Transcriptomic Profiling of Fe-Responsive lncRNAs and Their Regulation in Rice

Shoudong Wang
Zhejiang University - Zijingang Campus: Zhejiang University
Shuo Sun
Zhejiang University - Zijingang Campus: Zhejiang University
Runze Guo
Zhejiang University - Zijingang Campus: Zhejiang University
Wenying Liao
Zhejiang University - Zijingang Campus: Zhejiang University
Huixia Shou (huixia@zju.edu.cn)
Zhejiang University - Zijingang Campus: Zhejiang University

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Abstract

Background Iron (Fe) plays a vital role in various cellular processes in plants, including biosynthesis of chlorophyll, photosynthesis and respiration. Fe deficiency directly affects crop growth and development, ultimately resulting in reduced crop yield and quality. Long non-coding RNAs (lncRNAs) have recently been demonstrated to play critical regulatory roles in a multitude of pathways across numerous species. However, systematic screening of lncRNAs responding to Fe deficiency in plants has not been reported.

Results In this work, lncRNAs responsive to Fe deficiency were identified across the rice genome by strand-specific RNA sequencing. In total, 6,477 lncRNAs were identified. In Fe-deficient conditions, 47 lncRNAs were up-regulated and 33 lncRNAs were down-regulated in shoots, while 89 lncRNAs were up-regulated and 32 lncRNAs were down-regulated in roots, compared to normal conditions. Two lncRNAs (XLOC_010112 and XLOC_053944) were identified as potential miRNA precursors and another two (XLOC_012715 and XLOC_054182) as miRNA target mimics that may participate in Fe regulation. A number of differentially expressed lncRNAs (DE-lncRNAs) are likely to modulate the expression of Fe-related genes via a *cis*- or *trans*-regulation mode, including 3 DE-lncRNAs (XLOC_034336, XLOC_037283 and XLOC_043545) located nearby *OsbHLH156* and *OsHRZ2* genomic regions. Seventy-six DE-lncRNAs were found to be regulated by bHLH156 at the transcriptional level.

Conclusions This study provides a first profile of lncRNA expression as well as identifies the lncRNAs likely to play important roles in the regulation of Fe homeostasis. This identification and characterization form an important basis for understanding Fe regulatory networks in rice.

Background

Iron (Fe) is an essential micronutrient for plants but is often limited due to low availability in the soil [1]. To overcome Fe deficiency, plants have evolved two strategies to optimize Fe acquisition and uptake, i.e., the reduction strategy (Strategy I) for non-gramineous plants and the chelation strategy (Strategy II) for gramineous plants [2, 3]. Rice, which is adapted to grow submerged in a paddy where the reduced form of Fe is available, utilizes both Fe uptake strategies I and II [4-6]. A large number of genes are known for their involvement in Fe uptake and homeostasis, including those that encode transcription factors for regulating expression of downstream Fe-responsive genes, enzymes for synthesis of phytosiderophores (MAs), and transporters of MA-Fe(III) or Fe(II) in rice [3, 4, 7-14]. However, regulation of Fe-responses by long noncoding RNAs (lncRNAs) has not been reported.

LncRNAs are transcripts of more than 200 nucleotides in length but without coding potential that have recently gained widespread attention [15]. LncRNAs play roles in numerous crucial biological processes across many species by regulating expression of mRNAs at epigenetic, transcriptional, post-transcriptional, translational and post-translational levels [15-17]. LncRNAs are classified as sense, antisense, intronic, and intergenic, according to their position in relation to neighboring coding genes [18, 19]. In plants, lncRNAs were reported to be involved in development and stress responses [20, 21]. For

instance, the lncRNAs COLDAIR (Cold-Assisted Intronic noncoding RNA) and COOLAIR (Cold-Induced Long Antisense Intragenic RNA) are both located in the *Flowering Locus C(FLC*) gene, which regulates flowering time. COLDAIR and COOLAIR regulate expression of *FLC* at the epigenetic level by interacting with an evolutionarily conserved repressive complex, PRC2 (Polycomb Repressive Complex 2) [22-24]. Another lncRNA, the 1,236-nucleotide long LDMAR (Long-day-specific male-fertility–associated RNA), regulates photoperiod-sensitive male sterility (PSMS) in rice [25]. A number of lncRNAs have been reported to regulate phosphate homeostasis. IPS1 (Induced by phosphate starvation 1) reduces phosphorus acquisition by inhibiting the activity of miR399, through the target mimicry mechanism [26]. The *cis*-natural antisense RNA (*cis*-NATPHO1;2), transcribed from *OsPHO1;2*, was found to be a translational enhancer of its sense gene (*OsPHO1;2*) [27]. In yeast, *prt* (*pho1*-repressing transcript), generated from the promoter region of the *pho1* gene, regulates expression of *pho1* responding to different phosphate levels [28]. These two studies show that a complicated network involving lncRNAs regulates phosphate homeostasis. In contrast, there are no reported lncRNAs responding to Fe deficiency. In this study, the transcriptome of rice was surveyed to systematically identify and characterize any lncRNAs that respond to Fe deficiency.

Results

Genome-wide Identification of LncRNAs

To systematically identify and characterize lncRNAs in rice, ssRNA sequencing (ssRNA-seq) was performed on shoot and root samples from rice seedlings grown in Fe-sufficient and -deficient conditions. After 10 days of Fe-deficient growth, rice plants showed significant chlorosis and lower chlorophyll content in the young leaves (Fig. 1a and 1b). The expression of typical Fe-deficiency responsive genes, such as the iron-related bHLH transcription factor 2 (*IRO2*), nicotianamine synthases 1 and 2 (*NAS1/2*), Fe(III)-DMA transporters (*YSL15/16*) and Iron-Regulated Transporter 1 (*IRT1*), were significantly increased (Fig. 1c), indicating that the rice seedlings were under iron deficiency at the sampling time.

The pipeline for lncRNA identification and characterization is shown in Figure 2a (see methods). Using this pipeline, approximately 700 million 150-bp, pair-end reads were assembled into 31,947 transcripts using Cufflinks. The Coding Potential Calculator (CPC) was used to evaluate the protein-coding potential of the transcripts to distinguish protein coding transcripts and lncRNAs. Transcripts more than 200 bp in length with CPC scores <0 were defined as lncRNAs, the remaining transcripts were classified as protein-coding transcripts (mRNAs). Using this method, 25,470 mRNAs and 6,477 lncRNAs were identified. Based on their relative position to protein-coding genes, lncRNAs can be classified into three types: Intergenic lncRNAs have no overlap with any protein-coding sequences, while sense lncRNAs and anti-sense lncRNAs overlap with one or more exons of another transcript on the same or opposite DNA strand, respectively [20]. Among the 6,477 lncRNAs identified in this work, 3,730 (58%) were intergenic lncRNAs, 1,696 (26%) were *cis*-lncRNAs, and 1,051 (16%) were antisense lncRNAs (Fig. 2b).

Fe-deficiency responsive LncRNAs and mRNAs in rice shoot and root

To identify the lncRNAs and mRNAs that are differentially expressed in response to Fe deficiency, the normalized expression levels (in fragments per kilobase of exon per million fragments mapped, FPKM) of the lncRNAs or the mRNAs were compared between the Fe-deficient and Fe-sufficient treatments. In shoots, 80 DE-lncRNAs were identified. Among them, 47 lncRNAs were up-regulated and 33 were down-regulated under Fe deficiency (Fig. 2c; Table S1a). In roots, 89 lncRNAs were up-regulated and 32 were down-regulated under Fe deficiency (Fig. 2c; Table S1b). In addition, 394 and 841 mRNAs were differentially expressed in either roots or shoots due to Fe deficiency, respectively. In shoots, 240 mRNAs were up-regulated and 154 were down-regulated (Fig. 2c; Table S1c), while in roots, 536 mRNAs were up-regulated and 305 mRNAs were down-regulated (Fig. 2c; Table S1d).

The DE-lncRNAs and -mRNAs were used to generate a heat map (Fig. 3). Classes I and III contained lncRNA and mRNA transcripts that were expressed significantly higher in Fe-sufficient than in Fe-deficient conditions in either roots (Class I) or shoots (Class III), respectively. In contrast, transcripts in Classes and had higher expression under Fe-deficient conditions in roots or shoots, respectively. Transcripts in Class were more highly expressed in both shoots and roots under Fe-deficient conditions. Among the five groups, Class (the transcripts induced in Fe-deficient roots) contained the largest number of both lncRNAs (Fig. 3a) and mRNAs (Fig. 3b). In total, 171 lncRNAs and 1,001 mRNAs were differentially expressed under the different Fe supply conditions (Fig. 3; Table S2).

