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The Effects of Exogenous Iron on the Photosynthetic Performance and Transcriptome of Rice under Salt–Alkali Stress

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Abstract: Saline-sodic stress induces iron deficiency in rice, reduces leaf photosynthetic performance, and inhibits yield enhancement. In this study, we investigated the effects of exogenous Fe on the photosynthetic performance and transcriptomics of two different tolerant rice cultivars CB9 (Changbai9: saline tolerant cultivar) and TH899 (Tonghe899: saline sensitive cultivar) with 4-week-old Fe-deficient rice seedlings under saline stress, Fe deficiency stress, and both co-stresses. The results showed that under saline and alkaline stress, spraying exogenous iron favored the growth of the two cultivars of rice, with a 32.68% and 39.82 increase in fresh weight, a 2.20-fold and 2.16-fold increase in pigment, respectively, and an 80.28% and 100.00% increase in net photosynthetic rate, respectively, as compared with the iron-deficiency treatment. Transcriptome analysis showed that we found a higher number of differentially expressed genes (7785 differentially expressed genes) in response to exogenous Fe spraying in the soda-salt sensitive variety TH899. The differentially expressed genes that are common to the two cultivars are primarily enriched in metabolic pathways, including plant hormone signal transduction (map04075) and phenylpropanoid biosynthesis (map00940). Specifically, among these genes, 14 are differentially expressed in the carotenoid biosynthetic metabolic pathway. The differentially expressed genes specific to the salinity-tolerant variety CB9 were mainly enriched in the metabolic pathways of glyoxylate and dicarboxylic acid methyl metabolism (map00630), and carbon fixation in photosynthetic organisms (map00710), among which 20 genes were significantly expressed in the pathway for carbon fixation in photosynthetic organisms (map00710). The research results offer specific theoretical support for enhancing the salt tolerance of rice.

Keywords: rice; saline-sodic; iron; photosynthesis; transcriptomics

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1. Introduction

Rice (*Oryza sativa* L.) is one of the world's staple food crops, and the safe production of rice affects world food security and stability [1]. The western section of the Songnen Plain in northeastern China is recognized as one of the world's top three regions with extensive saline-sodic soil. The presence of sodium bicarbonate ($NaHCO_3$) and sodium carbonate (Na_2CO_3) in the soil, combined with elevated pH levels exceeding 8.5, significantly impede crop development [2]. Cultivation of rice reduces the salinity of the topsoil and improves the physical properties and nutrient status of the soil. Therefore, rice cultivation is one of the most effective biological measures for the improvement and utilization of soda saline land [3]. However, rice is a moderately salt-sensitive plant, and soda saline rice is directly subjected to ionic stress, osmotic stress, high pH stress, and oxidative stress caused by saline-sodic conditions [4]. In addition, high pH stress promotes rice Na^+ accumulation, exacerbates Na^+ toxicity under high salinity conditions [5], and leads to deficiencies in trace elements, such as iron (Fe), which results in deficient stress on plants and greatly limits the yield and economic benefits of rice [6,7].

Photosynthesis is one of the most sensitive physiological processes in plants and the basis for crop growth and yield formation [8]. Scientific investigations have demonstrated that in a saline-sodic ecosystem, plants respond by partially closing their leaves as a mechanism to minimize transpiration and uphold osmotic equilibrium, Consequently, this adaptive response results in a decline in carbon dioxide (CO_2) assimilation and a reduction in stomatal conductance [9]. In addition, salinity stress inhibits photosynthetic pigment synthesis on the one hand and disrupts the lamellar structure of chloroplast basal lamellae on the other hand, decreasing the chlorophyll and carotenoid content in leaves [10]. High pH also precipitates Fe-phosphate complexes with iron (Fe) [11], which reduces the effectiveness of Fe and leads to iron deficiency in plants, further inhibiting normal plant growth and development [6,7].

Studies have shown that alkali stress induces leaf chlorosis, which is thought to be a symptom of iron deficiency due to reduced iron uptake or reduced iron effectiveness [12]. Iron is an important cofactor for many enzymes, and iron deficiency affects physiological and biochemical processes throughout the plant, such as reduced leaf chlorophyll and carotenoid concentrations and decreased efficiency of cellular respiration and photosynthesis, leading to reduced yield and quality [12–14]. Liu et al. showed that the addition of moderate amounts of Fe to the soil could increase stomatal conductance and soil and plant analyzer development (SPAD) values by promoting stomatal opening and chlorophyll synthesis [15]. Yoon et al. showed that the application of nano zero-valent iron increased the chlorophyll content of Arabidopsis thaliana, promoting an increase in the rate of CO_2 assimilation, interstitial CO_2 concentration, transpiration rate, and stomatal conductance, while it also enhanced photosynthesis [16].

Transcriptome analysis can be used to study changes in gene expression in different organisms and improves our understanding of plant response mechanisms under different stress types. In response to salt stress, plants employ various regulatory mechanisms to enhance their salt tolerance. These mechanisms involve the modulation of reactive kinases, which include calcium-regulated phosphatase B-like proteins (CBL) and the associated protein kinases (CIPKs), as well as mitogen-activated protein kinase (MAPK) cascades and calcium-dependent protein kinases (CPKs), such as OsCPK4, OsCPK12, or OsCPK21 in rice. Activation of these kinases significantly contributes to the augmentation of salt tolerance in plants [17,18]. Transcription factors, such as AP2/ERF, bHLH, and NAC, also actively respond to changes caused by salt stress [18,19]. Research examining iron deficiency in plants reveals that the process of iron absorption, conveyance, and storage within plant structures necessitates a methodical collaboration among tissues and organelles. This intricate process is finely orchestrated by iron chelators, transport proteins, and an array of regulatory factors [20]. Wang and colleagues deduced that the gene expression pertaining to the superfamily (MFS), ABC transporter superfamily, natural resistance-associated macrophage protein (NRAMP) family, and oligopeptide transporter protein (OPT) family significantly influences the adaptation of wheat to iron-deficiency stress [20].

Studies have shown that foliar spraying of iron is a practical, inexpensive, and convenient technique for fortifying grains that can effectively alleviate iron deficiency symptoms in plants [21]. Currently, studies on rice under saline-sodic conditions and its induced iron deficiency are mainly focused on physiology, with fewer studies at the molecular level. Therefore, the present study was proposed to elucidate the effect of spraying exogenous Fe on the growth and photosynthetic performance of Fe-deficient rice under saline-sodic stress. Our aim was to reveal the feasibility of exogenous Fe in improving the photosynthetic efficiency and salinity tolerance of rice, to further improve the regulatory network of growth and photosynthetic characteristics of rice under saline-sodic stress by using transcriptomics, to provide more knowledge on the gene regulatory network of rice responding to exogenous Fe under saline-sodic stress, and to identify the possible important hubs that regulate these networks, so as to provide certain theoretical basis for this technique. We also aimed to further refine the regulatory networks of rice growth and photosynthetic characteristics under saline-sodic stress using transcriptomics technology, to provide more insights into

the gene regulatory networks of rice in response to exogenous iron under saline-sodic stress, and to identify possible important hubs regulating these networks. The study provides some theoretical basis for constructing high-yield rice cultivation techniques.

2. Materials and Methods

2.1. Experimental Design

The saline tolerant cultivar Changbai 9 (CB9) and saline sensitive cultivar Tonghe 899 (TH899) with 130 d and 131 d of fertility, respectively, were used as the test materials. Sodium iron ethylenediamine-di-o-hydroxyphenyl large acetate (EDDHA/Fe, 99.99%, China) containing ≥6.0% iron was used as the exogenous iron. We immersed seeds of a uniform size in a 5% sodium hypochlorite solution for 10 min to disinfect them. Subsequently, we initiated the germination of the disinfected seeds in distilled water at 30 °C under dark conditions for 2 days, then transferred them to a lighted environment at 30 °C for an additional 2 days to promote growth. Sprouted seedlings were transferred to water for 3 d, and then to 1/2 nutrient solution (0.04 mmol L^{-1} Fe, ICRISAT Rice Nutrient Solution [22]) for 3 d. They were then moved to a full nutrient solution without Fe for three weeks. This was followed by treatment with Fe deficiency culture (CK), exogenous iron spray treatment (+Fe): 0.8% EDDHA/Fe, saline-sodic treatment (SA): NaCl:Na_2SO_4:Na_2CO_3:$NaHCO_3$ = 1:9:1:9 mixed saline [23] with a Na^+ concentration of 50 mmol L^{-1}, pH approximately 8.5, and saline-sodic plus exogenous iron spray treatment (SA + Fe) with a concentration of 0.8% EDDHA/Fe and 50 mM of the saline mixture. An optimum iron concentration of 0.8% exogenous iron was selected for treatment following the findings of Gao et al. [4]. EDDHA/Fe solution formulated with 0.1% Tween-20 (polysorbate 20) was used for exogenous iron spraying. During spraying, the iron needed to uniformly adhere to the front and back of the leaf without dripping. Samples were collected 7 days post-treatment and were either immediately analyzed or rapidly frozen in liquid nitrogen and stored at −80 °C. The hydroponic experiments employed a photoperiod of 14 h of light at 28 °C followed by 10 h of darkness at 22 °C, while sustaining a relative humidity of around 70%. The nutrient solution was replaced every 3 days during the study, and the pH levels were regulated to 5.5 or 8.5 on a daily basis using 0.1 M hydrochloric acid or KOH.

