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An *ALMT1* Gene Cluster Controlling Aluminum Tolerance at the *Alt4* Locus of Rye (*Secale cereale* L.)

N. C. Collins,*[,2] N. J. Shirley,* M. Saeed,*[,1] M. Pallotta* and J. P. Gustafson†

**Australian Centre for Plant Functional Genomics, School of Agriculture, Food and Wine, University of Adelaide, Glen Osmond, SA 5064, Australia and* †*United States Department of Agriculture–Agricultural Research Service, Plant Genetic Research Unit, University of Missouri, Columbia, Missouri 65211*

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ABSTRACT

Aluminum toxicity is a major problem in agriculture worldwide. Among the cultivated Triticeae, rye (*Secale cereale* L.) is one of the most Al tolerant and represents an important potential source of Al tolerance for improvement of wheat. The *Alt4* Al-tolerance locus of rye contains a cluster of genes homologous to the single-copy *Al-activated malate transporter* (*TaALMT1*) Al-tolerance gene of wheat. Tolerant (*M39A-1-6*) and intolerant (*M77A-1*) rye haplotypes contain five and two genes, respectively, of which two (*ScALMT1-M39.1* and *ScALMT1-M39.2*) and one (*ScALMT1-M77.1*) are highly expressed in the root tip, typically the main site of plant Al tolerance/susceptibility. All three transcripts are upregulated by exposure to Al. High-resolution genetic mapping identified two resistant lines resulting from recombination within the gene cluster. These recombinants exclude all genes flanking the gene cluster as candidates for controlling *Alt4* tolerance, including a homolog of the barley *HvMATE* Al-tolerance gene. In the recombinants, one hybrid gene containing a chimeric open reading frame and the *ScALMT1-M39.1* gene each appeared to be sufficient to provide full tolerance. mRNA splice variation was observed for two of the rye *ALMT1* genes and in one case, was correlated with a ∼400-bp insertion in an intron.

SOIL acidity and associated Al toxicity represents a major limitation to crop production. Acidity causes Al-containing compounds to release the phytotoxic Al^{3+} cation into the soil solution where it can cause root stunting, restricting the ability of crops to acquire water and nutrients. Over 50% of the world's arable land is acidic (pH < 5.0) (Von Uexküll and Mutert 1995; Bot *et al.* 2000). In addition to liming, the use of Al-tolerant germplasm offers a strategy for minimizing yield losses due to soil acidity.

The main documented mechanisms of Al tolerance in plants involve detoxification of Al^{3+} cations by chelation to organic acids (carboxylate compounds) such as malate and citrate, either within the symplast or after exudation of these compounds from root tips (Kochian *et al.* 2005). A role for organic acid exudation as a mechanism of Al tolerance has been highlighted by the recent cloning of four Al-tolerance genes that all encode organic acid transporters. The *TaALMT1* gene controlling tolerance at the Alt_{BH} (*Alt2*) locus on wheat (*Triticum aestivum* L.) chromosome arm 4DL encodes the *al*uminum-activated *m*alate *t*ransporter-1 (TaALMT1) protein representing the founding member of a family of plant-specific membrane-integral proteins (Sasaki *et al.* 2004; Delhaize *et al.* 2007). In Arabidopsis, a homolog of *TaALMT1* (*AtALMT1*) makes a major contribution to the Al tolerance of wild-type plants (Hoekenga *et al.* 2006). In sorghum (*Sorghum bicolor* L.), the *SbMATE* Al-tolerance gene from the Alt_{SB} locus encodes a member of the large *m*ultidrug *a*nd *t*oxic compound *e*xtrusion (MATE) family of membrane transporters (Magalhaes *et al.* 2007) whose members in prokaryotes and eukaryotes transport a variety of small molecules (Omote *et al.* 2006). The *HvMATE* (*HvAACT1*) Al-tolerance gene from the barley (*Hordeum vulgare* L.) *Alp* locus on chromosome 4H also encodes a MATE transporter (Furukawa *et al.* 2007; Wang *et al.* 2007). TaALMT1 and AtALMT1 are malate transporters and HvMATE and SbMATE are citrate transporters, which provide Al tolerance in tolerant genotypes by facilitating Al-triggered excretion of the respective Al-chelating organic acids from the root tips (Sasaki *et al.* 2004; Hoekenga *et al.* 2006; Furukawa *et al.* 2007; Magalhaes *et al.* 2007).

Within the cultivated Triticeae, tolerant genotypes of rye (*Secale cereale* L.) are equally or more Al tolerant than the most tolerant wheats and triticales (× Triticosecale Wittmack), while tolerant barleys are the least Al tolerant (Mugwira *et al.* 1976; Aniol and Gustafson 1984; Kim *et al.* 2001). Sets of wheat–rye addition lines, wheat chromosome translocation lines, and triticale

[1]*Present address:* National Institute for Biotechnology and Genetic Engineering, PO Box 577 Jhang Rd., Faisalabad, Pakistan.

[2]*Corresponding author:* Australian Centre for Plant Functional Genomics, School of Agriculture, Food and Wine, University of Adelaide, Waite Campus, PMB1, Glen Osmond, SA 5064, Australia.
E-mail: nick.collins@acpfg.com.au

substitution lines have demonstrated the existence of rye genes that can contribute Al tolerance in the presence of a wheat genetic background (Aniol and Gustafson 1984; Ma *et al.* 2000). Therefore, rye represents an important potential source of Al tolerance that could be used to improve wheat and perhaps other cereals through the use of chromosome engineering and/or genetic transformation approaches.

In rye, Al-tolerance loci have been identified by linkage mapping on chromosome arms 6RS and 7RS (*Alt1* and *Alt4* loci, respectively) (Gallego *et al.* 1998; Matos *et al.* 2005). An Al-tolerance locus originally referred to as *Alt3* and reported to be on chromosome 4R was mapped using M39A-1-6 (tolerant) × M77A-1 (intolerant) F_2 and RIL mapping populations (Miftahudin *et al.* 2002, 2004a, 2005). However, Benito *et al.* (2005) claimed that this locus was actually on 7RS and therefore the same as the *Alt4* locus Matos *et al.* (2005) had mapped on 7RS using Ailés × Riodeva crosses. A rye homolog of *TaALMT1* (*ScALMT1-1*) has been proposed as a candidate for the rye *Alt4* tolerance gene, on the basis that it was closely linked to *Alt4* and because its expression was found to be highest in the root tips, upregulated by Al, and higher in a tolerant line than an intolerant line (Fontecha *et al.* 2007). However, the *Alt4* chromosome segment may also be related to the barley 4HL region containing the *HvMATE* (*Alp* locus) gene (Tang *et al.* 2000), raising the possibility that *Alt4* is a *MATE* gene rather than an *ALMT1* gene. The current study confirms that the locus segregating in the M39A-1-6 × M77A-1 cross (hereafter referred to as *Alt4*) is located on 7RS and reveals that the parents contain clusters of *ALMT1* homologs at the *Alt4* locus. The structure and function of these *ALMT1* homologs are explored, together with the colinearity of *ALMT1* and *MATE* genes, in the Triticeae and rice (*Oryza sativa* L.).

MATERIALS AND METHODS

Plant lines and DNA extraction: Recombinants used for fine mapping had been selected from an F_2 population of a cross between the rye inbred lines M39A-1-6 (Al tolerant) and M77A-1 (Al sensitive) (Miftahudin *et al.* 2005). This cross shows monogenic segregation for Al tolerance determined by the *Alt4* locus and does not segregate for tolerance at any of the other Al-tolerance loci known in rye. The recombinants for the *Alt4* interval were kindly provided by Miftahudin (Bogor Agricultural University). F_2 recombinants had been grown in a glasshouse containing no other rye plants. F_3 progeny of F_2 recombinants were grown in the University of Adelaide quarantine glasshouse without isolating the heads to prevent outcrossing. However, F_4 progeny of these plants used in tolerance assays were scored for PCR markers closely flanking the respective recombination points. Of the 186 F_4 individuals assayed, 16 (8.6%) were found to have been derived from outcrossing and were removed from the analysis. F_5 seed, which were also used in tolerance assays, were obtained exclusively from isolated heads. An F_4 family that was derived from the M39A-1-6 × M77A-1 cross and segregating for *Alt4* was analyzed with markers and individuals that were homozygous tolerant or intolerant were used to make tolerant and intolerant DNA bulks, respectively (10 individuals per bulk).

Genomic DNA for PCR and Southern analyses was extracted from leaf tissue using the method of Rogowsky *et al.* (1991). The method was also carried out in racks of 96 × 1.2 ml collection microtubes (Qiagen, Australia). Ball bearings (3.0 mm) were used to crush frozen samples by shaking in a MM300 Mixer Mill (Retsch, Germany) prior to removal of the balls with a magnet. Pellets were resuspended in RNaseA (40 ng/μl) (Sigma-Aldrich, St. Louis) and A_{260} measured to calculate total nucleic acid content.

Lines of Chinese Spring wheat containing individual chromosomes or chromosome arms of Imperial rye or Betzes barley used to assign markers to chromosomes were kindly provided by Ian Dundas and Rafiq Islam of the University of Adelaide, respectively. The presence of rye chromatin in individual wheat–rye addition line plants was checked by DNA dot-blot analysis using a probe for the R173 rye-specific repeat sequence (Rogowsky *et al.* 1992; nucleotides 2–442 of accession no. X64103). A rye line that was supplied to us as cv. Imperial often showed marker patterns different from those observed in the wheat–rye addition lines and therefore was not the same as the Imperial used to make the addition lines. Marker lanes representing this line, where included, are labeled "rye." Seed of the barley Galleon × Haruna Nijo doubled-haploid population (Karakousis *et al.* 2003) were kindly provided by Peter Langridge (University of Adelaide).