Verification of LncRNAs responding to Fe deficiency using RT-qPCR

Quantitative real-time PCR (RT-qPCR) was performed to verify the accuracy of the RNA-seq data for the lncRNAs. Nine intergenic lncRNAs responding to Fe-deficiency were picked for the verification. The RT-qPCR results showed that lncRNAs XLOC_006153 and XLOC_028199 from Class IV were induced in shoots but not detected in roots regardless of the Fe supply status. LncRNAs XLOC_052823 and XLOC_007199 from Class II were up-regulated by Fe deficiency in the roots. The remaining 5 lncRNAs belonged to Class , which were induced upon Fe deficiency in both shoots and roots (Fig. 4). The strong correlation between the RNA-Seq and RT-qPCR result indicated the reliability of our transcriptomic profiling data.

Identification of LncRNAs as potential miRNA precursors and miRNA target mimics

MicroRNAs (miRNAs) regulate key aspects of development, cell signaling, and responses to various biotic and abiotic stresses via binding to specific complementary transcripts, including protein coding or non-coding sequences, resulting in the degradation or translational repression of the target. LncRNAs have been shown to function as precursors of miRNA in many studies [17, 29]. By aligning miRNA precursors to the 171 DE-lncRNAs, 2 of the lncRNAs, XLOC_010112 and XLOC_053944, were identified as potential miRNA precursors, namely of miR398a and miR164f, respectively (Fig. 5a). XLOC_010112 is located in a region that overlaps with a coding gene (LOC_Os10g18150) that so far seems to not be expressed (Fig. 5a). Under Fe deficiency, XLOC_010112 was down-regulated in shoot and up-regulated in root (Fig. 5b). XLOC_053944 is an intergenic lncRNA (Fig. 5a), specifically expressed in root and significantly induced by Fe starvation (Fig. 5b). Interestingly, miR398a and miR164f are both involved in regulation of Fe

homeostasis in *Arabidopsis* [30, 31]. The predicted target genes of miR398a and miR164f include LOC_Os06g23650, LOC_Os06g46270 (*ONAC11*; *OsY37*; *OMTN4*), LOC_Os12g41680 (*OMTN3*; *OsNAC60*), and LOC_Os07g11360 (*RAL3*) (Table 1). Among them, *OsNAC11* was significantly induced, while *OsNAC60* was suppressed in root under Fe deficiency (Fig. 5c). We speculated that XLOC_053944 is induced in rice root under Fe-deficiency, and consequently generates miR164f. The increased amount of miR164f would reduce the transcript abundance of *OsNAC11* and *OsNAC60*, which regulate Fe-related genes as transcription factors (Fig. 5d).

In addition to generating miRNAs, lncRNAs are also targets of miRNAs. In this case, lncRNAs function as target mimics of the sequestered transcript, known as an endogenous target mimic (eTM), to inhibit miRNA activity [26]. In order to further verify whether miRNA target mimicry is involved in Fe regulation in rice, the potential interactions between the Fe-responsive lncRNAs and known Fe-related microRNAs were investigated. Two endogenous target mimics (eTMs), eTM159 and eTM408, were identified. The lncRNAs XLOC_012715 (up-regulated in shoot and down-regulated in root under iron deficiency) and XLOC_054182 (only expressed in shoot, and slightly induced by Fe starvation), were predicted to bind miR159 and miR408, respectively (Fig. 5e-f). The potential target genes of miR159 / miR408 are listed in Table 1 and include the MYB transcription factors *OsGAMYB* and *OsGAMYBL1* and the calmodulin-like protein *OsCML27*. The results demonstrated that target mimicry might be a part of the regulation of Fe homeostasis.

Interactions of DE-LncRNAs with mRNAs

Recent studies have shown that lncRNAs regulate the expression of protein-coding genes in two ways, those that are encoded nearby the coding genes (*cis*-regulation) on the same chromosome and those that are encoded elsewhere (*trans*-regulation) [32]. The genomic locations of the DE-lncRNAs and DE-mRNAs were mapped to each chromosome of the rice genome. The results indicated that both DE-lncRNAs and DE-mRNAs were evenly distributed to each chromosome, other than two regions on chromosome 9 and 12 that showed higher degrees of clustering of DE-lncRNAs. It is interesting that there were also many Fe-related DE-mRNAs that mapped near the region on chromosome 9 (Fig. 6a). For *cis*-target analysis, 12 DE-mRNAs spaced less than 10 kb away from 15 DE-lncRNAs in shoot (Table S3a) and less than 10 kb away from 37 DE-lncRNAs in root (Table S3b). The coding genes nearby Fe-regulated/responsive lncRNAs included bHLH transcription factors, E3 ubiquitin ligases and tyrosine protein kinases. Interestingly, 3 lncRNAs were found to be located nearby *OsbHLH156* and *OsHRZ2* (*Oryza sativa* haemerythrin motif-containing really interesting new gene (RING)- and zinc-finger protein 2), which are two important regulators involved in Fe homeostasis in rice (Fig. 6b) [14, 33]. LncRNA XLOC_037283 and XLOC_034336 are located in or nearby *OsbHLH156.* XLOC_037283 is likely a natural antisense transcript (NAT) located near part of promoter and coding region of *OsbHLH156* in the opposite orientation, while XLOC_034336 is within 8 kbs upstream of the start codon of *OsbHLH156*. Both lncRNAs showed similar expression patterns to *OsbHLH156* under Fe deficiency (Fig. 6c). XLOC_043545 is within 8 kbs upstream of the start codon of *OsHRZ2* and was mainly expressed in rice root under iron deficiency, while *OsHRZ2* was induced in both the shoot and root (Fig. 6d). The results demonstrated that lncRNAs might play roles

in the Fe signaling pathway as *cis*-regulators and that they are likely involved in transcriptional or post-transcriptional regulation of *OsbHLH156* and *OsHRZ2*.

For *trans*-target analysis, 478 interaction nodes between DE-lncRNAs and DE-mRNAs in shoot and 1,516 in root were inferred according to the complementary pairing of bases (Table S4). GO enrichment analysis was performed to identify the potential functions of the *trans*-target genes. As shown in Figure 7a, we found 14 GO terms that were significantly enriched in root, but only 2 in shoot. Among them, “Response to iron ion”, “Metal ion transport”, “Iron ion transport” and “Iron ion homeostasis” were all associated with response to Fe-deficient stress. Interaction nodes among the DE-lncRNAs and Fe-related genes were built into interaction networks. There were 6 DE-lncRNAs that were predicted to interact with more than 5 Fe-related genes (Fig. 7b). The results implied that a complex regulation network between lncRNAs and mRNAs might contribute to Fe homeostasis regulation in rice.

DE-LncRNAs were transcriptionally regulated by the transcription factors *bHLH156* and *IRO2*

To test whether expression of DE-LncRNAs could be regulated by Fe-related transcription factors, an ssRNA-seq was performed on shoot and root samples of a knock-out mutant of *bHLH156* grown in Fe-deficient and -sufficient conditions. bHLH156 acts as a core transcription factor in regulating Fe homeostasis together with IRO2 [14]. The number of DE-lncRNAs in *bhlh156* shoot (145 up-regulated and 89 down-regulated) and *bhlh156* root (419 up-regulated and 177 down-regulated) was greater than that in WT under normal conditions (Fig. 8a). Under Fe deficiency, 495 lncRNAs were up-regulated and 168 were down-regulated in *bhlh156* root when compared with WT (Fig. 8a). The lncRNAs responding in an antagonistic manner in rice roots under Fe-deficiency condition are most likely regulated by bHLH156. In comparison to WT, Fe-deficiency-induced lncRNAs in shoots (14) and in roots (50) were suppressed in the *bhlh156* mutant. Conversely, lncRNAs down-regulated in response to Fe deficiency in shoots (3) and roots (12) of wild type showed significantly higher expression in *bhlh156* (Figure 8b and 8c). To verify that these lncRNAs were truly regulated by bHLH156, four (XLOC_011962, XLOC_018668, XLOC_043504 and XLOC_056321) were chosen for analysis by RT-qPCR in both the *bhlh156* and *iro2* mutants. IRO2 is a necessary interacting partner for bHLH156 to activate downstream genes [14]. XLOC_011962, XLOC_018668, XLOC_043504 and XLOC_056321 were all specifically expressed in root and dramatically induced under Fe-deficiency in WT, but were barely detectable in either the *bhlh156* or *iro2* mutants (Fig. 8d). The results demonstrated that a number of DE-LncRNAs could be regulated by bHLH156 and IRO2 at the transcriptional level.

