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Drought tolerance induced by the overexpression of the nuclear *rbcL* gene in rice

Abstract – The objective of this work was to determine whether the overexpression of the nuclear Rubisco large subunit (*rbcL*) improves the drought tolerance of the genetically modified (GM) BRSMG Curinga upland rice (*Oryza sativa*) cultivar. GM and non-genetically modified (NGM) plants of the same cultivar were compared under the two following water treatments: well watered (WW) and water deficit (WD). The performance of the agronomic traits of GM plants, including grain yield, was superior to that of NGM plants in both treatments. By quantitative polymerase chain reaction, GM plants show a significantly higher expression of the *rbcL* gene in both WW and WD, as well as a larger amount of abscisic acid. With the RNAseq analysis, almost three times more upregulated genes are identified in GM plants in stage 2 after water restriction, indicating a greater protection against water deficit. The higher expression of genes related to the protection of the cellular metabolism and a series of physiological alterations may be involved in the increase in the drought tolerance of GM rice plants overexpressing the *rbcL* gene.

Index terms: *Oryza sativa*, abscisic acid, genetic engineering, quantitative PCR, Rubisco.

Tolerância à seca induzida pela superexpressão do gene nuclear *rbcL* em arroz

Resumo – O objetivo deste trabalho foi determinar se a superexpressão da grande subunidade nuclear da Rubisco (*rbcL*) aumenta a tolerância à seca da cultivar geneticamente modificada (GM) BRSMG Curinga de arroz (*Oryza sativa*) de terras altas. Plantas GMs e não geneticamente modificadas (NGMs) da mesma cultivar foram comparadas nos dois seguintes tratamentos de água: irrigação (WW) e déficit hídrico (WD). O desempenho dos caracteres agronômicos das plantas GMs, incluindo a produção de grãos, foi superior ao das NGMs em ambos os tratamentos. Por reação em cadeia da polimerase quantitativa, plantas GMs apresentam expressão significativamente maior do gene *rbcL*, tanto sob WW como sob WD, além de maior quantidade de ácido abscísico. Com a análise de RNAseq, quase três vezes mais genes regulados positivamente são identificados nas plantas GMs no estágio 2 após restrição hídrica, o que indica maior proteção contra o déficit hídrico. O aumento da expressão de genes relacionados à proteção do metabolismo celular e uma série de alterações fisiológicas podem estar envolvidos no aumento da tolerância à seca em plantas de arroz GMs que superexpressam o gene *rbcL*.

Termos para indexação: *Oryza sativa*, ácido abscísico, engenharia genética, PCR quantitativa, Rubisco.

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Introduction

Drought affects about 50% of global rice (*Oryza sativa* L.) production and, therefore, is a challenge for the development and release of commercial cultivars for most breeding programs (Chatterjee et al., 2017). Although hundreds of genes involved in the response to water deficit have already been identified in rice, it is difficult to develop drought-tolerant cultivars with a high yield potential since drought, a quantitative trait, is regulated by multiple genes, has a low heritability and high environmental effect, and may interact with other abiotic stresses (Baldoni, 2022). In this scenario, the development of genetically modified (GM) plants that overexpress genes of potential interest could pave the way for more drought-tolerant rice cultivars.

Under water deficit, the first physiological responses of the plant include a decrease in leaf area, the stimulation of leaf abscission, root growth towards wetter zones of the soil, and the induction of stomatal closure (Kollist et al., 2019). In the process of stomatal closure, the hormone abscisic acid (ABA) plays an important role, ultimately affecting photosynthesis, the oxidative state of the stroma, and senescence (Mullineaux et al., 2020).

Water stress, as well as other environmental signals, such as a high light incidence and temperature variations, is detected by chloroplasts or plastids, which are key organelles that produce most of the reactive oxygen species (ROS) in leaf cells (Qi et al., 2018). These organelles evolved from the endosymbiosis of cyanobacteria 1.2 billion years ago (Martin, 2003). Since then, there has been a massive transfer of genes from the chloroplast to the nucleus, typically maintaining 120 to 130 genes in the plant nuclear genome (Daniell et al., 2016). In rice, chloroplasts contain about 76 genes and have a genome size of 134.5 Kb (Xiong et al., 2009). The genes transferred from ancestral chloroplasts to the nucleus acquired their own expression and specific signals, causing proteins to be synthesized in cytosolic ribosomes and reintroduced into plastids with the aid of transit peptides, as is the case of the genes involved in Rubisco small subunit synthesis (Ivanova et al., 2017). About 80% of the proteins required by chloroplasts are encoded by the nucleus, translated in the cytosol with precursors extended at the N-terminus, and imported into the plastid via an adenosine triphosphate-dependent process (Gutteridge, 2018).

