

Full Length Research Paper

## Phylogenetic relationship among Kenyan sorghum germplasms based on aluminium tolerance

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Eighty nine (89) sorghum lines sourced from various parts of Kenya were used to determine phylogenetic relationships based on 10 DNA fragments at  $Alt_{SB}$  loci with SbMATE, ORF9 and MITE primers. Nine lines of varying aluminium tolerance levels were selected to compare their SbMATE gene expression via the real-time PCR quantification of SbMATE gene expression. The sorghum line MSCRO2 expressed a thousandfold more SbMATE gene activity than the sensitive lines (MSCRM49, MSCRN84 and MSCRN61) under Al treatment. Analysis was done by agarose gel electrophoresis stained with ethidium bromide. The objective of this study was to assess the level of phylogenetic relationships among the Kenyan sorghum germplasms at a known Al tolerance locus. Hierarchical cluster analysis joined at 70% simple matching coefficient using average linkage similarity level produced nine groups in which 67 lines fell in three major clusters of 39, 15 and 13 lines each. The three Al tolerant lines MSCRO2, MSCRC1 and MSCRN60 were clustered together. Lines MSCRO2, MSCRC1 and MSCRN60, screened to be Al tolerant were genetically related at 70% average linkage similarity level and therefore recommend their further development as a food security measure in Kenya.

**Key words:** Aluminium (Al) toxicity, *Sorghum bicolor*, Sorghum bicolor multi-drug, toxic extrusion compound (SbMATE) gene expression.

### INTRODUCTION

Aluminum (Al) is a mineral without a known biological function to plants except at pH below 5.5 when  $Al^{3+}$  is solubilized (Kochian et al., 2005) and becomes toxic to plants including sorghum. Approximately 40% of the earth's arable land comprise acid soils (Ma et al., 2001) covering about 4 billion hectares (Virupax, 2006). In Kenya, 13% of the arable land comprise acid soils (Kanyanjua et al., 2002), limiting crop production and therefore demanding urgent attention. Response to aluminium toxicity among genotypes has been reported to

have a genetic basis (Hoekenga et al., 2006). Scientists at Embrapa, Brazil identified sorghum line SC283 as tolerant and BR007 as susceptible and mapped  $Alt_{SB}$  gene on chromosome 3, responsible for 80% of the aluminium tolerance phenotype in sorghum mapping population (Magalhaes et al. 2004). Further studies have shown that the elite  $Alt_{SB}$  alleles cause a marked increase in sorghum aluminium tolerance (Caniato et al., 2007) thereby improving yields in acid soils. Overexpression of the Al-inducible malate transporter (ALMT1) improved Al

tolerance in barley (Delhaize et al., 2004) while overexpression of the *SbMATE* protein, a putative citrate transporter, improves Al tolerance in *Arabidopsis* and wheat (Magalhaes et al., 2007).

Morphological characteristics co-inherited with the desired trait have previously been used to estimate relatedness between cultivars. In wheat, barley and rye, genetic variation has been evaluated by simple Mendelian analysis, but in rice (Wu et al., 2000) and maize (Ninamango-Cardenas et al., 2003), natural genetic variation appears to be inherited quantitatively.

Estimates of the physical size of the sorghum genome range from 700 (Arumuganathan and Earle, 1991) to 772 Mbp (Peterson et al., 2002) allowing for variation within the genome for Al tolerance. Genetic diversity in sorghum has been estimated using various markers such as allozymes (Djè et al., 1998), restriction fragment length polymorphism (Deu et al., 2006), randomly amplified polymorphic DNA (Uptmoor et al., 2003; Agrama and Tuinstra, 2003), amplified fragment length polymorphism (Uptmoor et al., 2003; Menz et al., 2004), simple sequence repeats (Smith et al., 2000; Uptmoor et al., 2003; Agrama and Tuinstra, 2003; Menz et al., 2004; Casa et al., 2005), single-nucleotide polymorphism (Kruglyak, 1997) and intron length polymorphism (Choi et al., 2004). In each studies, major interest focused on a specific subset of sorghum germplasm. Despite wide use of molecular markers to estimate genetic diversity among sorghum genotypes (Uptmoor et al., 2003), little has been reported about relatedness among Kenyan sorghum germplasm based on Al tolerance forming the thrust of this study.

