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Validation of quantitative trait loci for aluminum tolerance in Chinese wheat landrace FSW

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Abstract Aluminum (Al) toxicity is one of the major constraints for wheat production in acidic soils worldwide and use of Al-tolerant cultivars is one of the most effective approaches to reduce Al damage in the acidic soils. A Chinese landrace, FSW, shows a high level of tolerance to Al toxicity and a mapping population of recombinant inbred lines (RILs) was developed from a cross between FSW and Al-sensitive US spring wheat cultivar Wheaton to validate the quantitative trait loci (QTL) previously identified in FSW. The mapping population was evaluated for net root growth (NRG) during Al stress in a nutrient solution culture and hematoxylin staining score (HSS) of root tips after Al stress. After 132 simple sequence repeat (SSR) markers from three chromosomes that were previously reported to have the QTLs were analyzed in the population, two QTLs for Al tolerance from FSW were confirmed. The major QTL on chromosome 4DL co-segregated with the Al-activated malate transporter gene (*ALMT1*), however, sequence analysis of the promoter region (Ups4) of *ALMT1* gene indicated that FSW contained a marker allele that is different from the one that was reported to condition Al tolerance in the Brazilian source. Another QTL on chromosome 3BL showed a minor effect on Al tolerance in the population. The two QTLs accounted for about 74.9% of the phenotypic variation for HSS and 72.1% for NRG and demonstrated an epistatic effect for both HSS and NRG. SSR markers closely linked to the QTLs have potential to be used for marker-assisted selection (MAS) to improve Al tolerance in wheat breeding programs.

Keywords Chinese landrace • aluminum tolerance • simple sequence repeats • QTL mapping

Introduction

Aluminum (Al) toxicity is a major limiting factor for crop production in acidic soils worldwide. When soil pH is lower than 5, exchangeable Al^{3+} is released into the soil solution, inhibiting normal root growth and function (Samac and Tesfaye 2003) and, therefore causes a significant reduction in crop yield. Over 40% of the world's potentially arable lands are acidic (von Uexküll and Mutert 1995; Bot et al. 2000) with up to 60% of them in developing countries (Kochian et al. 2005). Due to extensive crop production, the area of acidic soils is quickly increasing (Guo et al. 2010). Although irrigation or application of lime to acidic soils can increase soil pH to relieve Al toxicity, the high cost associated with transportation of lime to destination limits widespread adoption of this practice. Fortunately, significant genetic variation in Al tolerance has been reported in wheat (Stodart et al. 2007; Zhou et al. 2007a; Hu et al. 2008), and growing Al-tolerant cultivars is the most cost-effective approach to improve wheat production in acidic soils.

Inheritance of Al tolerance in wheat has been extensively studied especially from Brazilian source such as BH1146 and Atlas 66 (Kochian et al. 2005; Samac and Tesfaye 2003; Tang et al. 2002, Ma et al. 2005). However, results on number and locations of genes/QTLs for Al tolerance in wheat are still equivocal. Several studies indicated that Al tolerance in wheat was under monogenic control (Raman et al. 2005; Riede and Anderson 1996), whereas others suggested that multiple genes might be involved in enhancing Al tolerance in some wheat genotypes (Berzonsky 1992; Cai et al 2008; Zhou et al. 2007b). Also, the Al tolerance in Asian accessions might not be

64 the same as that from Brazilian sources (Hu et al. 2008; Raman et al. 2008; Zhou et al.
65 2007a). For example, a Chinese wheat landrace, FSW, showed a similar level of Al
66 tolerance to Atlas 66 as measured by hematoxylin staining, but it had a different
67 haplotype pattern for the markers derived from *ALMT1* (Hu et al. 2008), a gene
68 encoding an Al-activated malate transporter cloned from the Brazilian source (Sasaki
69 et al. 2004). In addition, different genetic backgrounds may affect expression of
70 tolerance genes that are from the same source. In Atlas 66, a QTL on chromosome
71 4DL was mapped in both populations of Atlas 66/Century and Atlas 66/Chisholm, but
72 a minor QTL on chromosome 3BL was detected only in Atlas 66/Chisholm (Ma et al.
73 2005; Zhou et al. 2007b).

