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ORIGINAL ARTICLE

Differential expression of photosynthesis-related genes and quantification of gas exchange in rice plants under abiotic stress

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Abstract Abiotic stresses caused by excessive cold, iron, and salt are among the main growth and yield-limiting factors of rice. Among the metabolic processes, photosynthesis stands out for being closely related to crop yields, causing a yield decline by reduced photosynthetic capacity in plants under abiotic stresses. The purpose of this study was to evaluate the differential expression of chloroplast genes involved in photosynthesis by RNA sequencing (RNA-seq) and quantify gas exchanges in leaves of rice plants exposed to cold, iron, and salt stress for 24 h. Of all genes expressed in each stress, cold had the highest number of differentially expressed genes (DEGs) related to light and chloroplast reactions, with 535 mostly down-regulated genes. Salt and iron stress was associated with 309 and 115 genes, respectively, involved in light reactions and chloroplast. The three stresses had transcripts with GOs related to light reactions, all with more than ten different GO terms. With regard to chloroplast, cold and salt stress had 12 terms of GO, and iron stress has 9 terms of GO. For gas exchange, only the parameter net assimilation rate differed significantly between stresses, with the lowest mean under cold stress. The results showed that cold stress affects photosynthesis most drastically, both at the molecular and the physiological level, and despite decreases in the net assimilation rate, changes under iron and salt stress were minor, which may be related to the tolerance of cultivar BRS Querência.

Keywords Cold stress · Iron stress · Salt stress · RNA-seq · *Oryza sativa* L. · PSII

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Introduction

Rice (*Oryza sativa* L.), the second most cultivated cereal and staple food for over half the world's population is sensitive to various environmental factors, such as salinity, low temperatures, and soils with excessive iron (Lafitte et al. 2004). In this context, these three abiotic stresses belong to the main factors growth and yield-limiting of rice.

Due to the sessile growth habit of rice plants, over time, mechanisms developed at the molecular, biochemical, and physiological level, allowing survival under adverse conditions (Nakashima et al. 2012). These mechanisms are complex and involved in the expression of several genes, as well as severe changes in metabolic processes, including protein synthesis (Cramer et al. 2011), activity of antioxidant enzymes (Suzuki et al. 2012), photosynthesis (Parveen and Ashraf 2010; Adamski et al. 2011), respiration (Moud and Maghsoudi 2008), nitrogen, and carbohydrate metabolism (Chatterjee et al. 2006; Polesskaya et al. 2006), changes in membrane (Yamaguchi-Shinozaki and Shinozaki 2006), osmotic potential (Munns and Tester 2008), and changes in the phytohormone balance (Ashraf et al. 2010; Rahman 2013).

Among the metabolic processes, photosynthesis stands out for being closely related to crop yields, with a decline in productivity due to reduced photosynthetic capacity in plants exposed to abiotic stresses (Saleem et al. 2011). An initial effect of salt stress on photosynthesis is related to decreased CO_2 availability, due to stomatal closure limiting the diffusion to the mesophyll, changes in activity and decreased contents of enzymes, such as ribulose-bisphosphate carboxylase oxygenase-RuBisCo, and changes in Photosystem II (PSII) (Netondo et al. 2004; Flexas et al. 2007; Yang et al. 2009).

Approximately 80 % of iron in plants is contained in photosynthetic cells, where iron excess causes a reduction in chlorophyll content, decreases in CO_2 assimilation, disorder of photosynthetic complexes, increased stomatal resistance, and reduction in transpiration rate (Sharma 2007; Timperio et al. 2007; Nenova 2009). In addition, low temperatures also reduce the photosynthesis rate in plants by reducing stomatal conductance and changing the thylakoid membranes, decreasing the chlorophyll content and its fluorescence, and damaging and impairing the photosystems and chloroplast development (Farooq et al. 2009; Zhu et al. 2010).

At the molecular level, the plant responses to abiotic stresses involve the modulation of the expression of several genes and crosstalk between many molecular pathways (Takahashi et al. 2004). Several studies using the technique of microarrays report changes in the expression of many genes involved in photosynthesis of plants under abiotic stress, which was thus proved to be a process highly responsive to environmental factors (Seki et al. 2002a, b; Rabbani et al. 2003; Matsui et al. 2008). With the development of the next-generation sequencers, the RNA-seq technique enabled the elucidation of transcriptional profiles at higher accuracy than the microarray technique (Wang et al. 2010). Thus, these methodologies along with physiological parameters deepen the understanding of the different altered biological processes and thereby contribute to the development of strategies to produce tolerant plants.

In this context, this study was conducted to elucidate the differential gene expression of chloroplast and of genes involved in photosynthesis by RNA sequencing and quantification of gas exchange in rice leaves (cv. BRS Querência), in stage V3, subjected to cold, salt, and iron stress for a period of 24 h.

