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A new allele of acid soil tolerance gene from a malting barley variety

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Abstract

Background: Acid soil is a serious limitation to crop production all over the world. Toxic aluminium (Al) cations in acid soil inhibit root growth and reduce yield. Although a gene tolerant to acid soil has been identified, it has not been used in malting barley breeding, which is partly due to the acid soil tolerance gene being linked to unfavorable malting quality traits.

Results: A Brazilian malting barley variety Br2 was identified as tolerant to acid soil. A doubled haploid (DH) population was developed from a cross between Br2 and the Australian acid-sensitive cultivar Hamelin. The DH population was tested for acid soil tolerance in native acid soil and a hydroponic system with pH 4.2, pH 4.2 + Al or pH 6.5, and genotyped using SSR, DArT and gene-specific markers. A single QTL was detected for all parameters related to acid soil tolerance. The QTL was mapped to the known *HvMATE* location on chromosome 4H. Sequence alignment of the *HvMATE* gene identified 13 INDELs and 87 SNPs, where one SNP coded for a single amino acid difference between the two varieties. A gene-specific marker was developed to detect the single nucleotide polymorphism between Hamelin and Br2. This marker co-segregated with aluminium tolerance and accounted for 79 % of phenotypic variation for acid soil tolerance.

Conclusion: The present study identified a novel source of acid soil/Al tolerance from a Brazilian malting barley cultivar Br2. This variety tolerated Al toxicity but was sensitive to low pH which is similar to most other Al-tolerant varieties. A gene-specific marker Cit7 was developed based on the *HvMATE* gene sequence. Cit7 will improve the efficiency of molecular-assisted selection of new barley varieties with tolerance to acid soil. Multiple alleles exist for the acid soil tolerance gene on chromosome 4H, so a malting barley variety that tolerates acid soil could be developed by selecting suitable tolerant alleles. Tolerance to low pH may play an important role for barley to adapt to acid soils.

Keywords: Malting barley, Citrate transporter, Aluminium tolerance, Gene-specific marker

Background

Acid soil is a major limiting factor to plant production worldwide. It accounts for 30 % of the total land area and 50 % of the arable land [1] with loss of production equating to more than 600 million US dollars annually [2]. Aluminium (Al) toxicity limits growth and productivity of barley (*Hordeum vulgare* L.) on acid soils and thus restricts barley as a crop in many agricultural areas

[2, 3]. The initial toxic effect of acid soil stunts and shortens root growth. The toxic aluminium (Al) cation in acid soil may also restrict water uptake and nutrient absorption, which eventually reduces plant production. Thus, root length is often selected as the phenotypic trait for aluminium toxicity tolerance [4, 5].

Genes related to Al tolerance are involved in multiple metabolic processes, including cell elongation and division, cell wall formation, oxidative stress, iron metabolism, signal transduction and other cellular mechanisms [6–8]. The expression of several Al-induced genes, such as AtBCB, parB, NtPOX and NtGDI1 in transgenic *Arabidopsis* plants resulted in better relative root growth under Al stress [9]. The secretion of organic anions such as citrate and malate from root apices plays an important role in excluding and

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detoxifying Al [10–12]. Of the many Al-induced genes, ALMT and MATE— controlling malate and citrate extrusion respectively—have been reported as the Al-tolerant genes in many plants [13].

Barley is one of the most sensitive species to Al toxicity among small-grain crops [14, 15] but differences in Al tolerance exist among varieties. Al tolerance is controlled by one single gene or several QTLs depending on materials [2]. The major tolerance gene on chromosome 4H has been given various names according to its origin or tolerance to Al^{3+} toxicity or low pH, which include *Pht* (low soil pH) [16], *Alp* (Al^{3+} in Dayton) [17], *Alt* (Al^{3+} in WB229) and *Alp3* (Al^{3+} in Brindabella) [18, 19]. Ma et al. [20] reported that a QTL tightly linked to Al tolerance explained more than 50 % of phenotypic variation in citrate secretion in a cross between an Al-resistant cultivar (Murasakimochi) and an Al-sensitive cultivar (Morex). This QTL was located at the same position of the major tolerance gene with the molecular marker Bmag353 tightly linked with citrate secretion [20]. Fine mapping combined with microarray analysis identified that *HvAACT1*, a MATE gene (also known as *HvMATE),* was responsible for the Al-activated citrate secretion [21]. Heterologous expression of *HvAACT1* in *Xenopus* oocytes showed transport activity for citrate and transgenic tobacco also showed higher citrate secretion when treated with Al [21]. Further study demonstrated that the relative expression of the *HvMATE* gene in the *Alp* locus on chromosome 4H was 30-fold higher in Dayton (tolerant) than Gairdner (susceptible) [22]. The expression marker exhibited complete linkage with the *Alp* locus in the DH population, accounting for 72 % of the variation for Al tolerance based on relative root growth under Al^{3+} stress [22]. These results further supported that *HvMATE*, a gene encoding a multidrug and toxic compound extrusion protein, is the candidate gene controlling Al tolerance on chromosome 4H.

Despite progress in identifying the tolerance gene, development of malting barley variety that tolerates acid soil has been slow. This is partly due to the acid soil tolerance gene being linked to unfavorable malting quality traits. No acid soil tolerant malting barley cultivar has been reported. In the present study, we identified a malting barley variety from Brazil that tolerates Al toxicity. The tolerance gene was mapped to the same location as the *HvMATE* gene. Further sequencing and phenotyping analysis demonstrated that the tolerance gene is a new allele. One gene-specific molecular marker was developed and confirmed in the Hamelin/Br2 DH population. The phenotypic variation determined by the marker was also compared with other markers currently used for molecular-assisted selection.