We examined the impact of supplementary iron on the expression of genes that are regulated differently in rice seedlings experiencing iron deficiency and saline stress. We performed transcriptome analysis of rice leaves under saline-sodic treatment, noted as CB_SA and TH_SA, respectively, and saline-sodic and sprayed exogenous iron, noted as CB_SA_Fe and TH_SA_Fe, respectively, of Changbai 9 (CB9) and Tonghe 899 (TH899). We conducted the transcriptome sampling following the method described by Li et al. [24]. Treated leaves were preserved on dry ice and submitted to Majorbio (Shanghai, China) for transcriptome sequencing. Three replicates were conducted for each treatment, resulting in a total of 12 samples.

2.2. Measurement Indicators

2.2.1. Plant Dry Weight and Fresh Weight

The plants underwent a 5-min immersion in a saturated $CaSO_4$ solution followed by rinsing with distilled water to eliminate extrinsic iron from the plant exterior. Subsequently, the fresh weight was determined post-absorption using absorbent paper. The specimens were subjected to 30 min of heat treatment at 105 °C for termination purposes, after which they were dehydrated at 80 °C until a consistent weight was achieved, and the corresponding weight was documented, facilitating the subsequent computation of moisture content.

2.2.2. Pigment Content

The leaves were soaked in saturated $CaSO_4$ solution for 5 min, and the leaf surface was gently wiped with filter paper. After rinsing with distilled water approximately 3~5 times, the leaf surfaces were blotted dry with absorbent paper. The chlorophyll content was

determined using the method of Nam et al. (2021) with colorimetry at 470, 649, and 665 nm, respectively [25].

2.2.3. Gas Exchange Parameters

The leaves were soaked in saturated $CaSO_4$ solution for 5 min, rinsed approximately 3~5 times using distilled water, and the surface water was blotted with absorbent paper. After sufficient light acclimation on the leaves, the parameters of net photosynthetic rate (A), stomatal conductance (Gs), photosynthetic water use efficiency (WUE), and intercellular CO_2 concentration (Ci) of the uppermost expanded leaves of rice seedlings were measured using a portable photosynthesizer, CIRAS-III (PP Systems, Amesbury, MA, USA). Photosynthetic parameters were set with an artificial light intensity of 1200 μmol m^{-2} s^{-1}, a CO_2 concentration of 380 μmol mol^{-1}, and a relative humidity of 70%.

2.2.4. Raw Sequencing Data Quality Control and Differentially Expressed Gene Processing

Raw reads were obtained using Illumina sequencing. After removing splice sequences and low-quality reads, clean data were differentiated for analysis. The clean data were aligned to the rice reference genome (IRGSP-1.0) using HiSat2. The expression levels of genes were quantified using RSEM software (v1.1.17) and utilize transcripts per million reads (TPM) to standardize the levels of gene expression across the sample.

Based on the TPM values, the read count data of the genes compared were analyzed for differential expression using DESeq2 software (1.42.0), based on the negative binomial distribution model. The established threshold for determining significantly differentially expressed genes is a false discovery rate (FDR) of less than 0.05 (DESeq2 < 0.001 (DEGseq)) and $|\log_2 FC| \geq 1$. When a gene meets both conditions, it is considered a differentially expressed gene (DEG). The gene is then compared with COG (Clusters of Orthologous Groups), GO (Gene Ontology), and DEG. The DEGs selected were annotated by comparing them with the COG (Clusters of Orthologous Groups), GO (Gene Ontology), and KEGG (Kyoto Encyclopedia of Genes and Genomes) databases. The GO and KEGG databases were used to analyze key DEGs. Calculations were performed using Fisher's exact test with corrected *p*-values, that is, P_{adjust}, at a threshold of 0.05 for GO enrichment analysis using the Goatools software (v1.3.1)and KEGG enrichment analysis with KOBAS software (3.0), respectively.

2.2.5. RT-qPCR Quantitative Reverse Transcription PCR Analysis

Purified RNA extracted during sequencing was reverse-transcribed into cDNA using the cDNA Synthesis SuperMix for qPCR (One-Step gDNA Removal) kit from Beijing TransGen Biotech. The differential genes screened based on transcriptome analysis were used in the NCBI database (https://www.ncbi.nlm.nih.gov/, accessed on 12 January 2024), and RT-qPCR specific primers were designed based on their sequences, with three replicates each for the sample and the internal reference gene (ACTIN) and 2 NTC (no template control) replicates. RT-qPCR was performed using the PerfectStart® Green qPCR SuperMix (https://www.transgen.com/qpcr/1848.html, accessed on 12 January 2024) kit in the QuantStudio™ (Thermo Fisher Scientific, Waltham, MA USA) Real-Time PCR System. Parameters included the denaturation phase (94 °C 30 s, 94 °C 5 s, 60 °C 34 s) and annealing and extension phase (95 °C 15 s, 60 °C for 1 min, 95 °C for 15 s) for 40 cycles. Quantitative expression analysis was performed using the algorithm $2^{-\Delta\Delta CT}$ method [26]. The primers used are detailed in Appendix A.

2.3. Data Analysis

Excel 2021 was used for data processing. We performed a multi-factor variance statistical analysis on the physiological indicators of rice using SPSS 27. We also evaluated the statistical significance of the differences using Duncan's multiple range test ($p < 0.05$). Sigmaplot 14 software were used for graphing.

3. Results

3.1. The Impact of Supplemental Iron on the Development of Young Rice Plants Subjected to Saline-Sodic Conditions

Spraying exogenous Fe was beneficial in increasing the fresh weight, dry weight, and water content of rice seedlings (Figure 1). Under non-saline-sodic stress, the +Fe treatment significantly increased the fresh weight (56.68% and 53.91%), dry weight (25.42% and 65.51%), and water content (19.56% and 21.22%) of rice cultivars CB9 and TH899 compared to the CK treatment. SA treatment aggravated the inhibition of growth in Fe-deficient seedlings in rice compared to the CK treatment. However, spraying exogenous iron under saline-sodic stress was beneficial in increasing the fresh weight (32.68% and 39.82%), dry weight (29.46% and 38.41%), and water content (8.72% and 10.70%) of rice seedlings and in restoring the growth of rice Fe-deficient seedlings. The trend for both cultivars was more consistent; under contrasting and saline–alkali conditions, spraying iron enhances the growth of salt-sensitive variety TH899 more than the growth of the salt-tolerant variety CB9.

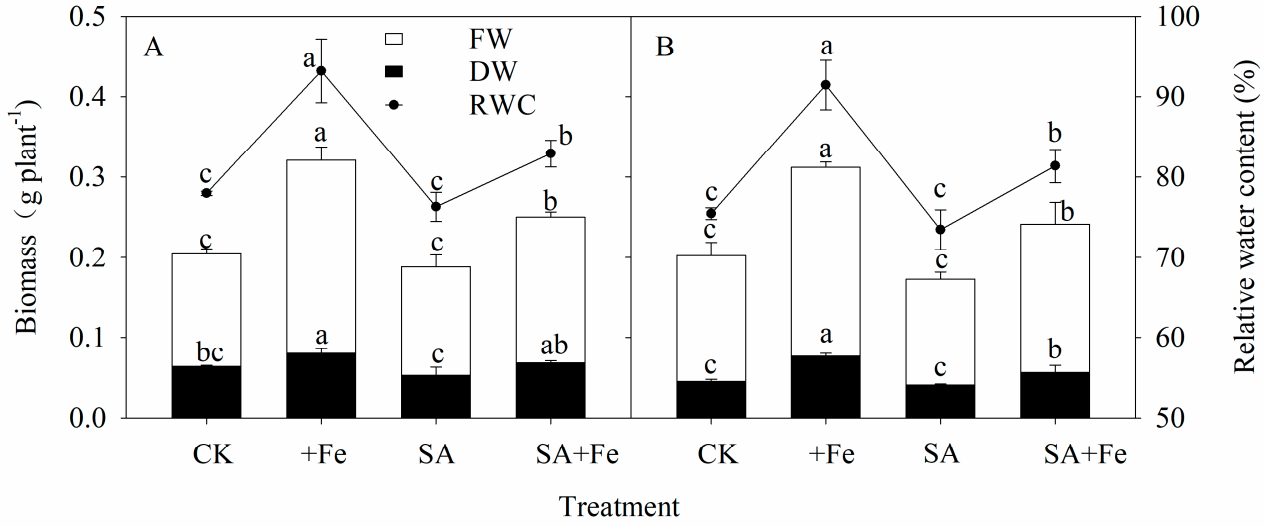

Figure 1. Effect of exogenous iron on the growth of (**A**) CB9 and (**B**) TH899 rice seedlings under saline-sodic stress. Note: Values marked with different lowercase letters in the same column indicate significant differences between treatments at the 0.05 level.

