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Identification of OsbHLH133 as a regulator of iron distribution between roots and shoots in *Oryza sativa*

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ABSTRACT

Iron (Fe) is an essential micronutrient element for plant growth. Regulation of Fe-deficiency signalling networks is one of the many functions reported for basic helix-loop-helix (bHLH) transcription factors in plants. In the present study, *OsbHLH133* was found to be induced by Fe-deficiency conditions in *Oryza sativa*. Insertional inactivation of *OsbHLH133* (*bhlh133*) resulted in growth retardation, with enhanced Fe concentration seen in shoots, and reduced Fe concentration in roots. Overexpression of *OsbHLH133* had the opposite effect, that is resulted in an enhanced Fe concentration in roots and reduced Fe concentration in shoots and also in xylem sap. Microarray analysis showed that some of the genes encoding Fe-related functions were up-regulated under Fe-sufficient conditions, in *bhlh133* mutant plants compared to wild-type plants. Significant differential expression of a number of signalling pathways, including calcium signalling, was also seen in *bhlh133* plants compared to wild-type plants, independent of Fe conditions.

Key-words: bHLH; Fe distribution; Fe homeostasis; regulator; rice (*Oryza sativa*); transcription factor.

INTRODUCTION

Iron (Fe) is an essential mineral element for both plants and animals. Although it is abundant in soil, the low solubility in neutral to alkaline soil limits its uptake and utilization. Either Fe excess or deficiency is harmful to plants (Halliwell & Gutteridge 1992). Thus, Fe homeostasis must be precisely maintained in plants (Hindt & Guerinot 2012; Kobayashi & Nishizawa 2012). Plants have two distinct strategies for Fe acquisition. Strategy I is used by non-graminaceous plants such as *Arabidopsis thaliana* (*Arabidopsis*). In these species, ferric iron (Fe^{3+}) is reduced to the more soluble ferrous iron (Fe^{2+}) by the ferric-chelate reductase FRO2 (Robinson *et al*. 1999). Subsequently, the major metal transporter, iron-regulated transporter (IRT1), takes up Fe^{2+} into plant roots (Eide *et al*. 1996; Vert *et al*. 2002). Graminaceous plant species use the strategy II mechanism. In these species, mugineic acids (MAs) are synthesized and secreted from plant roots by transporters, that is OsTOM1 in rice (Ishimaru *et al*. 2006; Nozoye *et al*. 2011). MAs can chelate Fe^{3+} to form the Fe^{3+}–MA complexes, which are transported into roots by a specific family of transporters called Yellow Stripe transporters, which were first characterized in *Zea mays* (maize) as ZmYS1, and later in a variety of other plant species such as barley (HvYS1) and rice (OsYSL15) (Curie *et al*. 2001; Murata *et al*. 2006; Inoue *et al*. 2009). Although rice has the molecular components to take up Fe using both strategies (Ishimaru *et al*. 2006; Cheng *et al*. 2007), it primarily utilizes strategy I, because the synthesis and diffusion of MAs is limited in rice (Ishimaru *et al*. 2006). A Fe^{2+} transporter, OsIRT1, is found to mediate the transport of Fe^{2+} from the external solution to the root cells in rice (Bughio *et al*. 2002; Ishimaru *et al*. 2006).

A number of genes, including transcription factors, have been identified to be involved in the regulation of Fe acquisition and homeostasis in *Arabidopsis* and rice (Hindt & Guerinot 2012; Kobayashi & Nishizawa 2012). Basic helix-loop-helix (bHLH) proteins comprise one of the largest transcription factor families in eukaryotic organisms. In total, there are 158 bHLH genes in *Arabidopsis* and 173 bHLH genes in rice that have been identified; however, only a few of these have been functionally characterized (Li *et al*. 2006; Pires & Dolan 2010). The regulation of signalling networks during Fe deficiency is one of the many functions reported for bHLH transcription factors in plants. The first bHLH transcription factor related to Fe supply was identified in tomato and named *FER* (Ling *et al*. 2002). A *fer* mutant failed to activate the strategy I pathway of Fe acquisition and displayed severe chlorosis in tomato (Ling *et al*. 2002; Brumbarova & Bauer 2005). The *Arabidopsis* protein Fe-deficiency-induced transcription factor (FIT) is the ortholog to FER (Yuan *et al*. 2005; Bauer, Ling & Guerinot 2007) and forms heterodimers with other bHLH proteins, encoded by *AtbHLH38* or *AtbHLH39* (Yuan *et al*. 2008). The complex of FIT/AtbHLH38 or FIT/AtbHLH39 positively regulates the epidermal expression of the Fe reductase gene *FRO2*, and further induces expression of the Fe^{2+}

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transporter IRT1 (Yuan *et al*. 2008). Another bHLH transcription factor in *Arabidopsis*, named POPEYE (PYE), interacts with the bHLH transcription factor IAA-Leu Resistant3 (ILR3) to regulate metal ion homeostasis in *Arabidopsis* (Rampey *et al*. 2006; Long *et al*. 2010). *PYE* positively regulates the growth and development of *Arabidopsis* under Fe-deficient conditions, under which the mutant *pye* displayed inhibition of root growth, and severe chlorosis (Long *et al*. 2010). In addition, the Fe-chelate reductase activity and acidification of rhizospheres in the *pye* mutant were decreased (Long *et al*. 2010).

In rice, regulation of the strategy II MA pathway has been shown to be under the control of the bHLH protein OsIRO2 (Ogo *et al*. 2007). *OsIRO2* is induced by Fe deficiency and positively regulates the expression of genes involved in MA biosynthesis, including nicotianamine (NA) synthase genes *OsNAS1/2*, NA aminotransferase gene *OsNAAT1*, deoxymugineic acid synthase gene *OsDMAS1*, metallothionein-like gene *OsIDS1* and the Fe^{3+}-DMA transporter *OsYSL15* (Inoue *et al*. 2003; Bashir *et al*. 2006; Ogo *et al*. 2006, 2007; Cheng *et al*. 2007). A previous study in our laboratory identified a negative regulator, involved in Fe homeostasis, named OsIRO3, and overexpression of *OsIRO3* was seen to suppress in the expression of genes normally induced under Fe deficiency in rice (Zheng *et al*. 2010). Overexpression of *OsIRO3* also led to hypersensitivity to Fe deficiency, with lower chlorophyll content and Fe concentration observed in the rice shoots (Zheng *et al*. 2010).

Two other transcription factors, IDEF1 and IDEF2, which specifically bind the iron deficiency-responsive element 1 (IDE1) and 2 (IDE2), respectively, are constitutively expressed in rice roots and shoots (Kobayashi *et al*. 2003, 2007; Ogo *et al*. 2008). IDEF1 is essential for the early response to Fe deficiency and positively regulates a number of Fe-responsive genes (Kobayashi *et al*. 2009). Recently, it was reported that IDEF1 directly binds to divalent metals, enabling the Fe nutritional status to be 'sensed' (Kobayashi *et al*. 2011).

In this study, we characterized a novel bHLH transcription factor, named OsbHLH133 (LOC_Os12g32400.1) in rice (Li *et al*. 2006). This transcription factor is strongly up-regulated under Fe-deficiency conditions and functional analysis suggests that it acts as an important regulator of Fe distribution in rice plants.

MATERIALS AND METHODS

Plant materials

The rice cultivar Nipponbare (NIP) and Dongjin (DJ) served as the wild-type (WT) controls for the overexpression lines (OE) and *bhlh133* mutant, respectively. The seeds of OE lines and the WT counterparts were harvested from the winter nursery, Hainan province, China. Seeds of mutant and DJ were harvested from the Zhejiang province, China.

To construct the overexpression vector of *OsbHLH133*, the full length of cDNA sequence was amplified using the primer pairs of 5′-ATGGAATGCAGCTCCTTTGAAGCA-3′ and 5′-ACGGATCCCTACTCTTGTGACAGAGAGTTCTG-3′. The amplified fragment was cloned into pTF101-ubi at the *Bam*HI site (Zheng *et al*. 2010). Then, *Agrobacterium*-mediated rice transformation was carried out as described previously (Chen *et al*. 2003). To verify the transgenic plants, quantitative RT-PCR (qRT-PCR) analysis was conducted.

