ALUMINUM TOLERANCE IN BARLEY: MOLECULAR MAPPING ANALYSES

TOLERÂNCIA AO ALUMÍNIO EM CEVADA: ANÁLISES DE MAPEAMENTO MOLECULAR

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ABSTRACT

Southern Brazil is characterized by acid soils with high levels of aluminum (Al), which difficult the total growth of the plant root, and barley is, among the cereal species, the most sensitive to this metal. Development of Al tolerant cultivars may be an effective solution to this problem. In the present study, RFLP, microsatellite, and AFLP markers were analyzed in a F_2 progeny originated from a single cross between the cultivars 'FM-404' (moderately Al tolerant) and 'Harrington' (Al tolerant) to identify molecular markers linked to the Al tolerance gene. Al tolerance was identified by the hematoxylin staining method. Al tolerance was found to be controlled by a single gene in 'FM-404' and the RFLP marker Xwg464, located on the long arm of the chromosome 4H, was found to be located 21.6 centiMorgans from this gene.

Key words: AFLP, microsatellites, RFLP, linkage mapping.

RESUMO

O sul do Brasil é caracterizado por solos ácidos com altos níveis de alumínio (Al) que impedem o crescimento total da raiz das plantas. A cevada é a espécie mais sensível entre os cereais para este metal. O desenvolvimento de cultivares tolerantes ao Al pode representar uma solução efetiva para este problema. No presente trabalho, foram analisados marcadores RFLPs, microsatélites e AFLPs em uma progênie F_2, originada um único cruzamento entre as cultivares 'FM-404' (moderadamente tolerante ao Al) e 'Harrington' (tolerante ao Al) para identificar marcadores moleculares associados ao gene de tolerância ao Al. A tolerância foi identificada pelo método de coloração com hematoxilina. Os resultados indicaram que a tolerância é controlada por um único gene em 'FM-404' e o marcador RFLP Xwg464, localizado no braço longo do cromossomo 4H, foi mapeado a 21,6 centiMorgans deste gene.

Palavras-chave: AFLP, microssatélites, RFLP, mapa de ligação.

INTRODUCTION

Southern Brazil is characterized by acid soils associated to aluminum (Al), and this condition represents a significant restriction to crop production (ANIOL, 1990; BENNET & BREEN, 1991). The initial and most dramatic symptom of Al toxicity is inhibition of root growth, and the reduction in its growth is detectable within minutes after Al addition (JONES & KOCHIAN, 1995; DEGENHARDT et al., 1998). The damage caused in the root system may lead to both nutrient deficiencies and water stress.

The literature contains frequent reports about the basis of Al tolerance in plants and some hypothesis have been proposed. For example, some species accumulate high concentrations of Al and must possess effective mechanisms for detoxifying the Al internally. This can be related to Al complexation by organic ligands, pH effects to reduce the concentration of Al^{3+}, compartmentation into vacuole, exudation of various compounds, and development of Al-resistant proteins (TAYLOR, 1991). Another Al tolerance mechanism involves exclusion of Al from the root apex, which may be attained either by the release of Al-chelating ligands or root-induced increases in rhizosphere pH (PELLET et al., 1994; RYAN et al., 1995; TAYLOR, 1988).

Conventional liming or other soil management practices are frequently inefficient for avoiding Al phytotoxicity (RAO et al., 1993). The lime application to the soil is ineffective in the subsoil and deep lime incorporation is technically difficult and expensive. For these reasons, development of cultivars adapted to acid soil is a promising alternative and has been the most effective strategy for crop production in this kind of soil.

The genetics of Al tolerance has been studied in several important crop plants. According to the species, Al tolerance can be monogenic or multigenic. In wheat (Triticum aestivum L. Thell), ANIOL (1990) found that Al tolerance is a complex character, controlled by several major genes, minor modifying genes, and probably by genes controlling suppression of Al tolerance genes; although DELHAIZE et al. (1993) have showed that it is controlled by a single locus. In rye (Secale cereale L.) and maize (Zea mays L.), two major dominant and independent loci controlling the Al tolerance were found (GALLEGO & BENITO, 1997; SIBOV et al., 1999). In rice (Oryza sativa L.), Al tolerance is a multigenic trait (KATIWADA et al., 1996), and in soybean (Glycine max (L.) Merrill), it was found to be associated with quantitative trait loci (BIANCHI-HALL et al., 2000). In barley (Hordeum vulgare ssp. vulgare L.), MINELLA & SORRELLS (1992) indicated that the Al tolerance is monogenic with expression of tolerance dependent on Al concentration and allele dose. ECHART & CAVALLI-MOLINA (2002) and RAMAN et al. (2002) had also observed monogenic inheritance although REID (1971) had suggested, in addition to the major gene, the presence of minor gene effects interfering in the Al tolerance.

