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A search for RFLP markers to identify genes for aluminum tolerance in maize*

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ABSTRACT

The objective of this study was to identify restriction fragment length polymorphism (RFLP) markers linked to QTLs that control aluminum (Al) tolerance in maize. The strategy used was bulked segregant analysis (BSA) and the genetic material utilized was an F_2 population derived from a cross between the Al-susceptible inbred line L53 and Al-tolerant inbred line L1327. Both lines were developed at the National Maize and Sorghum Research Center - CNPMS/EMBRAPA. The F_2 population of 1554 individuals was evaluated in a nutrient solution containing a toxic concentration of Al and relative seminal root length (RSRL) was used as a phenotypic measure of tolerance. The RSRL frequency distribution was continuous, but skewed towards Al-susceptible individuals. Seedlings of the F_2 population which scored the highest and the lowest RSRL values were transplanted to the field and subsequently selfed to obtain F_3 families. Thirty F_3 families (15 Al-susceptible and 15 Al-tolerant) were evaluated in nutrient solution, using an incomplete block design, to identify those with the smallest variances for aluminum tolerance and susceptibility. Six Al-susceptible and five Al-tolerant F_3 families were chosen to construct one pool of Al-susceptible individuals, and another of Al-tolerant, herein referred as "bulks", based on average values of RSRL and genetic variance. One hundred and thirteen probes were selected, with an average interval of 30 cM, covering the 10 maize chromosomes. These were tested for their ability to discriminate the parental lines. Fifty-four of these probes were polymorphic, with 46 showing codominance. These probes were hybridized with DNA from the two contrasting bulks. Three RFLPs on chromosome 8 distinguished the bulks on the basis of band intensity. DNA of individuals from the bulks was hybridized with these probes and showed the presence of heterozygous individuals in each bulk. These results suggest that in maize there is a region related to aluminum tolerance on chromosome 8.

INTRODUCTION

A major constraint to maize production in the tropics is excessive acidity of the soils. In Brazil, acid savannas with low cation exchange capacity and high saturation of toxic aluminum cover 205 million hectares, of which 112 million hectares are suitable for agricultural production (Olmos and Camargo, 1976). In most of this area, deficiencies of P, Ca, Mg and Zn are common and saturation of toxic Al and fixation of P by soil particles are usually high (Lopes and Cox, 1977).

Most of the available maize cultivars are susceptible to toxic aluminum in the soil, and decreases in yield due to Al toxicity have been extensively reported in the literature (Brenes and Pearson, 1973; EMBRAPA, 1980). Although technology for topsoil acidity correction is widely used in the tropics, there is no easy way to remove the effects of toxic aluminum in the subsoil. Therefore, to exploit the soil in aluminum-rich areas it

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is important to develop breeding programs aimed at generating aluminum-tolerant cultivars (Goedert *et al.*, 1980). Maize breeders have identified genetic variability for aluminum tolerance and germplasm suitable for selection is available (Rhue *et al.*, 1978; Magnavaca, 1982; Furlani *et al.*,1986; Lopes *et al.*, 1987).

Several techniques based on evaluations under field conditions and in nutrient solutions have been developed to screen for aluminum tolerance in maize (Howeler, 1991). Although maize breeders have traditionally relied on evaluations on soils with high aluminum saturation, the use of nutrient solutions with high aluminum concentrations has proven to be an effective way to complement field tests. The nutrient solution technique is rapid, allows screening of many progenies in small areas and a better control of environmental variations, which is more difficult to achieve under field conditions. Among the several criteria utilized to evaluate maize aluminum tolerance in nutrient solutions, seminal root growth seems to be the most reliable (Rhue and Grogan, 1977; Garcia Jr. *et al.*, 1979; Magnavaca, 1982; Sawasaki and Furlani, 1987). Using this criterion, several authors have shown that the trait is quantitatively inherited, with a predominance of additive genetic effects (Magnavaca, 1982; Lopes *et al.*, 1987; Sawasaki and Furlani, 1987). However, due to its high heritability, the character is expected to be controlled by a small number of genes (Prioli, 1987).