Discussion

LncRNAs have roles in a wide range of biological processes, including development, stress responses, and plant nutrition. In this work, lncRNAs that respond to Fe deficiency in rice roots and shoots were identified. The results generated in this study promote our understanding of how rice plants respond to Fe deficiency.

LncRNAs arise from intergenic, intronic, or coding regions in the sense and antisense directions but at lower expression levels than mRNAs. Thus, identification of lncRNAs requires the use of an ssRNA-seq strategy. In this study, 6477 lncRNAs were identified and characterized (Fig. 2a). Differentially expressed lncRNAs and mRNAs were identified by comparing their expression levels between +Fe and –Fe conditions. The expression patterns divided the differentially expressed (DE) RNA molecules into five classes (Fig. 3). Class , the molecules up-regulated in rice root with Fe deficiency, had the greatest number of transcripts. Among the differentially expressed RNA molecules, the number of lncRNAs and mRNAs responding to Fe deficiency showed a similar trend, with more lncRNAs and mRNAs up-regulated under Fe deficiency in roots. Moreover, a greater number of lncRNAs and mRNAs were detected in roots in Fe-deficient condition.

MiRNAs are small RNAs that regulate target genes at both the transcriptional and post-transcriptional levels and that are generated by sequential cleavage of long precursor transcripts. Some lncRNAs could also act as primary transcripts of miRNAs [29]. In this study, two miRNA precursors were identified as generated from lncRNAs, of which XLOC_053944 might produce miR164f, which degrades *OsNAC* mRNA (Fig. 5a-d). The targeting of NAC by miR164 acts as a negative regulator of drought tolerance in rice [34]. In addition, NAC genes have been found to play important roles in Fe homeostasis [10]. Further investigation should be conducted to verify whether miR164-targeting of NAC also participates in Fe regulation, which might serve as a link between drought tolerance and response to Fe deficiency.

LncRNAs have been shown to regulate phosphate homeostasis in plants by a novel mechanism called target mimicry [26, 35]. Two endogenous lncRNA target mimics (eTMs) were identified in rice, namely eTM159 and eTM408, which target two Fe-related miRNAs, miR159 and miR408 (Fig. 5e) [31]. Therefore, a target mimicry mechanism similar to the IPS1-mi399 regulation of phosphate homeostasis might also exist in Fe regulation. We speculated that iron deficiency in rice would prompt XLOC_012715 and XLOC_054182 to target miR159 and miR408, thus preventing them from degrading their target coding genes, including a zinc finger protein, calmodulin-like protein 27, *OsGAMYB*, and *OsGAMYB* (Table 1). Most target genes identified were not Fe-related, except for *OsCML27* (LOC_Os03g21380) which was up-regulated in root. It is possible that this target mimicry might only happen in specific cell types, and any changes in expression levels of target genes could not be detected using whole shoot or root tissue samples.

LncRNAs have been shown to either regulate expression of adjacent genes via recruitment of regulatory complexes through RNA-protein interactions or to correlate with expression of neighboring genes through acting as local regulators [36, 37]. In order to study whether this mechanism is involved in Fe regulation, we compared the locations of DE-lncRNAs to the locations of 64 known Fe-related genes (up to 10 kb up- and down-stream) (Table S5). Three DE-lncRNAs were identified nearby *OsbHLH156* and *OsHRZ2* (Fig. 6b), which are two important regulators involved in Fe signaling. *OsbHLH156* regulates Strategy II iron acquisition as a core transcription factor [14], and *OsHRZ2* is a putative iron-binding sensor that negatively regulates iron acquisition under Fe sufficiency [33]. Both *OsbHLH156* and *OsHRZ2* were strongly induced by Fe deficiency, however the transcriptional and post-transcriptional regulation of these

two genes are largely unknown. Two of the DE-lncRNAs, XLOC_034336 and XLOC_043545, were located upstream of *OsbHLH156* and *OsHRZ2*, respectively. XLOC_037283 is a NAT that overlapped within the genomic sequence of *OsbHLH156* and showed a synchronous expression pattern with *OsbHLH156* (Fig. b-d). A series of case studies have shown that NATs can either positively or negatively regulate expression of a cognate loci [38]. For example, an *Arabidopsis* gene of unknown function named *SRO5,* which overlaps the *P5CDH* (Δ^1-pyrroline-5-carboxylate dehydrogenase) gene in the antisense orientation, generates both 24-nt and 21-nt siRNAs that regulate *P5CDH* at the post-transcriptional level. The *P5CDH-SRO5* gene pair defines a mode of siRNA function that may be applied to other *cis*-antisense gene pairs [39]. For example, the Flowering Locus C (FLC) contains multiple cold-induced long antisense intragenic RNAs (COOLAIR) that are transcribed in the antisense orientation in relation to FLC. The COOLAIR antisense lncRNAs have an early role in the epigenetic silencing of the FLC gene, acting to silence FLC transcription transiently [22]. In mammals, NATs can increase mRNA stability by forming a duplex with the sense gene, similar to what occurs at the BACE1 locus [40]. To determine if a similar regulation mechanism exists between XLOC_037283 and *OsbHLH156*, a more detailed analysis of the spatio-temporal expression of XLOC_037283 and *OsbHLH156* upon Fe deficiency and of the methylation level at the *OsbHLH156* locus should be conducted. These DE-lncRNAs will enrich the further study of *OsbHLH156* and *OsHRZ2* under iron deficient condition and our understanding of the total Fe regulation network in rice.

The DE-LncRNAs described above mainly function as upstream regulators of Fe-related genes, however the transcriptional regulation of these Fe-deficiency responsive lncRNAs remains unknown. To study whether DE-lncRNAs could also be regulated by Fe-related genes, an additional ssRNA-seq was performed using the *bhlh156* mutant. A total of 76 DE-lncRNAs (in shoot, root, or both) were identified whose expression might be activated or inhibited by the transcription factor bHLH156 (Fig. 8c), which, in conjucntion with IRO2, is required for induction of nearly all Strategy II iron acquisition genes in rice [14]. RT-PCR was performed to verify the expression of these DE-lncRNAs in the *bhlh156* and *iro2* mutants. The results indicate that bHLH156 and IRO2 might also regulate Fe homeostasis via activating downstream lncRNAs.

Conclusions

Our study provides insight into the potential functions and regulatory interactions of mRNA and lncRNA molecules when rice plants are grown in Fe-deficient conditions. We believe our study will serve as an initial reference for understanding the function of lncRNAs in regulating iron homeostasis in *Oryza sativa* and provides the genic identities needed to design the next wave of experiments aimed at understanding this additional layer of regulation.

Methods

Plant growth condition

Oryza sativa L. cv. Nipponbare (Nip) obtained from BIOGLE GeneTech (Hangzhou, China) was used in this study as the wild type (WT). The WT seeds were germinated in the dark for 3 days, and then placed on a net floating on a solution with or without iron (1.43 mM NH_4NO_3, 1.0 mM $CaCl_2$, 0.32 mM NaH_2PO_4, 1.64 mM $MgSO_4$, 0.51 mM K_2SO_4, 0.13 mM $CuSO_4$, 9.0 mM $MnCl_2$, 0.08 mM $(NH_4)_6Mo_7O_{24}$, 0.02 mM H_3BO_3, 0.15 mM $ZnSO_4$, 0.25mM Na_2SiO_3 and 0 or 125 mM EDTA-Fe( ), pH 5.5-5.6). The nutrient solution was exchanged every 3 days. Rice plants were grown in a growth chamber at 30 ℃ during the day (16 hours) and 22 ℃ (8 hours) at night.

