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Salt Induced Change of Gene Expression in Salt Sensitive and Tolerant Rice Species

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Abstract

The aim of this study was to quantify the expression of the *SalT*, *LTP-Plant*, *CDPK* and *PP1* genes, described as responsive to salinity in rice genotypes, describe the promoter region of these genes and identify possible *cis*-elements that may be involved in the induction of gene expression under salinity. The cultivars BRS Bojuru (tolerant) and BRS Agrisul (sensitive) were subjected to 0, 12, 24, 36 and 48 h of exposure to salt. For the analysis of qRT-PCR, two detoxification/defense genes and two cell signal transduction genes were selected. In the identification *of cis*-elements, a region spanning 1.000 bp in the promoter region of genes was analyzed. The *Os01g0348900-SalT* gene presented an increase in quantification of the relative expression (QR) of 2.081 times in the sensitive and 63.7 times in the tolerant cultivar. The *Os03g0251000-LTP* gene showed a contrasting increase of the expression between the cultivars BRS Agrisul (QR = 11.0) and BRS Bojuru (QR = 1.54). The genes *Os03g0688300*-CDPK and *Os02g0820000-PP1* apparently do not show response to salinity, since in most treatments, the QR values were lower than the control. Thirty-eight *cis*-elements distributed in the four analyzed genes were identified. Of these, six were found only in the *Os01g0348900-SalT* gene promoter, suggesting a possible involvement of these *cis*-elements in the induction of expression of this gene under salinity. Based on these results, it can be concluded that these genes do not maintain a direct relation with salinity tolerance, but with mechanisms that allow acclimation to this condition.

Keywords: *Oryza sativa* L., *cis*-elements, genomics, transcription, salinity

1. Introduction

Rice, *Oryza sativa* L. is a glycophytic plant, and its exposure to high concentrations of salt is extremely detrimental to its growth and development. Regarding biochemical and physiological levels, salinity changes, among other factors, the functioning of the electron transport chain in mitochondria and chloroplasts, causes increasing in the formation of reactive oxygen species (ROS), which, in excess, cause damage to the membranes (lipid peroxidation), protein oxidation and DNA fragmentation (Mishra, Bhoomika, & Dubey, 2011).

Genetic control of tolerance to salinity is quantitative, involving a gene network of multiple loci distributed in different regions of the genome, which, once activated, can mitigate the effect of stress. Many research groups have used strategies, such as subtractive hybridization, microarray and SAGE, aiming at identifying a large number of genes expressed in response to salinity, which are deposited in public databases (Cotsaftis et al., 2011; Fan et al., 2013). Kumari et al. (2009) when evaluating the transcriptome of rice genotypes with contrasting tolerance to salinity identified 953 genes responsive to salt. However, the results obtained by microarray technique are quite broad, and some genes are selected as "candidates" to be validated in more specific tests for quantification of expression in response to stress. In order to validate some genes responsive to salinity, the

genes *SalT*, *LTP-Plant*, *CDPK* and *PP1* were selected, which are involved in processes of detoxification/cellular defense and signal transduction. In the present work, these genes had their relative expression under salt stress quantified by Real Time PCR.

The *SalT* gene encodes for a group of proteins that bind to carbohydrate (lectins). Some authors indicate that the binding of lectins to sugars has an important role in signaling response to abiotic stress, modulating signals in cell membranes and cytoplasm (Van Damme, Lannoo, Fouquaert, & Peumans, 2004). In rice, this gene was isolated and characterized in plants grown under salt stress, confirming its participation in plant adaptation to conditions of osmotic adversity (Zhang et al., 2000).

Lipid Transfer Proteins (LTPs) have been associated with the movement of lipids though membranes, responses to the attack of various pathogens (Chae et al., 2010), response to cold, abscisic acid, drought and salinity. In tomato, *LTP-like* gene is expressed specifically in stems, when plants are treated with ABA and NaCl.

