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Identification of new molecular markers linked to maize stalk rot disease resistance (*Fusarium moniliforme*) in maize

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Abstract

A segregating population (170 individual plants) from a cross between the resistant line Gm1021 and the susceptible line Gm1002 was made to identify molecular markers linked to maize stalk rot disease resistance. In total, 38 random amplified polymorphic DNA (RAPD) primers, 25 inter-simple sequence repeat (ISSR) primers, 22 simple sequence repeat (SSR) primers and 8 STS primers combined with bulked segregant analysis were tested for polymorphism among parental genotypes and F_2 population. Quantitative trait locus (QTL) for resistance to *Fusarium moniliforme* was associated with 2 RAPD markers (OPA02 and Pr 11), 1 ISSR marker (AD8), 4 SSR markers (SSR93, SSR105, SSR225, and SSR337) and 1 STS marker (STS03) which explained 59.3 to 89.2 % of the phenotypic variation. The genetic distance between stalk rot resistance QTL and OPA02 and Pr 11 were 4.9 and 2.9 cM, respectively (LOD scores of 27.4 and 33.4, respectively). The genetic distance between the trait and AD8 was 4.8 cM (LOD score 28.7). The markers SSR93, SSR105, SSR225, and SSR337 had genetic distances of 2.5, 1.8, 8.3 and 4.4 cM, respectively (LOD scores of 31.5, 35.6, 20.8 and 46.2 respectively). The genetic distance between STS03 and stalk rot resistance QTL was 3.9 cM (LOD score 33.8). Therefore, the RAPD, ISSR, SSR and STS markers linked to the QTL for resistance to *Fusarium moniliforme* can be further used in breeding for stalk rot resistance in maize.

Keywords: *Fusarium moniliforme*, maize, molecular markers, QTL, stalk rot disease.
Abbreviations: BSA_bulked segregant analysis; ISSR_inter-simple sequence repeat; QTL_quantitative trait loci; RAPD_random amplified polymorphic DNA; SSR_simple sequence repeat; STS_sequence tagged site.

Introduction

Maize (*Zea mays* L.) is one of the most important food and feed crops in the world. Maize provides nutrients for human and animals, and serves as basic raw materials for industry such as in starch, oil, protein, alcoholic beverages, food sweeteners, seasonings, fuel, etc. The green plant has been used with much success as feed for livestock. In Egypt, it is grown for food, feed, fodder and industrial purposes. In addition, the development of techniques for ensilage of the whole plant has made maize the major summer fodder crop for ruminants. Egypt imports approximately 35% of its maize needs. It is important to develop high–yielding and disease resistant hybrids to meet the country's demands.

The fungus *Fusarium* can cause stalk, root, ear and kernel rot. *F. graminearum* and *F. moniliforme* are pathogenic on maize stalk and root. *Fusarium* kernel or ear rot is the most widespread disease of corn ears. Losses result from reduced ear weight, poor grain quality, and mycotoxins that may contaminate feeds and food. It is caused by *F. moniliforme* and the closely related *F. moniliforme* var. subglutinans. Most corn inbreeds are sensitive to *F. moniliforme* but greater susceptibility occurs in high-lysine, brown midrib, cms-T male sterile maize and sweet corn (Ooka and Kommedahl, 1977; Tomov and Ivanova, 1990; Ledencan et al., 2003; Afolabi et al., 2008).

Breeding for genetic resistance to stalk rot is the most efficient, cost effective and environmentally friendly approach to reduce yield loss. Substantial numbers of maize germplasm have been evaluated for stalk rot resistance, and some have demonstrated high levels of resistance (Wang, 2001; Ledencan et al., 2003; Afolabi et al., 2008). This would allow for identifying potential resistance genes/ QTLs for resistance to stalk rot in maize by marker-assisted selection (MAS).

