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ORIGINAL PAPER

Genetic diversity for aluminum tolerance in sorghum

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Abstract Genetic variation for aluminum (Al) tolerance in plants has allowed the development of cultivars that are high yielding on acidic, Al toxic soils. However, knowledge of intraspecific variation for Al tolerance control is needed in order to assess the potential for further Al tolerance improvement. Here we focused on the major sorghum Al tolerance gene, Alt_{SB}, from the highly Al tolerant standard SC283 to investigate the range of genetic diversity for Al tolerance control in sorghum accessions from diverse origins. Two tightly linked STS markers flanking Alt_{SB} were used to study the role of this locus in the segregation for Al tolerance in mapping populations derived from different sources of Al tolerance crossed with a common Al sensitive tester, BR012, as well as to isolate the allelic effects of Alt_{SB} in near-isogenic lines. The results indicated the existence not only of multiple alleles at the Alt_{SB} locus, which conditioned a wide range of tolerance levels, but also of novel sorghum Al tolerance genes. Transgressive segregation was observed in a highly Al tolerant breeding line, indicating that potential exists to exploit the additive or codominant effects of distinct Al tolerance loci. A global, SSR-based, genetic diversity analysis using a broader sorghum set revealed the presence of both multiple Alt_{SB} alleles and different Al tolerance genes within highly related accessions. This suggests that efforts toward broadening the genetic basis for Al tolerance in sorghum may benefit from a detailed analysis of Al tolerance gene diversity within subgroups across a target population.

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Introduction

The genetic basis governing variation in plant tolerance to aluminum (Al) toxicity has been extensively described in the literature (Aniol and Gustafson 1984; Borgonovi et al. 1987; Magnavaca et al. 1987; Khatiwada et al. 1996; Garvin and Carver 2003; Kochian et al. 2004) and also has been exploited in plant breeding efforts to generate crops adapted for agriculture on the large areas of acidic soils throughout the world.

Inheritance patterns consistent with the segregation of one or two major Al tolerance genes have been described in wheat (Kerridge and Kronstad 1968; Camargo 1981), a species in which the cultivar BH1146 harbors a major Al tolerance gene, Alt_{BH}, that was

mapped to the long arm of chromosome 4D and was found to control nearly 85% of the phenotypic variation for Al tolerance (Riede and Anderson 1996). Interestingly, although the repeated detection of Al tolerance loci on chromosome arm 4DL from wheat accessions such as BH1146 (Riede and Anderson 1996), Chinese Spring (Aniol and Gustafson 1984; Luo and Dvorak 1996) and Atlas (Ma et al. 2005) suggests that functional Alt_{BH} alleles are widespread in wheat, an observed decrease in tolerance associated with the loss of other chromosome arms (Aniol and Gustafson 1984; Aniol 1990; Papernik et al. 2001) points toward some complexity for Al tolerance in this species. A likely explanation for these results is associated with the physiological mechanism of Al tolerance in wheat that is based on the chelation of Al in the rhizosphere by malate, which is released from root apices in response to Al stress, thus preventing the metal from reaching sensitive sites within the root (Delhaize et al. 1993a, b; Ma et al. 2001). The gene underlying this Al tolerance mechanism has been found to encode for an Al activated malate transporter (*ALMT1*, Sasaki et al. 2004) and is likely to correspond to the Alt_{BH} locus (Raman et al. 2005). Papernik et al. (2001) found that three ditelosomic lines of Chinese Spring exhibited decreased Al tolerance relative to the euploid parent, and this was due to the loss of genes on different chromosome arms (including 4DL) independently influencing Al-activated root malate release, rather than controlling distinctly different Al tolerance mechanisms. Additionally, the very Al tolerant wheat cultivar Atlas 66 appears to harbor at least one more Al tolerance gene in addition to Alt_{BH} (Camargo 1981; Tang et al. 2002), and others might also be present in its genome (Berzonsky 1992; Ma et al. 2005). Therefore, the studies in wheat indicate the presence of a single major Al tolerance gene, Alt_{BH}, with the involvement of at least one other gene at an as yet unidentified mapping location. Also, there may be other minor genes acting epistatically to Alt_{BH} in the pathway leading to Al-activated malate release, in addition to the possible involvement of genetic background effects in the expression of the wheat Al tolerance phenotype (Johnson et al. 1997).

The Alt_{BH} gene also seems to be a major component of Al tolerance throughout the grass family, as comparative studies have suggested that allelic variation at putatively orthologous loci contribute to Al tolerance in barley (chromosome 4H, Tang et al. 2000), oat (linkage group F, Wight et al. 2006) and rice (chromosome 3, Wu et al. 2000; Nguyen et al. 2003). Resulting from multiple translocations, the long arm of rye chromosome 4 where a major Al tolerance gene, *Alt3*, was detected (Aniol and Gustafson 1984; Miftahudin et al. 2002, 2005) comprises a proximal segment with homoeology to the short arms of the wheat group 7 chromosomes whereas the distal end of 4RL shows homoeology with the short arms of the wheat group 6 chromosomes (Devos et al. 1993). However, Devos and coworkers found that an RFLP marker located on the long arm of the wheat homoeologous group 4 chromosomes including 4DL, which harbors Alt_{BH}, is located on 4R rather than 7R. This indicates that a small portion of the wheat 4L chromosomes remain on 4R rather than on 7R. Because the *Alt3* locus in rye is tightly linked to *Xbcd1230* (Miftahudin et al. 2005), as is Alt_{BH} on wheat 4DL, the putatively orthologous gene series located on wheat 4DL, barley 4H, oat linkage group F and rice chromosome 3 possibly comprises rye *Alt3*. In addition, a functional *ALMT1* homolog was recently found in the dicot, *Arabidopsis*, which also employs Al-activated root malate exudation as a major Al tolerance mechanism (Hoekenga et al. 2006; Magalhaes 2006).

In sorghum, we have recently mapped the major Al tolerance gene, Alt_{SB}, to the terminal region of chromosome 3 in a population derived from the Al tolerance standard, SC283 (Magalhaes et al. 2004), and Alt_{SB} was also found to confer Al tolerance in a second sorghum line of distinct origin, SC566. While Alt_{SB} appears to be distinct from the putatively orthologous series at Alt_{BH}, a major Al tolerance QTL that has been repeatedly detected on rice chromosome 1 across widely different genetic backgrounds (Wu et al. 2000; Nguyen et al. 2001, 2002, 2003; Mao et al. 2004), in addition to Al tolerance gene(s) on rye 3R (Aniol and Gustafson 1984; Ma et al. 2000), are possibly orthologous to Alt_{SB} in sorghum (see detailed comparative analysis in Magalhaes et al. 2004). Overall, comparative studies indicate that Al tolerance in plants is largely influenced by putatively orthologous series of at least two major loci that are inherited as major Al tolerance genes in wheat and sorghum. However, the detection of QTL in apparently non-conserved positions in the genomes of maize (Sibov et al. 1999; Ninamango-Cárdenas et al. 2003), rice (Wu et al. 2000; Nguyen et al. 2001, 2002, 2003; Mao et al. 2004) and *Arabidopsis* (Kobayashi and Koyama 2002, Hoekenga et al. 2003; Kobayashi et al. 2005), as well as evidence for additional Al tolerance genes on rye 6RS (Aniol and Gustafson 1984; Gallego and Benito 1997; Gallego et al. 1998a, b) and 7RS (Matos et al. 2005), indicate that other novel Al tolerance genes may also play a role in plant Al tolerance.

