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Article

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Abstract: Water deficit greatly affects global crop growth and productivity, particularly in water-limited environments like upland rice cultivation, leading to reduced grain yield. Plants activate various defense mechanisms during water deficit, involving numerous genes and complex metabolic pathways. Exploring orthologous genes, linked to enhanced drought tolerance, can aid functional validation in target species using genomic data from model organisms. Here, we studied the *OsCPK5* gene in upland rice, an *AtCPK6* ortholog *from A. thaliana*, by overexpressing it in the BRSMG Curinga cultivar. Transformants were assessed under two conditions: water deficit applied 79 days after seeding, lasting 14 days, followed by 7 days of irrigation at 80% field capacity. Physiological data and samples were collected at R3, R6, and R8 stages. GM plants consistently exhibited higher *OsCPK5* gene expression across stages, peaking during grain filling. During this phase, GM plants displayed reduced stomatal conductance, photosynthetic rate, and increased water use efficiency compared to non-GM plants under drought. GM plants also exhibited higher filled grain percentage in both irrigation conditions. Their drought susceptibility index was 0.9 times lower than NGM plants, and they maintained a higher chlorophyll a/b index, indicating sustained photosynthesis. NGM plants under water deficit exhibited more leaf senescence, while *OsCPK5*-overexpressing plants retained green leaves. Overall, *OsCPK5* overexpression induced diverse drought tolerance mechanisms, hinting at potential for future development of more drought-tolerant rice cultivars.

Keywords: water use efficiency; gene expression; senescence; drought

1. Introduction

The water deficit is considered one of the main environmental stresses, and many regions of the world already face substantial water scarcity, with a consequent reduction in agricultural production (Raza et al., 2019). Most of the efforts of rice breeders have historically been devoted to increasing yield potential and, currently, due to the problem of climate change, they are focusing on developing materials that are more resistant to abiotic stresses (Zampieri et al., 2023). The initial physiological responses to water deficit include decrease in leaf area, stimulation of leaf abscission, directional root growth towards moister soil regions and induction of stomatal closure. In this context, the abscisic acid hormone (ABA) plays an important role in preventing excessive water loss through transpiration (Yang et al., 2022). Stomata are important gas exchange organs in plants, playing an essential role in

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regulating photosynthesis, respiration, transpiration and temperature (Martin-St Paul et al., 2017). Furthemore, stomata are also the water-regulating organs of plants, being essential for water conservation, a decisive factor for the survival of plants in conditions of water deficit (Fei et al., 2019). Water deficit can also cause physiological imbalance due to an excess of reactive oxygen species (ROS), resulting in oxidative stress. In response, plants produce a variety of antioxidant enzymes, such as superoxide dismutase (SOD), peroxidase (POD) and enzymes antioxidants (Gill & Tuteja, 2010), in order to maintain its metabolic stability in situations of environmental stress.

According to Serraj et al. (2011), genes associated with water deficit tolerance encode proteins for cellular adaptation, including chaperones, transcription factors or enzymes involved in signal transduction, such as protein kinases. Numerous studies have been conducted in rice to elucidate the roles of different genes in water stress tolerance. Certain genes that have led to enhaced water deficit tolerance in rice include *OsEPF1* (Caine et al., 2018), *EDT1* (Wu et al., 2019), *OsRab7* (El-Esawi & Alayafi, 2019), *OsMYB6* (Tang et al., 2019), *OsTF1L* (Bang et al., 2019), *OsbZIP42* (Joo et al., 2019), and *OsNAR2.1* (Chen et al., 2019). It is interesting to note that some of the mentioned genes have demonstrade significant improvements in grain yield and plant biomass accumulation in the species. However, the complexity of responses to water deficit suggests the involvement of a substantial number of genes that require further investigation for a more comprehensive understanding of stress tolerance mechanisms.

An increase in intracellular calcium concentration occurs in response to various stimuli, such as the accumulation of the hormone abscisic acid (ABA) in leaves during water deficit conditions. Upon reaching the plasma membrane, ABA binds to membrane receptors, leading to an increase in cytosolic calcium that can occur either by the transient entry of Ca^{2+} ions into the intracellular environment, or by the release of these ions from internal reservoirs, such as the endoplasmic reticulum and the vacuole. Such variation plays an important role in triggering signaling cascades in response to plant acclimatization (Tang & Luan, 2017). This process involves calcium-binding proteins that transmit the calcium signal, thereby inducing specific cellular and physiological responses (Dodd et al., 2010).

Three main classes of Ca^{2+} binding proteins have been characterized in plants, namely, calcium-dependent protein kinases (CDPKs), calmodulins and B-like calcineurin proteins (Asano et al., 2005). Among these classes, calcium-dependent protein kinases, commonly referred to as CPK or CDPK, are of particular significance due to their capacity to bind and transmit intracellular Ca^{2+} signals via a single gene product (Hamel et al., 2014). Such a combination may have originated in ancestral organisms through the fusion of protein kinase and calmodulin genes, wherein the former can modify other proteins by adding phosphate groups, while the latter can bind calcium (Zhang & Choi, 2001). Thus, CPKs can directly activate and regulate target proteins containing Serine and Threonine (Ser/Thr) residues upon detecting intracellular Ca2+ signals, facilitated by specific domains within their structural composition.

In plants, CPKs are widely distributed across various tissues, including roots, stems, leaves, and flowers (Boudsocq & Sheen, 2013). At cellular level, they are abundant in meristems, xylems, pollen, guard cells and embryonic cells (Simeunovic et al., 2016). Subcellularly, they are found in the cytosol, nucleus, tonoplasts, mitochondria, chloroplasts and peroxisomes, with the plasma membrane being the location where most CPKs are found in the Arabidopsis model plant. The diverse presence of CPKs in different regions of plants suggests their involvement in various signal transduction pathways (Shi et al., 2018). It is well- known that CPK protein kinases plays multiple roles in plant biology, including senescence and cell death, hormone signal transduction, stress and defense responses, growth and development, carbon and nitrogen metabolism, cytoskeletal formation, regulation of ion channels, among others (Lei et al., 2007). Protein kinases are species-specific and are encoded by a multigene family (Zhang et al., 2019). For instance, through genomic analysis, 34 CPK genes have been identified in Arabidopsis (Hrabak et al., 2003) and 31 CPK genes in rice (Ray et al., 2007).

Some CPKs have been functionally characterized in relation to water deficit tolerance in Arabidopsis. Transgenic plants of this species overexpressing the *AtCPK6* gene exhibited increased

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tolerance to water and salt deficits, along with elevated transcription levels of this gene under stress conditions (Xu et al., 2010). Some CPK genes appear to play a role in regulating stomatal opening and closing in Arabidopsis, a phenomenon also also observed in maize with the *ZmCPK4* gene, which increased water deficit tolerance by influencing stomatal closure through abscisic acid (ABA)-mediated pathways (Jiang et al. al., 2013). In Arabidopsis, the overexpression of *CPK10* led to enhanced water deficit tolerance through its participation in ABA and calcium-mediated stomatal movements (Zou et al., 2010). It is important to note that stomatal closure, despite reducing transpiration, it also limits gas exchange. This decrease in carbon dioxide assimilation generally results in reduced biomass and productivity in environments subject to water deficit.

Functional studies involving different CPKs in relation to water deficit tolerance have already been conducted in rice**.** The *OsCPK4* gene has been associated to enhanced water retention capacity in plants overexpressing this gene (Campo et al., 2014). Bundó & Coca (2017) observed that the *OsCPK10* gene, under water deficit conditions, increased the hydrogen peroxide detoxification capacity in rice plants. Overexpression of the *OsCPK9* gene led to improved stomatal closure and plant osmotic adjustment capacity, along with enhanced pollen viability and increased spikelet fertility (Wei et al., 2014). Given that the functions of CPK genes in rice, particularly in relation to water deficit tolerance, are not yet fully understood, studies involving gene overexpression in genetically modified plants (GMOs) compared to non-genetically modified plants can contribute to a better comprehension of the roles these proteins play in plant adaptation to adverse water conditions. Such research also has the potential to aid in the development of commercial cultivars with increased drought tolerance. The objective of this work was to study the effect of the overexpression of *OsCPK5* in genetically modified (GM) upland rice plants BRSMG Curinga in comparison to the BRSMG Curinga non-genetically modified (NGM) subjected to water deficit.

