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Characterization of markers linked to resistance motifs against maize lethal necrosis in Tanzanian maize germplasm

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Among the biological constraints facing maize production in Tanzania is a severe occurrence of maize lethal necrosis disease (MLN) raising an urgent need for application of new approaches. A pool of 22 maize genotypes with promising resistance and susceptibility to MLN infection were evaluated by Amplified fragment length polymorphism (AFLP) DNA fingerprinting analysis to detect genetic variation in the selected lines. Eleven AFLP primer combinations were screened and resulted in the identification of 95 polymorphic AFLP allelic fragments. Genetic similarities among the selected Tanzanian maize landraces and other maize lines were estimated by Unweighted Pair Group of Arithmetic Mean (UPGMA) and genotypes were clustered in three primary groups according to their reaction to MLN disease. Promising resistant and tolerant genotypes were grouped in cluster I and susceptible genotypes in clusters II and III. Landraces were grouped according to agro-ecological locations where they were collected. Unambiguous polymorphic AFLP fragments were eluted, purified and sequenced. Sequencing and nucleotide alignment on Basic Local Alignment Search Tool (BLAST) analysis showed similarities of fragments consistent with transcripts involved in disease resistance and stress responses. Further studies will explore the potential application of the identified AFLP markers and their significant association to MLN disease resistance genes in maize.

Key words: *Zea mays,* AFLP, maize lethal necrosis disease, Tanzania.

INTRODUCTION

Maize (*Zea mays* L.) is one of the world's major cereal crops and the third most important crop after wheat and rice (CIMMYT, 1990; Legesse et al., 2006). In recent years, maize has been ranked the first crop in production among other major cereals due to increased global demand for maize both as a major staple food and as an industrial raw material (FAOSTAT, 2016). In Tanzania, maize is the major cereal produced that contributes to

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about 60% of dietary carbohydrates for human consumption and provides more than 50% of utilizable protein for the Tanzanians growing population (Bisanda et al., 1998; Suleiman and Rosentrater, 2015).

Among the biological constraints facing maize production in Tanzania is the occurrence of maize lethal necrosis disease (MLN). MLN is a disease synergism caused by the infection of maize with maize chlorotic mottle virus (MCMV) and any of the potyvirus infecting cereal (Uyemoto et al., 1981). In Africa, MLN was first reported in Kenya in September 2011 and quickly spread to Tanzania in 2012 where it was locally reported as an unknown disease in Mwanza near Lake Victoria area and Arusha (CIMMYT, 2013). This disease has become a major setback in maize growing areas of East Africa (Wangai et al., 2012); hence standing out as the greatest threat to African food security crop (maize). MLN causes serious yield losses of up to 100% depending on the stage of growth of maize plant when it is attacked and particularly when the disease is not effectively controlled (CIMMYT, 2013).

To keep pace with the increased demand of maize due to the expanding population, the development of varieties with enhanced tolerance to biotic and abiotic constraints is thus a significant objective to attain (Boomsma and Vyn, 2008). Effective screening on Tanzanian's maize populations is vital to enhance the identification of genetic resistance for MLN. Currently, there is no published report showing resistance for MLN in Tanzanian maize core germplasms, however, research conducted by International Maize and Wheat Improvement Center (CIMMYT) in Kenya has revealed some promising inbred lines and pre-commercial hybrids with moderate resistance to MLN (CIMMYT, 2013). This underscores an urgent need for application of new approaches such as the use of molecular markers to screening for MLN genetic resistance in Tanzania's maize populations.

To date, a variety of molecular marker techniques have been developed for identifying polymorphisms in plant materials; restriction fragment length polymorphisms (RFLPs) were the first widely used DNA hybridization based molecular markers. Other PCR based techniques include random amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLPs), single nucleotide polymorphisms (SNPs), simple sequence repeats (SSRs) and other emerging techniques (Melchinger, 1990; Stevens, 2008; Wang et al*.,* 2011).

Amplified fragment length polymorphism relies on the use of polymerase chain reaction (PCR) for amplification of DNA. This approach offers several advantages over other DNA markers as it combines the advantages of PCR based technique in terms of efficiency, high throughput and amenability to automation with the specificity and robustness of RFLP based technique (Bhat et al., 2004). AFLP analysis can be applied in any plant species without previous knowledge of DNA sequence (Sigh et al., 2010). Although the technique is laborious and time-consuming, it is highly reliable due to its ability to detect many polymorphic bands in a single lane rather than high levels of polymorphism at each locus as compared to other marker methods such as microsatellite markers (Garcia et al., 2004; He and Prakash, 1997).

Studies that apply molecular markers to access genetic variability and phylogenetic relationships in Tanzanian maize populations are still limited. In this study, we used a powerful molecular technique that does not rely on previously known genes (the AFLP) to screen a diverse set of Tanzania maize germplasm, including landraces and inbred lines. The aim was to identify and characterize genetic markers that may be linked to resistance genes against infection by MLN disease-causing pathogens. A better understanding of resistance to MLN disease in maize and deploying the identified resistance genes in commercial maize varieties could facilitate the genetic control of MLN in Tanzania which would contribute to more practical and effective solutions for small-holder farmers.

