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Full Length Research Paper

DNA cytosine methylation alterations associated with aluminium toxicity and low pH in *Sorghum bicolor*

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Aluminium (Al) toxicity stress reduces crop yields in acidic soils. In this study, we investigated the level of DNA Cytosine methylation polymorphism caused by 150 µM Al^{3+} (89.97% free Al^{3+}) induction, low pH (4.0) and continuous application of 150 µM Al^{3+} in Sorghum inbred lines using methylation sensitive amplified polymorphism analysis (MSAP) and searched for homologies using the BLASTN program. We found that 150 µM Al^{3+} toxicity and low pH induced a persistent moderate DNA cytosine (C) methylation changes at early seedling stage. Analysis of DNA CG and CNG methylation levels showed variations amongst the inbreeds with more changes occurring at the CNG level than in the CG level. Morphological observations showed that Al and low pH stress triggered crop yield changes. BLAST analysis suggested that possible polymorphisms occurred in the regulatory regions of Al tolerance genes mainly via histone deacetylase, methyltransferase, peptide signals and transcription factors and could have definite roles in allelic effects which influence gene expression and an increase in Sorghum stress resistance.

Key words: Aluminium toxicity, DNA methylation, epigenetics, methylation sensitive amplified polymorphism analysis (MSAP), sorghum.

INTRODUCTION

Aluminum (Al^{3+}) toxicity, which is pH-dependent, is a major limitation in the production of sorghum, rice and maize in acid soils (Chang et al., 1998; Godbold and Jentschke, 1998). Recent studies show that there seems to be a multiple tolerance alleles which confer a range of tolerance levels in sorghum (Caniato et al., 2007). Hence, although mRNA expression levels of the cloned Al tolerance genes may indicate better quantitative information of the individual tolerance allele, coupling it with regulation stability at the DNA cytosine methylation level may contribute to Al screening programs by considering the heritability of cytosine methylation in plants.

Genes are not usually expressed in isolation and some have been known to act together in families while others are regulated through other mechanisms like cytosine DNA methylation, histone modification and even others involve chromatin rearrangement. Polymorphisms in the regulatory regions of the Al tolerance gene like *Alt_{SB}* have the possibility of contributing to large allelic effects, and hence are likely to influence the gene expression in the root apex of tolerant sorghum genotypes (Magalhães et al., 2007). Some of these polymorphisms, especially cytosine DNA methylation have been known to be epigenetic in their functions. Moreover, abiotic stresses can induce DNA cytosine hypomethylation or hypermethylation which influence gene expression in plants (Finnegan et al., 1998). Hence, morphological and epigenomic correlation study can aid in exploiting stress variations and influences in the Al tolerance gene loci. In addition, studies by Kochian et al. (2005) found longer exposures to Al^{3+} toxicity in *Sorghum bicolor* for 5 to 6

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Table S1. Shows the concentrations of free metal, free ligand, and total mol/L of ligand complexed by each metal in ASS medium as generated by the GEOCHEM-PC Version 1.0 program (Shaff et al., 2008).

Parameter	FREE MET	PO_4^{3-}	Cl^-	NO_3^-	SO_4^{2-}	NH_3	OH^-
FREE LIG		18.33	4.36	4.43	5.42	10.54	9.99
Al	6.19	8.80	-	18.98	8.19	-	7.17
K	5.23	-	10.30	-	9.78	-	15.74
Ca	4.78	10.18	10.17	14.04	7.96	-	13.50
Mg	5.42	12.53	9.83	14.11	8.69	-	12.93
Na	4.43	-	8.78	-	9.18	-	14.63
H^+	3.99	6.82	16.76	9.83	7.45	5.53	-

days resulted in significant increase in the Al resistance when compared to exposure of Al^{3+} in root growths after 2 to 4 days. However this increase in resistance did not cause an increase in the usually expected citrate exudation but, rather a decrease suggesting another mechanism should be responsible for the increase in Al^{3+} resistance. We suggested epigenetic mechanisms role through some moonlighting enzymes activities (Sriram et al., 2005). In this paper two inbred lines (YN336 and YN267 hereby designated A and D respectively) of *S. bicolor* L. whose F_1 have routinely been used in heterotic grain production were morphologically and genetically screened for Al^{3+} toxicity at 150 µM and at pH 4.0 with an aim of detecting the lowest level of sensitivity of the inbred lines. Previous screening of *S. bicolor* at 400 µM Al could not allow any plant to survive (Smith et al., 1983). Studies by Zhang et al. (2009) predicted a high susceptibility of the sorghum pure lines to epigenetic changes but a low susceptibility in the hybrids. Sorghum is grown alongside maize in acidic and Al toxic soils in many tropical countries. Al toxicity had been known to reduce yield in maize up to 40%, hence genetic and epigenetic evaluation of sorghum in similar environmental stresses is crucial. In this study we focused on root, leaf, panicle size and plant yield alongside the possible DNA cytosine methylation polymorphisms. This was followed with the sequencing and functional searches of the affected loci in the BLASTN databases. The study was carried at low pH (4.0) and Al stress (150 µM) separately so as to establish whether the two stresses are epigenetically independent during stress conditions. A modified methylation sensitive amplification polymorphism (MSAP) analysis method which uses the differential endogenous enzymes *Msp*I and *Hpa*II was applied (Dong et al., 2006).

Environmental and hormonal or peptide endogenous signals have been known to influence target genes through histone modifications like deacetylation and acetylation levels in response to biotic and abiotic stresses (Chinnusamy and Zhu, 2009).

MATERIALS AND METHODS

The two Sorghum inbred lines, YN336 (A) and YN267 (D), which were used in this study were produced and donated by the Institute of Cereal Crops, Jilin Academy of Agricultural Sciences, Changchun, China. After an initial induction in half strength Murashige & Skoog (MS) nutrient solution of Al^{3+} at 150 µM and also separately at pH 4.0 at the genomic sensitive emergence-transition stage between heterotrophic and autotrophic mode of nutrition of the seedlings (Murashige and Skoog, 1962; Boye and Marcarian, 1985; Gourley et al., 1990; Miller et al., 1992), the seedlings were later transferred into uniformly prepared soil mixture in pots where further observations were made. However, the control and one batch of the 150 µM Al induced plants in each inbred line were subjected to only inorganic nutrient mixture solution without Al, while the other batch of the Al^{3+} induced inbred lines of sorghum was subjected to a continuous 150 µM Al application (herein referred to as aluminium sensitive solution (ASS)) up to maturity.

Preparation and adjustment of the aluminium sensitive solution (ASS)

The aluminium ions were obtained from $AlCl_3{\cdot}6H_2O$ while pH was adjusted to 4.0 using 1.0 M HCl solution (Kinraide, 1991). We prepared an aluminium sensitive solution (ASS) for the later continuous application (conc. Al) in the soil containing 148 /µmol L^{-1} of Al from $AlCl_3.6H_2O$, 64 /µmol L^{-1} of P from NaH_2PO_4, 7.4 mmol L^{-1} of Ca from $CaCl_2$, 1.6 mmol L^{-1} of Mg from $MgSO_4$, 3.9 mmol L^{-1} of K from KCl and 24.7 mmol L^{-1} of N from a mixture of NH_4Cl and $NaNO_3$ at a ratio of 8:1(Furlani and Clark, 1981). Mineral composition of the ASS using GEOCHEM-PC Version 1.0 showed that ASS had 89.97% free Al^{3+}, 0.22% had complexed with PO_4^{3-}, 0.90% had complexed with SO_4^{2-}, and 8.91% had complexed with OH^- in solution, the amount of free ions and ligands in the solution is shown in Table S1.