However, the organellar DNA integrated into the nuclear genome is considered foreign genetic material, which can result in instability, inducing a series of mechanisms for its disposal (Zhang et al., 2020). According to Bock (2017), organelle genes transferred to the nuclear genome have two possible destinations: their rare functionalization, initiated by the capture of a promoter and likely followed by a long phase of evolutionary optimization to complete conversion from a prokaryotic to eukaryotic gene; or their gradual degradation through the accumulation of mutations if functionalization does not occur while the organelles are still intact. However, even if the remaining sequences are not functional, gene relocation can occur, contributing to an increase in genetic diversity (Kleine et al., 2009). This is interesting for rice due to the intact coding sequences of more than 50% of the photosynthesis-related genes (Chen et al., 2015). In addition, some organelle DNA sequences can also be integrated into introns and associated with changes in gene regulation (Zhang et al., 2020).

Under extreme water deficit conditions, a reduced Rubisco (EC 4.1.1.39) activity is the main limitation for a decreased photosynthesis (Abid et al., 2016). This is explained by the fact that Rubisco is a key enzyme in photosynthesis and the most abundant leaf protein, responsible for catalyzing two reactions: CO_2 fixation in photosynthesis and 2-phosphoglycolate production in the photorespiratory pathway (Suzuki & Makino, 2013). In taller plants, Rubisco consists of eight small subunits encoded by a nuclear multigenic family (RBCS) and eight larger subunits (RBCL) encoded by a multicopy gene in the chloroplast genome (Ichikawa et al., 2008). The *rbcS* precursors are processed when entering the chloroplast and then assembled with *rbcL* to produce the photosynthesis holoenzyme.

In the rice nuclear genome, the *rbcL* is one of the genes transferred from the chloroplast and integrated into the nucleus, but that has no promoter sequence (Chen et al., 2015). However, despite its apparent lack of biological function, the gene has been preserved throughout genome evolution.

The objective of this work was to determine whether the overexpression of the nuclear Rubisco large subunit (*rbcL*) improves the drought tolerance of the GM BRSMG Curinga upland rice cultivar.

Materials and Methods

The 3,916 base pair (bp) sequence of the homologous gene of the Rubisco large subunit (*rbcL*) precursor, located in nuclear genome LOC_Os12g10580, was used in the transformation of the BRSMG Curinga rice cultivar, as described by Dedicova et al. (2015). In this study, the rice transformation generated 23 independent events (E1 to E23), from which the evaluated plants were obtained, and generation advance was carried out from T1 onwards. Simultaneously, plants with desirable agronomic attributes were selected in order to meet rice breeder requirements. Three of the events (E2, E8, and E10) resulted in plants with superior drought tolerance. Of these, E8, of the T5 generation, was selected due to its additional desirable agronomic attributes for a commercial cultivar, such as plant height, adequate number of days to heading, and high grain yield.

The drought experiment was carried out from October 2018 to March 2019 in a greenhouse, located at the experimental station of Embrapa Arroz e Feijão, in the municipality of Santo Antônio de Goiás, in the state of Goiás, Brazil (49°17'W, 16°28'S, at 779 m of altitude). The plants were grown in hollow PVC columns (40 cm height x 30 cm width) filled with a Latossolo Vermelho Distrófico (Santos et al., 2018), i.e., an Oxisol, with a clayey texture. GM and non-genetically modified (NGM) plants of cultivar BRSMG Curinga were subjected to the two following water treatments: WW, well watered control or irrigated treatment, with full irrigation throughout the experiment at 90% field capacity; and water deficit (WD), starting at the R3 reproductive stage (panicle initiation) and ending at R7 (grain expansion and maturity) according to the rice growth staging system (Counce et al., 2000). The experimental design was randomized complete blocks with four replicates per water treatment and per genotype. A scale was placed at the base of each PVC column to control the replacement of evapotranspirated water twice a day. Water deficit lasted 14 days and involved completely suspending irrigation until each column lost 4.0 kg of water in relation to the final day of irrigation, meaning that the mass of each column after 4.0 kg of water loss was maintained for 14 days from the onset of suspended irrigation. Afterwards, irrigation was resumed.

