A Genetic Analysis of Aluminium Tolerance in Cereals

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Summary

Aluminium (Al) toxicity is a major threat to agricultural production world wide wherever acid soil exists. Wheat and barley, the major food and feed crops, are severely affected and this necessitates investigations that could help to improve the yield by utilising the available genetic diversity for Al tolerance with the aid of several molecular platforms. We investigated the quantitative trait loci (QTL) conferring tolerance to Al toxicity in three different mapping populations of wheat and barley. Using a set of D genome (*Ae. tauschii*) introgression lines, a major Al tolerance locus was assigned to chromosome arm 4DL, explaining 31% of the phenotypic variation displayed by the population. A second major QTL was mapped to chromosome arm 3BL using a set of doubled haploid progeny lines. This major QTL, $Qalt_{CS}$.*ipk-3B*, originated from 'Chinese Spring' accounted for 49% of the variation in the population. The inheritance for Al tolerance in barley was dissected based on a genetic map constructed with genic markers. QTLs were identified on chromosomes 2H, 3H and 4H. A sequence homology search was used to derive the putative function of the genes linked to the QTL, in order to identify potential candidate genes for Al tolerance. Some of these candidates are implicated in stress/defence responses, in particular, stress signal transduction, transcription regulation factors and cell metabolism.

Key words

Aegilops tauschii, Expressed sequence tags, *Hordeum vulgare* L., Microsatellites, *Triticum aestivum* L.

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Introduction

Aluminium toxicity restricts crop productivity in acidic soils, which prevail over 30-40% of the world's arable surface (Von Uexkull & Mutert, 1995). It is the single largest limiting factor for about 38% of farmland in Southeast Asia, 31% in Latin America and 20% in East Asia, Sub-Saharan Africa, and North America (Wood et al., 2000). At a pH below 5, Al cations (especially the highly phytotoxic Al^{3+}) become readily soluble in soil water, inhibiting root growth with knock-on effects on water and mineral acquisition by the plant (Kochian et al., 2005).

Studies in wheat cultivars points that Al tolerance is under the control of a single dominant gene located on chromosome 4DL, Alt_{BH} gene in 'BH1146' and *Alt2* in 'Chinese Spring' ('CS') (Luo & Dvorak, 1996; Riede & Anderson, 1996). The evidence that more than one gene is present in some wheat cultivars includes the observation that the tolerance level is reduced in lines lacking several different chromosomes (Aniol, 1990; Ma et al., 2006), and from non-monogenic segregation ratios in populations bred from Al tolerant x sensitive crosses (Berzonsky, 1992; Zhou et al., 2007). The objectives of the first part of the present study were to map Al tolerance QTLs based on sets of plant material that have been derived from the cultivar 'CS', of Asian origin, using molecular markers (SSRs).

In case of barley, generally considered to be more sensitive to acidic and Al toxic soils than are wheat, rye, maize, rice or oats (Bona et al., 1993; Ishikawa et al., 2000) the pattern of inheritance has been explored by both QTL mapping and trisomic analysis, and has led to the identification of a major gene (*Alp*) located on chromosome 4H (Minella and Sorrels, 1997). This locus is known to condition Al tolerance in different genetic backgrounds as well (Raman et al., 2005; Wang et al., 2006). Minor gene effects for Al tolerance have also been reported by Raman et al. (2005) on chromosomes 3H, 4H, 5H and 6H. In the second part of the paper we document the results of QTL analysis based on the Oregon Wolfe Barley (OWB) mapping population. We exploit a genetic map populated with functional (genic) markers, in order to identify, via genetic linkage, potential candidate genes underlying the Al tolerance QTL.

Materials and methods

Plant materials

In wheat, two populations were investigated. The first series of experiments were focused on two sets of materials: (1) seven whole chromosome substitution lines, in which the individual chromosomes of the D genome donor ('Synthetic 6x') replaced their homologs in cv. 'CS' (Law & Worland, 1973). 'Synthetic 6x' was obtained from a cross of tetraploid emmer and wild grass *Aegilops tauschii* (*T. dicoccoides* var. *spontaneovillosum* x *Ae. squarrosa* ssp. *eusquarrosa*) (McFadden & Sears, 1947). Therefore, the D-genome substitution lines represent *T. aestivum* / *Ae. tauschii* replacements; and (2) a set of 84 introgression lines, each of which contain a specific sub-chromosomal segment of one *Ae. tauschii* chromosome in the homozygous state in a cv. 'CS' background. These lines were developed using the 'CS' ('Synthetic 6x') D-genome substitution lines and have previously been characterised at the genetic level by Pestsova et al. (2006).

