

Correlation of proline content and gene expression involved in the metabolism of this amino acid under abiotic stress

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Abstract This study evaluated the metabolism of proline in rice based on the expression pattern of genes involved in biosynthesis and catabolism of amino acid and determined the correlation with the proline content produced under stress. In stage V4, genotypes with a differentiated response to salinity and low-temperature stresses were exposed separately to two test conditions: 150 mM NaCl and 13 °C. Proline increased in both genotypes and stresses. The expression of *P5CS1* was increased in both genotypes and conditions, while *P5CS2* was responsive in the sensitive genotype under salinity. High correlations between proline content/*P5CS1*, proline content/*P5CS2*, *P5CS1/P5CR*, and *P5CS2/P5CR* were observed for the tolerant genotype under salt stress. The correlation between proline content/*PDH* was 0.42 and -0.13 (tolerant genotype) and -0.54 and 0.15 (sensitive genotype) under salinity and cold, respectively. *P5CDH* was positively correlated with proline content under salinity for both genotypes. Under salt stress, there was a positive correlation between proline content/*OAT* in the tolerant genotype. It was concluded that the increase in proline content is greater for the tolerant genotype. Under salt stress, the levels of transcript genes are more correlated with the

proline content in the tolerant genotype, while at 13 °C, the correlation of the sensitive genotype was higher.

Keywords *Oryza sativa* L. · Abiotic stress · Osmotic adjustment · RT-qPCR

Introduction

The response of plants to abiotic stress is of great interest for research, and genotypes with higher yields in harsh environments are constantly being sought (Monteiro et al. 2014). When grown under stress, the plants are affected by metabolic and physiological damages harmful to their full growth and development (Qin et al. 2015). Thus, studies dealing with this matter are necessary for a better understanding of the adaptation mechanisms of plants to unfavorable environments of cultivation, which, due to constant climate change, are increasingly common (Fariduddin et al. 2013).

Drought, high salinity, and low temperature are some of the environmental conditions with adverse effects on the productivity of most crops, which is particularly true for rice (*Oryza sativa* L.) (Yamaguchi-Shinozaki and Shinozaki 2006). Salt stress inhibits the growth and development of plants causing: osmotic stress due to the reduction in water availability; ion toxicity, by sodium accumulation and nutritional imbalance; and oxidative stress, by the formation of reactive oxygen species and hormonal imbalance (Silva-Ortega et al. 2008). Cold stress is a problem that affects rice plants in 25 countries, with negative effects on productivity (Cruz et al. 2013). Low temperatures cause chlorosis, necrosis, changes in chlorophyll and fluorescence contents, and changes in the lipid structure and composition of the cell membrane, aside from

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increasing the levels of reactive oxygen species (ROS) and of malondialdehyde (MDA), causing oxidative cell damage (Theocharis et al. 2012). Aside from the above-mentioned effects, both stresses, salinity and cold, cause osmotic stress, causing the plants to trigger the osmotic adjustment to maintain the water, turgor, and cell homeostasis potential.

Osmotic adjustment is a physiological mechanism established by the accumulation of compatible solutes, such as proline, glycine betaine, trehalose, and sucrose in the vacuole or the cytosol. This contributes to the maintenance of the water balance and preservation of the integrity of proteins, enzymes, and cell membranes under stress (Ashraf et al. 2011; Marijuan and Bosch 2013). The amino-acid proline is the most studied compatible solute because of its sensitive response to adverse conditions, such as salinity, drought, and cold (Ashraf et al. 2011; Verbruggen and Hermans 2008).

In plants, proline is synthesized from two routes: the route of glutamate and route of ornithine. As part of the glutamate pathway, the enzymes pyrroline-5-carboxylate synthetase (P5CS) and P5C reductase (P5CR; EC 1.5.1.2) reduce the glutamate to semialdehyde glutamate and sequentially pyrroline-5-carboxylate (P5C) to proline, respectively (Szabados and Savaouré 2010). Proline biosynthesis generally occurs constitutively in the cytosol, and is controlled by gene *P5CS2*. On the other hand, under stress, proline biosynthesis is increased in the chloroplasts and is controlled mainly by gene *P5CS1* (Székely et al. 2008). Alternatively, proline can be synthesized from ornithine, which is transaminated by delta-ornithine aminotransferase (OAT) (Szabados and Savaouré 2010).

In contrast, the proline catabolism occurs in the mitochondria by the catalytic action of enzymes proline dehydrogenase (PDH; EC 1.5.99.8) which, in most species, is encoded by two genes and 1-pyrroline-5-carboxylate dehydrogenase (P5CDH; EC 1.5.1.12), which converts P5C to glutamate (Ribarits et al. 2007).

According to Xue et al. (2009), proline production is activated and its catabolism repressed during stress. Once the ideal condition is reestablished, the opposite regulation is triggered. However, in some species, data do not always indicate a positive correlation between proline accumulation and adaptation to stress. Some authors suggest that proline accumulation may be related to the tolerance degree, while others claimed that the accumulation of this amino acid is a “symptom” of the injuries, rather than a tolerance indicator (Hien et al. 2003).

The purpose of this study was to evaluate proline metabolism in rice plants by the expression pattern of genes involved in biosynthesis and catabolism of amino acid and check whether the quantity of transcripts is directly related to the proline content under salinity and

low temperature. Although there are several studies on the role of proline in response to abiotic stresses, it is noteworthy that this study addresses the simultaneous expression of all genes involved in the proline pathway, rather than that of single genes.

Materials and methods

Plant material and stress

The experiments were carried out with two Brazilian rice genotypes that have a differentiated response to salt and cold stress: BRS Pampa (subspecies *indica*—sensitive) and BRS Bojuru (subspecies *japonica*—tolerant).

The seeds were germinated on germitest paper rolls, at 25 ± 2 °C for 10 days, in an air-conditioned growth chamber. To simulate salt stress, the seedlings were transferred to a greenhouse at 28 ± 2 °C in a floating hydroponic cultivation system with trays containing 20 L of nutrient solution of Yoshida et al. (1976). Per genotype, three trays with 50 seedlings were grown until the plants had four leaves (stage V4). Thereafter, 150 mM NaCl was added to the nutritive solution, as proposed by Singh et al. (2010), and maintained under these conditions for 72 h for data collection.

For low-temperature stress, the seedlings were transferred to plastic trays (3 L) containing commercial soil fertilized as recommended for irrigated rice. Per genotype, three trays with 50 seedlings were used, which were placed in a greenhouse at 28 ± 2 °C and irrigated with nutrient solution Yoshida et al. (1976) until the plants had grown four leaves (stage V4). Thereafter, the plants of the control treatment were harvested and the remaining trays placed in a growth at 13 °C for 72 h for data collection.

The collection of material for analysis (leaves) was performed as follows for both stresses: C1 = 0 h (sampling 1) = plants unaffected by cold or salt stress; C2 = 6 h of stress; C3 = 24 h of stress; C4 = 48 h of stress, and C5 = 72 h of stress. The experimental design was completely randomized (DIC) in a factorial 2×5 (2×5 genotypes to stress exposure periods), with three replications per treatment. The experimental units consisted of a bulk of ten plants. Statistical analyses of the stresses were performed separately.