Materials and methods

Plant material and stress

Rice seeds (cv. BRS Querência) were germinated in a BOD growth chamber, with a 16-h photoperiod and temperature of 25 ± 2 °C, and maintained for 7 days. After this period, the seedlings were transferred to plastic trays (3 L) containing pre-washed sand with distilled water and maintained in a greenhouse at 26 ± 2 °C, relative humidity of about 60 %, and manual irrigation (every 2 days) with Yoshida nutrient solution (Yoshida et al. 1976) and water. Cultivar BRS Querência was chosen for being characterized by EMBRAPA (Brazilian Agricultural Research Corporation) as highly productive and characterized by us as moderately tolerant to salt and iron stress, and sensitive to low temperature (Supplementary Material 1).

For salt stress and Fe^{+2} toxicity, the plants were irrigated with Yoshida solution with 150 mM NaCl, as described by Singh et al. (2010), and 300 mg L^{-1} Fe^{+2}, as proposed by Elec et al. (2013), respectively. For low-temperature stress, plants were irrigated with Yoshida standard solution and maintained in a BOD incubator at 13 °C and a photoperiod of 16 h, as proposed by Cui et al. (2005). The control treatment was conducted in a greenhouse and irrigated with Yoshida solution.

The experimental unit consisted of a bulk of 100 plants, from which leaf tissues were collected, deep-frozen in liquid nitrogen at −80 °C for later RNA extraction. The control plants were collected at the same time as the stressed plants.

Preparation of cDNA and sequencing

Total RNA was extracted from 100 mg leaves (bulk of 50 plants) using Purelink® Plant RNA Reagent (Invitrogen™). Their integrity and quality was checked by NanoDrop ND-1000® spectrophotometer (GE Healthcare™) and electrophoresed in 1 % agarose gel. Libraries were prepared by the TruSeq RNA Sample 2 Preparation® V kit (Illumina™), according to the manufacturer's recommendations. The library quality was assessed with an Agilent 2100 BioAnalyzer® (Agilent Technologies™) and the Agilent DNA kit 1000® (Agilent™). Paired-end libraries were sequenced (data of 2× 100 pb) on a HiSeq 2500® platform (Illumina™).

Quality analysis and cleaning of reads

For the analysis and visualization of quality of a read, the software FastQC (vers. 0.1.1.2—(http://www.bioinformatics.babraham.ac.uk/projects/fastqc) was used. Thereafter, the software Trimmomatic (vers.0.32) (Bolger et al. 2014) was used to remove low-quality bases and adapters from each library. The parameters for this step were: PE—indicating paired-end format of the sequences; ILLUMINACLIP—to remove any possible adapter sequences; SLIDINGWINDOW: 4:25—to remove bases with a quality below 25 within a window of 4 bases; LEADING: 10 and

TRAILING: 10—to remove bases with a quality below 10, on both the sides of the reads; and MINLEN: 90—to maintain the reads with a minimum of 90 bases, discarding those below this after cleaning.

Identification of differentially expressed genes

The reads were mapped against the reference genome of *Oryza sativa* cv. Nipponbare (build 1.0 of IRGSP obtained from the Rice Annotation Project Database [RAP-DB (http://rapdb.dna.affrc.go.jp/index.html)] by the software TopHat vers. 2.0.11 (Trapnell et al. 2009). The software HTseq version 0.5.3 (Anders et al. 2015) was used to count reads.

Differentially expressed genes (DEGs) were identified with the software R v3.1.0 (http://www.r-project.org) with the edgeR package version 3.8.5 (Robinson et al. 2010), obtained from the Bioconductor repository (Gentleman et al. 2004). Expression levels were normalized by the RPKM method (reads per kilobase of transcript per million mapped reads), considering differentially expressed genes with CPM (Counts per Million) > 1 and $p < 0.01$ by EdgeR test.

The DEGs were subjected to annotation analysis using the software BLAST2GO version 2.7.2 (Conesa et al. 2005). After making the functional annotation of DEGs, genes related to chloroplast and, photosynthesis and light reaction were identified by the detection of GO terms related to these ontologies.

Quantitative real-time PCR (RT-qPCR) validation

Eight stress-responsive genes (Os01g0558850; Os02g0506600; Os02g0788500; Os03g0157900; Os04g0487200; Os07g0476900; Os07g0558300; Os10g0501500) identified by RNA-seq and were selected for validation by RT-qPCR (Supplementary Material 2).