2. Results

When comparing the harvest index (HI) of GM and NGM plants under different irrigation treatments, it was observed that the GM plants subjected to the control treatment exhibited a HI 11.1% higher than the NGM plants in the same treatment ($p<0.05$; Table 1). Conversely, in the water deficit treatment, no significant difference between the genotypes was observed. Statistical difference ($p< 0.05$) was observed for each genotype (GM and NGM plants) across different irrigation treatments for the traits: grain yield, number of filled grains, number of empty grains, and fresh mass (the latter only for the GM). On the other hand, no significant difference was observed for the traits: tiller number, panicle number, flag leaf length and width, and dry mass (Table 1).

Table 1. Average agronomic performance of OsCPK5_E4 (GM) and BRSMG Curinga (NGM) rice plants: grain yield, number of filled grains, harvest index, tiller number, panicle number, flag leaf length and width, dry and fresh mass, in control and water deficit (WD) irrigation treatments.

Trait	Genotype	Control	WD
Grain yield (g plant^{-1})	GM	42.7 Aa	20.2 Ab
	NGM	43.5 Aa	19.5 Ab
Number of filled grains	GM	357 Aa	155 Ab
	NGM	338 Aa	186 Ab
Number of empty grains	GM	47.4 Ab	189.8 Aa
	NGM	86.7 Ab	221.8 Aa
Harvest Index	GM	46.9 Aa	26.5 Ab
	NGM	41.7 Ba	25.8 Ab
Tiller Number	GM	20.7 Aa	22.6 Aa
	NGM	23.4 Aa	22.8 Aa
Panicle Number	GM	20.7 Aa	22.5 Aa
	NGM	23.4 Aa	22.2 Aa
Flag leaf length (cm)	GM	18.6 Aa	17.4 Aa
	NGM	17.8 Aa	18.5 Aa

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Flag leaf width (mm)	GM	16.2 Aa	15.4 Aa
	NGM	15.5 Aa	14.7 Aa
Dry Mass	GM	48.6 Aa	58.6 Aa
	NGM	59.3 Aa	57.8 Aa
Fresh Mass	GM	185.4 Aa	131.6 Ab
	NGM	169.7 Aa	158.3 Aa

Uppercase letters indicate a comparison between GM (genetically modified) and NGM (not genetically modified) plants for the same treatment (columns), and lowercase letters a comparison of water treatments for the same genotype (lines) (Tukey's HSD test at 5% probability and n = 4).

GM plants subjected to water deficit showed a drought susceptibility index (DSI) of 0.97, being considered relatively tolerant to drought stress, according to Grzesiak et al. (2019). Hence, the overexpression of the OsCPK5 gene in GM plants was found to confer relative tolerance to water deficit, suggesting reduced drought impact on upland rice performance. The DSI of 1.08 for BRSMG Curinga (NGM) indicates that this cultivar straddles a delicate balance between tolerance and sensitivity to water deficit, as observed by Lanna et al. (2021).

After 14 days of water deficit in the reproductive phase (Stage 2), the transpiration rate (E) of GM plants was 42.1% lower than that of NGM plants ($p< 0.05$; Table 2). In this stage, both GM and NGM plants exposed to water deficit exhibited respective decreases of 44.5% and 23.2% in transpiration rate, compared to their corresponding control plants ($p<0.05$).At the R6 stage, the decline in transpiration rate (E) was the result of the reduction in stomatal conductance (gs), reaching 51.2 and 33.0%, for GM and NGM plants, respectively (Table 2). It is worth mentioning that GM plants exhibited a stomatal conductance 46.7% lower than NGM plants subjected to water deficit. Regarding carboxylation efficiency (A/Ci), no differences were evident between GM and NGM genotypes under any irrigation treatment, at any period of collect. However, it was observed that GM plants maintained a consistent A/Ci across all periods of collect, in both irrigation treatments (Table 2).

Table 2. Values of photosynthetic rate (A), stomatal conductance (Gs), instantaneous water use efficiency (iWUE), intrinsic water use efficiency (WUEintr), transpiration rate (E), internal carbon concentration (Ci) and carboxylation efficiency (A/Ci) in NGM plants (wild-type) and GM plants (overexpressing the *OsCPK5* gene) throughout stages R3 (79 DAS - beginning of water deficit), R6 (93 DAS - end of water deficit) and R8 (101 DAS - 7 days after return to normal irrigation).

Genotype	**Stage 1 (79 DAS)**		**Stage 2 (93 DAS)**		**Stage 3 (101 DAS)**	
	Control	**WD**	**Control**	**WD**	**Control**	**WD**
A (GM)	8.25 Aa	8.18 Aa	6.71 Aa	4.37 Ab	7.89 Aa	7.07 Aab
A (NGM)	9,99 Aa	9.85 Aa	4.83 Ab	5.96 Ab	10.19 Aa	5.59 Ab
gs (GM)	0.185 Aa	0.162 Aa	0.082 Aa	0.040 Bb	0.207 Aa	0.147 Ab
gs (NGM)	0.175 Aa	0.157 Aa	0.112 Aa	0.075 Ab *	0.205 Aa	0.180 Aa
iWUE (GM)	2.2 Aa	2.2 Aa	2.6 Aa	3.1 Aa	2.1 Aa	2.4 Aa
iWUE (NGM)	2.5 Aab	2.6 Aa	1.5 Bb *	2.4 Aa	2.9 Aa	1.7 Ba
WUEintr (GM)	44.6 Ab	50.5 Ab	81.8 Aa	109.2 Aa	38.1 Ab	48.1 Ab
WUEintr (NGM)	57.1 Aa	62.7 Aab	43.1 Ba *	79.5 Aa	49.7 Aa	31.1 Ab
E (GM)	3.76 Aa	3.68 Aa	2.56 Aa	1.42 Bb	3.71 Aa	3.00 Aa
E (NGM)	3.94 Aa	3.83 Aa	3.19 Aa	2.45 Aa *	3.52 Aa	3.27 Aa

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Ci (GM)	300.5 Aa	279.8 Aa	274.0 Aa	236.0 Aa	291.0 Aa	278.0 Aa
Ci (NGM)	274.0 Aa	260.3 Aa	291.0 Aa	245.5 Aa	284.5 Aa	290.0 Aa
A/Ci (GM)	0.027 Aa	0.029 Aa	0.024 Aa	0.019 Aa	0.027 Aa	0.025 Aa
A/Ci (NGM)	0.036 Aa	0.039 Aa	0.017 Aa	0.024 Aa	0.036 Aa	0.019 Ab

* Significant differences between GM and NGM genotypes in each irrigation condition and collect period separately. The capital letter indicates statistical difference between plants of the same genotype in different irrigation conditions in each collect period. The lowercase letter indicates statistical difference between plants of the same genotype and in the same irrigation condition in the three collect periods (Tukey Test, $p < 0.05$ and $n = 4$).

Regarding the intrinsic water use efficiency (iWUE), under control treatment, GM plants presented iWUE 40.7% higher than NGM plants in the second collect period (R6 stage) (Table 2). Comparing GM and NGM plants grown under water deficit and control treatments at each data collecting stage, it was observed that in the third collect period (7 days after the return of normal irrigation), GM plants under water deficit did not show a significant difference in iWUE in comparison to corresponding plants in control treatment. NGM plants cultivated under water deficit presented iWUE 41.4% lower than the corresponding plants in control treatment.