Strand-specific RNA library construction and sequencing

Shoots and roots were separately collected from seedlings grown hydroponically for 10 days after germination with or without Fe, and immediately frozen in liquid nitrogen. Three biological replicates were used for each sample. Total RNA was extracted from these tissues using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Strand-specific RNA (ssRNA) library construction and RNA sequencing were performed by the Beijing Genomics Institute in Shenzhen (BGI, Shenzhen, China). To construct the ssRNA library, rRNA was removed with the Ribo-Zero Gold rRNA Removal Kit (Epicentre, Madison, WI, USA) from the pooled RNA. The RNA was fragmented into 200 - 500 nts in length using fragmentation buffer. After synthesis of first-strand and second-strand cDNA, adapters were added to both sides of the short fragments. The second strand was degraded by Uracil-N-Glycosylase. The resulting single strand cDNA was PCR amplified and then sequenced by Illumina HiSeq PE151. The RNA-seq data is available in the NCBI (Accession number: PRJNA527175).

Identification and characterization pipeline of LncRNAs

The raw data obtained by Illumina sequencing was filtered into clean data by removing adaptor sequences, low-quality reads and rRNA-containing reads with SOAPnuke and SOAP [41]. The dataset was aligned to the rice genome (Rice Genome Annotation Project) using the improved TopHat v 2.0 [42]. Cufflinks was used to reconstruct the transcripts. After filtering background noise transcripts, the final expression data was produced [43]. Transcripts shorter than 200 bp were discarded. For the remaining sequences, the transcript coding potential values were predicted by the Coding Potential Calculator (CPC) [44]. Each transcript with a CPC score <0 was considered a long non-coding RNA. mRNA transcripts (CPC scores >0) were also identified from the transcriptome in this work. Differentially expressed mRNAs or lncRNAs were identified using the R package NOISeq 2.31 (https://bioconductor.org/packages/release/bioc/html/NOISeq.html).

Validation of several LncRNAs using RT-qPCR

Tissues were collected from shoots and roots grown with or without iron for 10 days. Total RNA was isolated using TRIzol (Invitrogen). cDNA was synthesized from total RNA using a cDNA Synthesis Kit (TIANGEN), and RT-qPCR was performed on a LightCycler480 machine (Roche) with SYBR Green Supermix (CWBIO). ACTIN mRNA was used as the internal control for sample normalization. Means ± SD

were calculated by three biological repeats. The RT-qPCR primers (synthesized by TSINGKE) are shown in Table S8.

Prediction of the LncRNA-derived miRNAs and target genes

For miRNA precursor analysis, the miRNA sequences and their locations in the genome were acquired from PmiREN (http://www.pmiren.com/) [45]. An miRNA was defined as a lncRNA-derived miRNA if the pre-miRNA region in the genome was located within a lncRNA. The online software psRNATarget (http://plantgrn.noble.org/psRNATarget/) was used to predict target genes of miRNAs with a maximum expectation of 2.0 [46]. Less than two mismatches and G/U pairs were allowed within the mRNA and miRNA pairing regions.

Prediction and annotation of DE-LncRNA targets

The potential target genes of the differentially expressed lncRNAs (DE-lncRNA) were predicted based on the possibilities of *cis* and *trans* interaction nodes between the lncRNAs and mRNAs. For *cis*-target analysis, we searched the coding genes located within 10 kb upstream or downstream of the DE-lncRNAs. For *trans*-target analysis, interactions between DE-lncRNA and DE-mRNA were predicted thorugh complementary pairing of bases. The LncTar [47] tool was used for predicting target genes of the lncRNAs. The free energy and standard free energy of paired sites were calculated, and the target genes with standard free energy threshold <−0.1 were considered *trans*-target genes, while those <−0.2 were considered *cis*-target genes of lncRNAs. The online software agriGO (http://systemsbiology.cau.edu.cn/agriGOv2) was used to do the GO enrichment, and only those biological process terms with p <0.001 were considered as significantly enriched GO terms. The iron-deficiency, lncRNA-mRNA response networks were built using Cytoscape [48], which only contained the DE-lncRNAs and the *trans*-targets which had been reported as important Fe regulators.

Abbreviations

COLDAIR Cold-Assisted Intronic noncoding RNA

COOLAIR Cold-Induced Long Antisense Intragenic RNA

CPC Coding Potential Calculator

DE-lncRNA Differently Expressed LncRNA

eTM Endogenous Target Mimics

FLC *Flowering Locus C*

FPKM Fragments Per Kilobase of Exon Per Million Fragments Mapped

GO Gene Ontology

LncRNA Long Non-coding RNA

NCBI National Center for Biotechnology Information

PSMS Photoperiod-Sensitive Male Sterility

RT-qPCR Quantitative Real-Time PCR

SPAD Soil Plant Analysis Development

ssRNA Strand-Specific RNA

WT Wild Type

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All sequencing data generated inthis study has been submitted to NCBI database (Accession number: PRJNA527175).

Competing interests

The authors declare no conflict of interest.

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Author's Contributions

SW and HS conceived the project, designed the experiments, and wrote the manuscript. SW and SS carried out most experiments and all bioinformatics analysis. RG and WL performed RT-qPCR experiments. All authors have read and approved the manuscript.

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Tables

Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.

Supplementary Materials

Table S1. LncRNAs and mRNA significantly up- or down-regualted by Fe deficiency in shoot and root.

Table S2. All differently expressed lncRNAs and mRNAs.

Table S3. *Cis*-targets of DE-lncRNAs in shoot and root.

Table S4. Trans-targets of DE-lncRNAs in shoot and root.

Table S5. List of Fe-related genes.

Table S6. LncRNAs significantly up- or down-regualted by Fe deficiency.

Table S7. List of DE-lncRNAs in shoot used for constructing heatmap.

Table S8. Primers used in this study for validation of lncRNAs.