Quantification of free proline content

To determine the proline content, the method described by Bates et al. (1973) has been used, with some modifications. Approximately 0.5 g of leaf tissue were blended with 2 mL of aqueous solution of sulfosalicylic acid 3% (w/v) and then centrifuged at 4000g for 20 min. The test tubes were

filled with 1 mL of the supernatant, 2 mL of glacial acetic acid, 2 mL of ninhydrin acid, and 2 mL of distilled water, and maintained in a water bath at 100 °C for 60 min. After this period, the tubes were cooled in an ice bath and 4 mL of toluene added. The samples were shaken vigorously and allowed to reach room temperature and then read in a spectrophotometer at 520 nm. The absorbance values obtained were compared with the standard proline curve and the results expressed in $\mu\text{mol proline g}^{-1}$ FW (fresh weight). The data were subjected to analysis of variance (ANOVA) and Tukey's test (5%), using the statistical program SAS 9.3 (SAS Institute, Cary, NC).

RNA extraction, cDNA synthesis, and primer design

Total RNA was extracted from 0.1 g of leaves using the PureLink[®] Kit (Invitrogen[™]). For complete removal of DNA residues, the samples were treated with DNase I (Invitrogen[™]). The quality, concentration, and purity of total RNA were assessed by electrophoresis on 1% agarose gel and NanoDrop ND-1000. Each sample was reverse transcribed into cDNA using the commercial kit SuperScript First-Strand System for RT-PCR (Invitrogen[™]).

Primers were designed in the transcribed region of rice genes involved in the biosynthesis (*P5CS1*, *P5CS2*, *P5CR*, and *OAT*) and proline catabolism (*P5CDH* and *PDH*) using the sequences deposited in the *RAP-DB* database and the program PerlPrimer v. 1.1.21. To normalize RT-qPCR data internally, gene *UBQ10*, previously tested for the experimental conditions, was used (Morales et al. 2015). Only primers with a dissociation curve with a single peak and an

amplification efficiency near 100% were used in this study (Table 1).

RT-qPCR reactions

The total volume of the reactions was 12 μL , with 6.25 μL of the reagent FastStart Universal SYBR Green Master (Roche[®]), 0.25 μL (10 mM) of each primer (sense and antisense), 1 μL cDNA (1:5 dilution, previously defined), and 4.25 μL ultra pure water. The reactions were performed with a real-time BIO-Rad CFX-96 thermal cycler and the following amplification parameters: 95 °C for 10 min, 40 cycles at 95 °C for 15 s, and 60 °C for 1 min with insertion of melting curve from 65 to 95 °C, with an increment of 5 °C at each fluorescence measurement. Three technical repetitions were performed for each biological repetition, including samples for the control treatment as template-free controls. The relative quantification of the expression (RQ) of each gene was obtained, as described by Livack and Schmittgen (2001). The data were subjected to analysis of variance (ANOVA) and Tukey's test (5%), using the statistical program SAS 9.3 (SAS Institute, Cary, NC).

Correlation analysis

To measure the intensity of correlation between the expression of each gene of proline metabolism and free proline content, the Pearson correlation coefficient was calculated for each genotype within each stress, and represented in heat maps. Analyses were performed using the

Table 1 Primer sequence of proline biosynthesis and catabolism used for gene expression analysis by RT-qPCR. Annealing temperature 60 °C

Gene	RAP-DB	Sense/antisense	Amplicon (pb)
<i>P5CS1</i>	Os05t0455500-01 (AK101985)	5'-AGAGATTGTTCAAGGCATCTACAG-3' 5'-AACCAAAGGCTTCTCATATCCAG-3'	161
<i>P5CS2</i>	Os01t0848200-01 (AK101230)	5'-GGTTGATTATCCAGCAGCGT-3' 5'-CCCGCCATAGATAACTACCC-3'	150
<i>P5CR</i>	Os01t0948400-01 (AK070184)	5'-GTTGGTCTGGTCATCGAAGATT-3' 5'-CATTCTCAGTAGCCATCTCCC-3'	104
<i>P5CDH</i>	Os05t0536400-01 (AK121765)	5'-AGCAGAGGATGTGGACTTCA-3' 5'-GCCTTTCAAATCAGCAGCC-3'	132
<i>PDH</i>	Os10t0550900-01 (AK121010)	5'-GGTTCCAGGTGAGCAAG-3' 5'-AAGGAGGAAGACGAGAGCA-3'	106
<i>OAT</i>	Os03t0643300-01 (AK108010)	5'-TGCTGTGTATCAAACCAGGA-3' 5'-CTTTCAACGAGACCTTCATCTG-3'	160
<i>UBQ10</i>	Os02t0161900-01 (AK101547)	5'-TGGTCAGTAATCAGCCAGTTTGG-3' 5'-GCACCACAAATACTTGACGAACAG-3'	105

SAS v.9.3 software (SAS Institute Inc., Cary, NC) with the procedures *proc corr* and *proc sgrender*.

Results

Free proline content

According to the results of analysis of variance, there was a significant effect of interaction between the factors (2 genotypes \times 5 stress exposure periods) for the free proline content under both cold and salt stress. The proline content increased after 6 h of stress and reached the highest level after 72 h under both genotypes, in response to the salt treatment. For the (sensitive) genotype BRS Pampa, values ranged from 1.62 to 3.53 $\mu\text{mol proline g}^{-1}$ FW, with a 2.2-fold increase compared to the control, while in genotype BRS Bojuru (tolerant), the increase was 3.2-fold after 72 h of stress, compared to the control. In a comparison of the genotypes, there was a statistical difference between the stress periods of 6 and 48 h.

Under cold stress, there was a 1.3- and 2.2-fold increase in proline content in a period of 72 h over the control for BRS Pampa and BRS Bojuru, respectively, although this increase was statistically not significant, compared to the control plants. For BRS Pampa, the period of 6 and 48 h differed from the 72-h stress period, while for BRS Bojuru, the 24-h stress period differed statistically from the control treatment and from 48 h (Fig. 1).

Expression of proline metabolism genes

The analysis of variance for relative expression of the genes showed that the values were significantly different ($p \leq 0.05$) to the times of the exposure in NaCl and low temperature, except for *PC5S2* (BRS Bojuru) and *PDH* (BRS Pampa) under salinity (Tables 2, 3).

The increase in proline content observed in the two genotypes under salinity and low temperature probably occurred due to the increase in the expression of genes *P5CS1* and *P5CR* involved in the biosynthesis of this amino acid, accompanied by minor changes in the expression of *P5CDH* and *PDH* involved in mitochondrial proline catabolism (Figs. 2, 3).