For the molecular analysis and gas exchange measurements, leaf materials were collected at the three following plant development stages: stage 1 (R3), on the first day of suspended irrigation; stage 2 (R7), on the last day of water restrictions; and stage 3 (R8), on the seventh day after resuming irrigation. The collected leaves were immediately stored in an ultra-low-temperature freezer at -80°C. Gas exchange rate readings were taken from 8:00 to 11:00 a.m., from the middle portion of the flag leaf on the main stem of each plant, using the LCpro+ portable infrared gas-exchange analyzer (ADC BioScientific Ltd, Hoddesdon, United Kingdom). The following parameters were measured: photosynthetic rate (*A*), expressed in μmol m^{-2} s^{-1}; transpiration (*E*), expressed in mmol m^{-2} s^{-1}; stomatal conductance (*gs*), expressed in mol m^{-2} s^{-1}; and intracellular CO_2 concentration (*Ci*), expressed in μmol mol^{-1}. The photosynthetic photon flux density used was 1,200 μmol (quanta) m^{-2} s^{-1}. The leaf reading area was adjusted by multiplying the average leaf width by 2.5. Intrinsic water use efficiency (WUE_{intr}), in μmol CO_2 mol^{-1} H_2O, was calculated as [WUEintr = A/gs] (Tambussi et al., 2007), and instantaneous carboxylation efficiency (*A*/*Ci*), in μmol m^{-2} s^{-1}/(μmol mol^{-1})$^{-1}$, as [ACi = A/Ci].

In order to confirm the presence of the transformed gene, total genomic DNA was extracted from the leaf tissue of GM and NGM (control) rice plants, using the cetyltrimethylammonium bromide extraction method. The genomic DNA concentrations were adjusted to 10 ng μL^{-1}. Amplification reactions with a specific primer annealing in the ubiquitin promoter (forward primer) and the *rbcL* gene (reverse primer) were performed using the commercial 2× Multiplex PCR Kit (QIAGEN GmbH, Hilden, Germany). The used thermal cycler was GeneAmp 9700 (Thermo Fisher Scientific, Waltham, MA, USA), with an initial denaturation at 95°C for 2 min, 40 cycles each at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 10 min. Electrophoresis of a 561 bp fragment was performed on 2% agarose gel stained with SYBR green (Thermo Fisher Scientific, Waltham, MA, USA), as shown in Figure 1.

Total RNA was extracted from leaf tissues using the commercial RNeasy Plant Mini Kit (QIAGEN GmbH, Hilden, Germany) and, in line with the manufacturer's instructions, was followed by RNA treatment using DNase I (Thermo Fisher Scientific, Waltham, MA, USA). The RNA samples were quantified with the Qubit HS Assay Kit in the Qubit

2.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Complementary DNA (cDNA) samples of three and four biological replicates for NGM and GM, respectively, were synthesized from an RNA template via SuperScript II Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The quantitative polymerase chain reactions (qPCRs) were conducted using diluted cDNA (10 ng μL^{-1}) on two technical replicates. The primer targeting the *rbcL* and the superoxide dismutase (*MnSOD*) genes, as well as the *rbcL* and promoter region, was designed in the OligoPerfect Designer software (Thermo Fisher Scientific, Waltham, MA, USA). The reference genes used were the eukaryotic elongation factor-1α (*EEF-1α*),

