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USE OF RFLPs TO IDENTIFY GENES FOR ALUMINIUM TOLERANCE IN MAIZE*

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Abstract

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The objective of this study was to identify restriction fragment length polymorphism (RFLP) markers linked to quantitative trait loci that control Al tolerance in maize. The strategy used was bulked segregant analysis, which is based on selecting for bulk bred true F_2 individuals. The genetic material used consisted of an F_2 population derived from a cross between Al susceptible (L53) and Al tolerant (L1327) maize inbred lines. Both lines were developed in the maize breeding programme of the Centro Nacional de Pesquisa de Milho e Sorgo. The relative seminal root length (RSRL) index was used as the phenotypic measure of tolerance. The frequency distribution of RSRL showed continuous distribution, which is typical of a quantitatively inherited character, with a tendency towards Al susceptible individuals. The estimated heritability $[(\sigma^2_{F2} - \sigma^2_E)/\sigma^2_{F2}]$ was found to be 60%. This moderately high heritability value suggests that, although the character has a quantitative nature, it may be controlled by a small number of genes. Those seedlings of the F_2 population that scored the highest and lowest values for RSRL were subsequently selfed to obtain the F_3 families. These families were evaluated in nutrient solution to identify those that were not segregating. On the basis of the results, five individuals were chosen for each bulk. Sixty-five probes were selected at an average interval of 30 cM, covering all ten maize chromosomes. For the hybridization work, a non-radioactive labelling system, using dig-dUTP and alkaline phosphatase, proved to be quite efficient and reliable, resulting in Southern blots with good resolution and allowing the membranes to be stripped and reprobed at least three times. Twenty-three markers showed a co-dominant effect, identifying 40 RFLP loci that could distinguish the parental inbred lines. These 23 probes are now being hybridized with DNA from the two contrasting bulks. Also, a search for other informative markers is being carried out to increase genome coverage.

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1. INTRODUCTION

A major constraint to maize production in the tropics is the problem of excess acidity in the soils. Oxisoils, which are strongly weathered and generally acidic, cover 8.1% of the world's land area [1] and 22% (about 1.1×10^9 ha) of the land in the tropical belt of the world, mainly in the savannah regions of South America and Central Africa [2]. In Brazil alone, acid savannahs with a low cation exchange capacity and high toxic aluminium saturation cover 205×10^6 ha, of which 112×10^6 ha are suitable for agricultural production. Oxisoils exhibit major mineral deficiencies and toxicity. In most of this area, deficiencies in P, Co, Mg and Zn are common, and toxic Al saturation and P fixation by soil particles are usually high [3–5].

Most of the maize cultivars available are susceptible to toxic Al in the soil, and decreases in yield as a result of Al toxicity have been extensively reported in the literature [3–8]. Although technology for topsoil acidity correction is widely used in the tropics, there is no easy means of removing the effects of toxic Al in the subsoil. Therefore, to exploit the soil in Al rich areas it is important to develop breeding programmes aimed at generating Al tolerant cultivars [9]. Maize breeders have identified genetic variability for Al tolerance, and germplasm suitable for selection is available [6, 10–17].

Several techniques based on field and nutrient solutions have been developed for screening Al tolerance in maize [13–15, 17]. Although maize breeders have traditionally relied on evaluations of soils with high Al saturation, assessments of tolerance in nutrient solutions with high Al concentrations have proved to be an effective way of complementing field trials. The nutrient solution technique is rapid and allows screening of thousands of progeny in small spaces as well as better control of environmental variations, which is more difficult to achieve under field conditions.

Among the several parameters used to evaluate maize Al tolerance in nutrient solutions [11, 12, 18], seminal root growth under high Al concentrations seems to be the most reliable. Using this parameter, several authors have shown that the character is quantitatively inherited, with a predominance of additive genetic effects [13–16, 18]. However, owing to its high heritability, this character is thought to be controlled by a small number of genes [19].

Several different mechanisms have been proposed to explain Al tolerance in cultivated plants [20–22], but the mechanisms of Al tolerance in maize have not been described in detail, and its genetic control is still poorly understood. The advent of molecular biology, with all the accompanying techniques for genetic and biochemical analyses, has generated hope that the genetic complexity and molecular control of Al tolerance in maize can be unravelled. This knowledge would offer help in designing better breeding methods for efficient utilization of the existing genetic variability for cultivar development.

