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Identification of Molecular Markers Linked to Northern Corn Leaf Blight Resistance in Yellow Population of Maize

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ABSTRACT

The objectives of this study were to identify RAPD, ISSR and SCAR markers linked to northern corn leaf blight (NCLB) resistance genes in an F_2 population of yellow maize (*Zea mays* L.) using bulked segregant analysis and to map NCLB resistance genes in F_2 populations of maize. The F_2 yellow population of maize was developed from a cross between the resistant line Gm1021 and the susceptable line Gm1002. Bulked segregant analysis with RAPD, ISSR and SCAR markers was conducted to identify markers that were linked to the *Helminthosporium turcicum* (*Ht*) gene. The genetic distance between RAPD markers ($OPC04_{220\ bp}$, $OPC04_{450\ bp}$ and $OPE20_{270\ bp}$) and NCLB *Ht* resistance genes were 2.1, 7.8 and 13.4 cM, respectively, with logarithm of odds (LOD) scores of 34.2, 23.1, and 23.5, respectively. Therefore, these three RAPD markers were linked to the quantitative trait loci (QTLs) for the NCLB *Ht* resistance gene. The genetic distance between the SCAR marker ($SCE20_{270\ bp}$), which was derived from the converted $OPE20_{270\ bp}$ RAPD marker, and the NCLB *Ht* resistance gene, was 2.1 cM with an LOD score of 34.2. This confirmed that the SCAR marker targeted the same locus as the RAPD marker and that it too was linked to the QTL for the NCLB *Ht* resistance gene. The genetic distance between an ISSR marker ($HB13_{200\ bp}$) and the NCLB *Ht* resistance gene was 1.4 cM with an LOD score of 32.5. Therefore, this ISSR marker was linked to the QTL for the NCLB *Ht* resistance gene. The present study indicates that RAPD, ISSR and SCAR markers, combined with bulked segregant analysis, can be used to identify molecular markers linked to the NCLB resistance gene in maize. Once these markers are identified, they can be used to detect the QTLs linked to NCLB resistance in breeding programs, as a selection tool in early generations.

Keywords: bulked segregant analysis, *Helminthosporium turcicum* (Pass.), ISSR analysis, QTLs, RAPD analysis, SCAR analysis

INTRODUCTION

Maize (*Zea mays* L.) is an important cereal crop for food and feed in many parts of the world. In Egypt, maize is grown for food, feed, fodder and industrial purposes. Egypt imports approximately 35% of its maize needs (Khalifa and Zein El-Abedeen 2000). It is important to develop high-yielding and disease-resistant hybrids to meet the country's demands.

Northern corn leaf blight (NCLB) disease, caused by *Helminthosporium turcicum* (Pass.) or *Ht*, is one of the important foliar diseases of maize in temperate areas of the world, including Egypt. The disease is sporadic in occurrence, depending on environmental conditions (Bentolila *et al.* 1991). It is favoured by moderate temperature and high humidity (Shurtleff 1980). In Egypt, the disease is mostly found in the northern and northwestern regions of the Delta in late summer (off-season) maize planting, where favorable weather conditions prevail at that time of the year (El-Assiuty *et al*. 1987; Gouda 1996). Moreover, sprinkler irrigation systems, which are widely used in the newly reclaimed lands, provide high air moisture in the field throughout the season, which allows for disease incidence in these areas. When infection becomes severe, high yield losses can occur, especially when susceptible lines or hybrids are grown in these areas. If the disease is established before silking, grain yield reductions of more than 50% may occur (Raymundo and Hooker 1981). In Egypt, maize leaf blight caused a significant loss in grain yield, estimated at 30% or more in the Northern Delta region where the climatic conditions favour the disease development (Khalifa and Zein El-Aabedeen 2000).

The most efficient, effective, environmentally safe and economical means to control NCLB of maize is by use of resistant cultivars. Most maize breeders, therefore, prefer the use of quantitative NCLB resistance in their cultivar development programs. Several qualitative genes of resistance are known namely, *Htl*, *Ht2*, *Ht3*, and *HtN*, referring to their locus designations (Gevers 1975; Hooker 1977). *Htl*, *Ht2* and *Ht3* result in small chlorotic lesions and the amounts of necrotic tissue, fungal sporulation and inoculum for secondary infections are all reduced (Hooker and Kim 1973). The *HtN* gene results in a delay in lesion development until after flowering (Gevers 1975).

Current breeding programs predominantly rely on resistance, which exhibits a quantitative inheritance pattern. This type of resistance is effective against all presently known races of *Ht*, in contrast to loci with qualitative effects (Smith and White 1988). With a quantitative inheritance pattern, resistance seems to be manifested, primarily, by a reduction in lesion number and a broad range of levels of resistance has been observed (Hooker and Kim 1973).

