

Expression of the Genes *OsNRT1.1*, *OsNRT2.1*, *OsNRT2.2*, and Kinetics of Nitrate Uptake in Genetically Contrasting Rice Varieties

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Abstract

Four genetically contrasting rice varieties (IAC-47, Bico Ganga, Arroz de Revenda and Manteiga) according to Random Amplified Polymorphic DNA (RAPD) analysis were assessed regarding expression of the genes *OsNRT1.1*, *OsNRT2.1* and *OsNRT2.2* and the nitrate uptake kinetics parameters (K_m and V_{max}). Up to 250-fold increases in the induction of gene expression after nitrate re-supply were observed for the high-affinity transporter (*OsNRT2.1* and *OsNRT2.2*). However, no significant variations in V_{max} among the varieties were obtained. The lower value of K_m of the IAC-47 cultivar in relation to the Arroz de Revenda variety suggests a greater role of high-affinity transporter genes. These results indicate that closer attention should be paid to the expression levels of these genes in selecting varieties aiming to enhance nitrogen uptake efficiency.

Keywords

RAPD, Nitrogen, Nitrogen Use Efficiency, *Oryza sativa* L.

1. Introduction

Rice is a staple food for over half the world's people, especially in Asia, Africa and Latin America, being especially important for the sustenance of poor people [1]. However, its widespread cultivation in tropical soils with

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low availability of nitrogen (N) causes the need to develop varieties that are more efficient in the uptake and use of this element, to reduce the need for application of nitrogen fertilizers.

In studies of the nitrogen use efficiency, nitrate (NO_3^-) has great importance, because in aerated soils, it is the predominant form available to plants. The plant's capacity to uptake and accumulate NO_3^- rapidly in the initial development phase can provide a greater stock of N for metabolism in the grain-filling phase [2]-[4]. Previous articles have reported that genetically contrasting rice varieties present variations in the uptake and use of NO_3^- , affecting the nitrogen use efficiency [4] [5]. Therefore, characterization of the kinetics parameters and expression levels of the NO_3^- transporter genes is important to guide selection of varieties suitable for various growing conditions.

The objective of this study was to select, among local varieties traditionally grown in the Brazilian state of Maranhão, four genetically contrasting ones for determination of the N uptake kinetics parameters and analysis of the expression of the genes that encode transporter proteins of nitrate of low (*OsNRT1.1*) and high-affinity (*OsNRT2.1* and *OsNRT2.2*).

2. Materials and Methods

2.1. Genotypes Selection by RAPD (Random Amplified Polymorphic DNA)

Seeds of 66 rice varieties (56 landraces and 10 improved), both Brazilian and imported (**Electronic Supplementary Material**), were disinfested with sodium hypochlorite (2%) and germinated on gauze in distilled water. Five days after germination (DAG), the seedlings were collected and used to extract total genomic DNA [6]. For the polymerase chain reactions (PCRs), 26 primers were selected from those described by Araújo *et al.* [7], Areias *et al.* [8], Bhuyan *et al.* [9] and Rabbani *et al.* [10].

After tests and amplification condition adjustments, seven primers (OPA 04, OPA 07, OPA 19, OPD 10, OPE 17, OPH 05, OPP 02) were chosen based on the number of polymorphic bands and band reproducibility profile. The PCRs were performed in 20 μL of material, containing 25 ng of genomic DNA, 2.3 mM of MgCl_2 , 0.2 mM of dNTP, 0.2 μM of each primer, one unit of Taq recombinant polymerase DNA (Invitrogen) and 2 μL of the enzyme's buffer, with the volume completed by adding ultrapure water (Invitrogen), at temperatures according to Areias *et al.* [8].

The PCR products were revealed by electrophoresis in 1.5% agarose gel, prepared using TBE 1/2 X, for two hours at 100 V and then stained in an ethidium bromide solution (10 $\mu\text{g}\cdot\text{mL}^{-1}$). The gel images were analyzed using the GelCompar II software (Applied Maths) and the band profiles were used to generate a genetic similarity matrix using the coefficient of Jaccard [11]. Finally, a genetic similarity dendrogram was plotted using the unweighted pair group method with arithmetic mean algorithm (UPGMA).