Sorghum is a highly diverse species and the patterns of genetic diversity in sorghum have been found by

marker analysis to be mostly influenced by racial and geographic origins (Tao et al. 1993; Deu et al. 1994, 2006; Oliveira et al. 1996; Agrama and Tuinstra 2003). Particularly, the thorough RFLP analysis of a highly diverse sorghum core collection recently published by Deu et al. (2006) indicated the existence of varying levels of diversity within specific morphological races, suggesting that the quest for new, agronomically useful variability for important agronomic traits such as Al tolerance may profit from genetic diversity information across a target population.

Focusing on the Alt_{SB} gene, the objectives of the current study were to (i) investigate the range of intraspecific variation for Al tolerance control in a panel of sorghum lines that encompasses different sorghum morphological races and collection sites, (ii) clarify the role of allelic variation at Alt_{SB} on the expression of the Al tolerance phenotype, (iii) assess the distribution of functional Alt_{SB} alleles across a diverse germplasm set and make inferences regarding possible patterns of common Alt_{SB} ancestry in related accessions. For the first two objectives, a genetic analysis was undertaken with two sequence-tagged site (STS) markers tightly linked to Alt_{SB}, whereas objective (iii) involved an analysis of genetic diversity using an expanded sorghum panel.

Materials and methods

Genetic stocks

Table S1 (electronic supplementary material, ESM) details a panel of 47 sorghum accessions including elite inbred lines, landraces, wild species and breeding derivatives that were used for the genetic diversity study. The 12 lines in bold in Table S1 were used for the genetic analysis of Al tolerance. With the exception of the two Al tolerant lines, SC283 (primary source of Alt_{SB}) and SC566, and the sensitive line, BR007, which were previously used to map Alt_{SB} (Magalhaes et al. 2004), the genetic control of Al tolerance was not known for the other nine lines. Thus, eight lines were crossed to a common Al sensitive tester, BR012, and a single F1 plant derived from each cross was self-pollinated to generate different F_2 populations. Individual F_2 plants for CMS225 × BR012 were self-pollinated for progeny testing of Al tolerance on $F_{2:3}$ families. An additional F_2 population, BR012 × BR007, was also generated to study the genetic nature of the higher levels of Al tolerance in BR012 relative to the sensitive standard BR007. To study the phenotypic effects of the Alt_{SB} alleles from BR012, 3DX and CMS225 on Al tolerance, BC_3(F5)-derived near-isogenic lines (NILs) were developed taking the two Al tolerant parents as donors and the Al sensitive line BR012 as the recurrent parent.

Hydroponic analysis of Al tolerance

Inhibition of seminal root growth elicited by Al in nutrient solution was used to quantify Al tolerance, using the basal nutrient solution described in Magnavaca et al. (1987) at pH 4.0. Seeds were gently sand-scarified inside cloth bags, surface sterilized with 0.525% NaOCl for 10 min under continued stirring and finally rinsed eight times with 18 mΩ H_2O. Seeds were then allowed to germinate for 4 days on moistened germination paper rolls in a growth chamber with 27°C day and 20°C night temperatures, a light intensity of 330 μmol photons m^{-2} s^{-1} and a 12-h photoperiod. The seminal roots from the seedlings were inserted through the mesh bottoms of polyethylene cups placed into polyethylene containers filled with 8.5 l of nutrient solution under continuous aeration (49 seedlings/container).

Al tolerance analysis on inbred lines and near-isogenic lines

An Al toxicity dose response curve using total Al concentrations of 0, 60, 110, 148 and 222 μM was generated with the 12 lines in bold in Table S1, to define the level of Al to be used in the genetic studies. These concentrations correspond to free Al^{3+} activities of {0}, {11}, {20}, {27} and {39} μM Al^{3+} (brackets indicate Al^{3+} activity), respectively, as estimated with the speciation software program, GEOCHEM-PC (Parker et al. 1995). The Al activity of {27} μM Al^{3+} detected a wide continuum of Al tolerance across this highly diverse subset, which contained standards for Al tolerance and sensitivity, and was thus chosen to screen an expanded sorghum set. The expanded sorghum panel of 47 accessions listed in Table S1, including the same 12 lines that were subjected to the Al dose response curve, was thus screened for Al tolerance at a single Al activity of {27} μM Al^{3+} for 5 days. Seven lines that did not show root growth inhibition at this Al activity (5DX, ATF14, CMS225, SC283, CMS227, CMS226, SC566) along with additional lines used for comparative purposes, were subsequently screened at {37} and {58} μM Al^{3+} at pH 4.2. Experiments for evaluating Al tolerance of inbred lines used a completely randomized design with three replications and

seven plants per replication. Seedlings were given a 48-h acclimation period in nutrient solution lacking Al ({0} μM Al^{3+}), after which the *i*nitial *l*ength of each seedling's root growing in *c*ontrol solution (*ilc*) was measured. The solution was then replaced by nutrient solution of identical composition but containing either no Al or Al supplied as $AlK(SO_4)_2{\cdot}12H_2O$ at the desired final Al activities. *F*inal root *l*engths under *Al* treatment (*flAl*) or *c*ontrol solution (*flc*) were obtained after 5 days of exposure to Al. For each inbred line, *r*elative percent values of *n*et *r*oot *g*rowth inhibition (*RNRG*) at each Al activity of {11}, {20}, {27} and {39} μM Al^{3+} relative to the control root growth in {0} μM Al^{3+} were estimated by dividing the net root growth under Al treatment (*flAl–ilc*) by the net root growth without Al (*flc–ilc*).

