The bHLH Transcription Factor OsbHLH057 Regulates Iron Homeostasis in Rice

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Abstract: Many basic Helix-Loop-Helix (bHLH) transcription factors precisely regulate the expression of Fe uptake and translocation genes to control iron (Fe) homeostasis, as both Fe deficiency and toxicity impair plant growth and development. In rice, three clade IVc bHLH transcription factors have been characterised as positively regulating Fe-deficiency response genes. However, the function of OsbHLH057, another clade IVc bHLH transcription factor, in regulating Fe homeostasis is unknown. Here, we report that OsbHLH057 is involved in regulating Fe homeostasis in rice. OsbHLH057 was highly expressed in the leaf blades and lowly expressed in the roots; it was mainly expressed in the stele and highly expressed in the lateral roots. In addition, OsbHLH057 was slightly induced by Fe deficiency in the shoots on the first day but was not affected by Fe availability in the roots. OsbHLH057 localised in the nucleus exhibited transcriptional activation activity. Under Fe-sufficient conditions, OsbHLH057 knockout or overexpression lines increased or decreased the shoot Fe concentration and the expression of several Fe homeostasis-related genes, respectively. Under Fe-deficient conditions, plants with an OsbHLH057 mutation showed susceptibility to Fe deficiency and accumulated lower Fe concentrations in the shoot compared with the wild type. Unexpectedly, the OsbHLH057-overexpressing lines had reduced tolerance to Fe deficiency. These results indicate that OsbHLH057 plays a positive role in regulating Fe homeostasis, at least under Fe-sufficient conditions.

Keywords: rice; bHLH transcription factor; osbhlh057 mutants; Fe deficiency; OE lines; gene expression

1. Introduction

Iron (Fe) is an essential mineral nutrient for plant growth and development as it is responsible for numerous redox and electron transfer reactions, including chlorophyll synthesis and photosynthesis. Although abundant in the earth’s crust, Fe precipitates as insoluble Fe(III) oxides and hydroxides in aerobic or alkaline soils, making Fe unavailable to plants [1,2]. Fe deficiency stress results in leaf chlorosis and growth reduction and hence becomes a limiting factor for crop production and quality [3]. Excess Fe in plant cells, instead, could generate hydroxyl radicals which are toxic to cells via the Fenton reaction, leading to retarded growth [4,5]. Meanwhile, one billion people suffer from Fe deficiency anemia, particularly those who rely on plants for dietary Fe [6]. Thus, discovering sophisticated mechanisms by which plants control Fe homeostasis may profoundly impact crop yield and human nutrition.

Plants use two distinct Fe-uptake strategies, namely the reduction strategy (Strategy I) and the chelation strategy (Strategy II), which are employed by non-graminaceous species and graminaceous species, respectively [7]. In Strategy I plants, such as Arabidopsis (Arabidopsis thaliana), the solubility and mobility of insoluble Fe³⁺ in the rhizosphere are firstly improved by H⁺-ATPase AHA2 pumping protons to lower the pH of rhizosphere and PLEIOTROPIC DRUG RESISTANCE 9/ATP-BINDING CASSETTE G37 (PDR9/ABCG37) secreting coumarins. Then, FERRIC REDUCTASE OXIDASE 2 (FRO2) reduces the Fe³⁺...
at the root cell surface to \( \text{Fe}^{2+} \), which finally is absorbed by IRON REGULATED TRANSPORTER 1 (IRT1) [8–11]. In Strategy II plants, such as rice (Oryza sativa L.), TRANS-PORTER OF MUGINEIC ACID FAMILY PHYTOSIDEROPHORES 1 (TOM1) secretes 2′-deoxymugineic acid (DMA) to chelate \( \text{Fe}^{3+} \), thus enhancing the solubility of \( \text{Fe}^{3+} \) [12]. Then, the \( \text{Fe}^{3+} \)-DMA complex is taken up by YELLOW STRIPE 1-LIKE 15 (OsYSL15) [13]. Rice (Oryza sativa L.) can also directly acquire \( \text{Fe}^{2+} \) via OsIRT1 [14,15].