GM plants also exhibited notable intrinsic water use efficiency (WUEintr). In the second collection period (stage R6), GM plants under the control treatment displayed a WUEintr that was 40.2% higher than that of NGM plants under the same irrigation condition (Table 2). The higher WUEintr value in GM plants occurred in the second collection period (stage R6), across irrigation treatments. Both genotypes showed higher WUEintr 14 days after the irrigation cutoff, relative to the collection periods. However, GM plants were 40.2% more eficiente in using water as compared to the corresponding NGM plants under water deficit.

Discrepancies in the chlorophyll a and b proportions were observed between the GM and NGM genotypes during the first collection period (79 DAS, stage R3). In this period, GM plants exhibited a chlorophyll a/b ratio that was 25% and 23.1% higher than that of NGM plants under control and water deficit conditions, respectively (Table 3). In the second collection period (93 DAS, stage R6), no statistical differences between the genotypes were observed in either irrigation treatment. However, during the third collection period (101 DAS, stage R8), GM plants displayed chlorophyll a/b ratios that were 10.7% and 12% higher than those of NGM plants under control and water deficit treatments, respectively. No significant differences were observed in the chlorophyll a/b ratio during the first and second collection periods between plants of the same genotype grown under control conditions and those grown under water deficit treatment. However, during the third collection period, NGM plants exhibited a notable difference of 13.7% in the chlorophyll a/b ratio between plants grown under control and water deficit treatments.

Table 3. Chlorophyll a/b ratio in NGM plants (wild-type) and GM plants (overexpressing the *OsCPK5* gene) throughout the stages R3 (79 DAS- before cutting irrigation), R6 (93 DAS- after cutting irrigation) and R8 (101 DAS- 7 days after returning to normal irrigation) and the percentage reduction in plants grown under water deficit conditions (Decr.%). WD: water deficit.

Genotype	Stage R3 (79 DAS)			Stage R6 (93 DAS)			Stage R8 (101 DAS)		
	Control	WD	Decr. (%)	Control	WD	Decr. (%)	Control	WD	Decr. (%)
GM	2.4 Aa	2.6 Aa	-	2.7 Aa	2.4 Aa	8.4	2.8 Aa	2.5 Aa	8.1
NGM	1.8 Ab *	2.0 Ab *	-	2.9 Aa	2.4 Aa	14.8	2.5 Ab	2.2 Bb	13.7
Variation (%)	25	23.1					10.7	12	

The capital letter indicates statistical difference between plants of the same genotype in different irrigation conditions in each collect period. The lowercase letter indicates statistical difference between plants of different genotypes in the same irrigation condition. * Significant differences between GM and NGM genotypes under the same irrigation condition and collect time. Decr. (%) indicates the percentage decrease in the proportion of

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chlorophyll a and b in plants of the same genotype under water deficit compared to control (Tukey test, $p < 0.05$ and $n = 4$).

Through visual comparisons of the general appearance of the GM and NGM plants submitted to water deficit (Figure 1), it was observed that in the second collection period (93 DAS, stage R6), 14 days after irrigation cutoff, GM plants sustained the green aspect of their leaves. Conversely, the NGM plants exhibited numerous senescent leaves from the second collection period, a condition that persisted into the third collection period (101 DAS, stage R8), even following the restoration of irrigation.

.	GM	NGM
R3 stage		
R6 stage		

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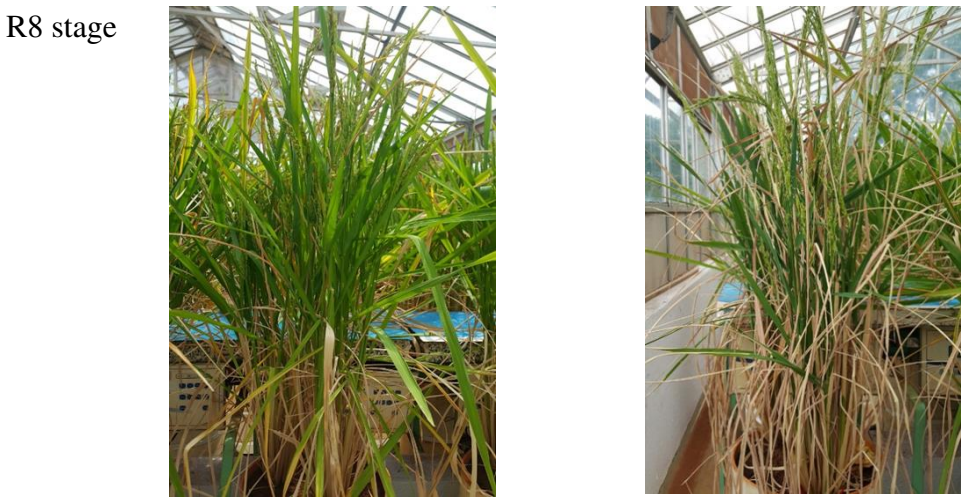

Figure 1. Phenotypic comparison between GM and NGM plants conducted across three developmental stages.

GM plants showed higher *OsCPK5* gene expression compared to NGM plants at all collect periods and in both irrigation treatments (Figure 2). In the control treatment, the GM plants showed the highest *OsCPK5* gene expression during period 2 (stage R6), and this pattern persisted in period 3 (stage R8). For GM plants subjected to water deficit treatment, the highest expression of the *OsCPK5* gene was observed in period 3 (stage R8), 7 days after the return of normal irrigation. No significant difference in *OsCPK5* gene expression was observed between NGM plants under control irrigation treatment and those under water deficit treatment at any of the collection periods. Furthermore, in the NGM plants there was no significant difference in the expression of the *OsCPK5* gene between the three collect periods, under either irrigation treatments.

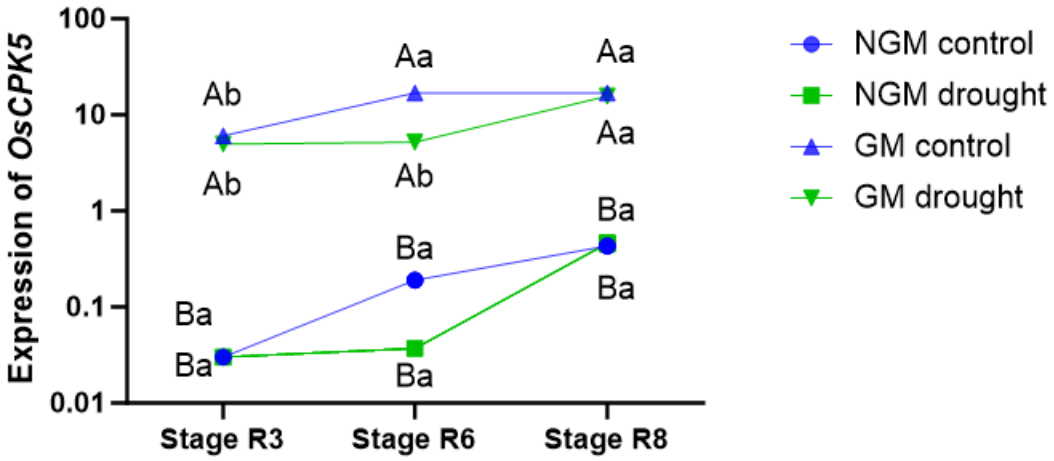

Figure 2. *OsCPK5* gene expression in NGM (wild-type) and GM (overexpressing *OsCPK5*) plants. The lowercase letter indicates statistical differences of the genotype over the collect periods (stages R3, R6 and R8); capital letter indicates differences between NGM and GM genotypes at each collect period (Tukey test, $p < 0.05$ and $n = 4$).

