Theor Appl Genet (2002) 105:458–464
DOI 10.1007/s00122-002-0934-0

H. Raman · J. S. Moroni · K. Sato · B. J. Read
B. J. Scott

Identification of AFLP and microsatellite markers linked with an aluminium tolerance gene in barley (*Hordeum vulgare* L.)

Received: 2 November 2001 / Accepted: 12 November 2001 / Published online: 11 June 2002

Abstract Barley is the most sensitive among the cereals to aluminium (Al) stress and breeding for more tolerant cultivars is a priority. To enhance selection efficiency for Al tolerance in barley, PCR-based AFLP and microsatellite markers linked to a locus conferring tolerance to aluminium were identified. The study used F_2 progeny derived from a single cross between Yambla (moderately tolerant of Al) and WB229 (tolerant of Al) and developed hydroponic pulse-recovery screening methods to assess tolerance of phenotypes based on root growth. The segregation ratios of tolerant and sensitive genotypes and F_3 progeny testing suggest that a single major gene controlled Al tolerance (*Alt*). In order to determine the chromosomal location of the *Alt* gene, we used the AFLP technique coupled with bulk segregant analysis. We evaluated tolerant and sensitive bulks using 30 combinations of *Eco*RI/*Mse*I primers, and 12 of these permitted differentiation of the sensitive and tolerant bulks. More than 1,000 amplified fragments were obtained, and 98 polymorphic bands were scored. AFLP analysis of wheat-barley chromosome addition lines indicated that the *Alt* gene was located on barley chromosome *4H*. Four chromosome *4H*-specific microsatellite markers (Bmac310, Bmag353, HVM68 and HVMCABG) were tightly linked to *Alt*. The large allelic variation detected with microsatellite marker Bmag353 allowed us to implement this marker for routine marker-assisted selection for Al tolerance, and 396 plants could be screened on a single gel.

Communicated by B.S. Gill

H. Raman (✉) · J.S. Moroni · B.J. Read · B.J. Scott
NSW Agriculture, Wagga Wagga Agricultural Institute, PMB, Pine Gully Road, Wagga Wagga, NSW 2650, Australia
e-mail: harsh.raman@agric.nsw.gov.au
Fax: +61-2-69381822

K. Sato
Research Institute for Bioresources, Okayama University, Kurashiki, 710-0046, Japan

Keywords AFLP · Microsatellites · Bulk segregant analysis · Linkage mapping · Aluminium tolerance · Marker-assisted selection

Introduction

Acid soils are commonly found in various parts of the world and include 1.5 billion hectares of cultivated lands (Baier et al. 1996). In Australia, soil acidity affects in excess of 90 million hectares of agricultural land and accounts for economic losses between $300 and $400 million (Watson 1998). In very acid soils (pH_{cacl_2}. <5.0), aluminium (Al) is the dominant element of concern, and the yield potential cannot be achieved because of Al toxicity caused by Al^{3+} ions (Kochian 1995; Scott et al. 1997). The toxic ions are released into solution and inhibit root cell division and cell elongation and DNA synthesis; this damage to roots impedes water and nutrient acquisition, transport of essential nutrients and, as a result, grain yield and quality (Foy 1984; Scott et al. 1997). Furthermore, Al toxicity can accentuate a problem with drought even in well-managed soils. Several approaches have been used to increase productivity on these soils, including soil and crop improvement. Liming can easily ameliorate soil acidity. However, while this is cost-effective for ameliorating acid surface soils, it is not feasible to lime strongly acid subsoils (Foy 1992). Selection and development of varieties tolerant to Al is a less expensive alternative or additional strategy (Foy 1996).

Among the winter cereals, barley is the most sensitive to Al (Mugwira et al. 1976; Scott and Fisher 1993). However, a wide range of genetic variability for tolerance of Al occurs (Foy et al. 1965) and has been exploited by conventional breeding (Read and Oram 1995). To expedite the transfer and selection of gene(s) for Al tolerance, a number of methods of screening for Al tolerance have been used with varying degrees of reliability; these methods include pot assays (Foy 1996), hydroponic methods (Berzonsky and Kimber 1986; Ma et al. 1997), a chlorophyll fluorescence test (Moustakas et al.

1993) and haematoxylin staining (Polle et al. 1978; Minella and Sorrells 1992; Cancado et al. 1999; Gallardo et al. 1999). Most of these methods are labour-intensive, time-consuming, can handle only small populations and are not able to discriminate heterozygotes. Hence, there is a need to develop a rapid, reliable and cost-effective screening system for large-scale screening of Al-tolerant germplasm of barley. Molecular markers are highly regarded as an efficient selection tool to indirectly select traits linked to them.

Although the inheritance of Al tolerance in barley is reported to be under the control of a single gene (Reid et al. 1971; Minella and Sorrells 1992), varying segregation ratios have been observed (Minella and Sorrells 1992). The gene conferring tolerance to Al (*Alp*) from the variety Dayton has been located on barley chromosome *4H* (Minella and Sorrells 1997) and has recently been mapped using an F_2 population of Dayton (tolerant)/Harlan Hybrid (moderately tolerant) with restriction fragment length polymorphism (RFLP) markers (Tang et al. 2000).