2.2. Evaluation of Gene Expression of Nitrate Transporter

To analyze the expression of the nitrate transporter genes with low (*OsNRT1.1*) and high affinity (*OsNRT2.1* and *OsNRT2.2*) and to determine the uptake kinetics parameters, four genetically contrasting rice varieties were selected (IAC-47, Bico Ganga, Arroz de Revenda and Manteiga) from the genetic similarity dendrogram obtained by Random Amplified Polymorphic DNA (RAPD) analysis (**Figure 1**). The seeds were disinfested and germinated as described previously. Fourteen days after sowing, the seedlings, measuring about 10 cm in height, were transferred to plastic pots (four seedlings each) containing 600 mL of the nutrient solution of Hoagland and Arnon [12] modified to 2 $\text{mmol}\cdot\text{L}^{-1}$ of NO_3^- -N and other nutrients, with one-fourth the normal ionic strength (IS). Four days later, the nutrient solution was modified to 1/2 IS, while the NO_3^- -N was maintained at 2 $\text{mmol}\cdot\text{L}^{-1}$. The solution was renewed every three days thereafter. On 30 DAG, the plants were subjected to 72 h of total N deprivation, while maintaining the other nutrients at 1/2 ionic strength. After the deficiency period, the N supply was resupplied to one group of plants, with 0.2 $\text{mmol}\cdot\text{L}^{-1}$ of nitrate, while the other group was kept in the solution without N. Root samples were collected 3, 6 and 9 h after resupply and stored at -80°C for subsequent total RNA extraction.

The total RNA was extracted according to Gao *et al.* [13] and the synthesis of the first cDNA strand was carried out from 0.5 μg of RNA, in a 96-well thermocycler (Eppendorf Mastercycler) using the High-Capacity cDNA Reverse Transcription Kit according to the manufacturer's recommendations. The PCRs in real time were performed in duplicate in a Step One Plus Real Time PCR System using the SYBR[®] Green PCR Master Mix kit

