Molecular characterization and mapping of ALMT1, the aluminium-tolerance gene of bread wheat (Triticum aestivum L.)

Harsh Raman, Kerong Zhang, Mehmet Cakir, Rudi Appels, David F. Garvin, Lyza G. Maron, Leon V. Kochian, J. Sergio Moroni, Rosy Raman, Muhammad Imtiaz, Fiona Drake-Brockman, Irene Waters, Peter Martin, Takayuki Sasaki, Yoko Yamamoto, Hideaki Matsumoto, Diane M. Hebb, Emmanuel Delhaize, and Peter R. Ryan

Abstract: The major aluminium (Al) tolerance gene in wheat ALMT1 confers an Al-activated efflux of malate from root apices. We determined the genomic structure of the ALMT1 gene and found it consists of 6 exons interrupted by 5 introns. Sequencing a range of wheat genotypes identified 3 alleles for ALMT1, 1 of which was identical to the ALMT1 gene from an Aegilops tauschii accession. The ALMT1 gene was mapped to chromosome 4DL using 'Chinese Spring' deletion lines, and loss of ALMT1 coincided with the loss of both Al tolerance and Al-activated malate efflux. Aluminium tolerance in each of 5 different doubled-haploid populations was found to be conditioned by a single major gene. When ALMT1 was polymorphic between the parental lines, QTL and linkage analyses indicated that ALMT1 mapped to chromosome 4DL and cosegregated with Al tolerance. In 2 populations examined, Al tolerance also segregated with a greater capacity for Al-activated malate efflux. Aluminium tolerance was not associated with a particular coding allele for ALMT1, but was significantly correlated with the relative level of ALMT1 expression. These findings suggest that the Al tolerance in a diverse range of wheat genotypes is primarily conditioned by ALMT1.

Key words: aluminium, tolerance, genetic marker, Triticum aestivum, QTL, deletion mapping.

Résumé : Les auteurs ont déterminé la structure d’ALMT1, un gène conférant la tolérance à l’aluminium (Al) chez le blé et qui code pour un transporteur de malate activé par l’Al. Ce gène est composé de 6 exons et il est interrompu par 5 introns. Le séquençage de ce gène chez une gamme de génotypes a permis d’identifier 4 allèles d’ALMT1, dont un était identique au gène ALMT1 chez une accession de l’Aegilops tauschii. Ce gène a été localisé sur le chromosome 4DL à l’aide d’une série de délétants chez le blé ‘Chinese Spring’. La perte d’ALMT1 coïncidait avec la perte de tolérance à l’Al et de l’exportation de malate stimulée par l’Al. La tolérance à l’Al chez 5 populations différentes d’haploïdes doublés s’est avérée déterminée par un seul gène majeur. Lorsque le gène ATML1 était polymorphe entre les 2 parents, des analyses QTL et de liaison génétique ont montré qu’ATML1 était situé sur 4DL et montrait une co-ségrégation avec une plus forte capacité d’exportation du malate stimulée par l’Al. Chez 2 des populations examinées, la tolérance à l’Al a aussi co-ségrégé avec une capacité accrue d’exportation du malate. La tolérance à l’Al n’était pas associée à un allèle codant particulier, mais elle était corrélée avec le niveau relatif d’expression d’ALMT1. Ces observations suggèrent que la tolérance à l’Al chez une gamme de génotypes du blé est largement déterminée par ALMT1.
Introduction

Soil acidity currently limits crop production on over 30% of the world’s arable land (Haug 1983). While acid soils present a number of challenges to plant growth, the major limit to production is aluminium (Al) toxicity, since micromolar concentrations of the trivalent Al cations can rapidly inhibit root growth (Foy et al. 1978; Carver and Owny 1995). Many plants have evolved mechanisms to help them tolerate Al stress, and a significant variation in Al tolerance is present within some species, such as wheat and maize (Kochian et al. 2003).

Several studies have demonstrated that Al tolerance is simply inherited in some genotypes of wheat (Kerridge and Kronstad 1968; Camargo 1981; Bona et al. 1994; Somers and Gustafson 1995). However, in other genotypes, Al tolerance involves the action of more than 1 gene. For instance, F2 populations from a cross between the Al-tolerant cultivar ‘Atlas 66’ and several different Al-sensitive parents exhibit a 15:1 segregation pattern, suggesting that 2 dominant genes contribute to Al tolerance in those populations (Camargo 1981; Berzonsky 1992). A multigenic model for tolerance is supported by studies showing that the backcross introgression of a major Al tolerance gene from 1 genotype into the genetic background of an Al-sensitive variety can produce near-isogenic lines (NIL) that are less tolerant than the Al-tolerant donor (Delhaize et al. 1993a; Johnson et al. 1997; Tang et al. 2002). Additionally, the range of Al tolerance in wheat exhibits a continuous distribution, which is also consistent with the involvement of more than a single Al tolerance gene (Mead and Slookmaker 1969; Baier et al. 1995; Ryan et al. 1995).