The second series of experiments used a set of 57 doubled haploid progeny obtained from the cross between cv. 'CS' and a whole chromosome (3B) substitution line in a cv. 'CS' background ['CS'('Synthetic' 3B)]. The donor of the substituted chromosome was the same as that exploited to create the D genome introgression lines. This population was used to construct a microsatellite (SSR)-based linkage map from which the location of the QTLs associated with Al tolerance were derived.

The OWB mapping population is a set of 94 spring barley doubled haploid lines developed by pollinating the F_1 hybrid OWB_{DOM} x OWB_{REC} with *H. bulbosum* (Costa et al., 2001). Characteristics of OWB_{DOM} and OWB_{REC}, which were selected as dominant and recessive morphological marker stocks, are described by Wolfe & Franckowiak (1991).

Phenotypic evaluation

Al tolerance was assessed using both root tolerance index (RTI) and hematoxylin staining. The protocols adopted were modified slightly from those described by Hede et al. (2002). For the former, the seed was first surface sterilized in 3% w/v sodium hypochlorite for 5 min, rinsed thoroughly in distilled water and then laid on moist filter paper first for 84 h at 7°C, and subsequently for a further ca. 36 h at 24°C. Six seedlings with similar root lengths were transferred to a frame floating on an aerated standard nutrient solution (Nawrot et al., 2001) containing appropriate Al concentration and pH. Control seedlings were exposed to the same solution without Al at pH 6.5. The seedlings were maintained at 24°C, under a 16 h light/8 h dark regime. The nutrient solution was replaced daily to avoid fluctuation in pH and Al concentration. After 4 d, the lengths of the two longest roots of each seedling were measured, and the mean of these were used to calculate the RTI (given by the ratio of mean root length in the presence of Al (RLA) to mean root length in its absence (RLC) x100); scoring scheme: RTI <40%-sensitive, 40-49%-moderately sensitive, 50-65%-moderately tolerant, >65%-tolerant.

For the hematoxylin assay, ten seedlings per line were exposed to a nutrient solution lacking Al for 24 h, after which they were transferred for a further 24 h to a nutrient solution containing 80 µM Al, pH 4.5. The roots were washed thoroughly with distilled water and immersed in 0.2% w/v aqueous hematoxylin for 15 min. Excess dye was removed by washing and the seedlings were returned to nutrient solution lacking Al for 24 h. At the end of this period, root re-growth was observed. Seedlings with all roots showing continued root re-growth were rated as tolerant (T), those showing re-growth on some roots were rated moderately tolerant (MT) whereas those with no roots showing re-growth were rated sensitive (S). All experiments were replicated twice.

Wheat – substitution lines comparison, SSR marker and linkage analysis

The substitution lines were compared with 'CS' (control) statistically by ANOVA followed by Dunnett's t-test. For microsatellite marker analysis, DNA was isolated from leaves of 10-14 d old seedlings. PCR reactions and fragment detection were performed as described by Röder et al. (1998). Amplicons were separated by denaturing polyacrylamide electrophoresis using the short gel cassettes provided by the Automated Laser

Fluorescence (ALF) express. Fragment sizes were calculated by 'Fragment Analyser v.1.02' software. MAPMAKER software (Lander et al., 1987) was used to establish a linkage map, and recombination frequencies were converted to cM using the Kosambi mapping function (Kosambi, 1944). Linkage between the markers were declared by setting the logarithm of odd (LOD) threshold at 3.0 and maximum recombination fraction at 0.4. Goodness of fit between the expected and observed segregation ratios was tested by χ^2 analysis. QTL analysis was performed using the single marker and simple interval mapping options provided by QGENE software (Nelson, 1997). A LOD threshold of 3.0 was set to claim the QTL to be significant.

Barley - linkage analysis and function assignment

Stein et al. (2007) constructed a genetic map of barley based on markers developed from 1,032 expressed sequence tags (ESTs). About 650 of these markers were informative in the OWB population. The markers were designated GBR, GBM and GBS (Gatersleben barley RFLP, microsatellite and SNP, respectively). QTL mapping was performed using the single marker analysis of QGENE software (Nelson, 1997). The function of ESTs linked to the Al tolerance QTL was derived from a BLASTX search against the public non-redundant protein database NRPEP (June 2008 version, ftp://ftp.ncbi.nih.gov/blast/db/FASTA/nr.gz). Candidate homologs and orthologs were defined as those giving a high scoring pair and significant E-value. The nucleotide sequences of the barley ESTs are available in v1.5 of the IPK Crop EST database (http://pgrc.ipk-gatersleben.de/cr-est).