Figures

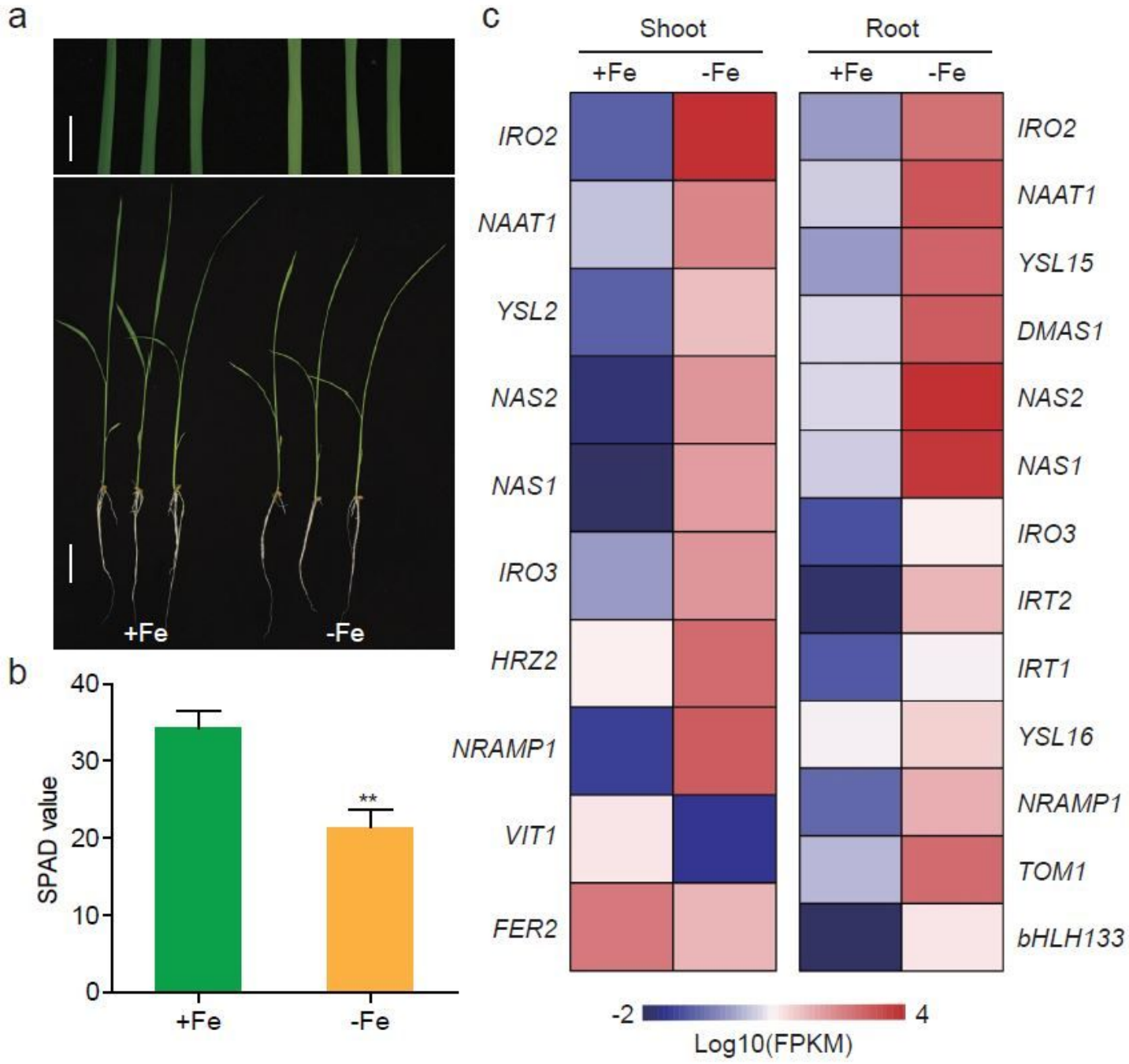

Figure 1

Physiological responses of rice seedlings to iron deficiency and expression pattern of Fe-related marker genes in RNA-seq. a Phenotypes of Nipponbare grown with 125 μM EDTA-Fe (II) (+Fe) or no Fe (-Fe) for 10 days. (Upper bar represents 1 cm, lower bar represents 3 cm). b SPAD (Soil Plant Analysis Development) values representing chlorophyll content of leaves. Data represent means ± SD, n = 4; **P <0.01, one-way ANOVA followed by Tukey test. c Heatmap of Fe-related marker genes in rice shoot and root under iron deficiency.

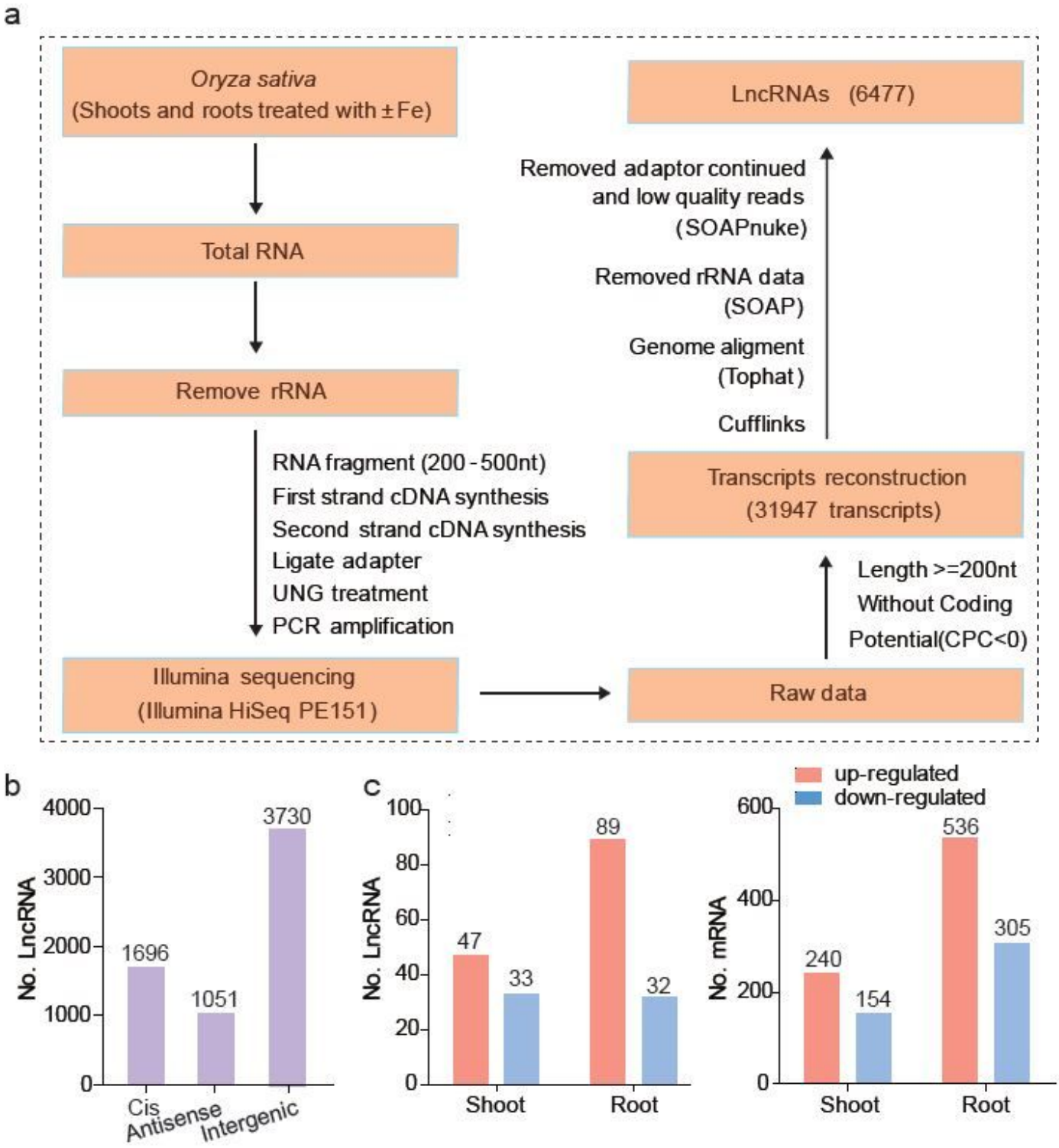

Figure 2

Identification and characterization of lncRNAs. a The pipeline for identification and characterization of known and novel lncRNAs responding to iron deficiency by strand-specific RNAseq (ssRNA-seq) (See method). b Location of lncRNAs relative to the nearest protein-coding genes. lncRNAs located on the antisense strand of coding transcripts were defined as antisense lncRNA. Other lncRNAs without overlap with coding transcripts were classified as intergenic lncRNAs, cis-lncRNAs were close (<=500 nt) and on

the sense strand with the adjacent genes. c Total number of differentially expressed lncRNAs and mRNAs. The number of up-regulated (Log 2 (fold change) >1; FPKM > 2; Probability > 0.8) and down-regulated (Log 2 (fold change) <−1; FPKM >2; Probability >0.8) lncRNAs and mRNAs in response to Fe deficiency in shoot and root.