Polymerase chain reaction (PCR)-based techniques have been used to identify molecular markers associated with various traits of agronomic importance. The amplified fragment length polymorphism (AFLP) technique (Vos et al. 1995) has been considered to be the most powerful in revealing the highest level of DNA polymorphism and is extensively being used to map qualitative and quantitative trait loci. AFLP markers have a high multiplex ratio as compared to other available marker systems (Powell et al. 1996). Bulk segregant analysis (BSA) permits efficient screening for molecular markers on bulked DNA pools rather than using DNA from individual plants (Michelmore et al. 1991) and has been extensively used to develop markers in various crops.

The investigations reported here were conducted to (1) determine the genetic control of Al tolerance and its chromosomal location in the Australian line WB229, derived from an Al-tolerant New Zealand cultivar, Kaniere and (2) to identify tightly linked molecular markers suitable for routine marker-assisted selection.

Materials and methods

Mapping and validation population

Two genotypes of two-row barley, Yambla (moderately tolerant to Al) and WB229 (advanced breeding line with tolerance to Al, derived from O'Connor/Kaniere) were used to map Al tolerance. A single Yambla/WB229 F_1 plant was selfed, and 67 randomly chosen F_2 plants were used for linkage mapping. After scoring for Al tolerance in solution culture, the F_2 plants were transplanted into pots and allowed to self to produce F_3 families for progeny testing. The usefulness of the microsatellite markers linked with the Al-tolerance gene was investigated using a validation population of 98 F_2 plants derived from WB229/Mimosa from a single F_1 plant.

Screening of the mapping population for aluminium tolerance

Seedlings of both parents, WB229 and Yambla, and their F_2 progeny were screened for Al tolerance in a solution culture system using a modified pulse-recovery method (Berzonsky and Kimber 1986). The screening process is outlined in method 1 in Table 1. Seeds were surface-sterilised in 1.2% sodium hypochlorite for 20 min and germinated overnight at room temperature in an aerated nutrient solution. Germinated seeds were laid on a plastic mesh, crease down, suspended over 10 l of a complete nutrient solution and were grown for 6 days at 21 °C. The nutrient solution contained (μ*M*): Ca, 1,000; Mg, 400; K, 1,000; NO_3, 3,400; NH_4, 600; PO_4, 100; SO_4, 401.1; Cl 78; Na, 40.2; Fe, 20; B, 23; Mn, 9; Zn, 0.8; Cu, 0.30; Mo, 0.1. Iron was supplied as Fe-EDTA prepared from equimolar amounts of $FeCl_3$ and Na_2EDTA. For the first 3 days of germination the container was covered with black plastic. Seven days after germination, four seedlings of the parents and 67 F_2 seedlings were transferred to a 45-l tank containing the complete nutrient solution. Each seedling was mounted with strips of polyurethane foam on a plastic frame, which covered the tank. The frame consisted of a 1.2-cm plastic grid covered with a thick polyethylene sheet white on one side and black on the other. The white surface of the plastic was facing upward, thus shielding the nutrient solution from light to inhibit algal growth. Holes were made on the sheet equi-distantly to accommodate each seedling separately. Plants were grown in a heated and evaporatively cooled greenhouse at a day/night temperature of 24/18 °C under a 16/8-h (day/night) photoperiod. Solution temperatures were maintained at 19 °C by immersing the tank in a common water bath, which was being cooled by a refrigerated unit. When required, supplementary light was provided by 4 HID high-pressure sodium lamps located 1.3 m above the plant bases.

Upon transfer of the seedlings, 100 μ*M* of Al was superimposed over the basal nutrient solution for the pulse step. The solution was adjusted to pH 4.0, and the pulse stress maintained for 4 days. For the recovery step, 12 days after germination, the solution was replaced with a nutrient solution containing an Al concentration of 50 μ*M*. Except for the first day of the pulse stress, all nutrient solutions were adjusted to pH 4.3 with HCl, and solutions were constantly aerated. Nutrient solutions were adjusted periodically with deionised water to compensate for water loss by evaporation and transpiration. Seventeen days after germination, plants were scored three times for root recovery. The plants showing both re-growth of seminal roots and new lateral roots were scored as Al-tolerant (+), whereas the plants failing to show any re-growth or new growth were scored as Al-sensitive (–). Plants having seminal root re-growth but not new lateral roots or plants showing new lateral roots but not re-growth of seminal roots were designated as intermediate in tolerance to Al (+/–).