Southern analysis: Southern blots, hybridizations, and autoradiography were performed using standard methods. The rye *ALMT1* cDNA probe fragment was obtained by reverse transcription (RT)–PCR from rye RNA using primers Alm-3 and Alm-5 (supplemental Table 7) and corresponds to nt 218–1357 of the *ScALMT1-M39.1* ORF. RNA and cDNA were prepared as described in the *ScALMT1* sequencing section. The fragment was cloned into pCR8/GW/TOPO TA vector (Invitrogen, Carlsbad, CA), reamplified from bacterial cells of a single colony, purified using the QIAquick PCR purification kit (Qiagen), sequenced using the BigDye version 3.1 kit (Applied Biosystems, Foster City, CA) to confirm identity, and the purified fragment used as template to make [α-^{32}P]dCTP-labeled probes by random priming.

Markers: For generation of new PCR markers, genes from the *Alt4*-related region of rice chromosome 3 were used in BLASTn searches against ESTs at NCBI (http://www.ncbi.nlm.nih.gov/) or the TIGR plant transcript assemblies (http://plantta.tigr.org/) to identify putative orthologs from rye or wheat. Primers matching rye/wheat exons and spanning a 0.5- to 1.5-kb interval in rice comprising mostly intron sequence were used to amplify the corresponding gene fragments from the rye mapping parents M39A-1-6 and M77A-1. PCRs were performed with hot-start immolase DNA polymerase (Bioline, Australia) in reactions of 20 μl containing ∼3.0 μg of total nucleic acid, using primers and conditions indicated in supplemental Table 1. Fragments were purified and sequenced in both directions. Sequences were aligned using Pregap4 and Gap4 programs (Bonfield *et al.* 1995) and restriction enzymes recognizing polymorphisms identified using the NEBcutter V2.0 program (http://tools.neb.com/NEBcutter2/index.php) or by analyzing ClustalW alignments (http://www.ebi.ac.uk/Tools/clustalw/) with CapsID (http://bbc.botany.utoronto.ca/capsid/input.spy). For scoring of *c*leaved *a*mplified *p*olymorphic *s*equence (CAPS) markers, 2–5 μl of unpurified PCR product were digested for 3–5 hr in 12-μl reactions containing 0.5 mg/ml acetylated BSA, 2 units of restriction enzyme, and 1× reaction buffer and the fragments visualized by agarose gel electrophoresis. The only polymorphism found in the STP fragment did not alter a restriction site and was therefore scored by direct sequencing of fragments

amplified from recombinant plants. For markers *B1*, *RPE*, *B11*, *B26*, *B6*, and *B4*, new primers internal to the initial sequencing primers were made to make amplification more reliable by reducing amplicon size. All PCR markers used in linkage analysis are described in supplemental Table 2.

Primers and conditions used for PCR marker analysis of wheat–rye addition lines are shown in supplemental Table 6. Primers were based on rye sequences obtained in this study, the *SCIM819-1434* marker sequence (AY587501), and rye ESTs and extended wheat EST contigs corresponding to RFLP probe sequences. PCR products were separated on 2–3% agarose gels. When PCR products of equal size were amplified from rye and wheat, the products were digested separately with a range of restriction enzymes (as for CAPS markers) to identify ones that would give rye-specific fragments.

Al-tolerance assays: Tolerance assays were performed using F_4 and F_5 seed of families homozygous or segregating for recombinant chromosomes. All individuals in tolerance assays were also scored for PCR markers closely flanking the respective recombination points to assist in data interpretation. Al-tolerance assays were carried out using the Eriochrome Cyanine R method (Aniol 1983). Seeds were pregerminated on moist paper for 2–3 days and transferred to an aerated hydroponics system in a greenhouse. Growth solution was as described by Aniol (1983), except that 0.01 mM instead of 0.1 mM $(NH_4)_2SO_4$ was used. The 1× solution was adjusted to pH 4.0 with HCl. Seedlings were grown without added aluminum for 4 days, then $AlCl_3$ was added to a concentration of 150 μM (4 ppm). After 24 hr in Al, roots were thoroughly rinsed in water, stained for 10 min in a 0.1% solution of Eriochrome Cyanine R (Sigma-Aldrich), rinsed again, and the plants returned to Al-free growth solution. The dye mainly stains the root tips. After 48 hr in Al-free solution, each seedling was assessed for regrowth in the longest root following relief from Al stress by measuring the distance from the middle of the stained section to the end of the root.

Mapping: The order of markers on the high-resolution map of the *Alt4* region was determined from the graphical genotypes. The side of the *Alt4* interval where the *MATE/GAB* marker pair was located was determined by three-point analysis in the M39A-1-6 × M77A-1 derived recombinants. The frequency of recombination between *MATE/GAB* and *B1/B4* in the Blanco × M39A-1-6 rye F_2 mapping population was converted to cM distance using the Kosambi mapping function.

***ScALMT1* sequencing:** The primers Alm-1, -2, -3, -4, -6, and -7 (supplemental Table 7), based on exons 1 and 3 of the wheat *TaALMT1* gene (Figure 2A), were used to amplify genomic fragments from rye *ALMT1* genes. PCRs were performed with immolase DNA polymerase (Bioline) in 20 μl reactions containing 3.0 μg of total nucleic acid, using a program comprising (after an initial step of 95° for 7 min to activate the hot-start enzyme) 20–40 cycles of 95° for 10 sec, 60° for 30 sec, and 68° for 2 min, followed by a final extension step of 68° for 10 min. Amplification of multiple homologous DNA fragments with a single primer pair is known to produce a high proportion of recombinant fragments during the latter cycles of PCR (Judo *et al.* 1998). Therefore, PCR amplifications were performed in 5-cycle increments and the reactions that were found to give the first visible products on an agarose gel were the ones used for cloning. Products were cloned into the pCR8/GW/TOPO TA vector (Invitrogen) and clone insert fragments amplified from bacterial cells of individual colonies using the original primer pairs. Clone insert fragments were purified using the QIAquick PCR purification kit (Qiagen) and sequenced. Alternatively, some bands were gel-purified using a QIAquick gel extraction kit (Qiagen) and direct sequenced.

Examples of the genomic PCRs are illustrated in Figure 2B. Product banding patterns obtained from recombinants 859 and 1135 resembled those obtained from M77A-1 and M39A-1-6, respectively (not shown). A total of 48, 40, 20, and 20 clones of the ∼0.5-kb size were sequenced from each of the M39A-1-6, M77A-1, 859, and 1135 lines, respectively. Half of the ∼0.5-kb clones sequenced from each line were derived from the Alm-6/Alm-7 primer combination and the other half from the Alm-2/Alm-3 combination. Six clones corresponding to the ∼0.9-kb doublet were sequenced from each of the M77A-1 and 859 lines. Additionally, the ∼0.9-kb doublets amplified from M77A-1 and 859, and the ∼0.27-kb band amplified from M77A-1 using the Alm-1/Alm-4 primers (Figure 2B), were gel purified and direct sequenced. Sequences obtained more than once from a given rye line were deemed genuine, while single-base differences unique to single clones were regarded as PCR errors. As a result of the minimum number of PCR cycles employed, PCR errors were detected in only a few clones.

Each total RNA sample for RACE–PCR and amplification of complete *ScALMT1* ORFs was prepared from the end 3.0 mm of the root tips of 10 seedlings grown as for the tolerance assays, harvested 24 hr after the addition of 150 μM Al to the hydroponics solution. Root tips were collected in 2-ml Eppendorf tubes containing two 3.0-mm steel ball bearings, snap frozen in liquid nitrogen, and reduced to powder by shaking in a MM300 mixer mill. The RNA was extracted from plant tissues using Trizol reagent (Invitrogen). 5′- and 3′-RACE were performed using the SMART RACE cDNA amplification kit (Clontech, Mountain View, CA) with PowerScript reverse transcriptase (Clontech), according to the manufacturer's instructions. 5′-RACE was performed as a seminested PCR using the Alm-15 then the Alm-16 gene-specific primer while 3′-RACE was performed using specific primer Alm-17 (supplemental Table 7). To obtain entire *ScALMT1* ORFs, first-strand cDNA was synthesized using an oligo-dT (17-mer) primer and SuperScript III (Invitrogen) reverse transcriptase according to the manufacturer's recommendations, except that a 50°-incubation temperature and DMSO at 6.0% was used to obtain efficient synthesis across the GC-rich 5′ ends of *ScALMT1* transcripts. Full-length *ScALMT1* ORFs were amplified with primers Alm-23 and Alm-27 (supplemental Table 7) in the presence of 5% DMSO, with (after 7 min at 95°) 25–40 cycles of 96° for 10 sec, 60° for 30 sec, and 72° for 2 min 30 sec, followed by a final extension step of 68° for 10 min. Products obtained with the minimum number of PCR cycles necessary to produce visible products on a gel were used for cloning. RACE and other RT–PCR products were gel purified using a QIAquick gel extraction kit (QIAGEN) and cloned and sequenced as for the genomic fragments, except that 5.0% DMSO was included in reactions to sequence 5′-RACE and full-length ORF products.

Expression analyses: Plants were grown as for tolerance assays. Seven days after sowing, Al was added to the growth solution to 150 μM. Prior to the addition of Al ("0 hr") and at 6 and 24 hr after adding Al, tissues were taken from 10 seedlings for each RNA sample. These tissues included root tip, root section, and leaf, as defined in the Figure 4 legend. Total RNA was extracted using Trizol reagent and treated with DNAse (DNA-free, Ambion, Austin, TX). First-strand cDNA was made in 20-μl reactions from 1.5 μg of total RNA, using an oligo-dT (17-mer) primer and SuperScript III reverse transcriptase (Invitrogen), according to the manufacturer's suggestions. cDNAs were diluted to 100 μl total volume. The reported expression levels refer to transcript copies per microliter of this cDNA dilution.