MATERIALS AND METHODS

Plant materials

Twenty-two maize genotypes were used as genetic materials in this study that included 12 landraces and 2 lines selected as tolerant and sensitive under artificial MLN disease evaluation at Naivasha MLN screening facility in Kenya by Ritte et al. (2017), 4 CIMMYT maize lines and 4 maize lines of U.S. origin with known MLN disease reaction backgrounds. Landraces and maize lines used to represent Tanzanian maize germplasm were provided by the National Plant Genetic Resources Center (NPGRC) and Selian Agricultural Research Institute (SARI) respectively, both located in Arusha-Tanzania. CIMMYT lines were provided by CIMMYT-Kenya whereas the US maize lines were donated by the University of Nebraska Lincoln and the United States Department of Agriculture (USDA). The US lines were used for preliminary AFLP experiments at Tuskegee University and genomic DNA of these materials was shipped for experiments conducted in Tanzania. Descriptions of these materials are shown in Table 1.

Genomic DNA extraction

Seeds samples of plant materials used in this study were germinated in a screen house at the department of crop science and horticulture, Sokoine University of Agriculture, Morogoro – Tanzania. Young maize leaves were sampled from seedlings of each maize landrace/line at four to five leaf growth stages. Samples were transported on ice to the laboratory and stored at -20°C followed by genomic DNA extraction as described in Egnin et al. (1998). The quality of DNA was assessed on 0.8% agarose gel electrophoresis and the concentration was determined by a known amount of λ DNA as standard. Agarose gel electrophoresis confirmed that the DNA was of high molecular weight with no contaminating RNA or degradation.

Table 1. List of 22 maize germplasm subjected to MLN AFLP screening.

S/N	Genotype ID	Response to MLN	Source
1	CML 494	Promising resistant	CIMMYT
2	CLYN 261	Promising resistant	CIMMYT
3	CLYN 231	Promising resistant	CIMMYT
4	TZA-3567	Tolerant	NPGRC
5	TZA-2793	Tolerant	NPGRC
6	TZA-3585	Tolerant	NPGRC
7	TZA-3543	Tolerant	NPGRC
8	TZA-4505	Tolerant	NPGRC
9	N 218	General Resistance	Nebraska
10	OH 7B	Tolerant	USDA
11	TZA-4320	Moderately Susceptible	NPGRC
12	TZA-5171	Moderately Susceptible	NPGRC
13	TZA-2292	Moderately Susceptible	NPGRC
14	CL-G2620	Susceptible	CIMMYT
15	TZA-5200	Susceptible	NPGRC
16	TZA-4043	Susceptible	NPGRC
17	TUX 5-50-1-3-1-1	Susceptible	SARI
18	KS 03-OB15-111	Very Susceptible	SARI
19	TZA-2264	Very Susceptible	NPGRC
20	TZA-1758	Very Susceptible	NPGRC
21	A635	Very Susceptible	USDA
22	OH 43	Very Susceptible	USDA

AFLP analysis

AFLP analysis procedure was performed with modifications of the protocol of Vos et al. (1995) supplied with the AFLP Analysis System I kit (Invitrogen, USA). About 500 ng of genomic DNA was digested with two restriction enzymes, *Eco*R I and *Mse* I (Invitrogen, USA), at 37°C for 2 h and 30 min followed by incubation at 70°C for 15 min to inactivate the restriction enzymes. *EcoRI* and *MseI* adapters were ligated to the digested fragments at 20°C for 2 h to generate template DNA for amplification. A four-fold dilution was performed on ligated DNA. Pre-selective amplification was performed with 5.5 µl of diluted ligated DNA template, 40 µl of pre-amp primer mix I (*Eco*R I-A/*Mse* I-C), 5 µl of 10X PCR buffer plus $MgCl_2$ and 0.5 µl of *Taq* DNA polymerase (5 U/µl) in 0.2 ml PCR tube. The PCR amplification conditions were set as, 94°C for 30 s, 56°C for 1 min and 72°C for 1 min or 20 cycles in MyCycler Thermal Cycler (*Bio-Rad*, USA). A 4-fold dilution was performed on the pre-amplified reaction in 1X TE buffer and stored at -20°C until ready for use.

