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ORIGINAL PAPER

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Molecular mapping of a quantitative trait locus for aluminum tolerance in wheat cultivar Atlas 66

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Abstract Genetic improvement of aluminum (Al) tolerance is one of the cost-effective solutions to improve wheat (*Triticum aestivum*) productivity in acidic soils. The objectives of the present study were to identify quantitative trait loci (QTL) for Al-tolerance and associated PCR-based markers for marker-assisted breeding utilizing cultivar Atlas 66. A population of recombinant inbred lines (RILs) from the cross Atlas 66/Century was screened for Al-tolerance by measuring root-growth rate during Al treatment in hydroponics and root response to hematoxylin stain of Al treatment. After 797 pairs of SSR primers were screened for polymorphisms between the parents, 131 pairs were selected for bulk segregant analysis (BSA). A QTL analysis based on SSR markers revealed one QTL on the distal region of chromosome arm 4DL where a malate transporter gene was mapped. This major QTL accounted for nearly 50% of the phenotypic variation for Al-tolerance. The SSR markers *Xgdm125* and *Xwmc331* were the flanking markers for the QTL and have the potential to be used for high-throughput, marker-assisted selection in wheat-breeding programs.

Keywords *Triticum aestivum* · Aluminum tolerance · SSR marker · QTL mapping

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Introduction

Acid soils have been estimated to occur on approximately 30% of all the land presently under cultivation and on more than 50% of the potentially arable lands worldwide (Von Uexkull and Mutert 1995). Many types of soils used for agriculture, particularly those in developing countries where forests have been cleared, are considered detrimentally acidic. Furthermore, modern farming practices, such as the extensive use of ammonia fertilizers and continuous removal of forage and grain from the same field, are creating more acid soils from previously neutral ones in developed countries such as the United States (Jackson and Reisenauer 1984).

When the soil pH value decreases below 5, aluminum (Al) is solubilized as the phytotoxic Al^{3+} species from nontoxic Al silicates and oxides (Hoekenga et al. 2003). Al-sensitive plants have limited root-growth and development, and thus are unable to acquire water and nutrients from the soil (Kochian 1995). Root stunting directly translates into reduced plant vigor and grain yield. Al-toxicity is the primary limitation of crop production for 38% of the farmland in Southeast Asia, 31% in Latin America, and approximately 20% in East Asia, Sub-Saharan Africa, and North America (Wood et al. 2000). Use of Al-tolerant cultivars contributes greatly to increased crop productivity of many crop species on acid soils, and impacts future agricultural expansion onto acidic soils (Carver and Ownby 1995; Samac and Tesfaye 2003).

Inheritance of Al-tolerance in wheat has been studied by classical, cytogenetic, and molecular approaches. Wheat cultivars typically used for studying Al-tolerance include Atlas 66, Carazinho, BH1146, Waalt, Neepawa, Chinese Spring (CS), and others. Reports on inheritance of Al- tolerance in wheat have been controversial. Some indicated that Al-tolerance in wheat was under monogenic control (Somers and Gustafson 1995; Riede and Anderson 1996; Basu et al. 1997), whereas others

reported that multiple genes might be involved in enhancing Al-tolerance in some wheat genotypes (Camargo 1981; Aniol 1990; Berzonsky 1992; Papernik et al. 2001).

Molecular markers may provide new resources for identifying Al-tolerance genes in breeding populations. Marker-assisted selection (MAS) will reduce, or may even eliminate, the need of phenotypic assays. Riede and Anderson (1996) identified an Restriction Fragment Length Polymorphism (RFLP) marker (*Xbcd1230*) tightly linked to the Alt_{BH} gene on the chromosome 4DL by using a population of 91 recombinant inbred lines (RILs) from the cross 'Anahuac'/'BH 1146'. Further studies using the same RIL population showed that Alt_{BH} was confined to a 5.9-cM interval between the markers *Xgdm125* and *Xpsr914* (Rodriguez and Gustafson 2001). In another study, Luo and Dvorak (1996) identified the *Alt2* gene from the same chromosome region of CS by using disomic substitution lines having the chromosomes of the D genome of *T. aestivum* L. cultivar CS individually substituted for their homoeologues in *T. turgidum* L. cultivar Langdon. Sasaki et al. (2004) recently cloned a wheat Al-activated malate transporter gene (*ALMT1*) that co-segregates with Al-tolerance in F_2 and F_3 populations derived from two near-isogenic lines (NILs) ET8 and ES8. Two different alleles of the *ALMT*1 were found in the two parents. But the chromosomal location of *ALMT1* was not determined.

