



Development, Validation and Allele Diversity of Microsatellite Markers closely linked with the *Alp* Locus associated with Aluminium Tolerance in Barley

H. Raman¹, A. Karakousis², S. Moroni¹, R. Raman¹, B. Read¹

¹NSW Agriculture, Agricultural Institute, Private Mail Bag, Wagga Wagga NSW 2650

²University of Adelaide, Adelaide

Abstract

Aluminium (Al) toxicity is one of the main factors restricting barley production in acid soils. The utilisation of barley cultivars tolerant to Al is one of the most economic strategies for expanding barley production in these soils. Among barley genotypes, Dayton has been reported to be one of the most tolerant to Al. The locus conferring Al tolerance (*Alp*) in Dayton has been mapped recently on *4H* using RFLP markers. RFLP markers are very costly, laborious and involve the use of radioisotopes and hence are not suitable for routine marker assisted selection (MAS). To increase selection efficiency for Al tolerance, we have used an F₂ population of Dayton/Harlan Hybrid to map a number of PCR based microsatellite markers closely linked with the *Alp* locus. The *Alp* locus is flanked by the microsatellite loci HVM68 and Bmag353. The microsatellite markers were validated using two F₂ populations derived from Dayton/Kearney and Dayton/F6ant-28. The marker correctly predicted Al tolerance with >90% accuracy in the latter cross. To further reduce the cost to assay the microsatellite markers, leaf tissue as template was employed for PCR amplifications; this enabled us to identify tolerant and sensitive alleles associated with Al tolerance. The high polymorphic informative content (PIC=0.47 to 0.86) of the Bmac30, Bmac186, Bmac299, Bmac310, Bmag353, Bmag375, EBmac775, EBmac906, GMS89 and HVM68 microsatellites makes them highly useful in barley breeding programs.

Introduction

Aluminium (Al) toxicity is commonly found in highly weathered acid soils of various parts of the world and limits the cultivation of important crops including wheat, barley, rice and maize (Rajaram and Villegas 1990, Baier *et al.* 1996, Mugiwara *et al.* 1976, Torres *et al.* 1997, Wu *et al.* 1997). Among the winter cereals, barley is the most sensitive to Al (Scott and Fisher 1993). The utilisation of barley cultivars tolerant to

Al is one of the most economic strategies for expanding barley production in acidic soils. A wide range in genetic variability for tolerance to Al exists in barley germplasm (Foy *et al* 1965, Ma *et al* 1997) and has been exploited by conventional breeding methods (Read and Oram 1995). Among barleys, Dayton is widely known for its Al tolerance. Single gene inheritance of Al tolerance in Dayton has been reported (Minella and Sorrells 1992) and this *Alp* locus has been mapped on the long arm of chromosome 4H by using trisomic analysis (Minella and Sorrells 1997) and recently with RFLP markers (Tang *et al* 2000).

To expedite selection of the *Alp* gene conferring Al tolerance, molecular markers have been recognised as an alternative to the conventional methods of selections. The current methods of routine selection of barley germplasm for Al tolerance are not efficient. The haematoxylin staining method is not satisfactory and reliable in barley. RFLP based marker assisted selection is very time-consuming, labour intensive and costly, as it require high amounts of DNA. To increase selection efficiency for Al tolerance, development of PCR based markers suitable for routine marker assisted selection would be very important to monitor introgression of *Alp* into cultivated germplasm. PCR based microsatellite markers detect more alleles per locus as compared to RFLPs and provide greater resolution for genetic analysis (Becker and Heun 1995, Olufowote *et al* 1997).

The objectives of the present study were to identify microsatellite markers closely linked to the *Alp* locus, validate these markers and determine their allelic variations in barley germplasm for their effective use in the breeding programs.

Materials and methods

Mapping Population: Forty-eight F₂ plants derived from a cross between Dayton and Harlan Hybrid (moderately Al sensitive) were used for the mapping of the *Alp* locus. These parents and their F₂ population were screened for Al tolerance as described elsewhere (Tang *et al* 2000).

DNA extraction: The DNA of the mapping population was extracted at Cornell State University, Ithaca as described (Tang *et al* 2000) and was provided by Dr. DF Garvin (USDA Cornell). For the validation populations and microsatellite diversity analyses, DNA was isolated from the young leaves (8-10 cm long) collected from 21 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.