An analysis of ditelosomic and nullisomic-tetrasomic lines of ‘Chinese Spring’ suggested that genes located on 6AL, 7AS, 4BS, 2DL, 3DL, 4DL, and 7D are important for conferring Al tolerance (Aniol and Gustafson 1984). Nevertheless, it is unclear whether all these loci identified from the deletion lines contribute to the natural variation in Al tolerance found in wheat germplasm (Riede and Anderson 1996; Luo and Dvorak 1996).

Al tolerance in many species is associated with an Al-activated efflux of organic anions, such as malate, citrate, and oxalate (Ryan et al. 2001). Several authors have argued that the organic anions protect roots by chelating the toxic Al3+ species in the soil surrounding the root apex (Delhaize and Ryan 1995; Ma et al. 2001; Kochian et al. 2004). In wheat, there is compelling evidence that Al tolerance is caused by the Al-activated efflux of malate from root apices (Delhaize et al. 1993b; Ryan et al. 1995b). Investigation of NILs of wheat (‘ES8’ and ‘ET8’) that differ in Al tolerance at a single genetic locus, Alt1, identified an Al-activated anion channel in root cells that facilitated greater malate release from the roots of the Al-tolerant genotype ‘ET8’ than the Al-sensitive genotype ‘ES8’ (Ryan et al. 1997; Zhang et al. 2001). These findings were used to develop a hypothesis that the gene for Al tolerance in wheat encodes either the channel protein itself or a protein that controls the activity of such a channel (Delhaize and Ryan 1995; Ryan et al. 2001). Recently, Sasaki et al. (2004) used the ‘ET8’ and ‘ES8’ lines to isolate a wheat cDNA (ALMT1) encoding a membrane-bound protein with properties consistent with a role in Al tolerance. ALMT1 cosegregates with Al tolerance in populations derived from a cross between ‘ET8’ and ‘ES8’, and expression of ALMT1 in transgenic barley, rice, and tobacco and in Xenopus oocytes conferred an Al-activated efflux of malate. The finding that transgenic barley plants and tobacco-suspension cells expressing ALMT1 were significantly more tolerant to Al stress further supports ALMT1 being the main Al tolerance gene in wheat (Delhaize et al. 2004; Sasaki et al. 2004).

The coding region of ALMT1 has 6 single-nucleotide polymorphisms (SNPs) between ‘ET8’ and ‘ES8’, which indicates that at least 2 alleles (ALMT1-1 and ALMT1-2) exist for this gene. The same polymorphisms occur between the Al-tolerant cultivar ‘Atlas 66’ (ALMT1-1) and the Al-sensitive cultivar ‘Scout’ (ALMT1-2) (Sasaki et al. 2004), and the moderately Al-tolerant cultivar ‘Chinese Spring’ possesses the ALMT1-2 allele. Another study of the ditelosomic lines of ‘Chinese Spring’ by Papernik et al. (2001) found that lines lacking the chromosome arms 4DL, 5AS, or 7AS exhibit reduced Al tolerance and reduced capacity for Al-activated malate release. This is consistent with several genes independently influencing a single Al tolerance mechanism that relies on malate efflux.

In this study we determined the structure and chromosomal location of ALMT1 by physical mapping with both ‘Chinese Spring’ deletion lines and several doubled-haploid (DH) mapping populations derived from parental genotypes that differ in Al tolerance. The relation between Al tolerance and ALMT1 expression in a diverse range of wheat genotypes was also investigated.

Materials and methods

ALMT1 gene sequencing

The published cDNA sequences of ALMT1 from ‘ET8’ and ‘ES8’ (GenBank accessions AB081803 and AB081804) were used to obtain the full genomic sequences of ALMT1 in 13 wheat genotypes that varied in Al tolerance (Table 1). The closest ortholog of ALMT1 in rice (accession number CAD40928) was used to predict and amplify the introns of ALMT1-1 and ALMT1-2 (Sasaki et al. 2004), and the moderately Al-tolerant cultivar ‘Chinese Spring’ was used to confirm the introns of ALMT1-2 allele. Another study of the ditelosomic lines of ‘Chinese Spring’ by Papernik et al. (2001) found that lines lacking the chromosome arms 4DL, 5AS, or 7AS exhibit reduced Al tolerance and reduced capacity for Al-activated malate release. This is consistent with several genes independently influencing a single Al tolerance mechanism that relies on malate efflux.

In this study we determined the structure and chromosomal location of ALMT1 by physical mapping with both ‘Chinese Spring’ deletion lines and several doubled-haploid (DH) mapping populations derived from parental genotypes that differ in Al tolerance. The relation between Al tolerance and ALMT1 expression in a diverse range of wheat genotypes was also investigated.
tubs were refilled with the same solution containing 200 µmol AlCl³/L. Root exudates were collected following the method of Papernik et al. (2001) and organic matter released into the nutrient solution was measured, and the solutions were replaced. Control tubs were grown in 8-L tubs containing 80 µmol AlCl³/L, pH 4.3) as described by Raman et al. (2001). The pH was monitored regularly and adjusted as needed. The growth tubes were kept in the dark for the first 3 d and then grown under conditions of 16-h photoperiod/8-h night for the duration of the experiment. After 4 d, 8 seedlings were selected for uniformity, and the length of their longest root was measured. Four seedlings were floated in a 40-L tub of a control nutrient solution, and the other 4 were floated in a 40-L tub of the same nutrient solution containing 80 µmol Al/L. After 24 h of growth, the longest root of each seedling was measured once again. Genotypic response to Al treatment was presented as either net root growth (NRG) or RRG as described earlier.