Assessing DNA cytosine methylation stability using MSAP analysis

Sorghum genomic DNA was extracted from the leaves using a modified high-salt cetyl trimethyl ammonium bromide (CTAB) method (Tel-Zur et al., 1999). The DNA quality and quantity was determined using the agarose gel electrophoresis. The methylation-sensitive amplified polymorphism (MSAP) analysis which reveals genome-wide cytosine DNA methylation changes (Dong et al., 2006; Zhang et al., 2009) and *Hpa*II together with *Msp*I which was purchased from New England Biolabs Inc. (http://www.neb.com). These isoschizomers which recognize the 5'CCGG site but have differential cytosine methylation sensitivity were used in the procedure. The *Hpa*II enzyme does not cut the DNA fragment if the cytosine in the double strand is methylated, but the *Msp*I is blocked from cutting if the external cytosine base is fully or hemi-methylated. The MSAP amplification products were then resolved by denaturing and in polyacrylamide gel electrophoresis (PAGE) before silver

Table S2. The Sorghum sequences of adaptors, pre-amplification primers and selective amplification primer combinations used in the MSAP analysis in this paper

Adapters	
*Eco*RI-adapter I	5'-CTCGTAGACTGCGTACC-3'
*Eco*RI-adapter II	5'-AATTGGTACGCAGTC-3'
*Mse*I-adapter I	5'-GACGATGAGTCCTGAG-3'
*Mse*I-adapter II	5'-TACTCAGGACTCAT-3'
H/M-adapter I	5'-GATCATGAGTCCTGCT-3'
H/M-adapter II	5'-CGAGCAGGACTCATGA-3'
Pre-selective primers	
E-A	5'-GACTGCGTACCAATTCA-3'
M-C	5'-GATGAGTCCTGAGTAAC-3'
H/M-0	5'-ATCATGAGTCCTGCTCGG-3'
Selective primers	
*Mse*I primers	
1. M-CAA	5'-GATGAGTCCTGAGTAACAA-3'
2.M-CAC	5'-GATGAGTCCTGAGTAACAC-3'
3.M-CAG	5'-GATGAGTCCTGAGTAACAG-3'
4.M-CAT	5'-GATGAGTCCTGAGTAACAT-3'
5.M-CTA	5'-GATGAGTCCTGAGTAACTA-3'
6.M-CTC	5'-GATGAGTCCTGAGTAACTC-3'
7.M-CTG	5'-GATGAGTCCTGAGTAACTG-3'
8.M-CTT	5'-GATGAGTCCTGAGTAACTT-3'
9. M-CCA	5'-GATGAGTCCTGAGTAACCA-3'
H/M primers	
1. H/M-TCT	5'-ATCATGAGTCCTGCTCGGTCT-3'
2. H/M-TCG	5'-ATCATGAGTCCTGCTCGGTCG-3'
3. H/M-TCC	5'-ATCATGAGTCCTGCTCGGTCC-3'
4. H/M-TTC	5'-ATCATGAGTCCTGCTCGGTTC-3'
5. H/M-TTG	5'-ATCATGAGTCCTGCTCGGTTG-3'
6. H/M-TTA	5'-ATCATGAGTCCTGCTCGGTTA-3'
7. H/M-TGA	5'-ATCATGAGTCCTGCTCGGTGA-3'
8. H/M-TGT	5'-ATCATGAGTCCTGCTCGGTGT-3'
9. H/M-TGC	5'-ATCATGAGTCCTGCTCGGTGC-3'
10. H/M-TAC	5'-ATCATGAGTCCTGCTCGGTAC-3'
***Eco*RI primers**	
a. E-AAC	5'-GACTGCGTACCAATTCAAC-3' (combined with 1, 4, 6, 8 and 10 H/M primers)
b. E-AAG	5'-GACTGCGTACCAATTCAAG-3' (combined with 4 and 5 H/M primers)
c. E-ACA	5'-GACTGCGTACCAATTCACA-3' (combined with 3, 4, 5, 8 and 10 H/M primers)
d. E-ACC	5'-GACTGCGTACCAATTCACC-3' (combined with 4 and 6 H/M primers)
e. E-ACG	5'-GACTGCGTACCAATTCACG-3' (combined with 3, 4, 5, 6 and 10 H/M primers)
f. E-AGC	5'-GACTGCGTACCAATTCAGC-3' (combined with 1and 4 H/M primers)
g. E-AGG	5'-GACTGCGTACCAATTCAGG-3' (combined with 1, 3, 4, 5, 6 and 10 H/M primers)
h. E-AGA	5'-GACTGCGTACCAATTCAGA-3' (combined with 4, 5, 6 and 8 H/M primers)
i. E-ATC	5'-GACTGCGTACCAATTCATC-3' (combined with 1, 4 and 5 H/M primers)

staining visualization and finally cutting of the variant bands. The CG, CNG and simultaneous CG/CNG hyper- and hypomethylation of both cytosines were analyzed as absence (0) or presence (1) of a band from a particular treatment relative to the control using 12 pairs of *Eco*RI + *Hpa*II/*Msp*I primer combinations (Table S2).

Identifying the variant bands and approximating their chromosomal distribution and functional relevance

In estimating possible chromosomal distribution and identifying the sequences, which were influenced by the various stresses. The

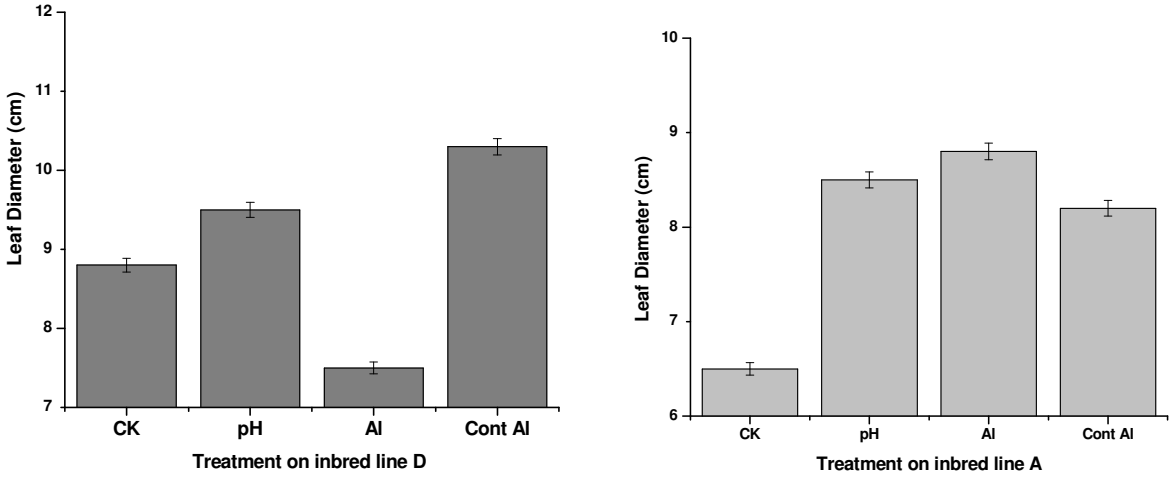

Figure 1. The leaf diameter measurements from Sorghum inbred line A and D after the various abiotic treatments.