For gene *P5CS1*, with preferential protein accumulation in the chloroplast when under stress, a gradual increase in mRNA levels was observed during the salt exposure period, peaking at 8.78 and 11.28 in the tolerant and sensitive genotypes, respectively, after 72 h of stress (Fig. 2a, c). In the cold treatment, the expression of gene *P5CS1* increased in all stress periods. However, the highest values of relative quantification (RQ) were 4.75 (tolerant genotype) and 3.49 (sensitive genotype) times higher than of the control after

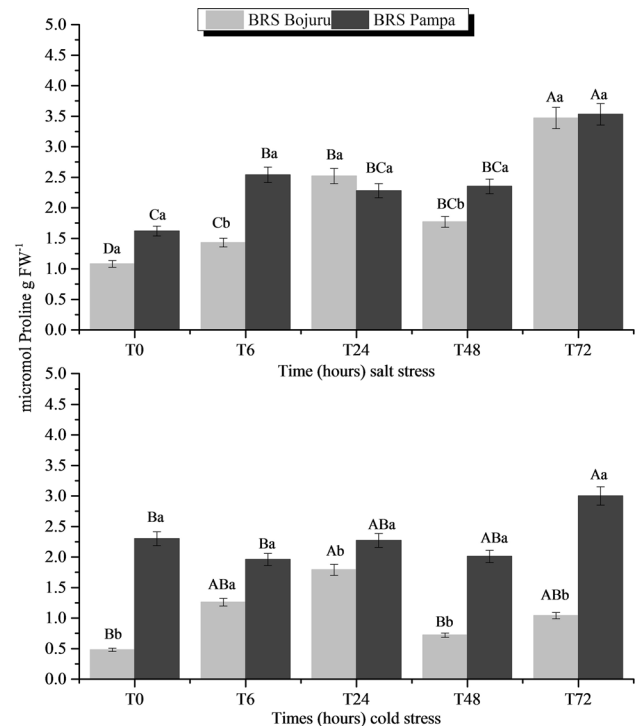


Fig. 1 Free proline content in two rice genotypes under salt stress (150 mM NaCl) and low temperature (13 °C). The values are represented by the mean \pm SD for each treatment ($n = 3$). Means followed by the *same capital letters* are not statistically different in the stress period for each genotype and equal *lowercase letters* do not differ among the genotypes within each stress period

72 h (Fig. 3a, c). The gene *P5CR*, which reduces P5C to proline, increased expression with 4.22 and 2.93 times higher RQ values in tolerant and sensitive genotypes under salinity, respectively, and 2.58 and 2.82 under cold in the last sampling (Figs. 2a, c, 3a, c). However, under cold in sensitive genotype, the amount of *P5CR* transcripts was lower than of the control after 6, 24, and 48 h, increasing until 72 h of stress (RQ = 2.82) (Fig. 3c).

The genes P5C dehydrogenase (*P5CDH*) and proline dehydrogenase (*PDH*) involved in proline catabolism were less responsive under stress application than the synthesis genes, with *P5CDH* and *PDH* values between 1.12–1.85 and 0.92–1.98 for BRS Bojuru and 1.20–1.88 and 0.73–1.10 for BRS Pampa (Fig. 2b, d). During cold stress, the *P5CDH* expression in BRS Bojuru varied between 1.1 and 1.98, similar to salt stress, while *PDH* was below the control at all sampling times. In BRS Pampa, the expression was between 0.91 and 1.86 (*P5CDH*) and 1.12 and 1.31 (*PDH*) (Fig. 3b, d).

In addition, proline biosynthesis occurs by the catalytic activity of the isoform gene *P5CS2* and via ornithine by the enzyme activity of ornithine delta aminotransferase (OAT). The gene expression of *P5CS2*, which accumulates protein preferentially in the cytosol, was higher in the salt-sensitive

Table 2 Relative quantification (RQ) of gene expression of proline biosynthesis (*P5CS1*, *P5CS2*, *P5CR*, and *AOT*) and catabolism (*P5CDH* and *PDH*) in rice genotypes under salinity (150 mM NaCl)

Genes	Genotypes	Times (h) salt stress				
		T0	T6	T24	T48	T72
<i>P5CS1</i>	BRS Bojuru	1.00 ^C	1.64 ± 0.20 ^C	1.90 ± 0.38 ^C	3.54 ± 0.3 ^B	8.78 ± 0.31 ^A
	BRS Pampa	1.00 ^D	1.45 ± 0.25 ^D	6.95 ± 0.37 ^C	9.86 ± 0.39 ^B	11.28 ± 0.28 ^A
<i>P5CS2</i>	BRS Bojuru	1.00 ^A	0.99 ± 0.26 ^A	1.13 ± 0.37 ^A	1.12 ± 0.40 ^A	1.61 ± 0.29 ^A
	BRS Pampa	1.00 ^C	1.95 ± 0.30 ^{BC}	4.14 ± 0.48 ^{AB}	4.33 ± 0.37 ^A	5.95 ± 0.43 ^A
<i>P5CR</i>	BRS Bojuru	1.00 ^D	2.04 ± 0.31 ^B	2.20 ± 0.49 ^B	1.66 ± 0.41 ^C	4.22 ± 0.37 ^A
	BRS Pampa	1.00 ^B	2.15 ± 0.44 ^A	2.01 ± 0.52 ^{AB}	2.05 ± 0.37 ^{AB}	2.93 ± 0.40 ^A
<i>P5CDH</i>	BRS Bojuru	1.00 ^B	1.12 ± 0.52 ^B	1.62 ± 0.40 ^{AB}	1.35 ± 0.50 ^{AB}	1.85 ± 0.41 ^A
	BRS Pampa	1.00 ^B	1.88 ± 0.30 ^A	1.60 ± 0.50 ^{AB}	1.51 ± 0.35 ^{AB}	1.20 ± 0.46 ^{AB}
<i>PDH</i>	BRS Bojuru	1.00 ^{BC}	0.92 ± 0.32 ^C	1.02 ± 0.41 ^{BC}	1.98 ± 0.36 ^A	1.70 ± 0.24 ^{AB}
	BRS Pampa	1.00 ^A	1.10 ± 0.21 ^A	0.81 ± 0.34 ^A	0.82 ± 0.28 ^A	0.73 ± 0.32 ^A
<i>AOT</i>	BRS Bojuru	1.00 ^A	1.35 ± 0.48 ^A	1.84 ± 0.27 ^A	3.24 ± 0.37 ^B	3.58 ± 0.24 ^B
	BRS Pampa	1.00 ^B	1.47 ± 0.32 ^A	1.40 ± 0.44 ^A	1.69 ± 0.36 ^A	1.36 ± 0.37 ^{AB}

Mean ± SD, $n = 3$. Significant differences ($p \leq 0.05$, Tukey's test) between times (h) salt stress for each gene and genotype are indicated by different letters

Table 3 Relative quantification (RQ) of gene expression of proline biosynthesis (*P5CS1*, *P5CS2*, *P5CR*, and *AOT*) and catabolisms (*P5CDH* and *PDH*) in rice genotypes at low temperature (13 °C)