**Table 1. Wheat genotype by Al tolerance phenotype, ALMT1 coding allele, and GenBank accession number.**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Phenotype</th>
<th>Allele</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>‘ET8’</td>
<td>Tol</td>
<td>ALMT1-1</td>
<td>DQ072260</td>
</tr>
<tr>
<td>‘Tan’</td>
<td>Tol</td>
<td>ALMT1-1</td>
<td>DQ072270</td>
</tr>
<tr>
<td>‘Diamondbird’</td>
<td>Tol</td>
<td>ALMT1-1</td>
<td>—</td>
</tr>
<tr>
<td>‘Halberd’</td>
<td>Tol</td>
<td>ALMT1-2</td>
<td>DQ072265</td>
</tr>
<tr>
<td>‘Chinese Spring’</td>
<td>Tol</td>
<td>ALMT1-2</td>
<td>DQ072262</td>
</tr>
<tr>
<td>‘Maringa’</td>
<td>Tol</td>
<td>ALMT1-2</td>
<td>DQ072267</td>
</tr>
<tr>
<td>‘Embrapa’</td>
<td>Tol</td>
<td>ALMT1-1</td>
<td>DQ072264</td>
</tr>
<tr>
<td>‘Currawong’</td>
<td>Tol</td>
<td>ALMT1-2</td>
<td>—</td>
</tr>
<tr>
<td>‘Cranbrook’</td>
<td>Sens</td>
<td>ALMT1-1</td>
<td>DQ072263</td>
</tr>
<tr>
<td>Aegeitis tauschii</td>
<td>Sens</td>
<td>ALMT1-1</td>
<td>DQ072271</td>
</tr>
<tr>
<td>‘Spica’</td>
<td>Sens</td>
<td>ALMT1-2</td>
<td>DQ072268</td>
</tr>
<tr>
<td>‘Sunco’</td>
<td>Sens</td>
<td>ALMT1-2</td>
<td>DQ072269</td>
</tr>
<tr>
<td>‘Janz’</td>
<td>Sens</td>
<td>ALMT1-2</td>
<td>DQ072266</td>
</tr>
<tr>
<td>‘CDB7’</td>
<td>Sens</td>
<td>ALMT1-2</td>
<td>—</td>
</tr>
<tr>
<td>‘ES8’</td>
<td>Sens</td>
<td>ALMT1-2</td>
<td>DQ072261</td>
</tr>
</tbody>
</table>

*As determined by CAPS marker (Sasaki et al. 2004).

Research Inc., Waltham, Mass., USA) and an ABI 3700 capillary sequencer (Applied Corporation, Norwalk, Conn.). PCR products that proved difficult to sequence (especially intron 3) were purified and subcloned into the pGEM®/T Easy vector according to the manufacturer’s instructions (Promega Corporation, Madison, Wis., USA) before sequencing. Evaluation of sequences and the construction of contigs was performed with the SeqEd software package (http://www.biology.wustl.edu) or Vector NTI (http://www.invitrogen.com/content.cfm?pageID=10352). All sequences were aligned to the cDNA sequence of ALMT1, and polymorphisms were detected.

**Physical mapping of Al tolerance and the ALMT1 gene using segmental deletion lines**

**Plant material and phenotyping**

Seeds of the ‘Chinese Spring’ ditelosomic lines carrying deletions on chromosome arm 4DL (Endo and Gill 1996) were grown in 8-L tubs containing 200 µmol CaCl²/L, pH 4.5, on a laboratory bench as described by Papernik et al. (2001). After 2 d the longest root of each seedling was then measured, and the solutions were replaced. Control tubs were grown in 8-L tubs containing 200 µmol CaCl²/L, pH 4.5, and treatment tubs were refilled with the same solution containing 2.5 µmol AlCl³/L. Seedlings were grown for 2 more days, and the longest root of each seedling was remeasured. The relative Al tolerance of each line was expressed as relative root growth (RRG), which is (root elongation in Al) × 100 / (root elongation in control). Root exudates were collected following the method of Papernik et al. (2001) and organic anions were analyzed with a capillary electrophoresis system (P/ACE 5510, Beckman Instruments, Fullerton, Calif., USA) as described by Piñeros et al. (2002).

**Southern blot analysis**

Membranes for Southern blotting were prepared by digesting 20 µg of genomic DNA with 20 units of each restriction enzyme. The digested DNA was separated on a 0.8% agarose gel and transferred to Hybond N+ membranes (Amersham, Arlington Heights, Ill., USA) according to the manufacturer’s recommendations. Probes were prepared using either the full coding region of ALMT1-1 or a 440-bp fragment at the 3’ end of the cDNA beginning at a PstI digestion site in exon 6. A cloned insert of ALMT1 was isolated from plasmid cDNA by restriction digestion and labeled with [³²P]-deoxyctydine-5'-triphosphate by random priming. Membranes were then hybridized and washed according to Sambrook et al. (1989).