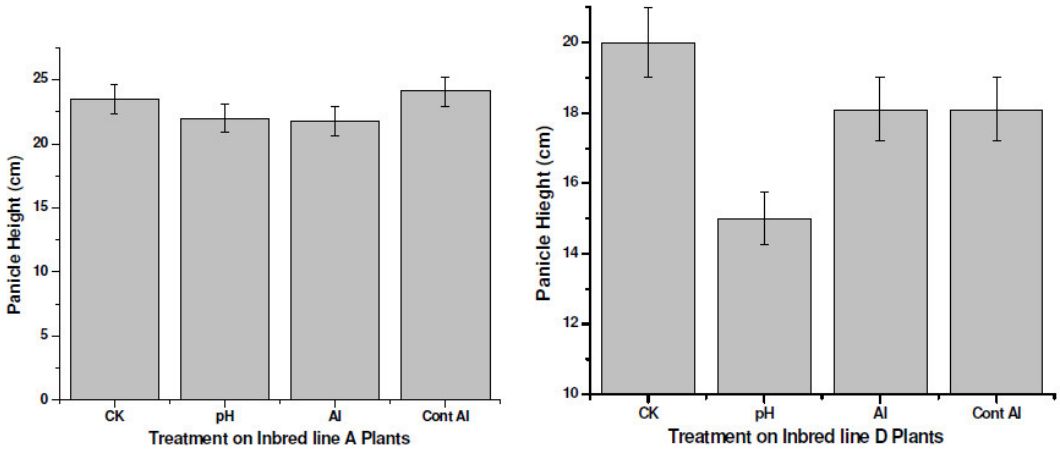

Figure S1. Variations in the sorghum panicle heights of inbred lines A and D.

variant bands were isolated from the silver stained MSAP gels and PCR re-amplified; the products were then cloned into the pMD18-T vector (Takara Biotechnology Inc., http://www.takara-bio.com) and sequenced using automated vector primers. Then a BLASTN search of the differential MSAP bands was done against the sorghum genomic sequence (http://www.gramene.org/Multi/blastview?species=Sorghum_bicolor). These were then used to investigate the functional, synteny, homology and orthology of the variant sequences underlying the DNA cytosine changes.

RESULTS

Morphological observations of sorghum

After four months, observations of the leaves below the panicle (flag leaf) had accumulated more anthocyanins than other parts of the plant in most of the Al and low pH treated plants. The plant height was not significantly different in inbred A plants in the three treatments, but observations in the inbred D showed the Al-stressed plants were taller and thinner than the plants treated with low pH alone and those under continuous ASS treatment. This Al induced inbred line D had also thinner leaves compared to the rest of the plants (Figure 1). Observations in both inbred lines showed a development of more brace roots in the Al continuously treated plants than in the other treatments. The least number of roots were observed in the controls. However, the control plants (CK) matured earlier than the other plants in both inbred lines followed by the ones in low pH, then the Al treated and lastly the Al continuously treated plants. Moreover, the continuously treated plants showed more yellowing of leaves compared to the other plants.

Analysis of inbred sorghum yield

There was a reduction of grain yield in the Al treated plants in D but an increase in yield at low pH exposed and in the continuously Al treated plants. This variation was reflected vaguely by the size of the panicles, for example, even though the size of the Al treated plants in inbred line D was larger (Figure S1), the yield change

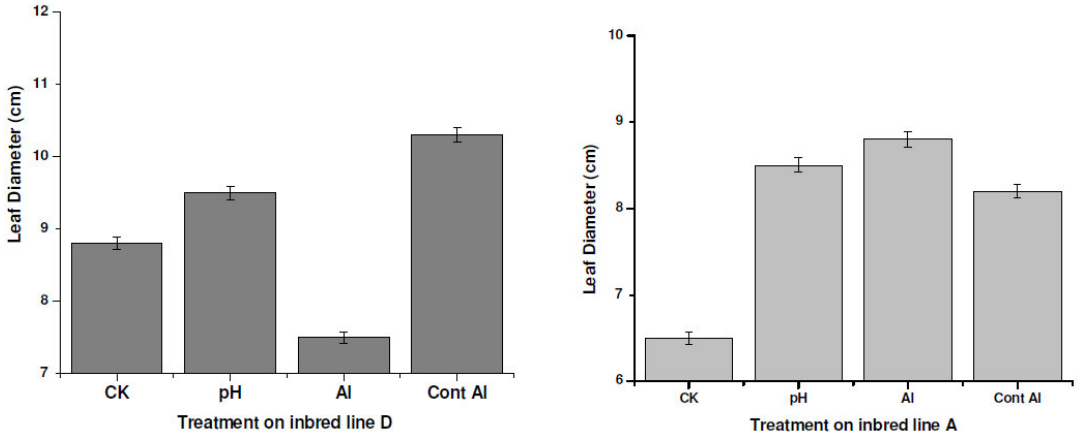

Figure S2. The average weights of sorghum dry seeds and panicle from inbred line A and D.