Al tolerance analysis with segregating populations

Different levels of Al tolerance were observed among the parents when subjected to increasing levels of Al toxicity in nutrient solution. Thus, Al activities for screening the segregating populations were chosen based on the growth response curves of the parents to maximize the phenotypic differences between the various tolerant sources and the sensitive tester BR012. Aluminum activities that caused up to 30% root growth inhibition (RNRG ≥ 70%) for the tolerant parents and at least 45% (RNRG ≤ 55%) for BR012 were used for screening the respective segregating populations. Hence, the BR012-derived populations with SC112 and IS8577 were screened at {20} μM Al^{3+}, the populations with SC175, SC549, 5DX, 9DX and 3DX as the tolerant parent were screened at {27} μM Al^{3+}, and {39} μM Al^{3+} was used for the BR012 × CMSXS225 cross. For the cross BR012 × BR007, {11} μM Al^{3+} elicited only a 13% root growth inhibition (RNRG) in BR012 while inhibiting the sensitive standard BR007 by 55%, and was thus selected for screening this derived segregating population. Due to the genetically heterogeneous nature of individuals within F_2 populations, an independent control lacking Al cannot be employed. Thus, intrinsic root growth rates in the absence of Al were assessed during a 24-h growth period in control solution, on an individual plant basis. Accordingly, after the 4-day germination period, seedlings were allowed to acclimate in control nutrient solution lacking Al for 24-h, at which time the *i*nitial *l*ength of each seedling's root in *c*ontrol solution (*ilc*) was measured. *F*inal *l*engths in *c*ontrol solution (*flc*) were recorded 24-h later, following replacement of the control nutrient solution with a solution of identical composition but containing Al. *F*inal root *l*engths under *Al* treatment (*flAl*) were obtained after 5 days of exposure to Al. The degree of *r*oot *g*rowth inhibition caused by Al over the 5-day exposure period *r*elative to the control root growth was calculated as RRG (% relative root growth) = $[(\text{flAl} - \text{flc})_{5d}/(\text{flc} - \text{ilc})_{1d} \times 5] \times 100$

DNA isolation and marker analysis

Genomic DNA was isolated from approximately 500 mg of leaf tissue from inbred lines and F_2 individuals for each segregating population using the protocol described by Saghai-Maroof et al. (1984). As part of a positional cloning effort to isolate the Alt_{SB} gene, we have identified two STS markers designated CTG29 (CTG29F: 5′-HEX-ATGCAGTATCTGCAGTATCA TTT and CTG29R: AATCCGTCAGGTCAGCAATC) and M181 (M181F: 5′-6FAM-AAGGCAA CAACTG AGGCACT, M181R: TCGCTAGAGTG GTGCAA GAA), which flanked the Al tolerance gene at genetic distances of 0.5 and 0.1 cM, respectively (the linkage distances were estimated with 2,085 F_2 individuals derived from a cross between SC283 and BR007; data not shown). These markers were used for the genetic analysis of Al tolerance in a multiplex format according to the following protocol. In a 20 μl PCR reaction, 1.75 pmol of each primer, 0.5 mM dNTP, 1 U of Taq polymerase; 20 mM Tris–HCl (pH 8.4); 50 mM KCl; 2 mM $MgCl_2$ and 30 ng of genomic DNA were used. Amplifications proceeded with an initial denaturation step of 95°C for 1 min followed by 30 cycles at 94°C for 30 s, 55°C for 1 min, 72°C for 1 min, and a final extension step at 72°C for 5 min. The reactions were diluted tenfold with ultrapure water and 2 μl were mixed with 1 μl of formamide HI-DI (Applied Biosystems), 0.5 μl of loading buffer (50 mg/ml blue dextran; 25 mM EDTA) and 0.2 μl of size standard (GS500 ROX, Applied Biosystems). The mixture was denatured at 95°C for 5 min and kept on ice until 2 μl of each reaction mixture were loaded onto a 5% (p/v) polyacrylamide gel (Long Ranger Gel Solution, Cambrex) containing 6 M urea. Electrophoresis was carried out on an ABI377 Prism sequencer (Applied Biosystems) at 3,000 V for 1.5 h in 1× TBE buffer.

For the genetic diversity study, three multiplex sets containing five SSR loci each (Dean et al. 1999), were used following the same PCR protocols described above, using 30 ng of DNA and 2.5 pmoles of each primer. These SSRs have been found to be inherited in a Mendelian fashion and are distributed throughout nine of the ten sorghum chromosomes (see chromosomal locations on Dean et al. 1999 and mapping positions for some SSR loci on http://www.sorgblast3.tamu.edu,

linkage maps), providing a comprehensive coverage of the sorghum genome. Only one SSR (Sb5-236) is located on sorghum chromosome 3, but it is not linked to Alt_{SB}.

Statistical analysis of Al tolerance data

One-way analysis of variance (ANOVA) of RNRG data at each Al activity followed by the Scott–Knott test (Scott and Knott 1974) were undertaken to cluster the accessions into homogeneous groups of RNRG means. For the NIL set and the parents, statistical significance for all pairwise RNRG differences was estimated by the Tukey procedure (Tukey 1953).

Linkage analysis

Individual Chi-square tests for goodness-of-fit to a 1:2:1 segregation ratio on F_2 populations were performed for the STS markers CTG29 and M181 for each mapping population. Marker-trait associations were analyzed by single-factor ANOVA of the RRG values (F_2 populations) or RRG means ($F_{2:3}$:CMS225:BR012) using the three marker genotypic classes as a classification variable. For each population, the portion of the phenotypic variance for Al tolerance explained by the markers was assessed by estimating the R^2 values of linear regression analyses between the marker genotypic classes and the RRG data.

Genetic diversity analysis

Dice similarity coefficients (S_D, Dice 1945) among all pairs of accessions were determined as $S_D = \frac{2v_{ij}}{2v_{ij}+w_{ij}+x_{ij}}$, where v_{ij} is the number of bands shared between both accessions, w_{ij} is the number of bands present in accession i and absent in accession j and x_{ij} is the number of bands absent in accession i and present in accession j. Genetic dissimilarities were calculated as $d_D = 1 - S_D$ (Nei and Li 1979). Associations among accessions were estimated using the UPGMA method (Sneath and Sokal 1973) and support for hierarchical clustering was estimated by 10,000 bootstrap resampling steps. Polymorphism information content (PIC) values were calculated as $PIC = 1 - \sum^n f_i^2$, where f_i^2 is the squared frequency of the ith allele.

Results

Genetic control of Al tolerance

A root growth dose response curve for Al^{3+} activities ranging from {11} to {39} μM for the nine sorghum lines used for the genetic analysis of Al tolerance, as well as the parents of our mapping populations used in Magalhaes et al. (2004) (SC283, SC566, and BR007), revealed a high degree of phenotypic variation for Al tolerance (Table 1). Using an Al tolerance threshold of 70% RNRG, groups of sorghum lines with homogeneous and discrete patterns of tolerance and sensitivity to Al were uncovered as the Al activity in the nutrient solution increased (see standard errors of the means in Table S2). The response curve was effective in detecting phenotypic variation for Al tolerance in the panel as the root growth of the Al sensitive standard BR007 was severely inhibited at the lowest Al^{3+} activity ({11} μM) whereas root growth in the tolerant lines SC283 and SC566 remained unaffected after exposure to {39} μM Al^{3+}.

