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*ARTICLE*

# Sequencing and gene expression studies for Al tolerance in contrasting genotypes of tropical maize

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**Abstract:** *The aims of this study were to evaluate the homology of the ZmMATE1 and ZmMATE2 gene sequences with those deposited in the international banks of sequences and quantify the differential expression of the genes ZmMATE1, ZmMATE2, and ZmNrat1 in germplasms of landrace and hybrid maize under aluminum (Al) stress in minimal solution. Fifty-two hybrids from different companies and 50 landrace varieties were genotyped for the genes ZmMATE1 and ZmMATE2. For studies of gene expression, the tolerant (H 44 and V 18) and the sensitive (H 22 and V 25) genotypes were exposed to minimal solution containing 4 mg $L^{-1}$ of Al for different periods (0, 1, 3, 6, 9, 12, 24, and 48 h). The results showed greater differential expression in the tolerant V 18 landrace variety of the gene ZmMATE1, indicating that exudation of citrate may be the main mechanism of Al tolerance in this genotype.*

**Keywords:** *Homologous sequences, ZmMATE1, ZmMATE2, ZmNrat1.*

## INTRODUCTION

Toxicity caused by aluminum (Al) represents one of the greatest obstacles to growing plants in acid soils. In these soils, in which pH is normally below 5.0, Al is found in the soluble form $Al^{+3}$, which is easily taken up by plant roots. The root tip is the first site of the toxic activity of the element, which interferes not only in cell division, but also in elongation of root cells, causing reduction in root growth and consequent losses in crop yield (Doncheva et al. 2005). An important physiological mechanism of Al tolerance in plants involves exudation of organic acids, activated by the presence of the stress factor. In maize, the exclusion of Al through citrate exudation by the root tip is widely recognized as the main mechanism of Al tolerance. However, Piñeros et al. (2005) describe that in addition to exudation of citrate by the roots, other physiological mechanisms, such as complexation of Al in the cell vacuoles and modification of the pH of the rhizosphere, may be involved in tolerance to the element.

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In an attempt to clarify the molecular basis of Al tolerance in maize, Maron et al. (2010) identified and characterized two members of the MATE (Multidrug and Toxic Compound Extrusion) family, *ZmMATE1* and *ZmMATE2*, as genes of greater effect on Al tolerance in maize. The gene *ZmMATE1* is a functional homolog of the Al tolerance gene in sorghum, *SbMATE*. Furthermore, characterization of

*ZmMATE2* showed that this gene is different from *ZmMATE1* and from other MATEs involved in Al tolerance in various aspects. Guimarães et al. (2014) identified the gene *ZmNrat1*, a homolog to the gene *OsNrat1* that codifies a specific Al transporter previously involved in tolerance in rice. Studies showed that expression of the gene *ZmNrat1* is rapidly induced in tolerant maize genotypes, whereas in sensitive genotypes, it has late expression.

Local races or landraces are important genotypes for breeding programs as they may provide for adaptation to specific environmental conditions. Coelho et al. (2016) investigated Al tolerance in hybrid maize and maize landraces. Comparison of the germplasms confirmed the superiority of the maize landrace germplasm regarding Al tolerance. According to the authors, the differences observed in Al tolerance may reflect the continuous cycles of natural and artificial selection that these varieties have undergone in their respective origin/growth environments.

The study of expression of genes responsible for Al tolerance is of extreme importance, and genomic approaches may make a considerable contribution toward understanding the mechanisms involved in tolerance. In this respect, the hypothetical expectation is that genotypes with greater Al tolerance have greater expression of genes for tolerance to this element compared to expression of these genes in sensitive genotypes. A further expectation is that differences in Al tolerance among germplasms (landrace and hybrid varieties) may be related to distinct genomic regions associated with tolerance in these tropical germplasms. In this sense, the aims of this study were to quantify differential expression of the genes *ZmMATE1*, *ZmMATE2*, and *ZmNrat1* in genotypes of tropical maize with contrasting levels of tolerance to Al, seeking to correlate the differences among the genotypes to distinct genomic regions associated with tolerance to this element.