Molecular markers that are closely linked with target alleles present a useful tool in plant breeding since they can help to detect the resistant genes of interest without the need of carrying out a field disease test. Also, they allow for screening large number of breeding materials at early growth stages and in a short time. Molecular markers, including RAPD, ISSR, STS and SSR, are useful tools for identifying and mapping resistance gene in maize. A major gene for Gibberella stalk rot resistance has been reported on chromosome 6 (Pè et al., 1994; Yang et al., 2004). They reported that at least 4 loci provide moderate levels of resistance to Gibberella stalk rot. Jung et al. (1999) reported one major QTL for resistance to anthracnose stalk rot, which explained over 50% of the variation in an $F_{2:3}$ populations. Bulked segregant analysis (BSA), which involves pooling of entries at the two extremes for a segregating trait (Michelmore et al., 1991), has been

effectively used for identifying molecular markers associated with disease resistance genes in a number of species (Reiter et al., 1992; William et al., 2006). BSA and linkage mapping in wheat has enabled identification of molecular markers associated with genes that condition resistance to northern corn leaf blight resistance (Barakat et al., 2009).

The main objectives of the present study were to identify RAPD, ISSR, STS and SSR markers linked to stalk rot resistance gene in F_2 maize population and mapping QTL linked to stalk rot resistance gene conferring to resistant under field condition.

Results

RAPD markers

Out of 38 arbitrary primers screened for polymorphisms between the two parental genotypes, GM1021 (resistant) and GM1002 (susceptible), 23 RAPD primers (60.1 %), provided polymorphic bands suitable to differentiate between the two parents. Of these 23 RAPD primers, OPA02 (5'- TGCCGAGCTG -3') and pr 11(5'- CAATCGCCGT -3') primers were produced a single and strong polymorphic band at 350 and 750bp, respectively. These were present only in the GM1021 (resistant parent) and resistant bulk, but absent in the GM1002 (susceptible parent) and susceptible bulk (Fig 1). These bands were selected for screening DNA bulks and their parental DNA. The primers OPA02 and pr11 generated the polymorphic fragments at 350 and 750bp, respectively, which were present only in *fusarium moniliforme* resistant bulk and GM1021 (resistant parent) and were missing in susceptible bulk and Gm1002 (susceptible parent). These RAPD markers (OPA02 and pr11) were regarded as candidate markers linked to *fusarium moniliforme* resistance gene in maize.

Linkage between RAPD markers (OPA02 and primer Pr11) were further investigated using a segregating F_2 population, derived from the cross between the resistant and the susceptible parent. Among the RAPD markers, OPA02, worked in 53 of 170 (31.17 %) individuals of the F_2 population. It exhibited the amplified polymorphic fragments (350bp), whereas, the remaining did not show any polymorphism. The ratio fitted the expected Mendelian ratio, 3:1 (χ^2 = 3.4, P <0.5) (Table 1). The RAPD marker Pr11 worked in 50 of 170 (29.41 %) individuals of population. It exhibited the amplified polymorphic fragment (750bp), whereas the remaining did show any polymorphism. The ratio fitted the expected Mendelian ratio, 3:1 (χ2 = 1.76, P<0.5) (Table 1).

ISSR markers

Out of 25 ISSR primers screened for polymorphisms between the resistant and susceptible parents, 17 ISSR primers (68.0 %) produced polymorphic bands. Of these 17 ISSR primers, AD8 primer ((AGC) 6G) produced a single and strong polymorphic band at 410bp, that was present only in the GM1002 (susceptible parent), but absent in GM1021 (resistant parent) (Fig. 1). This band was selected for screening DNA bulks and their parental DNA. The primer AD8 generated the polymorphic fragment at 410_{bp}, and was present only in susceptible bulks and susceptible parent and was missing in resistant bulks and resistant parent (Fig. 1). This ISSR marker (AD8) was regarded as candidate marker linked to *fusarium moniliforme* susceptibility gene in maize. This AD8 was further used to check its association with the stalk rot susceptible gene using the segregating F_2 population previously described. When analyzing the individual plants of F_2 population, the AD8 fragment was amplified in the DNA from F_2 susceptible plants. 118 of 170 (69.4 %) individuals in the F_2 population exhibited the amplified polymorphic fragments (410bp). The ratio fitted the expected Mendelian ratio, 3:1 (χ^2 = 2.87, P <0.1) (Table 1).

SSR markers

Out of 22 SSR primers screened for polymorphisms between the two tested genotypes, only four SSR primers (SSR93, SSR105, SSR255 and SSR337) amplified polymorphic bands (Table 1). Three SSR primers (SSR93, SSR105 and SSR255) were produced 3 strong polymorphic bands at 210, 200 and 200bp, respectively, which present only in the susceptible bulk and susceptible parent. These SSR markers (SSR93, SSR105 and SSR255) were regarded as candidate marker linked to the susceptibility gene for *fusarium moniliforme* in maize.