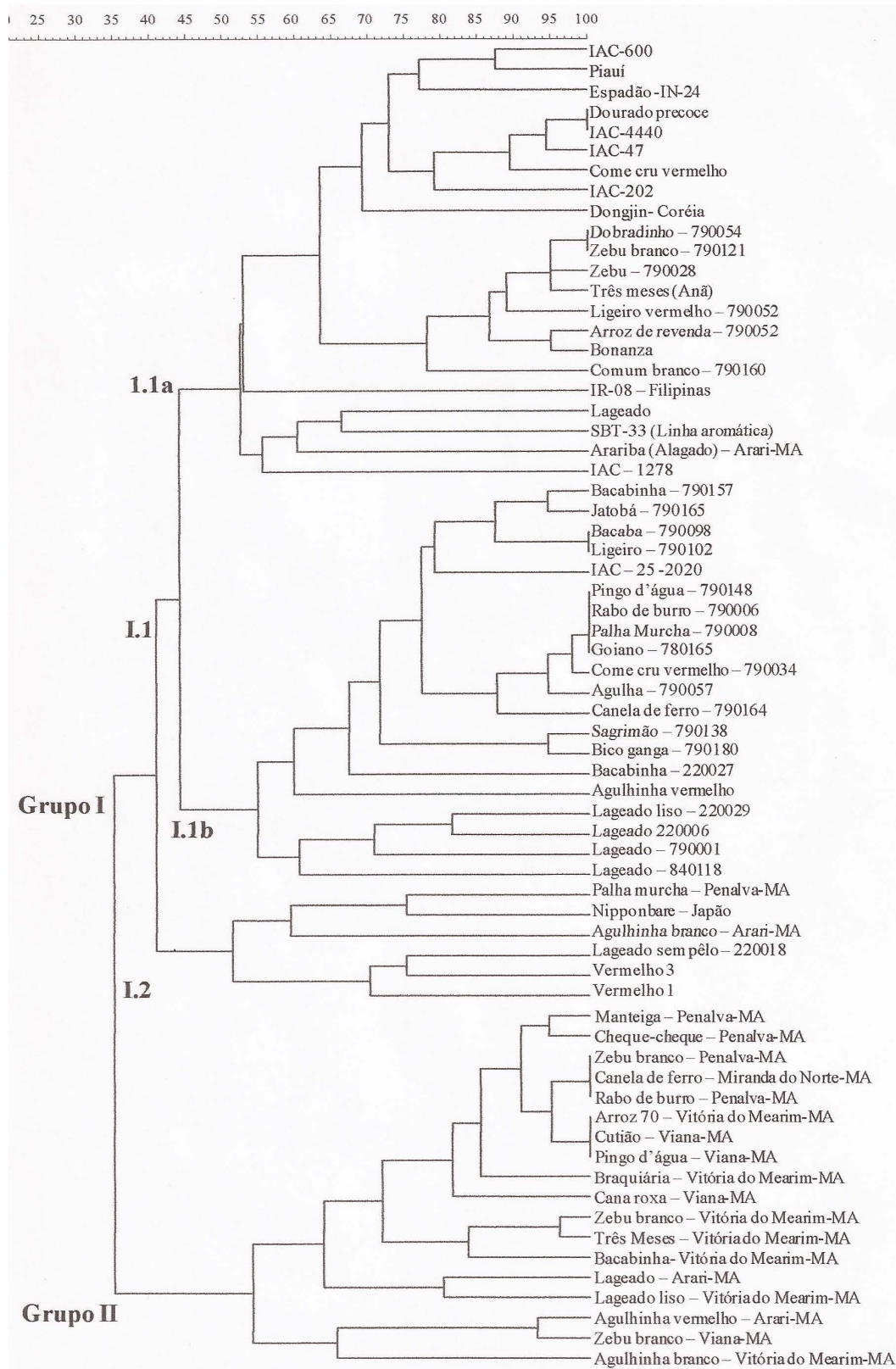


Figure 1. Genetic similarity dendrogram based on the amplified DNA products of 66 rice varieties by RAPD, according to the Jaccard similarity coefficient.

(Applied Biosystems), according to the manufacturer's recommendations. The sequences of the primers defined by Sperandio *et al.* [14] were used to amplify the nitrate transporters genes. Actin gene (*OsAct*) was used as an endogenous control [15]. The relative expressions were calculated according to Livak and Schmittgen [16] by relativization of the expression levels of the plants after resupply in function of the levels of those without resupply, so as to measure the levels of induction by the NO_3^- ion.

2.3. Nitrate Uptake Kinetics Assay

For the nitrate uptake kinetics assay, the Manteiga variety was excluded due to the absence of viable seeds. The germination of seeds of the other three varieties and the cultivation conditions in the growth chamber were the same as for the gene expression analysis. Immediately after N resupply and then each 30 minutes for the next 8 hours, 0.5-mL aliquots of nutrient solution were collected from each pot. At the end of this period, the volume of solution remaining in each pot was measured and the plants were collected and placed to dry at 60°C in a forced-air chamber, after which the dried material was weighed.

The nitrate concentrations in the solution were determined according to Cataldo *et al.* [17] and were used to plot ion depletion curves and to determine the nitrate uptake kinetics parameters (K_m and V_{max}) through the mathematical graphing method proposed by Ruiz [18], using the Cinética Win software developed by Ruiz and Fernandes Filho [19]. The values of K_m and V_{max} obtained for each replicate were submitted to analysis of variance and were used to estimate the NO_3^- influx rates, using the Michaelis-Menten equation, as described by Epstein and Bloom [20].

3. Results

3.1. Selection of Contrasting Genotypes by RAPD

The genetic similarity dendrogram obtained from the RAPD analysis presented two large groups (I and II) (Figure 1). Group I was highly heterogeneous regarding the origin of the varieties analyzed, including improved Brazilian and imported cultivars, new accessions to germplasma banks and a few from crops grown in the state of Maranhão.

The Piauí variety showed 88% similarity with IAC-600, a well-known variety called “black rice” developed by the Agronomic Institute of Campinas (São Paulo, Brazil). Although heterogeneous in its magnitude, Group I contained successive subdivisions that significantly separated the varieties included in it. Subgroup I.1a contained varieties with 100% similarity: Dourado Precoce and IAC-4440; Dobradinho and Zebu Branco. The improved variety IAC-47 was 95% similar to Dourado Precoce and IAC-4440. All the improved varieties were clustered in Group I. Most of the varieties with the name Lajeado from germplasma banks were also in Group I (Figure 1). It should be highlighted that Lageado Liso-220,029 and Lageado-220,006 had similarity greater than 80%, as also observed by Areias *et al.* [8].

In turn, Group II presented the particular characteristic that it only contained varieties from crops grown in the county of Arari, Miranda do Norte, Penalva, Vitória do Mearim and Viana in Maranhão, suggesting a close genetic base for the varieties grown in that region (Figure 1).

Therefore, the similarity dendrogram allowed selecting four genetically contrasting rice varieties (IAC-47, Bico Ganga, Arroz de Revenda and Manteiga) for analysis of expression of the genes *OsNRT1.1*, *OsNRT2.1* and *OsNRT2.2* and determination of the nitrate uptake kinetics parameters.