To confirm Al tolerance of the mapping population a progeny test of F_3 lines derived from each selected F_2 plant was performed following the methodology described above but with minor modifications, as shown in method 2 in Table 1. F_3 seed samples from the 17 Al-tolerant and 17 Al-sensitive F_2 plants selected for the BSA were used for the progeny testing. After surface sterilisation, the seeds were germinated overnight in a solution of the systemic fungicide Vitavax (0.005 g l^{-1}) to prevent fungal growth during screening. Six days after germination, the seedlings were transferred to four 45-l tanks containing a complete nutrient solution. Each tank contained, randomly distributed, five seedlings of each parent and three seedlings from each F_3 line. Thus, a total of 12 seedlings per F_3 line were tested. For the Al pulse step, 50 μ*M* of Al was superimposed over the basal nutrient solution and the pH adjusted to 4.3. Four days later, a further 50 μ*M* of Al was added, bringing the total Al concentration to 100 μ*M*. For the recovery step, 12 days after germination, the solution was replaced with a nutrient solution containing an Al concentration of 10 μ*M*. Fourteen days following germination, the plants were scored for Al tolerance using the criteria described above.

Screening of the validation population for aluminium tolerance

The WB229/Mimosa F_2 population consisted of 98 randomly selected individuals and was screened in similar growing conditions but with some modifications as outlined in method 3 of Table 1 to

Table 1 Solution culture pulse-recovery screening methods

Stage	Method 1		Method 2		Method 3	
	Day	WB229/Yambla F_2 population	Day	WB229/Yambla F_3 families	Day	WB229/Mimosa F_2 population
Seed sterilisation	0	1.2% NaOCl for 20 min	0	As in method 1	0	As in method 1
Germination	0	Overnight at room temperature (21 °C) in aerated deionised water	0	Overnight at 18°–20 °C in an aerated solution of Vitavax (0.005 g l^{-1})	0	As in method 2
Pre-treatment growth	1–6	On mesh in contact with complete nutrient solution, at 21 °C; under black plastic for 3 days	1–6	As in method 1	1–4	On mesh in contact with complete aerated nutrient solution, at 21 °C; under black plastic for 2 days
Growth conditions for treatment and recovery	7	Transplanted to plastic frames on tanks of complete aerated nutrient solution at 19 °C. Glasshouse at 18°/24 °C (night/day); 16/8-h (day/night) photoperiod	7	As in method 1		Glasshouse at 16°/24 °C (night/day) temperature, 16/8-h (day/night) photoperiod. Seedlings were not transplanted
Treatment pulse	7–11	Add 100 μ*M* Al to pH 4.0; on day 8 adjusted to pH 4.3	7–10	Add 50 μ*M* Al and adjust to pH 4.3	4	Add 50 m*M* Al and adjust to pH 4.3
			11	Al adjusted to 100 μ*M*	5	Roots stained with haematoxylin
Recovery	12–17	Complete nutrient solution plus 50 μ*M* Al at pH 4.3	12–14	Complete nutrient solution plus 10 μ*M* Al and adjusted to pH 4.3	5–8	Complete nutrient solution plus 10 μ*M* Al and adjusted to pH 4.3
Assessment	17	Tolerant: both re-growth of seminal roots and new lateral roots Sensitive: no re-growth nor new laterals Intermediate: either re-growth or new laterals	14	As in method 1	6–8	Daily measurements of root re-growth used to calculate relative root growth and ranked to give tolerance classes

optimise differences between parents. After 4 days of growth, a 24-h Al pulse step consisting of 50 μ*M* Al was imposed on the seedlings. This was followed by 3 days of recovery in a solution consisting of 10 μ*M* Al. Roots were stained with a haematoxylin solution prior to being transferred into the recovery solution, a procedure which enabled a better determination of the position from which root re-growth occurred. Daily measurements of root re-growth were taken on 3 consecutive days and used to determine the relative root growth rate (RRG) of each individual seedling. Ranking of RRG was used to classified genotypes as tolerant, sensitive or intermediate with respect to response to Al.

DNA extraction

DNA was isolated from the young leaves (8–10 cm long) collected from 10- to 14-day-old seedlings of each genotype in 2-ml round-bottom Eppendorf tubes as described earlier (Raman and Read 2000). A single plant was used to represent a genotype.

Bulk segregant analysis (BSA)

To identify the molecular markers linked with the Al-tolerance gene, we formed two DNA pools, each consisting of 17 F_2 plants displaying either the tolerant or sensitive reaction to Al, and carried out BSA (Michelmore et al. 1991) using the AFLP technique (Vos et al. 1995). The bulks were made by pooling equal amounts of 17 pre-amplified DNAs obtained from tolerant or sensitive progeny of Yambla/WB229. Thirty primer combinations of five E-primers with ACA, AGG, ACG, ACC and ACT and six M-primers with CAA, CAC, CAT, CTT, CTA and CTC were used to study polymorphism between the tolerant and sensitive bulks. Twelve primer combinations exhibiting polymorphism among bulks were further analysed using each single plant DNA from the respective F_2s to estimate their inheritance and linkage distances.

