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Molecular Characterization of Maize Inbred Lines against Stalk Rot Complex of Maize (*Zea mays* L.)

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A B S T R A C T

Stalk rot complex of maize (*Zea Mays* L.) caused by *Macrophomina phaseolina* (Tassi) Goid and *Fusarium verticilloides* (Sacc) Nirenberg reduces yield directly by affecting the physiological activity of the plants and finally cause lodging, which is the main reason of economic losses. Screening of Indian maize inbreds for the resistance to pathogen was done using 34 Simple Sequence Repeats (SSR) markers available in database. Among these SSR markers, two markers *viz.* SSRZ 135, and SSRZ 319 showed polymorphism for resistance to stalk rot. The marker SSRZ 319 located on chromosome 1 distinguished the resistant lines H 109, P 503, P 408 and E 618 from the susceptible lines *viz.*, H-8, P 320, P 373 and 18834. Resistant genotypes identified in the study would serve as potential donors in the stalk rot resistance breeding programme. Further, QTL *qRfg2* with most likely presence in the Indian maize inbred lines can be transferred to elite inbreds using marker-assisted selection.

Introduction

Stalk rot is a major disease of maize causing appreciable damage to the standing crop and may infect all types of corn (Renfro and Ullastrup, 1976). According to Sharma *et al.,* (1993), maize production in India is severely limited due to the incidence of a soil borne disease commonly called Post- flowering stalk rot (PFSR).

Apart from grain yield, PFSR reduces the fodder quality. The disease is caused by pathogen complex comprising three fungi, *viz., Cephalosporium acremonium,*
Macrophomina phaseolina, Fusarium moniliformae and one bacterial *Erwinia carotovora* var. *zeae* (Shankar, 2003). In India, the disease is prevalent in most of the maize growing areas *viz.*, Jammu and Kashmir, Punjab, Haryana, Delhi, Rajasthan, Madhya Pradesh, Uttar Pradesh, Bihar, West Bengal, Andhra Pradesh, Tamil Nadu and Karnataka, where water stress occurs after flowering stage of the crop (Singh *et al.,* 2012). PFSR is basically a soil borne disease for which fungicidal control of stalk rot is not much effective. Hence, discovery and

utilization of resistance genes to improve maize tolerance to stalk rot is a cost effective and environment friendly approach to reduce the grain yield loss (White, 1999). Through the extensive research done at Directorate of Maize Research, sources of resistance against the PFSR of maize have been identified (Kumar and Shekhar, 2005; Sekhar *et al.,* 2010; Hooda *et al.,* 2012). Parallelly, a large number of efforts are being diverted towards development of biotechnological tools for identification and tagging of genes conferring resistance to PFSR. For Fusarium stalk rot resistance a major gene (Yang *et al.,* 2004) and five QTL (Pe *et al.,* 1993) have been reported on the different chromosomes. Yang *et al.,* (2010) detected two loci QTL$_S$ *qrfg1* and *qrfg 2,* conferring resistance to Fusarium stalk rot. There are four genomic regions exist in the maize genome involved in the determination of resistance to *M. phaseolina*. In contrast to this progress, study on the genetics of resistance to charcoal rot or PFSR complex under Indian scenario is lagging behind and also information like molecular markers linked to stalk rot resistance, genetic diversity in maize inbreds related to PFSR complex have not been generated. Owing to this reason, present study was conducted for molecular characterization of some important Indian maize inbred lines with respect to the PFSR complex resistance.

Materials and Methods

Screening of maize genotypes in field

Preparation of inoculum

Isolation of *F. verticilloides* from the infected maize plants and preparation of inoculum from its pure cultures in PDA.

Raising and screening of genotypes

Twenty four maize genotypes collected from Winter Nursery Centre (WNC), Hyderabad were raised in field during *kharif*, 2013 following all agronomic practices during the cropping season. Plants of 45-50 days old were inoculated by the toothpick method (Payak, 1985) just after flowering. Disease scoring was done at the time of harvesting in field on a 1-9 scale (Payak and Sharma, 1983).

Molecular characterization of the genotypes with specific markers

Leaf sampling

Leaf samples were collected from ~30-days old field grown plants (3-4 leaf stage), wrapped in marked aluminium foil and then frozen in LN$_2$ before storing in -80°C.