For selective amplification, 11 primer combinations (*Eco*R I/*Mse* I) were employed. Each primer pair reaction mix was prepared by combining 5 µl of *Eco*R I primer (27.8 ng/µl) and 45 µl of *Mse* I primer (6.7 ng/µl) to obtain "Mix 1" sufficient for 10 AFLP reactions. Mix 2 reaction mixture enough for 10 AFLP reactions was prepared by pipetting in 1.5 ml Eppendorf tube 79 µl of distilled water, 20 µl of 10X PCR buffer plus $MgCl_2$ [200 mM Tris-HCl (pH 8.4), 15 mM $MgCl_2$, 500 mM KCL], and 1 µl of *Taq* DNA polymerase (5 U/µl). 5 µl of diluted pre-amplified DNA, 5 µl of "Mix 1" and 10 µl of "Mix 2" were combined in a 0.2 ml PCR tube, then subjected to PCR at the following conditions; incubation at 94°C for 30 seconds and one cycle at: 94°C for 30 s; 65°C for 30 ss and 72°C for 1 min, followed by 13 cycles of touchdown PCR where the annealing temperature was lowered by 0.7°C during 12 cycles. This was followed by 23 cycles at 94°C for 30 s, 56°C for 30 s and 72°C for 1 min. The reaction products were stored at -20°C and used in denaturing polyacrylamide gel electrophoresis (PAGE) analysis.

Denaturing PAGE of amplified AFLP fragments

Denaturing PAGE was performed with modification of the protocol by Summer et al. (2009). An equal volume of 2X TBE-Urea Dye (89 mM Tris base, 89 mM Boric acid, 2 mM EDTA pH 8.0, 10% Glycerol, 0.01% Bromophenol Blue, 0.02% Xylene Cyanol FF, 7 M Urea) was added to each PCR reaction. Samples were denatured by heating at 90°C for 3 min and immediately cooled on ice. PCR products from selective amplification were size separated by horizontal electrophoresis in denatured 6% polyacrylamide gels. Electrophoresis was performed in pre-chilled 1X TBE buffer at constant power (70 V) until Xylene cyanol was about 2-3 cm from the bottom of the gel. After electrophoresis, the gel was post-stained in a 0.5X TBE buffer and ethidium bromide (0.5 µg/ml) with gentle agitation for 25 min, followed by 2 min water rinse, then visualized and images captured by Canon Power Shot A650 (Canon Inc., China) on a UV trans-illuminator (254 nm, with orange filter).

Data analysis

Gel images with amplified fragments were scored in a dominant manner for presence or absence of unambiguous bands as 1 and 0