For the adaptive fluctuation of Fe availability, plants have evolved a sophisticated regulatory mechanism of Fe homeostasis in which conserved basic Helix-Loop-Helix (bHLH) transcription factors (TFs) play a predominant role [16,17]. Studies in the model plants rice and Arabidopsis have revealed that the Ib, IIIa, IVb, and IVc clades of bHLH TFs form a precise regulatory network that contains two interconnected regulatory modules [17]. The first module acts upstream of the Fe uptake and transport genes. Clade lb bHLH, bHLH38, bHLH39, bHLH100, and bHLH101 form heterodimers with clade IIIa bHLH TF FIT/bHLH29 to directly control the uptake of Fe [18–21]. OsIRO2, an Ib TF, can also interact with a clade IIIa bHLH TF Oryza sativa FER-LIKE FE DEFICIENCY-INDUCED TRANSCRIPTION FACTOR (OsFIT)/OsbHLH156. The complex of OsFIT/OsbHLH156 and OsIRO2 directly controls the expression of Strategy II Fe uptake-related genes [22–24]. Unlike the above bHLH TFs, which act as positive regulators, rice OsIRO3 and Arabidopsis PYE, belonging to the IVb clade bHLH, act as negative regulators of some Fe homeostasis-related genes [25–29]. The second module, composed of the IVb and IVc clade bHLH TFs, acts upstream of the first module. It has been demonstrated that three rice clades, IVc bHLH TFs [POSITIVE REGULATORY OF IRON HOMEOSTASIS 1 (OsPRI1)/OsbHLH060, OsPRI2/bHLH058, OsPRI3/OsbHLH059] and Arabidopsis IVc clade bHLH TFs [IRON DEFICIENCY TOLERANT 1 (IDT1)/bHLH034, IAA-LEUCINE RESISTANT 3(ILR3)/bHLH105, bHLH104, and bHLH115], play a positive role in the regulation of Fe deficiency responses by directly regulating lb gene expression [30–36]. Previous studies have suggested that clade IVc bHLH TF activities are regulated by post-transcriptional regulation. HEMERYTHRIN MOTIF-CONTAINING REALLY INTERESTING NEW GENE (RING)- AND ZINC-FINGER PROTEIN 1 (OsHRZ1) in rice and their ortholog BRUTUS (BTS) in Arabidopsis, which are the putative rice Fe sensors, can degrade OsPRI1/OsbHLH060, OsPRI2/bHLH058, and OsPRI3/OsbHLH059 and Arabidopsis IVc clade bHLH TFs [IRON DEFICIENCY TOLERANT 1 (IDT1)/bHLH034, IAA-LEUCINE RESISTANT 3(ILR3)/bHLH105, bHLH104, and bHLH115], play a positive role in the regulation of Fe deficiency responses by directly regulating lb gene expression [30–36]. Previous studies have suggested that clade IVc bHLH TF activities are regulated by post-transcriptional regulation. HEMERYTHRIN MOTIF-CONTAINING REALLY INTERESTING NEW GENE (RING)- AND ZINC-FINGER PROTEIN 1 (OsHRZ1) in rice and their ortholog BRUTUS (BTS) in Arabidopsis, which are the putative rice Fe sensors, can degrade OsPRI1/OsbHLH060, OsPRI2/bHLH058, and OsPRI3/OsbHLH059 and (ILR3)/bHLH105 and bHLH115, respectively [30,32,37,38]. OsbHLH061, another member of clade IVb bHLH, has been proven to be a negative regulator of Fe homeostasis by interacting with OsPRI1 and recruiting TOPLESS/TOPLESS-RELATED (TPL/TPR) repressors [39]. OsIRO3 can also inhibit the transcriptional activity of OsPRI1 by recruiting OsTPL/TPRs [29]. In Arabidopsis, clade IVb bHLH TF bHLH011 also negatively regulates Fe homeostasis by recruiting TPL/TPRs to inhibit clade IVc bHLH TFs [40]. Another clade IVb bHLH TF, bHLH21, forms a complex with clade IVc bHLH TFs to positively regulate Fe homeostasis [41–43]. Thus, the regulatory framework of Fe homeostasis in plants has been proposed to comprise HRZ/BTS \( \rightarrow \) IVc/IVb bHLHs \( \rightarrow \) lb/IVb bHLHs \( \rightarrow \) Fe uptake and transport-related genes. OsbHLH057 is the fourth member of the rice clade IVc bHLH TFs [25]. Recently, functional analysis of OsbHLH057 suggested that it positively regulates disease resistance and drought tolerance [44]. However, to date, there is still a lack of information on the role of OsbHLH057 in the regulation of Fe homeostasis. Therefore, the aim of the present study was to determine whether and how OsbHLH057 is involved in regulating Fe homeostasis.

Here, using a reverse genetic method, we demonstrate that OsbHLH057 is a critical factor that helps maintain Fe homeostasis. We showed that OsbHLH057’s transcript abundance is not changed by Fe deficiency in the roots but is induced in the shoots early in Fe deficiency. OsbHLH057 is a transcription activator localised in the nucleus. The knockout of OsbHLH057 decreased Fe deficiency tolerance, shoot Fe concentration, and some Fe homeostasis-related gene expression. Furthermore, overexpression of OsbHLH057
increased the shoot Fe concentration and the expression of several Fe homeostasis-related genes under Fe-sufficient conditions.

2. Results

2.1. Expression Pattern of OsbHLH057

Sequence analysis revealed that the genomic DNA of OsbHLH057 was composed of 3126 base pairs (bp), containing five exons and four introns (Figure S1A). The protein encoded by OsbHLH057 with 256 amino acids has a typical bHLH-ZIP domain and one OsHRZ-interacting domain identified by Peng et al. (2022) [45] in the C-terminal (Figure S1B). Therefore, we examined whether OsbHLH057 binds to OsHRZ1 and OsHRZ2 by use of a yeast-two-hybrid assay. The results of this assay showed that OsbHLH057 interacts with the C-terminal segments of OsHRZ1 and OsHRZ2 (Figure S2A), which is consistent with previous studies [45]. Moreover, a split luciferase complementation imaging assay showed that OsbHLH057 interacts with OsHRZ1 and OsHRZ2 but not with the negative control (Figure S2B), suggesting that the interaction between OsbHLH057 and OsHRZ1 or OsHRZ2 takes place in planta.