The quantitative trait loci (QTL) controlling several important agronomic characters have been successfully studied with molecular markers, using techniques such as restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) [23–26]. However, the commonly used strategies to map QTL are laborious and time consuming, and require a large number of individuals. Bulked segregant analysis (BSA) is a much more efficient technique for scoring molecular markers to specific regions of the genome [27]. This methodology has been shown to be very efficient in the study of traits controlled by a small number of genes, but it can also be used to study major QTL with great effects on the phenotype [28]. Taking into consideration previous studies that have reported Al tolerance in maize as a quantitatively inherited trait possibly controlled by a small number of genes, we chose the BSA strategy to identify the RFLP markers linked to the QTL affecting this trait. In this paper, we describe our partial mapping results using seminal root growth in nutrient solutions as the parameter for evaluating Al tolerance.

2. MATERIALS AND METHODS

2.1. Phenotypic evaluation

The genetic materials used in this study consisted of an F_2 population derived from a cross between the maize inbred lines L53 and L1327 developed in the maize breeding programme of the Centro Nacional de Pesquisa de Milho e Sorgo. L53 and L1327 had previously been identified as Al susceptible and Al tolerant, respectively.

Seven hundred and fifty randomly drawn F_2 seeds, 100 seeds of the F_1 and 100 seeds of each of the two inbred parents, were germinated for 7 d in rolled paper towels moistened with tap water. Six hundred F_2 seedlings and 21 seedlings from each of the other genetic materials were chosen at random. After measuring the initial lengths of the seminal roots, the seedlings were transferred to plastic plates (49 seedlings per plate) and grown in a greenhouse for 7 d in 8 L of aerated nutrient solution containing 6 ppm Al in the form of $KAl(SO_4)_2$ [29]. During harvesting, the final seminal root length was measured and the plants were returned to the nutrient solution. Data relative to the initial and final seminal root lengths were used to calculate the relative seminal root length (RSRL) value. The RSRL indices were established by determining the values of the final seminal root length (FSRL) minus the initial seminal root length (ISRL), and by dividing these values by the ISRL (FSRL − ISRL/ISRL).

The 60 seedlings that scored the highest RSRL values (Al tolerant) and the 60 with the lowest RSRL values (Al susceptible) were transplanted from the nutrient solution to field conditions, grown and selfed to obtain the F_3 families. Thirty days

after planting, the leaf tissue of each F_2 individual was collected, frozen in liquid nitrogen, lyophilized and stored at −20°C for DNA analysis. Fifty-four of these F_3 families (27 tolerant and 27 susceptible) were grown in nutrient solution using the same procedure as that described for F_2 evaluation. 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 opposite end) for bulking, and to eliminate the heterozygous families. The 54 families were distributed in 18 randomized block design experiments with three treatments and two replications, making 36 plates each with 49 seedlings. Each replication contained an F_1 hybrid in order to estimate environmental variance. The genetic variance of each family was calculated from the weighted average of the variances estimated from each replication (total variance minus environmental variance).

2.2. Probe selection and labelling

The set of RFLP markers used in this study corresponds to PstI digested genomic DNA cloned into PUC 19 plasmids, which were obtained from D. Hoisington of the Centro Internacional de Mejoramiento del Maíz y Trigo, Mexico, and S. Chao of the University of Missouri, United States of America. These markers had previously been shown to identify polymorphism in maize and are available along with linkage map data as a public set of maize RFLP probes. Sixty-five probes were selected at an average interval of 30 cM in such a way as to cover all the maize genomes.[1] These probes were then tested for their ability to identify RFLPs between the parental inbred lines (L53 and L1327) when the DNA was digested with EcoRI, BamHI or HindIII.