**Northern blot analysis**

Root mRNA was extracted using the mRNA extraction kit (Ambion, Inc., Austin, Tex., USA) according to the manufacturer’s recommendations. The mRNA (1 µg for each sample) was separated by electrophoresis on formaldehyde-agarose gels, transferred to nylon membranes (Hybond N+, Amersham) by capillary blot, fixed at 80 °C for 2 h, and hybridized according to Sambrook et al. (1989).

**Analysis of the doubled haploid (DH) populations**

**Al tolerance**

Five DH wheat populations derived from ‘Diamondbird’/‘Janz’, ‘Currawong’/‘CDB7’, ‘Spica’/‘Maringa’, ‘Sunco’/‘Tan’, and ‘Cranbrook’/‘Halberd’ (Table 1) were evaluated for Al tolerance using root growth and (or) haematoxylin staining. The growth conditions and Al treatments used to analyse these populations varied, because the experiments were performed in separate laboratories. However, in all cases, the conditions used clearly distinguished the parental genotypes for Al tolerance.

(i) ‘Diamondbird’/‘Janz’: seeds of the DH populations derived from ‘Diamondbird’/‘Janz’, ‘Currawong’/‘CDB7’, ‘Spica’/‘Maringa’, ‘Sunco’/‘Tan’, and ‘Cranbrook’/‘Halberd’ (Table 1) were evaluated for Al tolerance using root growth and (or) haematoxylin staining. The growth conditions and Al treatments used to analyse these populations varied, because the experiments were performed in separate laboratories. However, in all cases, the conditions used clearly distinguished the parental genotypes for Al tolerance.

(ii) ‘Sunco’/‘Tan’ and ‘Cranbrook’/‘Halberd’: seeds of these DH populations were germinated in aerated, deionized water for 3 d on trays and transferred to aerated nutrient solutions containing the following: 4 mmol CaCl²/L; 0.1 mmol (NH₄)₂SO₄/L; 6.5 mmol KNO₃/L; 2.5 mmol MgCl₂·6H₂O/L; 0.4 mmol NH₄NO₃/L; 13 µmol NaH₂PO₄/L; 2 µmol MnSO₄·H₂O/L; 0.3 µmol CuSO₄·5H₂O/L; 0.8 µmol ZnSO₄·7H₂O/L; 10 µmol H₃BO₃/L; 0.1 µmol Na₂MoO₄·2H₂O/L; 10 µmol FeSO₄·7H₂O/L; and 440 µmol AlCl₃·H₂O/L. The solution was maintained between pH 4.1 and 4.2. After 7 d in the treatment solutions, the length of the main seminal root of 4 plants per individual DH line was mea-
sured. Genotypes with known responses to Al were grown at the same time to compare growth among trays and among experimental trials.

(iii) ‘Currawong’/‘CD87’, ‘Spica’/‘Marina’ and ‘Diamondbird’/‘Janz’: 3 DH populations derived from these parental lines were screened for Al tolerance by the haematoxylin-staining assay (Polle et al. 1978). Seeds were germinated and grown as described for ‘Diamondbird’/‘Janz’. Four days after germination, 50 µmol AlK(SO₄)₂/L was added to the nutrient solution. The Al concentration was chosen to discriminate between Al tolerant and sensitive genotypes, based on previous dose–response curves (data not shown). After 24 h in the Al solution, haematoxylin staining was performed using the assay described by Polle et al. (1978). Root tips (the distal 1–3 cm) exhibiting dark blue/purple staining were considered Al sensitive, whereas unstained or lightly stained seedlings were scored as Al tolerant. Parental lines and other wheat lines with known responses to Al were included in all of the assays as controls.

**Malate efflux**

Al-activated malate efflux was measured from the individuals of 2 DH populations derived from ‘Diamondbird’/‘Janz’ and ‘Currawong’/‘CD87’ using an enzyme assay described by Delhaize et al. (1993b). The locus involved in Al-activated malate efflux in DH populations from ‘Diamondbird’/‘Janz’ and ‘Currawong’/‘CD87’ was designated Xme.

**Molecular and linkage mapping**

Genetic linkage analysis was used to determine the chromosomal locations of ALMT1 and Al tolerance loci in the 5 DH populations and to compare these with the previously mapped Al tolerance loci Ati₂h (Riede and Anderson 1996) and A1r2 (Luo and Dvorák 1996). Full linkage maps of ‘Cranbrook’/‘Halberd’ and ‘Sunco’/‘Tasman’ DH populations developed by the Australian National Wheat Molecular Marker program (Chalmers et al. 2001) were used to identify quantitative traits loci (QTLs) for Al tolerance. QTL analysis was carried out using Mapmanager (Manly et al. 2001) and QGENE (Nelson 1997) software as described by Cakir et al. (2003). For the remaining 3 DH populations (‘Diamondbird’/‘Janz’, ‘Currawong’/‘CD87’, and ‘Spica’/‘Marina’) simple sequence repeat (SSR) markers previously shown to map on 4D (Röder et al. 1998; Pestova et al. 2000; Gupta et al. 2002; http://www.shigen.nig.ac.jp/wheat/konugi/maps/) were tested on the parental lines to determine whether they were polymorphic. Sequences of some of these SSR markers were accessed from IPK (Gatersleben, Germany). SSR and linkage analysis was carried out as described previously (Raman et al. 2005). M13-tailed oligos were labelled with a fluorescent dye (D2, D3, or D4, Beckman Coulter Inc., Fullerton, Calif., USA) and subsequently used for analysis of DH populations as described by Raman et al. (2005).