To investigate the underlying factors contributing to the delayed leaf senescence in GM plants, we examined the gene expression of *MnSOD*, an important component in the defense against oxidative stress in rice plants subjected to water deficit conditions. It was observed that GM plants exhibited higher transcription levels of this gene starting from the first collection period (stage R3), and this elevated expression persisted compared to NGM plants ($p<0.05$) until the end of the water deficit phase, corresponding to the second collection period (stage R6). After seven days of the return

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of irrigation (stage R8), the *MnSOD* expression in GM plants cultivated under water deficit conditions equaled that of NGM plants in the same irrigation treatment (Figure 3).

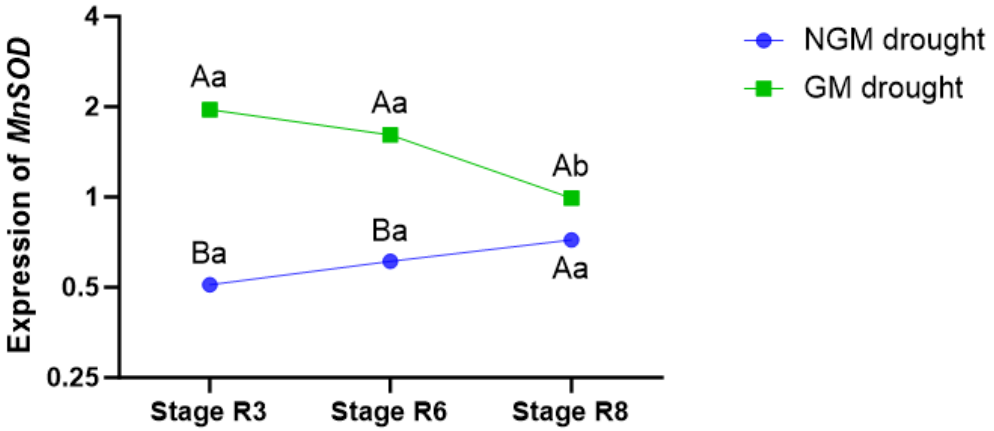

Figure 3. Gene expression of the *MnSOD* gene in NGM (wild-type) and GM plants (overexpressing *OsCPK5*). Lowercase letters denote significant differences within each genotype across the collection periods (stages R3, R6, and R8), while capital letters indicate disparities between NGM and GM genotypes for each collection period (Tukey test, $p < 0.05$ and $n = 4$).

RNA-seq generated an average of 43.6 Gbp of clean reads, of which 41.7 Gb (95.6%) were aligned to the rice reference genome (Table 4). Five billion reads were aligned to exons (89.3%), with the highest alignment achieved in well-annotated reference genomes. The 10 most expressed genes, determined by the abundance of transcripts mapped to the genome, are described in Table 5. For illustrative purposes, the transcript of the *OsCPK5* gene used in the transformation (Os02g0685900) has been added to Table 5.

Table 4. Description of the sequenced libraries (RNA-seq) derived from leaf tissues in the Douradão and Primavera cultivars.

Libraries	Raw reads avg.	Clean reads avg.	Reads Aligned avg.	Reads aligned to exons
GM drought	46,065,473	45,329,575	43,432,707 (95.8%)	5,841,166,813 (89.9%)
GM control	43,492,722	42,764,401	40,969,707 (95.8%)	5,473,640,936 (89.3%)
NGM drought	43,542,120	42,639,242	40,547,019 (95.1%)	5,422,323,506 (89.3%)
NGM control	44,357,608	43,611,743	41,666,443 (95.5%)	5,537,551,774 (88.8%)
Average	44,364,481	43,586,240	41,653,969 (95.6%)	5,568,670,757 (89.3%)

Table 5. Counting the average number of most abundant RNA-seq transcripts.

Gene Id	GM drought	GM control	NGM drought	NGM control	Total	Description(1)
Os11g0707000	861,550	861,372	783,893.5	709,240	3,216,055.5	Rubisco activase
Os11g0171300	415,106	403,158.5	388,764.5	284,405.5	1,491,434.5	Chloroplast aldolase
Os12g0190000	68,818	137,986	130,697.5	120,118	457,619.5	GDP-L-galactose phosphorylase
Os08g0157600	85,963	86,221.5	100,490	107,916.5	380,591	Circadian clock associated 1
Os09g0346500	114,615.5	147,237.5	75,065.5	37,457	374,375.5	Chlorophyll a/b binding protein

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Os01g0200700	136,507	101,727.5	66,638.5	58,841	363,714	Metallothionein i-3a
Os08g0139700	15,126	31,237.5	140,959.5	161,352	348,675	Terpene synthase 29
Os08g0200300	107,531	94,602	77,955.5	53,800.5	333,889	Photosystem II subunit PsbR3
Os05g0202800	95,020.5	106,567.5	40,654.5	66,918.5	309,161	Metallothionein 3b
Os07g0637300	58,555	53,547	70,199.5	73,397	255,698.5	Pyruvate dehydrogenase kinase
Os02g0685900*	2.5	2.5	1.5	2	8.5	Calcium-dependent protein kinase 5

* Transcript of the *OsCPK5* gene, overexpressed in GM plants, and present in the table for comparison. Overall, it was the 22,850th most expressed transcript. (1) According to the Rice Annotation Project (RAP).

Gene expression level analysis stands as a central task in an RNA-seq experiment, computed through the count of mapped reads. When contrasting the libraries of GM and NGM plants exposed to drought conditions, the FPKM count for the Os02g0685900 transcript (*OsCPK5*) reached 233,193,538,410,951 (upregulated) for GM, whereas for NGM, it amounted to only 0.52 (Table 6). Conversely, the GM control libraries exhibited upregulation relative to GM drought conditions, analogous to the upregulation seen in the NGM control libraries when compared to GM drought conditions.

Table 6. Contrasting the FPKM values for the Os02g0685900 (*OsCPK5*) transcript in RNA-seq libraries. The initial value corresponds to the library in the row, while the subsequent value corresponds to the library in the column.

	GM control	**NGM drought**
GM drought	23,371,037,618,603//264,976,737,934,358	233,193,538,410,951//0.52
NGM control	179,833,986,804,252//277,541,258,971,447	180,057,622,291,041//0.54

A functional classification of GM vs NGM sequences from drought libraries, based on Gene Ontology (GO) analysis, revealed two groups of significantly upregulated genes: biological process (BP) encompassing 13 classes; with "response to the oxygen-containing compound" showing the highest gene count at 22, and molecular function (MF), presenting four categories, with "unfolded protein binding" hosting 10 genes. Considering significantly downregulated genes, three GO groups were identified: BP, with 89 classes, where "defense response" featured 43 genes), MF comprising 19 classes, with "carbohydrate binding" harboring 34 genes), and cellular component (CC) with 1 class, where "region extracellular" comprised 45 genes.

Examining the upregulated genes within the BP category, the classes most relevant to water deficit included "response to hydrogen peroxide" (GO:0042542), with 6 heat shock protein genes (hsp), "response to reactive oxygen species" (GO:0000302) comprising 5 hsp, 2 peroxidases and 1 aminotransferase, "osmotic stress response" (GO:0006970, see Table 7), and "oxidative stress response" (GO:0006979) with 5 hsp, 2 peroxidases, 1 aminotransferase, 1 HLH protein and two unknown proteins). Notably, the last two classes were absent in the GO analysis of the GM x NGM control libraries. Among the downregulated genes in the BP category, the most directly related was "stress response" (GO:0080134, see Table 8). This class also did not appear in the GO analysis of the GM x NGM control libraries.

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Table 7. Differentially expressed genes upregulated in the "response to osmotic stress" class, category BP, in rice plants genetically modified with *OsCPK5* gene (GM) compared to the non-genetically modified BRSMG Curinga (NGM).