3.2. The Expression Levels of Nitrate Transporter

Three hours after the resupply of nitrate, increases were observed of between 40 and 250 times in the expression of the *OsNRT2.1* and *OsNRT2.2* genes in relation to the plants maintained without nitrogen (Figure 2(a) and Figure 2(b)), while for *OsNRT1.1* the expression levels were two times higher (Figure 2(c)). In general, *OsNRT2.1* and *OsNRT2.2* were strongly induced with the resupply following the suppression after 3 hours, while interestingly *OsNRT1.1* presented higher expression in some varieties 6 and 9 hours later, suggesting a slower induction response (Figure 2(c)).

In the Arroz de Revenda and Manteiga varieties, the induction of expression of the *OsNRT2.1* and *OsNRT2.2* genes was greater with the resupply and was longer lasting (Figure 2(a) and Figure 2(b)), suggesting better adaptation to conditions of low soil nitrate levels.

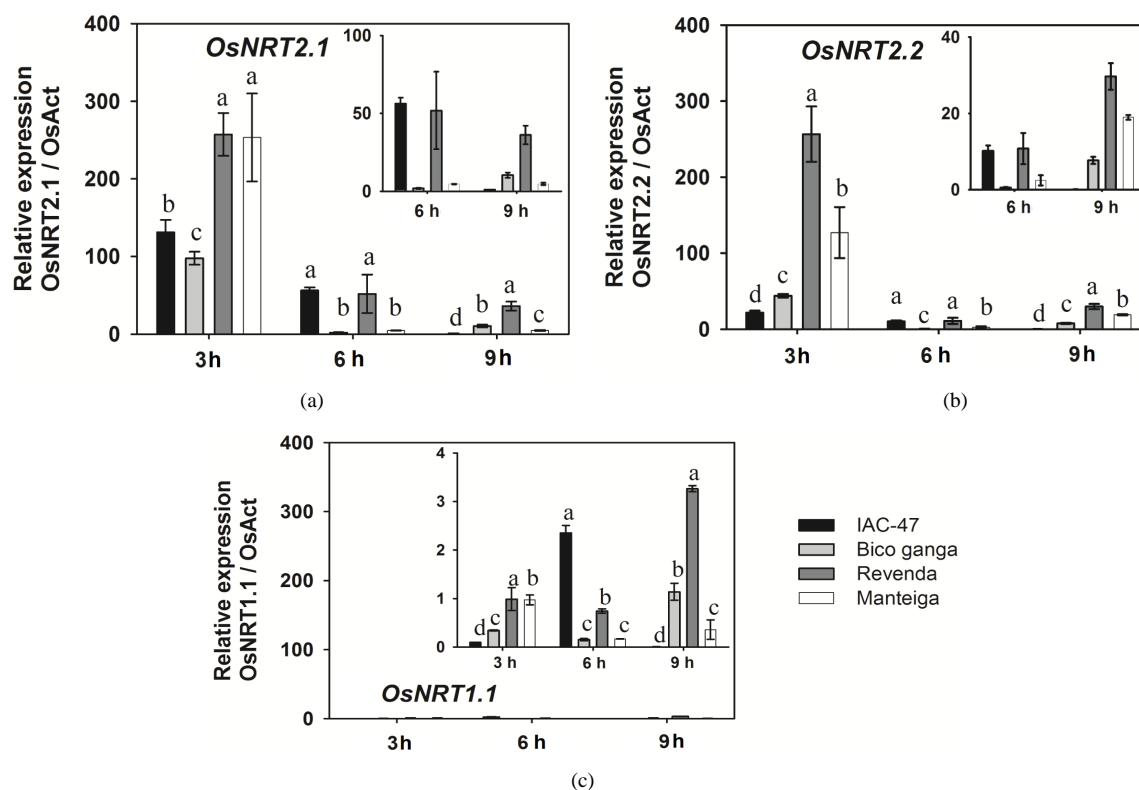


Figure 2. Relative expression of the nitrate transporters *OsNRT2.1* (a), *OsNRT2.2* (b) and *OsNRT1.1* (c) in the roots of four rice varieties at 3, 6 and 9 h after resupply of 0.2 mmol·L⁻¹ of NO₃⁻-N. The expression levels are in relation to the reference gene (*OsAct*) and the plants maintained without N. The inserts show expanded details of the graphs. Vertical bars indicate the standard deviation from the mean of three replicates.