## MATERIAL AND METHODS

### Genotyping with the genes *ZmMATE1* and *ZmMATE2*

Germplasms of hybrid maize, composed of 50 hybrids from different companies (Monsanto, Pioneer, Syngenta, and Dow AgroSciences) and of landrace maize (50 varieties collected from the Southeast and South regions of Brazil), were previously characterized for Al tolerance in minimal solution. A randomized block experimental design was used with three replications, and 12 seedlings of each genotype were evaluated per replication. After seed germination, the initial length (IL) of the main root was measured. After that, the seedlings were placed in a tank containing 280 L of a solution with 4 mg $L^{-1}$ Al ($AlCl_3$ $6H_20$) + 40 mg $L^{-1}$ Ca ($CaCl_2$ $2H_20$), essential for root growth. After 48 hours of exposure, the length of the main root was measured again (FL – final length). The difference between IL and FL (FL – IL) in cm was calculated (called DIF – growth difference) (Coelho et al. 2015). Using the DIF data, the relative aluminum tolerance index (RATI) was estimated by $RATI = \left[\frac{DIF_x - DIF_S}{DIF_T - DIF_S} \text{ x } 4.0\right] + 1.0$, were: $DIF_x$ = DIF for each genotype; $DIF_S$ = DIF for sensitive control; $DIF_T$ = DIF for tolerant control. The sensitive and tolerant hybrid controls (H 22 sensitive and H 44 tolerant) received RATI values of 1.0 and 5.0, respectively (Coelho et al. 2016).

This panel was genotyped in the current study to check for the presence and possible polymorphism among maize genotypes for the genes *ZmMATE1* and *ZmMATE2*. With the aim of correlating the phenotypic data with the genotypic, the RATI and polymorphism data were standardized, and the dissimilarity matrix among the genotypes was obtained by the generalized Mahalanobis distance. Based on the genetic dissimilarity index, cluster analysis was carried out by the UPGMA (Unweighted Pair-Group Method Using Arithmetic Average). Analyses were made on the R version 3.1.3 software (R Core Team 2013).

### Molecular detection of the genes *ZmMATE1* and *ZmMATE2*

Genomic DNA was extracted from a sample of young leaves of 15 seedlings from each genotype analyzed (landrace maize and hybrid maize). The samples were quantified in the Nanovue GE Healthcare® spectrophotometer device and diluted to a concentration of 10 ng $mL^{-1}$. The genomic regions surrounding the oligonucleotides that represent the genes *ZmMATE1* and *ZmMATE2* identified by Maron et al. (2010) were used to detect the presence of amplified fragments through polymerase chain reaction (PCR) in the two maize germplasms studied. The forward primer 5'CCGGATGTTTGCTGGATTTT3' and the reverse primer 5'TGGCCAAA TCGACCATGATT3' were used in the respective germplasms for detection of the gene *ZmMATE1*. The gene *ZmMATE2* was amplified using the pair of the forward primer 5'GCAGTTCGTACGTAGTGGTG3' and the reverse primer 5'AGTACGTAGCTAGGCGATGC3'.

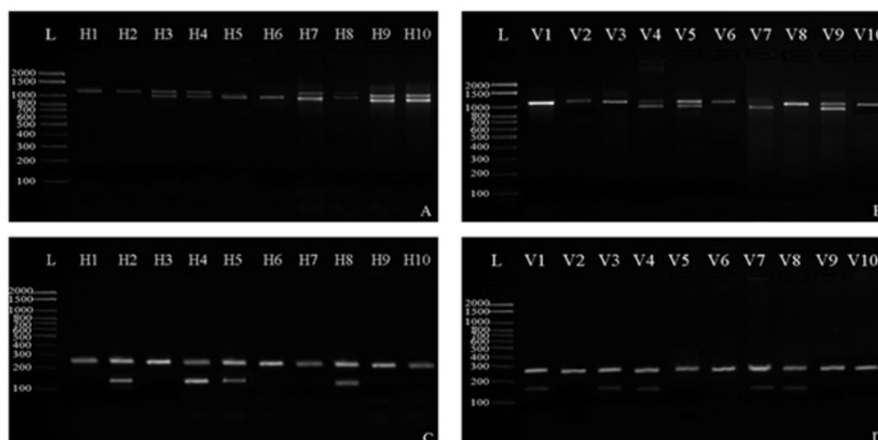


***Figure 1.*** Agarose gel (2%). Genotyping view of the part of (A) hybrids with the primer *ZmMATE1*; (B) landraces with the primer *ZmMATE1*; (C) hybrids with the primer *ZmMATE2*; and (D) landraces with the primer *ZmMATE2*. L = 100 bp step ladder.

The PCRs were prepared for a final volume of 20 mL containing 1X Green GoTaq® Flexi buffer, 2.0 mM $MgCl_2$, 0.1 mM dNTP, 0.2 mM primer (forward and reverse), 0.75 U Taq DNA polymerase (Promega, 5 U $mL^{-1}$), and 40 ng of template DNA. The PCR products were resolved in 2% agarose gel in a horizontal electrophoresis cube for 2 h at 80 V with a 100 bp step ladder (Figure 1). The homozygous state (1166 or 1000 bp) and heterozygous state (1166 and 1000 bp) of the *ZmMATE1* alleles were observed for the set of hybrids and landrace varieties (Figure 1A, B). The homozygous state (280 or 175 bp) and heterozygous state (280 and 175 bp) were likewise observed for the *ZmMATE2* gene (Figure 1C, D). After that, the PCR products of these genes were purified using the Axy Prep PCR Cleanup Kit (Axygen, Bioscience), following manufacturer's instructions. The purified samples were sent for automatic sequencing (ABI-PRISM 3100 Genetic Analyzer) set up with 50 cm capillary lengths and POP6 polymer (Applied Biosystems). ClustalW v 1.6 software was used for alignment of the nucleotide sequences of the genes *ZmMATE1* and *ZmMATE2*. The BLAST (Basic Local Alignment Search Tool) software was used for homology analysis of the nucleotide sequences at the MaizeGDB (Portwood et al. 2018), and the ideogram of chromosomes 5 and 6 (location of *ZmMATE2* and *ZmMATE1*, respectively) of each genotype was obtained by the CViT - Chromosome Viewing Tool (Cannon and Cannon 2011).