These polymorphic markers (SSR93, SSR105 and SSR255) were further evaluated as previously described using F_2 population. When analyzing the individual plants of F_2 population, the SSR93, SSR105 and SSR255 fragments were amplified in only F_2 susceptible individuals. For the SSR markers; the SSR93, SSR105 and SSR255, 132 of 170, 118 of 170 and 121 of 170 individuals in the F_2 population, respectively, exhibited the amplified polymorphic fragments of 210 bp, 200 bp and 200 bp, respectively. The ratio fitted the expected Mendelian ratio, 3:1 (χ^2= 0.628, 2.82 and 1.32, respectively) (Table 1).

A typical amplification pattern generated by SSR337 was shown in Fig. 1. Among the most susceptible F_2 lines, three had profiles of the susceptible parent; Gm 1002 and eight were heterozygotes. Among the most resistant F_2 lines, four had profiles of the resistant parent; Gm 1021 (Fig. 1). The SSR337 allele from the susceptible parent was smaller than resistant parent. This locus was inherited in a Mendelian co-dominant manner. There were clear co-segregations between the amplification of the smaller SSR337 allele and the F_2 plants showing the susceptible phenotypes. In the homozygous resistant F_2 plants, only the large SSR337 allele was amplified. In a proportion of susceptible F_2 plants, both the larger and the smaller alleles were amplified. These plants were presumably heterozygous. The co-dominant microsatellite marker SSR337 was able to identify the heterozygotes. The segregation ratio was 1 (49 resistant homozygote): 2 (73 susceptible heterozygote): 1(48 susceptible homozygote) in the genotyping F_2 plants. The ratio fitted the expected Mendelian ratio, 1:2:1 (χ2 = 3.397) (Table 1).

STS markers

Eight STS markers, namely, STS01, STS378, STS414, STS444, STS434, STS03, STS04 and STS06 (Yang et al., 2010) were used to detect the fusarium stalk rot resistances gene in the F_2 maize population (GM1021 x GM1002). Out of 8 primers screened for polymorphisms between the two parental genotypes, GM1021 (resistant) and GM1002 (susceptible) and one STS primer STS03, revealed polymorphisms when annealing temperature was 56°C. The STS03 primer produced a polymorphic band of 300 bp. This band was present only in the susceptible parent (Fig. 1).

Table 1: Significant association between stalk rot resistance and markers (RAPD, ISSR, SSR and STS) in the 170 F_2 plant population (Gm1021 × Gm1002) detected, using Chi – square (χ^2).

Tool	Marker	Sequence of primer (5'–3')	Fusarium stalk rot severity			Expected Ratio	χ^2
			S	R			
RAPD	OPA-02	TGCCGAGCTG	117	53		3:1	3.4 ^{ns}
	Pr.11	CAATCGCCGT	120	50		3:1	1.7 ^{ns}
ISSR	AD8	$(AGC)_6GC$	118	52		3:1	2.8 ^{ns}
SSR	SSR93	F: CGCCGTACAGACTGCTATGA R: CACATGCTACGACTGCGATG	132	38		3:1	0.62 ^{ns}
	SSR105	F: GTTCATCCTGATTCCCATCC R: CAGCCTTGCTTCTACACCAC	118	52		3:1	2.82 ^{ns}
	SSR255	F: TCGACGAGATACGCGACTAC R: CAGTACAAAGCCGATCCAAG	121	49		3:1	1.3 ^{ns}
	SSR337	F: CACCAGCTTAATTGTCCTGT R: CCACCGTAACAACTCGTACT	48H	73He	49H	1:2:1	3.4 ^{ns}
STS	STS03	F: CTTGTATCATCAGCTAGGGCATGT R: GTGATCTGAACGCCAACCTC	118	52		3:1	2.8 ^{ns}

S= susceptible plants, R= Resistant plants. ns: not-significant at 0.5 level of probability. H; homozygous susceptible plants. He; heterozygous susceptible plants.

