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Transcriptional modulation of genes encoding nitrate reductase in maize (*Zea mays*) grown under aluminum toxicity

Talita Cantú, Crislaine Emidio Vieira, Rafaélla David Piffer, Giovanna Carneiro Luiz and Silvia Graciele Hülse de Souza*

Laboratory of Molecular Biology, University Paranaense, Umuarama, 87502-210, Brazil.

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The free aluminum (Al) content in soil can reach levels that are toxic to plants, and this has frequently limited increased productivity of cultures. Four genes encoding nitrate reductase (NR) were identified, named *ZmNR1–4*. With the aim of evaluating NR activity and the transcriptional modulation of the *ZmNR1*, *ZmNR2*, *ZmNR3,* and *ZmNR4* genes in leaves, 30-day-old hybrid maize BRAS 3010 plants were irrigated with a solution of $Al_2(SO_4)_3.18H_2O$ for 16 days. The transcriptional levels of *ZmNR2*, *ZmNR3,* and *ZmNR4* and NR activity will exhibit standard changes similar in the leaves, where, from the second week of stress onwards, there was a decrease in enzymatic activity and in the accumulation of transcripts. An increase *ZmNR1* mRNA levels were observed, indicating that this gene may be associated with other metabolic pathways. This study resulted in the identification and characterization of different genes that encode NR and are involved in nitrogen metabolism in maize, in which the *ZmNR2*, *ZmNR3,* and *ZmNR4* genes regulate the activity of NR in response to aluminum stress. The characterization of these genes may help in our understanding of the genetic-molecular and physiological mechanisms of maize subjected to aluminum stress.

Key words: Abiotic stress, Al, gene expression, nitrogen, metal toxicity, mineral nutrition.

INTRODUCTION

Maize (*Zea mays*) is the main cereal produced in Brazil. In 2015/2016 season, the cultivated area of maize was 15.7 million hectares with a total production of 76.2 million tons of grain (CONAB, 2016). Nowadays, Brazil is the world's third largest producer of maize, only behind the United States and China (USDA, 2016). The economic importance of maize is due to the different ways it is used, including its wide consumption as food

*Corresponding author. E-mail: silviahulse@unipar.br.

and its use in technological industries as a raw material for films, biodegradable packages, and biofuel (Boone et al., 2016).

Toxicity induced by aluminum (Al) is the main factor limiting agricultural productivity in acidic soils (pH< 5.5) (Garzón et al., 2011). Estimates have revealed that approximately 50% of global arable land that is potentially usable for food and biomass production is acidic and, thus, subjected to Al^{3+} toxicity (Kochian et al., 2004; Ma, 2007).

The primary manifestation of Al phytotoxicity is the inhibition of root growth and interference in the absorption, transportation, and use of water, and in mineral nutrition and metabolic changes, such as organic acid and nitrogen metabolism (Matsumoto, 2000; Azmat and Hasan, 2008; Azmat et al., 2015). As a consequence, important enzymatic processes are affected, among which the activity of nitrate reductase (NR), and consequently nitrogen absorption, are notable. Nitrogen is an essential nutrient required in large amounts by plants (Zhao et al., 2016). This element acts as an essential nutrient and regulates root development, leaf expansion and expression of genes encoding enzymes involved in carbon and nitrogen metabolism (Wang et al., 2003). Nitrogen (N) is used to form glutamine, which is considered as a precursor for the synthesis of many amino acids, nucleic acids, enzymes, and proteins, as well as secondary metabolites (Bagh et al., 2004; Kusano et al., 2011). Despite the high levels of N in the Earth's atmosphere, most living organisms do not have the capacity to absorb this element in a gaseous form. This explains why plants use nitrate and ammonia as inorganic sources of nitrogen, which are absorbed by the roots (Wickert et al., 2007).

The varied forms and concentrations of nitrogen present in the soil have led plants to evolve strategies to locate and absorb this mineral in better ways. To regulate the demands of N incorporation in plants, gene families encode products with functions that are directed by enzymes. In this case, nitrate reductase (NR, EC1.6.6.1), which is the first enzyme is involved in nitrogen assimilation (Liseron-Monfils et al., 2013). Nitrate (NO_3^-) is reduced to nitrite (NO_2^-) in the cytosol, and is catalyzed by NR. It is then converted to ammonium (NH_4^+) by the nitrite reductase enzyme (NiR, EC 1.7.7.1) and incorporated into amino acids by glutamine synthetase (GS, EC 6.3.1.2) and glutamate synthetase (GOGAT, EC 1.4.1.14), forming glutamine and glutamate, which are essential to amino acids and are required for protein synthesis (Crawford, 1995; Debouba et al., 2013).