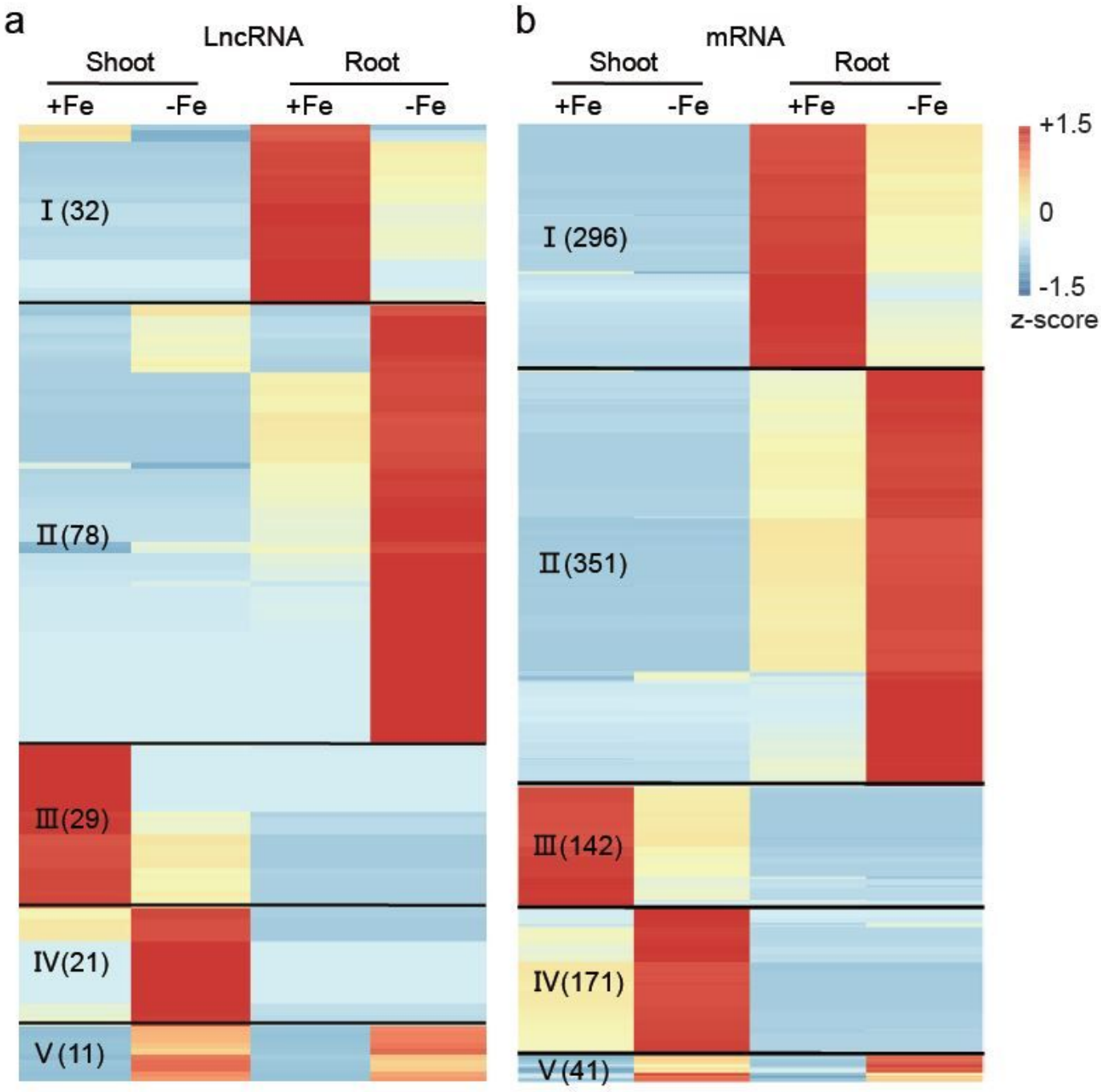

Figure 3

Heatmap of differentially expressed lncRNAs and mRNAs under ±Fe condition in both shoot and root. a-b The differentially expressed lncRNAs (a) and mRNAs (b) were divided into five classes based on their expression patterns. Class , highly expressed in root and down-regulated under Fe deficiency; , up-

regulated under Fe deficiency in root; , highly expressed in shoot and down-regulated under Fe deficiency; , up-regulated under Fe deficiency in shoot; , up-regulated in both shoot and root. The number of molecules in each class are listed in parentheses.

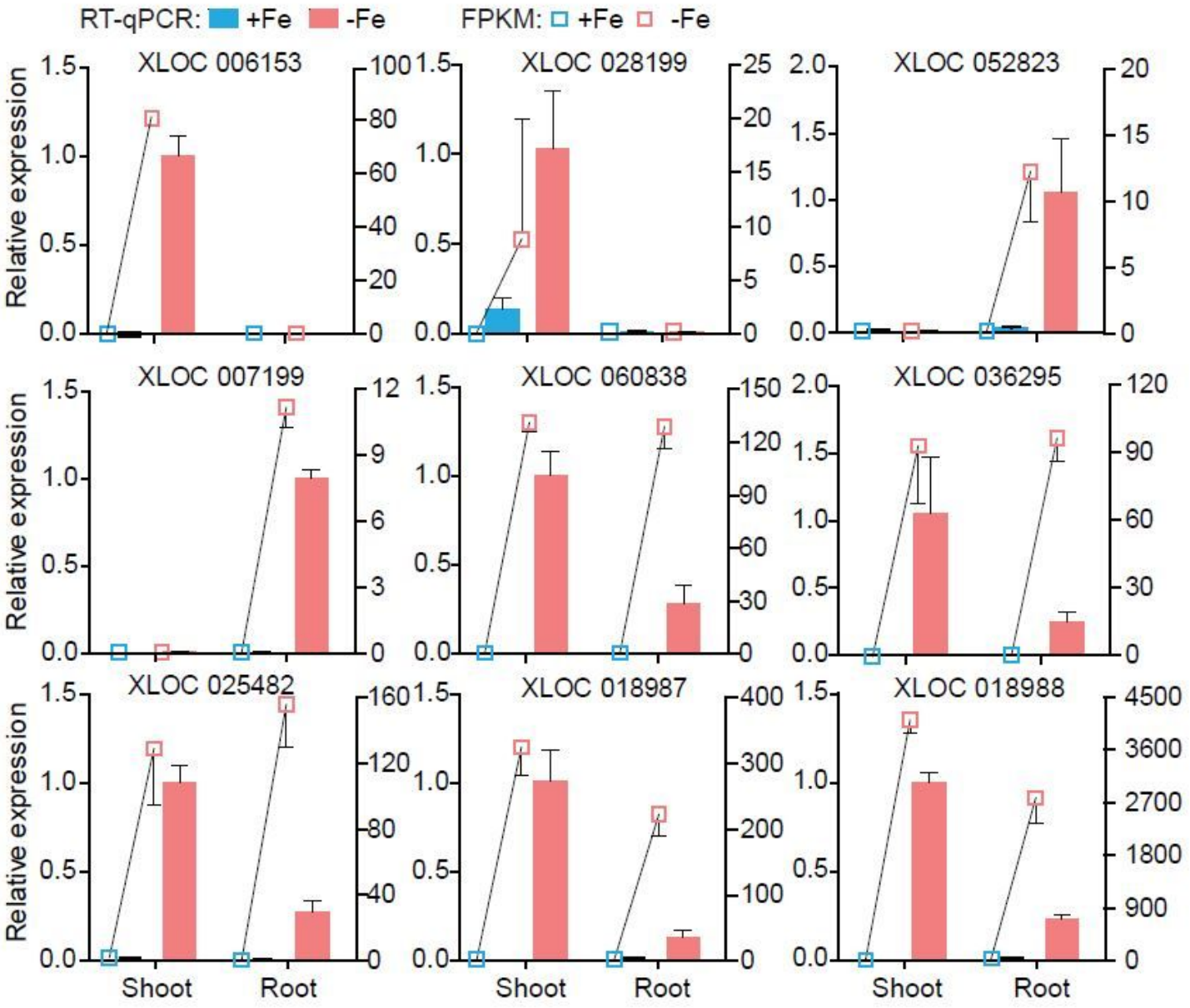

Figure 4

RT-qPCR validation of nine lncRNAs labeled as responding to Fe deficiency in the RNA-seq transcriptome. Nine lncRNAs were chosen for RT-qPCR analysis. The relative expression level of the lncRNAs is presented in the bar graph with the left axis, the right shows the FPKM value (open squares) from the RNA-seq transcriptome. Actin was used as a reference gene, Means ± SD were determined from three biological repeats.