A cleaved amplified polymorphic sequence (CAPS) marker was used to distinguish between the 2 ALMT1 alleles as described by Sasaki et al. (2004).

**ALMT1 expression**

ALMT1 expression and Al tolerance of the parental lines of the 5 DH populations as well as ‘ET8’, ‘ES8’, and ‘Chinese Spring’ were measured using the procedures described by Delhaize et al. (2004). Expression levels were estimated with real-time reverse transcriptase (RT)-PCR and expressed relative to expression of the wheat PT-1 gene (GenBank accession AF110180).

Total RNA was prepared (Qiagen RNasey Plant Mini Kit; Qiagen, Hilden, Germany) from 20 root tips (3 mm long) collected from the various genotypes with 3 biological replicates for each line. The RNA extraction included an on-column DNase step to degrade any contaminating genomic DNA. cDNA was prepared from total RNA (2 µg) as described previously (Schenk et al. 2000) with the exception that the final elution from the spin column was diluted to 100 µL. Levels of ALMT1 and control gene expressions were determined by real-time quantitative RT-PCR on Rotor-Gene 3000 Real Time Cyclers (Corbett Research, Sydney, Australia). One-tenth dilutions were used as template for the quantitative RT-PCR in a total volume of 10 µL as follows: 5 µL SYBR Green JumpStart Taq ReadyMix (Sigma Chemical Co., St. Louis, Mo., USA), 0.5 µL primer mix (50:50 mix of forward and reverse primers at 10 pmol/µL each) and 4.5 µL template. The primers 5’-CGTGAAGACGGAAAGCC-3’ and 5’-CCCTCGACTCAGGTTACTAACAGC-3’ were used for amplification of ALMT1 transcript, 5’-GAAGGACATCTTTACGGGATTCT-3’ and 5’-CACGGCCATGAAGAAGG-3’ for amplification of PT-1. Cycling conditions were as follows: 5 min at 94 °C followed by 45 cycles of 15 s at 94 °C, 15 s at 55 °C, and 20 s at 72 °C. At the end of the cycling, the samples were incubated at 40 °C for 5 min then at 55 °C for 1 min; this was followed by a melting curve program (55°C to 99 °C in 1-degree increments with a 5-s hold at each temperature). The Corbett software was used to calculate comparative ALMT1 expression levels using the PT-1 gene as the control.

**Results**

**Structure of the ALMT1 gene**

The 1388-bp coding region of ALMT1 is interrupted by 5 introns ranging from 0.1 to 1.8 kb (Fig. 1A). This structure was verified in an ALMT1 sequence obtained directly from a BAC clone of A. tauschii (Accession AUS18913). The ALMT1 sequences obtained from 13 genotypes of wheat fell into 1 of 2 haplotypes (ALMT1-1 and ALMT1-2), which were distinguished by at least 44 SNPs or small insertions/deletions (indels) (Fig. 1B). The presence of 1 additional SNP detected in Marina indicates that at least 3 ALMT1 alleles exist for ALMT1. Polymorphisms in the introns supplement the 6 SNPs previously described in the exons (Sasaki et al. 2004). Two of the 6 SNPs located in exons result in amino acid changes, and 1 of these, in exon 4, was used to develop a CAPS marker to distinguish ALMT1-1 from ALMT1-2 (Sasaki et al. 2004). Table 1 indicates that the ALMT1 allele does not determine the relative Al tolerance of wheat, because tolerant genotypes can possess either ALMT1-1 or ALMT1-2 (Table 1).
Physical mapping of Al tolerance and ALMT1

A set of deletion lines for chromosome arm 4DL derived from ‘Chinese Spring’ was evaluated for Al tolerance. Figure 2A shows that the wild type ‘Chinese Spring’ genotype (euploid) and the deletion line with the most distal breakpoint on chromosome arm 4DL (4DL-14) had similar levels of Al tolerance. Relative root growth was inhibited to a greater extent in all other deletion lines, including 4DL-2, which has the breakpoint closest to that in 4DL-14. Similarly, the levels of Al-activated malate efflux from the roots of 4DL-2, and all subsequent deletion lines, were approximately 10% of the rates from the 4DL-14 line and the euploid parent. Several SSR markers mapped to the same bin (fraction length 0.71 to 0.86) delimited by the breakpoints in 4DL-14 and 4DL-2 (Fig. 2A).