For each sample, 1 µg of total RNA removed DNA by DNase Amplification Grade I (Invitrogen™) treatment was converted into cDNA using the kit iScript® cDNA Synthesis Kit (Bio-Rad Laboratories, Inc.™). The total volume of the RT-qPCR reactions was 12 µL, consisting of 6.25 µL SYBR green fluorophore (Applied Biosystems™, USA), 0.25 µL 10 mM of each primer (forward and reverse), 1 µL cDNA, and 4.25 µL ultrapure water. The reactions were run on a Bio-Rad CFX Real-Time Thermal Cycler®, using the following amplification conditions: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min, and 72 °C for 1 min with the insertion of the melting curve of 65–95 °C, with an increment of 5 °C for each fluorescence measurement. For each biological replicate, three technical replicates (triplicates) were performed. The rice *Ubiquitin10* (Os02g0161900-UBQ10) gene was used as a reference (Moraes et al. 2015). Relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Gas exchange

The gas exchange was measured in fully expanded leaves, using a portable infrared gas analyzer (LI-6400XT; LI-COR™, Lincoln, NE, USA), at a photon flux density of photosynthetically active 1500 µmol m^{-2} s^{-1}, CO_2 concentration within the chamber of 400 µmol mol^{-1} controlled by a CO_2 injection system, and relative humidity between 20 and 58 %. The leaf temperature was 27 ± 2 °C under salt and iron stress, and 14 ± 2 °C for cold stress, and the air-leaf vapor pressure deficit between 1 and 2 kPa. All measurements were made and stored after reaching the steady state (coefficient of variation <2 %), with six readings per treatment. The following variables were analyzed: CO_2 net assimilation rate [A (mol CO_2 m^{-2} $_{\text{leaf area}}$ s^{-1})], stomatal conductance [gS (H_2O mol m^{-2} $_{\text{leaf area}}$ s^{-1})], intercellular CO_2 concentration [Ci (µmol CO_2 mol^{-1})], transpiration rate [Tr (mmol H_2O m^{-2} $_{\text{leaf area}}$ s^{-1})], and the efficient use of water [UEA, (mmol H_2O m^{-2} s^{-1})$^{-1}$] was also calculated, by dividing photosynthesis by transpiration.

Statistical analysis

For the three stresses, the experimental design was completely randomized, with three replications per treatment, where each experimental unit (replication) consisted of two trays with 50 plants each, i.e., a total of 100 plants per replication. Data were analyzed by the analysis of variance (ANOVA) and the means compared by the Tukey test ($p \leq 0.05$), using the software SAS 9.3 (SAS Institute, Cary NC).

Results

Sequencing of mRNA and alignment against the reference genome

The sequencing generated around 186 M reads, in four condition (control, cold, iron, and salt). Reads were mapped to the reference genome (cv Nipponbare-IRGSP-1.0); the mapping mean was 88.9 %, ranging from 88.47 to 89.21 % for iron and control, respectively (Table 1). From the total reads mapped, 6.60–25.27 % were multiple alignments. The multi-alignment parameter is a significant barrier in transcriptomic studies using the short-read sequencing methodology for being a major source of errors and coverage reduction in these studies. This parameter is

Table 1 Total results of reads from rice plants (cv. Querência BRS) exposed to cold, iron, and salt stress for 24 h, mapped against the reference genome

Condition	Input	Mapped	Mapped %	Multiple alignments	Multiple alignments %
Control	46,291,402	41,295,053	89.21	9,460,112	22.91
Cold	47,724,928	42,483,780	89.02	2,805,725	6.60
Iron	41,268,406	36,509,050	88.47	9,225,840	25.27
Salt	51,365,782	46,688,598	88.95	4,355,103	9.53

influenced by both length and complexity of the reads, so that these multi aligned reads were excluded from further analysis to reduce error probability. Technical quality between biological replicates of each treatment was performed by PCA analysis (Supplementary Material 3).

Differentially expressed genes related to photosynthesis

Of the total of genes expressed for each stress, the number of DEGs was highest for cold, with 7905, of which 535 genes (6.76 %) were related to photosynthesis and light reactions (LR), and chloroplast. To salt stress 2092, DEGs were identified, with 309 genes (14.77 %) related to light reactions and chloroplast. Only 630 DEGs were detected for iron stress, with 115 genes (18.25 %) related to photosynthesis and light reactions and, chloroplast components (Fig. 1) (List of DEGs—Supplementary Material 4).

The highest number of photosynthesis-related DEGs in common to two stresses occurred between cold and salt stress, with 125 genes, although the expression pattern was very different, with a greater number of down-regulated genes for cold stress. Salt and iron stress presented 27 genes in common, with very similar expression pattern. The lowest number of DEGs in common was between cold and iron stress, with 10 genes.