## Quantification of the differential expression of the Al tolerance genes

The hybrids H 22 (sensitive) and H 44 (tolerant) and the landrace varieties V 25 (sensitive) and V 18 (tolerant) were used in quantification of the differential expression of the genes *ZmMATE1*, *ZmMATE2*, and *ZmNrat1*. The genotypes were exposed to minimal solution [40 mg $L^{-1}$ of $Ca^{2+}$ + 4 mg $L^{-1}$ of $Al^{3+}$, according to Coelho et al. (2016)] for periods of 0, 1, 3, 6, 9, 12, 24, and 48 h. The seedlings were placed in expanded polystyrene trays and deposited on the treatment solution. The solutions were kept under constant aeration and pH between 4.2 and 4.5. After each exposure period, 12 seedlings of each genotype were taken from the solution for extraction of total RNA. The total RNA of each treatment was extracted from the tip (1.0 cm) of 12 roots using the RNAspin Mini RNA Isolation Kit (GE Healthcare UK Limited), following the protocol of the manufacturer after fragmentation of the tissue in liquid nitrogen. The total RNA was quantified in a Nanovue GE Healthcare® spectrophotometer. To visualize the integrity of the total RNA, 2.0 µL aliquots of the extracted RNA were added to loading buffer with formamide for electrophoresis in 1% agarose gel. The samples of total RNA were stored in an ultrafreezer (-80 ºC) until use. The cDNA (complementary DNA) was synthesized from approximately 1.5 µg of total RNA by reverse transcription using the commercial First-Strand cDNA Synthesis Kit (GE Healthcare UK Limited), following the technical recommendations of the manufacturer. Electrophoresis in 1% agarose gel was performed to verify the integrity of the cDNA samples, which were then stored in a freezer (-20 ºC).

Analysis of gene expression was performed by qRT-PCR trials in the Mx3005P™ Real Time q-PCR System (Stratagene®). The LUG gene was used as internal control of the expression experiments, according to previously obtained results

(Manoli et al. 2012). The forward primer 5'TGTGAGTTTGGCGGATGTGT3' and reverse primer 5'TCACAATCTAGGCCAGTA CAACAGA 3' were used for the gene *ZmMATE1*, and the forward primer 5'CCTGAGCGAGCGAGC T3' and reverse primer 5'CCATCGTGCGTGTATATATATATTACGTGTA3' for the gene *ZmMATE2*, as described by Maron et al. (2010). The forward primer 5'CGCGCTTCTGATCCAAACA3' and reverse primer 5'GCGAGATGCTTGCCTGTCTT3' were used for the gene *ZmNrat1*, as described by Guimarães et al. (2014).

For the amplification reactions, the following reagents were used: SYBR® Green Master Mix 1X (Life Technologies), 10 µM of the forward/reverse primers, and ultrapure sterile water to complete 20 µL of reaction mixture. The quantity of 2 µL of cDNA of each sample was used after standardization of the concentrations to 10 ng $\mu L^{-1}$. A negative control (water) was included in each qRT-PCR experiment so as to confirm the absence of contaminants in the reaction. The equipment was programmed for the following cycling: 10 minutes at 95 ºC and 40 cycles of 15 seconds at 95 ºC, 30 seconds at 65 ºC, and 30 seconds at 72 ºC. The dissociation curve was composed by the following programming: 1 minute at 94 ºC, 30 seconds at 55 ºC, and 10 seconds at 94 ºC. The levels of differential expression of each gene were calculated using the ddCT method (Livak and Schmittgen 2001) through the GenEx Enterprise program (version 6.0.5 Demo). The data obtained was analyzed by one way ANOVA and Tukey's post-hoc test at 5% probability.

## RESULTS AND DISCUSSION

### Screening of the *ZmMATE1* and *ZmMATE2* genes

With the aim of correlating the genotyping data of the gene *ZmMATE1* with the prior characterization of the genotypes (RATI), cluster analysis was performed for the germplasm both of the hybrids and of the landrace varieties, using a cutoff point of 1.0 in dissimilarity. Cluster analysis of the hybrids showed the formation of eight groups (Figure 2A). G1 clustered eight genotypes, which exhibited a mean RATI of 2.56 and an amplification standard of one fragment of approximately 1000 bp (Table 1 and Figure 1A).