Southern blot analysis was performed on DNA extracted from 4DL-14 and 4DL-2 lines using a probe prepared from a 440-bp fragment derived from the 3’ end of the ALMT1 coding region (Fig. 2B). A single intense band was detected in the DNA from 4DL-14, whereas no bands were detected for any of the enzymes in 4DL-2. This indicates that the 3’ sequence of ALMT1 is unique to chromosome 4D. Furthermore, the loss of a band in 4DL-2 compared with 4DL-14 shows that ALMT1 localizes to the same chromosomal region on 4DL that conferred Al tolerance and malate efflux. When the same filter was hybridized against a probe prepared from the entire coding region of ALMT1, multiple bands were observed for many of the enzymes (data not shown). This is consistent with the results of Sasaki et al. (2004) and confirms that additional genes similar to ALMT1 are also present, presumably located on the A and B genomes.

Genetics of Al tolerance and malate efflux in the DH populations

Root growth and haematoxylin staining were used to score for Al tolerance in several DH populations. The frequency distributions of root growth for the individual lines in 3 populations tested (‘Diamondbird’/‘Janz’, ‘Sunco’/‘Tasman’, and ‘Cranbrook’/‘Halberd’) resulted in bimodal distributions...
consistent with a major gene controlling Al tolerance in each population (Fig. 3). Similarly, when haematoxylin staining was used to score for Al tolerance in DH populations derived from ‘Diamondbird’/‘Janz’, ‘Currawong’/‘CD87’, and ‘Spica’/‘Maringa’, the segregation ratios did not differ significantly from 1:1 (Table 2), which is consistent with the presence of a major gene controlling Al-activated malate efflux. Furthermore, Al tolerance scored on the basis of haematoxylin staining cosegregated with the capacity for Al-activated malate efflux in the 155 individual lines of the ‘Diamondbird’/‘Janz’ DH population and in the 163 lines of the ‘Currawong’/‘CD87’ DH population (data not shown).

**QTL and linkage analysis**

We investigated linkage between ALMT1 and Al tolerance in 3 of the 5 DH populations whose parental genotypes were polymorphic for ALMT1 as determined with the previously
described CAPS marker (Sasaki et al. 2004). The Al-tolerant parental lines ‘Diamondbird’ and ‘Tasman’ possessed the ALMT1-1 allele, whereas the corresponding Al-sensitive parental genotypes ‘Janz’ and ‘Sunco’ possessed the ALMT1-2 allele (Table 1). By contrast, the Al-tolerant genotype ‘Halberd’ had the ALMT1-2 allele, and Al-sensitive ‘Cranbrook’ possessed the ALMT1-1 allele. Parents of the other DH populations (‘Currawong’/’CD87’ and ‘Spica’/’Maringa’) all possessed the ALMT1-2 allele, which prevented us from scoring ALMT1 segregation with the CAPS marker.

QTL analysis with the ‘Sunco’/’Tasman’ and ‘Cranbrook’/’Halberd’ DH populations showed that ALMT1 and a single major QTL for Al tolerance mapped to the same region on chromosome 4D (Fig. 4). In the ‘Sunco’/’Tasman’ population, this QTL (logarithm of the odds (LOD) score = 62.35) accounted for 80% of the variation in Al tolerance, and in the ‘Cranbrook’/’Halberd’ population the same QTL had a LOD score of 44.54 and explained 93% of the variation.

Linkage analysis showed that in the ‘Currawong’/’CD87’ and ‘Diamondbird’/’Janz’ DH populations ALMT1 maps close to the SSR marker WMC331, which has previously been mapped to 4DL in the ‘W7984’/’Opata 85’ population (Gupta et al. 2002). No recombinants were identified between Al tolerance (haematoxylin staining), malate efflux, and ALMT1 in the ‘Diamondbird’/’Janz’ population (Fig. 5). Single-marker regression analysis indicated that ALMT1 detected 92% of the total phenotypic variance for Al tolerance with a likelihood ratio statistic (LRS) value of 385.2 (LOD = 83.7).

Despite the availability of a large number of SSR markers mapped on 4D, only GDM129, GW129, GWM194, CFD23, WMC331, GW165, WMC52, WMC48b, and GWM1302 were polymorphic and could be mapped on chromosome 4D in different populations. The order of SSR markers around the Al tolerance gene derived from 3 of the DH populations is shown in Fig. 5. The closely linked SSR marker WMC331 had a LOD score of 32.5 with Xalt and explained 92% of total phenotypic variance for Al tolerance scored on the basis of root growth (LRS = 208.7) in the ‘Diamondbird’/’Janz’ population. The GWM1302 marker exhibited a significantly skewed segregation in the ‘Spica’/’Maringa’ population (71:39) but gave a LOD score of 19.1 (P = 0.001) with the Xalt locus. Marker GDM129 also showed a skewed segregation in the ‘Currawong’/’CD87’ population (110:51). ‘CD87’ produced a 138-bp allele with this marker, and ‘Currawong’ produced a 140-bp allele with the same marker; both alleles were present in a “stutter”, making it difficult to score reliably. Markers GWM1302 and GDM129 were not included in the linkage analysis. The STS marker for RFLP BCD1230, which is tightly linked with Al tolerance (Riede and Anderson 1996; Rodriguez-Milla and Gustafson 2001), was not polymorphic in any of the 5 DH populations used here.