The objectives of our research were to: (1) identify quantitative trait loci (QTL) controlling Al-tolerance from Atlas 66, (2) develop high-throughput PCR-based markers for Al-tolerance QTL from Atlas 66, (3) compare the locations of QTL in Atlas 66 with those reported from other cultivars, and (4) determine the QTL for Al-tolerance in two NILs.

Materials and methods

Plant materials and evaluation of Al-tolerance

A total of 118 F_5 RILs derived from the cross of Atlas 66 × Century were advanced from different F_2 plants by single seed descent. Atlas 66 (Frondoso//Redhart 3/Noll 28) is a cultivar with a high level of Al-tolerance released in 1948 from North Carolina. Century (Payne//TAM W-101/Amigo) is an Al-sensitive cultivar released from Oklahoma. Two Al-tolerant NILs, OK91G105 and OK91G106, each carrying Al-tolerance gene or genes from Atlas 66 in a Century background (Caver et al. 1993), were evaluated for markers associated with Al-tolerance in the RILs.

Al-tolerance in RILs and their parents was evaluated by staining wheat roots with hematoxylin and measuring the root growth after Al stress (Polle et al. 1978). Wheat seeds were placed on moist paper in a petri dish. The seeds were incubated at 4°C for 24 h and then moved to room temperature (22–25°C) for 24 h. Three germinated seeds with similar root lengths were selected and transferred onto a nylon-net at the bottom of a plastic cup with the bottom removed. Cups with germinated seeds were placed in a 30-well plastic cup holder. The cup holder floated on 7-l of deionized water in a 27-l plastic tray. Two bubble rods in the bottom of the tray were connected to an air pump for aeration during hydroponics culture. After 48 h, the deionized water was replaced with a nutrient solution consisting of 4 mM $CaCl_2$, 6.5 mM KNO_3, 2.5 mM $MgCl_2{\cdot}6H_2O$, 0.1 mM $(NH4)_2SO_4$, and 0.4 mM NH_4NO_3 (Polle 1978). The PH of the nutrient solution was monitored and adjusted daily.

The seminal root of each seedling was measured after the seedlings were incubated in the nutrient solution for 24 h at 22°C, with 16 h of fluorescent light. The seedlings were then transferred to a fresh nutrition solution, with the addition of 0.36 mM $AlK(SO_4)_2{\cdot}12H_2O$ at pH 4.0. After 48 h of Al stress, the same seminal root in each seedling was measured again. The same procedure was followed for control plants, except without addition of $AlK(SO_4)_2{\cdot}12H_2O$ in the nutrition solution. Root elongation during 48 h Al stress was referred to as the stress root growth (SRG). Root elongation in the control solution (0 mM $AlK(SO_4)_2{\cdot}12H_2O$) during 48 h was described as control root growth (CRG). The root-tolerance index (RTI) (%) for each line in each replication was calculated as 100 × SRG/CRG. The experiment was repeated three times, arranged in a complete randomized-block design.

Roots of Al-treated seedlings were also subjected to hamatoxylin stain after they were measured. To rinse excess Al^{3+}, roots contained by the cups were immersed in deionized water for 1 h, with water replacement two to three times. Clean roots were then immersed in a hematoxylin solution consisting of 0.2% hematoxylin (w/v) and 0.02% (w/v) KIO_3 for 15 min, then the roots were rinsed with deionized water four times. Root tips of each RIL and parents were visually scored for the degree of hematoxylin staining. Three grades of stain were scored: no stain on root tips as grade 1, light stain as 2, and heavy stain as 3.

Bulked segregant analysis

Leaf tissue of the same plants from phenotyping experiments was used for DNA isolation according to the cetyltrimethyl ammonium bromide (CTAB) method (Saghai-Maroof et al. 1984). A bulked segregant analysis (BSA) strategy was used to screen SSR markers associated with Al-tolerance (Michelmore et al. 1991). Equal amounts of DNA from five Al-tolerant and five Al-sensitive genotypes from the RIL population were pooled separately. The two bulks and the parents were used initially to screen 797 SSR primer pairs for polymorphism, including 119 GWM primers (Roder et al. 1998), 14 GDM primers (Pestsova et al. 2000), 492 BARC primers (Song et al. 2005), 32 WMC primers (Gupta et al. 2002), and 140 CFD or CFA primers

(Guyomarc'h et al. 2002; Sourdille et al. 2003). Polymorphic markers between the parents and the two bulks were further analyzed in the RIL population from Atlas 66/Century.

