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AFLP markers tightly linked to the aluminum-tolerance gene *Alt*3 in rye (*Secale cereale* L.)

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Abstract Rye (*Secale cereale* L.) is considered to be the most aluminum (Al)-tolerant species among the Triticeae. It has been suggested that aluminum tolerance in rye is controlled by three major genes (*Alt* genes) located on rye chromosome arms 3RL, 4RL, and 6RS, respectively. Screening of an F_6 rye recombinant inbred line (RIL) population derived from the cross between an Al-tolerant rye (M39A-1–6) and an Al-sensitive rye (M77A-1) showed that a single gene controls aluminum tolerance in the population analyzed. In order to identify molecular markers tightly linked to the gene, we used a combination of amplified fragment length polymorphism (AFLP) and bulked segregant analysis techniques to evaluate the F_6 rye RIL population. We analyzed approximately 22,500 selectively amplified DNA fragments using 204 primer combinations and identified three AFLP markers tightly linked to the *Alt* gene. Two of these markers flanked the *Alt* locus at distance of 0.4 and 0.7 cM. Chromosomal localization using cloned AFLP and a restriction fragment length polymorphism (RFLP) marker indicated that the gene was on the long arm of rye chromosome 4R. The RFLP marker (BCD1230) cosegregated with the *Alt* gene. Since the gene is on chromosome 4R, the gene was designated as *Alt3*. These markers are being used as a starting point in the construction of a high resolution map of the *Alt3* region in rye.

Keywords Aluminum tolerance · Rye · AFLP · Linkage

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Introduction

Aluminum (Al) is recognized as the major problem for crop production on acid soils. The primary effect of Al toxicity is root growth inhibition that results in plants having reduced water and nutrient uptake (Foy et al. 1978). In the long term, this inhibition leads to reduced crop production.

Cereal crops exhibit variation in Al tolerance, and rye (*Secale cereale* L.) is the most Al tolerant cereal (Mugwira et al. 1978). Since rye is useful as a component of triticale (×*Triticosecale* Wittmack) and as a source of new genes for wheat (*Triticum* ssp.) through wheat-rye introgression, elucidating the mechanism controlling Al tolerance in rye will provide fundamental knowledge that can be used to increase Al tolerance in wheat.

Aluminum-tolerance (*Alt*) genes are considered to be the main factors controlling the Al-tolerance trait in rye. It has been suggested that there are at least three genes that reside on rye chromosome arms 6RS (*Alt1*), 3RL (*Alt2*), and 4RL (*Alt3*) controlling the trait (Aniol and Gustafson 1984). Gallego and Benito (1997) reported isozyme loci located on chromosome 6 that were linked to *Alt1*. Gallego et al. (1998) identified two random amplified polymorphic DNA (RAPD) markers, which they converted to sequence-characterized amplified region (SCAR) markers, that flanked the gene located on the short arm of chromosome 6, 5.5 cM and 2.1 cM from the gene. In this paper, we report on three amplified frag-

ment length polymorphism (AFLP) markers tightly linked to the *Alt3* gene in rye.

Materials and methods

Plant materials

An F_6 recombinant inbred line (RIL) rye population (145 lines) derived from a cross between two long-term inbred lines (at least 15 generations of selfing) produced by Dr. G.J. Scoles, University of Saskatchewan, were used as parents. M39A-1–6 is an Al-tolerant line, while M77A-1 is an Al-sensitive line (Scoles, unpublished). In addition, the Chinese Spring/Imperial (CS/I) wheat-rye addition lines from the USDA-Sears collection were used to verify the chromosome location of the selected marker.

Aluminum screening of F_6 rye RIL population

Screening of the F_6 rye RIL population for Al tolerance was performed based on the 'relative tolerance index' (RTI) method (Somers et al. 1996). Seeds (approximately 15–20) of each line within the RIL population were treated with 1.25% sodium hypochlorite for 10 min before germination. After washing with distilled water, the seeds were germinated in petri dishes and incubated at 4°C for 24 h followed by incubation at room temperature (21°C) for next 24–48 h. Six germinated seeds of each line that had a similar root length (about 5 mm) and seed size were used for the Al tolerance screening test.