In several previous studies, quantitative trait loci (QTLs) associated with general resistance to NCLB, were identified on all 10 maize chromosomes (Freymark *et al.* 1994; Dingerdissen *et al.* 1996; Schechert *et al.* 1999; Welz and Geiger 2000; Brown *et al.* 2001; Huang *et al*. 2002; Yin *et al*. 2003; Ogliari *et al*. 2005; Wisser *et al*. 2006; Ogliari *et al*. 2007; Khampila *et al.* 2008; Poland *et al*. 2009). All of these studies reported that molecular markers associated to NCLB resistance are potentionlly useful for the identification of genotyped individuals carrying NCLB resistant traits in breeding programs. The use of molecular markers can increase the efficiency of conventional plant breeding by identifying markers linked to the trait of interest, which are difficult to evaluate and/or are largely affected by the environment (Tanksley *et al.* 1989). Hence, there is a need to develop a rapid screening method to select for resistance

Received: 13 May, 2009. Accepted: 16 July, 2009.

Original Research Paper

to NCLB. Tight linkage between molecular markers and genes for disease resistance can be of great benefit to disease resistance breeding programs by allowing the investigator to follow the DNA markers (PCR-based markers) through early generation rather than waiting for phenotypic expression of the resistance genes (Lefebure and Chevre 1995). 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 field disease test. Also, it allows for screening big number of breeding materials at early growth stages and in short time.

The objectives of this investigation were to identify RAPD, ISSR and SCAR markers linked to NCLB resistance genes in F_2 populations of maize, using bulked segregate analysis and to map NCLB resistance genes in F_2 population of maize.

MATERIALS AND METHODS

Plant materials and disease evaluation

Identification of RAPD, ISSR and SCAR markers linked to NCLB disease resistance were carried out on a segregating F_2 population derived from a cross between two yellow lines namely, the resistant line Gm1021, and the susceptible one Gm1002 and their parents. Gm1002 is a NCLB susceptible, yellow dent and inbred line. Gm1021 is a NCLB resistant, yellow dent and inbred line. These inbred lines were obtained from the Agriculture Research Center, Giza, Egypt. These inbred lines were crossed in 2005 growing season to produce F_1 population. The F_1plants were selfed in 2006 growing season to produce the F_2 population.

For evaluating against NCLB, an F_2 population (139 individual plants), and their parents were planted under field conditions in the late summer of 2007 at the Experimental Farm Station, Faculty of Agriculture, Alexandria University, Alexandria, Egypt, where environmental conditions allow for uniform disease infection.

Artificial infection was done to enhance natural infection, using an *Ht* T-13AS isolate, which was a single spore culture grown in Petri dishes containing potato dextrose agar medium for 10 days at 25 ± 2°C. Spore suspensions were prepared by adding sterilized distilled water (SDW) 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/ml using SDW. Plants were inoculated at the three-to-five leaf stage of growth, in the evening, using a spore suspension. The severity of NCLB, as a percentage of the infected leaf area (% average lesion size), was assessed after the flowering growth stage around 8 weeks after the inoculation and readings were classified, according to Elliot and Jinkins (1946) as follows:

Rating scale	Leaf area infected (%)	Resistance level
0.5	<5	Highly resistant (HR)
1.0	6-10	Resistant (R)
2.0	11-25	Moderately resistant (MR)
3.0	26-50	Moderately susceptible (MS)
4.0	51-75	Susceptible (S)
5.0	>75	Highly susceptible (HS)

DNA extraction

Genomic DNA was extracted from fresh leaves (14 weeks old) of individual F_2 plants and their parents, using the Saghai-Maroof *et al*. (1984) CTAB method. RNA was removed from the DNA preparation by adding 10 µl of RNAase (10 mg/ml) and then incubated for 30 min at 37°C. DNA sample concentration was quantified by using a spectrophotometer (Beckman Du-65). The reagents were obtained from Pharmacia Biotech (Amersham Pharmacia Biotech Ltd., UK).

PCR amplification

Thirty-eight RAPD primers (**Table 1**) and 25 ISSR primers (**Table 2**) were used in the present investigation to amplify the template DNA. The PCR reaction mixture consisted of 20-50 ng genomic DNA, 1×PCR buffer, 2.0 mM $MgCl_2$, 100 µM of each dNTP, 0.1 µM primer and 1U *Taq* polymerase in a 25 µL 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% (for RAPD analysis) or 2% (for ISSR analysis) agarose gel.