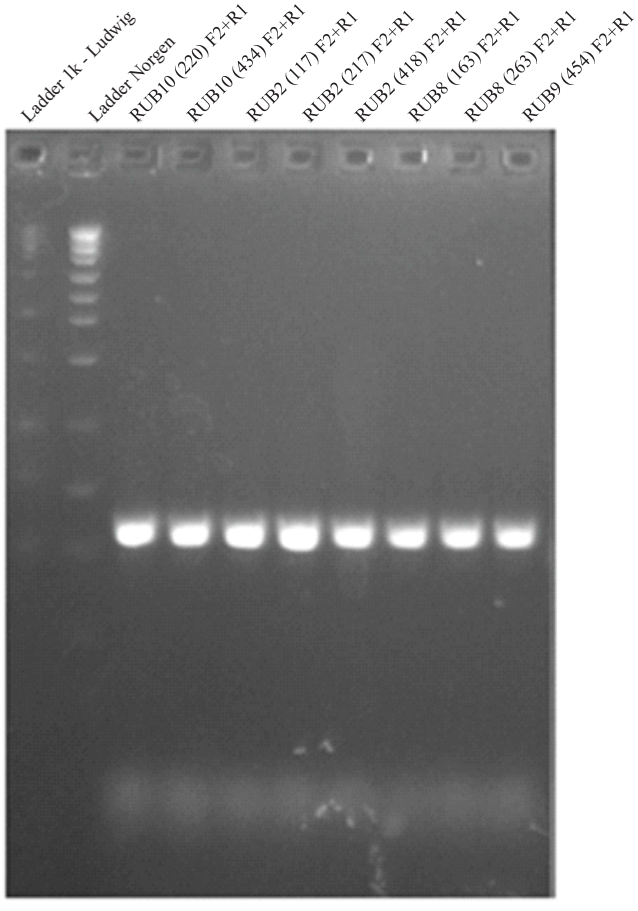

Figure 1. Result of the polymerase chain reaction identifying the presence of the gene of interest, at 561 bp in 2% agarose gel, in genetically modified plants of the BRSMG Curinga rice (*Oryza sativa*) cultivar obtained from three transformation events (E2, E8, and E10).

actin (ACT) (Zhang et al., 2009), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (Kim et al., 2003). The stability value of the reference genes was determined by the Normfinder software (MOMA: Department of Molecular Medicine, Aarhus N, Denmark). The reactions were carried out using the 2x PowerUp SYBR Green Master Mix detection system (Thermo Fisher Scientific, Waltham, MA, USA) in a final volume of 20 µL. Thermocycling was performed in the 7500 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA), with one cycle at 94°C for 2 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The raw data were exported from the Sequence Detection Software v.2.0.5 (Thermo Fisher Scientific, Waltham, MA, USA) to the LinRegPCR software (Ruijter et al., 2009), which calculates the initial amplicon concentrations of the target gene expressed in arbitrary units of fluorescence (N0) using the equation: N0 = Nq_threshold/(Eff_mean^Cq), where Nq_threshold is the fluorescence threshold set to determine Cq, which is the number of cycles needed to reach the Nq_threshold; and Eff_mean is the mean PCR efficiency for the amplicon that is amplified in the current sample (Ruijter et al., 2009). The N0 values of the endogenous and exogenous *rbcL* and *MnSOD* genes were normalized by the geometric mean of the N0 values of the reference genes. The normalized N0 values were used for the analysis of variance and Tukey's test, at 5% probability, through the R software (R Core Team, 2013).

ABA was quantified for GM and NGM plants using the ABA ELISA Kit plant hormone (Elabscience, Houston, TX, USA). A 500 mg sample of leaf tissue was macerated in liquid nitrogen, transferred to a falcon tube containing 4.5 mL of the extraction buffer, and mixed vigorously by inversion. The tube was incubated overnight at 4°C in the dark, shaken gently, and then centrifugated at 5,000 rpm for 30 s. The upper layer was retrieved and diluted with distilled water at a 1:10 ratio. The subsequent steps followed the manufacturer's instructions. The reaction was stopped by adding 50 µL of Stop Solution to each well, and absorbance at 450 nm was read in the Biotek Epoch Microplate Spectrophotometer (Agilent, Santa Clara, CA, USA). The calibration curve was generated using the average absorbance values for each set of standards and samples. The hormone concentration of

the samples was determined automatically from the calibration curve.

For the RNAseq analysis, the upregulated *rbcL* identified and used was LOC_Os12g10580 for rice transformation and the homologous LOC_Os10g21268 for NGM plants.