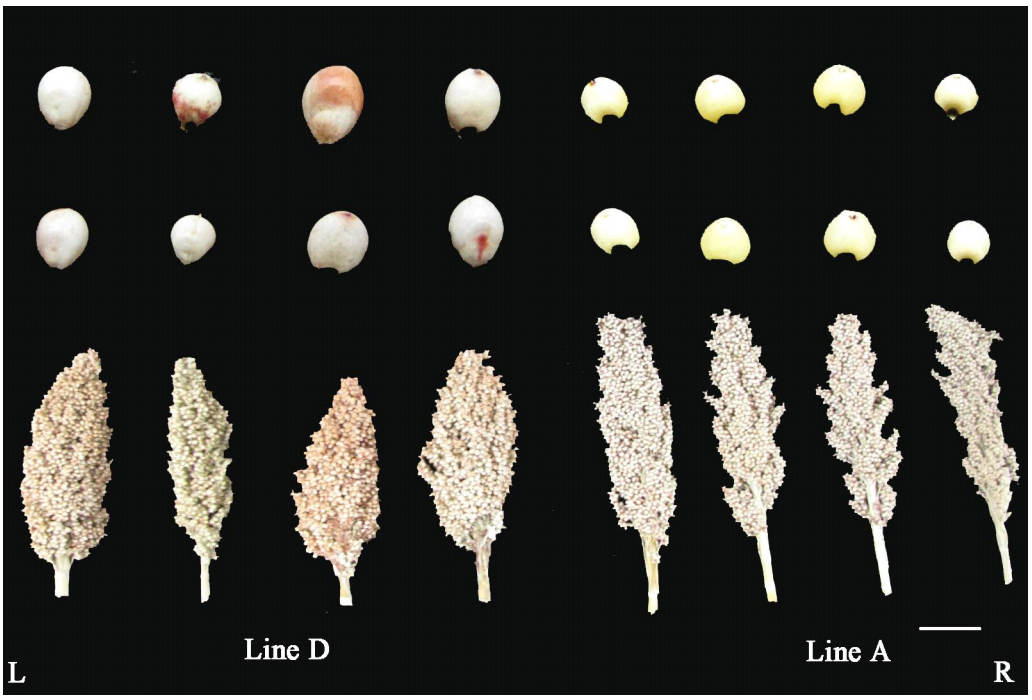

Figure 2. Various seed sizes and shapes compared to the corresponding panicles below from the sorghum inbred lines A and D respectively after various treatments. From the extreme left (L) are treatment from continuous Al, induced Al, low pH and the controls on the extreme right (R) the low pH caused pink coloration and increase in size in inbred line D, while inbred line had size increase in all stress compared to the control. The white bar is 20 cm.

was not reflected afterwards. The sizes of the seeds were positively correlated with the weight of the grain and panicle weight (Figure S2). The seed size and grain yield in inbred A increased with the increase of stress, with highest yield and size being at the continuously Al treated plants. In addition, the accumulation of the anthocyanins seemed to advance towards the leaves adjacent to flag leaf but, later observations of the seeds of inbred line D showed that they were pinker and bigger than those in the control, at continuous Al and at seedling Al induced plant, respectively (Figure 2). During harvest it was observed that the lines grown under continuous application of ASS were harder to be separated from the panicles compared to the control and the ones grown under low pH. Hence, most of them had the seeds still attached to their glumes. This observation was more in inbred D than in inbred A. There was a positive correlation between the leaf diameter and the panicle weight, showing their probable photosynthetic influence in yield.

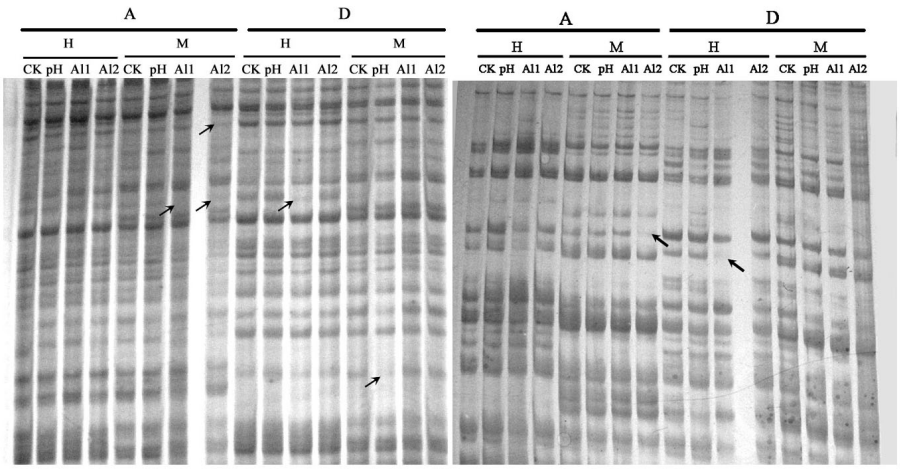

Figure 3. A sample of the MSAP bands obtained by using the primer combination EcoRI+AGG/GACTGCGTACCAATTCAGG and H/M-TCG/5'-ATCATGAGTCCT GCTCG GTCG. The letters on the figure indicate the various treatments from the control (CK). Al1 is stress at 150 µMAl while Al 2 is continuous 150 µM Al stress. H and M represent the endonucleases *Hpa*II and *Msp*I, respectively. The black arrows show the variant bands at particular loci.

Table 1. Relative levels of cytosine methylation at the CCGG sites in the sorghum inbred A and D at various stresses.

Sorghum line	Stress on plant	Levels of DNA Methylation (%)	
		CG	CNG
Inbred A	Control	7.65	3.67
	pH 4.0	6.73	4.28
	150 µM Al	7.65	4.89
	Cont 150 µMAl	7.03	4.28
Inbred D	Control	6.42	4.59
	pH 4.0	6.73	3.67
	150 µM Al	6.73	3.36
	Cont 150 µMAl	7.03	2.75

DNA cytosine methylation analysis

Analysis of the DNA cytosine levels in the two inbred lines

A total of 554 clear and reproducible bands were obtained and used for the MSAP analysis, see samples in Figure 3. The results showed that the level of CG methylation at 150 µM Al was lower in inbred line D (6.73%) compared to 7.66% in inbred line A while the level of CG methylation after continuous application of ASS Al solution was the same in both inbred lines (7.03%). The percentage CG methylation change at low pH was also the same (6.73%) in both inbred lines. This indicates that the inbred line A was more stable at the CG level compared to inbred line D. Comparatively, the largest individual CG methylation change in inbred A was at 150 µM Al treatment, while in D it was at continuous 150 µM Al treatment. However, in the percentage CNG level inbred A at 150 µM Al had 4.89% change compared to a lower 3.36% in inbred line D. At the continuous application of ASS solution, the CNG methylation level in inbred line D was even lower at 2.75% while that in inbred line A increased to 5.20% (Table 1). At low pH, the CNG level difference from the control was 0.61% in inbred line A but higher (0.92%) in inbred line D. This shows that inbred line A was more stable than inbred line D at the CNG level. This consequently shows that the observed morphological differences are mainly due to polymorphisms at the CNG rather than in the CG level.

Analysis of the DNA cytosine patterns in the two inbred lines

The total CG hypermethylation in inbred line A was about 1.2% at 150 µM Al but 0.0% at low pH. This was smaller