3.2. Impact of Supplemental Iron on Foliar Pigment Concentration in Rice Seedlings Exposed to Saline–Sodic Conditions

Saline sodic conditions effected all factors except for chlorophyll b content, while iron treatments significantly inhibited chlorophyll a, chlorophyll b, carotenoids, and the total pigment content of rice seedling leaves (Figure 2). Only the chlorophyll b content was significantly affected between cultivars, suggesting that spraying iron is more beneficial for the accumulation of chlorophyll b content in CB9. There was a highly significant interaction between saline-sodic conditions and Fe stress on photosynthetic pigments, except for chlorophyll b. Compared with the CK treatment, +Fe treatment significantly increased chlorophyll a (3.01 and 3.08-fold), chlorophyll b (1.93 and 1.47-fold), carotenoids (2.61 and 2.10-fold), and total pigment content (2.79 and 2.65-fold) in rice seedlings of both cultivars. Saline-sodic stress aggravated the damage to photosynthetic pigments. Under saline-sodic stress, spraying exogenous Fe significantly increased the pigment content of rice seedling leaves by 2.41, 2.06, 1.67, and 2.20-fold (CB9), and 2.42, 1.35, 1.95, and 2.16-fold (TH899), respectively.

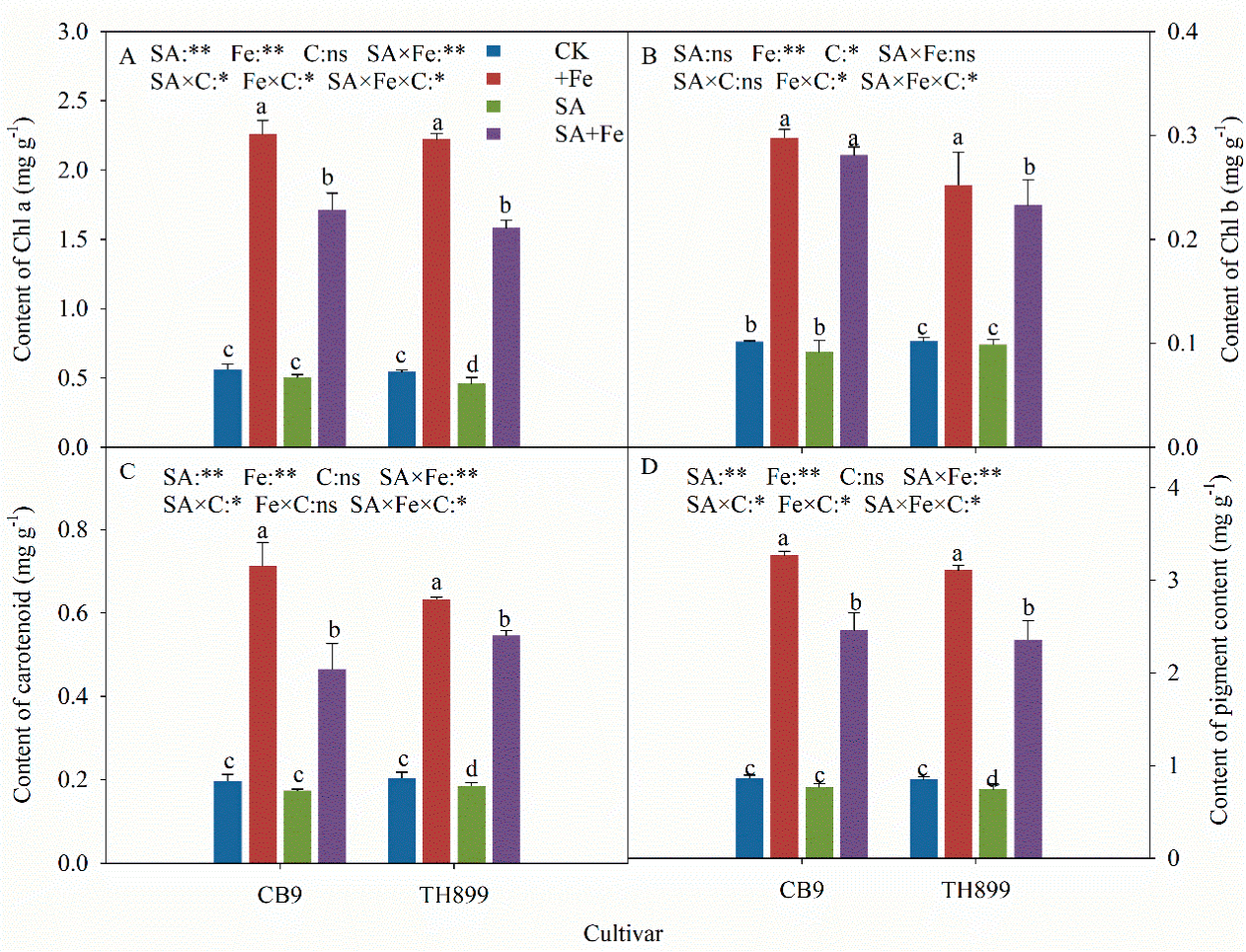

Figure 2. Effect of exogenous iron on leaf pigment content of rice seedlings under saline-sodic stress: (**A**) chlorophyll a, (**B**) chlorophyll b, (**C**) carotenoids, (**D**) total pigment content. Note: Values marked with different lowercase letters in the same column indicate significant differences between treatments at the 0.05 level; ns: not significant. * and ** indicate ANOVA significant at the 0.05 and 0.01 levels, respectively. SA: Saline sodic treatment; Fe: Iron treatment; C: Cultivar; SA × C: Intercropping between saline sodic treatment and cultivars; SA × Fe: Intercropping between saline sodic and iron treatment; Fe × C: Intercropping between iron treatment and cultivars; SA × Fe × C: Intercropping between saline sodic, iron treatment and cultivars.

3.3. Impact of Supplemental Iron on Gas Exchange Parameters of Rice Seedling Leaves Experiencing Saline-Sodic Stress

Cultivar, saline-sodic, and iron treatments had significant effects on gas exchange parameters (except Ci, as in Table 1); compared to TH899, CB9 maintains a higher net photosynthetic rate (A) under salt–alkali stress. However, the improvement in the net photosynthetic rate (A) of TH899 from iron spraying is greater than that of CB9. The interaction of saline-sodic and Fe treatments had a significant effect on Gs, A, and E. Compared with CK, +Fe treatment improved the gas exchange capacity of rice leaves with a significant increase in Gs, A, and E, and a significant decrease in Ci. SA treatment significantly inhibited the net photosynthetic rate A and transpiration rate E of rice seedlings, resulting in intercellular CO_2 concentration Ci. Meanwhile, it had no significant effect on stomatal conductance Gs. Under saline-sodic stress, spraying exogenous Fe was beneficial in increasing the photosynthetic capacity of seedling leaves in both cultivars, with a significant increase in Gs (23.44% and 20.69), A (80.28% and 100.00%), and E (45.16% and 32.26%) of both rice cultivars treated with SA + Fe compared to the SA treatment. There was a significant decrease in Ci (27.82% and 25.08%).

Table 1. Effect of exogenous iron on gas exchange parameters of rice seedling leaves under saline-sodic stress.

Cultivars	Treatment	C_i (μmol mol^{-1})	G_s (mmol H_2O m^{-2} s^{-1})	A (μmol CO_2 m^{-2} s^{-1})	E (mmol H_2O m^{-2} s^{-1})
CB9	CK	417.3 ± 6.0 b	266.0 ± 6.2 c	8.9 ± 0.7 c	3.8 ± 0.2 c
	+Fe	265.3 ± 10.1 d	360.0 ± 7.0 a	19.9 ± 0.6 a	6.3 ± 0.1 a
	SA	439.3 ± 8.1 a	256.0 ± 8.0 c	7.1 ± 0.6 d	3.1 ± 0.1 d
	SA + Fe	317.0 ± 3.6 c	316.0 ± 11.1 b	12.8 ± 0.6 b	4.5 ± 0.1 b
TH899	CK	373.0 ± 11.5 b	264.3 ± 10.6 c	7.7 ± 0.7 c	4.0 ± 0.1 b
	+Fe	308.3 ± 13.7 d	350.3 ± 17.6 a	17.7 ± 0.3 a	5.7 ± 0.1 a
	SA	446.0 ± 9.2 a	245.0 ± 10.1 c	5.6 ± 0.6 d	3.1 ± 0.2 c
	SA + Fe	334.0 ± 6.6 c	295.7 ± 7.0 b	11.2 ± 0.6 b	4.1 ± 0.2 b
F-value	C	2.259	6.418 *	46.854 **	12.585 *
	SA	134.51 **	57.758 **	349.345 **	357.505 **
	Fe	521.293 **	297.839 **	785.964 **	644.787 **
	SA × C	2.831	1.410	0.102	0.000
	SA × Fe	1.414	16.946 *	106.517 **	54.202 **
	Fe × C	43.205 *	1.059	1.058	21.305 *
	SA × Fe × C	26.855 **	0.006	0.786	1.281

Note: Values marked with different lowercase letters in the same column indicate significant differences between treatments at the 0.05 level. * and ** indicate ANOVA significance at the 0.05 and 0.01 levels, respectively. SA: saline-sodic treatment; Fe: iron treatment; C: cultivar; SA × C: intercropping between saline-sodic treatment and cultivars; SA × Fe: intercropping between saline-sodic and iron treatment; Fe × C: intercropping between iron treatment and cultivars; SA × Fe × C: intercropping between saline-sodic, iron treatment, and cultivars.