Marker selection of plants for the second expression experiment was performed on *Alt4* segregating families derived from the M39A-1-6 × M77A-1 cross at 6 days after sowing, using *Alt4*-flanking CAPS markers. After taking samples for RNA extraction, a second round of DNA extraction and

marker analysis was performed to verify genotypes of the sampled plants.

ScALMT1 gene expression was measured by quantitative RT–PCR (Q-PCR) using primer pair Alm-6/Alm-10 (supplemental Table 7). Sequences corresponding to the 9 bases at the 5′ end of primer Alm-6 were not obtained from genes *ScALMT1-M39.3*, *-M39.4* and *-M39.5*, and *-M77.2*. Otherwise, the genomic and full-length ORF sequence information that became available verified that all the *ScALMT1* genes in M39A-1-6 and M77A-1 were identical at the primer binding sites. Primer Alm-10 spanning the splice sites of intron 2 (supplemental Figure 4) provided specificity for cDNA by being unable to prime from genomic DNA. Primers for Q-PCR of rye "housekeeping" genes, encoding glyceraldehyde-3-phosphate dehydrogenase, elongation factor 1α, cyclophilin, and actin were developed to provide controls for normalization (supplemental Table 9). Q-PCR from cDNA was carried out essentially as described by Burton *et al.* (2004), except that an RG 6000 real-time thermal cycler (Corbett Research, Australia) was used with half-scale reactions, employing a program comprising 3 min at 95°, followed by 45 cycles of 95° for 1 sec, 55° for 1 sec, and 72° for 30 sec, followed by a single step for 15 sec at the optimal acquisition temperature (supplemental Table 9). Normalization was performed as described by Vandesompele *et al.* (2002). Due to the substantial control gene expression pattern differences observed between the different tissues, data from the different tissues were normalized separately, using the three control genes showing the lowest internal control gene instability measure (M) for that particular tissue.

For selected cDNA samples used in Q-PCR, the relative expression levels of individual *ScALMT1* genes in these samples were determined. PCR reactions of 20 μl were performed using 1 μl of cDNA template and primers Alm-6 and Alm-10. The PCR program comprised (after 7 min at 95°) 20–50 cycles of 95° for 10 sec, 60° for 30 sec, and 68° for 1 min 30 sec, followed by a single step of 68° for 10 min. The minimum number of PCR cycles required to give a visible product were identified and those reactions used in cloning with the pCR8/GW/TOPO TA vector (Invitrogen). Individual clone inserts were PCR amplified from cells of single bacterial colonies and digested with a set of restriction enzymes to determine which *ScALMT1* transcript each fragment was derived from (supplemental Table 8). Digestion conditions were the same as those used for CAPS markers. One clone derived from each of the M39A-1-6 leaf-24-hr and root-tip-0-hr samples from the first experiment showed a novel combination of restriction patterns. Upon sequencing, these clones were found to be hybrids of multiple gene sequences. These likely represented artifacts of PCR that occur during amplification of mixtures of homologous sequences (Judo *et al.* 1998) and were removed from the data set. Each of the remaining clones gave restriction fragment profiles consistent with one of the known *ScALMT1* gene sequences.

RESULTS

Refining the map of the *Alt4* region: Of the rye *Alt4* genetic markers previously identified by colinearity to the corresponding interval on the rice (*Oryza sativa* L.) chromosome-3 sequence, PCR (CAPS) markers for the *B1* and *B4* genes (Miftahudin *et al.* 2004a) were modified, and the *RPE*, *B11*, *B26*, and *B6* genes assayed by RFLP (Miftahudin *et al.* 2005) were converted to easy-to-use codominant PCR markers. PCR markers were also developed using 11 additional genes from the rice *B1-B4* interval, the rye homolog of *HvMATE*, and a rye homolog of a neutral α-glucosidase AB precursor (*GAB*) gene, which is located close to the *MATE* gene in rice and barley (Wang *et al.* 2007). The details of the polymorphism screen undertaken here for marker development are summarized in supplemental Table 1. More than 17,500 bp of sequence was compared between the mapping parents M39A-1-6 and M77A-1, revealing 321 single nucleotide polymorphisms (SNPs) and 42 insertion/deletions (InDels), corresponding to an average frequency of one polymorphism every 48 bp. Details of all the developed PCR marker assays are provided in supplemental Table 2.

From a population of 1123 M39A-1-6 × M77A-1 F_2 plants, Miftahudin *et al.* (2005) had reported 15 recombinants for the marker interval *B1-B4* containing *Alt4*. In the current study, we recovered 13 of the reported recombinants (recombinants 257 and 965 were not recovered), plus an additional unreported recombinant (1135) isolated from the same population. All available PCR markers were scored in several F_3 progeny of each F_2 recombinant, enabling the genotypes for each recombinant chromosome to be determined as shown in supplemental Figure 1. Except for the *KEL* gene, which was found to be unlinked to the *Alt4* region, all new markers from the rice *B1-B4* rice interval mapped in a colinear fashion in the vicinity of *Alt4*, allowing construction of the revised molecular marker map shown in Figure 1. The rye *MATE* and *GAB* genes were found to be outside of the *B1-B4* interval, consistent with their more distant location in rice. To more accurately determine their positions, the *B1*, *B4*, *MATE*, and *GAB* markers were mapped in a cv. Blanco × M39A-1-6 rye F_2 mapping population of 96 individuals. In this population, no recombination was observed between *B1* and *B4* or between *MATE* and *GAB*, while the two pairs of markers mapped 27.5 cM (48 recombinants) from one another (Figure 1). Close linkage between the *MATE* and *GAB* gene, as observed in barley (Wang *et al.* 2007), confirmed that the *MATE* gene mapped in rye was the direct ortholog of the barley *HvMATE* (*HvAACT1*) Al-tolerance gene (Furukawa *et al.* 2007; Wang *et al.* 2007).

The *Alt4* genotypes of all the recombinants were checked using the Eriochrome Cyanine R method (Aniol 1983), which measures tolerance on the basis of the degree of root regrowth following relief from Al stress (Aniol 1983; supplemental Figure 2). Supplemental Tables 3–5 summarize the results from the tolerance assays, performed using F_4 or F_5 families either homozygous or segregating for the recombinant chromosomes. *Alt4* tolerance behaved as a dominant trait. Tolerant and intolerant individuals showed regrowth after 2 days of 10–24 mm and 1–5 mm, respectively. Individual seedlings assayed for tolerance were also scored for PCR markers closely flanking the recombination sites to

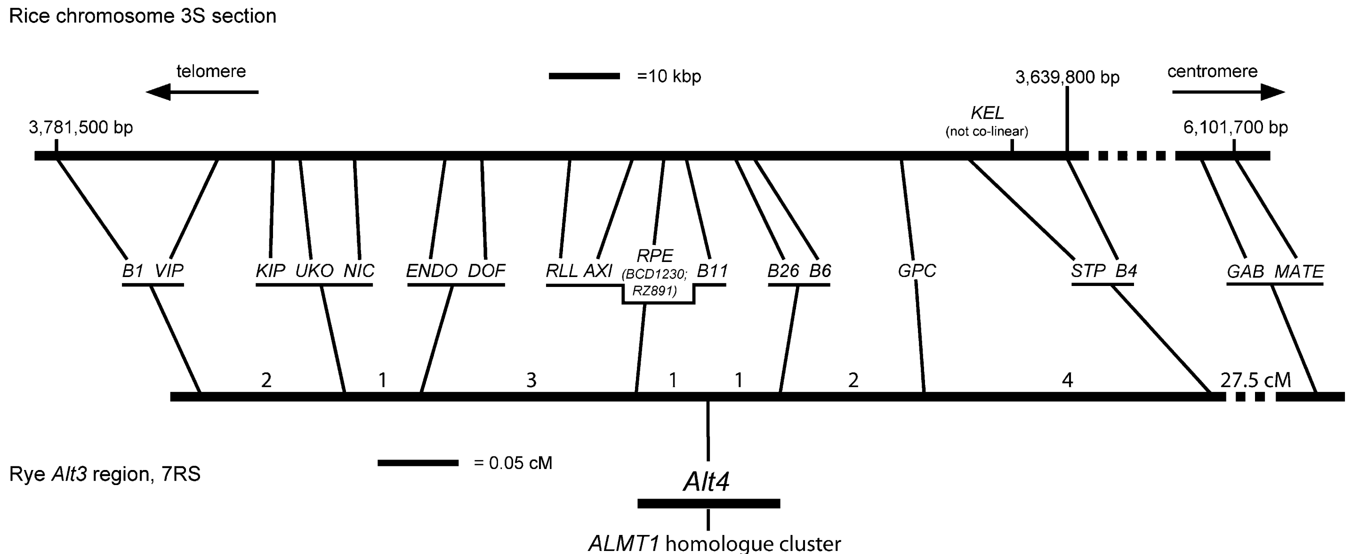

FIGURE 1.—Genetic map of the *Alt4* region of rye and colinearity with rice. Positions of genes on rye 7RS and their corresponding orthologs in a section of the short arm of rice chromosome 3 are shown by the lines connecting the maps. Numbers on the rye map indicate recombinants observed in each interval in the mapping population of 2246 gametes, except for the *B4* to *GAB/MATE* interval, which was measured using a different population. Numbers above the rice sequence refer to nucleotide positions in chromosome 3 sequence assembly AP008209.1. The *B1-B4* interval is contained entirely within the rice BAC clone OSJNBa0091P11 (AC073556). The *ALMT1* gene cluster was not assigned a precise genetic position due to recombination observed within the cluster. The orientation of the mapped section with respect to the centromere of rye chromosome 7R was not determined.

confirm when variation in tolerance was controlled by inheritance of the recombinant chromosome and to verify that individuals of informative genotype had been sampled. *Alt4* genotypes agreed with those determined previously (MIFTAHUDIN *et al.* 2005), except that recombinant 862, previously reported to be homozygous tolerant, was found to be *Alt4* heterozygous (supplemental Table 3). All families used in tolerance assays were also scored for the *MATE* marker. For the majority of the families the *Alt4* tolerance genotype contrasted with the *MATE* marker genotype (supplemental Tables 3–5), demonstrating that the rye *MATE* gene does not determine rye *Alt4* tolerance/intolerance, at least in this cross. The genotype data (supplemental Figure 1) combined to place *Alt4* in the 0.09-cM interval between marker groups *RLL/AXI/RPE/B11* and *B26/B6*, with one recombination event separating *Alt4* from these groups of markers on each side (Figure 1). The corresponding 4.3-kb *B11-B26* interval in rice contains a predicted gene for an unknown protein (TIGR locus *LOC_Os03g07270*; RiceGAAS predgene 21 of BAC clone OSJNBa0091P11), plus a sequence identified as a potential gene by Genescan only (RiceGAAS Predgene 20 of OSJNBa0091P11), which is repetitive in the rice genome and which matched no rice EST sequences in the database.

