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High-resolution mapping of the *Alp* locus and identification of a candidate gene *HvMATE* controlling aluminium tolerance in barley (*Hordeum vulgare* L.)

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Abstract Aluminium (Al) tolerance in barley is conditioned by the *Alp* locus on the long arm of chromosome 4H, which is associated with Al-activated release of citrate from roots. We developed a high-resolution map of the *Alp* locus using 132 doubled haploid (DH) lines from a cross between Dayton (Al-tolerant) and Zhepi 2 (Al-sensitive) and 2,070 F_2 individuals from a cross between Dayton and Gairdner (Al-sensitive). The Al-activated efflux of citrate from the root apices of Al-tolerant Dayton was 10-fold greater than from the Al-sensitive parents Zhepi 2 and Gairdner. A suite of markers (ABG715, Bmag353, GBM1071, GWM165, HvMATE and HvGABP) exhibited complete linkage with the *Alp* locus in the DH population accounting 72% of the variation for Al tolerance evaluated as relative root elongation. These markers were used to map this genomic region in the Dayton/Gairdner population in more detail. Flanking markers HvGABP and ABG715 delineated the *Alp* locus to a 0.2 cM interval. Since the HvMATE marker was not polymorphic in the Dayton/Gairdner population we instead investigated the expression of the *HvMATE* gene. Relative expression of the *HvMATE* gene was 30-fold greater in Dayton than Gardiner. Furthermore, *HvMATE* expression in the $F_{2:3}$ families tested, including all the informative recombinant lines identified between HvGABP and ABG715 was significantly correlated with Al tolerance and Al-activated citrate efflux. These results identify *HvMATE*, a gene encoding a multidrug and toxic compound extrusion protein, as a candidate controlling Al tolerance in barley.

Keywords Aluminium tolerance · Barley · Citrate efflux · Comparative mapping · *MATE* · Molecular markers

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Introduction

Aluminium (Al) toxicity is a major factor limiting crop production on acidic soils, which include about 40% of the arable land in the world (Kochian 1995). At low pH (<5), dissolution of Al-containing compounds is enhanced and the release of toxic Al cations (particularly Al^{3+}) into soil solution can rapidly inhibit root growth. Barley (*Hordeum vulgare* L. $2n = 2x = 14$) is one of the most Al-sensitive members of the *Poaceae* family (Mugwira et al. 1976). Most Al sensitive cultivars either die within a few weeks after germination on acidic soils or yield very poorly. Nevertheless, significant genetic variation for Al tolerance exists in the barley germplasm (Minella and Sorrells 1992; Reid et al. 1969) and this has been exploited in breeding programs to develop high-yielding varieties with greater tolerance to Al toxicity (Read and Oram 1995). The mechanism

of tolerance, at least in some genotypes of barley, appears to rely on the Al-activated release of citrate from the roots (Ma et al. 2004). These citrate anions are thought to protect plants from Al toxicity in the same way that malate efflux protects wheat: by binding to the harmful Al^{3+} cations in the apoplasm and detoxifying them (Delhaize and Ryan 1995).

Dayton is one of the most Al-tolerant barley genotypes (Minella and Sorrells 1992) and a single locus (*Alp*) on the long arm of the chromosome 4H (4HL) conditions Al tolerance in populations derived from Dayton/Harlan Hybrid and Dayton/F6ant28B48-16 (Raman et al. 2003; Reid 1970; Tang et al. 2000). The same locus conditions Al tolerance in other populations including those generated from Yambla/WB229, Mimosa/WB229 (Raman et al. 2002), Harrington/Brindabella (Raman et al. 2001), Murasakimochi/Morex (Ma et al. 2004), Ohichi/F6ant28B48-16 (Raman et al. 2005a) and F6ant28B48-16/Honen (Wang et al. 2006). Minor gene effects for Al tolerance have also been suggested (Echart et al. 2002; Raman et al. 2005a; Reid 1970).

The SSR markers currently being used for marker-assisted selection (MAS) of Al tolerance in Australian barley breeding programs map at least 2.1 cM from *Alp* (Langridge and Barr 2003; Raman et al. 2003). Considering an average value of 4.4 Mb/cM (megabase/centimorgan ratios) calculated for the barley genome (Künzel et al. 2000), a genetic distance of 2.1 cM corresponds to a physical distance of 9.24 Mb. These markers are not 'diagnostic' for predicting Al tolerance and are of limited value for map-based cloning of the *Alp* gene. Furthermore, all the mapping studies conducted so far employed relatively small populations comprising 48–100 individuals (Raman et al. 2003; Tang et al. 2000). Therefore, a high-resolution map of the *Alp* genomic region is needed both for improving the efficiency of MAS and to identify candidate genes that encode for Al tolerance.

Comparative mapping studies using RFLP markers have revealed extensive synteny or co-linearity among the genomes of rice, wheat, barley, rye, oat, maize, and sorghum (Devos and Gale 2000). Wheat chromosome 4D, barley chromosome 4H, rye chromosome 4R and rice chromosome 3 possess regions syntenic with one another (Miftahudin et al. 2004; Namuth et al. 1994; Van Deynze et al. 1995) and all harbour loci for Al tolerance (Fontecha et al. 2007; Luo and Dvorak 1996; Miftahudin et al. 2004; Nguyen et al. 2003; Raman et al. 2005b; Riede and Anderson 1996). Of these loci only the Al-tolerance gene from wheat, *TaALMT1*, has been isolated to date (Sasaki et al. 2004). *TaALMT1* encodes an Al-activated transporter of malate and Magalhaes (2006) suggested that the orthologous loci in the other species also possess *TaALMT*-like genes. The synteny between the *Alp* locus in barley and other cereals can be assessed with markers from those species and the information can be used to develop new markers for high-resolution mapping.

In the present study, we exploited the synteny between barley, wheat, rye and rice to map the *Alp* genomic region in barley. From a high-resolution map of the *Alp* region constructed with recombinant lines selected from a large F_2 population we identified a candidate gene designated as *HvMATE* controlling Al tolerance in barley.

