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Full Length Research Paper

Application of simple sequence repeats (SSRs) markers to study the resistance of locally adapted maize hybrids to damaging maize streak virus disease

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Simple sequence repeat markers were used to study S6 recombinant inbred lines originating from a single cross between two inbred lines OSU 23i and EM12-210, for resistance to maize streak virus disease. A total of 115 recombinant inbred lines belonging to three families previously selected using conventional methods were screened using 52 SSR markers contained between Bin 1.04 and 1.05 of maize chromosome 1. Of these, only three markers were chosen on the basis of their polymorphism content for use in the study. This paper discusses the results of combined selection with both artificial inoculation and the three selected SSR markers. The use of artificial inoculation detected 78 resistant lines and 36 susceptible lines. On the other hand, the SSR markers detected 40 resistant lines, and 74 susceptible lines. But a combination of both the artificial inoculation and SSR marker selection reduced the number of resistant lines to 29 and increased the number of susceptible lines to 85. With these findings it was concluded that, a conventional maize breeder requires the use of molecular markers in order to improve selection intensity and maximize genetic gain.

Key words: simple sequence repeats (SSRs), maize streak virus disease, resistance, marker assisted selection

INTRODUCTION

The core breeding strategy that has resulted in major genetic improvement of maize grain yield and other desirable agronomic traits in Kenya, in the recent years was the use of pedigree breeding, combined with extensive multi-location testing which was designed to assess the phenotypic performance of new genotypes across a large sample of target environments. This led to the coordinated ecosystem breeding (CEB), an approach which aims at breeding materials resistant/tolerant to priority stresses at a given agro-ecological zone.

Maize streak virus disease (MSVD) is a priority stress in the humid lowlands, mid altitudes and highlands of Kenya. In some of these regions, the disease results in up to 100% yield loss especially when susceptible genotypes are infected. The disease is characterized, by yellow streaks running parallel to leaf veins and in susceptible genotypes, severe infection may result in stunting, inter-veinal necrosis, chlorosis, and death. MSVD is endemic to Africa and its adjacent Indian Ocean Islands, and is caused by maize streak virus (MSV) (Fuller, 1901; Storey, 1924, 1932a) . Sporadic epidemics have been experienced in Kenya (Bock et al., 1974: Theuri and Njuguna, 1988) . MSVD severity depends on the age at which the plant is infected, on the genotype, and the virus strain (Storey and McClean, 1930; McClean, 1947). The virus is known to exist in many forms and vary according to agro-ecology, season and vector dynamics (Rybicki, 1994). The virus is transmitted by leafhoppers of the genus *Cicadulina*, and the most prevalent and efficient in transmission is by *Cicadulina mbila* which exists in populations of both active and inactive vectors.

Management of MSVD is difficult partly due to the variability of the virus, and partly due to the susceptibility of locally adapted maize lines and the unpredictable vector migratory and survival patterns. The development

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Figure 1. Two viruliferous leaf hoppers are placed in the plastic bottle and a leaf is inserted in the bottle and closed with cotton wool (A) and one leaf was inoculated per plant (B).

and deployment of resistant genotypes is the most appropriate and cost-effective approach to controlling MSVD. However, breeding for resistance requires efficient tools that can reduce the time taken to develop hybrids, and enable breeders to pyramid genes against different strains of the virus. Previously, Kenya Agricultural Research Institute (KARI) obtained resistant sources from International Centre for Wheat and Maize Improvement, Carretera, Mexico (CMMYT), International Co-operation Centre for Agronomic Research for Development, Paris, France (CIRAD), and International Institute of Tropical Agriculture, Ibadan, Nigeria (IITA) which were screened against the MSVD. One of the lines OSU 23i sourced from CIMMYT was found to score better than other lines, and was chosen for the conversion of one locally adapted line EM12-210. The cross between the two was inbred up to S6 generation through conventional breeding methods. Since conventional methods take breeders over 8 years to be able to release a variety with good levels of MSV resistance, there is need to develop, apply and adopt DNA markers in selection as an additional tool. The use of markers has higher chances of success in reducing the duration taken to release hybrids. DNA Markers detects the recovery of the recurrent genome in backcross breeding, and are more efficient in selection of genomes that have recombinant events close to the target gene.