The screening was conducted in a growth chamber under controlled environmental conditions [25°C/20°C (day/night), 16/8 h (light/dark), 80% relative humidity]. Seedlings of each line were grown on plastic trays with the roots submerged in a minimal nutrient solution without added phosphorous (0.4 m*M* $CaCl_2 \cdot 2H_2O$, 0.65 m*M* KNO_3, 0.25 m*M* $MgCl_2 \cdot 6H_2O$, 0.01 m*M* $(NH_4)_2SO_4$ and 0.04 m*M* NH_4NO_3) at pH 4.0 for 24 h before being screened with Al stress. Control treatments (three seedlings) received fresh minimal nutrient solution, and the Al stress treatments (three seedlings) received minimal nutrient solution with the addition of 2 ppm of Al in the form of $AlCl_3$. During the experiment, the solutions were aerated and changed every 24 h to maintain a constant pH. After 72 h, the length of the longest root was measured in both treatments, and an RTI was calculated as the ratio of the root length of seedlings grown at 2 ppm Al to the root length of seedlings grown at 0 ppm Al.

DNA isolation and screening for closely linked AFLP markers

High-molecular-weight DNA was isolated from leaf tissue of 4-week old individual plants of each line based on the standard cetyltrimethyl ammonium bromide (CTAB) method (Saghai-Maroof et al. 1984).

Bulked segregant analysis (BSA)

Screening for the AFLP markers closely linked to the *Alt* gene/s was based on the BSA method developed by Michelmore et al. (1991). Two DNA pools of 11 Al-tolerant and 11 Al-sensitive lines of the rye RIL population were used in BSA to detect AFLPs. The DNA from individual plants of each bulk was used to confirm the polymorphism of potential markers. The selected markers then were mapped to the F_6 rye RIL population.

AFLP analysis

Templates for AFLP reactions were prepared based on the method of Vos et al. (1995) using 500 ng DNA for restriction digests with *Eco*RI and *Mse*I and ligation of the respective adapters. Each of these reactions was incubated for 3 h at 37°C.

Selective amplifications were performed using various combinations of *Eco*RI (E) primers with three selective nucleotides and *Mse*I (M) primers with three selective nucleotides (E-NNN/M-NNN). Reaction mixture volumes were 10 µl and contained 2.5 µl of diluted pre-amplification products, 0.25 U *Taq* polymerase, 15 ng M-NNN primer, 12.5 ng [^{33}P]-labeled E-NNN primer, 10 m*M* Tris-HCl pH 8.3, 15 m*M* $MgCl_2$, 50 m*M* KCl, and 0.2 m*M* each of all four dNTPs. The selective amplifications were carried out using 36 cycles of a 30-s denaturation at 94°C, a 30-s annealing and a 60-s extension at 72°C. The annealing temperature was 65°C for the first cycle and then was reduced by 0.7°C for each of next 12 cycles; for the remaining 23 cycles the annealing temperature was 56°C.

The AFLP reaction products were resolved on denaturing gels containing 5% polyacrylamide, 7.5 *M* urea, and 1×TBE (89 m*M* Tris, 89 m*M* boric acid, 2 m*M* EDTA). A loading buffer (10 µl) consisting of 98% deionized formamide, 10 m*M* EDTA pH 8.0, 1 mg/ml bromophenol blue and 1 mg/ml xylene cyanol was added to each of the selective amplification products prior to gel loading. This mixture was heated at 94°C for 5 min, then quickly cooled on ice before 3.5 µl of each sample was loaded on the gel. Electrophoresis was carried out on a Bio-Rad sequencing gel apparatus (Bio-Rad, Hercules, Calif.) using 1×TBE running buffer, with running parameters of 75 W (2,174 V, 34.5 mA) and a plate temperature of 45°C, for about 2 h. Prior to exposing the gel to X-ray film for 24–48 h, it was dried with a gel drier at 80°C for 1.5 h.