OsbHLH057 expression was investigated in different tissues at different growth stages. The results of reverse transcription quantitative PCR (RT-qPCR) showed that OsbHLH057 was ubiquitously expressed and primarily expressed in the leaf blades and leaf sheaths at all growth stages (Figure 1A). To further investigate whether the expression of OsbHLH057 was affected by Fe availability, we exposed rice plants to Fe deficiency over 7 d and subsequently resupplied them with Fe for 3 d. The expression of OsbHLH057 was unaffected by Fe deficiency in the roots (Figure 1B) and was only slightly induced in the shoots by Fe deficiency at 1 d (Figure 1C).

![Figure 1](image_url)

**Figure 1.** Expression profiling of OsbHLH057 in rice. (A) Reverse transcription quantitative PCR (RT-qPCR) analysis of the expression levels of OsbHLH057 in various rice tissues of different growth stage. (B, C) RT-qPCR analysis of the expression levels of OsbHLH057 in the root (B) and shoot (C) under different Fe supply conditions. (D) GUS staining of various tissues in ProOsbHLH057::GUS transgenic plants, including root tips (RT), basal mature zone of root (BMZ), lateral roots (LR), transverse section of BMZ (TBMZ), transverse section of TR (TLR), leaf (L), leaf sheaths (LS), ligule and auricle (LA), basal node (BN), transverse section of BN (TBN), and transverse section of L (TL). Red line in BN indicates the place of section. Scale bars = 50 μM in TBMZ, TLR, and TL; Scale bars = 1 mm in other pictures.

To further investigate the expression pattern of OsbHLH057 in different tissues, a 2175-bp promoter of OsbHLH057 was used to drive beta-glucuronidase (GUS) expression
in rice plants. GUS staining results suggested that OsbHLH057 was mainly expressed in the meristematic zone of root tips and the stele of the basal mature zone of the root and was highly expressed in the lateral roots (Figure 1D). In the shoot, OsbHLH057 was highly expressed in leaf blades and sheaths but had no expression in leaf ligule and auricle (Figure 1D). In the leaf blades, OsbHLH057 was highly expressed in vascular tissues and mesophyll cells (Figure 1D). Furthermore, the transverse section of the basal node showed that the vascular bundles had strong OsbHLH057 expression (Figure 1D).

2.2. OsbHLH057 Is a Nucleus-Localised Transcription Activator

To explore the subcellular localisation of OsbHLH057, the 35S::OsbHLH057-GFP vector was transiently co-transformed into rice mesophyll protoplasts with the 35S::NLS-mCherry vector. The GFP signal of the OsbHLH057-GFP fusion protein was detected only in the nucleus and overlapped with the mCherry signal of the NLS-mCherry protein, a nuclear marker (Figure 2A). This observation suggests that OsbHLH057 is a nucleus-localised protein, which agrees with the predicted function of OsbHLH057 as a TF. To further examine the transcription activity of OsbHLH057, a dual luciferase reporter assay was performed. In this system, the firefly luciferase (LUC) under the control of five repeats of the GAL4 binding cis-element with mini 35S was used as the reporter construct, in which Renilla luciferase (REN) driven by a constitutive cauliflower mosaic virus (CaMV) 35S promoter was used as an internal control. An effector plasmid was constructed by fusing OsbHLH057 to the GAL4 DNA binding domain (BD), which was driven by the CaMV 35S promoter, and the blank vector 35S::BD was used as the control effector (Figure 2B). As expected, the fusion protein BD-OsbHLH057-transfected tobacco leaves had a much higher value of LUC to REN than the control effector, indicating that OsbHLH057 had transcription activation activity. These results suggest that OsbHLH057 is a nucleus-localised transcription activator.

![Figure 2](image_url)
Two OsbHLH057 gene sequences in the first and second exons were selected as mutation sites (Figure S1A). The two homozygous osbhlh057 mutants (osbhlh057-1 and osbhlh057-2) were identified by sequencing. osbhlh057-1 and osbhlh057-2 had a deletion of ‘C’ and an insertion of ‘A’, respectively (Figure S3A), both of which resulted in the appearance of a frameshift mutation and a premature stop codon (Figure S3B). Then, we compared the growth performance of wild type (WT) and osbhlh057 mutants under both Fe-sufficient and -deficient conditions. Regardless of Fe availability, the growth performance of the osbhlh057 mutants was apparently poorer than that of the WT, which was consistent with the lower biomass of roots and shoots in the osbhlh057 mutants compared with the WT (Figure 3A,D,E). After one week of Fe deficiency treatment, the new leaves from plants under Fe-deficient conditions showed chlorosis, a typical phenotype of Fe deficiency. The new leaves of the osbhlh057 mutants had more chlorosis than the WT (Figure 3B). Consistent with this observation, the leaf soil and plant analyzer development (SPAD) values of the osbhlh057-1 and osbhlh057-2 mutants were significantly lower than those of the WT (Figure 3C).