A T-DNA insertion mutant *bhlh133* was obtained from the rice T-DNA insertion sequence database (http://signal.salk.edu/cgi-bin/RiceGE) on DJ genotype (Jeon *et al*. 2000; Jeong *et al*. 2006). The insertion site of the T-DNA is in the start codon of the gene, just between the A and T base of the start (ATG) codon (Supporting Information Fig. S2a). The insertion was confirmed using PCR with T-DNA left boarder primer (5′-ACGTCCGCAATGTGTTATTAA-3′) and gene-specific primers (5′-TAGTGTCACTCTCCTGTGCT-3′ and 5′-CGAAGCTATCATCTTGGTAA-3′). qRT-PCR analysis was performed to further confirm the effect of T-DNA insertion on the expression of *OsbHLH133*.

Growth conditions

Rice seeds were germinated in water for 3 d and then transferred to culture solution containing 1.425 mM NH_4NO_3, 0.323 mM NaH_2PO4, 0.513 mM K_2SO4, 0.998 mM $CaCl_2$, 1.643 mM $MgSO_4$, 0.009 mM $MnCl_2$, 0.075 μM $(NH_4)_6Mo_7O_{24}$, 0.019 mM H_3BO_3, 0.155 μM $CuSO_4$, 0.152 μM $ZnSO_4$, 100 μM or 2 μM ethylenediaminetetraacetic acid (EDTA)-Fe (II) (Yoshida *et al*. 1976). Plants were grown in a growth chamber with 12 h 30 °C light/12 h 22 °C dark. The pH of the nutrient solution was adjusted to 5.5 with 2 N HCl, and the solution was renewed every two days.

Fe-deficiency treatments were performed as described before (Zheng *et al*. 2010). For time-course analysis, 2-week old seedlings were transferred to Fe-deficient nutrient solution. Plants were harvested on days 0, 1, 2, 3, 4, 5 and 6 of Fe deficiency. On day 6 of Fe deficiency, Fe was re-supplied by adding 100 μM EDTA-Fe (II), and plants were harvested 3 d later. To investigate the phenotypes of overexpressing and mutant plants, seedlings were germinated and grown on the culture solution supplied with 100 μM or 2 μM EDTA-Fe (II). After 3 weeks of treatment, shoot lengths and SPAD value (total chlorophyll content) were measured. For Fe concentration measurement, seedlings were grown in the normal nutrient solution for 3 weeks, and then were transferred to nutrient solution with or without EDTA-Fe (II) for 10 d.

Measurement of Fe concentration

To determine the concentration of Fe in plants, samples were dried for 3 d at 80 °C before being weighed. Afterward, they were digested in 5 mL of 11 N HNO_3 for 5 h at 150 °C.

For xylem sap collection, rice plants were cut at a height of 3 cm above the roots, a column filled with filter paper was placed on the cut end. Xylem sap was collected for 1 h and centrifuged into new tubes. Ten individual plants were pooled into one sample and samples were digested with 0.5 mL of 11 N HNO_3 at 80 °C for 60 min. After digestion, samples were diluted to a 3 mL volume.

Fe concentration was measured using inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7500ce, Santa Clara, CA, USA).

Measurement of chlorophyll content

A portable chlorophyll meter (SPAD-502; Konica Minolta Sensing, JP) was used to measure the SPAD value (total chlorophyll content) of the fully expanded youngest leaves.

Histochemical β-glucuronidase (GUS) staining assay

To investigate the expression pattern, 2009 bp upstream of the ATG codon of the *OsbHLH133* gene was amplified using the primers pair: 5′-ATCTCCGTTAGGCCATGT CCAAC-3′ and 5′-GCAGATTGCTTCAAAGGAGCTG CA-3′. The promoter was cloned into pBI101.3, connected to a *GUS* gene (Jefferson, Kavanagh & Bevan 1987) to produce a pOsbHLH133:GUS reporter construct. Transgenic plants were generated via *Agrobacterium*-mediated transformation. For the histochemical GUS staining assay, T_1 transgenic seeds were germinated and grown under Fe-replete and Fe-deprivation solutions for 10 d. Roots and leaves were subjected to GUS-staining buffer containing 100 mM sodium phosphate (pH 7.0), 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronidase), 1 mM $K_4[Fe(CN)_6]$, 1 mM $K_3[Fe(CN)_6]$, 0.5% (v/v) TritonX-100 and 20% (v/v) methanol. After staining, roots and leaves were imbedded in 5% (w/v) low-melting-point agarose (Sigma, St Louis, MO, USA). Sections of 50 or 60 μm thickness were cut by vibrating microtome (VT 1000 S, Leica, Bensheim, Germany) and the images were examined under a microscope (Eclipse 90i, Nikon, Tokyo, Japan).

Affymetrix microarray analysis

Seedlings were germinated and grown on the culture solution supplied with 100 μM or 2 μM EDTA-Fe (II) and after 3 weeks of treatment, roots of the seedlings were sampled, snap frozen in liquid nitrogen and stored at –80 °C until RNA isolation. The RNA isolation and microarrays were performed according to manufacturers' instructions, as described in (Zheng *et al*. 2009). Three biological replications were used for the analysis. All CEL files were first normalized by MAS5 to determine present/absent calls and only those genes present in = >2 replicates were used for further analysis. Data were normalized by GeneChip Robust Multiarray Averaging (GC-RMA) using the Partek Genomics Suite software (version 6.5) and differential expression analysis was carried out using Cyber-T (Baldi & Long 2001; Choe *et al*. 2005). Genes were defined as significantly differentially expressed if the fold-change had an associated P value of <0.05 and false discovery rate was posterior probability of differential expression (PPDE) >0.96, as carried out in (Narsai, Castleden & Whelan 2010; Narsai *et al*. 2011). Significant over-representation of functional categories was determined by a z-score analysis as described in (Narsai *et al*. 2009). Over-representation of functional categories was determined using Fisher's test (ORA Cut-off value = 1) within the Pageman software (Usadel *et al*. 2006).

qRT-PCR

Total RNA was extracted from root or leaf samples using Trizol reagent according to manufacturer's instruction (Invitrogen, Carlsbad, CA, USA). RNA samples were then treated with RNase-free DNase I (TaKaRa Biomedicals, Tokyo, Japan) to remove the residues of genomic DNA. First-strand cDNA was synthesized using M-MLV reverse transcriptase (Promega, Madison, WI, USA). cDNA was amplified by PCR in LightCycler 480 machine (Roche Diagnostics, Basel, Switzerland) with SYBR Premix Ex Taq (Perfect Real Time) Kit (TaKaRa Biomedicals). Triplicate quantitative assays were performed on each sample and reference gene *OsActin* was used as an internal control. Transcript levels were calculated relative to *OsActin* using the formula $2^{-\Delta Ct}$ or $2^{-\Delta\Delta Ct}$. All primer sequences used for the PCR reactions are shown in Supporting Information Table S1.

Statistical analysis of data

For comparisons of treatments in Figs 3–5, significance was determined by a one-way analysis of variance and for differences between groups, with least significant difference (LSD) t-test ($P \leq 0.05$).