Four RNA-seq libraries were prepared for each of the two GM and NGM plants in stage 2, with two biological replicates per sample, i.e., two biological replicates each for the WD and WW treatments. The libraries were subjected to paired-end sequencing using the HiSeq 2000 system (Illumina Way, San Diego, CA, USA). Paired-end read quality was first visualized with the FastQC software v.0.11.3 (Babraham Bioinformatics, 2015) and, subsequently, trimmed to eliminate adapters and low-quality bases with the Trimmomatic v.0.35 software (Bolger et al., 2014). The high-quality paired-end sequences were mapped to the reference genome (cultivar Nipponbare of *O. sativa* ssp. *Japonica* released in MSU Rice Genome version 7.0) using the Bowtie2 software (Langmead & Salzberg, 2012), and the expression level of the genes was calculated with the RSEM 1.3.3 software (Li & Dewey, 2011). Differentially expressed genes were identified by between-sample comparison using the EdgeR bioconductor package (Robinson et al., 2010). The false discovery rate was set at <0.01, and Fold Change>2 was used as a threshold to evaluate the significance of differences in gene expression.

Results and Discussion

In the WD treatment, NGM plants showed a significant decrease of 56% in grain yield, whereas that of GM plants was only 8% (Table 1). Therefore, under WD, the grain yield of NGM plants was 41% lower than that of GM plants; however, in the WW treatment, there were no differences between GM and NGM plants for this attribute. Furthermore, when comparing both treatments, the total number of filled grains decreased significantly only in NGM plants. In the literature, there are reports that drought during anthesis typically results in a smaller number of filled grains due to photosynthesis inhibition, leading to male sterility (Ullah et al., 2018) and reduced grain filling (Sehgal et al., 2018). Ullah et al. (2018) added that, under water stress, plants are usually smaller and have a lower leaf area, which reduces the amount of photosynthetically active radiation absorbed by the leaves and, ultimately, decreases yield.

The unfavorable conditions due to drought may be caused by damage to the light-harvesting complex of the leaves, which is important since more than 70% of the carbohydrates directed to the rice grain come from the flag leaf (Abid et al., 2016). In the present study, GM plants showed higher grain yields, which could be attributed to the longer average flag leaf length that could have increased their photosynthetically active area.

Under WD, stomata were more closed in NGM plants in stage 2, suggesting a limited CO_2 acquisition (Table 2). However, in both water treatments, NGM plants in stage 3 showed more-open stomata and a higher concentration of intercellular CO_2 than GM plants, likely in order to mitigate the impact of a greater oxidative stress, indicated by the higher expression of the SOD enzyme in stage 2 (Carmody et al., 2016). GM plants in stage 3 exhibited a greater water use efficiency (WUE) and were better at regulating *gs* during water stress, ensuring a superior WUE when compared with NGM plants. The decline in gs positively affects WUE, which may improve vegetative development and grain yield. According to Sinclair (2018), increased water availability reduces the impact of drought and maintains plant physiological activity. By extending this characteristic from plants to the canopy, a better WUE and higher atmospheric

Table 1. Average agronomic performance of genetically modified (GM) and non-genetically modified (NGM) plants of the BRSMG Curinga rice (*Oryza sativa*) cultivar subjected to well-watered (WW) and water deficit (WD) irrigation treatments in 2019, in the municipality of Santo Antônio de Goiás, in the state of Goiás, Brazil[(1)].

Trait	Genotype	WW	WD
Grain yield (g per plant)	GM	35.6Aa	32.9Aa
	NGM	44.7Aa	19.5Bb
Number of filled grains	GM	359Aa	306Aa
	NGM	345Aa	186Bb
Flag leaf length (cm)	GM	21Aa	21Aa
	NGM	17Ba	18Ba
Flag leaf width (mm)	GM	16Aa	16Aa
	NGM	15Ba	15Ba

[(1)]Uppercase letters indicate a comparison between GM and NGM plants for the same treatment (columns), and lowercase letters, a comparison of water treatments for the same genotype (lines) by Tukey's honestly significant difference test, at 5% probability, and n = 4.

CO_2 levels would prevent future drought events due to a greater water availability in the soil (Souza et al., 2019).