MATERIALS AND METHODS

Identification and screening of the inbred line populations

Seeds from each family (families 114, 118, and 118) were sown in the field in Muguga (20 seeds per line and 10 lines per family). Each plant was inoculated with MSV using two viruliferous *Cicadulina mbila* leaf hoppers at 3-4 leaf stage (21 days; Figure 1). The experiments were laid out in a randomized complete block design with 3 replicates. For each block, there were two rows of plants which were not inoculated with MSV. Di-ammonium phosphate (DAP) fertilizer was applied at the rate of 3 g per hill. Furadan (soil pesticide) was applied at the rate of 3 g per hill of un-inoculated plants. CAN fertilizer was applied at the rate of 2 g per hill, 32 days post planting. MSV disease scores were taken at 21 days and at 60 days post inoculation, using a modified scale of 0-5 (Kyetere et al., 1999) as follows: 0 = no symptoms, 1 = very few streaks on leaves, 2 = light streaking on older leaves, gradually decreasing on young leaves, 3 = moderate streaking on old and young leaves, and slight stunting, 4 = severe streaking on about 60% of leaf area and stunted plants, 5 = severe streaking on over 75% of leaf area, severely stunted, and 6 = 100% streaking or dead plants

Genomic DNA extraction from maize

DNA extraction was done using a modified protocol as described by Hoisington et al. (1994). Eight leaves were sampled from young maize (ten-leaf stage) and separately placed in small perforated bags, transported to the laboratory and stored at -80°C until ready for DNA extraction. Each sample was chopped into half -inch segments with a sterilized pair of scissors and placed in a pre-chilled mortar. Liquid nitrogen was added to quickly freeze-dry the samples before grinding to fine powder with pestle. The ground material was placed in a 15 ml polypropylene centrifuge tube and stored at -20°C. When ready to extract, 0.3 g of the ground material was weighed into new 15 ml polypropylene centrifuge tube. Immediately, 9 ml of pre- warmed (65°C) CTAB extraction buffer (700 mM sodium chloride, 100 mM Tris HCl pH 7.5, 50 mM EDTA pH 8.0, 140 mM β-mercaptoethanol and 1% cetyl trimetyl ammonium bromide, CTAB) was added to lyse the cells. After cell lysis, 10 µl ice-cold proteinase K (20 mg/ml) was added, mixed briefly and incubated at room temperature for 1 h. To the homogenate, 4.5 ml chloroform/octanol mixture (24:1) was added tubes capped tightly, mixed by gentle rocking with a medium circular motion using a shaker for 15 min. This formed a thick emulsion between the DNA phase and the chloroform/octanol phase. The homogenate was centrifuged at 4000 rpm for 15 min at room temperature, before the upper aqueous layer was siphoned out into a new 15 ml polypropylene centrifuge tube. To the supernatant, 4.5 ml of chloroform/octanol mixture (24:1) was added, rocked gently for 10 min on a shaker and centrifuged at 4000 rpm for 10 min at room temperature. The upper layer was pipetted out and transferred to a new 15 ml polypropylene centrifuge tube and 20 µl pre-boiled RNAse A (10 mg/ml) was added, mixed by gentle inversion and incubated for 30 min at room temperature. The DNA was precipitated by adding 2.5 ml of 2.5 M sodium acetate followed my 6 ml of ice-cold iso-propanol (or ice-cold absolute ethanol) and mixed gently on a shaker for 30 min. The precipitated DNA was hooked with a sterile glass pasteur pipette hook and placed in a 1.5 ml micro-centrifuge tube containing 500 µl of wash 1 (consisting of 76% ethanol and 0.2 M sodium acetate) and washed for 5 min. Wash 1 was poured out while holding the DNA on the glass hook,

Table 1. SSR markers used to study differences in the maize families. (Source: http://www.maizegdb.org)