RESULTS

Fe deficiency specifically induces expression of *OsbHLH133*

Previous microarray analyses showed that the transcript abundance of *OsbHLH133* was significantly induced by Fe deficiency in rice roots (Zheng *et al*. 2009). To confirm this expression pattern, 3-week-old rice seedlings were transferred to Fe-, Mn-, Cu- or Zn-deprived nutrient solutions for 10 d. qRT-PCR analysis showed that the transcript abundance of *OsbHLH133* was specifically induced under Fe and copper (Cu) deficiency conditions in roots, but not by other mineral nutrient deficiencies (Fig. 1a). A 60-fold induction of *OsbHLH133* expression was observed in response to Fe deficiency, with a 4.6-fold induction was observed also in response to Cu deficiency in roots (Fig. 1a). The transcript level of *OsbHLH133* in roots was higher than that in leaves regardless of Fe supplies (Fig. 1b). In leaves, the basal level of *OsbHLH133* expression was very

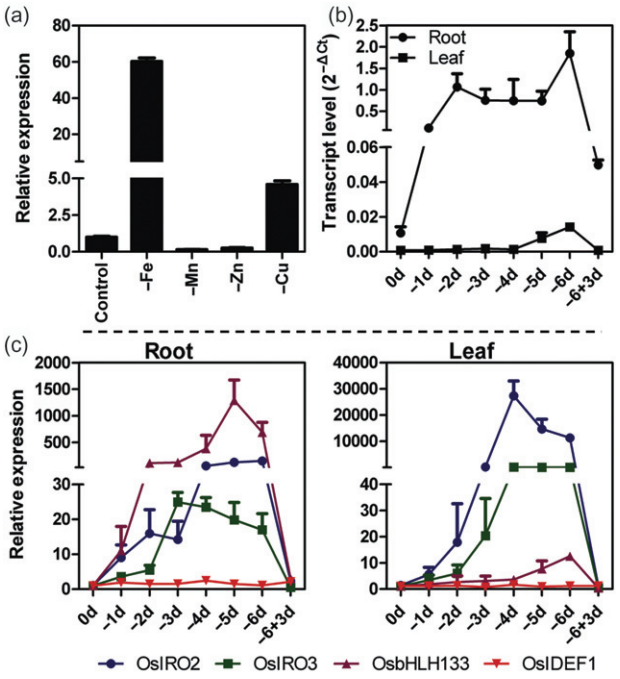


Figure 1. Expression analysis of *OsbHLH133* transcript abundance in WT rice plants. (a) Transcript abundance of *OsbHLH133* was determined by qRT-PCR under different nutrient deficient conditions. All values are expressed relative to the expression level under nutrient-sufficient conditions (control-set to 1.0) as appropriate. (b) Transcript levels of *OsbHLH133* relative to *OsActin* in root and leaf during Fe-deficiency treatment over 6 d, followed by a return to Fe-sufficient conditions for 3 d. (c) Kinetic analysis showing the induction of *OsIRO2*, *OsIRO3*, *OsbHLH133* and *IDEF1* during Fe-deficiency treatment.

low under normal conditions. However, under Fe-deficient conditions, the expression of *OsbHLH133* was seen to be significantly induced in both leaves and roots (Fig. 1b).

As a transcription factor, OsbHLH133 was targeted to the nucleus (Supporting Information Fig. S1). Kinetic analysis of *OsbHLH133* induction and the induction of three other transcription factors involved in Fe-deficiency response in rice, *OsIRO2*, *OsIRO3* and *IDEF1*, were carried out to gain insight into the role of these during Fe deficiency. In roots, *OsbHLH133* was induced approximately 10-fold after just 1 d of Fe-deficiency treatment and reached peak expression at 5 d post-treatment (Fig. 1c). In leaves, the transcript abundance of *OsbHLH133* was very low under Fe-sufficient conditions, and was slightly induced under Fe-deficiency treatment (Fig. 1c). Notably, the pattern of *OsbHLH133* induction was seen to be comparable to that seen for *OsIRO2* (Fig. 1c).

Growth performance of *OsbHLH133* overexpression lines and T-DNA insertion mutants

To examine the function of OsbHLH133 in rice plants, a T-DNA insertion mutant *bhlh133* was obtained from the rice T-DNA insertion sequence database (Jeon *et al*. 2000; Jeong *et al*. 2006). Transgenic lines that constitutively overexpress the *OsbHLH133* gene in the Nipponbare genotype (NIP) were also developed to gain further insight into its function. Using qRT-PCR analysis, two independent overexpression lines (OE) of *OsbHLH133* were confirmed and named OE-1, OE-2 (Fig. 2). As expected, the transcript levels of *OsbHLH133* in the OE lines were significantly higher than the WT counterparts, while no *OsbHLH133* transcript was detected in the *bhlh133* mutant, regardless of Fe supply (Fig. 2).

Under Fe-sufficient (+Fe) conditions, *bhlh133* mutant plants displayed a small but significantly ($P < 0.05$) reduced shoot length (Fig. 3a, Supporting Information Fig. S2b,c). Seed set from *bhlh133* plants was reduced to 30% compared to WT under Fe-sufficient conditions (Supporting Information Fig. S2d). In contrast, when seeds were germinated and grown in Fe-deficient conditions for 3 weeks, no significant differences in shoot length were observed between the *bhlh133* mutant plants and WT plants (Fig. 3a). Interestingly, under Fe-sufficient conditions, no significant differences in growth performance were observed between the *OsbHLH133*-OE and the WT seedlings (Fig. 3b). However, under Fe-deficient conditions, the shoot length of the *OsbHLH133*-OE lines was significantly shorter than the WT (Fig. 3b). To assess the degree of leaf chlorosis caused by Fe deficiency in the *OsbHLH133*-OE lines, the chlorophyll content (in SPAD units) of the youngest fully expanded leaves of the WT, *bhlh133* mutant and *OsbHLH133*-OE plants was measured. Results showed that the chlorophyll content in the *bhlh133* mutant was significantly ($P < 0.05$) higher than the WT, while the chlorophyll content in the *OsbHLH133*-OE lines was significantly ($P < 0.05$) lower than that of the WT plants (Fig. 3c). These results suggest that overexpressing *OsbHLH133* leads to hypersensitivity to Fe deficiency, while the *bhlh133* knockout plants show improved tolerance to Fe deficiency in rice.

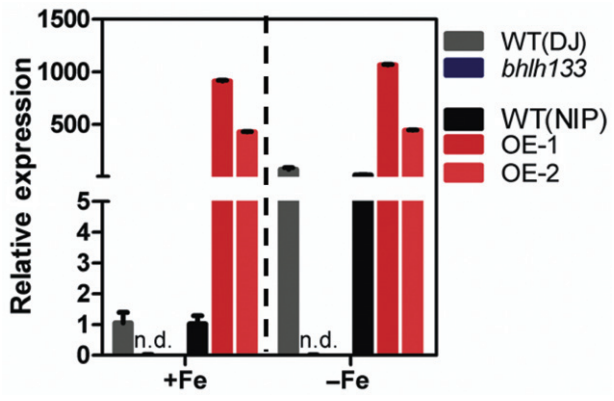


Figure 2. Expression of *OsbHLH133* in the root of overexpression (OE) and mutant (*bhlh133*) plants in Fe-sufficient (+Fe) and -deficient (–Fe) conditions. As expected, no transcripts encoding OsbHLH133 could be detected in the *bhlh133* mutant plants and over 100-fold induction is seen in the OE plants.