Conventional PCR showed the presence of the exogenous *rbcL* gene in all studied GM plants. In addition, GM plants showed a higher *rbcL* expression than their NGM counterparts in all three stages ($p<0.05$) and both treatments (Figure 2). As observed by Chen et al. (2015), the *rbcL* gene has no promoter sequence in the rice nuclear genome, meaning that its expression in NGM plants is related to the *rbcL* gene synthesized in plastids. Regarding homologous genes, such as LOC_Os10g21268 in the present study, Cheng (2013) found that it is often difficult to distinguish between transcripts derived from organelles and from the nucleus when mapping against the total transcriptome. In other words, the transcript identified as upregulated in NGM plants was not from the nuclear genome, but from the chloroplast as shown by the high homology of 99.8% between the studied genes. The greater *rbcL* gene expression in GM plants in all three stages may be explained by the fact that the promoter used for the gene of interest is ubiquitin, which, due to its constitutive nature, allows of the gene to be synthesized throughout the plant life cycle. Although this did not affect the yield of GM plants when compared with their NGM counterparts under the WW treatment, the larger number of *rbcL* transcripts in the former was likely the main reason for the higher yield of 68% in the WD treatment.

The raw sequencing data generated 28.64 Gb of RNA-Seq data, with an average of 7.16 Gb per library. The average number of mapped reads was 87.2%, assembled into 30,079 genes, using Nipponbare as the reference genome. The total number of upregulated transcripts was 3,598 and 1,352 for the GM_WD and NGM_WD libraries, respectively.

Considering the annotated genes, the RNAseq analysis identified almost three times more upregulated genes in GM than in NGM rice in stage 2 after 14 days of WD. The number of expressed protective proteins and kinase genes identified in GM plants was more than four and seven-fold higher than in their NGM counterparts, respectively. Protein kinases are known to regulate most cell pathways, especially those involved in signal transduction (Lehti-Shiu & Shiu,

Table 2. Photosynthetic rate (*A*), stomatal conductance (*gs*), transpiration rate (*E*), intrinsic water use efficiency (WUE_{intr}), sub-stomatal CO_2 concentration (*Ci*), and carboxylation efficiency (*A*/*Ci*) of genetically modified (GM) and non-genetically modified (NGM) plants of the BRSMG Curinga rice (*Oryza sativa*) cultivar subjected to well-watered (WW) and water-deficit (WD) irrigation treatments in three growth stages, in 2019, in the municipality of Santo Antônio de Goiás, in the state of Goiás, Brazil[(1)].

Variable	Stage 1 (77 DAS)		Stage 2 (90 DAS)		Stage 3 (97 DAS)	
	WW	WD	WW	WD	WW	WD
A (GM)	12.1	12.5	5.7	6.1	10.4	10.5
A (NGM)	11.1	10.4	4.3	5.2	10.7	9.0
gs (GM)	0.195	0.185	0.105	0.073	0.165B	0.13B
gs (NGM)	0.165	0.18	0.132a	0.07b	0.207Aa	0.18Ab
E (GM)	5.5A	5.6A	2.4B	1.7	4.1	3.6
E (NGM)	3.9B	4.0B	3.0Aa	2.1b	3.6	3.4
WUEintr (GM)	66.8	67.4	56.3	72.3	63.8A	88.2A
WUEintr (NGM)	63.7	61.8	39.8b	71.9a	51.5B	50.0B
Ci (GM)	234.0	232B	295.3	294.3	261.8	218.8B
Ci (NGM)	266.5	261.2A	325	266.7	283.5	281.1A
A/*Ci* (GM)	0.054	0.053A	0.02	0.023	0.041	0.051A
A/*Ci* (NGM)	0.040	0.041B	0.016b	0.025a	0.038	0.032B

[(1)]Uppercase letters indicate a comparison between GM and NGM plants for the same treatment (columns), and lowercase letters, a comparison of water treatments for the same genotype (lines) by Tukey's honestly significant difference test, at 5% probability, and n = 4. DAS, days after sowing.