In *Arabidopsis,* two genes, NIA1 (At1g7760) and NIA2 (At1g37130), have been identified and responsible for nitrate reduction. NIA2 is the main gene, responsible for 90% of the enzymatic activity (Wilkinson and Crawford, 1991; Zhouand Kleinhofs, 1996; Debouba et al., 2013). Light is known to induce the expression of the NR genes (Tischner, 2000; Lillo et al., 2001). Liseron-Monfils et al. (2013) identified four expression clusters of the genes that encode the NR enzyme, where each one of these genes has a specific function during the plant's life. Studies have shown that aluminum can inhibit root growth in maize and cause protein oxidation, which triggers changes in various biochemicals, physiological, and genetic processes (Boscolo et al., 2003; Purcino, et al., 2003; Souza et al., 2016). There is insufficient Information in the literature in respect to the transcriptional regulation of nitrate and nitrogen assimilation in maize subjected to aluminum stress. The aims of the present study were to evaluate the expression of genes encoding NR and to investigate the modulation of NR activity in maize leaves exposed to aluminum toxicity.

MATERIALS AND METHODS

Identification of nitrate reductase (NR) genes in maize

NR protein sequences have been identified in the model plant *Arabidopsis thaliana* (At1g37130 and At1g77760) and were obtained from the TAIR database (http://www.arabidopsis.org/) and was used for studies on the Phytozome Database of *Z. mays* (https://phytozome.jgi.doe.gov/pz/portal.html), using the program BlastP (Altschul et al., 1997). The sequences were blasted against others deposited in the National Center for Biotechnology Information (NCBI) GenBank Database (http://www.ncbi.nlm.nih.gov), using the programs BlastP and BlastX, to confirm their identity. The sequences deduced from amino acids were obtained using the program Open Reading Frame Finder (ORF Finder; NCBI, http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The protein sequences were aligned utilizing Clustal Omega algorithm version 2.0.3 (Sievers et al., 2011).

Characteristics of predicted NR proteins in maize

The physical and chemical characteristics of NRs in maize were determined using the tool ProtParam online (http://web.expasy.org/protparam), including the number of amino acids (AA), molecular weight (MW), and theoretical isoelectric point (PI).

Phylogenetic analysis

Phylogenetic analysis was performed by aligning NR protein sequences using the algorithm Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Searches for similar proteins were performed in the program BlastP (http://www.ncbi.nlm.nih.gov). The phylogenetic tree was constructed using the neighbor-joining (NJ) method and also using the pair-wise deletion option with help from MEGA version 6.0 (Tamura et al., 2013). One-thousand bootstrap replicates were utilized to test for confidence.

Table 1. Sequences of primers used for semi-quantitative RT-PCR analysis of nitrate reductase (NR) genes.

Genes	Forward primer 5'-3'	Reverse primer 5'-3'	Tm (˚C)	N˚ of cycles
ZmNR1	GAGTCCGACAGCTACTACCA	GATCACCGAGTTGATGTTGAG	60	30
ZmNR2	CCGAGTCCGACAATTACTACC	CGTTATCACCGAGTTTATGTTCAG	60	30
ZmNR3	TACGATCAAAGGATACGCATACTC	CTTGCCGTACTTGGTTGGAC	60	30
ZmNR4	CGACAACTACTACCATTACAAGGA	CGTTATCACCGAGTTTATGTTCAG	60	30
ZmUBQ	TGGTTGTGGCTTCGTTGGTT	GCTGCAGAAGAGTTTTGGGTACA	60	30

Vegetal material

Seeds of hybrid maize BRAS 3010 were planted in pots with a 1-kg capacity containing soil and vermiculite at 1:1, and were watered weekly with the nutritive solution as proposed by Hoagland and Arnon (1950). The experiment was conducted in a greenhouse. Thirty-day-old plants were irrigated with a solution of aluminum sulfate (Al_2 $[SO_4]_3.18H_2O$) on the first and second days at a concentration of 50 and 100 µM, respectively. From the third day until the end of the experiment, the plants were irrigated daily with 150 µM aluminum sulfate (Pimenta et al., 1989). Simultaneously, the same amount of pots with material been irrigated with water (control) was kept. Samples were obtained on days 0, 2, 4, 8, and 16. At each sampling (control and stress), leaves were collected (three pools of leaves from different plants; under the same stress conditions where each pool constituted a biological repetition). The collected materials were immersed immediately in liquid N and stored in a freezer at -80°C.