The probes were labelled via amplification by polymerase chain reaction (PCR) using digoxigenin-11-dUTP (Boehringer Mannheim, Germany). Each reaction mixture consisted of 1 × PCR (10 μL) buffer; 50 μM of dNTPs (dATP, dCTP, dGTP); 48.7 μM of dTTP; 1.25 μM of dig-dUTP; 1.6 units of Taq polymerase; 0.2 μM of CV72 and CV76 primers; 100 ng of plasmid containing the probe fragment; and the final volume was made up to 100 μL with H_2O. Mineral oil (75 μL) was added to the top of each reaction mixture to avoid evaporation during temperature cycling. The amplification cycles were as follows: one 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. A final cycle of 72°C for 1 min completed amplification. The quality of the amplifications was visualized in an agarose gel (0.8%) stained with ethidium bromide.

[1] cM = centimorgan. Morgan is a unit of relative distance between genes on a chromosome. One morgan represents a crossover value of 100%; a crossover value of 1% is a centimorgan.

2.3. Detection of RFLPs

The DNA of the parental inbred lines and their F_1 hybrid was purified from the lyophilized leaf tissue. The DNA was quantified visually in an agarose gel (0.8%) by comparing it with standards of known concentration, and then redissolved to a concentration of 1 μg/μL in TE (10 mM Tris, pH8.0; 1 mM EDTA). Approximately 50 μg of genomic DNA were digested with each of the three restriction enzymes using 2.5 units of enzyme per microgram of DNA for 12 h. The digested DNA was loaded on to a 0.8% (wt/vol.) agarose gel prepared with 1 × TAE buffer (40 mM Tris acetate, pH8.0; 10 mM EDTA) and electrophoresed overnight at 50 V.

The gel was rocked for 1 h in a denaturing solution (0.4 M NaOH; 0.6M NaCl) and then neutralized for 1 h in 0.5M Tris, pH7.5; 1.5M NaCl. The DNA was transferred from the gel on to a nylon membrane in the presence of transfer buffer (1M ammonium acetate; 20 mM NaOH) for a period of 24 h. The membrane was then washed in 2 × SSC for 5 min, in 5 × SSPE for 10 min, blotted dry and baked at 95°C for 3 h.

All the membranes were hybridized in sealed plastic bags at 65°C. Prehybridization was performed for 3 h in 30 mL of hybridization solution (5 × SSC; 0.1% blocking reagent (Boehringer Mannheim, Germany)). Hybridization was carried out for 15–18 h in 30 mL of hybridization solution containing 2400 ng of the probe previously denaturated for 10 min in boiling water. The membranes were washed twice at room temperature in 0.15 × SSC, 0.1% SDS for 5 min, and three times for 15 min at 65°C. The membranes were then incubated in buffer 2 (0.01M Tris-HCl, pH7.5; 0.15M 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 of buffer 2) for 1 h, washed three times for 10 min in buffer 2 (0.01M Tris-HCl, pH7.5; 0.15M NaCl) and once for 5 min in buffer 3 (100 mM Tris-HCl; 100 mM NaCl; 50 mM $MgCl_2$). The membranes were exposed to the substrate for alkaline phosphatase (AMPPD, 10 μL/mL of buffer 3) for 1 h, wrapped in saran wrap and exposed to X ray films for 15–18 h. The membranes were stripped through washes in 2 × SSC for 10 min at room temperature, 0.2M NaOH, 0.1% SDS for 10 min at 37°C, 5 min in TE at room temperature, and then stored in TE at 4°C until use.

3. RESULTS AND DISCUSSION

The frequency distribution of the RSRL values obtained for the F_2 population is shown in Fig. 1. The mean RSRL values of the parental inbred lines (L53 and L1327) and their F_1 hybrid were 0.13, 1.13 and 1.42, respectively, and their variances were 0.011, 0.076 and 0.176, respectively. The F_2 population showed an average of 0.688 and a variance of 0.175. The figure shows continuous distribution,

which is typical of quantitatively inherited traits, with a tendency towards more susceptible individuals. This is in agreement with the information that tolerance to Al is a quantitatively inherited trait.

The environmental variance was estimated from the average of the inbred lines and the F_1 hybrid variances. The estimated heritability $[(\sigma^2_{F2} - \sigma^2_E)/\sigma^2_{F2}]$ was found to be 60%. This moderately high heritability value indicates that, although the character has a quantitative nature, it may be controlled by a small number of genes.