Calcium is a secondary messenger involved in the response to many stimuli and participates as an effector in a large group of proteins described as calcium sensors". Among these proteins, calmodulin, calcium-dependent protein kinases (CDPK) are included, which act interconnecting networks of signal transduction. Within the family of genes that encode CDPK proteins, each isoform often have different role in the biology of the organism. In rice, 29 CDPK genes with different physiological functions were identified (Holder, Ridzuan, & Green, 2012). Thus, studies are necessary to identify CDPKs responsive to salt stress and/or other types of stress. Furthermore, the phospho protein phosphatase (PPP) have an important role in the regulation of metabolism, cell growth and division, and participate in signaling and gene activation in response to light, hormones and stress (Xu, Jing, Mao, Jia, & Chang, 2007).

Characterization of the promoter region of plant genes usually begins by the identification and characterization of genes expressed in a particular tissue or under conditions of physiological stress, through studies of gene expression. From this functional characterization, structural studies can be started from the upstream region of the gene (Potenza, Aleman, & Sengupta-Copalan, 2004). Each gene has a unique combination of *cis*-element associated to transcription factors (TFs), making the temporal and spatial control of transcription specific. Bureau et al. (1996), when studied the promoter region of SalT gene in rice, identified sequences similar to transposable elements like Castaway and Stowaway, which could have influenced the response of expression of this gene. However, further studies are needed to advance in the identification of *cis*-elements and TFs involved in the response to salinity.

Therefore, the aim of this study was to quantify the relative mRNA expression level of *Os01g0348900 SalT, Os03g0251000-LTP-Plant*, *Os03g0688300-CDPK*- and *Os02g0820000-PP1* genes in rice genotypes with contrasting tolerance to salinity, as well as describe the promoter region of these genes, identify potential *cis*-elements that may be involved in the induction of gene expression under salt stress and indicate molecular markers for assisted selection for salt tolerance.

2. Material and Methods

2.1 Candidate Genes: Selection

The selection of candidate genes responsive to salinity was made from published data of comparative transcriptome of Pokkali (tolerant) and IR64 (sensitive to salt stress) rice cultivars (Kumari et al., 2009). For a representation of different points of stress metabolism, transcripts EF575947 and EF576356 annotated with ontologies related to detoxification/cellular defense, referring to genes *Os01g0348900-SalT* induced protein and *Os03g0251000-LTP*- Plant lipid transfer protein were selected, respectively. For the function of signals transduction, transcripts EF576188 and EF576186, referring to genes, Os03g0688300-CDPK-Putative-Calcium-dependent protein kinase and Os02g0820000-PP1-Putative Serine/threonine protein phosphatase were selected, respectively.

2.2 Primers

Sequences from transcribed regions for each gene were obtained from the updated annotation of the rice genome (RAP-DB v.5) and in the Primer Express 3.0 software (Applied Biosystems®) was used top design primers (Table 1). Prior to the design of primers, alignments were made among the possible isoforms of gene families under study to ensure the specificity of each primer set. As the reaction normalizing gene *OsActina* (Os03g0718100/AK100267) was chosen which is suitable for rice plant under abiotic stress (Zhang et al., 2009). Subsequently, the stability of the expression of this gene under salt stress was verified and confirmed by the database Genevestigator (https://www.genevestigator.com/gv/). In this database it was not found alterations in

expression level of the selected genes in conditions of stress and no stress as well as genotypes tolerant and sensitive to salinity.

Table 1. List of primers used in the reactions of qRT-PCR and amplicon size (bp)

Gene/Transcript	Sequence	*Amplicon*
SalT	F: 5'TCTGGAACGCTTATCGACGC 3'	
Os01g0348900/AK062520	R: 5'TGGGAATCAAGGGTGGACG 3'	50
LTP	F: 5'TATATATCCGCCAGCAGCATGC 3'	
Os03g0251000/AK104253	R: 5'CCTCCATGAAGCACGGCAC 3'	50
CDPK	F: 5'AGGATGGGCGGATCAGCTAC 3'	
Os03g0688300/AK105102	R: 5'TCCCGGATTTCATCATCGC 3'	50
Ser/thr PP1	F: 5'ATCTGCTGGCGCATGTCTTC 3'	
*Os02g0820000/A*K120439	R: 5'GCGAAAATGCCGGTTAGTCG 3'	50
Actina 1	F: 5'CAGCCACACTGTCCCCATCTA 3'	
Os03g0718100/AK100267	R: 5'AGCAAGGTCGAGACGAAGGA 3'	66

*Tm: 60°C.