Activity of NR

In vitro determination of NR activity in maize leaves was performed according to the methods proposed by Jaworski (1974) and Carelli et al. (1990). Incubation solution (buffer phosphate 0.1 M pH 7.5, KNO_3, n-propanol 3%) was added to the materials collected *in vivo*, and samples were then filtered by vacuum and incubated in a warm bath at 30°C for 40 min. After that, sulfanilamide and n-naftil-etilene-di-amino solution was added and the concentration of the nitrite formed was determined using a spectrophotometer at 540 nm.

Amino acids

The concentration of amino acids was determined using the method proposed by Praxedes et al. (2006) with a few modifications. A sample of 20 mg vegetal material was weighted, 250 µL 80% ethanol was added, and the samples were incubated in a warm bath at 80°C for 20 min in a microcentrifuge, following which the supernatant was removed. This procedure was repeated twice. The samples were then placed on ice. To prepare the standard curve, 60% ethanol, sodium citrate, and ninhydrin were mixed and this solution was placed in a warm bath at 90°C for 20 min. Then, 100 µL of each sample was added to 150µL 60% ethanol, 250 µL sodium citrate (0.2% ascorbic acid pH 5.2%), and 500 µL ninhydrin, placed in a warm bath and cooled on ice before being read in a spectrophotometer at 570 nm.

RNA extraction and cDNA synthesis

Total RNA was extracted from maize leaves utilizing the SV Total RNA Isolation System commercial kit (Promega, Madison, USA) following the manufacturer's instructions. RNA integrity was analyzed by electrophoresis in 1.2% agarose gel. The first cDNA was synthesized utilizing 2 µL of the total purified RNA and the GoScript™ Reverse Transcription System commercial kit (Promega, Madison, USA) and oligo primers $(dT)_{15}$following the manufacturer's instructions.

Semi-quantitative analysis by RT-PCR

The sequences of primers utilized for semi-quantitative RT-PCR analysis of *ZmNR* and *ubiquitin* (*ZmUBQ*) (Christensen and Quail, 2005), which was used as a control, given in Table 1. RT-PCR was performed in a final volume of 15µL, containing: 1.5 µL 10× PCR buffer, 0.8 µL $MgCl_2$ (50 mM), 0.5 µL dNTPs (10 mM), 0.5 µL each primer (10 mM), 0,4 µL*Taq DNA polymerase* (5U),and 2 µL cDNA diluted 1:10. The reactions were performed in a thermocycler model AG 22331 (Eppendorf, Hamburg, Germany), with the following conditions: one initial cycle of 94°C for 5 min and 30 cycles of: 94°C for 30 s for denaturing, 60°C for 40 s for annealing, 72°C for 30 s for extension, and one final cycle of 72°C for 10 min (Table 1). The quantification of gene transcripts was standardized using the constitutively expressed gene *ZmUBQ* as normalizer. The amplified PCR product was subjected to electrophoresis in agarose gel 1.2% stained with ethidium bromide. The gel images were captured by a photo documenting systems, L-PIX Molecular Imaging (Loccus Biotecnologia, Cotia, Brazil) and analyzed by densitometry (Freschiet al., 2009). IMAGEJ 1.46 (http://rsbweb.nih.gov/ij/download.html) software was used to quantify band intensities.

Statistical analysis

The results were evaluated and submitted to analysis of variance (ANOVA) and the averages were compared by Tukey's test ($p \leq 0.05$) using the statistical software SISVAR (Ferreira, 1999).

RESULTS AND DISCUSSION

Nitrogen assimilation is crucial for plants, and the enzyme NR plays an important role in this pathway. In higher plants, this enzyme has been structurally, biochemically, and genetically well defined (Ovečka and Takáč, 2014). Based on *in silico* analyses of the Phytozome Database, four NRs were identified and named *Zea mays* NR (*ZmNR1–4*). The complete cDNA sequences of Zm*NR1* (GRMZM2G568636), Zm*NR2* (GRMZM2G428027),

Table 2. Physicochemical characteristics of *ZmNR* genes.

Genes	Nucleotide CDS bp	Amino acids	Molecular weight	Isoelectric point
ZmNR1	2733	910	101,57	6,37
ZmNR2	2634	877	97,59	6,36
ZmNR3	2673	890	98,52	6,39
ZmNR4	2802	933	103,04	6,32

AA, amino acid; bp, base pair; CDS, coding sequence; MW, molecular weight (kDa); pI, isoelectric point.

