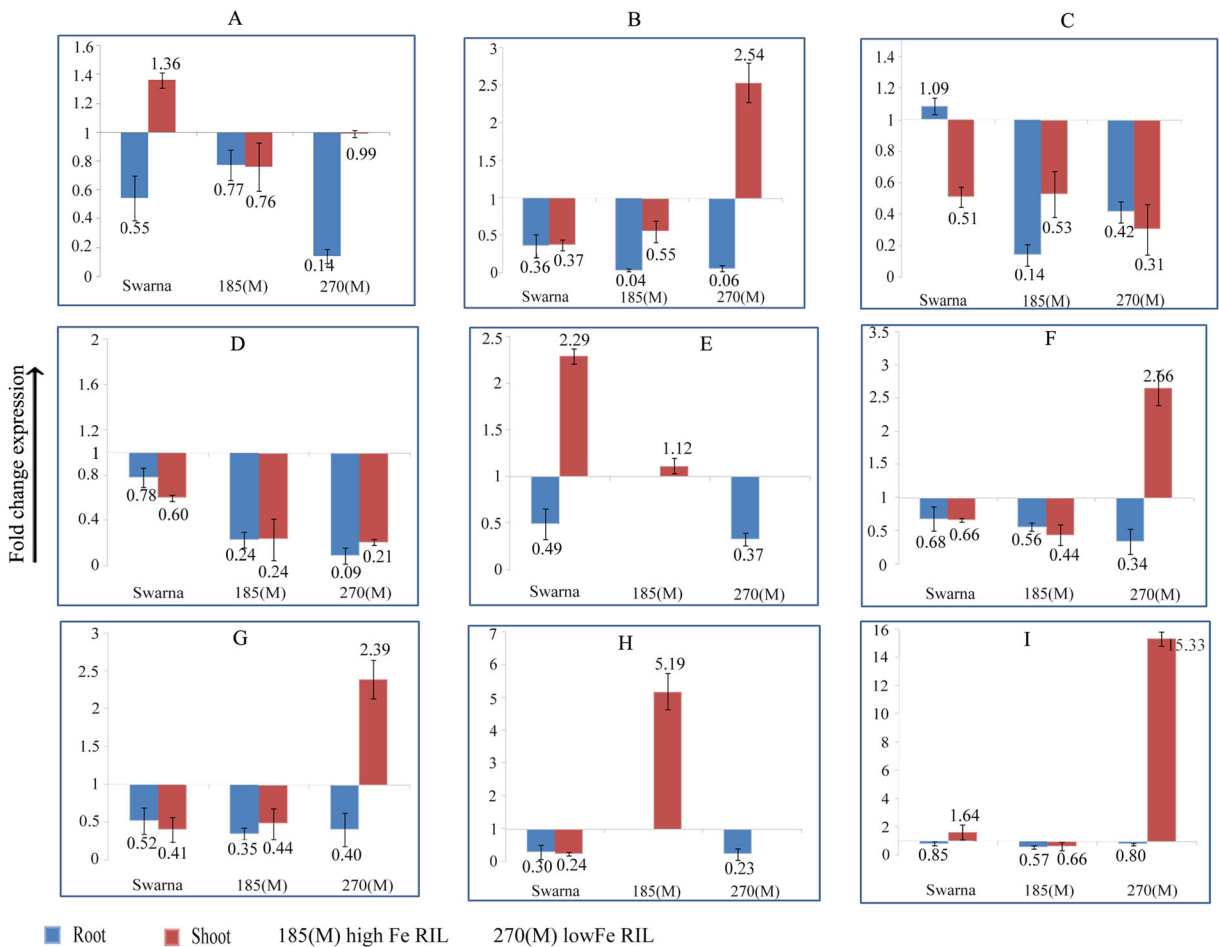
Fig. 1 Fold change expression of microRNAs in –Fe in comparison to+Fe growth medium in root and shoot of 17 days old rice seedling. Seven days old rice seedlings of high iron line (HL), low iron line (LL) and Swarna were transferred to –Fe and+Fe nutrient solution for 10 days. For+Fe treatment, Fe-EDTA was present in the nutrient solution and for –Fe treatment, Fe-EDTA was not added. Seedlings were grown in hydroponics in a growth chamber at 30 °C/22 °C (day/night) temperatures with a 12-h-light/12-h-dark regime. Small RNA was extracted from root and shoot tissue of 17 days old rice seedlings grown in –Fe and+Fe medium. Real time PCR expression analysis in recombinant inbred lines (HL, LL) and Swarna rice genotypes was carried out using U6 internal control as reference gene. Fold difference was calculated from $2^{-\Delta\Delta Ct}$ method. Note: Y axis shows the fold expression and the scale is different in each graph. A- miR156; B- miR162; C-miR167; D- miR168; E- miR169; F- miR171; G- miR172; H-miR395; I- miR398