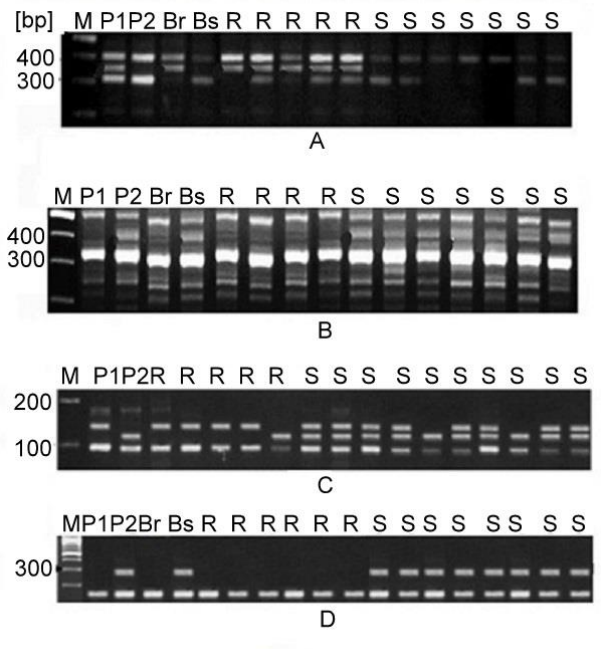

Fig 1. *A* - RAPD fragments produced by OPA-02; *B* – ISSR fragments produced by AD8; *C* - SSR fragments produced by SSR 337; *D* - STS fragments produced by STS-03. M - Molecular mass marker, P1 and P2 – parents Gm1021 and Gm1002, respectively. Br - bulk resistant, Bs - bulk susceptible, R and S - F_2 resistant (R) and susceptible (S) individuals in the cross Gm1021 × Gm1002.

The STS03 primer was selected for screening DNA bulks and their parental DNA. The STS03 primer, generated the polymorphic fragment at 300 bp, which was present only in susceptible bulk and Gm 1002 (susceptible parent) and was missing in resistant bulk and the Gm 1021 (resistant parent) (Fig. 2). When analyzing the individual plants of F_2 population, the STS03 fragment was amplified in only F_2 susceptible individuals. For the STS marker STS03, 118 of 170 individuals, in the F_2 population, exhibited the amplified polymorphic fragment (300 bp), whereas the remaining did not show any polymorphism. The ratio fitted the expected Mendelian ratio, 3:1 (χ^2 = 2.82 and 0.05, P > 0.01, respectively) (Table 1).

Linkage analysis

To check potential for co-segregation of DNA fragments and resistant phenotypes, multiple regression analysis was carried out to confirm associations between the molecular markers and the resistance to fusarium stalk rot in all 170 F_2 plants. The relationships between RAPD markers; OPA02 and Pr11 and resistance to fusarium stalk rot were highly significant and explained 69 and 75% of the variation, respectively. Also, the ISSR marker (AD-8) was significantly (P <0.01) associated with the resistance to fusarium stalk rot and explained 67 % of the variation. Also, the SSR93, SSR105, SSR225 and SSR337 markers

Table 2: Location of QTL's affecting host- plant response to *F. moniliforme* in F_2 population (Gm1021 × Gm1002).

Locus	Map (cM)	LOD	R^2%	Additive effect
RAPD: $OPA02_{350bp}$	4.9	27.4	68.1	0.08
RAPD: $Pr11_{700bp}$	2.9	33.4	75.1	0.06
ISSR: $AD8_{410bp}$	4.8	28.7	65.4	-0.08
SSR: $SSR93_{210bp}$	2.5	31.5	78.2	-0.09
SSR: $SSR105_{200bp}$	1.8	35.6	89.2	-0.22
SSR: $SSR225_{200bp}$	8.3	20.8	59.3	-0.12
SSR: SSR337	4.7	46.2	82.1	-0.09
STS: $STS03_{300bp}$	1.8	33.3	87.2	-0.08

were significantly ($P < 0.01$) associated with the resistance to fusarium stalk rot and explained 78, 89, 59 and 82 % of the variation, respectively. In addition, the STS marker (STS03) was significantly ($P < 0.01$) associated with the resistance to fusarium stalk rot and explained 87 % of the variation (Table 2). This indicates that the RAPD, ISSR, SSR and STS markers were associated with the resistance to Fusarium stalk rot as an indicator for stalk rot resistance gene.