AFLP analysis

AFLP marker analysis was conducted using the Large Genome AFLP System 1 (Life Technologies, Gaithersburg, MD.) essentially as described by Vos et al. (1995). About 200 ηg DNA of each line was digested with *Eco*RI and *Mse*I enzymes, and the restriction fragments were ligated with double-stranded *Eco*RI/*Mse*I adapters. Primers with one additional nucleotide (*Eco*RI primer + A and *Mse*I primer + C) were used for pre-amplification, whereas primers with three additional nucleotides were used for selective amplifications. The ligated DNA fragments were amplified in a 25-μl volume. *Eco*RI primers were end-labelled using γ-[^{33}P]ATP (Amersham Pharmacia, UK) and T4 polynucleotide kinase (Promega, Madison, Wis.). Selective amplifications were carried out in a total reaction volume of 20 μl containing 6 ηg labelled *Eco*RI primer, 30 ηg unlabelled *Mse*I primer, 1×PCR buffer (20 m*M* Tris HCl pH 8.4, 50 m*M* KCl), 1.5 m*M* $MgCl_2$ and 1 U *Taq* polymerase (Promega). Amplifications were performed in an Omni E thermocycler with Hot-Lid using the following modified conditions for selective amplification: one cycle at 94 °C for 30 s, 65 °C for 30 s, 72 °C for 60 s, followed by nine cycles, each decreasing by 1 °C; finally by 23 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 60 s.

The amplified products were equally mixed with formamide loading buffer [98% formamide, 10 m*M* EDTA pH 8.0, 0.25% bromophenol blue and 0.25% xylene cyanol FF (w/v)] and denatured at 90 °C for 3 min. The denatured PCR products (3 μl) were loaded on the gels (0.4 mm). Electrophoresis was carried out at a constant temperature and wattage (50 °C, 120 W) for 90–120 min using 1× TBE buffered 6% polyacrylamide gels (19:1 acrylamide:bis acrylamide; Bio-Rad, Hercules, Calif) containing 7 *M* urea, 1× TBE buffer (Maniatis et al. 1982). The gels were transferred onto Whatman 3 MM filter paper and dried at 85 °C for 2 h on a gel-drier (Bio-Rad). The dried gels were exposed at room temperature for 16–20 h to X-ray sheets (Biomax-MR, Kodak) using a low-energy screen (Integrated Science) and developed.

Microsatellite analysis

Since Minella and Sorrells (1997) mapped the *Alp* gene on chromosome 4H, we decided to test 4H as a potential source of aluminium tolerance genes from WB229. Microsatellites already mapped on barley chromosome 4H (Liu et al. 1996; Ramsay et al. 2000) were synthesised by Life Technologies (Gibco-BRL, Australia) from published sequences and analysed as follows. The forward primer of each pair was end-labelled using 1,850 Bq of γ-[^{33}P]ATP (Amersham-Pharmacia). Amplifications were performed in 12.5-μl reactions, each containing 50 ng DNA, 6 ρmol of both primers, 200 μ*M* each dNTP, 1.5 m*M* $MgCl_2$, 1× PCR buffer A (Promega) and 0.5 U *Taq* DNA polymerase (Promega) in 0.5-ml thin-walled microfuge tubes (Integrated Science). The PCR products were analysed on denaturing 6% polyacrylamide gels (Bio-Rad; 19:1, acrylamide:bis acrylamide) in 1× TBE buffer (Maniatis et al. 1982). We employed multiple loadings of PCR products onto the sequencing gel allowing 396 samples to be analysed concurrently. The gels were run at a constant temperature and wattage (50 °C, 120 W) for 90 min. Drying of the gel and autoradiography was performed as described previously.

Linkage mapping and statistical analysis

AFLP and microsatellite markers were scored manually either as "A" (homozygous, for WB229 allele) "B" (homozygous, Yambla allele) or "H" (heterozygous), and missing data were scored as "-". Linkage analysis was performed on F_2 segregation data using MAPMANAGER QTX07 (Manly and Olson 1999). Genetic distances were calculated using the Kosambi function. The closely linked AFLP markers were mapped onto specific chromosomes using wheat-barley disomic addition lines (kindly supplied by Dr. A. K. Islam, University of Adelaide, Australia and by CSIRO, Canberra) for chromosomes 2H, 3H, 4H, 5H, 6H, 7H and ditelosomic addition lines for 1H, 4H (Islam et al. 1981; Shepherd and Islam 1981). The relative distance between the gene conferring Al tolerance and the markers was further estimated using microsatellites. A Chi-square test was performed to check for goodness of fit between the expected Mendelian ratio and observed AFLP and microsatellite segregation data. Markers showing distorted segregation for dominant and co-dominant loci (not 3:1 or 1:2:1) were excluded from the linkage mapping. Two loci were considered linked if the likelihood ratio statistic was greater than 30 using linkage criterion $P = 0.0001$.

Validation and routine marker assisted selection for Al tolerance

To expedite routine simple sequence repeat (SSR) analysis suitable for marker-assisted selection (MAS), we used two approaches: (1) radiolabelled primers were used for PCR amplifications and multiple (four) loadings were performed on a sequencing gel, and (2) unlabelled primers (6 ρmol of each primer pair) were used for PCR amplifications and the PCR products were either separated on 6% denaturing acrylamide gels at 300 V for 120–150 min or on 3% TAE buffered agarose gels (2% low-melting, high-resolution agarose and 1% normal DNA grade agarose; Progen Industries, Australia). The gels were stained with ethidium bromide (0.5 μg ml^{-1}), visualised under a UV transilluminator and photographed with Polaroid type 667 film.