increase in expression of miR398 (15.3 fold) was observed in shoot. Unlike in HL, miR169 and 395 were down-regulated in root and did not express in shoot of LL. While analysing the expression of these miRNAs in parent Swarna, all miRNAs except miR167 showed down-regulation in root under -Fe. The shoot showed down-regulation of 6 miRNAs (miR 162, 167, 168, 171, 172 and 395) and up-regulation of miR156, 169, and 398. Interestingly, −Fe root showed down-regulation of most of the miRNAs in all rice genotypes i.e., HL, LL and Swarna when compared with+Fe.

Differential response of miRNAs in HL, LL compared to Swarna

9 miRNAs were used for expression analysis to demonstrate their expression change in various comparisons viz. –Fe vs+Fe; high iron line vs low iron line; RILs vs Swarna, to understand miRNAs expression profile in genotype and nutrient specific manner. This is important to understand the differential expression of miRNAs in Fe treatments and treatment×genotype interactions.

Swarna vs HL

To investigate the differential expression of miRNAs, HL was compared with Swarna (Supplementary Fig. 1). Under+Fe condition, expression of miR156, miR162, miR167 and miR168 was up-regulated in root and down-regulated in shoot of HL in comparison to Swarna. The miR171, miR172 and miR398 showed up-regulation in both root and shoot. Expression of miR169 and miR395 was not detected in root. Under – Fe condition, expression of miR156, miR162, miR167, miR168 and miR169 was up-regulated in root but down-regulated in shoot of HL when compared with Swarna. Expression of miR171, miR172, miR395 and miR398 was up-regulated in both root (1 to 9.5 fold) and shoot (1 to 3 fold). In comparison to Swarna, HL showed up-regulation of all the miRNAs in root under -Fe. In shoot of HL, down-regulation of most miRNAs was observed except miR171, miR172, miR395 and miR398 under -Fe.

Swarna vs LL

Differential expression of miRNAs in LL as compared to Swarna was analysed under+Fe and -Fe condition, (Supplementary Fig. 1). Under+Fe condition, expression of miR156, miR162, miR167, miR168, miR169, miR171, miR172 and miR395 was 1.9 to 122 fold up-regulated in root while expression of these miRNAs was down-regulated in shoot of LL compared to Swarna. Only miR398 showed up-regulation in both root (17.7 fold) and shoot (1.1 fold). Under –Fe condition, expression of miR162, miR171, miR172 and miR398 was up-regulated by 1.7 to 24 fold in root and 1.7 to 10.6 fold in shoot of LL compared to Swarna. However, the expression of miR156, miR167 and miR168 was down-regulated both in root and shoot. miR169 and miR395 showed 1.3 fold and 6.1 fold increased expression in root but no expression in shoot of LL. In root and shoot, miR171, miR172, miR395 and miR398 showed considerably higher expression in LL compared to Swarna under -Fe.

LL vs HL

miRNA expression in LL seedlings was compared with that in HL seedlings in both+Fe and –Fe to test if it was affected by level of seed Fe concentration (Supplementary Fig. 2). Under+Fe condition,

expression of miR162, miR167, miR169 and miR398 was down-regulated in root and shoot of LL in comparison to HL. In+Fe, the most prominent difference was the 12-fold upregulation of miR395 in shoot of LL compared to HL. The next was 5-fold up-regulation of miR172 and about 2-fold up-regulation of miR156 and 168 in root of LL compared to HL. The change in expression of other 5 miRs was not considerable. In –Fe, miR172 was upregulated ~14-fold in LL root compared to HL root. miR395 was upregulated 4-fold in LL root. miR162, 172 and 398 were upregulated 2 to 3 fold in shoot of LL compared to shoot of HL. The expression of other 5 miRNAs was about the same as HL in root/shoot. It is important to note that miR172 expression in –Fe in LL root (14 fold) compared to HL was about 3 times more than its expression in+Fe in LL (5 fold) root compared to HL.