Several different mechanisms have been proposed to explain aluminum tolerance in cultivated plants (Foy and Fleming, 1978), although for maize no mechanism has been described in detail, and the genetic control of aluminum tolerance is still poorly understood. The advent of molecular biology with all the accompanying techniques for genetic and biochemical analysis generated great expectations to unravel the genetic complexity and molecular control of aluminum tolerance in maize. This knowledge would help in designing improved breeding methods for efficient utilization of the existing genetic variability for cultivar development.

Quantitative trait loci (QTLs) controlling several important agronomic characters have been successfully studied through the use of molecular markers, utilizing techniques such as RFLP and random amplified polymorphic DNA - RAPD (Nienhuis *et al.*, 1987; Reiter *et al.*, 1991; Diers *et al.*, 1992; Edwards *et al.*, 1992). However, the commonly used strategies to map QTLs are laborious, time consuming and require large number of individuals. Bulked segregant analysis (BSA) was developed as an efficient technique to score molecular markers to specific regions of the genome (Michelmore *et al.*, 1991). This methodology has been shown to be very efficient in studying traits controlled by a small number of genes, and may also be utilized to study major QTLs with large effects on the phenotype (Wang and Paterson, 1994). Considering the evidence that aluminum tolerance in maize is a quantitatively inherited trait, possibly controlled by a small number of genes, the objective of this study was to identify RFLP markers linked to QTLs affecting this trait by using bulked segregant analysis.

MATERIAL AND METHODS

Phenotypic evaluation

The genetic material utilized in this study consisted of an F_2 population derived from a cross between the maize inbred lines, L53 and L1327, developed by the maize breeding program of the National Maize and Sorghum Research Center - CNPMS/EMBRAPA, Brazil. These two lines have more than 10 generations of selfing. The aluminum susceptible line (inbred L53) was derived from the open pollinated variety CMS11 (Pool 21) and the aluminum-tolerant parent (inbred L1327) was derived from Cateto Colômbia.

Three thousand randomly drawn F_2 seeds and 300 seeds of each of the two inbred parents were germinated for seven days in rolled paper towels moistened with tap water. One thousand, five hundred and fifty-four F_2 seedlings and 130 seedlings from the parental inbred lines were randomly chosen. After measurement of the initial seminal root length (ISRL), these seedlings were transferred to plastic plates (49 seedlings per plate) and grown in a greenhouse for seven days in eight liters of aerated nutrient solution containing 6 ppm of aluminum in the form of $KAl(SO_4)_2$ (Magnavaca, 1982). There were three seedlings from the parental inbred lines on each plate. During harvesting, final seminal root length (FSRL) was measured and the plants transferred back to the nutrient solution. ISRL and FSRL were used to calculate the relative seminal root length (RSRL), where RSRL = (FSRL - ISRL)/ISRL.

The 60 seedlings with the greatest RSRL values (Al-tolerant) and the 60 seedlings with the smallest RSRL values (Al-susceptible) were transplanted from the nutrient solution to field conditions, grown and then selfed to obtain F_3 families. Thirty of these F_3 families (15 tolerant and 15 susceptible) were grown in nutrient solution using the same procedure described for the F_2 individuals. The objective was to identify F_3 families breeding true for the selected trait (Al susceptibility at one end of the distribution and Al tolerance at the other end) for bulking and eliminate the heterozygous ones.

The 30 F_3 families and six checks were distributed in an incomplete block design with three replications, making 36 plates of 49 seedlings. Each plate contained three treatments (14 seedlings) and seven seedlings of an Al-susceptible inbred line, L19, as a control. The six checks used were: the two parents, their F_1, the open pollinated variety CMS36 (synthetic variety obtained by recombination of 16 aluminum-tolerant inbred lines), the double cross hybrid BR201 (hybrid adapted to acid soil which associates intermediate levels of aluminum tolerance with phosphorus efficiency) and the inbred line L726 (aluminum susceptible line derived from the open pollinated variety BR105-Swan1). The genetic variance within F_3 families was calculated by the weighted average of the variances estimated from each replication (phenotypic variance minus the environmental variance).