Materials and methods

Genetic materials

A doubled haploid (DH) barley population consisting of 132 lines was constructed from a single F_1 derived from a cross between Dayton (Al-tolerant) and Zhepi 2 (Al-sensitive) using pollen culture and was used as a low-resolution mapping population. For high-resolution mapping, 236 recombinants were selected from 2070 F_2 individuals derived from the cross between Dayton (Al-tolerant) and Gairdner (Al-sensitive) using Bmag490 and Bmac310 markers flanking the *Alp* locus. The F_2 recombinants were raised in the glasshouse and the F_2 derived F_3 seeds were subsequently used for Al tolerance testing.

Assessment of Al tolerance

DH lines and their parents were assessed for Al tolerance using a nutrient solution culture method. The composition of the nutrient solution is described by Raman et al. (2002). A spatial complete block design with two replicates was employed. Each replicate consisted of two aerated 40 L tubs containing circulated nutrient solution. The treatment tub contained 15 μM Al as $AlK(SO_4)_2$ (+Al) while Al was omitted from the control tub (−Al). The tubs were divided into replicate blocks of lines, each within an incomplete 9×16 array. Seedlings were allocated to positions within blocks using DiGGer (Coombes 2002) to give an efficient spatial design allowing for row and column effects and positive correlations between locations within a tub.

Sixteen seeds of uniform size from each of the parents and 132 DH lines were surface sterilized with 1% NaClO solution for 10 min and thoroughly rinsed. After 3 days of germination (2 days on moist filter paper in petri dishes in a refrigerator at 4°C and 1 day in nutrient solution at 25°C) and 1 day pre-growth in a 16/8 h (day/night) photoperiod, eight seedlings of each line were selected and the initial lengths of the longest roots were measured. Seedlings were then floated in the control and treatment solutions in polyurethane foam strips and the length of the longest root on each seedling were measured after a further 48 h. The predicted mean root elongation in control (RE − Al) and treatment (RE + Al) solutions were spatially analysed in Genstat using the restricted maximum likelihood method.

The relative root elongation (RRE) was calculated as RRE (%) = (RE + Al)/(RE − Al) × 100. Roots of the DH lines of the Al-treated seedlings were stained with 0.1% erichrome cyanine R (ECR) after 3 days under Al stress (15 μM) and scored sensitive or tolerant as previously described (Wang et al. 2006).

For high-resolution mapping, 28 F$_3$ individuals per F$_2$ recombinant (F$_{2:3}$) were assessed for Al tolerance using the same protocol (Wang et al. 2006). Parental lines were included as checks to ensure correct scoring.

Estimates of Al tolerance for the data in Fig. 6 were measured under different conditions. Seeds were germinated on moist filter paper for 2 days. The lengths of the longest root were recorded before the seedlings were divided between two 20 L tubs holding aerated nutrient solution containing (μM) 500 KNO$_3$, 500 CaCl$_2$, 500 NH$_4$NO$_3$, 150 MgSO$_4$, 2 KH$_2$PO$_4$, 2 FeCl$_3$, 11 H$_3$BO$_3$, 2 MnCl$_2$, 0.4 ZnCl$_2$, 0.2 CuCl$_2$ (pH 4.5). One tub contained no added Al and the other tub contained 5 μM AlCl$_3$. Net root elongation was measured after 4 days and RRE was calculated as described above.

Molecular marker analysis

Genomic DNA was isolated from young leaves of the Dayton/Zhepi 2 DH lines, from F$_2$ individuals of the Dayton/Gairdner population and from bulk leaf samples of the F$_{2:3}$ recombinant families. A framework map of the DH population constructed using 531 DArT, SSR and STS markers

(Wenzl et al. 2006) were employed for mapping Al tolerance. The *Alp* locus was further saturated with markers (Table 1) previously mapped near the Al tolerance loci from chromosome 4H of barley (Künzel et al. 2000; Raman et al. 2002), from chromosome 4D of wheat (Raman et al. 2005b), and from chromosome 4R of rye (Miftahudin et al. 2004). Molecular analyses were performed following the recommended PCR profiles. Primers generating amplicons below 400 bp were tailed with the M13 sequence (Schuelke 2000). Capillary electrophoresis, data collection and analysis were performed according to Raman et al. (2005c). Primers generating amplicons over 400 bp were used as standard oligos (untailed) and PCR products were separated on 2% (w/v) agarose gels. Monomorphic amplicons generated using primer pairs of ABG715, MWG0948, MWG2134, MWG2268, and B1 markers were sequenced using the Applied Biosystems BigDye^{TM} Terminator method on AB 3730XL capillary sequencer (Australian Genome Research Facility, Brisbane, Australia) to detect the presence of single nucleotide polymorphisms (SNPs). Sequences were compared and analysed for restriction enzyme sites using BioEdit software (http://www.mbio.ncsu.edu). The digested products were resolved on 2% (w/v) agarose gels.

Development of markers using the synteny between the barley and rice genomes

To identify the genomic region in rice syntenic with barley *Alp*, the sequence of the co-segregating marker ABG715

Table 1 A summary of markers used for mapping of the *Alp* locus

	Marker type	Marker name	Reference	M13 tail
Barley	SSR	Bmac181, Bmac186, Bmac310,	Liu et al. (1996)	Tailed
		Bmag353, Bmag375, Bmag490, Bmag740, Bmag808, EBmac635, EBmac701, HVM3, HVM67, HVM68, HVRCABG, HVM77, HVM13	Ramsay et al. (2000)	
	EST-SSR	GBM1003, GBM1028, GBM1044, GBM1067, GBM1071, GBM 1020	Thiel et al. (2003) kindly supplied by Drs. Andreas Graner and Martin Ganal (IPK)	Tailed
	STS	MWG542, MWG793, MWG880,	Künzel et al. (2000)	Tailed
		MWG939, MWG0948, MWG2036, MWG2110, MWG2134, MWG2163, MWG2224, MWG2268		
		ABG715, ABG484, MWG2135		Un-tailed
Wheat	SSR	GWM165, GWM194, GWM 251,	Röder et al. 1998	Tailed
		GWM497, GWM538, GWM608, GWM609, GWM 624, CFD23, CFD89, BARC98, BARC225, WMC48, WMC331, WMC399	http://www.wheat.pw.usda.gov	
	CAPS	ALMT1	Sasaki et al. 2004	Un-tailed
Rye	STS	B1, B4	Miftahudin et al. 2004	Un-tailed