Expression analysis of miRNA targets under Fe deficiency

To investigate whether the expression of putative target genes of Fe-responsive miRNAs was also influenced by Fe deficiency, expression pattern of 9 target genes corresponding to 9 miRNAs was studied using RT-PCR in root and shoot (Fig. 2). Under –Fe, HL showed increased expression of most of the targets in root and shoot tissues when compared with+Fe. SCL transcription factor (target of miR171) showed down-regulation in shoot while fold change expression of DCL1 (target of miR162), AP2 TF (target of miR172) and Cu/Zn-SOD (target of miR398) was not major in root of HL. Swarna showed up-regulation of all target genes in root and down-regulation in shoot under Fe deficiency. LL showed varied expression in root and shoot tissues. In LL, Cu/Zn-SOD (target of miR398), SCL transcription factor (target of miR171) and Auxin responsive factor (target of miR167) showed up-regulation in root and down-regulation in shoot in –Fe when compared with+Fe. The Argonaute (target of miR168) and nuclear transcription factor-Y (target of miR169) showed up-regulation in shoot and root respectively. SPL transcription factors (target of miR156) and AP2 TF (target of miR172) showed up-regulation in shoot and root while DCL1 (target of miR162) and ATP sulfurylases (target of miR395) did not show considerable change in expression. Interestingly, SCL transcription factor (target of miR171) expression was high in root and low in shoot of all genotypes tested here under –Fe in comparison to+Fe.
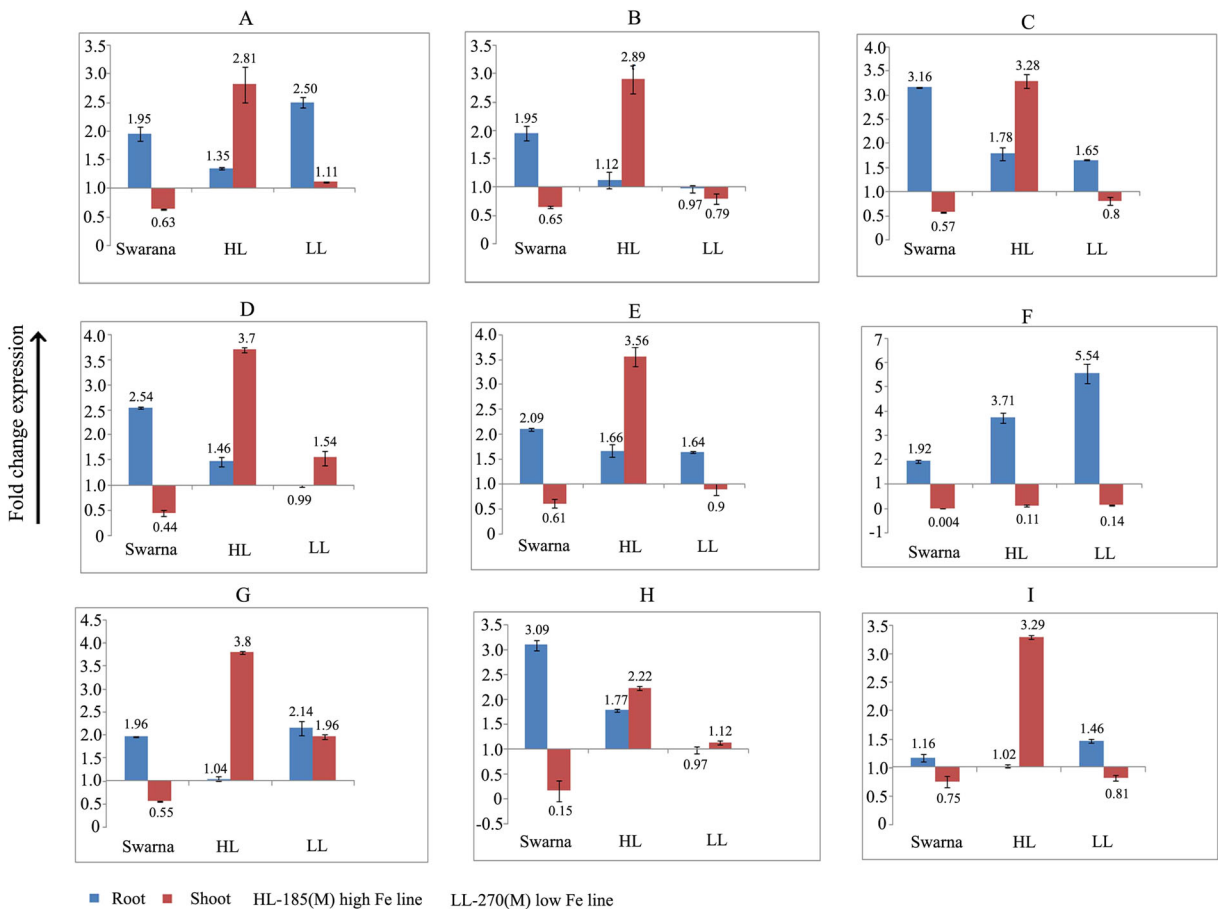
Fig. 2 Fold change expression of microRNA targets in –Fe in comparison to+Fe growth medium in root and shoot of 17 days old rice seedling. Seven days old rice seedlings of high iron line (HL), low iron line (LL) and Swarna were transferred to –Fe and+ Fe nutrient solution for 10 days. For+Fe treatment, Fe-EDTA was present in the nutrient solution and for –Fe treatment, Fe-EDTA was not added. Seedlings were grown in hydroponics in a growth chamber at 30 °C/22 °C (day/night) temperatures with a 12-h-light/12-h-dark regime. RNA was extracted from root and shoot tissue of 17 days old rice seedlings grown in –Fe and+Fe medium. Real time PCR expression analysis in recombinant inbred lines (HL, LL) and Swarna rice genotypes was carried out using *OsAct1* internal control as reference gene. Fold difference was calculated from $2^{-\Delta\Delta Ct}$ method. Y axis shows the fold expression and the scale is different in each graph. A- SPL TF; B-DCL1; C-*OsARF12*; D- AGO1; E- NFYA; F- SCL TF; G- AP2 TF; H-APS; I- Cu/ZnSOD