2.3 Sample Preparation and Statistical Design

Seeds of BRS Bojuru (subspecies japonica) and BRS Agrisul (subspecies indica) rice genotypes were used as plant material, characterized as tolerant and sensitive to salinity, respectively (Benitez et al., 2010).

The experiment was conducted in a greenhouse with 70% relative humidity and temperature of 25 ± 2 ºC. The seeds were germinated in 2 L plastic containers with washed sand as substrate and irrigated with Hoagland nutritive solution (Hoagland & Arnon, 1938). Fourteen days after the date of sowing (vegetative stage), the plants started to be irrigated daily, alternating nutrient solution and 100 mL of nutrient solution containing 300 mM NaCl. After 0, 12, 24, 36 and 48 h of exposure to salt stress, the leaves of both genotypes were collected and stored at -80 ºC in ulltrafreezer for subsequent RNA extraction. The experiment followed a completely randomized experimental design in a 2x5 factorial arrangement (2 genotypes x 5 salinity exposure times) with three biological replicates consisting of five plants per pot, totaling 15 plants per treatment.

2.4 qRT--PCR Analyzis

Total RNA was extracted from 0.1 g of leaf tissue obtained from plants exposed to different treatments with NaCl, using Trizol Reagent (Invitrogen ™). The quantity of total RNA was measured by spectrophotometer at 260 and 280 nm, which provides an estimate of the relative purity of the RNA. The quality and integrity of the nucleic acid were evaluated by electrophoresis on 1.5% agarose gel.

The single strand cDNA synthesis for each sample was produced from the mRNA by using oligo (dT) primer and other reagents from SuperScript FirstStrand System for RT-PCR (Invitrogen™). Finally, single-stranded cDNA quality was confirmed by amplification of transcripts of *OsActina* using PCR and electrophoresis on 1.5% agarose gel.

The qRT-PCR analysis were performed on 7500 Real-Time PCR System (Applied Biosystems ®) using SYBR® Green (Invitrogen™). Initially, each set of primer was evaluated by dissociation curve, and only those primers with specific amplicons (with a single peak of dissociation from the strands of the PCR products) and with efficiencies: 100%, 95%, 102%, and 96% for SalT, LTP, CDPK and PP1, respectively, were kept, according to the formula:

$$E = [10^{-1/slope}]$$

PCR reactions were performed in triplicate, each with a total volume of 20 µL. The cycling conditions were: renaturation of the samples for 2 min at 50 °C and activating *Taq* DNA polymerase for 10 min at 95 °C. Subsequently, 40 cycles were performed with three steps (95 °C for 30s, 60 °C for 1 min, and 72 °C for 1 min) and finally a last step at 72 °C for five min.

2.5 Data Analysis

Relative quantification of differential expression was performed using the comparative threshold cycle method (Livack & Schmittgen, 2001) using the equation $QR = 2^{-\Delta\Delta CT}$.

In this paper, instead of using a graph showing only means and errors, we decided to use analysis of variance to verify the existence of differences between the samples analyzed in relation to different QRs. Thus, analysis of variance was performed from the replicates of each treatment. The results were considered statistically significant when $P \leq 0.05$. The mean values were compared by Tukey test at 5% probability. For the analyzes, the statistical software SAS Learning Edition (2002) was used.

2.6 Cis-elements Analysis

The identification of putative *cis*-elements was made by alignments to the database *O. sativa* deposited in www.microsatellite.org. For this, 1000 bp upstream of the presumed start site of transcription of each gene were analyzed, using as reference the sequences deposited for cultivar Nipponbare at The MSU Rice Genome Annotation Project Database and Resource (http://rice.plantbiology.msu.edu/). *Cis-elements* with $P \leq 5$ by Z-score test were considered significant.