Genes	Genotypes	Times (h) cold stress				
		T0	T6	T24	T48	T72
<i>P5CS1</i>	BRS Bojuru	1.00 ^C	1.33 ± 0.28 ^C	2.18 ± 0.35 ^B	1.79 ± 0.41 ^C	4.75 ± 0.27 ^A
	BRS Pampa	1.00 ^B	1.75 ± 0.45 ^B	2.91 ± 0.41 ^A	1.76 ± 0.45 ^B	3.49 ± 0.30 ^A
<i>P5CS2</i>	BRS Bojuru	1.00 ^A	0.76 ± 0.26 ^{AB}	0.49 ± 0.27 ^B	0.46 ± 0.34 ^B	0.57 ± 0.29 ^B
	BRS Pampa	1.00 ^A	0.49 ± 0.28 ^B	0.52 ± 0.32 ^B	0.69 ± 0.24 ^{AB}	0.83 ± 0.38 ^{AB}
<i>P5CR</i>	BRS Bojuru	1.00 ^B	2.34 ± 0.31 ^A	2.36 ± 0.39 ^A	2.37 ± 0.43 ^A	2.58 ± 0.40 ^A
	BRS Pampa	1.00 ^B	0.53 ± 0.22 ^C	0.81 ± 0.33 ^{BC}	0.74 ± 0.27 ^{BC}	2.82 ± 0.38 ^A
<i>P5CDH</i>	BRS Bojuru	1.00 ^B	1.98 ± 0.52 ^A	1.01 ± 0.38 ^B	1.29 ± 0.45 ^{AB}	1.86 ± 0.41 ^A
	BRS Pampa	1.00 ^{AB}	0.90 ± 0.48 ^B	1.37 ± 0.29 ^{AB}	1.86 ± 0.47 ^A	1.20 ± 0.33 ^B
<i>PDH</i>	BRS Bojuru	1.00 ^A	0.29 ± 0.32 ^C	0.87 ± 0.21 ^{AB}	0.53 ± 0.36 ^{BC}	0.80 ± 0.24 ^{AB}
	BRS Pampa	1.00 ^B	1.31 ± 0.39 ^A	1.17 ± 0.28 ^{AB}	1.12 ± 0.42 ^{AB}	1.26 ± 0.31 ^A
<i>AOT</i>	BRS Bojuru	1.00 ^C	0.80 ± 0.28 ^C	1.86 ± 0.40 ^B	2.73 ± 0.37 ^A	2.46 ± 0.24 ^A
	BRS Pampa	1.00 ^{AB}	0.69 ± 0.42 ^C	0.92 ± 0.44 ^B	1.24 ± 0.38 ^A	0.90 ± 0.35 ^B

Mean ± SD, $n = 3$. Significant differences ($p \leq 0.05$, Tukey's test) between times (h) salt stress for each gene and genotype are indicated by different letters

genotype, reaching the highest mRNA level after 72 h of stress (RQ = 5.95). On the other hand, the *OAT* gene was also more responsive in the tolerant genotype after 72 h (RQ = 3.58) (Fig. 4a, b). In cold treatment, the *P5CS2* expression values were lower than the control after all stress periods in both genotypes. However, the same was not true for *OAT*, which was more responsive in tolerant genotype after 48 h of stress (RQ = 2.73) (Fig. 4c, d).

Correlation

Under salt stress, the amplitude of variation of correlations was 0.42–0.99 between the proline content and gene expression, for BRS Bojuru and –0.54 to 0.97 for BRS

Pampa (Fig. 5a, b). Thus, for the tolerant genotype, all correlations between proline content and gene expression were significant and highly correlated with *P5CDH*, *P5CS2*, and *P5CR* and low correlation with *PDH*. Considering the correlation coefficients between the genes of proline metabolism, *P5CS2* was highly correlated with *P5CS1* (0.98) and *P5CR* (0.93), respectively. Similar values were found for the pairs *P5CS1/P5CR* (0.91) and *OAT/PDH* (0.92), whereas the lowest correlation was observed for the pair *PDH/P5CR* (0.37) (Fig. 5a).

Contrasting responses were observed in sensitive genotype, for which he was found negative correlation value between proline content and *PDH* gene (–0.54), while the *P5CS2* gene showed the most positive correlation with

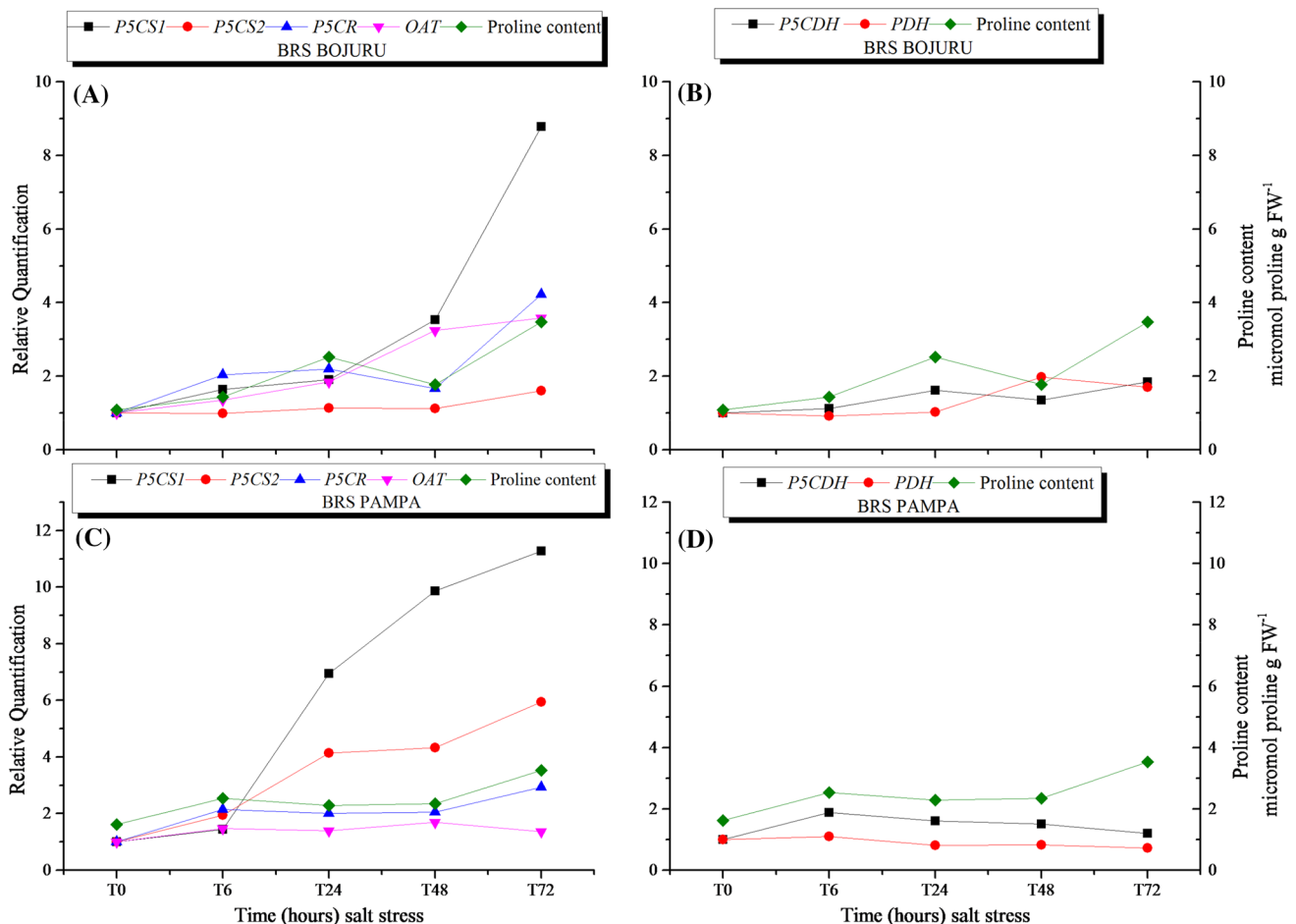


Fig. 2 Relative quantification (RQ) of gene expression of proline biosynthesis (*P5CS1*, *P5CS2*, *P5CR*, and *AOT*) and catabolism (*P5CDH* and *PDH*) and quantification of free proline content in rice