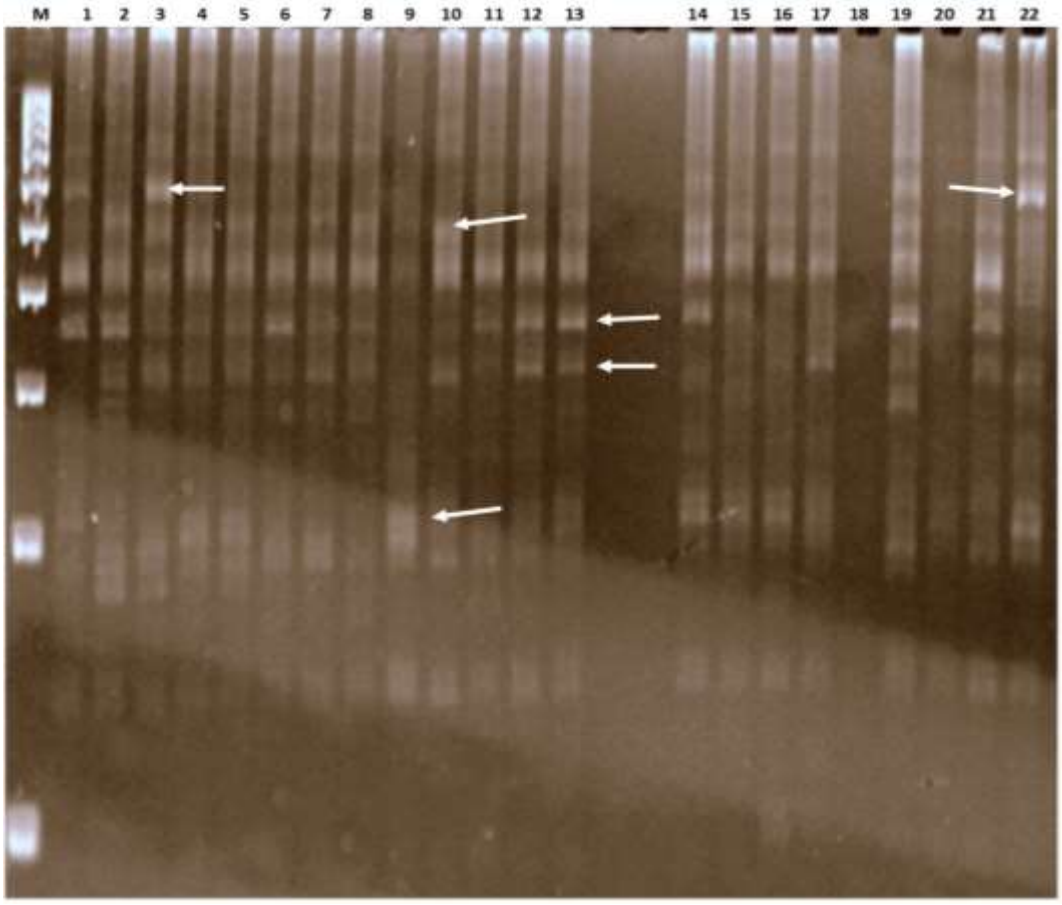

Figure 1. AFLP marker profiles of 22 maize genotypes on 6% polyacrylamide gel. 1, CML494; 2, CLYN261; 3, CLYN231; 4, TZA-3567; 5, TZA-2793; 6, TZA-3585; 7, TZA-3543; 8, TZA-4505; 9, N218; 10, OH7B; 11. TZA-4320; 12, TZA-5171; 13, TZA-2292; 14, CL-G2620; 15, TZA-5200; 16, TZA-4043; 17, TUX5-50-1-3-1-1; 18, KS03-OB15-111; 19, TZA-2264; 20, TZA-1758; 21, A635 and 22. OH43 obtained using primer combination M-CAA/E-ACG. Lane identified by "M" contains 100 bp size DNA ladder ((EZload, Bio Rad®). The arrows indicate polymorphic bands.

respectively to prepare binary matrix. The resulted 1/0 data matrix were exported into a spreadsheet calculated based on the genetic similarity matrix (Nei and Li, 1979) and analyzed using Numerical Taxonomy System (NTSYSpc) software as according to Rohlf (2000). Based on AFLP DNA marker polymorphism data, genetic similarities among the selected Tanzanian maize landraces and other maize lines used in the study were estimated by Unweighted Pair Group of Arithmetic Mean (UPGMA) procedure in cluster analysis and a dendrogram was developed from the similarity matrix (Sneath and Sokal, 1973).

Sequencing and MLN associated AFLP marker development

Unambiguous fragments with strong intensities that were significantly polymorphic in resistant and susceptible genotypes were eluted from gels and purified. Ten microliters of each of eluted fragment were re-amplified with the corresponding primer pairs followed by confirmation on a 2% agarose gel. The confirmed eluted fragments were sent out for sequencing services (Beckman Coulter Genomics Incorporation). Sequence data were uploaded in Bio Edit software version 7.2.5 for sequence editing (Hall, 1999). Edited sequence data were analyzed and compared with sequences of *Zea mays* L. available in the public database using MEGA Software, version 6 by performing nucleotide blast search at the National Center for Bioinformatics (NCBI) Website http://blast.ncbi.nlm.nih.gov/Blast.cgi by using BLASTn program.

RESULTS

AFLP polymorphism

A total of 127 amplified AFLP fragments were revealed among the 22 maize genotypes, 95 of which were polymorphic (Figure 1). The number of amplified AFLP bands ranged from 9 with primer combination (M-CAA/E-AGC) to 17 with primer combination (M-CTG/E-ACA),

Table 2. List of AFLP primer combinations (*Mse* I/*Eco*R I), number of scored AFLP allelic fragments, polymorphic fragments, monomorphic fragments and the percent polymorphism.

S/N	Primer combination	Number of scored allelic fragments	Polymorphic allelic fragments	Monomorphic allelic fragments	Polymorphism (%)
1	M-CTC/E-AAC	13	9	4	69.23
2	M-CTG/E-AAG	10	7	3	70.00
3	M-CTC/E-AAG	11	8	3	72.72
4	M-CAT/E-ACC	10	9	1	90.00
5	M-CAT/E-ACA	12	10	2	83.33
6	M-CTG/E-ACA	17	12	5	70.58
7	M-CAA/E-ACG	13	11	2	84.61
8	M-CAA/E-ACT	10	6	4	60.00
9	M-CTT/E-AGG	12	10	2	83.33
10	M-CTA/E-ACG	10	6	4	60.00
11	M-CAA/E-AGC	9	7	2	77.77
Total		127	95	32	74.68*

*Average polymorphism percentage.

respectively, with sizes ranging from 100 to 800bp. In contrast, the percentage of polymorphism varied from 60% with primer combination (M-CTA/E-ACG) to 90% with primer combination (M-CAT/E-ACC), respectively, and average percentage of polymorphism was 74.7% (Table 2).

Cluster analysis

Binary matrix data that were scored as presence and absence (1/0) of allelic bands were used to construct a dendrogram using unweighted pair group of arithmetic mean (UPGMA) method based on similarity values (Figure 2). The dendrogram revealed three major clusters in which cluster I was further divided into three sub-clusters. The sub-cluster 1.1 included MLN promising resistant CIMMYT lines (CML494, CLYN261, and CLYN231), the sub-cluster 1.2 consisted of the tolerant landraces (TZA-3567, TZA-2793, TZA-3543 and TZA-3585), and the third sub-cluster 1.3 contained tolerant landrace TZA-4505 and the US line N218. Cluster II grouped together the tolerant USDA line OH7B and other MLN moderately susceptible Tanzanian maize landraces. Genotypes TZA-4320, TZA-5171 and TZA-2292 were included in the sub-cluster 2.1. While the sub-cluster 2.2 composed of susceptible CIMMYT line CL-G2620, susceptible landraces TZA-5200, TZA-4043, TZA-1758 and susceptible USDA line A635. The sub-cluster 2.3 grouped together two SARI lines TUX 5-50-1-3-1-1 and KS 03-OB15-111 and the susceptible landrace TZA-2264. The USDA susceptible line OH43 was isolated in cluster III.