3. Results and Discussion

3.1 Analysis of variance

The analysis of variance showed significant statistic difference ($P \leq 0.05$) in the relative quantification of the expression of all genes analyzed at different exposure times to stress in both genotypes**.**

3.2 Relative Quantification (RQ)

For *SalT* gene, the highest increase in relative quantification (RQ) of expression in both genotypes was observed. In BRS Agrisul, the increase in the expression was proportional to the increase of time of exposure to stress. With 48 hours of induction, there was a significant increase of expression estimated at 2,081 times the control treatment (0 h). In the tolerant cultivar, there was an increase by 63.7 times in *SalT* gene expression in plants subjected to 24 hours of stress, followed by a decrease in gene expression in other induction times, but the QR values observed were also higher but not statistically significant to those observed in the control treatment (Figure 1).

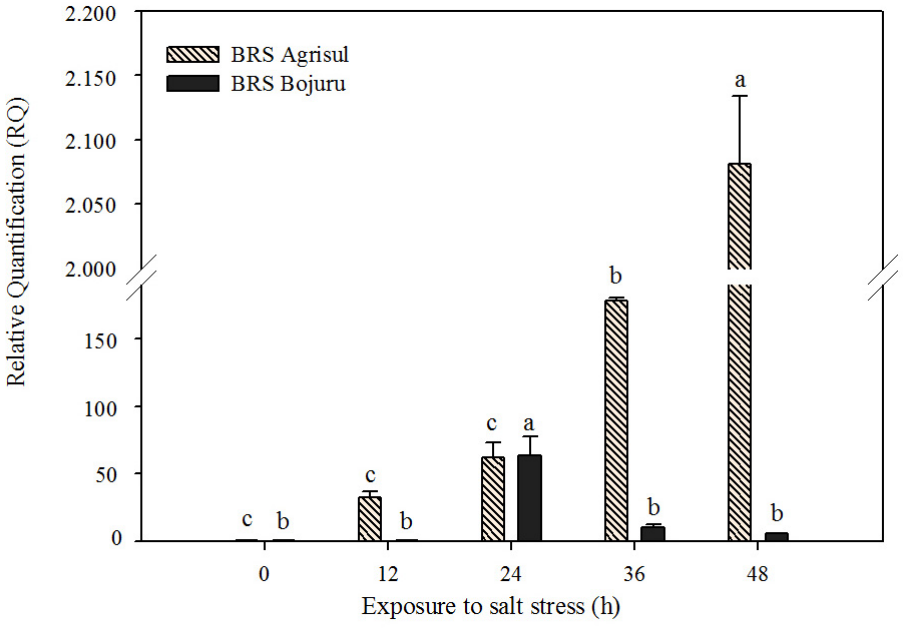

Figure 1. Relative quantification (RQ) of the differential expression of the *Os01g0348900 SalT* gene, in five periods of exposure to 300 mM NaCl in BRS Bojuru (tolerant) and BRS Agrisul (sensitive) cultivars. Vertical bars represent the standard deviation of three replicates. Same letter do not differ significantly by Tukey test at 5% probability

Although this gene was expressed at a higher level in the sensitive cultivar, it was increased 63 times in the tolerant cultivar, which means that both plants are using this gene in their process of acclimatization. The difference lies in the fact that in the tolerant plant this gene is required only after 24 hours of stress, whereas in the sensitive plant the expression increases proportionally as the stress becomes more severe. Similar results to those found in this work have been reported by Garcia et al. (1998) who observed an increase of *SalT* gene expression in rice cultivar of *indica* subspecies, which are typically characterized as sensitive to salinity.

According to Claes et al. (1990), it is not possible to attribute a specific function to the *SalT* protein, but its function is likely to be of osmoprotection similar to proline, sugars and some small organic acids. Subsequently, Garcia et al. (1998) and Zhang et al. (2000) isolated and characterized the protein SalT in rice plants subjected to salinity, reinforcing the hypothesis that this protein is involved in osmotic adaptation to adverse conditions. Thus, the results of this study demonstrating the increase in *SalT* gene expression may contribute to the evidence described previously.