Advances in DNA marker technology have added a new dimension to the study of genetic traits since the past decade, offering hope that marker technology can clarify the genetics of Al tolerance and aid practical breeding (BIANCHI-HALL et al., 2000). The breeder can use markers for marker-assisted selection (MAS). In addition, genetic information and molecular markers obtained for a trait in one species may be exploited in related species, giving more precise information about a trait through comparative mapping. Although Al tolerance is important for growing barley in many parts of the world, the

gene localization of this trait was not investigated until recently. TANG et al. (2000) reported three RFLP markers tightly linked to the *Alp* gene (Al tolerance gene) in the cultivar 'Dayton': Xbcd1117, Xwg464, and Xcdo1395, and RAMAN et al. (2002) described four microsatellite markers (Bmac310, Bmag353, HVM68, and HVMCABG) tightly linked to this gene in the cultivar 'Yambla'.

The objective of the present work was to obtain data about the location of major Al tolerance genes in barley analyzing a F_2 population obtained from a cross involving a Brazilian Al tolerant ('FM-404') cultivar.

MATERIAL AND METHODS

Genetic material: Cultivars 'FM-404' and 'Harrington' were crossed to obtain a segregating F_2 population. 'FM-404' is a Brazilian two-rowed spring barley, which represents one of the most Al tolerant genotypes developed under acid soil conditions in Brazil, although MINELLA & SORRELLS (1992) had considered it as only moderately tolerant. The pedigree of 'FM-404' is uncertain, but according to ARIAS et al. (1983), 'Alpha' (which is derived from the cross 'Manchuria'/'Champion of Vermont') is one of the parents. This cultivar was developed and supplied by AmBev Brewery, Navegantes Malting Subsidiary, Porto Alegre, Rio Grande do Sul (RS), Brazil. 'Harrington' is an Al sensitive Canadian two-rowed spring barley. It was supplied by Embrapa-Trigo, Passo Fundo, RS, Brazil. The pedigree of 'Harrington' is 'Klages'/3/'Gazelle'/'Betzes'//'Centennial'.

The cross of 'FM-404' with 'Harrington' was made in a greenhouse of AmBev Brewery in Encruzilhada do Sul, RS, Brazil, in 1998. F_2 progeny was obtained from selfing of one F_1 plant, and each F_2 plant was selfed to obtain the F_3 generation. Selfing of F_1 was made in Encruzilhada do Sul, in 1999, and those of F_2 plants were made in a greenhouse at Cornell University, Ithaca, NY, USA, in 2000.

Aluminum tolerance screening: Al response was evaluated in the parents and F_2 generation. To confirm the genotype of F_2 plants, 12 individuals from each F_3 progeny, obtained from individual F_2 plants selfed, were also analyzed. Al tolerance analysis was assayed by the hematoxylin staining technique as previously described by POLLE et al. (1978) with some modifications. Disinfected seeds were germinated in Petri dishes and 24 hours later, the seedlings were placed in hydroponic solution following the protocol described by TANG et al. (2000) with $30\mu M$ $AlCl_3$ that best distinguished the genotypes. The seedlings were visually scored based on the hematoxylin staining of the root tips, as complete (C), partial (P), and non-stained (N).

DNA extraction: DNA was extracted from leaf tissue of parents and F_2 progeny according to the method described by REIDE & ANDERSON (1996).

Microsatellites (SSRs) analysis: Thirty-seven primer pairs (LIU et al., 1996) were used to analyze microsatellite sequences placed in the seven chromosomes of barley (Table 1). Polymerase chain reaction (PCR) amplifications were performed in a thermal cycler (Perkin-Elmer Corporation, model 480, Norwalk, CT, USA). The reaction mixture was that described by SAGHAI-MAROOF et al. (1994) except that the mixture contained $200\mu M$ each deoxynucleotide without radiolabel. Depending on the primer pair used, amplification of the SSRs was performed using different conditions: the conditions 1, 2, and 3 were as described by LIU et al. (1996), while the conditions 4 and 5 were as described by BECKER & HEUN (1995) and RÖDER et al. (1995), respectively (Table 1). PCR products were denatured, separated by 6% denaturing polyacrylamide (sequencing) gels containing 8M urea at 60W constant current and revealed using silver staining.

