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Abscisic acid, stress and ripening (ASR) proteins play a role in iron homeostasis in rice (*Oryza sativa* L.)

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ABSTRACT

Iron (Fe) is essential for plant growth, playing a key role in photosynthesis, respiration, and nitrogen fixation. Despite its abundance, Fe is not easily available for uptake by roots, making Fe deficiency a common issue that reduces crop yields. The ASR (Abscisic acid/Stress/Ripening) proteins are known to be responsive to different abiotic stresses. Two ASR proteins from rice (*Oryza sativa* L.), OsASR1 and OsASR5, were described as transcription factors involved in aluminum (Al) toxicity response, and were shown to be partially redundant in their function. Here we explored a possible role of ASR proteins in Fe deficiency in rice plants. We showed that rice plants silenced for ASR genes (named OsASR5-RNAi) had increased sensitivity to Fe deficiency, with early and more severe chlorosis, as well as reduced photosynthesis, stunted growth, reduced seed set and altered ionome in roots, leaves and seeds. Transcriptomic analysis indicated that roots of OsASR5-RNAi plants had similar expression of Fe uptake genes, such as *OsIRT1* and *OsYSL15*. However, long distance phloem transporter *OsYSL2* was up-regulated in OsASR5-RNAi roots to a larger extent compared to WT, suggesting ASR proteins negatively regulate *OsYSL2* expression. We also identified other interesting candidate genes, such as *OsZIFL2* and *Thionins*, that are dependent on ASR proteins for regulation under Fe deficiency. Our work demonstrated that OsASR proteins are important for proper Fe deficiency response in rice.

1. Introduction

Iron (Fe) is essential for plants. It is present in the structure of several proteins and complexes that participate in electron transfer reactions during photosynthesis and respiration, including photosystems I and II, ferredoxins and cytochromes (Przybyla-Toscano et al., 2021). Fe is also a critical cofactor for nitrogenase used in symbiotic nitrogen fixation by rhizobia (Liu et al., 2023; Sankari et al., 2022). However, despite being abundant in the Earth's lithosphere, Fe is not readily available for uptake by plant roots. Consequently, crops tend to be susceptible to Fe deficiency (Trofimov et al., 2024). Often manifested as leaf chlorosis, Fe deficiency impairs photosynthesis, resulting in stunted growth, delayed flowering and yield losses (Huang and Suen, 2021; Krohling et al., 2016). On other hand, Fe excess can be harmful, since Fe can produce reactive oxygen species (ROS) by Fenton's reactions, leading to toxicity (Becana et al., 1998; Wairich et al., 2024). Therefore, Fe homeostasis must be finely regulated to ensure adequate Fe concentrations in different plant tissues.

Plants have two strategies for Fe uptake, known as Strategy I and Strategy II (Rodrigues et al., 2023; Wairich et al., 2022). Strategy I is performed by non-grass plants and is based on the solubilization of ferric ion (Fe^{3+}) by the release of H^+ protons into the rhizosphere; reduction of Fe^{3+} to ferrous ion (Fe^{2+}) by ferric chelate reductases, such as FRO2 (Robinson et al., 1999; Connolly et al., 2003); and Fe^{2+} uptake by high

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affinity plasma membrane transporters, such as IRT1 (Iron-Regulated Transporter 1) (Eide et al., 1996; Korshunova et al., 1999; Vert et al., 2002). On the other hand, Strategy II is performed by grasses and is based on the release of Fe^{3+} chelators, named phytosiderophores (PSs), into the rhizosphere (Carrillo and Borthakur, 2021; Nozoye et al., 2011). The intact Fe^{3+}-PS complexes are then taken up by YS1/YSL15 (Yellow-Stripe Like) transporters (Curie et al., 2001; Inoue et al., 2009; Lee et al., 2009). Plants within the *Oryza* genus, including rice (*Oryza sativa* L.), use the Combined Strategy, which consists of Strategy II coupled with the up-regulation of an IRT-like transporter, allowing these species to uptake both Fe^{3+}-PS complexes and Fe^{2+} ions (Ishimaru et al., 2006; Wairich et al., 2019).

Aluminum (Al^{3+}) toxicity is another common disorder for plants, resulting in significant yield losses. Al toxicity is typically observed in acidic soils, particularly in pH lower than 5.5. Under these conditions, Al is highly soluble and readily available for uptake by roots (Bojórquez-Quintal et al., 2017). Because of that, plants have developed mechanisms to tolerate Al toxicity. Al-tolerant plant species are known to release organic acids into the rhizosphere, such as citrate, malate and oxalate, which chelate Al^{3+} ions and prevent their entry into roots (Yang et al., 2019). In parallel, to minimize Al toxic effects on vital cellular processes, some Al-tolerant plants also show enhanced antioxidant defense systems, including the production of enzymes, such as superoxide dismutase, catalase, and peroxidases, to scavenge ROS (Ranjan et al., 2021). Furthermore, uptake by plasma membrane and subsequent vacuolar detoxification of Al^{3+} in root and/or shoot cells is also described (Yan et al., 2022).

Rice has been used as a model plant for studying Fe homeostasis due to its genetic and physiological similarities with other important cereal crops such as bread wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.) and maize (*Zea mays* L.) (Kim et al., 2014; Song et al., 2018). The presence of the combined strategy in rice allows comparisons with Strategy I plants, with several homologous proteins playing similar roles in upstream regulatory networks and uptake mechanisms (Grillet and Schmidt, 2019, Wairich et al., 2025), as well as with Strategy II plants, for which rice is a good model given its small genome and availability of tools for molecular genetics. Since rice is usually cultivated in acidic soils, it provides an ideal system to investigate the adaptive responses and tolerance mechanisms of plants under Al toxicity (Arenhart et al., 2015; Zhang et al., 2019). Moreover, rice is considered the most Al-tolerant cereal (Famoso et al., 2010). Interestingly, Al has been shown to affect Fe homeostasis in rice as the supply of Al alleviated the chlorotic phenotype of Fe-deficient plants (Alves et al., 2025). It was suggested that Al could be causing P deficiency, which is known to alleviate Fe deficiency-induced chlorosis (Nam et al. 2021; Therby-Vale et al., 2022). However, little is known about the underlying mechanisms, and which proteins could be involved in regulating both Al toxicity and Fe deficiency responses.

The *ASR* (*Abscisic acid/Stress/Ripening*) genes are plant-specific proteins known to be involved in fruit ripening, sugar metabolism and to be responsive to different abiotic stresses, such as drought, salinity and cold (Jia et al., 2016; Li et al., 2018; Parrilla et al., 2022; Yacoubi et al., 2022). There are six *ASR* genes in the rice genome, and since *ASR* genes are not present in the *Brassicaceae* family, rice is the model of choice to understand the function of these proteins in plants (Parrilla et al., 2022; Philippe et al., 2010). Two members of the *OsASR* family, namely *OsASR1* and *OsASR5,* were linked to Al stress response in rice (Arenhart et al., 2013, 2014, 2016). Both genes are responsive to Al stress (Arenhart et al., 2013). OsASR5 was shown to control several Al-responsive genes by directly binding to their promoters (Arenhart et al., 2014). It was shown that OsASR1 and OsASR5 can functionally compensate for each other at Al responsive target gene promoters, suggesting that these genes have at least partial functional redundancy (Arenhart et al., 2016). Among other Al-responsive genes, OsASR5 is able to directly bind to *Sensitive To Al Rhizotoxicity 1* (*OsSTAR1*) promoter and is involved in *OsSTAR1* up-regulation in Al toxicity (Arenhart et al., 2014; Huang et al., 2009). *OsSTAR1* is also named *Efflux transporter of NA 2* (*OsENA2*), which has a role in Fe homeostasis (Nozoye et al., 2011). Moreover, *OsASR* transcripts have been found to be responsive to Fe in transcriptomic studies (Stein et al., 2019; Wairich et al., 2019, 2021). However, whether these proteins play a role in Fe homeostasis has hitherto remained unexplored.

Here we evaluated how knockdown rice plants of *ASR* genes respond to Fe deficiency. We found that down-regulation of the *OsASR* genes increases sensitivity to low Fe, with early onset and more pronounced leaf chlorosis, and decreases photosynthesis, growth and seed set. We also found that the mineral element composition of seeds, root and leaf tissues are altered. Transcriptomic analyses in roots indicate that OsASR5-RNAi phenotype is not associated with the main Fe deficiency pathway, i.e., regulating OsYSL15 and OsIRT1 transporters. However, *OsYSL2* is strongly up-regulated in OsASR5-RNAi plants, suggesting that Fe distribution might be involved in the observed phenotype. Our work demonstrates that ASR proteins play a role in Fe homeostasis.

2. Material and methods

2.1. Plant materials and growth

We used *Oryza sativa* L. cv Nipponbare and the previously described OsASR5-RNAi line (Arenhart et al., 2013). OsASR5-RNAi line have been characterized in detail in several other papers of our group (Arenhart et al., 2013, 2014, 2016). The construct decreases expression of several *OsASR* transcripts (see data below; Fig. 8).

Seeds of both genotypes were germinated in Petri dishes with filter paper and distilled water for 7 days and transferred to vermiculite. After two weeks, plants were transferred to a hydroponic system. The nutrient solution consisted of 700 μM K_2SO_4, 100 μM KCl, 100 μM KH_2PO_4, 2 mM $Ca(NO_3)_2$, 500 μM $MgSO_4$, 10 μM H_3BO_3, 0.5 μM $MnSO_4$, 0.5 μM $ZnSO_4$, 0.2 μM $CuSO_4$, 0.01 μM $(NH_4)_6Mo_7O_{24}$, and 100 μM Fe^{3+}-EDTA. The pH of the nutrient solution was adjusted to 5.4 and the solution was renewed twice a week. Plants were exposed to treatments after two weeks of acclimation. For Fe deficiency treatment (−Fe), Fe was omitted from nutrient solution, while control plants were cultivated in complete nutrient solution. Plants were kept under treatment or under control condition (CC) for up to 24 days. Growth conditions were 27 °C ± 1 °C under photoperiod of 16h/8h light/dark and irradiance of 150 μmol photons m^{-2} s^{-1}. Plants grown until maturity were cultivated in greenroom conditions with photoperiod of 12h/12h light/dark and irradiance of 200 μmol photons m^{-2} s^{-1}.