RFLP detection

The set of 113 RFLP markers used in this study corresponds to *Pst*I-digested genomic DNA cloned into PUC19 plasmids, obtained from Dr. David Hoisington (International Maize and Wheat Improvement Center - CIMMYT, Mexico) and from Dr. Theresa Musket (University of Missouri, Columbia). These markers have been previously shown to identify polymorphism in maize and are available with linkage map data as a public set of maize RFLP probes. One hundred and thirteen probes were selected at an average interval of 30 cM in such a way to cover all the maize genome. These probes were then tested for their ability to identify RFLPs between the parental inbred lines (L53 and L1327), when their DNAs were digested with *Eco*RI, *Bam*HI or *Hind*III.

The probes were labeled via amplification by polymerase chain reaction using digoxigenin-11-dUTP (Boehringer Mannheim). Each reaction consisted of 1x PCR buffer (10 µl), 50 µM dNTPs (dATP, dCTP, dGTP), 48.75 µM dTTP, 1.25 µM dig-dUTP, 1.6 U Taq-polymerase, 0.2 µM CV 72 and CV 76 primers, 100 ng plasmid containing the probe fragment and H_2O to a final volume of 100 µl. Mineral oil (75 µl) was added to each reaction mixture to avoid evaporation during temperature cycling. Amplifications were performed in an EZ cycler (Ericomp Inc., San Diego, CA) programmed as follows: 1 cycle at 94°C for 1 min, 25 cycles at 94°C for 1 min, 55°C for 2 min and 72°C for 2 min and a final cycle at 72°C for 1 min. Amplifications were visualized in 0.8% agarose gels stained with ethidium bromide.

DNA from the parental inbred lines and the F_1 was purified from lyophilized leaf tissue using the method described by Saghai-Maroof *et al.* (1984). The DNA was quantified visually on 0.8% agarose gels by comparison with standards of known concentration, and redissolved to a concentration of 1 µg/µl in TE (10 mM Tris, pH 8.0, 1 mM EDTA). Approximately 30 µg of genomic DNA was digested with each of the three restriction enzymes using 2.5 U of enzyme per µg of DNA for 18 h at 37°C. Digested DNA was loaded onto 0.8% agarose gels prepared with 1x TAE buffer (40 mM Tris acetate, pH 8.0, 10 mM EDTA) and electrophoresed overnight at 50 V.

The gel was stained with ethidium bromide to permit visualization of the DNA, rocked for 1 h in a denaturing solution (0.4 M NaOH, 0.6 M NaCl) and then neutralized for 1 h in 0.5 M Tris, pH 7.5, 1.5 M NaCl. The DNA was transferred from the gel onto a nylon membrane in the presence of transfer buffer (1 M ammonium acetate, 20 mM NaOH) for a period of 24 h. The membrane was then washed in 2x SSC (3 M NaCl, 0.3 M Na-citrate) for 5 min, in 5x SSPE (NaCl, NaH_2PO_4, EDTA) for 10 min and baked at 95°C, in vacuo, for 3 h.