The linkage relationship between the RAPD markers (OPA02 and pr 11) and stalk rot resistance was estimated using F_2 population derived from the cross, Gm1021X Gm1002. The genetic distance between RAPD markers (OPA02 and Pr11) and stalk rot resistance were determined to be 4.9 and 2.9 cM, respectively, with LOD scores of 27.4 and 33.4, respectively (Fig. 2 and Table 2). Therefore, the RAPD markers (OPA02 and Pr11) were linked to the QTL for stalk rot resistance gene in maize.

After linkage analysis on the F_2 population, the genetic distance between ISSR marker (AD08) and stalk rot resistance gene was determined to be 4.8 cM with LOD scores of 28.7 (Fig. 2 and Table 2). Therefore, ISSR marker (AD-8) was also linked to QTL for stalk rot resistance. The linkage relationship between the four SSR markers (SSR93, SSR105, SSR225, and SSR337) and stalk rot resistance trait were determined to be 2.5, 1.8, 8.3 and 4.4 cM, respectively, with LOD scores of 31.5, 35.6, 20.8 and 46.2 respectively (Fig. 2 and Table 2). Therefore, these SSR markers were also linked to the QTL for the resistance to fusarium trait as an indicator for stalk rot resistance gene.
The genetic distance between STS marker (STS03) and stalk rot resistance gene was determined to be 1.8 cM, with LOD scores of 33.3 (Fig. 2 and Table 2). Therefore, STS marker STS03 was also linked to the QTL for stalk rot resistance gene in maize.

Two RAPD, one ISSR, four SSR and one STS markers covering the distance of 8.3 cM on this linkage group were linked to the fusarium stalk rot resistance gene (Fig. 2). After linkage analysis (Map Manger) on the F_2 population, these markers were linked in one group. Based on the information available for SSR and STS markers, we are able to assign the RAPD markers OPA02 and Pr11 and the ISSR marker AD-8 for the GM1021 × GM1002 F_2 population on the chromosome 10 (Fig. 2).

The positive additive effects by OPA02 and Pr11 markers indicated higher values for the traits conferred by the alleles from the resistant parent Gm1021 (Table 2). Meanwhile, the six QTLs (AD8, SSR93, SSR105, SSR225, SSR337 and STS03) were detected with negative additive effects, indicating higher values conferred by susceptible parent 'Gm1002' (Table 2). Therefore, it will be feasible to transfer favoured alleles from both the parents to elite maize varieties as recurrent lines. Hence, the QTL mapping

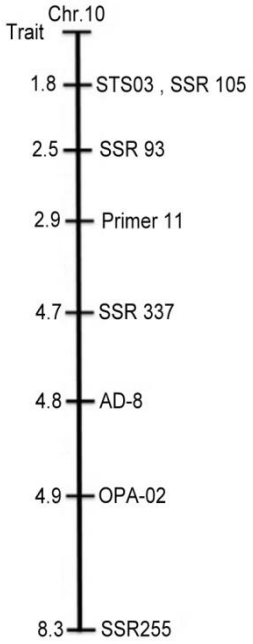

Fig 2. Polymorfic RAPD marker (OPA-02 and Pr.11), ISSR marker (AD8), SSR marker (SSR93, SSR105, SSR225, and SSR337) and STS marker (STS-03) were located on the 10 chromosome. All distances from QTL are expressed in cM (*Map Maker*). F_2 population of 170 hybrids (Gm1021 × Gm1002) was used.

population will be very useful in transferring favoured alleles from both the parents by further backcrossing and marker assisted breeding.

Discussion

The resistance breeding is heavily dependent on the genetics of resistance, which may be difficult to understand to come up with conclusive results. Consequently, genetic gains due to selection are also slow. The advent of DNA markers has opened avenues for plant breeders to exploit new approaches to plant breeding that are cost effective (Dreher et al., 2000). Marker Assisted Selection (MAS) and DNA finger printing techniques can increase the efficiency of conventional plant breeding by speeding up the time of varietal development (Welz and Geiger, 2000). However, identifying molecular markers associated with important genes or traits in most instances requires screening of a relatively large number of individuals in developed to overcome this difficulty because comparing bulk samples is easier than evaluating many individuals in different populations (Altinkut and Gozukirmizi, 2003; Barakat et al., 2009, 2010).