2012), which occurs within the cells and throughout the plant after stress recognition. The transduction of environmental signals generally results in the expression of altered genes at the cellular level, increasing or repressing gene expression in response to stress, which can affect plant metabolism and development (Kollist et al., 2019). Far larger numbers of upregulated genes from the Ca^{2+}-dependent protein kinase/calmodulin enzyme class (CAMK) family, activated by increased intracellular calcium ion (Ca^{2+}) and calmodulin concentrations, have been identified in GM plants (Swulius & Waxham, 2008). According to these authors, activated CAMK is involved in the phosphorylation of transcription factors and, consequently, in the regulation of the expression of response genes, also contributing to the regulation of the cell life cycle. However, more dehydrins (DHN), a multi-family of plant proteins produced in response to cold and drought stress (Verma et al., 2017), were upregulated in NGM plants. This is an interesting finding since GM plants did not require a greater synthesis of genes from this DHN family, but used other mechanisms, such as the interaction between *rbcL* transcripts and ABA synthesis. In addition, plant hormones, as ABA, jasmonic acid, ethylene, and salicylic acid, are synthesized in response to drought and involved in osmotic adjustment and other drought-related processes (Ullah et al., 2018). In the present study, significantly upregulated transcripts were found in GM and NGM plants subjected to water stress, and both libraries identified an ABA precursor.

The number of upregulated genes associated with the abovementioned hormones was about three times higher in GM plants, indicating a greater protection against water deficit in these plants. The larger repertoire of upregulated genes was essential in the superior drought tolerance of these plants and, consequently, in their higher grain yield under stress. This shows that the evaluated hormones played a vital role in reducing oxidative stress in GM plants, which, due to their overexpression of *rbcL*, had a lower level of *MnSOD* in stage 2 after 14 days of water restriction in comparison with NGM plants, which were undergoing a greater oxidative damage due to drought (Figure 3). This means that the expression of *MnSOD* was significantly higher (44%) in NGM plants in stage 2 on the last day of water restrictions ($p<0.05$). In order to reverse this damage, ROS that accumulate in the

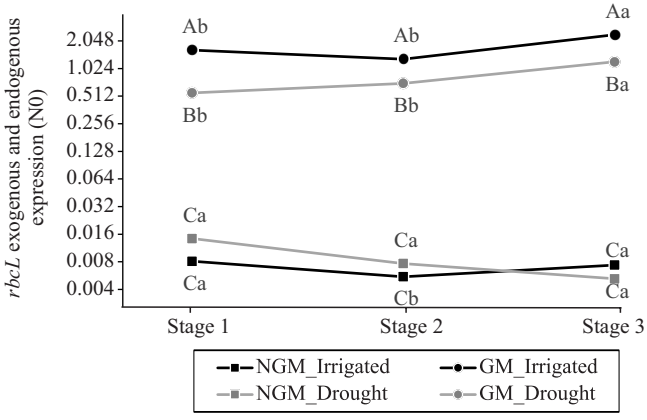

Figure 2. Expression (N0) of *rbcL* genes in genetically modified (GM) and non-genetically modified (NGM) plants of the BRSMG Curinga rice (*Oryza sativa*) cultivar evaluated in 2019 in the municipality of Santo Antônio de Goiás, in the state of Goiás, Brazil. Lowercase letters indicate a comparison between genotypes throughout the following sampling stages: stage 1, at 77 days after sowing (DAS); stage 2, at 90 DAS; and stage 3, at 97 DAS. Uppercase letters indicate a comparison between genotypes at each sampling stage by Tukey's honestly significant difference test, at 5% probability, and n = 3.

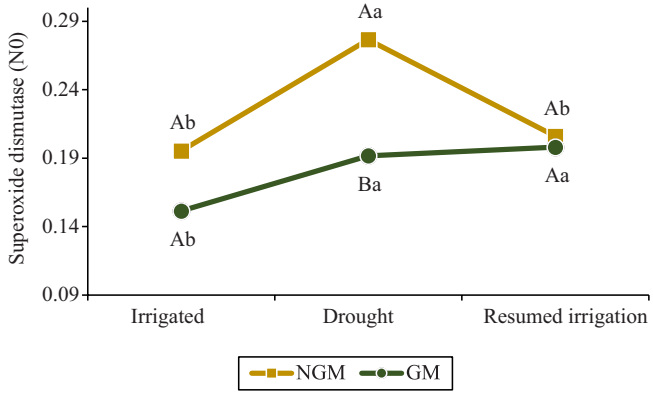

Figure 3. Expression (N0) of the superoxide dismutase gene (*MnSOD*) in genetically modified (GM) and non-genetically modified (NGM) plants of the BRSMG Curinga rice (*Oryza sativa*) cultivar evaluated in 2019 in the municipality of Santo Antônio de Goiás, in the state of Goiás, Brazil. Lowercase letters indicate a comparison between treatments (lines), whereas uppercase letters indicate a comparison between genotypes (columns) within the same treatment by Tukey's honestly significant difference test, at 5% probability.

plastids due to exposure to biotic and abiotic stresses act as signals of gene expression in the nucleus (Cullis et al., 2009). The presence of these signals in the cytosol of GM and NGM plants caused by water stress and the larger number of *rbcL* transcripts observed in the former may have been responsible for altering plant metabolism, culminating in the greater drought tolerance of GM plants. When irrigation resumed in stage 3, *MnSOD* returned to its normal level, a finding previously reported in rice (Wang et al., 2019).