Marker name	Repeat	Forward primer and Reverse primer sequence (5'-3')
p-bnlg 1811	AG(16)	ACACAAGCCGACCAAAAAAC//GTAGTAGGAACGGGCGATGA
p-umc 1144	(CT)8	ATGGCCCACTCATCATATCTCTGT//TGTGTTGATTAGCAGCGGATAAAA
p-umc 1917	(CTG)6	ACTTCCACTTCACCAGCCTTTTC//GGAAAGAAGAGCCGCTTGGT

and washed with 500 µl wash 2 (consisting of 76% ethanol and 10 mM ammonium acetate) and washed for 5 min. This second wash was poured out, and the DNA dried inside the fume hood for 30 min and re-suspended in 300 µl of 0.1X TE at 37°C for 1 h while mixing every 15 min by gentle inversion to help speed up the process.

The quality of genomic obtained from the above methods was assessed by agarose gel electrophoresis. The 0.8% agarose gel was prepared by weighing 0.8 g of agarose in a 250 ml beaker containing 100 ml of 1X TAE buffer (160 ml 2.5 M Tris HCl, 0.23 ml glacial acetic acid, and 0.4 ml 0.5 M EDTA) and swirled to mix. The mixture was boiled in a microwave oven and allowed to cool to about 45°C. The molten gel was stained by adding 1 µl Ethidium bromide (10 mg/ml) solution before pouring into a mini gel mold with two 14-well combs. The gel was allowed to solidify (i.e. when the gel turned cloudy and hard) before being used. The solid gel was then placed in a gel box containing 1X TAE buffer ensuring the gel was completely submerged prior to removing the combs. Each DNA sample was mixed with 10X bromophenol blue loading dye at a ratio of 5 µl of DNA to 2 µl of dye, loaded into the wells, before electrodes of the gel box were hooked up (black to black and red to red), and power turned on to 100 volts. After running for about 1 h, the gel was removed, visualized on a UV box and photographed. From the photographs, the quality of DNA was checked and Sheared DNA samples were re- precipitated with ice-cold iso-propanol, while traces of RNA were removed by incubating the DNA with 5 µl of RNAse A (10 mg/ml) for 1 h at 37°C

The concentration of the genomic DNA was determined on the basis of optical density readings. From each stock DNA sample, a 15 µl aliquot diluted in 735 µl of 1X TE buffer, and its optical density (ODs) read at wavelengths 260 nm and 280 nm. The concentration of DNA in samples was determined as follows: 1 OD unit is approximately 50 µg double stranded DNA per ml, and 15 µl of sample in 750 µl cuvette is a dilution of 50 times. After calculating the concentrations in µg/µl, dilutions were made to a uniform stock concentration of 0.3 µg/µl for amplification.

Polymerase chain reaction (PCR) amplification

The PCR master mix for each of the three primers (primers p-bnlg1811, p-umc1144, and p-umc1917) was prepared by adding 1X PCR reaction buffer, 1.5 mM $MgCl_2$, 0.8 mM dNTP mix, 0.5 µM primer mix, and 0.125 U Taq polymerase in a 1.5 ml micro-centrifuge tube on ice. The sequences of the primers used are given in Table 1. In a 20 µl PCR reaction volume, 18 µl of mastermix was mixed with 2 µl of 100 ng DNA. The reaction mixture was vortexed gently, centrifuged briefly before placing on a thermocycler. The DNA was initially denatured in a one cycle of 5 min at 95°C, then 30 cycles of 94°C for 30 s, 58°C for 45 s, and 72°C for 30 s. This was followed by one cycle at 72°C for 8 min, and an indefinite hold at 4°C. After amplification, the products were stored at –20°C until ready to use. PCR products were checked in a 1.8% agarose gel.

Scoring of genotypic data

Each locus was scored for parental alleles where AA represents resistant parent, BB for susceptible parent, and AB representing both. In some cases there was an additional allele which was designated as non-parental and was scored as C. Genotypic and phenotypic data were analyzed using the statistical program 'Popgene'.