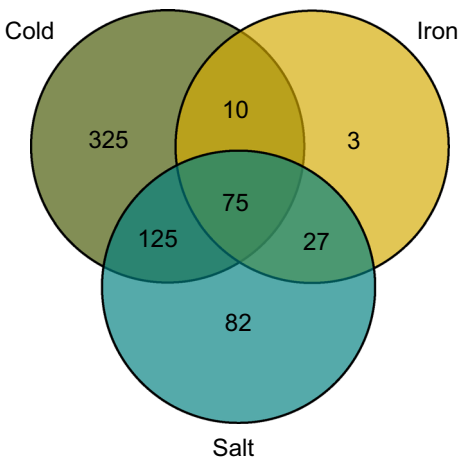

Fig. 1 *Venn diagram* of differentially expressed genes related to photosynthesis and light reaction, and chloroplasts in rice plants (cv. BRS Querência) subjected to cold, iron, and salt stress for 24 h

The highest number of unique DEGs was related to cold stress, with 325 genes, most down-regulated. On the other hand, salt stress had unique 82, mostly up-regulated DEGs and three unique genes for iron toxicity (Os04g0690800—*chloroplast chlorophyll a-b-binding protein*; Os02g071200—*protease do-like 7-like*; Os02g0475400—*uncharacterized sodium-dependent transporter yocs-like*), with the two last negatively regulated. In addition, 75 DEGs were common to all three stresses (Fig. 2).

Characterization of GO terms involved with chloroplast and photosynthesis

Under the different sets of DEGs, the three stresses had transcripts with GOs related to light reactions and photosynthesis, with salt stress presenting 13 terms of GO, followed by cold stress with 12 and iron with 10. For chloroplast-related GOs, cold and salt stress had 12 terms of GO, and iron stress has 9 terms of GO. The main LR and photosynthesis-related GO terms for three stresses are associated with photosynthesis (GO:0015979) at high level followed by photosystem II assembly (GO:0010207) and photosynthesis, light reaction (GO:0019684). The main chloroplast-related GO terms were chloroplast (GO:0009507) at high level followed by chloroplast envelope (GO:0009941) and chloroplast stroma (GO:0009570). The GO-term thylakoid (GO:0009579) also had importance to stress by cold and salt, with 160 and 88 DEGs, respectively (Table 2).

For genes with GOs related to light reactions and photosynthesis, cold stress had 147, salt stress 77, and iron stress 42 DEGs. For chloroplast-related GO terms, cold, salt, and iron stress had 553, 317, and 114 DEGs, respectively. Fig. 3 shows the DEGs by class (photosynthesis and light reaction, and chloroplast) commonly to the three stresses.

Real-time quantitative PCR (RT-qPCR) validation

To validate the RNA-seq results, some genes were selected for analysis in RT-qPCR. The correlation between the results was evaluated using the values of RNA-seq fold change and relative RT-qPCR quantification. The high Pearson correlation ($r = 0.88$) indicated that the RT-qPCR values are similar to those resulting from RNA-seq,