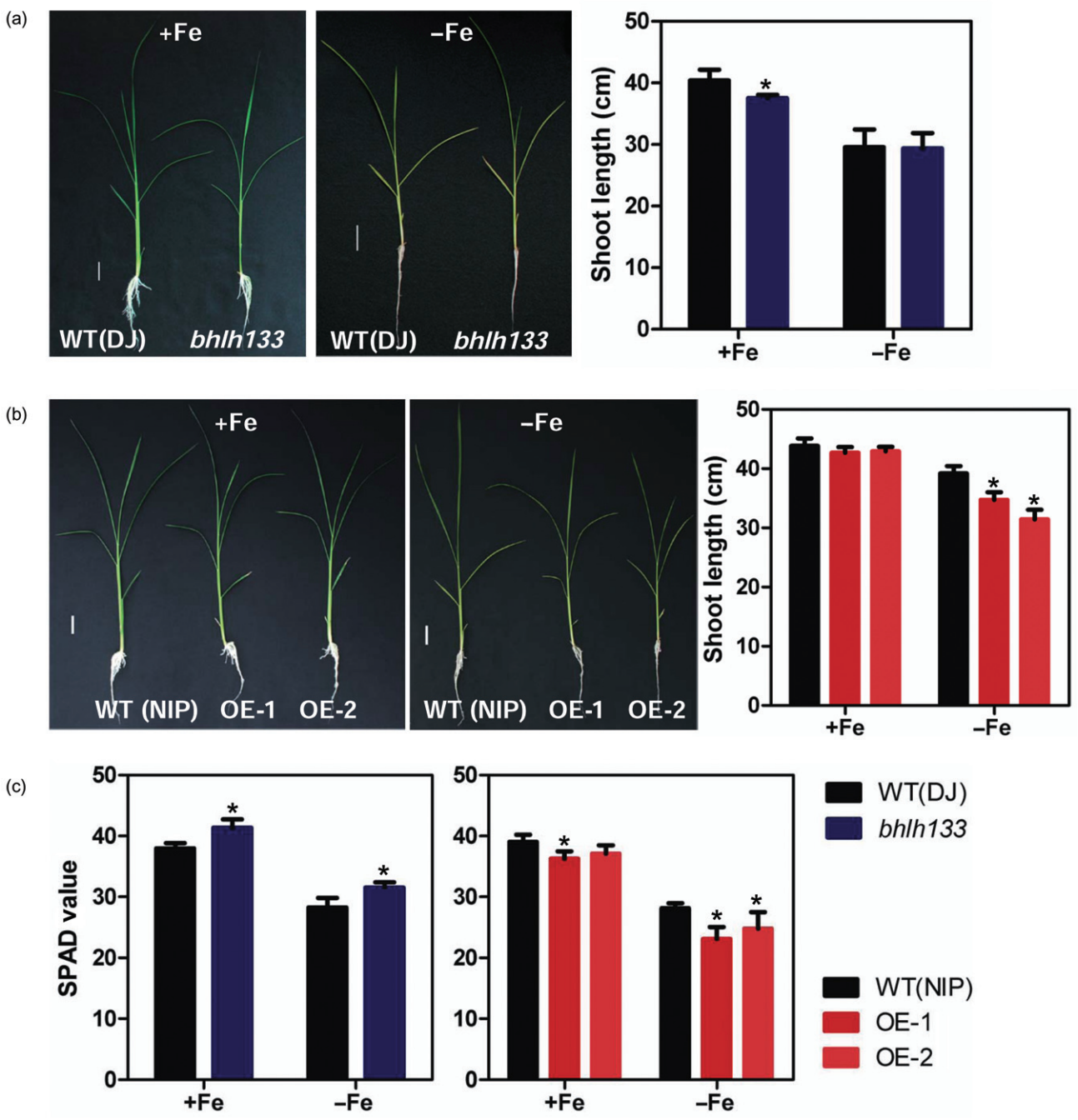


Figure 3. Growth performance of overexpression (OE) and mutant (*bhlh133*) plants in Fe-sufficient (+Fe) and -deficient (–Fe) conditions. Seedlings were germinated and grown on the culture solution supplied with 100 μM (+Fe) or 2 μM EDTA-Fe (II) (–Fe) for 3 weeks. (a) Growth performance and shoot length of mutant plants under Fe-sufficient (+Fe) and -deficient (–Fe) conditions. (b) Growth performance and shoot length of OE lines under Fe-sufficient (+Fe) and -deficient (–Fe) conditions. (c) SPAD value of the youngest and fully expanded leaves. Bar = 3 cm. Data shown as the mean ± SD ($n = 5$). Significance ($P < 0.05$) of differences in (longer/shorter or higher/lower) compared to WT is indicated by an asterisk.

Fe concentrations of *OsbHLH133* overexpression lines and T-DNA insertion mutants

To observe the effect of *OsbHLH133* abundance on Fe concentrations in plants, the Fe concentrations in the leaves, stems and roots of the *OsbHLH133* overexpression lines, *bhlh133* mutant and WT plants were analysed (Fig. 4). Under Fe-sufficient conditions, the Fe concentrations in the leaves and stems of the *OsbHLH133*-OE plants were significantly lower than WT (Fig. 4a). In contrast, the Fe concentration in the roots of the OE-1 and OE-2 plants was

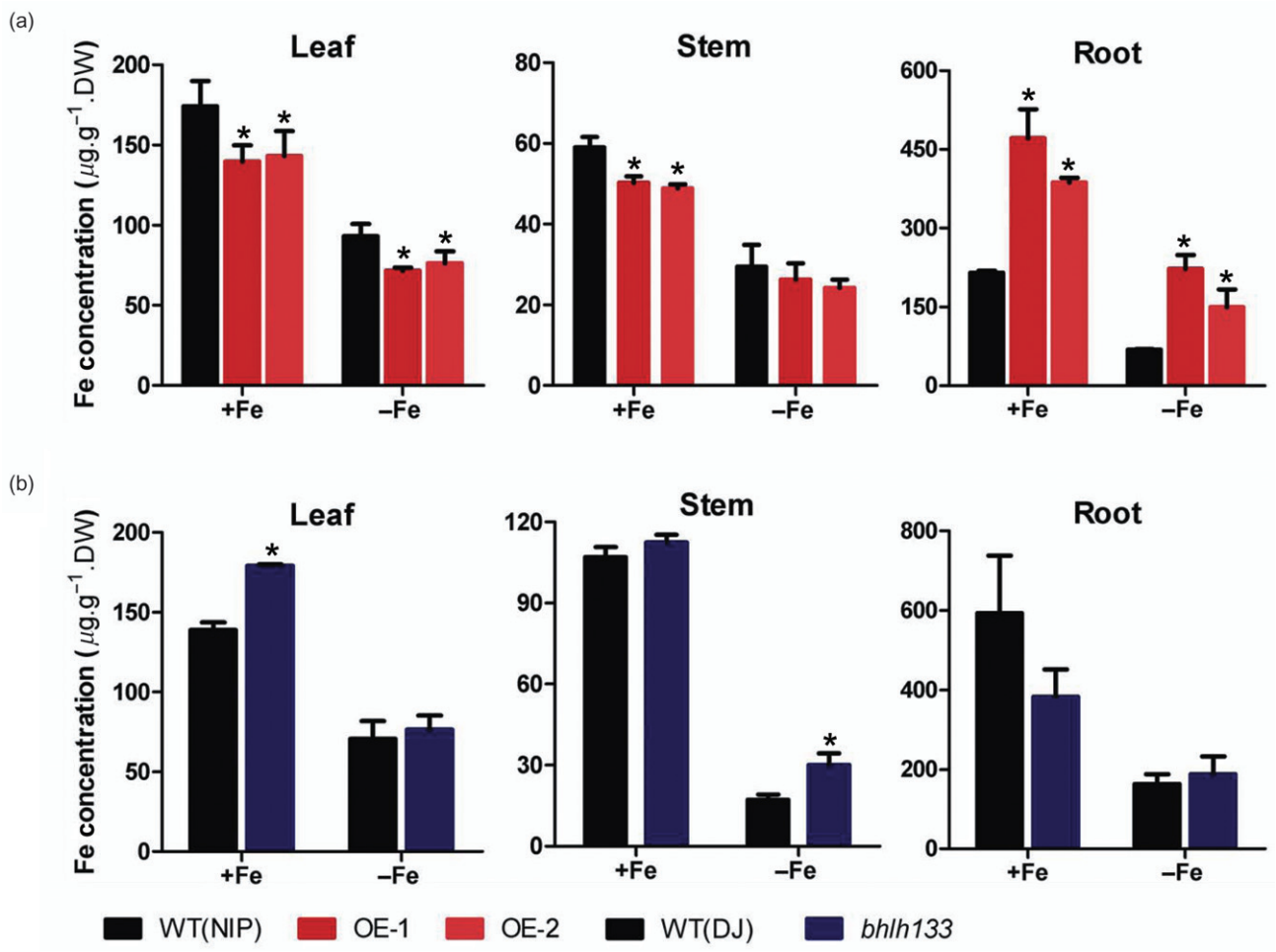


Figure 4. Analysis of Fe concentration in *OsbHLH133*-OE and mutant plants. Three weeks old seedlings were transferred to nutrient solutions containing 100 μM (+Fe) or 0 μM (–Fe) EDTA-Fe (II) and after 10 d of treatment, Fe concentrations in leaves, stems and roots were measured by ICP-MS. Data are shown as the mean ± SD ($n = 3$). Significance ($P < 0.05$) of differences in Fe concentration (higher/lower) compared to WT is indicated by an asterisk respectively.

significantly higher than WT, 2.2- and 1.8-fold, respectively (Fig. 4a). Notably, under Fe-deficient conditions, *OsbHLH133*-OE seedlings showed the same overall pattern of Fe distribution between roots and leaves, as seen under Fe-sufficient conditions (Fig. 4a).