Results

Phenotyping and inheritance of Al tolerance in barley

The Hamelin/Br2 DH population was assessed using acid soil and three hydroponic experiments: pH 4.2, pH 4.2 + Al and pH 6.5. The two parents differed significantly in their Al tolerance. In the acid soil treatment, the female parent Hamelin had an average root length of 123 mm, while the male parent Br2 had 191 mm. In the hydroponic experiments, root growth of the two parents was similar in the pH 6.5 treatment, with average root lengths of 88 and 92 mm for Hamelin and Br2, respectively. The solution pH of 4.2 significantly inhibited root growth of both varieties with average root lengths reduced to 47 and 41 mm for Hamelin and Br2, respectively. Addition of Al to the pH 4.2 solution significantly reduced root growth in the sensitive cultivar (Hamelin) but had little effect on the tolerant cultivar (Br2): average root length of Hamelin decreased by more than 50 % to 22 mm, while Br2 only decreased by about 10 % to 36 mm.

Acidity and Al also had a significant effect on root growth of the DH lines with root length ranging from 46 to 405 mm in acid soil, 34 to 116 mm in the control solution (pH 6.5), 24 to 79 mm at pH 4.2 and 14 to 41 mm at pH 4.2 + Al (Fig. 1). The root lengths of DH lines showed continuous distributions in all the hydroponic treatments. In the acid soil treatment, root lengths of DH lines could be divided into two separate groups, indicating one major
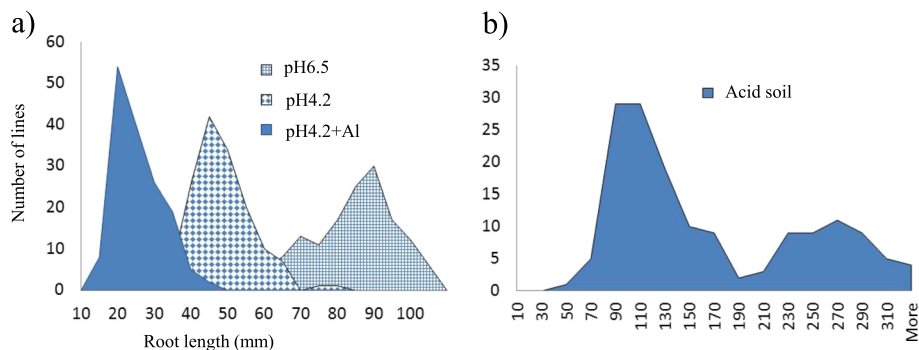

Fig. 1 Frequency distributions of root length for the Hamelin/Br2 DH population under acid soil, pH 6.5, 4.2 and pH 4.2 + Al treatments. **a** pH 6.5, pH 4.2 and pH 4.2 + Al treatments. **b** Acid soil treatment

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gene controlling acid tolerance. Chi-square analysis showed that the segregation did not fit the 1:1 ratio, due to the significant distortion of markers in the region for the Al tolerance gene (Additional file 1).

Root lengths of DH lines in solution pH 4.2 + Al had a significant correlation with those in the acid soil, explaining 62.6 % of the phenotypic variation in root length in the acid soil treatment. This result suggests that the hydroponic experiment with Al was consistent with the acid soil experiment. However, root lengths of DH lines grown at solution pH 4.2 had no correlation with those in the acid soil ($P = 0.025$, $R^2 = 0.0328$).

A scatter graph was constructed using the phenotypic data from the acid soil and pH 4.2 + Al treatments (Fig. 2). All DH lines could be clearly classified into two groups, with red scatters signifying the sensitive group and blue scatters representing the tolerant group (102 red, 52 blue, four data missing). However, Chi square analysis showed that the segregation did not fit the 1:1 ratio ($\chi 2 = 16.23$, $\alpha = 5$ %). An additional regression analysis also showed that the phenotype data collected from acid soil and pH 4.2 + Al treatments were significantly correlated with each other ($P < 0.0001$, $R^2 = 0.626$). This suggests that the acid soil/Al tolerance in Br2 is probably controlled by a major gene.

Linkage map construction and QTL analysis

Thirty-seven lines selected from the DH population were genotyped using DArT. In total, 446 commonly-used SSR markers and 388 DArT markers were tested for polymorphism, in which44 SSR markers and 258 DArT markers were mapped to seven chromosomes (Additional file 2). One major QTL for acid soil tolerance was detected on chromosome 4H by preliminary QTL analysis (data not shown). Based on the preliminary analysis, six

polymorphic markers in the QTL region were selected to map the whole population and the marker order was similar to the previously reported consensus map (Fig. 3) [23]. QTL analysis showed that the same QTL controls root growth in acid soil and pH 4.2 + Al solution (Fig. 4b). The nearest marker Bmag353 explained 67.5 % of the phenotype variation in root length at pH 4.2 + Al and 72.4 % in acid soil, 78.1 % of average root length at pH 4.2 + Al and in acid soil, and 55.4 % of relative root length at pH 4.2 + Al (Table 1). When DH lines were grouped into tolerant and sensitive, 88.1 % of the variation in tolerance was explained by Bmag353 using single marker regression analysis (P < 0.001). Based on previous studies, Bmag353 and Bmac310 were the common markers associated with the *HvMATE* acid soil tolerance gene [24, 22]. Thus, the tolerant cultivar Br2 may share the same tolerance gene identified in previous studies.