AFLP analysis: The AFLP Analysis System I Kit (GIBCO BRL) was used for AFLP analysis following the manufacturer's recommendations. *Eco*RI and *Mse*I endonucleases were used to generate the restriction fragments. The pre-amplification was performed with two AFLP primers, each having one selective nucleotide, and the selective amplification with two AFLP primers, each containing three selective nucleotides. The *Eco*RI primers were end-labelled with ^{33}P using T4 polynucleotide kinase. One hundred and forty four combinations (8 *Eco*RI x 18 *Mse*I primers) were used for the analysis. Products from selective amplification were separated on 6% denaturing polyacrylamide (sequencing) gel. After drying, gels were exposed to X-ray film.

RFLP analysis: Ten restriction enzymes (*Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, *Hae*I, *Hind*III, *Pst*I, *Sst*I, *Xba*I, and *Xho*I) were used to digest DNA samples and survey filters were hybridized with 24 wheat, barley and oat (*Avena sativa* L.) genomic and cDNA clones (Xbcd1117, Xcdo1395, Xrz517, Xwg464, Xabc321, Xbcd1092, Xabg3, Xcdo795, Xcdo541, Xabg472, Xcdo586, Xwg114, Xcdo669, Xcdo465, Xbcd15, Xwg181, Xcdo1312, Xcdo38, Xmwg542, Xbcd1230, Xabg394, Xmwg634, Xcdo650, and Xbcd1092) that have been mapped to barley chromosome 4 (GRANER et al., 1991; HEUN et al., 1991; KLEINHOFS et al., 1993; TANG et al., 2000). This chromosome was more intensively investigate because there was previous indication of presence of at least one barley Al tolerance gene. The filters were sequentially washed with procedures previously described by TANG et al. (2000) and then were placed against X-ray film to obtain autoradiographs.

Molecular polymorphism analysis: The polymorphism screening was performed in the parental cultivars for RFLP and SSR markers. For the AFLP technique, the BSA method (MICHELMORE et al., 1991), with two bulks constructed from extreme phenotypes of the F_2 population was used, aiming to reduce false positive polymorphisms between parents. Eighty-four plants of F_2 generation were analyzed individually with the polymorphic RFLP, SSR, and AFLP markers.

Linkage analysis: Linkage analysis was conducted with the MAPMAKER version 3.0 (LANDER et al., 1987), using Kosambi mapping function, a LOD score of 5.0 and a recombination fraction of 0.30.

Table 1- Primer sequences, PCR conditions, chromosomal location of barley SSRs, and polymorphism occurrence.

SSR	Repeat	PCR Condition[1]	Barley Chromosome	Polymorphism
HVM2	$(GA)_{11}$	1	7	no
HVM3	$(AT)_{29}$	3	4	no
HVM4	$(AT)_9$	3	1	yes
HVM5	$(GT)_6(AT)_{16}$	3	1	no
HVM6	$(GA)_9$	1	7	no
HVM13	$(GA)_{6,}(GA)_{6,}(GA)_6$	1	4	no
HVM14	$(CA)_{11}$	1	6	no
HVM15	$(GA)_8$	1	3	no
HVM20	$(GA)_{19}$	1	5	yes
HVM22	$(AC)_{13}$	1	6	no
HVM23	$(GA)_9$	2	2	no
HVM26	$(CA)_{11}$	2	2	no
HVM27	$(GA)_{14}$	1	3	no
HVM30	$(CA)_8$	2	7	yes
HVM33	$(CA)_7$	1	3	no
HVM34	$(GA)_{10}$	2	6	yes
HVM36	$(GA)_{13}$	1	2	yes
HVM40	$(GA)_{6,}(GT)_{4,}(GA)_7$	1	4	yes
HVM43	$(CA)_9$	1	5	yes
HVM44	$(GA)_8$	1	3	no
HVM49	$(CA)_{12}$	1	1	no
HVM50	$(GA)_9$	3	2	no
HVM54	$(GA)_{14}$	1	2	no
HVM60	$(AG)_{11,}(GA)_{14}$	1	3	yes
HVM62	$(GA)_{11}$	1	3	no
HVM65	$(GA)_{10}$	1	6	yes
HVM67	$(GA)_{11}$	1	4	no
HVM68	$(GA)_{22}$	1	4	yes
HVM70	$(CA)_8$	3	3	yes
HVM74	$(GA)_{13}$	3	6	yes
HVM77	$(CA)_7$	1	4	no
HVBKASI	$(C)_{10,}(A)_{11}$	4	2	no
HVCMA	$(AT)_9$	4	1	no
HVCSG	$(CA)_{4,}(C)_{17}$	4	2	no
HVDHN7	$(AAC)_5$	4	7	no
HVDHN9	$(AC)_6$	4	7	yes
HVLEU	$(ATTT)_4$	4	7	no