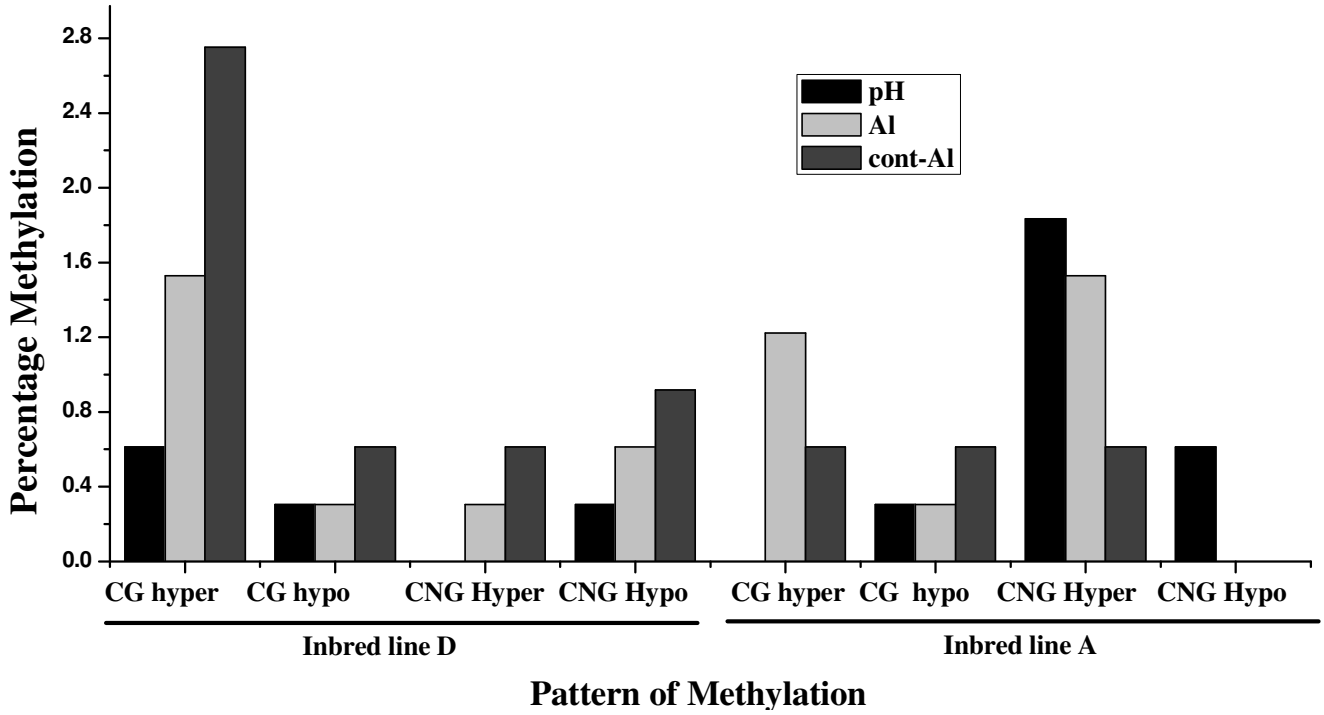

Figure 4. The pattern of cytosine methylation levels of the two Sorghum inbred lines A and D.

compared with inbred line D which had 1.5% CG hyper methylation at 150 µM Al and 0.6% at low pH. The difference was more pronounced in the continuous ASS application with more CG hyper methylation taking place in D (2.75%) than in inbred line A (0.61%). This showed inbred line A epigenome to be more stable than that of D. This was also reflected at low pH (Figure 4). Suggesting that some tolerant genes might have been activated by the stress as shown by the hypomethylation or others where silenced via hypermethylation. Generally, this indicated that the morphological stability of A was either based on shutting down some high metabolic consuming genes or activating only key stress tolerant genes as shown by the CG and CNG methylation patterns.

Analysis of the CCGG deviations due to the Al and low pH stress

The NTSYSpc version 2.10e software distinguished the two inbred lines clearly (Figure 5) and then showed a uniform deviation pattern from the controls in both *Hpa*II cuttings. However, the inbred line D was affected earlier in both *Hpa*II and *Msp*1 CCGG cutting patterns. The high Al stress in inbred A tended to push the line towards the control probably due to the activation of stress resistance genes. The patterns seems to indicate that low pH seems to cause more changes in inbred A than in D, although the changes in inbred D seems to be more stochastic and with a more deviation from the control.

Variant bands and approximate chromosomal distribution and functional relevance

Using the prediction method we found the MSAP band variants to have been from areas whose domains are concerned with epigenetic, cell membranes, cytosol and mitochondrial membranes related transcriptional and chromatin modifying regulators. This seemed to specifically form interactions with histone deacetylases, transcription factors and non coding proteins (see Table S3). This analysis of the bands showed that the effects of Al toxicity altered cytosine DNA methylation through complex pathways for example; CHR1 (CHROMATIN REMODELING 1); ATPase/ helicase; SWI2/SNF2 like chromatin remodeling proteins, chromatin remodeling factor DDM1a. The DDM1 appeared to act as a chromatin-remodeling ATPase involved in cytosine methylation in the CG and non-CG contexts; it is indeed involved in gene silencing and maintenance of DNA methylation and histone methylation.

The DNA cytosine methylation enzyme methyl-transferase was also shown to have been triggered alongside transposons proteins like the gypsy-like retrotransposon family, transcriptional repressors, repeat sequence proteins and ATCSLD4 — a cellulose synthase/ transferase, transferring glycosyl groups which encodes a gene similar to cellulose synthase. Other morphologically related band sequence results included the possible effects of Al toxicity influence on the outer membrane like the OMP85 family protein, which is

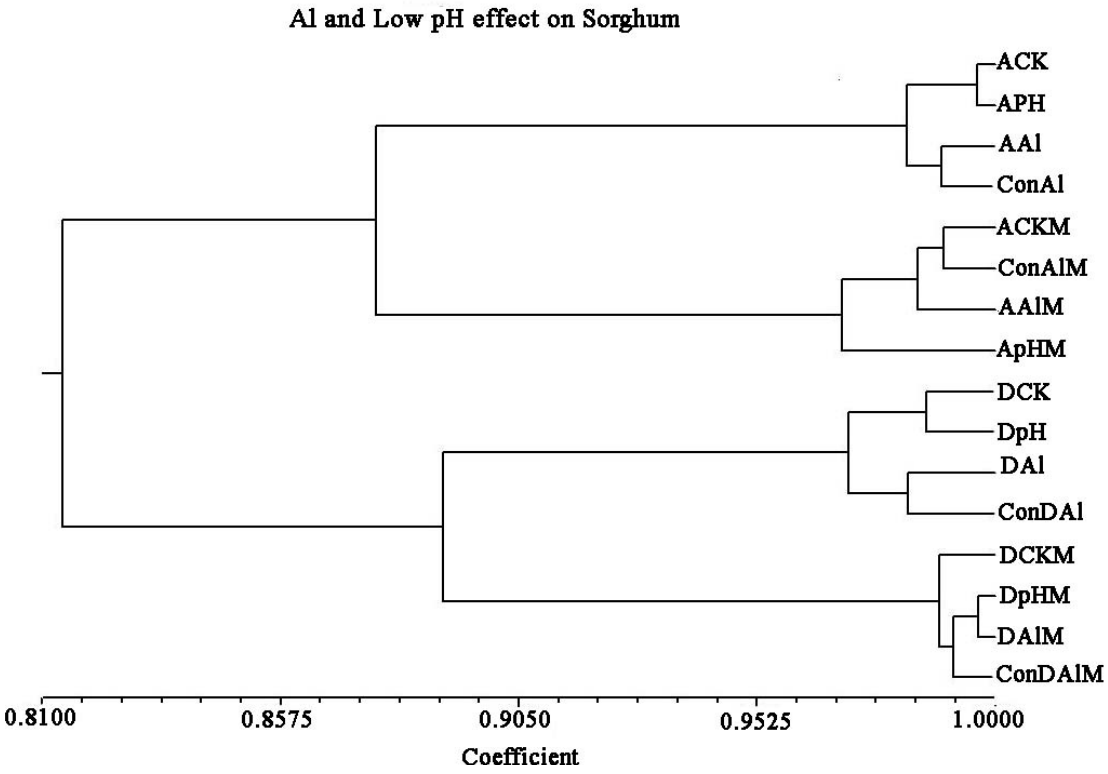

Figure 5. The clustering of the A and D inbred lines MSAP data using NTSYSpc version 2. The upper part of each dendrogram cluster show the *Hpa*II cutting while the lower part show the CCGG cutting from the *Msp*I (indicated with a "M"). The MSAP distinguished the two inbred lines and shows a pattern of the way the stresses made the plant to deviate from the controls, ACK and DCK.

expressed in callus formation. The anthocyanin related Anthocyanidin 5, 3-O-glucosyltransferase, putative, was indicated to have been expressed in the inbred lines during the abiotic stresses. The low pH triggered also ion related proteins like the zinc knuckle domain containing protein and mitochondrial substrate carrier family protein, which functions in binding, calcium ions involved in cytosol transport located in the mitochondrial inner membrane. This is well connected to the citrate organic acid release due to the Al resistance genes in sorghum.