Correlation between miRNAs and target genes expression

To analyze the expression correlation between miRNAs and their target genes under Fe deficiency, the relative expression values of genes and miRNAs were plotted together for all three genotypes (Fig. 3). Here, root tissue of all the genotypes showed negative correlation between expression of miRNAs and their target genes. As expected, target genes of all miRNAs showed increased expression while corresponding miRNAs showed decreased expression in root. Shoot of HL and Swarna also showed expected negative correlation of most of the miRNAs and targets expression, while LL showed varied correlation.

Discussion

Understanding the molecular basis of Fe homeostasis in plants is essential to elucidate the mechanism of Fe acquisition and metabolism. In spite of several reports demonstrating important role of miRNAs in metal homeostasis, it is still not known if miRNAs are involved in the regulation of Fe metabolism in rice. In this study,
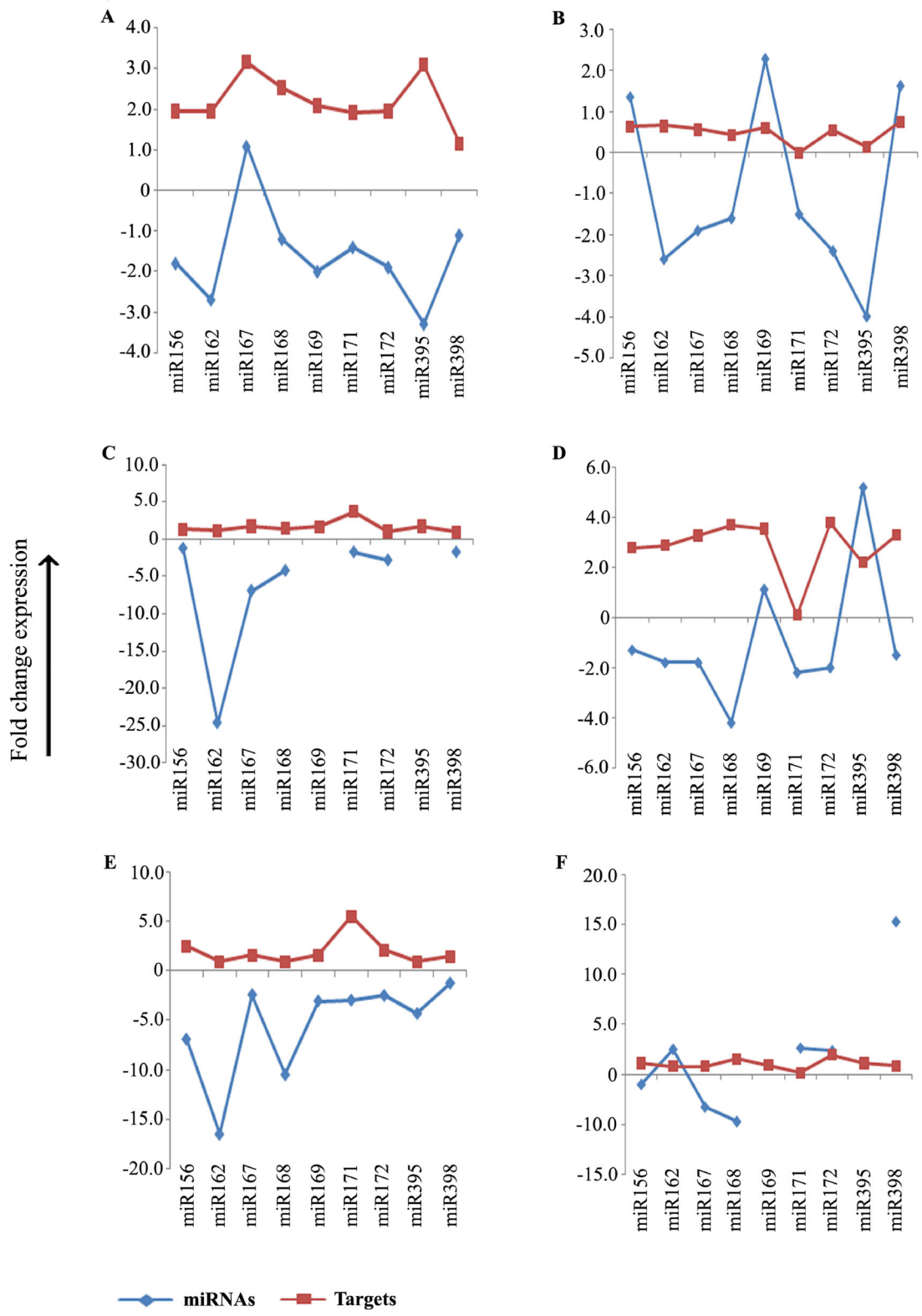
◀ **Fig. 3** Expression correlation of miRNAs and their target genes in roots and shoots of Swarna (**a**, **b**), HL (**c**, **d**), and LL (**e**, **f**) under Fe deficiency. Opposite expression pattern for almost all of the miRNAs and corresponding genes was observed in roots (**a,c,e**) except for miR167 in Swarna. Expression pattern of miRNAs and their corresponding genes varied in shoots (**b**, **d**, **f**) of all lines. Y axis was set to zero to show the fold up or down expression and the scale is different in each graph