## MATERIALS AND METHODS

### Root germination and treatment

Eighty nine (89) Kenyan sorghum lines were screened alongside the tolerant check variety SC283, and the susceptible check variety BR007, making a total of 91. Seeds were germinated for three days; six uncontaminated and nearly same length seedlings per line per treatment were selected and transplanted to trays containing eight liters of nutrient solution prepared according to procedures described by Magnavaca et al. (1987). Trays were wrapped with black polythene skirts to shield light from weakening the iron chelate. The experiment was laid out in a randomized complete block design (RCBD) each time a number of lines were screened. Three replications per line were used. Seminal roots were inserted through a hole at the bottom of polythene cups, supported by black beads and kept under continuous aeration in a growth chamber at 26°C day and 23°C night temperatures, a light intensity of 330  $\mu\text{mol photons m}^{-2} \text{sec}^{-1}$  and a 14 hr photoperiod. After 24 h of acclimatization at pH 4.0, seminal roots were measured and recorded; nutrient solution replaced either with 0  $\mu\text{M}$  Al for control set or with one supplemented with 148  $\mu\text{M}$  [27]  $\text{Al}^{3+}$ , pH 4.0 added as  $\text{AlK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$  for the treated set. Each line was replicated six times per treatment.

### Determination of genetic diversity at *AltsB* locus

Eighty nine (89) Kenyan sorghum lines were used in this study to estimate the overall genetic diversity in Kenyan sorghum lines:

Ajabsido and SC 22 were used as controls for Miniature Inverted Repeat Transposable Elements (MITEs) assay, BTx623 and SC283 for *SbMATE* and BTx623 with SC 373 for open reading frame (ORF 9) assay.

DNA was extracted from five leaves of each line which were collected and immediately frozen in liquid nitrogen. Six days after germination, they were ground using a Geno Grinder for 30 s at 400 rpm following the Integrated DNA Technologies (IDT) protocol modified by KebLlanes et al. (2002). DNA concentration was determined with Nano Drop® ND-1000 Spectrophotometer and by fluorescence spectroscopy using Pico Green Tris-EDTA dye (pH 7.5) via TACO software. Multiplex PCR were performed in a total volume of 15- $\mu\text{l}$  containing; 3  $\mu\text{l}$  of template DNA, 0.75  $\mu\text{l}$  of taq polymerase, 1  $\mu\text{l}$  of each primer (MJ 23 and MJ 26 for the MITE, HB30 and HB37 for the *SbMATE*, and HB1 and HB2 for the ORF9) used in the assay, except HB23 and HB24 used in the *SbMATE* (0.1  $\mu\text{l}$  each). 0.5  $\mu\text{l}$  dimethylsulfoxide (DMSO) and double distilled water was added to make up the 15  $\mu\text{l}$  volume mark.

The PCR reaction was programmed to run for 4 min at 94°C, 1 min at 94°C, 1 min at 50°C, 1 min at 72°C, then 1 min at 94°C for 35 cycles, 7 min at 72°C and 10°C to the end for both ORF and *SbMATE* assay. The reaction for MITE assay was programmed for 2 min at 95°C, 1 min at 94°C, 1 min at 55°C, 2 min at 72°C, then 1 min at 94°C for 30 cycles, 10 min at 72°C and stopped at 10°C.

Amplification products were resolved by gel electrophoresis in a 1.5 % agarose gel stained with 7  $\mu\text{l}$  of 10 mg/ml ethidium bromide run in 1xTBE buffer at pH 7.5, for 2½ h at 135V, 55 mA. The DNA fragments were detected by UV trans-illumination and photographed under UV using a Polaroid 667 camera. Each primer band was considered an independent allele and assigned numbers in the order of increasing molecular weight. The size of each band was estimated against DNA molecular weight marker. The bands were scored for polymorphism by visual inspection. To secure reliable scores, only the unambiguous bands ranging from 19 to 6000 base pairs were scored for each primer set. A polymorphic DNA band was read as "1" (present at a locus) or "0" (absent at a locus) into a binary data matrix for each sorghum entry for all the three primer combinations.

### Determination of aluminium induced expression of *SbMATE*

#### RNA extraction

Due to the need to quantify alterations in mRNA levels using real-time PCR among lines, total RNA was extracted. Nine selected Kenyan sorghum lines used based nutrient solution screening as Al sensitive (RNRG < 10%), moderately tolerant (30- 45% RNRG) and Al tolerant (RNRG > 70%). The two check varieties SC283 and BR007 were also germinated and grown in Magnavaca nutrient solution containing 148  $\mu\text{M}$  Al for treated set and 0  $\mu\text{M}$  Al for control. 1 cm root apices of 15 seedlings per line per treatment were excised and immediately frozen in liquid nitrogen and stored at -80°C. Total RNA was extracted using a QIAGEN RNeasy Plant Mini Kit according to manufacturer's instructions (QIAGEN, 4<sup>th</sup> ed. 2006). The quality of RNA was checked by electrophoresis on 1.0% agarose gel and then quantified using a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE).