74 Malate release from root tips has been considered as the major mechanism of Al
75 tolerance in wheat (Sasaki et al. 2004). The major QTL on 4DL cosegregated with
76 *ALMT1* in several populations (Ma et al. 2005; Raman et al. 2005; Sasaki et al. 2004).
77 Several markers (ALMT1-CAP, SSR3a, and SSR3b) were developed from the
78 gene-coding region for marker-assisted selection (MAS) of the 4DL QTL (Raman et
79 al. 2006). However, these markers were only effective in some crosses but not others
80 (Zhou et al. 2007b). A new marker has been developed from the promoter region of
81 *ALMT1* and reported as a diagnostic marker for Al tolerance on 4DL (Sasaki et al.
82 2006; Raman et al. 2008). In FSW, QTLs were initially mapped on 4DL, 3BL and 2A
83 in a population from a cross between FSW and a Chinese line ND35 (Cai et al. 2008).
84 However, these QTLs have not been validated in other populations. The objectives of
85 this study were to validate, in FSW, the effect of Al tolerance QTL that have been

previously identified in other sources, to investigate haplotype patterns of *ALMT1* marker alleles and to develop high-throughput PCR-based markers for MAS of Al tolerance in wheat breeding programs.

Materials and methods

Plant materials and evaluation of Al tolerance

A mapping population of 217 F_6 recombinant inbred lines (RILs) was derived from the cross FSW / Wheaton by the single-seed-descent method. FSW is an Al-tolerant landrace from China, and Wheaton is an Al-sensitive cultivar from Minnesota, U.S.A.

To evaluate Al tolerance of the RILs, wheat seeds were germinated on wet filter papers in petri dishes at 4°C for 72 h. Three germinating seeds with similar appearance were transferred onto a nylon wire net on open bottom of a plastic cup. A plastic cup holder was used to support the cups floating on deionized water at room temperature (20-23°C) with a 16 h photoperiod using fluorescent lights. Two bubble rods in the water connected to an air pump provided aeration during the culture period. After 48 h of hydroponic culture, the deionized water was replaced with nutrient solution (pH 4.0) consisting of 4 mM $CaCl_2$, 6.5 mM KNO_3, 2.5 mM $MgCl_2.6H_2O$, 0.4 mM NH_4NO_3, 0.1 mM $(NH_4)_2SO_4$, and 0.36 mM $AlK(SO_4)_2.12\ H_2O$. Reactions of parents and RILs to Al stress were evaluated by measuring root growth during Al stress and degree of hematoxylin staining of Al-treated root tips. The principal root of each seedling was measured twice after two days of hydroponic culture and

three-days of Al treatment in nutrient solution to calculate root length difference between the two measurements as net root growth (NRG). Root hematoxylin stain measures the Al amount that entered into plant roots during Al treatment and has been widely used to measure plant Al tolerance (Ma et al, 2005; Polle et al. 1978). After the second measurement of root length, excess Al^{3+} on the root surface was rinsed off in de-ionized water for 1 h, with three replacements. Clean roots were then submerged in a hematoxylin solution containing 0.2% hematoxylin (w/v) and 0.02% (w/v) $NaIO_3$ for 15 min, followed by rinsing the roots with de-ionized water three to four times. The stained root tips of each stained seedling were visually scored as hematoxylin stain score (HSS) using a 1-3 grading scale: no stain on root tips as 1, lightly stained as 2, and heavily stained as 3 (Ma et al. 2005). The experiments were repeated twice with three and four replicates (cups), respectively, using a randomized complete block design. In each experiment, an additional replication was used as control in which the culture solution did not contain any Al^{3+}.

After hematoxylin staining, wheat seedlings were grown in a greenhouse for one week to harvest leaf tissue of each seedling for DNA isolation. Leaf tissue was collected in a 1.5-mL tube and dried in a freeze drier (Thermo Fisher, Waltham, MA, USA) for 2 d. Tubes containing dried tissue were shaken at 25 times/s for 4 min in a Mixer Mill (Retsch GmbH, Haan, Germany) with a 3.2 mm stainless steelbead in each tube.