**Table 2.** Phenotypic segregation for Al tolerance and Al-activated malate efflux in DH populations of wheat. T, tolerant; S, sensitive; E, efflux; NE, no efflux.

<table>
<thead>
<tr>
<th>DH population</th>
<th>Total DH lines</th>
<th>Tolerant or Efflux‡</th>
<th>Sensitive or No Efflux</th>
<th>χ² (1:1)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>(I) Al tolerance*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>‘Diamondbird’/’Janz’</td>
<td>155</td>
<td>86 (T)</td>
<td>69 (S)</td>
<td>1.86 (n.s)</td>
</tr>
<tr>
<td>‘Currawong’/’CD87’</td>
<td>163</td>
<td>92 (T)</td>
<td>71 (S)</td>
<td>2.70 (n.s.)</td>
</tr>
<tr>
<td>‘Spica’/’Maringa’</td>
<td>110</td>
<td>48 (T)</td>
<td>62 (S)</td>
<td>1.78 (n.s.)</td>
</tr>
<tr>
<td>(II) Al-dependent malate efflux</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>‘Diamondbird’/’Janz’</td>
<td>155</td>
<td>86 (E)</td>
<td>69 (NE)</td>
<td>1.86 (n.s)</td>
</tr>
<tr>
<td>‘Currawong’/’CD87’</td>
<td>163</td>
<td>92 (E)</td>
<td>71 (NE)</td>
<td>2.70 (n.s.)</td>
</tr>
</tbody>
</table>

*Scored by means of haematoxylin staining.
†n.s. not significantly different from 1:1 (P_{stat}).
‡Plants that scored positive for malate efflux showed rates of 0.4 to 1.6 nmol apex–1 h–1. No malate efflux was detected from other plants.

Al tolerance correlates with ALMT1 expression

Real-time quantitative RT-PCR was used to measure the expression of ALMT1 in 13 genotypes included in this study (see Table 1). The Al tolerance of these genotypes was also compared to determine the relation between ALMT1 expression and Al tolerance. Figure 6 shows that a positive correlation exists between Al tolerance and the relative expression

© 2005 NRC Canada
**Fig. 5.** Map location of aluminium tolerance gene (Alt) on chromosome 4DL. Aluminium tolerance (Xalt) was scored using haematoxylin staining in the DH populations shown. Numbers indicate cumulative distances (cM) between marker positions. The location of ALMT1 (Xalmt1) is shown for the ‘Diamondbird’/’Janz’ population, because these parents possess different alleles of ALMT1 (see Table 1). The Al-activated malate efflux phenotype was measured in the ‘Diamondbird’/’Janz’ and ‘Currawong’/’CD87’ populations and is indicated as Xme.

of ALMT1 in root apices ($r^2 = 0.86$). There was no relation between the level of Al tolerance and the ALMT1 allele being expressed.

**Discussion**

Two haplotypes for the ALMT1 gene were identified in divergent wheat germplasm that encompassed a wide range of Al tolerance levels. The occurrence of the ALMT1-1 and ALMT1-2 haplotypes may predate the evolution of hexaploid wheat, since the ALMT1-1 haplotype was present in the single A. tauschii accession included in this study. While this is consistent with the notion that hexaploid wheat independently arose on more than 1 occasion, further work is required to determine whether the ALMT1-2 haplotype is present in other accessions of A. tauschii. Whether this model can also explain the variation in sequence that presumably underlies the different levels of ALMT1 expression remains to be determined.

Examination of 5 DH wheat populations developed from parental genotypes with contrasting Al tolerance led us to conclude that tolerance is controlled by a single major QTL in all populations. Examination of the ‘Chinese Spring’ deletion lines showed that ALMT1 is located near the distal end of chromosome 4DL. This finding is consistent with previous studies that mapped the Alt2 locus from ‘Chinese Spring’ (Luo and Dvorák 1996) and the AltBH locus from ‘BH1146’ (Riede and Anderson 1996; Rodriguez-Milla and Gustafson 2001) to the long arm of 4D. The genetic mapping data from the 5 DH populations were also consistent with a major gene for Al tolerance located on 4DL. It is likely that the ALMT1 gene recently isolated from ‘ET8’ (Sasaki et al. 2004) corresponds to the same genetic locus where Alt1, Alt2, and AltBH from ‘Carazinho’, ‘Chinese Spring’, and ‘BH1146’, respectively, reside. SSR markers closely linked with Al tolerance were also identified and have been mapped to the long arm of chromosome 4D on a composite map of wheat.