All the membranes were hybridized in sealed plastic bags at 65°C. Prehybridization was performed for 3 h in hybridization solution (5x SSC, 0.1% blocking reagent, Boehringer Mannheim, 0.1% sarkosyl, 10% SDS). Hybridization was carried out for 15 to 18 h in hybridization solution containing the probe, previously denatured for 10 min in boiling water, at a concentration of 80 ng probe/ml of solution. The membranes were washed twice, at room temperature, in 0.15x SSC, 0.1% SDS for 5 min, and thrice for 15 min at 65°C. The membranes were then incubated in Buffer 1 (0.01 M Tris, pH 7.5, 0.15 M NaCl) for 5 min and in Buffer 2 (0.01 M Tris, pH 7.5, 0.15 M NaCl, 0.1% blocking reagent) for 30 min at room temperature, followed by incubation in Buffer 2 containing anti-dig antibody conjugated with alkaline phosphatase (1 µl/15 ml Buffer 2) for 1 h. After this the membranes were washed twice for 15 min each in Buffer 2, twice in Buffer 1 for 15 min and once for 5 min in Buffer 3 (0.10 M Tris, pH 9.5, 0.10 M NaCl, 0.05 M $MgCl_2$). Membranes were exposed to alkaline phosphatase (AMPPD - 10 µl/ml Buffer 3) substrate for 1 h, wrapped in Saran Wrap and exposed to X-ray film (Kodak) for 15-18 h. Membranes were stripped through washes in 2x SSC for 10 min at room temperature, then in 0.2 M NaOH, 0.1% SDS for 10 min at 37°C and finally in TE (Tris-EDTA) at room temperature for 5 min and stored in TE at 4°C until use.

Bulk analysis

The RFLP procedures used for the bulk analysis were the same as described for probe selection. The

Al-susceptible and Al-tolerant bulks were made up of six and five F_2 individuals, respectively. Leaf tissue of 28 seedlings from each selected F_3 family were mixed to represent the F_2 plants. DNA was extracted from this tissue mixture, quantified and bulked in such a way that all F_2's were equally represented in each of the two bulks.

Fifty-four RFLP markers previously chosen for their ability to distinguish the parental inbred lines, and their F_1's were used to identify polymorphism between the contrasting bulks. Eleven individuals (six Al-susceptible and five Al-tolerant) selected to constitute the bulks were also genotyped in relation to the RFLPs that distinguished the bulks.

RESULTS AND DISCUSSION

Phenotypic response to Al stress

The RSRL value frequency distribution obtained for the F_2 population is shown in Figure 1. The mean and standard error for RSRL values of the parental inbred lines (L53 and L1327) were 0.15 ± 0.005 and 0.75 ± 0.028, respectively. The F_2 population showed an average RSRL of 0.43 .± 0.006 and a variance of 0.058. Figure 1 shows a continuous distribution, which is typical of quantitatively inherited traits, with a tendency to have more susceptible individuals. This trend towards a larger number of susceptible F_2's can be confirmed by the coefficients of skewness and kurtosis, estimated as 1.29 and 2.39, respectively. However, the recovery of parental phenotypes suggests that aluminum tolerance may not be a complex trait, and is in agreement with past evidence that tolerance to aluminum is a quantitatively inherited trait controlled by a small number of genes (Prioli, 1987).

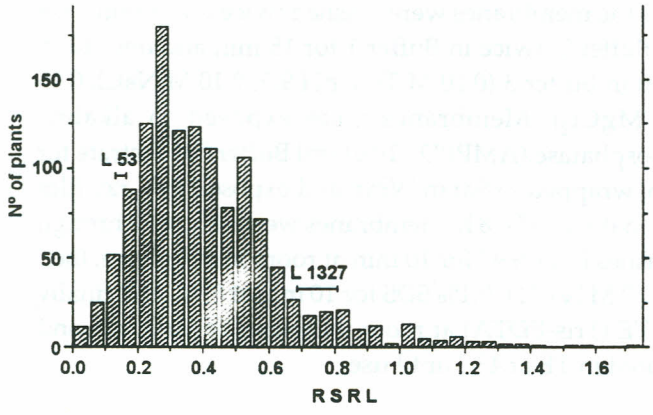

Figure 1 - Histogram of relative seminal root length (RSRL) value frequency distribution obtained from the F_2 population grown in nutrient solution containing a toxic concentration of aluminum. Ninety-five percent confidence intervals are: 0.15 ± 0.01 (L53) and 0.75 ± 0.06 (L1327).