DNA isolation, purification and quantification

DNA isolation from the leaf samples was carried out using the modified CTAB method (Saghai-Maroof *et al.,* 1984) optimized at Maize Genetics Unit, Division of Genetics, IARI. The DNA was dissolved in Tris-EDTA buffer (1 M Tris: 0.5 M EDTA) and quantified using a Spectrophotomer (Bio-Tek Instruments, USA). The quality of DNA was checked using 0.8% agarose gel electrophoresis, followed by dilution with Tris-EDTA buffer to the concentration 10 ng/µl, the final concentration for PCR reaction. DNA quantification was also done directly by loading 1µl of DNA in Nanodrop spectrophotometer (Thermo Scientific Model).

PCR amplification

A set of 30 SSR (Simple Sequence Repeat) markers (Table 1, Sl. No. 1-30) were selected carefully from all 10 chromosomes depicted in public domain MaizeGDB; http://www.maizegdb.org. Another four SSR (Sl. No. 31-34) and two CAPS (Sl. No. 35-36) markers were taken from R gene specific marker reported by Zhang *et al.,* (2012). Using

all the 34 markers, PCR amplifications were performed in a BIORAD Thermal Cycler with a final reaction volume of 10 µl having ~30-40 ng of genomic DNA.

The protocol for the PCR amplification consisted of an initial denaturation at 94°C for 4 min, followed by about 35 cycles of 94°C for 30 sec (denaturation), X°C for 30 sec (annealing), and 72°C for 45 sec followed by extension at 72°C for 7 min. The X°C refers to the annealing temperature which varied (ranging from 52- 62°C) with each primer.

Resolution of PCR amplified products and scoring of marker profiles

The PCR amplified product for each SSR marker was resolved through gel electrophoresis in a horizontal gel system using 1.0X TBE buffer (Sambrook *et al.,* 1989). Ethidium bromide (10 mg/ml) was used for staining, 4% Biorose agarose gel was used. At both ends of the gel, 50 bp DNA ladder (MBI-Fermentas) was loaded and images were recorded using a Gel Documentation System (Alpha Innotech, USA), followed by scoring of marker profiles.

Results and Discussion

Phenotyping of maize inbred lines for resistance to stalk rot

In order to characterize the maize inbred lines for resistance to Fusarium stalk rot, a set of 24 maize inbred lines *viz.*, H-8, H-62, H-61, BML-6, H-139, 14933, H109, H10, P503, H75, P320, H182, H103, P408, H37, P364, H68, 14982, E613, P373, E618, 18527, 18768 and 18834 were used in the current study. The inbred lines were evaluated under artificial epiphytotic condition created by toothpick method of inoculation. Among the inbred lines studied, four inbred lines H 109, P 503, P 408 and E 618 were found to be resistant and H-8, P 320, P 373 and 18834 were found to be

highly susceptible (Table 2). Majority of the lines namely H-62, BML-6, H-139, 14933-2, 14933-1, H 75, H 37, H 68, 14982-5, 18527 and 18768 were found to be moderately resistant and seven lines namely H-61, H10, DMSC-I, H 182, H 103, P 364 and E 613 were moderately susceptible.

Molecular characterization of maize inbred lines for stalk rot resistance

Out of the 34 SSR markers used for characterization of maize genotypes, 32 yielded monomorphic band across 24 genotypes. CAPS markers *viz.*, CAPSZ406 and CAPSZ459 also showed monomorphic band in all the genotypes. Two SSR markers flanking the QTL *qRfg2 viz.,* SSRZ135 and SSRZ319 located at physical position of 227.4 Mb and 261.65 Mb, respectively on chromosome 1 showed polymorphism among the 24 germplasm used in the current study. The marker SSRZ135 showed two allele of 180bp and 200bp size (Fig. 1), whereas 160bp and 150bp alleles could be resolved by using the marker SSRZ319. The marker SSRZ319 distinguished all the four stalk rot resistant genotypes *viz.,* H 109, P 503, P 408 and E 618 each of them possessing 150bp allele (Fig. 2), while susceptible genotypes *viz.,* H-8, P 320, P 373 and 18834 possessed 160bp allele (Table 3). Although the other marker SSRZ135 revealed polymorphic bands, but it failed to distinguish between the resistant and susceptible inbred lines. Fusarium stalk rot is one of the devastating diseases of Maize in India. Therefore, understanding the genetics of the resistance is of utmost importance in order to develop high yielding disease resistance maize varieties. Of the several Fusarium stalk rot resistant QTLs identified, the QTL *qRfg2* located on chromosome 1 explaining the phenotypic variance of ~8.9% has been identified in resistant maize inbred line '1145' (Zhang *et al.,* 2012). The QTL *qRfg2* is flanked by the markers SSRZ319 and SSRZ135.