Results

Wheat cv. 'CS' / Ae. tauschii substitution and introgression lines

The parents 'CS' and 'Synthetic 6x', the seven D genome substitution lines and the whole set of 84 introgression lines were characterised in 30µM Al at pH 4.5. In the presence of Al stress, the RTI of 'CS' was 76% (scored as tolerant), whereas that of the other parent of the substitution lines ('Synthetic 6x') was only 53% (moderately tolerant) (Fig. 1). Among the substitution lines the least tolerant was 'CS' ('Synthetic 4D') which was followed by 'CS' ('Synthetic 5D'). Comparing the single lines with 'CS' (ANOVA followed by Dunnett's t-test) the most significant difference ($p<0.05$) was observed for chromosome 4D substitution line.

When the same set of lines was evaluated by hematoxylin staining under 80 µM Al and pH 4.5, both 'Synthetic 6x' and 'CS' ('Synthetic 4D') were classed as sensitive, but all the other substitution lines and 'CS' showed significant root re-growth.

QTL analysis was performed with the whole set of 84 introgression lines. A major QTL was associated with the SSR loci *Xgdm125* and *Xgwm976*, both mapping to the centromeric region of the long arm of chromosome 4D (Fig. 2).

This QTL was highly significant ($p<0.0001$) with LOD score 6.69 and explained about 31% of the phenotypic variation (PV) for RTI. The same QTL was observed for RLA but not for RLC. A root re-growth QTL was located in the same region, although with a higher LOD score (30.54) and PV (82%). The Al tolerance was inherited from the 'CS' parent in both methods.

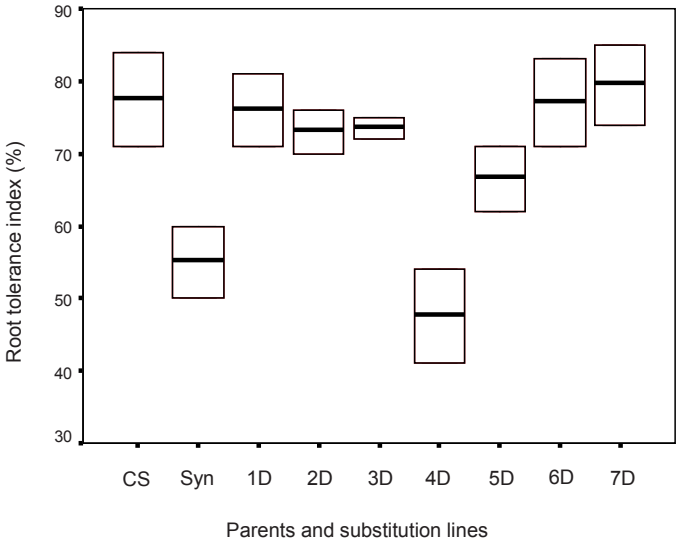

Figure 1. Compares RTI of 'Chinese Spring' ('CS') with 'Synthetic 6x' and the set of derived D genome substitution lines under 30µM aluminium, pH 4.5.

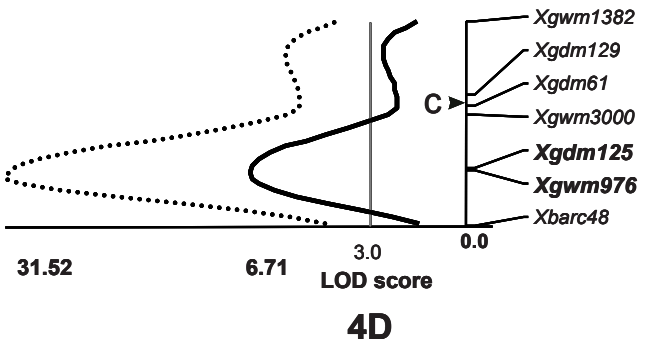

Figure 2. Profile for interval analysis along chromosome 4D based on RTI (bold lines) and hematoxylin staining (dashed lines). C: centromere. Closely linked markers are highlighted.