#### Complementary DNA synthesis

This involves copying of mRNA into cDNA using reverse transcriptase that is more temperature stable than mRNA and also the SYBR Green 1 dye binds only to double strand DNA. First-strand cDNA was synthesized using 1  $\mu\text{g}$  of total RNA in a total volume of 8  $\mu\text{l}$  with DEPC water treated with 1  $\mu\text{l}$  RNase free DNase I in 10X DNase buffer and 1  $\mu\text{l}$  of 25 mM EDTA and then incubated at 65°C

**Table 1.** Sorghum lines used for diversity studies and their Al tolerance status.

Code number	Identity	% RNRG	Code number	Identity	% RNRG	Code number	Identity	% RNRG
1	A3	30	31	M11	09	61	N35	11
2	A4	32	32	M17	20	62	N4	12
3	C1	73	33	M20	13	63	N51	28
4	C13	15	34	M24	10	64	N52	13
5	C19	57	35	M3	29	65	N57	09
6	C26	30	36	M32	09	66	N6	09
7	E8	12	37	M33	47	67	N60	88
8	F13	16	38	M33a	27	68	N61	09
9	F15	69	39	M37	17	69	N68	10
10	F16	15	40	M38	33	70	N77	13
11	F2c	40	41	M40	15	71	N80	06
12	F3	16	42	M42	18	72	N81	12
13	F6	12	43	M43	23	73	N84	08
14	F8	08	44	M44	53	74	N85	09
15	G2	33	45	M45	32	75	N85c	13
16	H3	23	46	M46	28	76	N88a	09
17	I19	20	47	M47	06	77	O2	75
18	I4	46	48	M49	05	78	P1	11
19	J1a	50	49	M5	15	79	P3	41
20	J2	15	50	M69	09	80	P5	26
21	K1	08	51	M79f	21	81	Q2	31
22	K12	16	52	M87	22	82	Q3	16
23	K4	07	53	N102	10	83	Q4	43
24	K5e	16	54	N103	10	84	R1	17
25	K6	10	55	N119	16	85	R3	21
26	K8	08	56	N120	32	86	R7	08
27	L1	07	57	N13	14	87	T53	24
28	L5	52	58	N140b	63	88	T54	14
29	L6	37	59	N157a	17	89	T55	34
30	M1	06	60	N24	09			

Each line identity is preceded by the prefix 'MCSR'. The RNRGs were obtained after six days of growth in Magnavaca (1987) nutrient solution with 0 Al or 148  $\mu$ M [27] Al. Line identities were arranged alphabetically.

for 10 min. Subsequently, 10  $\mu$ l of the total RNA per sample was reverse transcribed using 1  $\mu$ l of random primer and 1  $\mu$ l Super-Script III reverse transcriptase, 4  $\mu$ l of 5X reverse transcriptase (RT) buffer, 2  $\mu$ l of 10 mM dNTPs, 0.5  $\mu$ l of RNase inhibitor (RNase OUT) and 1.5  $\mu$ l of DEPC water. The mix was incubated at 37°C for 90 min, 70°C for 10 min and then the reaction was stopped at 4°C. The 20  $\mu$ l first-strand cDNA synthesized was added to 80  $\mu$ l Tris EDTA and stored at -20°C. The SbMATE transcript was amplified from the first-strand cDNA using the forward primer JL 470 (5'CAGCCATTGCCCATGTTCTTT-3') and the reverse primer JL 471 (5'ACCAGCTTGCTCAGCATTATCA -3'). The 18S gene used as internal reference was amplified using JL477 (AATCCCTTAACGAGGATCCATTG) forward primer and JL 476 (CGCTATTGGAGCTGGAATTACC) reverse primer in a total volume of 10  $\mu$ L. A dilution of 10<sup>-3</sup> was used for the endogenous template and the amplicons were detected by SYBR green I. Two-primer worksheet was used in loading samples, primers and probes. Relative Quantitation (RQ) = 2<sup>-ddC<sub>T</sub></sup>, where: dC<sub>T</sub> (sample) = C<sub>T</sub> target - C<sub>T</sub> endogeneous, ddC<sub>T</sub> = dC<sub>T</sub>sample - dC<sub>T</sub>reference (ddC<sub>T</sub> = expression of each target gene less calibrator expression), C<sub>T</sub>-Threshold cycle.