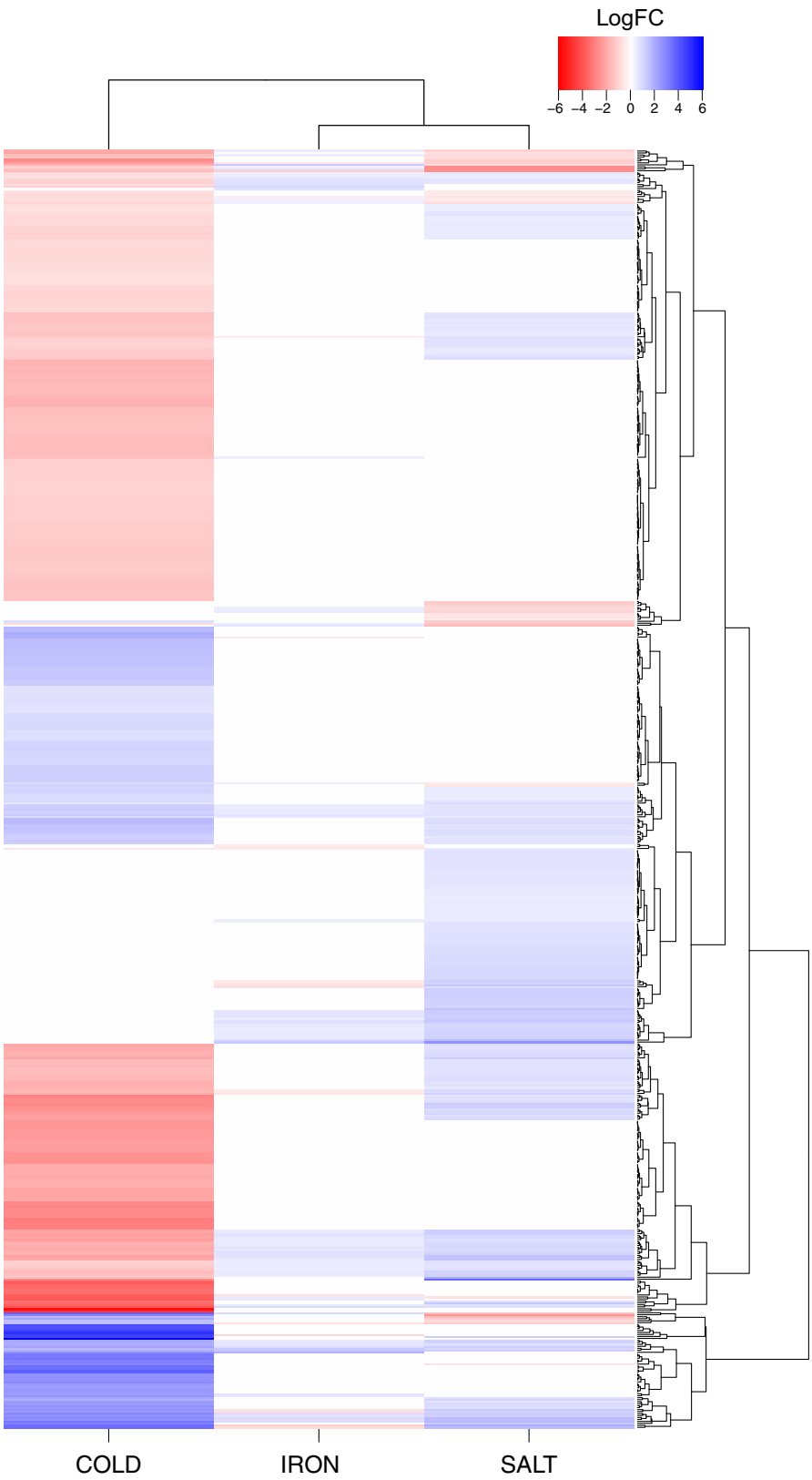

Fig. 2 *Heatmap* of differentially expressed genes related to photosynthesis and light reaction, and chloroplasts in rice plants (cv. BRS Querência) exposed to cold, iron, and salt stress for 24 h

Table 2 Summary of GOs occurrence related to photosynthesis and chloroplasts in rice plant (cv. BRS Querência) subjected to cold, iron, and salt stress for 24 h

	GO accession	GO-term description	Cold	Iron	Salt
LR and photosynthesis	GO:0015979	Photosynthesis	135	37	69
	GO:0009765	Photosynthesis, light harvesting	2	0	2
	GO:0019684	Photosynthesis, light reaction	16	4	6
	GO:0030076	Light-harvesting complex	0	0	1
	GO:0009643	Photosynthetic acclimation	0	0	1
	GO:0009767	Photosynthetic electron transport chain	6	2	4
	GO:0009773	Photosynthetic electron transport in photosystem I	9	4	6
	GO:0009772	Photosynthetic electron transport in photosystem II	1	1	1
	GO:0048564	Photosystem I assembly	3	0	4
	GO:0010207	Photosystem II assembly	32	11	21
	GO:0009517	PSII associated light-harvesting complex II	1	1	0
	GO:0010304	PSII associated light-harvesting complex II catabolic process	3	2	3
	GO:0010109	Regulation of photosynthesis	3	1	2
	GO:0045156	Electron transporter, transferring electrons within the cyclic electron transport pathway of photosynthesis activity	2	1	1
Chloroplast	GO:0009507	Chloroplast	244	35	139
	GO:0009941	Chloroplast envelope	95	29	56
	GO:0042644	Chloroplast nucleoid	3	0	3
	GO:0044434	Chloroplast part	5	1	3
	GO:0030095	Chloroplast photosystem II	4	2	3
	GO:0009902	Chloroplast relocation	23	8	16
	GO:0009573	Chloroplast ribulose-bisphosphate carboxylase complex	2	0	2
	GO:0009570	Chloroplast stroma	115	40	96
	GO:0009534	Chloroplast thylakoid	17	4	10
	GO:0009579	Thylakoid	160	0	88
	GO:0010027	Thylakoid membrane organization	51	15	38
	GO:0042793	Transcription from plastid promoter	23	6	21

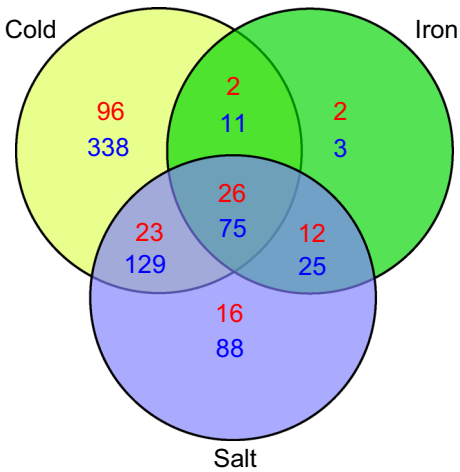

Fig. 3 *Venn diagram* of DEGs related to photosynthesis and light reaction, and chloroplast in rice plants (cv. BRS Querência) subjected to cold, iron, and salt stress for 24 h. *Blue color* is related to chloroplast GO terms, and *red color* to photosynthesis and light reaction

confirming the accuracy and reproducibility of the sequencing results (Fig. 4).