Gene-specific marker development and association analysis

The *HvMATE* gene contains 13 exons and 12 introns with a full length of 12,257 bp (Fig. 5a). Forty-four pairs of primers covering the whole gene were designed to amplify the fragments of *HvMATE* gene in Hamelin and Br2 (Additional file 3). The PCR product size of these primers varied from 200 bp to 500 bp. Sixteen pairs of these primers were polymorphic between Hamelin and Br2 (Table 2). One marker Cit7 (Cit7F: 5-GCAGCC AAGACCTTGAGAAAGC-3 and Cit7R: 5-GCCTGAAC TAGCCCGAGAAATG-3) designed from the coding region of *HvMATE* gene and 54 other polymorphic SSR markers were used to construct the linkage map of the full population. The results showed that the gene-specific marker Cit7 was located between SSR markers Bmag353 and Bmac310 on chromosome 4H (Fig. 3). When Cit7
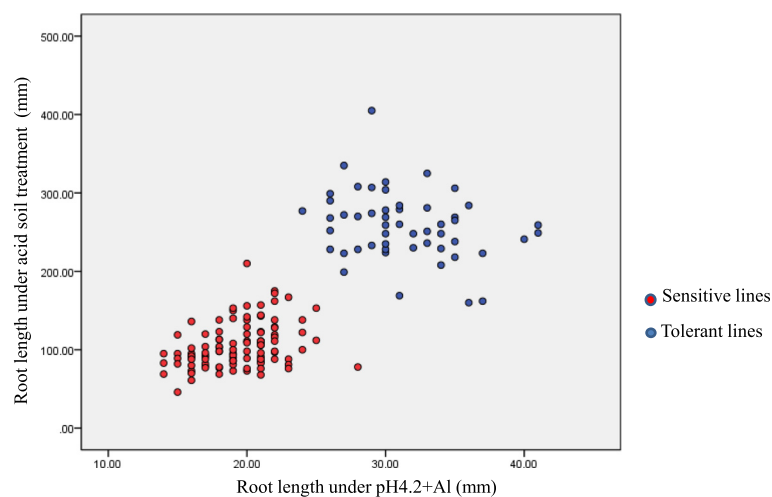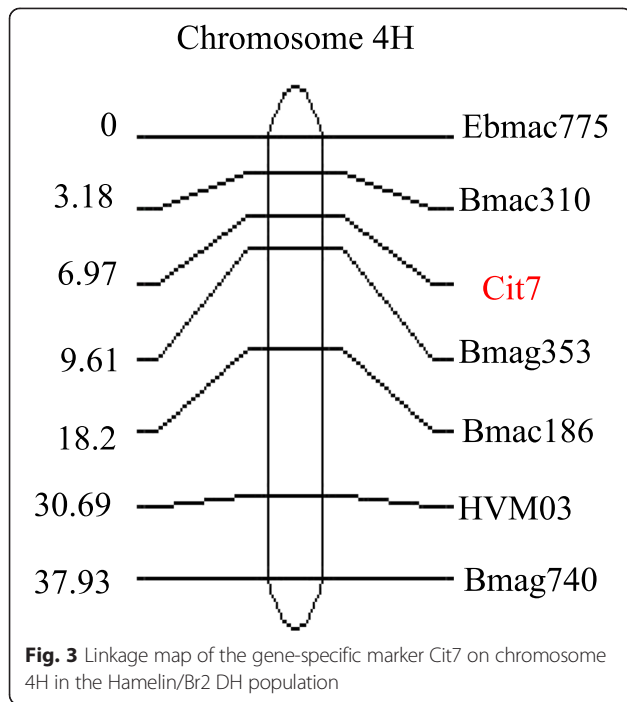

Fig. 2 Scatter graph showing Hamelin/Br2 DH lines could be classified into two groups

Fig. 3 Linkage map of the gene-specific marker Cit7 on chromosome 4H in the Hamelin/Br2 DH population

was integrated into the chromosome 4H linkage map, the phenotypic variation explained by the QTL increased for all traits (Fig. 4a and Table 1). For example, 82 % of the variation in root length in acid soil was explained by the QTL compared to 73.4 % by other markers previously (Table 1).