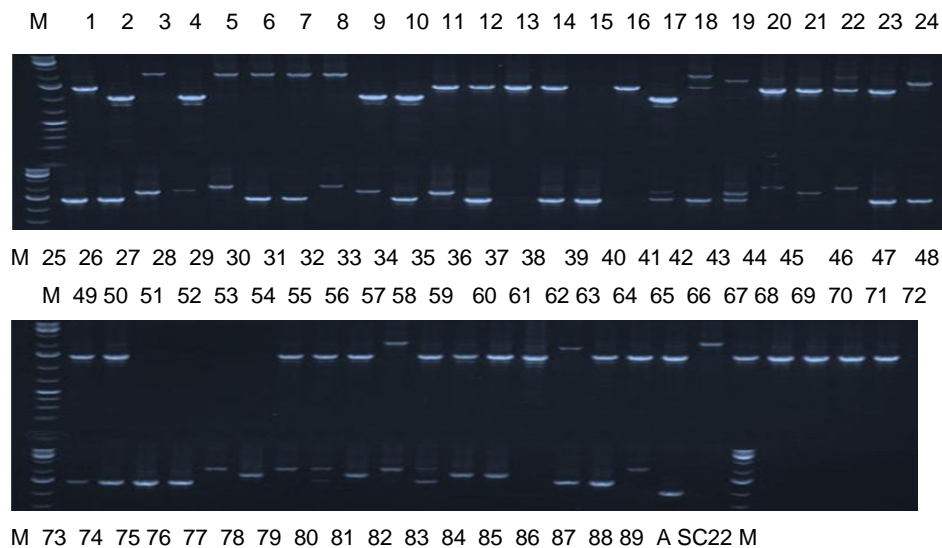
### Statistical analysis

Relative quantification of SbMATE gene expression were calculated by C<sub>T</sub> method; 18S rRNA was used as an endogenous control and the expression of BR007 under 148  $\mu$ M Al was used as calibrator. Data analysis was done using Applied Biosystems 7500 Real-Time PCR system, 7900 mode, SDS version 1.4 software and means separated by Duncan's multiple range test. Genotyping data matrix was analysed using Genstat Discovery Edition 3.0 software and hierarchical clusters generated using average linkage with genetic similarities determined by simple matching coefficients as described by Gower (1971).

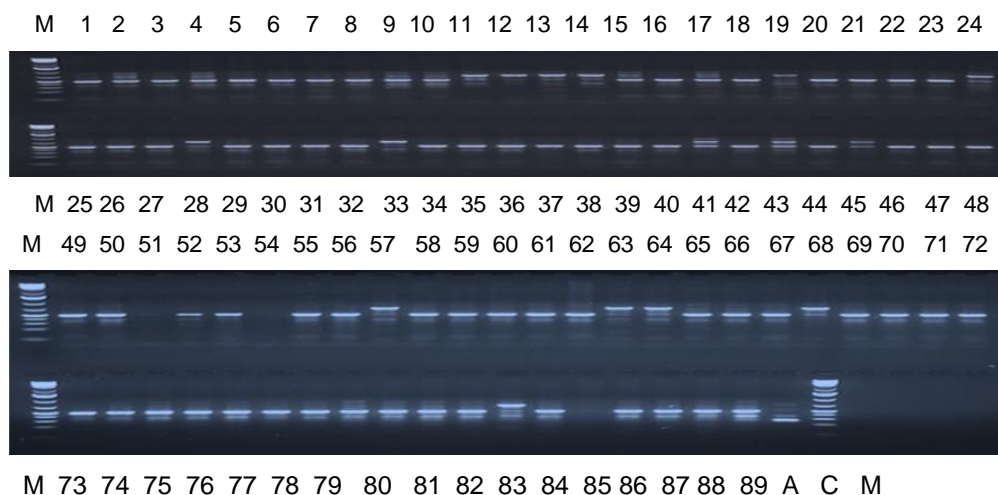
## RESULTS AND DISCUSSION

### Determination of genetic diversity using aluminium tolerance loci

For each of the genomic DNA extracted and analysed from the 89 sorghum lines (Table 1), none of the primers