Specific-PCR was performed using SCAR primers, which were synthesized by Pharmacia Biotech. The sequences of pair primer SCE20 was F/R 5'-GTG GCT GGT TTG CTG TGA-3' (18 bases)/5'-TTG TAT CGC CAC TTC CAC CA-3' (20 bases) (Khampila *et al*. 2008). Amplification of genomic DNA, with the SCAR primers, consisted of 4 min pre-denaturation at 94°C, followed by 45 cycles of 1 min denaturation at 94°C, 1 min annealing, and 2 min extension at 72°C, with a final extension period of 4 min at 72°C, and a hold at 4°C until recovery. A series of annealing temperatures were utilized from 56 to 60°C in 2°C increments in the PCR reaction conditions in an effort to identify an annealing temperature that would yield single, strongly amplified bands of the expected size. The SCAR primers designed were used to amplify DNA, obtained from the Gm1021 and Gm1002 parental lines, the resistant and susceptible bulks, and each F_2 population. Amplification products were electrophoretically resolved on 1.5% aga-

Table 1 Number of amplifications and polymorphic products of 38 primers used to screen the polymorphism between the two inbred lines (Gm1021 and Gm1002).

Primer	Nucleotide sequence (5'-3')	№ of amplification products	№ of polymorphic products
Pr1	CAGGCCCTTC	0	0
Pr2	TGCCGAGCTG	0	0
Pr3	AGTCAGCCAC	8	3
Pr4	AATCGGGCTG	4	1
Pr5	AGGGGTCTTG	6	0
Pr6	GGTCCCTGAC	0	0
Pr7	GAAACGGGTG	4	1
Pr8	GTGACGTAGG	0	0
Pr9	GGGTAACGCC	0	0
Pr10	GTGATCGCAG	0	0
Pr11	CAATCGCCGT	6	1
Pr12	TCGGCGATAG	4	3
Pr13	CAGCACCCAC	4	1
Pr14	TCTGTGCTGG	3	3
Pr15	TTCCGAACCC	5	2
Pr16	AGCCAGCGAA	5	0
Pr17	GACCGCTTGT	3	1
Pr18	AGGTGACCGT	0	0
Pr19	CAAACGTCGG	4	0
Pr20	GTTGCGATCC	4	2
UBC321	ATCTAGGGAC	0	0
UBC475	CCAGCGTATT	4	3
UBC532	TTGAGACAGC	5	1
OPA02	TGCCGAGCTG	4	4
OPA06	GGTCCCTGAC	0	0
OPA07	GAAACGGGTG	4	1
OPB8	GTCCACACGG	7	1
OPB9	TGGGGGACTC	4	1
OPB13	TTCCCCCGCT	5	3
OPC04	CCGCATCTAC	6	4
OPC15	GACGGATCAG	8	7
OPE20	AACGGTGACC	0	0
OPF15	CCAGTACTCC	0	0
OPJ 04	CCGAACACGG	0	0
OPJ10	AAGCCCGAGG	5	5
OPU06	ACCTTTGCGG	10	2
OPZ03	CAGCACCGCA	8	2
OPH13	GACGCCACAC	8	3

Table 2 Number of amplifications and polymorphic products of 25 ISSR primers used to screen the polymorphism between the two inbred lines (Gm1021 and Gm1002).

Primer	Nucleotide sequence (5'-3')	№ of amplification products	№ of polymorphic products
AD1	(GA)9C	8	3
AD2	(AGC)6G	7	1
AD3	(ACC)6G	14	4
AD4	(AGC)6C	0	0
AD5	(CA)10C	7	1
AD6	GT(CAC)7	10	4
AD7	(AG)9C	8	4
AD8	(AGC)6G	6	1
AD9	(AC)9G	7	2
M-1	(AC)8CG	9	2
M-2	(AC)8CC-T	7	0
M-6	(CAC)5	7	3
M-7	(CAG)5	10	3
M-8	(GTG)5	9	2
M-9	(GACAC)4	0	0
M-10	(CA)6A/GG	8	0
M-11	(CA)6A/G	7	2
M-12	(CA)6RY	7	1
M-13	(AGC)3Y	0	0
HB13	(GAG)3GC	8	2
HB14	(CTC)3CG	4	3
HB15	(GTG)3GC	7	1
PO2	AT(CGAT)3CG	0	0
D12	(GAGA)3CG	0	0
D14	(CAC)3GC	12	3

rose gels containing 0.1 µg/ml ethidium bromide and photographed on a UV transilluminator.

Bulked segregant analysis

Bulked–segregant analysis (BSA) was used in conjunction with RAPD, ISSR and SCAR 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/µl from each of 14 susceptible and 14 resistant F_2 plants selected, based on phenotypic assessments (**Fig. 1**). PCR was carried out on the bulks and parental DNA samples using RAPD, ISSR and SCAR 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 NCLB resistance alleles. Based on the evaluations of DNA bulks, individual F_2 plants were analyzed with cosegregating primers to confirm RAPD, ISSR and SCAR markers linkage to the NCLB resistance genes.