Results

Phenotype of F_2s derived from Yambla/WB229

The pulse-recovery method discriminated between Al-tolerant, -sensitive and -intermediate genotypes. Root growth of all plants ceased during the pulse stage. During the recovery stage, however, the Al-tolerant WB229 showed seminal root re-growth as well as growth of lateral roots, while the moderately Al-tolerant Yambla did not show any root growth (Fig. 1). Thus, WB229 was scored as Al-tolerant, while Yambla was scored as Al-sensitive at this concentration of Al. Among the 67 F_2 plants of Yambla/WB229 screened, 17 were scored as Al-tolerant, 33 as intermediate in tolerance and 17 as sensitive to Al, when seminal and lateral re-growth was used as criteria for selection. This distribution fitted a 1:2:1 Mendelian ratio for monogenic segregation and suggested that a single gene controlled Al tolerance in the F_2 progeny from the Yambla/WB229. However, Al tolerance was found to vary with the parameters of scoring. When seminal root re-growth was taken as the sole criterion, the F_2 progeny segregated into 3:1 ratio, as seminal roots of intermediates grew to the same degree of those of the tolerants.

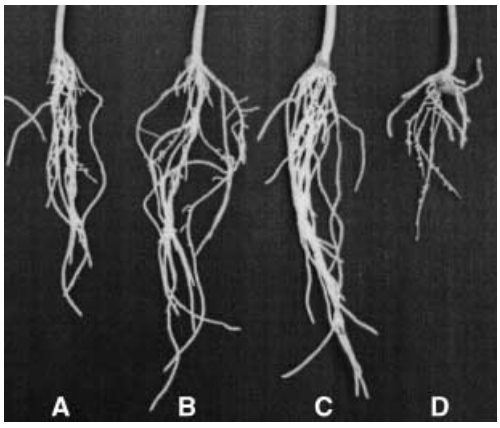

Fig. 1 Root growth of Yambla, WB229 and their F_2s in the nutrient solution after the recovery from Aluminium stress. *A* Yambla, *B* WB229, *C* F_2 tolerant, *D* F_2 sensitive

To confirm Al-tolerance gene expression in the F_2s of the Yambla/WB229 population, F_3 progeny testing was conducted. Root growth of all plants ceased during the pulse stage. During the recovery stage, the Al-tolerant WB229 showed consistent seminal root re-growth as well as growth of lateral roots, while the Al-sensitive Yambla did not show any root growth. Significant discrimination between tolerant and sensitive response to Al stress was observed between parents and F_3 lines. The F_2 families did not exhibit significant segregation within themselves ($\chi^2 = 0.006$, $P > 0.995$). Among the 17 Al-tolerant F_2 lines, only one showed a 1:1 segregation ratio in F_3 for Al-tolerant and Al-sensitive plants, indicating that the F_2 parent was wrongly classified as tolerant instead of intermediate. Despite this mis-classification, the segregation data clearly showed that a single gene controlled Al tolerance in WB229.

Table 2 Chi-square test for the segregation of AFLP markers in F_2 population of Yambla/WB229 (*E Eco*RI, *M Mse*I)

Primer combination	Bands amplified (no.)	Polymorphic[a] segregating bands (%)
E-ACC/M-CAA	115	6.1
E-ACC/M-CAC	77	1.3
E-ACC/M-CTA	104	2.9
E-ACC/M-CTC	104	2.9
E-AGG/M-CTT	83	2.4
E-AGG/M-CTC	70	4.3
E-ACG/M-CAA	89	3.4
E-ACG/M-CAC	85	5.9
E-ACG/M-CAT	82	3.7
E-ACG/M-CTT	57	5.3
E-ACA/M-CTC	103	4.8
E-ACT/M-CTA	82	6.1