When the tolerant lines in Table 1 (other than SC283 and SC566) were crossed to BR012, which was used as the Al sensitive common parent, the frequency distributions for RRG values observed across F_2 populations did not exhibit clear discontinuities (data not shown) as previously observed for the F_2 populations derived from BR007 × SC283 and BR007 × SC566 (Magalhaes et al. 2004). To study the influence of the

Table 1 Root growth response curve to different Al^{3+} activities for the sorghum lines that were crossed with BR012 for the genetic analysis of Al tolerance, as well as for the parents of our initial Al tolerance mapping populations (SC283, SC566, and BR007)

Sorghum lines	RNRG(%)			
	{11} μM Al^{3+}	{20} μM Al^{3+}	{27} μM Al^{3+}	{39} μM Al^{3+}
BR007	45 a	21 a	16 a	9 a
BR012	***87 b***	53 b	35 b	20 a
IS8577	***113 b***	***79 c***	54 b	29 a
SC112	***106 b***	***82 c***	45 b	18 a
SC549	***100 b***	***91 c***	***74*** c	50 b
3DX	***116 b***	***95 c***	***70*** c	52 b
9DX	***99 b***	***82 d***	***70*** c	63 c
5DX	***125 b***	***113 d***	***96*** d	56 b
SC175	***105 b***	***100 d***	***85 c***	***78 c***
CMS225	***94 b***	***109 d***	***109 d***	***82 c***
SC566	***123 b***	***104 d***	***105 d***	***98 d***
SC283	***103 b***	***114 d***	***112 d***	***108 d***
CV(%)	14.3	8.6	15.2	20.0

Percent Relative Net Root Growth (RNRG) values are the means of three replications (seven plants per replication). Numbers in italic indicate Al activities beyond which a 70% RNRG tolerance threshold was broken (RNRG dropped below 70%), except for highly tolerant lines that sustained RNRG means of ~80 to 100% at the highest Al activity of {39} μM Al^{3+}. Lines whose RNRG means are followed by the same lower-case letters within each of the four Al activities constitute homogeneous RNRG groups by the Scott–Knott test ($P < 0.05$). Standard error of the means for these data are shown in Table S2. CV: coefficient of variation

BR012 background on the shape of the frequency distribution for RRG, we subsequently tested an F_2 population derived from BR012 × SC283 for Al tolerance in nutrient solution (Figure 1). A χ^2 analysis for goodness-of-fit to a 3 (Al tolerant):1(Al sensitive) model using the 40–50% RRG class as a threshold (Figure 1) indicated that the frequency distribution was also mostly influenced by the segregation of a single major gene ($\chi^2 = 0.037$, $P[\chi^2 \geq 0.037] = 0.85$) as previously observed for BR007 × SC283 (Magalhaes et al. 2004). Thus, the genetic background of the sensitive tester (BR012 vs. BR007) did not have a major influence on the lack of bimodal segregation patterns in crosses with SC283.

Because clear discontinuities in the RRG frequency distributions were not observed for most of the BR012 crosses, Al tolerance was treated as a quantitative trait for mapping purposes. The markers CTG29 and M181, which were previously found to flank Alt_{SB} at distances of 0.5 and 0.1 cM respectively, were subsequently used to elucidate the role of the Alt_{SB} locus on Al tolerance in the different Al tolerant sources depicted in Table 1. Significant marker-trait associations were found for all mapping populations except for those derived from SC112 and 5DX (Table 2), indicating that Alt_{SB} plays a role in controlling Al tolerance in most of the sorghum lines that were studied. However, the *P* value for linkage for F_2:BR012 × SC112 strongly suggested that gene(s) distinct from Alt_{SB} contributed to Al tolerance in SC112. Although not significant at 1 and 5%, the *P* value for F_2:BR012 × 5DX was considerably lower than that observed for F_2:BR012 × SC112, advising caution when ruling out the involvement of a weak Alt_{SB} allele on Al tolerance in 5DX. The portion of the phenotypic variance explained by the markers ranged from 9% for F_2:BR012 × 9DX up to 79% for $F_{2:3}$:BR012 × CMS225. A striking 60% increase of the R^2 value was observed upon progeny testing of the F_2:BR012 × CMS225, reaching almost $R^2 = 80\%$ when the derived $F_{2:3}$ families were analyzed (Table 2). A considerable decrease of the experimental error when RRG was expressed in terms of family means was probably responsible for the higher R^2 value in $F_{2:3}$ families. This demonstrates the lack of precision for estimates of the amount of the phenotypic variance for Al tolerance explained by the markers, which is often underestimated when the phenotype is based on single F_2 plant measurements of Al-induced root growth inhibition in nutrient solution.

As shown in Table 1, RNRG for BR012 was significantly higher than that of BR007 at all activities up to {27} μM Al^{3+} and tended to be superior even at the highest Al level, where root growth in both sensitive lines was dramatically inhibited. To investigate the genetic nature of this superior Al tolerance in BR012, an F_2 population derived from BR012 × BR007 was

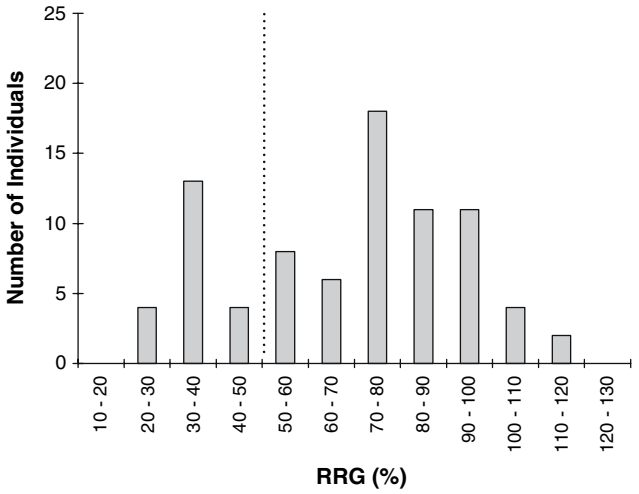

Fig. 1 Percent relative root growth (*RRG*) frequency distribution for F_2 progeny derived from a cross of BR012 × SC283. Eighty-one F_2 individuals were grown in nutrient solution containing {27} μM Al^{3+} for 5 days. RRG mean values and standard errors were 88 ± 3.93 for SC283 ($n = 40$) and 29 ± 1.61 for BR012 ($n = 21$). Each interval depicted in the *x*-axis comprises progeny with RRG conforming to lower limit ≤ progeny RRG ≤ upper limit. The *dashed line* depicts a threshold for Al sensitivity (RRG ≤ 50%) and tolerance (RRG > 50%)

Table 2 Single-marker analysis for M181 and CTG29 and percent relative root growth (RRG)