Table.1 List of primers used for molecular characterization maize inbred lines

S. No.	Markers	FORWARD PRIMER	REVERSE PRIMER	Location
1	umc1335	ATGGCATGCATGTGTTTGTTTTAC	ACAGACGTCGCTAATTCCTGAAAG	1.06
2	umc1280	AAAATCCATGGCTTCTTTCTTTCC	AACAGCCAGTTTTGGGCTGTATAA	10.05
3	umc1590	CAGAGTCTGATAGTCCGAACCCAG	GTAAAGCTCACAGCTTCCGACAG	1.04
4	umc1035	CTGGCATGATCACGCTATGTATG	TAACATCAGCAGGTTTGCTCATTC	1.06
5	umc1245	TGGTTATGTGCATGATTTTTCCTG	CATGCGTCTGATCTTCAGAATGTT	1.07
6	mmc0041	AGGACTTAGAGAGGAAACGAA	TTTATCCTTACTTGCAGTTGC	1.08
7	umc1396	TTCGATTATTCCATTGAGCCTCTG	CTCCTAACGCAGGAGACAAGAGAG	1.06
8	umc1358	AGAACCTCCCGCTTGACGAC	ACCTCAACCTCGACCTCTGCAT	1.07
9	umc1570	CAGGAGATGATGAGCGGGAG	GTCGTAGAGGTGGTGCTGCTG	9.04
10	umc1571	GCACTTCATAACCTCTCTGCAGGT	CACCGAGGAGCACGACAGTATTAT	9.04
11	STS	CGCAATTCACCACATCATTTTA	CAACTACGTCGGATAGAACAA	-
12	HM1	CGGATTCGTCTGCTGGTGGGTGTGC	GATGTCGAGGTGAGGGAAC	-
13	umc1920	GGTTCGGGTTTGCTACGTGTT	ACGAGACAACACAACCAAGACAAA	3.04
14	umc2000	CTGTTGTCAAGCCAAGCCAGT	AGGCTTGTGAGACTCAGCAGTTTT	
15	umc1634	TCCGTTGAGGACACTCGAATTTAT	GTAGCCTGCAAAACATCCAAGAAC	9.03
16	umc1505	TTACACAGAAGCCCATTTGAAGGT	GGATGGTTGTTGGTGGTGTAGAAT	9.08
17	umc1813	CTGTACATGGATATGGCATTGGTG	GCATATACACCACCTTGGACAACA	3.09
18	umc1926	ATGCCAGCATTCTTCATCCTACAT	TGAGGCTTGGTCCACTAAAGAAAG	4.03
19	umc1142	CCGAAAACCCATTCTTCTAGCATC	GTGCGGTGTTCTCTCTTTCACTCT	4.05
20	umc1656	AGTTTTGACCGCGCAAAAGTTA	GTACGAGCAGGCCATTAACCC	6.02
21	y1	CAAGAAGAGGAGAGGCCGGA	TTGAGCAGGGTGGAGCACTG	6.01
22	umc56	CAACTCATCTTTGATAGGGCAACC	ACCCAGCTCCATTAATAACCCAAT	7.03
23	phi091	ATCTTGCTTCCATAAGATGCACTGCTCT	CTCAGCTTCGGTTCCTACACAGT	7.03
24	umc1245	TGGTTATGTGCATGATTTTTCCTG	CATGCGTCTGATCTTCAGAATGTT	1.07
25	bmc1556	ACCGACCTAAGCTATGGGCT	CCGGTTATAAACACAGCCGT	1.07
26	umc1947	GGATCTCACCCCCTGCTGTC	ATCACGCGCTCACTCTCCTCT	2.08
27	umc1943	GTGCTGCAGAATTCAACTCCTTC	ACCATTTCTGCGTTTCCACAGT	4.02
28	bnlg1931	GGGATGCTCGTAGTAGGGGT	ACGCACACAACAAAGAGACG	3.07
29	bnlg2244	CAGGAAAACGAAAACCCAGA	CTACGCGGGTCTCATCTCAT	4.08
30	bnlg1456	CTCTAGGTGGTTAAGATTAACTCATT	TTCATGAGGACCGTGTTGAA	3.05
31	SSRZ135	CCGATCCTCCTCCTTCAG	CTGACGTAGTGCTGCGA	1.09
32	SSR334	TTCGAGCATGCCAAAGAA	GGTGCACACAGACATGG	-
33	SSRZ319	CACCTTCCTCTTGCTGTC	CTGCACCTGCTAGTCCTG	1.09
34	SSR58	GACGCTGCACAATAGGTT	TCATTATACACCGACGACC	-
35	CAPSZ459	GCAATCGGAATTTAGGG	GCATAACTCGGCTGGCAT	-
36	CAPSZ406	GATACATGCACAGAAG	GTCCATTGTCACCACTGA	-