Marker analysis

Genomic DNA was extracted using the Cetyltrimethyl ammonium Bromide (CTAB) method (Saghai-Maroof et al. 1984). A total of 132 pairs of SSR primers from the chromosomes that were previously reported to have QTLs for Al tolerance were selected to screen parents (Cai et al. 2008; Zhou et al. 2007b; Ma et al. 2005) and polymorphic primers were further analyzed in the F_6 RIL population.

For SSR analysis, a 10-μL PCR mixture contained 40 ng of template DNA, 2.5 mM $MgCl_2$, 200 μM each of dNTPs, 50 nM of forward tailed primer, 100 nM of reverse primer and 50 nM of M13 fluorescent-dye labeled primer, 1×PCR buffer, 1 U of *Taq* polymerase. A touchdown PCR program was used for PCR amplification, in which the reaction mixture was incubated at 95 ℃ for 5 min, then continued for 5 cycles of 1 min of denaturing at 96 ℃, 5 min of annealing at 68 ℃ with a decrease of 2 ℃ in each subsequent cycle, and 1 min of extension at 72 ℃ . For another 5 cycles, the annealing temperature started at 58 ℃ for 2 min with a decrease of 2 ℃ for each subsequent cycle. Then, reactions went through an additional 25 cycles of 1 min at 96 ℃, 1 min at 50 ℃, and 1 min at 72 ℃ with a final extension at 72 ℃ for 5 min. PCR products were analyzed in an ABI PRISM 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

Data analysis

Marker data collected from the ABI DNA Analyzer were further processed using GeneMarker version 1.5 (SoftGenetics LLC, State College, PA, USA) and rechecked twice manually for accuracy. Genetic linkage groups of SSR markers were

constructed using JoinMap3.0 (Van Ooijen and Voorrips 2001). Recombination frequencies were converted into centiMorgans (cM) using the Kosambi function (Kosambi 1944). The threshold value of logarithm of odd (LOD) score was set at 3.0 to claim linkage between markers with a maximum fraction of recombination at 0.4. WinQTLCart2.5 (Wang et al. 2007) was used for QTL mapping. Genome-wide LOD threshold values for declaring a significant QTL at $P<0.05$ were obtained by running 1,000 times of permutations separately for NRG and HSS traits (Doerge and Churchill 1996). Analysis of variance, heritability of Al tolerance traits and determination coefficient (R^2) were calculated using SAS system Version 9.1 (SAS Institute, Inc., 2003, Cary, NC, USA).

Results

Variation in root responses of RILs and their parents to Al stress

The roots of FSW were longer (3.3 cm) than those of Wheaton (0.6 cm) after 72 h of hydroponic culture in a nutrient solution containing 0.36 mM Al^{3+}. After 3 d of Al treatment, the root tips of Wheaton were fully stained by hematoxylin (grade 3), whereas those of FSW were not stained (grade 1). In non-Al controls, Wheaton and FSW showed similar root lengths and hematoxylin stain scores. Therefore, the Al concentration used in this study was appropriate for differentiating the tolerant genotypes from the sensitive genotypes by measuring either NRG or HSS.

The frequency distribution of NRG of the RILs under Al stress was continuous

with the major peak toward Wheaton (Fig. 1). A similar distribution was observed for HSS (Fig. 2). A highly significant correlation coefficient ($r=0.87$, $P<0.01$) was observed between NRG and HSS in the mapping population. The correlations between untreated root length and NRG, HSS were low ($r=0.21$ and 0.20, respectively) and not significant in the RIL population. Therefore, NRG and HSS were independent of variation in root growth under non-Al-stressed conditions among RILs. Variance analysis showed that the effects of RILs were significant in both NRG and HSS (Table 1). Heritability was high for both NRG (0.88) and HSS (0.87), and thus, only a few genes may be involved in Al tolerance in the population.