Sequence assembly, alignment and new allele identification

The sequences of Br2, Hamelin and Svanhals were assembled and aligned using software BioEdit (http://www.mbio.ncsu.edu/BioEdit) with default parameters. The sequences were compared with sequences from Morex, Murasakimochi and Haruna Nijo [25, 21]. There were 14 indels and 86 SNPs between the tolerant and sensitive cultivars (Additional file 4). Of which, 87 variations differed from these alleles reported elsewhere [25, 21]. The sequence variations varied from 1 SNP to 29 bp. Six indels located in the 5′ upstream region, one indel (12619–12620 bp) and one SNP (12660 bp) are located on the 3′ UTR. Five indels are located on the downstream of
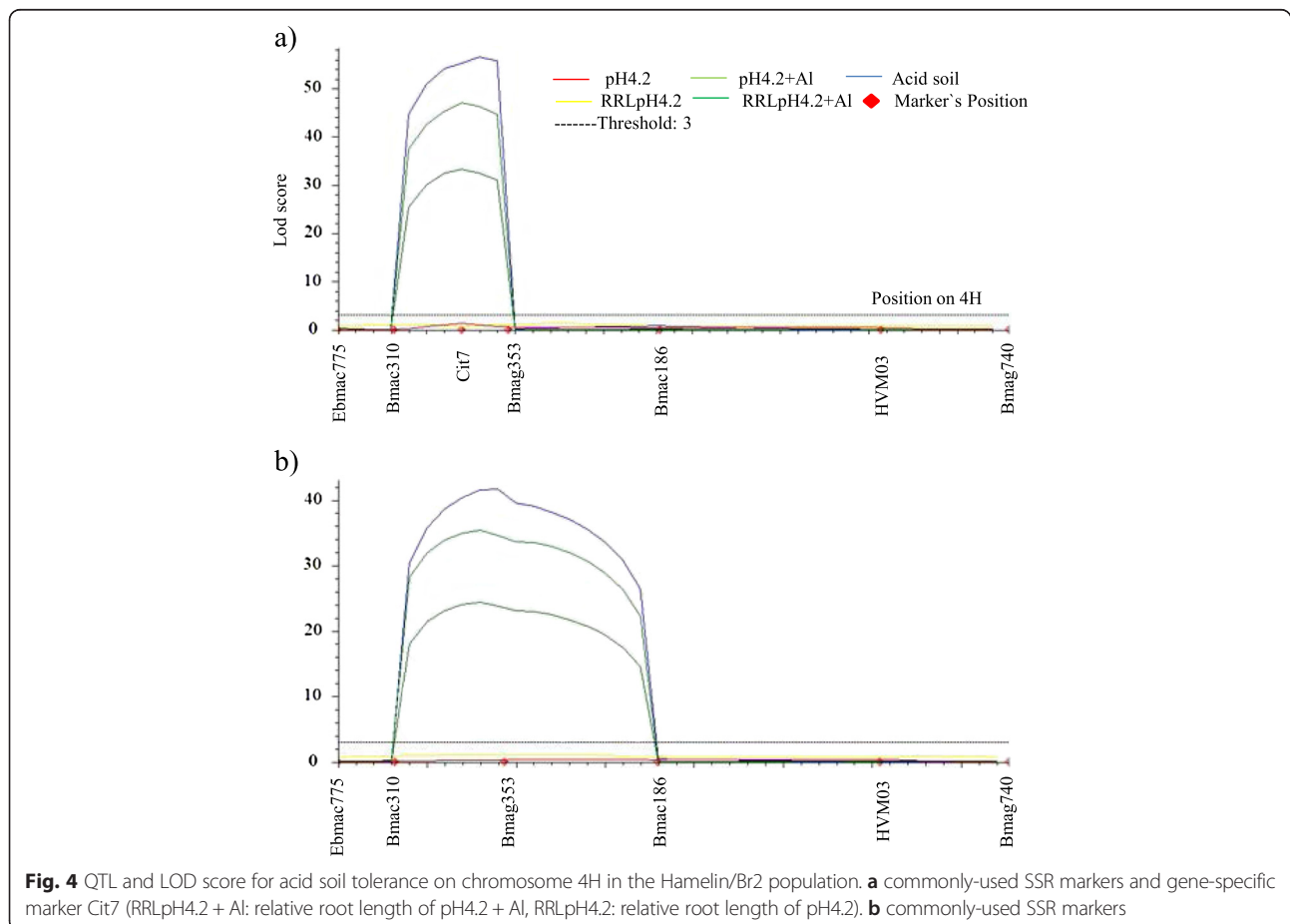

Fig. 4 QTL and LOD score for acid soil tolerance on chromosome 4H in the Hamelin/Br2 population. **a** commonly-used SSR markers and gene-specific marker Cit7 (RRLpH4.2 + Al: relative root length of pH4.2 + Al, RRLpH4.2: relative root length of pH4.2). **b** commonly-used SSR markers

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Table 1 Comparison of Cit7 and Bmag353 on explained phenotypic variations (EPV) on acid soil, pH4.2 + Al and relative root length of pH4.2 + Al (RRL) based on interval QTL analysis and single marker regression analysis

Traits	Before add Cit7		After add Cit7		Cit7	Bmag353
	LOD	EPV (%)	LOD	EPV (%)	EPV (%)	EPV (%)
pH 4.2 + Al	35.4	68.5	47.0	75.5	75.7	67.5
Acid soil	41.6	73.4	56.5	82.0	79.0	72.4
RRL	24.4	55.5	33.3	63.0	63.0	55.4

3′ UTR. Thus, the tolerance gene from Br2 represents a new allele.

Further sequence analysis demonstrated that Cit7 covers part of the sequence of the *HvMATE* gene exon 3 (7255–7461 bp) and part of the sequence of exon 4 (7572–7765 bp) (Fig. 5). One SNP (T (sensitive)–G (tolerant)) was detected between sensitive and tolerant cultivars (Fig. 6). Online translation software, expasy-translate tool (available from http://web.expasy.org/translate/), was used for DNA translation under default parameters. The results showed that the SNP coded for a single amino acid difference (L (172)–V) between the sensitive and tolerant cultivars (Fig. 6).