2.2. Growth measurements and agronomic traits

Measurements of shoot height and root length of individual plants (n = 5) were performed using a graduated ruler. Shoot and root samples were dried in a forced ventilation oven at 60 °C for one week. The dry mass values were obtained using an analytical precision balance. At maturity, plants were evaluated for total number of panicles and number of filled and empty seeds (n = 12 to 24).

2.3. SPAD, gas exchange and fluorescence analyses

The relative chlorophyll content were measured with the Soil Plant Analysis Development (SPAD) chlorophyll meter SPAD 502 (Konica Minolta, Inc., Tokyo, Japan) from the 11th to the 21st day of treatment every two days. Measurements were performed in the central region of the fifth fully expanded leaf of the same plant. Data was obtained from five different plants (n = 5).

Gas exchange and chlorophyll *a* fluorescence was measured in the youngest fully expanded leaves from both WT and OsASR5-RNAi lines under control condition or −Fe. The measurements were performed at the 7th, 14th, and 21st days of treatment. CO_2 assimilation rate (*A*; μmol CO_2 m^{-2} s^{-1}), stomatal conductance (g_s; mol H_2O m^{-2} s^{-1}), internal CO_2

concentration (C_i; μmol CO_2 mol air^{-1}) and transpiration (E; mmol H_2O m^{-2} s^{-1}) were measured with a LCpro-SD portable InfraRed Gas Analyzer system (IRGA; ADC Bioscientific, Hoddesdon, England). The internal parameters in the IRGA chamber during gas exchange measurements were: 27 °C, 1000 μmol photons m^{-2} s^{-1}, 420 ppm CO_2 concentration, ambient humidity (80 % ± 5 %). Each sample corresponded to the normalized value of two leaves from different plants and the measurement recordings were performed 15 min after placing the samples inside the measuring chamber with the internal parameters described previously. The water use efficiency (μmol CO_2 mmol H_2O^{-1}) was calculated as A/E and the carboxylation efficiency was calculated as A/Ci. Normalized data is used for figures presentation.

In vivo chlorophyll *a* fluorescence was measured using an OS-30p chlorophyll fluorometer (Opti-Sciences, Hudson, NH, USA). The fluorescence parameters were measured by the saturation pulse method (Schreiber et al., 1995). The maximum and the minimum fluorescence (Fm and Fo, respectively) were measured in 30-min dark-adapted plants and the maximum quantum yield of photosystem II (PSII) [Fv/Fm; (Fm–Fo)/Fm] was calculated.

2.4. Mineral element analyses

Whole root or leaf samples were dried at 65 °C and weighed in polytetrafluoroethylene tubes. Samples were digested with concentrated HNO_3 (67−69 %; Bernd Kraft) and pressurized in a high-performance microwave reactor (UltraCLAVE IV, MLS GmbH). The digested samples were diluted with deionized water (Milli-Q Reference A+, Merck Millipore). Elemental analysis was carried out by high resolution inductively coupled plasma mass spectrometry (HR-ICP-MS) (ELEMENT 2, Thermo Fisher Scientific, Germany).

Elemental concentration of seeds was determined using an inductively couple plasma mass spectrometer (ICP-MS; Elan DRCe, PerkinElmer; or NexION 300D, PerkinElmer) as described previously (Zhang et al., 2014). The radar charts were plotted using the online tool available at https://www.bioinformatics.com.cn/en (Tang et al., 2023).

2.5. RNA extraction, library preparation and RNA-seq analyses

For the RNA-seq analyses, plants were cultivated as described above, and roots from 4 plants per sample were collected after five days of treatment. RNA extraction was performed with the Direct-zol™ RNA MiniPrep Plus Kit (Zymo Research). For each treatment and genotype, three libraries (n = 3) were sequenced using the Illumina® NovaSeq6000 system, generating 100 bp paired-end reads.

Pre-processing and analysis of RNA-seq datasets were carried out according to Vitoriano and Calixto (2021). Briefly, residual adaptor sequences were removed from raw paired-end reads using Trimmomatic version 0.39 (Bolger et al., 2014). To generate quantitative transcript-specific expression data, the trimmed reads were pseudo-aligned to the rice reference transcriptome (all cDNAs) available at the MSU Rice Genome Annotation Project Database, Osa1 Release 7 (Kawahara et al., 2013) using Salmon version 1.4.0 (Patro et al., 2017). Full differential gene expression and differential alternative splicing analysis were carried out with the 3D RNA-seq app (Guo et al., 2021).

For this, we firstly generated read counts and Transcript per Million reads (TPM) information from each Salmon output file using tximport R package version 1.10.0 and the lengthScaledTPM method. Next, transcripts with less than 1 count per million (CPM) in ≥11 out of 12 samples were removed. Subsequently, the normalization of read counts was carried out across the samples using the weighted Trimmed Mean of M-values (TMM) method. Lastly, the voomWeights pipeline of limma R package was used for differential expression analysis, setting the following thresholds: absolute $\log_2$-fold change (L2FC) ≥ 1 and adjusted P-value (FDR) ≤ 0.01. In these analyses, the contrast groups were set as OsASR5-RNAi CC x WT CC, WT −Fe x WT CC, OsASR5-RNAi −Fe x OsASR5-RNAi CC, and OsASR5-RNAi −Fe x WT −Fe, in which "CC" means control condition and "−Fe" as the Fe deficiency treatment. Raw data can be found at Sequence Read Archive (SRA) database at NCBI (National Center for Biotechnology Information), accession number PRJNA1210584.

Hierarchical clustering was used to partition the differentially expressed genes (DEGs) into 10 clusters with euclidean distance and ward.D clustering algorithm (Saraçli et al., 2013). ComplexHeatmap R package version 2.16.0 was used to make the heatmap (Gu, 2022).

For the gene ontology (GO) analysis, DEGs were separated into up- and down-regulated genes, taking into account the Fe deficiency treatment in the same genotype [i.e. WT (−Fe x CC) and OsASR5-RNAi (−Fe x CC)]. To identify enriched gene ontologies (Fisher's test, $P \leq 0.05$), we used the web-based platform Comprehensive Annotation of Rice Multi-Omics (CARMO) (Wang et al., 2015). The Venn diagrams and GO enrichment bubble graphics were plotted using the online tool available at https://www.bioinformatics.com.cn/en (Tang et al., 2023).

2.6. Statistical analyses

Our data were tested for normality and homogeneity of variance using the Shapiro-Wilk test and Levene test, respectively. Afterwards, we used Student's -test or One-Way ANOVA, followed by Tukey's test, if both assumptions were met. If the data were not normal, we followed with the Mann-Whitney U test (for independent samples) or the Kruskal-Wallis test, followed by Dunn's test for pairwise comparisons (for multiple comparisons). If the data were normal but did not meet the assumption of homogeneity of variance, we performed Welch's ANOVA, followed by the Games-Howell post-hoc test.

3. Results

3.1. Plants silenced for ASR genes are more sensitive to Fe deficiency

Transgenic rice plants expressing a construct to silence *OsASR5* (hereafter OsASR5-RNAi) were previously generated in *Oryza sativa* cv. Nipponbare (hereafter WT) background. The data presented in the original paper and here (see below) show that the expressed construct leads to knockdown of ASR proteins (Arenhart et al., 2013; see below). Since these genes can substitute one another at the promoter binding sites of their target genes and compensate for each other's loss of function (Arenhart et al., 2016), this transgenic line is excellent for exploring the role of ASR proteins in rice mineral nutrition.

We exposed 30-day-old plants of WT and OsASR5-RNAi genotypes to either control (CC) or Fe deficiency (−Fe) treatments for up to 21 days. Fe deficiency-induced chlorosis was observed in leaves of WT plants but was clearly more pronounced in OsASR5-RNAi (Fig. 1A). To better quantify the differences in Fe deficiency-induced changes in leaf color in WT and OsASR5-RNAi plants, we used SPAD to measure chlorosis in the fifth leaf of both genotypes in a time-course −Fe experiment (Fig. 1B). Measurements started after 11 days after the onset of treatment, since the fifth leaf was the last to fully expand during treatment in both genotypes. While SPAD values were slightly lower in OsASR5-RNAi plants under control condition compared to WT, OsASR5-RNAi plants showed considerably lower values compared to WT control under Fe deficiency plants (Fig. 1B). These data further demonstrate that the knockdown of the *OsASR* genes lead to more pronounced leaf chlorosis under −Fe.