3.4. Transcriptome Data Quality Assessment

In this study, we aimed to investigate the molecular response mechanism of Fe-deficient seedlings of CB9 and TH899 rice cultivars to exogenous Fe spraying under saline-sodic stress. mRNA analysis of rice leaves of CB9 and TH899 under saline-sodic stress (CB_SA and TH_SA) and saline-sodic stress with exogenous iron spraying (CB_SA_Fe and TH_SA_Fe) treatments was performed using transcriptome sequencing. Table 2 summarizes the results of the RNA-seq analysis on three biological replicates for each treatment. The clean data from each sample reached over 6.52 Gb, the percentage of Q20 bases was over 96.93%, the percentage of Q30 bases was over 91.65%, and the percentage of total mapped was over 94.99%, which ensured data availability.

Table 2. Transcriptome data quality assessment.

Sample	Raw Reads	Clean Reads	Error Rate (%)	Q20 (%)	Q30 (%)	GC Content (%)	Total Mapped	Uniquely Mapped
CB_SA_1	62204786	61643158	0.0272	97.21	92.25	53.29	58739919 (95.29%)	56782102 (92.11%)
CB_SA_2	55700666	55161400	0.0268	97.41	92.62	53.19	52684428 (95.51%)	50908597 (92.29%)
CB_SA_3	61435748	60853462	0.0271	97.26	92.37	53.45	58047116 (95.39%)	55705397 (91.54%)
CB_SA_Fe1	52790506	52296848	0.0278	96.99	91.73	53.67	49824516 (95.27%)	48155861 (92.08%)
CB_SA_Fe2	56852140	56316326	0.0274	97.14	92.09	53.69	53658569 (95.28%)	51790933 (91.96%)
CB_SA_Fe3	67354178	66715724	0.0279	96.93	91.65	53.54	63376544 (94.99%)	61058034 (91.52%)
TH_SA_1	57918838	57380932	0.0276	97.06	91.89	53.33	54786641 (95.48%)	52270723 (91.09%)
TH_SA_2	46366348	45959544	0.0278	97	91.72	52.93	43875231 (95.46%)	41465762 (90.22%)
TH_SA_3	44643230	44243696	0.0269	97.33	92.44	52.45	42453058 (95.95%)	40891765 (92.42%)
TH_SA_Fe1	57172238	56631350	0.0277	97.03	91.81	53.31	53875797 (95.13%)	51863259 (91.58%)
TH_SA_Fe2	59909992	59455624	0.0266	97.45	92.7	53.24	56676702 (95.33%)	54589706 (91.82%)
TH_SA_Fe3	50289926	49706494	0.0276	97.04	91.88	53.62	47420063 (95.4%)	45904726 (92.35%)

3.5. Differentially Expressed Genes in Rice Fe-Deficient Seedlings under Saline-Sodic Stress in Response to Exogenous Fe Spraying

By comparing whether the same rice cultivar was sprayed with exogenous Fe under saline-sodic stress, we constructed a total of two comparison groups: CB_SA_Fe vs. CB_SA and TH_SA_Fe vs. TH_SA. Screening for differentially expressed genes (DEGs) was per-

formed according to $P_{adjust} < 0.05$ and $|\log_2 FC| \geq 1$. Figure 3 illustrates that in this study, there were 5703 differentially expressed genes (3238 upregulated and 2465 downregulated) in the CB_SA_Fe vs. CB_SA comparison group, and 7785 differentially expressed genes (4311 upregulated and 3474 downregulated) in the TH_SA_Fe vs. TH_SA comparison group. (Figure 3). The results showed that the number of differentially expressed genes was higher in TH899, a soda salt-sensitive variety, in response to exogenous iron spraying. To investigate the effect of exogenous iron spraying on the molecular mechanism of iron-deficient rice leaves under saline-sodic stress. In this study, 3590 differentially expressed genes were found to be common to the two comparison groups of CB_SA_Fe vs. CB_SA and TH_SA_Fe vs. TH_SA (Figure 3, DEGs A). Among them, 1498 genes were upregulated and 2092 genes were downregulated (CB), and 1414 genes were upregulated and 2176 genes were downregulated (TH). There were 2113 specific differentially expressed genes in the leaves of Fe-deficient seedlings of the CB9 cultivar in response to exogenous Fe spraying under saline-sodic stress (Figure 3 DEGs B). Among them, 967 genes were upregulated, and 1146 genes were downregulated.

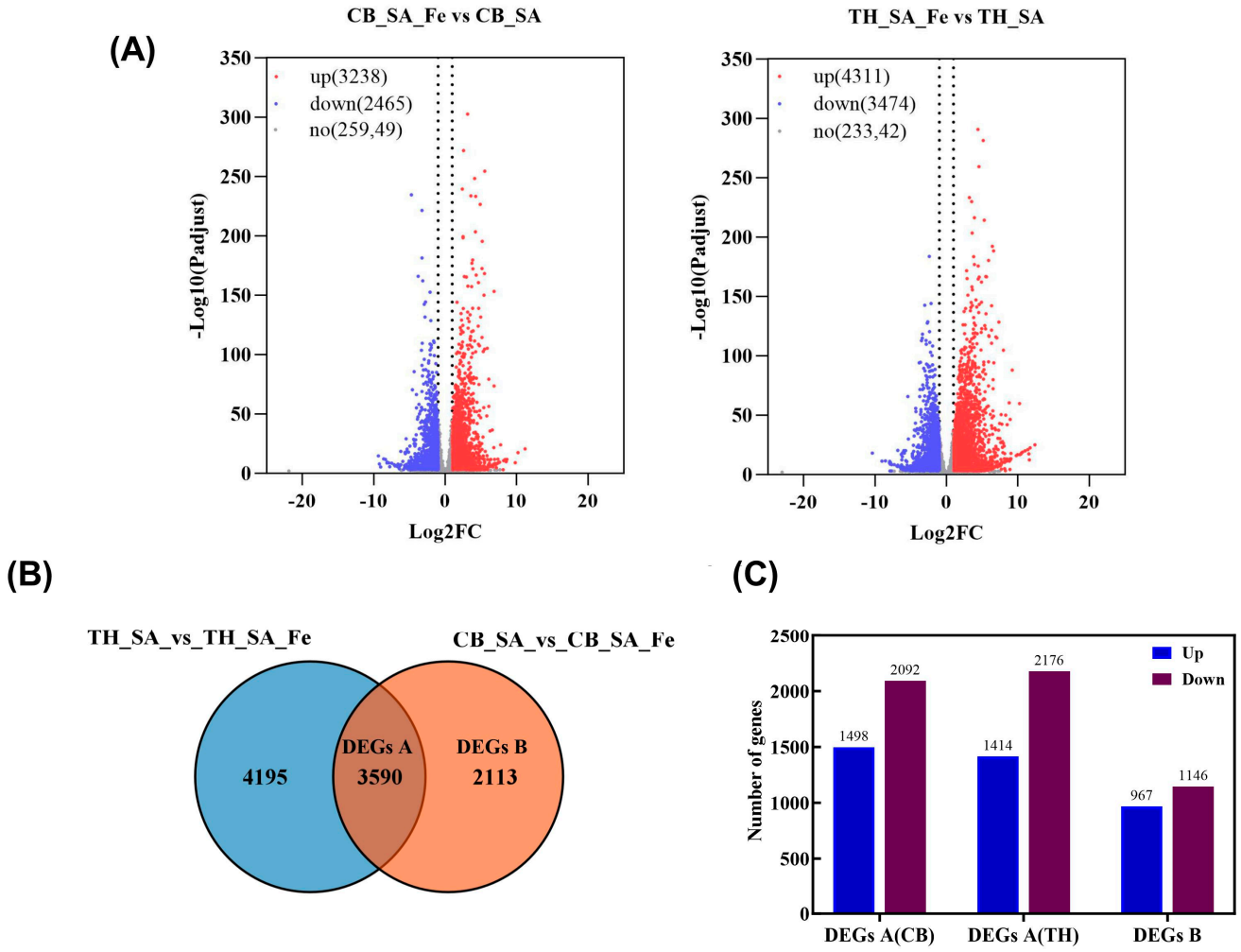

Figure 3. Volcano plot of expression differences (**A**) and Venn diagram of differential genes (**B**) and the number of up- and downregulated genes in gene DEGs A and DEGs B (**C**). Note: On both sides of the black dotted line are differentially expressed genes (**A**).

3.6. Expression Analysis of Differential Genes in Rice Fe-Deficient Seedlings under Saline-Sodic Stress in Response to Exogenous Fe Spraying

GO enrichment analysis was conducted on the differentially expressed genes (gene set DEGs A) in rice Fe-deficient seedlings subjected to exogenous iron spraying under saline-

sodic stress, as depicted in Figure 4. The analysis revealed that the top five enriched GO terms by DEGs A were the regulation of jasmonic acid-mediated signaling pathway (GO: 2000022), iron ion transport (GO: 0006826), iron ion homeostasis (GO: 0055072), cellular amino acid catabolic process (GO: 0009063), and the carboxylic acid catabolic process pathway (GO: 0046395). This indicated that DEGs A is mainly enriched in transmembrane transport related to ion transport and plays a role in regulating ion transport.