Gene ID	Description(1)	pValue
Os01g0136200	16.9 KDA CLASS I HEAT SHOCK PROTEIN 2	1,78E-10
Os03g0266300	17.9 KDA CLASS I HEAT SHOCK PROTEIN 1	1,83E-02
Os03g0626500	UNKNOWN	3,93E-01
Os01g0136100	16.9 KDA CLASS I HEAT SHOCK PROTEIN 1	1,96E+03
Os09g0110300	CYCLASE-LIKE 4	7,67E+05
Os03g0643300	ORNITHINE AMINOTRANSFERASE	1,07E+07
Os01g0667200	GLYOXALASE II-1	1,72E+07
Os10g0471100	WAX-DEFICIENT ANTHER 1	2,40E+09
Os03g0267000	18.0 KD CLASS I HEAT SHOCK PROTEIN	0.02364
Os03g0281900	ABC TRANSPORTER G FAMILY MEMBER 5	0.03731
Os07g0517100	HSP18.8	0.04134

(1) According to the Rice Annotation Project (RAP).

Table 8. Differentially expressed genes dowregulated in the "regulation of response to stress" class, category BP, in rice plants genetically modified with *OsCPK5* gene (GM) compared to the non-genetically modified BRSMG Curinga (NGM).

Gene ID	Description(1)	pValue
Os09g0417800	WRKY GENE 62	1,91E-44
Os03g0402800	TIFY GENE 10A	7,15E-31
Os09g0439200	TIFY GENE 10C	1,56E-24
Os10g0392400	TIFY GENE 11D	6,93E-23
Os01g0130200	NRR	9,62E-16
Os07g0615200	TIFY GENE 10B	1,44E-15
Os03g0180900	TIFY GENE 11C	6,10E-13
Os01g0508500	NRR REPRESSOR HOMOLOGUE 2	5,80E-08
Os03g0181100	TIFY GENE 11B	8,51E-05
Os11g0195500	phytoalexin deficient 4	4,10E-02
Os01g0194300	NPR1 HOMOLOG 1	2,21E+03
Os01g0221100	JASMONYL-L-ISOLEUCINE SYNTHASE 2	4,40E+06
Os04g0395800	TIFY GENE 9	6,34E+07
Os06g0603600	*Oryza sativa* SYG/PHO8/XPR1 (SPX) DOMAIN GENE	2,03E+09
Os03g0180800	TIFY GENE 11A	4,14E+09
Os12g0197500	SUPPRESSOR OF GENE SILENCING 3	4,18E+09
Os03g0285800	MULTIPLE STRESS RESPONSIVE MAP KINASE 2	0.002180
Os01g0508100	NRR REPRESSOR HOMOLOGUE 3	0.000275
Os05g0368100	UNKNOWN	0.000859
Os10g0391400	TIFY GENE 11E	0.000886

(1) According to the Rice Annotation Project (RAP).

Enrichment analysis identified two statistically significant pathways (of KEGG) associated with differentially expressed genes comparing GM versus NGM transcripts (libraries from drought treatment) (Table 9). None of these pathways were identified in the GM versus NGM libraries of the control treatment, but "Ribosome", "Photosynthesis - antenna protein", "Ribosome biogenesis in eukaryotes", "Phenylpropanoid biosynthesis" have been found.

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Table 9. Differentially expressed genes associated with two drought-related KEGG metabolic pathways.

Pathway	Gene ID	Description[(1)]
Plant Hormone Signal Transduction (osa040705); pValue: 0.003	**Upregulated**	
	Os02g0796500	B-TYPE RESPONSE REGULATOR 3
	Os10g0564500	STRESS/ABA-ACTIVATED PROTEIN KINASE 3
	Os08g0176900	b-ZIP TRANSCRIPTION FACTOR 64
	Os03g0297600	REGULATORY COMPONENTS OF ABA RECEPTOR 4
	Downregulated	
	Os03g0402800	TIFY GENE 10A
	Os09g0439200	TIFY GENE 10C
	Os10g0392400	TIFY GENE 11D
	Os07g0615200	TIFY GENE 10B
	Os03g0180900	TIFY GENE 11C
	Os03g0667100	NPR1-like gene 3
	Os03g0181100	TIFY GENE 11B
	Os11g0143300	A-TYPE RESPONSE REGULATOR 9
	Os12g0139400	A-TYPE RESPONSE REGULATOR 10
	Os01g0194300	NPR1 HOMOLOG 1
	Os01g0221100	JASMONYL-L-ISOLEUCINE SYNTHASE 2
	Os04g0395800	TIFY GENE 9
	Os04g0673300	A-TYPE RESPONSE REGULATOR 6
	Os01g0382000	PATHOGENESIS-RELATED GENE 1B
	Os03g0180800	TIFY GENE 11A
	Os10g0391400	TIFY GENE 11E
	Os01g0221000	-
MAPK signaling pathway (osa04016); pValue: 0.04	**Upregulated**	
	Os10g0564500	STRESS/ABA-ACTIVATED PROTEIN KINASE 3
	Os03g0297600	REGULATORY COMPONENTS OF ABA RECEPTOR 4
	Os04g0556000	HEAVY METAL ATPASE 5
	Donwregulated	
	Os03g0132900	CHITINASE 11
	Os06g0726200	CHITINASE 1
	Os09g0438000	RESPIRATORY BURST OXIDASE HOMOLOG G
	Os03g0743500	CALMODULIN-LIKE PROTEIN 4
	Os10g0542900	CHITINASE 8
	Os01g0382000	PATHOGENESIS-RELATED GENE 1B
	Os04g0578000	ACC SYNTHASE 2
	Os03g0285800	MULTIPLE STRESS RESPONSIVE MAP KINASE 2
	Os05g0474800	WRKY GENE 70

[(1)] According to the Rice Annotation Project (RAP).

3. Discussion

This study highlights the impact of *OsCPK5* gene overexpression in GM plants on the regulation of stomatal closure, resulting in a decrease in stomatal conductance (gs). Stomatal closure serves as the primary leaf response to drought conditions, minimizing water loss by decreasing transpiration rate (E) and increasing water use efficiency (WUE) (Ashraf & Harris, 2013). Notably, significant differences in gs and E were observed between GM and NGM plants during the second collect period

(stage R6), a critical phase when water loss intensifies due to grain filling in the panicles. Despite the identification of this important physiological difference the two genotypes, where the GM plants were more efficiente in water use, both GM and NGM presented the same grain yield. Certainly, this outcome might arise from the intricate nature of grain productivity, influenced by an array of metabolic pathways and genetic interactions.

To deal with water deficit, plants close their stomata to maintain cell turgor and metabolism, which can affect photosynthetic rate (Zampieri et al., 2023). Photosynthesis is highly sensitive to drought stress and is the foremost process that is altered by such condition. Decreased production of photoassimilates reduces leaf growth and crop yield (Ahmadi-Lahijani & Emam, 2016). The enhancement in stomatal regulation, observed as a result of the overexpression of *OsCPK5* is also reported in studies involving other genes of the CPK family. For instance, Jiang et al. (2013) investigated the impact of overexpressing the corn *ZmCPK4* in *Arabidopsis thaliana*, finding an amplified sensitivity of the plant to the hormone abscisic acid (ABA), increasing stomatal closure in response to water deficit. Wei et al. (2014) observed that, under normal growth conditions, transgenic rice plants overexpressing *OsCPK9* exhibited no significant differences in stomatal opening compared to control plants, however, following a period of water deficit, a higher proportion of fully closed stomata was observed in the transformed plants, as opposed to control plants, suggesting that the *OsCPK9* gene influences stomatal movement under water deficit conditions.