We also measured shoot height, root length and their respective dry masses of WT and OsASR5-RNAi plants under control condition and −Fe. The shoot height from the two genotypes were similar in control condition, while both shoot height and shoot dry mass were lower in OsASR5-RNAi under −Fe (Fig. 1C and D). While the WT roots were shorter in −Fe than in control, OsASR5-RNAi had roots longer than WT regardless of treatment (Fig. 1E and G). Despite longer roots, OsASR5-RNAi plants showed lower root dry mass compared to WT under −Fe, while being similar in control condition (Fig. 1F and G), suggesting that OsASR5-RNAi plants partition less carbon to roots. Taken together, these

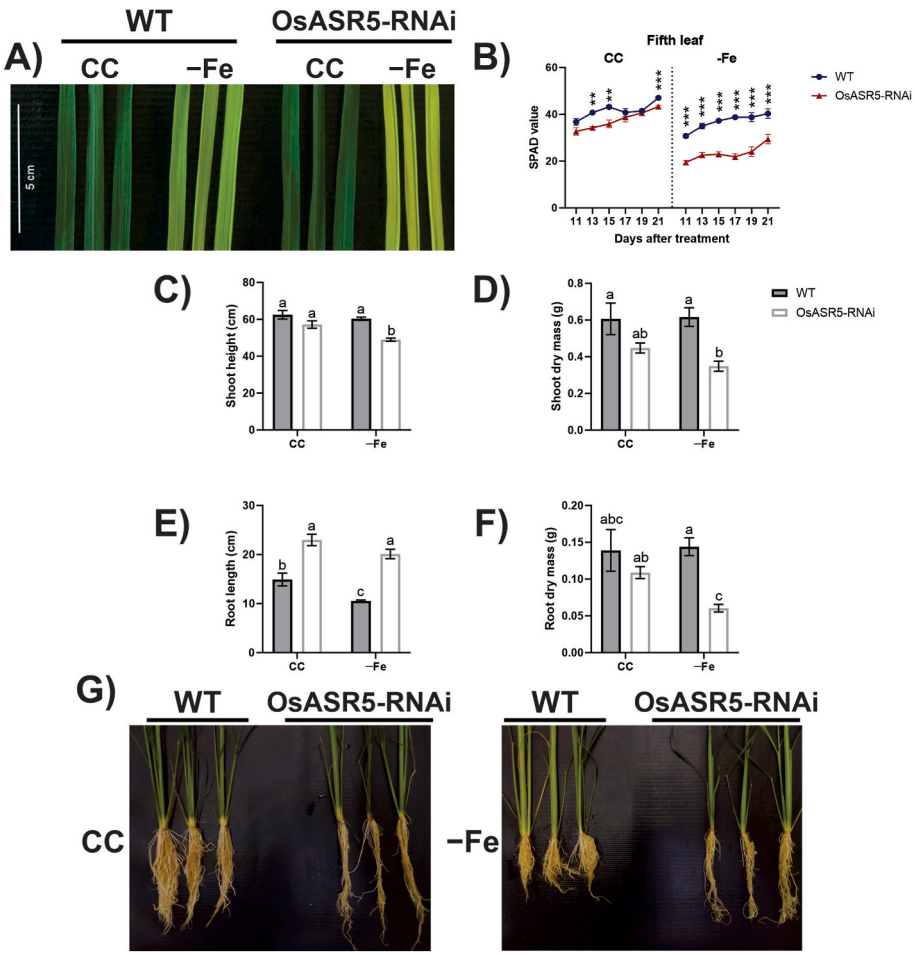

Fig. 1. The phenotype of OsASR5-RNAi plants under Fe deficiency treatment (−Fe). (A) Representative photograph of the fifth leaf of WT and OsASR5-RNAi plants after 21 days of treatment. (B) SPAD values on the fifth leaf of both genotypes during the treatment period. The asterisks denote statistically significant differences between the two genotypes within the same treatment. (C) Shoot height, (D) shoot dry mass, (E) root length and (F) root dry mass of WT and OsASR5-RNAi plants after 23 days of treatment. (G) Representative photographs of the root systems of WT and OsASR5-RNAi plants after 21 days of treatment. Error bars indicate standard error. Statistically significant differences are denoted by asterisks (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$) or by distinct letters. CC = control condition.

results suggest that OsASR5-RNAi plants are more sensitive to −Fe, showing earlier chlorosis and decreased shoot and root biomass accumulation when Fe is low.

3.2. *OsASR5-RNAi plants photosynthesis is reduced under Fe deficiency*

To quantify possible differences in photosynthesis associated with increased chlorosis in OsASR5-RNAi under −Fe, we quantified photosynthetic parameters at 7, 14 and 21 days of treatment (Fig. 2). −Fe clearly decreased the CO_2 assimilation rate in WT plants at 7 and 21 days, and in OsASR5-RNAi plants at 7 and 14 days (Fig. 2A). Interestingly, the overall values for OsASR5-RNAi under control condition was similar to WT plants exposed to −Fe, while OsASR5-RNAi under −Fe showed the lowest values in all three time points. There were no differences comparing time points within each genotype/treatment (Fig. 2A).

Stomatal conductance was similar to all genotypes at 7 days of treatment (Fig. 2B). At 14 days, stomatal conductance showed to be lower in OsASR5-RNAi under −Fe compared to both OsASR5-RNAi under control condition and WT under −Fe. At 21 days, stomatal conductance of OsASR5-RNAi under −Fe slightly increased but it was still lower compared to WT plants under −Fe (Fig. 2B). Consistently, OsASR5-RNAi plants under −Fe showed the highest internal CO_2 concentration (Fig. 2C). Transpiration rate was similar when comparing most genotype-treatment interactions, but OsASR5-RNAi plants exposed to −Fe showed reduction at 14 and 21 days, both compared to 7 days after treatment (Fig. 2D). Water use efficiency (Fig. 2E) and carboxylation efficiency (Fig. 2F) showed similar patterns as found for the CO_2 assimilation rate (Fig. 2A), with OsASR5-RNAi plants with the lowest values when exposed to −Fe.

We also measured chlorophyll *a* fluorescence. Interestingly, Fv/Fm values decreased at 14 days of treatment in OsASR5-RNAi plants under −Fe and recovered at the 21st day (Fig. 2G). WT plants under −Fe, on the other hand, only showed a slight decrease at the 21st day (Fig. 2G). Few differences were observed for Fm values (Fig. 2H), while Fo was clearly increased in OsASR5-RNAi plants under −Fe at 14 days of treatment (Fig. 2I). Taken together, these data strongly suggest that while OsASR5-RNAi plants have defects in photosynthesis, −Fe exacerbates these phenotypes.

3.3. *Elemental profiling of leaves and roots of OsASR5-RNAi plants*

To understand whether down-regulation of *OsASR* genes affected the ionome, we quantified the concentrations of elements in leaves and roots of WT and OsASR5-RNAi plants under control condition and −Fe (Fig. 3). In both treatments, we observed increases in concentrations of some elements in leaves of OsASR5-RNAi plants compared to WT, such as: calcium (Ca; 72 %), copper (Cu; 81 %), manganese (Mn; 67 %), phosphorus (P; 28 %) and Fe (46 %) under control condition; and Cu (32 %), P (55 %), potassium (K; 16 %), sulphur (S; 20 %) and zinc (Zn; 31 %)

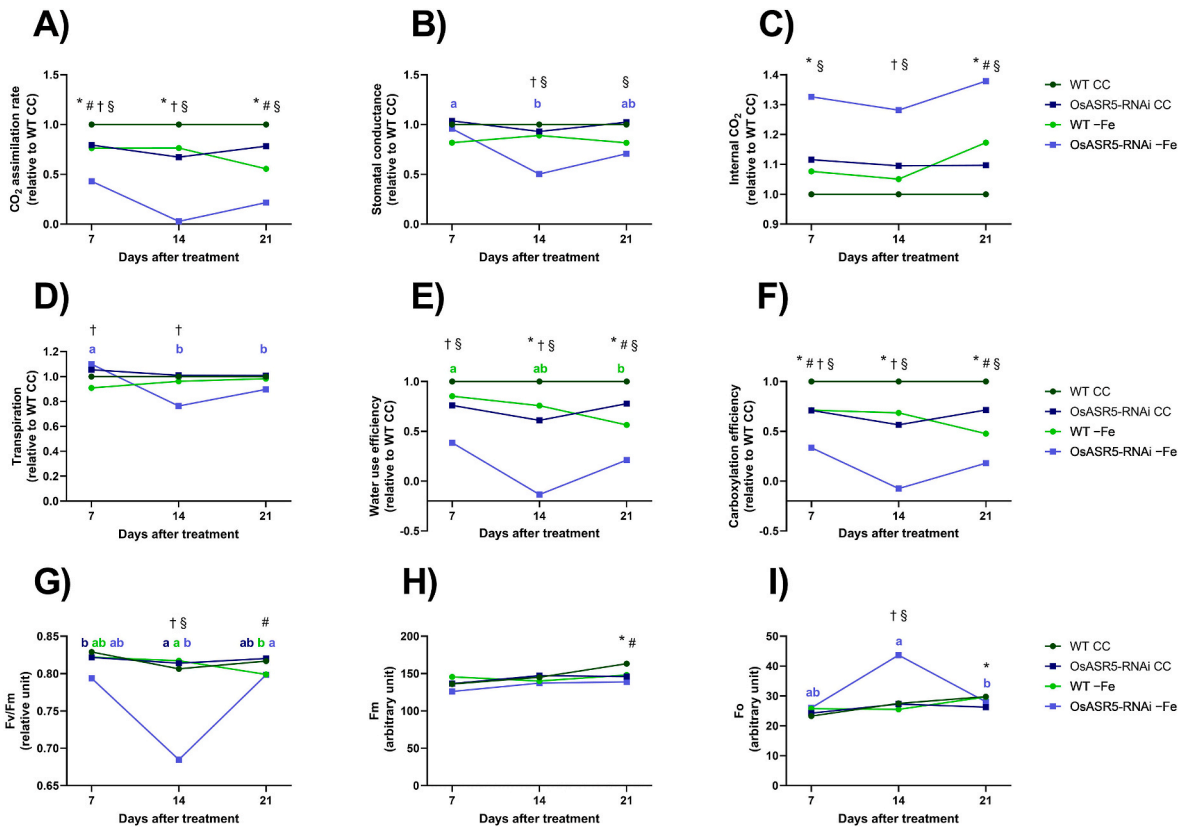

Fig. 2. The impact of *OsASR* genes knockdown and Fe deficiency treatment on photosynthesis. Changes in (A) CO_2 assimilation rate, (B) stomatal conductance, (C) internal CO_2 concentration, (D) transpiration rate, (E) water use efficiency, (F) carboxylation efficiency, (G) maximum quantum efficiency of PSII photochemistry (Fv/Fm), and (H; Fm) maximal and (I; Fo) minimal fluorescence of WT and OsASR5-RNAi rice plants under control condition (CC) or Fe deficiency treatment (−Fe) for 7, 14, and 21 days. All values were normalized relative to WT CC. The symbols indicate statistically significant differences determined by Student's t-test ($P \leq 0.05$) between WT CC and OsASR5-RNAi CC (*), WT CC and WT −Fe (#), OsASR5-RNAi CC and OsASR5-RNAi −Fe (†), or WT −Fe and OsASR5-RNAi −Fe (§) in each time of treatment. Different letters with the same color mean statistically significant difference comparing treatment times within the genotype-treatment group represented with the corresponding color. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