genotypes BRS Bojuru (a, b) and BRS Pampa (c, d) under salinity (150 mM NaCl). The points are represented by the mean for each treatment ($n = 3$)

proline content (0.97). Among the genes involved in proline biosynthesis, had *P5CS1* correlation with *P5CS2* and 0.73–0.10 with *P5CR*. For the *PDH* gene, except for *PDH/P5CR*, negative correlation estimates were observed between *PDH* and all other genes, with values ranging from -0.28 (*PDH/OAT*) and -0.94 (*PDH/P5CS1*), indicating contrasting response expression in each gene pair. By analyzing the correlations between the other combinations, it was found that proline content/*P5CR*, *P5CS1/P5CR*, and *P5CDH/P5CR* was no correlation. Moreover, the correlation between estimated proline content/*P5CS2* *P5CS1* and/*P5CDH* highly frequent (0.97) (Fig. 5b).

In the cold treatment, the correlations between the tested genes and the proline content were low for the tolerant genotype. The highest value was observed for *P5CR*/proline content (0.60), while gene *P5CS2* was least correlated with the proline content (-0.51). Analyzing the gene combinations, high negative correlations were observed with gene *P5CS2*, namely: *P5CS2/P5CR* (-0.85), *P5CS2/OAT* (-0.81), and *P5CS2/P5CS1*

(-0.48). No correlation was found for *P5CS2/P5CDH* (-0.07), *OAT*/proline content (-0.01), *OAT/P5CDH* (-0.05), *OAT/PDH* (0.09), and *P5CDH*/proline content (0.10). On the other hand, the other combinations correlated positively, but with lower values than those found in the NaCl treatment (Fig. 6a).

For the sensitive genotype, the proline content was positively correlated with the genes *P5CS1* (0.70) and *P5CR* (0.97), whereas with *OAT*, the value was negative (-0.48). Genes involved in proline biosynthesis were not closely correlated with each other, since for *P5CS1/P5CS2*, the value was -0.26 , for *P5CS1/P5CR* 0.68 and for *P5CS2/P5CR* 0.48. Most negative correlations were observed in combinations with the *OAT* gene and no correlation in combinations with *P5CDH* (Fig. 6b). The responses of the two genotypes were contrasting, especially with regard to the proline content and expression of proline biosynthesis genes (*P5CS1*, *P5CS2*, and *P5CR*), for which the correlations of combination had different trends (Fig. 6a, b).

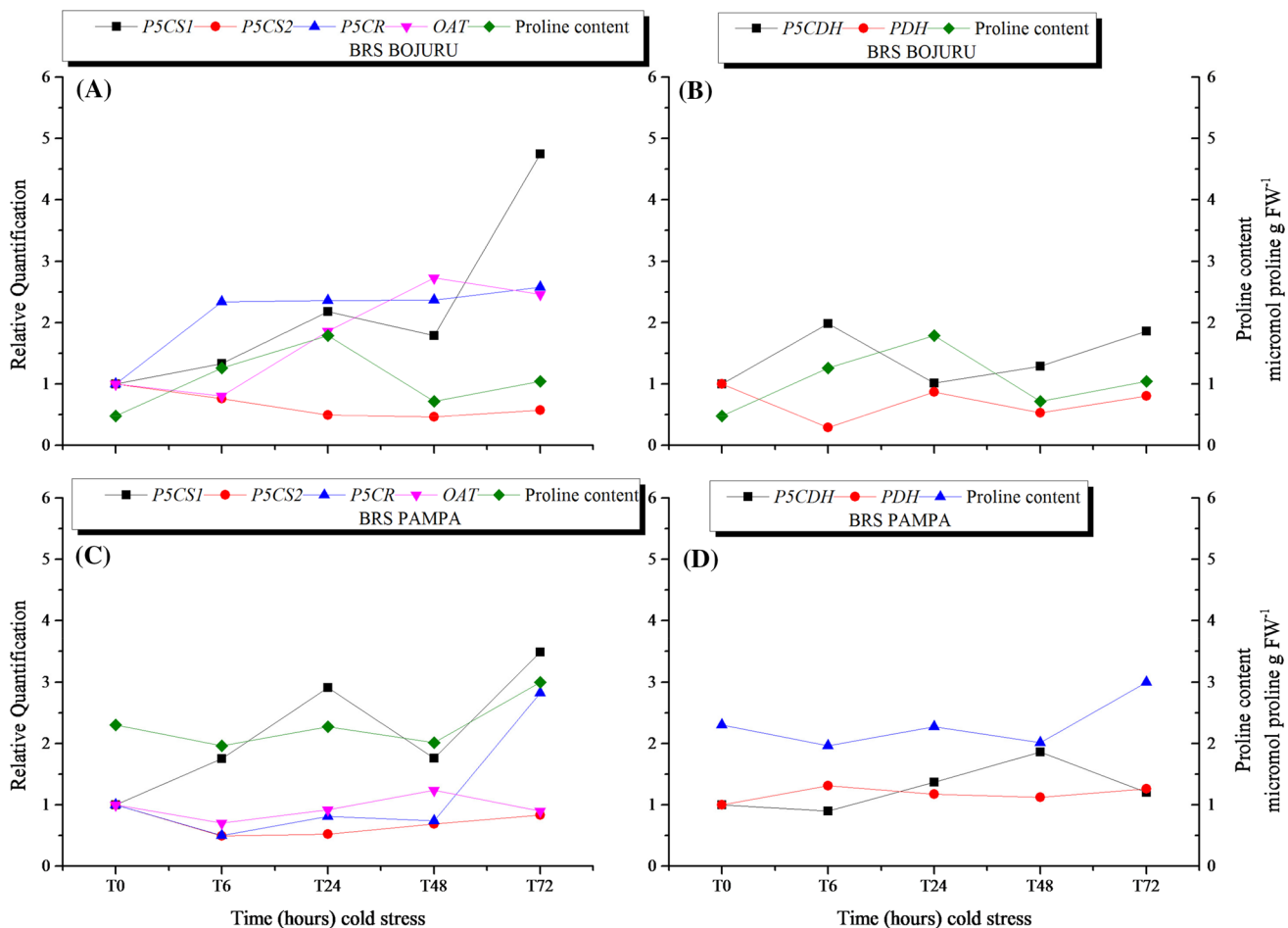


Fig. 3 Relative quantification (RQ) of gene expression of proline biosynthesis (*P5CS1*, *P5CS2*, *P5CR*, and *OAT*) and catabolism (*P5CDH* and *PDH*) and quantification of free proline content in rice

genotypes BRS Bojuru (a, b) and BRS Pampa (c, d) at low temperature (13 °C). The points are represented by the mean for each treatment ($n = 3$)

Discussion

The osmotic adjustment is one of the most effective physiological mechanisms for the maintenance of cell turgor under conditions of low water potential in the soil caused, among others, by drought, salinity, and low atmospheric temperature (Marijuan and Bosch 2013). This mechanism is established by the accumulation of compatible solutes (proline, glycine betaine, trehalose, sucrose, polyamines, mannitol, and pinitol) that contribute to the maintenance of the intracellular water balance and preservation of the integrity of proteins, enzymes, and cell membranes (Ashraf et al. 2011). In this study, proline metabolism in rice plants was evaluated based on the expression pattern of genes involved in biosynthesis and catabolism of amino acid. It was found that the amount of transcripts is directly related to the proline content produced under salinity and cold stress.