DISCUSSION

Reliable development of high yielding sorghum cultivars in Al toxic and acidic soils can be achieved through the exploitation of the intraspecific genetic and epigenetic variation for aluminum (Al) tolerances. There is increasing evidence to support the suggestions that some genomic DNA changes or mutations are triggered by environmental stresses and are adaptive in nature, and hence they are not random (McClintock, 1978, 1984, reviewed by Metzgar and Wills, 2000; Rando and Verstrepen, 2007). This phenotypic plasticity in stress makes the plants to adjust to the reprogramming of cell differentiation especially in reproductive development during stress (Chinnusamy and Zhu, 2009). DNA cytosine methylation has been shown to be epigenetic in function due to its sensitivity and responsiveness (Boyko and Kovalchuk, 2008). Plants strive to maintain a strict balance between the advantageous response and the deleterious genomic changes triggered by environmental stresses in order to have an evolutionary relevance (Zhang et al., 2009). Because developmental stability is related to canalization or the robustness at the phenotypic level (Gibson and Dworkin, 2004), we deduced that mildness in Al and low pH toxicity levels could be used by plant to stably fine tune gene and epigenetic expressions in stressful conditions during plant breeding.

Cytosine DNA methylation plays an important central role in coordinating other epigenetic mechanisms like histone modification and small RNAs via enzymes such as DNA methyltransferases and 5-methylCytosine glycosylases (Zhang et al., 2009). Hence the identify-cation of a highly QTL trait plastic inbred line in stress conditions can be a valuable resource in plant breeding. Past researches had identified that aluminium tolerance in sorghum is controlled by a large gene on chromosome 3, Alt_{SB}, which is homologue to chromosome 1 of rice

Table S3. The genes which were identified as putative orthologues from the variant MSAP bands obtained after low pH and Al stresses; described as "1" orthologues if only one copy is in each species, "2" if there are many homologues genes in both cases and 3 if there are many homologues genes in many species . The list below shows the identified Novel Ensembl predictions from http://www.gramene.org/Multi/blastview.

Species	Type	Ensembl identifier	External ref.
Oryza sativa	1	LOC–Os11g10510 Target % identity (id) : 92; Query % id: 92	Dehydrogenase, putative, expressed
	2	LOC–Os09g08930 Target %id: 22; Query %id: 33	Transposon protein, putative, CACTA, En/Spm sub-class
	1	LOC–Os08g19420 Target %id: 62; Query %id: 64	O-methyltransferase, putative, expressed
Arabidopsis thaliana	3	AT4G08200-TAIR-G Target %id: 29; Query %id: 22	Transposable element gene; similar to unknown protein [*Arabidopsis thaliana*] (TAIR:AT1G43722.1); similar to Zinc transporter, putative [*Asparagus officinalis*] (GB:ABB55331.1); contains domain gb def: SI:dZ173M20.16 (Novel transposase) (PTHR22930:SF4); contains domain family not named (PTHR22930)
	3	AT2G05720-TAIR-G Target %id: 13; Query %id: 24	Transducin family protein / WD-40 repeat family protein; transducin family protein / WD-40 repeat family protein; functions in: nucleotide binding; involved in: biological_process unknown; located in: CUL4 RING ubiquitin ligase complex; expressed in: shoot apex, petal, cauline leaf; expressed during: petal differentiation and expansion stage; contains InterPro DOMAIN/s: WD40 repeat-like (InterPro:IPR011046), WD40 repeat, region (InterPro:IPR017986), WD40/YVTN repeat-like (InterPro:IPR015943), WD40 repeat (InterPro:IPR001680);
	1	AT1G80880-TAIR-G Target %id: 37; Query %id: 39	Pentatricopeptide (PPR) repeat-containing protein; pentatricopeptide (PPR) Repeat-containing protein; involved in: biological_process unknown; expressed in: flower; expressed during: 4 anthesis; contains InterPro DOMAIN/s: Pentatricopeptide repeat (InterPro:IPR002885).
Oryza sativa	1	LOC–Os08g17080 Target %id: 74; Query %id: 75	PPR repeat domain containing protein, putative, expressed
Arabidopsis thaliana	2	AT2G23990-TAIR-G Target %id: 34; Query %id: 38	Plastocyanin-like domain-containing protein;plastocyanin-like domain-containing protein; functions in: electron carrier activity, copper ion binding; located in: anchored to membrane; expressed in: embryo; expressed during: C globular stage; contains interpro domain/s: Plastocyanin-like (InterPro:IPR003245), Cupredoxin (InterPro:IPR008972);

Table S3. Contd.

	2	AT3G16520-TAIR-G Target %id: 37; Query %id: 36	UDP-glucoronosyl/UDP-glucosyl transferase family protein; UDP-GLUCOSYL TRANSFERASE 88A1 (UGT88A1); functions in: in 6 functions; involved in: metabolic process; located in: cellular_component unknown; expressed in: 22 plant structures; expressed during: 13 growth stages; contains interpro domain/s: UDP-glucuronosyl/UDP-glucosyltransferase (InterPro:IPR002213)
Oryza sativa	3	LOC–Os05g45100 Target %id: 72; Query %id: 73	Anthocyanidin 5,3-O-glucosyltransferase, putative, expressed
	3	LOC–Os04g16270 Target %id: 8; Query %id: 13	Nucleic acid binding protein, putative
	3	LOC–Os04g20610 Target %id: 21; Query %id: 25	Zinc knuckle domain containing protein
	3	LOC–Os05g16080 Target %id: 11; Query %id: 20]	Retrotransposon protein, putative, unclassified
	3	LOC–Os09g07000 Target %id: 5; Query %id: 15	Retrotransposon protein, putative, Ty3-gypsy subclass
Oryza sativa	1	LOC–Os08g17160 Target %id: 54; Query %id: 51	Plastocyanin-like domain containing protein, putative, expressed
Oryza sativa	3	LOC–Os12g19580 Target %id: 9; Query %id: 19]	Photosynthetic reaction center protein, putative
Arabidopsis thaliana	1	AT4G38190-TAIR-G Target %id: 74; Query %id: 72	ATCSLD4; cellulose synthase/ transferase, transferring glycosyl groups; encodes a gene similar to cellulose synthase
Oryza sativa	1	LOC–Os08g25710 Target %id: 89; Query %id: 87	CSLD3 - cellulose synthase-like family D, expressed
Arabidopsis thaliana	3	AT1G36610-TAIR-G Target %id: 15; Query %id: 10	Transposable element gene; gypsy-like retrotransposon family, has a 9.1e-283 P-value blast match to GB:AAD27547 polyprotein (Gypsy_Ty3-element) (Oryza sativa subsp. indica)
	1	AT4G34490-TAIR-G Target %id: 55; Query %id: 64	ATCAP1 (ARABIDOPSIS THALIANA CYCLASE ASSOCIATED PROTEIN 1); actin binding; CYCLASE ASSOCIATED PROTEIN

Table S3. Contd.