Table 3. Comparison among the predicted complete sequences of NR Amino acids of *Zea mays* (*ZmNR*) cDNAs. The identity values are over the diagonal whereas the similarity values are under the diagonal.

Genes	Identity and Similarity of Amino acids Percentage (%)			
	ZmNR1	***ZmNR2***	***ZmNR3***	***ZmNR4***
ZmNR1	1.00	0.74	0.74	0.86
ZmNR2	0.85	1.00	0.93	0.72
ZmNR3	0.85	0.86	1.00	0.71
ZmNR4	0.90	0.81	0.81	1.00

Zm*NR3* (GRMZM5G878558), and Zm*NR4* (GRMZM2G076723), contain 2733, 2634, 2673, and 2802 bp and encode proteins with 910, 877, 890, and 933 amino acid residues, respectively. The molecular weights were estimated to be 101.57, 97.59, 98.52, and 103.04 kDa, with isoelectric points of 6.37, 6.36, 6.39, and 6.32 for *ZmNR1* to *4*, respectively (Table 2). The molecular weights determined by *in silico* analyses in the present study are close to values reported previously in the literature, demonstrating that the native enzyme is a homodimer ranging from 100 to140 kDa in size depending on the species (Kuo et al., 1982; Crawford et al., 1988). The prediction of amino acid sequences of *Z. mays* in relation to the complete sequence is indicated in percentage of identity and similarity (Table 3). The identity ranged from 71% (*ZmNR3* and *ZmNR4*) to 93% (*ZmNR2* and *ZmNR3*) are presented and average as 78,3%. For similarity, the variation was 81% (*ZmNR2* and *ZmNR4; ZmNR3* and *ZmNR4*) and 90% (*ZmNR1* and *ZmNR4*) and the average of 84,7 % in all *ZmNR* studied in our work. The high level of variation in the characteristics of this protein indicates the high complexity of NR genes. The dissimilarity between the proteins may be indicative in distinct metabolic functions.

A phylogenetic analysis was performed based on the NR amino acid sequence in order to determine the relationship between the maize *NR* gene and that from other plants species, which have previously been characterized (Figure 1). The following sequences were included in the analysis: three sequences from rice (*Oryza sativa*), one each from citrus (*Citrus clementina*), sorghum (*Sorghum bicolor*), millet (*Setaria italica*), sesame (*Sesamum indicum*), barley (*Hordeum vulgare*), and vine (*Vitis vinifera*), and two from *Arabidopsis* (*A. thaliana*), in addition to the four NR sequences identified in maize. Figure 1 shows that genes encoding *ZmNR1* to *4* are in the same group as the NR genes of other monocotyledon species, while those from dicotyledonous species are in a second group. Sequences of paralogous genes were identified in the genomes of maize, sorghum, rice, barley, and millet. Phylogenetic analysis identified homologous proteins of NR from monocotyledons in dicotyledonous species. Although a comparison between mono- and dicotyledonous species is not always possible, the structure of NR genes in cereals appears to have evolved in a different way, becoming more complex than those found in dicotyledonous species (Plett et al., 2010; Buchner and Hawkesford, 2014). This may be due to the fact that, based on non-synonymic substitution rates, the estimated divergence between monocotyledon and dicotyledonous species occurred approximately 340 million years ago when fungi and plants or algae and plants were used as reference points (Zhou and Kleinhofs, 1996).

In general, functional domains are conserved in genes from the same group, and for this reason, they probably share the same functions and have a common ancestor (Kranz et al., 1998). The sequences that encode NR, *ZmNR1* to *4*, were found to contain three functional domains (Figure 2). Crawford et al. (1988) identified