Reports disagree as to whether the rye Al-tolerance locus mapped using the M39A-1-6 × M77A-1 cross is located on rye 4R or 7RS (MIFTAHUDIN *et al.* 2002; BENITO *et al.* 2005). To resolve this issue, PCR markers for *RPE* and *B4* flanking this locus (Figure 1), *PSR163* on 7RS, *PSR129* on 7RL, and *PSR167* on 4RL (DEVOS *et al.* 1993), and *SCIM819-1434* located 7.4 cM from the Al-tolerance locus on 7RS described by MATOS *et al.* (2005), were tested on lines of wheat cv. Chinese Spring containing individual chromosomes or chromosome arms from rye cv. Imperial. Details of the marker assays are provided in supplemental Table 6 and the results illustrated in supplemental Figure 3. *PSR163*, *PSR129*, and *PSR167* markers identified sequences unique to the 7RS, 7RL, and 4RL addition lines, respectively, confirming that the identities of those lines were correct. *RPE*, *B4*, and *SCIM819-1434* identified products unique to the 7RS addition line. Therefore, at least according to the chromosome arm definitions of DEVOS *et al.* (1993), the Al-tolerance locus segregating in the M39A-1-6 × M77A-1 cross is located on 7RS, and this is the same chromosome arm that contains the locus of MATOS *et al.* (2005).

The RFLP probe *BCD1230*, homologous to the *RPE* marker gene (Figure 1), had been used to detect a locus close to the *Alt4* locus in rye as well as a locus close to the Alt_{BH} Al-tolerance locus in wheat, leading to the proposal that these two tolerance loci may reside on related chromosome segments (MIFTAHUDIN *et al.* 2002). To test this possibility further, we located additional *Alt4*-linked markers in wheat, using deletion mapping data (MIFTAHUDIN *et al.* 2004b; http://wheat.pw.usda.gov/cgi-bin/westsql/map_locus.cgi). Three genes mapped close to *Alt4* in rye (*KIP*, *GPC*, and *B4*; Figure 1) were found by BLASTn searches to be highly homologous to

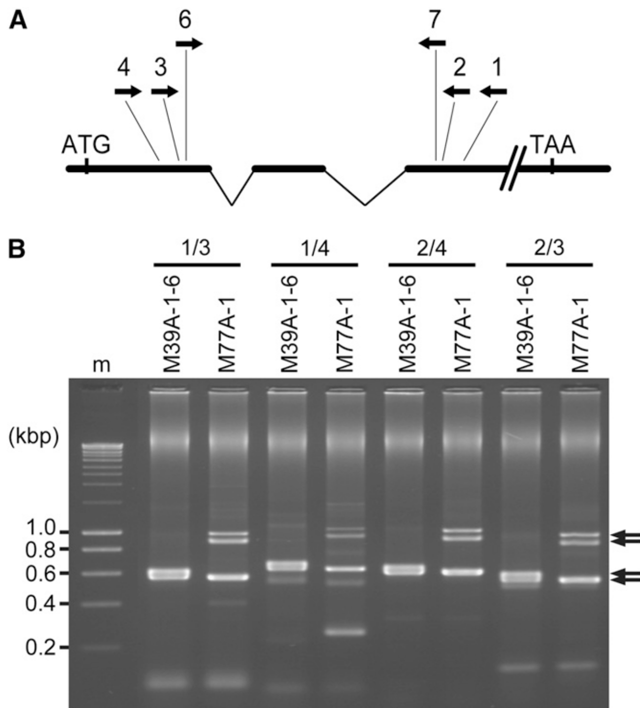

FIGURE 2.—PCR amplification of rye *ALMT1* gene partial genomic fragments. (A) Positions of primers used for PCR amplification of rye *ALMT1* gene fragments in the wheat *TaALMT1* gene sequence, relative to the first two introns and translation start/stop codons. (B) PCR amplifications from DNA of Al-tolerant parent M39A-1-6 and Al-intolerant parent M77A-1 using different primer pairs. Arrows indicate bands shown by cloning and sequencing to represent *ALMT1* homologs. The two uppermost bands represent alternative secondary structure variants of the *M77.1* gene fragment caused by the presence of the inverted-repeat MITE in this fragment (not shown). Sizes of molecular weight marker (m) bands are indicated.

wheat ESTs BF474257, BM134287, and BE499019, respectively, which had all been located to deletion 4DL12-0.71-1.00. *BCD1230/RPE* was also found to be homologous to ESTs (BE497160/BE405275) mapped to this same deletion bin. This deletion bin, representing the distal third of 4DL, also contains the Alt_{BH} locus (RAMAN *et al.* 2005). Hence, it was confirmed that *Alt4* and Alt_{BH} are located on related chromosome regions in the rye and wheat genomes.

A cluster of *ALMT1* genes at the rye *Alt4* locus: Although the corresponding interval in rice lacks an *ALMT1* homolog, the existence of an *ALMT1* gene in the vicinity of *Alt4* was expected from the relationship between the *Alt4* and Alt_{BH} chromosome regions and the fact that rye and wheat are close relatives. When primers from exons 1 and 3 of *TaALMT1* were used in PCRs from M39A-1-6 and M77A-1 DNA, similar patterns of multiple products were obtained using the different primer combinations (Figure 2, A and B). Gel purification and direct sequencing of the ∼0.27-kb band amplified from M77A-1 using the Alm-1/Alm-4 primers (Figure 2B) revealed it to be a sequence unrelated to *TaALMT1*. Cloning and sequencing of the ∼0.5-kb products revealed a number of *TaALMT1*-related sequences. Direct sequencing and sequencing of clones showed the doublet at ∼0.9 kb, amplified from M77A-1 (Figure 2B), to be comprised of a single *ALMT1* sequence. This fragment appeared as a doublet because of secondary structure (data not shown). The number of *ALMT1* genes identified in M39A-1-6 and M77A-1 was five (named *ScALMT1-M39.1* to *-M39.5*) and two (*ScALMT1-M77.1* and *-M77.2*), respectively. An alignment of the partial genomic sequences is shown in supplemental Figure 4. The gene represented by the ∼0.9-kb doublet, designated *ScALMT1-M77.1*, contained a ∼400-bp insertion in intron 2, which was unique among the *ScALMT1* sequences. This insertion contained a 227-bp miniature inverted repeat transposable element (MITE).

In PCRs on DNA bulks of homozygous tolerant or intolerant individuals from the mapping population, the ∼0.9-kb doublet was amplified from the intolerant bulk only (not shown), indicating that the *ScALMT1-M77.1* gene was linked to the *Alt4* locus. Likewise, use of a PCR primer pair specific for *ScALMT1-M39.4* (Alm-28 and Alm-29; supplemental Table 7; supplemental Figure 4), and digestion of Alm-6/Alm-7 genomic PCR product mixtures with a set of restriction enzymes that gave diagnostic restriction fragments for particular gene copies (supplemental Table 8), gave banding patterns from the tolerant and intolerant DNA bulks that were consistent with all seven *ScALMT1* sequences being closely linked to *Alt4* (not shown).

In Southern blots with a rye *ALMT1* cDNA probe and the restriction enzyme *Eco*RV, all restriction fragments detected in M39A-1-6 were distinguishable in size from those detected in M77A-1 (Figure 3). In bulks of homozygous tolerant or intolerant individuals from the mapping population, and in individuals homozygous for the recombinant chromosomes, one weakly hybridizing fragment derived from M39A-1-6 (arrow in Figure 3) appeared unlinked to the *Alt4* locus. Recombinants 859 and 1135, representing the closest recombination events to each side of *Alt4* (supplemental Figure 1), showed novel banding patterns as a result of recombination within the *ALMT1* gene cluster (see below). Otherwise, hybridization patterns in the homozygous recombinants were the same as those in M39A-1-6 or M77A-1 (Figure 3) and corresponded with the *Alt4* tolerance allele present on each of the recombinant chromosomes. In this way, the *ALMT1* gene cluster was located to the same 0.9-cM region as the *Alt4* locus (Figure 1). PCR amplifications and Southern analysis with the wheat–rye addition lines (supplemental Figure 5, A and B) confirmed the presence of an *ALMT1* gene cluster on 7RS of rye cv. Imperial and revealed no homologs on any other rye chromosomes. Hence, both Al-tolerant and Al-intolerant rye lines contain clusters of *ALMT1* gene homologs at the *Alt4* Al-tolerance locus.