Significant differences for RSRL values were found among F_3 families. The adjusted means and genetic variance of the RSRL values obtained for the F_3 families are shown in Table I and for the checks and the control are shown in Table II. F_3 families from each extreme (five with the highest and six with the lowest RSRL values) associated with a low variance were chosen to build two bulks with contrasting phenotypes (Al-tolerant and Al-susceptible).

RFLP analysis

Of the 113 selected probes, 54 were able to distinguish the parental lines and their F_1, with 46

Table I - Adjusted means and genetic variance of relative seminal root length values obtained for the F_2:F_3 Al-susceptible and Al-tolerant families. Al-susceptible families (1 to 15) and Al-tolerant families (16 to 30).

F_3 families	Average	Variance[1] ($x10^3$)	F_3 families	Average	Variance ($x10^3$)
1^S	**0.3536**	**9.470**	16	0.5814	15.126
2^S	**0.3290**	**14.021**	**17^T**	**0.6459**	**13.452**
3	0.6340	9.568	18	0.6152	10.109
4	0.4967	25.924	**19^T**	**0.6355**	**23.322**
5	0.6047	34.224	20	0.5431	17.189
6^S	**0.3441**	**7.836**	21	0.5517	39.269
7	0.4675	11.881	22	0.5517	8.041
8	0.4535	1.097	23	0.4606	44.184
9	0.4113	17.432	24	0.5789	21.952
10^S	**0.2461**	**4.304**	**25^T**	**0.6397**	**21.267**
11^S	**0.3261**	**13.342**	26	0.6166	45.824
12	0.7529	2.771	27	0.6052	6.969
13	0.4218	13.954	28	0.5993	16.475
14^S	**0.1206**	**2.319**	**29^T**	**0.7311**	**20.845**
15	0.6747	102.115	**30^T**	**0.7802**	**5.847**

[1] $\sigma^2_G = (n_1 - 1)\,\sigma^2_{G1} + (n_2 - 1)\,\sigma^2_{G2} + (n_3 - 1)\,\sigma^2_{G3} / (n_1 + n_2 + n_3 - 3)$; $n_{1\text{-}3}$ = number of plants in replication 1-3; σ^2_{G1}·σ^2_{G2} and σ^2_{G3} = genetic variance in each replication.
^{S}Susceptible; ^{T}Tolerant.

Table II - Adjusted means and variance of relative seminal root length values obtained for the checks (L53, L1327, F_1, BR201, CMS36 and L726) and the control (L19).

Checks	Average	Variance ($x10^3$)
L53	0.1172	0.000
L1327	0.8067	28.448
F_1	0.6815	3.368
BR201	0.4080	7.852
CMS36	0.7139	0.238
L726	0.2324	0.000
L19*	0.3054	8.037

*Control; means of 36 plates (7 seedlings per plate) and pooled variance.

probes showing codominance and eight showing a complex pattern. Some probes were polymorphic with more than one enzyme, thus a total of 73 RFLP marker loci were identified. This number of polymorphic probes was not sufficient to provide the desired genome coverage since after selection they turned out to be non uniformly distributed along the 10 maize chromosomes.

Among the 54 markers used to analyze the contrasting bulks, three markers on chromosome 8 (UMC48, UMC103 and CSU155) revealed polymorphisms, as a result of differences in band position and intensity (Figure 2).

Hybridization of DNA from individual members of the bulks with the three polymorphic markers showed that about 50% of the members in each bulk were heterozygous (Figure 3). When we compared the banding pattern of individuals 1 to 6 with the banding pattern of the Al-susceptible bulk we verified that individuals 1 and 4 were homozygous for the three markers, individuals 3, 5 and 6 were heterozygous and individual 2 varied from one marker to another. Individual 7 did not present a good resolution for interpretation and therefore has not been used. When the banding pattern of individuals 8 to 11 was compared with that of Al-tolerant bulk it was found that individuals 9 and 10 were homozygous, individual 11 was heterozygous for the three markers and individual 8 varied.