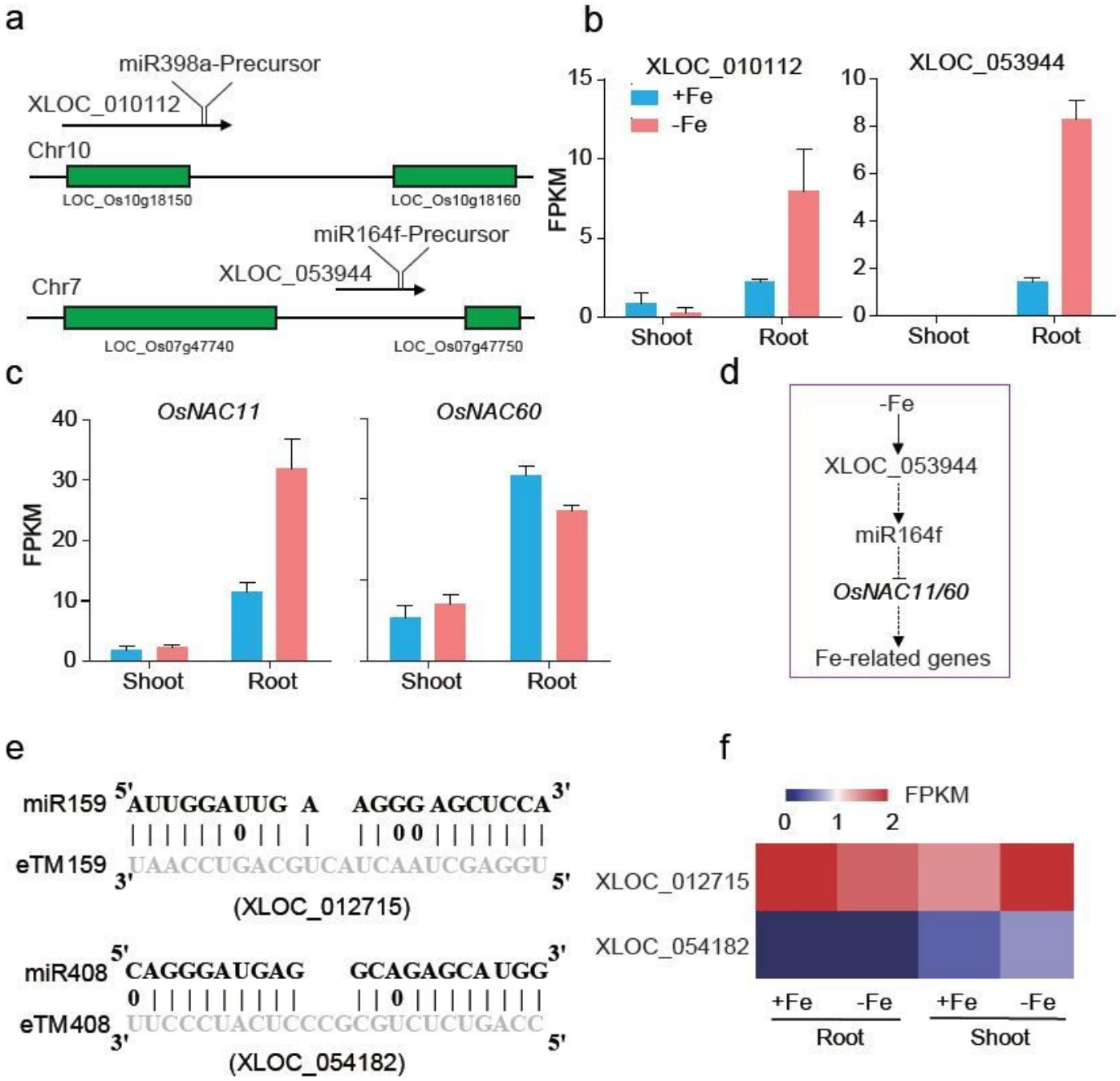

Figure 5

Predicted lncRNAs function as miRNA precursors and endogenous target mimics. a Schematic of two miRNA precursors encoded within two lncRNAs in the rice genome. B The expression value of the lncRNAs XLOC_010112 and XLOC_053944 in shoot and root of rice. c The expression value of OsNAC11 and OsNAC60 in shoot and root. d Predicted working model for XLOC_053944. e and f Two endogenous target mimics (eTMs), osa-eTM159 and osa-eTM408, respond to iron deficiency in rice. The predicted base-pairing pattern between osa-miR159 and osa-miR408 and their eTMs. Base pairing between the miRNAs and their lncRNA target mimic are shown, in which a vertical line means a Watson-Crick pair, two

dots represent a G-U pair, and 0 means a mismatch. f The expression levels of the eTMs osa-eTM159 and osa-eTM408 from the ssRNA-seq data.

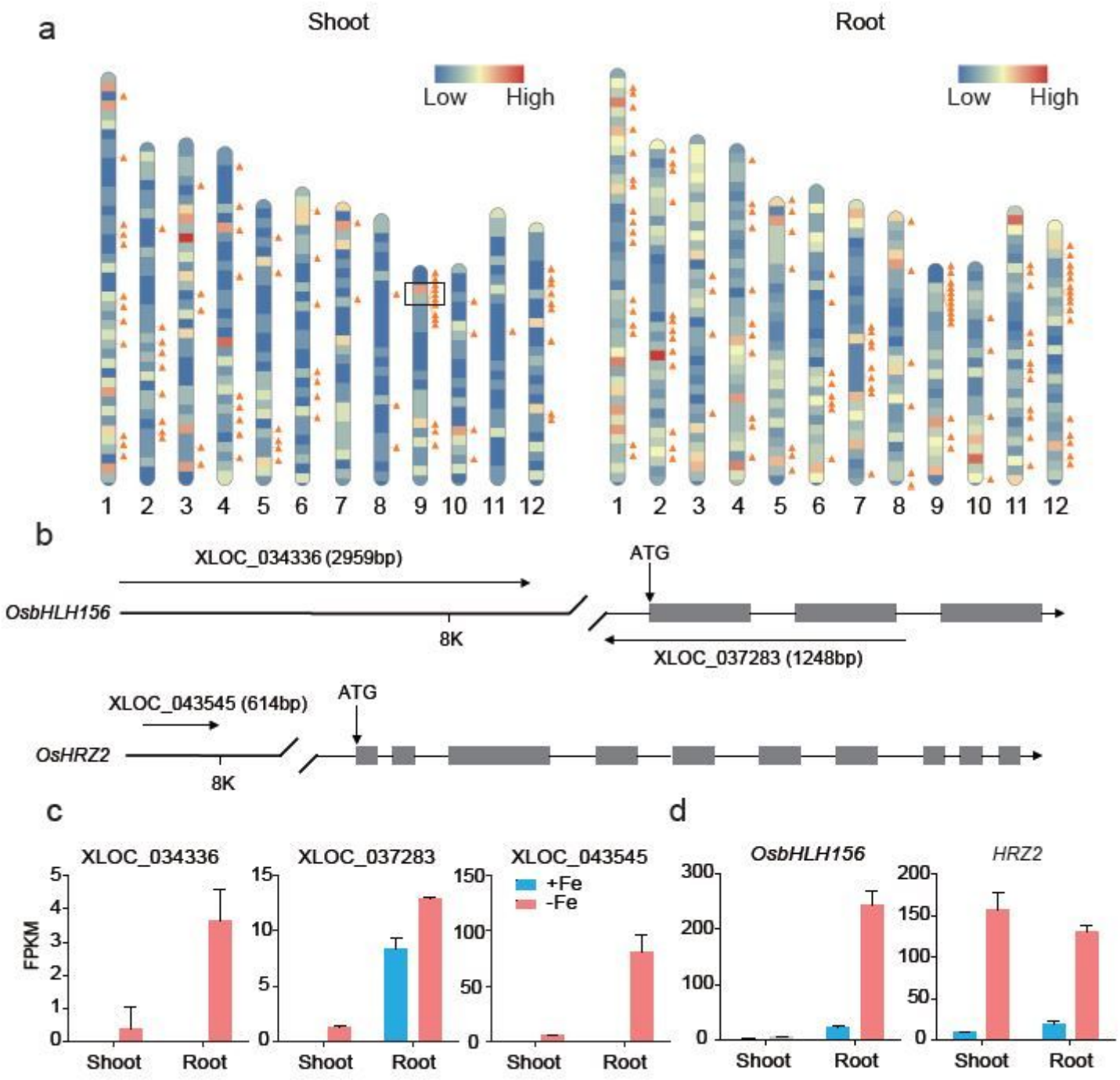

Figure 6

Predicted lncRNAs function as cis-regulators of nearby protein-coding genes. a Distribution of DE-lncRNAs and DE-mRNAs on the 12 rice chromosomes. The transcripts that were differentially expressed in shoots and roots between +Fe and -Fe conditions were separately mapped to the chromosomes for clarity. Different colors represent different densities of mRNAs on the chromosome, with red color denoting a high density and blue denoting a low density. Each triangle represents one lncRNA identified in

this study. b Schematic diagram of three DE-LncRNAs at genomic regions of OsbHLH156 and HRZ2. c-d Expression value of three LncRNAs (c) and bHLH156 and HRZ2 (d) under ±Fe conditions.