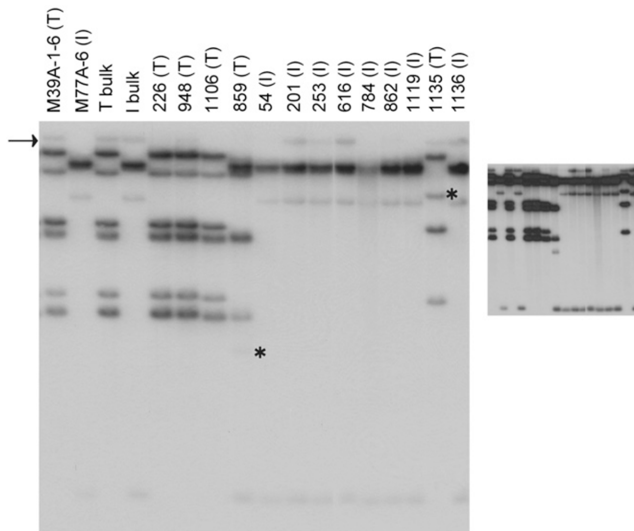

FIGURE 3.—Southern blot analysis of rye *ALMT1* homologs. Rye genomic DNAs cut with *Eco*RV were probed with a rye *ALMT1* cDNA. Included are the inbred mapping parents M39A-1-6 (tolerant) and M77A-1 (intolerant), bulks of homozygous tolerant or intolerant segregants, and lines homozygous for *Alt4*-region recombinant chromosomes. I, intolerant; T, tolerant. The arrow indicates a fragment not linked to *Alt4*. Asterisks indicate novel-sized restriction fragments in 859 and 1135. A longer exposure of the same blot (inset) more clearly illustrates weakly hybridizing fragments.

This contrasts with previous reports in which solitary *ALMT1* gene copies were described for the tolerance and intolerance alleles of the rye *Alt4* locus and wheat Alt_{BH} locus (SASAKI *et al.* 2004; FONTECHA *et al.* 2007).

***ScALMT1* expression patterns, full-length ORFs, and splice variants:** *ScALMT1* gene expression was monitored by Q-PCR. We also developed Q-PCR primers for rye genes encoding glyceraldehyde-3-phosphate dehydrogenase, elongation factor 1α, cyclophilin, and actin to provide controls for normalization (supplemental Table 9). Q-PCR from tissues of M39A-1-6 (tolerant) seedlings, 0, 6, and 24 hr after exposure to toxic levels (150 μM = 4 ppm) of Al revealed low, intermediate, and high expression in leaves, root midsections, and root tips, respectively, with expression in root tips and root midsections being upregulated upon exposure to Al (Figure 4A). Using a set of restriction enzymes that could distinguish potential RT–PCR products derived from any of the *ScALMT1* genes (supplemental Table 8), individual RT–PCR fragments cloned from leaf-24-hr, root-tip-0-hr, and root-tip-24-hr amplification product mixtures were assigned to particular M39A-1-6 gene copies to estimate the relative expression levels of each gene in each of the samples. *ScALMT1-M39.1* and *-M39.2* were the only transcripts detected in the root-tip-0-hr and root-tip-24-hr samples and were present in roughly equal proportions in both samples (Figure 4B), indicating that these genes were equally upregulated by Al exposure. RT–PCR product mixtures amplified from M77A-1 root tips were also subjected to cloning and restriction analysis. All 96 clones analyzed were derived from *ScALMT1-M77.1* transcripts, indicating that *M77.1* was the only gene expressed in root tips from the *M77A-1* haplotype.

In another experiment (Figure 4, C and D), seedlings that were homozygous for the *M39A-1-6* or *M77A-1 Alt4* locus haplotypes, or heterozygous, were selected with PCR markers from segregating families, and plants of the three genotypes were analyzed separately for *ScALMT1* gene expression in roots at 0, 6, and 24 hr after exposure to 150 μM Al. In M77A-1 root tips, total *ScALMT1* gene expression (representing the *M77.1* gene) was upregulated by Al exposure (Figure 4C). Samples from heterozygotes provided the opportunity to test the relative expression of the *ScALMT1* genes in a common (tolerant) background. In heterozygotes, total *ScALMT1* expression increased approximately sixfold after Al exposure (Figure 4C). By 24 hr, the proportions of *ScALMT1* transcripts that were derived from the *M39.1* and *M39.2* genes remained relatively unchanged, while the proportion derived from the *M77.1* gene appeared to decrease slightly (by 50%; Figure 4D), suggesting that the *M77.1* gene may undergo a lower fold increase in expression level following Al stress than the other two genes. In heterozygotes, transcript expression levels of *M39.2* appeared to be reduced relative to those of *M39.1* (to ∼50% of *M39.1* levels; Figure 4D). In both homozygotes and heterozygotes, transcript levels of *M77.1* appeared to be a half to a quarter of those of *M39.1* (Figure 4, C and D).

RACE–RT–PCR performed on M39A-1-6 root-tip-24-hr RNA using primers based on published *ALMT1* sequences from wheat (SASAKI *et al.* 2004) and rye (FONTECHA *et al.* 2007) yielded complete 3′-UTR sequence and partial 5′-UTR sequence for *ScALMT1-M39.1* and *-M39.2*. Primers Alm-23 and Alm-27 based on the UTR sequences were then used in RT–PCR amplifications of full open reading frames of the *ScALMT1-M39.1* and *-M39.2* and *-M77.1* genes. Comparison of the cereal *ALMT1* cDNA sequences (supplemental Figure 6) revealed identities of 98–99% between the rye sequences. All the rye sequences have the same 21-bp in-frame deletion relative to the wheat *TaALMT1* ORF and are otherwise ∼91% identical with the wheat sequence. The transcripts from each rye gene, including *M77.1*, all encode predicted full-length proteins with high levels of similarity to the published rye and wheat sequences (supplemental Figure 7). Hydrophobicity plots for the rye proteins were essentially identical to those of the TaALMT1 protein (not shown), which has seven hydrophobic domains, only six of which are membrane spanning (MOTODA *et al.* 2007). In the TaALMT1 topology model described by MOTODA *et al.* (2007), the seven-amino-acid region of TaALMT1 that is deleted in the rye proteins occurs entirely within the N-terminal extracellular tail, at the junction between the

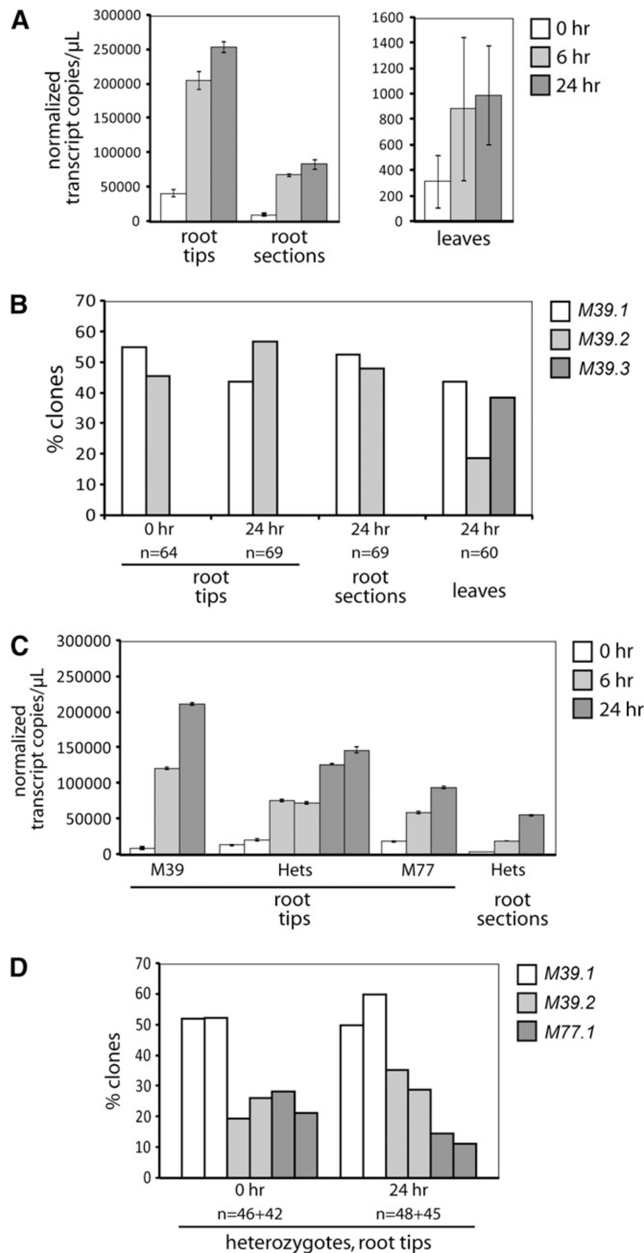

FIGURE 4.—Expression analyses of rye *ALMT1* homologs. A and B represent one experiment performed with M39A-1-6 plants only, while C and D represent another experiment performed using sib lines heterozygous for *Alt4* (two replicates) or homozygous for the *M39A-1-6* or *M77A-1 Alt4* haplotypes (one replicate). The end 3 mm of the root ("root tip"), 7-mm root sections taken from just below the seed ("root sections"), and the 7-mm section of coleoptiles plus rolled up leaf within taken from just above the seed (leaves) were sampled from seedlings at 0, 6, or 24 hr after exposure to toxic (150 μM) Al. Total *ScALMT1* gene expression was measured by Q-PCR with the primer pair Alm-6/Alm-10 that can potentially amplify from all *ScALMT1* copies (A and C). Alm-10 spans the intron-2 splice site and would not be expected to amplify *M77.1* splice variants lacking exon 2. Shown are means and standard deviations of normalized transcript copy numbers per microliter cDNA obtained from three replicate Q-PCR amplifications from the same cDNA. Q-PCR data from the different tissues were normalized independently (see MATERIALS AND METHODS). The proportions of different *ScALMT1* gene transcripts present in selected RNA samples were determined by cloning RT–PCR product mixtures and subjecting the indicated numbers of clone inserts to restriction analysis to identify from which gene each insert was derived (B and D).

first hydrophobic (extracellular) domain and the second hydrophilic domain.