QTL for Al tolerance in FSW

After 132 SSR primers were screened, 35 were polymorphic between parents and further analyzed in the F_6 RIL population. A total of 24 markers were mapped in the 3 linkage groups spanning 138.7 cM of genetic distance. The first group had 9 SSRs spanning 41.8 cM on chromosome 3BL, the second had 12 SSRs spanning 88.2 cM on chromosome 4DL, and the third had only 3 SSRs spanning 8.7 cM on chromosome 2A. These three linkage maps were used for further QTL analysis. Interval mapping identified two QTLs for Al tolerance on chromosomes 4DL and 3BL. The QTL on 4DL showed a major effect on both NRG and HSS, whereas the QTL on 3BL had a minor effect on NRG and HSS (Fig. 3). The QTL on 4DL co-segregating with *Xwmc331* was flanked by the markers *Xups4* and *Xgdm125*, with R^2 values of 65.7% for NRG and 70.1% for HSS and LOD values 57.8 for NRG and 64.9 for HSS. The

196 QTL on 3BL was flanked by the markers *Xbarc344* and *Xbarc164*, with R^2 values of
197 3.7% for NRG and 2.7% for HSS, with LOD value 7.8 for NRG and 6.7 for HSS
198 (Table 2).

199 To analyze the effect of the two QTLs on Al tolerance, the closest markers
200 *Xwmc331* on 4DL and *Xbarc344* on 3BL were selected to represent the two QTLs
201 (Fig. 4). Four possible combinations of the two QTLs are: 4DL+/3BL+, 4DL+/3BL-,
202 4DL-/3BL+, 4DL-/3BL-, in which 4DL+ and 3BL+ represent Al tolerance marker
203 alleles of QTLs from 4DL and 3BL of FSW, respectively, and 4DL- and 3BL-
204 represent corresponding Al-sensitive marker alleles from Wheaton. Mean
205 comparisons of these genotype combinations indicated that combination of these two
206 QTLs increased NRG by 2.6 cm and decreased HSS by 1.8 relative to the genotype
207 with Al-sensitive haplotype of the marker alleles on both 4DL and 3BL. In the
208 presence of the Al-tolerance marker allele on 4DL, the 3BL marker allele associated
209 with Al tolerance increased NRG by 1 cm, whereas it only increased about 0.2 cm
210 without the 4DL marker allele linked to Al tolerance. Similarly, the tolerance allele on
211 3BL decreased by 0.6 in HSS in the presence of 4DL allele and very little when
212 marker allele associated with Al tolerance on 4DL was absent (Fig. 4). These two
213 QTLs for Al tolerance appeared to have epistatic effect on NRG and HSS.

214 Two *ALMT1*gene markers, *Xups4* and *Xssr3a*, were polymorphic between the two
215 parents and they were used to analyzed the RILs. *Xups4* amplified two different sizes
216 of amplicons between Al-tolerant FSW and sensitive Wheaton. The size of 471 bp
217 allele was associated with Al-tolerant genotypes, whereas the 440 bp allele was

associated with Al-sensitive genotypes in the population. The correlation coefficient of the *Xups4* allele with HSS and NRG were 84% and 83%, respectively, in the RIL population. *Xssr3a* amplified a 225 bp fragment in FSW and a 223 bp fragment in Wheaton. The correlation coefficient of the Xssr3a allele with HSS and NRG are 83% and 82%, respectively, in the RIL population. *Xssr3b* did not amplify any alleles in two parents and the RILs, and thus it was not analyzed further.

Discussion

Evaluation of Al tolerance

Al tolerance of wheat is usually evaluated in acidic soils under field conditions. Inconsistent phytotoxicity and pH value among the plots may induce significant environmental variations (Ma et al. 2005). Thus field tests may not provide consistent results for the proper comparison. An alternative method for evaluating Al tolerance using hydroponic culture provides a strict control in nutrient solution containing a toxic level of Al and pH, and can provide non-destructive measurements in large populations. Therefore, it has been widely used in genetic studies (Polle et al. 1978; Ma et al. 2006; Guo et al. 2007; S. Navakode et al. 2009). With this method, net root growth of Al-stressed seedling has been measured to reflect plant tolerance to Al toxicity in several studies (Parker and Pedler 1998; Zhou et al. 2007a). Hematoxylin staining can measure the extent of Al accumulation in root cells and has been widely used to evaluate Al tolerance in several crops (Delhaize et al. 1993; Cancado 1999; Anas 2000). In this study, both NRG and HSS were used to measure Al tolerance of parents and the RIL mapping population. A high correlation between the two traits was observed ($r=0.87$, $P<0.01$).