Cloning the selected marker

An AFLP band associated with the selected marker was excised from dried polyacrylamide gels and incubated in 50 µl water at 4°C for 1 h. The fragment was then amplified using the same reaction and conditions as in the selective AFLP amplification procedure. The amplification products were then electrophoresed in 1.0% low-melting-point agarose gel in 1×TAE (Tris-acetate EDTA). After staining the gel in a 0.5-µg/ml ethidium bromide solution, we excised the fragment and cloned it to pGEM-T Easy Vector (Promega, Madison, Wis.) according to the manufacturer's procedures. In order to isolate the correct clone, we analyzed candidate clones with restriction enzymes and Southern hybridization.

Chromosomal localization of the selected AFLP marker

Two approaches were used in this study, hybridization of a cloned marker to DNA of the CS/I wheat-rye addition lines and verification of the marker location by using anchor probes for the indicated rye chromosome. One anchor probe, BCD1230, which showed single-copy and polymorphism between the two parents, was then hybridized to DNA of the F_6 rye RIL population to analyze its segregation.

Probe hybridization

DNA from CS/I wheat-rye addition lines and the F_6 rye RIL population were digested with restriction enzymes and separated on a 0.7% agarose gel in 1×TAE buffer. The DNA was then transferred onto Hybond N^+ nylon membrane (Amersham Pharmacia Biotech, Piscataway, N.J.) under alkaline conditions (Sambrook et al. 1989). Selected probes were randomly labeled with [^{32}P] isotope using the High Prime kit (Roche Molecular Biochemicals, Indianapolis, Ind.).

Hybridizations were performed in solutions containing 6% SSC, 5×Denhardts, 0.5% SDS and 100 µg/ml denatured salmon sperm DNA at 65°C for 12–16 h. After hybridization, membranes were washed two times in 2×SSC and 0.5% SDS for 15 min at room temperature and 0.1% SSC and 0.1% SDS for 15–60 min at 65°C and then exposed to X-ray film at –80°C for 4–5 days.

Data analysis

Segregation analysis was performed using the Chi-square method with a critical *P* value of 0.05. A total of 140 lines showing homozygosity for the trait were analyzed. Clearly readable AFLP bands were scored as dominant markers. Potential AFLP markers showing polymorphism in BSA were mapped to the F_6 rye RIL population. The data was analyzed using MAPMAKER 3.0 software (Lander et al. 1987) to construct the linkage maps with an LOD score of 5.0. The map distance was calculated using the Kosambi function (Kosambi 1944).

Results

Segregation of the *Alt* gene on the F_6 rye RIL population

The RTI values of Al-tolerant and -sensitive parents at 2 ppm of Al were 0.93 and 0.23, respectively. Since this concentration of Al clearly distinguished the RTI of both parents, we decided to screen the RIL population at 2 ppm of Al in order to ascertain the segregation pattern of the gene/s controlling Al tolerance.

The 145 lines of the F_6 rye RIL population were screened at 0 and 2 ppm of Al for 3 days. Of these, 140 lines in the population could be clearly classified as either tolerant or sensitive; the other five lines exhibited high variability in RTI, possibly indicating that they were segregating for the gene of interest. We excluded those lines from any further analysis.

The RTI values of the 140 lines were bimodally distributed (Fig. 1) and slightly skewed toward the sensitive direction (61 tolerant vs. 79 sensitive), suggesting that a single major gene was involved in Al tolerance in this population. A Chi-square test showed that the Al tolerance segregated in the ratio of 1 (tolerant):1 (sensitive) (χ^2: 2.314; *P*: 0.12819), confirming single-gene inheritance.