[a] Non-significant at $P \leq 0.05$

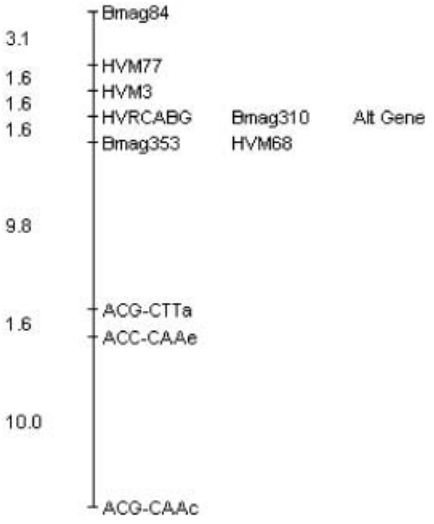

Fig. 2 Linkage map of aluminium-tolerance locus on 4H in Yambla/WB229

Identification of AFLP markers linked to the Al-tolerance locus

Among 30 *Eco*RI and *Mse*I primer combinations, 98 polymorphic loci were found between bulks of tolerants and sensitives. Twelve different primer combinations enabled differentiation of tolerant and sensitive bulks, and 1,068 selectively amplified fragments were obtained (Table 2). All the polymorphic loci exhibited normal Mendelian segregation. Forty-three loci were found to be linked with Al tolerance. However, eight AFLP markers – E-ACC/M-CAAa, E-ACC/M-CAAc, E-ACC/M-CAAf, E-ACC/M-CAAg, E-AGG/M-CTTa, E-AGG/M-CTTb, E-ACG/M-CATa and E-ACT/M-CTAb – were tightly linked with the Al-tolerant locus as no recombination between the markers and Al tolerance was detected. The alleles E-AGG/M-CTTa, E-AGG-M/CTTb, E-ACC/M-CAAa, E-ACC/M-CAAe and E-ACC/M-CAAf were linked with sensitivity to Al. However, the other markers E-ACC/M-CAAc, E-ACG/M-CAAa, E-ACT/M-CTAb, E-ACG/M-CACc were linked with the allele associated with Al tolerance. These AFLP markers were associated in *cis* (coupling phase) with the dominant Al-tolerance allele and no co-dominant AFLP bands were detected.

Chromosomal location and mapping of Al-tolerance locus

To determine the chromosomal location of the Al-tolerance locus, we mapped the linked AFLP fragments on di-telosomics and wheat-barley addition lines. Among the 43 AFLP markers linked to Al tolerance, only three loci detected with E-ACG/M-CAAa, E-ACC/M-CAAf and E-ACT-CTAb could be mapped on the 4H chromosome of barley. To find markers associated in the repulsion phase with the Al-tolerance locus and suitable for routine MAS, we employed co-dominant microsatellite markers and sequence-tagged site (STS) markers already mapped on barley chromosome 4H to study polymorphisms. Among 15 microsatellites markers, ten markers – HVM3, HVM67, HVM68, HVM77, HVRCABG, Bmac84, Bmag353, Bmac310, EBmac906 and GMS89 – were polymorphic for Yambla and WB229 and showed normal Mendelian segregation (3:1). However, the microsatellites HVM77, EBmac906, GMS89, HVRCABG showed small variations in allele size. The other markers – HVM13, Bmac30, Bmac181, WMS6 and Bmag384 – were not polymorphic. The three STS markers, ABG472, WG464 and ABG319, also did not yield any polymorphisms. The HVM67 microsatellite did not show any linkage with the Al-tolerance gene in WB229 but has been mapped previously on the linkage map of Steptoe/Morex on the distal end of 4H (Liu et al. 1996). However, the other microsatellite markers HVM68, Bmag353, Bmac310 and HVRCABG were closely linked with Al tolerance. All eight AFLPs and microsatellite markers Bmac310 and HVRCABG co-segregated with Al tolerance and map in a cluster. On the basis of available data on AFLP and microsatellite markers, the order of markers around the Al-tolerance gene was determined as: Bmac84 (3.1±1.6)-HVM77 (1.6±1.1)-HVM3 (1.6±1.1)-E-ACT/M-CTAb//E-AGG/M-CTTa//E-AGG/M-CTTb//E-ACC/M-CAAg//Bmac310//Al-tolerance gene//HVRCABG//E-ACC/M-CAAc/E-ACC/M-CAAf (1.6±1.1)-Bmag353/HVM68-(9.8±2.9)-E-ACG/M-CTTa 1.6±1.2)-E-ACC/M-CAAe (10.0±3.0)-E-ACG/M-CAAc. All of the microsatellite markers linked to the Al-tolerance locus in this investigation map near the centromere of 4H and cluster within 2–5 cM (Fig. 2). Unlike previous reports, we have found that the microsatellites HVM68, EBmac906, GMS89, HVM67, HVRCABG, HVM3, Bmag353, Bmac310 and Bmac0084 were dominant and unable to distinguish heterozygous genotypes in the F_2 population of Yambla/WB229. Among the heterozygous genotypes, primers were observed to have the tendency to amplify the microsatellite allele associated with Al

Table 3 Prediction of genotypes of F_2 plants based on the flanking microsatellite markers for the Al-tolerance locus in the WB229/Mimosa population

Marker	Genotype[a]	Marker-assisted selection (Plant no.)	Solution culture (plant no.)	Prediction accuracy (%)
Bmag353	T	24	26	92.3
	H	50	48	96.0
	S	24	24	100.0
Bmac310	T	24	24	100
	H	50	50	100
	S	24	24	100

[a] T, Tolerant; H, heterozygote; S, sensitive

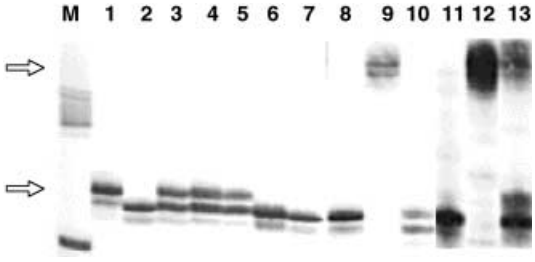

Fig. 3 Segregation pattern of microsatellite marker Bmag353 among F_2 derivatives of Yambla/WB229. *Lanes: 1–7* F_2 derivatives of Yambla/WB229, *8* heterozygote of Yambla/WB229, *9* Yambla (sensitive to Al), *10–11* WB229 (tolerant to Al), *12* Mimosa, *13* heterozygote of WB229/Mimosa

tolerance and not the allele associated with sensitivity. This observation was confirmed by template mixing of genotypes of known tolerants and sensitives (data not shown). However, the microsatellite marker Bmag353 detected the heterozygous genotypes in the validation F_2 population of WB229/Mimosa (Fig. 3). Furthermore, heterogeneity between individual loci within Al-tolerant genotypes was also found among derivatives of Yambla/WB229 and WB229/Mimosa (Fig. 3).