RESULTS

Characteristics of the parental lines used

The resistant inbred line OSU 23i has flint kernels and is a medium maturing genotype. The resistance levels were confirmed through successive testing and selection using the 0- 6 scale and only those scoring 0 were selected and fixed. The susceptible line is a locally adapted line EM12-210 with semi dent kernels and is also a medium maturing genotype.

Inoculation of maize seedlings with the maize streak virus

The inoculated plants began to show symptoms from two weeks post inoculation. The three families exhibited different levels of resistance/tolerance to the disease. Family 114 had the lowest mean symptom score rating of 0.3, followed by family 118 with 0.46, and family 141 with 0.52. This correlated negatively with the disease incidence and severity which was shown by calculating the mean rating for plants exhibiting symptoms, where family 141 had the lowest rate of 1.60, followed by family 118 with the rate of 1.96, and family 114 with the rate of 2.23. However, in terms of resistance, family 141 was 77.2% resistant, family 118 was 60.8% resistant, and 114 was 58.6% resistant, and this corroborated with the mean rating for plants exhibiting symptoms.

Microsatellite analysis

Three microsatellite primers targeting three loci of chromosome 1 were used in this study. The primers amplified all the assayed lines and gel electropherograms

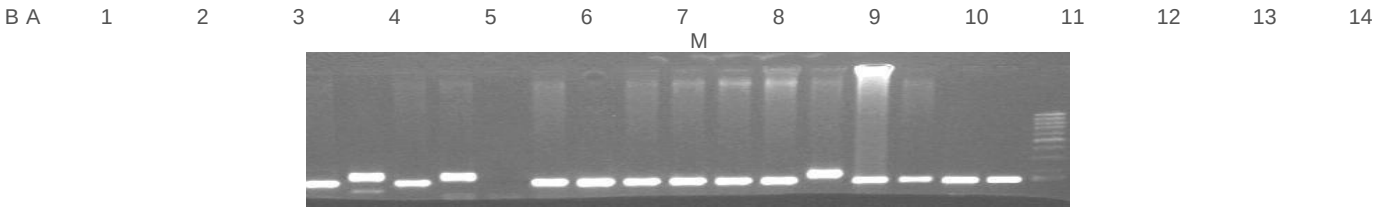

Figure 2. Amplification products using primer p-umc1917 and resolved on a 1.8% agarose gel. 'A' represents the susceptible parent while 'B' represents the resistant parent and the numbered samples were the S6 progeny.

Table 2. Chi-square test for Hardy-Weinberg equilibrium used to analyse the three maize families.

Locus	Family114			Family118			Family141		
	df	P=0.05	χ^2	df	P=0.05	χ^2	df	P=0.05	χ^2
p-bnlg1811	3	7.82	9.622303	1	3.84	10.372054	1	3.84	11.756586
p-umc1144	3	7.82	13.840427	1	3.84	0.731460	1	3.84	7.614521
p-umc1917	1	3.84	5.069930	1	3.84	0.007527	3	7.82	0.798175

Table 3. Allele frequencies between the three maize families using 3 SSR markers.

Locus	Allele	Family114	Family118	Family141
p-bnlg1811	A	0.4833	0.3667	0.8065
	B	0.4833	0.6333	0.1935
	C	0.0333		
p-umc1144	A	0.4000	0.3667	0.5484
	B	0.5833	0.6333	0.4516
	C	0.0167		
p-umc1917	A	0.4500	0.5167	0.7097
	B	0.5500	0.4833	0.2742
	C			0.0161

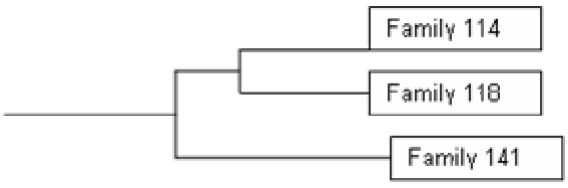

Figure 3. Dendrogarm showing how the three maize families clustered.