Only two hybrids were placed in G2, with a RATI of 2.84 and amplification of one fragment of 1166 bp. In G3, 12 hybrids were clustered. These genotypes exhibited a mean RATI of 3.46 and two fragments (1166 and 1000 bp) amplified. In G4, only H 40 was grouped, with a RATI of 3.62 and amplification of the fragment of 1000 bp. Four hybrids with

***Table 1.*** Mean values of the relative Al tolerance index (RATI) and the size of the amplified fragment (AF) in base pairs (bp) for the respective groups formed from cluster analysis for the germplasm of hybrid and landrace varieties of maize

| ***ZmMATE1*** | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Hybrids** | | **AF** (bp) | | **Varieties** | | **AF** (bp) | |
| **Groups** | **RATI** | **1166** | **1000** | **Groups** | **RATI** | **1166** | **1000** |
| G1 | 2.56 | - | + | G1 | 1.00 | + | + |
| G2 | 2.84 | + | - | G2 | 3.41 | + | + |
| G3 | 3.46 | + | + | G3 | 4.14 | - | + |
| G4 | 3.62 | - | + | G4 | 6.56 | + | - |
| G5 | 4.79 | - | + | G5 | 4.78 | + | - |
| G6 | 1.00 | + | + | | | | |
| G7 | 2.49 | + | - | | | | |
| G8 | 2.69 | + | + | | | | |
| ***ZmMATE2*** | | | | | | | |
| **Hybrids** | | **AF** (bp) | | **Varieties** | | **AF** (bp) | |
| **Groups** | **RATI** | **280** | **175** | **Groups** | **RATI** | **280** | **175** |
| G1 | 4.54 | + | - | G1 | 1.00 | + | - |
| G2 | 4.55 | + | + | G2 | 5.74 | + | - |
| G3 | 3.31 | + | - | G3 | 4.25 | + | - |
| G4 | 3.41 | + | + | G4 | 3.75 | + | + |
| G5 | 1.00 | + | - | | | | |
| G6 | 2.26 | + | + | | | | |
| G7 | 2.24 | + | - | | | | |

greater tolerance were placed in G5, namely, H 44, H 27, H 38, and H 41, with a mean RATI of 4.79, and all amplified the fragment of 1000 bp. G6 was formed of only H 22, which exhibited the greatest sensitivity to Al, with a RATI of only 1.00 and amplification of the two fragments (1166 and 1000 bp). In G7, three hybrids clustered, with a mean RATI of 2.49 and one fragment of 1166 bp. G8 clustered the greatest number of genotypes (21), which exhibited a mean RATI of 2.69 and amplification of the two fragments (Table 1 and Figure 1A).

For the set of landrace varieties, cluster analysis formed five groups (Figure 2B). The hybrid H 22, with a RATI of 1.00 (greatest sensitivity) and amplification of the two fragments (1166 and 1000 bp) was isolated in G1, and had the greatest genetic dissimilarity from the rest of the genotypes. In G2, 15 landrace varieties were clustered, which obtained a mean RATI of 3.41 and amplified the fragments of 1166 and 1000 bp (Table 1 and Figure 1B). G3 included 18 varieties and the tolerant hybrid (H 44), which exhibited RATI of 4.14 and the amplified fragment of 1000 bp. The varieties V 18, V 6, and V 23, considered the most tolerant to Al, were grouped in G4, with the highest mean RATI of 6.56 and the fragment of 1166 bp. And finally, in G5, 14 landrace varieties were clustered, with a RATI of 4.78 and one fragment of 1166 bp (Table 1 and Figure 1B).

The amplification pattern of the control varieties tolerant and sensitive to *ZmMATE1* (H 44 and V 18, and H 22 and V 25, respectively) allows inferences to be made regarding possible differences of tolerance (RATI) associated with the polymorphism among them. The polymorphism between the tolerant genotypes (H 44 and V 18) could explain the superiority in tolerance of the landrace variety in relation to the hybrid. While H 44, used here as a control, exhibited a