**Figure 3.** Phenotypes of OsbHLH057 loss-offunction mutants. 12-day-old seedlings of WT, osbhlh057-1, and osbhlh057-2 were shifted in nutrient solution with or without Fe for 7 d. (A) Pictures of 19-d-old seedlings. Scar bars, 5 cm. (B) New leaves from seedlings under Fe-deficient condition. Magnification of part leaves in the dotted line in (A) with 5 times. (C) The SPAD of the third leaves. (D,E) Root and shoot biomass. Root (D) and shoot (E) dry weight. (F,G) Fe concentration in the WT, osbhlh057-1, and osbhlh057-2. Root (F) and shoot (G) Fe concentration. Data in (C–E) and (F,G) represent the means ± standard deviation (SD) of six and three biological replicates, respectively. Means with different letters are statistically significant differences as determined by one-way ANOVA followed by Duncan’s multiple-range test (p < 0.05).

Furthermore, we measured the Fe concentration in the osbhlh057 mutants. Under both Fe-sufficient and -deficient conditions, the root Fe concentrations of the osbhlh057 mutants were similar to that of the WT (Figure 3F). The shoot Fe concentrations in the osbhlh057 mutants were significantly lower than that in the WT control, regardless of external Fe availability (Figure 3G).

2.4. Overexpressing OsbHLH057 Enhances Fe Accumulation in the Shoot under Fe-Sufficient Conditions

To further clarify the functions of OsbHLH057 in the regulation of Fe homeostasis, we generated OsbHLH057 overexpression transgenic plants containing the full-length coding sequence of OsbHLH057 driven by a CaMV 35S promoter. RT-qPCR analysis indicated that the transcript abundance of OsbHLH057 was significantly higher in the two inde-
dependent overexpression plants (OsbHLH057-OE-2 and OsbHLH057-OE-4) than in the WT control (Figure S4). Compared with the WT plants, both OsbHLH057 overexpressing lines displayed shorter roots and shoots with less biomass under Fe-sufficient and -deficient conditions (Figure 4A,D,E). Under Fe-sufficient conditions, the Fe concentration in the shoots of OsbHLH057-OE-2 and OsbHLH057-OE-4 was higher than that of the WT, as expected (Figure 4G). Additionally, the root Fe concentration was significantly lower in OsbHLH057-OE-2 than in the WT and decreased in OsbHLH057-OE-4 compared to the WT, but there was no statistical difference (p-value = 0.0739) (Figure 4F). Under Fe-deficient conditions, the new leaves of OsbHLH057-OE-2 and OsbHLH057-OE-4 plants showed more chlorosis, and lower SPAD values than the WT. The shoot Fe concentrations of the OsbHLH057-OE-2 and OsbHLH057-OE-4 plants were lower than that of the WT, but there was no statistical difference between the WT and OsbHLH057-OE-4 plants, except for those that were unaccepted (Figure 3A–C,G). In addition, there was no substantial alteration in the root Fe concentration between the WT and OsbHLH057-overexpressing lines under Fe-deficient conditions (Figure 4G). These observations indicate that OsbHLH057 overexpression results in Fe overaccumulation in the shoot under Fe-sufficient conditions but causes hypersensitivity to Fe deficiency.

2.5. Expression of Fe Homeostasis-Related Genes in the WT and OsbHLH057 Knockout Mutant or Overexpression Lines

Based on these results, OsbHLH057, as a transcription factor, was expected to maintain Fe homeostasis by controlling the expression of many Fe homeostasis-related genes. Therefore, we examined gene expression changes in the WT and osbhlh057 mutant plants. In rice, OsNAS1, OsNAS2, TOM1, OsYSL15, and OsNATT1 are representative Fe-deficiency response genes involved in Fe uptake and transport. In our RT-qPCR assay, the expression of these genes was strongly induced by Fe deficiency, as expected, and showed similar expression levels in Fe-deficient roots of WT and osbhlh057 mutant plants under Fe-deficient conditions (Figure 5). However, the transcript abundances of OsNAS1, OsNAS2, TOM1, OsYSL15, and OsNATT1 in the osbhlh057 mutant were significantly repressed compared with the WT under Fe-sufficient conditions (Figure 5). The rice bHLH protein OsIRO2 was a...
vital regulator of Fe homeostasis that controls the expression of OsNAS1, OsNAS2, TOM1, OsYSL15, and OsNATT1. Therefore, we quantified the expression level of OsIRO2 in the WT and osbhlh057-1. Similar to OsNAS1, OsNAS2, TOM1, OsYSL15, and OsNATT1, the OsIRO2 expression level in osbhlh057-1 was lower than in the WT under Fe-sufficient conditions but identical to the WT under Fe-deficient conditions (Figure 5).

We further investigated the gene expression changes of OsNAS1, OsNAS2, TOM1, OsYSL15, OsNATT1, and OsIRO2 in OsbHLH057-overexpressing transgenic plants. Compared to the WT, the expression levels of these genes were strongly upregulated in the roots of the OsbHLH057-overexpressing lines under Fe-sufficient conditions (Figure 6). Under Fe-deficient conditions, whereas the expression levels of OsNAS2 and OsYSL15 were significantly downregulated in the OsbHLH057-overexpressing lines compared with the WT, the expression levels of TOM1, OsYSL15, OsNATT1, and OsIRO2 were no different between the WT and OsbHLH057-overexpressing lines, except that OsTOM1 expression was lower in the OsbHLH057-OE-4 than in the WT (Figure 6).