Population	*n*	Marker	*F*	*P*(<)	R^2
F_2:BR012 × 3DX	100	M181	5.42	0.0059	0.10
	100	CTG29	5.42	0.0059	0.10
F_2:BR012 × 5DX	87	M181	1.55	0.2186	–
	87	CTG29	1.55	0.2186	–
F_2:BR012 × 9DX	88	M181	4.41	0.0150	0.09
	84	CTG29	5.57	0.0054	0.12
F_2:IS8577 × BR012	90	M181	6.98	0.0015	0.13
	88	CTG29	7.11	0.0014	0.14
F_2:BR012 × SC175	96	M181	12.38	0.0000	0.21
	96	CTG29	12.74	0.0000	0.21
F_2:BR012 × SC112	84	M181	0.66	1.0000	–
	82	CTG29	0.97	1.0000	–
F_2:BR012 × SC549	91	M181	11.99	0.0000	0.21
F_2:BR012 × BR007	98	M181	18.79	0.0000	0.28
F_2:BR012 × SC283	75	CTG29	7.08	0.0016	0.16
F_2:BR012 × CMS225	87	CTG29	41.77	0.0000	0.50
$F_{2:3}$:BR012 × CMS225	45	CTG29	79.32	0.0000	0.79

F_2 individuals for each cross were grown in nutrient solution using the Al^{3+} activities described in the Material and methods. RRG values for $F_{2:3}$:BR012 × CMS225 were the means based on 14 individuals per family (*n*: population size for each cross)

Note Crosses in which data for only one maker are shown were monomorphic for the second marker

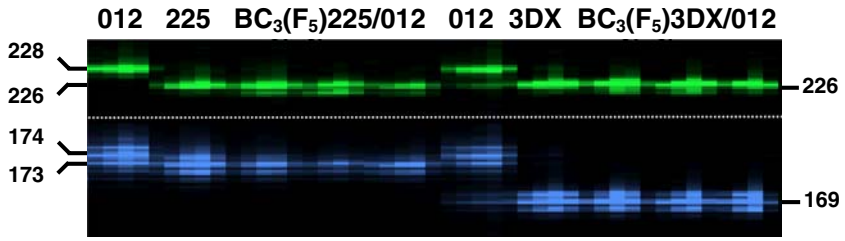

Fig. 2 Amplification profile of BR012 (012), CMS225 (225), three $BC_3(F_5)$225/012 true breeding progeny, BR012 (012), 3DX, and three $BC_3(F_5)$3DX/012 true breeding progeny with the STS markers CTG29 (*green*) and M181 (*blue*). *Numbers* to the left and right indicate the base pair (*bp*) sizes of the amplification products

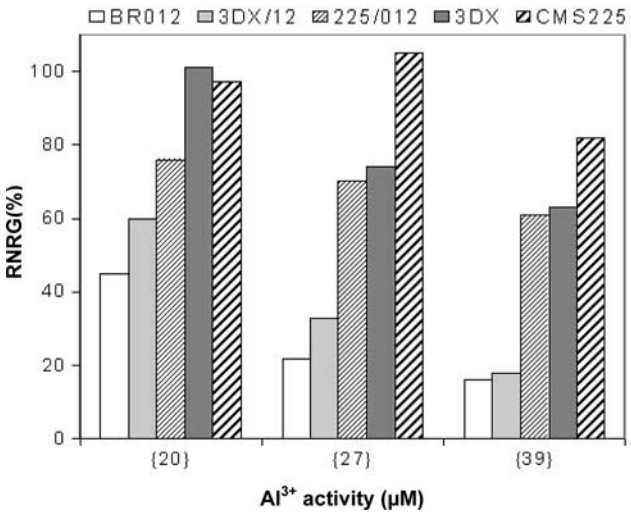

Fig. 3 Root growth response curve to Al for the near-isogenic lines (*NILs*) BR012, 3DX/012, 225/012, and donors 3DX and CMS225. Percent Relative Net Root Growth (*RNRG*) values are means of three replications (seven plants per replication, statistical analysis and standard error of the means shown on Table S3). The Al^{3+} activity of {11} μM was omitted because this level was not sufficient to inhibit root growth in any of the lines

evaluated for Al tolerance at {11} μM Al^{3+}, an activity at which BR012 was only slightly affected by Al (RNRG = 87%), whereas BR007 experienced a strong inhibition of root growth (RNRG = 45%, Table 1). The *P* value for marker linkage to RRG was highly significant for F_2:BR012 × BR007, and almost 30% of the phenotypic variance was explained by the segregation of M181 in this population. This result indicated that part of the higher Al tolerance exhibited by BR012 relative to BR007 is due to allelic variation at the Alt_{SB} locus, although other Al tolerance genes with minor phenotypic effects likely influenced the rather normal distribution for RRG observed in F_2:BR012 × BR007 (data not shown).

A comparison of only F_2 populations highlighted a wide range of R^2 values, which reached a maximum of 0.5 for the F_2 population derived from the BR012 × CMS225 cross. When only the populations that showed significant marker associations to Al tolerance were analyzed, the wide range of Al tolerance for the tolerant parents (Table 1) and a significant variation of the R^2 values suggested the influence of allelic variation at the Alt_{SB} locus. Nonetheless, because Al tolerance segregated as a quantitative trait, it is also reasonable to speculate that Al tolerance genes distinct to Alt_{SB} are partly responsible for the RNRG differences in Table 1. To isolate the allelic effects for Alt_{SB} on Al tolerance from those of other genes, the lines 3DX and CMS225, which are two representatives of the tolerant RNRG classes in Table 1 (RNRG of 70 and 109%, respectively at {27} μM Al^{3+}), were used to generate near-isogenic lines (NILs) by backcrossing into the sensitive tester BR012 genetic background. Accordingly, we identified $BC_3(F_4)$ lines homozygous for the Alt_{SB} alleles donated by 3DX and CMS225 using marker assisted selection at both STS flanking loci, and generated true breeding $BC_3(F_5)$ progeny (Fig. 2). Due to the tight genetic linkage (0.6 cM) between CTG29 and M181, the probability of a double crossing over event in the Alt_{SB} region is extremely low. Thus, one can reasonably expect the NILs, which carry on average 93.75% of the BR012 genome, to reflect differences in Al tolerance due to different donor alleles of Alt_{SB}. Figure 3 shows that the RNRG means of the NIL set comprised of the common recurrent parent BR012, $BC_3(F_5)$3DX/012 (3DX/012) and $BC_3(F_5)$225/012 (225/012), and the donor parents 3DX and CMS225, differed from each other across the Al levels used. The RNRG mean of the NIL, 3DX/012, was higher than that of BR012 at {20} and {27} μM Al^{3+}, whereas both lines were highly intoxicated at an Al^{3+} activity of {39} μM (Fig. 3). The NIL 225/012 was by far the most tolerant one, as its RNRG mean was higher than that of BR012 and 3DX/012 at {20}, {27} and {39} μM Al^{3+} (Table S3). These results strongly indicate that the range of Al tolerance across the sorghum lines in Table 1 is under the influence of an allelic series at the Alt_{SB} locus, which could be classi-