under −Fe (Fig. 3A and B). Increases were also observed in roots of OsASR5-RNAi plants in both treatments: Ca (11 %), Cu (163 %), Mn (40 %) and Zn (19 %) under control condition; and Cu (94 %), Fe (94 %), Mn (48 %), P (29 %) and Zn (57 %) under −Fe (Fig. 3C and D). K was the only element to show reduced concentrations in OsASR5-RNAi roots in both treatments: 15 % in control condition and 28 % in −Fe (Fig. 3C and D). Furthermore, S showed a 13 % decrease only in the control condition in OsASR5-RNAi roots (Fig. 3C).

To understand how −Fe affect both genotypes, we also compared the ionomes of plants from the same genotype in control condition and −Fe in both leaves and roots (Fig. S1). There is an obvious decrease in Fe concentration in leaves and roots of both genotypes, as expected (Fig. S1). Interestingly, we found that P concentration is increased in OsASR5-RNAi leaves, but not in WT leaves, when plants are under −Fe (Figs. S1A and S1B). Zn, S, Ca, Mn and Mg decreased in WT leaves, whereas Cu and K increased (Fig. S1A). In OsASR5-RNAi leaves, Ca, Mn and Mg were decreased, whereas K increased by Fe deficiency (Fig. S1B). In roots, P and Mn is decreased in both genotypes, whereas Cu increased (Figs. S1C and S1D). Taken together, these results suggest that OsASR5-RNAi ionome changes differ from that of WT, which could be related to the −Fe chlorotic phenotype.

3.4. The knockdown of the OsASR genes reduces seed set and changes the seed ionome

We quantified seed set and panicle number in WT and OsASR5-RNAi plants. Panicle number did not differ between WT and OsASR5-RNAi (Fig. 4A). The total number of seeds produced by both genotypes was similar. However, OsASR5-RNAi plants showed higher number of empty seeds and consequently lower number of filled seeds (Fig. 4B). We next analyzed the ionome of seeds from OsASR5-RNAi plants compared to WT. We found that concentrations of several elements are increased in seeds of OsASR5-RNAi plants other than Fe (14 %), such as K (31 %), P (25 %), magnesium (Mg; 16 %), Zn (47 %), Mn (56 %), Cu (58 %) and cadmium (Cd; 254 %) (Fig. 4C). Therefore, these data suggest that the knockdown of *OsASR* genes affects seed set in rice plants, which may also result in increased concentration of several elements in filled seeds.

3.5. Transcriptomic analyses of Fe deficiency response in OsASR5-RNAi plants

To gain insight into possible changes in the transcriptome of OsASR5-RNAi plants that can be associated with increased −Fe sensitivity, we performed RNA-seq of root tissues of both WT and OsASR5-RNAi plants under control condition and −Fe (n = 3). An average of 24.2 million pairs of reads per sample were generated and used to obtain transcript-specific expression data using Salmon (Patro et al., 2017). Approximately 87 % of the 200-bp read pairs mapped to the rice reference transcriptome Osa1 Release 7 (Kawahara et al., 2013) (Supplementary Table S1), allowing for expression quantification of 55, 683 genes (Supplementary Table S2).

The R package 3D RNA-seq was used to perform analyses of differential gene expression (Guo et al., 2021). We identified 1412 differentially expressed genes (DEGs; Supplementary Table S3 and Fig. 5A). From these, 948 genes were regulated by −Fe in WT (406 up-regulated and 542 down-regulated), while 797 genes were regulated in

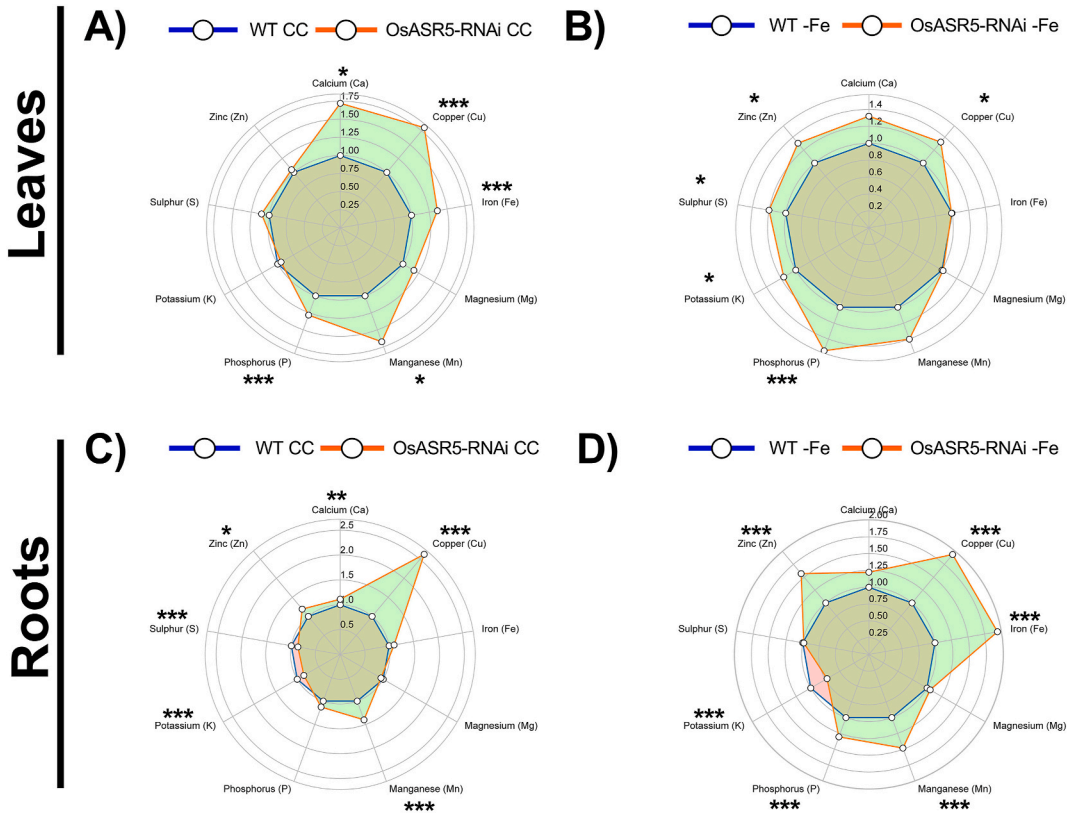

Fig. 3. Radar charts representing the average concentration (n = 6; μg g^{-1} dry mass) of calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), phosphorus (P), potassium (K), sulphur (S) and zinc (Zn) in the leaves and roots of the OsASR5-RNAi line relative to the wild-type (WT) under control condition (CC) or iron deficiency (−Fe) at 21 days of treatment. Statistically significant differences were determined by Student's t-test (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$).

OsASR5-RNAi plants (355 up-regulated and 442 down-regulated). Comparing the up-regulated gene sets, we found 288 genes commonly up-regulated in both genotypes, whereas WT plants showed 118 specific up-regulated genes, and OsASR5-RNAi plants showed 67 genes (Fig. 5B). Among the down-regulated genes, 247 were commonly down-regulated in both genotypes, whereas 295 and 195 were specific to WT and OsASR5-RNAi, respectively (Fig. 5C). These results confirm widespread transcriptomic changes in response to not only Fe deficiency but also knockdown of *ASR* genes in rice.

To gain insight into the pathways and functions potentially associated with the role of OsASR proteins under −Fe, we performed functional enrichment analysis of the DEG groups (Fig. 5B and C) using CARMO (Wang et al., 2015). A total of 22 statistically significant molecular function (MF) categories were detected for the group of genes up-regulated by Fe deficiency in both WT and OsASR5-RNAi genotypes (Fig. 6A). Among these, we found "Transporter activity", "Metal ion transmembrane transporter activity", "Nicotianamine synthase activity", "Metal ion binding", "Zinc ion transmembrane transporter activity", "Iron ion binding" and "Iron ion transmembrane transporter activity" (Fig. 6A and Supplementary Table S4). Interestingly, eight categories were specifically enriched among up-regulated genes in WT, including "Iron ion binding", "Oxidoreductase activity", "ATPase activity", "Heme binding", "Nucleoside-triphosphatase activity" and "Transporter activity" (Fig. 6B and Supplementary Table S4). The genes exclusively up-regulated in OsASR5-RNAi plants were only enriched for "Heme binding" and "Oxidoreductase activity" (Supplementary Table S4). These results confirm that −Fe significantly upregulated genes related to Fe metabolism and metals transport.