![Figure 5. Expression of Fe homeostasis-related genes in the WT and osbhlh057-1 mutant. 12-day-old seedlings of the WT and osbhlh057-1 mutant were transferred to 1/2 Kimura B solution containing 2 μM FeSO4 or 0 μM FeSO4 for 4 days. The roots were sampled for gene expression analysis. OsActin1 was used as an internal control. Data are means ± SD of three biological replicates. Asterisks indicate significant differences between WT and osbhlh057-1 based on two-tailed Student’s t-test (*p < 0.05).](image1)

![Figure 6. Expression of Fe homeostasis-related genes in the WT and OsbHLH057 overexpression lines. 12-day-old the WT and OsbHLH057 overexpression seedlings were transferred to nutrient solution with 2 μM FeSO4 or without FeSO4 for 7 days. The expression of OsNAS1 (A), OsNAS2 (B), OsTOM1 (C), OsYSL15 (D), OsNATT1 (E), and OsIRO2 (F) in the roots were analyzed. OsActin1 was used as an internal control. Data are means ± SD of three biological replicates. Means with different letters indicate statistically significant differences as determined by one-way ANOVA followed by Duncan’s multiple-range test (p < 0.05).](image2)
These results indicate that OsbHLH057 positively regulates the expression of many Fe homeostasis-related genes, at least under Fe-sufficient conditions.

3. Discussion

Plants could precisely sense and transmit Fe signals to TFs to properly regulate the expression of Fe homeostasis-related genes [30–32]. In rice, three clade IVc bHLH TFs, OsPRI1/OsbHLH060, OsPRI2/bHLH058, and OsPRI3/OsbHLH059, facilitate Fe homeostasis by positively regulating the expression of Fe uptake and transport-related genes. As one of the clades IVc bHLH TFs, whether OsbHLH057 is involved in regulating Fe homeostasis in rice is unknown. Determining how OsbHLH057 maintains iron homeostasis benefits a comprehensive understanding of the rice Fe homeostasis network and breeding of Fe-fortified rice. In the present study, we show that OsbHLH057 is an essential regulator in controlling Fe homeostasis.

Through RT-qPCR analysis, we found that OsbHLH057 expression was unaffected by external Fe availability in the roots but was slightly induced by Fe deficiency and was highly expressed in the leaves (Figure 1B,C). Previous studies showed that OsbHLH057 expression was somewhat influenced by Fe shortage in both roots and leaves [31] and was elevated in the leaves compared to the roots. The slight difference in the results of OsbHLH057 expression in the roots under Fe-deficient conditions may be attributed to different growth conditions. For other IVc bHLH genes, Zhang et al. (2017, 2020) reported that the gene expression of OsbHLH058/059/060 was unaffected by deficiency [30,32]. Kobayashi et al. (2019) found that OsbHLH060 expression was slightly induced by Fe deficiency. OsbHLH059 expression was unaffected by Fe deficiency, and OsbHLH058 was repressed by Fe deficiency [31]. These results suggest that IVc bHLH genes are not easily changed by Fe deficiency at the transcript level.