identified from low-resolution map (see Results) was queried in BLASTn searches against the rice non-redundant database in NCBI (http://www.ncbi.nlm.nih.gov/). ABG715 identified the rice BAC clone OJ1193C08 and the sequences on this clone and two adjacent BAC clones OSJNBb0085F02 and OSJNBa0090D11 (http://www.tigr.org/tigr-scripts/e2k1/irgsp_orderedBAC.spl?db=osa1&chr=3) were used as queries in further BLASTn searches of the barley EST database in NCBI. Barley EST sequences identified this way were used to design EST-specific primers using primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The presence of repeats of dinucleotide tri-, tetra-, penta- and hexanucleotide motifs were searched and targeted for primer design as described previously (Raman et al. 2006).

Low-resolution mapping of the *Alp* locus

All the marker data from the DH mapping population were analysed to test the goodness of fit using Chi-square tests ($p = 0.05$). These tests were also applied to determine the goodness of fit of segregation patterns to Mendelian ratios for phenotypes scored with ECR staining. All the marker and phenotypic data were imported into Map Manager QTX version b20 (Manly et al. 2001) and used for linkage analysis using the Kosambi (1944) mapping function. Measurements on RE-Al, RE + Al and RRE were used for QTL analysis with Map Manager using the framework map of the DH population from Dayton/Zhepi2 (Wenzl et al. 2006). "Suggestive", "significant", and "highly significant" critical likelihood ratio statistics (LRS) were established by 1,000 permutation tests at $p = 0.05$ (Churchill and Doerge 1994) and used to declare QTLs.

High-resolution mapping of the *Alp* locus

Two markers (Bmag490 and Bmac310) flanking the *Alp* locus in the DH population from Dayton/Zhepi2 were used to select 236 recombinants from the 2070 F_2 individuals derived from a Dayton/Gairdner. The recombinant lines were selfed and their $F_{2:3}$ progeny were assessed for Al tolerance using ECR staining. Any markers co-segregating with *Alp* in the low-resolution DH population that were polymorphic in the Dayton/Gairdner population were used to construct a high-resolution map among the recombinants. The genotypic data were also integrated into the Map Manager and map distance was calculated using Kosambi (1944) function.

Citrate efflux

Seeds were germinated on moist filter paper for 2 days and transferred to the same nutrient solution described above

for Fig. 6. After 4 days growth under laboratory lighting three replicates of ten root apices (3–4 mm) were excised from six to eight plants per line. The tissue was transferred to 5 ml sterilised glass vials containing 1 mL 0.2 mM $CaCl_2$, pH 4.3). The tubes were sealed with Parafilm^{TM} and placed on a shaker ($\sim$60 rpm) for 40–60 min. The vials were removed from the shaker, the tissue rinsed twice with 1 mL of the same $CaCl_2$ solution and then 1 mL of treatment solution (25 μM $AlCl_3$ and 0.2 mM $CaCl_2$, pH 4.3) was added to each vial and returned to the shaker for 4 h. The solution was collected, dried down in a rotary evaporator and made up with 100 μL assay solution. The assay solution was prepared by mixing 1 mL H_2O, 120 μL 1 M TrisCl (pH 8.0), 10 μL NADH solution (prepared by dissolving 20 mg NADH and 20 mg $NaHCO_3$ in 2 mL H_2O), 1 μL malate dehydrogenase (10 mg/mL, Sigma-Aldrich), 1 μL lactate dehydrogenase (5 mg/mL, Sigma-Aldrich) and 12 μL 0.1 M sodium isocitrate. The dissolved sample was transferred to a micro-cuvette and a stable absorbance at 340 nm was obtained before and after adding 2 μL citrate lyase ($\sim$0.2 units, Sigma-Aldrich). The disappearance of NADH is proportional to the citrate present and the initial citrate content in each sample was calculated from a standard curve.

Quantitative RT-PCR

HvMATE expression in root apices was assayed by quantitative real-time reverse transcriptase PCR (qRT-PCR) using procedures described previously (Delhaize et al. 2004; Raman et al. 2005b) except that the cDNA preparations were not passed through a spin-column but were diluted 100-fold and used directly in the assay. The forward primer was 5′-CGTTCGTGTTCGACGGCATC-3′ and the reverse primer was 5′-AGCTGATCCATCACTTCCGG-3′. Expression was quantified relative to the expression of the barley glyceraldehyde-3-phosphate dehydrogenase gene (Burton et al. 2004) using the forward primer 5′-GTGAGGCTGGTGCTGATTACG-3′ and reverse primer 5′-TGGTGCAGCTAGCATTTGAGAC-3′.

Results

Inheritance of Al tolerance

Aluminium tolerance was assessed in the 132 DH lines derived from Dayton/Zhepi2 using the ECR staining method and measurements of root growth. The roots of the Al-tolerant parent Dayton were unstained after Al treatment and had normal lateral roots, whereas roots of the Al-sensitive parent Zhepi2 were stained purple with stained nubs along the seminal roots where lateral roots would normally
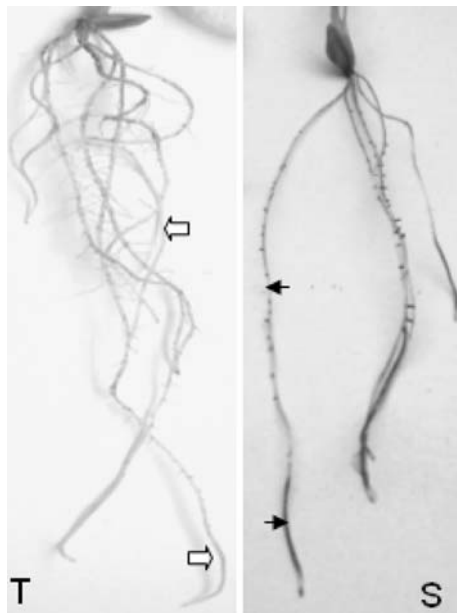
Fig. 1 Level of Al tolerance as indicated by eriochrome cyanine R staining of roots after 3 days in 15 μM Al. (*T* tolerant plant; *S* sensitive plant; *open right arrow* Unstained root tips; *open left arrow* Lateral roots developed; *right arrow* Root tips heavily stained; *left arrow* heavily stained nubs along the seminal roots)

