

# Comparative Transcriptomics of Lowland Rice Varieties Uncovers Novel Candidate Genes for Adaptive Iron Excess Tolerance

Saradia Kar<sup>1,2,†</sup>, Hans-Jörg Mai<sup>1,†</sup>, Hadeel Khalouf<sup>1</sup>, Heithem Ben Abdallah<sup>1</sup>, Samantha Flachbart<sup>3</sup>, Claudia Fink-Straube<sup>4</sup>, Andrea Bräutigam<sup>5</sup>, Guosheng Xiong<sup>6</sup>, Lianguang Shang<sup>6</sup>, Sanjib Kumar Panda<sup>2,7</sup> and Petra Bauer<sup>1,8,\*</sup>

<sup>1</sup>Institute of Botany, Heinrich Heine University, Universitätsstr. 1, Düsseldorf 40225, Germany

<sup>2</sup>Plant Molecular Biotechnology Laboratory, Department of Life Science and Bioinformatics, Assam University, Silchar, India

<sup>3</sup>Institute of Plant Biochemistry, Heinrich Heine University, Universitätsstr. 1, Düsseldorf 40225, Germany

<sup>4</sup>Leibniz Institute for New Materials, Saarbrücken 66123, Germany

<sup>5</sup>Faculty of Biology, Bielefeld University, Universitätsstr. 27, Bielefeld 33615, Germany

<sup>6</sup>Agricultural Genomics Institute at Shenzhen, Chinese Academy of Agricultural Sciences, Shenzhen 518120, China

<sup>7</sup>Department of Biochemistry, School of Life Sciences, Central University of Rajasthan, Ajmer, Rajasthan 305817, India

<sup>8</sup>Cluster of Excellence on Plant Science (CEPLAS), Heinrich Heine University, Düsseldorf, Germany

<sup>†</sup>These authors are joint first co-authors.

\*Corresponding author: E-mail, petra.bauer@hhu.de; Fax, +49-211-81-12881.

(Received 2 October 2020; Accepted 29 January 2021)

Iron (Fe) toxicity is a major challenge for plant cultivation in acidic waterlogged soil environments, where lowland rice is a major staple food crop. Only few studies have addressed the molecular characterization of excess Fe tolerance in rice, and these highlight different mechanisms for Fe tolerance. Out of 16 lowland rice varieties, we identified a pair of contrasting lines, Fe-tolerant Lachit and -susceptible Hacha. The two lines differed in their physiological and morphological responses to excess Fe, including leaf growth, leaf rolling, reactive oxygen species generation and Fe and metal contents. These responses were likely due to genetic origin as they were mirrored by differential gene expression patterns, obtained through RNA sequencing, and corresponding gene ontology term enrichment in tolerant vs. susceptible lines. Thirty-five genes of the metal homeostasis category, mainly root expressed, showed differential transcriptomic profiles suggestive of an induced tolerance mechanism. Twenty-two out of these 35 metal homeostasis genes were present in selection sweep genomic regions, in breeding signatures, and/or differentiated during rice domestication. These findings suggest that Fe excess tolerance is an important trait in the domestication of lowland rice, and the identified genes may further serve to design the targeted Fe tolerance breeding of rice crops.

**Keywords:** Iron toxicity • Iron uptake • Leaf bronzing • Metal homeostasis • *Oryza sativa* • Oxidative stress • Rice • RNA sequencing • Susceptible • Tolerant •

## Introduction

Iron (Fe) is a vital cofactor for many redox processes in plants. This micronutrient is needed during photosynthesis,

respiration, nutrient assimilation and in many other metabolic pathways. Fe is very abundant in the soil. Since it is mostly bound to ferric hydroxides, Fe is however often not readily bioavailable in the soil, and plants actively mobilize Fe. Therefore, plants acidify the soil, chelate Fe by small organic molecules and reduce ferric (Fe<sup>3+</sup>) to ferrous Fe (Fe<sup>2+</sup>) (strategy I, found in most plant species) or they chelate Fe<sup>3+</sup> with phytosiderophores (strategy II, in Poaceae) (reviewed by Brumbarova et al. 2015). Some environmental conditions, on the other hand, favor the dissolution of high amounts of Fe<sup>2+</sup> in a way that Fe is toxic to plants. Fe excess toxicity occurs in acidic soils with an anaerobic environment through heavy irrigation, rainfall or reduced drainage, further intensified by organic matter, low cation exchange, richness in Fe oxides and sulfate in the soil (Mahender et al. 2019).

Rice (*Oryza sativa*) is among the top most widely consumed staple foods (Das et al. 2010). More than 700 million tonnes of rice grains are produced on a yearly basis, about 90% in Asia and less in Africa (Muthayya et al. 2014). Despite being a strategy II plant, rice acquires Fe directly as Fe<sup>2+</sup> in paddy field conditions (Ishimaru et al. 2006). Northeast India has high potential for the cultivation of diverse lowland rice varieties. However, its soils are strongly acidic with measured pH ranges from 4.4 to 6 (Reza et al. 2012, Baruah et al. 2013, Barthakur 2018). In this region, bioavailable Fe is abundant leading to Fe toxicity as a major constraint affecting the annual yield (Baruah et al. 2007, Ahmed et al. 2011). The available Fe amounts in Northeast Indian waterlogged soils have been measured to be very high in the range of 100–2,000 mg (=1–35 mmol) per kg (Mahender et al. 2019). High soil Fe

bioavailability of 5 mM Fe has also been found in South America (Stein et al. 2014).

A typical symptom of Fe toxicity under acidic conditions is leaf bronzing with stress-induced anthocyanin pigmentation and necrotic lesions leading in severe cases even to plant failure (Tanaka et al. 1966). Fe toxicity is caused by oxidative stress.  $\text{Fe}^{3+}$  and  $\text{Fe}^{2+}$  stimulate the generation of hydroxyl and lipid alkoxyl radicals, through the Haber–Weiss and Fenton reactions. These radicals attack proteins, DNA and lipids, modify their reactive groups and cause decomposition of these biomolecules and mutations (Le et al. 2019).

Rice is among the most tolerant cereal crops, perhaps also due to its domestication and selection on hypoxic conditions. Hydroponic Fe toxicity experiments have been performed with rice as long-term studies with concentrations ranging between 1 and 10 mM  $\text{Fe}^{2+}$  during 2–4 weeks of exposure, or as pulse stress treatments for 1–5 d with concentrations of 5–15 mM  $\text{Fe}^{2+}$  (Wu et al. 2014, Müller et al. 2015, Stein et al. 2019). Few concrete breeding target loci for Fe toxicity tolerance are known (Zhang et al. 2017). Analysis of genome-wide transcriptional response patterns revealed genotype–environment interactions and Fe tolerance response clusters and genes, which are partly at the origin of high Fe tolerance mechanisms described for rice at the morpho-physiological level. Several rice varieties tolerant to Fe toxicity exclude Fe, chelate or eliminate it, e.g. by repressing Fe acquisition in the root, forming an effective root plaque barrier to Fe and promoting root cell wall lignification, preventing Fe translocation to shoots, sequestering Fe in the cell wall and vacuole and/or chelating Fe by metal chelator nicotianamine (Tripathi et al. 2014, Wu et al. 2014, Aung et al. 2019, Stein et al. 2019). Exclusion and sequestration lower free cytoplasmic Fe levels and are sometimes complemented by a second level of tolerance, which is to prevent the cellular Fe and reactive oxygen species (ROS) toxicity through the action of antioxidants (Tripathi et al. 2014, Wu et al. 2014, Aung et al. 2019, Stein et al. 2019). Other effects may also occur, e.g. potassium transport may block root-to-shoot transport of Fe under high Fe stress (Wu et al. 2019, Diop et al. 2020). These studies show that different mechanisms contribute to high Fe tolerance in different accessions of rice. To improve Fe excess tolerance in plants, it is necessary to collect more data about genes associated with this condition and importantly investigate the relevance of such tolerance genes during domestication and selection.

Novel mutations become fixed in a population if their effects are beneficial for the population, referred to as selective sweep. Genome research in rice has uncovered genomic regions important during domestication and breeding (Xie et al. 2015). Wild rice *Oryza rufipogon* is the immediate ancestor of modern rice, and domestication sweeps have occurred in the history of *O. sativa* (Zhang et al. 2012). Genomic resources and tools are available to determine the relevance of genes for domestication (Chen et al. 2019).

Here, we screened 16 lowland rice varieties from Northeast India for excess Fe tolerance in terms of root and shoot growth to identify Fe-tolerant Lachit and susceptible Hacha with contrasting abilities at the molecular–physiological level to cope

with short-term excess Fe stress. Comparative RNA sequencing (RNA-seq) identified 35 metal homeostasis genes and physiological pathways associated with excess Fe tolerance. Two-third of these genes were under selection during rice domestication, indicating their functional relevance. These findings can be used to design the targeted Fe tolerance breeding of rice crops.

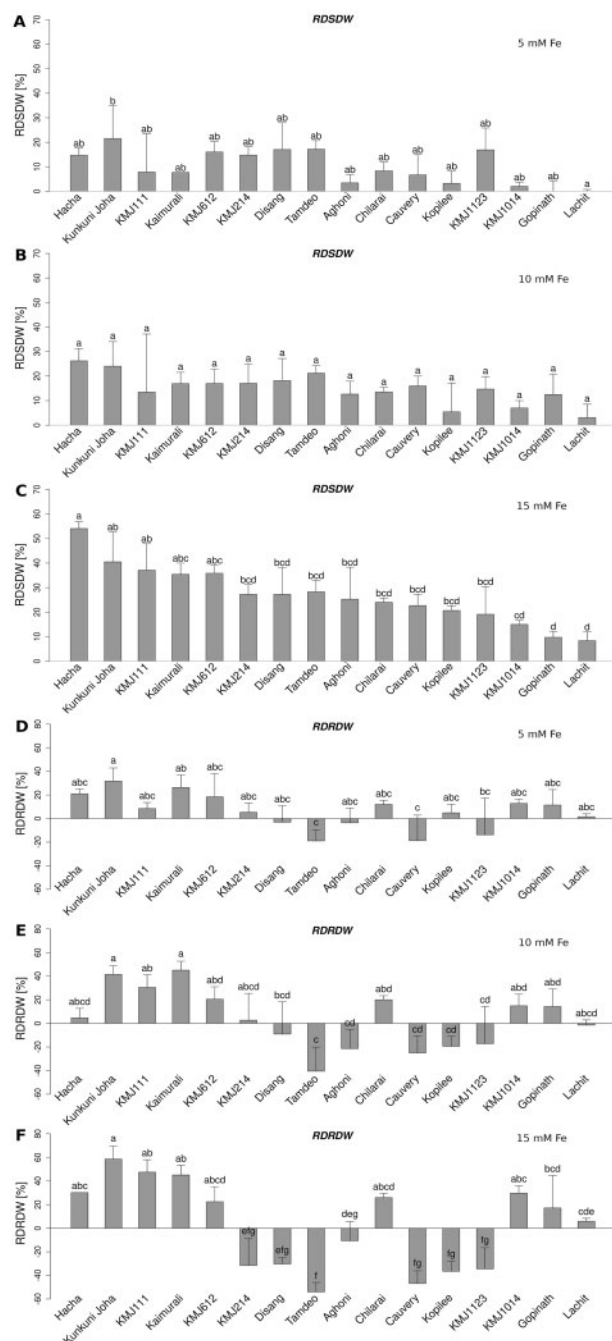
## Results

### Lowland Indica rice varieties Hacha and Lachit have contrasting abilities to deal with excess Fe stress

Sixteen lowland *O. sativa* Indica varieties from northeast India were screened under excess Fe (5, 10 and 15 mM  $\text{Fe}^{2+}$ ) under low pH and control conditions. The high Fe and low pH condition mimicked the natural dissolution of  $\text{Fe}^{2+}$  under low pH, being responsible to large extent for  $\text{Fe}^{2+}$  toxicity in acidic conditions, and is hereafter termed ‘excess Fe’. A spectrum of relative decrease in shoot dry weight (RDSDW) upon excess Fe stress compared with the control was noted (Fig. 1A–C). Differences among the lines were rather low at 5 and 10 mM Fe supply, but significant at 15 mM (Fig. 1A–C). Lachit displayed a lowest RDSDW, indicating best tolerance, while Hacha showed a highest RDSDW and was a most susceptible tested line (Fig. 1A–C). Hacha and Lachit shoot fresh weights differed only in the treatment conditions but not in the control situation (Supplementary Fig. S1). Moreover, Hacha and Lachit did not differ with respect to root fresh weights (Fig. 1D–F) or relative decrease in root dry weight (RDRDW) scores (Supplementary Fig. S2). For this reason, we selected Lachit and Hacha as contrasting pair and studied their further responses at the 15 mM excess Fe condition (Fig. 1A–C).

Plants of the two lines were exposed to 48-h excess Fe combined and control treatments and their response patterns recorded. Recovery from excess Fe stress was tested in a revival experiment that lasted up to 6 d following the treatments, when plants were reexposed to control conditions (Fig. 2). Under control treatment, Hacha and Lachit L2 (second leaf from base) and L3 (third leaf from base) leaves increased in size steadily for 1–1.3 cm during the 6 d (Fig. 2). L3 leaves grew more than L2 leaves. Hacha L3 leaves gained an average of  $2.5 \pm 0.49$  cm (value  $\pm$  SD), and Lachit L3 leaves gained nearly  $6 \pm 1.6$  cm during this time in the control condition, compared with  $0.9 \pm 0.8$  and  $1.3 \pm 0.6$  cm in the L2 leaves, respectively (Fig. 2B, D compared to Fig. 2A, C). Following excess Fe stress, Hacha L2 and L3 leaves did not grow further during the 6 d (Fig. 2). Lachit, on the other hand, was able to recover and survived the Fe stress. Lachit L2 and L3 leaves grew 0.5 and 0.4 cm, respectively, during the 6-d recovery period after the stress, but did not reach the sizes of control leaves (Fig. 2). Although the length increases in Lachit leaves were not statistically different from those of Hacha leaves (Fig. 2), Lachit plants had grown further and ultimately had survived, in contrast to Hacha. Thus, based on their growth phenotypes, Lachit tolerated the high Fe treatment but not Hacha.