3.3. Kinetic Parameters of the Nitrate Uptake

Due to the large differences regarding the induction of the NRTs, especially observed 3 hours after resupply, we also expected to see large differences in the nitrate uptake capacity between the varieties. However, no difference was observed between the varieties with respect to the curves of nitrate depletion in the nutrient solution and the uptake isotherms (Figure 3). There were no significant differences of V_{max} between the treatments. The K_m value of the Arroz de Revenda variety was higher than that of IAC-47, which had the lowest value of this parameter (Table 1).

4. Discussion

All the varieties studied presented higher expression of the *OsNRT2.1* and *OsNRT2.2* genes 3 hours after resupply. This result does not agree with that observed by Araki and Hasegawa [21] for the Nipponbare rice cultivar, for which these genes were induced by nitrate and exhibited peak expression about 3 hours after resupply. However, Hu *et al.* [22], studying *Arabidopsis thaliana*, observed that the peak induction for *AtNRT2.1* and *AtNRT1.1* was reached only 30 minutes after resupply of nitrate, suggesting that the response time of the orthologues of NRTs can vary greatly from one species to another.

It is interesting to observe that the varieties with the highest levels of *OsNRT2.1* and *OsNRT2.2* transcripts during the study period were in nearly all cases those with the highest levels of *OsNRT1.1* transcripts, principally at the intervals of 6 and 9 hours for the IAC-47 and Arroz de Revenda varieties. This relation can be due to the role of NRT1.1 as a constituent of the signaling mechanism for recognizing the presence of nitrate in the solution, as demonstrated in *Arabidopsis* [23].

According to Girin *et al.* [24], the expression of NRT2.1 is strongly suppressed by nitrate assimilation products. Therefore, the effects of induction by nitrate, free of suppression by feedback, explain the explosive

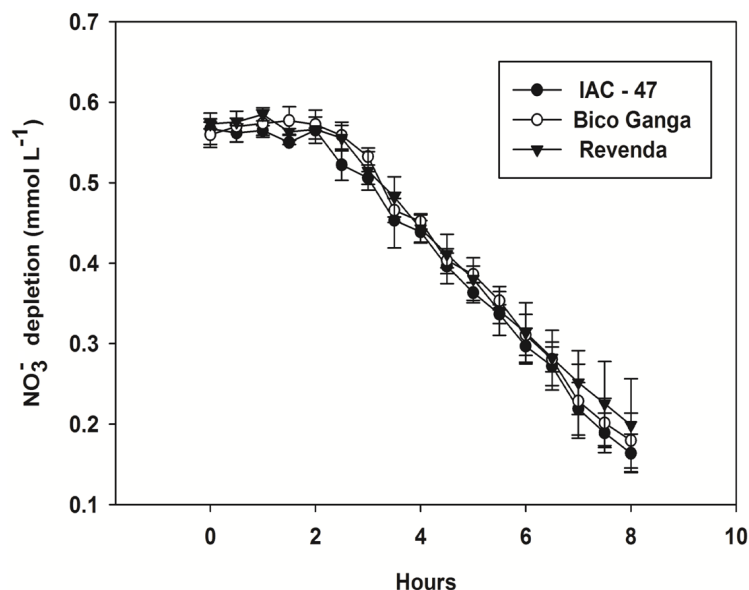


Figure 3. Nitrate depletion of the nutrient solution with $0.5 \text{ mmol}\cdot\text{L}^{-1}$ of NO_3^- -N in three rice varieties. Each point represents the mean of three replicates. Vertical bars indicate the standard deviation from the mean of three replicates.

Table 1. Nitrate uptake kinetics parameters of three rice varieties after resupply with $0.5 \text{ mmol}\cdot\text{L}^{-1}$ of NO_3^- -N.

Varieties	Kinetics parameters	
	V_{max} ($\mu\text{mol}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$)	K_m ($\mu\text{mol}\cdot\text{L}^{-1}$)
IAC-47	123.28 a	106.23 b
Bico Ganga	137.22 a	129.38 ab
Arroz de Revenda	148.53 a	201.32 a

Equal letters in the column do not differ between each other at 5% probability by the Tukey test. The values are the means of three replicates.

response of the *OsNRT2* genes (induction of up to 250 times) in the first hours. According to Wirth *et al.* [25], the *OsNRT2.1* transporter is responsible for absorption of up to 75% of the available nitrate in *Arabidopsis thaliana*, while the huge differences regarding the induction response levels of the *NRT2s*, as observed for the varieties evaluated here, are not reflected in significant differences between the nitrate depletion curves (Figure 3), or between the V_{max} values (Table 1) found in the second experiment. Regarding K_m , the lower value obtained for the IAC-47 cultivar in relation to Arroz de Revenda (Table 1) suggests greater action of transporters with higher affinity. However, as demonstrated by Feng *et al.* [26], it should be considered that to promote the absorption of nitrate, the *OsNRT2.1/2.2* carriers require the participation of the protein NAR codified by the *OsNAR2.1* gene, which was not evaluated in this study.