The *Os03g0251000-LTP* gene had an increase in expression after the plants were subjected to 12 hours of stress in both, the sensitive and the tolerant cultivars. Although this increase was significant in both genotypes, it was higher in the sensitive cultivar, similar to the *SalT* gene, however, to a lesser extent, 11 times compared to control plants. In the tolerant cultivar the increase observed was 1.5 times in the same period of stress exposure (Figure 2). Therefore, these results do not indicate that this gene locus as a major genetic marker of tolerance to saline stress, since it was expressed at a higher level in the sensitive cultivar, however, there are other *LTPs* loci in the genome of rice, which may have different responses against salt in tolerant genotypes.

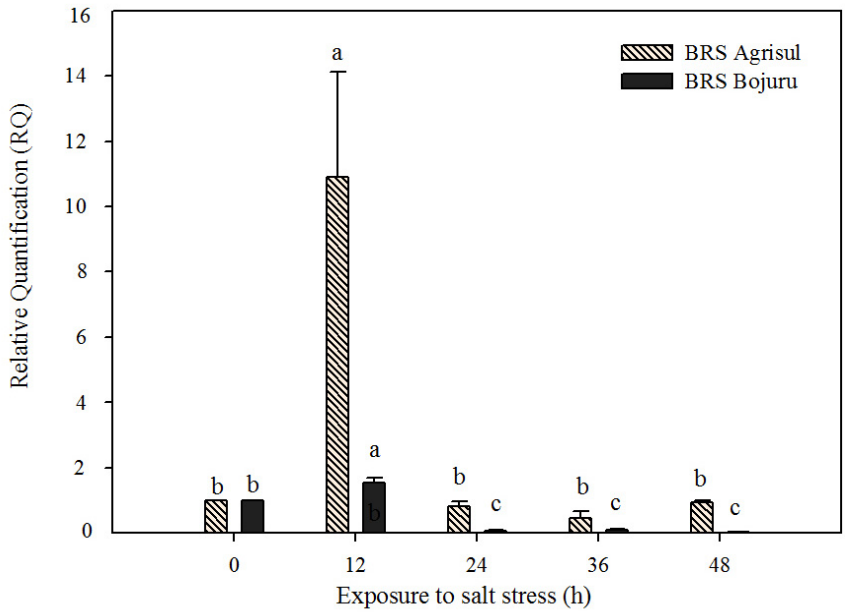

Figure 2. Relative quantification (RQ) of the differential expression of the *Os03g0251000-LTP* gene in five periods of exposure to 300 mM NaCl in BRS Bojuru (tolerant) and BRS Agrisul (sensitive) cultivars. Vertical bars represent the standard deviation of three replicates. Same letter do not differ significantly by Tukey test at 5% probability

The data obtained in this study with the locus *Os03g0251000-LTP* agree with those found by Wang et al. (2012). When evaluating the expression of different *LTP* loci of rice genome in plants irrigated with 250 mM NaCl, they observed a significant increase in the expression of *Os11g24070, Os04g33920* and *Os05g06780* genes within 12 hours of exposure to stress. Moreover, the same authors showed an increase in *Os01g60740* gene expression under low temperatures and no changes in transcript levels of this gene under salinity. Thus, this set of information indicates that the *LTP* gene family plays an important role in rice under adverse environmental conditions, among them salinity.