Wheat cv. 'CS' / 'CS (Synthetic 3B)' DH lines

Analysis of a set of 'CS/Synthetic 6x' single chromosome substitution lines (data not shown), figured out chromosome 3B as affecting Al tolerance. The 'CS' ('Synthetic 3B') substitution line had a lower tolerance (52%) than 'CS' (62%). The RTI of the population ranged from 48-70%. A set of doubled haploid progeny derived from the cross 'CS' ('Synthetic 3B') x 'CS' was exploited to construct a linkage map based on microsatellite markers.

Linkage map of chromosome 3B was constructed from the 14 informative SSR markers (Fig. 3). Five loci segregated in an expected 1:1 ratio, while remaining nine skewed in favour of 'CS'. A highly significant QTL ($p<0.0001$) was found to be associated with the markers *Xgwm1029* and *Xgwm1005* (mapping to the centromeric region of the long arm) for both RLA and RTI. The RTI QTL explained 49% of the phenotypic variance, with

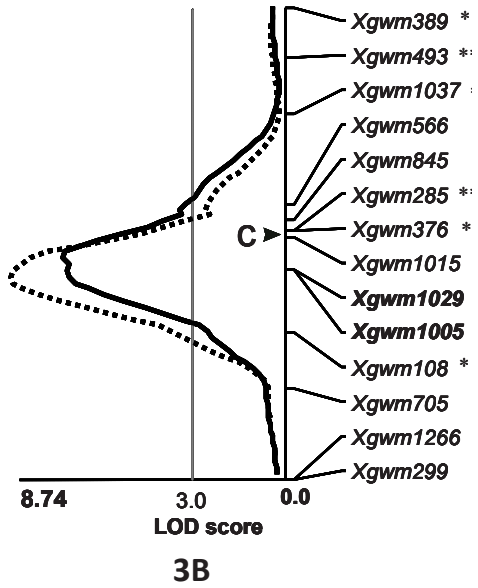

Figure 3. Profile for interval analysis along chromosome 3B for Root tolerance index (RTI). C: centromere. Regular and dotted lines represent the two separate replicates. Closely linked markers are highlighted. Segregation distortion of marker loci: * P<0.05; ** P<0.01; **** P<0.0001

the allele for tolerance originating from 'CS'. Since no RLC QTL was apparent at this location, this QTL appears to be specific for Al stress. This is the second major Al tolerance QTL detected in wheat and is designated as *Qalt*$_{CS}$*.ipk-3B*.

Barley OWB population

Parent OWB$_{DOM}$ had a higher RTI (83% at 10μM Al^{3+}, 68% at 20μM) than OWB$_{REC}$ (RTIs of 67% and 57%). Transgressive segregation was observed in the OWB population, with some progeny performing better than OWB$_{DOM}$, and a few worse than OWB$_{REC}$. The range in RTI among the members of the mapping population was 43-87% at the lower stress level (moderately sensitive to highly tolerant), and 36-86% at the higher level. Genetic analysis revealed that chromosomes 2H, 3H, and 4H all carry Al tolerance QTL. Distinct QTL were identified at each of the two Al concentrations (Tab. 1, Fig. 4).

The biological function of selected ESTs (Tab. 2) was derived mostly from rice, although a few were also available from wheat and from barley itself. These functions included several involved in the global response to either abiotic or biotic stress. They belonged to various functional groups, including stress/defence signal transducers, secondary messengers, transcription regulators, controllers of cell structure and biogenesis, and cell metabolism. Some encoded proteins were with unknown function.

Table 1. Single marker analysis for Al tolerance QTL

Marker	Chr.	Source allele	LOD score	R^2 value
10 μM Al, pH 4.7				
GBR441	4H	DOM	1.87	0.11
GBM1251	2H	REC	1.69	0.09
20 μM Al, pH 4.7				
GBM1233	3H	DOM	2.15	0.11
GBM1043	3H	DOM	1.69	0.09