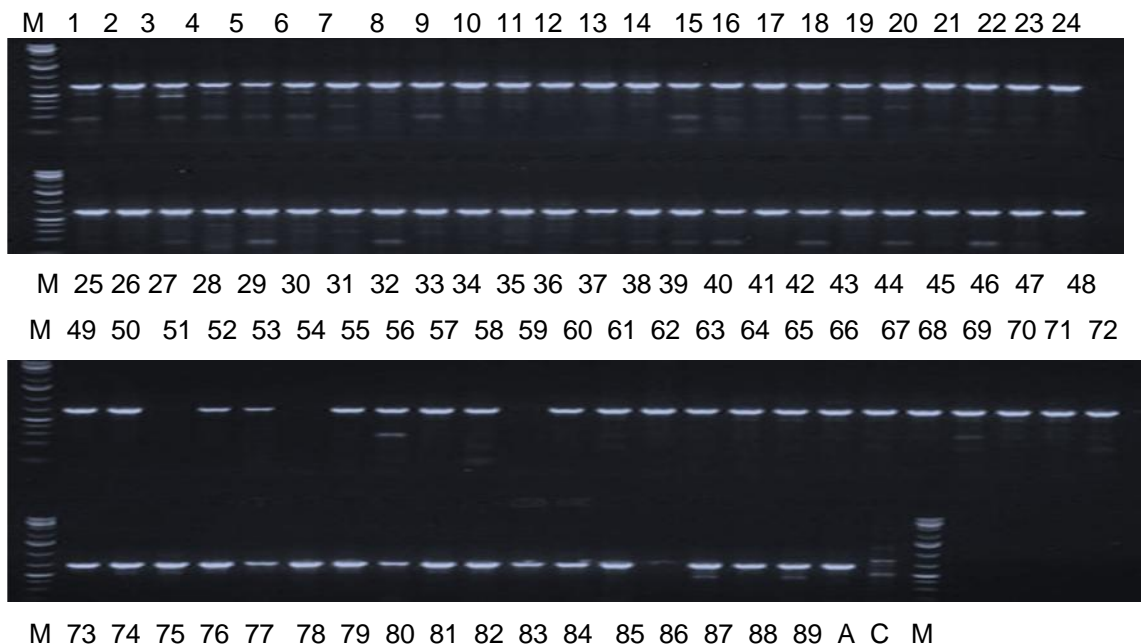
**Figure 1.** Amplification product for genomic DNA of 89 sorghum samples using MITE primer. Five alleles were scored; 1, Ajabsido, 2, BTx623, 3, IS 3620 C, 4, Macia; 5, SC 22. Alleles ran from 456, 1184, 1544, 1912 and 2280 bp respectively. For each allele, presence was read as '1' and absence read as '0'. Only unambiguous fragments were scored.



**Figure 2.** Amplification product for genomic DNA of 89 sorghum samples using SbMATE primer. Two alleles A (259 bp) and C (483 bp) were tested. 16 line samples MSCR (F3, F6, F8, H3, J1a, K5e, L5, M20, M40, M43, M45, N13, N51, N52 and R1) had an insertion G which could not be primed at C (483 bp) but slightly above, hence scored separately. For each allele, presence was read as '1' and absence read as '0'. 'A' confers tolerance to aluminium as in SC 283 and 'C' confers susceptibility. Most of the Kenyan sorghum had the C allele. MSCRM79f, MSCRN103 and MSCRR1 samples were not amplified by this primer. Only unambiguous fragments were scored.

used in the study amplified DNA fragments from MSCRM79f. The site specific primers for SbMATE gene amplified DNA bands in the rest of the lines. Various DNA fragments were obtained and easily gave clear similarity and differences among lines. A total of ten fragments were scored; five alleles for the MITE primer, three for SbMATE and two alleles for ORF9. The MITE and SbMATE alleles were polymorphic and therefore useful in determining diversity. The ORF9 alleles were monomor-

phic and did not contribute to the assessment of diversity. The MITE primer produced bands at 456 base pairs for (Ajabsido), at 1184 base pairs (BTX623), at 1544 base pairs (IS3620 C), at 1912 base pairs (Macia) and at 2280 base pairs (SC22) (Figure 1). The SbMATE primer amplified fragments at 259 base pairs (A), 483 base pairs (C) and at 619 base pairs which contained an insertion of a base (G) (Figure 2) while ORF9 produced fragments only at 19 base pairs in all the accessions (Figure 3).



**Figure 3.** Amplification product for genomic DNA of 89 sorghum samples using ORF-9 primer. The open reading frame 9 (ORF9) of SbMATE gene in sorghum was also used to amplify DNA fragments. As can be seen the fragments were clearly present in all lines. The two alleles (indel 19 bp) indicating presence of insertion of a dominant marker conferring susceptibility while absence indicate deletion. There were no amplifications for DNA fragments of MSCRM79f, MSCRN103, MSCRN24 and MSCRR7 although they were sensitive. For each allele, presence was read as '1' and absence read as '0'.

### Cluster analysis

The degree of similarity between the 89 sorghum lines as assessed using the simple matching coefficient and hierarchical clustering determined using the average linkage, showed diversity among Kenyan sorghum for the Al tolerance loci. The moderately tolerant clustered with both tolerant and sensitive lines indicating heterogeneity among lines. Out of the three primers, ORF-9 showed a monomorphic band that could not contribute to the understanding of diversity of these lines. The cluster analysis based on aluminium tolerant loci, MITE and SbMATE grouped Kenyan sorghum into two major groups (1 and 2) joined at 54 % similarity level (Figure 4).