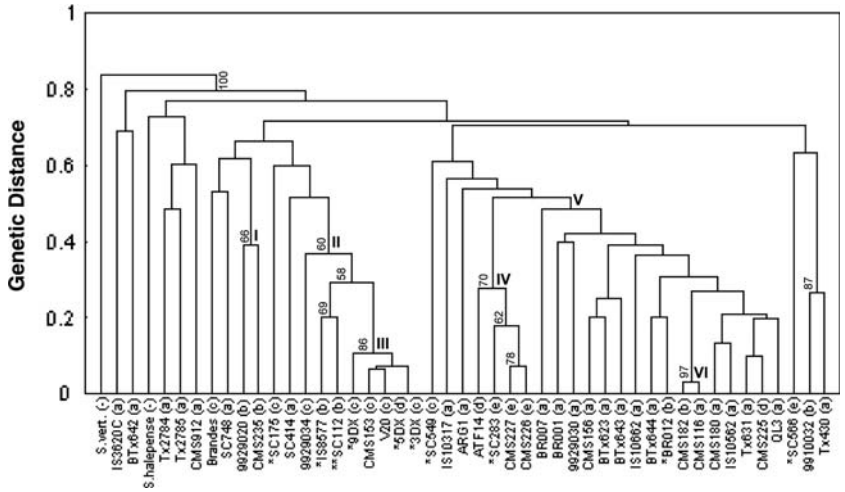

Fig. 4 Dendrogram depicting associations among 47 sorghum accessions as revealed by UPGMA analysis of a Nei and Li (1979) matrix of genetic dissimilarities. Bootstrap values (expressed in percentages) that exceeded 55% are shown on the respective branches. *Letters in parenthesis* indicate homogeneous percent relative net root growth (*RNRG*) groups by the Scott–Knott test at $P < 0.05$. RNRG: 10–30 (**a**), 30–50 (**b**), 60–80 (**c**), 90–105 (**d**), 115–135 (**e**). Values are means of three replications (seven plants per replication and accession means and standard errors of those means are shown in Table S4). *SC175, IS8577, 9DX, 5DX, 3DX, SC549, SC283, BR012 and SC566 were found to carry functional Alt_{SB} alleles based on the genetic analysis. The line 5DX was included in this set due to its intermediate *P* value for linkage to Alt_{SB} and close relatedness to 3DX (see Discussion). **SC112: tolerance of this line beyond the level observed for BR012 is due to Al tolerance genes distinct from Alt_{SB}

fied in ascending order of individual phenotypic effects as BR012 < 3DX < CMS225. Because highly significant linkage of CTG29 and M181 to RRG as well as high R^2 values were detected for the F_2:BR012 × BR007 population (Table 2), the implication is that the Alt_{SB} allele for BR012 is different and stronger in its phenotypic effect than the most sensitive Alt_{SB} allele from BR007. However, because we did not compare the BR007 allele with those of 3DX and CMS225 across the same genetic background, we cannot rule out the possibility that the BR007 allele is the same as either of those from the tolerant lines, but shows reduced expression in BR007 due to a genetic background effect.

SSR analysis of genetic diversity

All 15 SSR loci used in this study were polymorphic and produced a total of 130 alleles for the 47 sorghum lines listed in Table S1, with the number of alleles per SSR locus ranging from 2 (Sb5-256) to 12 (Sb5-206, Sb4-32 and Sb6-36) and an average of 8.7 alleles per locus. The PIC values varied from 0.04 (Sb5-256) to 0.81 (Sb6-57), with a mean value of 0.62 across loci. In this study, relatedness at the pedigree level was likely responsible for several groupings among the sorghum accessions (Fig. 4). Associations due to pedigree relatedness that were highly supported by bootstrap values were clusters I (accessions derived from the same random mating population), IV (SC283 and derivatives) and VI (SC326-6, i.e., CMS116 in Table S1, and its derivative CMS182). Geographic origin was probably responsible for relatedness on cluster III, within which accessions 9DX, CMS153, V20, 5DX and 3DX share a common origin from Uganda. Accessions IS8577 and SC112, are respectively from Kenya and Ethiopia, revealing a possible regional commonality within cluster II as Uganda, Kenya and Ethiopia are all eastern African countries. The remaining accession on cluster II, 9929034, is derived from a random mating population and may have shown association to other accessions in this cluster due to pedigree relatedness of the random mating parents. Cluster V was formed by accessions mostly derived from the USA and Brazil, but some associations within this cluster were rather loose.

Sixty-six percent of the sorghum accessions were highly or moderately Al sensitive (RNRG groups a and b, 10–30 and 30–50%, respectively, Fig. 4 and Table S4). Most of the highly (RNRG group a) or moderately (RNRG group b) Al sensitive accessions were found within Cluster V (one exception was the highly tolerant accession CMS225) and among lines that were loosely similar to IS3620C or *S. halepense*. Highly Al tolerant lines (RNRG groups c, d and e) showed a

scattered distribution throughout the dendrogram except for a more pronounced representation on cluster II. Accessions that were found to harbor functional Alt_{SB} alleles encompassed highly divergent lines found in distinct clusters, representing diverse geographical origins and morphological races (Fig. 4; Table S1). Sorghum accessions that could not be discriminated with regard to their degree of Al tolerance at {27} μM Al^{3+} (RNRG groups d and e) were subsequently grown on nutrient solution containing Al activities of {37} and {58} μM Al^{3+}. Table S4 shows that the most Al tolerant lines were SC566 and CMS227, which sustained appreciable root growth rates at the extremely high Al activity of {58} μM (RNRG ~65%), which significantly exceeds the already high Al tolerance level in the standard, SC283.

Discussion

A comparison of the germplasm set used in the current study to a broad sorghum array of similar size that was evaluated using the same SSR loci (Smith et al. 2000) indicated a higher number of alleles per locus in the former and similar PIC values between the two datasets. The maintainer (B) and fertility restorer (R) lines studied by Smith et al. (2000) included sorghum breeding germplasm that encompassed lines developed in several different geographic areas and with diverse maturity ranges, kernel colors and plant heights. Thus, although the sorghum accessions used in the current study included elite breeding lines and derivatives, the resulting panel that also included lines from different origins, landraces and wild relatives still retained significant sorghum genetic diversity. *S. verticilliflorum* was the most divergent accession of the panel, consistent with the idea that cultivated sorghum is derived from the arundinaceum (Aldrich and Doebley 1992) or the aethiopicum (Sun et al. 1994) races of *S. bicolor* subsp. *verticilliflorum* . Among other highly divergent accessions are the wild relative *S. halepense* and IS3620C which, although belonging to the race guinea of subspecies *bicolor*, shows an extremely grassy phenotype more typical of the wild subspecies, representing a possible case of introgression (Cui et al. 1995).