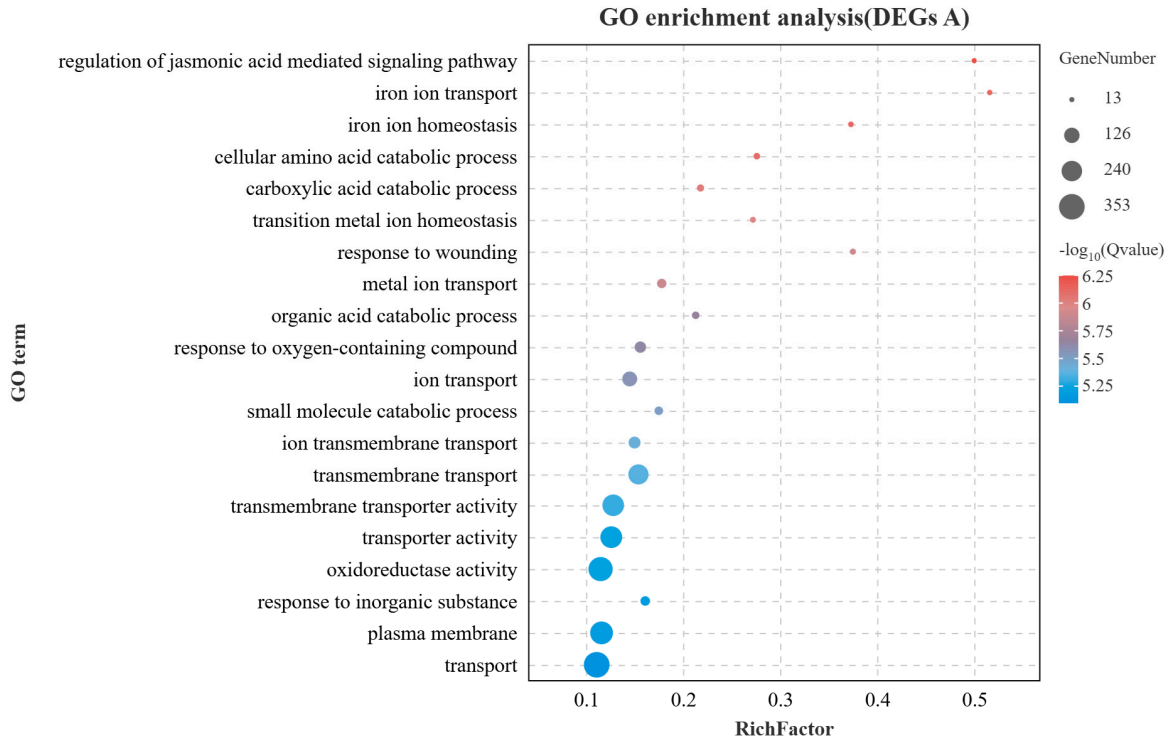

Figure 4. GO enrichment analysis of differentially expressed genes in response to exogenous iron spraying in Fe-deficient seedling of two rice varieties under saline-sodic stress.

The KEGG enrichment analysis (Figure 5) revealed that the primary differentially expressed genes (DEGs A) were notably enriched in various metabolic pathways. These pathways include plant hormone signal transduction (map04075), phenylpropanoid biosynthesis (map00940), the MAPK signaling pathway—plant (map04016), carotenoid biosynthesis (map00906), and glycerolipid metabolism (map00561). This observation indicates that DEGs A predominantly participate in signaling cascades, particularly in phytohormone signaling, thereby exerting regulatory functions in signal transduction processes.

3.7. Exogenous Fe Spraying Significantly Affects Metabolic Pathways of Carotenoid Biosynthesis of Rice Fe-Deficient Seedlings under Saline-Sodic Stress (DEGs A)

Carotenoids are one of the key components of plant photoprotection and act as co-pigments in photosynthesis. Carotenoids are also precursors for the synthesis of signaling molecules, such as abscisic acid (ABA), solanum lactone, and β-cyclocitral. Fourteen differentially expressed genes were enriched in the metabolic pathway of carotenoid biosynthesis (map00906) by comparing CB_SA_Fe vs. CB_SA and TH_SA_Fe vs. TH_SA. Eleven differentially expressed genes were upregulated in the biosynthetic metabolic pathway of carotenoids by comparing CB_SA_Fe vs. CB_SA, mainly focusing on the synthesis of 9-cis-epoxycarotenoid dioxygenase (*Os12g0617400*, *Os07g0154100*, *Os03g0645900*), Phytoene synthase *(Os12g0626400*, *Os09g0555500*), β-carotene Hydroxylase (*Os04g0578400*, *Os03g0125100*), and others. Three differentially expressed genes were downregulated, mainly related to the synthesis of abscisic acid 8′-hydroxylase 1 (*Os02g0703600*) and aldehyde oxidase (*Os07g0282300*, *Os07g0282500*). Unlike the CB9 variety, the gene regulating

abscisic acid 8′-hydroxylase 1 synthesis (*Os02g0703600*) was upregulated in TH_SA_Fe vs. TH_SA. Most of these differentially expressed genes are associated with carotenoid-ABA synthesis, which may be promoted by spraying exogenous Fe to improve rice tolerance (Table 3). In addition, the differentially expressed genes involved were also associated with the bioavailability of Fe.

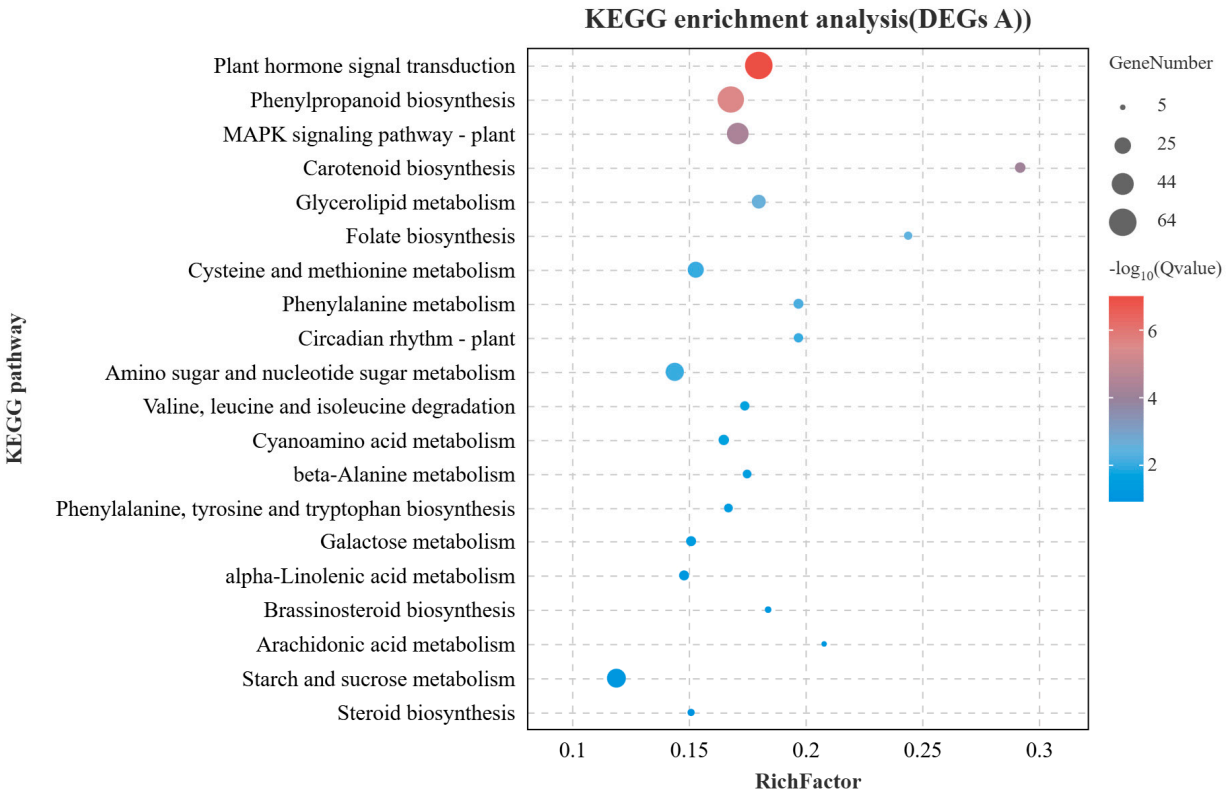

Figure 5. KEGG enrichment analysis of differentially expressed genes in response to exogenous iron spraying in Fe-deficient seeding of two rice varieties under saline-sodic stress.

Table 3. Gene sets (DEGs A) enriched in carotenoid biosynthetic metabolic pathways.