Excess Fe caused leaf rolling (Fig. 3A). After 2 d of excess Fe, Hacha showed stronger leaf rolling symptoms than Lachit (Fig. 3B). Leaf rolling might be coupled to drought. Therefore,



**Fig. 1** Selection of two rice varieties with contrasting abilities to sustain growth under excess Fe stress. (A–C) Relative decrease in shoot dry weight (RDSDW) and (D–F) Relative decrease in root dry weight (RDRDW) are shown. 16 lowland *Oryza sativa* Indica varieties from northeast India were screened. Data were obtained under excess Fe (5, 10 and 15 mM Fe<sup>2+</sup>) under low pH versus control conditions. (A) RDSDW at 5 mM Fe treatment; (B) RDSDW at 10 mM Fe treatment; (C) RDSDW at 15 mM Fe treatment; (D) RDRDW at 5 mM Fe treatment; (E) RDRDW at 10 mM Fe treatment; and (F) RDRDW at 15 mM Fe treatment. Different letters, indicative of significant difference ( $P < 0.05$ ;  $n = 3$ ; ANOVA with Tukey HSD).

we assessed the fresh weight-to-dry weight (DW) ratios to possibly link the dehydration and high Fe stress. The fresh weight-to-DW ratios were lower upon excess Fe treatment than the control in both lines; however, the ratios remained comparable

between Lachit and Hacha (Fig. 3C). Taken together, leaf rolling was a symptom of high Fe stress, more pronounced in Hacha than Lachit. However, different degrees of leaf rolling had minor effects on the overall fresh weight-to-DW ratios.

Fe causes oxidative stress in cells with the formation of ROS, such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and lipid peroxides, involved in the Fe excess-induced leaf bronzing (Aung and Masuda 2020). H<sub>2</sub>O<sub>2</sub> generation was qualitatively determined by diaminobenzidine (DAB) staining. Excess Fe-stressed plants showed stronger DAB staining in leaves of both lines than controls (Fig. 4A). Quantitative measurements of H<sub>2</sub>O<sub>2</sub> confirmed higher levels in leaves upon excess Fe vs. the controls but did not uncover differences between the lines (Fig. 4B). Malondialdehyde (MDA) is a byproduct of polyunsaturated fatty acid breakdown, occurring upon lipid peroxidation, catalyzed by Fe. MDA contents were elevated upon excess Fe in leaves of both varieties, whereby MDA contents were lower under control and excess Fe stress conditions in Lachit compared with Hacha (Fig. 4C). Taken together, ROS generation is coupled to high Fe in both lines, and MDA generation is higher in each condition for Hacha than Lachit.

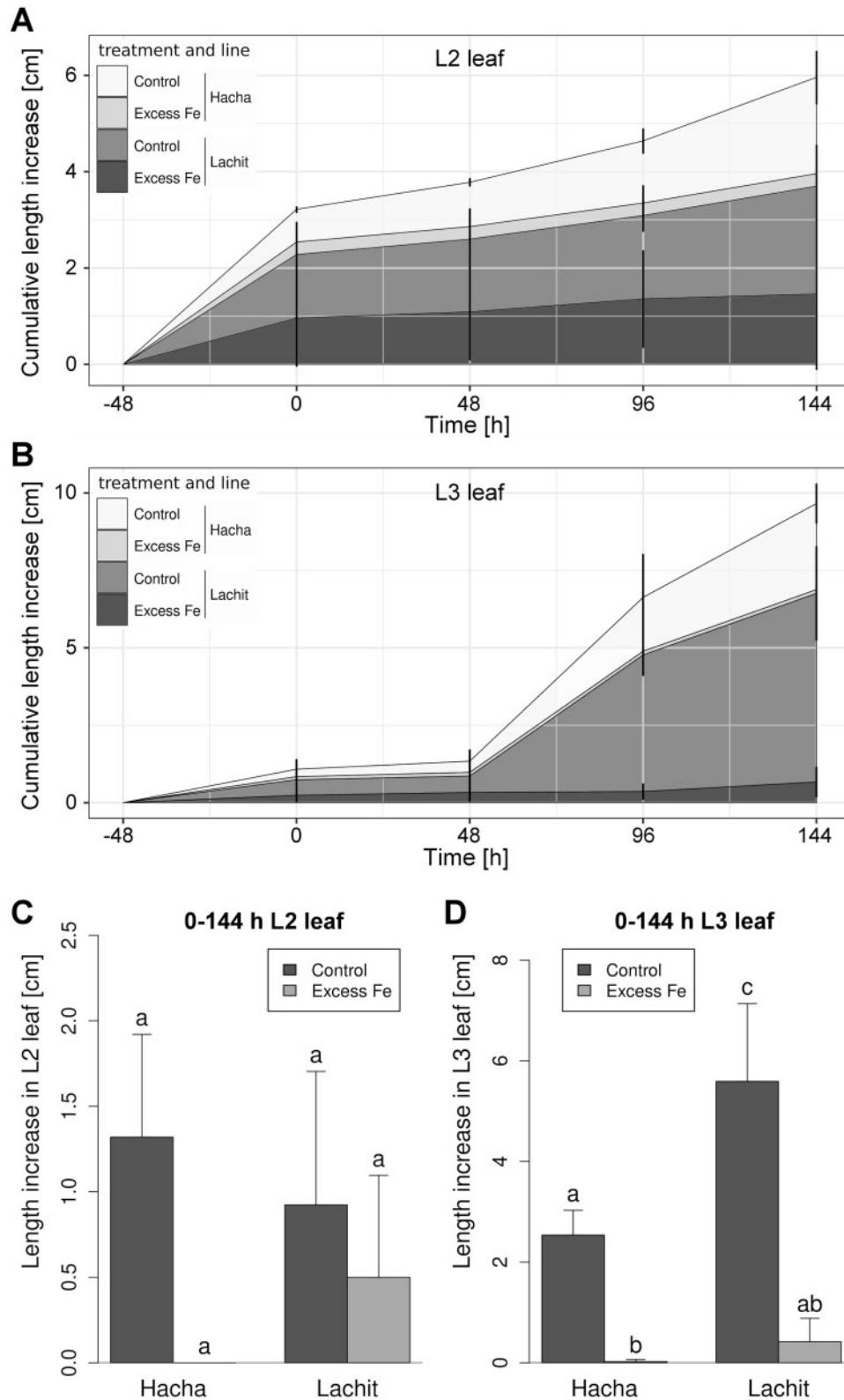
The reactions of leaves to high Fe treatment indicate that excessive Fe must have been transported from roots to leaves to some degree in both genotypes. Under control conditions, Hacha and Lachit had similar Fe, zinc (Zn), manganese (Mn) and copper (Cu) contents (Fig. 5A–D). As expected, Fe-stressed Hacha leaves had an almost 300 times higher Fe content than control leaves and Fe-stressed Lachit leaves had about 200 times higher Fe contents than control leaves (Fig. 5A). Lachit leaves had lower Fe levels than Hacha plants, in response to excess Fe (Fig. 5A). Under excess Fe, Lachit had also lower levels of Zn, Mn and Cu contents compared with Hacha (Fig. 5B–D). Cu contents were slightly increased in Hacha under excess Fe compared to the control (Fig. 5D). Taken together, metal content measurements strongly suggest that lower contents of all four metals in leaves of Lachit contributed to the lower metal toxicity phenotype observed in this line.

Fe deposits can be localized in distinct tissues via Perls Fe staining. Hacha and Lachit did not differ in their Fe localization patterns in leaves and roots (Fig. 5E). Fe deposits were found in parenchyma cells that have photosynthetic functions, in vascular bundles and in sclerenchyma cell rows present on either side of the veins underneath the epidermis. In roots, Fe was deposited in cells of the central cylinder (Fig. 5E).

In summary, Lachit tolerated the Fe stress condition, leaves grew more and plants survived the stress, in contrast to Hacha. Hacha was more susceptible to excess Fe and had enhanced Fe levels compared with Lachit. Fe tolerance in Lachit can be explained by a lower uptake or root-to-shoot translocation of Fe, Zn, Mn and Cu, and consequently resulting in less oxidative stress seen as lipid peroxidation.

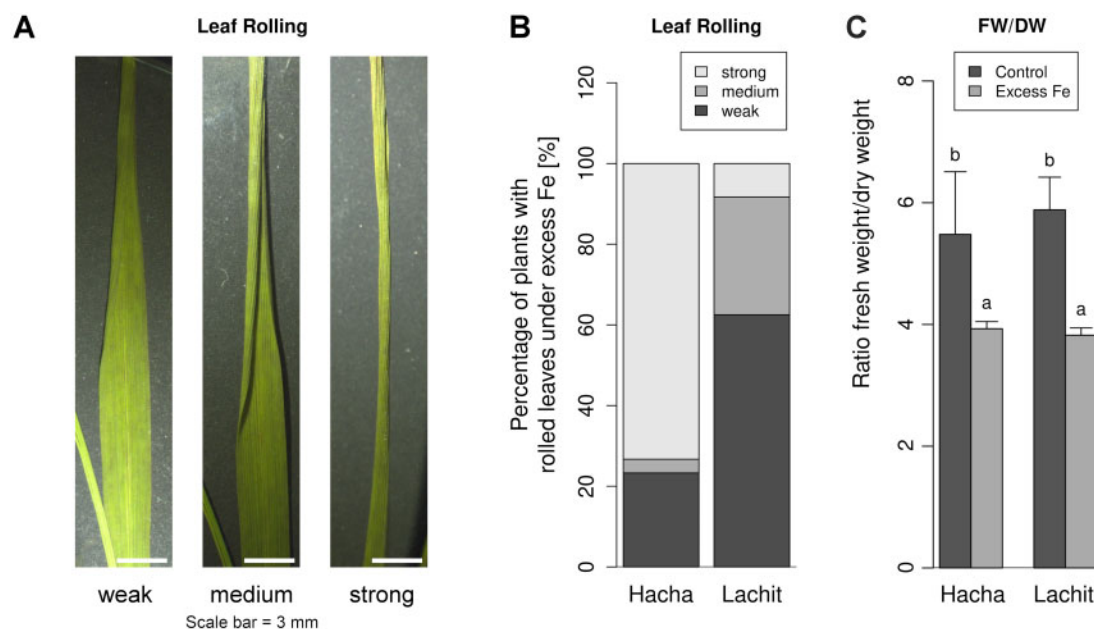
### Comparative RNA-seq transcriptome analysis of Lachit and Hacha uncovers key regulatory patterns to excess Fe stress

Comparative transcriptomics via RNA-seq is an approach to uncover biological mechanisms based on gene expression



**Fig. 2** Excess Fe stress and revival experiment. Stacked area plots of the cumulative length increases of (A) L2 (second leaf from base) and (B) L3 (third leaf from base) leaves in Hacha and Lachit, recorded from the beginning of excess Fe stress (–48 h) to the end of excess Fe stress (0 h) and during a subsequent revival phase after the end of the stress period (up to 48, 96 and 144 h) and respective controls. Stem diagrams representing length increases of (C) L2 leaves and (D) L3 leaves, between 0 and 144 h after the end of the excess iron and control treatments. Different letters indicate a significant difference between the respective groups ( $P < 0.05$ ;  $n = 3$ ; ANOVA with Tukey HSD).





**Fig. 3** Leaf rolling and fresh weight-to-DW ratios of L2 (second leaf from base) leaves. Leaf rolling symptoms of excess Fe stress with (A) photos and scale of weak to strong leaf rolling phenotypes and (B) proportions of weak, medium and strong leaf rolling symptoms in Hacha and Lachit exposed to excess Fe stress. (C) Fresh weight (FW)-to-DW ratios of L2 leaves. Different letters indicate significant differences between samples ( $P < 0.05$ ;  $n = 3$ ; ANOVA with Tukey HSD).

pattern differences between genetic variants and treatments. Hacha and Lachit were exposed to the 2-day excess Fe stress and control conditions. Roots and L2 leaves were used for the RNA-seq analysis pipeline (Supplementary Fig. S3).

Principal component analysis (PCA) and hierarchical clustering (HC) served to control the quality of samples and assess the different responses of Hacha and Lachit. The most significant principal component (PC1) separated the tissue types, roots vs. leaves, with 52% of the variation (Fig. 6A). The second principal component (PC2) separated excess Fe treatment vs. control, with 22% of the variation (Fig. 6A). The third principal component (PC3) resolved cultivar-specific differences accounting for 7% of the variation (Fig. 6B). For both leaf and root samples, the Fe effect was different between lines. The excess Fe-treated Lachit samples were closer to their controls than the respective Hacha samples (Fig. 6A). HC confirmed the PCA results. HC resolved the same three parameters in the same order of significance (Supplementary Fig. S4A).