Phenotypic screens for Al tolerance based on haematoxylin staining and root growth are efficient in screening large populations in wheat breeding, but they are unable to differentiate homozygous from heterozygous plants. Markers are able to identify heterozygotes, and therefore they can be used to monitor the introgression of Al tolerance genes, especially for F1 gene-enrichment and early generation selection. The coding region of ALMT1 can be used as a molecular marker for Al tolerance with the CAPS marker described previously (Sasaki et al. 2004), but its use is limited to populations in which the parental lines possess different alleles. While this limitation is also true for WMC331, the SSR marker closely linked to Al tolerance, it would be a cost-effective alternative to the CAPS marker for high-throughput genotyping platforms because it does not require an additional digestion step. Diverse germplasm could also be evaluated for Al tolerance on the basis of ALMT1 gene expression, which shows a strong relation with Al tolerance.

Al tolerance loci in other members of the Triticeae have been located on chromosomes that are homoeologous to 4D in wheat. For example, the Alt3 locus of rye (Secale cereale L.) has been mapped to 4RL (Miftahudin et al. 2002) and the Alt/Alp locus of barley (Hordeum vulgare L.) to 4HL (Tang et al. 2000; Raman et al. 2002; Raman et al. 2003). One interpretation of this pattern is that a similar gene underlies Al tolerance among members of the Triticeae, as previously suggested by Garvin and Carver (2003). There is some physiological support for this idea from barley, because, not only do barley plants exhibit an Al-activated efflux of organic anions from their roots but also the magnitude of this response is correlated with Al tolerance (Ma et al. 2004).
The closest ortholog to ALMT1 in the database appears in rice and maps to chromosome 4 where, so far, no QTLs for Al tolerance have been described. A single major locus for Al tolerance, *Al.ts1*, was recently mapped in sorghum (*Sorghum bicolor*), a member of the Poaceae family that is outside of the tribe Triticeae (Magalhães et al. 2004). *Al.ts1* mapped to chromosome 3, which is not orthologous to the group 4 chromosomes of the Triticeae. Therefore, either the genes encoding Al tolerance in sorghum are different to *ALMT1*, or chromosomal rearrangements have disrupted the microsynteny of orthologous chromosomes. Interestingly, chromosome 3 in sorghum is orthologous to chromosome 1 in rice, where other QTLs for Al tolerance have also been identified (Wu et al. 2000; Ma et al. 2002; Nguyen et al. 2001, 2002, 2003). This might indicate that these non-Triticeae grasses have orthologous genes that encode for alternative mechanisms of Al tolerance.

Sasaki et al. (2004) observed that Al tolerance in wheat was not associated with a particular allele of *ALMT1*. Indeed, they demonstrated that the expression of either of these alleles in tobacco suspension cells could enhance the cells’ tolerance to Al. We have confirmed that the *ALMT1* alleles are not diagnostic for Al tolerance and have shown a significant correlation between *ALMT1* expression and Al tolerance in a diverse range of genotypes. This supports previous suggestions that Al tolerance in wheat is controlled by the Al-activated efflux of malate, which is encoded by *ALMT1* (Delhaize et al. 1993b; Ryan et al. 1995b; Sasaki et al. 2004). Since Al tolerance in wheat is strongly correlated with the capacity for Al-activated malate efflux (Ryan et al. 1995a) it is possible that the additional tolerance loci detected in some genotypes (see Introduction) might function by modulating *ALMT1* expression and therefore malate efflux. Support for this model comes from Papiernik et al. (2001), who showed that the 5AL and 7AL distelosomic lines of ‘Chinese Spring’ exhibit reduced Al tolerance and reduced Al-activated malate efflux compared with the euploid parent. This indicates that the loss of 1 or more genes on chromosomal arms 5AS or 7AS can lower Al tolerance by reducing malate efflux (encoded by *ALMT1* on 4DL), but it does not necessarily follow that genes on 5AS and 7AS contribute to the natural variation in Al tolerance among genotypes. Nevertheless, Tang et al. (2002) showed that the incomplete transfer of Al tolerance from ‘Atlas 66’ to other lines was associated with the incomplete transfer of capacity for Al-activated malate efflux. Therefore, this model can accommodate a multigenic basis for Al tolerance in wheat acting through a single mechanism encoded by *ALMT1*.

The data presented here suggest that Al tolerance is controlled by the expression of *ALMT1*, which is located on 4DL. We hypothesise that the level of *ALMT1* expression is regulated either by the promoter region upstream from *ALMT1* or by a sequence that is physically and genetically linked very tightly with *ALMT1*. This hypothesis is currently being tested.

**Acknowledgements**

The authors thank Meredith Carter and Tim Setter (Department of Agriculture, WA), Zdenka Tomes, and Donna Seberry (New South Wales (NSW) Department of Primary Industries) for experimental assistance. This research work was supported by NSW Agricultural Genomics Centre funded by BioFirst Initiative of NSW Government, Grains Research and Development Corporation, and Commonwealth Scientific and Industrial Research Organisation. Thanks are due to M. Ganal, M. Röder (IPK, Gatersleben, Germany) and P. Sourdille (France) for providing primers and Daryl Mares (University of Adelaide) for supplying seeds of ‘Spica’/‘Marina’ DH population. We are grateful to Evans Lagadah for supplying a BAC library of *Aegilops tauschii*.

**References**


© 2005 NRC Canada