Amplicon sequencing

The results of AFLP amplicons sequencing revealed that, out of 63 amplicons sent for sequencing, 32 amplicons were successfully sequenced while 31 amplicons were not. Sequence homology BLAST search at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) using BLASTn program revealed different gene functions (Figure 3) and out of 32 amplicons 22 were homologous to *Z. mays* L. reference genome in the database, whereas 10 amplicon sequences were related to other species. Among the 22 amplicons that were in homology with *Z. mays* L. 15 were associated with plant response to biotic and abiotic stresses (Supplementary materials Table 1).

DISCUSSION

The results of this study showed the efficiency of the AFLP technique for determination of molecular polymorphism in maize germplasm. The AFLP primer combination M-CTG/E-ACA yielded the highest number of 17 amplified DNA fragments and M-CAA/E-AGC with the lowest (9). Primer combination M-CAT/E-ACC showed significant molecular polymorphism percent (90%). While more laborious and time-consuming (Garcia et al., 2004), AFLP can lead to the detection of large numbers of bands in a single lane of the AFLP gel, which in turn increases the chance of finding polymorphic markers per lane (He and Prakash, 1997).

These results are in line with those of Maheswaran et al. (1997) who detected a substantial number of

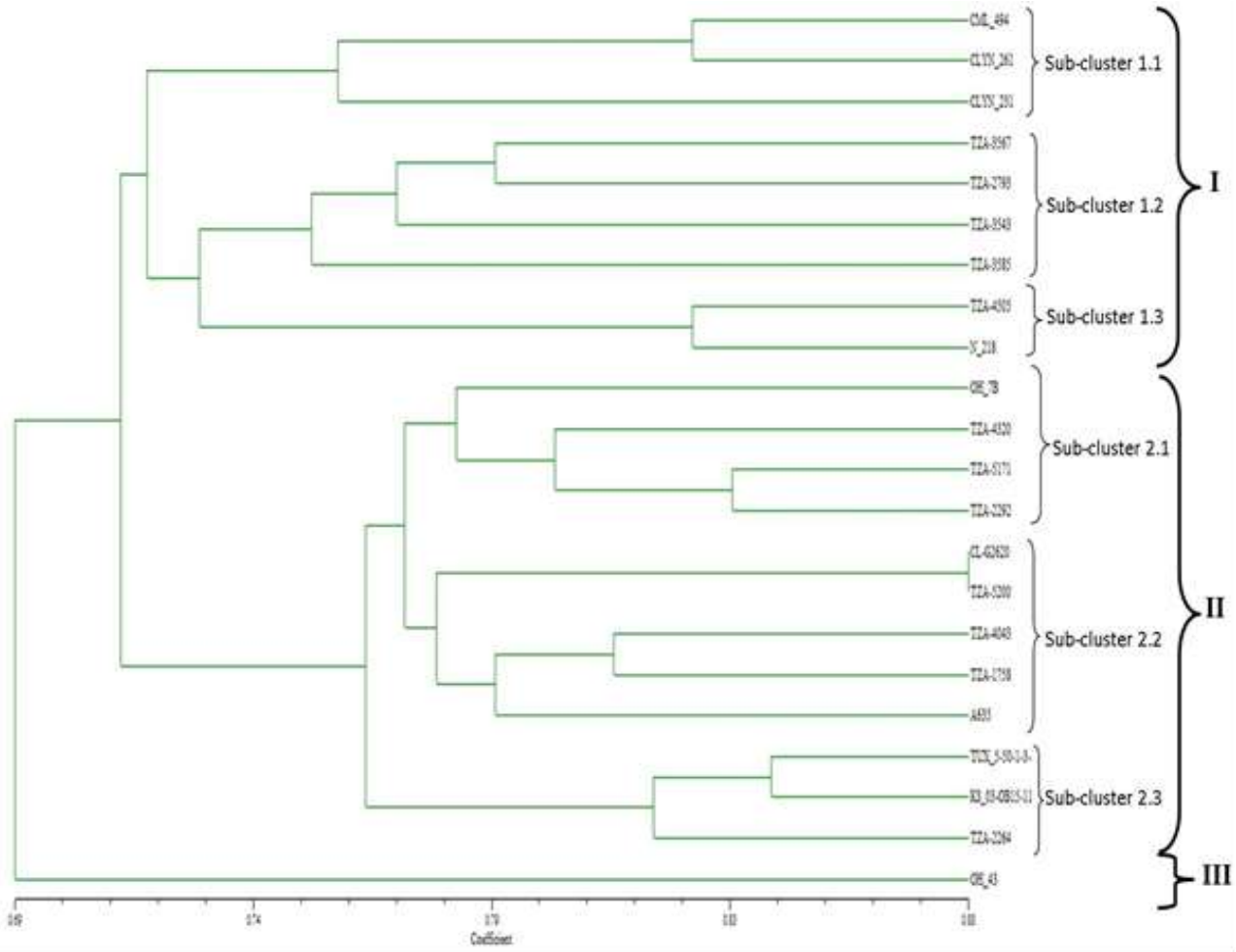

Figure 2. UPGMA dendrogram showing genetic relationships among the 22 maize genotypes generated based on Jaccard's coefficients and AFLP binary matrix data. I, II and III indicate major groups.

polymorphic AFLP bands in studies involving *Oryza sativa*. In this study, low polymorphism was noted on primer pairs M-CTA/E-ACG and M-CAA/E-ACT which attained the polymorphism of 60% respectively. This could be linked to the type of primer combinations used or scoring method applied while conducting the present investigation, as only consistent bands were scored and suspicious bands were not included (Vos et al., 1995). Cluster analysis showed that