BSA and AFLP analysis

Approximately 22,500 selectively amplified DNA fragments using 204 primer combinations were analyzed. All

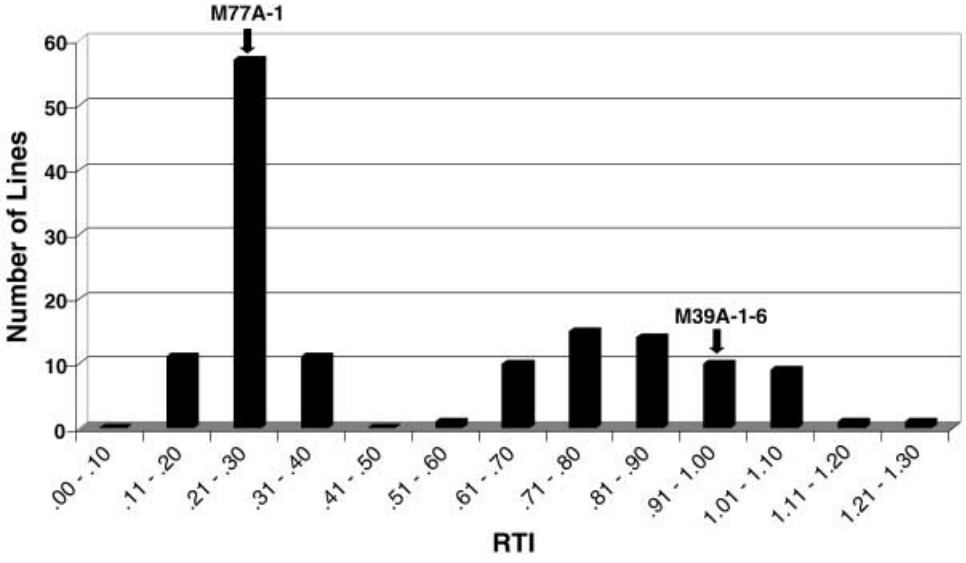

Fig. 1 Distribution of RTI values among 140 lines of the F_6 RIL rye population after 3 days stress at 2 ppm of Al in a nutrient solution. The *arrows* show the RTI mean of the Al-tolerant (M39A-1–6) and Al-sensitive (M77A-1) parents

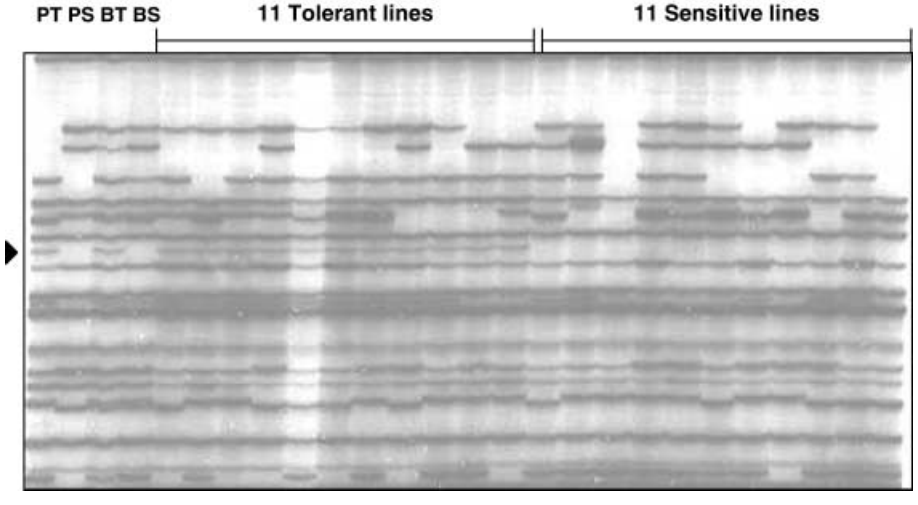

Fig. 2 Autoradiogram of bulked segregant analysis of 11 Al-tolerant and 11 Al-sensitive lines using the AFLP technique. *PT* Al-tolerant parent, *PS* Al-sensitive parent, *BT* Al-tolerant bulk, *BS* Al-sensitive bulk. The *arrow* indicates the potential marker (AMAL4) linked to the *Alt* gene