We found 27 statistically significant categories commonly enriched in down-regulated genes between WT and OsASR5-RNAi (Fig. 6C and Supplementary Table S4). Among these, we found "Oxidoreductase activity", "Heme binding", "Iron ion binding", "Calcium ion binding", "Metal ion binding", "Nutrient reservoir activity" and "Transporter activity". In WT, 15 categories were specific considering the down-regulated genes, including categories such as "Iron ion binding" and "Metal ion binding" (Fig. 6D and Supplementary Table S4). In OsASR5-RNAi, 19 categories were enriched considering the down-regulated genes, including "ATP binding", "ADP binding", "Kinase activity", "Nucleotide binding", "Protein kinase activity" and "Protein serine/threonine kinase activity" (Fig. 6E and Supplementary Table S4). Taken together, these results demonstrate that, despite similarities, the two genotypes have some differences in how they respond to −Fe.

3.6. *Differentially expressed genes under Fe deficiency in OsASR5-RNAi plants*

To better understand how OsASR5-RNAi plants differ in their response to Fe deficiency compared to WT plants, we performed hierarchical clustering of all 1412 DEGs based on expression patterns, and separated them into 10 clusters (Fig. 5A and Supplementary Table S5).

Among these, we identified two clusters that contained mostly genes up-regulated by −Fe, regardless of the genotype: clusters 4 and 7. Cluster 4 has 196 genes, including the transcription factors *OsIRO2/OsbHLH056* (Ogo et al., 2006, 2011), *OsIRO3/OsbHLH063* (Li et al., 2022a; Zheng et al., 2010), *OsbHLH133* (Wang et al., 2013) and *OsFIT/OsbHLH156* (Wang et al., 2020); E3 ubiquitin ligase *OsHRZ2* (Guo et al., 2022; Kobayashi et al., 2013); *OsOPT7* (Bashir et al., 2015); *OsIMA1* (*IRONMAN* (Kobayashi et al., 2021; Peng et al., 2022)); *OsMIR* (de Oliveira et al., 2020; Ishimaru et al., 2009); the nicotianamine (NA)/deoxymugineic acid (DMA) transporter *OsZIFL4/OsTOM1* (Nozoye et al., 2011; Ricachenevsky et al., 2011); the two efflux transporters of NA *OsENA1* and *OsENA2/OsSTAR1* (Huang et al., 2009; Nozoye et al., 2019); and several other transporters such as *OsNRAMP1* (Curie et al., 2000), *OsIRT2* (Nakanishi et al., 2006), *OsYSL13* (Ponte

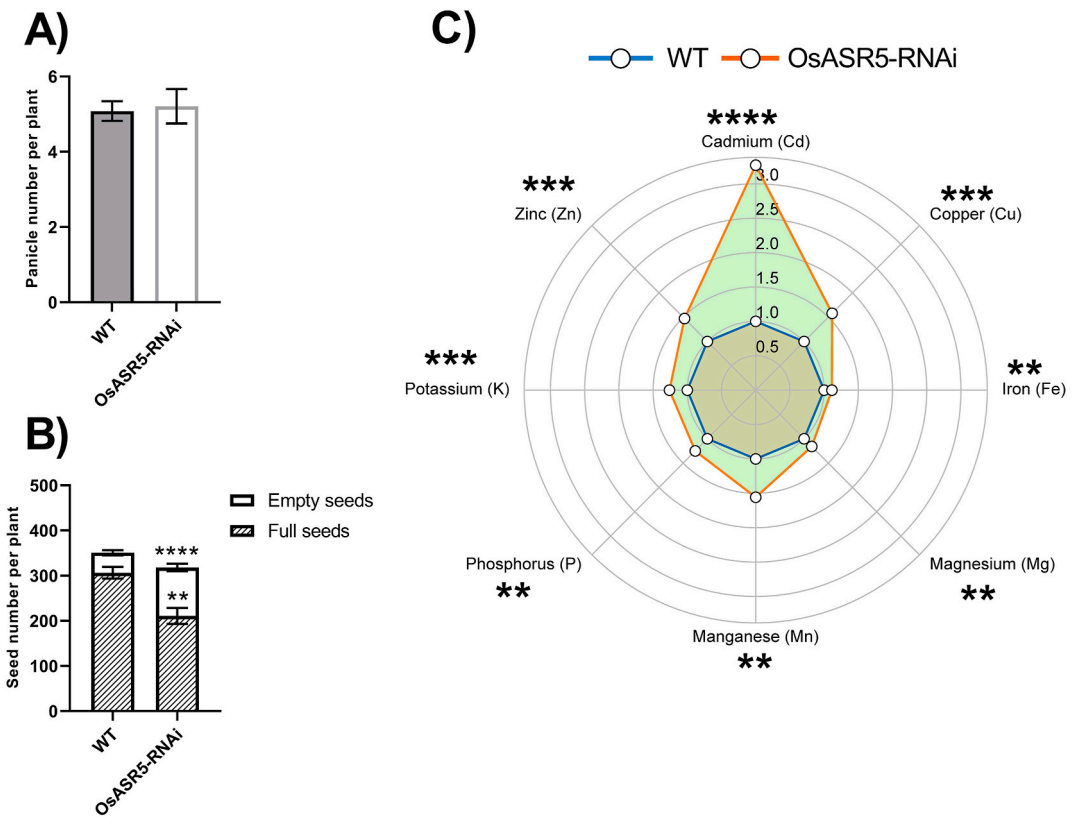

Fig. 4. The impact of *OsASR* genes knockdown on seed set and ionome composition. (A) Panicle number per plant and (B) number of filled and empty seeds per plant between WT and OsASR5-RNAi genotypes. (C) Radar chart representing the average concentration (n = 5; μg g^{-1} dry mass) of cadmium (Cd), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), phosphorus (P) and potassium (K) in seeds of the OsASR5-RNAi line relative to the wild-type (WT). Statistically significant differences were determined by Student's t-test (**$P \leq 0.01$; ***$P \leq 0.001$).

et al., 2023; Zhang et al., 2018) and *OsZIFL12/OsVMT* (Che et al., 2019; Ricachenevsky et al., 2011) (Fig. S2 and Supplementary Table S5). Genes encoding transporters for other nutrients were also identified, such as the Cu transporter *OsCOPT7* (Guan et al., 2024; Tang et al., 2024), the sulphate transporter *OsSULTR3;3* (Zhao et al., 2016) and the Zn transporter *OsZIP4* (Ishimaru et al., 2005). In addition, the arsenic (As)-responsive transporters *OsABCB11* (LOC_Os01g50100) (Wang et al., 2017) and *OsMATE2* (Das et al., 2018) were also present (Fig. S2 and Supplementary Table S5). Cluster 7 has 191 genes, including other well-known genes responsive to −Fe such as the E3 ubiquitin ligase *OsHRZ1* (Kobayashi et al., 2013), and important transporters, such as *OsIRT1* (Bughio et al., 2002; Lee and An, 2009), *OsYSL15* (Inoue et al., 2009; Lee et al., 2009), *OsYSL9* (Senoura et al., 2017) and *OsPEZ2* (Bashir et al., 2011) (Fig. S3 and Supplementary Table S5).

While all genes in Cluster 4 are similarly regulated in WT and OsASR5-RNAi plants, 15 genes in Cluster 7 show differences in expression levels comparing the two genotypes, being either not up-regulated by −Fe in OsASR5-RNAi plants or up-regulated to a lower extent compared to WT (Supplementary Table S5). Among these genes, we found the pleiotropic drug resistance (PDR) ABC transporter *OsPDR16/OsABCG32* (*LOC_Os01g24010*) (Crouzet et al., 2006; Verrier et al., 2008); a hypothetical protein encoding a small peptide (*LOC_Os02g37260*), a terpene synthase (*LOC_Os08g07120*) and the laccase gene *OsLAC23* (*LOC_Os11g42200* (Liu et al., 2017);) (Fig. 7A−7D). Another eight genes are located in chromosome 7, with five located within ~200 kb. These include one transposon gene (*LOC_Os07g25030*) and four thionin-like genes, organized in two in tandem pairs (*LOC_Os07g24820* and *LOC_Os07g24830*; *LOC_Os07g25050* and *LOC_Os07g25060*) (Fig. 7E−7I). Moreover, *OsZIFL2* was up-regulated by −Fe in WT but not in OsASR5-RNAi (Fig. 7J). These genes seem to be directly or indirectly dependent on OsASR proteins for up-regulation under −Fe.

In Cluster 10, we found genes that are negatively regulated by OsASR proteins, since their expression was up-regulated under −Fe only in OsASR5-RNAi plants (Fig. 5A and Supplementary Table S5). Among them, we found *OsYSL2*, a known Fe-NA transporter located in the phloem that is important for long distance Fe translocation. *OsYSL2* is induced by −Fe in roots of WT plants, in agreement with data from the literature (Ishimaru et al., 2010; Koike et al., 2004). Interestingly, OsASR5-RNAi plants show expression levels under control condition comparable to WT under −Fe, and much more pronounced up-regulation under −Fe (Fig. 8A). The result suggests that OsASR5-RNAi might not be able to adequately regulate long-distance Fe transport.

Cluster 5 contained genes down-regulated in response to −Fe in both genotypes (Fig. 5A and Supplementary Table S5), including *OsFER1* and *OsFER2* (Stein et al., 2009), which are known to be positively regulated by Fe excess (Wairich et al., 2021) and negatively by −Fe (Wairich et al., 2019) (Fig. 8B and C). We also observed that *OsASR* genes are grouped in Cluster 3 and Cluster 9 (Supplementary Table S5 and Supplementary Table S6). *OsASR1* and *OsASR2* are in Cluster 3. They were both down-regulated by −Fe in WT, alongside *OsASR3* (Fig. 8D−8F). Intriguingly, *OsASR1* was the only *OsASR* gene to be not down-regulated in OsASR5-RNAi under the control condition (Fig. 8D). *OsASR3* and the three remaining *OsASR* genes, located in Cluster 9, were down-regulated in OsASR5-RNAi in both treatments (Fig. 8F−8I), which demonstrates that the construct is decreasing the expression of transcripts from this gene family. Altogether, these results demonstrate that *OsASR1*, *OsASR2* and *OsASR3* transcripts tend to be down-regulated in roots of WT plants under −Fe, and that the OsASR5-RNAi plants have decreased expression of *OsASR* genes, except for *OsASR1*.