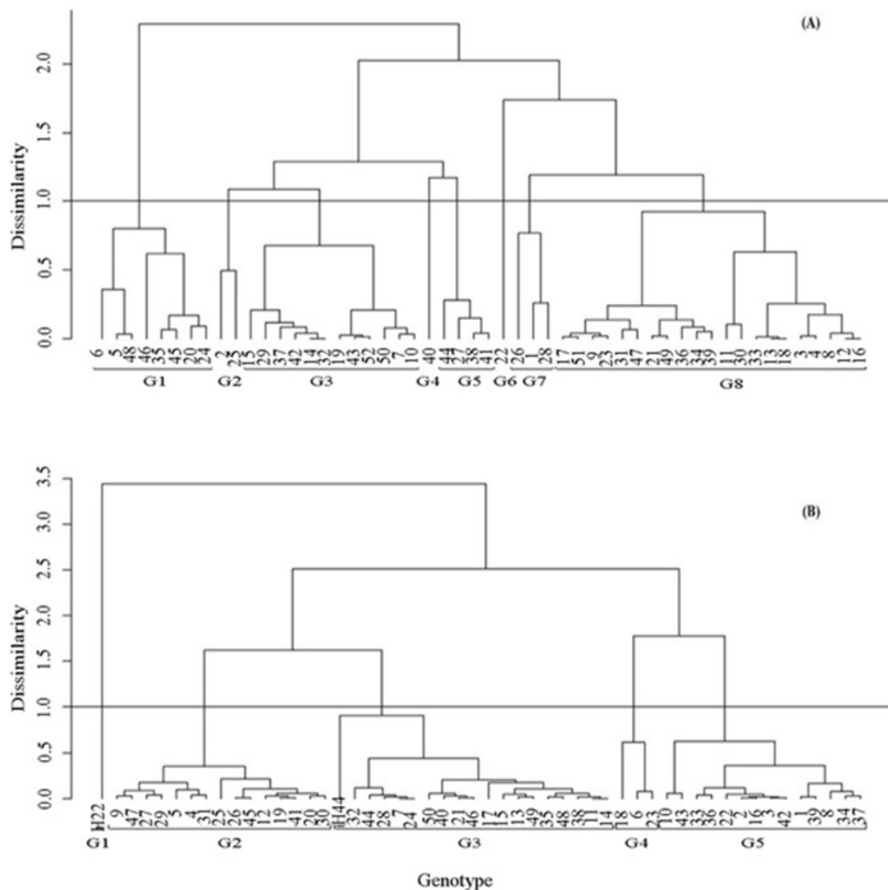


***Figure 2.*** Clustering according to the relative Al tolerance index (RATI) values and of the amplified fragments for the primer ZmMATE1. (A) germplasm of hybrid maize and (B) germplasm of landrace varieties of maize plus the tolerant and sensitive control treatments (H 44 and H 22, respectively).

RATI of 5.00, the landrace variety V 18 had a RATI of 6.96. The superior tolerance of V 18 may reflect the presence of more effective tolerance genes since this genotype comes from agricultural regions of low technological level, characterized by environments with low natural fertility and high Al saturation (Coelho et al. 2016).

Studies developed by Maron et al. (2010), Guimarães et al. (2014), Matonyei et al. (2017), and Matonyei et al. (2020) revealed that this genomic region (*ZmMATE1*) explains most of the phenotypic variation of Al tolerance in Cateto maize. The greater tolerance observed in the germplasm of landrace maize in the present study could also be directly related to the presence of the same genes coming from the Cateto breed of maize, a germplasm that is very common in the region these landrace varieties of maize were collected (Coelho et al. 2018).

Cluster analysis with the genotyping data for *ZmMATE2* in relation to the hybrid germplasm showed the formation of seven groups (Figure 3A). Eight hybrids were clustered in G1, which obtained a mean RATI of 4.54 and amplification of one fragment of 280 bp (Table 1 and Figure 1C). G2 clustered three hybrids, with a RATI of 4.55 and two fragments (280 and 175 bp) amplified. Fifteen hybrids were placed in G3, with a RATI of 3.31 and one fragment of 280 bp. G4 clustered eight hybrids with two fragments amplified and a mean RATI of 3.41. Only the sensitive hybrid, H 22, was placed in G5, with the lowest RATI and one fragment of 280 bp. Five other hybrids with two fragments amplified were clustered in G6, with a RATI of 2.26. The 12 other hybrids amplified one fragment of 280 bp and RATI of 2.24, which were placed in