Cloning and sequencing of the RT–PCR products revealed mRNA splice variants for the *ScALMT1-M39.2* and *-M77.1* genes. A quarter (2 of 8) of the clones obtained for *M39.2* were precisely missing exon 4 (113 bp), resulting in a predicted frameshift and truncated product (EU146238), which is the same as the full-length ScALMT1-M39.2 protein up until the 232nd amino acid (in boldface type and underlined in supplemental Figure 7) and ends in a novel 68-amino-acid C-terminal extension. Approximately half (15 of 24) of the clones obtained for *M77.1* were precisely missing exon 2 (150 bp) and would encode a predicted protein (EU146240) with an in-frame deletion of 50 amino acid residues (underlined in supplemental Figure 7). The deleted region begins in the second transmembrane domain and ends in the fourth transmembrane domain (third to fifth hydrophobic domains, respectively) of the full-length protein, as defined by the membrane topology model of MOTODA *et al.* (2007). The *M77.1* splice variant was visible as a smaller product in RT–PCR amplifications from M77A-1 root tip RNA (supplemental Figure 8). The proportions of correctly and incorrectly spliced *M77.1* transcripts in root tips of M77A-1 plants did not appear to change after Al exposure, judging from the results of RT–PCRs that detected both splice variants at once (not shown).

The 859 and 1135 recombinant lines: Lines containing the homozygous 859 and 1135 recombinant chromosomes showed Southern blot *ALMT1* banding patterns unlike either of the parents (Figure 3). To explore the possibility that these lines were derived by pollen or seed contamination, all the marker fragments listed in supplemental Table 1, except for *DOF, GPC, STP, KEL, GAB*, and *MATE*, were amplified from each of the two homozygous recombinants and sequenced. The results were entirely consistent with these lines having arisen from single recombination events between M39A-1-6 and M77A-1 chromosomes. In line 859, the sequences of all markers to the *B1* and *B4* sides of *Alt4* were identical to M39A-1-6 and M77A-1, respectively, while in the 1135 line the reverse was true. The sequenced fragments totaled over 10.9 kb and covered 219 sites of polymorphism between M39A-1-6 and M77A-1, making it very unlikely that the 859 and 1135 recombinant chromosomes were derived from any rye haplotypes other than *M39A-1-6* and *M77A-1*.

The location of the 859 and 1135 recombination events within the smallest (*B11-B26*) *Alt4* marker interval (supplemental Figure 1) suggested that these lines may have arisen from recombination between the *ALMT1*

gene clusters. Therefore, the nature of these lines was investigated further by using the same procedure as was applied to the parents to determine the number and sequence of *ALMT1* gene copies present in the lines and to identify the *ALMT1* full-length ORFs expressed in their root tips after exposure to Al. The results of these analyses were all completely consistent with the 859 and 1135 recombinants having arisen from recombination between the M39A-1-6 and M77A-1 *ALMT1* gene clusters, as represented in Figure 5. In this model, a similar pairing of the M39A-1-6 and M77A-1 *ALMT1* clusters at meiosis was followed by recombination at similar locations to give rise to the 859 and 1135 recombinant chromosomes representing almost perfectly reciprocal products (Figure 5). For line 1135, recombination occurred between the 5′ ends of the *M77.1* and *M39.2* ORFs to produce an expressed gene (*ScALMT1-1135.1*) with a hybrid ORF comprised of the 5′ end of *M77.1* and 3′ end of *M39.2*. The hybrid ORF differed from the *M39.2* sequence at only two base positions near the 5′ end, only one of which encoded an amino acid difference (supplemental Figures 6 and 7). Among the Alm-6/Alm-7 genomic PCR fragment sequences obtained from the 1135 line were ones identical to the *M39.2* sequence shown in supplemental Figure 4, which were presumably derived from the hybrid gene. The combined cDNA and genomic sequence information enabled the recombination point in the *1135.1* hybrid gene to be located between the 14th base position of the ORF and the 11th base position of intron 1. For line 859, recombination appeared to have occurred 5′ of the *M77.1* and *M39.2* ORFs. In addition to the *M39.1* transcript, this line expressed a transcript identical to the *M77.1* sequence shown in supplemental Figure 6, locating the 859 recombination point 5′ of the first known position that is polymorphic between *M77.1* and *M39.2* (base position 18 in the 5′-UTRs as defined by supplemental Figure 6). The single novel-sized *ScALMT1*-hybridizing restriction fragment identified in the 1135 line (asterisk in Figure 3) most likely represents the hybrid *1135.1* gene, while the presence of another novel fragment in the 859 line (asterisk in Figure 3) suggests that the 859 recombination event also occurred close to or within an *ALMT1* ORF.

The same aberrant *M77.1* transcript lacking exon 2 observed in the M77A-1 parent line was also observed for the *M77.1* gene inherited by the 859 recombinant chromosome and was also visible as a smaller product in RT–PCR amplifications from this line (supplemental Figure 8). Likewise, the aberrant *M39.2* splicing event leading to omission of exon 4, seen in the M39A-1-6 line, was also seen for the *1135.1* hybrid gene comprised mostly of *M39.2* sequences. Evidently, the amount of correctly spliced transcript from this hybrid gene was sufficient to provide tolerance to the tolerant 1135 line.

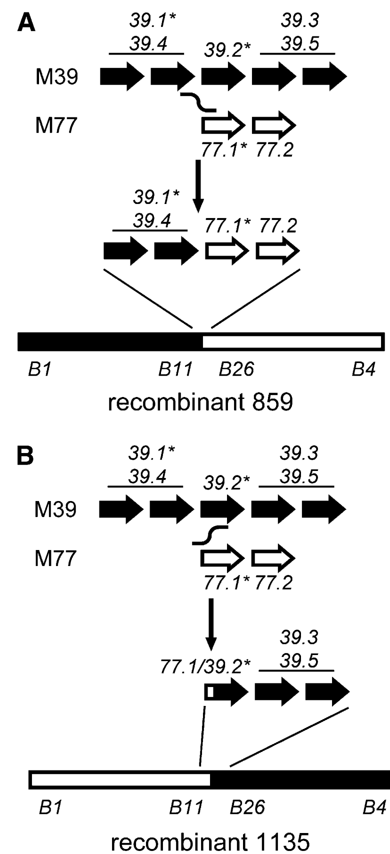

Figure 5.—Model for the origin of the (A) 859 and (B) 1135 recombinants. Positions and orientations of *ScALMT1* genes in the parent and recombinant haplotypes are represented by arrows. The relative orders of genes within the pairs *M39.1*/*M39.4* and *M39.3*/*M39.5* could not be inferred. The locations of the gene clusters within the overall structures of the recombinant chromosomes are shown. Black and white fill indicates genes and chromosome regions originating from the M39A-1-6 and M77A-1 parents, respectively. Asterisks mark genes transcribed in root tips. *M39.3* was also expressed in leaves.

Location of an *ALMT1* gene in barley: Fontecha *et al.* (2007) reported that barley contains a homolog of the wheat *TaALMT1* gene on chromosome 2H, whereas Wang *et al.* (2007) reported such a barley homolog only on chromosome 4H. To explore this possible discrepancy, we used the rye *ALMT1* cDNA probe in Southern blot analysis of Chinese Spring wheat lines containing individual chromosomes and chromosome arms of barley cv. Betzes. With four restriction enzymes, ≥1 hybridizing fragments were observed in Betzes barley, and the barley-specific fragments only appeared in the addition lines containing 2H or 2HL (not shown). A *Bam*HI RFLP was mapped in a Galleon × Haruna Nijo population of 112 doubled-haploids and cosegregated with the *ANP1* marker, near the centromere on 2HL (Figure 6). BLAST searches with nearby marker sequences established colinearity with the corresponding region on rice chromosome 4, showing that the *ALMT1* gene in barley was in a position corresponding to the closest homolog of *TaALMT1* in rice (TIGR locus

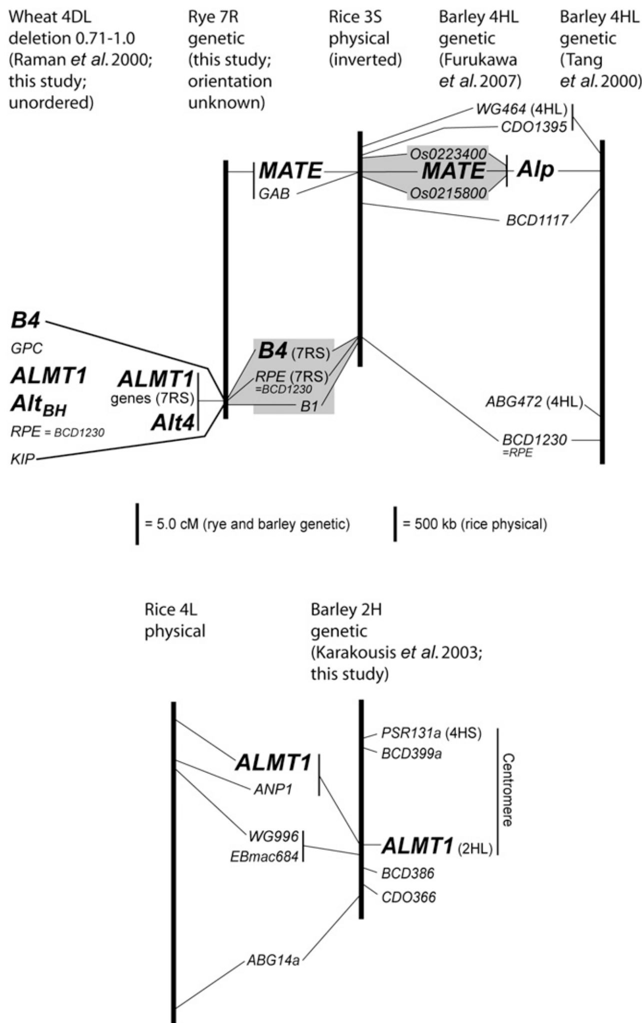

Figure 6.—Locations of genes and markers related to Al tolerance in rice, wheat, and barley. Lines connect markers and genes common to the illustrated chromosomes or chromosome arm segments. The orientation of the mapped section on rye chromosome 7R and the orders of genes and loci located to wheat 4DL deletion bin 0.71–1.0 relative to the centromeres are unknown. The rice 3S map is inverted to align with barley. Otherwise, all maps are oriented with the short arm toward the top. Shaded areas connect areas shown to have extensive colinearity (Furukawa *et al.* 2007; Wang *et al.* 2007; this study). The chromosome arm locations of markers are shown, where this has been determined using chromosome addition lines.