emerge (Fig. 1). Of the 132 DH lines tested, 64 were scored as Al-tolerant and 68 were scored as Al-sensitive which fits a 1:1, monogenic segregation ratio ($\chi^2 = 0.12$; $P = 0.73$). Quantitative measurement of RE + Al and RRE showed bimodal distributions consistent with the segregation pattern for a major gene (Fig. 2). Significant correlations were observed between RE + Al and RRE ($r = 0.83$), RE + Al and root staining ($r = 0.82$), and RRE and root staining ($r = 0.89$).

Parental polymorphism survey

Barley 4H markers

Several SSR markers tested were found to be polymorphic (13/16) between Dayton and Zhepi2. Among the STS markers (Table 1), only MWG2135, displayed a fragment length polymorphism. The amplicons of ABG715, MWG0948, MWG2134, and MWG2268 were monomorphic but subsequent sequencing revealed SNPs between Dayton and Zhepi 2 (Table 2) for all these except MWG2268, which allowed cleaved amplified polymorphic (CAPS) markers to be developed (Fig. 3).

Wheat 4D markers

Among 15 wheat SSR markers tested (Table 1), only GWM165 and GWM608 amplified fragments in the

parental lines of the DH population. Marker GWM165 was polymorphic between the parents and amplified a fragment of 238 bp in Zhepi 2 and 260 bp in Dayton. Marker GWM608 generated a 128 bp fragment, which was not polymorphic between the parents. The CAPS marker specific to *TaALMT1*, the gene conditioning Al tolerance in wheat (Sasaki et al. 2004), was not polymorphic in parental lines.

Rye 4R markers

The STS markers B1 and B4 linked to an Al tolerance locus in rye, *Alt3* (Miftahudin et al. 2004), were tested for their linkage with the *Alp* locus in barley. The B1 marker amplified an expected fragment of approximately 500 bp in both the parental lines. The amplicons were sequenced and showed a 100% match with a barley sucrose transporter 1 gene (*SUT1*; AJ272309) but no SNPs were detected between Dayton and Zhepi 2. Marker B4 generated four bands in Zhepi 2 and two bands in Dayton between 222–676 bp allowing B4 to be used for molecular mapping.

Markers developed from the syntenic region of rice

The genomic region of *Alp* of barley was syntenic to three rice BAC clones OSJNBb0085F02, OJ1193C08 and OSJNBa0090D11 assembled on chromosome 3. Genes annotated on these BACs were used to identify potential orthologs in barley. Fourteen primer pairs were designed from the barley ESTs identified and used to generate markers. Some of these markers as well as the alignment of other barley markers to rice BAC clones are shown in Table 3. Two markers generated in this way, HvGABP and HvMATE were polymorphic in the DH population. HvGABP was also polymorphic for the Dayton/Gairdner population as a CAPS marker (Fig. 4) but the sequences of HvMATE amplicons were not polymorphic between the Dayton and Gairdner parental lines.

Low-resolution mapping of the *Alp* locus

A Chi-square test revealed that most of the polymorphic markers showed a 1:1 segregation ratio in the DH population except for HVM67, EBmac701, and B4 markers (results not shown). The six markers ABG715, GBM1071, GWM165, Bmag353, HvGABP and HvMATE showed complete linkage with the *Alp* locus (Table 4; Fig. 5a). Interestingly, marker B4, which is tightly linked to the *Alt3* locus (4R) in rye (Miftahudin et al. 2004), did not map to 4H in barley but to chromosome 2H. The locus was flanked with DArT marker bPB-2219 and Bmac93 that mapped within 1.1 cM (see linkage map of Dayton/Zhepi 2. Addi-

Fig. 2 Frequency distribution for **a** control root elongation (RE − Al, mm); **b** root elongation under Al stress (RE + Al, mm); **c** relative root elongation (RRE, %). RE-Al, RE + Al and RRE of sensitive parent 'Zhepi 2' was 41.18 mm, 14.98 mm and 36.38% respectively; whereas the tolerant parent Dayton was 38.11 mm, 36.06 mm and 94.62% respectively
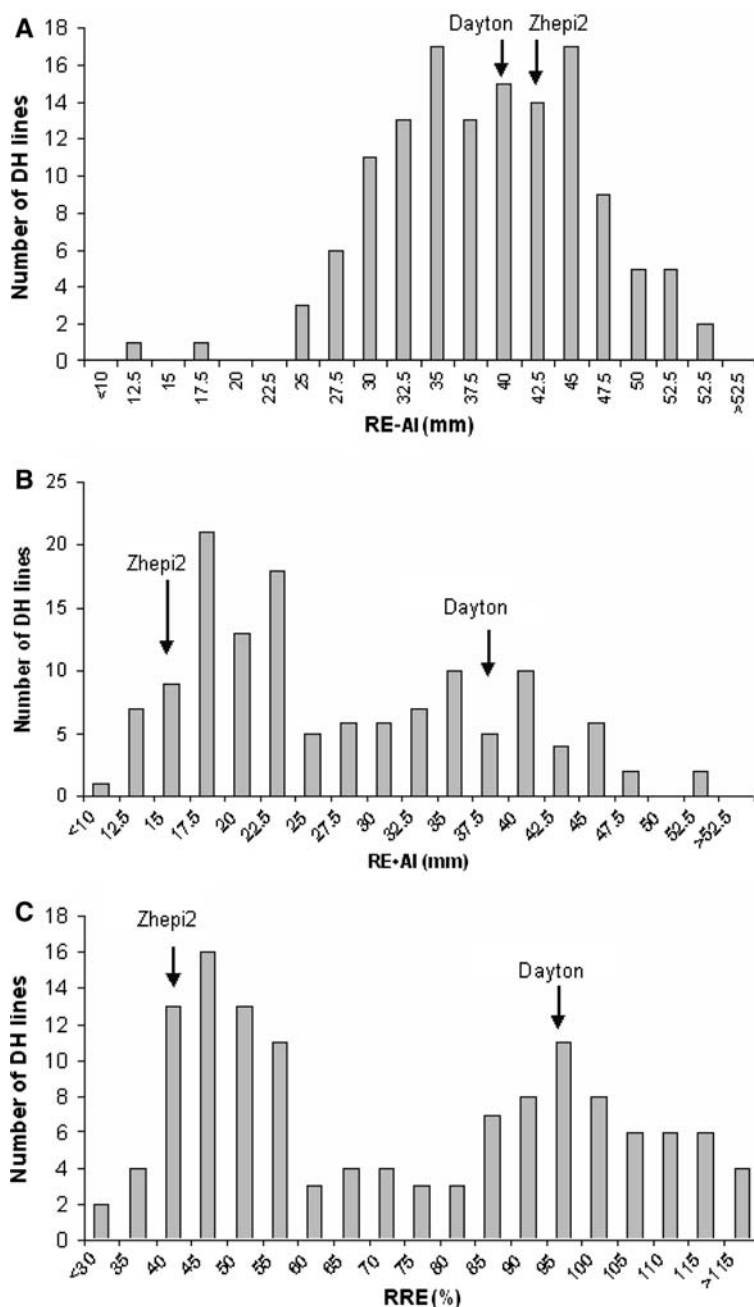
tional file 1, http://www.biomedcentral.com/1471-2164/7/206/additional/, Wenzl et al. 2006).