we identified miRNAs for target genes that underlie the QTLs for Fe and Zn concentration mapped previously (Anuradha et al. 2012). The expression analysis of few candidate genes present within the mapped QTLs was reported recently in HL, LL and Swarna (Agarwal et al. 2014). 15 genes were profiled which included: 4 genes from the ZIP family of divalent metal transporters, 3 genes of the YSL family of metal phytosiderophore transporters, 3 genes from the phytosiderophore biosynthetic enzyme family, 1 gene from the NRAMP family of metal transporters, 2 genes from iron regulated transporters and 2 related to metal homeostasis. Out of these 15 genes, 8 genes (*OsYSL1*, *OsNAS1*, *OsNAS2*, *OsNAS3*, *OsIRT1*, *OsZIP6*, *OsZIP8*, *OsNRAMP1*) underlie or were close to mapped QTL while 7 genes (*OsDMAS1*, *OsNAAT*, *OsIRT2*, *OsYSL2*, *OsYSL15*, *OsZIP1*, *OsZIP4*) were based on previous reports of Fe homeostasis associated genes. The study showed differential expression of genes in root and shoot tissue of high and low iron line. HL displayed exceptionally high upregulation of *OsNAS2* and *OsYSL2* in roots and *OsIRT2*, *OsDMAS1* and *OsYSL15* in shoots when exposed to Fe deficiency. These are important components involved in acquiring Fe^{2+} and Fe^{3+} from soil, and in long distance transport in shoots and loading Fe in seeds (Ishimaru et al. 2006, 2010; Inoue et al. 2009; Bashir et al. 2006). Four QTL associated genes- *OsZIP8*, *OsNAS3*, *OsYSL1* and *OsNRAMP1* also showed distinct allelic differences between HL and LL for markers flanking Fe QTLs. The study suggested that HL is more efficient in regulating its molecular machinery to maintain Fe homeostasis (Agarwal et al. 2014) which is further supported in this study by using miRNA expression profile. In the present study, target genes of miR156 (SPL TF), miR167 (ARF), miR398 (Cu/Zn SODs), miR172 (AP2 TF), and miR168 (AGO) were identified within the QTLs for Fe and Zn concentration (Table 1). Further, expression of miR169, 395 and 171 was also performed as they were reported to be Fe/nutrients responsive in *Arabidopsis* (Kong and Yang 2010). miR162 involved in DCL1 regulation was also included

to understand the miRNAs regulation in Fe homeostasis in rice. DCL1 is involved in biogenesis of miRNAs (Vazquez et al. 2004). Real Time PCR based expression analysis of these miRNAs and their target genes was analysed in root and shoot of two RILs and Swarna cultivar under+Fe and -Fe conditions. Quantitative PCR analysis has been utilized to study the nutrients responsive miRNAs in plants (Ding et al. 2011; Kong and Yang 2010).