To test whether one or both genotypes show excess Fe responses, differential transcript accumulation in roots and shoots was assessed (Fig. 7A). Overall, more changes were observed in roots compared to shoots (Fig. 7A). Hacha showed a higher degree of differential gene regulation than Lachit under excess Fe vs. the control in roots and shoots. The difference between Hacha and Lachit was more pronounced in leaves than in roots (Supplementary Fig. S4B). More genes were differentially regulated in Hacha than in Lachit especially in leaves (26.4% more in roots, 131.1% more in leaves) (Fig. 7A, excess Fe vs. control).

To dissect the difference in response between the genotypes, comparisons between Hacha and Lachit were examined. A total of 37.6% less genes were differentially abundant between Lachit

and Hacha under excess Fe in roots than under the control condition while this difference in roots was only 8.2% (Fig. 7B, Lachit vs. Hacha). Thus, the numbers of genes differently expressed between the two lines at a given Fe treatment were relatively low compared to the numbers of genes regulated within the lines upon excess Fe vs. control. A high number of genes were differentially expressed between excess Fe and the control condition within a line without being expressed at a different level between the two lines (e.g. 1,462, 497 and 291 genes up in leaves, and respective other genes down in leaves, or up and down in roots; Supplementary Fig. S5, Fig. 7B). Therefore, we predict that these genes do not likely explain the major tolerance of Lachit compared with Hacha.

We considered genes most interesting if they were differentially expressed between lines. First of all, some of these gene groups are differentially expressed between excess Fe and control and at the same time differentially expressed between Lachit and Hacha (e.g. 92, 2 and 14 genes up in leaves of Hacha, Lachit or both in response to the stress and up in Lachit vs. Hacha; similarly, respective genes with corresponding downregulation, as well as genes showing these regulation patterns in roots; Supplementary Fig. S5). A relatively large number of interesting genes were also differentially expressed between Hacha and Lachit either under the control, excess Fe or both conditions, but without being regulated by the stress itself (e.g. 446, 94 and 380 genes up in leaves, and respective other genes down in leaves, or up and down in roots; Supplementary Fig. S5). These genes that differentiate between Lachit and Hacha are most likely candidates for excess Fe tolerance of Lachit.

In summary, global transcriptome comparisons confirm that Hacha responded in a more drastic manner to excess Fe than

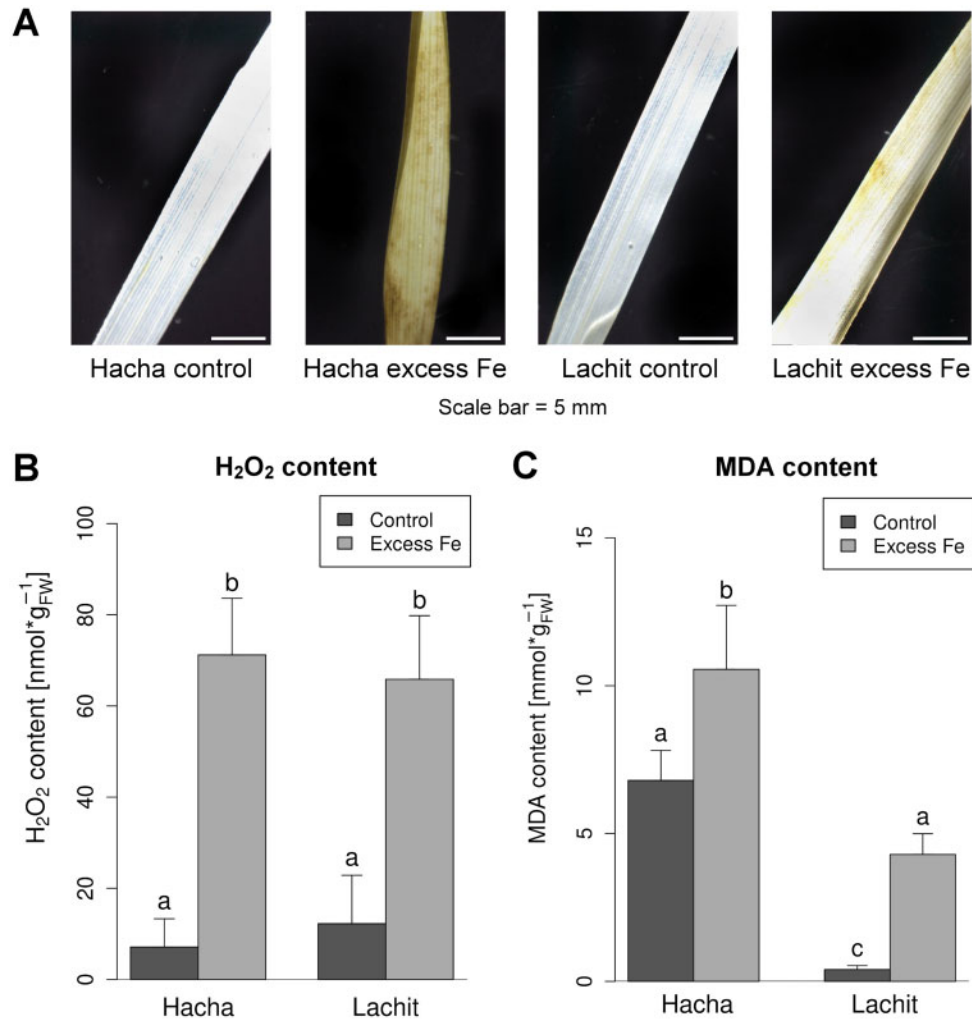


Fig. 4 Oxidative stress under excess Fe in L2 leaves. (A) Photos of DAB-stained leaves; (B) H<sub>2</sub>O<sub>2</sub> contents; and (C) MDA contents. Different letters indicate significant differences between samples ( $P<0.05$ ;  $n=3$ ; ANOVA with Tukey HSD).

Lachit. Leaf transcriptomes revealed more drastic responses to Fe stress in Hacha than in Lachit. However, the primary target of excess Fe in terms of total changes in gene expression patterns was roots rather than leaves. The transcriptome comparisons point to groups of genes, which may shed light on the tolerance mechanism in Lachit and explain the physiological phenotypes at the molecular level.

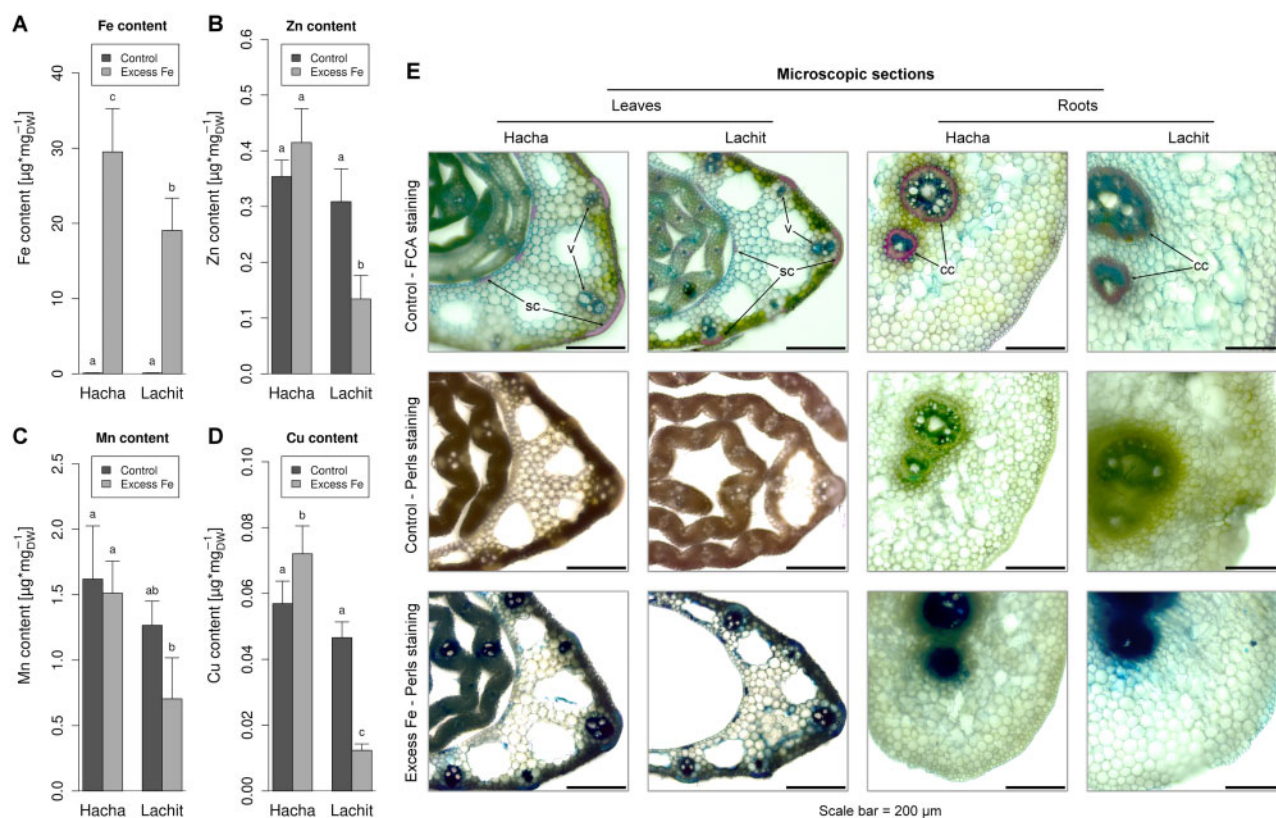
### Gene ontology term enrichment reflects morphological and physiological phenotypes of Hacha and Lachit in response to excess Fe

Multiple gene expression changes reflecting enriched gene ontology (GO) terms indicate cellular pathways associated with variations in growth conditions or genotypes.

At first, we investigated GO terms (Supplementary Tables S3–S14) related to plant performance. Since Hacha suffered more than Lachit under excess Fe and ultimately died, we expected an enrichment of GO terms for cell breakdown in Hacha rather than in Lachit, in the comparisons of excess Fe stress vs. the control. Indeed, we found an enrichment of 13 GO terms linked with cell death, organelle disassembly and

autophagy in roots of Hacha (Fig. 8A) and 16 such GO terms in leaves of Hacha (Fig. 8B). In Lachit, 12 GO terms linked with cell death, organelle disassembly and autophagy were enriched in roots (Fig. 8A) and none in leaves upon stress vs. control (Fig. 8B). In the direct comparison of Lachit vs. Hacha, six GO terms for cell death, organelle disassembly and autophagy were enriched in Hacha compared to Lachit in roots but none in leaves (Fig. 9). Ferroptosis in plants is facilitated by reactive unsaturated fatty acid-derived lipid peroxides, which are formed in the plasma membrane by lipoxygenases in the presence of Fe (Dixon et al. 2012, Distéfano et al. 2017, Conrad et al. 2018). Lipoxygenase genes (Supplementary Table S15) were significantly enriched under excess Fe in Hacha leaves (Fig. 7A; Fischer exact test,  $P=0.01978$ ) but not in Lachit leaves, or among any genes downregulated by excess Fe in leaves or in any of the root sample comparisons (Fig. 7A). This confirms at the molecular level that Hacha leaves had been undergoing cell death in response to excess Fe in leaves.

Since Lachit in contrast to Hacha survived the excess Fe treatment, we expected an enrichment of GO terms for photosynthesis and light responses among the genes in excess Fe-



**Fig. 5** Metal contents and Fe distribution. (A) Fe, (B) Zn, (C) Mn and (D) Cu contents per DW in L2 leaves of Hacha and Lachit in response to excess Fe and control. Different letters indicate significant differences between samples ( $P < 0.05$ ;  $n = 3$ ; ANOVA with Tukey HSD). (E) Microscopic images of cross-sections of Hacha and Lachit L2 leaves and roots following FCA (fuchsin-chrysoidine-astra blue) staining (general anatomy) and Perls staining (Fe deposits); v, veins (leaves); sc (leaves), sclerenchyma cell rows; and cc, central cylinder (roots) are designated by arrowheads. Scale bars: 200  $\mu\text{m}$ .

treated Lachit vs. Hacha leaves. Indeed, 13 enriched GO terms were associated with photosynthesis and light responses in this comparison and none in roots (Fig. 9). Thus, under excess Fe Lachit had continued to grow, as indicated by its responses to light and expressing photosynthesis functions.