Pathway	Genes ID	CB_SA_Fe vs. CB_SA			TH_SA_Fe vs. TH_SA		
		Significant	log_2FC	Regulate	Significant	log_2FC	Regulate
ID: map00906 (Carotenoid biosynthesis)	Os03g0645900	yes	6.60	up	yes	6.32	up
	Os12g0626400	yes	2.79	up	yes	2.50	up
	Os04g0578400	yes	1.35	up	yes	1.16	up
	Os12g0617400	yes	2.66	up	yes	2.46	up
	Os07g0282300	yes	−1.28	down	yes	−1.24	down
	Os02g0703600	yes	−2.27	down	yes	1.31	up
	Os07g0164900	yes	2.18	up	yes	1.45	up
	Os07g0154100	yes	4.05	up	yes	6.64	up
	Os09g0555500	yes	4.27	up	yes	2.78	up
	Os03g0125100	yes	4.14	up	yes	4.27	up
	Os12g0617250	yes	2.94	up	yes	2.86	up
	Os10g0546600	yes	1.78	up	yes	1.29	up
	Os01g0566500	yes	3.55	up	yes	10.10	up
	Os07g0282500	yes	−1.99	down	yes	−2.76	down

3.8. Enrichment Analysis of Specific Differentially Expressed Genes to CB9 Fe-Deficient Seedlings in Response to Exogenous Iron Spraying under Saline-Sodic Stress

GO enrichment analysis of CB9 cultivar-specific differentially expressed genes (gene set DEGs B, Figure 6) showed that the top five GO terms that were significantly enriched

were lipid metabolic process (GO: 0006629), oxidoreductase activity (GO: 0016491), catalytic activity (GO: 0003824), small molecule metabolic process (GO: 0044281), and photosynthesis, dark reaction (GO: 0019685). This suggests that DEGs are predominantly enriched in metabolic processes related to lipid metabolism and enzyme activity and are closely linked to crop stress resistance.

In the KEGG enrichment analysis (Figure 7), the top five differential metabolic pathways mainly enriched by gene set DEGs B were glyoxylate and dicarboxylate metabolism (map00630), carbon fixation in photosynthetic organisms (map00710), diterpenoid biosynthesis (map00904), tryptophan metabolism (map00380), and glycolysis/gluconeogenesis (map00010). This suggests that DEGs are predominantly enriched in metabolic pathways related to defense and photosynthesis.

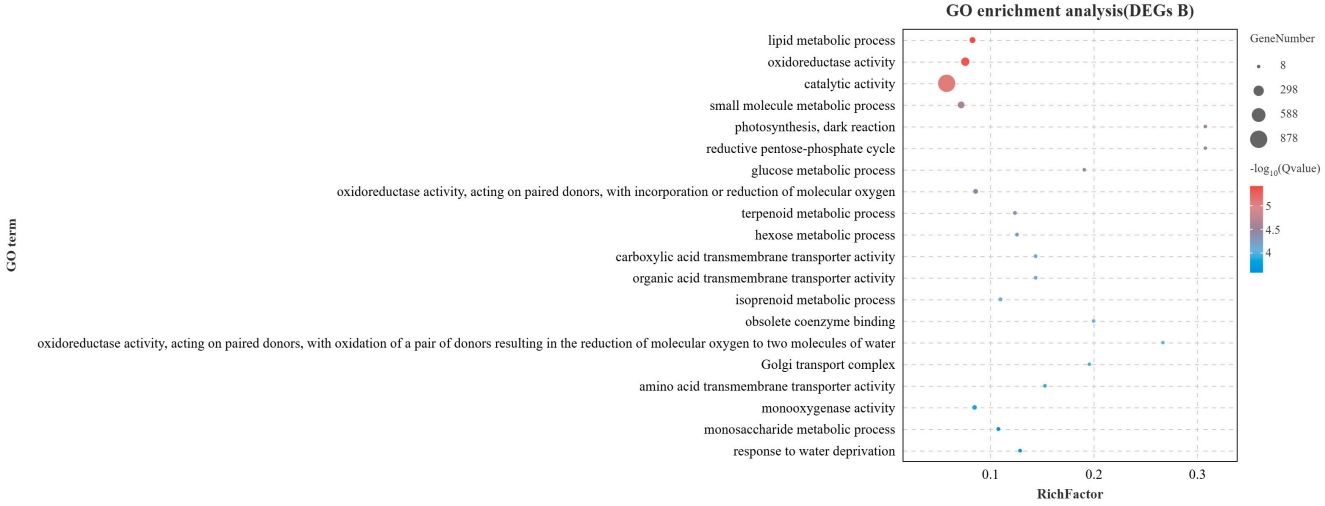

Figure 6. GO enrichment analysis on particular differentially expressed genes in CB9 Fe-deficient seedlings subjected to saline-sodic stress following treatment with exogenous iron application.

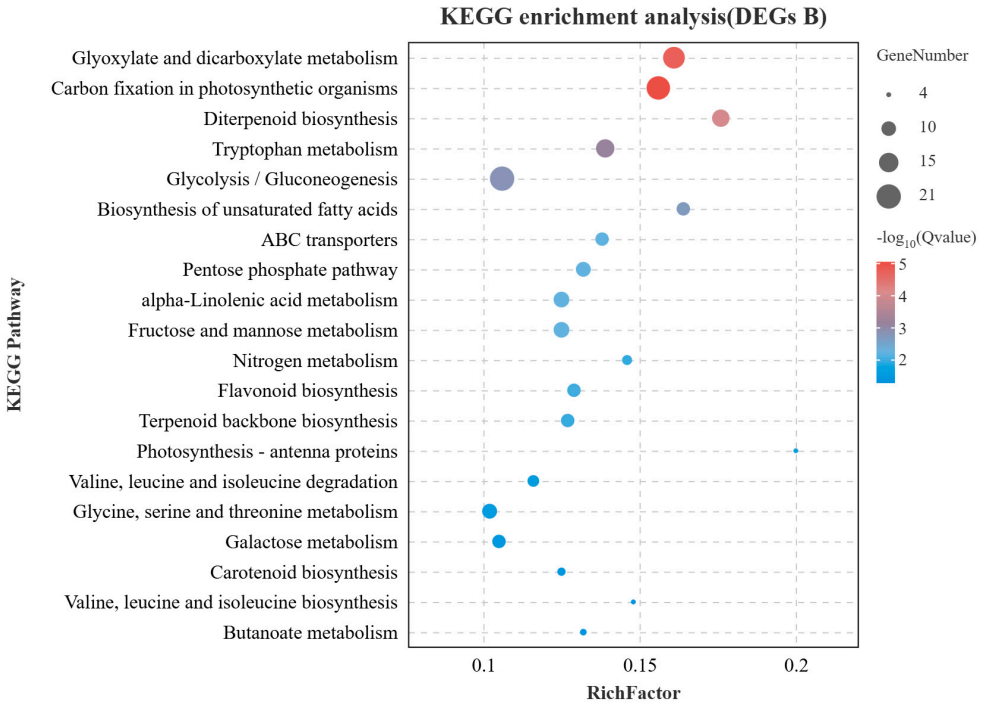

Figure 7. KEGG enrichment analysis on particular differentially expressed genes in CB9 Fe-deficient seedlings subjected to saline-sodic stress following treatment with exogenous iron application.

3.9. Exogenous Fe Spraying Significantly Affects Carbon Fixation Pathways in the Photosynthetic Biology of CB9 Fe-Deficient Seedlings under Saline-Sodic Stress (DEGs B)

Among the CB9 specific differentially expressed genes, a total of 20 genes were significantly enriched in carbon fixation metabolic pathways in photosynthetic organisms. A total of 16 genes were upregulated, mainly related to the synthesis of ribulose-1,5-bisphosphate (*Os07g0176900*, *Os12g0274700*), fructose-1,6-bisphosphatase (*Os01g0866400*, *Os06g0664200*), triosephosphate isomerase (*Os09g0535000*), phosphoglycerate kinase (*Os10g0442100*), and others. Four genes were downregulated, mainly associated with the synthesis of transketolase (*Os04g0266900*), glyceraldehyde-3-phosphate dehydrogenase (*Os02g0601300*), alanine aminotransferase (*Os10g0390500*), and NADP-malic enzyme (*Os01g0723400*) (Table 4). These genes may be related to the ability of the salinity-tolerant variety CB9 to maintain high photosynthetic efficiency under saline-sodic stress.

Table 4. Gene set (DEGs B) enrichment in carbon fixation metabolic pathways in photosynthetic organisms.

Pathway	Genes ID	CB_SA_Fe vs. CB_SA			TH_SA_Fe vs. TH_SA		
		Significant	log_2FC	Regulate	Significant	log_2FC	Regulate
ID: map00710 (Carbon fixation in photosynthetic organisms)	Os09g0535000	yes	1.45	up	no	0.64	up
	Os07g0176900	yes	1.29	up	no	−0.09	down
	Os12g0274700	yes	2.29	up	no	0.15	up
	Os01g0866400	yes	2.19	up	no	0.73	up
	Os12g0291733	yes	2.57	up	no	0.23	up
	Os01g0723400	yes	−1.09	down	no	−0.69	down
	Os10g0390500	yes	−1.54	down	no	−0.24	down
	Os01g0208700	yes	1.04	up	no	0.04	up
	Os10g0442100	yes	1.11	up	no	0.24	up
	Os12g0292301	yes	2.41	up	no	0.82	up
	Os02g0697900	yes	1.23	up	no	−0.90	down
	Os05g0496200	yes	1.13	up	no	0.39	up
	Os04g0266900	yes	−1.09	down	no	−0.24	down
	Os06g0664200	yes	1.10	up	no	−0.16	down
	Os12g0291100	yes	1.22	up	no	0.09	up
	Os07g0630800	yes	1.36	up	no	−0.56	down
	Os03g0267300	yes	1.41	up	no	0.12	up
	Os02g0601300	yes	−1.13	down	no	−0.15	down
	Os04g0459500	yes	1.30	up	no	−0.03	down
	Os12g0292400	yes	1.52	up	no	0.37	up

Note: To reflect the differences, we added the expression differences of relevant genes in the TH_SA_Fe vs. TH_SA to the Table 4.