Water deficit tolerant plants respond drought mainly through fine control of stomatal and mesophilic conductance (Yang et al., 2021), and under these conditions, impairment of CO_2 assimilation may occur, as well as restrictions on growth and metabolism (Zou et al., 2015). However, in this study, it was observed that the improvement in stomatal closure of GM plants in the stage 6 (grain filling phase), following a 14-days of water deficit, did not lead to a reduction in the productivity of this genotype, when compared to NGM plants cultivated under similar irrigation treatment. Some factors migh have contributed to decreasing the impact of water restriction on the productivity of GM plants relative to NGM plants, even after a substantial reduction in stomatal conductance and transpiration rate during the grain-filling phase. These factors include greater water use efficiency during the grain-filling phase, delayed leaf senescence, and higher levels of chlorophyll a and b in GM plants compared to NGM plants.

The stability in carboxylation efficiency observed in GM plants at the three collect stages, in both irrigation treatments, could potentially be linked to enhanced protection against the degradation of Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase). Another probable reason was that ribulose activase, the enzyme that regulates Rubisco activation (Waheeda et al., 2023), was the most expressed enzyme identified by RNAseq, with GM plants under water deficit having 9% more transcripts of this enzyme than plants NGM in the same irrigation condition, and 18% more than the NGM plants in the control treatment. Furthermore, the elevated protection against the degradation of this enzyme after re-irrigation in GM plants might have contributed to a better cellular redox balance, thereby creating a a more favorable cellular environment for its preservation and activity. The carboxylation efficiency was also noted by HU et al. (2010), when evaluating the effect of water deficit on Kentucky bluegrass grass (*Poa pratensis* L.), a C3 perennial grass. In that study, the drought-tolerant genotype demonstrated an enhanced activity and state of activation of Rubisco following re-irrigation, restoring metabolic activity to to levels comparable with control plants an this phenomenon was considered a plausible cause for the recovery of photosynthetic activity.

Water use efficiency (WUE) is associated to the ability of plants to deal with varying degrees of water deficit, playing a pivotal role in maintaining productivity even when water availabity is restricted (Schulz et al., 2020). During the critical water deficit period (R6 stage), GM plants overexpressing the *OsCPK5* gene exhibited higher iWUE compared to NGM plants. The iWUE after the water deficit period suggests that NGM plants were unable to restore their CO_2 assimilation machinery, leading to malfunction of the photosynthetic apparatus and degradation of pigments, compromising the different stages of photosynthesis (Kerry et al., 2018). The correlation between gs reduction and greater water use efficiency has been observed by Li et al. (2017) and Zhang et al. (2018), evaluating C3 plants (like rice), under moderate water deficit scenarios.

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Reducing gs per amount of CO_2 assimilated and increasing the rate of CO_2 assimilation can improve WUEintr (Blankenagel et al., 2018). In R6 stage, GM plants overexpressing the *OsCPK5* gene showed higher WUEintr than NGM plants, in both irrigation treatments. In fact, under water deficit, the GM plants exhibited a notable decrease in gs and E at 14-day after cutting off irrigation (stage R6), concomitant with an augmentation of WUEintr at this particular stage in contrast to NGM plants. In wheat, Li et al. (2017) reported negative correlations among photosynthesis, transpiration and stomatal conductance on water use efficiency, suggesting stomatal characteristics as factors responsible for regulating water use efficiency in these plants. Increasing the water use efficiency in rice is critical, as rice grown in the lowland system requires more than 2.5 kg of water for each grain of rice produced (Zampieri et al., 2023).

Visually, GM rice plants overexpressing the *OsCPK5* gene showed a greener leaf appearance in contrast to NGM plants during the grain filling phase after 14 days of irrigation restriction, while the leaves of the NGM plants turned yellow and senescent. The correlation between overexpression of a calcium-dependent protein kinase and the green appearance of leaves was also observed by Campo et al. (2014), when examining the overexpression of *OsCPK4* to increase tolerance to water deficit in rice plants of the cultivar Nipponbare (*Oryza sativa* Japonica). Visual differences between plants overexpressing the *OsCPK4* gene and control plants were observed from the 14th day without irrigation, when the control plants (wild-type) showed symptoms of damage induced by water deficit, such as leaf wilting, which remained until the last day of cutting irrigation (17th day). On the other hand, transgenic plants overexpressing *OsCPK4* remained healthy and green. The authors verified that after rehydration of the plants, approximately 90% of the transgenic plants recovered from the stress, while none of the control plants survived the water deficit treatment. In our experiment, a similar result occurred, as the return of irrigation for 7 days (stage R8) failed to rejuvenate NGM plants from the applied water deficit, increasing the senescente appearance of their leaves in comparison with GM plants, where the senescence was delayed. Plants with a stay-green trait are important in case of drought, as the extension of the photosynthesis period leads to greater grain productivity (Lee & Masclaux-Daubresse, 2021).

According to Wang et al. (2019), the relationship between the expression of CPK family genes and the senescence process in rice leaves was further underscored through the functional validation of the *OsCPK12* gene. In this context, they observed that mutant plants, in which the gene was was knocked out, exhibited yellowing, senescent leaves during the grain-filling stage, while wild-type plants exhibited a greener appearance. Investigations into the possible reasons for this difference showed that mutant plants showed greater accumulation of H_2O_2 and superoxide radicals in leaves, in addition to more cell death and apoptosis than wild-type plants. On the other hand, *OsCPK12* overexpression led to higher photosynthetic rates, increasing chlorophyll a and b content, as well as delaying leaf senescence and delaying plant growth period, providing positive effects for crop productivity.

There is a positive correlation between increased *SOD* gene expression and increased tolerance of plants to environmental stresses (Yang et al., 2022). In this work, the evaluation of the gene expression of *MnSOD*, important to prevent oxidative stress (Tripathi & Tripathi, 2014), revealed that GM plants overexpressing *OsCPK5* grown under water deficit treatment presented a rapid and strong activation of this defense response from the R3 stage, remaining until the end of the irrigation cut, during the reproductive phase. Sairam & Saxena (2000) considered that the levels of antioxidant metabolites and enzymes that regulate the cellular redox state increase under different stresses, concluding that a greater antioxidant activity confers greater tolerance of plants to these stresses. Sharma & Dubey (2005) also observed increased expression/activity of total superoxide dismutases (SODs) due to water deficit in rice. Furthermore, De Deus et al. (2015), when evaluating the enzymatic activity and gene expression levels of eight SOD isoforms in rice, observed that more SOD genes were expressed in the vegetative phase in relation to to the reproductive phase. However, the enzymatic activity of SOD increased only in the reproductive phase of the plants.

In the current study, despite the values of the Chlorophyll a/b ratio of GM and NGM not having been statistically different through the quantification in the greenhouse experiment, GM plants

presented 35% more transcripts of the Chlorophyll a/b binding protein gene than the NGM plants under water deficit, while GM plants had 75% more transcripts than NGM plants under control irrigated treatment. Light-harvesting chlorophyll a/b binding (Lhc) proteins, according to Wu et al. (2023), play a role in the efficiency of photosynthesis, in addition to participating in the response to abiotic stresses. As pointed out by Yang et al. (2022), an increased proportion of chlorophyll a and b is indicative of greater drought tolerance, which is further evidence that GM plants had a better response to drought. The relationship between overexpression of a calcium-dependent protein kinase and an increase in chlorophyll content was also observed by Wei et al. (2014) after water deficit treatment in rice plants overexpressing the *OsCPK9* gene (*Oryza sativa*, cultivar Nipponbare), in relation to plants with silencing of this gene and control plants. Wang et al. (2019), when investigating possible reasons for differences in chlorophyll content from overexpression or silencing of CPK family genes in *Oryza sativa* ssp. Indica, noticed that mutant plants of genes of the CPK family presented negative regulation of genes involved in the synthesis of chlorophylls and positive regulation of genes involved in the degradation of these pigments. This fact may indicate that overexpression of *OsCPK5* also act on the synthesis or degradation pathways of these pigments in upland rice, paving the way for new investigations into which genes and metabolic pathways related to the synthesis or degradation of chlorophylls were altered from overexpression of the *OsCPK5* gene.