the genotypes were grouped into three clusters based on their genetic differences, responses to MLN and the geographical origins where landraces were collected. Although the resulted groups were consistent with resistance traits, some mixtures were observed. A similar result has been reported earlier in sorghum accessions and breeding varieties by Uptmoor et al. (2003).

CIMMYT lines (CML494, CLYN261and CLYN231) reported to be MLN promising resistant were pooled to sub-cluster 1.1. Similarly, tolerant landraces TZA-3567, TZA-2793, TZA-3543, and TZA-3585 were as well clustered together in sub-cluster 1.2; landrace TZA-4505 and the resistant US line N218 were grouped in sub-cluster 1.3. In the cluster I, landraces were collected from similar agro-ecological zones in the same region(s), for example, landraces, TZA-3567, and TZA-3543 were collected from Morogoro district in Morogoro region which appears the same for landrace TZA-2793 which was also collected from Kilombero district in Morogoro region as well. Our results suggest that these landraces may have a similar genetic background because farmers usually tend to save and exchange seeds. This may be a reason to presume that similar landrace lines may be known in different vernacular names in the same region. This may apply for landraces TZA-3585 and TZA-4505, which were collected from Mtwara and Ruangwa districts respectively. These two districts are found in the same

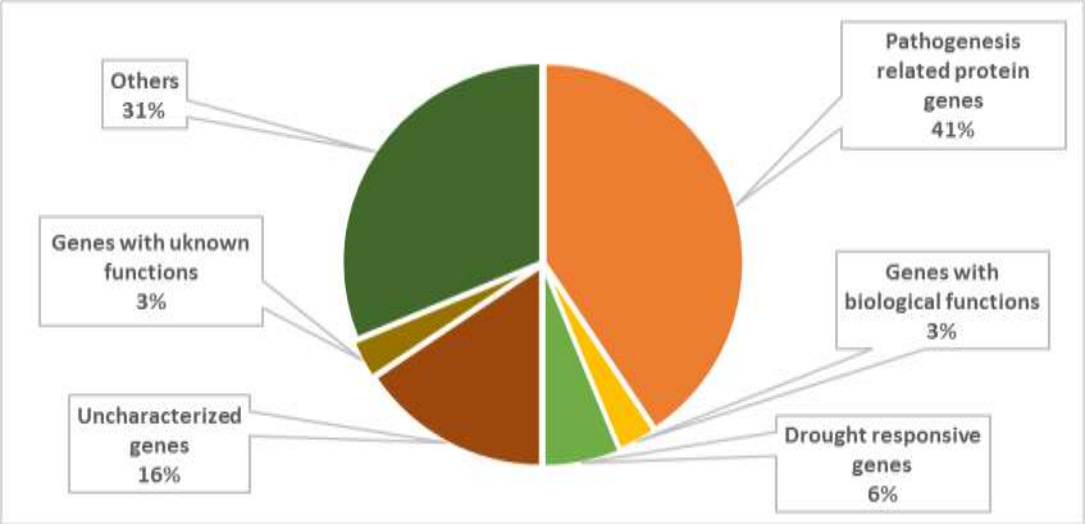

Figure 3. Functional classification of sequenced AFLP markers as they were revealed by the BLASTn program

agro-ecology zone.

The US line OH7B was clustered in cluster II, this maize line is reported to be resistant against MDMV (Roane et al., 1983), a potyvirus also implicated to induce MLN in synergy with MCMV. The reported findings suggest that resistance against any one of the causal viruses could significantly reduce crop damage CIMMYT (2013). Apparently, this line is mentioned to tolerate the incidence of MLN. The tolerance could be due to its ability to resist infection against MDMV.

