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SSR markers associated for late leaf spot disease resistance by bulked segregant analysis in groundnut (*Arachis hypogaea* L.)

D. Shoba · N. Manivannan · P. Vindhiyavarman · S. N. Nigam

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Abstract Late leaf spot (LLS) caused by *Phaeoisariopsis personata* is the major foliar disease that reduces the pod yield and severely affects the fodder and seed quality in groundnut. Molecular markers linked with LLS can improve the process of identification of resistant genotypes. In the present study, a LLS susceptible genotype (TMV 2) and the LLS resistant genotype (COG 0437) were crossed and their F_2 population was used for marker analysis. The phenotypic mean data on $F_{2:3}$ progenies were used as phenotype. Parents were surveyed with 77 SSR (Simple Sequence Repeat) primers to identify polymorphic markers. Among SSR markers, nine primers were found polymorphic between the parents TMV 2 and COG 0437. These markers were utilized for bulked segregant analysis (BSA). Among the polymorphic SSR markers, three primers viz., PM 375_{162}, pPGPseq5D5$_{220}$ and PM 384_{100} were able to distinguish the resistant and susceptible bulks and individuals for LLS. In single marker analysis, the markers PM 375, PM 384, pPGPseq5D5, PM 137, PM 3, PMc 588 and Ah 4-26 were linked with LLS severity score. The phenotypic variation explained by these markers ranged from 32 to 59 %. The markers identified through BSA were also confirmed with single marker analysis. While validating the three primers over a set of resistant and susceptible genotypes, the primer PM 384_{100} allele had association with resistance. Hence PM 384 could be utilized in the marker assisted breeding programme over a wide range of genetic background.

Keywords Groundnut · Late leaf spot · SSR markers · Bulked segregant analysis

D. Shoba · N. Manivannan (✉) · P. Vindhiyavarman
Department of Oilseeds, Tamil Nadu Agricultural University, Coimbatore 641 003, Tamil Nadu, India
e-mail: nmvannan@gmail.com

S. N. Nigam
ICRISAT, Patancheru 502 324, India

Introduction

Groundnut (*Arachis hypogaea* L.) is an important oilseed crop in the tropical and subtropical countries of the world. It is grown on approximately 24 million ha throughout the world (FAOSTAT 2007) and is mainly cultivated in developing countries of Africa and Asia, and 89 % of world production and 94 % of total area is confined to these continents. The most important groundnut growing countries are India, China, Nigeria, Sudan and USA. In India, it occupies an area of 6.41 million ha with a production of 9.36 million tonnes, which accounts for a productivity of 1460 kg/ha during 2007–2008 (Anonymous 2008). Groundnut kernel contains about 45–55 % oil and 25–30 % protein. The biological value of groundnut protein is among the highest of the vegetable proteins. Groundnut is a good source of all B vitamins except B_{12} and groundnut oil is

primarily used in the manufacture of vegetable oil (vanaspati ghee). The oil cake obtained after the extraction of the oil is a valuable organic manure and animal feed. Groundnut utilization pattern in India as percentage are, oil extraction (81 %), seed purpose (12 %), direct consumption (6 %) and Hand Picked Selections (HPS) (1 %). There are several biotic and abiotic stresses that adversely affect groundnut production. Among them, late leaf spot (LLS) is the major foliar disease that not only reduces pod yield but also severely affect the fodder and seed quality. LLS caused by *Phaeoisariopsis personata* and rust caused by *Puccinia arachidis* can together reduce the yield over 50 % loss to groundnut production (Subrahmanyam et al. 1985; Waliyar 1991). The symptoms of LLS are circular and darker spots appear on the lower surface of the leaves and also forms in stems and pegs resulting in severe yield loss to the groundnut growers. LLS also has an adverse influence on seed quality as well as on quality of haulms. The regular incidence of the above disease under late sown conditions warrants the development of resistant cultivars in groundnut. Though there are many chemical control methods available, development of disease resistant varieties is the best way to control the disease to improve production quality and reduce the adverse effects of chemicals on our ecosystem.