RFLP and Microsatellite Analysis: RFLP analyses were carried out at Cornell State University as described (Tang *et al* 2000) and the data on 3 RFLP markers (Xwg464, Xcd01395 and Bcd1117) closely linked to *Alp* were used. Twenty microsatellites already mapped on barley chromosome 4HL were synthesised by Life Technologies (Gibco-BRL, Australia) from published sequences and were analysed as described (Saghai-Marooof *et al* 1994, Becker and Heun 1995, Struss and Pleiske 1997, Liu *et al* 1996, Ramsay *et al* 2000). Some of the microsatellites were analysed using end-labelled forward primers with ³³P ATP as described previously (Martin *et al* 2000).

The F₂ segregation data for the RFLP and microsatellite markers were used for linkage analysis using MapManager QTX011b (Meer et al 2001).

Validation of microsatellite markers linked with Al tolerance: The effective use of the SSR markers linked with Al tolerance gene was investigated using 2 Dayton/Kearney F₂ populations and an F₃ progeny derived from Dayton/F₆ant. The seedlings of all the progenies were screened for Al tolerance in a solution culture system using a modified Al pulse-recovery method (Berzonsky and Kimber 1986) as described earlier (Raman *et al.* in press; Method 3).

The Al concentrations used with the F₂ Dayton/Kearney populations during the pulse and recovery stages were 50 and 10 µM, respectively. Seven seeds of each F₃ family of Dayton/F₆ant were randomly distributed on the rafts with 2 replicates. The Al concentrations used with the F₃ Dayton/F₆ant progeny during the pulse and recovery stages were 50 and 25 µM, respectively. For both sets of progenies during the recovery stage (7 to 9 days after germination) daily measurements of root length were taken consecutively to determine the root extension rate (RER) of each individual seedling. Analysis was performed using PROC REG (regression procedure) of the statistical software SAS/STATS (SAS Institute, 1989). Ranking of RER was used to classify the F₂ seedlings as tolerant, sensitive or intermediate in response to Al. RER was used to classified F₃ families as homozygous sensitive, homozygous tolerant or heterozygous in response to Al.

For wide applicability of microsatellite markers closely linked with *Alp*, 42 genotypes of barley, being used as parents in Australian barley breeding programs, were screened. The frequencies of alleles of 15 microsatellite loci mapped within 10cM from *Alp* locus were used to calculate diversity index (PIC values) as described (Weir *et al* 1990).

Results

Identification of microsatellite markers linked to *Alp* locus

Out of 22 primer-pairs screened, 17 detected the polymorphism between the tolerant parent Dayton and the sensitive Harlan Hybrid. Markers that showed good quality were further used for segregation analysis in the mapping population. All of these microsatellites have been previously mapped on chromosome 4H in the DH population derived from Lina/*H. spontaneum* Canada Park (Ramsay *et al* 2000). Most of the microsatellite markers showed normal Mendelian segregation ratios and inherited in a co-dominant manner (1:2:1). The marker EBmac669 did not show any linkage with rest of the mapped microsatellites. The markers Bmag353, HVM68, Bmac310, Xwg464, Xcd01395 and Bcd1117 and were closely linked with Al tolerance (Fig1).

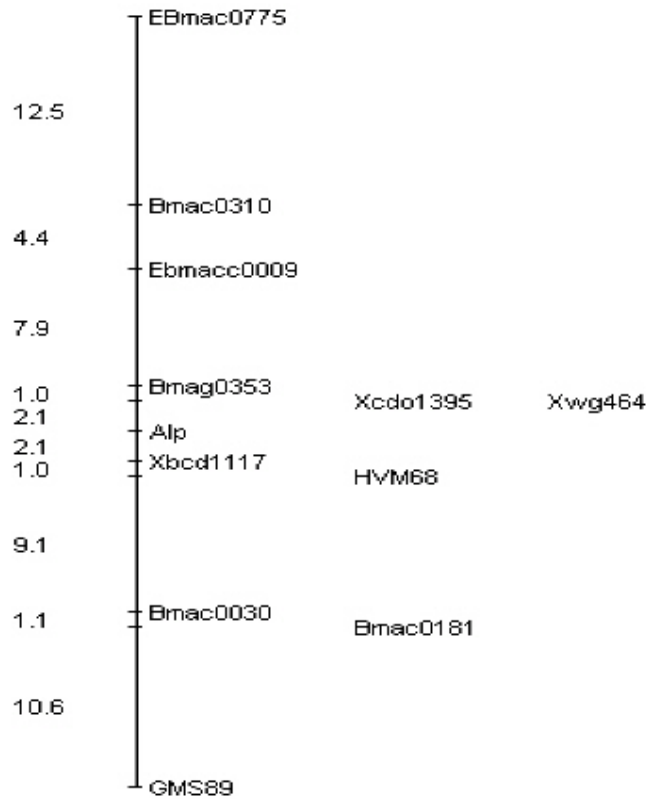


Fig 1: Linkage relationship between *Alp* and RFLP and microsatellite markers on the long arm of chromosome 4H.