Gas exchanges

Table 3 shows the results for liquid CO_2 assimilation rate [A (mol CO_2 m^{-2} $_{leaf\ area}$ s^{-1})], stomatal conductance [gS (H_2O mol m^{-2} $_{leaf\ area}$ s^{-1})], intercellular CO_2 concentration [Ci (μmol CO_2 mol^{-1})], transpiration rate [Tr (mmol H_2O m^{-2} $_{leaf\ area}$ s^{-1})], and the efficient use of water [UEA, (mmol H_2O m^{-2} s^{-1})$^{-1}$] of the rice plants subjected to control, cold, iron, and salt stress treatments.

For the parameter CO_2 net assimilation rate (A), the control treatment had the highest value, averaging 34.68 mol CO_2 m^{-2} $_{leaf\ area}$ s^{-1}, differing significantly from iron and cold stress, but not differing significantly from salt stress. Followed by the control, salt stress had a mean value of 32.33, but did not differ significantly from iron toxicity, with a mean of 29.85. The greatest decrease

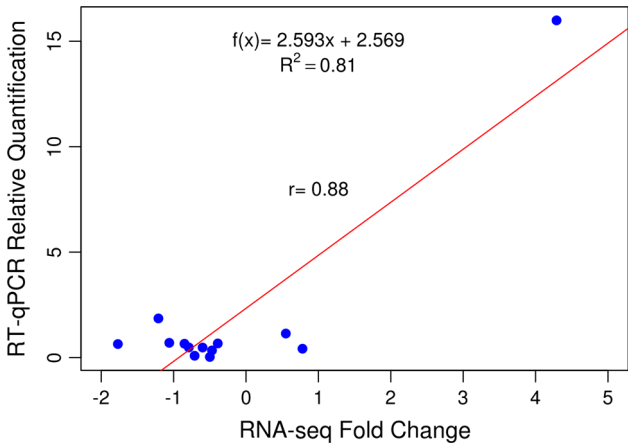

Fig. 4 Pearson correlation between fold change and relative quantification of data obtained by RNA-seq and RT-qPCR, respectively

Table 3 CO_2 net assimilation rate (A), stomatal conductance (gS), intercellular CO_2 concentration (Ci), leaf transpiration (Tr), and water use efficiency (UEA) in rice plants (cv. BRS Querência), exposed to iron, salt, and cold stress for 24 h

Stress	A	gS	Ci	Tr	WUE
Control	34.68 A*	0.5885^{ns}	270.25^{ns}	10.29^{ns}	3.46^{ns}
Salt	32.33 AB	0.4848	260.50	9.09	3.57
Iron	29.85 B	0.6514	293.00	11.14	2.77
Cold	24.20 C	0.4226	219.50	9.31	2.84
CV (%)	7.37	30.14	14.09	12.08	14.39

* Values followed by different letters in the column differ significantly by the Tukey test ($p \leq 0.05$)

^{ns} No significant difference by the Tukey test ($p \leq 0.05$)

in net assimilation rate was in cold stress, which differed significantly from all other treatments, with a mean of 24.20 mol CO_2 m^{-2} $_{leaf\ area}$ s^{-1}.

The variation in A was not accompanied by the parameters of stomatal conductance, intercellular CO_2 concentration, transpiration rate, and water use efficiency, with no statistically significant difference between treatments ($p \leq 0.05$).

Discussion

Due to the previous studies that demonstrated the photosynthesis as the main biological process affected by abiotic stress (data not published), we used RNA sequencing (RNA-seq) and the measurement of gas exchange in leaves of rice cultivar BRS Querência under cold, iron, and salt stress for 24 h, to analyze the main changes related to photosynthesis, at the molecular as well as the physiological level.

Typically, the number of DEGs is associated with tolerance or sensitivity of a genotype to a given stress. Genotypes with higher tolerance have fewer DEGs, while more sensitive genotypes have more, particularly down-regulated genes (Walia et al. 2005; Maron et al. 2008; Degenkolbe et al. 2009). In this study, cold stress has a higher number of DEGs, mostly down-regulated, which may be related to a greater tolerance of BRS Querência to iron and salt stress. Moreover, the heatmap analyses of DEGs common to two or three stresses showed a similar expression pattern of iron and salt stress, possibly due to the ionic nature common to both.