Under Fe-sufficient conditions, the Fe concentration in the leaves of the *bhlh133* mutant plants was significantly higher than that of the WT counterpart, while the root Fe concentration in the *bhlh133* mutant was lower than that found in WT plants (Fig. 4b). These observations in the *bhlh133* mutant plants are in agreement with the observations seen in the *OsbHLH133*-OE lines (Fig. 4).

Fe concentrations in xylem sap were also measured and the results showed that Fe concentration in the xylem sap from *OsbHLH133*-OE lines was decreased under both Fe-sufficient and deficient conditions (Fig. 5). The Fe concentration in the xylem sap of both OE-1 and 2 lines were either marginally significant ($P < 0.06$) or significantly ($P < 0.05$) less than that of the WT, respectively (Fig. 5). In *bhlh133* mutant plants, Fe concentration in the xylem sap

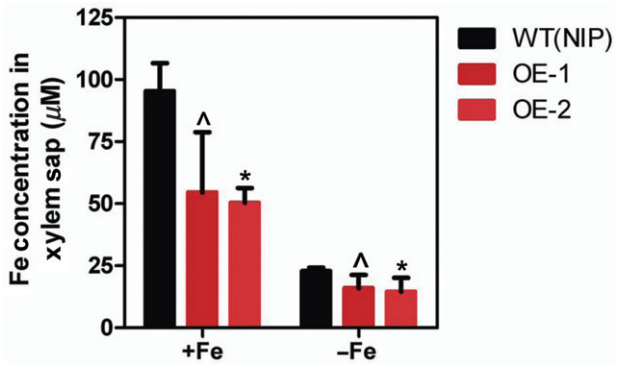


Figure 5. Fe concentrations in xylem sap of OE plants. Three-week-old seedlings were transferred to nutrient solutions containing 100 μM (+Fe) or 2 μM EDTA-Fe (II) (–Fe), after 10 d treatment, xylem sap was collected. Fe concentrations were measured by ICP-MS. Data are shown as the mean ± SD ($n = 3$). Asterisks indicates significantly lower Fe concentration ($P < 0.05$), and a hat (^) indicates marginally significantly lower Fe concentration ($P < 0.06$).

showed no significant difference from the WT counterpart under Fe-deficient, Fe-sufficient or excess Fe conditions, although a slight increase was seen under the excess Fe conditions (Supporting Information Fig. S3).

Note that there was no major difference in the zinc (Zn), manganese (Mn) or copper (Cu) concentrations among the leaves, roots or xylem sap of the mutant, *OsbHLH133*-OE lines and WT (Supporting Information Figs S4 & S5).

Spatial expression pattern of OsbHLH133

To determine the pattern of transcriptional regulation of OsbHLH133 in rice, we assayed the GUS expression of pOsbHLH133:GUS reporter construct in transgenic plants. Four independent transgenic lines were analysed. Under Fe-sufficient conditions, weak GUS expression was mainly observed in the maturation zone of the roots (Fig. 6a), but not at the root tip (Fig. 6c). Cross-sections of the roots exhibited that GUS activity was present in the endodermal cells of the maturation zone, which was 4 cm above the root tip (Fig. 6b), but not in the section of the zone 0.5 cm above the root tip (Fig. 6d). In contrast, under Fe-deficient conditions, GUS expression was observed throughout the roots, including the epidermis, exodermis, cortex, endodermis and the stele (Fig. 6e–h), indicating expression of *OsbHLH133* across these cell types. In leaves, GUS activity was barely detectable when plants were grown in the presence of Fe (data not shown). However, under Fe-deficient conditions, GUS expression was observed in the vascular bundles of the leaf blade and leaf sheath (Fig. 6i,j), confirming the induction of *OsbHLH133* expression under these conditions.

Microarray analysis of *bhlh133*

To examine the gene expression changes seen in the WT and *bhlh133* mutant plants, genome-wide expression analysis was carried out on root tissues using Affymetrix rice genome microarrays. As expected, both the qRT-PCR and microarray analysis showed that the expression of *OsbHLH133* was significantly ($P < 0.05$, PPDE >0.96) suppressed in the roots of the *bhlh133* mutant plants, and induced under Fe deficiency in the WT plants (Table 1). In the WT and *bhlh133* mutant comparisons, only 809 genes were seen to be differentially expressed under Fe-sufficient conditions (Fig. 7a, orange circle; Supporting Information Table S2), compared to nearly double this number of genes (1597 genes) differentially expressed under Fe-deficient conditions (Fig. 7a, dark pink circle, Supporting Information Table S2), thereby supporting a specific role for OsbHLH133 under Fe-deficient conditions in roots.

In the *bhlh133* mutant plants, 377 genes (321 up-regulated and 56 down-regulated genes) were seen to be differentially expressed compared to the WT, independent of Fe conditions (Fig. 7a, orange and dark pink overlap, Supporting Information Table S2). Closer examination of these revealed that the 321 up-regulated genes were over-represented in transcripts encoding proteins across a

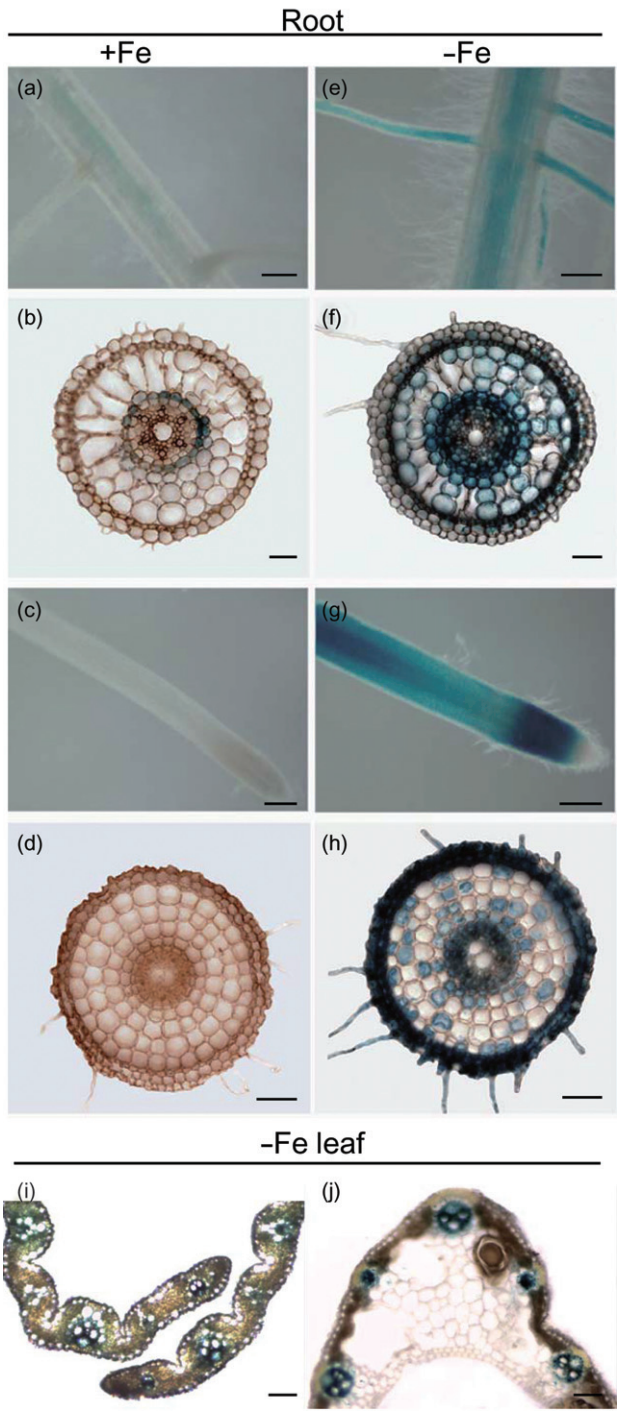


Figure 6. Spatial expression patterns of OsbHLH133 in roots and leaves. Histochemical GUS staining was performed using 10-day-old seedlings grown on Fe-sufficient (a, b, c, d) or Fe-deficient (e, f, g, h, i, j) nutrient solutions. (a, b, e, and f), Gus staining on the maturation zones of pbHLH133:GUS transgenic roots; (c, d, g, and h), Gus staining on the zones of pbHLH133:GUS transgenic root tips; (i and j), Gus staining on Fe-deficient leaf blade and leaf sheath, respectively. (b, d, f and h), images of the root sections that were 0.5 cm away from the root tip (d, h) and 4 cm away from the root tip (b, f). Scale bars = 500 μm for (a, c, e, g) and 100 μm for (b, f, d, h, i, j).