We observed that some of the microsatellites such as Bmac30 and Bmag375 exhibited dominant inheritance and were unable to distinguish heterozygous genotypes in the F₂ population of Dayton/Harlan Hybrid. These microsatellite markers detected only bands associated with AI tolerance among heterozygous genotypes and thus the mapping F₂ population segregated into 3:1 ratio (tolerant: sensitive). This may be attributed to alteration of nucleotide sequence within the priming recognition sites. These alterations may be due to point mutations, insertions, deletions or inversions, which lead to lack of primer matching, and thus an absence of the corresponding band.

Validation of microsatellite markers for AI tolerance

The pulse-recovery assay discriminated between AI tolerant, sensitive and intermediate genotypes. Root growth of all plants ceased during the pulse stage. During the recovery stage, however, AI tolerant Dayton showed extensive seminal root regrowth as well as growth of lateral roots while AI sensitive Kearney and F6ant

showed very little growth. Thus significant discrimination between tolerant and sensitive response to Al stress was observed between the parents and among seedlings of the F₂/F₃ families. The distribution of the phenotypic expression of Al tolerance fitted a 1:2:1 Mendelian ratio for monogenic segregation in all the three populations (Table 1). This indicated that, under our screening conditions, a single gene controlled Al tolerance in the Dayton/Kearney and Dayton/F₆ant crosses.

The microsatellite marker Bmag353 allowed selection of individuals on the basis of their genotypes and correctly predicted the Al tolerance phenotypes of the seedlings as confirmed by F₃ progeny testing using solution culture. Two markers closely linked to *Alp* (Bmag353 and Bmac310) were not either polymorphic or were difficult-to-score in Kearney/Dayton and hence EBmac669 was used. The large allele size variation between Dayton and F₆ant allowed us to assay this marker on agarose gels (2% low melting and 1% normal) and thus is well suitable for high-throughput. To reduce further the cost to assay the microsatellite markers, leaf tissue as template from both the Dayton/Kearney and Dayton/F₆ant populations was employed successfully for PCR amplifications. This enabled the rapid identification of tolerant and sensitive alleles associated with Al tolerance.

Table 1: Phenotypic Expression of Al Tolerance in the Validation Populations

Name	Validation Population	Population size	Al Tolerant	Al Intermediate	Al Sensitive	χ^2 (1:2:1)	P
A	Dayton/Kearney	76	20	41	15	1.13	0.57
B	Dayton/Kearney	62	18	33	11	1.84	0.40
C	Dayton/F ₆ ant	96	22	50	24	0.25	0.88

Allele Diversity

The polymorphic informative content (PIC) of the 15 microsatellites mapped within 10cM from *Alp* locus, varied from 0.05 to 0.86. The microsatellites (Bmac30, Bmac186, Bmac299, Bmac310, Bmag353, Bmag375, EBmac775, EBmac906, GMS89 and HVM68) revealing high PIC value (0.47 to 0.86) makes them highly useful in barley breeding programs. The information on level of polymorphism for these microsatellite markers may allow us to (a) design crosses aiming to introgress Al tolerance and develop strategies for marker assisted selection (MAS), (b) implement markers rapidly in the breeding program (c) identify barley genotypes efficiently, and (d) to study association genetics.

The identification of microsatellite markers closely linked with the *Alp* locus conferring Al tolerance in Dayton barley are useful to expedite the transfer of Al tolerance from Dayton. These are currently being used in NSW Agriculture's barley

breeding program (for details see Rehman *et al* 2001, this issue) and should speed-up development of Al tolerant cultivars.

Acknowledgments

The authors are thankful to Drs LV Kochian, ME Sorrells and DF Garvin from Cornell State University Ithaca and NSW Agriculture, the Acid Soil Action Initiative, and the Grains Research and Development Corporation, Australia for providing financial support for this research.

References

1. Berzonsky W.A., and Kimber G (1986) *Plant Breed* 97: 275-278
 2. Minella E., Sorrells, M.E. (1992) *Crop Sci* 32: 593-598
 3. Raman H., Moroni, J.S., Sato, K., Read, B.J., Scott, B.J. (2001) *Theor Appl Genet* (in press).
 4. Ramsay L., Macaulay M., Ivanissivich S., MacLean K., Cardle L., Fuller J., Edwards K., Tuveesson S., Morgante M., Massari A., Marmiroli N., Sjakste T., Ganai M., Powell W., Powell W., Waugh R. (2000) *Genetics* 156:1997-2005
 5. Tang Y., Sorrells M.E., Kochian L.V., Garvin D.F. (2000) *Crop Sci* 40: 778-782
-