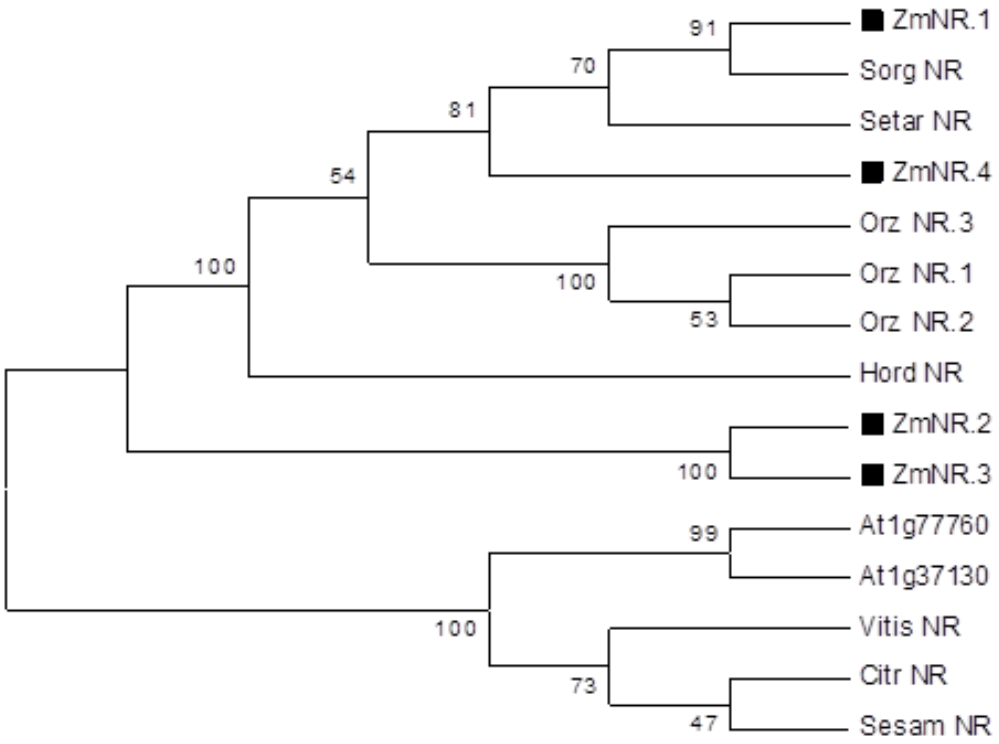

Figure 1. Phylogenetic analysis of nitrate reductase (NR)in *Zea mays* and other plants. Protein names with their respective accession numbers are: *A. thaliana* (AT1G37130, AT1G77760), *S. bicolor* SORG_NR (gi|242079443), *S. itálica* SETAR_NR (514796865), *O. sativa japonica* ORZ_NR1 (Os08g0468100), *O. sativa japonica* ORZ_NR2 (Os08g0468700), *O. sativa* ORZ_NR3 (gi|27527625), *H. vulgare* HORD_NR (326494090), *V. vinifera* NITIS_NR (526117535), *S. indicum*SENS_NR (747046092), and*C. clementina* CITR_NR (567883105). Protein sequences of maize NR are marked with ■

molybdenum binding domain in the N-terminal region of the protein, a heme domain in the central region, and a FAD binding domain in the C-terminal region of the protein. In higher plants, in NR, a molybdoheme-Flavo protein catalyzes the first and rate-limiting step of velocity in the nitrogen assimilation of the plant. NR comprises an enzymatic complex constituted by a mini electron transport chain, in which the FAD binding domain accepts two electrons from NADH or NADPH (Campbell and Smarelli 1986; Campbell and Kinghorn, 1990). The electrons are displaced by the heme domain to the molybdenum complex, which are then transferred to nitrate. The mechanisms of transcription, post-translation, and regulation are extremely complex, and are controlled by various endogenous and environmental factors (Viegas and Silveira, 2002).

NR activity in the leaves of the control plants was kept constant, and did not change significantly among the treatment days throughout the experimental period (Fig 3A). However, following the addition of $Al_2(SO_4)_3.18H_20$ to the soil, an increase in NR activity was observed up to the eighth day of treatment; however, this activity was lower by 50% than that of the control by the 16th day of treatment. The effects of aluminum toxicity on NR activity are varied, and studies have shown that plants respond differently to the same kinds of stress. A reduction in the NR activity was observed in grasses such as wheat (Foy and Fleming, 1982) and rice (Sharma and Dubey, 2005) and in the legumes, where high concentrations of Al decreased the efficiency of the nitrogen fixation (Gomes et al., 2002; Fernandes, 2006).