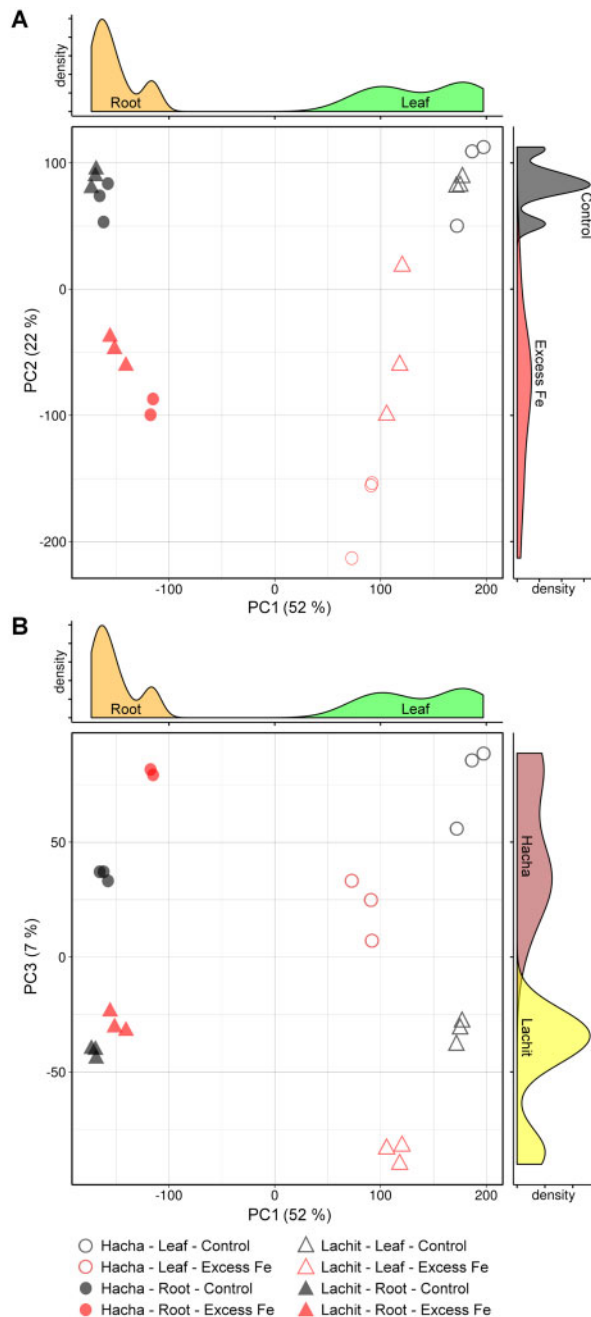
Hacha showed more signs of oxidative stress than Lachit under excess Fe. We therefore expected a molecular signature of oxidative stress in the comparison of Hacha and Lachit. In Hacha roots and leaves, 15 and 10 oxidative stress terms were enriched in response to excess Fe among upregulated genes (Fig. 8A, B), and two among downregulated genes in leaves (Fig. 8B). In the respective Lachit comparisons, eight and seven terms among upregulated genes and three GO terms among downregulated genes were enriched (Fig. 8A, B). Hence, Hacha enriched more oxidative stress-related GO terms than Lachit in roots and leaves. In the comparison between the lines, two oxidative stress terms were enriched among genes that were upregulated in Hacha vs. Lachit leaves, and none were enriched in Lachit vs. Hacha (Fig. 9). However, in roots, three such terms were enriched among upregulated genes in Lachit roots compared to Hacha and one term was downregulated in roots (Fig. 9).

Leaf rolling can be the result of sclerenchyma formation and drought (Cal et al. 2019). Hence, we expected lignin biosynthesis, cell wall, phenylpropanoid, abscisic acid and drought-related GO terms to be enriched in some of the comparisons.

Indeed, in excess Fe-treated leaves vs. control leaves, nine GO terms of these kinds were enriched in Hacha and eight in Lachit leaves (Fig. 8B). In the comparison of leaves under excess Fe treatment, six GO terms of these types were enriched among genes upregulated in Hacha vs. Lachit and none among genes upregulated in Lachit vs. Hacha (Fig. 9), whereby mostly cell wall thickening-related terms were enriched among genes expressed at a higher level in Hacha leaves compared to Lachit leaves (Fig. 9). In the corresponding root comparisons, there were two GO terms related to lignin enriched among genes upregulated in Lachit under stress, compared to three GO terms enriched among genes upregulated in Hacha (Fig. 9).

Leaf metal contents are the result of metal transport and allocation in the plant. The lower leaf metal contents in Lachit under excess Fe may stem from differential metal homeostasis gene expression in roots and leaves. In Hacha roots, 11 metal homeostasis-related GO terms were enriched under excess Fe among upregulated genes and 2 among downregulated ones (Fig. 8A), while in Lachit roots, there were 13 and 0 GO terms enriched in respective comparisons (Fig. 8A). In leaves, Hacha enriched eight metal homeostasis-related GO terms among upregulated genes and five among downregulated genes, whereas Lachit enriched only two such terms among upregulated and three among downregulated genes (Fig. 8B). Hence, Hacha reacted more vividly to excess Fe than Lachit in leaves with respect to metal homeostasis-related GO terms. No metal

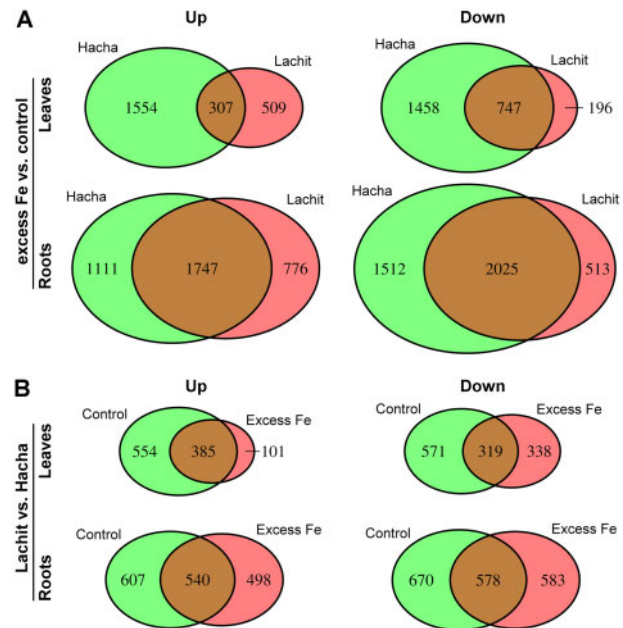




**Fig. 6** Principal component analysis (PCA) biplots of RNA-seq samples and marginal density plots. The marginal density plots serve to illustrate the separation between the respective plant lines, organs or treatments in the biplots. (A) PC1 vs. PC2; (B) PC1 vs. PC3. PC1, separation of roots and leaves; PC2, separation of excess Fe and control; PC3, separation of Hacha and Lachit.

homeostasis-related GO terms were enriched among differentially expressed genes in leaves, but three were enriched among genes upregulated in Lachit vs. Hacha in roots (Fig. 9). This indicates that metal homeostasis genes expressed in roots might be involved in the Fe tolerance mechanism in Lachit.

Overall, enrichment of GO terms related to plant performance and cell death, oxidative stress, leaf rolling and drought as well as metal homeostasis reflect the different



**Fig. 7** Two-way Venn diagrams with total numbers of regulated genes. (A) Excess Fe vs. control and (B) Lachit vs. Hacha. The areas of the diagram partitions reflect the numbers of up- and downregulated (up, down) genes in the respective partitions.

morphological and physiological responses of Hacha and Lachit to excess Fe.

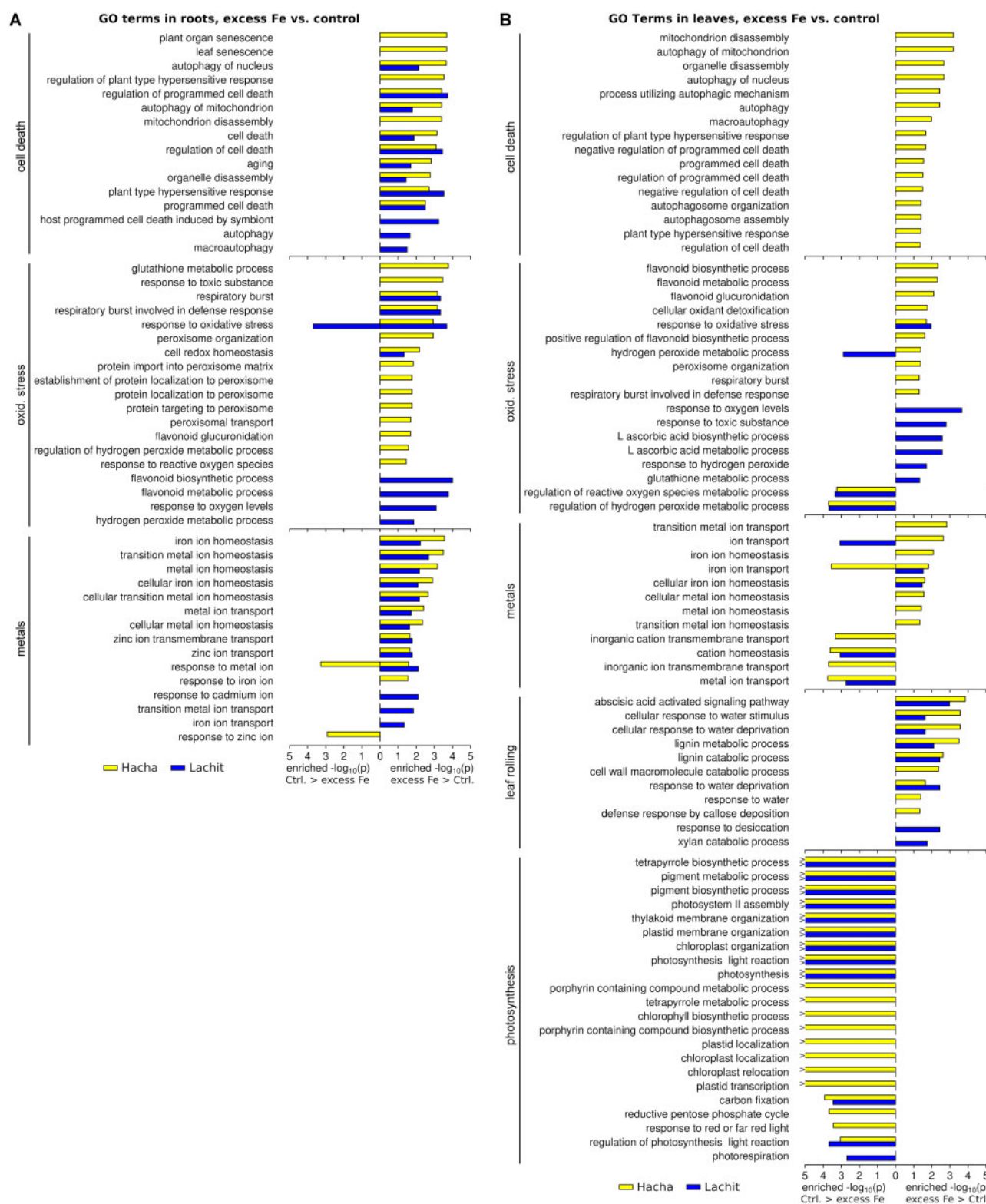
### Individual genes differentially expressed between Lachit and Hacha under excess Fe

To identify potential genes for a metal tolerance mechanism, we closely inspected oxidative stress (Supplementary Table S16) and metal homeostasis-related genes (Supplementary Table S17) that were differentially regulated in Lachit compared with Hacha. We used several of these genes to validate the transcriptome set using reverse transcription-quantitative PCR (RT-qPCR) (Supplementary Fig. S4).

Increased survival of Lachit may be caused by reduced root-to-shoot translocation, by decreased Fe uptake of the root, by increased tolerance of the stress or by a combination of these. We examined the potential Fe transport within the plant. In leaves, *YSL10* and *Os07g0516600* were upregulated in Lachit vs. Hacha (Fig. 10 top left, Supplementary Table S18) while *HMA9*, *IPB1.2* and *PDR3* and *PDR5* were downregulated (Fig. 10 top right, Supplementary Table S19). The regulation patterns of these genes suggest that they could be involved in keeping leaf metal contents at a lower level in Lachit than in Hacha.

In excess Fe-stressed roots, 17 metal homeostasis genes were expressed at a higher level in Lachit than in Hacha, including four *HMT/D* genes, an *OPT* gene, a *MATE* transporter gene, *RBOHG*, *RBOHB*, *PAL02*, *ROMT16*, *NRAMP4*, *ZIP1*, *ZIP2*, *FER1*, *OsNodulin-like2* (similar to *AtVIT homolog 4*), *CDT2*, *CDT3* and *CDT5* (Fig. 10 bottom left, Supplementary Table S20), while 13 genes were expressed at a lower level, like *PDR17*, *ABCG7*, two *HMT/D* genes, *MATE2*, *YLS2*, *OPT7*, *NRAMP1*, *NAAT1*, *HMA2*, *HMA9* and *TOM2* (Fig. 10 bottom right, Supplementary Table





**Fig. 8** Gene ontology (GO) term enrichment of selected GO terms under excess Fe vs. the control. (A) Roots. (B) Leaves. Enrichment values are  $-\log_{10}(P)$  values;  $>$ ,  $>1E-30$  P-value. GO term enrichment analysis was performed with R: topGO (see also [Supplementary Tables S3–S10](#)).

**S21**). Therefore, the regulation patterns of these genes suggest that they might contribute to the exclusion of metals from root and from root-to-shoot transport in Lachit rather than in Hacha.