BSA was firstly reported by Michelmore et al. (1991) to identify RAPD markers tightly linked to genes for resistance to lettuce downy mildew. Molnar et al. (2000) reported that BSA is an effective approach for developing molecular markers for genes that confers resistant to pathogens, causing foliar diseases such fusarium stalk rot and accounts for a significant proportion of the phenotypic variation. This method has been used with RAPD markers to identify seven markers in barley associated with resistance to *Pyrenophora teres* f.*maculata*. Recently, identification of new microsatellite marker linked to the grain filling rate as indicator for heat tolerance genes in F_2 wheat population combined with bulked segregant analysis have been reported (Barakat et al., 2011). Several types of molecular markers associated with flag leaf senescence using bulked segregant analysis in wheat under water-stressed conditions were identified (Milad et al., 2011; Barakat et al., 2013).

In this study, mapping and identifying quantitative trait loci (QTL) for fusarium stalk rot resistance gene in maize were described in the population of maize hybrid (GM1021 × GM1002) using RAPD, ISSR, SSR and STS markers. Using BSA, we were able to identify two RAPD markers (OPA02 and pr 11), one ISSR marker (AD8), four SSR markers (SSR93, SSR105, SSR225, and SSR337) and one STS marker (STS03) linked to the fusarium stalk rot resistance gene in the GM 1021 × GM 1002 F_2 population. Previously, molecular mapping of QTLs for resistance to northern corn leaf blight (NCLB) in F_2 population of maize derived from a cross between two white lines, the resistant line Sids-63 (Sd-63) and the susceptible line Sids-7 (Sd-7) have been studied (Barakat et al., 2010). They reported that the Pr11 primer, which generated two polymorphic fragments at 180 and 300bp, were present only in NCLB-susceptible bulk and Sd7 (susceptible parent) and were missing in NCLB-resistant bulk and Sd63 (resistant parent). They also reported that the Pr11 markers were linked to the quantitative trait loci (QTL) for (NCLB) resistance Ht1 gene. Yang et al. (2010) reported that the SSR markers (SSR93, SSR105, SSR225 and SSR337) and STS marker (STS03) were located on chromosome 10. The result of the present study showed that these five markers were associated with the QTL for the resistance to fusarium stalk rot. Hence, we can conclude that the QTL for fusarium stalk rot resistance is present on chromosome 10. QTLs were associated with above mentioned markers and explained from 59.3 to 89.2 % of the phenotypic variation for fusarium stalk rot resistance gene in maize. Therefore, these markers should be useful for marker-assisted selection since they can help to detect the resistant genes of interest without the need of carrying out field evaluation. They also allow screening large breeding material at early growth stages and in a short time.

Materials and Methods

Plant materials and disease evaluation

A segregating F_2 population derived from the cross between two maize white lines, the resistant line Gm1021 (resistance to stalk rot disease) and the susceptible line Gm1002 (susceptible to stalk rot disease) was used in this study. The cross was made during the season of 2008 and was selfed in 2009 to produce the F_2 population. For evaluating against stalk rot disease, F_2 population (170 individual plants) and their parents were planted under field conditions, in the late summer of 2010 at the Experimental Farm Station, Faculty of Agriculture, Alexandria University, Alexandria, Egypt, where environmental conditions allow for a uniform disease infection. The artificial infection was done to enhance the natural infection, using an isolate of *Fusarium moniliforme* obtained from Department of maize and Sugar Crops Disease Research, Agri. Res. Cent. Giza, Egypt. The obtained isolates were identified at the Maize Pathology Research Section, Plant Disease Research institute, Agricultural Research Center (ARC), Giza, Egypt, according to several investigators (Ellis, 1971; Barnett and Hunter, 1972). Such isolate was a single spore culture, grown on potato dextrose agar medium (PDA) for ten days at 25 ± 2°C. Spore suspensions were prepared by adding sterilized distilled water over fungal growth, which was scraped off, using a sterilized needle. The suspensions were, then, strained through a sterilized cheese-cloth. Spore concentration was adjusted at 2.5×10^3 spores cm^3, using sterilized distilled water. Plants (40-d-old) of the inbred lines; Gm 1002, Gm 1021 and F_2 were injected with 5 cm^3 of Fusarium filtrate between the first and second lower node of the stem. The number of resistant and susceptible plants was recorded after two weeks.