Some studies have shown that high concentrations of aluminum in the soil stimulate NR activity, as observed in the leguminous forage crop *Stylosanthes macrocephala* (Amaral et al., 2000) and in beans (Wang et al., 2010). Other authors have revealed that there is no change in NR activity in response to aluminum toxicity in the aerial parts of young plants of sugar cane cultivar IAC91-5155 (Carlin et al., 2012). In the present study, exposure to aluminum stress during 16 days of treatment may have negatively affected the nutrition and nitrogen assimilation in leaves. The NR activity (Figure 3A) in leaves was

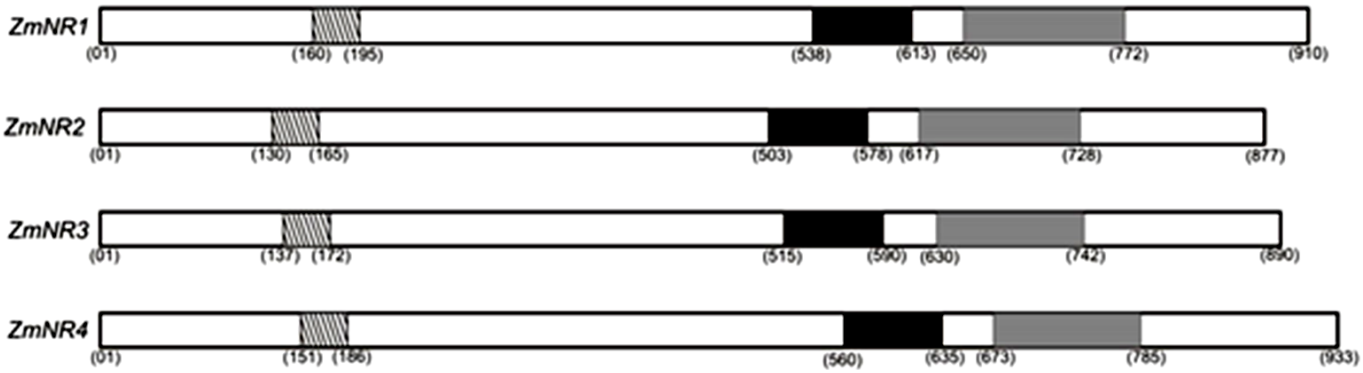

Figure 2. Structure of *ZmNR* proteins (*ZmNR1*, *ZmNR2*, *ZmNR3,* and *ZmNR4*). The stripes, black, and grey regions represent the molybdenum-pterin-binding domain, a heme-binding domain, and an FAD-binding domain, respectively. The numbers below the boxes indicate the positions of the amino acid residues.

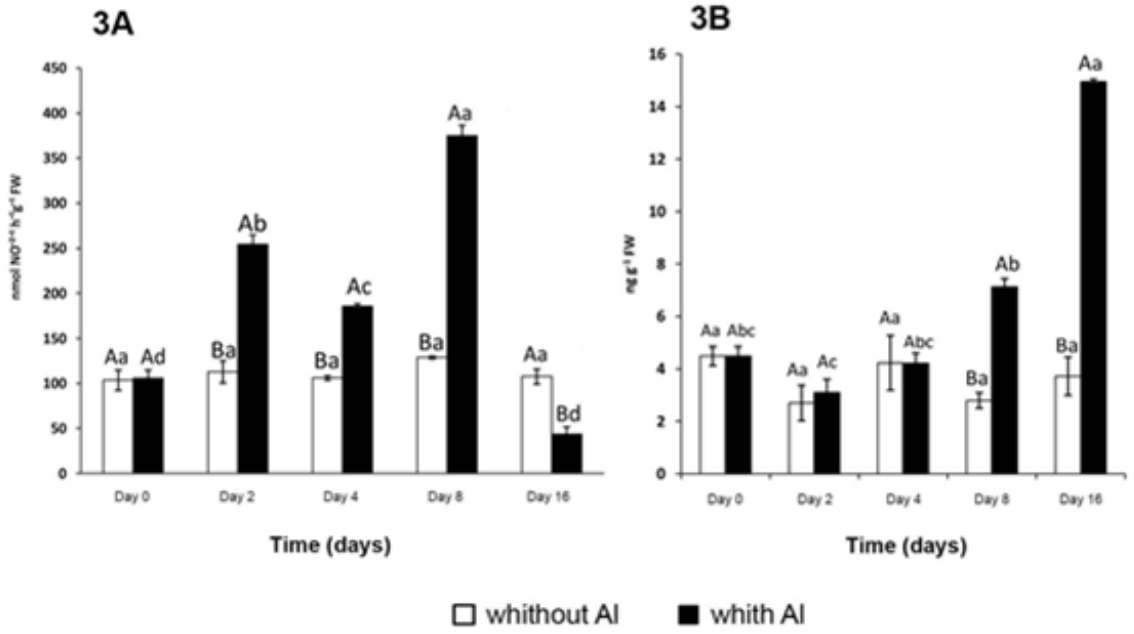

Figure 3. NR activity (3A) and Amino acid levels (3B) in maize leaves in the BRAS 3010 cultivar. Uppercase letters compare control and aluminum-stressed plants and low case letters compare the treatments days. Different letters represent significant between means at P≤ 0.05 level determined by Tukey multiple comparison procedure. The values represent the averages ± standard deviation of three biological replications.