Finally, we tested differences in the stress responses. We assembled a list of oxidative stress-related genes in Venn diagrams ([Supplementary Fig. S7](#), [Supplementary Tables S16 and S22–S25](#)). Different members of gene families coding for

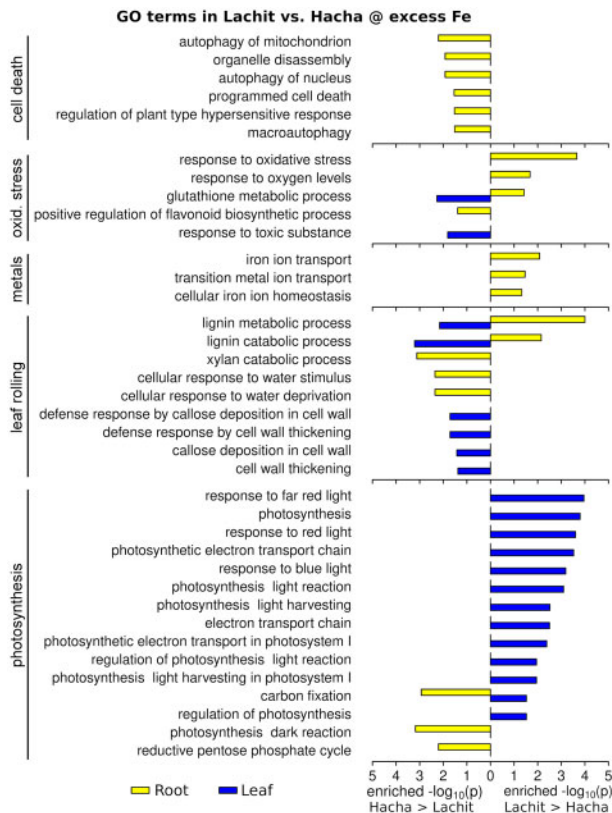


Fig. 9 Gene ontology (GO) term enrichment of selected GO terms in Lachit vs. Hacha under excess Fe. Enrichment values are  $-\log_{10}(P)$  values;  $> 1E-30$  P-value. GO term enrichment analysis was performed with R: topGO (see also [Supplementary Tables S11–S14](#)).

isozymes involved in antioxidant reactions frequently were found expressed in opposite manner. For example, one AO (ascorbate oxidase), one PRX (peroxidase) and one DHAR gene (dehydroascorbate reductase) were expressed at a higher level in Lachit compared to Hacha leaves, whereas an MDAR (monodehydroascorbate reductase) stayed neutral, one TRX-PRX (coding for thioredoxin-peroxiredoxin) and six GST genes (glutathione-S-transferases) were expressed at a lower level in Lachit compared to Hacha leaves. A clear regulation pattern of such gene functions was therefore not apparent.

Altogether, close inspection of metal homeostasis- and oxidative stress-related genes strongly indicates a tolerance mechanism that is based on diminished uptake and retention of Fe and other bivalent metals in the root tissues and enhanced local Fe detoxification rather than a tolerance mechanism based on antioxidant genes. A root-borne metal homeostasis mechanism may be in place because of the higher number of differentially expressed genes related to this category in Lachit. The identified 6 and 30 metal homeostasis genes expressed differentially between Lachit and Hacha in leaves or roots (a total of 35 different genes; [Table 1](#)) were thus potential targets for metal tolerance in Lachit.

### Enrichment of metal homeostasis candidate genes during rice domestication

Beneficial genes for agronomic purposes are likely targets during domestication and breeding. To obtain functional hints

about the aforementioned 35 genes reflecting metal tolerance in Lachit ([Table 1](#)), we examined their presence among genes that had been selected during breeding and speciation of *O. rufipogon* and *O. sativa*. Interestingly, we found that eight genes were included in the selective sweep regions ([Huang et al. 2012](#)), namely Os01g0125600 (heavy metal transport/detoxification protein domain-containing protein), NRAMP4, YSL10, OsNodulin-like2, CDT5, HMA9, RBOHG and Os12g0144600 (heavy metal transport/detoxification protein domain-containing protein) ([Table 1, Supplementary Table S26](#)). Furthermore, two genes showed breeding signatures of rice improvement ([Xie et al. 2015](#)), IBP1 and YSL2 ([Table 1, Supplementary Table S26](#)). We separately performed single-nucleotide polymorphism (SNP) haplotype analysis of 147 rice accessions against *O. rufipogon*, as described ([Wang et al. 2020](#)) and found that four genes were included in selective sweep regions in the speciation of *O. rufipogon* and *O. sativa*, Os01g0125600 (heavy metal transport/detoxification protein domain-containing protein), NRAMP4, YSL10 and HMA9 ([Table 1, Supplementary Table S26](#)).

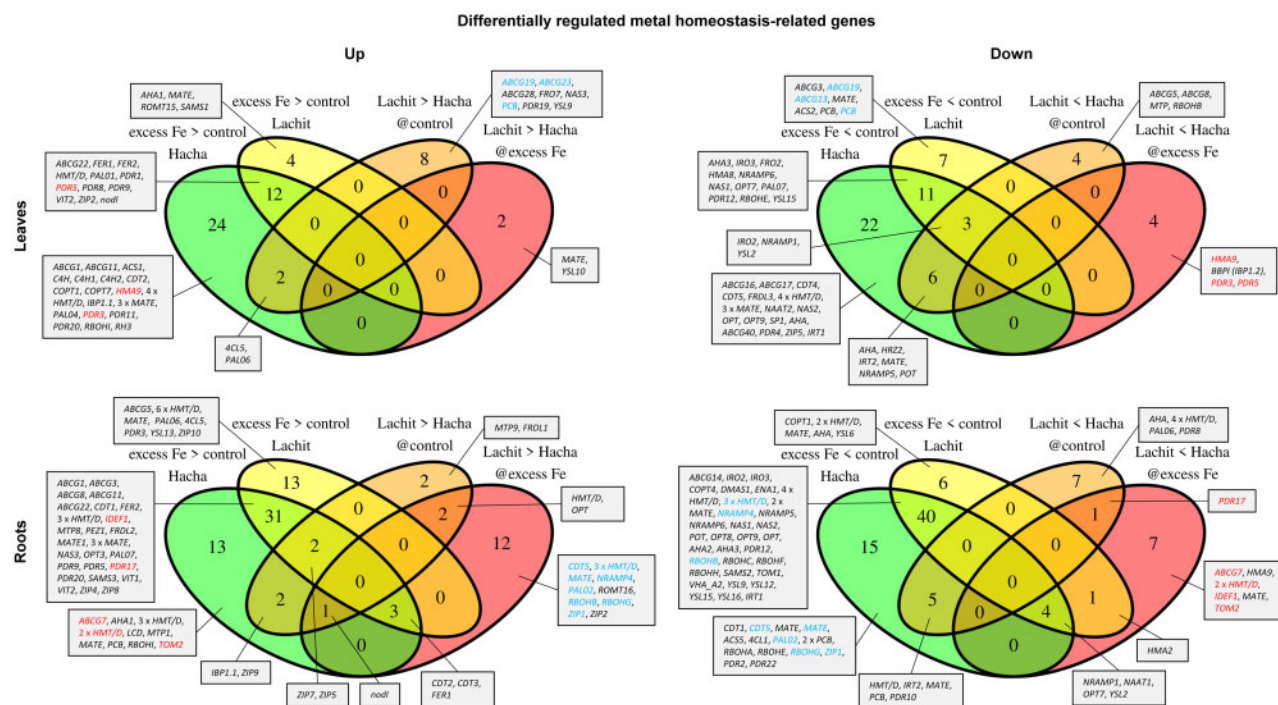
Since we investigated Indica varieties and our results differed from previous studies in which Japonica varieties had been investigated ([Wu et al. 2017](#)), we asked whether the potential Fe tolerance genes found in this study were subject to differentiation between Indica and Japonica rice varieties. Phylogenetic SNP analysis of the 35 genes was performed, which showed that 20 of them were subject to differentiation between Indica and Japonica varieties. These genes include heavy metal transport/detoxification protein domain-containing proteins (Os03g0819400, Os07g0682000, Os08g0403300, Os09g0272000 and Os12g0144600), CDT3, CDT5, RBOHB, NRAMP4, NAAT1, YSL2, ZIP2, YSL10, OsNodulin-like2, an Oligopeptide transporter (OPT) domain-containing gene (Os06g0239300), HMA9, a multi-antimicrobial extrusion protein MatE gene (Os07g0516600), IDEF1 and RBOHG ([Table 1, Supplementary File S1](#)).

Taken together, 22 different genes out of the 35 metal homeostasis candidates whose expression is induced by iron excess conditions were found to be important in the domestication of wild rice.

### Discussion

We report a root-borne tolerance mechanism of an Indica rice variety to high Fe stress related to metal homeostasis. For that, we selected and characterized a pair of Indica rice varieties with contrasting abilities to cope with excess Fe. Identified excess Fe-regulated genes indicative of tolerance genes were selected during rice domestication.

In both Hacha and Lachit, excess Fe caused reduction in leaf growth and leaf rolling phenotypes. Excessive amounts of Fe were transported to leaves where Fe accumulated to high levels, particularly in the vascular bundles and in sclerenchyma tissue. Excess Fe acted as a redox-reactive ion and caused H<sub>2</sub>O<sub>2</sub> generation and lipid peroxidation. More than 7,000 genes were differentially expressed in roots and leaves of excess Fe-treated plants vs. control plants, showing the profound effect



**Fig. 10** Four-way Venn diagrams of metal homeostasis-related genes. Left, upregulated genes (up); right, downregulated genes (down); upper part, leaves; lower part, roots. The respective comparisons are indicated. Metal homeostasis genes are indicated in gray boxes with gene name, gene family abbreviation or function abbreviation; blue gene names, expressed at higher level in Lachit than in Hacha (left) and downregulated in Hacha (right); red gene names, expressed at lower level in Lachit than in Hacha (right) and upregulated in Hacha (left). Arbitrary family or function abbreviations used above are: PCB, phytochelatin biosynthesis; HMT/D, heavy metal transport/detoxification; nodl, nodulin-like. For detailed information on all the genes in this figure, see [Supplementary Tables S17–S21](#).

of this stress on plant growth and physiology also at the molecular level. These molecular patterns explain the physiological phenotypes we observed. Oxidative stress and Fe transport GO terms were induced in the excess Fe stress-treated plants, showing that plants had perceived and responded to Fe and ROS signals. Out of 15 genes previously described to be induced or repressed in response to excess Fe and reflecting different defense mechanisms against Fe toxicity and oxidative stress (Quinet et al. 2012, Aung et al. 2018, Aung and Masuda 2020), more than half were found regulated in expected manner in our Fe excess datasets, such as rice ferritin genes, *OsVIT2*, *OsNAS3*, *OsFER1* and *OsFER2* being upregulated by the Fe excess stress in leaves and/or roots and *OsIRT1*, *OsYSL15*, *OsTOM1*, *OsNAS1* and *OsNAS2* being downregulated in roots. Upregulated genes serve Fe mitigation in the upper parts of the plant and Fe retention in roots, while downregulated genes serve to decrease Fe acquisition from soil to root (Aung et al. 2018, Aung and Masuda 2020). This finding along with increased MDA contents as described before (Quinet et al. 2012) clearly shows that plants experienced Fe excess and defended against it. Leaf rolling could be related to dehydration stress, as regulated GO terms for ABA and drought responses were apparent. On the other hand, cell wall modification and lignification may also be relevant for leaf rolling and similarly resulted in an excess Fe-dependent changes in GO terms for phenylpropanoid metabolism and cell wall modification (Cal et al. 2019). Plant performance was severely affected and the

lower growth and compromised survival under excess Fe correlated with lower photosynthesis-related gene expression and cell death-related GO term regulation. These symptoms were expected because of the cytotoxic redox effects of elevated Fe in cells and plants, reported before (Tewari et al. 2013).

A very interesting question was to explore the differences between Hacha and Lachit. We noted that, with regard to all Fe stress symptoms, Hacha had responded in a more severe manner than Lachit to the stress (Table 2). We interpret the findings as follows: Hacha transports more Fe, Zn, Mn and Cu from root to leaves than Lachit under excess Fe stress. Consequently, leaves respond in a stronger manner by leaf rolling and lipid peroxidation. Interestingly, in previous studies with excess Fe treatment of susceptible Japonica cultivars (Quinet et al. 2012, Finatto et al. 2015, Aung et al. 2018), leaf Cu contents did not decrease to the extent that we observed in excess Fe-tolerant Lachit. Furthermore, in two of these previous studies, leaf Zn contents barely decreased or tended to increase under excess Fe treatment (Finatto et al. 2015, Aung et al. 2018) while decreased Zn contents as we observed in Lachit under excess Fe have also been reported before (Quinet et al. 2012). With the drastically reduced leaf Cu contents under excess Fe, Lachit appears to be different from Hacha and the above-mentioned Japonica cultivars. To which extent and how this might contribute to a possible tolerance mechanism remains elusive. The toxic effects of Fe are stronger in Hacha than in Lachit, ultimately leading to the plant growth failure of Hacha but not of Lachit. These