Leaves of GM plants overexpressing the endogenous and exogenous *OsCPK5* gene showed higher transcription of this gene compared to NGM plants in both irrigation treatments at the three evaluated collection periods (stages R3, R6 and R8). A similar result was obtained by Xu et al. (2010) when examining the transcription levels of the *AtCPK6* gene, ortholog of *OsCPK5*, in *A. thaliana*, in which the levels of the *AtCPK6* gene in transformed plants were higher than in wild-type plants, and associated with increased drought tolerance. The *OsCPK5* gene was poorly expressed in relation to the other transcripts identified by RNAseq analysis. Even so, the amount of transcription of the *OsCPK5* gene was slightly higher in GM plants than in NGM, which indicates that the best alternative to monitor the presence of specific genes is quantitative PCR. Ray et al. (2007), when evaluating CPK family genes in *Oryza sativa* ssp. Indica using microarray and RT-qPCR, observed that fourteen CPK genes were upregulated during the panicle development stage and six during the seed development stage, evidencing that CPK expression is also regulated by the rice development stage.

The majority of the upregulated transcripts in GM drought libraries identified by GO analysis were "heat shock proteins" (HSPs), which have important roles in transducing cell signaling, regulating apoptosis, and protecting cells against biotic and abiotic stresses (Haq et al., 2019). Conversely, the downregulated transcripts were mainly TIFY genes, which have a regulatory function in plant development and responses to biotic and abiotic stresses (Zhang et al., 2020), exhibiting a similar function to HSPs. The WRKY transcription factor, which plays a key role in transmitting and responding to drought stress signals, was also found to be downregulated (Yang et al., 2022), that is, WRKY expression was higher in NGM plants. According to the KEGG analysis, TIFY proteins were identified in the "plant hormone signal transduction" pathway, showing higher expression in NGM plants compared GM plants under water deficit. The other pathway identified by KEGG was the "MAPK signaling pathway", associated with the mitogen-activated protein kinase (MAPK) cascade, a defense mechanism induced against abiotic stresses in plants (Majeed et al., 2023). Most of the genes in this pathway were upregulated in NGM plants, supporting the hypothesis that these plants are more adversely affected by the consequences of water deficit than the GM plants.

4. Material and Methods

The *OsCPK5* gene (LOC_Os02g46090 – TIGR ID; Os02g0685900 – RAPDB ID), utilized in rice transformation, was initially identified from the sequence of its orthologue in Arabidopsis, the *AtCPK6* gene (AT2G17290). The company DNA Cloning Service (Hamburg, Germany) carried out the cloning of the *OsCPK5* gene in the p7i2x-Ubi binary vector, which has the 35S promoter for the bar gene, conferring tolerance to ammonium glufosinate and the Ubiquitin promoter (Ubi-1) for the *OsCPK5* gene. Rice transformation for overexpression of the *OsCPK5* gene was detailed by Dedicova et al. (2015) utilizing *Agrobacterium tumefaciens* (EHA 105 strain). The cultivar employed for the

transformation was BRSMG Curinga, commercially released by Embrapa in 2005. The selection of the transformed calluses was conducted using the herbicides bialaphos and phosphinothricin (PPT), in culture medium, both of which contain ammonium glufosinate as an active ingredient. The calli that proliferated in the presence of this selection agent were subcultured, originating T0 plants that were subsequently subjected to PCR to amplify the bar gene to identify genetically modified (GM) plants. Then, the Southern blot technique was performed to estimate the number of copies inserted in the genome. Furthermore, to confirm the activity of the bar gene in the transformed rice, the chlorophenol red test was carried out using leaves from transgenic tillers of the T0 generation. The 17 independent genetic transformation events obtained were evaluated for water deficit tolerance and ideal plant type traits as they advanced through the generations (T1 to T3). Additionally, the leaves were treated with a 2% ammonium glufosinate herbicide solution, in order to select plants tolerant to this herbicide and, consequently, carrying the gene of interest. Ultimately, the genetically modified (GM) event 4 (OsCPK5-E4, generation T4) was chosen for conducting the experiment both with and without water deficit, in order to evaluate its physiological performance, productivity and the expression profile of the *OsCPK5* and *MnSOD* genes in comparison to the non-genetically modified (NGM) cultivar BRSMG Curinga.

The experiment took place in the 2018/2019 season on a semi-automated phenotyping platform, situated at the Embrapa Arroz e Feijão experimental station, in Santo Antônio de Goiás City, Brazil (49° 17′ W, 16° 28′ S, 779 m altitude). The employed experimental design was a randomized blocks with four replications, for both control and water deficit treatments. The plots consisted of PVC columns 40 cm in height and 30 cm in diameter, filled with Dystrophic Red Clay Oxisol, a soil type typical of the Cerrado Biome, with pH and fertility adjusted according to technical recommendation (Fageria, 2006). Within each column, two plants from event 4 were sown, following the same procedure for NGM plants. The columns were placed on scales to determine when 80% of the soil's field capacity was reached, as well as to monitor water lost and the necessary irrigation to maintain water content. Plants were irrigated once a day, in the morning, to reach the desired weight in each column. The irrigation treatments were: (1) irrigated (or control), with irrigation close to 80% of the soil's field capacity, and (2) drought or water deficit, with restricted irrigation during the reproductive phase, starting 79 days after sowing and lasting 14 days. This limited irrigation approach aimed to maintain the column weight of the column after loosing 4 kg of water. Over the 14 day period, the amount of water replaced in the columns subjected to water deficit was adusted to 80% of the field capacity, minus 4 kg. This replacement was necessary to induce plant response to water deficit for a period of two weeks, and after this period, irrigation returned to normal, as in the control treatment.

The phenotypic performance of GM plants overexpressing the *OsCPK5* gene, in the T4 generation, was acessed in comparison to the NGM BRSMG Curinga. The physiological data and plant material from both GM and NGM plants were collected in three periods: Period 1 - Beginning of water deficit (79 days after sowing, at the panicle emission - stage R3); Period 2 - end of the water deficit (93 days after sowing, in the grain filling - stage R6) and Period 3 - seven days after the return of irrigation to 80% of the soil field capacity (101 days after sowing, at the beginning of physiological maturation - stage R8). Physiological data were obtained from the middle segment of the flag leaf of the main stem of each plant, between 8:00 am and 11:00 am, across the three collection periods, using a portable leaf chamber analyzer (IRGA, model LCpro-SD, ADC BioScientific). The measured parameters were photosynthetic rate (A) (μmol CO_2 m^{-2} s^{-1}), stomatal conductance (gs) (mol H_2O m^{-2} s^{-1}), transpiration rate (E) (mmol H2O m^{-2} s^{-1}) and intracellular concentration of CO_2 (Ci) (μmol mol^{-1}). The employed photosynthetic active photon flux density (PFFD) used was 1200 μmol [quanta] m^{-2} s^{-1}. The carboxylation efficiency was calculated as [A/Ci] for each plant, the water use efficiency and instantaneous iWUE (μmol CO_2 mol^{-1} H_2O) was calculated as [iWUE=A/E], and the intrinsic water use efficiency WUEintr (μmol CO_2 mol^{-1} H_2O) was calculated as [WUEintr=A/gs]. The estimated content of chlorophyll content a and b was evaluated in fully expanded flag leaves of each plant, at each of the three collect periods, using a portable chlorophyll meter ClorofiLOG® model CFL 1030 (Falker Automação Agrícola, Brazil). The obtained chlorophyll content was employed to calculate the

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chlorophyll a and b ratio as Cl.a/Cl.b. These results were statistically analyzed by ANOVA and Tukey's test ($p< 0.05$), in the R software.