Discussion

Tolerance to low pH and Al toxicity

Acid soil limits plant growth through pH toxicity and metallic toxicity, especially Al [12]. To investigate possible toxicity mechanisms in the tolerant cultivar used in this experiment, three hydroponic treatments were employed to evaluate the effects of low pH and low pH + Al on root growth. The results showed that low pH had a significant impact on root growth. The combination of low pH and Al had even larger effects on root growth (Fig. 1). Our results are consistent with those previously reported [14, 26, 27]. Both parents were sensitive to low pH and root length decreased from about 90 to 40 mm when the solution pH decreased from 6.5 to 4.2. However, Br2 had higher Al tolerance with only 10 % further reduction in root length after the addition of Al compared to a 50 % reduction in Hamelin. Root growth in DH lines showed similar trends with low pH affecting root growth of nearly all the lines and Al only affecting sensitive lines. The difference in low pH tolerance and Al tolerance has also been reported elsewhere. For example, Kidd and Proctor [28] reported that *Holcus lanatus* L. and *Betula pendula* Roth races collected from different sites differed in pH tolerance and Al tolerance. The authors concluded that the races collected from acid organic soil were tolerant to low pH, while
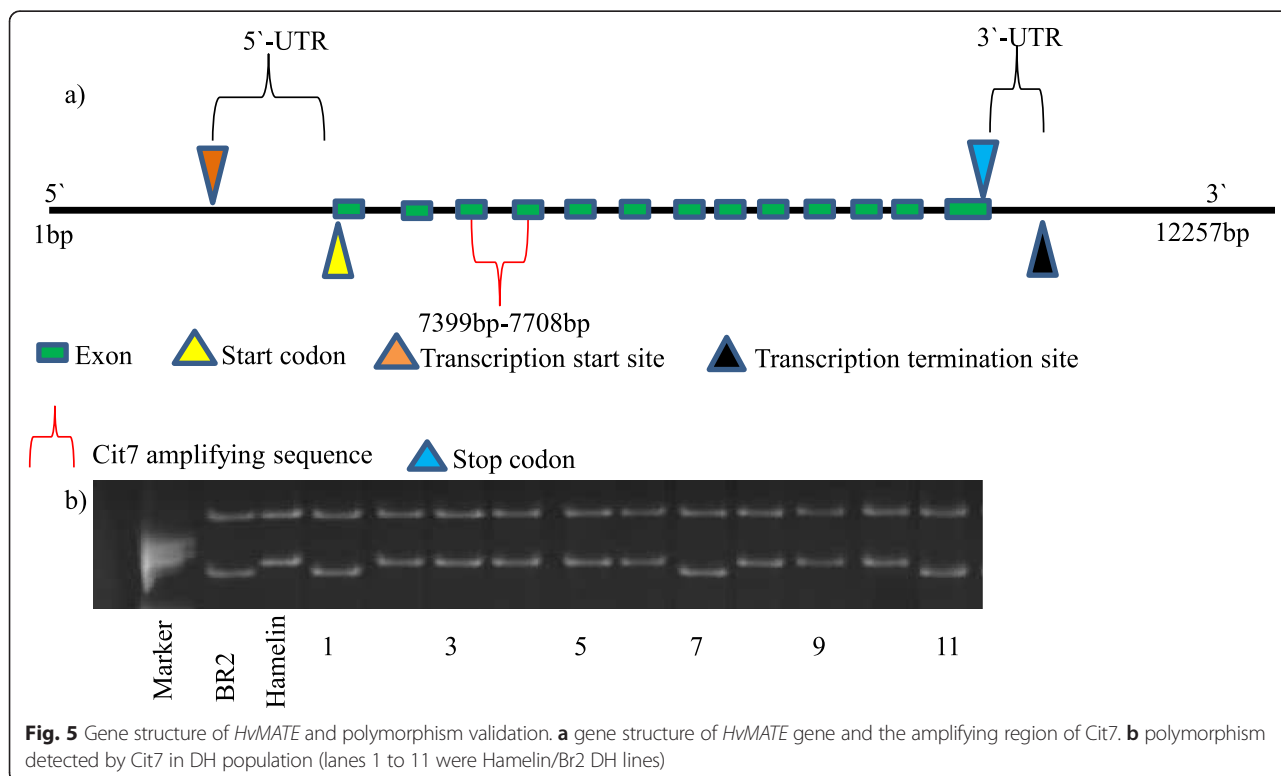

Fig. 5 Gene structure of *HvMATE* and polymorphism validation. **a** gene structure of *HvMATE* gene and the amplifying region of Cit7. **b** polymorphism detected by Cit7 in DH population (lanes 1 to 11 were Hamelin/Br2 DH lines)

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Table 2 Polymorphic gene-specific markers derived from the *HvMATE* gene between Hamelin and Br2