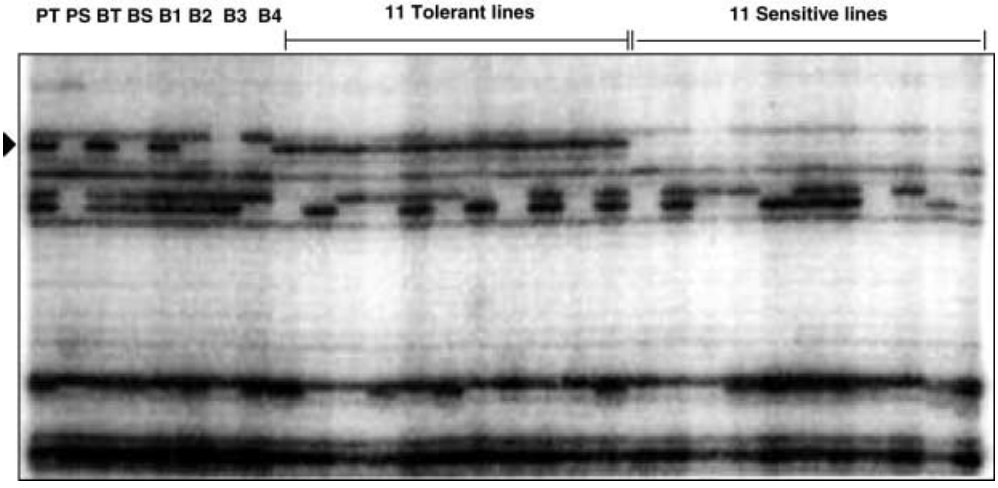

Fig. 3 Autoradiogram of additional bulked segregation analysis using the AFLP technique. The *first four columns* show the regular analysis. *PT* Al-tolerant parent, *PS* Al-sensitive parent, *BT* Al-tolerant bulk, *BS* Al-sensitive bulk. The *second four columns* are additional bulks. *B1* Recombinant Al-tolerant bulk, *B2* recombinant Al-sensitive bulk, *B3* original Al-sensitive bulk without one recombinant line, *B4* Al-sensitive line 166 showed recombination for other markers. The *arrow* indicates the potential marker (AMAL5) located between the *Alt* gene and marker AMAL2

of the primer combinations assayed exhibited 21–53% polymorphism between the two parents; however, after BSA screening of primer combinations and verification of the potential bands to all individual lines within the two bulks (Fig. 2), only five primer combinations produced AFLP bands specific to the tolerant parent. In order to identify markers tightly linked to the *Alt* gene, we investigated further only potential markers which showed no recombination among individual lines within the two bulks (Fig. 2). In addition, to reduce error when estimating genetic distance, only the potential markers showing coupling to Al tolerance were mapped for the entire F_6 rye RIL population. Five markers potentially linked to the *Alt* gene (AMAL1, AMAL2, AMAL3, AMAL4, and AMAL5) were identified.

In the case of marker AMAL5, two additional bulks were made from five tolerant and four sensitive lines showing recombination between the *Alt* gene and the AMAL2 marker. Figure 3 shows the potential AMAL5 marker identified using this approach. The band was present in the Al-tolerant parent and was also present in the Al-tolerant bulk as well as in the recombinant Al-tolerant bulk. AFLP analysis of the individual lines within the tolerant bulk also showed the presence of the corresponding band without any recombination. Absence of the corresponding band was observed in the Al-sensitive parent as well as in the Al-sensitive bulk and recombinant Al-sensitive bulk. No recombination occurred among the individual lines of the Al-sensitive bulk. In mapping of the marker AMAL5 (Fig. 3) to the entire F_6 rye RIL population, only two recombinant lines were identified.