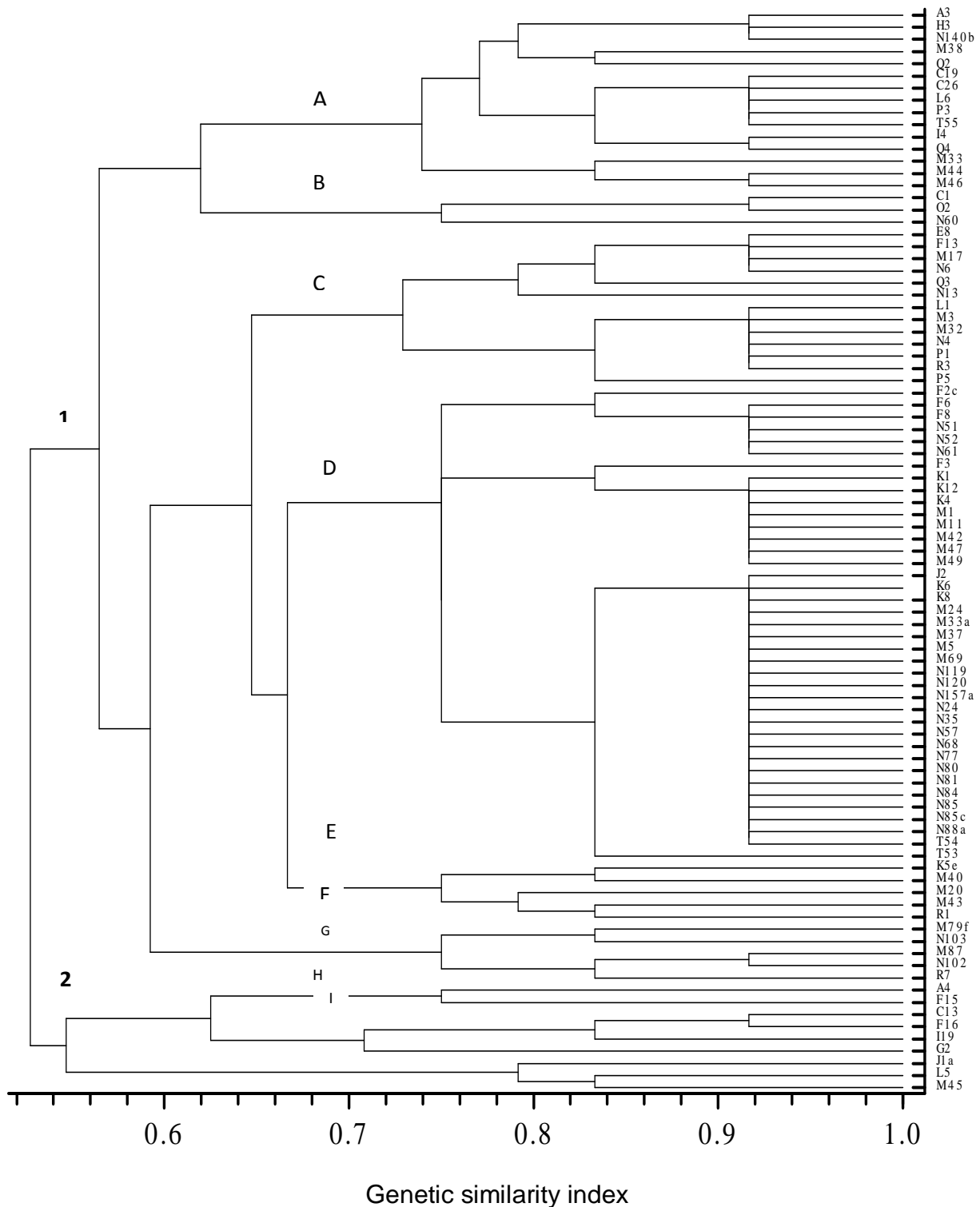
Group 1 consisted of 80 lines of varying tolerance status. Group 2 composed of nine moderately aluminium tolerant lines and included the aluminium tolerant F15. The MITE and SbMATE primers were polymorphic with 3 to 5 bands ranging from 259 to 2280 bp and were used for cluster analysis producing nine hierarchical sub-clusters joined at 70 % similarity level.

Although most amplified bands were common to all of the 89 sorghum lines, as indicated by simple matching coefficient of 0.70 or greater, the dendrogram constructed using average linkage grouped 67 lines into

three major clusters: A (15 lines), C (13 lines), and D (39 lines). The remaining 22 lines were grouped into six small clusters each with between 2 and 5 lines. Cluster 'B' comprised three aluminium tolerant lines MSCRC1, MSCRO2 and MSCRN60. Cluster G comprised of two lines, tolerant MSCRF15 and moderately tolerant MSCRA4.