[1] PCR conditions: 1,2, and 3 (LIU et al., 1996); 4 (BECKER & HEUN, 1995); 5 (RÖDER et al., 1995)

RESULTS AND DISCUSSION

To identify the Al tolerant and sensitive genotypes, the hematoxylin staining procedure was used. The technique developed by POLLE et al. (1978) consists of a visual estimation of the stain ability of the root tips of young plants by hematoxylin following growth in the presence of Al. According to the authors, the test was developed based on the evidence that Al enters primarily at near the root tip. Tolerant genotypes tend to accumulate less Al in their roots than sensitive ones, and hematoxylin produces permanent staining upon reaction with Al in the root cells (WRIGHT & DONAHUE, 1953; VOSE & RANDALL, 1962; FOY et al., 1967; HENNING, 1975). The method has given results consistent with those based on root growth measurements (CARVER et al., 1988; POLLE et al., 1978), and has been used in genetic studies, breeding programs, and mapping studies in cereals (TAKAGI et al., 1983; SCOTT & FISHER, 1986; LARKIN, 1987; CARVER et al., 1988; MINELLA & SORRELLS, 1992, 1997; SIBOV et al., 1999; TANG et al., 2000; ECHART & CAVALLI-MOLINA, 2002). This method was chosen because it is simple, accurate

and non-destructive and the seedlings can grow to maturity.

The hematoxylin stain procedure allowed classifying the barley plants analyzed into three groups in relation to Al tolerance, according to previous nomenclature described by MINELLA & SORRELLS (1992): Al sensitive plants as C (completely stained), equal to 'Harrington'; Al tolerant plant as N (non stained), such as 'FM-404'; and those with an intermediate phenotype similar to 'FM-404' but with a fainter staining in the root apical region as P (partially stained). This classification was confirmed by analysis of the descendants from each F_2 plant, since no segregation was observed in progenies from F_2 plants classified as homozygotes for tolerance (non stained) or sensitivity (completely stained), while 1:2:1 (N:P:C) segregation was observed in plants previously considered heterozygotes (partially stained). Among 84 F_2 plants analyzed, 24 could be identified as N, 18 as C, and 42 plants were classified as P. The segregation 1:2:1 (N:P:C) observed in F_2 (χ^2= 0.43, P= 0.513) indicated the presence of a single gene for Al tolerance segregating in this population. Monogenic inheritance for Al tolerance has already been reported by MINELLA & SORRELLS (1992), TANG et al.

(2000), ECHART & CAVALLI-MOLINA (2002).

Thirteen microsatellite markers, corresponding to 38% of total analyzed, revealed differentiation between the parents and were used to analyze the mapping population (Table 1). On the other hand, RFLPs were less polymorphic, with only two markers (Xwg464 and Xbcd15) revealing differences between the parents. The AFLP analysis also detected a low polymorphism, where only five primer combinations were suitable for segregation analysis in the mapping population (E-AAC/M-CCA, E-AGC/M-CCT, E-AAG/M-CGT, E-ACC/M-CAG, and E-ACA/M-ACT).

Many factors are extremely important in map construction. The genetic distance between parental cultivars has direct effects on the ability to detect polymorphic markers which differentiate them. Although many studies have shown that molecular markers, including microsatellites (BECKER & HEUN, 1995; BIANCHI-HALL et al., 2000), AFLPs (YIN et al., 1999; PIERRE et al., 2000), and RFLPs (GRANER & BAUER, 1993; LEISTER et al., 1999), are efficient for mapping genes, the cultivars 'Harrington' and 'FM-404' exhibited a low level of inter-cultivar molecular differentiation. The low level of polymorphism observed made more difficult to find molecular markers linked to barley Al tolerance gene. However, a common genetic origin of these cultivars cannot be the explanation for this similarity. 'FM-404' is a Brazilian cultivar described as originated by selection from 'Alpha', which is derived from the cross 'Manchuria'/ 'Champion of Vermont' (ARIAS et al., 1983), while 'Harrington' is a Canadian cultivar originated by crosses of 'Klages'/3/'Gazelle'/ 'Betzes'//'Centennial'. Relatively low differentiation between barley lines has been previously reported (GRANER et al., 1991; HEUN et al., 1991; SAGHAI-MAROOF et al., 1994) and has made the development of barley maps fairly laborious.