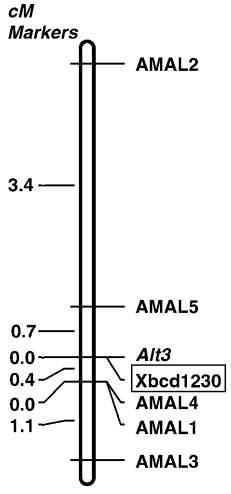

Fig. 4 Genetic linkage map of AFLP and BCD1230 markers linked to the *Alt* gene. Only the markers linked to the gene in coupling were mapped with MAPMAKER 3.0 (Lander et al. 1987). Genetic distances in centiMorgans were calculated using the Kosambi function (Kosambi 1944)

AFLP markers linked to the *Alt* gene

To confirm the segregation and linkage of five potential markers to the *Alt* locus, we screened 140 individual lines of the F_6 rye RIL population with the same AFLP primer combinations used to identify those potential markers. The results showed that most of the Al-tolerant lines had the potential AFLP marker (band) originating from the Al-tolerant parent and that most of the Al-sensi-

Table 1 AFLP markers and their segregation among 140 lines of the F_6 rye RIL population (*PT* Al-tolerant parent, *PS* Al-sensitive parent)

Primer designation	Primer combinations	AFLP marker designation	Approximate size of markers (bp)	Number of lines having		$\chi^2_{1:1}$	*P* value
				PT band	PS band		
E24	EACA/MCCT	AMAL1	240	62	78	1.829	0.176
C10	EAAG/MAGC	AMAL2	150	60	80	2.857	0.091
B6	EAAC/MACC	AMAL3	800	61	79	2.314	0.128
H6	EACT/MACC	AMAL4	230	62	78	1.829	0.176
U2	ECCA/MAAC	AMAL5	100	61	79	2.314	0.128

tive lines as well as the Al-sensitive parent were missing the respective band. In addition, all of the markers segregated for the Al-tolerance gene in a 1:1 ratio of presence to absence, respectively (Table 1). These results indicated that the AFLP markers were linked to the *Alt* gene. The linkage analysis of those markers using MAPMAKER with the LOD score of 5.0 showed that all of the AFLP markers could be mapped to the same linkage group as the *Alt* locus (Fig. 4).

We identified five AFLP markers specific to the Al-tolerant parent and closely linked to the *Alt* locus (Fig. 4). Three of the markers, AMAL1, AMAL4 and AMAL5, were tightly linked and flanked the gene. Two markers, AMAL1 and AMAL4, which co-segregated with each other, were located 0.4 cM from the gene, whereas the AMAL5 marker was located 0.7 cM distant, on other side of the gene. We identified one recombinant between AMAL1 and AMAL4 and the *Alt* locus, with both being present in the same recombinant line. The other two markers, AMAL2 and AMAL3, were 4.1 cM and 1.5 cM from the gene, respectively, and flanked the gene. The AMAL2 marker was on the same side as AMAL5 marker, whereas the AMAL3 marker was located on the same side as AMAL1 and AMAL4.

Cloning of the AFLP marker AMAL4

Theoretically, all AFLP markers can be cloned into a particular vector; however, several factors were considered in this investigation in order to get the correct clones. We decided to clone the AMAL4 marker for three reasons. Firstly, the marker was tightly linked to the gene and therefore, utilization of the clone to localize the marker on rye chromosome would be more valid. Secondly, the marker band showed good separation with the surrounding AFLP bands on a 5% polyacrylamide gel that avoided cross contamination with other bands when cutting the band from the gel. Finally, the fragment was large enough to be easily cloned (240 bp).

We cloned the marker into pGEM-T Easy Vector (Promega) and used it to localize the marker to a specific chromosome. Both the polymerase chain reaction (PCR) analysis, utilizing the same selective amplification primers used in generating the AFLP marker, and a restriction digest of the recombinant plasmid showed the same insert size as the original AFLP band. We were not able to remap the clone in our population either using PCR or RFLP analysis due to lack of polymorphism between the two parents. However, RFLP analysis indicated that the clone was exhibited as a single copy in rye genomic DNA. We therefore decided to use this clone to perform chromosomal localization using CS/I wheat-rye addition lines.

Chromosomal localization of the *Alt* gene

Four restriction enzymes (*Eco*RI, *Eco*RV, *Bam*HI, and *Hin*dIII) were used to localize the marker; however, only restriction enzyme *Eco*RI showed a single rye band. The band present in both parents was also present in the rye Imperial (I), the CS/I, as well as the Chinese Spring 4R addition line (CS+4R). This indicated that the marker was located on rye chromosome 4R.