This was generated by simple matching coefficient and average link similarity based on frequency of amplified bands of SSR (MITE) and SNP (SbMATE). The horizontal scale is the measure of simple matching genetic similarity coefficient according to Gower (1971).

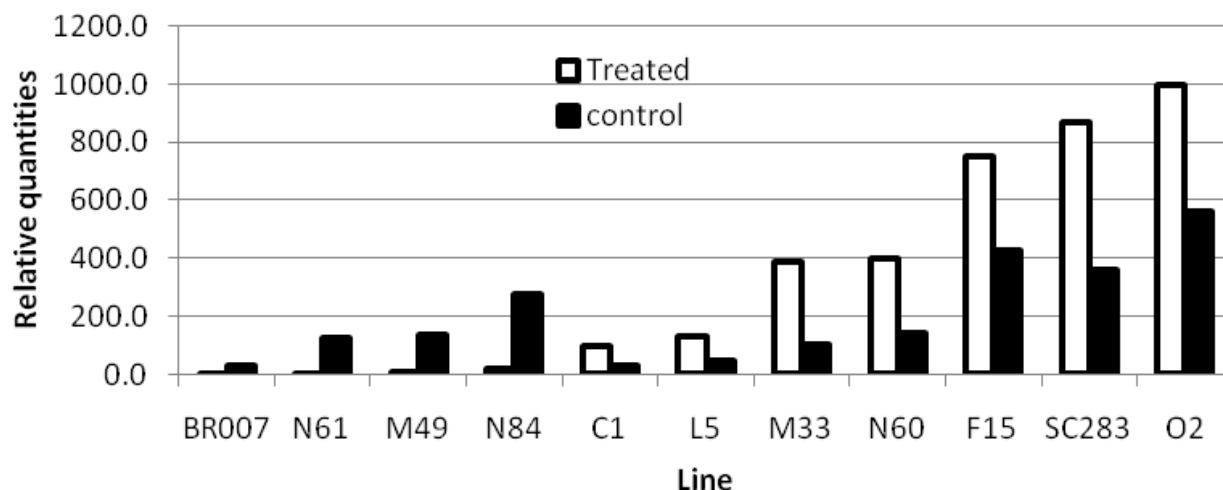
The cluster membership of the 89 Kenyan sorghum lines was defined by MITE and SbMATE primers. Cluster B consisted of Al tolerant lines, MSCRO2 and MSCRN60 and the ICRISAT Al tolerant standard, MSCRC1. Members of cluster D contained 39 susceptible lines. Cluster G had the least membership composed of moderately tolerant lines (Figure 4). The aluminium tolerance loci used to genotype Kenyan sorghum lines revealed prevalence of susceptibility alleles except in four lines (MSCRO2, MSCRN60, MSCRC1 and MSCRF15). Most of the susceptible lines were closely related hence clustered together by aluminium tolerance loci, SbMATE and MITE. Unlike the susceptible lines, tolerant lines were diverse at the same loci. ORF primer amplification products were monomorphic therefore had no contribution to genetic diversity implying that the gene



**Figure 4.** Dendrogram showing phylogenetic relationship among the 89 sorghum lines collected from various provinces of Kenya.

has limited variability among sorghum lines. The moderately tolerant A4 and the tolerant F15 clustered together suggesting that either there was poor discrimination by the primers or as an indication of

marked genetic differences between the lines as suggested by Chakauya and Tongoona (2008). Similar cases applied to the clustering together of susceptible and moderately tolerant lines.



**Figure 5.** SbMATE gene expression among sorghum lines after six days in Al treatment. Aluminium tolerant line O2 expressed SbMATE more than the tolerant check SC283 as Al sensitive check BR007 expressed the least quantities.

The failure of all primers to amplify DNA from M79f suggested that either there were serious deletions at the aluminium tolerance loci or low purity of its DNA fragments. Some susceptible lines related closely with moderately tolerant cultivars implying that there seem to be more loci determining Al tolerance. Incidentally, insertion of an extra nucleotide base (G) at the SbMATE locus conferred Al susceptibility in sixteen lines. Despite the insertion, there seem to be other factors contributing to Al tolerance among sorghum lines as postulated by Kochian et al (2004). The Alt SB locus control Al tolerance in sorghum (Magalhaes, 2004) and variation at the locus seems to be determining sorghum genetic diversity. The SbMATE gene at the Alt SB locus encodes a plasma membrane-citrate transporter belonging to the multi-drug and toxic compound extrusion (MATE) family (Magalhaes et al., 2007) plays an important role in conferring Al tolerance among Kenyan sorghum.