Samples of healthy leaf tissue were collected at Period 1 (stage R3), Period 2 (stage R6), and Period 3 (stage R8), seven days after the return of irrigation. These collected samples were promptly wrapped in aluminum foil, flash-frozen in liquid nitrogen, and preserved in an ultra freezer (-80°C) for subsequent utilization in gene expression analysis via RT-qPCR. Thirty days after the beginning of flowering, the treatments were ready for harvest. At that moment, the length and width of three flag leaves from distinct tillers of each plant were measured, coinciding with the IRGA readings. A ruler was used to measure the length and a caliper to measure the width of the leaves. Plant height (in centimeters) was determined from the base of the stem to the apex of the highest panicle. Upon harvest, the number of tillers and panicles of each plant was counted. The panicles were harvested individually for the purpose of weighing and quantifying thegrains within each plant. Plants with removed panicles were trimmed near the soil's surface, placed within labeled envelopes, and weighed to obtain their fresh mass. Then, they were subjected to a drying process within an oven set at 60ºC for 72 hours, being weighed again to obtain the dry mass.

To determine the yield data, the total weight of grains per plant was measured. Furthermore, the number of filled and empty grains of three random panicles of each plant was counted. Additionally, the drought susceptibility index (DSI) was determined according to the methodology of Fischer & Maurer (1978), obtained through the formula:

$$ISS = \frac{1-(Yd/Yp)}{D}$$

Where Yd is the yield value of the genotype under water deficit; Yp is the yield value of the genotype under normal irrigation; D is the average yield (total average weight of grains per plant) of all genotypes under water deficit condition/average yield (total average weight of grains per plant) of all genotypes under irrigation condition. According to Grzesiak et al. (2019), genotypes are classified water deficit tolerant when DSI values are below than 1, whereas genotypes with DSI values exceeding 1 are considered susceptible to water deficit.

The harvest index (HI), representing the grain yield relative to the total dry matter of the plant, reflects the physiological capacity of the plants to allocate photosynthate for grain filling in panicles. It was determined according to the formula proposed by Bijalwan and Dobriyal (2014):

$$HI\ (\%) = \frac{\text{grain yield}}{\text{biological yield}}\ x\ 100$$

The grain yield signifies grain weight per plant, while biological yield accounts for grain weight per plant plus dry mass weight, computed for each irrigation treatment. Statistical analysis was performed using ANOVA and Tukey's test ($p<0.05$) within the R software.

Total RNA extraction was performed from the leaf tissue of four GM plants (two from the control treatment and two from the water deficit treatment), as well as four NGM plants. Leaf samples were collected at Periods 1, 2 and 3. The extractions were carried out using the RNeasy Plant Mini kit (Qiagen), according to the manufacturer's instructions, followed by DNase treatment of total RNA (RNase Free DNA, Qiagen). The extracted RNA was resuspended in 50µL of RNase-free water and kept at -80ºC.

The RNA samples were evaluated in the Qubit® 2.0 Fluorometer for quantify total RNA. The integrity of the RNA molecules was evaluated using the Agilent RNA 6000 Nano kit (Agilent Technologies), on the Bioanalyzer 2100 device. A reverse transcription reaction (RT) was conducted to generate cDNA molecules from 1µg of RNA using the GoScript Reverse Transcription System kit (Promega), as per the manufacturer's guidelines. Random primers were used to cDNA generation. Subsequently, cDNA samples were adjusted to a concentration of 10 ng x μL^{-1} and stored at -20°C. A pair of primers (forward and reverse) targeting the *OsCPK5* gene was designed at an exon-exon junction to increase the amplification specificity of the transcribed gene sequence. The OligoPerfect™ Designer program (Invitrogen, http://tools.lifetechnologies.com/content.cfm?pageid=9716) was used for primer design.

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Primer concentrations for the reference genes and the *OsCPK5* gene were adjusted for the sample set. Amplification efficiencies for these genes were determined from the slope in efficiency curves on CT plots against the logarithm base two of initial template concentrations. Efficiency values (E) were calculated using the equation [E = 10(-1/slope) – 1] × 100 (Bustin et al., 2009). Correlation coefficients values (r^2) were aldo determined signifying the the standard curve´s linearity; values nearing 1 are ideal, as are values of the standard curve's slope, which should approximate -3.32, indicating a PCR reaction with 100% efficiency. Each reaction had a final volume of 10.0µL, comprising 5 ng of cDNA, a pair of primers (forward/reverse) with adjusted concentrations for the sample, and 5µL of PowerUp™ SYBR® Green Master Mix (ThermoFisher). Amplification conditions were as follows: 50°C for 2 min (UDG incubation); 95°C for 2 min (cDNA denaturation); 40 cycles of 95°C for 15s and 60°C for 30s (annealing and extension). RT-qPCR reactions were conducted and analyzed on the 7500 Real Time PCR Systems (Applied Biosystems®). The stability of the reference genes actin (*ACT*) and eukaryotic elongation factor-1α (*eEF-1α*), used for data normalization, was determined using the NormFinder software (Andersen et al., 2004).

In gene expression analysis, the comparative CT method (ΔΔCT) was used to determine the relative quantification (RQ) using the DataAssist program version 3.01 (Life Technologies). Relative quantification of the OsCPK5 gene was conducted with three technical replicates comparing the normalized target quantity in each sample (within its respective treatment) to the normalized target quantity in the control plants BRSMG Curinga (NGM). The ΔCt value for each sample was obtained by subtracting the Ct values of the reference genes from the Ct values of the gene of interest. The ΔΔCt value was calculated using the formula: ΔΔCt = ΔCt (sample) - ΔCt (normalizing sample). Subsequently, the formula 2^(-ΔΔCt) was applied to obtain the relative expression value of the gene under investigation. The normalized Ct values of the target gene underwent ANOVA and Tukey's test ($p<0.05$), accessible within the R software.

For transcriptome analysis, eight leaf tissue samples were collected from GM and NGM plants (two biological replicates for each irrigation treatment) at stage R6. RNA extraction was carried out using a PureLink® RNA Mini Kit (Ambion® CA, USA) following the manufacturer's protocol. RNA quantity and quality were assessed using *Qubit*® 2.0 Fluorometer (GE Healthcare UK Ltd, England) and BioAnalyzer 2100 (Agilent Technologies CA, USA), respectively. The transcriptome sequencing (RNA-seq) was performed on Illumina Hi-Seq 2000 platform (Genone Ltd., Rio de Janeiro, Brazil). Raw data in fastq was initialy processed removing adapter-containing and low-quality reads. The reference genome and gene model annotation files were directly sourced from the genome website (Oryza sativa ssp Japonica, Nipponbare - MSU Rice Genome version 7.0). An index of the reference genome was constructed, and paired-end clean reads were aligned to it using Hisat2 v2.0.5 (Mortazavi et al., 2008).

Mapped reads from each sample were assembled using StringTie v1.3.3b (Pertea et al., 2015). Gene expression levels were quantified using FeatureCounts v1.5.0-p3 (Liao et al., 2014), which count the mapped reads per gene. Subsequently, FPKM (Fragments Per Kilobase of transcript sequence per Million base pairs sequenced) for each gene was calculated based on gene length and the mapped read count.Differential expression analysis, involving two conditions/groups (with two biological replicates per condition), was conducted through the DESeq2R package version 1.20.0 (Love et al., 2014). Genes with an adjusted P-value of ≤ 0.05 were identified as differentially expressed. Gene Ontology (GO) enrichment analysis of these differentially expressed genes was performed using the clusterProfiler R package with gene length bias correction (Young et al., 2010). GO terms with a corrected P-value of ≤ 0.05 were considered significantly enriched. Further GO enrichment analysis was carried out using the Singular Enrichment Analysis tool (SEA, http://www.broadinstitute.org/gsea/index.jsp) with a false discovery rate (FDR) and P-value ≤ 0.05. Additionally, differentially expressed genes were placed within metabolic pathways utilizing the Kyoto Encyclopedia of Genes and Genomes (KEGG), available at http://www.genome.jp/kegg/.

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