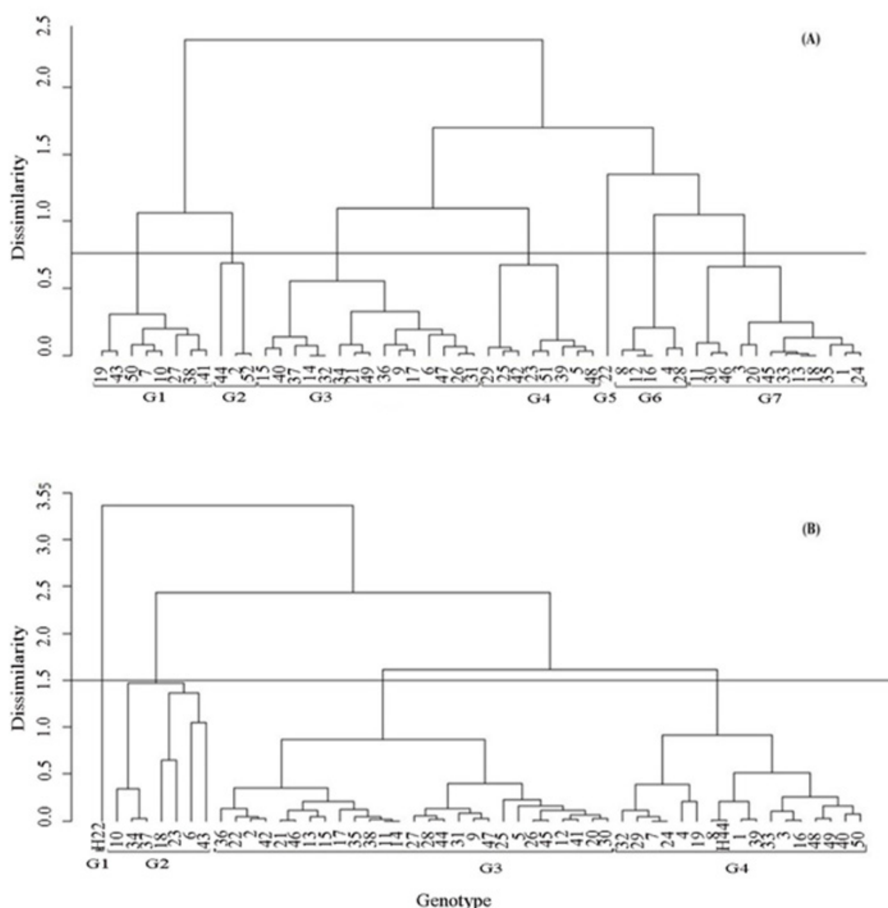


***Figure 3.*** Clustering according to the relative Al tolerance index (RATI) values and of the amplified fragments for the primer ZmMATE2. (A) germplasm of hybrid maize and (B) germplasm of landrace varieties of maize plus the tolerant and sensitive control treatments (H 44 and H 22, respectively).

G7 (Table 1 and Figure 1C).

Cluster analysis for the landrace germplasm led to the formation of four groups from a cutoff point in the dendrogram at 1.5 of dissimilarity (Figure 3B). Once more, the H 22 hybrid (G1) was the most dissimilar among the genotypes evaluated (greatest sensitivity) (Table 1 and Figure 1D). G2 was composed of seven landrace varieties, which exhibited a mean RATI of 5.74 and an amplified fragment of 280 bp. Most the varieties were clustered in G3 (27), with a mean RATI of 4.25 and one amplified fragment of 280 bp. The tolerant hybrid H 44 was placed in G4, together with 10 varieties. This set of genotypes exhibited a mean RATI of 3.75 and two fragments of 175 and 280 bp (Table 1 and Figure 1D). These results confirm that molecular polymorphism is not associated with tolerance or sensitivity to Al, which is in partial agreement with the data obtained by Guimarães et al. (2014), who showed no polymorphism for the gene *ZmMATE2* between a sensitive line and a tolerant variety of Cateto maize.

Homology analysis was carried out using the database of Maize GDB. This analysis resulted in the ideogram representation of chromosomes 5 and 6, indicating the possible regions in which the sequences studied would be more strongly clustered (Figure 4). The most strongly clustered regions are indicated in the respective chromosomes by the color red, and the weakest clustered regions by the color green. The sequences related to the gene *ZmMATE2* are in chromosome 5, and those related to the gene *ZmMATE1* are in chromosome 6 (Figure 4). These results confirmed that the sequences have high identity with the respective Al tolerance genes studied, as well as localization in the genomic regions described in the literature. They were therefore validated by the analyses.

### Quantification of the differential expression of the genes *ZmMATE1*, *ZmMATE2*, and *ZmNrat1*

The patterns of expression of the genes *ZmMATE1*, *ZmMATE2*, and *ZmNrat1* were verified through use of quantitative real time PCR (Figure 5). Throughout the periods of root exposure to Al, expression of *ZmMATE1* was constant at time