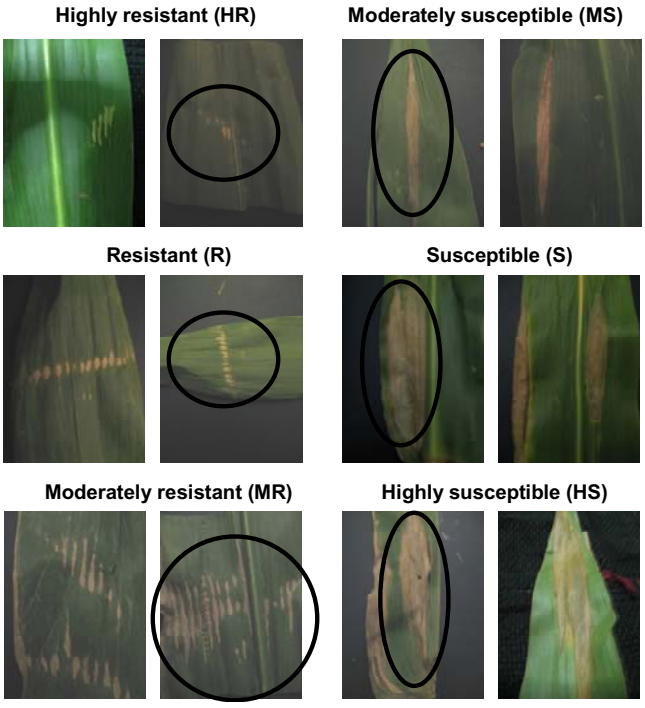

Fig. 1 Infection types (Highly resistant HR, Resistant R, Moderately resistant MR, Moderately susceptible MS, Susceptible S, Highly susceptible HS) developed on maize cultivars as a response to infection with isolate of *Helminthosporium turcicum*.

Data analysis

Goodness of fit to a 3: 1 ratio was calculated for RAPD, ISSR and SCAR markers by the χ^2 test. The association between molecular markers and resistance to NCLB trait was assessed with correlation and simple regression analysis, using PROC REG of SAS version 9.1 software packages (SAS Institute, Cary, NC, 2007). Magnitude of the marker associated phenotypic effect was described by the coefficient of determination (r^2) and simple correlation (r), which represented the fraction of variance explained by the polymorphism of the marker.

Linkage analysis

Map manager QTX Version 0.22 (Meer *et al.* 2001) was used to analyze the linkage relationship of RAPD, ISSR and SCAR markers detected from bulked segregate analysis. Linkage was detected when a log of the likelihood ratio (LOD) threshold of 3.0 and maximum distance was 50 cM. Kosambi's mapping function (Kosambi 1944) was used.

RESULTS

RAPD markers analysis

Thirty-eight primers of arbitrary nucleotide sequence (**Table 1**) were used to screen the polymorphism between the highly susceptible inbred line, Gm1002, and the highly resistant inbred line, Gm1021, to NCLB. Out of 38 primers, 23 RAPD (60.05%) gave polymorphic bands that were suitable to differentiate between the two parents. A total of 138 bands were amplified using 38 RAPD primers, producing an average of 3.63 bands per primer. The number of RAPD fragments that were amplified ranged from three to 10 and the sizes ranged from about 140 to 1400 bp. Of these 23 RAPD primers, OPC04, which produced a single and strong polymorphic band at 450 bp, that was present only in the resistant parent (Gm1021) but absent in the susceptible parent (Gm1002), was selected for screening DNA bulks and their parental DNA. On the other hand, the same primer (OPC04) produced a single and strong polymorphic band at 220 bp that was present in only Gm1002 (the susceptible parent), but absent in Gm1021 (the resistant parent) (**Fig. 2A**). Primer OPC04 generated the 450-bp polymorphic fragment, which was present only in the resistant bulked DNA (Br: resistant bulks) and Gm1021 (resistant parent) and was missing in the susceptible bulked DNA (Bs: susceptible bulk) and Gm1002 (susceptible parent). On the other hand, primer OPC04 generated a polymorphic fragment at 220 bp, which was present only in in Bs and Gm1002 (susceptible parent), but was missing in Br and Gm1021 (resistant parent) (**Fig. 2A**). These RAPD markers ($OPC04_{220\ bp}$ and $OPC04_{450\ bp}$) were regarded as candidate markers linked to the NCLB resistance gene in maize. In addition, OPE20 primer produced a strong polymorphic band at 270 bp that was present in only the susceptible parent (Gm1002). This band was also present in Bs, but not in Br and resistant parent Gm1021 (**Fig. 2B**).