The sorghum germplasm used for this study included 16 accessions of strict African origin, which were from Uganda (5 accessions), Ethiopia (4), Nigeria (3), Sudan (2), Tanzania (1), and Kenya (1), with 4 accessions of unknown origins. Conversely, 27 accessions were breeding derivatives from Brazil (13), US (13) and Australia (1). Most of the breeding derivatives (19) were found within cluster V and ~80% of those were highly or moderately Al sensitive (groups a and b in Fig. 4). The exception was cluster IV, which was formed by the tolerant standard SC283 and three derivatives. As reported by Foy et al. (1993), Al tolerance is a rather rare trait in sorghum, being possibly a derived state rather than a natural characteristic of the species as proposed for wheat by Garvin and Carver (2003). The presence of only a few highly tolerant, unrelated lines within the germplasm set (see, for example, SC283, SC566 and 5DX, groups d and e in Fig. 4), reflects the fact that Al tolerance was intentionally introgressed within the breeding programs to cope with a specific constraint posed by Al toxicity for sorghum production on acid soils.

The patterns of genetic diversity in sorghum are mostly influenced by racial and geographic origins (Tao et al. 1993; Deu et al. 1994, 2006; Oliveira et al. 1996; Agrama and Tuinstra 2003). However, the strong prevalence of breeding derivatives in the present study, in conjunction with insufficient representatives of accessions from different geographical origins in Africa or of known morphological races did not allow us to observe clear consistencies between patterns of genetic diversity and origin/race. One exception was cluster III, which contains only Ugandan lines. Therefore, pedigree relatedness was the major factor underlying patterns of genetic diversity in the present study, as detailed in the Results session.

An in-depth view of genetic diversity for traits that are relevant for agriculture can serve a variety of purposes. Plant breeders can refer to such knowledge to assess the potential in adopting recombination-based breeding strategies aimed at exploiting the additive effects of superior alleles at distinct loci. In addition, the extent at which non-identical phenotypes are produced either by different alleles at one major locus (i.e. allelic heterogeneity) or by several different genes (i.e. non-allelic heterogeneity) yields insights into the potential of using comparative mapping to select and combine in one crop orthologs found in related species. Accordingly, although the simultaneous presence of both allelic and non-allelic heterogeneity can be expected to be the rule for most of the phenotypes, rather than the occurrence of either of those alone, the balance between them may shift, and this knowledge is important to define the most effective breeding strategies.

The first important finding in this study was the presence of non-allelic heterogeneity, with the following evidence supporting this assertion. First, except for the cross BR012 × SC283, all other mapping populations showed normal frequency distributions for Al tolerance, suggesting the contribution of distinct loci.

Second, we did not detect marker-trait association in a mapping population derived from the reasonably tolerant accession, SC112, indicating that this accession harbors a relatively weak Alt_{SB} allele, similar in its phenotypic effect to that from BR012. However, the higher RNRG of SC112 relative to BR012 across all Al^{3+} activities (Table 1) indicated that the superior tolerance in this sorghum line is conditioned by loci distinct to Alt_{SB}. Third, the Al tolerance (RNRG means) for the NILs 3DX/012 and 225/012 were significantly lower than those of the parents 3DX and CMS225, particularly at the higher Al^{3+} activities of {27} and {39} μM Al^{3+} (RNRG means were reduced by ~70 and 25%, respectively at {39} μM Al^{3+}, Fig. 3 and Table S3). Particularly for 3DX/012, this very large reduction in Al tolerance resulted in only a slightly higher tolerance for the NIL over the sensitive parent BR012. However, RNRG for the NIL 225/012 was still 67 and 74% of the RNRG for CMS225 at {27} and {39} μM Al^{3+}, respectively, suggesting that different Al tolerance genes are likely to have a higher impact on Al tolerance in 3DX in comparison with CMS225. Incomplete transfer of Al tolerance has also been observed in wheat, where NILs having Atlas 66 as the tolerance donor were less tolerant than Atlas 66 itself, which was considered additional evidence for the existence of a second Al tolerance gene in this wheat cultivar (Tang et al. 2002).

The last evidence that points toward the existence of significant Al tolerance gene diversity in sorghum comes from a practical observation. The sorghum line CMS227 is a selection from the cross SC283 × SC326 (i.e., CMS116 in Table S1), and the tester line BR012 is derived from a SC748 × SC326 cross. The RNRG mean for SC326 at {27} μM Al^{3+} was 27 ± 5.6% (Table S4), similar to that of BR012 (35.0 ± 4.0%), whereas the RNRG for SC748 was significantly lower (12 ± 1.1%) than that of BR012 and similar to the RNRG mean of the Al sensitive standard BR007 (14 ± 0.7%). The tester BR012 was found to harbor a relatively weak Alt_{SB} allele but our data also suggests that other minor Al tolerance genes in its background contribute to the normal segregation pattern observed for the F_2 population BR012 × BR007 and also to the higher Al tolerance of BR012 with respect to BR007. These findings suggest that Al tolerance in BR012 was inherited from SC326, and that the superiority of CMS227 with respect to SC283 at {58} μM Al^{3+} (Table S4) is probably the result of the additive or codominant effects of the Alt_{SB} allele from SC283 in conjunction with other minor genes that were also inherited from SC326. As detailed in the Introduction, evidence for multigenic control of Al tolerance have been found in wheat (Camargo 1981; Aniol and Gustafson 1984; Aniol 1990), but the genes located on chromosome arms 4DL, 5AS and 7DS all appear to condition Al-activated root malate release (Papernik et al. 2001) encoded by the *ALMT1* gene (Sasaki et al. 2004). Interestingly, Atlas, which was found to possess a second Al tolerance gene different than Alt_{BH} (Camargo 1981), was found to be less tolerant than BH1146, the primary source of Alt_{BH} (Riede and Anderson 1996), suggesting that non-additive effects may take place in Atlas. It has also been hypothesized that different genes act together in Atlas to enhance Al-induced malate release (Papernik et al. 2001; Tang et al. 2002), which could result in non-additive effects. Other evidence for multigenic control of Al tolerance have been found in rye (Aniol and Gustafson 1984; Gallego and Benito 1997; Gallego et al. 1998a, b; Ma et al. 2000; Matos et al. 2005), maize (Sibov et al. 1999; Ninamango-Cárdenas et al. 2003), rice (Wu et al. 2000; Nguyen et al. 2001, 2002, 2003; Mao et al. 2004), oat (Wight et al. 2006) and *Arabidopsis* (Kobayashi and Koyama 2002; Hoekenga et al. 2003; Kobayashi et al. 2005). Therefore, it is important to clarify the physiological mechanism(s) of Al tolerance that take place in sorghum lines that show non-allelic heterogeneity such as SC112, as well as in progeny derived from crosses between those lines, in order to assess the actual potential for additional Al tolerance improvement in sorghum by recombination-based strategies aimed at exploiting transgressive segregation. Nevertheless, as discussed above for CMS227, this strategy seems promising in sorghum.