Aluminium tolerant Kenyan sorghum lines possessed the MITE alleles; allele 4 (Macia) at 1912 base pairs and allele 3 (IS3620 C) at 1544 base pairs. This implied that these loci confer Al tolerance whereas most susceptible lines possessed the MITE alleles; allele 2 (BTX623) at 1184 base pairs conferring susceptibility. None of the Kenyan lines possessed allele 1 (Ajabsido) at 456 base pairs. All the lines possessed SbMATE alleles A (259 base pairs) that confer Al tolerance present in SC283. The AltSB loci appear to be polygenic with several genes expressed uniquely when exposed to aluminium. Thus, there seems to be variation within the AltSB loci among lines.

Molecular markers developed for examining genetic diversity and indirect selection of traits linked to Al tolerance loci (Raman et al., 2002) have proved to be efficient. Genetic diversity in sorghum has been estimated using several types of molecular markers, including restriction fragment length polymorphism (Deu

et al., 2006), randomly amplified polymorphic DNA (Uptmoor et al., 2003; Agrama and Tuinstra, 2003), amplified fragment length polymorphism (Uptmoor et al., 2003; Menz et al., 2004), simple sequence repeats (Menz et al., 2004; Casa et al., 2005), single-nucleotide polymorphism (Kruglyak, 1997) and intron length polymorphism (ILP) (Choi et al., 2004) have contributed immensely to evaluation of genetic diversity among various lines.

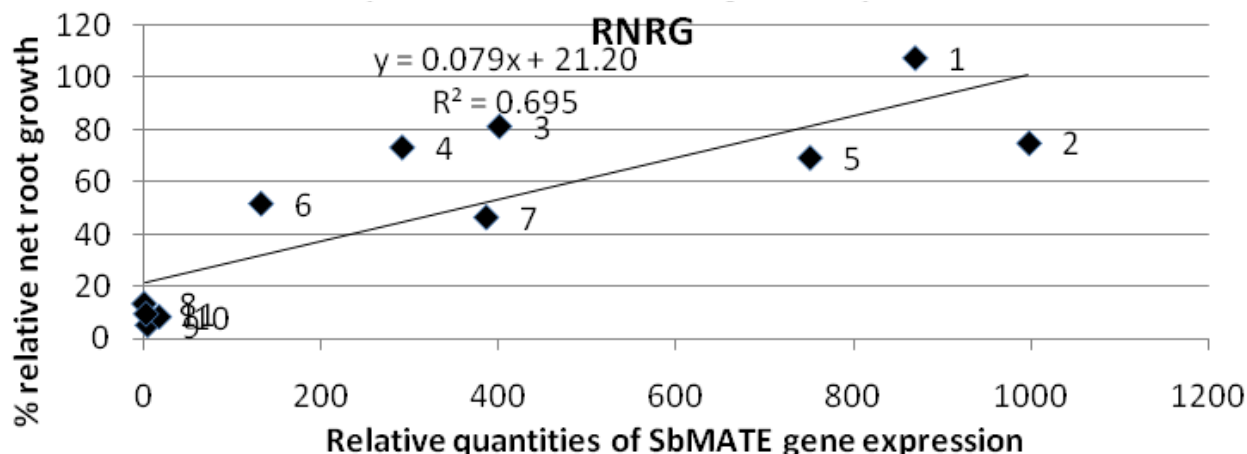
#### Aluminium induced SbMATE gene expression

The selected sorghum lines tested varied significantly in their SbMATE gene expression under Al stress. Relative quantities of SbMATE gene expression of tolerant lines O2, C1, N60 and F15 under Al treatment was 400 to 900 times that of the sensitive lines M49, N61 and N84 (Figure 5). The sensitive lines expressed SbMATE gene more times in the control than the treated due to their lack of Al induced mechanism, but tolerant lines expressed SbMATE gene more times under treatment than in the controls. The lines O2 expressed SbMATE gene more than the others including the witness line SC283. There existed a strong and significant positive regression between SbMATE gene activity and relative net root growth ( $R^2 = 0.70$ ) (Figure 6).

#### Conclusion

Despite the fact that only nine Kenyan sorghum lines were selected for real-time PCR analysis, the hierarchical cluster analysis showed that SbMATE gene plays a major role in tolerance to aluminium. There was genetic segregation for aluminium tolerance among Kenyan sorghum populations grouping together lines of similar





**Figure 6.** Regression between SbMATE gene expression and % RNRG. The codes (1-11) represent 1-SC 283, 2-O2, 3-N60, 4-C1, 5-F15, 6-L5, 7-M33, 8-BR007, 9- M49, 10- N84 and 11-N61. SbMATE gene expression explains about 70% of the mechanisms influencing activities averting aluminium toxicity.

tolerance status. SbMATE gene activity varied among lines thereby dictating genetic diversity and relatedness in their tolerance status to toxic aluminium.

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