Studying osbhlh057 loss-of-function mutants revealed that OsbHLH057 is essential for the response to Fe deficiency and for maintaining Fe homeostasis (Figure 3). Compared with the WT, OsbHLH057 knockout resulted in more chlorosis in new leaves under Fe-deficient conditions and decreased the Fe concentration in the shoots under both Fe-sufficient and -deficient conditions (Figure 3). In addition, the Fe concentration in the OsbHLH057-overexpressing lines further supports the idea that OsbHLH057 acts as a positive regulator of Fe homeostasis. The shoot Fe concentration in the plants overexpressing OsbHLH057 was increased by 33.8–46.8% compared with the WT when grown under Fe-sufficient conditions (Figure 4G). Furthermore, we explored the mechanism by which OsbHLH057 positively regulates Fe homeostasis. OsNAS1, OsNAS2, OsTOM1, OsYSL15, OsNAAT1, and OsIRO2 were representative of Fe uptake- and transport-related genes and Fe deficiency-induced genes. In our assay, the expression levels of OsNAS1, OsNAS2, OsTOM1, OsYSL15, OsNAAT1, and OsIRO2 were strongly induced by Fe deficiency, but this process was unaffected by the osbhlh057 mutants. However, the expression of these genes was repressed in the osbhlh057 mutants but enhanced in the OsbHLH057-overexpressing lines under Fe-sufficient conditions (Figures 5 and 6). Consistent with this finding, we demonstrated that OsbHLH057 exhibited transcriptional activation activity (Figure 2B,C). Moreover, OsIRO2 expression was regulated by OsbHLH057, indicating that OsbHLH057 acts upstream of OsIRO2. Recently, OsbHLH057 was found to interact with OsHRZ1 and OsHRZ2 in a yeast-two-hybrid assay. We demonstrated that OsbHLH057 physically interacted with OsHRZ1 and OsHRZ2, which are situated upstream of the Fe homeostasis network, in plants and yeast (Figure S2). These data suggest that OsbHLH057 acts upstream of the Fe homeostasis network. Therefore, OsbHLH057 is crucial for Fe uptake and transport-related gene expression and hence facilitates Fe homeostasis under Fe-sufficient conditions.
To our surprise, the OsbHLH057-overexpressing lines displayed chlorotic leaves and decreased Fe concentration in the shoots when grown in low-Fe conditions; no statistical difference was observed between the WT and OsbHLH057-OE-4 (Figure 4). This unexpected result may be attributed to the secondary effect of OsbHLH057 driven by the CaMV 35S promoter. First, the ubiquitous expression of the 35S promoter changes the tissue-specified expression of OsbHLH057, which may be critical for OsbHLH057 function under Fe-deficient conditions. Secondly, bHLH34/104/105/115, Arabidopsis IVc bHLH TFs, can form homo- or heterodimers, affecting their regulation activity [34–36]. Therefore, rice IVc bHLH TFs may also form homo- or heterodimers, and the high expression level of OsbHLH057 may disorder the balance of the dimerization process, especially under Fe-deficient conditions where OsbHLH057 is likely not easily degraded by OsHRZ1/2. Another explanation for the susceptibility to Fe deficiency in OsbHLH057-overexpressing lines may be attributed to possessing some targets that differ from their paralogs. In Arabidopsis, the overexpression of bHLH105 (ILR3) also showed chlorotic leaves, which may contribute to the downregulation of genes encoding chloroplast proteins, and AtNEET, which functions as a Fe-S/Fe donor in chloroplasts [46]. Thus, OsbHLH057 may indirectly downregulate some genes encoding chloroplast-related proteins to influence Fe uptake and transport under Fe-deficient conditions.

Our data indicate that the effects of OsbHLH057 on regulating Fe uptake and transport are not entirely similar to their paralogs. Previous studies have shown that all mutants of OsPRI1/OsbHLH060, OsPRI2/bHLH058, and OsPRI3/OsbHLH059 accumulate higher levels of Fe in the roots but lower levels in the shoots, suggesting that Fe translocation from root to shoot is impaired [30,32]. For the osbhlh057 mutants, although the Fe concentration in the roots was not significantly different, the Fe concentration in the shoots was considerably lower than in the WT (Figure 3F,G). This result supports that the translocation of Fe from root to shoot is disrupted in the osbhlh057 mutants, which is further supported by the higher Fe concentration in the shoots but lower Fe concentration in the roots of the OsbHLH057-overexpressing lines under Fe-sufficient conditions (Figure 4F,G). Although there was no significant difference (p-value = 0.0739) based on statistical analysis, the root Fe concentration in OsbHLH057-OE-4 decreased 17% compared to the WT. The expression of OsbHLH057 in the roots was mainly expressed in the stele (Figure 1D), which is also related to the function of OsbHLH057 in regulating the translocation of Fe to the shoot. Unlike the lower Fe concentrations in the roots of the OsbHLH057-overexpressing lines, the OsPRI2/bHLH058- and OsPRI3/OsbHLH059-overexpressing transgenic plants contained higher Fe concentrations in both the roots and shoots [32]. The differences among the lines overexpressing OsbHLH057, OsPRI2/bHLH058, and OsPRI3/OsbHLH059 suggests that their effects on regulating Fe uptake and transport have some nuance. A slight difference also appeared in the regulation of Fe homeostasis-related genes compared with the WT; the expression of Fe homeostasis-related genes was repressed in the osbhlh057 mutants only under Fe-sufficient conditions (Figure 5) but repressed in the mutants of OsPRI1/OsbHLH060, OsPRI2/bHLH058, and OsPRI3/OsbHLH059 under both conditions [30,32].

In conclusion, we developed a schematic function model for OsbHLH057 (Figure 7). Under Fe-sufficient conditions, loss-of-function or overexpression of OsbHLH057 did not affect the SPAD value but decreased or increased the shoot Fe concentration and the expression of Fe homeostasis-related genes; overexpression of OsbHLH057 led to a slight decrease in the root Fe concentration. Under Fe-deficient conditions, both loss-of-function and overexpression of OsbHLH057 resulted in a decreased SPAD value and shoot Fe concentration but no change in root Fe concentration and gene expression compared with the WT.
Figure 7. A schematic of OsbHLH057 in regulating Fe homeostasis. The effect of loss-of-function and overexpression of OsbHLH057 on increasing (up arrows) or decreasing (down arrows) physiological processes in the roots and shoots under Fe-sufficient (+Fe) and Fe-deficient (−Fe) conditions. Black arrows, SPAD value; pink, Fe concentration; white, gene expression.