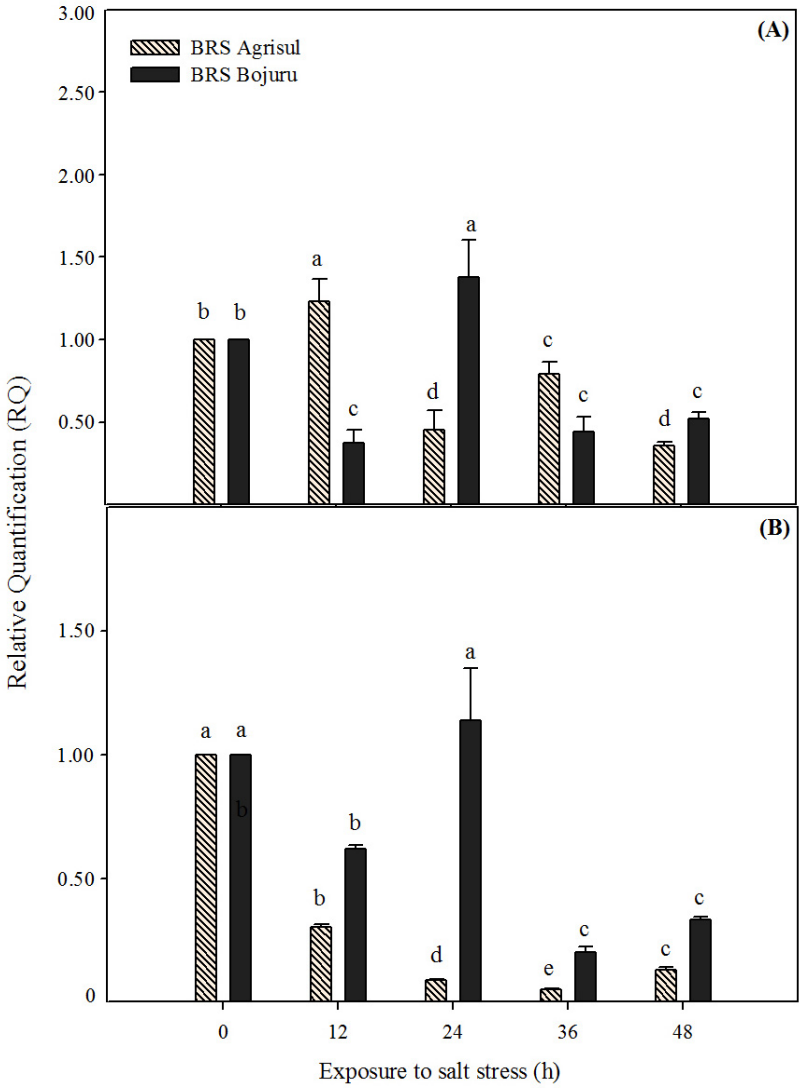

Figure 3. Relative quantification (RQ) of the differential expression of *Os03g0688300-CDPK* (A) and *Os02g0820000-PP1* (B) genes in five periods of exposure to 300 mM NaCl in BRS Bojuru (tolerant) and BRS Agrisul (sensitive)cultivars. Vertical bars represent the standard deviation of three replicates. Same letter do not differ significantly by Tukey test at 5% probability

The data obtained from analysis of *Os03g0688300-CDPK* gene expression indicate that apparently this locus is not related to salt stress, since in most treatments QR values are lower than those observed in the control treatment in both genotypes evaluated. In tolerant cultivar after 24 hours of stress, there was a slight increase in the expression, RQ = 1.3 and the same behavior was observed in the sensitive cultivar after 12 hours of exposure to salt (Figure 3A).

Moreover, Asano et al. (2011) found that in rice *Os04t0584600 (OsCDPK12)* over expression increased the plants tolerance to salt stress and reduced the accumulation of hydrogen peroxide (H_2O_2), along with increased expression of OsAPx2 and OsAPx8 enzymes, suggesting that this gene promotes salt tolerance by reducing the accumulation of reactive oxygen species. Also, locus *Os03t0128700 (OsCDPK7)* over expression showed increased tolerance to cold, salt and dehydration stress (Saijo, Hata, Kyozuka, Shimamoto, & Izui, 2000). These results associated with those obtained in this experiment indicate that although there is a large number of copies of these genes in the rice genome, possibly not all copies are important to salt stress.