The adjusted mean values and the variance of RSRL obtained for the F_2:F_3 families are shown in Table I. Five individuals from each extreme (highest and lowest RSRL values) associated with a low variance were chosen to build two bulks of contrasting phenotype (Al tolerant and Al susceptible).

The non-radioactive labelling system using dig-dUTP and alkaline phosphatase proved to be quite efficient and reliable, resulting in blots with good resolution and

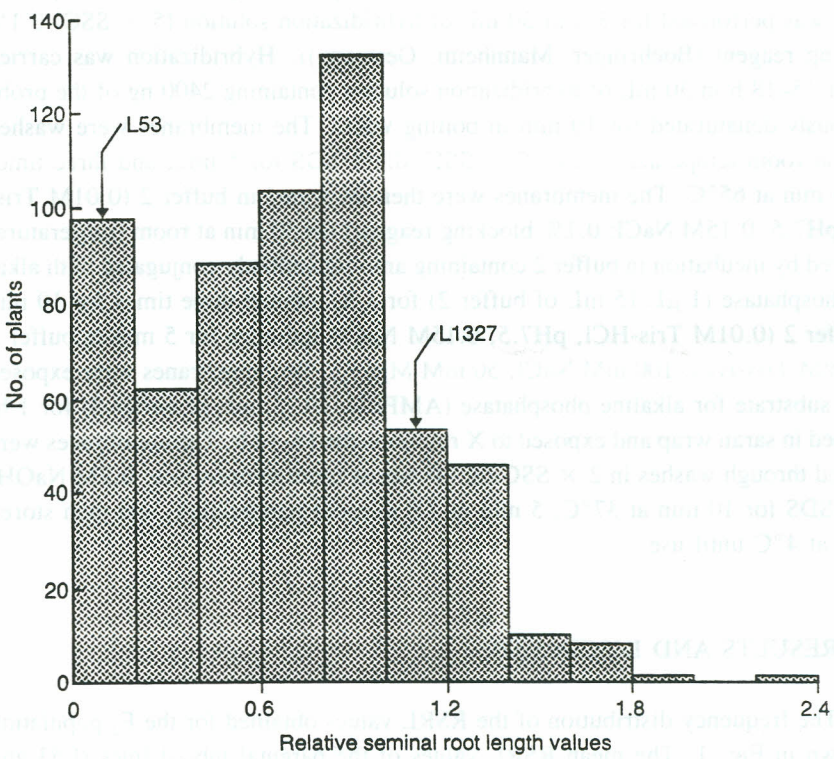

FIG. 1. Histogram of the distribution of RSRL values obtained for the F_2 population grown in nutrient solution containing toxic Al.

TABLE I. ADJUSTED MEANS AND VARIANCE OF RSRL OBTAINED FOR THE F_2:F_3 FAMILIES

(Numerals in bold indicate the materials used to build the two bulks) [a]

F_3 families	Average (RSRL)	Variance[b] ($\times 10^{-3}$)	F_3 families	Average (RSRL)	Variance ($\times 10^{-3}$)
1	0.2151	3.20	28	0.2102	13.97
2	0.1555	2.16	29	0.2585	9.67
3	0.1195	0.00	30	0.1486	2.58
4	0.1311	0.48	31	0.5025	4.95
5	0.1281	1.02	**32^T**	**0.3543**	**4.50**
6	0.1685	3.77	33	0.1704	5.62
7	0.1141	0.00	34	0.1331	3.15
8	0.1328	0.12	35	0.1094	0.83
9	0.1252	0.74	36	0.2010	7.81
10^T	**0.2527**	**14.21**	37	0.2366	6.26
11	0.1437	5.07	**38^T**	**0.2495**	**12.56**
12	0.2264	4.94	39	0.1400	0.33
13	0.1285	1.89	40	0.1169	0.04
14^S	**0.1421**	**3.95**	41	0.1851	4.85
15	0.1410	0.00	42	0.1723	31.75
16^T	**0.2002**	**13.66**	43	0.1899	23.96
17	0.1710	0.00	**44^S**	**0.1435**	**0.82**
18	0.1510	3.39	45	0.2027	20.67
19	0.3190	39.24	46	0.1596	6.42
20	0.1460	3.72	**47^S**	**0.0994**	**0.00**
21^S	**0.1260**	**0.00**	48	0.1483	0.43
22	0.1930	3.08	**49^S**	**0.1005**	**0.00**
23	0.1680	21.36	50	0.1946	5.75
24	0.4290	29.17	51	0.1807	4.28
25	0.1403	0.05	52	0.2349	6.63
26	0.1132	0.73	53	0.1056	25.28
27	0.0947	0.01	**54^T**	**0.3708**	**17.17**

[a] ^{T} = tolerant; ^{S} = susceptible.