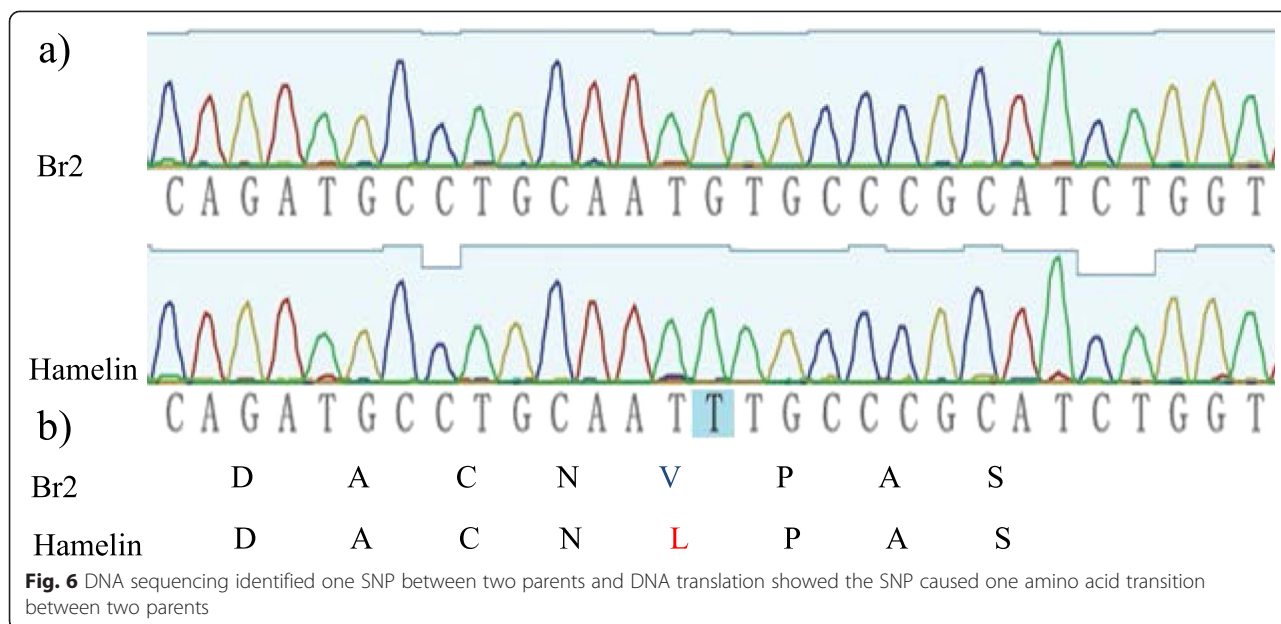| Marker name	Product length (bp)	Annealing temperature (°C)	Polymorphic type	Primers
HvMATE-21indel	497	62	Agarose	F: GCTAGGGCTTGAAAACTGTTTG
				R: GACGAACTGTACGATGATGATGC
D0	352	55	Agarose	F: GCCACGCCTTACAGTAAAGAAC
				R: CCTAGCTATCTCAAGTTGGCTTAC
Cit7	312	67	SSCP	F: GCAGCCAAGACCTTGAGAAAGC
				R: GCCTGAACTAGCCCGAGAAATG
Cit14	225	58	SSCP	F: TCGGGTATTGGAGTTAGAAGGG
				R: CGGGCACATTTGATGCAAGGAT
Cit16	205	55	SSCP	F: CCCGAGTTATGTCATTTTTCCTCTC
				R: GGGCCTGGTTGGGCCTTAT
Cit6	225	55	SSCP	F: ACCTTCCGTGACATCTGCTCTA
				R: ATCGGTGAGTCCTGGAATAGTG
U5	491	55	SSCP	F: CACACAACTGGAAAACAACTACC
				R: GGATAAAACTTCAGTGCGACG
Cit1501	332	55	SSCP	F: GAAGGGGCCTATTGCTTCAC
				R: CACCCATAAGTTGTGGTTCGG
Cit19	355	67	SSCP	F: TGGTGAAACGGGCATGTCTC
				R: GAAACCAGGTATATTGCAAGAGC
U12f + U11r	726	55	Agarose	F: TCGTCAATCGCAACTCTCAGA
				R: CATATCGTTTGTCGTATCACGC
U4	404	55	SSCP	F: CAAGTGTGAAATAGAGAGTCGGTAG
				R: CGCAAGAACATTTTTGTCACG
D5	453	55	SSCP	F: CGGGTATTGGAGTTAGAAGGG
				R: GCTATAAAGTCCACGCTATGCAG
D3	448	58	SSCP	F: CTCCTGCGAGGCAGATGAG
				R: CTCGCTCTCCCTAATGGTGG
D01R	362	60	SSCP	F: GCTCAACCAGACTCAGGTAAGC
				R: CCAAACAGGGCCTAAGCTTC
D202rr	474	60	SSCP	F: GTCTTCAACAGCATGATTAAGGTC
				R: CAAACCTAGCACTATTCGGGTG
D4FF	440	58	SSCP	F: CAATCCTTGCATCAAATGTGC
				R: GGCCCTAAGATAGAAGCACAAG

those from acid mineral soils were tolerant to Al but not necessarily tolerant to low pH [28].

Tolerance to acid soil may be a combination of low pH tolerance and Al tolerance. In the present study, all of the DH lines were severely affected by low pH and no QTL was detected for root length. The major difference between DH lines was Al tolerance with a major QTL being detected for root length and relative root length in the pH 4.2 + Al treatment. The QTL was located in the same position as that for root growth under acid soil conditions. The results indicate that, compared with low pH, Al toxicity is better for differentiating DH lines in this population. Since most of the current acid soil tolerant

varieties tolerate Al toxicity but not necessarily low pH, the search for germplasm tolerant to low pH may be the key for future breeding programs targeting acid soil tolerance.

A single gene controls tolerance to Al toxicity in the tolerant cultivar Br2

It is still not fully understood whether Al tolerance in plants is a quantitative or qualitative trait [12]. In some species such as rice [29–31], maize [32] and triticale [33], the tolerance has been identified as quantitative, while in other species such as wheat [34], pea [35] and chickpea [36], Al tolerance was inherited monogenetically. Within

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Fig. 6 DNA sequencing identified one SNP between two parents and DNA translation showed the SNP caused one amino acid transition between two parents

different genotypes in same species, the inheritance can differ [37]. Most studies have shown that Al tolerance in barley is controlled by a single gene [2, 17, 16]; however, there are some reports that suggest that Al tolerance in barley is a quantitative trait. For example, Navakode et al. [38] reported that Al tolerance was controlled by three QTLs located on chromosomes 2H, 3H and 4H. Two of these QTLs (on chromosomes 2H and 4H) were detected under 10 μM Al, while one was found on chromosome 3H at 20 μM Al [38]. In the present study, the scatter graph (Fig. 2) showed two distinct groups, indicating that Al tolerance in the tolerant cultivar Br2 was a qualitative trait. Our results support previous studies that suggest that only one gene is responsible for Al tolerance [17, 16]. Segregation distortion is a common phenomenon in barley which skews the frequency of alleles from their Mendelian expectations [39]. The distortion of markers in the region of the tolerance gene (Additional file 1) in this DH population caused the biased segregation ratio of the tolerant to the sensitive, which did not fit 1:1 ($\chi^2 = 16.23$).

QTL analysis and marker efficiency

Aluminium/acid soil toxicity is caused by excessive exposure to soluble toxic metallic elements and lack of sufficient essential elements in low pH conditions [14]. Bmac310 and Bmag353 are the most commonly-used SSR markers in barley acid soil studies. Raman et al. [40] reported that these two markers were tightly linked with the *Alp* locus. The authors also pointed out that Bmac310 and Bmag353 could be broadly used in marker-assisted selection for breeding [40]. Wang et al. [22] also validated a candidate gene *HvMATE* on chromosome 4H and found that markers ABG715, Bmag353, GBM1071, GWM165 and

HvGABP had complete linkage with the locus. The same gene was validated by Furukawa et al. [21]. Compared with the commonly-used SSR markers Bmac310 and Bmag353, the new marker Cit7 is more precise in explaining phenotypic variation under acid soil treatments (Table 1).