Other factors also might be involved in these varieties, balancing the capacity to uptake nitrate in detriment of differences in the levels of the transporters. Liu *et al.* [27], studying the relative contribution of size and root activity for uptake of N between two contrasting strains of corn regarding N uptake efficiency, observed that the inefficient variety presented higher and more persistent expression levels of *ZmNRT1.1*, *ZmNRT2.1*, *ZmNRT2.2* and *ZmNAR2.1*, while greater uptake efficiency was attributed to larger size and stronger growth response of the root system. Therefore, a more extensive root system in a variety with a lower *NRT* expression level could balance its capacity to absorb nutrients versus the other with high expression level. Nevertheless, we found no significant differences between the varieties regarding root dry mass. Therefore, the selection of genotypes for use in programs to improve N uptake efficiency also should consider other factors, such as the root system mor-

phology.

Taken together, the data presented here demonstrated that expression of the high-affinity nitrate transporter genes of genetically contrasting rice varieties increased after resupply with this ion, with the highlight being the Arroz de Revenda variety, for which the expression induction was up to 250 times.

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Electronic Supplementary Material

List of rice varieties, including 56 landraces and 10 improved, used in the study with their accession number and origin, if known.

Rice varieties	Accession number	Origin	Rice Varieties	Accession number	Origin
Agulhinha branco	-	Arari-MA	Pingo d'água-48	790148	-
Agulhinha vermelho	-	Arari-MA	Rabo de Burro	790006	-
Zebu branco	-	Viana-MA	Come cru vermelho-34	790034	-
Lageado	-	Arari-MA	Palha murcha-08	790008	-
Agulhinha branco	-	Vitória do Mearim-MA	Agulha	790057	-
Cana roxa	-	Viana-MA	Goiano	780165	-
Arroz 70	-	Vitória do Mearim-MA	Lageado-18	840118	-
Braquiária	-	Vitória do Mearim-MA	IAC-25*	2020	-
Cutião	-	Viana-MA	Ligeiro	790102	-
Manteiga	-	Penalva-MA	Sagrimão	790138	-
Zebu branco	-	Penalva-MA	Bico Ganga	790180	-
Rabo de burro	-	Penalva-MA	Comum branco	790160	-
Cheque-cheque	-	Penalva-MA	IAC-1278*		IAC-SP
Canela de Ferro	-	Miranda do Norte-MA	Dobradinho	790054	-
Pingo d'água	-	Viana-MA	Ligeiro vermelho	790052	-
Bacabinha	-	Vitória do Mearim-MA	Zebu branco-21	790121	-
Zebu branco	-	Vitória do Mearim-MA	Arroz de revenda	790088	-
Lageado liso	-	Vitória do Mearim-MA	Zebu	790028	-
Três meses	-	Vitória do Mearim-MA	IR-08*	-	Filipinas
Palha murcha	-	Penalva-MA	Três meses anã	-	-
Nipponbare	-	Japão	Lageado	-	-
Vermelho 01	-	-	Bonanza	-	-
Lageado sem pêlo	220018	-	Come cru vermelho	-	-
Vermelho 03	-	-	IAC-202*	-	IAC-SP
Agulhinha vermelho	-	-	IAC-47*	-	IAC-SP
Lageado-01	790001	-	Dourado Precoco	-	-
Lageado liso	220029	-	IAC-4440*	-	IAC-SP
Bacabinha	790157	-	IAC-600*	-	IAC
Jatobá	790165	-	Espadão	IN-24	-
Bacaba	790098	-	SBT-33 (aromático)*	-	-
Bacabinha	220027	-	Arariba (Alagado)	-	Arari-MA
Canela de Ferro	790164	-	Dongjin*	-	Coréia
Lageado	220006	-	Piauí	-	-

Varieties used in previous studies Souza *et al.* (1998), Ferraz Junior *et al.* (2001), Araújo *et al.* (2003), Areias *et al.* (2006) and Santos *et al.* (2007) or provided by ou fornecidas pelo CENARGEM (Embrapa), CNPAF (Embrapa) or collected in the Maranhão State (Brazil). The accession number of germplasm bank is presented when available. *Improved varieties.

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