The 20 polymorphic loci data set evaluated did not allow the construction of a linkage map. Only one short linkage group belonging to chromosome 4 was detected (Figure 1). The co-dominant RFLP marker Xwg464, located on the long arm of chromosome 4H (KLEINHOFS et al., 1993), showed linkage to Al tolerance gene with 21.6 centiMorgans (cM) of distance (LOD score 5.0). The microsatellite HVM68, mapped in the same chromosome 4 of barley (LIU et al., 1996), was approximately 21.9 cM from marker Xwg464; in other words, more than 40cM from Al tolerance gene. Those two markers segregated in 1:2:1 ratio in the F_2 population which is in agreement with the segregation of co-dominant loci (Xwg464 - χ^2=0.43, P=0.513; HVM68 - χ^2=0.79, P=0.375). No other markers showed linkage to each other or to Al tolerance gene.

The marker Xwg464 had already been found linked to Al tolerance gene in the cultivar 'Dayton', but distant 2.1 cM from that. It is possible to presume that the Al tolerance gene in 'FM-404' is the gene *Alp* of 'Dayton' based on MINELLA & SORRELLS (1992) results, which showed no segregation for this trait in a cross between 'FM-404' and 'Dayton' (both Al tolerant cultivars). Differences in the map positions of low and multicopy probes obtained in other mapping populations have occasionally been reported (BEAVIS & GRANT, 1991; SHERMAN et al., 1995). SCHÖNFELD et al. (1996), mapping RFLPs for resistance genes to powdery mildew in barley, found seven low-copy clones mapped in positions that differed from previously published barley RFLP maps. They suggested that the low-copy characteristic of these probes together with the differences in linkage between them and the target gene are indicating that these RFLP loci may have originated from independent duplication events. Nevertheless, duplication of the RFLP Xwg464 marker cannot be the explanation for the difference observed for the distance between this locus and the Al tolerance loci in the cultivars 'Dayton' and 'FM-404', because this RFLP marker is a single copy clone.

Other facts that may explain the different distances found in the present analysis and in the research of TANG et al. (2000) would be the occurrence of simple or double crossing-over in different frequencies or the occurrence of chromosomal rearrangements. The occurrence of double crossing-over between the two target genes would underestimate the distance observed between these genes because it would reduce the number of recombinant individuals. However, due to the large difference observed is difficult to affirm that double crossing-over can explain this result. Otherwise, the deletion of a chromosomal segment between these two genes would shorten the distance between them, and the addition of a segment would enlarge the distance, wich could explain the difference observed between the distance of the barley Al tolerance gene and the molecular marker.

Due to the distance between the Al tolerance gene and the molecular marker Xwg464, this marker is not appropriated to be used in breeding programs that aim the selection of Al tolerance. Therefore, other crosses with Brazilian cultivars and other molecular markers must be investigated to find a marker tightly linked to Al tolerance gene in Brazilian barley genotypes.

CONCLUSIONS

Al tolerance was found to be controlled by a single gene and mapped on the long arm of the chromosome 4H, located 21.6 centiMorgans from the RFLP marker Xwg464 in the Brazilian barley cultivar 'FM-404'.

ACKNOWLEDGEMENTS

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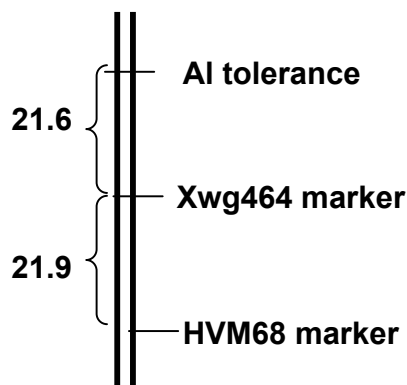

Figure 1 - Map of chromosome 4 region with the Al tolerance gene and two linked molecular markers obtained from the cross between Al tolerant 'FM-404' and Al sensitive 'Harrington' cultivars.

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