Explanations for the presence of heterozygous individuals in both bulks could be the occurrence of recombination between the QTL(s) and the region where the markers are located, or possibly the phenotypic evaluation was not sensitive enough to discriminate contrasting homozygous individuals.

Although both bulks presented heterozygous individuals, the results showing polymorphism between the bulks at three marker loci on chromosome 8 are promising when we consider that it has been already reported that there is a region on chromosome 8 that is highly correlated with root growth when maize is subjected to low-phosphorus stress (Reiter *et al.*, 1991).

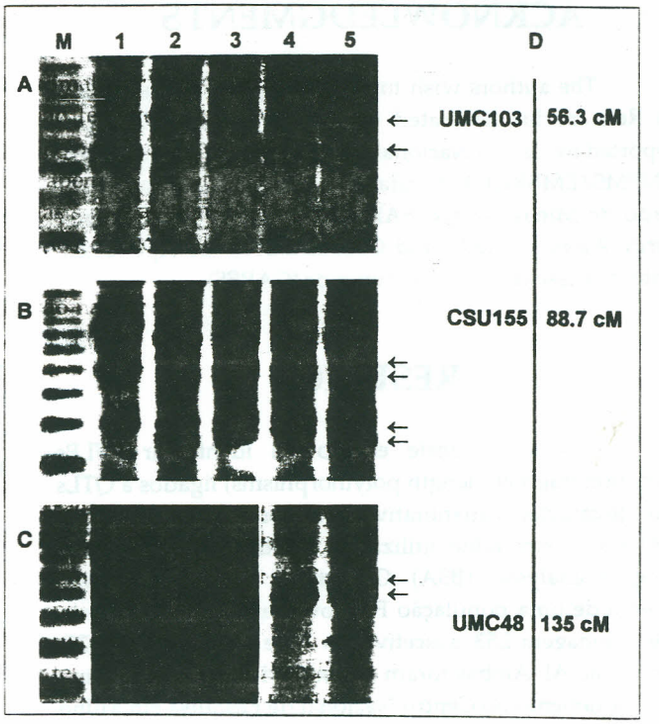

Figure 2 - Southern blots of parental and bulked DNA samples digested with *Eco*RI and probed with UMC103 **(A)**, CSU155 **(B)** and UMC48 **(C)**. Lane 1, L53 (Al-susceptible parent); lane 2, L1327 (Al-tolerant parent); lane 3, F_1; lane 4, Al-susceptible bulk; lane 5, Al-tolerant bulk; M, molecular weight marker. Arrows indicate the polymorphic regions. **(D)** Schematic representation of polymorphic markers location on chromosome 8 (Chao *et al.*, 1994).