As observed for the *Os03g0688300-CDPK* gene, no relation with tolerance or adaptive mechanisms to salinity was found for *Os02g0820000-PP1* gene. There was a reduction in the expression of this gene for both cultivars,

except for 24 hours, where the BRS Bojuru cultivar had values close to those found at 0 hours of stress induction (Figure 3B).

In rice, the binding of a protein called RSS1 (Rice Salt Sensitive 1) to the catalytic site of PP1 protein activates/deactivates the cell division and progression of tissue under salt stress, enabling cell growth in contact with the salt. Mutant plants to *rss1* were classified as hypersensitive to salt, possibly due to the non-activation of PP1 proteins and the stop in cell growth in contact with the salt (Ogawa et al., 2011). The results obtained in this study showed a reduction in the expression of *Os02g0820000-PP1*, indicating that possibly for the cultivars analyzed, this gene is not related to the salt tolerance, however, the possibility that this relations can be observed in other genotypes, organs or periods of exposure to salt cannot be ruled out.

3.3 Analysis cis-elements

The analysis of *cis*-elements allowed the identification of differences in promoter sequences of *Os01g0348900-SalT*, *Os03g0251000-LTP, Os03g0688300-CDPK* and *Os02g0820000-PP1* genes. A total of 38 *cis*-elements, distributed along the sequence were identified (Figure 4). From the total *cis*-elements identified, six (AACACOREOSGLUB1; ACGTABOX; AGCBOXNPGLB; CRTDREHVCBF2; REBETALGLHCB21 and WBOXATNPR1) were found only in the promoter region of the *Os01g0348900 SalT* gene, which, according to analysis of relative quantification of the expression, showed the highest QR value after exposure to the salt.

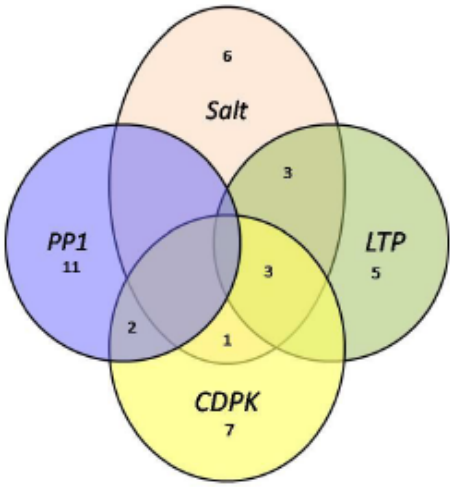

Figure 4. Venn diagram illustrating the distribution of *cis*-elements in the promoter region (1000 bp) of *Osg0348900-SalT, Os03g0251000-LTP, Os03g0688300-CDPK* and *Os02g0820000-PP1* genes

According to information in the PLACE database, none of these six *cis*-elements is related to the direct regulation of genes under salt stress. The AGCBOXNPGLB *cis*-element was identified in *Arabidopsis* as a sequence that binds to AtERF type proteins, which are responsive to stress conditions. On the other hand, CRTDREHVCBF2 *cis*-element was identified as a DNA sequence regulated by low temperatures. However, it cannot be ruled out that the presence of these *cis*-elements modulates in a positive and indirect way the *Os01g0348900-SalT* gene expression in response to salt stress. However, for this assumption further analyzes are needed, for example, analysis of mutant plants.

According to Lee et al. (2002), the interaction of *cis*-elements with transcription factors leads to the activation or repression of gene expression in response to environmental or developmental factors. For the *Os03g0251000-LTP* gene, sequences corresponding to the following *cis*-elements were found: GCN4OSGLUB1; INRNTPSADB; SEF3MOTIFGM; POLASIG1 and RYREPEATLEGUMINBOX. These *cis*-elements were not detected in the other genes analyzed in this study.

As possible changes in the sequences of the promoter of this gene are unknown in the BRS Bojuru and BRS Agrisul cultivars, it cannot be ruled out the possibility that the absence of any of these *cis*-elements in the tolerant cultivar, BRS Bojuru, may be the cause of lower induction of this gene. Sequencing of the promoter

region of these cultivars to confirm this hypothesis should be carried out. In addition, further analyzes with *in silico* genes/*cis*-elements described in salt tolerance can bring more information on this context.