Microsatellite analysis

The PCR reactions were performed in a DNA Engine Tetrad® Peltier thermal cycler (MJ Research, Waltham, MA, USA). A 10-µl PCR mixture contained 40 ng of template DNA, 0.2 mM of each dNTP, one time PCR buffer, 2.5 mM $MgCl_2$, and 0.6 units of *Taq* polymerase (Promega, Madison, WI, USA). Forward primer was labeled with IRDye 700 or 800 (Li-Cor Inc., Lincoln, NE, USA). A touch-town program was used for PCR amplification, in which the reaction incubated at 95°C for 5 min, then continued for five cycles of 45 s of denaturing at 95°C, 5 min of annealing at 68°C with a decrease of 2°C in each of subsequent cycles, and 1 min of extension at 72°C. For another five cycles, the annealing temperature started at 58°C for 2 min with a decrease of 2°C for each of subsequent cycles. Then, PCR went through an additional 25 cycles of 45 s at 94°C, 2 min at 50°C, and 1 min at 72°C with a final extension at 72°C for 5 min.

A Li-Cor 4200 DNA analyzer was used to separate the amplified PCR fragments with 6.5% Gel Matrix (Li-Cor Inc.) in one-time triborate–EDTA (TBE) buffer (50 mM Tris, 50 mM boric acid, and 1 mM ethylentdiaminetetra acetate, EDTA). Gels were pre-run for 10 min before the 0.8-µl samples were loaded. The electrophoresis condition was set at 1500 V, 40 W at 45°C.

Mapping of the *ALMT1* gene

To determine the location of the Al-activated malate transporter gene (*ALMT1*), A cleavage amplified polymorphic sequence (CAPS) marker was used for mapping the RIL population from Atlas 66/Century as described previously (Sasaki et al. 2004).

Data analysis

Broad-sense heritability (H^2) was computed as $6_g^2/(6_g^2+6_e^2)$ based on the estimates of genetic and error variances derived from the analysis of variances (SAS Institute, 1989). Linkage analysis of the SSR markers was conducted using MAPMAKER, Version 2.0 for MacIntosh (Lander et al. 1987), with an LOD score of 3.0. The map distance was calculated by using the Kosambi function (Kosambi 1944). The QGENE software (Nelson 1997) was used for interval analysis and estimation of determination coefficient (R^2) for each marker.

Results

Sensitivity of RILs to Al stress

Sensitivity of each genotype to Al stress was assessed by comparing root-growth rates of wheat seedlings grown under Al stress versus those grown under normal conditions. The Al-tolerant parent Atlas 66 and Al-sensitive parent Century grew on equal rates in the absence of Al, but at significantly different rates under Al stress (Table 1). During 2 days of Al stress, the roots of Atlas 66 increased 2.7 cm, whereas Century increased only 0.77 cm. The 0.36 mM $AlK(SO_4)\cdot 2H_2O$ concentration clearly differentiated the two parents and, thus, was used to screen the RIL population.

The SRG differed significantly ($p=0.01$) among RILs of the wheat population at 0.36 mM Al^{3+} for 48 h (Table 1). The bimodal distribution of SRG (Fig. 1a) suggested that one major QTL or single gene involved in SRG in Atlas 66. Broad-sense heritability for SRG was 89.76%, indicating that SRG is a highly heritable trait.

Because large variation in root length existed among control seedlings of RILs (Fig. 1b), RTI may provide a more accurate measurement to reflect the effect of Al stress on root elongation. Individual RILs also differed significantly for RTI, ranging from 7.7% to 86.1%. Although the frequency distributions for RTI and SRG seemed different (data not shown), the correlation coefficient for RTI versus SRG ($r=0.90$) was highly significant (Fig. 2). The broad-sense heritability of RTI was also very high in the RIL population (H^2 = 91.02%).