To investigate the involvement of miRNA mediated pathways in Fe homeostasis in rice, miRNAs expression was obtained by measuring mature miR162 and miR168 levels in HL, LL and Swarna seedlings (root and shoot) under Fe sufficient and deficient condition. DCL1 and Argonaute (AGO) proteins, which are indispensable factors of miRNA biogenesis and functioning, were predicted to be targeted by miR162 and miR168, respectively (Vazquez et al. 2004). Expression of miR162 and miR168 was down-regulated in Fe deficient root and shoot compared to Fe sufficient root and shoot in all samples irrespective of grain Fe and Zn concentration. This suggests that AGO and DCL1 expression is regulated under Fe deficiency which in turn gives an ample scope to hypothesize the role of miRNAs in Fe homeostasis in rice. Since miRNAs expression in plants is governed by these two miRNAs through regulation of DCL1 and AGO expression which are key proteins involved in biogenesis of miRNAs, this led us to study the expression of other miRNAs.

Reactive oxygen species (ROS) are reactive radicals produced in aerobic cellular processes and more so under any stress and can cause severe damage to cells. Superoxide dismutases (SOD) are important enzymes involved in detoxifying superoxide radicals (O_2^-), catalyzing their dismutation to hydrogen peroxide and O_2. In copper (Cu) deficiency, expression of Cu/Zn SOD protein is down-regulated and it is replaced by Fe-SOD. This is regulated by miR398 in *A. thaliana*, by targeting the degradation of Cu/Zn-SOD transcript when Cu is limited (Yamasaki et al. 2007). In Swarna and LL, miR398 was down-regulated in root while up-regulated in shoot under -Fe compared to+Fe. As expected, its target gene Cu/Zn SOD was up-regulated in root and down regulated in shoot. Under –Fe condition, HL showed decreased expression of miR398 in both root and shoot. The down-regulation of miR398 under –Fe in this study suggests the increased expression of Cu/Zn SOD, a major copper protein in the cytoplasm of plant cells. Real time PCR quantification also showed

increased expression of Cu/Zn-SOD in tissues where miR398 was down-regulated. Earlier studies have demonstrated that Fe-deficient plants accumulate additional Cu in monocots and dicots (Waters and Troupe 2012; Chaignon et al. 2002; Welch et al. 1993), suggesting that down-regulation of miR398 and up-regulation of Cu/Zn-SOD under Fe deficiency in rice may be an important strategy to maintain Fe-Cu homeostasis. Further, HL showed better coordination in expression of miR398 and its target in root and shoot as compared to LL and Swarna, suggesting HL has more efficient mechanism to maintain Fe-Cu homeostasis. miR398 down-regulated by Fe deficiency, suggesting that it could be involved in plant adaptation to Fe limitation (Waters et al. 2012).

Members of the miR169 family are reported to be regulated by high salinity, ABA-mediated responses, and cold stress in *Arabidopsis* (Liu et al. 2008, 2009; Zhou et al. 2009; Sunkar and Zhu 2004). Analysis of the expression of miR169 precursor showed that miR169a was substantially down-regulated in both roots and shoots by N starvation (Zhao et al. 2011). Down-regulation of miR169 is likely to be involved in relief of oxidative stress which accompanies nutrient deficiency and other stresses. Its target nuclear transcription factor-Y was induced by nitrogen (N) starvation and also activates several drought-responsive genes (Zhao et al. 2011; Li et al. 2008; Sunkar et al. 2006). Down regulation of miR169 under –Fe in roots of LL and Swarna but no expression in HL roots indicates that miR169 is regulated by Fe deficiency especially in roots. Its target showed higher expression in root of all lines and shoots of HL in Fe deficient condition. Thus, we show that miR169 is differentially regulated in root and shoot tissue under Fe deficiency which was not previously reported in rice.

MicroRNA395 is a regulator of a low-affinity sulfate transporter and ATP sulfurylases which are key proteins involved in sulfur homeostasis (Liang et al. 2010). Fe is found in the center of Fe-S clusters which act as electron acceptors and donors in several cellular processes including photosynthesis, respiration, sulfate assimilation and ethylene biosynthesis (Lim et al. 2005). In this study, miR395 was down-regulated in root of Swarna and LL and up-regulated in shoot of HL under Fe deficiency. The expression of miR395 is previously reported to be down-regulated under Fe deficiency in *Arabidopsis* (Kong and Yang 2010). The down-regulation of miR395 during Fe deficiency is associated with up-regulation of its target genes which may result

in an increase in sulfate assimilation and translocation (Hsieh et al. 2009). This study suggests the differential expression of miR395 in HL and LL under Fe deficiency.