Discussion

In studying markers linked to the Al-tolerance gene in rye, BSA has been used in conjunction with the RAPD technique (Gallego et al. 1998). The use of BSA in combination with the AFLP technique to identify molecular markers linked to an *Alt* gene in rye has not previously been reported. In this study, we employed BSA in conjunction with AFLP techniques to successfully identify three AFLP markers tightly linked to the *Alt3* gene.

Linkage analysis indicated that the markers AMAL1 and AMAL4, which co-segregated with each other, were located 0.4 cM apart from the gene (Fig. 4). For AMAL3, four lines were recombinants between the marker and the gene, and this marker was located on the same side of the *Alt3* gene as AMAL1 and AMAL4. One Al-sensitive line (line 166) was recombinant with respect to all three markers and was used for locating markers with respect to the gene. We also identified two additional markers located on the other side of the *Alt3* gene. For AMAL2, generated from primer combination EAAG/MAGC, nine lines were recombinants between this marker and the gene, indicating that the marker was not very tightly linked with the gene. Since line 166 was not found among the recombinants, it can be suggested that this marker was on the other side of the *Alt3* gene. Linkage analysis proved that the marker flanked the *Alt3* gene and was 4.1 cM from the gene (Fig. 4). To generate a marker more closely linked to the *Alt3* gene in that region, we utilized two additional bulks containing the recombinant lines identified by analyzi ng for the AMAL2 marker. Those recombinant bulks were used together with the original bulks and both parents to screen more primer combinations (Fig. 3). To identify a marker that would be on the same side as the AMAL2 marker, we also included line 166 in the analysis (Fig. 3). This approach helped to speed up the screening process by ignoring markers located on the opposite side of the gene. After screening 45 additional *Eco*RI/*Mse*I primer combinations, one AFLP marker (AMAL5) was identified that was located in the region of interest with a distance of 0.7 cM from the *Alt3* gene (Fig. 4).

To locate the markers to a specific chromosome of rye for further use either in the construction of a high-resolution map or for facilitating gene transfer to other species, AFLP marker AMAL4 was cloned into pGEM-T Easy Vector. Restriction enzyme analysis of 32 clones, which were randomly sampled, with *Alu*I indicated that there were three different types of clones produced from the single AFLP band. This result was in agreement with Shan et al. (1999) who reported that inserts present in the colonies from a single transformation event are were typ-

ically not identical. To determine the correct clone, we sequenced and compared all three types of clones. Sequence analysis indicated that type-I clones were 99% identical to the type-III clone, the only difference being 1 bp at one of the two *Alu*I sites. Since only one type-III clone was identified among 32 samples analyzed, the difference was probably due to base-pair mismatch during re-amplification; therefore that clone was discarded. Sequence comparisons between type-I and type-II clones indicated that they had different sequences. To determine the correct clone, we used both clones as probes. The analysis indicated that the type-II clone was a high-copy-number sequence. Therefore, a type-I clone was used to perform the chromosomal localization studies.

BCD1230 co-segregated with the *Alt3* gene on rye chromosome 4RL (Fig. 4) and has been reported to be closely linked to a major Al-tolerance (Alt_{BH}) gene in wheat (Riede and Anderson 1996) and also weakly linked to an Al-tolerance (*Alp*) gene in barley (Tang et al. 2000). Comparison of the Al-tolerance gene among Triticeae showed that the Al-tolerance gene in those Triticeae members was located on the long arm of homoeologous chromosome 4. This confirms the suggestion of Tang et al. (2000) regarding the close relationship between Alt_{BH} and *Alp*; it is likely that the *Alt3*, Alt_{BH}, and *Alp* genes are orthologous loci because of the high level of synteny among chromosome arms 4RL, 4DL, and 4HL, and they may share a common function, even though they were identified at different levels of Al toxicity.

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