4. Materials and Methods

4.1. Plant Materials and Growth Conditions

Using a CRISPR-Cas9 genome editing system [47], we generated OsbHLH057-knockout mutants (osbhlh057-1 and osbhlh057-2) in the Oryza sativa cv. Nipponbare background. Guide RNA (gRNA1) and gRNA2 sequences were selected and ligated into the SK-gRNA vector, and then the SK-gRNA1 and SK-gRNA2 vectors were cut with Kpn I and Bgl II. Finally, the segments containing gRNA1 and gRNA2 were ligated into the pC1300-Cas9 vector cut with Kpn I and BamHI, respectively. To test the role of OsbHLH057, we created two OsbHLH057-overexpressing lines in the Nipponbare background in which two independent lines (OsbHLH057-OE-2 and OsbHLH057-OE-4) were used for the subsequent analysis. For the construction of the OsbHLH057-overexpressing vector, the full-length CDS of OsbHLH057 was cloned from the cDNA of Nipponbare and first inserted into the vector pDONR221 and finally recombined into the vector pGWB2 [48]. To construct the ProOs-bHLH057::GUS vector, a 2175-bp genomic DNA was amplified by 2×Hieff Canace® Plus PCR Master Mix (Yeasen, Shanghai, China) and recombined into the vector pCAMBIA1300-GUS using ClonExpress® II One Step Cloning Kit (Vazyme, Nanjing, China). The above resultant plasmids were transferred to Agrobacterium tumefaciens EHA105. Transformations to the callus of Nipponbare were carried out as described previously [49]. All primers used for the construction of vectors are listed in Supplemental Table S1.

After germination in water for two days at 37 °C, the seeds were transferred to a net floating on 0.5 mM CaCl₂ solution and kept dark for three days. At the fourth day, CaCl₂ solution was replaced by one-half-strength Kimura B solution containing 0.18 mM (NH₄)₂SO₄, 0.27 mM MgSO₄, 0.09 mM KNO₃, 0.18 mM Ca(NO₃)₂, 0.09 mM KH₂PO₄, 0.50 μM MnCl₂, 3.00 μM H₃BO₃, 1.00 μM (NH₄)₆Mo₇O₂₄, 0.40 μM ZnSO₄, 0.20 CuSO₄, and 2.00 μM FeSO₄. The solution pH was adjusted to 5.5 and renewed every two days. Seedlings were grown in a greenhouse with 14 h 30 °C: 10 h 25 °C, light: dark cycles. For Fe deficiency treatments, the FeSO₄ was removed from the solution.

4.2. RNA Isolation and RT-qPCR

To examine the tissue-specific expression of OsbHLH057 in different growth stages, different tissues, including root, basal node, leaf blade, leaf sheath, node I, panicle, and seed, from rice plants at vegetative growth, flowering, or grain filling were sampled, as described previously [50]. To investigate the Fe deficiency response of OsbHLH057, two-week-old seedlings were treated without Fe for seven days (7 d) and then resupplied with 2 μM FeSO₄ for 3 d. The roots and shoots were collected at 1, 3, 5, and 7 d in Fe deficiency treatment and 1 and 3 d in the Fe resupply stage. For examining the relative expression of OsbHLH057 in the OsbHLH057-overexpressing lines, roots of the wild type (WT) and
OsbHLH057-overexpressing lines cultivating in the solution with 2 µM FeSO₄ was collected and stored at −80 °C.

For analyzing gene expression influenced by OsbHLH057, wild type, OsbHLH057-knockout mutants, and OsbHLH057-overexpressing lines were planted in solution with or without Fe for one week, and the roots were collected for RNA extraction. Total RNA was extracted using TaKaRa Universal RNA Extraction Kit (TaKaRa, Dalian, China) and then synthesized to cDNA using TaKaRaPrimeScript™ 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China). The subsequent cDNA was used for real-time quantitative PCR (RT-qPCR) using ChamQ™ SYBR® Color qPCR Master Mix (Vazyme, Nanjing, China) on a Mastercycler® ep realplex real-time PCR system (Eppendorf, Hamburg, Germany). OsActin1 was amplified as an internal control. The relative gene expression level was calculated by the Equation 2

\[ \Delta\Delta C_t \]. All primers used for RT-qPCR are listed in Supplemental Table S1.

4.3. Subcellular Localization Analysis

For subcellular localization, the coding sequence (CDS) without stop codon of OsbHLH057 was cloned into the N terminus of GFP in the pYL322-d1-eGFP vector using ClonExpress® II One Step Cloning Kit (Vazyme, Nanjing, China) to generate 35S::OsbHLH057-GFP vector. The 35S::NLS-mCherry vector was used as a nuclear marker. The co-transformed 35S::OsbHLH057-GFP and 35S::NLS-mCherry vectors were transiently expressed in rice protoplasts, as described previously [51]. As a negative control, 35S::GFP and 35S::NLS-mCherry vectors were also co-transformed. The fluorescence signals were observed using a laser confocal microscope (UltraVIEW VOX, PerkinElmer, Waltham, MA, USA). Primers used for subcellular localization are listed in Supplemental Table S1.