From stage 1 to 2, ABA content increased 0.615 and 0.278 μg mL^{-1}, respectively, in GM and NGM plants under water stress. In stage 2, the obtained value was three-fold higher ($p<0.05$) for GM plants. However, in stage 3, ABA content decreased in GM plants to the same level as that of their NGM counterparts (Figure 4).

The significant increase in ABA in GM plants in stage 2 may be a response to *rbcL* overexpression, which resulted in the high grain yield observed under WD. One hypothesis to support this interaction between *rbcL* and ABA content is that the accumulation of this gene in the cytosol can be interpreted as the result of chloroplast degradation, which would induce a series of reactions to protect the plant cell. As such, increased ABA synthesis may be a protective measure for maintaining cellular metabolism, since plants

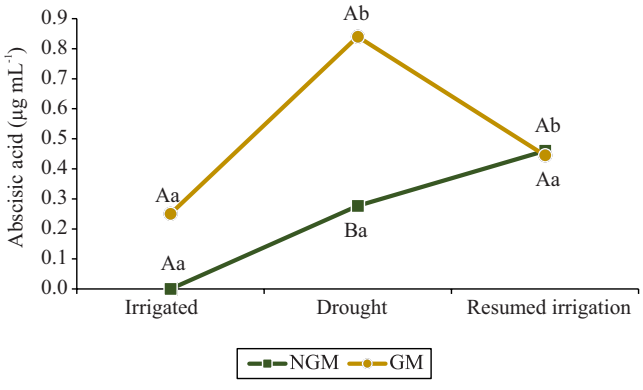

Figure 4. Abscisic acid concentration in genetically modified (GM) and non-genetically modified (NGM) plants of the BRSMG Curinga rice (*Oryza sativa*) cultivar evaluated in 2019 in the municipality of Santo Antônio de Goiás, in the state of Goiás, Brazil. Lowercase letters indicate a comparison between treatments for each genotype, whereas uppercase letters indicate a comparison between genotypes for the same treatment by Tukey's honestly significant difference test, at 5% probability.

have different strategies for mitigating the effects of water stress. Ullah et al. (2018) highlighted that, as a signaling hormone, ABA triggers a series of changes in plants to protect them from damage caused by stress, such as drought, being synthesized in plastids and the cytoplasm.

According to Cullis et al. (2009), changes in the chloroplasts are better coordinated when the transferred genes are in the nuclear genome. As such, the nucleus depends on chloroplast signaling, allowing modifications in nuclear gene expression as plant adaptive responses to stress, depending on the status of the chloroplasts, as observed in *Haberlea rhodopensis* Friv., whose drought tolerance and rapid recovery after rehydration were attributed to its specific chloroplast characteristics, and whose *rbcL* gene was identified as having 13 positive selection sites (Ivanova et al., 2017). This reinforces the hypothesis regarding the role of *rbcL* in improving drought tolerance in GM plants. Therefore, the finding of the present study that the overexpression of *rbcL* induces a greater drought tolerance in GM rice plants may be a starting point for the development of more drought-resistant cultivars.

Conclusions

1. The higher expression of genes related to the protection of cellular metabolism and a series of physiological alterations may be involved in the increase in the drought tolerance of genetically modified (GM) rice (*Oryza sativa*) plants overexpressing the nuclear Rubisco large subunit (*rbcL*).

2. GM plants show a significantly higher expression of the *rbcL* gene under both well watered and water deficit treatments, as well as a larger amount of abscisic acid, when compared with non-genetically modified (NGM) plants.

3. With the RNAseq analysis, almost three times more upregulated genes are identified in GM plants after water restriction, indicating a greater protection against water deficit.

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