Table 1. Differential expression (in roots) of genes encoding Fe-related functions

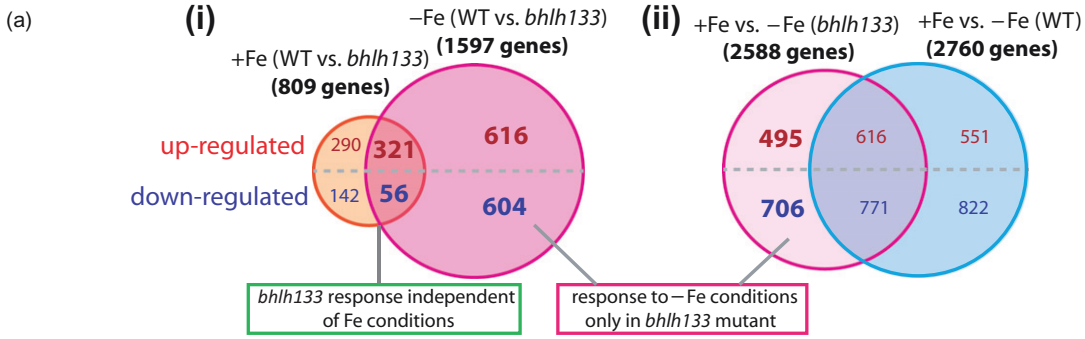
		Fold-change			
		bhlh133 versus WT		–Fe versus +Fe	
TIGR identifiers	Gene name	+Fe	–Fe	WT	*bhlh133*
LOC_Os12g32400.1	OsbHLH133	X	-15.15	8.97	X
Strategy II					
DMA biosynthesis					
LOC_Os03g19427.1	OsNAS1	1.65	X	3.13	1.92
LOC_Os03g19420.1	OsNAS2	1.38	X	2.36	1.74
LOC_Os07g48980.1	OsNAS3	1.15	1.26	1.22	1.33
LOC_Os02g20360.1	OsNAAT1	1.29	X	2.60	2.06
LOC_Os03g13390.1	OsDMAS1	1.36	X	3.38	2.58
LOC_Os06g02220.1	OsMTN	X	X	1.74	1.76
LOC_Os04g57400.1	OsMTK	X	X	1.57	1.64
LOC_Os01g22010.1	OsSAM2	X	X	1.23	1.26
LOC_Os12g39860.1	OsAPT1	X	X	1.25	1.28
LOC_Os04g24140.1	OsRPI	1.33	X	3.13	2.56
LOC_Os11g29370.1	OsDEP	X	X	1.83	1.80
LOC_Os09g28050.1	OsIDI4	X	X	1.74	1.71
Transporter					
LOC_Os11g04020.1	OsTOM1	2.29	X	7.22	3.25
LOC_Os02g43410.1	OsYSL15	2.75	1.14	7.21	2.99
Transcription factor					
LOC_Os01g72370.1	OsIRO2	X	X	13.93	7.78
LOC_Os03g26210.1	OsIRO3	X	X	33.78	35.06
Strategy I					
LOC_Os03g46470.1	OsIRT1	X	X	5.50	4.78
LOC_Os03g46454.1	OsIRT2	X	X	7.59	8.09
Fe transport and mobilization					
LOC_Os07g15460.1	OsNramp1	1.77	X	6.01	3.48
LOC_Os04g38940.1	OsVIT1;2	3.14	X	11.83	3.97
LOC_Os02g43370.1	OsYSL2	1.64	X	4.16	2.61
LOC_Os12g18410.1	OsMIR	X	X	2.90	2.36
LOC_Os11g05390.1	OsENA1	1.37	X	3.80	2.78
LOC_Os06g48060.1	OsENA2	X	X	1.30	1.25
LOC_Os03g11734.1	OsFRDL1	1.59	X	4.57	2.95

Fold-changes are shown, 'X' indicates no significant differential expression (significance was determined by a *P*-value of <0.05 and false discovery rate was PPDE >0.96).

number of functional categories including major CHO metabolism, lipid metabolism, stress and signalling functions (green boxes; Fig. 7b). Interestingly, these signalling processes largely included transcripts encoding receptor kinases and calcium signalling (green boxes; Fig. 7b), suggesting that the intercellular communication processes are altered in the *bhlh133* mutant, independent of Fe conditions. It was also seen that of the 56 down-regulated genes responsive in the mutant, independent of Fe conditions, there was a significant enrichment of transcripts encoding secondary metabolism functions, protein synthesis and elongation functions (green box; Fig. 7b).

Two sets of genes were identified as exclusively responsive to Fe-deficient conditions in the *bhlh133* mutant (Fig. 7ai, exclusive genes in dark pink circle; 616 up-regulated genes and 604 down-regulated genes) and light pink circles (Fig. 7aii, 495 up-regulated genes and 706 down-regulated genes, Supporting Information Table S2). The over-represented functional categories are shown for these genes, which were responsive to Fe-deficient conditions only in the *bhlh133* mutants (Fig. 7b, pink boxes). This analysis revealed that under Fe-deficient conditions, an up-regulation of genes encoding amino acid metabolism functions, secondary metabolism functions and ammonium transporters is observed only in the *bhlh133* mutant (pink box, Fig. 7b), while the down-regulated genes were significantly enriched in photosynthesis-related functions, tetrapyrrole synthesis, ribosomal proteins, as well major CHO synthesis and lipid transfer functions (Fig. 7b, pink boxes, Supporting Information Table S2). In addition, three genes encoding chloroplast-localized ferredoxin and ferredoxin reductases were also seen to be down-regulated exclusively in the *bhlh133* mutant in response to Fe-deficient conditions, and this was not seen in the WT (Supporting Information Table S2).

Under Fe-sufficient conditions, some of the genes encoding Fe-related functions were up-regulated in *bhlh133* mutant plants (Table 1, Supporting Information Table S2).


(a)
(i)
+Fe (WT vs. bhlh133)
(809 genes)
−Fe (WT vs. bhlh133)
(1597 genes)
up-regulated
down-regulated
290
321
142
56
616
604
bhlh133 response independent of Fe conditions
(ii)
+Fe vs. −Fe (bhlh133)
(2588 genes)
+Fe vs. −Fe (WT)
(2760 genes)
495
706
616
771
551
822
response to −Fe conditions only in bhlh133 mutant