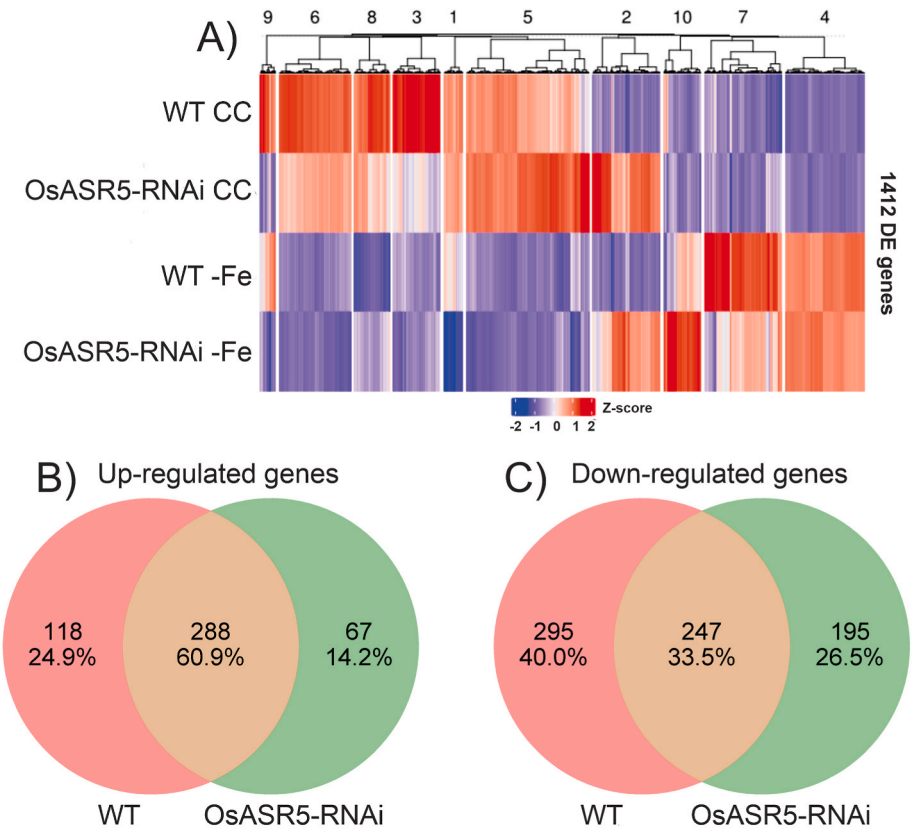

Fig. 5. Differentially expressed genes (DEGs) under Fe deficiency (−Fe) in both WT and OsASR5-RNAi genotypes. (A) Heatmap of 1412 DE genes between WT and OsASR5-RNAi plants under control condition (CC) or Fe deficiency (−Fe). (B and C) Venn diagrams showing the specific and common up- or down-regulated genes between the two genotypes under −Fe.

4. Discussion

ASR proteins are known to play a role in plant responses to abiotic stresses, such as drought, salinity and cold, as well as in fruit ripening regulation (Jia et al., 2016; Li et al., 2018; Parrilla et al., 2022; Yacoubi et al., 2022). Two ASR proteins from rice, OsASR1 and OsASR5, are well characterized as transcription factors and are positive regulators of genes involved in aluminum (Al) tolerance (Arenhart et al., 2013, 2014, 2016). In these studies, OsASR5-RNAi plants were more sensitive than WT to Al toxicity, as well as showing decreased transcript levels for *OsASR* genes (Arenhart et al., 2013). The latter is in accordance with our RNA-seq data (Fig. 8D−I; Supplementary Table S2). *OsASR3* to *OsASR6* showed lower expression levels in OsASR5-RNAi plants regardless of the treatment (Fig. 8F−8I; Supplementary Table S6). *OsASR2* showed lower expression levels in OsASR5-RNAi only under the control condition (Fig. 8E; Supplementary Table S6). In addition, *OsASR1*, *OsASR2* and *OsASR3* showed to be down-regulated by −Fe in WT plants (Fig. 8D−8F; Supplementary Table S6). Thus, our data indicate that the expression of some *OsASR* genes is altered regarding the Fe status of the plant.

We observed that OsASR5-RNAi plants are more sensitive to −Fe, showing decreased shoot growth, more pronounced chlorosis and decreased photosynthetic activity (Figs. 1 and 2). Under control condition, OsASR5-RNAi plants have increased Fe concentration in leaves, whereas under −Fe, OsASR5-RNAi plants leaf Fe concentration was similar to WT (Fig. 3A and B). Roots of OsASR5-RNAi plants have higher Fe concentration under −Fe compared to WT (Fig. 3D). Therefore, it seems that Fe distribution in OsASR5-RNAi is disturbed, with roots retaining Fe under −Fe, which may be linked to lower shoot growth. It is interesting to note that P concentration is increased in OsASR5-RNAi leaves in both treatments (Fig. 3A and B), and in roots under −Fe (Fig. 3D). Fe deficiency is known to increase P concentration in roots and shoots of rice plants (Alves et al., 2025), and antagonistic interaction of Fe and P homeostasis has been extensively studied (Guo et al., 2022; Lay-Pruitt et al., 2022; Nam et al., 2021; Therby-Vale et al., 2022; Yang et al., 2024; Wairich et al., 2025). In *Arabidopsis thaliana*, −Fe leads to the accumulation of ROS in the chloroplast, which triggers the down-regulation of *AtbZIP58*, a basic leucine zipper transcription factor that controls the expression of several photosynthesis-related genes (Nam et al., 2021). This results in the occurrence of −Fe-induced chlorosis in leaves, which is dependent on P bioavailability. On the other hand, under combined Fe and P deficiency (−Fe −P), ascorbic acid accumulates in the chloroplast via the inorganic phosphate (Pi) transporter AtPHT4; 4, preventing the accumulation of ROS and consequently blocking the down-regulation of *AtbZIP58*. Thus, −Fe-induced chlorosis is not observed in −Fe −P (Nam et al., 2021). In addition, a reciprocal inhibitory module was also described between the negative regulators of Fe homeostasis, OsHRZ1 and OsHRZ2, and the central regulators of Pi signaling, OsPHR1 and OsPHR2 (PHOSPHATE STARVATION RESPONSE). Under −Fe, the HRZs are up-regulated both transcriptionally and translationally, which triggers the degradation of PHRs. This is supposedly a mechanism to prevent Pi accumulation from interfering with Fe root-to-shoot translocation (Guo et al., 2022). Furthermore, *phr2* and *phr1 phr2* plants were more chlorotic and had lower Fe shoot/root rates under −Fe and also in −Fe −P. Opposite results were observed in *phr2 hrz2* plants, indicating that OsPHRs positively promote the −Fe response by indirectly promoting the degradation of OsHRZs (Guo et al., 2022). Our results indicate that OsASR5-RNAi plants have higher P concentrations, which could be involved in the increased susceptibility to −Fe-induced chlorosis. Further work will be necessary to explore if the mechanism involving OsPHR2/OsHRZs are linked to ASR proteins.