In Arabidopsis, miR172 and its target AP2 (APETALA2-related) TF play a major role in regulating stem cell fate in the floral meristem, organ identity, flowering time and photoperiod (Zhu and Helliwell 2010; Jung et al. 2007; Zhao et al. 2007; Chen 2004; Aukerman and Sakai 2003). Common bean AP2 TFs respond to phosphorus starvation in roots and nodules (Hernandez et al. 2007, 2009). In rice, five AP2-like genes (*SNB*, *OsIDS1*, *SHAT1*, *Os05g03040*, and *Os06g43220*) contain miR172 target sites (Zhu et al. 2009; Sunkar et al. 2005). We show that one of these AP2-like genes *Os05g03040* - which is close to *qFe5.1* is regulated under Fe deficiency by miR172 . Here, down-regulated expression of miR172 was detected in roots and shoots of all rice lines except LL shoot, while its target *Os05g03040.2* showed up-regulation in roots and shoots of all lines except shoots of Swarna under Fe deficiency compared to Fe sufficient condition, suggesting iron as an important element of key developmental processes by regulating one AP2 TF. It is interesting to note that AP2 was major transcription factor responding to iron stress in soybean roots and leaves (Lauter et al. 2014). Further, expression comparison of miR172 in LL and HL showed that LL root had 14 fold higher expression than HL root under –Fe, suggesting that miR172 is an important determinant in differential response of HL and LL under Fe deficiency.

miR167 targets ARF (auxin response factor) to control auxin signalling and regulates lateral root or root hair development, a typical symptom of Fe deficiency (Zhou et al. 2012). Here, miR167 was down-regulated in root and shoot of HL and LL under Fe deficiency and expression analysis of *OsARF12* showed increased expression in root of all lines. *OsARF12* regulates root elongation and affects iron accumulation in rice and its expression is regulated by osa-miRNA167d (Qi et al. 2012). Likewise, miR156 showed down regulation in root and shoot of all lines except Swarna shoot under Fe deficiency. As expected, its target squamosa promoter binding protein like transcription factor (SPL TF) showed increased expression in root and shoot of all lines except shoot of Swarna. miR156 was reported to be down-regulated by cadmium (Cd) stress in *Brassica napus* and rice (Ding et al. 2011; Xie et al. 2007). In another study, miR156a was shown to be up-regulated

in root of *B. napus* under sulfate deficiency (Huang et al. 2010). The differential regulation of miR156 under different metal stresses suggests that this miRNA may have important role in iron homeostasis in rice. We thus provide evidence for the involvement of these two miRNAs - miR156 and miR167 in response to Fe deficiency in rice which has not been reported previously.

Cross talk of miRNAs under Fe deficiency

After analyzing the expression of miRNAs and their targets, a hypothetical model is proposed on cross talk of these miRNAs and genes regulated under Fe deficiency in rice roots (Fig. 4). Under Fe deficiency, miR156 gets down-regulated in roots of all lines and shoot of HL. Shoot of Swarna showed increased expression of miR156 while LL shoot showed minor down-regulation. Correspondingly, increased or decreased expression of its target SPL TF was observed in root and shoot of HL, LL, and Swarna. In a recent study, reduced growth of the *spl7* mutant of *Arabidopsis* in Cu deficient medium was partially rescued by Fe supplementation. A defect in root-to-shoot Fe movement in Cu-deficient *spl7* mutant plants is associated with a loss of ferroxidase activity, suggesting the involvement of multicopper oxidases in Fe homeostasis of vascular plants (Bernal et al. 2012). Therefore, miR156 might be regulating Fe homeostasis in rice through SPL mediated regulation of copper which seems to be more efficient in HL in comparison to LL and Swarna, as evident from expression pattern of miR156 and SPL in root and shoot. The increase in Cu is needed to replace the Fe as cofactor

of SOD under Fe deficiency. As shown in the model, miR398 gets down regulated and its target Cu/ZnSOD is up-regulated in rice roots. It suggests that miR156 and 398 work in coordination under Fe deficiency. Fe–Cu cross-talk has been demonstrated in *Arabidopsis* where FeSODs are down-regulated and the proteins are functionally replaced by Cu/ZnSODs by increased expression of CSD1 and CSD2 under Fe deficiency (Waters et al. 2012).