**Table 1** Single nucleotide polymorphism (SNP) analysis of 35 metal homeostasis-related and putative iron tolerance genes with regard to selection during rice domestication and breeding

| Gene            | MSU ID       | RAP ID         | 1   | 2   | 3   | 4   |
|-----------------|--------------|----------------|-----|-----|-----|-----|
| IBP1            | Os01g0124401 | LOC_Os01g03360 |     | YES |     |     |
| HMT/D protein   | Os01g0125600 | LOC_Os01g03490 | YES |     | YES |     |
| CDT3            | Os01g0178300 | LOC_Os01g08300 |     |     |     | YES |
| RBOHB           | Os01g0360200 | LOC_Os01g25820 |     |     |     | YES |
| ZIP1            | Os01g0972200 | LOC_Os01g74110 |     |     |     |     |
| NRAMP4          | Os02g0131800 | LOC_Os02g03900 | YES |     | YES | YES |
| NAAT1           | Os02g0306401 | LOC_Os02g20360 |     |     |     | YES |
| YSL2            | Os02g0649900 | LOC_Os02g43370 |     | YES |     | YES |
| ZIP2            | Os03g0411800 | LOC_Os03g29850 |     |     |     | YES |
| OPT7            | Os03g0751100 | LOC_Os03g54000 |     |     |     |     |
| HMT/D protein   | Os03g0819400 | LOC_Os03g60480 |     |     |     | YES |
| MatE protein    | Os03g0839200 | LOC_Os03g62270 |     |     |     |     |
| ABCG7           | Os03g0859500 | LOC_Os03g64200 |     |     |     |     |
| PAL2            | Os04g0518100 | LOC_Os04g43760 |     |     |     |     |
| YSL10           | Os04g0674600 | LOC_Os04g57840 | YES |     | YES | YES |
| OsNodulin-like2 | Os04g0686800 | LOC_Os04g59020 | YES |     |     | YES |
| CDT5            | Os05g0178300 | LOC_Os05g08554 | YES |     |     | YES |
| MATE2           | Os05g0554000 | LOC_Os05g48040 |     |     |     |     |
| CDT2            | Os06g0143100 | LOC_Os06g05120 |     |     |     |     |
| ROMT16          | Os06g0165800 | LOC_Os06g06980 |     |     |     |     |
| OPT protein     | Os06g0239300 | LOC_Os06g13200 |     |     |     | YES |
| HMA9            | Os06g0665800 | LOC_Os06g45500 | YES |     | YES | YES |
| HMA2            | Os06g0700700 | LOC_Os06g48720 |     |     |     |     |
| NRAMP1          | Os07g0258400 | LOC_Os07g15460 |     |     |     |     |
| MatE protein    | Os07g0516600 | LOC_Os07g33310 |     |     |     | YES |
| PDR5            | Os07g0522500 | LOC_Os07g33780 |     |     |     |     |
| HMT/D protein   | Os07g0682000 | LOC_Os07g48390 |     |     |     | YES |
| IDEF1           | Os08g0101000 | LOC_Os08g01090 |     |     |     | YES |
| HMT/D protein   | Os08g0403300 | LOC_Os08g31140 |     |     |     | YES |
| HMT/D protein   | Os09g0272000 | LOC_Os09g09930 |     |     |     | YES |
| RBOHG           | Os09g0438000 | LOC_Os09g26660 | YES |     |     | YES |
| FER1            | Os11g0106700 | LOC_Os11g01530 |     |     |     |     |
| TOM2            | Os11g0135000 | LOC_Os11g04030 |     |     |     | YES |
| PDR3            | Os11g0587600 | LOC_Os11g37700 |     |     |     |     |
| HMT/D protein   | Os12g0144600 | LOC_Os12g05040 | YES |     |     | YES |

SNP patterns of the 35 metal homeostasis-related genes (red ovals in Fig. 10) were investigated for their presence (designated as YES) in selective sweep regions (column 1) according to Huang et al. (2012), in breeding signatures (column 2) according to Xie et al. (2015), in speciation between *O. sativa* and *O. rufipogon* (column 3) and in the Indica and Japonica rice differentiation (column 4).

morphological and physiological outcomes are triggered by gene expression changes. The total number of differentially regulated genes in leaves and roots that we found was about 50% more elevated in Hacha compared with Lachit. These genes, being differentially expressed in Hacha vs. Lachit upon the stress, reflected several GO terms for poor plant performance, such as cell disassembly and cell death, lipoxigenase, drought and oxidative stress. On the other side, the better

**Table 2** Summary of responses of Hacha and Lachit to excess Fe

| Parameter                                  | Lachit vs. Hacha |
|--------------------------------------------|------------------|
| RDSDW                                      | <                |
| L2, L3 growth, revival                     | >                |
| Leaf rolling                               | <                |
| Leaf Fe content                            | <                |
| Leaf Zn, Mn and Cu content                 | <                |
| Leaf MDA content                           | <                |
| Leaf H <sub>2</sub> O <sub>2</sub> content | =                |
| Leaf regulation lipoxigenase genes         | <                |
| Leaf regulation photosynthesis GO terms    | >                |
| Regulation ROS homeostasis genes           | =                |
| Regulation metal tolerance genes in roots  | >                |

growth of Lachit was mirrored by an enrichment of GO terms related to photosynthesis.

When scanning regulated genes between Lachit and Hacha under excess Fe, we could detect differences in 35 metal homeostasis genes, part of which may explain tolerance. The most striking genes we found regulated were *FER1* (up in Lachit vs. Hacha roots), *NAAT1*, *YSL2* and *TOM2* (down in Lachit vs. Hacha roots). Ferritins detoxify Fe by sequestering free Fe in the cavity of its 24-mers (Briat et al. 2010). Thereby, Fe is isolated from the cytosol and prevented from inducing damage in root cells and from being further transported to the shoot. Hence, enhanced *FER1* expression may contribute to Fe tolerance in Lachit by sequestration and detoxification of Fe in the root. *YSL2* is a nicotianamine transporter and plays a role in root-to-shoot Fe translocation (Ishimaru et al. 2010). The phytosiderophore efflux transporter *TOM2* is also important for Fe distribution from the root to the shoot (Nozoye et al. 2015). Hence, downregulation of both transporters probably contributes to reduced root-to-shoot Fe translocation. Downregulation of these genes in Lachit may occur due to reduced expression of *IDEF2* in Lachit vs. Hacha roots. *IDEF2* has been proposed to positively regulate Fe translocation genes, such as *YSL2* (Ogo et al. 2008). The function of *NAAT1* has been well studied. *NAAT1* (nicotianamine amino transferase) catalyzes the first crucial step in phytosiderophore deoxymugineic acid (DMA) and mugineic acid (MA) biosyntheses from nicotianamine in rice (Kobayashi et al. 2008). Since phytosiderophores of the MA family are essential for iron uptake in strategy II plants, downregulation of DMA and MA biosyntheses may contribute to less iron uptake by the root. This is supported by recent findings, where, in reverse, induction of DMA and MA biosyntheses leads to enhanced tolerance to Fe deficiency in rice (Masuda et al. 2019).

Therefore, we hypothesize that enhanced expression of *FER1* and reduced expression of *YSL2* and *TOM2* under high iron stress are the mechanism to sequester, detoxify and retain iron in the root in a stronger manner in Fe excess-treated Lachit than Hacha. This is strongly supported by our metal measurements, in which Lachit not only displayed less iron in the shoots than Hacha but also other divalent metals, such as Cu and Zn. Downregulation of *NAAT1* indicates that this mechanism is



accompanied by reduced Fe uptake in Lachit due to decreased phytosiderophore biosynthesis. Downregulation of phytosiderophore biosynthesis and other metal transporters would also explain lower contents of Cu and Zn in Lachit leaves, as such mechanisms were previously reported (Widodo et al. 2010, Nozoye et al. 2015). Whether the lower levels of Cu and Zn indicate secondary deficiencies of these metals remains unclear. However, in combination with lower Fe levels in leaves, lower levels of Cu and Zn appear beneficial or at least less harmful in Lachit. Decreased Zn, Mn and Cu contents in response to high Fe were reported before (Tanaka et al. 1966, Shao et al. 2007), but it is unexpected that this metal interference with the retention of metals in the root occurred in Lachit but much less in Hacha.

In this study, we did not determine whether any of the identified genes indeed contribute to tolerance because of their level of expression nor did we determine the origin of differential expression, e.g. whether it is caused by *cis*-regulatory effects, *trans*-acting factors that affect transcription rates and transcript stabilities. In addition, the genes from Hacha and Lachit may also encode proteins that differ in their protein activity due to sequence changes.

Among the metal homeostasis genes, which were regulated differently in Lachit than in Hacha, we also observed a number of poorly characterized genes. Most notably, *OsNodulin-like2*, an ortholog of *AtVTL4*, was expressed at a higher level in Lachit than in Hacha roots under iron stress. As a putative vacuolar Fe transporter, *OsNodulin-like2* could contribute to the detoxification and retention of Fe in the root by the sequestration of Fe in the vacuole complementing the above-mentioned mechanism of Fe retention in the root. Therefore, *OsNodulin-like2* is a good candidate for further research. *NRAMP1* has been shown to act in cellular Cd uptake and root-to-shoot distribution (Takahashi et al. 2011), is induced under Fe deficiency (Bashir et al. 2014) and might also play a role in Fe redistribution to shoots. Hence, downregulation of *NRAMP1* in Lachit vs. Hacha might add to the retention mechanism in Lachit roots. NADPH oxidase/respiratory burst oxidase homolog (RBOH) proteins generate localized ROS increases to regulate developmental and stress responses (Chapman et al. 2019) and downregulation in roots upon high Fe may prevent excessive ROS signaling.

Other metal homeostasis-related genes that were differentially regulated between Lachit and Hacha under excess Fe are not well-enough characterized or annotated in the context of Fe homeostasis to allow for further assumptions, such as *HMA2*, *HMA9*, *OPT7*, *NRAMP4*, *ZIP1* and *ZIP2*. However, phenylalanine ammonia lyase *PAL02* and caffeoyl-CoA-O-methyl transferase *ROMT16* (*CCoAOMT-1*) were expressed at a higher level in Lachit than in Hacha roots under excess Fe. Both act in concert in the coumarin biosynthesis pathway. Coumarins play a role in Fe uptake in strategy I plants (Fourcroy et al. 2014, Rajniak et al. 2018) but their role in strategy II plants in Fe homeostasis is unclear.

Remarkably, three out of four rice CDT (*CADMIUM TOLERANT*) genes were expressed at higher levels in Lachit vs. Hacha roots under excess Fe in our study. Although the exact function of these proteins is not yet known, CDTs may bind metal ions due to a cysteine-rich C-terminal domain. *OsCDT2.2* (transcript variant of the *CDT2* locus formerly named *CDT1*)

may chelate Cd in the apoplast, impeding Cd uptake into the cell (Kuramata et al. 2009). *OsCDT3* has Al-binding activity, which may occur at the inner side of the plasma membrane (Xia et al. 2013). Since Cd and Al are mainly bi- and trivalent metals, respectively, and Fe can be present in its bivalent (ferrous) or trivalent (ferric) oxidative form, CDT proteins might also sequester Fe in the apoplast or in the cell as proposed for Cd and Al (Kuramata et al. 2009, Xia et al. 2013). Although not investigated in the context of Fe homeostasis, their striking transcriptional regulation makes the CDTs a very interesting target for future research.

Selection sweep region, breeding signature and SNP haplotype analyses suggest a certain importance of 22 out of the 35 potential Fe tolerance genes. These observations indicate functional significance of metal homeostasis genes identified in our study due to the correlation with lowland rice breeding. Beneficial traits are usually selected during the speciation of wild and cultivated rice and breeding of agriculturally important varieties. In addition, analysis of phylogenetic tree differentiation revealed that 20 out of the 35 Fe tolerance genes are substantially different in Indica and Japonica rice accessions. This suggests that metal homeostasis genes for excess Fe tolerance play an important role in rice differentiation and breeding.

In summary, our study highlights new candidate genes for excess Fe tolerance in the lowland rice variety Lachit. The most noteworthy gene functions standing out from our data comprise reduced mobilization as well as increased retention of Fe and other bivalent metals in the root tissues. This mechanism is most likely accompanied by reduced Fe uptake. Our metal measurements strongly support these conclusions. In addition, we observed a number of new genes that might be able to add to the mechanisms, such as *OsNodulin-like2*, *NRAMP2*, *ZIP1*, *ZIP2* and the CDTs since they all code for metal transporters or metal-binding proteins with distinct transcriptional regulation patterns in Lachit. This makes these genes targets for future research on Fe toxicity or Fe homeostasis in general. These new candidate genes can be explored as targets for the breeding of improved lowland rice crops.

In the perspectives of this work, the importance of individual genes and gene sequences can be further confirmed in genome sequencing, transgenic and genetic experiments. In particular, it would be interesting to determine why and how these genes are regulated differently in the cultivars.

## Materials and Methods

### Plant material

A total of 16 lowland *O. sativa* L. ssp. Indica varieties were obtained from the Regional Rice Research Station (RARS), Karimganj, Assam, India, and the ICAR-NRRI-Regional Rainfed Lowland Rice Research Station (RRLRRS), Gerua, Guwahati, Assam, India. Hacha is Fe excess intolerant (derived from a cross of CRM 53 X IR 64), while Lachit is Fe excess tolerant (derived from a cross between CRM 13-3241 and Kalinga 2) (Awasthi et al. 2017).

### Plant growth

Plant growth was conducted in a controlled growth chamber, with a 16-h light/8-h dark cycle, 70% humidity, a light intensity of 220  $\mu\text{mol}/\text{m}^2/\text{s}$  and a day/night temperature of 26°C. Rice seeds were disinfected using 1% sodium hypochlorite for 15 min. Subsequently, seeds were soaked in water for 1 d at 4°C and

transferred for 3 d in the dark to 30°C for germination. Homogenously grown seedlings were transplanted into 1-l pots filled with half-strength-modified Hoagland solution for 2 d. Then, half-strength nutrient solution was replaced with a full-strength solution containing 1 mM calcium nitrate, 2.5 mM potassium nitrate, 2.86 mM ammonium nitrate, 1 mM potassium dihydrogen phosphate, 1 mM magnesium sulfate, 0.045  $\mu$ M boric acid, 0.009  $\mu$ M Mn chloride, 0.666  $\mu$ M Zn sulfate, 0.4  $\mu$ M Cu sulfate, 0.0176  $\mu$ M sodium molybdate and 50  $\mu$ M Fe sodium EDTA ( $\text{Fe}^{2+}$ ), pH 6.2, which also represented the control treatment condition. The nutrient solutions were renewed every second day. After another 6 d, an  $\text{Fe}^{2+}$  stress treatment combined with low pH was applied, mimicking the natural dissolution of  $\text{Fe}^{2+}$  in an Fe-rich waterlogged acidic soil, termed throughout the text 'excess Fe'. For that, full-strength Hoagland medium plus Fe sulfate ( $\text{Fe}^{2+}$ ) at pH 5.2 was provided, as indicated as 2-day treatment with 15 mM Fe sulfate ( $\text{Fe}^{2+}$ ). In Fig. 1, plants were alternatively also treated with 5 and 10 mM Fe sulfate ( $\text{Fe}^{2+}$ ) at pH 5.2. For revival experiments in Fig. 2, the plants were reexposed to control medium for the indicated times after the 2-day stress condition and again the medium was renewed every second day. For the RNA-seq experiment, eight plants were pooled for a biological sample. In total, three biological replicates were prepared. The revival experiment was conducted with eight plants per condition. For physiological and morphological experiments, three to eight plants were grown as indicated in the figure legends.