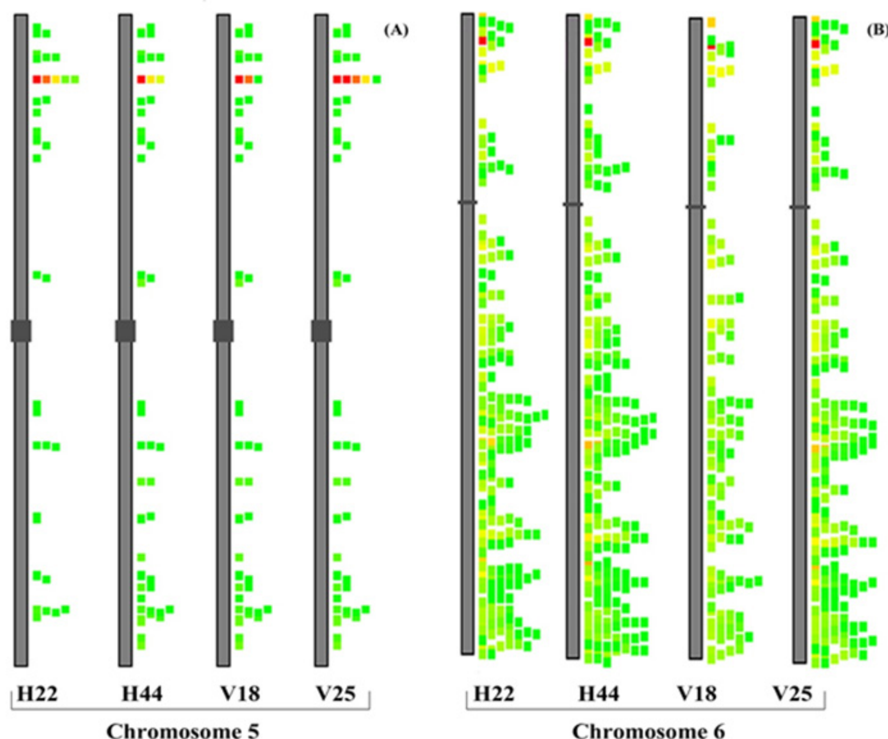


*Figure 4.* Ideogram of chromosomes 5 and 6, indicating the regions where the sequences obtained for each maize genotype (H 22, H 44, V 18, and V 25) are more closely linked by the BLAST analysis. A) Sequence obtained for the primer ZmMATE2. B) Sequence obtained for the primer ZmMATE1.

0 (absence of the element), but was strongly regulated by the presence of Al. There was an increase in expression after 1 h of exposure in all the genotypes evaluated, with a notable response of the tolerant landrace variety V 18, which reached superior and differential expression beginning at 3 h. In general, there was an increase in expression of the gene with the increase in the periods of exposure. Nevertheless, the superiority of the variety V 18 can be seen, which reached peak expression of 8.1 after 12 h of exposure, whereas the other genotypes exhibited maximum expression of 2.3 (Figure 5A).

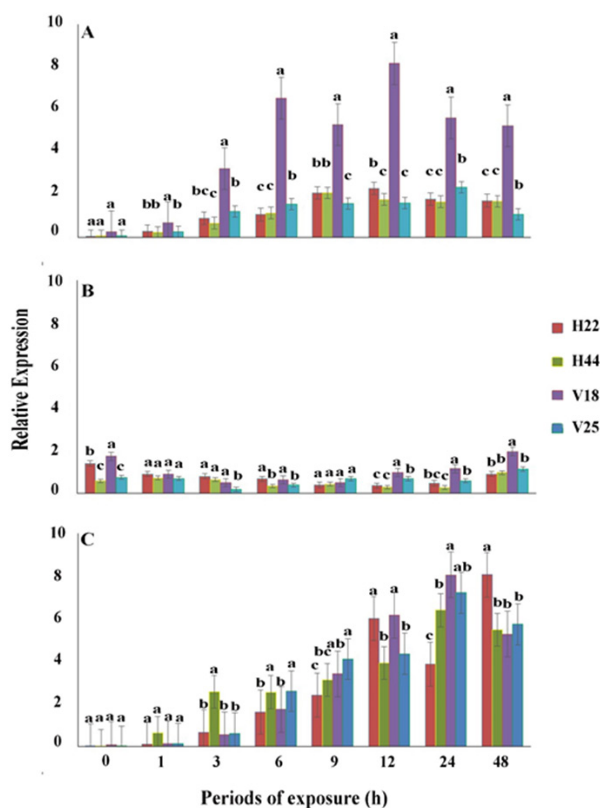


***Figure 5.*** Relative expression of the genes ZmMATE1 (A), ZmMATE2 (B), and ZmNrat1 (C) in the hybrids (H 22 and H 44) and in the landrace varieties (V 18 and V 25) of maize, according to the periods of exposure to Al (0, 1, 3, 6, 9, 12, 24, and 48 h).

Analysis of differential expression of the Al tolerance genes showed a pattern of expression similar to that found in the literature. The levels of expression of the *ZmMATE1* gene in the presence of Al were much higher in the landrace variety V 18 compared to the other genotypes studied. This result confirms that this gene may be contributing more significantly to expression of Al tolerance in this landrace variety of maize in relation to the other genomic regions involved in expression of this trait. Maron et al. (2010) observed high levels of expression of this gene in the root tip of two varieties of Cateto maize, and its expression was regulated by the presence of Al. In addition, the authors found high exudation of citrate in the root tip of these same genotypes, correlating expression of the gene *ZmMATE1* with the mechanism of tolerance through exclusion of Al by means of exudation of organic acids. However, the tolerant hybrid H 44 exhibited the same level of expression as the sensitive genotypes (H 22 and V 25), indicating that the mechanism of tolerance in this hybrid may be related to another gene. Mantonyei et al. (2020) reported that the *ZmMATE1* gene did not play a significant role in Al tolerance in Kenyan maize germplasm. According to the authors, maize homologs to genes previously involved in Al tolerance in other species (*ZmNrat1*, *ZmMATE3*, *ZmWRKY*, and *ZmART1*) were highly expressed in the tolerant genotype.