4.4. Transcription Activity Analysis

For the GAL4-dependent chimeric transactivation assay, transient dual-luciferase expression assays were performed. The full-length CDS of OsbHLH057 was cloned and fused into the effector vector pCAMBIA1300-BD, creating 35S::BD-OsbHLH057. The reporter plasmid 5×GAL4-mini35S::firefly luciferase (LUC) containing 35S::renilla firefly luciferase (REN) internal control was used before [39]. Combinations of these effector vectors (35S::BD-OsbHLH057 and 35S::BD) and reporter vectors were transformed into tobacco leaves using Agrobacterium-mediated transformation, as described previously [39]. In Agrobacterium-mediated transformation, the bacteria expressing the corresponding vector were cultured, harvested, and re-suspended in Murashige and Skoog-MES medium containing 10 mM MES, 0.2 mM acetosyringone, and 10 mM MgCl₂ (pH = 5.6) to the highest concentration of OD600 = 0.5. Then, the Agrobacterium were mixed and infiltrated into N. benthamiana leaves. The infiltrated leaves were sampled for measuring LUC and REN activities using a Dual-Luciferase Reporter Assay Kit (Yeasen, Shanghai, China). The activity of LUC to REN under BD control was set to 1. The primers used for transcription activation assays are given in Supplemental Table S1.

4.5. Yeast-Two-Hybrid Assay

For the yeast-two-hybrid-assay, the full-length CDS of OsbHLH057 was cloned and fused into the vector pGADT7 (AD) to generate AD-OsbHLH057. The C terminus of OsHRZ1 and OsHRZ2 were inserted into the vector pGBK7T (BD) to form BD- OsHRZ1C and BD-OsHRZ2C, respectively. These AD and BD vectors were transformed into AH109 cells. After culturing on the synthetic dropout nutrient medium lacking tryptophan and leucine and the synthetic dropout nutrient medium lacking tryptophan, leucine, histidine, and adenine plates at 30 °C for 2 d. The yeast cells could grow on both selective mediums, which indicated protein–protein interactions. The primers used for the yeast-two-hybrid-assay are listed in Supplemental Table S1.
4.6. Split-LUC Complementation Assay

The C terminus of OsHRZ1 or OsHRZ2 without a stop codon was amplified from rice cDNA and inserted into pCAMBIA1300-nLUC [52] vector, and the full-length coding sequence of OsbHLH057 was amplified and fused with cLUC in the vector of pCAMBIA1300-cLUC [52] through homologous recombination using the ClonExpress II One Step Cloning Kit (Vazyme, Nanjing, China). Combination proteins, OsHRZ1C-nLUC or OsHRZ2C-nLUC and cLUC-OsbHLH057, cLUC-OsbHLH057 and nLUC, OsHRZ1C-nLUC or OsHRZ2C-nLUC and nLUC, and cLUC and nLUC were transformed into tobacco leaves via Agrobacterium-mediated transformation, as described above transcription activity assay. The transinfected leaves were sampled for LUC signal detection using a Tanon 5200 Multi automatic chemiluminescence/fluorescence image analyzer (Tanon, Shanghai, China). The reconstruction of the LUC signal indicated the occurrence of interaction between proteins. The primers used for the construction of vectors are listed in Table S1.

4.7. Measurement of SPAD Values and Fe Concentrations

12-day-old seedlings of the WT and OsbHLH057-knockout and -overexpressing were transferred in nutrient solution containing 2 or 0 µM FeSO₄ and grown for 7 d. The portable chlorophyll meter (SPAD-502; Konica Minolta Sensing, Osaka, Japan) was used to measure the SPAD values of the new fully expanded leaves. The roots and shoots were collected for Fe concentration analysis. The method used to digest the roots and shoots was according to Dong et al., 2018 [53]. The Fe concentration was examined using inductively coupled plasma mass spectrometry (ICP-MS; NexION 300X; Perkin-Elmer, Waltham, MA, USA).

4.8. Histochemical GUS Staining

Histochemical GUS staining was performed in the ProOsbHLH057::GUS transgenic rice plants. Various organs of seedlings grown in nutrient solution containing 2 µM FeSO₄ were harvested and subjected to GUS staining as described previously [24]. After vacuum treatment for 30 min, the samples were incubated at 37 °C overnight and decolorized with 95% ethanol. Photographs were taken with a stereo microscope (Nikon, Tokyo, Japan). Sections of 20 µm thickness were cut and photographed using a vibratome (VT1200S, Leica, Nussloch, Germany) and a microscope (DM500, Leica, Nussloch, Germany), respectively.

4.9. Statistical Analysis

Data analysis was performed using SPSS v.20.0 (IBM Corp, Armonk, NY, USA). Data were shown as means ± SD. Differences in the means between two groups were compared using a two-tailed Student’s t-test and among three or more groups using one-way ANOVA followed by Duncan’s multiple-range test.

4.10. Accession Numbers

Sequence data from this article can be found in the Rice Genome Annotation Project database under the following accession number: OsbHLH057 (LOC_Os07g35870), Os-PRI1 (LOC_Os08g04390), OsPRI2 (LOC_Os05g38140), OsPRI3 (LOC_Os02g02480), OsIRO2 (LOC_Os01g72370), OsNAS1 (LOC_Os03g19427), OsNAS2 (LOC_Os03g19420), OsNAAT1 (LOC_Os02g20360), OsTOM1 (LOC_Os11g04020).

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