The *Os01g0348900-SalT* and *Os03g0251000-LTP* genes share three *cis*-elements in common in their promoter region, one of them, MYB2AT, is involved in the regulation of genes that are responsive to water deficit. The *Os03g0688300-CDPK* and *Os02g0820000-PP1* genes, which were not induced by salt stress, have two *cis*-elements (NODCON2GM and OSE2ROOTNODULE) present in their promoter regions. All *cis*-elements identified in this study are listed in Table 2.

Table 2. List of cis-elements found in the promoter region (1000 bp) of Os01g0348900 SalT, Os03g0251000-LTP, Os03g0688300-CDPK and Os02g0820000-PP1 genes of *Oryza sativa*

Cis-element	*SalT*	*LTP*	*CDPK*	*PP1*
AACACOREOSGLUB1	1 (1.9)*			
ACGTABOX	1 (3.8)			
AGCBOXNPGLB	1 (0.1)			
CRTDREHVCBF2	1 (1.9)			
REBETALGLHCB21	1 (1.1)			
WBOXATNPR1	3 (2.7)			
AMYBOX2	1 (0.1)	1 (0.1)		
MYB2AT	1 (2.8)	1 (2.8)		
TATCCAYMOTIFOSRAMY3D	1 (1.3)	1 (1.3)		
RYREPEATBNNAPA	2 (2.0)	2 (2.0)	2 (2.0)	
RYREPEATGMGY2	1 (3.8)	2 (0.0)	1 (3.8)	
RYREPEATVFLEB4	1 (0.1)	1 (0.1)	1 (0.1)	
NODCON2GM			5 (0.1)	4 (1.2)
OSE2ROOTNODULE			5 (0.1)	4 (1.2)
TATABOX3	1 (4.8)		1 (4.8)	
GCN4OSGLUB1		1 (0.0)		
INRNTPSADB		3 (1.0)		
POLASIG1		4 (0.5)		
RYREPEATLEGUMINBOX		2 (0.1)		
SEF3MOTIFGM		2 (0.1)		
ARFAT			1 (3.8)	
CAREOSREP1			2 (0.1)	
CBFHV			3 (0.6)	
DOFCOREZM			11 (4.2)	
DRE2COREZMRAB17			1 (1.5)	
LTRECOREATCOR15			7 (2.1)	
TATAPVTRNALEU			1 (0.6)	
BP5OSWX				1 (0.0)
CACTFTPPCA1				13 (3.6)
CURECORECR				6 (5.2)
GTGANTG10				9 (0.4)
MRNASTA2CRPSBD				1 (0.1)
P1BS				1 (7.8)
QARBNEXTA				1 (0.0)
RAV1AAT				6 (0.0)
SEF4MOTIFGM7S				3 (2.5)
T/GBOXATPIN2				1 (2.0)
TRANSINITDICOTS				1 (0.9)

* Values outside the parentheses indicate the number of times that the *cis*-elements were found in the promoter region and the figures in brackets indicate the value of *P* according to Z-test score.

4. Conclusions

Under the experimental conditions tested, the *Os01g0348900-SalT* and *Os03g0251000-LTP* genes apparently are not directly related with salinity tolerance, since they were induced at a higher level in the sensitive cultivar. However, possibly both genotypes require the expression of these genes in different intensities and periods for the process of acclimatization to salinity.

The *Os02g0820000-PP1 and Os03g0688300-CDPK* genes apparently are not related to tolerance or acclimatization to salinity.

The presence of the *cis*-elements AACACOREOSGLUB1, ACGTABOX, AGCBOXNPGLB, CRTDREHVCBF2, REBETALGLHCB21 and WBOXATNPR1 may be related to the *Os01g0348900-SalT* gene expression in response to salt stress, however, further analysis in silico with genes and *cis*-elements described in tolerance to salt stress, as well as analyzes with mutant plants, can bring more information about this context.

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