The two parents showed a large contrast in NRG and HSS. Significant variations in NRG and HSS were observed among the RILs with high heritability of both measurements. QTL for HSS and NRG were mapped on the same chromosome positions. The two QTLs on 4DL and 3BL together accounted for about 74.9 % of the

248 phenotypic variation for HSS and 72.1% for NRG. Results suggested that both HSS
249 and NRG were reliable measurements for the mapping study of Al tolerance.
250
251 Inheritance of Al tolerance in wheat
252 Wheat is the best-characterized species and genetic system for analyzing Al tolerance
253 (Kochian et al. 2004). Several studies that used the Brazilian sources of tolerance such
254 as BH1146 and Atlas 66 postulated that Al tolerance segregated as a single dominant
255 locus. Riede and Anderson (1996) first mapped the gene as Alt_{BH} on 4DL of BH1146
256 using restricted fragment length polymorphism and concluded that this gene was fully
257 responsible for Al tolerance in BH1146. Ma et al. (2005) identified a QTL for Al
258 tolerance on the same chromosome region of Atlas 66 using a RIL population from
259 Atlas 66/Century. However, several other studies suggested that at least two loci
260 might be involved in Al tolerance in Atlas 66 (Garvin and Carver 2003; Tang et al.
261 2002; Zhou et al 2007b). Several studies using wheat genetic stocks including
262 deletion lines, nullitetrasomics, and ditelosomics also supported multigenic controls
263 of Al tolerance (Aniol and Gustafson 1984; Aniol 1990; Ma et al. 2006; Papernik et al.
264 2001). In this study, two QTLs on 4DL and 3BL were identified, which agrees with
265 Zhou et al. (2007b). In that study, the minor QTL on 3BL of Atlas 66 accounted for
266 11.1% of the phenotypic variation for HSS and 8.6% for NRG. Cai et al. (2008) used a
267 population developed by crossing FSW to a Chinese dwarf line ND35 and reported
268 that the QTL on 3BL showed a major effect on Al tolerance with R^2= 47.0 % for HSS
269 and 41.7% for NRG. However, the QTL showed a much smaller effect on Al tolerance

in the Wheaton background (R^2=2.7% and 3.7% for HSS and NRG, respectively) in this study although the QTL on 3BL in this study was mapped on the same chromosome region as that reported by Cai et al. (2008). This 3BL QTL was detected in different sources of Al-tolerant germplasm and same source in different genetic backgrounds, and therefore is more likely a 'real' QTL. However this QTL appears to be less stable than the one on 4DL. The minor QTL for HSS and NRG on chromosome 2A reported by Cai et al. (2008) was not detected in this study although the markers linked to the QTL reported by Cai et al. (2008) were polymorphic in the current population. It is also possible that other minor genes may be involved in Al tolerance in the population because only three previously reported chromosome regions were screened in this study.

Marker allele for *ALMT1* in FSW

ALMT1 on 4DL has been considered a major contributor to Al tolerance in several germplasm lines (Raman et al. 2005; Ma et al. 2005; Sasaki et al. 2004; Zhou et al. 2007b) and it has been used as a major Al tolerance gene in MAS in breeding programs where Al tolerance is a major breeding objective. Raman et al. (2005) studied the structure and chromosomal location of *ALMT1* and concluded that Al tolerance in a diverse range of wheat genotypes is primarily conditioned by *ALMT1*.

In this study, the QTL with the largest effect on Al tolerance in FSW was also mapped to a similar location as that in Atlas 66 (Ma et al. 2005; Zhou et al. 2007b). *ALMT1* as represented by *Xups4* was also mapped in the QTL region in FSW that

292 confirmed the previous report (Cai et al. 2008). Interestingly, *Xwmc331* was the
293 closest marker for the QTL, not *Xups4*, and *Xgdm125* and *Xups4* flanked the QTL for
294 both traits, which agrees with Cai et al. (2008) who mapped the major QTL between
295 *Xgdm125* and *Xups4* in FSW/ND35 population. *Xups4* is a sequence upstream from
296 *ALMT1* in wheat.