It is also important to mention the increases observed in the

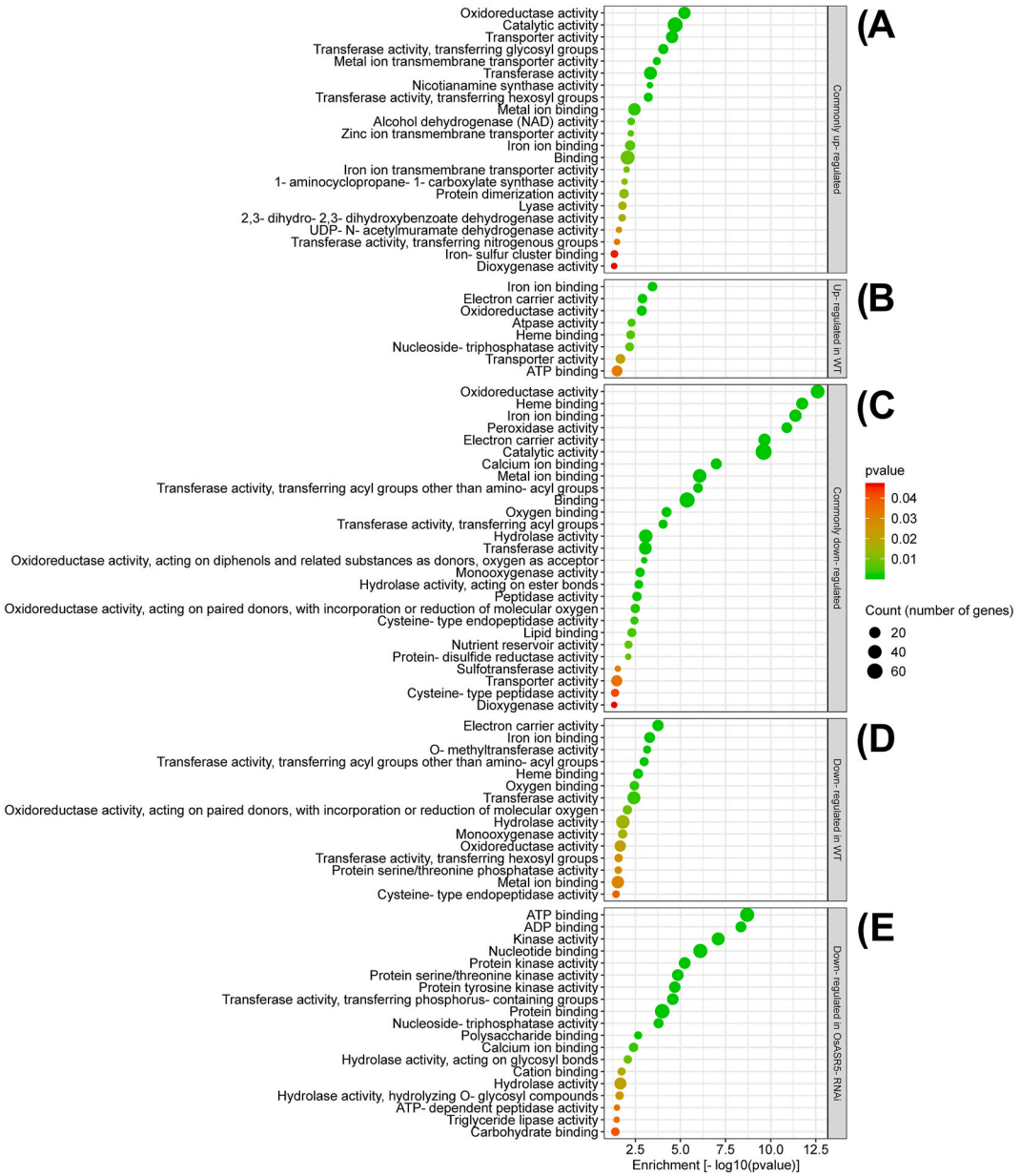

Fig. 6. Enrichment analysis of molecular functions (MF) categories of: (A) commonly up-regulated genes, (B) up-regulated genes in WT, (C) commonly down-regulated genes, (D) down-regulated genes in WT and (E) down-regulated genes in OsASR5-RNAi, comparing -Fe and control data for each genotype.

concentrations of other elements. Elemental homeostases are known to cross-talk with that of Fe by common regulatory pathways or simply by non-specific transport performed by induced divalent metal transporters (Rai et al., 2021; Wu et al., 2022; Wairich et al., 2025). Cu showed higher concentrations in the roots and leaves of OsASR5-RNAi plants under both treatments, when compared with the WT (Fig. 3). It was demonstrated that −Fe stimulates greater uptake and accumulation of Cu in different plant species (Garcia-Molina et al., 2020; Kastoori Ramamurthy et al., 2018; Waters et al., 2012; Waters and Armbrust, 2013), supposedly as a strategy for Cu/Zn superoxide dismustases (SODs) to compensate for the low levels of Fe-containing SODs in ROS scavenging (Waters et al., 2012). Regarding Zn, we observed higher concentrations in OsASR5-RNAi leaves and roots under −Fe compared to WT in the same conditions (Fig. 3B and D). Kastoori Ramamurthy et al. (2018) also observed higher Zn concentrations in Arabidopsis roots and rosettes under −Fe, but lower in comparison with the increases in Cu concentrations. It is worth noting that the Fe(II) transporter OsIRT1, which is up-regulated under −Fe, is also capable of transporting Zn, which may explain the increases in Zn concentrations in plants subjected to −Fe (Korshunova et al., 1999; Wu et al., 2022). However, *OsIRT1* was not differentially expressed between the two genotypes in our RNA-Seq data (Fig. S3B). It will be an interesting avenue to explore how other micronutrients might be involved in OsASR5-RNAi phenotype under Fe deficiency.

Our data suggest that the mechanism by which OsASR proteins might be affecting Fe homeostasis does not involve altered Fe uptake (Kobayashi and Nishizawa, 2012). Besides *OsIRT1*, we found that −Fe markers such as *OsYSL15*, *OsIRO2*, *OsHRZ1* and *OsHRZ2*, among others (Bughio et al., 2002; Inoue et al., 2009; Kobayashi et al., 2013; Lee et al., 2009; Lee and An, 2009), are similarly up-regulated in roots of both genotypes (Fig. S2 and Fig. S3). These data suggest that OsASR5-RNAi plants are able to properly induce the main regulatory network controlling Fe uptake, which is regulated by the transcription factor OsIDEF1 (Kobayashi et al., 2007; Kobayashi and Nishizawa, 2012). Therefore, this suggests that Fe homeostasis in OsASR5-RNAi plants is misregulated in other aspects, such as long-distance Fe transport.

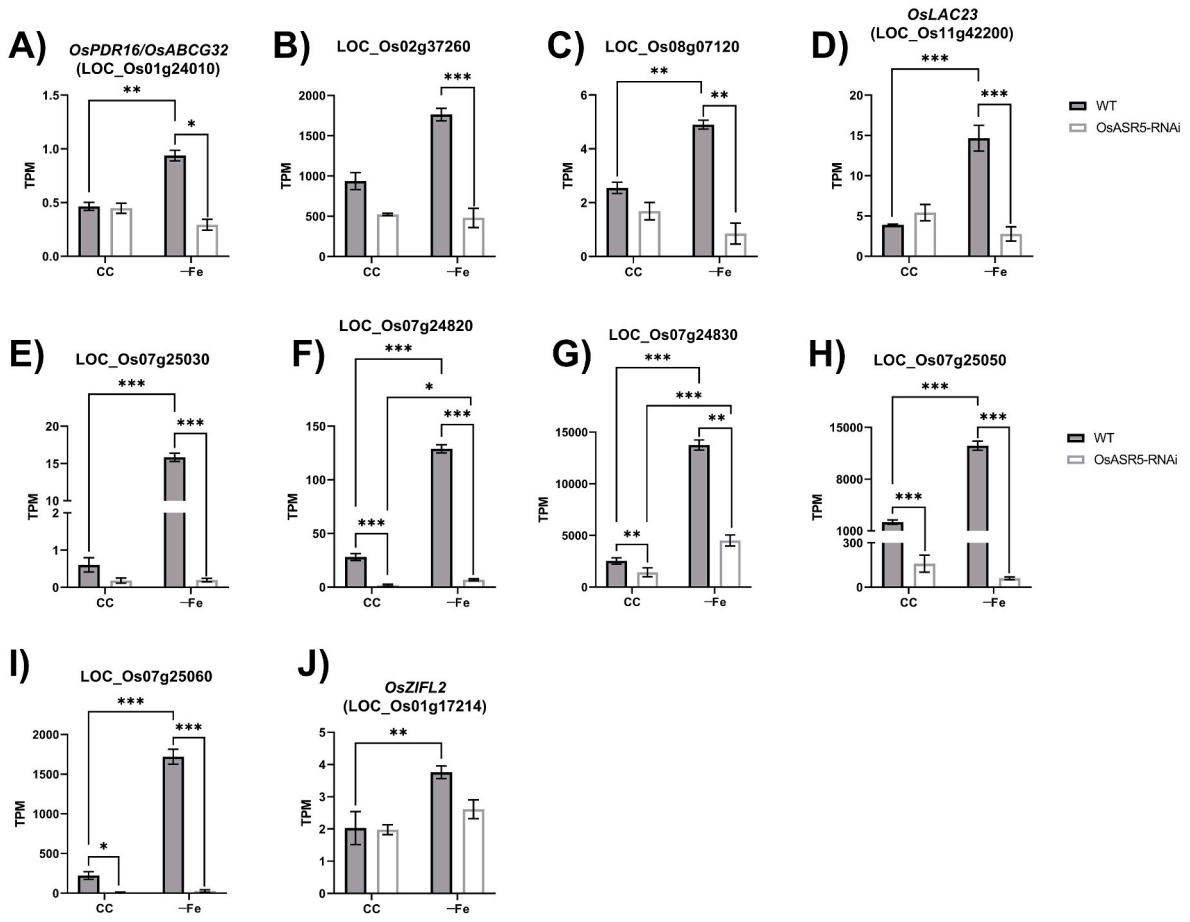

Fig. 7. Normalized expression levels from RNA-Seq of key genes located into cluster 7 under control condition (CC) or Fe deficiency (−Fe), such as (A) *OsABCG32/OsPDR16*, (B) a hypothetical protein encoding a small peptide (LOC_Os02g37260), (C) a terpene synthase (LOC_Os08g07120), (D) a laccase (LOC_Os11g42200), (E) a transposon gene (LOC_Os07g25030), (F to I) four thionin-like genes and (J) *OsZIFL2*. Statistically significant differences were carried out with the 3D RNA-seq app (* adjusted $P \leq 0.01$; ** adjusted $P \leq 0.001$; *** adjusted $P \leq 0.0001$). TPM, transcripts per million.

In agreement with that, *OsYSL2* expression was up-regulated in OsASR5-RNAi plants compared to WT, either in control condition or −Fe (Fig. 8A). OsYSL2 is a Fe(II)/Mn(II)-NA transporter that is involved in Fe phloem long-distance transport within the plant and Fe translocation into grains (Ishimaru et al., 2010; Koike et al., 2004; Nozoye et al., 2007). Its expression is directly regulated by the NAC transcription factor IDEF2, which is constitutively expressed regardless of the Fe status of the plant (Ogo et al., 2008). Since IDEF2-OsYSL2 regulatory module is distinct from OsIDEF1-OsIRO2-OsYSL15 module (Kobayashi et al., 2010; Kobayashi and Nishizawa, 2012), our data suggest that OsASR proteins might act as a regulator of this branch of Fe deficiency response in roots.