An increase in proline content was observed in both treatments and genotypes, but was greater in BRS Bojuru

(tolerant) under both experimental conditions. Under salt stress, changes in proline content already occurred at the beginning of stress. Peaks were reached under severe stress (72 h) in both genotypes, and the increase was greater in the tolerant genotype than the control. Under cold stress, it was also noted that the tolerant genotype had double the increase of sensitive genotype compared to control plants exposed to 72 h of cold (Fig. 1). Similar results were obtained by Hien et al. (2003), who observed a relative increase in proline content in saline-tolerant rice cultivars 48 h after stress application, whereas no increases were observed for the sensitive cultivar, even after 72 h of treatment with 200 mM NaCl. These different responses of genotypes may be associated with signaling cascades that regulate the proline metabolism, which are controlled by multiple cellular mechanisms and which should be further investigated (Bhaskara et al. 2015). Among the factors involved, abscisic acid (ABA) and calcium play a positive role in gene signaling and expression related to proline biosynthesis. On the other hand, phospholipase D (PLD)

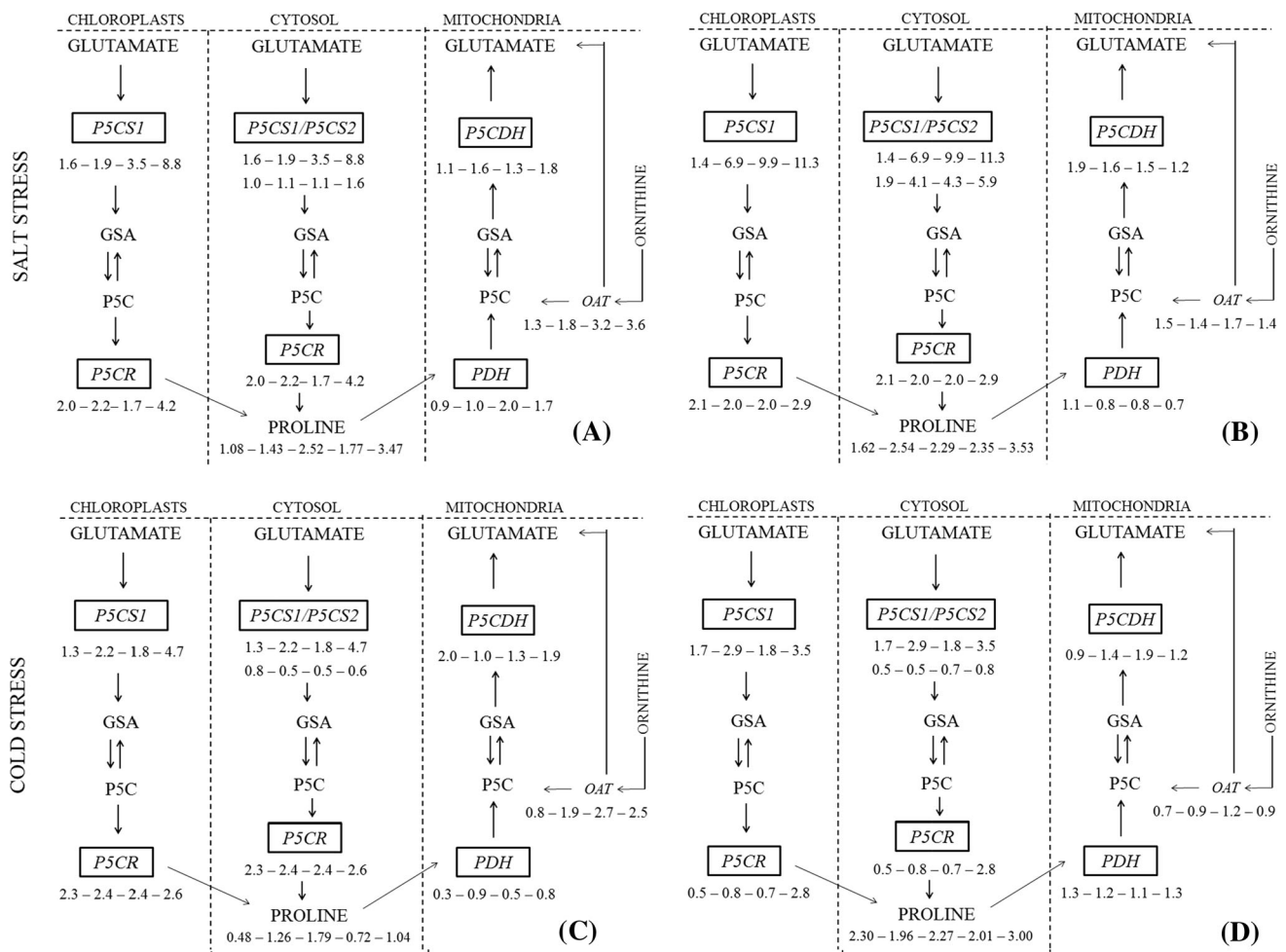


Fig. 4 Diagram of proline metabolism with the respective expression values in relation to the genes involved (starting from T6 on the left) and the proline content (starting from T0 on the left) in rice plants of genotypes BRS Bojuru (a, c) and BRS Pampa (b, d), under salt and cold stress

regulates the biosynthesis of amino acid negatively (Thiery et al. 2004). Thus, the factors that regulate this signaling are possibly first positively activated in plants tolerant to osmotic stress caused also by salinity and cold.

Two pathways were described for proline biosynthesis, the glutamate pathway and the ornithine pathway. From glutamate, proline is synthesized by pyrroline-5-carboxylate (P5C) by reactions catalyzed by the enzymes P5C synthase (P5CS) and pyrroline-5-carboxylate reductase P5CR, whose genes have been identified and isolated in many plant species (Satoh et al. 2002; Winter et al. 2015).

During the NaCl treatment in this study, the transcription of both isoforms of P5CS in genotype BRS Pampa (sensitive) was increased compared to the control. In genotype BRS Bojuru (tolerant), increases in the expression for *P5CS1* were also observed during stress, while the mRNA level of isoform *P5CS2* was similar between the sampling times. Although the transcript levels were lower for the tolerant genotype, some correlations were higher, and for proline content/*P5CS1*, the correlation was 0.85

and 0.98 for *P5CS1/P5CS2* (Fig. 5a). This behavior can be explained by the fact that the transcriptional level not always directly related to the enzymatic activity, since several regulatory systems are involved in the final protein availability and activity. In one of the first studies, Anderson and Seilhamer (1997) compared the levels of mRNA with the corresponding proteins, in which the correlation was only 0.43.