LOC_Os04g34010; Figure 6), which has been named *OsALMT5* (Delhaize *et al.* 2007).

DISCUSSION

***ALMT1* homologs as Al-tolerance genes in rye:** Solitary *ALMT1* genes were reported at the Alt_{BH} locus in wheat Al-tolerant or Al-intolerant lines (Sasaki *et al.* 2004; Raman *et al.* 2005). Similarly, single *ALMT1* genes at the rye *Alt4* locus of Al-intolerant (cv. Riodeva) and Al-intolerant (cv. Riodeva) genotypes were described (Fontecha *et al.* 2007). In contrast, we identified a cluster of *ALMT1* homologs at the *Alt4* locus of rye, in both the tolerant M39A-1-6 line (five genes) and the intolerant M77A-1 line (two genes). Therefore, different rye genotypes can contain different numbers of *ALMT1* homologs at the *Alt4* locus, ranging from perhaps one copy, up to at least five copies. The gene copy numbers in this study were determined using multiple primers on genomic and cDNA (including RACE–PCR) and sequencing of many clones, from both the parent lines and the 859 and 1135 recombinants. However, it is possible that these lines contained highly related (recently duplicated) copies that were identical in the amplified region. The latter possibility aside, we think it very unlikely that these lines contained additional *ALMT1* gene copies that went undetected by sequencing.

Fontecha *et al.* (2007) observed perfect cosegregation between a codominant marker for a rye *ALMT1* homolog and the dominant Al-tolerance trait encoded by the *Alt4* locus in a population of 381 F_2 plants. The current study tested this association to a higher level of genetic resolution. In the recombinants derived from a population of 1123 F_2 individuals, the *ALMT1* homologs mapped to the same 0.09-cM region as the *Alt4* locus. Furthermore, the 859 and 1135 recombinant chromosomes resulting from recombination within the *ALMT1* cluster carry reciprocal allele types for molecular markers flanking the *Alt4* locus yet both confer tolerance (Figure 5; supplemental Figure 1), essentially ruling out all genes that flank the cluster as candidates for encoding the *Alt4* tolerance. Consequently, these recombinants very strongly indicate that the *ALMT1* genes are responsible for controlling Al tolerance at the *Alt4* locus. Two of the genes from the tolerant *M39A-1-6* haplotype (*ScALMT1-M39.1* and *-M39.2*) showed mRNA expression in root tips (Figure 4, B and D) and encode full-length predicted proteins with high similarity to TaALMT1 (supplemental Figure 7). *M39.1* was inherited by the 859 recombinant chromosome, and a gene comprising most of the *M39.2* ORF and the active *M77.1* promoter was present in the 1135 recombinant chromosome (Figure 5). *M39.1* and *M39.2* therefore each appeared to be potentially active and sufficient to proved tolerance, offering an explanation as to why the two recombinants, carrying reciprocal flanking marker alleles and derived from recombination at nearly the same position within the *ALMT1* cluster (Figure 5), could both be tolerant.

In a group of 13 wheat accessions, *TaALMT1* mRNA expression levels showed a strong positive correlation with tolerance, suggesting that *ALMT1* gene expression levels may be an important determinant of tolerance/susceptibility (Raman *et al.* 2005). Wheat *TaALMT1* mRNA expression is not upregulated by Al, but the organic acid transporting activity of the TaALMT1 protein appears to be activated post-translationally by the presence of toxic levels of Al (Sasaki *et al.* 2004). In

rye, mRNA expression levels of the *M39.1* and *M39.2* genes increased in root tips in the first 6 hr after exposure to toxic levels of Al and increased again in the next 18 hr (Figure 4). Expression concentrated in the root tip, and Al-triggered transcriptional upregulation, were also reported for the previously described rye *ScALMT1-1* gene (Fontecha *et al.* 2007). The contrasting Al inducibility of the wheat and rye genes may be related to the different patterns of excretion of organic acids observed in wheat and rye. The Al-tolerant wheat cv. Atlas 66 exuded malate at high levels 2–10 hr after Al exposure, whereas malate and citrate exudation by rye cv. King increased at a steady state throughout this period, suggesting a different mode of regulation (Li *et al.* 2000). Therefore, rye may be slower to reach maximum rates of organic acid exudation following Al exposure because ScALMT1 proteins are present at low levels in the absence of Al and then increase upon exposure due to transcriptional upregulation, whereas tolerant wheat may be able to respond more quickly to Al because the Al-activated TaALMT1 transporter is expressed at a maximum level before exposure to Al.

In tolerant wheat and rye, Al-triggered excretion of organic acids occurs mainly in the first few millimeters of the root apex (Delhaize *et al.* 1993; Li *et al.* 2002), which is also the part of the roots that exhibit the most conspicuous Al-toxicity damage, as manifested by root-tip malformation and swelling, in intolerant genotypes (Delhaize *et al.* 2004; supplemental Figure 2). In wheat, *TaALMT1* expression was not detected in leaves and in roots was detected at severalfold higher levels in the apex than in the other parts (Sasaki *et al.* 2004). Rye *ALMT1* gene expression was found to be approximately threefold higher in root tips than in other parts of the roots, before and after exposure to Al, and was up to 130–250 times lower in leaves than in root tips (Figure 4, A and C). Therefore, the expression patterns of the rye *ALMT1* genes were consistent with a potential involvement in an Al-tolerance mechanism involving organic acid excretion from the root tips.

***ScALMT1* splice variants:** Around half of the transcripts produced by the *ScALMT1-M77.1* gene lacked exon 2 due to aberrant splicing, resulting in an in-frame 50-amino-acid deletion within the predicted product, which may or may not destroy the protein's activity. Results of the expression study using *Alt4* heterozygotes (Figure 4D) also suggested that the *M77.1* promoter may be less active and/or less Al inducible than the *M39.1* and *M39.2* promoters. If the interpretation of the 1135 recombinant (Figure 5) is correct, the only gene conferring Al tolerance in the 1135 line expresses a functional ORF comprising mostly *M39.2* coding sequence, driven by the *M77.1* promoter. No splice variants lacking exon 2 were detected for this hybrid gene, probably because the sequences causing this splice defect in *M77.1* were located 3′ of the recombination site and therefore were not inherited by the hybrid gene. Of the 18 F_4 individuals from the segregating 1135-1 family assessed for tolerance (supplemental Table 4) 9 contained the 1135 recombinant chromosome paired with the M77A-1 nonrecombinant chromosome and were fully tolerant, indicating that a single hemizygous copy of the hybrid gene driven by the *M77.1* promoter was sufficient for tolerance. Therefore, if a halving of the transcript abundance in the 1135 hemizygote did not affect tolerance, an effective halving of the amount of functional *M77.1* transcript as a result of the aberrant splicing would not appear to fully account for the failure of the *M77.1* gene to provide tolerance in homozygotes. Consequently, the M77.1 full-length protein encoded by the correctly spliced transcript may have lower activity than the M39.1, M39.2, and 1135.1 proteins. The full-length M77.1 protein has three amino acid substitutions relative to the M39.1, M39.2, and 1135.1 functional rye proteins (Figure 7; Cys^{125} to Tyr, Arg^{178} to Ile, Ala^{324} to Thr), which may potentially impair activity.

The aberrant *M77.1* transcript lacked exon 2, due to joining of the 5′ splice (donor) site of intron 1 to the 3′ splice (acceptor) site of intron 2. The sequence at the several bases surrounding splice sites is either essential or influential for splice-site choice in plants (Reddy 2007). However, *ScALMT1* genes showed no sequence variation around the splice sites of either intron 1 or 2 (supplemental Figure 4). The missplicing in *M77.1* could have been caused by the unique ∼400-bp insertion, including the 227-bp MITE in intron 2 of *M77.1* (supplemental Figure 4). This insertion may have interfered with the nucleophilic attack of the intron-2 5′ splice site by the branch point in intron 2, leading instead to occasional attack of the 5′ splice site of intron 1 by the intron-2 branch point. Various stresses are known to affect the relative frequencies of splice variants of particular genes (Reddy 2007). However, in RT–PCRs that detected both *M77.1* splice variants at once, the proportions of the two products appeared unchanged in root tips following exposure to Al stress (not shown). At the same point of the ∼400-bp insertion in *ScALMT1-M77.1*, the *-M39.5* and *-1* genes have 5-bp and 39-bp insertions unrelated in sequence to the insertion in *M77.1* (supplemental Figure 4), indicating that this may be a hot spot for sequence insertion and/or deletion. However, the functional significance of these other insertions is unknown, since no transcripts of any type were detected from the *M39.5* gene, and no alternative splicing was reported for *ScALMT1-1*.

Transcripts missing exon 4 were sometimes produced by the *M39.2* gene and by the hybrid *1135.1* gene comprised of *M39.2* sequence from the 5′ end of intron 1 onwards. The aberrant transcripts with the 113-bp deletion occurred at a lower frequency than the *M77.1* transcripts with the 150-bp deletion, based on the poor visibility of the smaller transcripts in RT–PCR amplifi-

cations from the M39A-1-6 and 1135 lines (supplemental Figure 8), and the smaller proportion of cDNA clones obtained for these variants (2/8 and 1/23 cDNA clones from *M39.2* and *1135.1*, respectively). Skipping of exon 4 causes a frameshift and severe truncation of the predicted product, most likely abolishing protein function. However, there was no evidence that this occasional splicing defect had an impact on Al tolerance, since the *1135.1* gene was shown to be sufficient to provide Al tolerance.