Single nucleotide polymorphism can affect gene function

After decades of studies on acid soil/Al tolerance in plants, several genes controlling Al tolerance have been detected, such as *TaALMT* in wheat [34], *ScAACT1* in rye [41], *AtALMT1* in *Arabidopsis* [42], *HvMATE* in barley [21] and *ZmMATE1* in maize [43]. However, it is still not clear which gene sequence variations affect gene expression. Sasaki et al. [44] reported that variation in the sequence upstream of *TaALMT* gene can affect gene expression. More recently, Fujii et al. [25] reported that one 1 Kb insertion upstream of the gene sequence was detected in some tolerant Asian accessions which was shown to have promoter activities. In contrast, Maron et al. [45] reported that the phenotypic variation between one Al-tolerant parent and one sensitive parent maize was caused by a different copy-number on the *ZmMATE1* gene.

In the present study, one SNP in the coding region of *HvMATE* was detected by the gene-specific marker Cit7 between the sensitive and tolerant cultivars and validated by DNA sequencing. In order to validate Cit7 in diverse germplasm, 56 other accessions (Additional file 5) collected from different parts of the world were used to conduct the association analysis. Result showed the polymorphism was significantly correlated with phenotypic variation under acid soil treatment ($P = 0.0057$, $R^2 = 0.129$). DNA translation identified one amino acid change between the sensitive and tolerant cultivars. However, the 1 Kb insertion in the

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upstream of the gene sequence for Al tolerance in some Asian tolerant accessions [25] was not detected in Br2. It is likely that the amino acid change affects gene function [46, 47], which has been confirmed in several studies [48, 49]. For example, Doyle and Amasino [48] reported one mutant clf-59, the protein of which contained one pro-to-ser amino acid transition in a cys-rich region. The mutant was reported to elevate levels of trimethylation on lysine 27 of histone H3 (H3K27me3) and repressed FLC (FLOWERING LOCUS C) during vernalization [48]. In barley, Yang et al. [50] validated that the Thr/Ala-233 and Ala/Ser-885 substitutions in the limit dextrinase gene were associated with enzyme thermostability using 60 barley genotypes from different parts of the world. More evidence is needed to prove its role in gene function.

Conclusions

In this study, one malting barley variety Br2 from Brazil was identified to be Al tolerance and the corresponding gene was validated to be *HvMATE* on chromosome 4H. Multiple sequence variations were identified and these novel variations provide rich resources for selection of tolerant alleles in the breeding programs. One gene-specific marker Cit7 was developed and validated to increase phenotypic variation efficiency explained by QTL. The marker could be used for marker-assisted selection in breeding of acid soil tolerant cultivars in the future.

Methods

Plant materials

A double haploid (DH) population derived from a cross of Hamelin/Br2 was generated from anther culture. This population consisted of 158 DH lines. The Al-sensitive female parent cultivar Hamelin is from the Western Australian barley breeding program and the male parent Br2 is a malting barley variety from Brazil. Preliminary screening demonstrated that Br2 tolerates acid soil. Additional fifty-six accessions were collected from different parts of the world for validation of the gene-specific markers (Additional file 5). This study does not involve humans, human data or animals.

Phenotyping for acid soil/aluminium toxicity tolerance

Soil method

Natural acid soil was collected from the 10–30 cm layer. Soil pH was 4.2 with soluble aluminium of 8.1 mg/kg. For the control treatment, lime was added to the same soil to adjust the pH to 6.5. Five seeds of each line were sown in pots containing acid soil or limed acid soil. Seedlings were removed from the soil for root length measurements one week after sowing. Root length was used as a parameter for acid soil tolerance. The experiment was conducted in a glasshouse and each sample was replicated three times.

Hydroponic method

Three hydroponic treatments were used to screen barley for acid/aluminium tolerance: (1) control treatment at pH 6.5, (2) acid treatment at pH 4.2, and (3) acid + aluminium treatment at pH 4.2 with 2 ppm aluminium. All three treatments contained the same nutrient solution with the following macronutrients (mM): $CaCl_2.2H_2O$, 4.0; $(NH_4)_2SO_4$, 0.1; KNO_3, 6.5; $MgCl_2.6H_2O$, 2.5; NH_4NO_3, 0.4 and the following micronutrients (μM): NaH_2PO_4, 13; $MnSO_4.H_2O$, 2; $CuSO_4.5H_2O$, 0.3; $ZnSO_4.7H_2O$, 0.8; H_3BO_3, 10; $Na_2MoO_4.2H_2O$, 0.1 and $FeSO_4.7H_2O$, 10. In the acid + aluminium treatment, Al was included as $AlCl_3.6H_2O$ at 74 μM (2 ppm Al). The solution pH was adjusted to either 6.5 or 4.2 using KOH or HCl and constantly monitored for pH changes.