Among the genes common to salt and cold stress, some responsive to abiotic stresses were down-regulated, e.g., Os01g0581300 (*lycopene epsilon cyclase*), Os05g0587200 (*ribulose-bisphosphate carboxylase oxygenase large subunit*), and Os09g0553100 (*Histone H4*) (Sahi et al. 2003; Hashimoto and Komatsu 2007; Kim et al. 2013). However, a large number of genes were down-regulated for cold stress and up-regulated for salt stress, as in the case of genes *superoxide dismutase; chloroplast iron-superoxide partial* (Os06g0143000) and *Carotenoid cleavage dioxygenase 8* (Os01g0566500). This last gene participates in several physiological processes of the plant, and overexpression of Carotenoid cleavage dioxygenase confers tolerance to cold and salt stress in *Arabidopsis* plants (Baba et al. 2015).

The *RNA polymerase sigma factor* gene (SIG-Os05g0589200), which expresses a subunit of RNA polymerase present in the chloroplast, common to salt and iron stress, was down-regulated only for iron stress. The SIG is involved in the transcription of several tRNA genes in chloroplasts, allowing protein translation as well as pigment synthesis in chloroplasts (Kanamaru and Tanaka 2004). Down-regulation of this gene may be one of the likely reasons for the decrease in net assimilation rate in iron stress.

Several studies have described PSII, the photosynthetic complex located in the thylakoid membrane and responsible for photosynthetic oxygen evolution, as a target of many abiotic stress conditions, such as heavy metals, photoinhibition, drought, and low temperatures (Allen and Ort 2001; Pérez-Bueno et al. 2004; Singh-Tomar et al. 2012). In a study on tomato genotypes with contrasting cold tolerance based on transcriptome analysis, Liu et al. (2012) reported greater inhibition of PSII in the sensitive genotype. Furthermore, the previous study showed that when rice seedling was treated with low temperatures, a large number of genes including those involved in photosynthesis were highly down-regulated (Nouri et al. 2015). In our study, some of the unique down-regulated genes in cold-stressed plants were related to PSII subunits (Os02g0129300; Os03g0747700; Os08g0200300;

Os11g0592350). Kohan-Baghkheirati and Geisler-Lee (2015) reported that *Arabidopsis* plants subjected to low temperatures showed more DEGS when compared to five other types of stress, most being down-regulated and many related to photosynthesis. Aside from reducing the expression of PSII-related genes, lower temperatures decrease the fluidity of the membrane, so that there is a decrease in the D1 and D2 protein turnover rate, due to a delay in diffusion of damaged proteins for degradation (Allen and Ort 2001). A gene encoding the smallest subunit of ribulose 1,5-bisphosphate carboxylase (Os12g0291400) and two that expresses a subunit of photosystem I (Os03g0731100 and Os07g0435300) were also down-regulated. Although PSI is target of stress under cold conditions, it has been shown that PSII is more sensitive to low temperature than PSI (Huang et al. 2010).

However, plants developed mechanisms to protect their photosynthetic apparatus, of which carotenoid is one of the most important. Lycopene gene *B-cyclase* (Os02g0190600), which expresses an enzyme located in the chloroplasts and responsible for carotenoid synthesis, was down-regulated for cold stress. Inhibition of enzyme Lycopene B-cyclase drastically reduces carotenoid synthesis, reducing protection and damaging the chloroplast membrane (La Rocca et al. 2007).

There are also reports that cold stress induces apoptosis in plant cells due to loss of the membrane function, chloroplast disintegration, enzyme inactivation, and chromatin fragmentation (Koukalova et al. 1997; Wang et al. 2001). Melatonin, aside from several functions in plant growth and development plays a role in inhibiting apoptosis induced by environmental stresses, such as cold (Lei et al. 2004). The protein family GNAT (GCN5-related N-acetyltransferase) is responsible for catalyzing the transfer of the acetyl group from acetyl coenzyme A to a number of molecules and is part of the melatonin biosynthesis route. Recently, many genes encoding the N-acetyltransferase enzyme, penultimate enzyme in the melatonin synthesis route in plants, have been characterized for rice (Kang et al. 2013). In this study, two genes (Os04g0465500 and Os08g0102000) responsible for the synthesis of this enzyme had a lower expression in cold stress than in the control. This demonstrates that for BRS Querência, cold stress inhibits the expression of genes related to the synthesis of protective molecules of the photosynthetic apparatus and membranes.