markedly decreased in response to aluminum stress from day 16 onwards. It is possible that this reduction was the result of aluminum toxicity in the cells of the vegetal tissues. In high concentrations, aluminum in the soil can prevent the assimilation of essential nutrients into the vegetal tissues; therefore, compromising metabolism and limiting the development of the plant (Martins et al., 2011; Souza et al., 2016). The results of the control treatments, which were irrigated with water, were observed, the activity of NR did not differ significantly over time, reinforcing the hypothesis that, the toxicity caused by aluminum negatively affects the assimilation of N and consequently, NR activity in maize.

The amino acid content in response to aluminum stress was similar in the leaves of control plants and in those that were stressed until the fourth day of treatment (Figure 3B). From the eighth day of treatment, a marked increase in the amino acid content occurred in the plants

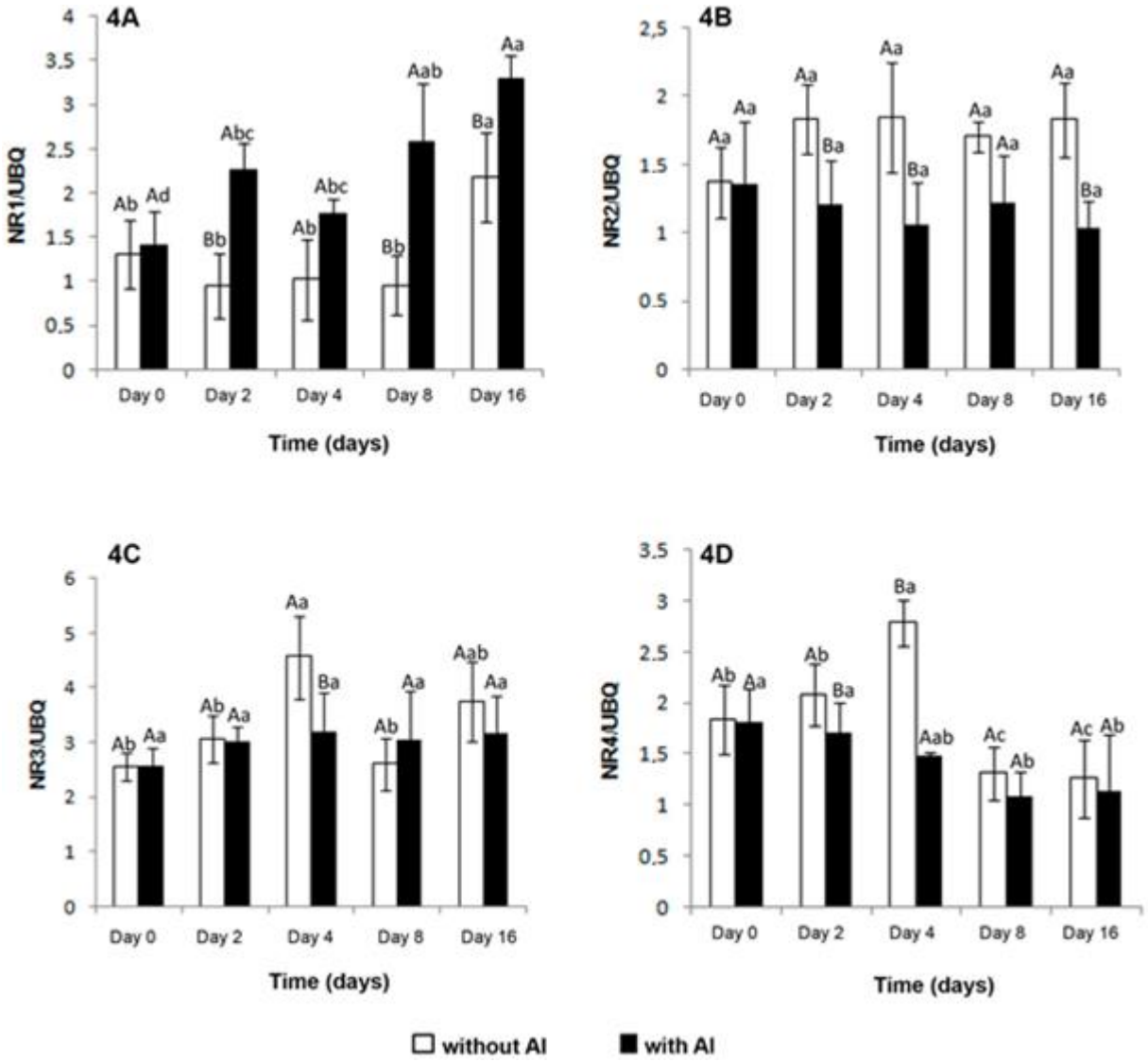

Figure 4. Expression of the *ZmNR1* (4A), *ZmNR2* (4B), *ZmNR3* (4C), and *ZmNR4* (4D) genes in response to aluminum stress in maize leaves. Plants were irrigated with water or $Al_2(SO_4)_3.18H_2O$ solution on days 0, 2, 4, 8, and 16. Uppercase letters compare control and aluminum-stressed plants and low case letters compare the treatments days. Different letters represent significant between means at P≤ 0.05 level determined by Tukey multiple comparison procedure. The values represent the averages ± standard deviation of three biological replications.

subjected to aluminum stress. The amino acid content in the leaves of plants subjected to aluminum stress was around 100 and 400% higher than that in the leaves of the control plants. The increased amino acid content in the leaves is possibly due to the relationship between amino acids and aluminum stress. Plants exposed to stress synthesize and accumulate multiple metabolites as a defense mechanism against the stress (Sharma and Dietz, 2006). Souza et al, (2016) attributed the, increase in total amino acids to an increase in protease activity, which broke the reserve proteins in the plants exposed to the aluminum toxicity, increasing the content of total soluble amino acids, aiming for osmotic adjustment. In addition, decreased NR activity also affects the concentrations of amino acids, particularly asparagine and glutamine (Prinsi et al., 2009).

To determine the effect of aluminum stress on NR genes, the corresponding RNA levels were quantified. The NR genes were found to be differentially expressed in response to stress (Figure 4). In maize, leaves subjected to aluminum stress has a decrease in the levels of *ZmNR2*, *ZmNR3,* and *ZmNR4* (Figure 4B, 4C