QTL analysis with the Dayton/Zhepi 2 DH population revealed that a single genomic region on 4HL encompassing the *Alp* locus was significantly associated with RE + Al (LRS score: 119.9) and RRE (LRS score: 170.4). This region was flanked by markers Bmag490 and HVM68 and accounted for 59% of the genotypic variation in RE + Al and 72% of the variation in RRE (Fig. 5a). Dayton contributed favourable alleles both for both RE + Al and RRE as expected.

High-resolution mapping of the *Alp* locus

Among the 2070 F_2 plants derived from Dayton/Gairdner, 236 recombinants were identified using two SSR markers flanking the *Alp* locus, Bmag490 and Bmac310 (Fig. 5a). Molecular analyses revealed that both markers exhibited a normal Mendelian segregation ratio as expected for a single gene. Where possible, the 236 recombinants were then genotyped with the other markers linked to *Alp* in the low-resolution DH population. These included the markers GWM165, ABG715, Bmag353 and HvGABP. The markers

Table 2 SNP detection and CAPS analysis of markers ABG715, MWG0948 and MWG2134 in the parental lines

Marker	Parents	Length (bp)	SNP	Restriction enzyme	Cleavage sites	Predicted fragments (bp)
ABG715	Dayton	534	AAA<u>A</u>TCC	*Apo*I	2	218, 177, 139
	Zhepi2	534	AAA<u>T</u>TCC	*Apo*I	3	177, 144, 139, 74
MWG0948	Dayton	302	ATTA<u>A</u>TAA	*Ase*I	1	207, 95
	Zhepi2	302	ATTA<u>G</u>TAA	*Ase*I	0	302
MWG2134	Dayton	278	GGAT<u>C</u>CAT	*Alw*I	3	131, 98, 36, 13
	Zhepi2	278	GGAT<u>T</u>CAT	*Alw*I	1	180, 98

PCR fragment length, partial sequence with SNP sites underlined, restriction enzyme, number of predicted cutting sites and predicted fragments in both parents are given

Fig. 3 CAPS analysis of markers ABG715, MWG0948 and MWG2134 resolved on 2% agarose gel (M-pGEM DNA marker, *PT*-tolerant parent, *PS*-sensitive parent). **a** Undigested PCR products of ABG715, **b** PCR products of ABG715 digested with restriction enzyme *Apo*I, **c** Undigested PCR products of MWG0948, **d** PCR products of MWG0948 digested with restriction enzyme *Ase*I, **e** Undigested PCR products of MWG2134, **f** PCR products of MWG2134 digested with restriction enzyme *Alw*I
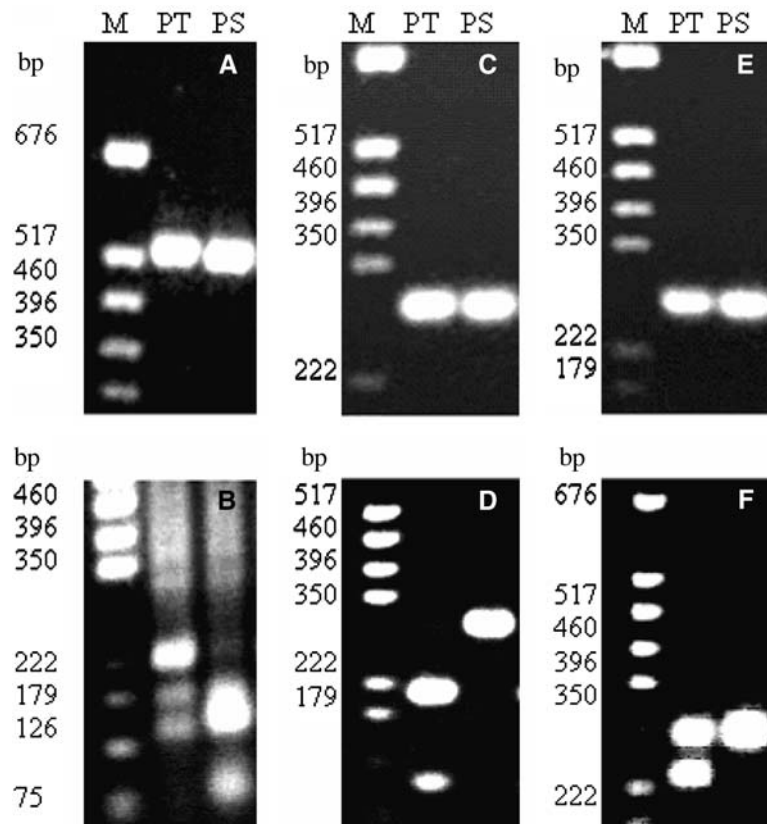
Table 3 The alignment of *Alp* linked STS and RFLP markers on barley chromosome 4H with rice BAC clones assembled on rice pseudomolecule 3