Other landraces that were identified as susceptible to MLN under artificial inoculation (TZA-4320, TZA-5171, 2292, TZA-5200, TZA-4043, TZA-1758 and TZA_2264) were grouped together in cluster II along with known susceptible CIMMYT line CL-G2620, susceptible US line A635 and two SARI lines TUX 5-50-1-3-1-1 and KS 03-OB15-111 which were also identified as susceptible under artificial inoculation. Line OH43 reported as highly susceptible to MDMV (Roane et al., 1983) was in its own cluster III.

When breeding for disease resistance in plants, two general types of resistance are recognized *viz.* the qualitative and quantitative resistances. The former resistance typically confers a high level of resistance which is usually race-specific and is based on single dominant or recessive genes. In contrast, the quantitative resistance in plants is typically partial and race-nonspecific in phenotype, oligogenic or polygenic in inheritance and is conditioned by additive or partially dominant genes (Wisser et al., 2006). However, it is easier to work with qualitative resistance in crop genetic studies and breeding, quantitative resistance is often the more useful in an agronomic context, due to its generally higher durability (Parlevliet, 2002).

In maize, the majority of disease resistance deployed in elite varieties in the field is quantitative in nature (Wisser *et al.,* 2006). Large number of plant resistance genes have been characterized and efficiently used in many crop breeding programs (Ali and Yan, 2012). A challenge remains to identify new resistance for diseases whose genetic resistance has not been identified and efficiently introgressed in existing germplasm to resist the emerging plant pathogens. For maize lethal necrosis, its genetics and inheritance is reported to be unknown and is expected to be very complicated due to the involvement of two viruses (Manje et al., 2015). However, its genetic resistance is suggested to be poligenically controlled (Nelson et al., 2011).

Genome-wide association analysis studies conducted by Manje et al. (2015) in tropical maize germplasm identified SNP markers that were considered to significantly associate with possible candidate genes for MLN disease resistance. In the same study, B73 maize genome reference sequence was used to identify putative candidate genes based on the SNPs associated with MLN resistance in which a set of putative candidate genes were identified based on their functions. In this work we identified 13 AFLP markers associated with plant defense responsive genes (Supplementary materials Table 1) these markers also had similar functional characteristics (Figure 3) as those reported in Manje et al. (2015). In that regard it is worthwhile to speculate that, the identified AFLP markers may also be associated with resistance of maize against MLN.

The polymorphic bands sequences and nucleotide BLASTn search revealed that the AFLP fragment (244bp)

amplified from the tolerant line OH7B by primer pair M-CAA/E-ACG showed high homology with nucleotide sequences of *Zea mays* presented in the NCBI database. A maximum identity of 97% (E value = 9e-37) was revealed between this polymorphic fragment sequences with *Zea mays* B73 pathogenesis-related protein 2 and GASA-like protein genes. Other AFLP polymorphic fragment sequences which had similar hits with other genes of *Zea mays* B73 were amplified from genotypes TZA-2292 (276 bp), TZA-4320 (279 bp), TZA-3585 (332bp and 386bp), line CLYN231 (276 bp and 281 bp), TZA-5171 (380 bp) and TZA-4043 (355 bp) (Supplementary materials Table 1).

On the other hand, genes of *Zea mays* rust resistance protein rp3-1 (rp3-1) gene, complete cds; and truncated rust resistance protein rp3-2t (rp3-2) gene, were also hit by the tested genotypes in this study. The analysis revealed a similarity of 83% (E value= 9e-13) of AFLP marker obtained from line CLYN261which was found to be homologous to *Z. mays* B73 serine/threonine kinase protein and RNA-dependent RNA polymerase (mop1) genes and a homology of 83% (E value = 2e-13) of *Z. mays* putative zinc finger protein of unknown genes (Supplementary materials Table 1). The identified loci such as pathogenesis-related (PR) proteins, rust resistance protein (rp3-1) gene and serine/threonine kinase proteins have been reported to be associated with disease resistance (Bhavani et al., 2013). PR proteins are constituted of highly complex gene families involved in pathogen defense as well as a wide range of normal developmental processes, because of that, they increase the resistance of the plant against pathogenic attack. Such PR proteins play an outstanding role in disease resistance, seed germination and help the plant to adapt to the environmental stress (Adrienne and Barbara, 2006).

The fragment sequences of lines CML494 (330bp) and OH43 (162bp) amplified by primer pairs M-CAA/E-ACG and M-CAA/E-ACG were observed similar with drought responsive lncRNA (complete sequence) from *Zea mays* isolate TCONS_00063399 with E-value 0.064 and 4e-45, respectively. Long non-coding RNAs (lncRNA) are novel molecules with important functions in a wide range of biological processes, which also include developmental regulations and stress responses. A report demonstrated that many lncRNAs participate in responses to a wide variety of biotic and abiotic stresses (Zhang et al., 2014). However, much details on mechanisms involved in these biological processes are not well understood (Kim and Sung, 2012).