These polymorphic markers, $OPC04_{220\ bp}$, $OPC04_{450\ bp}$ and $OPE20_{270\ bp}$, were further used to check their linkage to the NCLB resistance gene, using a segregating F_2 population, derived from the cross between the resistant parent (Gm1021) and the susceptible one (Gm1002). When ana-

Table 3 Significant association between NCLB resistance and markers (RAPD, ISSR and SCAR) in the 139 F_2 plant population of maize detected, using Chi-squared (χ^2) correlation (r), and coefficient of determination (r^2) analysis.

Tool	Markers	Tested plants			Expected Ratio	χ^2	r	r^2
		Total	R	S				
RAPD	PC04-450	139	97	42	3 : 1	2.6	-0.79**	0.61**
	PC04-220	139	102	37	3 : 1	0.06	-0.88**	0.74**
	PCE20270	139	95	44	3 : 1	3.2	-0.90**	0.82**
ISSR	HB13	139	97	42	3 : 1	2.01	-0.91**	0.83**
SCAR	SCE20	139	95	44	3 : 1	3.2	-0.90**	0.81**

R=Resistant plants S= susceptible plants ns,
** non significant and significant at .01 level of probability, respectively.

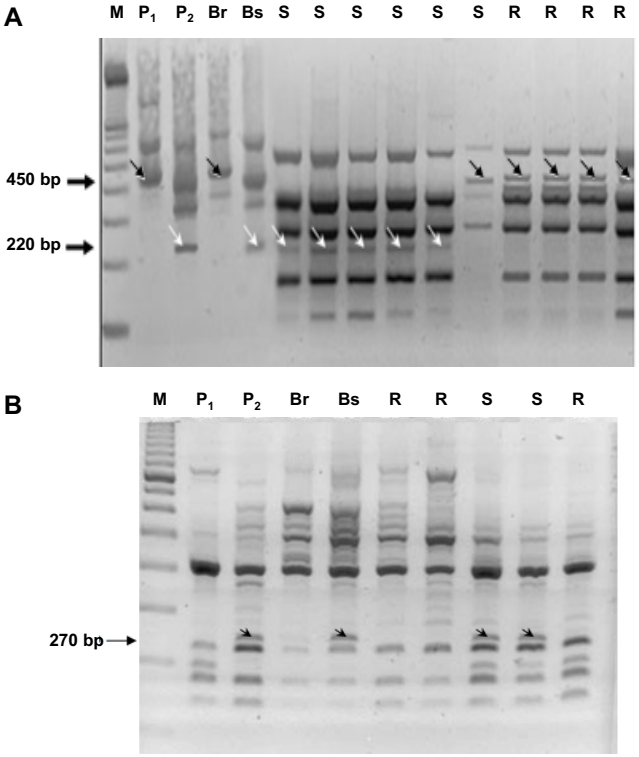

Fig. 2 RAPD fragments, produced by OPC04 primer (5′-CCGCATC TAC-3′) (A) and OPE20 primer (5′-AACGGTGACC-3′) (B). M: molecular weight, followed by P_1 and P_2 parents, Gm1021 and Gm1002, respectively. Br, bulk resistance; Bs, bulk susceptible, F_2 individuals in the cross, Gm1021 x Gm1002 (R resistant; S: susceptible).

lyzing the individual plants of the F_2 population, $OPC04_{450\ bp}$ and $OPC04_{220\ bp}$ as well as $OPE20_{270\ bp}$ fragments were amplified in the DNA obtained only from F_2 resistant and susceptible plants, respectively. For the RAPD marker, $OPC04_{450\ bp}$, 96 of 139 (69.1%) individuals in the F_2 population exhibited the amplified polymorphic fragments (450 bp) while the remainder did not (**Fig. 2A**). The ratio fitted the expected Mendalian ratio of 3: 1 (χ^2 = 2.6, P <0.1) (**Table 3**). In the RAPD marker, $OPC04_{220\ bp}$, 37 of 139 (26.6%) individuals in the population exhibited the amplified polymorphic fragment (220 bp), while, the remainder did not (**Fig. 2A**). The ratio fitted the expected Mendalian ratio of 3: 1 (χ^2 = 0.06, P<0.75) (**Table 3**). In addition, the RAPD marker, $OPE20_{270\ bp}$, 44 of 139 (31.6%) individuals in the population exhibited the amplified polymorphic fragment (270 bp) while the remaining did not (**Fig. 2B**). The ratio fitted the expected Mendalian ratio of 3: 1 (χ^2 = 3.2, P<0.75) (**Table 3**).

To check for potential co-segregation of DNA fragments and NCLB resistant phenotypes, correlation and simple regression analysis were carried out in order to confirm an association between the $OPC04_{220\ bp}$, $OPC04_{450\ bp}$ and $OPE20_{270bp}$ markers, and the resistant to NCLB in all 139 F_2 progenies. The results showed that the correlation and regression analysis for the relationship between the three markers ($OPC04_{220\ bp}$, $OPC04_{450\ bp}$ and $OPE20_{270\ bp}$), and the phenotypes of F_2 individuals were significant (r = -0.88, -0.79 and -0.90, respectively; r^2 = 0.74, 0.61 and 0.82, respectively; **Table 3**). This indicates that the three markers were linked with the NCLB resistant gene.