Al-treated roots of all RILs were also subjected to hematoxylin staining. Response of individual plants to hematoxylin stain was scored as highly tolerant, moderately tolerant, or sensitive, on the basis of the degree of stain in root tips. Root tips of Al-tolerant parent Atlas 66 did not exhibit any stain (grade 1), whereas the root tips of Century were thoroughly stained (grade 3). Hematoxylin-stained scores (HSS) of RILs ranged from grades 1 to 3. The broad-sense heritability of HSS was the same as those for SRG and RTI (Table 1), indicating that the HSS is also a highly inheritable trait for Al-tolerance in this population.

SSR marker analysis and QTL mapping

A total of 797 pairs of SSR primers were used to screen polymorphisms between the parents of the RIL population. Among those, 131 pairs exhibited polymorphisms between Atlas 66 and Century and were used to detect polymorphisms between two bulks. Seven primers generated one polymorphic band each between the bulks and were further analyzed in the RIL population. Four markers demonstrated significant association with Al-tolerance in the population on the basis of single-marker analysis, and were mapped to one linkage group. These

Table 1 Descriptive statistics of three variables measured from 118 RILs and the two parents in three replications

Trait	Line	Minimum	Maximum	Mean	CV (%)	$LSD_{0.05}$	H^2 (%)
Control root growth (cm)	Atlas 66			5.02	5.12		
	Century			5.02	8.47		
	RILs	2.55	6.77	4.76	17.54	1.37	69.67
Stress root growth (cm)	Atlas 66			2.7	22.97		
	Century			0.77	14.09		
	RILs	0.39	3.56	1.94	18.76	1.72	89.76
Root tolerance index (%)	Atlas 66			53.84	21.67		
	Century			15.29	14.82		
	RILs	7.68	86.12	41.79	25.62	14.38	91.02
Score of hematoxylin stain	Atlas 66			1.00	0		
	Century			3.00	0		
	RILs	1.00	3.00	1.73	22.20	0.58	90.34

markers were all located on wheat chromosome 4DL (Pestsova et al. 2000; Gupta et al. 2002; Song et al. 2005). Therefore, the Al-tolerance QTL should be on 4DL based on interval mapping (Fig. 3). A high LOD score and determination coefficient ($R^2 = 50\%$) indicated that the QTL has a major effect on Al-tolerance. Together with other markers, the genetic linkage group consists of eight SSR markers spanning 97.5 cM. The peak location of this QTL was at the interval between *Xwmc331* and *Xgdm125*, which are 8.4 cM apart (Fig. 3).

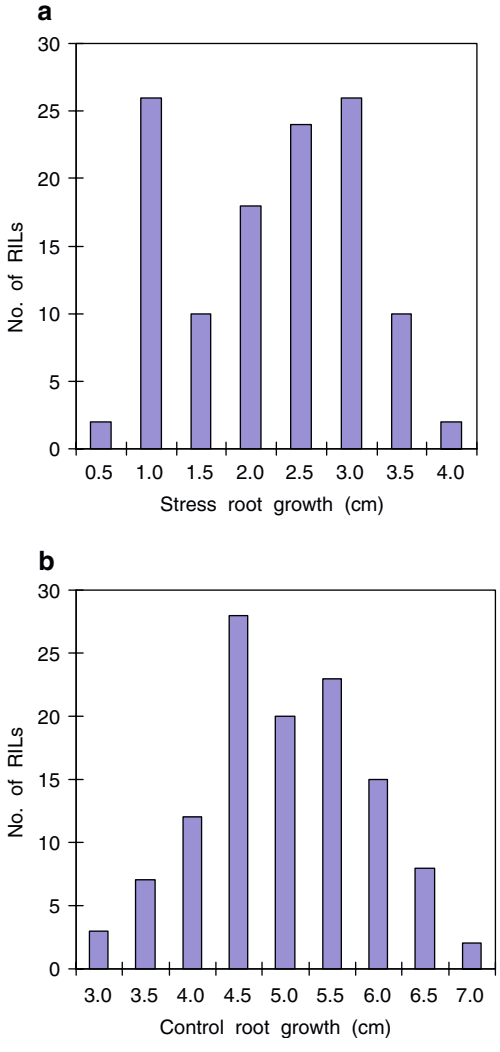

Fig. 1 Frequency distributions of stressed root length (*top*) and control root growth (*bottom*)

Analysis of polymorphism associated with the *ALMT1* gene

A CAPS marker previously developed by Sasaki et al. (2004) discriminated two alleles *ALMT1-1* and *ALMT1-2* associated with Al-tolerance and Al-sensitivity, respectively, in a RIL population from Atlas66/Century. The mapping result showed that the *ALMT1* gene is located between the SSR markers *Xwmc331* and *Xgdm125*, coincident with the peak of the Al-tolerance QTL (Fig. 3). Genetic distances between the *ALMT1* locus and SSR markers *Xwmc331*and *Xgdm125* were 6.2 and 2.2 cM, respectively.