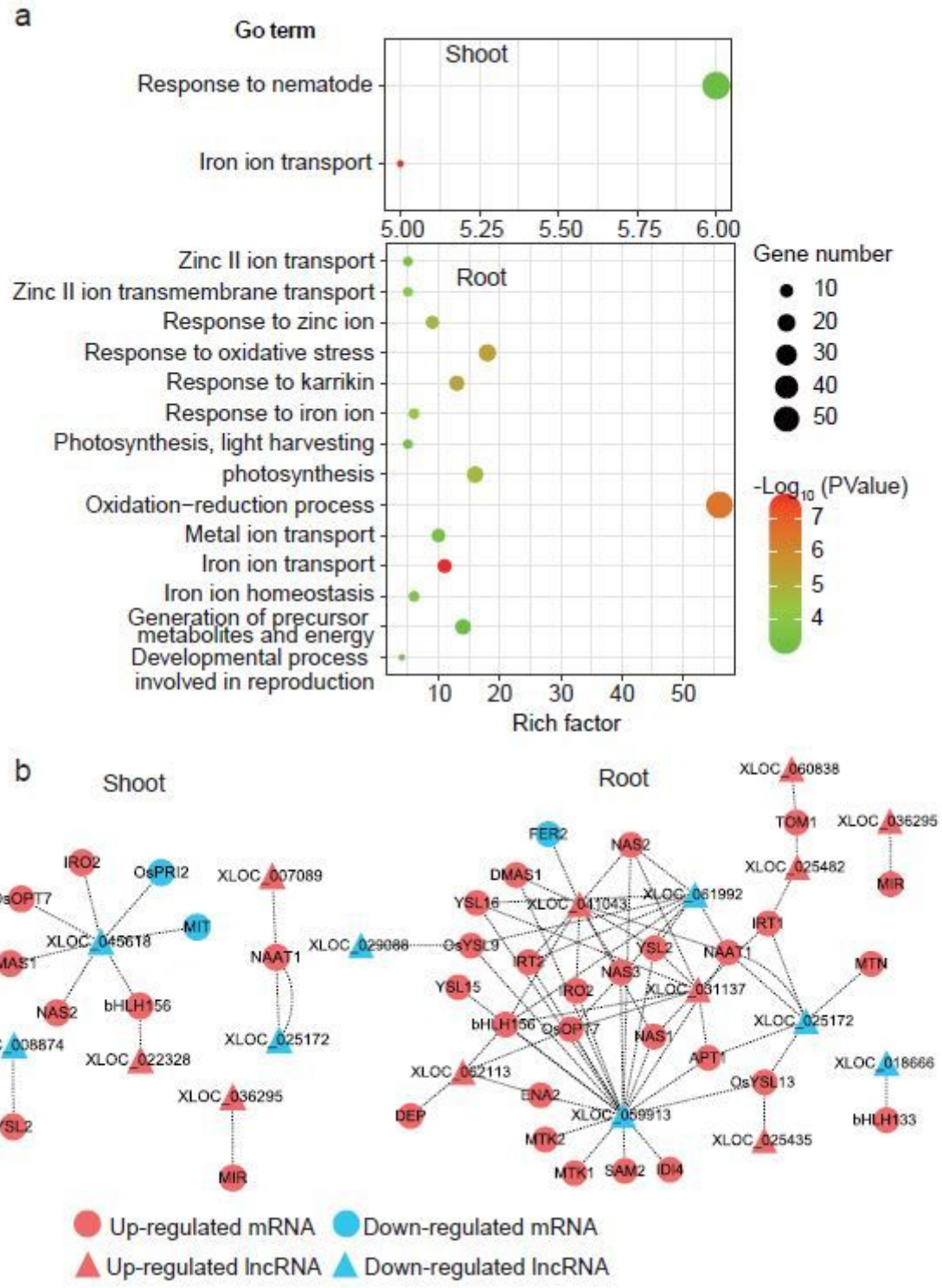

Figure 7

Interaction of DE-lncRNAs with DE-mRNAs. a Gene ontology (GO) enrichment of DE-mRNAs predicted to interact with DE-lncRNAs in shoot and root ($p < 0.001$). Only biological process terms are shown. b Interaction networks among DE-lncRNAs and Fe-related mRNAs based on complementary pairing of

bases in shoot and root. Circles represent Fe-related mRNAs and triangles represent DE-lncRNAs. Red and blue represent upregulated and down-regulated, respectively. Dotted line shows the potential interaction between mRNA and lncRNA.

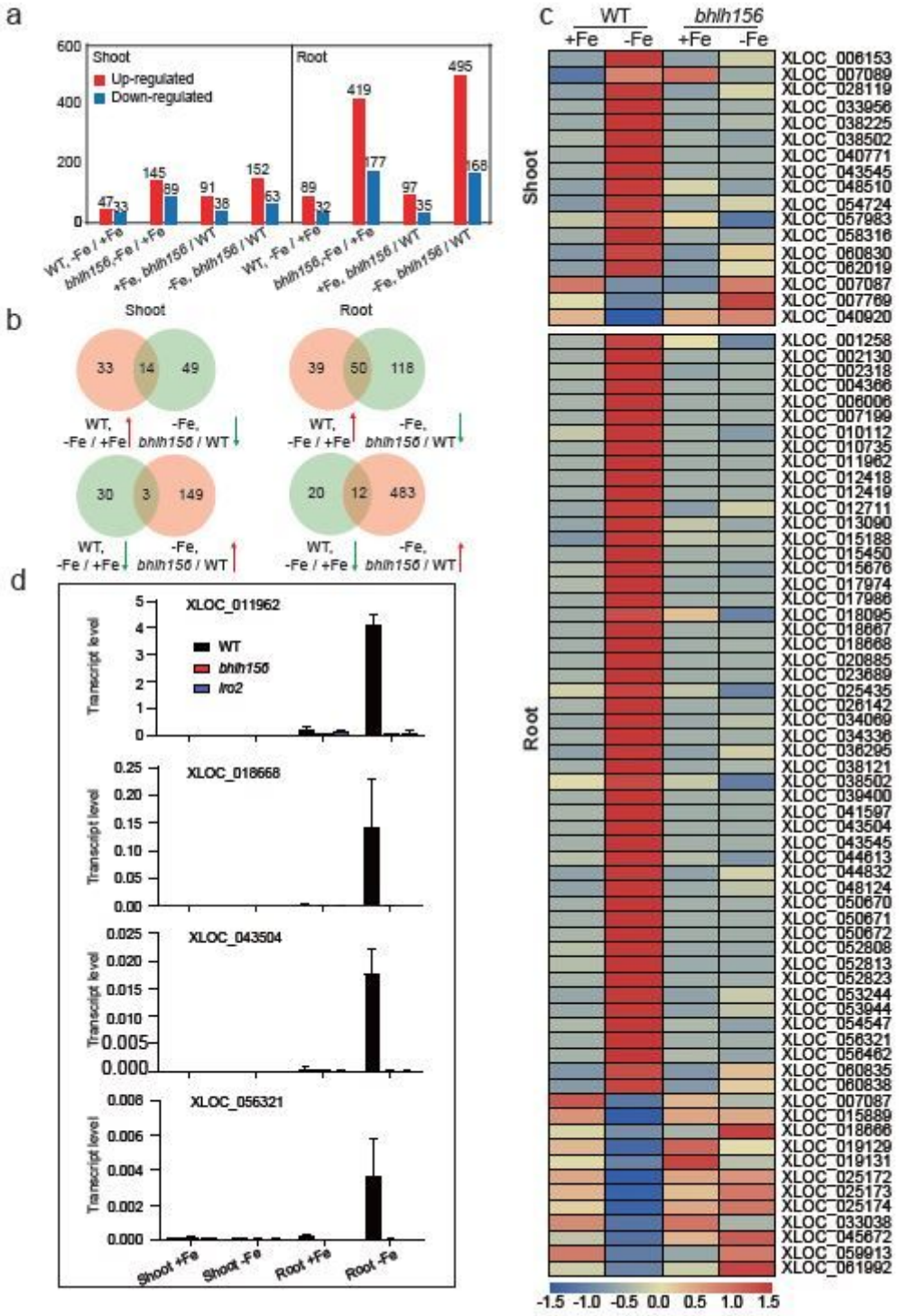

Figure 8

DE-LncRNAs regulated by bHLH156 and IRO2. a Number of DE-lncRNAs in shoots and roots of bhlh156 mutant under Fe deficiency. The filtering rules were the same as those in Figure 2c. b Venn diagram of

number of responded lncRNAs in regards to genotype and Fe supply in shoots (left) and roots (right) of rice. c Heatmap constructed of DE-lncRNAs responding in an antagonistic manner in rice roots under Fe deficiency. The color scale indicates the z-score associated with the DE-lncRNAs. d Transcript abundance of DE-lncRNAs in shoot and root of WT, bhlh156 and iro2 genotypes. Expression was detected by RT-qPCR. Transcript levels were calculated relative to OsActin.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS1.xlsx
- TableS2.xlsx
- TableS3.xlsx
- TableS4.xlsx
- TableS5.xls
- TableS7.xlsx
- TableS8.xlsx
- Table1.jpg