Significant at $p < 0.01$

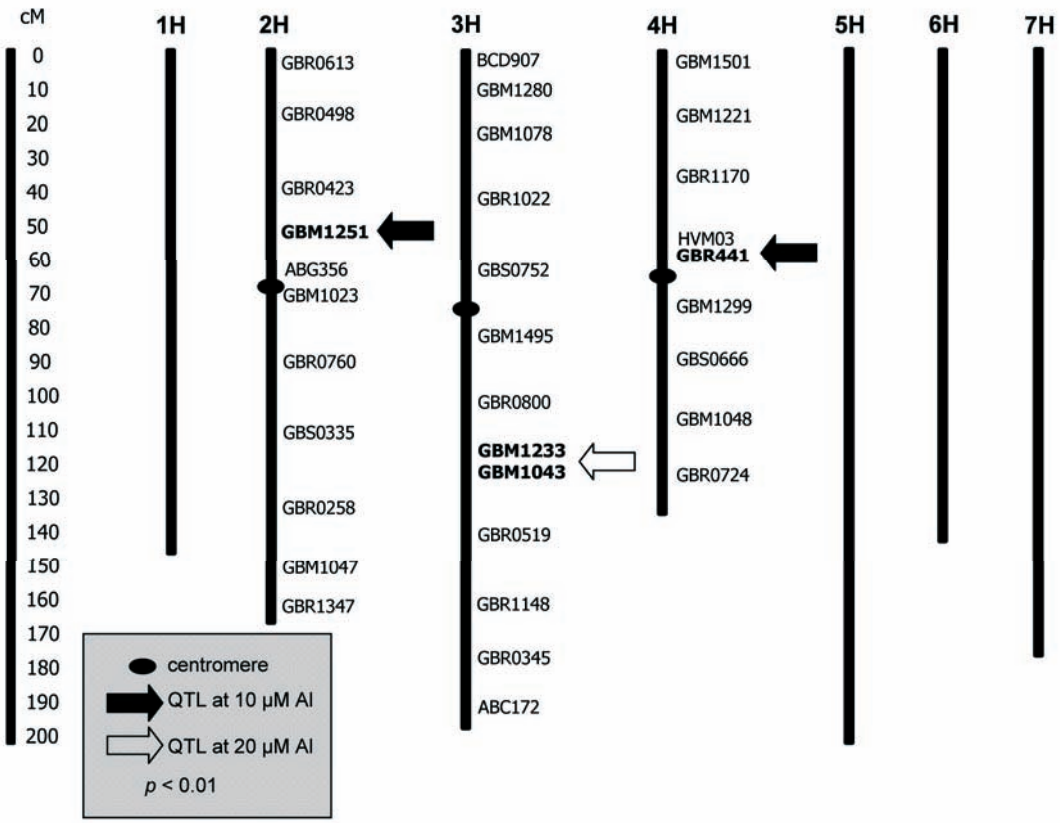

Figure 4.
Scheme of OWB barley linkage map, adopted from transcript map constructed by Stein et al. (2007).
Al tolerance QTL are indicated by arrows.

Table 2. Homology search for function of selected ESTs linked to Al tolerance QTL (further details see Navakode et al., 2009b)

EST-marker	Chr.	Functional annotation	Organism
GBR1306	2H	Peroxidase	*T. aestivum*
GBR1185	2H	Universal stress protein	*H. vulgare*
		Putative universal stress protein USP1	*O. sativa*
GBM1043	3H	Sedoheptulose-1,7-bisphosphatase, chloroplast precursor	*T. aestivum*
		Sedoheptulose-1,7-bisphosphatase precursor	*O. sativa*
GBM1452	4H	Zinc finger family protein, putative, expressed	*O. sativa*
GBR0026	4H	Leucine Rich Repeat, putative	*O. sativa*
		Protein kinase domain containing protein	*O. sativa*

Discussion

Wheat

Ae. tauschii, the D genome progenitor of hexaploid wheat, is generally considered as a source of novel genes to improve quantitative and qualitative traits (Börner et al., 2002; 2006). We investigated the influence of *Ae. tauschii* for Al tolerance by studying 84 introgression lines derived from the same on cv. 'CS' background. The poor performance of 'Synthetic 6x' compared to 'CS' implies that the accession of *Ae. tauschii* that donated the D genome appears to carry no positive factors for Al tolerance and/or the AB genomes of both parents are likely to be different. It is still a question, whether the negative alleles are harboured only by the accession in our study ('Synthetic 6x') or in the whole *Ae. tauschii* species originated from different areas.

A lower Al tolerance of 4D and 5D 'CS'/'Synthetic' substitution lines compared to 'CS', indicates that a tolerance gene(s) is present on the respective 'CS' D genome chromosomes and/or that the *Ae. tauschii* chromosomes carry a gene(s) promoting sensitivity to Al stress. By QTL analysis, the locus associated with Al tolerance was assigned to the long arm of chromosome 4D near the centromere tagged by SSR markers *Xgdm125* and *Xgwm976*, respectively, explaining 31% of the phenotypic variation (Navakode et al., 2009a). This locus was found to confer tolerance in Brazilian cv. 'BH1146' as well as in cv. 'Atlas 66' (U.S.A) (Riede & Anderson, 1996; Zhou et al., 2007). Since the introgression lines covered only the D genome, the rest of the variation, which is yet to be explained, may be contributed by the A and/or B genomes. Moreover, the probability of detecting QTL on chromosome 5D might not be possible due to the absence of polymorphic markers in those regions.