Photosynthesis in native tropical plants is substantially affected by cold, in a process called low-temperature photoinhibition (Allen and Ort 2001). In this study, the net assimilation rate was lowest under cold stress, confirming this phenomenon. However, this reduction was not due to a decline in the intercellular CO_2 concentration, since there is usually a relationship between low stomatal conductance and Ci with a low net assimilation rate, but in this study, these two parameters did not differ from the control under any stress, corroborating the findings of Savvides et al. (2012). Thus, these results suggest that gS and Ci are not limiting factors for A in a stress period of 24 h. In a similar study, Yan et al. (2006) also reported a decrease in CO_2 net assimilation rate in rice plants, with no changes in the parameters gS and Ci when exposed to 24 h of cold stress. This decrease in A is probably a result of other factors, such as Rubisco activity and ATP availability, which are both critically affected by low temperatures (Sage 2002; Sage and Kubien 2007).

The decreased expression of genes responsible for the synthesis of protective molecules of the photosynthetic apparatus and membranes, such as carotenoids and melatonin, respectively, at low temperatures demonstrates the vulnerability of photosystems exposed to cold stress. In addition, the down-regulation of genes for subunits of photosystem II shows that the repair of this photosystem is insufficient to maintain the liquid assimilation rate at low temperatures.

For salt stress, only seven unique genes were down-regulated. Among these is the genes *Lrr receptor-like serine threonine-protein kinase*, known to be a positive regulator of tolerance to salt stress (Sun et al. 2013), which shows that a 24-h stress period may have been little stressful for a moderately tolerant cultivar. Another unique down-regulated gene for salt stress was *chlorophyll a-b-binding protein family* (Os09g0296800), which are apoproteins of the PSII antenna complex and are required for stomatal responses to ABA, supporting the above hypothesis (Jansson 1994; Park et al. 2012; Xu et al. 2012). However, most unique DEGs for salt stress were up-regulated, and one of the most expressive were heat shock protein (Os05g0302916, Os05g0303000, and Os08g0487800), widely known to respond to stress (Wang et al. 2004). Other genes, such as *cytochrome c oxidase assembly factor 5* (Os09g0518800), *ribulose-bisphosphate carboxylase small chain chloroplast expressed* (Os12g0292400), and *violaxanthin de-epoxidase-related protein* (Os01g0716400), were also up-regulated.

The effects of salinity on photosynthesis are usually attributed to stomatal closure that limits gas diffusion, and consequently alters gas metabolism (Chaves et al. 2009). In addition, high NaCl concentrations cause degradation in photosynthetic pigments and reduce PSII activity (Boriboonkaset et al. 2013). In this study, although there was a slight decrease in mean A, there were no significant differences between salt stress and control for any of the parameters analyzed. However, Kawasaki et al. (2001) observed a significant drop in net assimilation rate in rice plants subjected to salt stress (150 mM) within minutes, in disagreement with the results of this study. The fact that

BRS Querência has a large number of up-regulated genes, some responsible for the homeostasis of ions and photosystem II, and small changes in photosynthetic metabolism after 24 h, may be associated with the tolerance to salt stress of this cultivar.

Iron toxicity caused no major changes at the molecular level, which is the stress with the fewest DEGs, all related to chloroplast. However, there was a reduction in net assimilation rate that differed significantly from the control treatment Müller et al. (2015) found similar results, where the authors affirm that the reduction in photosynthetic rate of rice plants under iron excess may have been due to the oxidation of constituents of the photosynthetic complexes. Iron toxicity studies also showed that at moderate concentrations (4 mM), a reduction in net assimilation rate is only attributed to stomatal limitations, while at higher doses (9 mM), both stomatal and non-stomatal limitations are responsible for this reduction (Pereira et al. 2013). However, our results confirmed those of Quinet et al. (2012), who observed an increase in stomatal conductance at the onset of iron stress, followed by a reduction after only 2 weeks, damaging the PSII and reducing the concentration of *a* and *b* chlorophyll. As in the case of salt stress, a high Fe^{+2} concentration for only 24 h seems to be insufficient to trigger stomatal closure, suggesting that the observed reduction in A would be mainly associated to damage to PSII.

Conclusions

The results showed that the largest number of down-regulated DEGs related to photosynthesis and chloroplasts as well as the greatest reduction in net assimilation rate were associated with cold stress. This demonstrates that within 24 h of stress, low temperatures affect photosynthesis drastically, at the molecular as well as the physiological level, mainly at photosystem II. Despite reductions in net assimilation rate, cultivar BRS Querência was less affected by iron and salt stress, which can be related to the tolerance of this cultivar. The changes in net assimilation rate under all three stresses were related to non-stomatal processes.

Author contribution statement Conceived and designed the experiments LCM, EJBB, and ACO. Wrote, edited and analyzed the data: MNA and LWPA. Revised the paper: DRF, LCB, EJBB, SD, and LCM. Conducted the experiments: MNA, LCB, RD, SD, and SFSS. All authors readed the paper and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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