Validation of microsatellite markers for Al tolerance

To validate the usefulness of the developed markers in a different genetic background, an F_2 population derived from WB229/Mimosa was used. The microsatellite markers allowed selection of individuals on the basis of their genotypes and correctly predicted the Al-tolerance phenotypes of the seedlings (Table 3).

Discussion

Conventional methods for Al-tolerance screening such as field evaluation, soil/pot assay and solution culture assays are resource demanding. The molecular markers that we have developed are very efficient and can handle large populations in a limited time and space. Furthermore, these markers are PCR-based and can employ leaf tissue or sap as template for allele detection (Raman and Read 1999), thus avoiding the high cost of DNA extraction. Our results on the validation of microsatellite markers linked to Al tolerance clearly demonstrate that these markers can be used in different genetic backgrounds. Bulk segregant analysis proved to be an efficient method to identify molecular markers especially linked with qualitative genes. The microsatellite markers HVM3, HVM77, Bmac310 and HVRCABG have been reported to be localised near the centromere of chromosome 4H on linkage maps of barley in Steptoe/Morex, Igri/Franka and Lina/Canada Park (Liu et al. 1996; Perovic et al. 2000; Ramsay et al. 2000). Heterogeneity between individual loci within Al-tolerant genotypes was also found among derivatives of Yambla/WB229 and WB229/Mimosa, which may be attributed to mutations. Variable mutation rate has been reported in some SSRs (Mahtani and Willard 1998).

Our results reveal that a single gene controls Al tolerance in WB229 and that it is probably derived from the Al-tolerant line Kaniere from New Zealand. Monogenic inheritance of Al tolerance has also been reported in Dayton barley, but the expression of tolerance was reported to be dependent upon Al concentration and allele dose (Minella and Sorrells 1992). We determined that the Al-tolerance gene in WB229 is located on the long arm of chromosome 4H. Minella and Sorrells (1997) mapped the *Alp* gene on 4H and Tang et al. (2000) confirmed this location. Likewise, Stoelen and Andersen (1978) have also mapped the gene (*Pht*) for tolerance to acid soils, possibly the same gene, on chromosome 4H. These studies indicated that the Al-tolerance gene(s) are conserved on the 4H chromosome. Aluminium tolerance in wheat (*AltBH*; Riede and Anderson 1996) was located on wheat chromosome 4DL and Al tolerance in rye (Alt3; Aniol and Gustafson 1984) was located on chromosome 4R. These chromosomal regions are homoloeogous and, hence, it is possible that these cereals may have derived from a common ancestor and have conserved genes associated with Al tolerance. Recently, Tang et al. (2000) reported that *Alp* is orthologous to the wheat *AltBH* gene as the relative position of *Alp* and *AltBH* is identical with respect to a common set of molecular markers.

The understanding of different alleles/genes associated with Al tolerance will allow the development of breeding strategies for manipulating and improving Al tolerance in barley germplasm. Furthermore, efficient screening of barley for Al tolerance and the subsequent selection of tolerant genotypes using tightly linked markers may also provide a means of extending its cultivation on to acid soils.

Acknowledgments The authors thank NSW Agriculture, the Acid Soil Action Initiative and the Grains Research and Development Corporation, Australia for providing financial support for this project. It is declared that the experiments comply with the current laws of Australia.

References

Aniol A, Gustafson JP (1984) Chromosome location of genes controlling aluminum tolerance in wheat, rye and triticale. Can J Genet Cytol 26:701–705

Baier AC, Somers DJ, Gustafson JP, Guedes-Pinto H (1996) Aluminum tolerance in triticale, wheat and rye. Dev Plant Breed 5:437–444

Berzonsky WA, Kimber G (1986) Tolerance of *Triticum* species to aluminum. Plant Breed 97:275–278

Cancado GMA, Logriercio LL, Martins PR, Parentoni SN, Paiva E, Borem A, Lopes MA (1999) Hematoxylin staining as a phenotypic index for aluminum tolerance selection in tropical maize (*Zea mays* L.). Theor Appl Genet 99:747–754

Foy CD (1984) Physiological effects of hydrogen, aluminium, and manganese toxicities in acid soil. In: Adams F (ed) Soil acidity and liming. American Society of Agronomy, Madison, pp 57–97

Foy CD (1992) Soil chemical factors limiting plant root growth. Adv Soil Sci 19:97–131

Foy CD (1996) Tolerance of barley cultivars to an acid, aluminum toxic subsoil related to mineral element concentrations in their shoots. J Plant Nutr 19:1361–1380

Foy CD, Armiger WH, Briggle LW, Reid DA (1965) Differential aluminum tolerance of wheat and barley varieties in acid soils. Agron J 57:413–417