A second major Al tolerance QTL, novel in wheat, was mapped by analyzing the progenies derived from 'CS' x 'CS (Synthetic 3B)'. The QTL designated *QAlt$_{CS}$.ipk-3B* explained 49% of the phenotypic variation observed in the population. A QTL with minor effect was detected by Zhou et al. (2007) on 3BL in addition to a major QTL on chromosome 4D in a set of progeny derived from 'Atlas 66'. 'CS' and 'Atlas 66' are unlikely to be related to one another, as the former is a Chinese landrace, and the latter's pedigree ('Frondosa'//'Redhart3'/'Noll28') involves Brazilian cultivars. Thus *Qalt$_{CS}$.ipk-3B* in our study probably represents either a different allele of the 'Atlas 66' minor QTL, or is a distinct locus.

Barley

Although the OWB population displays a large amount of phenotypic variation, both parents are tolerant / moderately tolerant at both the higher and the lower Al concentration used here. The major part of the tolerance present in both parents appears therefore to be contributed by the same gene(s), and it is likely that the genetic basis for this tolerance is associated with the *Alp* locus on chromosome 4H. Thus, it was only possible in these experiments to detect minor QTLs controlling this trait, and these were located on three of the seven barley chromosomes (2H, 3H, and 4H) (Navakode et al., 2009b). The analysis of the transgressive segregants showed that the favourable alleles at the minor QTL were dispersed between the two parents, and this therefore offers the opportunity to improve the level of Al tolerance by marker assisted selection targeted at these QTL.

QTL for root elongation under Al stress have been located on chromosomes 3H, 4H, 5H and 6H (Raman et al., 2005), but the literature contains no previous report of an Al tolerance QTL mapping on chromosome 2H. The position of the 3H locus has also not been mapped before. The 4H QTL in this study was identified to be associated with markers on the short arm of chromosome 4H near the centromere. Probably this can be distinct from the major effect locus on 4HL but needs further validation in other populations since marker HVM03 in this study is included in a suite of markers linked with this trait on 4HL by Raman et al. (2002).

Candidate genes for Al tolerance have been identified and studied in several grass species. In our study, among the markers mapped within 5cM of the QTL on chromosome 2H, GBR1306 and GBR1185 were associated with plant stress response. Stress proteins, such as the rice 'universal stress protein' and its barley ortholog(s) (GBR1185) may play an important regulatory role in the global plant stress response. The peroxidases (GBR1306 homolog in wheat) are a large family of enzymes, triggered by several biotic and abiotic stresses, which act to protect cells against oxidative damage caused by reactive oxygen species. A role for the peroxidases in countering Al stress is possible, since Al^{3+} induces oxidative damage and lipid peroxidation in plant tissues (Maron et al., 2008). Regarding the 3H QTL markers, the sequence of GBM1043 is highly homologous to the wheat and rice gene encoding the Calvin cycle enzyme sedoheptulose-1,7-biphosphatase (SBPase), the increased production of which has been reported to enhance photosynthesis and growth during early plant development (Lefebvre et al., 2005).

The significant genes associated with the chromosome 4H QTL were GBM1452 and GBR0026. The zinc finger family (rice ortholog of GBM1452) includes one member that has been shown to be essential for both acid and Al tolerance in *Arabidopsis thaliana* (Iuchi et al., 2007). Several genes have been identified as being implicated in cell wall structure and composition under Al stress (Maron et al., 2008). In the case of GBR0026, the rice ortholog encodes a protein containing leucine-rich repeats (LRRs), which have been reported to be associated with gene regulation or signalling during cell wall biosynthesis (Xu et al., 2008).

Conclusion

Our investigation reveals the inheritance of Al tolerance genetics in wheat and barley as polygenic. Markers tagging the wheat QTLs could be exploited for marker assisted selection or introgression for improving Al tolerance, in particular the 3B QTL, *Qalt$_{CS}$.ipk-3B*, which is novel in wheat. Most of the candidate genes for Al tolerance in barley, as revealed by homology search, were associated with stress responsive factors. They need further validation through physiological experiments.

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