	3	AT2G16230-TAIR-G Target %id: 37; Query %id: 42	catalytic/ cation binding / hydrolase, hydrolyzing O-glycosyl compounds; catalytic/ cation binding / hydrolase, hydrolyzing O-glycosyl compounds; functions in: cation binding, hydrolase activity, hydrolyzing o-glycosyl compounds, catalytic activity; involved in: carbohydrate metabolic process; located in: endomembrane system; expressed in: root; contains interpro domain/s: X8 (InterPro:IPR012946), Glycoside hydrolase, family 17 (InterPro:IPR000490), Glycoside hydrolase, catalytic core (InterPro:IPR017853), Glycoside hydrolase, subgroup, catalytic core (InterPro:IPR013781);
	2	AT5G66750-TAIR-G Target %id: 63; Query %id: 58	CHR1 (CHROMATIN REMODELING 1); ATPase/ helicase; Protein is similar to SWI2/SNF2 chromatin remodeling proteins. DDM1 is appears to act as a chromatin-remodeling ATPase involved in cytosine methylation in CG and non-CG contexts. Involved in gene silencing and maintenance of DNA methylation and histone methylation. Hypomethylation of many genomic regions occurs in ddm1 mutants, and can cause several phenotypic abnormalities, but some loci, such as BONSAI (At1g73177) can be hypermethylated in ddm1 mutants after several generations, leading to different phenotypes. DDM1 might be involved in establishing a heterochromain boundary. A line expressing an RNAi targeted against DDM1 shows some resistance to agrobacterium-mediated root transformation.
Homo sapiens	2	ENSG00000119969 Target %id: 34; Query %id: 34	Lymphoid-specific helicase (EC 3.6.1.-)(SWI/SNF2-related matrix-associated actin-dependent regulator of chromatin subfamily A member 6)(Proliferation-associated SNF2-like protein) [Source: UniProtKB/Swiss-Prot; acc: Q9NRZ9]
Oryza sativa	3	BGIOSIBCE030113 Target %id: 78; Query %id: 78	Chromatin remodeling factor DDM1a. [Source: Uniprot/SPTREMBL; acc: Q05KP6]
Oryza sativa and Saccharomyces cerevisiae	3	LOC_Os03g51230 Target %id: 79; Query %id: 80	SNF2 family N-terminal domain containing protein, expressed
Arabidopsis thaliana	2	AT5G05520-TAIR-G Target %id: 56; Query %id: 56	Outer membrane OMP85 family protein; located in: mitochondrion, plastid; expressed in: 24 plant structures; expressed during: 14 growth stages;
	2	AT5G07320-TAIR-G Target %id: 70; Query %id: 65	Mitochondrial substrate carrier family protein; functions in: binding, calcium ion binding; involved in: transport; located in: mitochondrial inner membrane, membrane; expressed in: 23 plant structures; expressed during: 15 growth stages; contains interpro domain/s: EF-HAND 1 (InterPro:IPR018247),

Table S3. Contd.

2	AT1G10450-TAIR-G Target %id: 39; Query %id: 37	SNL6 (SIN3-LIKE 6); Encodes SIN3-like 6, a homolog of the transcriptional repressor SIN3 (AT1G24190).
2	AT3G11070-TAIR-G Target %id: 55; Query %id: 54	Outer membrane OMP85 family protein; expressed in: callus; contains interpro domain/s: Bacterial surface antigen (D15) (InterPro:IPR000184), Surface antigen variable number (InterPro:IPR010827)
2	AT1G59890-TAIR-G Target %id: 41; Query %id: 39	SNL5 (SIN3-LIKE 5); SIN3-LIKE 5 (SNL5); involved in regulation of transcription, DNA-dependent; located in: nucleus; expressed in: 24 plant structures; expressed during: 15 growth stages; contains InterPro domain/s: Histone deacetylase interacting

from which region many QTLs for aluminum tolerance in rice have been identified (Magalhães et al., 2004). The complex mechanism by which the Al toxicity and low pH in acid soils could have caused differences in methylation at CG/CNG levels and CG/CNG patterns might be similar or linked to the one in which it induces the expression of oxidative stress, reactive-oxygen species (ROS) genes and promotes lipid per oxidation, all of which have been associated with epigenetic changes in plants (Yamamoto et al., 2001; Milla et al., 2002; Boscolo et al., 2003). The membrane transporters which are involved in Al tolerance could be damaged by ROS at gene level or at function level based on recent evidence in animals (Mena et al., 2009) who reported that ROS can cause protein, lipid, and DNA damage in cells.

The DNA cytosine methylation is maintained and coordinated by several enzymes like DNA methyltransferase 1 (*MET1)*, chromomethylase 3 (*CMT3*), and domain rearranged methyl-transferase 2 (*DRM2)*. It plays an important role in controlling gene expression. DNA cytosines are methylated mainly in transposable elements, repetitive sequences but recent studies have shown that DNA cytosines in promoters are also methylated and hence regulate transcription of genes (Lister et al., 2008; Li et al., 2008; Cokus et al., 2008). The present findings showed the activation of genes like membrane transporters, chromatin remodeling factor DDM1a. The DDM1 acts as a chromatin-remodeling ATPase which is involved in cytosine methylation in the CG and non-CG contexts; it is indeed involved in gene silencing and maintenance of DNA methylation and histone methylation. The methyltransferase which is involved in DNA cytosine methylation was activated in continuously Al^{3+} exposed sorghum plants along side transposons proteins like the gypsy-like retrotransposon family, transcriptional repressors. DNA methylation has been referred to as a repressive mark in chromatin gene promoters of the genome (Wang et al., 2007). This explains the DNA methylation CG and CNG pattern variations in the stresses. Al^{3+} toxicity action of disrupting the cell membrane was reflected by resistance plants activating the homologous ATCSLD4 a cellulose synthase/ transferase, transferring glycosyl groups which encodes a gene similar to cellulose synthase. Some tolerant species produce callus in cases of Al^{3+} injury, the resistance of sorghum to Al^{3+} could be reflected by the activation of an outer membrane like the OMP85 family protein which is usually expressed during callus formation. We could explain the anthocyanin production in Al^{3+} and low pH by considering the activation of the related anthocyanidin 5, 3-O-glucosyltransferase, putative.