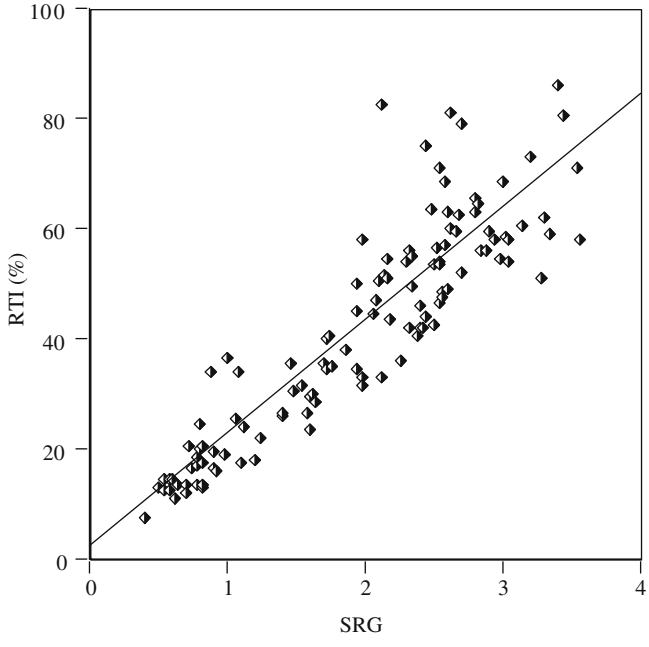

Fig. 2 Pairwise correlation between SRG and RTI for 118 RILs

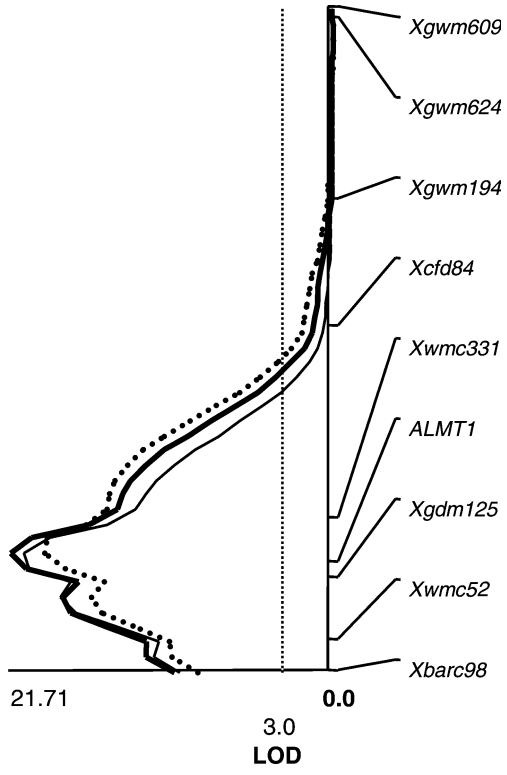

Fig. 3 Interval analysis of a chromosome region on 4DL associated with Al-tolerance based on SRG (*dashed line*), RTI (*regular line*) and hematoxylin stain score (*bold line*)

Characterization of two NILs by using molecular markers

Two NILs (OK91G105 and OK91G106) were evaluated with one CAPS and two SSR markers (*Xwmc331* and *Xgdm125*) for the presence of the Al-tolerance QTL. They are BC_3-derived NILs from the same cross that was used for the generation of the RIL population. Phenotypic data showed that the two NILs had relatively high SRG (2.81 ± 0.15 and 3.34 ± 0.22 cm) and RTI (41.3 ± 3.39 and $51.4 \pm 2.29\%$) values, indicating the extent of Al-tolerance of the two NILs was much greater than that of Century, but slightly less than that of Atlas 66 (Table 1). Results from the SSR analysis showed that banding patterns of these three markers were the same in the NILs as in Atlas 66 (Fig. 4). These results suggest that both the NILs carried the same major Al-tolerant QTL on the chromosome 4DL.