### Morphological examination of plants

The root and shoot fresh weights were determined after dissecting the root system. Root and shoot DWs (RDW, SDW) were determined after fully drying the materials at 120°C. The relative decrease in SDW (RSDSW) and relative decrease in RDW (RDRDW) scores were calculated in % as  $\text{SDW}_{\text{control-excess}} \times 100 / \text{SDW}_{\text{control}}$  or respective calculations with RDWs. Leaf lengths were measured from the base of the sheath to the tip of the blade. Leaves were weighed to determine the fresh weight. After fully drying the leaves at 120°C the leaf DWs were determined. Leaf rolling was assessed as weak, medium or strong as indicated in Fig. 3A.

### H<sub>2</sub>O<sub>2</sub> detection

DAB staining was used to visualize H<sub>2</sub>O<sub>2</sub> in vivo. L2 leaf blades were excised, vacuum infiltrated for 5 min in a solution of 1 mg/ml 3,3'-DAB tetrahydrochloride in 50 mM MES, pH 6.5, followed by a 1-h incubation in a shaker and washed with distilled water. The sample was placed into 80% ethanol and boiled at 80°C until the leaves were destained from chlorophyll. The reaction was stopped by washing three times with distilled water. The samples were observed under a microscope.

H<sub>2</sub>O<sub>2</sub> contents were quantified using the Amplex Red kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Fifty milligrams of plant sample was ground in 200  $\mu$ l of potassium phosphate buffer and 50  $\mu$ l of supernatant was used for horseradish peroxidase-based Amplex Red assay reactions, using appropriate controls, as described (Brumbarova et al. 2016). The absorbance at 560 nm was used to calculate the H<sub>2</sub>O<sub>2</sub> content based on a mass standard curve.

### Malondialdehyde content

Lipid peroxidation was determined using colorimetric detection via the thiobarbituric acid (TBA)–MDA assay, according to Zhang and Huang (2013). Fifty milligrams of plant material was homogenized with 2 ml of trichloroacetic acid (TCA, 0.1%). After centrifugation, 1 ml of supernatant was mixed with 2 ml of 0.5% (w/v) TBA in 20% (w/v) TCA and 10  $\mu$ l of 4% butylated hydroxytoluene. After 30-min boiling and cooling, the absorbance was determined at 532 and 600 nm. MDA concentrations were calculated after subtracting  $\text{OD}_{532} - \text{OD}_{600}$  and using the molar extinction coefficient of MDA (155 mM/cm).

### Determination of metal contents and Perls Fe staining

L2 leaves and root systems were air-dried and dried at 100°C for 10 h and finely powdered with an agate mortar and pestle. Metal contents (Fe, Zn, Mn, Cu) were determined following microwave digestion of dried samples with HNO<sub>3</sub> and H<sub>2</sub>O<sub>2</sub> (Multiwave 3000, Anton Paar GmbH, Ostfildern, Germany) by high-resolution continuum source atomic absorption spectrometry HR-CS AAS (contrAA 700, Analytik Jena AG, Jena, Germany) and calculated using reference metal contents.

Perls Fe staining was conducted with 50–100- $\mu$ m hand-made sections of L2 leaf sheaths and roots, according to Brumbarova and Ivanov (2014). Sections were destained and then subjected to Perls Prussian Blue staining with potassium ferrocyanide solution to yield a blue precipitate. The 50–100- $\mu$ m hand-made cross-sections were prepared. For general anatomy observations, the sections were incubated for 5–8 min in 1 mg/ml fuchsin, 1 mg/ml chrysoidine, 1 mg/ml astra blue and 0.025 ml/ml acetic acid staining (= FCA staining) solution. Sections were washed in ddH<sub>2</sub>O, twice in 30% ethanol, 70% ethanol, again twice in 30% ethanol and isopropanol and mounted in ddH<sub>2</sub>O on slides.

### RNA isolation and RNA-seq

L2 leaves and entire root systems from eight plants per sample were dissected from Hacha and Lachit exposed to a control growth condition or 2-day 15 mM excess Fe condition. Three independent biological replicates were harvested per condition and line. RNA was isolated from 100 mg of plant material using the RNeasy plant mini kit (Qiagen, Hilden, Germany). RNA was checked and sequenced according to (Brilhaus et al. 2016). The quality and quantity of RNA was analyzed following DNase digestion using the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, California, USA). RNA samples with intact bands and an RNA integrity number of >7.0 were used for the preparation of RNA-seq libraries with TruSeq RNA Sample Prep Kit v2 (Illumina Technologies, San Diego, California, USA). Double-stranded cDNA was purified for end repair, adaptor ligation and DNA fragment enrichment. The libraries were sequenced as 101-bp paired-end reads using Illumina HiSeq at the Biologisch-Medizinisches Forschungszentrum (Heinrich Heine University, Düsseldorf, Germany). Basecalling and demultiplexing were performed using bcl2fastq2. RNA-seq data are deposited in GEO accession number GSE150103.

### Analysis of RNA-seq data and of candidate genes

Paired reads were trimmed with trimmomatic (Bolger et al. 2014) and their quality was evaluated with fastqc (Andrews 2010). Using kallisto (Bray et al. 2016), the trimmed reads were mapped to the *O. sativa* transcriptome (Oryza\_sativa.IGRSP-1.0.cdna.all.fa, downloaded from EnsemblPlants) and quantified. *Arabidopsis thaliana* homologs were determined by blasting the rice transcriptome against the *A. thaliana* proteome using the rice CDS (Oryza\_sativa.IGRSP-1.0.cds.all.fa; downloaded from Gramene) and *A. thaliana* proteome sequences (TAIR10\_pep\_20101214\_updated; downloaded from TAIR) using the Blast+ suite (Altschul et al. 1990). Genes were called similar if the *e*-value was <1E-5. Among multiple hits, Arabidopsis proteins with highest score followed by lowest *E*-value were taken. Transcripts per million counts for all samples were used in R for PCA, HC and plots to visualize the biological replicate quality. Estimated counts were statistically analyzed using edgeR (Robinson et al. 2010, McCarthy et al. 2012) with Bonferroni correction. Fold-change values of gene expression were calculated in pairwise comparisons between Lachit and Hacha upon excess Fe stress or the control for roots and leaves, and within a line between excess Fe and control for roots and leaves. All data including absolute expression data, fold-change ratios, *P*-values and annotations are provided in Supplementary Table S1. Genes were considered differentially expressed if their adjusted *P*-value was <0.01 (Supplementary Table S2). GO analysis was carried out using topGO (Alexa and Rahnenfuhrer 2010) with the rice annotations (go\_ensembl\_oryza\_sativa.gaf; downloaded from Gramene), applying Fisher's exact test.

For the analysis of selection sweep regions, gene loci were compared to selection sweep regions and breeding signatures as described (Huang et al. 2012, Xie et al. 2015). We also performed diversity statistics ( $\pi$ ), population-differentiation statistics (*F*<sub>st</sub>) and cross-population likelihood (XP-CLR) analysis using 147 rice accession as described (Wang et al. 2020). Candidate targets were determined from SNP haplotype analysis and phylogenetic trees were constructed by FastTree software (Price et al. 2010) using selected 230 rice accessions in a representative mini core collection constructed by Zhang et al. (2011). Resequencing data of 23 *O. rufipogon* and 147 *O. sativa* lines were obtained as previously described (Wang et al. 2020). Briefly, reads were first trimmed with Trimmomatic version 0.32 (Bolger et al. 2014) with parameters 'ILLUMINACLIP:2:30:10 LEADING:20 TRAILING:20 SLIDINGWINDOW:4:20 MINLEN:36' and then aligned to the Nipponbare version 7 reference genome (<http://rice.plantbiology.msu.edu/>, last accessed February, 17, 2021) using BWA-MEM version 0.7.10 (Li and Durbin 2009). SNP calling and filtration were carried out with SAMtools version 1.6 (Li et al. 2009).

## Gene expression by RT-qPCR

RT-qPCR was conducted with RNA samples prepared for RNA-seq using three biological replicates. cDNA synthesis and qPCR were carried out as described (Ben Abdallah and Bauer 2016). Total RNA was used for cDNA synthesis using an oligo-dT primer. qPCR was conducted using the SYBR Green detection method. Each biological cDNA sample was tested in two technical replicates. Absolute starting quantities of templates were determined using mass standard curves. Normalized absolute expression data were obtained after normalization to the internal reference genes. The primer sequences (5'–3' direction) for reference genes were *OsTUB1* CTCATCAGTGGCAAGGAGGA and CTCCAA CAGCGTTGAACACA (OS11G0247300, tubulin 1) and *OsUBI1* CTTCACC TTGTCCTCCGTCT and TTGTCCTGGATCTTGGCCTT (Os02G0161900, ubiquitin 1). The primer sequences (5'–3' direction) for genes of interest were *OsFER1* TCTTCCAGCCATTCGAGGAG and TGACTCCATGCATAGCCACA (OS11G0106700, ferritin 1), *OsMT1g*, *OsMTP8* ACTTGCCCTCTACATAT ACTGCA and TCACTCAGACATACAGATTCCTC (OS02G0775100, metal tolerance protein 8), *OsPRX131* GCTAGATGACACCCCAACCT and AGGGAAGTG TCGATGTTGGT (Os11g0112200, peroxidase 131), *OsSALT* AGGCGTGACAA TCTACAGCT and ATCATAGACTGGGCCATGGG (Os01g0348900, SALT STRESS-INDUCED), *OsASR3* CAAGAAGGACGGGGAGGAG AND CACCTC CTCTTCACCTTGT (OS02G33820, ABIOTIC STRESS-RESPONSIVE), *OsNRAMP4* ACTGTGCAGGGAAGAGTACC and GGGGTCCAACCTTTCTTG CTG (OS02G0131800, *OsNRAMP4* metal transporter) and *OsYSL15* GCAAC ACAGCATCAGAGGAG and CCAGCGCTAAACTTCATGCA (OS02G0650300, *OsYSL15* phytosiderophore importer).

## Statistical analysis

Statistical analyses of morphological, physiological and RT-qPCR gene expression data were carried out using R. Comparison of means was performed using one-way analysis of variance (ANOVA) and Tukey honest significant difference (HSD) test. RNA-seq data were analyzed as described above and using exact tests for differences between two groups of negative-binomial counts (Robinson and Smyth 2007) with Bonferroni correction.

## Supplementary Data

Supplementary data are available at PCP online.

## Funding

This work received funding by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) under Germany's Excellence Strategy—EXC-2048/1 (project ID 390686111).

## Acknowledgments

The authors thank Elke Wieneke for technical assistance. We are thankful to Huixia Shou, Zhejiang University, China, for the discussions on the manuscript.

## Disclosures

The authors declare that they have no conflicts of interest.

## References

Ahmed, T., Chetia, S., Chowdhury, R. and Ali, S. (2011). *Status Paper on Rice in Assam. Rice Knowledge Management Portal*. pp. 1–49. Directorate of Rice Research, Hyderabad.

Alexa, A. and Rahnenfuhrer, J. (2010) topGO: Enrichment Analysis for Gene Ontology. R Package Version 2.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) Basic local alignment search tool. *J. Mol. Biol.* 215: 403–410.

Andrews, S. (2010) FastQC: A Quality Control Tool for High Throughput Sequence Data. <https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>.

Aung, M.S. and Masuda, H. (2020) How does rice defend against excess iron? Physiological and molecular mechanisms. *Front. Plant Sci.* 11. 10.3389/fpls.2020.01102, last accessed February, 17, 2021

Aung, M.S., Masuda, H., Kobayashi, T. and Nishizawa, N.K. (2018) Physiological and transcriptomic analysis of responses to different levels of iron excess stress in various rice tissues. *Soil Sci. Plant Nutr.* 64: 370–385.

Aung, M.S., Masuda, H., Nozoye, T., Kobayashi, T., Jeon, J.-S., An, G., et al. (2019) Nicotianamine synthesis by OsNAS3 is important for mitigating iron excess stress in rice. *Front. Plant Sci.* 10:

Awasthi, J.P., Saha, B., Regon, P., Sahoo, S., Chowra, U., Pradhan, A., et al. (2017) Morpho-physiological analysis of tolerance to aluminum toxicity in rice varieties of North East India. *PLoS One* 12: e0176357.

Barthakur, I.K. (2018) Soil pH as a phenotype determinant in humans: proposing a scientific hypothesis. *Open J Soil Sci.* 08: 36–46.

Baruah, B.K., Das, B., Medhi, C. and Misra, A.K. (2013) Fertility status of soil in the tea garden belts of golaghat district, Assam, India. *J. Chem.* 2013: 1–6.

Baruah, K.K., Das, S. and Das, K. (2007) Physiological disorder of rice associated with high levels of iron in growth medium. *J. Plant Nutr.* 30: 1871–1883.

Bashir, K., Hanada, K., Shimizu, M., Seki, M., Nakanishi, H. and Nishizawa, N.K. (2014) Transcriptomic analysis of rice in response to iron deficiency and excess. *Rice* 7: 18.

Ben Abdallah, H. and Bauer, P. (2016) Quantitative reverse transcription-qPCR-based gene expression analysis in plants. *Methods Mol. Biol.* 1363: 9–24.