and 4D) mRNA relative to that of the control, while *ZmNR1* levels increased throughout the experiment (Figure 4A). The levels of *ZmNR1* transcript increased quickly following the addition of aluminum to the soil from the second day of treatment (Figure 4A). At the end of the treatment (day 16), the transcript levels of *ZmNR2* was decreased by about 50% relative to that of the control leaves (Figure 4B). Generally, the accumulation of *ZmNR3* (Figure 4C) and *ZmNR4* (Figure 4D) transcripts in leaves was lower in the plants submitted to stress as compared with the control plants; however during the days of stress the answer was kept.

Analysis of the accumulation of NR transcripts (*ZmNR1* to *4*) revealed that the levels of *ZmNR2*, *ZmNR3,* and *ZmNR4* (Figure 4B, 4C and 4D), as well as NR activity (Figure 3A) were affected in a similar way in the leaves. Therefore, from the second week of aluminum stress, there was a significant decrease in both enzymatic activity and transcript accumulation. A decrease in the levels of *ZmNR2*, *ZmNR3,* and *ZmNR4* mRNA may be associated with a parallel inhibition of NR activity by aluminum in leaves. Conversely, an increase in the levels of *ZmNR1* mRNA (Figure 4A) was observed. It appears that NR activity in response to aluminum stress is predominantly associated with *ZmNR2*, *ZmNR3,* and *ZmNR4*. The expression of *ZmNR1* was increased by aluminum stress in maize leaves. It is possible that *ZmRN1* is not involved in NR regulation; however, this gene may be involved in other metabolic pathways. Debouba et al, (2013) suggested that the transcript levels of NIA2 (At1g37130) are more important in the regulation of NR by saline stress in leaves and roots than those of NIA1 (At1g77760). Desikan et al. (2002) suggested that NIA1, which encodes NR, assures that NO produced to close the stomata is generated via a pathway mediated by ABA.

The results of this study help to reinforce previous findings showing that aluminum toxicity and its effect on nitrogen assimilation cause irreversible harm to the plant. Four genes that regulate NR activity were identified, which are directly related to post-transcriptional changes. Characterization of these genes will aid understanding of the genetic-molecular and physiologic mechanisms of maize when subjected to aluminum stress. These results will assist in programs aimed at the genetic improvement of maize, in order to obtain new aluminum-tolerant varieties. This will help to increase productivity, grain quality, and the incorporation of new agriculture areas that have not previously been used.

Conflict of interests

The authors have not declared any conflict of interests.

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