Table.2 Markers, location on chromosome and their amplification

S. No.	Markers	Bin Location	Allelic Polymorphic
1	umc1335	1.06	Monomorphic
2	umc1280	10.05	Monomorphic
3	umc1590	1.04	Monomorphic
4	umc1035	1.06	Monomorphic
5	umc1245	1.07	Monomorphic
6	mmc0041	1.08	Monomorphic
7	umc1396	1.06	Monomorphic
8	umc1358	1.07	Monomorphic
9	umc1570	9.04	Monomorphic
10	umc1571	9.04	Monomorphic
11	STS	-	Monomorphic
12	HM1	-	Monomorphic
13	umc1920	3.04	Monomorphic
14	umc2000		Monomorphic
15	umc1634	9.03	Monomorphic
16	umc1505	9.08	Monomorphic
17	umc1813	3.09	Monomorphic
18	umc1926	4.03	Monomorphic
19	umc1142	4.05	Monomorphic
20	umc1656	6.02	Monomorphic
21	y1	6.01	Monomorphic
22	umc56	7.03	Monomorphic
23	phi091	7.03	Monomorphic
24	umc1245	1.07	Monomorphic
25	bmc1556	1.07	Monomorphic
26	umc1947	2.08	Monomorphic
27	umc1943	4.02	Monomorphic
28	bnlg1931	3.07	Monomorphic
29	bnlg2244	4.08	Monomorphic
30	bnlg1456	3.05	Monomorphic
31	SSRZ135	1.09	Polymorphism
32	SSR334	-	Monomorphism
33	SSR319	1.09	Polymorphism
34	SSR58	-	Monomorphism
35	CAPSZ459	-	Monomorphism
36	CAPSZ406	-	Monomorphism

Table.3 Genotypic score of 24 maize inbred line generated using markers linked to
Fusarium stalk resistance QTL *qRfg2*

Lane No.	Inbreds	Primer (SSRZ 135)	Primer (SSRZ319)	Reaction
2	H-62	180	150	MR
4	BML-6	200	NA	MR
5	H-139	200	150	MR
6	14933	200	160	MR
11	H 75	180	150	MR
16	H 37	200	160	MR
18	H 68	180	160	MR
19	14982,-5	200	150	MR
20	18527	200	160	MR
24	18768	200	150	MR
3	H-61	NA	160	MS
8	H 10	180	160	MS
13	H 182	180	150	MS
14	H 103	NA	150	MS
17	P 364	180	150	MS
23	E 613	200	150	MS
7	H 109	200	150	R
9	P 503	180	150	R
15	P 408	200	160	R
22	E 618	200	150	R
1	H-8	200	160	S
12	P 320	200	160	S
21	P 373	200	150	S
10	18834	200	160	S

NA: Not amplified

In the present study, SSRZ319 was efficient in distinguishing the resistant and susceptible maize inbred lines. This suggests that Indian maize inbred lines that plays role in governing resistance to Fusarium stalk rot in maize, is most likely to possess QTL *qRfg2*. However, the other flanking marker SSRZ135 could not distinguish the resistant and susceptible maize inbred lines. This could be attributed to usage of different sets of genotypes (used in the present study), where distance between the QTL and markers is more (Zhang *et al.,*

2012). Due to this, the possibility of high occurrence of recombination between the gene and the marker would be more, thereby leading to random presence of both the alleles among resistant and susceptible inbreds.

The present study, thus characterized a set of diverse inbreds for their reaction to Fusarium stalk rot. Resistant genotypes identified in the study would serve as donors in the resistance breeding programme. QTL *qRfg2* with most likely presence in the resistant inbreds can be

transferred to elite inbreds using marker-assisted selection. Breeding with the assistance of molecular marker have been reported to be successful in case of developing resistance against different stresses in maize such as common smut (Ding *et al.,* 2008), head smut (Li *et al.,* 2008), *Fusarium moniliforme* ear rot (Zhang *et al.,* 2006), banded leaf and sheath blight (BLSB) (Zhao *et al.,* 2006), Maize Dwarf Mosaic Virus (MDMV) disease (Liu *et al.,* 2006) and Sugarcane Mosaic Virus (SCMV) disease (Zhang *et al.,* 2003)

Acknowledgement

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