The exudation of organic acids like citrate and malate due to Al^{3+} toxicity could be confirmed by the triggered proteins like the zinc knuckle domains and mitochondrial substrate carrier family proteins, which functions in binding, calcium ions (Ca^{2+}) and are involved in cytosol transport some from the l mitochondrial inner membrane. This is well connected to the citrate organic acid release due to the Al resistance genes in Sorghum. Such findings using the MSAP analysis give a wealth of information which can aid in unlocking the reason why citrate influx could not explain the differences in Al tolerance amongst cultivars (Piñeros et al., 2005; Zheng et al., 2005).

Sorghum is more stable at high stresses than in low stresses

Nucleosome histone post-translational modifica-tions and sometimes DNA methylation often

determine gene expression based on developmental and stress cues (Zhu, 2008). Studies have shown that there is a resetting of most of the stress induced modification in later suitable conditions but some are retained and inherited as stress memories to enable plants to cope with similar future stresses (Chinnusamy and Zhu, 2009). Here we report that the sorghum genome seem to have mechanisms which make it have an epigenome which is stress resistant. This stability of sorghum under stress conditions may be the reason for its difficulty in many gene transformation programs. We found that sorghum performed even better under more mild Al toxicity. This seems to suggest the flexibility of the genome of sorghum towards harsh conditions and possible activation of adaptive tolerant genes in extreme conditions. Hence, supporting the idea that Al tolerance is basically not an inherent characteristic in some plants, but a derived state (Garvin and Carver, 2003) although, the activation of a gene which is triggered by deficiency of phosphorus could reflect on the effect of Al^{3+} mineral interaction in the ASS solution. These findings could explain the methylation changes after continuous application of Al in the Sorghum inbred lines A and D at CG/CNG levels and CG/CNG patterns.

In addition, although transposons are usually methylated and hence maintained in a repressive state, abiotic stress can activate transposons through cytosine DNA demethylation (Hashida et al., 2006). The low level of hypomethylation may indicate little transposon element activation in sorghum which reflects the stability of the sorghum epigenome. Studies have shown that environmental and endogenous protein signals can also repress gene expression through the deacetylation of histones like in transcriptional gene silencing (TGS) and RNA-directed DNA methylation (RdDM). These dynamic histone modifications can be converted into the stable DNA methylation (Chinnusamy and Zhu, 2009; Probst et al., 2004; Aufsatz et al., 2002). Recent studies by Rossi et al. (2007) showed that the down-regulation and over-expression of the maize Rpd3-type *hda101* histone deacetylase gene induced some morphological and developmental defects. Although the total levels of acetylated histones and histone acetylation of both repetitive and non-repetitive sequences were affected in *hda101* transgenic mutants, which altered only the transcript levels of genes but not the repeats.

This altered epigenetic status that is closely associated with chromatin remodeling in a particular stress condition can render a plant to be more stress tolerant. Furthermore, transcriptional activation has frequently been associated with local acetylation of histone proteins which are found near the promoter regions of genes (Finnegan, 2001). DNA methylation and histone deacetylation are linked in plants (Li et al., 2002); however the full understanding of how DMTases and HDACs target and regulate specific regions of the genome is limited (Aufsatz, 2002). The observed peptide signal could be hormonal signal which has been reported as being responsible for some loci specific chromatin modifications in plants (Nielsen, 1997; Sokol et al., 2007, Chinnusamy and Zhu, 2009). For example, the ROS3 is a RNA recognition motif that contains a protein. It binds to small RNAs and has been reported to be capable of directing sequence-specific demethylation by ROS1 and related DNA demethylases (Zheng et al., 2008). Moreover, *Bäurle* et al. (2007) reported that FCA and FPA proteins in *Arabidopsis,* which form an autonomous flowering pathway by down regulating flowering repressor FLC, have the ability to regulate DNA methylation. We propose that an increase and maintainace of low pH in soil could have triggered dissimilar DNA Cytosine alterations especially in inbred line A. Furthermore, this may seem to confirm the hypothesis that an excess of Al might still be the activator of the physiological exudation mechanism of Al^{3+} after combining with organic acids in tolerant plants as mediated by the anionic channel activity in the membranes (Ma et al., 2001; Jones and Ryan, 2003).

The low cytosine DNA methylation alteration and stability in sorghum could still be explained by the low LTRs in sorghum compared to other cereals for a particular locus. For example evolutionary deletions, insertions or alterations in sequences in a span of 78-kb in the locus *Adh1* (Tarchini et al., 2000) revealed that there is more than double (57%) conserved DNA with no LTRs in sorghum than in maize (22%) which had most of it being LTRs in the same locus (Tikhonov et al., 1999). This conservation of genes in sorghum necessitates a deeper search for homologs which may identify new sources in Al tolerance diversity (Magalhães, 2006), in which the polymorphic nature of the MSAP can be useful. This DNA cytosine stability results indicate that choosing the inbred Line A as a recurrent parent in breeding programs is more appropriate in backcrosses because it might have less background effects as an Al tolerant line (Hoekenga et al., 2006). Other epigenetic studies especially cytosine DNA methylation when coupled with map-based cloning may advance the exploration of the mechanisms of Al tolerance for food production in marginal soils in developing countries which could help in limiting agricultural encroachment into forest and water catchment areas.

Al effect on sorghum seeds and its future memory influences

The increase in seed size could possibly be explained by linking it to the effect of secondary stress signals; Based on earlier studies that had suggested that ABA accumulation which regulates seed development through epigenetic processes, we may postulate that ABA may be responsible for the sorghum seed size alteration in low pH and Al stress (Chinnusamy et al., 2008).

The observed early leaf senescence and anthocyanin accumulation due to the Al and pH stress, normally lead to reduced photosynthesis and less biomass accumulation. Studies by Tian and Chen (2001) showed that *Jasmonic acid (*JA) and ethylene responsive HDACs, HDA6 and HAD19 appear to control leaf senescence. Wu et al. (2008) further illustrated that the delayed senescence was associated with down regulation of senescence genes while the flowering delay was associated with enhanced expression of FLC. These observations agree with a phenomenon of epigenetic DNA methylation or histone modification in gene expression regulation. However, although epigenetic stress induced changes might have an adaptive advantage; the source of the seeds must be carefully selected as there is a possibility of intrinsic maintainace of a negative impact on crop yield in plant stress memory in the future when suitable conditions are provided.

Conclusions

Al^{3+} is eliciting a primary stress response from sorghum by triggering secondary stress signals like plant growth regulators (PGR), like ABA which in turn change the expression of epigenetic regulators like DNA methyltransferases, histone modifications enzymes, chromatin remodeling factors which influenced the alteration of cytosine DNA methylation and histone modifications causing most likely a non heritable change or mitotically heritable change in the sorghum plants.

The sorghum plant which is seemingly a very tolerant plant has, through the years of evolutions and adaptations, developed mechanisms to maintain the integrity of its genome in times of abiotic stresses. Further studies can be done to evaluate how the interspecies correlation between aluminium tolerant genes like Alt_{SB} and protein abundance relates to epigenetic mechanisms in Sorghum, using peptide antibodies at early transitions stages of seedling growth in root tips and other specific cell locations using immuno-localizations.

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