Discussion

Although wheat materials are usually evaluated for Al-tolerance in acidic soils under field conditions in wheat-breeding programs, inconsistent phytotoxicity among plots may significantly increase the environmental error and decrease the accuracy of the phenotypic data. In addition, non-stressed treatments are usually applied in a different field (normal soil pH), which may not provide a valid control for proper comparison. An alternative method is to evaluate wheat materials in a hydroponics system that provides a strict control over nutrient availability and pH, and provides non-destructive measurements in large populations. Therefore, it has been widely used in genetic studies (Baier et al. 1995; Carver and Ownby 1995; Samac and Tesfaye 2003). Controlling the Al^{3+} concentration is critical to accurate separation of genotypes. A concentration of 0.36 mM Al^{3+} was used for phenotyping of Al-tolerance in this study. With this method, root-growth rate during Al stress was measured to determine Al-tolerance of different genotypes. Two parameters were evaluated to measure Al effect on root growth: absolute root growth (SRG) of Al stress and RTI of Al-stressed plants during the period of Al stress, in comparison with that from control plants of the same RIL. A high correlation was observed between the two parameters ($r = 0.9$), and QTL for both parameters were mapped in the same region; therefore, they both could be used for genetic study of Al-tolerance in wheat in this study. Root growth of control RILs also showed a wide range of distribution, which may complicate the genetic effect of Al-tolerance in different genetic backgrounds. Therefore, RTI, as reflected by the percentage of root growth under Al stress, relative to that in the control, may provide a more reliable measurement of wheat root response to Al stress.

Hematoxylin staining of root tips in conjunction with hydroponics culture is also a widely accepted technique for the evaluation of Al-tolerance (Polle et al. 1978; Samac and Tesfaye 2003). The degree of hematoxylin staining in root tips provides a semi-quantitative measurement of Al content in root tips. In the present study,

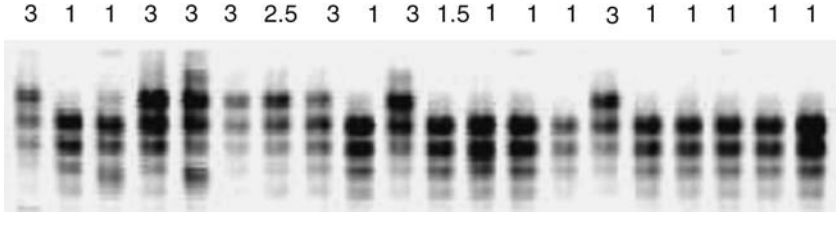

Fig. 4 An electrophoresis pattern of PCR products amplified by SSR primer XGDM 125 on Li-Cor 4200 DNA analyzer. The DNA samples from left to right are: Century (1); Atlas 66 (2); 16 RILs derived from the cross Atlas 66/Century (3–18); OK91G105 (19) and OK91G106 (20)

HSS was highly correlated with both SRG ($r = 0.91$) and RTI ($r = 0.87$). Because it is quick, inexpensive, and easy to score, hematoxylin staining is a very efficient way to evaluate large numbers of lines from segregating populations.

The inheritance of Al-tolerance in wheat has been extensively reported. Monogenic control with dominance effects was reported in some cultivars, while multigenic control was reported in others (Prestes et al. 1975; Camargo 1984; Somers and Gustafson 1995; Somers et al. 1996; Riede and Anderson 1996; Basu et al. 1997). Aniol and Gustafson (1984) used ditelosomic wheat lines to study Al-tolerance of wheat and reported that chromosome arms 5AS, 6AL, 7AS, 2DL, 3DL, and 4DL might have the genes for Al-tolerance. They indicated that Al-tolerance genes were mainly located in the A and D genomes which is consistent with Lafever and Campbell (1978) and Aniol (1990). Aniol (1990) reported the genes controlling Al-tolerance on 2DL, 4DL, and 5AS. Papernik et al. (2001) reported that the loss of chromosome arms 5AS, 7AS, or 4DL significantly reduced the Al-tolerance, indicating that three or more genes on these chromosome arms might contribute to Al-tolerance in CS. Prestes et al. (1975) reported a gene for Al-tolerance on chromosome 5D of Atlas 66.