The second important finding in this study was the high degree of allelic heterogeneity at the Alt_{SB} locus in sorghum. Our genetic analysis detected a wide range of phenotypic variation for Al tolerance that is controlled by multiple alleles of Alt_{SB}, which could be classified in ascending order of their phenotypic effects as BR012 < 3DX < CMS225. The two most tolerant sorghum cultivars, SC283 and SC566, have been previously found to rely on Alt_{SB} for their tolerance (Magalhaes et al. 2004), but the fact that SC566 was significantly more tolerant than SC283 at {58} μM Al^{3+} (Table S4), an extremely high Al^{3+} activity for sorghum, raises the possibility that the SC566 allele is distinct and stronger than the SC283 allele. Although F_2 populations derived from both SC566 × BR007 and SC283 × BR007 produced bimodal distributions for Al tolerance in nutrient solution (Magalhaes et al. 2004), we cannot at this point rule out the possibility that the superiority of SC566 is also due to other minor and/or modifying genes in its background. Nonetheless, because a possible elite Alt_{SB} allele in SC566 would make

this line the preferred choice for Al tolerance breeding in sorghum, NILs are being generated to shed light onto the relative effects of the Alt_{SB} alleles from SC283 and SC566 as well as to their possible superiority relative to the CMS225 allele.

A wide search for Al tolerance genes distinct to the barley Al tolerance gene, *Alp*, was conducted with 37 barley genotypes of diverse genetic and geographical origins (Minella and Sorrells 1992). These authors concluded that there is little potential for Al tolerance improvement using these sources, as multiple alleles controlling Al tolerance were reported as the primary mode of genetic control for this trait. In addition, Rhue et al. (1978) also reported multiple alleles for Al tolerance in maize but several other studies suggest complex inheritance patterns (Magnavaca et al. 1987; Pandey et al. 1994; Borrero et al. 1995; Ninamango-Cárdenas et al. 2003), indicating that distinct Al tolerance genes must exist in maize. Recently, Raman et al. (2005) examined five wheat double haploid populations developed from divergent parents with contrasting Al tolerance and found that a single major gene, which is likely to correspond to Alt_{BH} (Riede and Anderson 1996), was responsible for the Al tolerance control. This raises the possibility that an allelic series at the Alt_{BH} locus also occurs in wheat. Interestingly, the more balanced interplay between allelic and non-allelic heterogeneity that we observed in sorghum seems more similar to what has been reported in maize than in wheat and barley, which is consistent with the closer phylogenetic proximity between sorghum and maize than between sorghum and the Triticeae species (Gaut 2002). However, evidence for multigenic control for Al tolerance in rye is extensive, including two genes whose chromosomal locations on rye 7RS (Matos et al. 2005) and 4RL (Miftahudin et al. 2002, 2005) make orthology with wheat Alt_{BH} possible, one or more genes on 6RS (Aniol and Gustafson 1984; Gallego and Benito 1997; Gallego et al. 1998a, b), and a possible Alt_{SB} ortholog on 3RS (Aniol and Gustafson 1984; Ma et al. 2000; Magalhaes et al. 2004). This suggests that if simultaneous occurrence of allelic and non-allelic heterogeneity does not occur in individual Triticeae species such as barley, it does occur in the Triticeae tribe as a whole.

The genetic diversity analysis conducted here also has helped in better understanding the genetic control of Al tolerance in the sorghum line 5DX, another potential Al tolerance source with regards to molecular breeding schemes aimed at pyramiding tolerance genes in sorghum. Bootstrap analysis yielded strong support for cluster III (average genetic distance of ~0.1 and bootstrap value of 86% in Fig. 4), within which a common origin from Uganda for the accessions 9DX, CMS153, V20, 5DX and 3DX probably influenced their close relatedness. Some degree of pedigree relatedness among certain accessions in cluster III is also likely, particularly between 3DX and 5DX, which had a genetic distance of 0. All accessions within this cluster exhibited significant Al tolerance, and all belonged to RNRG group c in Fig. 4 (RNRG ranging from 63 to 80%) except for 5DX, which stood out in terms of Al tolerance, with a RNRG of approximately 100% in {27} μM Al^{3+} (Table 1 and Table S4) placing it in group d. The fact that the Alt_{SB} allele in 3DX is not markedly superior to that of the sensitive tester BR012 (Fig. 3), along with the genetic diversity data indicating that 3DX and 5DX most likely share a very recent common ancestor, suggests that a rather weak allele could have been co-inherited by both 3DX and 5DX. Thus, it is possible that an introgression of different Al tolerance gene(s), some of which could significantly increase tolerance, occurred in the ''DX-like'' genetic background, giving rise to the significantly higher level of Al tolerance in 5DX. The presence of other Al tolerance genes in 5DX may also have overridden the phenotypic effect detected by the markers flanking its most likely weak Alt_{SB} allele, thus explaining the intermediate *P* value for linkage (Table 2).

Interestingly, the highly Al tolerant line CMS225, is a selection from CMS153 (Al tolerant, group c in Fig. 4) × CMS110 (i.e. Tx430, highly Al sensitive, group a). Thus, tolerance from CMS225, which is strongly based on Alt_{SB} (Table 2), was inherited from the Ugandan line CMS153, which is highly related to 9DX, V20, 5DX and 3DX within cluster III. This indicates that a high degree of allelic heterogeneity, with a strong Alt_{SB} allele probably present in CMS153 and a rather weak Alt_{SB} allele present in 3DX (see NIL data for 225/012 and 3DX/012 in Fig. 3) occurs in cluster III. In addition, SC112 is also related to the cluster III accessions (average genetic distances of ~0.3 between IS8577/SC112 and cluster III, Fig. 4), and 5DX and SC112 were both found to rely on Al tolerance genes distinct from Alt_{SB} to express their Al tolerance. Overall, these results indicate that a high degree of both allelic and non-allelic heterogeneity for Al tolerance co-exist within highly related accessions, and that efforts to broaden the genetic basis for Al tolerance should not be based solely on genome-wide patterns of diversity. In addition, eastern African sorghums might be an interesting repository, not only of superior Alt_{SB} alleles but also of novel Al tolerance genes, although additional evidence is needed in order to verify this hypothesis.

The genetic control of Al tolerance that was found in this study indicates that both allelic and non-allelic heterogeneity are important factors for breeding Al tolerant sorghums. However, different to what was previously found in barley and to a lesser extent wheat, the combination of different Al tolerance genes in sorghum appears to be an effective strategy to originate highly tolerant transgressive segregants. Because the sorghum panel used for the genetic diversity analysis was rather small and significantly composed of breeding derivatives, additional studies are needed in order to reveal clear patterns that could orient the search and combination of superior Alt_{SB} alleles and different Al tolerance genes. To gain insights into that, a detailed study is now underway with a large and highly diverse sorghum core collection. Overall, the results of this study point toward a relatively unexplored and potentially rich breeding potential for Al tolerance in sorghum, both within and beyond the Alt_{SB} locus.

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