3.10. Transcription Factor Analysis of Differentially Expressed Genes in Rice Fe-Deficient Seedlings under Saline-Sodic Stress in Response to Exogenous Iron Spraying

A total of 384 transcription factors belonging to 31 different transcription factor families were identified in gene set DEGs A (Figure 8). Among these, the bHLH, ERF, MYB, WRKY, and NAC families had the most transcription factors. Gene set DEGs B identified a total of 136 transcription factors belonging to 27 different transcription factor families, among which WRKY, ERF, MYB, HB-other, and NAC families had the most transcription factors.

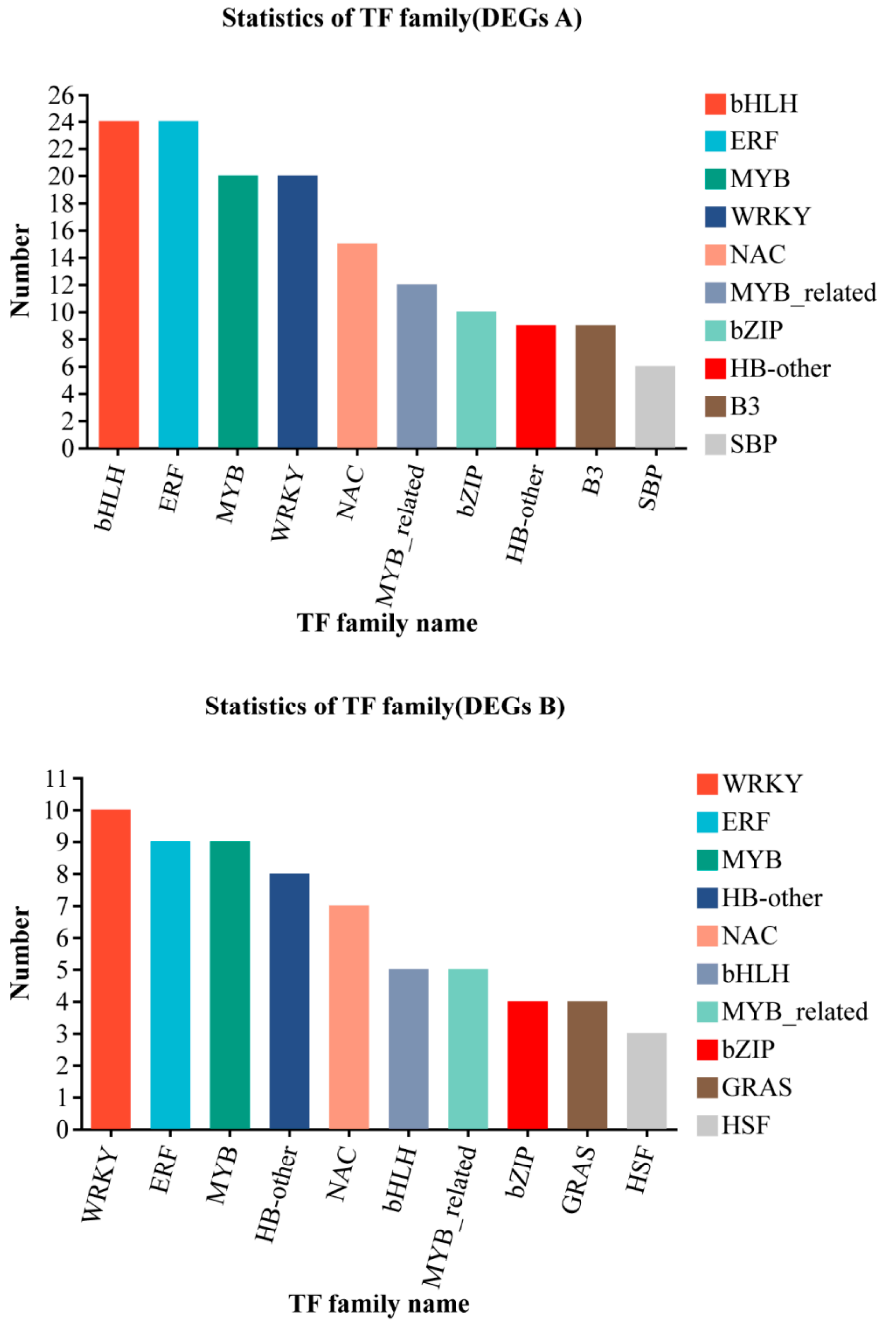

Figure 8. Transcription factor analysis of differentially expressed genes (DEGs A and DEGs B) in response to exogenous iron spraying in rice Fe-deficient seedlings under saline-sodic stress.

3.11. RT-qPCR Validation

The RNA-seq data's reliability was confirmed by randomly selecting 12 genes in each treatment for quantitative real-time PCR (RT-qPCR). The primer sequences are provided in detail in Appendix A, Table A1. As depicted in Figure 9, the relative expression patterns of the 12 randomly selected genes in RT-qPCR were largely consistent with the transcriptome data trends, validating the reliability of the transcriptome data in this study.

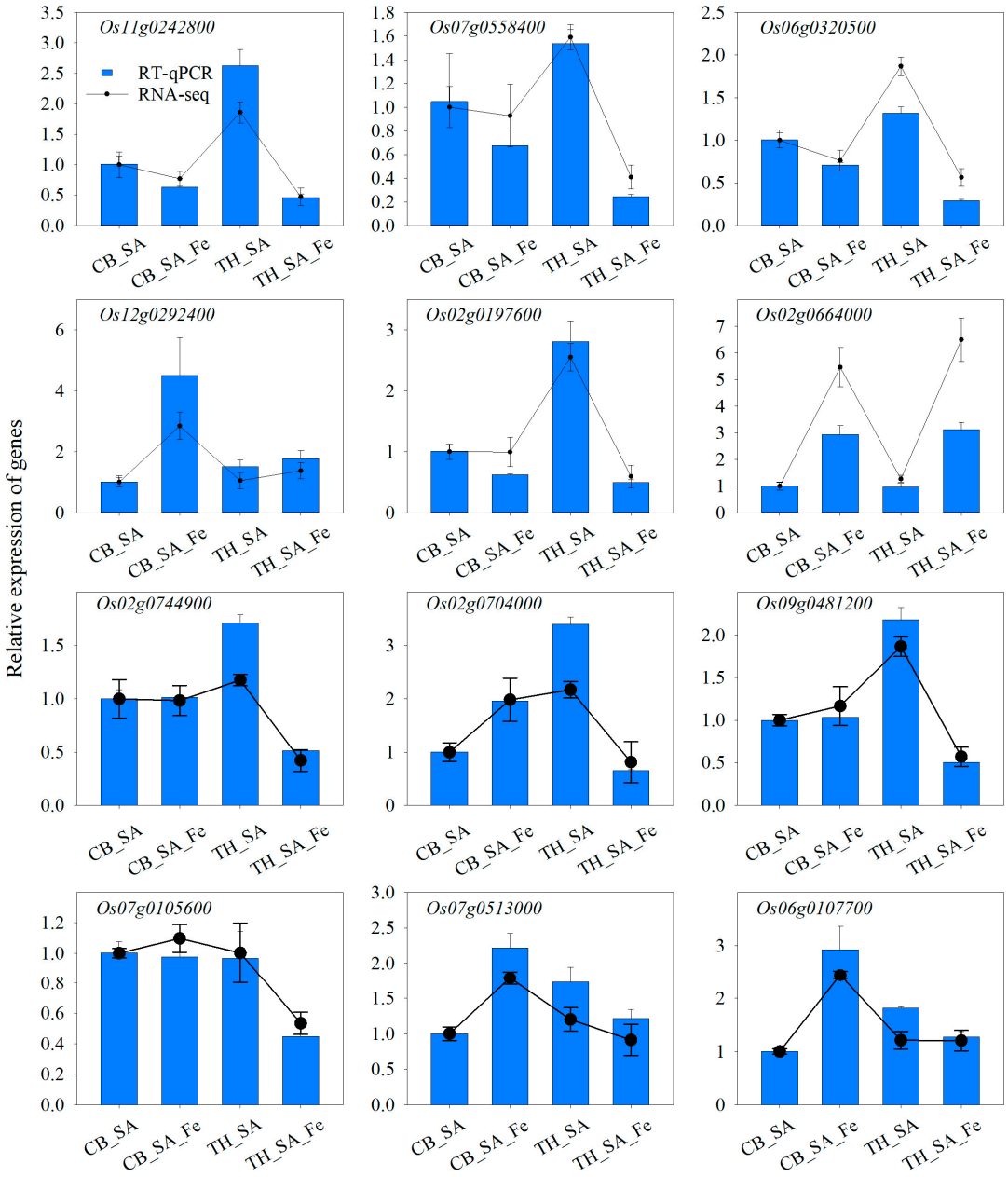

Figure 9. Relative expression of key genes in leaves of Fe-deficient rice seedlings under saline-sodic stress in response to exogenous iron spraying.