The rye *ALMT1* gene cluster: A characteristic of some clusters of tandemly repeated genes is the ability to align in a variable way during meiotic chromosome pairing (analogous to a slide rule), resulting in recombination products containing different numbers of repeats. This can result in spontaneous loss of the phenotypic trait when recombination generates a new haplotype containing only inactive gene copies. The maize *rp1-D* gene-cluster haplotype pairs with itself in a wide variety of ways (Sun *et al.* 2001), whereas evidence for only one type of pairing between the *Cf-4* and *Cf-9* haplotypes of the complex tomato *MW* locus was obtained (Wulff *et al.* 2004). The extent and distribution of homologies in intergenic regions are thought to be determinants of the recombination behaviors of different complex loci (Wulff *et al.* 2004). The 859 and 1135 recombinants resulted from the same alignment of the *M39A-1-6* and *M77A-1* haplotypes and recombination at a similar position, at or near the 5′ ends of the paired *M39.2* and *M77.1* genes (Figure 5), suggesting a bias in how *M39A-1-6* and *M77A-1* haplotypes recombine. However, further recombinants will need to be analyzed to determine if these haplotypes can recombine in other ways.

Recombination at *Alt4* can generate novel coding sequences, as demonstrated by the chimeric *ScALMT1-1135.1* gene. The patchwork pattern of sequence polymorphisms between *ScALMT1* genes also indicates a history of recombination between the ORFs. For example, the *ScALMT1-2* cDNA sequence is identical to *ScALMT1-M39.2* at four polymorphic base positions between the end of exon 1 and the start of exon 2, but then diverges from *ScALMT1-M39.2* and matches *ScALMT1-1* sequence for two polymorphic base positions before becoming different from *ScALMT1-1* again in exon 4 (supplemental Figure 6). Rye is an outcrossing, wind-pollinated species. Therefore, in heterogeneous rye populations, one would expect there to be frequent opportunities for recombination between different *Alt4* locus haplotypes with the potential to generate new variation in *ALMT1* copy number and coding sequence.

Differences in gene copy number and accompanying changes in expression levels are regarded as important determinants of trait variation, both within and between species (Dumas *et al.* 2007). The overall amount of *ScALMT1* mRNA produced by the two active *ScALMT1* copies from the *M39A-1-6* haplotype was threefold or more greater than that produced by the only gene transcribed from the *M77A-1* haplotype (Figure 4D). However, a reduced level of correctly spliced transcript produced by the *M77A-1* haplotype does not appear to account fully for its failure to provide Al tolerance, and the encoded M77.1 protein would appear to be at least partially defective. We also saw no evidence that the *859* and *1135* haplotypes, each carrying a single active *ScALMT1* gene copy, conferred any less Al tolerance than the parental *M39A-1-6* haplotype containing two active tolerance genes, even when the former were paired with the intolerant haplotype in *Alt4* heterozygotes. Further tests with higher levels of Al stress will need to be carried out to fully evaluate the effect of *ScALMT1* gene copy number on Al tolerance in rye.

Expression of gene *M39.3* was observed in the samples comprising part of the coleoptile and rolled-up leaf base taken from just above the seed ("leaves," Figure 4B), but was not detected in roots. It is possible that *M39.3* was expressed at similar low levels in the roots but was not detected there because high expression of *M39.1* and *M39.2* in roots prevented its detection. Expression of *M39.1* and *M39.2* was also detected in leaves. Metal homeostasis, Al tolerance in the apoplast, and many important biochemical processes in plants involve transport of organic acids such as malate, or their metal complexes, between subcellular compartments (Holtum *et al.* 2005; Kochian *et al.* 2005; De Angeli *et al.* 2007; Delhaize *et al.* 2007; Haydon and Cobbett 2007). Potentially, rye ALMT1 proteins, or other members of the ALMT protein family (Delhaize *et al.* 2007), could be involved in these processes in root tips and other tissues.

Relationships between cereal Al-tolerance genes: We showed that the *Alt4* Al-tolerance locus identified with the M39A-1-6 × M77A-1 cross is located on the short arm of rye chromosome 7R as it is defined on the RFLP map of Devos *et al.* (1993) (supplemental Figure 3). This agrees with the claim by Benito *et al.* (2005) that this locus was on 7RS, but disagrees with the chromosome 4R location originally determined by Miftahudin *et al.* (2002). The 4R location was determined by RFLP analysis using a probe cloned from a single-linked AFLP band. However, the AFLP band was reported to be comprised of a mixture of at least two different sequences, only one of which was low copy and could be used for mapping (Miftahudin *et al.* 2002). Hence, an incorrect chromosome assignment may have been obtained by mapping with the wrong cloned fragment. An Al-tolerance locus mapped using rye Ailés × Riodeva crosses was also located to 7RS (Matos *et al.* 2005). The common 7RS location of the loci detected in the different crosses led Benito *et al.* (2005) to propose that these rye loci were the same. Their equivalence is now confirmed by the finding that *ALMT1* genes reside at both loci. The locus identified using the M39A-1-6 × M77A-1 cross was originally named *Alt3*. However, in light of the current

findings, we suggest that the 7RS locus be referred to as *Alt4*, and that the name *Alt3* be reserved for the potential Al-tolerance gene Aniol and Gustafson (1984) identified on 4R by chromosome addition line analysis.

Both the rye *Alt4* locus and the Alt_{BH} Al-tolerance locus on wheat 4DL had been mapped close to RFLP loci detected with the probe *BCD1230* (*RPE*, Figure 1; Miftahudin *et al.* 2002). In the current study, analysis of additional markers confirmed that these loci were on related chromosome segments (Figure 6). The relationship between wheat 4L and rye 7RS is consistent with the known relationships between the wheat and rye genomes, including discontinuities in colinearity caused by chromosome translocations that occurred since the divergence of wheat and rye from a common ancestor. Relationships between rye 4R and wheat group 4 chromosomes are limited to the short arms, whereas relationships to wheat 4L are found only on rye 7RS and 5RL (Devos *et al.* 1993). Consistent with the occurrence of the wheat Alt_{BH} and rye *Alt4* loci on related chromosome segments, we demonstrated that tolerance at *Alt4* is controlled by genes homologous to the Alt_{BH} tolerance gene *TaALMT1*.

The barley chromosome 4H region containing the *Alp* Al-tolerance locus is also related to the rye *Alt4* and wheat Alt_{BH} regions (Tang *et al.* 2000; Figure 6). However, the *HvMATE* gene, which is completely unrelated to *TaALMT1*, controls Al tolerance at *Alp* (Furukawa *et al.* 2007; Wang *et al.* 2007). The rye ortholog of *HvMATE* was mapped 27.5 cM from the *Alt4* locus and recombined with *Alt4* tolerance in most of the rye families characterized for Al tolerance (Figures 1 and 6; supplemental Tables 3–5), clearly ruling out the rye *MATE* gene as a candidate for controlling Al tolerance at *Alt4*. Therefore, while rye contains an ortholog of the barley *HvMATE* gene close to the *Alt4* locus, there is no evidence that it contributes to Al tolerance in rye, at least in the lines investigated.

We identified a barley homolog of *TaALMT1* only on chromosome 2H, as did Wang *et al.* (2007). In contrast, Fontecha *et al.* (2007) reported identifying a barley *ALMT1* homolog only on chromosome 4H using the same Chinese Spring–Betzes barley chromosome addition lines that we used initially to locate a copy to 2H. While a 4H location is expected from the overall colinearity between group 4 chromosome regions carrying Al-tolerance loci in wheat, rye, and barley, the locus position we determined on 2H is the one expected from colinearity with rice (Figure 6). The chromosome 4H position was determined by PCR (Fontecha *et al.* 2007) and may require verification. The locations of *ALMT1* genes on rye 7RS and wheat 4DL do not fit with the overall colinearity with rice (Figure 6). Within the Triticeae, wheat and rye diverged from a common ancestor more recently than wheat/rye diverged from barley (Huang *et al.* 2002). Therefore, one interpretation would be that *ALMT1* on barley 2H represents the original location and that *ALMT1* moved to the chromosome group 4 position in the wheat/rye lineage after the divergence from the barley lineage. No Al-tolerance loci have been reported on barley chromosome 2H. Furthermore, Al-tolerance QTL have been reported on rice chromosome 4 (Nguyen *et al.* 2002; Xue *et al.* 2006), but not in the vicinity of the *TaALMT1* homolog (data not shown). Therefore, of the two *ALMT1* positions detected in cereals, the one represented by the wheat/rye copies appears to be the only one associated with an ability to confer Al tolerance.

In contrast to the *ALMT1* genes, the *MATE* genes were located in colinear positions in barley, rye, and rice (Figure 6). Al-tolerance QTL have been identified on rice 3S (Wu *et al.* 2000; Nguyen *et al.* 2003) in a region containing the closest rice homolog of the barley *HvMATE* citrate transporter gene. Al tolerance from the wild rice species *Oryza rufipogon* was most closely associated with markers *CDO1395* and *RG391* located only 0.5 cM apart, and the nearest flanking marker on the upper side (lower side in Figure 6) was located 11.0 cM away (Nguyen *et al.* 2003). The genetic distance between the *MATE* gene and the position corresponding to *Alt4* in rice (Figure 6) is 12.3–13.2 cM (http://rgp.dna.affrc.go.jp/cgi-bin/statusdb/irgsp-status.cgi). Hence, the position of this rice QTL corresponds well with the *MATE* gene. Rice roots excrete citrate in response to Al stress (Chen *et al.* 2006), which also fits with the known citrate transporting capability of the homolog in barley. Therefore, the *MATE* gene could be considered as a good candidate for the gene underlying the rice 3S QTL effect.

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