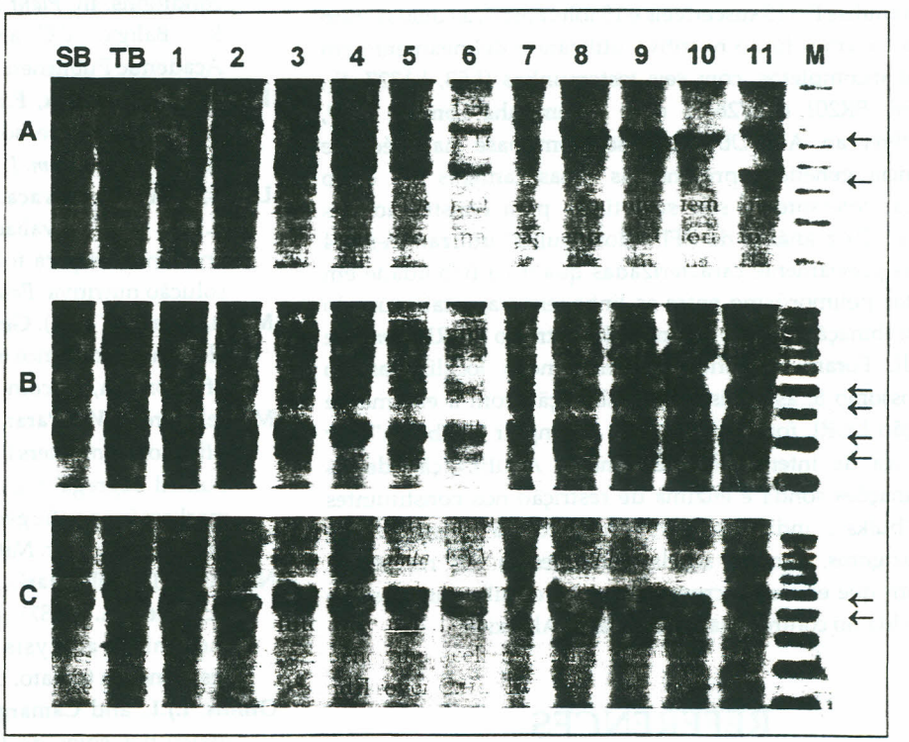

Figure 3 - Southern blots of bulked DNA samples and individual members of the bulks digested with *Eco*RI and probed with UMC103 **(A)**, CSU155 **(B)** and UMC48 **(C)**. **SB**, Al-susceptible bulk; **TB**, Al-tolerant bulk; **1** to **6**, Al-susceptible individuals; **7** to **11**, Al-tolerant individuals; **M**, molecular weight marker. Arrows indicate the polymorphic regions.

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RESUMO

O objetivo deste estudo foi identificar RFLPs (restriction fragment length polymorphisms) ligados a QTLs (locos de caracteres quantitativos) que controlam a tolerância ao Al tóxico em milho utilizando a estratégia de "bulked segregant analysis" (BSA). O material genético utilizado consistiu de uma população F_2 proveniente do cruzamento entre a linhagem L53, suscetível ao Al, e a linhagem L1327, tolerante ao Al. Ambas foram desenvolvidas pelo programa de melhoramento do Centro Nacional de Pesquisa em Milho e Sorgo - CNPMS/EMBRAPA. Avaliaram-se 1554 indivíduos F_2 em solução nutritiva contendo Al tóxico utilizando o índice comprimento relativo da raiz seminal (RSRL) como medidor fenotípico de tolerância. A distribuição de freqüência dos valores de RSRL foi contínua, unimodal com tendência para suscetibilidade. Plântulas F_2 que apresentaram os maiores e menores valores de RSRL foram transplantadas para o campo e autopolinizadas para obtenção de famílias F_3. Selecionaram-se 30 famílias F_3 (15 suscetíveis e 15 tolerantes), as quais foram avaliadas em solução nutritiva utilizando delineamento em blocos incompletos, com seis testemunhas (L53, L1327, F_1, CMS36, BR201 e L726) e uma testemunha comum (L19, suscetível ao Al). Obtiveram-se, com base na média e variância genética apresentadas pelas famílias F_3, cinco famílias tolerantes e seis suscetíveis para construção dos "bulks". Para análise de RFLP dos "bulks" utilizaram-se 54 sondas previamente caracterizadas quanto à habilidade em detectar polimorfismo entre as linhagens parentais quando em combinação com as enzimas de restrição *Eco*RI, *Bam*HI e *Hind*III. Foram identificadas três sondas localizadas no cromossomo 8, as quais, em combinação com a enzima de restrição *Eco*RI, foram capazes de distinguir os "bulks" por diferença de intensidade de bandas. A utilização destas combinações sonda e enzima de restrição nos constituintes dos "bulks", individualmente, mostrou a existência de heterozigotos, para as sondas em questão. Os resultados indicam que existe no cromossomo 8 do milho uma região envolvida no controle da tolerância ao Al tóxico.

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