297 Sasaki et al. (2006) further investigated the promoter structure of *ALMT1* and
298 concluded that expression of Al tolerance is mainly conditioned by the variation in
299 promoter size. The germplasm that amplified large fragments (706 to 1229 bp) by
300 *Xups4* from the promoter region of *ALMT1* were considered Al tolerant whereas the
301 germplasm that amplified 469 bp or smaller fragments were considered to be sensitive
302 to Al stress. In this study, FSW amplified a 471 bp amplicon, a sensitive allele based
303 on Sasaki et al. (2006), but showed Al tolerance. However, Sasaki et al. (2006) also
304 noticed that Japanese lines showed a weak correlation between *ALMT1* expression
305 and Al tolerance. This suggested that the mechanisms of Al tolerance in FSW might
306 be different from that of the Brazilian source. FSW may have a different mechanism
307 in regulating expression of *ALMT1* or the *ALMT1* promoter may not be the key
308 molecular regulator for the *ALMT1* expression in FSW. It is also possible that some
309 other factors may be involved in the control of malate efflux in addition to the level of
310 *ALMT1* expression (Sasaki et al. 2006; Raman et al. 2005).

311 All three 4DL markers (*Xwmc331*, *Xups4* and *Xgdm125*) that were polymorphic in
312 FSW/Wheaton population were mapped in the QTL region showing a very large
313 effect on Al tolerance. *Xwmc331* and *Xgdm125* are SSR markers and suitable for

high-throughput analysis, and therefore, they can be used for MAS. *Xups4* is a gene marker, and should be the best marker for MAS. Al tolerant FSW amplified a smaller fragment of *Xups4* (453bp after removal of a 18bp M13 tail) that was considered the allele associated with Al-sensitivity (Sasaki 2006) in Brazilian sources. Therefore the amplicon size of *Xups4* cannot be used as the only selection criterion for the 4DL QTL resistance allele. However, it still is an informative marker for the 4DL QTL if it is polymorphic in a breeding population. Previously, a cleaved amplified polymorphism (CAP) marker has been used as diagnostic marker for the 4DL QTL in marker-assisted breeding for Al tolerance (Zhou et al. 2007b; Ma et al. 2005), but it requires an additional step of restriction digestion after PCR amplification (Raman et al. 2006). Thus, it can be replaced with *Xwmc331* or *Xups4* when FSW is used as an Al-tolerant source.

In a summary, two QTLs for Al tolerance previously mapped in other populations were confirmed in a new FSW population. The major QTL on chromosome 4DL co-segregated with the Al-activated malate transporter gene (*ALMT1*), but it was a different allele from the one previously reported to condition Al tolerance, was identified in FSW. Another QTL on chromosome 3BL showed a minor effect on Al tolerance in the population. The two QTLs accounted for about 74.9% of the phenotypic variation for HSS and 72.1% for NRG. DNA markers closely linked to the QTLs should be useful for MAS to improve Al tolerance in wheat breeding programs.

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Figure 1

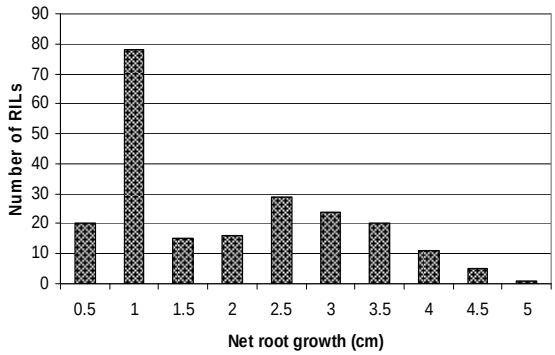


Fig. 1 Frequency distribution of net root growth (NRG) for 217 F6 recombinant inbred lines from the cross FSW/Wheaton after 72 h of Al stress. Arrows point to mean NRG for parents FSW (right) and Wheaton (left).