ISSR marker analysis

Twenty-five ISSR primers (**Table 2**) were used to screen the polymorphism between the highly susceptible inbred line, Gm1002, and the highly resistant inbred line, Gm1021, to NCLB. Out of 25 primers, 18 (72.0%) gave polymorphic bands that were suitable to differentiate between the two parents. A total of 162 bands were amplified, producing an average of 6.48 bands per primer. The number of ISSR fragments that were amplified ranged from 4 to 14 and the sizes ranged from about 160 to 1600 bp. Of these 18 ISSR primers, HB13 primer, which produced a single and strong polymorphic band at 200 bp, was present only in the resistant parent (Gm1021), but absent in the susceptible parent (Gm1002) (**Fig. 3**). This HB13 primer was selected for screening DNA bulks and their parental DNA. The primer, HB13 (5'[GAG]3 GC 3'), generated the polymorphic fragment at 200 bp, which was present only in NCLB resistant bulks and Gm1021 (resistant parent) and was missing in NCLB susceptible bulks and Gm1002 (susceptible parent) . This ISSR marker (HB13200 bp) was regarded as a candidate marker linked to NCLB resistance gene in maize.

This polymorphic marker, $HB13_{200\ bp}$, was further used to check linkage to the NCLB resistance gene using a segregating F_2 population derived from the cross between the resistant parent (Gm1021) and the susceptible one (Gm1002). When analyzing the individual plants of the F_2 population, the $HB13_{200\ bp}$ fragment was amplified in the DNA, obtained only from F_2 resistant plants. PCR amplification of the resistant parent (Gm1021), the susceptible parent (Gm1002), resistant bulk, susceptible bulk and F_2 resistant and F_2 susceptible individuals using $HB13_{200\ bp}$ primer is shown in **Fig. 3**.

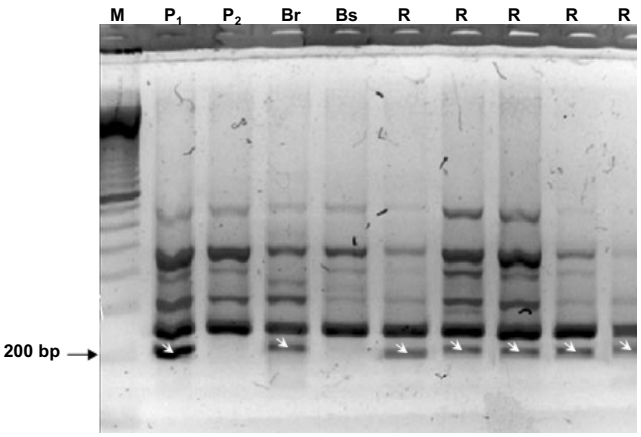

Fig. 3 ISSR fragments produced by HB13 primer. M: molecular weight, followed by P_1 and P_2 parents, Gm1021 and Gm1002, respectively. Br, bulk resistance; Bs, bulk susceptible, F_2 individuals in the cross, Gm1021 x Gm1002 (R: resistant).

For the ISSR marker, $HB13_{200\ bp}$, 97 of 139 (69.78%) individuals in the F_2 population exhibited the amplified polymorphic fragments (200 bp) while the remainder did not (**Fig. 3**). The ratio fitted the expected Mendalian ratio of 3: 1 ($\chi^2 = 2.01$, $P < 0.1$) (**Table 3**).

To check for potential co-segregation of DNA fragments and NCLB resistant phenotypes, correlation and coefficient of determination were determined in order to confirm the association between the $HB13_{200\ bp}$ marker and resistance to NCLB in all 139 F_2 progenies. The results show that the correlation and regression analysis for the relationship between the marker, $HB13_{200\ bp}$, and the phenotypes of F_2 individuals were significant ($r = -0.91$ and $r^2 = 0.83$, respectively; **Table 3**). This indicates that the marker was linked with the NCLB resistant gene.

The present study indicated that ISSR markers, combined with bulked segregate analysis, could be used to identify a molecular marker linked to NCLB resistance gene in maize. Once these markers are identified, they can be used to detect QTLs linked to NCLB resistance in maize breeding programs, as a selection tool in early generations.