4. Discussion

Chlorophyll serves as the primary site for photosynthesis. However, both salinity and iron deficiency stresses have been found to significantly decrease chlorophyll content. This reduction in chlorophyll content leads to a decrease in photosynthesis, ultimately inhibiting plant growth [27–29]. In this study, compared to the CK + Fe treatment, the CK, SA, and SA + Fe treatments all led to a varying degree of reduction in the photosynthetic pigment content and net photosynthetic rate of seedling leaves, consequently inhibiting seedling growth (Figure 2, Table 1). Among them, SA treatment inhibited the growth of rice seedlings most severely (Figure 1). However, spraying exogenous Fe could promote the increase in pigments content as well as the enhancement of photosynthesis and seedling growth in Fe-deficient rice under saline-sodic stress (Figures 1 and 2, Table 1). This may be due to the induction of saline and iron deficiency coercion to induce more active oxygen, which accelerated pigment degradation, and because saline-sodic conditions and

Fe deficiency inhibited pigment synthesis [29,30]. Furthermore, the decrease in pigment content subsequently affects photosynthesis, ultimately resulting in the inhibition of plant growth [31]. Iron (Fe) plays a crucial role in photosynthesis. Approximately 80% of iron is found in the chloroplasts in plant leaves, where it acts as a cofactor for cytochrome complexes, b6f, and iron–sulfur proteins. These molecules are directly involved with the two photosystems (PSII and PSI), the nutritional status of iron can affect chlorophyll synthesis and photosynthetic reactions [15]. Therefore, the application of exogenous iron enhances the salt and alkali tolerance of rice by improving related indicators, such as photosynthesis.

It was shown that seedling salinity tolerance was positively correlated with photosynthesis [32]. The net photosynthetic rate of CB9 leaves was higher than that of TH899 under saline-sodic and Fe deficiency stress (Table 1). This indicates that CB9 cultivars are more tolerant and maintain high photosynthetic performance under stress. By KEGG enrichment analysis of the CB9-specific differentially expressed gene DEGs B, we enriched the metabolic pathway for carbon fixation in photosynthetic organisms and found 20 significantly expressed related genes, such as *RBCS* (*Os12g0274700*), among them (Table 4). In addition, DEGs B were enriched for glyoxylate and dicarboxylate metabolism (map00630), diterpenoid biosynthesis (map00904), tryptophan metabolism (map00380), and glycolysis/gluconeogenesis (map00010) pathways (Figure 7). These pathways are mainly associated with plant defense and photosynthesis, which may also be an important reason for CB9's salinity tolerance and stronger photosynthetic performance under saline-sodic stress [33,34] Our study showed that the carotenoid content of two rice cultivars sprayed with exogenous iron significantly increased under saline-sodic stress. Carotenoids are not only important photoprotective components but are also prerequisite substances for abscisic acid synthesis. Carotenoids can promote the synthesis of abscisic acid, which is conducive to improving plant resistance [35]. We enriched the carotenoid biosynthetic metabolic processes in DEGs A by KEGG enrichment analysis (Table 3). This suggests that exogenous Fe spraying promoted carotenoid biosynthesis under saline-sodic stress. In addition, by analyzing the genes in the carotenoid biosynthetic metabolic pathway of the enriched carotenoids, we found that these genes are closely related to the synthesis of ABA, such as *Os03g0645900*, *Os02g0703600*, and so on. Meanwhile, plant hormone signal transduction (map04075) was significantly enriched in DEGs A. This also indirectly suggests that exogenous Fe spraying promotes the synthesis and signaling of hormones, such as ABA, which plays an important role in improving salinity tolerance in rice [32].

Molecular mechanisms associated with plant metal stress responses, that is, ion transport, chelation, and storage are key mechanisms involved in plant metal tolerance and metal homeostasis re-establishment [13]. GO-enriched entries of differentially expressed genes of two cultivars of Fe-deficient seedlings after exogenous iron spraying under saline-sodic stress were mainly focused on iron ion transport, iron ion homeostasis, and cellular amino acid catabolic processes. This indicates that rice seedlings regulate iron ion transport and other related pathways to enhance iron uptake and accumulation after exogenous iron spraying. Amino acid accumulation plays a protective role as an antioxidant in abiotic stresses, scavenging reactive oxygen species (ROS) and preventing damage to plant cells [36]. In the study by Selby-Pham et al. (2017) [13], iron deficiency changed the induction levels of histidine, proline, and asparagine. Enrichment of cellular amino acid catabolism provided evidence that iron is closely linked to amino acid metabolism, as in Kong et al. (2019) [37]. A meta-analysis of the transcriptome of different genotypes of rice under salt stress at the seedling stage revealed that the GO-enriched entries were all linked to abiotic stresses, particularly salt stress [37]. In this study, the specific differentially expressed genes to CB9 Fe-deficient seedlings under saline-sodic stress in response to exogenous iron spraying were mainly enriched in lipid metabolic processes, oxidoreductase activity, catalytic activity, and small molecule metabolic processes. This was associated with osmoregulatory and antioxidant effects and may also be related to the higher salinity tolerance of CB9 cultivars.

Transcription factors function as regulatory elements in plant reactions to abiotic stresses by binding to distinct cis-acting motifs within stress-responsive gene promoters, thereby modulating the transcription of genes involved in stress tolerance [38]. In this study, the major transcription factors in two rice cultivars under saline-sodic and iron deficiency stress included bHLH, ERF, MYB, WRKY, and NAC. These transcription factors play a role in regulating plant growth and metabolism in response to abiotic stresses as well as enhancing plant stress tolerance [17,28,39]. Among them, bHLH is the most abundant transcription factor in response to exogenous iron sprays (DEGs A). The current evidence aligns with prior research, which has identified the bHLH family as the primary group of transcription factors known to regulate genes responsive to iron deficiency and to maintain iron homeostasis in plants [20]. The main transcription factors detected in CB9-specific differentially expressed genes (DEGs B) were WRKY, ERF, MYB, HB-other, and NAC families. Relevant studies of all transcription factors except HB-other have shown that they are associated with plant responses to abiotic stress [17,28,39]. While less research has been conducted on HB-other transcription factors in response to abiotic stress, studies in *Populus* and ground tipped melon have shown that HB-other transcription factors are associated with drought tolerance regulation in plants [40,41]. Therefore, regulation of the transcription factor HB-other may be important for CB9 to resist osmotic stress caused by saline-sodic stress and to maintain the growth metabolism.

5. Conclusions

This study demonstrates that the application of exogenous iron (Fe) can alleviate iron deficiency symptoms in rice seedlings (CB9 and TH899) under saline–alkali stress and promote seedling growth. By comparing and analyzing the transcriptome data of two different salt-tolerant rice varieties, it was observed that the application of exogenous Fe promoted hormone and pigment metabolism pathways in rice. Furthermore, supplementation of this Fe enhanced the photosynthetic metabolism pathway in the salt-tolerant variety CB9, thereby increasing the tolerance and photosynthetic efficiency of rice seedlings.

Author Contributions: Conceptualization, D.G. and S.Z.; methodology, D.G.; software, R.H.; validation, D.G., S.Z. and R.H.; formal analysis, D.G.; investigation, L.G.; resources, Y.G.; data curation, S.Z.; writing—original draft preparation, D.G.; writing—review and editing, Y.G. and L.G.; visualization, D.G.; supervision, Y.G. and L.G.; project administration, Y.G. and L.G.; funding acquisition, Y.G. and L.G. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The supporting data for the findings of this empirical investigation can be acquired upon request directed to the author responsible for correspondence.

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Conflicts of Interest: The authors declare no conflicts of interest.

Appendix A

Table A1. qRT-PCR validation primer sequences.

Gene ID	Sequence (5′-3′ End)	Number of Bases
Os12g0292400	F: GTACTGGACCATGTGGAAACT	21
	R: AACGAAGGCATCAGGGTATG	20
Os02g0197600	F: ACCAGCCTCAAGTTCCATTAG	21
	R: CGCATACGCACGTACATACA	20
Os11g0242800	F: CTGGCTGGCCTCAAGTTAAT	20
	R: AACACATTCTTCCACCCTTCTC	22

Table A1. *Cont.*

Gene ID	Sequence (5′-3′ End)	Number of Bases
Os07g0558400	F: ACACCTTCTCCTCATCCTCTTA	22
	R: ATGACCTCTCCTCTCTCCATAC	22
Os06g0320500	F: CATCATCCCGAGAACCATCTAC	22
	R: ACAGTACGCACGCACATAA	19
Os02g0744900	F: GGTGGATCAGTGTGCATGTATAG	23
	R: GGTACACAGCCTCTCTCTCTTA	22
Os02g0664000	F: GTAGCCCTGCTGTGTAATGT	20
	R: GCAGTCGCAGGTCTCAATAA	20
Os02g0704000	F: CTCACTCACTCACCATGAAGTC	22
	R: GCGTTCTTCTTCCTGCCATA	20
Os09g0481200	F: ATCGTCGCCTACTACATCCT	20
	R: ACACCAGAGATCCATTCAGATTAC	24
Os07g0105600	F: GGCATCTACATAGCCGACATC	21
	R: GAGGTACTTGGTGACGTAGTTG	22
Os07g0513000	F: TCAATTCAGCGATGCCAAATG	21
	R: TACGGCGATATCCCTCCTAAT	21
Os06g0107700	F: GAGAACTTCCGGGTCGATTATG	22
	R: CACAGCTCTTCCTTGTACTCTG	22
ACTIN	F: GATCTGGCATCACACCTTCTAC	22
	R: CTGGGTCATCTTCTCACGATTG	22

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