Figure 2

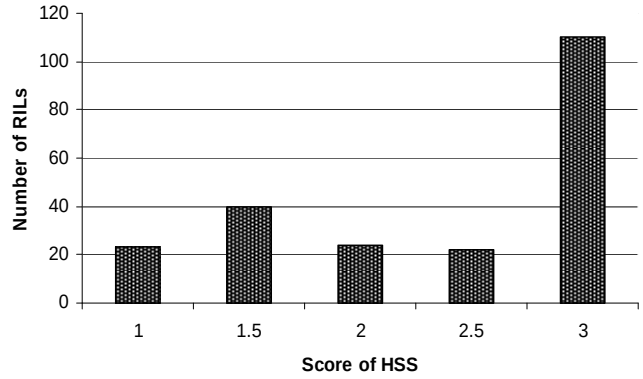
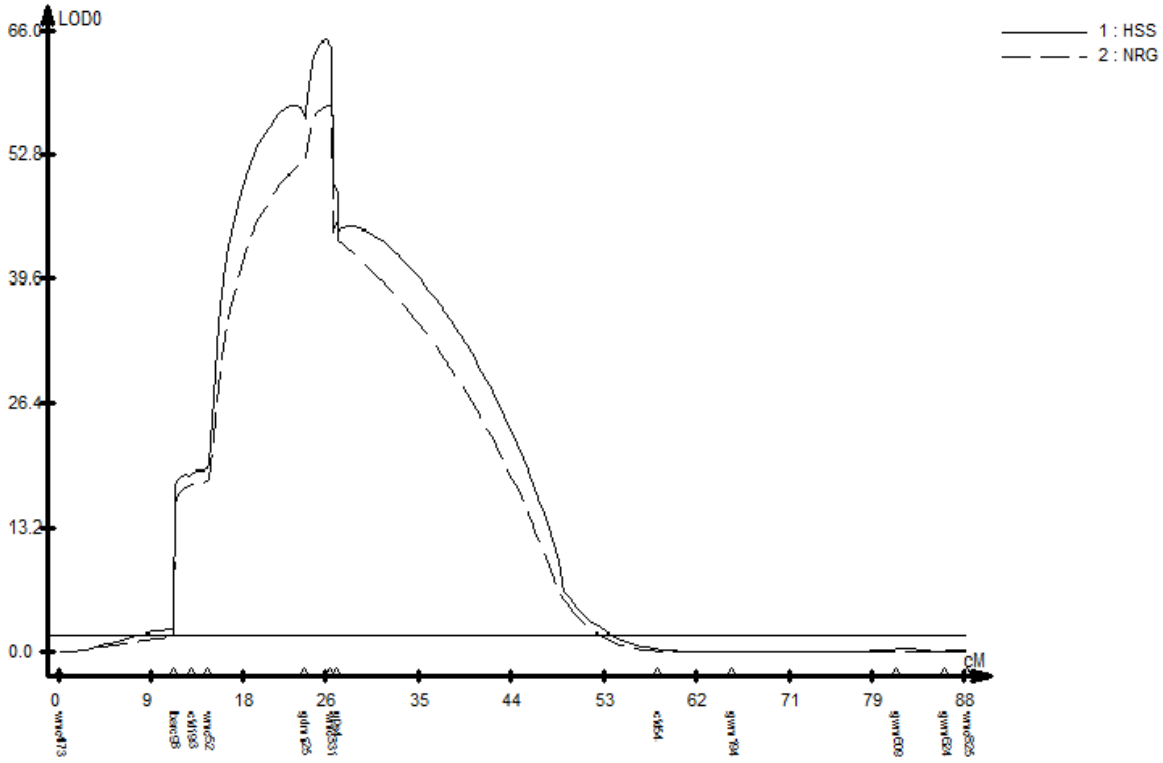


Fig. 2 Frequency distribution of hematoxylin stain score (HSS) for RILs from the cross FSW/Wheaton after 72 h of Al stress. Arrows point to mean HSS of HSS for parents Wheaton (right) and FSW (left).