Through analysis of expression of the gene *ZmMATE2*, a very different pattern of expression could be observed in relation to the gene *ZmMATE1* (Figure 5B). Expression of this gene remained nearly constant in the four maize genotypes and, in addition, it seems that this gene does not respond to the presence of Al. This pattern of expression is in agreement with the results reported by Maron et al. (2010) and Guimarães et al. (2014), who found absence of differential expression among tolerant and sensitive genotypes, and the levels of expression of the *ZmMATE2* gene were similar in both the roots and in the shoot tissues of maize. The characterization of the *ZmMATE2* gene, mapped on the telomeric region of chromosome 5, showed that this gene differs from *ZmMATE1* and from other MATEs in diverse aspects. Maron et al. (2010) found that *ZmMATE2* does not share the same identity of amino acids as other MATEs, suggesting that this gene does not mediate the flow of citrate. The nature of the exudation transported by *ZmMATE2* is still unknown. Piñeros et al. (2005) showed that although Al activates exudation of citrate by roots, Al tolerance in maize could not be explained solely by this mechanism.

For the gene *ZmNrat1*, an increase in expression could be seen over the periods of exposure to Al. With 3 h of exposure, the greatest expression was observed for the tolerant hybrid H 44. Beginning at 24 h of exposure, there was an increase in expression of the landrace varieties V 18 and V 25, followed by the hybrid H 44. At 48 h of exposure to Al, there was inversion in gene expression of the genotypes because those with high expression at the time 24 h showed

reduction in expression, whereas the sensitive hybrid H 22 exhibited a differential increase in expression of the gene *ZmNrat1* in relation to the other genotypes studied (Figure 5C).

Recently, results of genetic analyses of QTLs of Al tolerance in maize obtained by Guimarães et al. (2014) showed that *ZmMATE2* was constitutively expressed in tolerant and sensitive genotypes. In addition, the authors observed that the amino acid sequences of these genotypes are identical, indicating that *ZmMATE2* is probably not the candidate gene that controls tolerance in the QTL of chromosome 5. Some authors have associated this region with the gene *ZmNrat1*, which is a homolog of the gene *Nrat1* (Nramp aluminum transporter 1) in rice (Guimarães et al. 2014, Coelho et al. 2019, Mantonyei et al. 2020). *Nrat1* is a transporter located in the plasmatic membrane of the root apical cells, showing transport activity for $Al^{3+}$, but not for bivalent metals or for the Al-citrate complex. Expression of this gene is induced by Al and occurs specifically in the roots, in all the root cells, except in the epidermal cells.

The levels of expression of *ZmNrat1* increased with longer periods of exposure, and expression was regulated by the presence of Al. Nevertheless, after 3h of exposure, only the tolerant hybrid (H 44) exhibited greater expression in relation to the other genotypes. From 24h of exposure on, an increase was observed in expression of the tolerant landrace variety V 18, followed by V 25 and by the hybrid H 44. The sensitive hybrid H 22 showed late differential expression (48 h), whereas the other genotypes (V 18, V 25, and H 44) exhibited a decline in expression of this gene. These results corroborate the data of Guimarães et al. (2014), in which the maize genotype most tolerant to Al exhibited greater expression of the gene in the first hours of exposure, but was subsequently maintained and exceeded by the more sensitive genotype. According to Chen et al. (2020), the strategy of absorption of Al mediated by *Nrat1*, followed by complexation and sequestration into the vacuoles for final detoxification, is more effective in the detoxification process than the mechanism that permits accumulation of Al in the apoplast (mainly in the cell wall), because this accumulation may interfere in the extensibility of the cell wall and, consequently, reduce root growth.

The complexity of the Al tolerance mechanisms, the responses of citrate exudation activated by the presence of Al, and the probable occurrence of additional mechanisms of Al tolerance in maize are in agreement with genetic evidence that shows the involvement of various Al tolerance genes in different genomic regions in genetic control of this trait. The results obtained in this study confirm that exudation of citrate must be the main mechanism of Al tolerance found in the landrace variety of maize V 18 (highly tolerant), confirmed especially by the higher level of expression of the gene *ZmMATE1* and by the higher value of the RATI compared to the tolerant hybrid H 44. The results also show the possibility of another mechanism involved in the tolerance of the hybrid H 44, because it exhibited differential expression only for the gene *ZmNrat1* in the initial phase of exposure.

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