Previous studies have also demonstrated the correlation between the induction of *P5CS* gene and the accumulation of proline in sorghum under abiotic stresses and MeJA treatment (Su et al. 2011). For ryegrass, Li et al. (2014) found a high cause-effect relationship between the increase in transcripts of both isoforms of *P5CS* and the proline content, which was upregulated after stress. In rice plants, Hur et al. (2004) found that the expression of gene *P5CS1* was constant, while *P5CS2* was induced by mannitol and NaCl.

Under low temperatures, the levels of *P5CS1* expression increased in both genotypes, but less than under salinity.

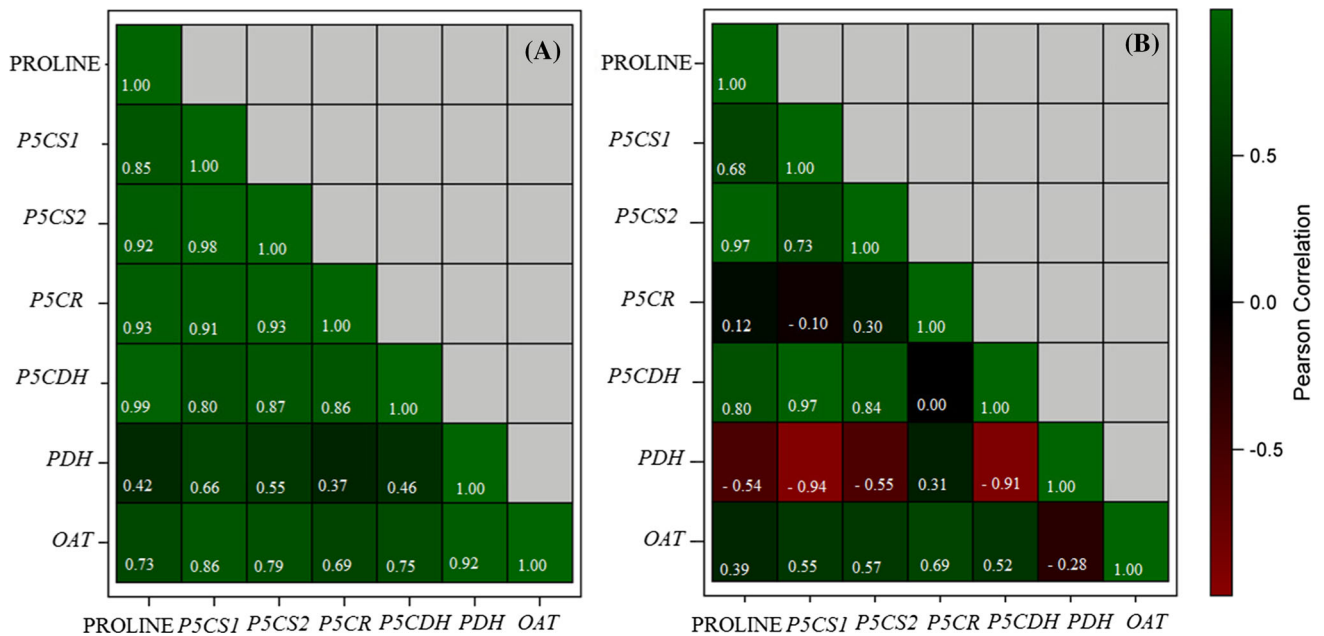


Fig. 5 Pearson correlation between the free proline content and expression of proline metabolism genes in rice genotypes BRS Bojuru (a) and BRS Pampa (b) under salt stress (150 mM NaCl)

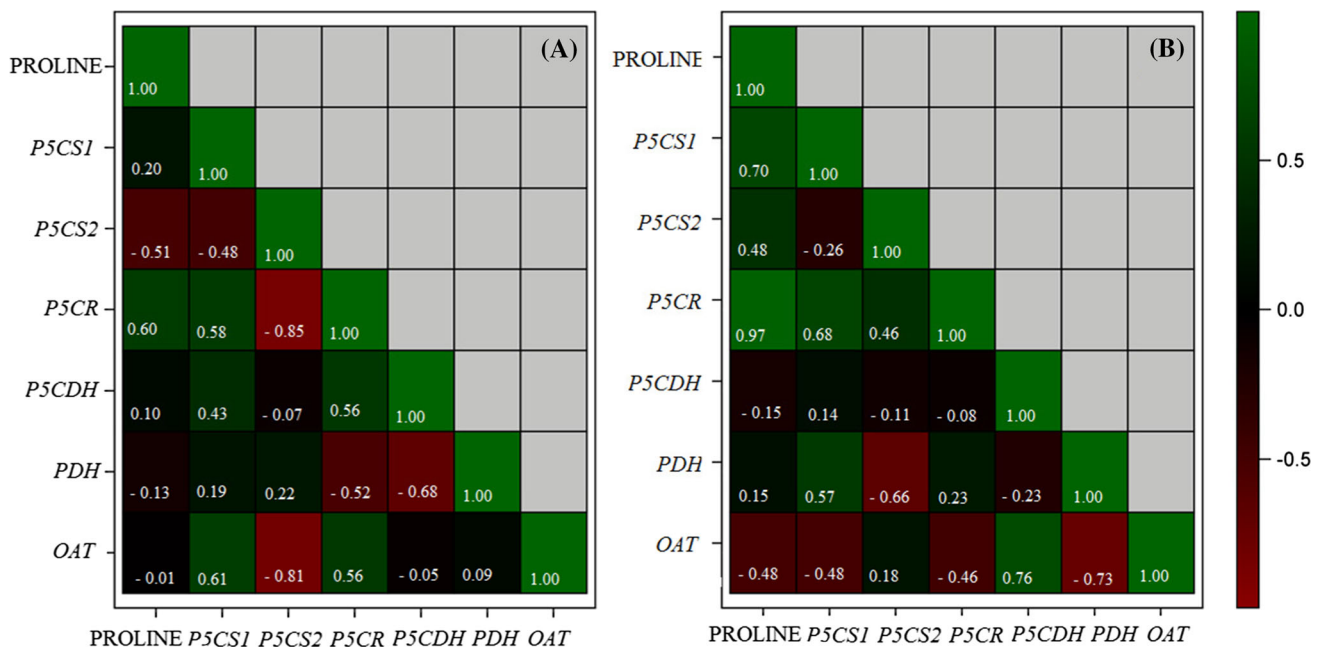


Fig. 6 Pearson correlation between the free proline content and expression of proline metabolism genes in rice genotypes BRS Bojuru (a) and BRS Pampa (b) under cold stress (13 °C)

Conversely, *P5CS2* was negatively regulated by cold, with measurement values below the control and correlation of 0.50 and 0.48 between proline content/*P5CS2* in the tolerant and sensitive genotypes, respectively (Figs. 4c, d, 6). According to Kavikishor et al. (2015), it is not yet clear how these genes interact with other genes through networks of cellular components. Thus, the availability of maps of

these networks could help understand the regulation of proline metabolism and justify the contrasting responses to the different tested conditions (salinity and cold).