Marker	Barley EST accession #		Length	Rice BAC	E-value (Identity)	Putative function
MWG2134	AJ234794	gDNA	376	OJNBa0032O09	4e-38 (116/126)	Calcium ATPase
CDO1395	AA231829	cDNA	340	OJ1743A09	2e-27 (195/238)	Nuclear pore complex protein
HvMATE	AV942930	cDNA	585	OSJNBa0090D11	4e-17 (86/99)	Multidrug and toxic compound extrusion (MATE) protein
HvGABP	AW983176	cDNA	809	OSJNBa0090D11	1e-63 (191/213)	Neutral alpha-glucosidase <u>AB</u> precursor
ABG715	Sequenced in this study	gDNA	1800	OJ1193C08	0.0 (875/1008)	Golgi nucleotide-sugar transporter
BCD1117	BE438923 (DN187816)	cDNA	226 (700)	OJNBa0014O06	1e-36 (125/140)	GDP-mannose pyrophosphorylase

Table 4 Primer sequences of the developed/identified STS or SSR markers linked to the *Alp* locus

Marker	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	PCR product size (bp)	Annealing temp (°C)
HvGABP	CTGCAGATTGGGATCACCTT	CAACCACAGAGGACGCATAA	~600	55
HvMATE	GGCACAGAACAACGACATGA	GATGGCGGTTCGTCTACAGT	242	55
ABG715	GGGCATGAGAGCAATTCAAG	GGGACGACTGTTAATGAGAT	~500	55
GWM165	TGCAGTGGTCAGATGTTTCC	CTTTTCTTTCAGATTGCGCC	259	55
Bmag353	ACTAGTACCCACTATGCACGA	ACGTTCATTAAAATCACAACTG	130	60
GBM1071	Kindly supplied by Drs.Andreas Graner and Martin Ganal (IPK)		240	55

Fig. 4 CAPS analysis of rice synteny-based marker HvGABP in the recombinant lines (PCR products were digested with *BanI*) (M-pGEM DNA marker; *PS-Al* sensitive parent; *PT-Al* tolerant parent; *S-Al* sensitive; *T-Al* tolerant; *H*-heterozygotes)
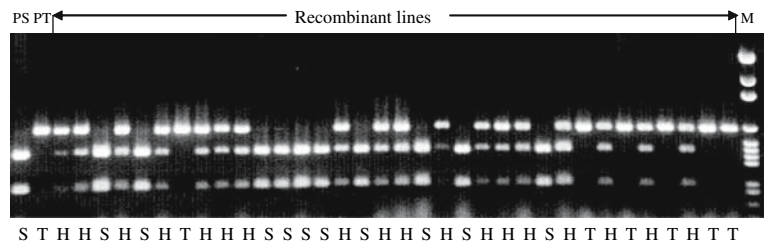
Fig. 5 Genetic linkage map of the *Alp* locus. **a** A low-density map generated in a DH population from Dayton/Zhepi 2. *Vertical lines* on the left side indicate the significantly associated genomic region for RRE and RE + Al. *Solid bars* indicate the peak locations. **b** A high-resolution map generated in 236 recombinant lines from Dayton/Gairdner. Genetic distance in centimorgans (cM) was calculated using the Kosambi (1944) function
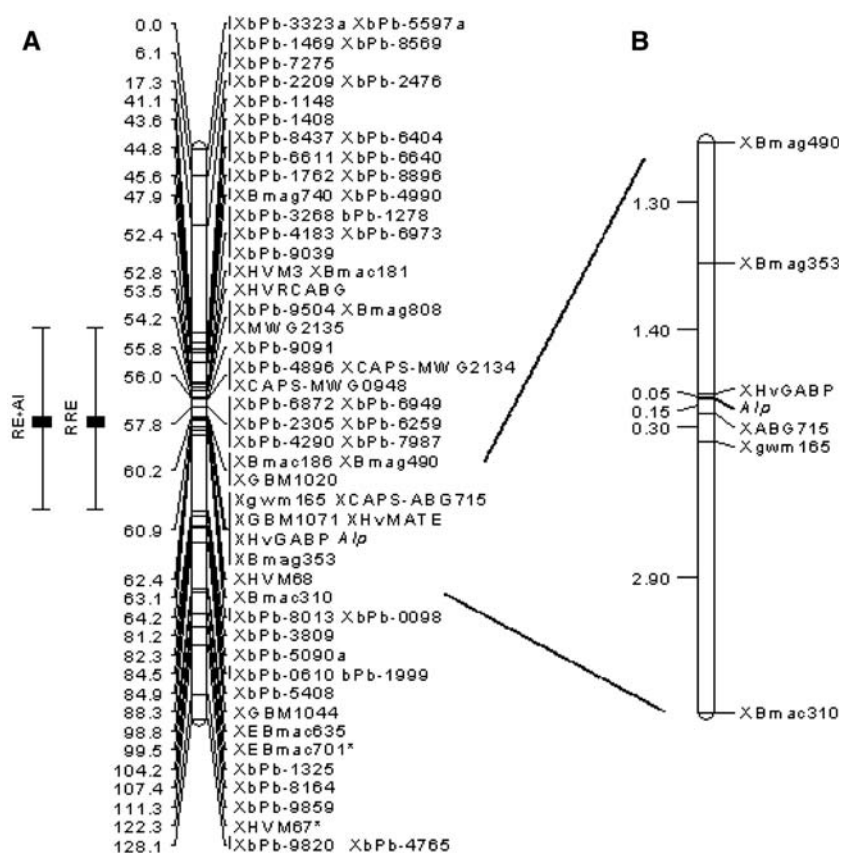
HvMATE and GBM1071 were monomorphic between Dayton and Gairdner. Two recombinants were identified between *Alp* and HvGABP and six were identified between *Alp* and ABG715 (Table 5). ABG715 mapped 0.15 cM distal to *Alp* and HvGABP mapped 0.05 cM proximal to

Alp (Fig. 5b). Marker HvMATE was not polymorphic for the Dayton/Gairdner population and subsequent sequencing of the genomic sequence defined by the HvMATE EST did not identify any polymorphisms (Genbank #EF489298, EF588043).