Bolger, A.M., Lohse, M. and Usadel, B. (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30: 2114–2120.

Bray, N.L., Pimentel, H., Melsted, P. and Pachter, L. (2016) Near-optimal probabilistic RNA-seq quantification. *Nat. Biotechnol.* 34: 525–527.

Briat, J.F., Duc, C., Ravet, K. and Gaymard, F. (2010) Ferritins and iron storage in plants. *Biochim. Biophys. Acta* 1800: 806–814.

Brillhaus, D., Bräutigam, A., Mettler-Altmann, T., Winter, K. and Weber, A.P. (2016) Reversible burst of transcriptional changes during induction of crassulacean acid metabolism in *Talinum triangulare*. *Plant Physiol.* 170: 102–122.

Brumbarova, T., Bauer, P. and Ivanov, R. (2015) Molecular mechanisms governing Arabidopsis iron uptake. *Trends Plant Sci.* 20: 124–133.

Brumbarova, T. and Ivanov, R. (2014) Perls staining for histochemical detection of iron in plant samples. *Plant Cell.*

Brumbarova, T., Le, C. and Bauer, P. (2016) Hydrogen peroxide measurement in Arabidopsis root tissue using Amplex Red. *Bio-protocol* 6: e1999.

Cal, A.J., Sanciango, M., Rebolledo, M.C., Luquet, D., Torres, R.O., McNally, K.L., et al. (2019) Leaf morphology, rather than plant water status, underlies genetic variation of rice leaf rolling under drought. *Plant. Cell Environ.* 42: 1532–1544.

Chapman, J.M., Muhlemann, J.K., Gayomba, S.R. and Muday, G.K. (2019) RBOH-dependent ROS synthesis and ROS scavenging by plant specialized metabolites to modulate plant development and stress responses. *Chem. Res. Toxicol.* 32: 370–396.

Chen, E., Huang, X., Tian, Z., Wing, R.A. and Han, B. (2019) The genomics of *Oryza* species provides insights into rice domestication and heterosis. *Annu. Rev. Plant Biol.* 70: 639–665.

Conrad M., Kagan V.E., Bayir H., Pagnussat G.C., Head B., Traber M.G., et al. (2018) Regulation of lipid peroxidation and ferroptosis in diverse species. *Genes. Dev.* 32:602–619.

Das, A., Tomar, J., Ramesh, T., Munda, G., Ghosh, P. and Patel, D. (2010) Productivity and economics of lowland rice as influenced by



- incorporation of N-fixing tree biomass in mid-altitude subtropical Meghalaya, North East India. *Nutr. Cycl. Agroecosyst.* 87: 9–19.
- Diop, B., Wang, D.R., Drame, K.N., Gracen, V., Tongoon, P., Dzidzienyo, D., et al. (2020) Bridging old and new: Diversity and evaluation of high iron-associated stress response of rice cultivated in W. Africa. *J. Exp. Bot.* 71: 4188–4200.
- Distéfano, A.M., Martin, M.V., Córdoba, J.P., Bellido, A.M., D'Ippólito, S., Colman, S.L., et al. (2017) Heat stress induces ferroptosis-like cell death in plants. *J. Cell Biol.* 216:463–476.
- Dixon, S.J., Lemberg, K.M., Lamprecht, M.R., Skouta, R., Zaitsev, E.M., Gleason, C.E., et al. (2012) Ferroptosis: an iron-dependent form of non-apoptotic cell death. *Cell* 149: 1060–1072.
- Finatto, T., DE Oliveira, A.C., Chaparro, C., DA Maia, L.C., Farias, D.R., Woyann, L.G., et al. (2015) Abiotic stress and genome dynamics: specific genes and transposable elements response to iron excess in rice. *Rice* 8: 13.
- Fourcroy, P., Siso-Terraza, P., Sudre, D., Saviron, M., Rey, G., Gaymard, F., et al. (2014) Involvement of the ABCG37 transporter in secretion of scopoletin and derivatives by Arabidopsis roots in response to iron deficiency. *New Phytol.* 201: 155–167.
- Huang, X., Kurata, N., Wang, Z.-X., Wang, A., Zhao, Q., Zhao, Y., et al. (2012) A map of rice genome variation reveals the origin of cultivated rice. *Nature* 490: 497–501.
- Ishimaru, Y., Masuda, H., Bashir, K., Inoue, H., Tsukamoto, T., Takahashi, M., et al. (2010) Rice metal-nicotianamine transporter, OsYSL2, is required for the long-distance transport of iron and manganese. *Plant J.* 62: 379–390.
- Ishimaru, Y., Suzuki, M., Tsukamoto, T., Suzuki, K., Nakazono, M., Kobayashi, T., et al. (2006) Rice plants take up iron as an Fe<sup>3+</sup>-phytosiderophore and as Fe<sup>2+</sup>. *Plant J.* 45: 335–346.
- Kobayashi, T., Nakanishi, H., Takahashi, M., Mori, S. and Nishizawa, N.K. (2008) Generation and field trials of transgenic rice tolerant to iron deficiency. *Rice* 1: 144–153.
- Kuramata, M., Masuya, S., Takahashi, Y., Kitagawa, E., Inoue, C., Ishikawa, S., et al. (2009) Novel cysteine-rich peptides from *Digitaria ciliaris* and *Oryza sativa* enhance tolerance to cadmium by limiting its cellular accumulation. *Plant Cell Physiol.* 50: 106–117.
- Le, C.T.T., Brumbarova, T. and Bauer, P. 2019. The Interplay of ROS and Iron Signaling in Plants. In *Redox Homeostasis in Plants, from Signalling to Stress Tolerance*. Edited by Panda, S. K. and Yamamoto, Y. Y. Springer, Cham, Switzerland, p. 43–66.
- Li, H. and Durbin, R. (2009) Fast and accurate short read alignment with Burrows–Wheeler transform. *bioinformatics* 25: 1754–1760.
- Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., et al.; 1000 Genome Project Data Processing Subgroup (2009) The sequence alignment/map format and SAMtools. *Bioinformatics* 25: 2078–2079.
- Mahender, A., Swamy, B., Anandan, A. and Ali, J. (2019) Tolerance of iron-deficient and-toxic soil conditions in rice. *Plants* 8: 31.
- Masuda, H., Aung, M.S., Kobayashi, T., Hamada, T. and Nishizawa, N.K. (2019) Enhancement of iron acquisition in rice by the mugineic acid synthase gene with ferric iron reductase gene and OsIRO2 confers tolerance in submerged and non-submerged calcareous soils. *Front. Plant Sci.* 10: 1179.
- Mccarthy, D.J., Chen, Y. and Smyth, G.K. (2012) Differential expression analysis of multifactor RNA-Seq experiments with respect to biological variation. *Nucleic Acids Res.* 40: 4288–4297.
- Müller, C., Kuki, K.N., Pinheiro, D.T., de Souza, L.R., Siqueira Silva, A.I., Loureiro, M.E., et al. (2015) Differential physiological responses in rice upon exposure to excess distinct iron forms. *Plant Soil* 391: 123–138.
- Muthayya, S., Sugimoto, J.D., Montgomery, S. and Maberly, G.F. (2014) An overview of global rice production, supply, trade, and consumption. *Ann. N.Y. Acad. Sci.* 1324: 7–14.
- Nozoye, T., Nagasaka, S., Kobayashi, T., Sato, Y., Uozumi, N., Nakanishi, H., et al. (2015) The phytosiderophore efflux transporter TOM2 is involved in metal transport in rice. *J. Biol. Chem.* 290: 27688–27699.
- Ogo, Y., Kobayashi, T., Itai, R.N., Nakanishi, H., Kakei, Y., Takahashi, M., et al. (2008) A novel NAC transcription factor, IDEF2, that recognizes the iron deficiency-responsive element 2 regulates the genes involved in iron homeostasis in plants. *J. Biol. Chem.* 283: 13407–13417.
- Price, M.N., Dehal, P.S. and Arkin, A.P. (2010) FastTree 2—approximately maximum-likelihood trees for large alignments. *PLoS One* 5.
- Quinet, M., Vromman, D., Clippe, A., Bertin, P., Lequeux, H., Dufey, I., et al. (2012) Combined transcriptomic and physiological approaches reveal strong differences between short-and long-term response of rice (*Oryza sativa*) to iron toxicity. *Plant Cell Environ.* 35: 1837–1859.
- Rajniak, J., Giehl, R.F., Chang, E., Murgia, I., Wirén, N. and Sattely, E.S. (2018) Biosynthesis of redox-active metabolites in response to iron deficiency in plants. *Nat. Chem. Biol.* 14: 442–450.
- Reza, S., Utpal, B., Bandyopadhyay, S., Dipak, S. and Dutta, D. (2012) Characterization of soil acidity under different land uses in Assam. *Agropedology* 22: 123–127.
- Robinson, M.D., McCarthy, D.J. and Smyth, G.K. (2010) edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26: 139–140.
- Robinson, M.D. and Smyth, G.K. (2007) Small-sample estimation of negative binomial dispersion, with applications to SAGE data. *Biostatistics* 9: 321–332.
- Shao, G., Chen, M., Wang, W., Mou, R. and Zhang, G. (2007) Iron nutrition affects cadmium accumulation and toxicity in rice plants. *Plant Growth Regul.* 53: 33–42.
- Stein, R.J., Duarte, G.L., Scheunemann, L., Spohr, M.G., De Araújo Júnior, A.T., Ricachenevsky, F.K., et al. (2019) Genotype variation in rice (*Oryza sativa* L.) tolerance to Fe toxicity might be linked to root cell wall lignification. *Front. Plant Sci.* 10.
- Stein, R.J., Lopes, S.I.G. and Fett, J.P. (2014) Iron toxicity in field-cultivated rice: contrasting tolerance mechanisms in distinct cultivars. *Theor. Exp. Plant Physiol.* 26: 135–146.
- Takahashi, R., Ishimaru, Y., Senoura, T., Shimo, H., Ishikawa, S., Arao, T., et al. (2011) The OsNRAMP1 iron transporter is involved in Cd accumulation in rice. *J. Exp. Bot.* 62: 4843–4850.
- Tanaka, A., Loe, R. and Navasero, S. (1966) Some mechanisms involved in the development of iron toxicity symptoms in the rice plant. *Soil Sci. Plant Nutr.* 12: 32–38.
- Tewari, R.K., Hadacek, F., Sassmann, S. and Lang, I. (2013) Iron deprivation-induced reactive oxygen species generation leads to non-autolytic PCD in Brassica napus leaves. *Environ. Exp. Bot.* 91: 74–83.
- Tripathi, R.D., Tripathi, P., Dwivedi, S., Kumar, A., Mishra, A., Chauhan, P.S., et al. (2014) Roles for root iron plaque in sequestration and uptake of heavy metals and metalloids in aquatic and wetland plants. *Metallomics* 6: 1789–1800.
- Wang, Y., Shang, L., Yu, H., Zeng, L., Hu, J., Ni, S., et al. (2020) A strigolactones biosynthesis gene contributed to the Green Revolution in rice. *Mol. Plant* 13: 923–932.
- Widodo, B., Broadley, M.R., Rose, T., Frei, M., Pariasca-Tanaka, J., Yoshihashi, T., et al. (2010) Response to zinc deficiency of two rice lines with contrasting tolerance is determined by root growth maintenance and organic acid exudation rates, and not by zinc-transporter activity. *New Phytol.* 186: 400–414.
- Wu, L.B., Holtkamp, F., Wairich, A. and Frei, M. (2019) Potassium ion channel gene OsAKT1 affects iron translocation in rice plants exposed to iron toxicity. *Front. Plant Sci.* 10: 579.
- Wu, L.B., Shadi, M.Y., Gregorio, G., Matthus, E., Becker, M. and Frei, M. (2014) Genetic and physiological analysis of tolerance to acute iron toxicity in rice. *Rice* 7: 8.

- Wu, L.B., Ueda, Y., Lai, S.K. and Frei, M. (2017) Shoot tolerance mechanisms to iron toxicity in rice (*Oryza sativa* L.). *Plant Cell Environ.* 40: 570–584.
- Xia, J., Yamaji, N. and Ma, J.F. (2013) A plasma membrane-localized small peptide is involved in rice aluminum tolerance. *Plant J.* 76: 345–355.
- Xie, W., Wang, G., Yuan, M., Yao, W., Lyu, K., Zhao, H., et al. (2015) Breeding signatures of rice improvement revealed by a genomic variation map from a large germplasm collection. *Proc. Natl. Acad. Sci. USA* 112: E5411–E5419.
- Zhang, H., Zhang, D., Wang, M., Sun, J., Qi, Y., Li, J., et al. (2011) A core collection and mini core collection of *Oryza sativa* L. in China. *Theor. Appl. Genet.* 122: 49–61.
- Zhang, J., Chen, K., Pang, Y., Naveed, S.A., Zhao, X., Wang, X., et al. (2017) QTL mapping and candidate gene analysis of ferrous iron and zinc toxicity tolerance at seedling stage in rice by genome-wide association study. *BMC Genomics* 18: 828.
- Zhang, L., Dai, W., Wu, C., Song, X. and Qiang, S. (2012) Genetic diversity and origin of Japonica-and Indica-like rice biotypes of weedy rice in the Guangdong and Liaoning provinces of China. *Genet. Resour. Crop Evol.* 59: 399–410.
- Zhang, Z. and Huang, R. (2013) Analysis of malondialdehyde, chlorophyll proline, soluble sugar, and glutathione content in *Arabidopsis* seedling. *Bio-protocol* 3: e817.