The enzyme pyrroline-5-carboxylate reductase (*P5CR*), encoded by gene *P5CR*, is a precursor of proline biosynthesis in plants. In this study, contrasting responses were observed again, between stresses as well as between

cultivars. In plants of the tolerant genotype (BRS Bojuru), the *P5CR* expression was increased and the correlation between proline content/*P5CR* under salinity was high. As mentioned above, proline biosynthesis can occur both in the cytosol and the chloroplast. Under stress, the preferred pathway is the chloroplast pathway, by the genes *P5CS1* and *P5CR*. The correlations of this study showed that proline biosynthesis may have occurred at both cell locations in the salt-stressed tolerant genotype, since high correlations between proline content/*P5CS1* and proline content/*P5CS2* were observed, aside from significant relations between *P5CS1/P5CR* and *P5CS2/P5CR* (Figs. 4a, 5a).

On the other hand, for the sensitive genotype, although increases in the *P5CR* expression were observed, the correlation of this gene with proline content was only 0.12. Analyzing the correlations, it is believed that the preferred pathway of proline biosynthesis in the sensitive genotype occurred by the cytosol, since higher relations were observed between proline content/*P5CS2* (0.97) and *P5CS2/P5CR* (0.30) versus 0.68 between proline content/*P5CS1* and -0.10 between *P5CS1/P5CR* (Figs. 4b, 5b). Similar results to the sensitive genotype were observed in *Arabidopsis thaliana* and rice under osmotic stress, where only the *P5CS* expression was correlated with proline concentration, and the *P5CR* expression was not correlated (Sripinyowanich et al. 2013).

Under cold stress, the response of *P5CR* gene had the same tendency as the proline content in sensitive plants, with significantly higher values than the control, explaining the high correlation between proline content/*P5CR* for genotype BRS Pampa. For BRS Bojuru, this correlation was lower, which can be explained by the significant changes in *P5CR* expression levels, differing from proline content, which changed during the period of cold stress. Unlike for salt stress, it is believed that the preferred proline biosynthesis pathway under cold stress is by the chloroplast for both genotypes, since higher correlations were observed between proline content/*P5CS1*, *P5CS1/P5CR* for BRS Pampa and proline content/*P5CS1*, *P5CS1/P5CR* for BRS Bojuru (Figs. 6a, b). It is well-documented that the regulation of *P5CS* and *P5CR* depends on the cofactor NADH or NADPH (Giberti et al. 2014). Thus, this adjustment possibly has a stronger effect on the chloroplast of both genotypes under cold stress.

Similar to the important role of proline biosynthesis for stressed plants, a rapid proline degradation is a way of minimizing stress effects, for providing reducing agents that support the mitochondrial oxidative phosphorylation and generate ATP for plant recovery from stress and repair of accumulated damage. Proline catabolism occurs in mitochondria by the sequential action of proline dehydrogenase (PDH) and P5C dehydrogenase (*P5CDH*), which

converts P5C to glutamate (Szabados and Savouré 2010). For the tolerant genotype, the data of the experiment with salt stress showed a correlation between proline content accumulation and *PDH* gene expression of 0.42, justified by the opposite response behavior after all stress periods. For the sensitive genotype, there was a negative correlation between these variables (Figs. 2, 5). In the cold treatment, the response was opposite to that to salinity; for the tolerant genotype, the correlation proline content/*PDH* was negative and for the sensitive genotype, the correlation was insignificant (Fig. 6a, b).

According to Satoh et al. (2002), the gene expression of *PDH* is repressed under dehydration, but is induced when plants are rehydrated after 10 h of stress. Therefore, deepening the understanding of *PDH* expression can be useful to shed light on the molecular process of plant recovery from osmotic stress. These same authors determined that the ACTCAT cis-element, present in the promoter region, is involved in the regulation of *PDH* gene expression under hypoosmolarity.

Pyrroline-5-carboxylate (P5C) is an intermediate product of both proline biosynthesis and degradation. It is synthesized in the mitochondria during catabolism by enzyme PDH and converted to glutamate by 1-pyrroline dehydrogenase-5-carboxylate (*P5CDH*) (Qamar et al. 2015). Although the *P5CDH* gene is involved in proline degradation, a high positive correlation between proline accumulation and the relative expression of *P5CDH* under salinity for both genotypes was observed in this study. For BRS Bojuru (tolerant), this high correlation is explained by the same pattern of response between the two variables, at 6, 24 and 72 h and decrease at 48 h stress. For BRS Pampa (sensitive), the correlation was due to the observed increase at 6 and 24 h in the proline content and expression of *P5CDH* and the difference between these variables after 48 and 72 h (Fig. 2b, d). On the other hand, under cold stress, there was no significant correlation between *P5CDH* and proline content for either genotype (Fig. 6a, b).

In many cases, the mRNA level of a gene does not follow the level of protein activity encoded by it, due to mechanisms of post-transcriptional regulation and/or post-translational modifications. In a study by Qamar et al. (2015), the *P5CDH* gene was post-transcriptionally downregulated by siRNAs under salt stress. The proline catabolism generates the byproduct H_2O_2 , thus activating the antioxidant signaling pathways (Zhang and Becker 2015). However, there are lines of research that state that P5C metabolism in the mitochondria increases the production of reactive oxygen species (ROS) in excess, leading to cell death (Nishimura et al. 2012).

One of the arginine hydrolysis products is arginine conversion to ornithine by the enzyme arginase (ARG), which is metabolized by the enzyme ornithine delta

aminotransferase or ornithine aminotransferase (OAT) to P5C, this to GSA and then to glutamate. The direct contribution of OAT for proline accumulation induced by stress is controversial (Winter et al. 2015). In the experimental conditions, there was a positive correlation between proline content and expression of the *OAT* gene in the tolerant genotype, while sensitive to the correlation was positive, but low, under salt stress. Moreover, under cold (13 °C), there was no correlation between these variables for the tolerant genotype negative correlation for the sensitive (Figs. 5, 6). Roosens et al. (2002) hypothesized that OAT plays an important role in proline accumulation during osmotic stress, which was proved in *Arabidopsis thaliana* plants by a positive correlation between the increase in free proline content, enzyme activity of OAT, and mRNA levels of the *OAT* gene in seedlings under salt stress. This hypothesis was reinforced by analysis of transgenic *A. thaliana* plants that overexpressed *OAT*, synthesizing more proline than the wild plants under osmotic stress (Roosens et al. 1998, 2002). In the same sense, the overexpression of *OAT* in rice (*Oryza sativa* L.) resulted in higher proline levels and activated the antioxidant defense, making the plants more tolerant to NaCl stress (You et al. 2012). However, more research is needed to understand how OAT contributes to increase free proline and whether the GSA produced by OAT can be used directly for proline synthesis or if it is compulsorily converted to glutamate by *P5CDH* (Winter et al. 2015).

Conclusions

From the results, it was concluded that: (1) the increase in proline content is highest in BRS Bojuru (tolerant) under the two tested stresses and (2) under salt stress, the transcripts levels of biosynthesis genes are more correlated to proline content in the tolerant genotype, while under cold, the correlation was higher for the sensitive genotype (BRS Pampa).

Author contribution statement Conceived and designed the experiments LCB and EJBB. Wrote, edited, and analyzed the data: LCB, ILV, PAA, MNA, GPM, and GSR. Revised the paper: LCB, LCM, AMMJ, and EJBB conducted the experiments: LCB, ILV, PAA, MNA, GPM, and GSR. 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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