SCAR markers analysis

RAPD markers need to be converted into SCAR markers (Cao *et al*. 1999). Therefore, specific-PCR analysis, using SCAR-SCE20 F/R (Khampila *et al*. 2008) was initially tested on individual maize inbred lines (Gm1021 (resistant parent), Gm1002 (susceptible parent)) as well as two individuals of the bulks at annealing temperatures of 60°C. The polymorphisms of the SCAR fragment were identified by the presence or absence of single bands, as shown in **Fig. 4**. Previously, Khampila *et al*. (2008) used RAPD OPE-20 primer to identify an $OPE_{20536\ bp}$ marker linked to the NCLB resistance phenotype. This marker was converted into a dominant SCAR $SCE_{20429\ bp}$ marker. However, in the present study, the primers, OPE20 and SCAR-SCE20 at 270 bp were successfully linked to NCLB resistance gene in the F_2 yellow population. The SCAR marker was then assayed in the F_2 population. All genotypes that were positive for the $SCE_{20270\ bp}$ marker, including the Gm1002 susceptible parent, the susceptible bulk and all F_2 plants, showed a single 270-bp fragment while all NCLB-negative genotypes, including the resistant parent Gm1021 and the resistant bulk, failed to generate any fragment (**Fig. 4**).

For the SCAR marker, $SCE20_{270\ bp}$, 44 of 139 (31.6%) individuals in the population exhibited the amplified polymorphic fragment (270 bp), while, the remainder did not (**Fig. 4**). The ratio fitted the expected Mendalian ratio of 3: 1 ($\chi^2 = 3.2$, $P < 0.75$) (**Table 3**).

To check for potential co-segregation of DNA fragments and NCLB resistant phenotypes, correlation and simple regression analyses were carried out in order to confirm the association between the $SCE20_{270\ bp}$ marker and resistance to NCLB in all 139 F_2 progenies. The results show that the correlation and regression analysis for the relationship between the marker, $SCE20_{270\ bp}$, and the phenotypes of F_2 individuals were significant ($r = -0.90$, $r^2 = 0.82$; **Table 3**). This indicates that the marker was linked with the NCLB resistance gene.

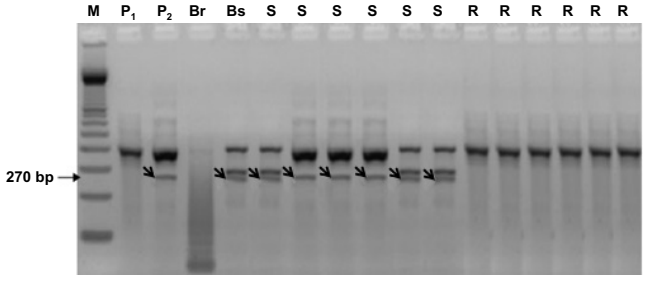

Fig. 4 SCAR fragments produced by SCE20 primer. M: molecular weight, P_1 and P_2 parents, Gm1021 and Gm1002, respectively. Br, bulk resistance; Bs, bulk susceptible F_2 individuals in the cross, Gm1021 x Gm1002 (R: resistant S: susceptible).

Mapping of genes resistance to NCLB

The linkage relationship between the RAPD markers $OPC04_{220\ bp}$, $OPC04_{450\ bp}$ and $OPE20_{270\ bp}$ and the NCLB *Ht* resistance gene were estimated using an F_2 population derived from the cross Gm1021 X Gm1002. The genetic distance between RAPD markers $OPC04_{220\ bp}$, $OPC04_{450\ bp}$ and $OPE20_{270\ bp}$ and the NCLB *Ht* resistance gene were determined to be 7.8, 13.4 and 2.1 cM, respectively, with LOD scores of 23.1, 23.5 and 34.2, respectively (**Fig. 5; Table 4**). Therefore, the RAPD markers ($OPC04_{220\ bp}$, $OPC04_{450\ bp}$ and $OPE20_{270\ bp}$) were linked to the QTL for the NCLB *Ht* resistance gene. After mapmaker linkage analysis of the F_2 population, the genetic distance between the SCAR marker ($SCE20_{270\ bp}$), which was derived from the converted $OPE20_{270\ bp}$ RAPD marker, and the NCLB *Ht* resistance gene, was determined to be 2.1 cM with an LOD score of 34.2 (**Fig. 5; Table 4**). These confirmed that the SCAR marker targeted the same locus as the RAPD marker. Therefore, the SCAR marker ($SCE20_{270\ bp}$) was linked to the QTL for the NCLB *Ht* resistance gene.

After mapmaker linkage analysis on the F_2 population, the genetic distance between ISSR marker ($HB13_{200\ bp}$) and the NCLB resistance *Ht* gene was determined to be 1.4 cM with an LOD score of 32.5 (**Fig. 5; Table 4**)**.** Therefore, ISSR markers ($HB13_{200\ bp}$) was linked to the QTL for NCLB *Ht* resistance gene.