The down-regulation of miR167 under Fe deficiency caused the up-regulation of its target auxin response factor (*ARF12*) in rice roots. The relationship between auxin response and Fe acquisition in rice has been shown earlier. Mutants of *osarf12* gene showed lower expression of the two genes, *OsIRT1* and *OsIRT2*, related to Fe content and acquisition (Qi et al. 2012). In our previous study, *OsIRT1* and *OsIRT2* showed very high expression in roots of LL under Fe deficiency (Agarwal et al. 2014) suggesting that expression of miR167, *OsARF12* and, *OsIRT1* and *OsIRT2* are tightly linked and the two biological functions Fe uptake and auxin response are closely associated (Qi et al. 2012). Further, down-regulation of miR395 was noticed in rice roots under Fe deficiency. It is important to note that miR395 regulates *SULTR* genes which are involved in transport of sulphur, low-affinity sulfate transporters, and ATP sulfurylases (Forieri et al. 2013; Liang et al. 2010; Chiou 2007). Sulphur is important component of Fe-S cluster present in many proteins, therefore, metabolism of these two metals is closely associated. The biosynthesis of Fe-S clusters requires simultaneously reduced S and chelated Fe that co-ordinates the uptake and

Fig. 4 A gene regulatory model proposed for interaction of miRNAs and their putative targets under Fe deficiency in rice roots. The red colour text signifies down-regulation and green colour text indicates up-regulation
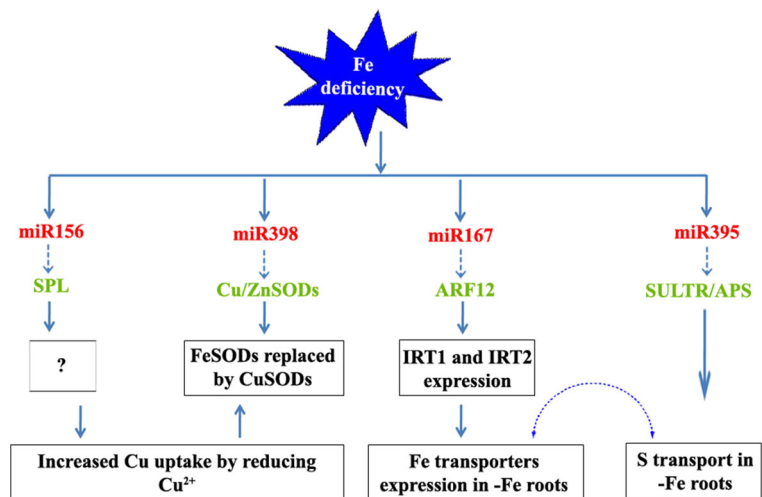
reduction of both nutrients (Vigani et al. 2013). Forieri et al. 2013 showed 70 fold increased expression of *SULTR* gene under Fe and S deficiency compared to the sufficiency of both nutrients in *Arabidopsis*. Fe shortage triggered expression of genes encoding high-affinity *SULTR* and several other genes of the S metabolic pathway in root of wheat (Ciaffi et al. 2013). In our study also, the link between sulphur and Fe metabolism is strengthened with the expression data of miR395 and its target in rice root. miR167 and 395 may work in co-ordination by regulating the transporters of Fe (*OsIRT1* and *OsIRT2*) and S (*SULTR*), respectively in rice roots. Overall, the cross talk of miRNAs and target genes suggests that regulation of miRNAs under Fe deficiency is closely linked with each other. The expression profile data of miRNAs also provides evidence of a link between regulation of Fe metabolism and other biological pathways such as auxin response and S metabolism.

In conclusion, down-regulation of miRNAs and consequent up-regulation of their putative targets observed in the present study under Fe deficiency indicates the involvement of miRNA mediated regulation of Fe homeostasis in rice. The largely negatively correlated expression patterns between miRNAs and their targets further validated the regulatory role of miRNAs under Fe deficiency in rice. In particular, down-regulation of most of miRNAs and corresponding up-regulation of their putative target genes in root tissue under –Fe in comparison to +Fe indicates that the roots are more responsive to Fe deficiency which is in agreement with our previous findings showing increased expression of Fe transporter genes in roots under -Fe (Agarwal et al. 2014). The observation that three prominent Fe deficiency down-regulated miRNAs, miR169, miR395, and miR398, are also reported as responsive to other nutritional deficiencies (e.g., nitrogen, sulfur and copper) suggests that these miRNAs may be involved in signal transduction pathways of several nutrients in general. The results also support the observation that miR156, miR171, miR169, and miR172 are involved in plant response to abiotic stresses, including iron deficiency stress. The cross talk of miRNAs and regulated targets helps in deciphering the molecular pathways associated with Fe metabolism and other biological pathways. It would be interesting to specifically evaluate the role of each of these miRNAs in Fe homeostasis in rice and other crops not only in seedlings but also during further development stages.

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