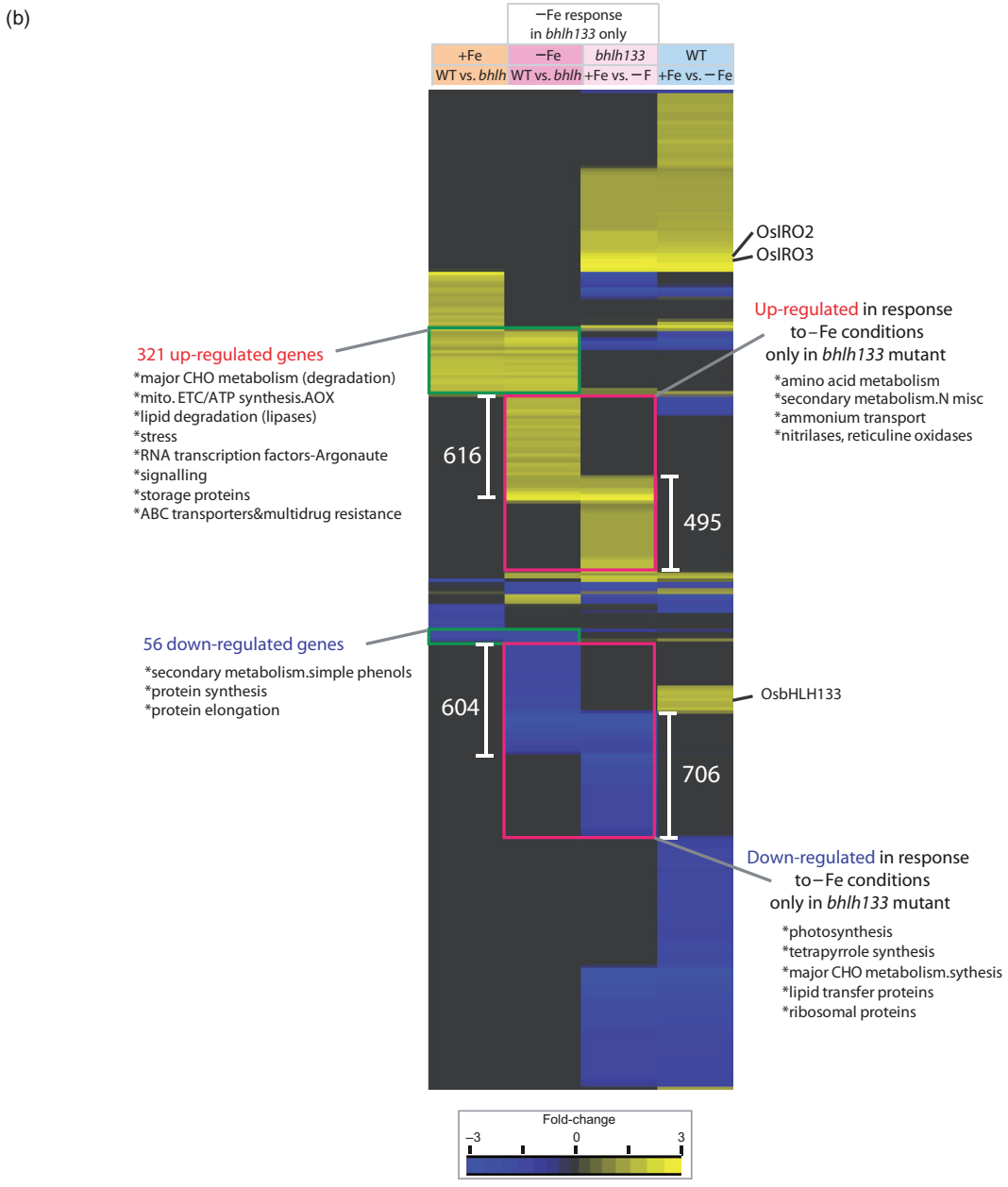

(b)
−Fe response in bhlh133 only
+Fe
WT vs. bhlh
−Fe
WT vs. bhlh
bhlh133
+Fe vs. −F
WT
+Fe vs. −Fe
OsIRO2
OsIRO3
Up-regulated in response to −Fe conditions only in bhlh133 mutant
*amino acid metabolism
*secondary metabolism.N misc
*ammonium transport
*nitrilases, reticuline oxidases
321 up-regulated genes
*major CHO metabolism (degradation)
*mito. ETC/ATP synthesis.AOX
*lipid degradation (lipases)
*stress
*RNA transcription factors-Argonaute
*signalling
*storage proteins
*ABC transporters&multidrug resistance
616
495
56 down-regulated genes
*secondary metabolism.simple phenols
*protein synthesis
*protein elongation
604
706
OsbHLH133
Down-regulated in response to −Fe conditions only in bhlh133 mutant
*photosynthesis
*tetrapyrrole synthesis
*major CHO metabolism.sythesis
*lipid transfer proteins
*ribosomal proteins
Fold-change
−3
0
3

Figure 7. Differential expression analyses in response to +/–Fe conditions in WT and *bhlh133* mutant. (a) (i) Venn diagram showing the number of significantly differentially expressed genes ($P < 0.05$, PPDE >0.96) in the *bhlh133* mutant compared to the WT in the presence of Fe (+Fe WT versus *bhlh133*-orange) and under Fe-deficient conditions (–Fe WT versus *bhlh133*-dark pink). (ii) Venn diagram showing the number of significantly differentially expressed genes ($P < 0.05$, PPDE >0.96) in response to Fe-deficient conditions in the *bhlh133* mutant (+Fe versus –Fe *bhlh133*-light pink) and WT (+Fe versus –Fe WT-blue). (b) Heatmap showing the fold-changes in expression, from each of the four comparisons in shown in the Venn diagrams in (a). Genes that were differentially expressed genes in *Osbhlh133* mutants, independent of Fe conditions, are indicated in the green boxes. Genes that were differentially expressed only in response to Fe-deficient (–Fe) conditions in the *Osbhlh133* mutant plants are indicated in the pink boxes. Significantly over-represented functional categories in these gene-sets [as determined by Pageman statistical analysis, Fisher's test (ORA cut-off value = 1)] are indicated with an asterisk.

Namely, Yellow Stripe-Like transporter genes *OsYSL2* and *OsYSL15*; the DMA transporter gene *OsTOM1*, as well as *OsNAAT1*, *OsNAS1*, *OsNAS2*, *OsNAS3* and *OsDMAS1*, which are essential for DMA biosynthesis. Three metal transporter genes, *OsNramp1* (natural resistance-associated macrophage protein) (Takahashi *et al*. 2011), *OsVIT1;2* (ortholog to the *Arabidopsis* vacuolar iron influxer AtVIT1) (Kim *et al*. 2006) and the rice citrate transport gene *OsFRDL1* (Yokosho *et al*. 2009), were all also up-regulated (Table 1). Two other transcription factors, known to be associated with transcriptional regulation under Fe-deficient conditions (OsIRO2, OsIRO3, Ogo *et al*. 2006, 2007; Zheng *et al*. 2010), were also up-regulated in response to Fe deficiency in both the *bhlh133* and WT plants (Fig. 7b; Table 1). Thirteen of the 28 iron-responsive genes (shown in Table 1) that are induced in response to Fe deficiency in the WT and *bhlh133* mutant were also induced in the *bhlh133* mutant plants under Fe-sufficient conditions. Thus, a Fe-deficiency signal appears to be initiated in the *bhlh133* plants (even in the presence of Fe), that is consistent with the lower Fe concentration seen in the *bhlh133* roots. Fe deficiency also appeared to result in the significant induction of these listed Fe-responsive genes in both the WT and *bhlh133* (Table 1). Notably, the 13 Fe-responsive genes induced in the *bhlh133* under Fe-sufficient condition were expressed at a similar level as that present in the WT under Fe-deficient conditions (Table 1).

DISCUSSION

Among all the predicted members of the bHLH-domain containing transcription factors (Li *et al*. 2006; Pires & Dolan 2010), the bHLH transcription factors of tomato (FER), *Arabidopsis* (FIT, AtbHLH38, AtbHLH39 and PYE) and rice (OsIRO2 and OsIRO3) have been previously reported to be involved in the Fe-deficiency response and homeostasis (Ling *et al*. 2002; Colangelo & Guerinot 2004; Ogo *et al*. 2006; Yuan *et al*. 2008; Long *et al*. 2010; Zheng *et al*. 2010). Similar to these known Fe-responsive bHLH transcription factors, the transcript abundance of *OsbHLH133* has also been observed as induced by Fe deficiency (shown in this study and in data from Zheng *et al*. 2009). Phylogenetic analysis showed that *Arabidopsis* bHLH transcription factors, AtRHD6 and AtRSL1 were closely related with OsbHLH133 (Supporting Information Fig. S6). In *Arabidopsis*, AtRHD6 and AtRSL are reportedly known to be involved in root hair formation (Masucci & Schiefelbein 1994; Menand *et al*. 2007; Yi *et al*. 2010). However, unlike the root hairless or short root hair phenotype seen in AtRHD6 or AtRSL, rice *bhlh133* mutant and the transgenic plants overexpressing *OsbHLH133* have normal root hair formation (data not shown). Physiological analysis showed that the alteration of *OsbHLH133* expression in the T-DNA insertional mutant plants or overexpressing plants affected the Fe distribution between shoots and roots. As OsbHLH133 is not closely related to other known Fe-responsive transcription factors (Supporting Information Fig. S6), it is likely that bHLH133 is involved in Fe homeostasis by, as yet, an unknown mechanism.