Gallardo F, Borie F, Alvear M, von-Baer E (1999) Evaluation of aluminum tolerance of three barley cultivars by two short-term screening methods and field experiments. Soil Sci Plant Nutr 45:713–719

Islam AKMR, Shepherd KW, Sparrow DHB (1981) Isolation and characterization of euplasmic wheat-barley chromosome addition lines. Heredity 46:161–174

Kochian LV (1995) Cellular mechanism of aluminum toxicity and resistance in plants. Annu Rev Plant Physiol Plant Mol Biol 46:237–260

Liu ZW, Biyashev RM, Saghai-Maroof MA (1996) Development of simple sequence repeat DNA markers and their integration into a barley linkage map. Theor Appl Genet 93:869–876

Ma JF, Zheng SJ, Li XF, Takeda K, Matsumoto H (1997) A rapid hydroponic screening for aluminium tolerance in barley. Plant Soil 191:133–137

Mahtani MM, Willard HF (1998) Physical and genetic mapping of the human X chromosome centromere: repression of recombination. Genome Res 8:100–110

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Manly KF, Olson JM (1999) MAPMANAGER QTX07. http://mcbio.med.buffalo.edu/mmQTX.html

Michelmore RW, Paran I, Kesseli RV (1991) Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. Proc Natl Acad Sci USA 88:9828–9832

Minella E, Sorrells ME (1992) Aluminum tolerance in barley: genetic relationships among genotypes of diverse origin. Crop Sci 32:593–598

Minella E, Sorrells ME (1997) Inheritance and chromosome location of *Alp*, a gene controlling aluminum tolerance in "Dayton" barley. Plant Breed 116:465–469

Moustakas M, Ouzounidou G, Lannoye R (1993) Rapid screening for aluminum tolerance in cereals by use of the chlorophyll fluorescence test. Plant Breed 111:343–346

Mugwira LM, Elgawhary SH, Patel KI (1976) Differential tolerances of triticale, wheat, rye and barley to aluminium in nutrient solution. Agron J 68:782–787

Perovic D, Smilde WD, Haluskova J, Waugh R, Sasaki T, Graner A (2000) Update of the Igri/Franka molecular marker map. Barley Genetics Newsl 30:15–19 http://wheat.pw.sda.gov/ggpages/bgn/30/a29.02.html)

Polle E, Konzak CF, Kittrick JA (1978) Visual detection of aluminum tolerance levels in wheat by hematoxylin staining of seedling roots. Crop Sci 18:823–827

Powell W, Morgante M, Andre C, Hanafey M, Vogel J, Tingey S, Rafalski A (1996) The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. Mol Breed 2:225–238

Raman H, Read BJ (1999) Efficient marker assisted selection for resistance against Barley Yellow Dwarf Virus in barley using leaf tissue and sap as templates in barley. Barley Genetics Newsl 29 (http://wheat.pw.sda.gov/ggpages/bgn/29/a29.02.html)

Raman H, Read BJ (2000) Molecular breeding for resistance to Russian Wheat Aphid in barley. J Agric Genomics 5:1–9 (http://www.ncgr.org/research/jag/papers00/paper100/indp100.html)

Ramsay L, Macaulay M, Ivanissevich S, MacLean K, Cardle L, Fuller J, Edwards KJ, Tuvesson S, Morgante M, Massari A, Maestri E, Marmiroli N, Sjakste T, Ganal M, Powell W, Waugh R (2000) A simple sequence repeat-based linkage map of barley. Genetics 156:1997–2005

Read BJ, Oram RN (1995) *Hordeum vulgare* (barley) cv. Brindabella. Aust J Exp Agric 35:425

Reid DA, Fleming AL, Foy CD (1971) A method for determining aluminium response of barley in nutrient solution in comparison to response to Al-toxic soil. Agron J 63:600–603

Riede CR, Anderson JA (1996) Linkage of RFLP markers to an aluminum tolerance gene in wheat. Crop Sci 36:905–909

Scott BJ, Fisher JA (1993) Comparing selection strategies for tolerance of acid soils. In: Proc Workshop Adaptation Plants Soil Stresses. INTSORMIL publication no. 94-2. University of Nebraska, Lincoln pp 103–116

Scott BJ, Conyers MK, Poile GJ, Cullis BR (1997) Subsurface acidity and liming affect yield of cereals. Aust J Agric Res 48:843–854

Shepherd KW, Islam AKMR (1981) Wheat: barley hybrids – the first eighty years. In: Evans LT, Peacock WJ (eds) Wheat science today and tomorrow. Cambridge University Press, New York, pp 107–128

Stoelen O, Andersen S (1978) Inheritance of tolerance to low soil pH in barley. Hereditas 88:101–105

Tang Y, Sorrells ME, Kochian LV, Garvin DF (2000) Identification of RFLP markers linked to the barley aluminum tolerance gene *Alp*. Crop Sci 40:778–782

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res 23:4407–4414

Watson B (1998) National soil acidification program: draft management strategy. In: Proc Nat Soil Acidification Conf. Queensland, Australia