are shown (Figure 2). Family 141 had a resistance of 33.3%, family 118 had 26% and family 114 had 42.9%. Hardy-Weinberg expectations were tested by three procedures using popgene version 1.31 (Yeh and Boyle, 1997). The three methods were, chi-square test based on total heterozygosity (Table 2), the likelihood ratio test (G-statistics) contrasting observed and expected frequencies, and the Monte Carlo method of the exact test (Guo and Thomson, 1992). The effective number of alleles (Ne) was also calculated to test reciprocal homozygosity (Kimura and Crow, 1963), and the unbiased estimates of heterozygosity were determined according to Nei (1979). Family 114 had significant deviations from the expected Mendelian ratios for all loci, while family 118 significantly deviated only for p-bnlg1811 locus. For family 141, there were significant deviations for two loci (Table 2). This corroborated with the allele frequency distributions, where the exact tests revealed significant differences in the allele frequencies between the three families (Table 3). A statistically significant deviation from the Hardy-Weinberg equilibrium was observed in all the families but not for all loci.

The effective number of alleles was assessed as a corollary to the expected haterozygosity. For any given number of alleles, the expected heterozygosity (gene diversity) is highest when all the allele frequencies are equal. The expected homozygosity and heterozygosity statistics were computed using Levene (1949) and also Nei's expected heterozygosity.

A neighbor-joining tree was constructed based on Nei's (1972) genetic distance using un-weighted pair group method with arithmetic mean averages (UPGMA). This resulted in two clusters, families114 and 118 clustering as one and family 141 clustering as another (Figure 3).

Table 4. Comparison of selection efficiency between MSV score, SSR marker and both MSV and SSR.

Family	Individuals screened	Selected with SSR marker	Selected with MSV sores	Selected with both MSV score and SSR marker
118	23	6	16	3
141	57	19	39	17
114	35	15	23	9
Total	**115**	**40**	**78**	**29**

DISCUSSION

SSR markers in this study were useful in its stringency of selection. All the three loci were polymorphic for the parental lines used to generate the recombinant inbred lines. The genetic basis of MSV resistance trait and the effect of chromosomal regions on the expression of this trait were revealed in this study. The allele representing the resistant parent was detected in different proportions across the three loci. The highest proportion was in family 141 with 69% percent resistance allele. In families 114 and 118, the proportions of the resistant parent allele were 44 and 41%, respectively. These results agree with the number of resistant lines selected per family. Two of the three markers, primer p-bnlg1811 which flanks the AG(16) repeat motif and p-umc1917 which flanks the (CTG)6 repeat motif were more polymorphic than the third marker p- umc1144 which flanks the (CT)8 repeat motif. The later marker had tight polymorphism, and was implicated in some cases for the detection of resistant parent allele in materials which showed symptoms of the disease. The families clustered into two implying that two families were more related than the third one in terms of resistance to MSV. It was evident that family 114 and 118 clustered together because they had high frequencies for the allele representing the susceptible parent.

From the results of artificial inoculation with MSV, all the lines that carried allele of either parent B or of both parents were found to be either susceptible or tolerant. In addition, a few individuals carrying the resistant parent allele had a score of one and were tolerant. Thus, the use of disease pressure and the markers reduced the number of selected individuals from 78 with inoculation and 40 with the markers to only 29 individuals (Table 4). This is indicative of possible presence of alleles with additive phenotypic effects that influence heritability differently.

The phenotypic expression with MSV pressure exhibits continuous variation with heterozygous parents not expressing the phenotype and even among individuals expressing it have differing degrees of expression (scores between 1 and 6). This is one possible reason why conventional breeding takes long to come up with genotypes with stable resistance to MSV. Therefore, conclusions made here are that a breeder needs to use marker assisted selection in conventional breeding. Relying on the marker alone has some degree of inefficiency also, and so the use of both increases the stringency of selection and genetic gain. The 29 lines selected as resistant will be used to convert lines selected for other major foliar diseases, common rust, grey leaf spot, and *Turcicum* blight.

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