[b] $\sigma^2_{G2} = (n_1 - 1)\,\sigma^2_{G1} + (n_2 - 1)/(n_1 + n_2 - 2)$, where n_1 is the number of plants in replication 1; n_2 is the number of plants in replication 2; and σ^2_{G1} and σ^2_{G2} are the genetic variance in each replication.

allowing the membranes to be stripped and reprobed at least three times. Figure 2 shows an example of a Southern blot obtained using the probe UMC 43 labelled with dig-dUTP. The enzymes used were EcoRI, HindIII and BamHI, and the DNA was extracted from the inbred lines L53 and L1327 and their F_1 hybrid.

From the 65 selected probes, 23 showed a co-dominant effect, identifying 40 RFLP loci that could distinguish the parental inbred lines. The tolerant and susceptible DNA bulks are now being hybridized with these 23 selected probes. Also, we are screening additional cDNA and genomic RFLP markers in order to search for other informative probes to increase genomic coverage.

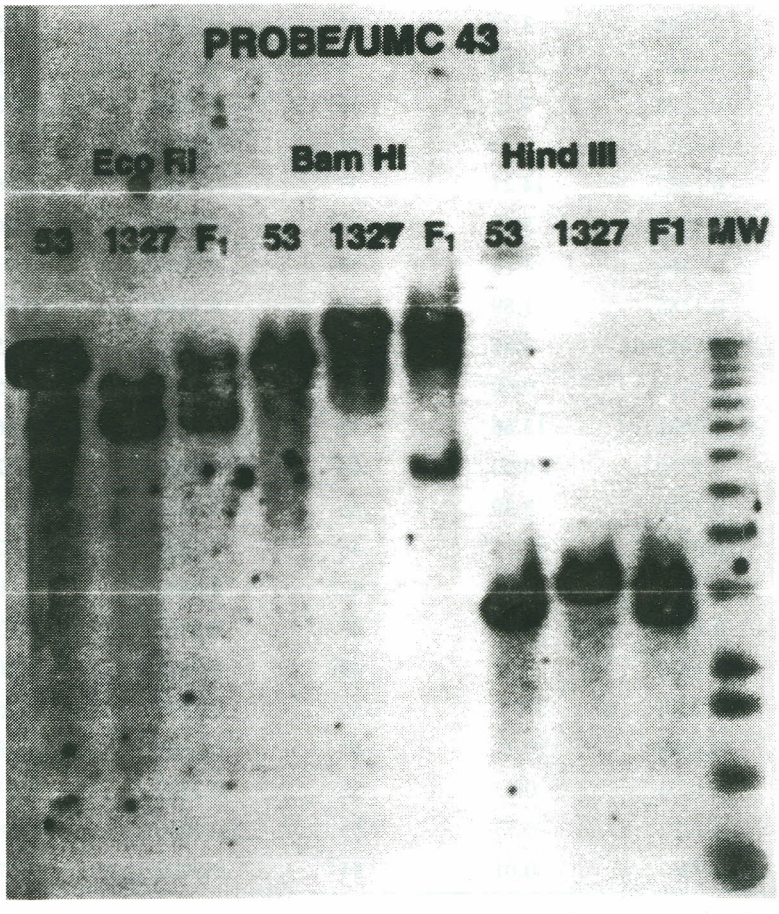

FIG. 2. Autoradiograph of the genomic DNA of inbred lines L53 and L1327 and their F_1 hybrid digested with EcoRI, HindIII and BamHI and probed with UMC 43 labelled with dig-dUTP.

The $F_{2(L53 \times L1327)}$:F_3 families were planted in the field to be selfed for three more generations in order to obtain recombinant inbred lines. These genetic materials will allow us to isolate and study the effect of the QTL identified as being involved in the control of Al tolerance.

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