It is also known that there is natural variation of *OsYSL2* in rice genotypes. A minor *OsYSL2* allele, named as *CF1*, was linked to Cd accumulation in grains indirectly by affecting Fe homeostasis (Li et al., 2022b). The authors observed that near isogenic lines with the high-expression, high Fe affinity *CF1*-allele, compared to the major allele, had higher concentrations of Fe in shoots and grains, while having lower concentrations of Cd. They suggested that since CF1 has no affinity to Cd-NA, Fe status is higher in plants with the *CF1* allele, which results in decreased expression of Fe and Cd transporter *OsNRAMP5* and *OsNRAMP1*, consequently decreasing Cd translocation to aerial tissues (Ishimaru et al., 2012; Takahashi et al., 2011, 2014; Zhang et al., 2024). Although we observed higher expression of *OsYSL2* in OsASR5-RNAi and increased chlorosis, but no change in *OsNRAMP5* or *OsNRAMP1*, it is possible that OsASR5-RNAi is interfering with the regulation of OsYSL2, changing Fe distribution and resulting in increased chlorosis. Moreover, we found that *OsASR* down-regulation changed the seed ionome, comparable to the phenotype observed in CF1 minor allele-harboring plants (Li et al., 2022b). For example, we observed a substantial increase in the concentration of Cd in the grains of *OsYSL2*-up-regulated OsASR5-RNAi plants, when compared to WT (Fig. 4C). Higher concentration of Cd was also observed in the grains of plants carrying the high Fe affinity CF1-allele, suggesting that OsASR5-RNAi plants misregulating of OsYSL2 might be linked to the observed seed phenotype. Taken together, these data suggest that OsASR proteins might be involved in the regulation of long-distance Fe transport through OsYSL2.

Another possible downstream candidate for OsASR proteins regulation is OsZIFL2 (ZINC-INDUCED FACILITATOR LIKE) (Ricachenevsky et al., 2011). *OsZIFL2* was up-regulated by −Fe in roots of WT plants, but not in OsASR5-RNAi plants (Fig. 7J). The data suggest that OsASR proteins might be positive regulators of *OsZIFL2*. AtZIF1 from *Arabidopsis thaliana* was the first ZIFL protein functionally characterized (Haydon et al., 2012; Haydon and Cobbett, 2007). It was shown to be a vacuolar transporter, affecting Fe and Zn homeostasis by changing NA distribution. Two paralogs, *AtZIFL1* and *AtZIFL2*, were also characterized, and it was shown that AtZIFL1 is at least partially redundant with AtZIF1, whereas AtZIFL2, albeit also affecting Fe and Zn homeostasis, does not functionally overlap with AtZIF1 (Lee et al., 2021). In rice and other monocots, the *ZIFL* gene family is extensively expanded (Ricachenevsky et al., 2011). This might be related to the diversification of substrates, since *Poaceae* species synthesize and use phytosiderophores (PSs) for metal uptake and movement within the plant. For

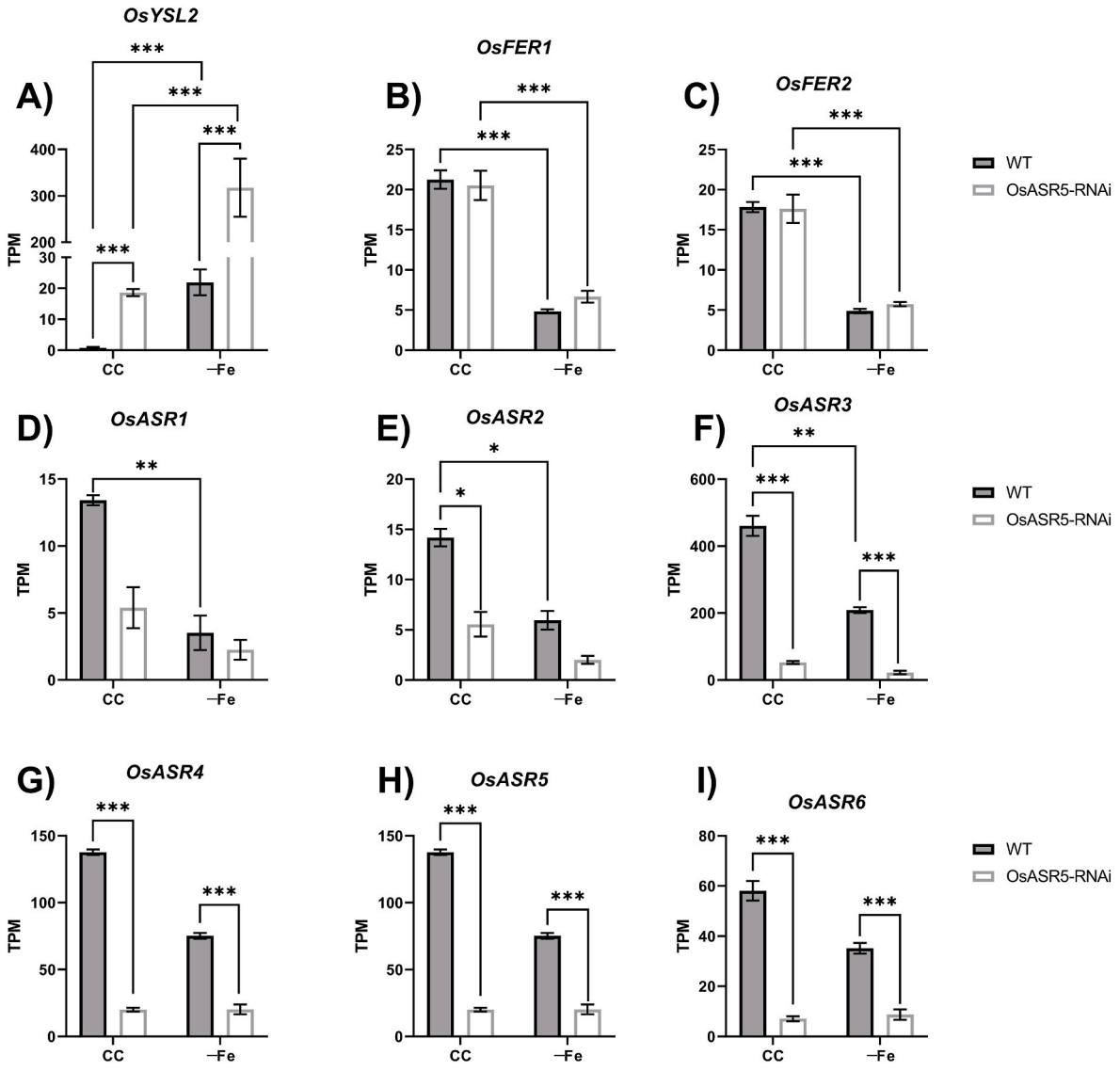

Fig. 8. Normalized expression levels from RNA-Seq of (A) *OsYSL2*, (B) *OsFER1*, (C) *OsFER2* and (D to I) the six rice *OsASR* genes under control condition (CC) or Fe deficiency (−Fe). Statistically significant differences were determined using the 3D RNA-seq app (* adjusted $P \leq 0.01$; ** adjusted $P \leq 0.001$; *** adjusted $P \leq 0.0001$). TPM, transcripts per million.

example, OsZIFL4/TOM1 is a plasma-membrane-localized transporter that secretes PSs to the rhizosphere (Nozoye et al., 2011), whereas OsZIFL12/OsVMT is a vacuolar NA/DMA transporter (Che et al., 2019). Knockdown or knockout of either gene affects Fe homeostasis. Yet, OsZIFL2 has no characterized function, but it is plausible that it may play a role in NA and/or PS transport within plant cells, and therefore might be involved in regulating Fe transport.

The four genes annotated as *thionins-like* (LOC_Os07g24820, LOC_Os07g24830, LOC_Os07g25050 and LOC_Os07g25060) also represent potential downstream targets of the OsASR proteins, since all were significantly less expressed in OsASR5-RNAi plants compared to the WT in −Fe (Fig. 7F−7I). To a lesser extent, the same was observed in the control condition for LOC_Os07g24820 and LOC_Os07g25050. Interestingly, LOC_Os07g24820 and LOC_Os07g24830 were expressed in OsASR5-RNAi under −Fe, when compared to the control condition. Thionins are small peptides that contain around 45–48 amino acid residues and have a characteristic cysteine-rich structure (Stec, 2006). They are typically known to have a broad-spectrum antimicrobial activity, which makes them important components of the plant's innate immune system against different pathogens, such as bacteria, fungi and nematodes (Höng et al., 2021). In this sense, it is known that leguminous plants secret small cysteine-rich peptides from roots to promote Fe sequestration. This triggers an increase in Fe uptake by bacteria, which stimulates nitrogenase, thus improving the nitrogen symbiotic fixation process (Sankari et al., 2022). In the rice genome, 44 genes coding for canonical thionins (OsTHIONs) were identified (Silverstein et al., 2007). To our knowledge, few rice thionin genes have been studied (Boonpa et al., 2019; Ji et al., 2015; Kitanaga et al., 2006). It was observed that the overexpression of *OsTHION9* resulted in Cd-sensitive yeast growth in media containing 10 μM or 20 μM Cd (Liu et al., 2023). *OsTHION9*-overexpressing rice plants showed higher yield per plant and reduced Cd levels in shoots and grains compared to the WT when grown in Cd-contaminated soil. The opposite results were observed in OsTHION9-knockout plants. Taken together, thionins and thionins-like seem to be interesting candidate genes for a possible role in Fe homeostasis and other metals.

5. Conclusions

Our work demonstrates that ASR proteins have a role in Fe

homeostasis. We observed decreased growth and photosynthesis, increase leaf chlorosis, and altered ionome in plants silenced for ASR genes. Transcriptomic data suggests that *OsYSL2* is misregulated in OsASR5-RNAi plants, suggesting that the phenotype might result from altered Fe long-distance transport. We also identified other candidate genes that might be involved in Fe deficiency sensitivity, such as putative thionin and thionin-like peptides.

Declaration of competing interest

The authors declare that there are no potential competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2025.109882.

Data availability

Data will be made available on request.

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