Table 5 Phenotypes and marker alleles of eight $F_{2:3}$ recombinant lines identified between *Alp* and the flanking markers HvGABP and ABG715

Locus	$F_{2:3}$ Recombinant lines							
	R1	R2	R3	R4	R5	R6	R7	R8
HvGABP	H	H	H	B	A	H	A	A
Alp	H	H	H	B	A	H	H	H
ABG715	B	B	B	H	H	A	H	H

R1–R8 Recombinant lines identified using flanking markers from the 236 $F_{2:3}$ lines from Dayton/Gairdner. *A* Dayton allele (Al-tolerant), *B* Gairdner allele (Al-sensitive) and *H* heterozygous for Dayton and Gairdner alleles
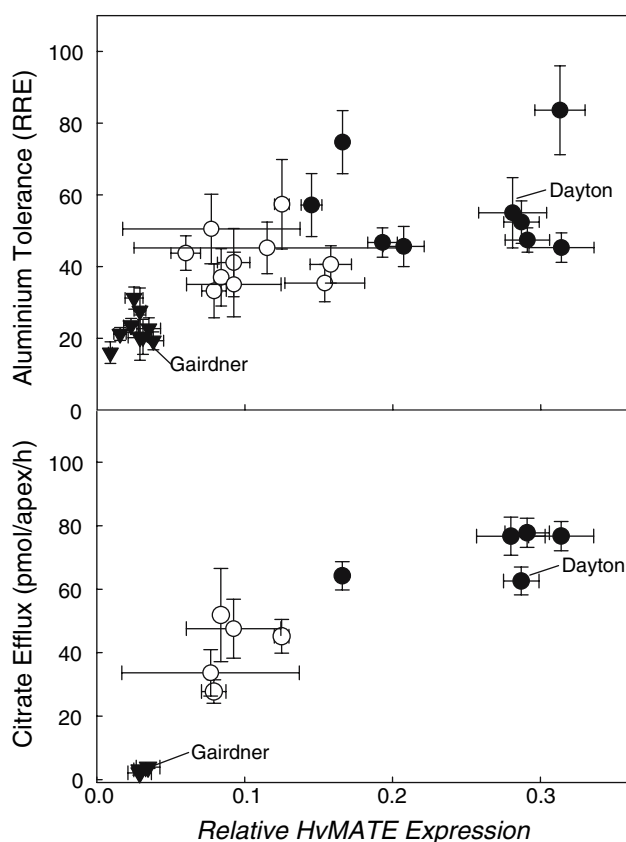

Fig. 6 Relationships between *HvMATE* expression and Al tolerance and Al-activated citrate efflux. Data represent the mean of values and standard errors (SE) for expression ($n = 3$), citrate efflux ($n = 3$ or 4) and Al tolerance ($n = 5–8$). To account for the accumulation of errors associated with deriving RRE the SEs were calculated as follows: $SE_{RRE} = RRG \ [(SE_x/x)^2 + (SE_y/y)^2]^{1/2}$ where x and y represent the mean root length in the control and aluminum treatments. The symbols represent whether the $F_{2:3}$ families tested were previously found to be segregating for Al tolerance when mapping *Alp* (*filled circle*) homozygous Al-tolerant, (*open circle*) segregating for Al tolerance, (*inverted filled triangle*) homozygous Al-sensitive. Also indicated are the parental lines Dayton (Al-tolerant) and Gairdner (Al-sensitive). The correlation coefficients are $r = 0.738$ ($P < 0.001$) for **a** and $r = 0.85$ ($P < 0.001$) for (**b**)

Citrate efflux and *HvMATE* expression

Since the Al-tolerance of other barley genotypes appears to rely on Al-activated citrate efflux from roots (Ma et al. 2004) we investigated whether citrate efflux differed between the parental lines in the populations used here. Citrate efflux from the root apices of Dayton was 63 ± 4 pmol/apex/h compared to 2 ± 1 pmol/apex/h from Gairdner and 5 ± 0 pmol/apex/h from Zhepi 2. We then examined the *HvMATE* gene further because the rice/barley synteny indicates that the *HvMATE* and *HvGABP* genes are likely to be adjacent to one another. Furthermore, other MATE proteins are known to facilitate citrate efflux from Arabidopsis and sorghum cells (Durrett et al. 2006; Magalhaes et al. 2007).

Expression of the *HvMATE* gene was measured in the root apices of the parental lines using qRT-PCR. Relative expression of the *HvMATE* gene in the Al-tolerant Dayton was 30-fold higher than Gairdner and no expression could be detected in Zhepi2. We then investigated whether expression of the *HvMATE* gene correlated with Al tolerance and Al-activated citrate efflux in selected $F_{2:3}$ families of the Dayton/Gairdner population including the eight informative recombinants identified with HvGABP and ABG715 markers (Table 5). Relative expression of *HvMATE* was significantly correlated with Al tolerance and Al-activated citrate efflux (Fig. 6). Of the 26 $F_{2:3}$ families assayed, *HvMATE* expression and citrate efflux were greater in the $F_{2:3}$ families from F_2 individuals previously scored as homozygous tolerant than in the $F_{2:3}$ families of F_2 individuals scored as homozygous sensitive. $F_{2:3}$ families from F_2 individuals previously scored as being heterozygous for Al tolerance were generally intermediate for expression and citrate efflux. However, the variability of the expression measurements was too large to reliably differentiate between those $F_{2:3}$ families that were homozygous for Al tolerance and those that were segregating for A1 tolerance. Nevertheless, *HvMATE* expression in the eight recombinant lines was consistent with the *Alp* alleles of these families predicted from the measurements of Al-tolerance and citrate efflux.