DNA extraction

Genomic DNA was extracted from fresh leaves of individual F_2 plants and their parents, using CTAB (Sagahi-Maroof et al. 1984). RNA was removed from the DNA preparation by adding 0.01 cm^3 of RNAase (10 mg cm^{-3}) and incubating for 30 min at 37ºC. DNA sample concentration was quantified by using a spectrophotometer (Beckman Du-65).

PCR amplification

Thirty-eight RAPD primers and twenty five ISSR primers (Barakat et al., 2010) were used in the present investigation to amplify the template DNA. The PCR reaction mixture consisted of 20-50 ng of genomic DNA, 1× PCR buffer, 2.0 mM MgCl2, 100 µM of each dNTP, 0.1 µM primer, and 1 U *Taq* polymerase in a 0.025 cm^3 volume. Template DNA was initially denatured at 94 °C for 4 min, followed by 45 cycles of 94 °C for 1 min, 36 °C (for RAPD analysis) or 50 °C (for ISSR analysis) for 1 min, and 72 °C for 1 min with a final extension at 72 °C for 10 min. Amplification products were fractionated on 1 % agarose gels (for RAPD analysis) or 2 % agarose gels (for ISSR analysis).

Twenty two pairs of SSR and eight STS primers (Yang et al., 2010**)** were also used. The PCR cycle included an initial denaturation at 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 1 min; annealing at 50, 55, or 60 °C (depending on the individual microsatellite primer) or annealing temperatures varied from 57 to 59°C (depending on STS primer) for 1 min; and extension at 72 °C for 2 min followed by a 17-min final extension at 72 °C. The amplification products were then electrophoresed in 2 - 3 % agarose gels.

Bulked segregant analysis

Bulked–segregant analysis (BSA) was used in conjunction with RAPD, ISSR, STS and SSR analysis (Michelmore et al., 1991) to find markers linked to genes of interest. Resistant and susceptible bulks were prepared from F_2 individuals by pooling aliquots, containing equivalent amounts of total DNA, approximately, 50 ng cm^{-3} from

each of ten susceptible and ten resistant F_2 plants selected, based on phenotypic assessments. PCR was carried out on the bulks and parental DNA samples using RAPD, ISSR, STS and SSR primers that were polymorphic between parents using the same conditions as described above. After analysis of the bulks for the presence or absence of various markers, individual F_2 plants forming the bulks were then tested to confirm a correlation with the stalk rot resistance alleles. Based on the evaluations of DNA bulks, individual F_2 plants were analyzed with cosegregating primers to confirm RAPD, ISSR STS and SSR markers linkage to the stalk rot resistance gene.

Data analysis

Goodness of fit to a 3:1 ratio was calculated for RAPD, ISSR, STS and SSR markers by χ^2- test. The association between molecular markers (RAPD, ISSR, STS and SSR) and the values of stalk rot resistance gene of the F_2 plants (Moreno and Gonzalez 1992) was assessed with correlation and simple regression analysis, using *PROC REG of SAS v*. 9.1 software packages (SAS Institute, Cary, NC, 1985). The magnitude of the marker associated phenotypic effect was described by the coefficient of determination (R^2) which represented the fraction of variance explained by the polymorphism of the marker.

Linkage analysis

Map manager *QTX v* 0.22 (Meer et al., 2002) was used to analyze the linkage relationship of RAPD, ISSR, STS and SSR markers detected from bulked segregant analysis. Linkage was detected when a log of the likelihood ratio (LOD) threshold was 3.0 and maximum distance was 20 cM. The chromosomal location of the QTLs for the stalk rot resistance gene of the F_2 plants (Morens- Gonzales, 1992) was further confirmed by the simple interval mapping method using *QGENE* program (Nelson, 1997).

Conclusion

The present study indicated that RAPD, ISSR, SSR and STS markers, combined with bulked segregant analysis, could be used to identify molecular markers linked to *F. moniliforme* resistance gene in maize. The markers presented in this study might be further considered in maize breeding programs for developing improved lines.

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