As little as 10 μM EDTA-Fe (II) is sufficient for healthy growth of wild-type rice and when plants were supplied with 10 times more Fe (II) (i.e. 100 μM EDTA-Fe (II)), most of the Fe was retained in the roots, while the shoot Fe concentration was strictly regulated (Zheng *et al*. 2010). These findings indicate that there is an important role for roots in preventing excess Fe uptake and translocation to shoots. However, the molecular mechanism(s) controlling these processes are largely unknown. In this study, our results from the physiological analysis of *OsbHLH133*-OE lines and *bhlh133* mutant plants indicate the involvement of OsbHLH133 in controlling Fe homeostasis or distribution between roots and shoots. Overexpression of *OsbHLH133* resulted in a lower shoot Fe concentration, higher root Fe concentration (Fig. 4a) and hypersensitivity to Fe deficiency (Fig. 3b,c). In contrast, the Fe concentration between leaf and root was altered in the *bhlh133* mutant plants, in an opposite manner to that seen in the *OsbHLH133*-OE lines (Fig. 4b).

The possible role of OsbHLH133 as a negative regulator of Fe translocation from roots to shoots, together with the specific induction of *OsbHLH133* seen under Fe deficiency, gives new insights into how plants respond to Fe deficiency at a physiological level. Nutrient deficiency in general, including under Fe deficiency, is known to result in altered root architecture and root hair formation (López-Bucio, Cruz-Ramirez & Herrera-Estrella 2003). These responses occur so that the plant can increase in surface area and explore the soil for additional nutrients. However, root and root hair growth requires Fe (Guerinot & Yi 1994; López-Bucio *et al*. 2003), and, thus, under limited Fe conditions, the available Fe is retained in the roots to allow plants to respond to Fe deficiency.

Under Fe-sufficient conditions, *OsbHLH133* is expressed at a low level in endodermal cells of the maturation zone of

roots, but not in root tips and shoots (Fig. 6a–d); supporting the idea that OsbHLH133 might be involved in the regulation of root to shoot long-distance transportation of Fe. Indeed, the Fe concentration in the xylem sap of *OsbHLH133*-OE lines was also seen to be lower than that in the WT plants (Fig. 5). Under Fe-deficiency conditions, *OsbHLH133* was strongly expressed in roots (Fig. 6e–h), not only in endodermal cells, but also in other cells, suggesting that OsbHLH133 may play a role other than in xylem transportation. In addition, in the aerial parts of the plant, *OsbHLH133* is only expressed in the vascular tissues under Fe-deficient conditions, suggesting that OsbHLH133 is involved in Fe translocation in Fe-deficient leaves.

To date, a number of transporters have been characterized to be involved in xylem and phloem Fe loading, including the *Arabidopsis* FRD3, which mediates efflux of citrate into the root vasculature (Durrett, Gassmann & Rogers 2007), the rice FRD3-like protein OsFRDL1 (Yokosho *et al*. 2009), phenolics efflux zero 1 (PEZ1) (Ishimaru *et al*. 2011), *Arabidopsis* ferroportin 1/iron regulated 1 (AtFPN1/AtIREG1) (Morrissey *et al*. 2009), YSLs, NA, IRT1, NRAMP and a number of others (see review: Guerinot 2010; Kobayashi & Nishizawa 2012). Analysis of global transcript expression changes in rice roots of the *bhlh133* mutant plants and the WT transcriptome in the presence and absence of Fe (Fig. 7a) was expected to detect the differential expression in these transporters. However, no significant difference in the expression of the above transporters genes was found between the WT and the mutant. It is possible that there is differential expression of one (or a few) transporter in a specific tissue or cell type; however, the microarray analysis involved the use of full root samples and may have masked the differences in cell type-specific expression. Further analysis using trace Fe analysis could clarify how OsbHLH133 affects Fe distributions.

A number of genes were altered in expression in the rice *bhlh133* mutant plants, irrespective of Fe growth conditions, and, among these, a significant enrichment of signalling functions were seen in these up-regulated genes. These up-regulated genes encoding signalling functions, (largely calcium signalling and receptor kinases) and represent a novel list of target genes which might be used to investigate how Fe homeostasis may be controlled in rice (Supporting Information Table S3). Furthermore, the reduction in the expression of genes encoding photosynthesis-related functions in *bhlh133* under Fe-deficient growth condition was notable. Plastids and chloroplasts have a high demand for Fe, as co-factors in variety of enzymes; thus, it is envisaged that these functions are limited, and, thus, expression is repressed, in the roots of *bhlh133* mutant plants under Fe-deficient conditions. The down-regulation of genes encoding proteins involved in tetrapyrrole metabolism is also consistent with the reduction in transcript abundance for genes encoding photosynthetic genes, and may also play a role in signalling the down-regulation of photosynthetic genes in this system.

In conclusion, this study has identified OsbHLH133 as a regulator of Fe homeostasis in rice, most likely by negatively regulating Fe transportation from root to shoot. It is shown that under Fe deficiency, the roots prioritize the retention of Fe. In addition, alterations in the expression of genes encoding proteins involved in calcium signalling in *bhlh133* mutant plants, under both Fe-sufficient and deficient conditions, suggest a role for calcium signalling in root-to-shoot translocation of Fe.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Subcellular localization of OsbHLH133. Onion epidermis cells expressing OsbHLH133-sGFP (a–c) and sGFP (d–f). Images were taken under fluorescence (a, d), under transmitted light (b, e) and overlay images (c, f). Bar = 50 μm.

Figure S2. Growth performance, shoot length and seeds set of WT and mutant plants. (a) Gene structure and T-DNA insertion site. (b) Growth performance of mutant plants after 2.5 months cultivation in Fe-sufficient nutrient solution. (c) Shoot length of the plants in the ripening stage. (d) Seeds number per plant. Bar = 3 cm. Data as means ± SD ($n = 3$). Asterisks indicate $P \leq 0.05$.

Figure S3. Fe concentration in xylem sap of mutant plants. Seedlings were grown in the normal nutrient solution for 3 weeks, and then were transferred to nutrient solution with 2 μM, 100 μM and 1000 μM EDTA-Fe (II) for 10 d. Data as means ± SD ($n = 3$).

Figure S4. Analysis of Cu, Zn and Mn concentrations in *OsbHLH133*-OE and mutant plants. Three-week-old seedlings were transferred to nutrient solutions containing 100 μM (+Fe) or 0 μM (–Fe) EDTA-Fe (II) and after 10 d of treatment, metal concentrations in leaves and roots were measured by ICP-MS. Data are shown as the mean ± SD ($n = 3$).

Figure S5. Cu, Zn and Mn concentrations in xylem sap of *OsbHLH133*-OE and mutant plants. Three-week-old seedlings were transferred to nutrient solutions containing 100 μM (+Fe) or 2 μM EDTA-Fe (II) (–Fe), after 10 d treatment, xylem sap was collected. Metal concentrations were measured by ICP-MS. Data are shown as the mean ± SD ($n = 3$).

Figure S6. Neighbour-joining phylogenetic tree of OsbHLH133-like bHLHs with known bHLH transcription factors which function in Fe-deficient response. The amino acid sequences of proteins were used to phylogenetic tree construction.

Table S1. Primer sequences for quantitative RT-PCR.

Table S2. Differentially expressed genes in response to +/–Fe conditions in the WT and *bhlh133* mutant.

Table S3. Genes encoding signalling functions in response to +/–Fe conditions in the WT and *bhlh133* mutant.