Table 4 Location of QTLs affecting host-plant response to *H. turcicum* in F_2 population of Gm1021 x Gm1002 cross.

locus *Ht*	№ of F_2 plants	Map(cM)	SE (+/-)	LOD
ISSR-HB13	139	1.4	1.0	32.5
SCARSCE20 270bp	139	0.7	0.7	34.2
OPE20-270bp	139	0.0	0.0	---
OPC04-220bp	139	5.7	2.1	23.1
OPC04-450bp	139	5.6	2.0	23.5

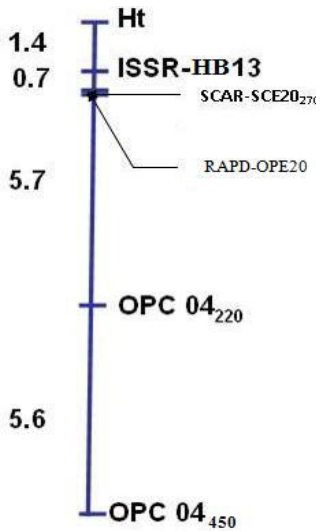

Fig. 5 RAPD, ISSR and SCAR markers ($OPC04_{220\ bp}$, $OPC04_{450\ bp}$, $OPE20_{270\ bp}$, $HB13_{200\ bp}$ and $SCE20_{270\ bp}$) were located through MAPMAKER-QTL analysis. All distances are given in cM using Kosambi's mapping function.

DISCUSSION

Using a method inspired by BSA, we are able to identify several types of molecular markers associated to NCLB resistance phenotype in maize. These markers should be useful for marker-assisted selection. The present results support the idea that BSA can provide fast detection of molecular markers linked to genes of interest. In previous

studies, Barakat *et al*. (2008a, 2008b, 2008c) detected several molecular markers linked to wheat rust resistance regions (*Lr34/Yr18* and *Lr46/Yr29*). They found three RAPD markers ($OPB13_{700\ bp}$, $Pr1_{400\ bp}$, $Pr2_{350\ bp}$) linked to the leaf rust resistance region *Lr34/Yr18* in wheat. They also reported the presence of a RAPD marker ($OPB8_{280}$ bp) linked to the stripe rust resistance region *Lr46/Yr29* in the F_3 population of wheat. Chromosomal locations of the maize *HtP* and *rt* genes that confer resistance to *Exserohilum turcicum* were reported by Ogliari *et al.* (2007). Five microsatellite markers were linked to the *HtP* resistance gene. Recently, identification of RAPD and SCAR markers linked to NCLB in waxy maize were reported by Khampila *et al.* (2008). They used bulked segregate analysis (BSA) to search for RAPD markers linked to NCLB resistance genes using an F_2 population. Four RAPD markers had been identified which were converted into dominant SCAR markers.

Random primers used in RAPD analysis usually anneal with multiple sites in different regions of the genome so that several genetic loci are amplified and also the markers are inherited as dominant genetic markers. This limits the application of this marker type, particularly in cases where one would like to distinguish homozygous from heterozygous genotypes. PCR amplification, which generates RAPD fragments of interest, is very sensitive to specific reaction conditions. Moreover, poor reproducibility can occur in RAPD analysis (Karp *et al*. 1997). In order to increase the specificity of the reaction and to simplify the use of markers linked to NCLB resistance in waxy maize breeding programs, a SCAR marker was derived from the RAPD marker $OPE20_{270\ bp}$, which was assigned $SCE20_{270\ bp}$. SCAR markers have resulted in a rapid screening of segregating progenies of maize for the NCLB resistance, as screening is based on the presence or absence of a single band in the agarose gel (Ohmori *et al*. 1996). Converting RAPD markers to SCAR markers have been reported to facilitate screening of genotypes for a particular trait as they are identified as distinct single bands in agarose gel. Moreover, in some reports this type of marker can be used to differentiate heterozygotes from homozygotes (Paren and Michelmore 1993). In the present study, the association of RAPD and SCAR markers to resistance to NCLB was successfully identified. SCAR markers may be useful for routine marker-assisted selection (MAS) for resistance to NCLB in maize breeding programs, permitting an early selection of resistant genotypes without the cumbersome steps of inoculation, symptom detection and avoiding the problem of NCLB changes from one pathotype to another (Arnedo-Andrés *et al.* 2002).

Breeding for NCLB resistant maize cultivars by conventional means is considered the most effective and feasible method to overcome yield losses due to NCLB. However, conventional breeding is laborious, time-consuming and dependent on environmental conditions. The use of molecular markers is an efficient alternative to the tedious work of phenotype evaluation for NCLB resistance and allows for an efficient selection of NCLB resistance genes. Future work may involve the use of SCAR markers for MAS in maize breeding programme and introgression of genes for resistance to NCLB in maize with superior agronomical performances.

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