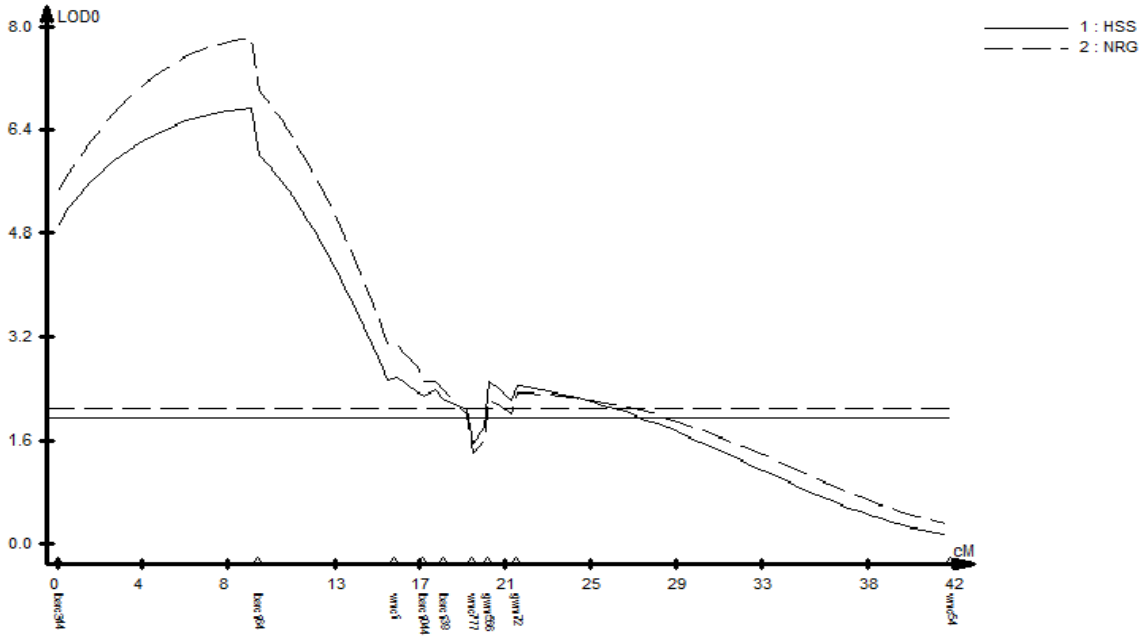
463 **Figure 3 A**



464

465 **Fig. 3** A. Major QTL on 4DL and B. a minor QTL on 3BL.

466 **Figure 3 B**



467

Figure 4

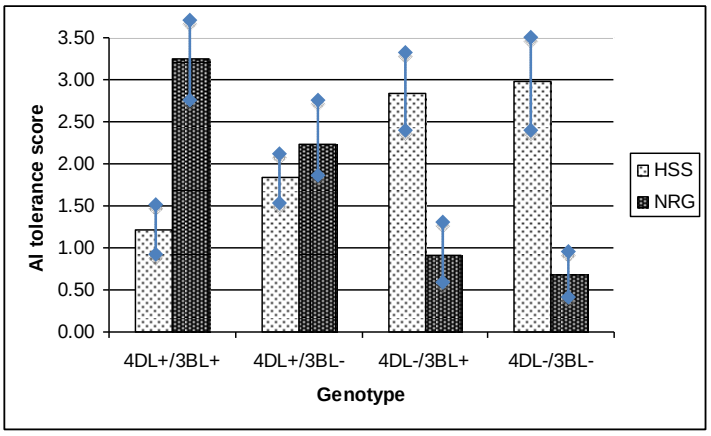


Fig. 4 Effect of 4DL and 3BL QTLs on Al tolerance in RIL population from the cross FSW/Wheaton. 4DL+ and 3BL+ represent Al resistant marker alleles of the QTLs from 4DL and 3BL of FSW respectively, and 4DL- and 3BL- represent Al sensitive marker alleles of the two QTLs from Wheaton, respectively. NRG and HSS represent net root growth (cm) and hematoxyin staining score, respectively. Lines are standard deviations.

Table 1 Variance components and heritability for net root growth (NRG) and hematoxylin stain score (HSS) in the recombinant inbred population derived from the cross FSW/Wheaton

Source	DF	SS	MS	F-value	h^2
NRG					
Experiment	1	97.39		97.39	298.89**
RILs	216	1929.00	8.93	27.41**	0.88
Experiment*RILs	216	236.69	1.10	3.36**	
Error	1057	344.41	0.33		
Total	1490	2607.39			
HSS					
Experiment	1	2.87	2.87	17.49**	
RILs	216	892.45	4.13	25.21**	0.87
Experiment *RILs	216	120.26	0.56	3.40**	
Error	1059	173.56	0.16		
Total	1492	1189.14			

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