The results of this study showed the sequences derived from AFLP polymorphic amplicons associated to disease resistance genes including the pathogenesis-related proteins genes, Serine/threonine kinase protein, rust resistance protein (rp3-1) gene and receptor kinases *Z. mays* putative zinc finger protein genes. Other AFLP amplicons were homologous with plant response to stress such as lncRNA. Therefore, cluster analysis using sequences related to the resistance to pathogens is beneficial towards the identification of resistant genotypes. Further studies will explore the potential application of the identified AFLP markers and their significant association to MLN disease resistance genes in maize.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Supplementary materials Table 1. Sequence homology results of sequenced AFLP markers as were revealed by BLASTn program.

S/N	Primer combination and genotype ID	Fragment size (bp)	Function	E-Value	Identity (%)
1	M-CAA/E-ACG **OH7B**	244	*Z. mays* B73 pathogenesis-related protein 2and GASA-like protein genes, complete cds Seq ID:gi/105990542/gb/DQ417752.1	9e-37	97
2	M-CAA/E-ACG **TZA-2292**	276	*Zea mays* B73 pathogenesis-related protein 2and GASA-like protein genes, complete cds Seq ID:gi/105990542/gb/DQ417752.1	3e-15	88
3	M-CAA/E-ACG **TZA-4320**	279	*Zea mays* B73 pathogenesis-related protein 2and GASA-like protein genes, complete cds Seq ID:gi/105990542/gb/DQ417752.1	8e-67	86
4	M-CAA/E-ACG **TZA-3585**	386	*Zea mays* B73 pathogenesis-related protein 2and GASA-like protein genes, complete cds	9e-119	92
			Zea mays rust resistance protein rp3-1 (rp3-1) gene, complete cds; and truncated rust resistance protein rp3-2t (rp3-2) gene, complete sequence	7e-105	91
5	M-CAA/E-ACG **CLYN231**	276	*Zea mays* B73 pathogenesis-related protein 2 and GASA-like protein genes, complete cds	2e-45	83

Supplementary materials Table 1. Continue.

S/N	Primer combination and genotype ID	Fragment size (bp)	Function	E-Value	Identity (%)
6	M-CAA/E-ACG TZA-5171	380	*Zea mays* B73 pathogenesis-related protein 2and GASA-like protein genes, complete cds	5e-91	99
7	M-CAA/E-ACG TZA-3585	332	Zea mays B73 pathogenesis-related protein 2 and GASA-like protein genes, complete cds	1e-55	84
			Zea mays rust resistance protein rp3-1 (rp3-1) gene, complete cds; and truncated rust resistance protein rp3-2t (rp3-2) gene, complete sequence	1e-54	83
8	M-CAA/E-ACG TZA-4043	355	Zea mays B73 pathogenesis-related protein 2 and GASA-like protein genes, complete cds	6e-76	95
			Zea mays rust resistance protein rp3-1 (rp3-1) gene, complete cds; and truncated rust resistance protein rp3-2t (rp3-2) gene, complete sequence	9e-71	93
9	M-CTC-E-AAG CLYN261	144	Zea mays putative zinc finger protein (Z438D03.1), unknown (Z438D03.5), epsilon-COP (Z438D03.6), putative kinase (Z438D03.7), unknown (Z438D03.25) and C1-B73 (Z438D03.27) genes, complete cds	2e-13	83
			Zea mays B73 serine/threonine kinase protein, expressed protein and RNA-dependent RNA polymerase (mop1) genes	9e-13	83

Supplementary materials Table 1. Continue.

S/N	Primer combination and genotype ID	Fragment size (bp)	Function	E-Value	Identity (%)
10	M-CAA/E-ACG CLYN231	281	Zea mays B73 pathogenesis-related protein 2 and GASA-like protein genes, complete cds	6e-09	95
11	M-CAA/E-ACG TZA-2292	279	Zea mays B73 pathogenesis-related protein 2 and GASA-like protein genes, complete cds	5e-39	88
12	M-CAA/E-ACG CML494	330	*Zea mays* isolate TCONS_00063399 drought responsive lncRNA, complete sequence	0.064	100
13	M-CAA/E-ACG OH43	162	*Zea mays* isolate TCONS_00063399 drought responsive lncRNA, complete sequence.	4e-45	92
14	M-CTC/E-AAG CLYN231	229	Zea mays rust resistance protein rp3-1 (rp3-1) gene, complete cds; and truncated rust resistance protein rp3-2t (rp3-2) gene, complete sequence	2e-19	86
15	M-CAA/E-ACG CL-G2620	393	Zea mays B73 pathogenesis-related protein 2 and GASA-like protein genes, complete cds	3e-49	84
			Zea mays rust resistance protein rp3-1 (rp3-1) gene, complete cds; and truncated rust resistance protein rp3-2t (rp3-2) gene, complete sequence	5e-40	82