Camargo (1981) demonstrated that several minor genes might confer Al-tolerance in Atlas 66. Berzonsky (1992) demonstrated that dominant genes on the D genome and other two genomes might determine Al-tolerance in Atlas 66. Tang et al. (2002) also suggested that more than one gene contributes to Al-tolerance in Atlas 66, based on the performance of different NILs derived from Atlas 66. In this study, using a population of RILs derived from the cross between Atlas 66 and Al-sensitive cultivar Century, we identified one major QTL for Al-tolerance that explained approximately 50% of phenotypic variance as expressed by SRG, RTI, and HSS. But a significant portion of the genetic variation still remains unexplained by the major QTL. Other loci with smaller effects may exist in Atlas 66. The SSR markers in these minor QTL regions are lacking, and other markers are needed to identify these QTL.

To compare the QTL identified in this study with Alt_{BH}, an attempt was made to map *Xbcd1230* marker (Rodriguez and Gustafson 2001) in the population of Atlas 66/Century. Unfortunately, polymophism was not detected between Atlas 66 and Century. However, the major locus for Al-tolerance was mapped on chromosome 4DL in the present study. This QTL accounts for approximately 50% of the phenotypic variation for SRG, RTI, and HSS after exposure of the wheat roots to 0.36 mM Al^{3+} for 48 h in hydroponics. The same chromosome region was associated with Al-tolerance in cultivars CS (Luo and Dvorak 1996) and BH1146 (Riede and Anderson 1996). The CS originated from China and not relates to either BH 1146 or Atlas 66, but BH 1146 (Grossal//Frontnra/Mentana) and Atlas 66 (Frondosa//Redhart3/Noll28) are related and share a common progenitor in their pedigree because Grossal was a selection from Polyssu, and Frondosa was derived from the cross of Polyssu/Alfred Chaves 6. Therefore, the major genes (QTL) for Al-tolerance from BH 1146 and Atlas 66 most probably are the same and are derived from Polyssu, a Brazilian wheat cultivar with a high level of Al-tolerance (Foy et al. 1965).

Sasaki et al. (2004) cloned a wheat gene *ALMT1* from ET8, which encodes an Al-activated malate transporter. But the chromosome location of *ALMT1* gene was not determined (Sasaki et al. 2004). In this study with Atlas 66/Century, we found that the *ALMT1* gene maps on the same position as that of the 4DL QTL in Atlas 66. Two SSR markers *Xwmc331* and *Xgdm125*, flanked this gene (Fig. 3), indicating that the *ALMT1* gene might be either the major Al-tolerance gene (*Alt1*) in wheat accessions ET8, BH1146, and Atlas 66, or one of several genes in the Al-tolerance gene cluster on 4DL. It is also possible that the *ALMT1* gene is very tightly linked to the gene for Al-tolerance if it is not the gene for Al-tolerance per se.

Marker-assisted selection is a powerful tool for rapid introgression of Al-tolerance into improved wheat cultivars. Although some RFLP markers linked to Al-tolerance in wheat have been identified (Luo and Dvorak 1996; Riede and Anderson 1996), RFLP analysis is still expensive and time consuming and is infeasible for broad-scale application in wheat-breeding programs. Rodriguez and Gustafson (2001) constructed a linkage map of chromosome 4DL with RFLP, SSR, and AFLP markers using the same RIL population used by Riede and Anderson (1996), identified SSR marker *Xgdm125* linked to Alt_{BH}, and developed a PCR-based marker from clone BCD1230 that co-segregated with Alt_{BH}. But *BCD1230* was not polymorphic between the two parents used in this study and the detail on the potential use of these PCR-based markers for MAS was not discussed (Rodriguez Milla and Gustafson 2001). The marker derived from the *ALMT1* gene has been mapped on the same location as that for the major Al-tolerance QTL and, therefore, is an ideal indicator of the *ALMT1* gene. One drawback of the CAPS is that analysis of this marker requires an additional step of digestion of PCR products, which does not meet the need of high-throughput marker implementation in marker-assisted breeding programs. In this study, we have identified two SSR markers, *Xwmc331* and *Xgdm125*, flanking the major QTL on 4DL. These markers can be analyzed in a high-throughput DNA analyzer and, thus, have the potential to be used as high-throughput markers for marker-assisted screening of Al-tolerance in breeding programs. These two SSR marker alleles for Al-tolerance, along with the *ALMT1* gene, were detected in two NILs derived from Atlas 66. The results indicated that the both NILs contain the 4DL major QTL for Al-tolerance.

Acknowledgements This paper reports the results of research only. Mention of trade names or commercial products in this article is

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