Approximately 50 seeds of each line were placed in a 9 cm Petri-dish with 5 ml deionised (DI) water. Extra seeds of each line were included (approximately double) to ensure there were enough seeds with similar root lengths for the experiment. The Petri-dishes were wrapped in Clingfilm in bundles of 10–20 to incubate the seeds in the dark for 45 h at 4 °C, followed by 47–48 h at 18 °C. Germinated seeds were placed into allocated positions on the grid trays ensuring that roots were kept moist. The seeds were positioned so that the roots faced downwards into the nutrient solution. Seeds with root lengths between 4 and 7 mm were selected preferentially. Replicate 1 was sown on the first day and Replicate 2 on the second day. Germinated seeds were stored at 4 °C between sowing dates. The grid trays containing germinated seeds were placed into the three treatment solutions and grown at 20/15 °C (day/night) in a controlled environment room with a 12-h day length. All solutions bubbled gently with air stones and aquarium pumps to aerate the solutions and prevent stagnation. The pH of the solutions was checked daily and adjusted as necessary using either KOH or HCl. Dosing meters, which dosed acid or alkali, were also used to maintain pH at desired levels. Root lengths were measured after 7 days. The pH effect was calculated as the percentage root length reduction at pH 4.2 over pH 6.5. The Al effect was calculated as the percentage root length reduction at pH 4.2 + Al over pH 4.2.

Genotyping the DH population

Forty DH lines were initially selected for genotyping based on phenotypic data with each 20 DH lines representing the tolerant and susceptible groups using Diversity Arrays Technology (DArT) (http://www.diversityarrays.com; barley version 2.0 array). Three DH lines with poor data quality were not included in further analyses. In addition, 446 commonly-used SSR markers were synthesized using information from previous publications [51–60, 23] and used to screen polymorphic markers to map acid soil

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tolerance using all 158 DH lines from the Hamelin/Br2 population.

Forty-four primers (Additional file 3) were synthesized from contig_51011 containing the *HvMATE* gene from the International Barley Genome Sequencing Consortium (http://webblast.ipk-gatersleben.de/barley/viroblast.php) using Primer Premier 5.0 (Premier Biosoft International, Palo Alto, CA).

DNA extraction, PCR reaction and sequencing

Young leaves from two-week-old seedlings were cut with scissors and stored at −80 °C. Genomic DNA was extracted from young leaves using the phenol/chloroform method [50]. PCR products were loaded on 2 % agarose, 6 % polyacrylamide and 12 % SSCP/TBE gels, stained with ethidium bromide and visualized under UV light to detect polymorphisms in different cultivars. The SSCP method followed previously described procedures [61, 62] (acrylamide/bisacrylamide ratio of 37.5:1) in cold 0.5 × TBE and run at room temperature for 24–36 h. PCR products were sequenced directly in both directions using the Big-Dye™ Terminator method on an Applied Biosystems 3730 DNA Sequencer (SABC, Murdoch University, Western Australia) after purification with a QIAquick PCR purification kit (Qiagen).

Data analysis

Root length in acid soil, pH 4.2, pH 4.2 + Al, relative root length of pH 4.2 (root length under pH 4.2 divided by root length under pH 6.5), relative root length of pH 4.2 + Al (root length under pH 4.2 + Al divided by root length under pH 6.5) were used for the QTL analysis. Association of the markers with traits was calculated by PASW Statistics v.18 (SPSS Inc., Chicago, USA). Geneious software package Geneious v5.1 (http://www.geneious.com) and BioEdit (http://www.mbio.ncsu.edu/BioEdit) with default parameters were used for DNA sequence editing, comparison and alignment. Linkage map construction and QTL analysis were performed using QTL IciMapping Version 3.2 at LOD = 3.0 and Kosambi map unit function [63–66].

Availability of supporting data

All the supporting data are included in 5 additional files. DNA sequencing data is deposited in GeneBank with Accession Numbers: KT168175, KT168176 and KT168177.

Additional files

> **Additional file 1: Segregation distortion of markers in the region of Al tolerance gene on 4H;** * means significance at 0.05, ** means significance at 0.01. (DOCX 34 kb)
>
> **Additional file 2: Linkage map of DArT and SSR markers in Hamelin/Br2 DH population.** (PPTX 814 kb)

> **Additional file 3: Primers' position and coverage of *HvMATE* gene (Primers' names are highlighted in red).** F: forward primer position (bp), R: reverse primer position (bp). (PPTX 369 kb)
>
> **Additional file 4: SNP and INDEL of *HvMATE* gene in 6 barley cultivars.** (DOCX 48 kb)
>
> **Additional file 5: Fifty-six barley accessions and their origins.** (DOCX 37 kb)

Abbreviations

Al: Aluminium; ALMT1: Aluminium-activated malate transporter 1; AtBCB: Arabidopsis blue copper binding protein; parB: Tobacco glutathione S-transferase; NtPOX: Tobacco peroxidase; NtGDI1: Tobacco GDP-dissociation inhibitor; clf-59: Curly leaf; FLC: Flowering locus C; L: Leucine; V: Valine; SNP: Single nucleotide polymorphism; HvAACT: *Hordeum vulgare* aluminium-activated citrate transporter; HvMATE: *Hordeum vulgare* multidrug and toxin extrusion; TaALMT: Wheat aluminium-activated malate transporter; ScAACT1: *Secale cereal* aluminium-activated citrate transporter; AtALMT1: *Arabidopsis* aluminium-activated malate transporter; PCR: Polymerase chain reaction; DH: Doubled-haploid; DArT: Diversity arrays technology; SSCP: Single-strand conformation polymorphism; SNP: Single nucleotide polymorphism; bp: Base pair; Alt: Aluminium tolerance; Pht: Tolerance to low pH; Alp: Tolerance to aluminium and low pH; Alp3: Tolerance to aluminium and low pH 3; UTR: Untranslated region.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MB participated in DNA sequencing, new marker development, data interpretation and provided the initial draft of the manuscript. JX conducted initial SSR molecular marker and QTL analysis. XZ conducted DarT marker analysis and phenotyping. SB developed the DH population and phenotyping. GZ participated in data analysis and finalized the paper. MZ conducted phenotyping and data analysis. GPZ, DS and CL participated in experimental design, data interpretation and critical revisions of the paper. All authors read and approved the final manuscript.

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