Discussion

A single *Alp* locus conditions Al tolerance

Aluminum tolerance in the barley lines was estimated staining the roots with ECR, RE + Al and RRE. Staining indicates whether exclusion of Al from the root tips is occurring (Ma et al. 2001), whereas measurements of root elongation could reflect a combination of mechanisms that affect tolerance. Statistical analysis revealed that all three assessments of tolerance were significantly correlated with

Fig. 7 Comparative mapping and colinearity in the Al tolerance genomic regions between barley and rice (same marker order and different orientation)
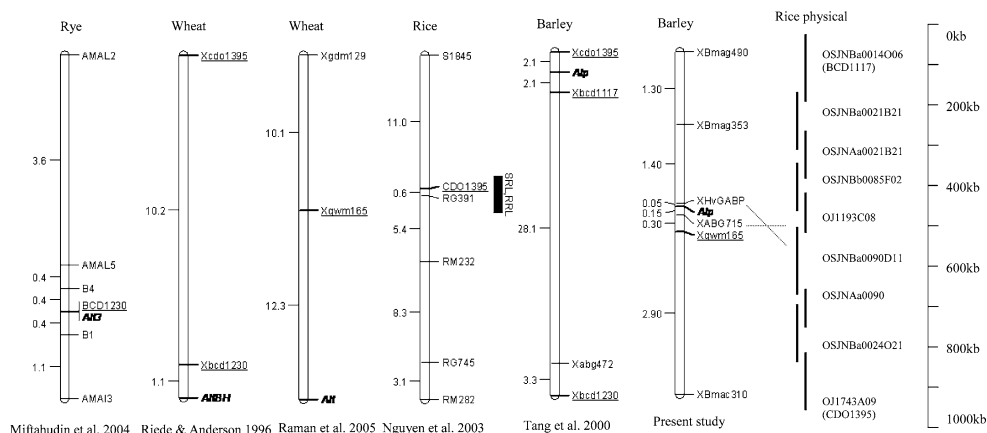
one another. The segregation analysis for root staining patterns and frequency distributions of RE + Al and RRE revealed that a single locus conditions Al tolerance in the barley cultivar Dayton. A single-gene model for Al tolerance scored on the basis of hematoxylin staining (Tang et al. 2000), root re-growth (Raman et al. 2002, 2003), ratio of root to shoot fresh weight (Ma et al. 2004), and ECR staining (Wang et al. 2006) has been reported previously for different barley populations. Linkage and QTL analysis confirmed that the major locus *Alp*, located on the long arm of chromosome 4H encodes Al tolerance in the populations from Dayton/Zhepi 2 and Dayton/Gairdner. Our results are consistent with previous reports that identified loci linked to acid-soil tolerance and Al tolerance in barley (Ma et al. 2004; Minella and Sorrells 1997; Raman et al. 2002, 2003; Stølen and Andersen 1978; Wang et al. 2006).

Comparative and high resolution mapping of *Alp*

We showed that the wheat SSR marker (GWM165-4DL) was located 0.45 cM from the *Alp* locus on the long arm of 4H. This marker has been located 1.5 cM apart from BCD1117 in the cMap of wheat chromosome 4D (http://www.rye.pw.usda.gov). Tang et al. (2000) have reported that BCD1117 and CDO1395 markers flank the *Alp* locus (Fig. 6). CDO1395 also explained approximately 20% of the genetic variation for Al tolerance in a rice population (Nguyen et al. 2003). BCD1230 exhibited tight linkage with the Al tolerance locus *Alt3* in rye (Miftahudin et al. 2004), and *Alt*$_{BH}$ in wheat (Riede and Anderson 1996) but was mapped 33 cM away from *Alp* locus in barley (Tang et al. 2000). Marker GWM165 tightly linked with Al tolerance in the present study was mapped about 12 cM apart from the Al tolerance locus in wheat (Raman et al. 2005b). Interestingly, rye marker B4, which is tightly linked with the *Alt3* locus on 4R (Miftahudin et al. 2004), mapped to chromosome 2H in barley instead of the expected 4H. It is possible that multiple copies of B4 may exist in the barley genome, or there may be some

conservation of genes between chromosomes 2H and 4R. Similarly, Gruber et al. (2006) identified an *ALMT1* homolog in barley (*HvALMT1*), which mapped in the vicinity of the B4 locus on 2H in the DH population from Dayton/Zhepi 2. Recently Fontecha et al. (2007) reported that an *ALMT1* homolog was located on barley 4H, which is consistent with the expected synteny between the wheat 4DL and barley 4HL. However this conclusion relied on PCR amplification and needs to be verified by Southern blot analysis.

The rice/barley synteny was exploited to develop additional markers for high-resolution mapping of the *Alp* locus. The locus was finally delineated to a 0.2 cM region in the Dayton/Gairdner population by the flanking markers HvGABP and ABG715 (Fig. 5b). The region of the rice genome found to be syntenic with the *Alp* locus spans approximately 120 kb on chromosome 3 (Fig. 7) and is flanked by Os03g11590 (ABG715) and Os03g11720 (HvGABP) (http//www.tigr.org/tdb/e2k1/osa1/). Within this region there are no homologs of *TaALMT1*, the Al tolerance gene of wheat. This result indicates that despite a similar chromosomal location for Al tolerance loci in wheat and barley, the genes are likely to encode different proteins and are therefore not orthologous.

Within the syntenic region of rice on BAC clone OSJNBa0090D11 a gene encoding a MATE stands out as a candidate homolog for the Al tolerance gene of barley. Members of the MATE family were recently shown to facilitate citrate efflux from Arabidopsis and sorghum cells (Durrett et al. 2007; Magalhaes et al. 2007) and it is conceivable that *HvMATE* encodes a protein responsible for the Al-activated efflux of citrate from Al-tolerant barley. Although the *HvMATE* marker was not polymorphic in the Dayton/Gairdner population, expression levels did differ between the parental genotypes and therefore could be used to score the $F_{2:3}$ families. The finding that *HvMATE* expression was correlated with citrate efflux and Al tolerance identifies *HvMATE* as a candidate for the gene controlling Al tolerance in barley.

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