Different sources of LLS have been reported as having digenic recessive basis (Tiwari et al. 1984). From LLS resistance studies, the resistance is complex and polygenic in nature and probably controlled by several recessive genes (Sharief et al. 1978; Nevill 1982; Green and Wynne 1986, 1987; Motagi 2001; Dwivedi et al. 2002). In addition, additive genetic variance seems to contribute predominantly to the resistance (Kornegay et al. 1980; Hamid et al. 1981; Anderson et al. 1986; Jogloy et al. 1987). Partial and polygenic nature of LLS makes the identification of resistant and susceptible lines cumbersome through conventional screening techniques (Leal-Bertioli et al. 2009). However, molecular markers associated with LLS would improve the process of identification of resistant genotypes. Identification of DNA markers associated with resistance to rust and LLS and their location on a genetic linkage map are pre requisites for the Marker Aided Selection (MAS) in groundnut (Mace et al. 2006). Low level of polymorphism in cultivated groundnut has been observed at the DNA level by using RFLPs (Halward et al. 1991), RAPDs (Dwivedi et al. 2001; Subramanian et al. 2000; Mondal et al. 2005), AFLPs (He and Prakash 2001; Krishna et al. 2004) and ISSRs (Raina et al. 2001). These results led to the generalization that *A. hypogaea* lack genetic variation and restricted the production of polymorphic profiles using DNA molecular marker techniques. By using advanced techniques such as SSRs substantial polymorphism at molecular level could be revealed (Singh et al. 1998).

Among the molecular markers, SSR has proved to be the most powerful tool for variety identification in groundnut of similar origin and has much potential in genetic and breeding studies (Wang et al. 2007). SSRs as DNA markers have many advantages than other markers (Morgante and Olivieri 1993) and have been used in the assessment of genetic variation in allotetraploid crops viz., *Triticum dicoccoides* (Li et al. 2003), *Brassica napus* (Tommasini et al. 2003) and *Gossypium hirsutum* (Guang and Xiong-Ming 2006). Recently, high level of polymorphism has been observed in cultivated groundnut by using SSR primers (Mace et al. 2006). Molecular markers and genetic linkage maps are pre- requisites for molecular breeding in any crop. Such tools would speed up the process of introgression of beneficial traits into preferred varieties. Considering the above points, the present study was undertaken to construct a genetic map and to identify molecular markers associated with late leaf spot disease resistance through bulked segregant analysis (BSA) for rapid identification of chromosomal regions responsible for LLS disease resistance in groundnut.

Materials and methods

Mapping population

The F_2 mapping population comprising of 120 $F_{2:3}$ lines developed from the cross TMV 2 × COG 0437 was used for this study. TMV 2 originated from mass selection from Gudiatham bunch (AH 32) and COG 0437 originated from the cross CO 2 × ICGV 94118. TMV 2 belongs to Spanish bunch and is a popular variety in southern parts of India and also has high susceptibility to LLS. The genotype COG 0437 belongs to Virginia bunch and highly resistant to LLS. The field experiments were carried out at Oilseeds farm, Department of Oilseeds, Tamil Nadu

Agricultural University, Coimbatore during the year 2007–2010. The F_2 population was used for genotyping and the F_3 population was used for phenotyping.

Phenotyping for late leaf spot disease resistance

Artificial screening was carried out on F_3 progenies and the parents. Each progeny was raised in a 4 m row spaced at 30 cm and intra row spacing was 10 cm. Augmented design I was used to raise the population during June–Sep, 2009. For effective screening, infector rows of highly susceptible cultivar for LLS, COGn 4 was raised after every fifth row. LLS symptoms usually appear between 50 days after planting so the infected leaf debris from the fields at harvest in the early season was collected in cloth bags, spore suspension prepared and sprayed on 50 days old plants. Haemocytometer was used to count spores to obtain desired inoculum concentration of approximately 10^6 spores/ml. When sufficient moisture is present, leaf spot infections occur quickly, and leaf wetness due to frequent irrigation along with high humidity are favourable for the infection. Mini sprinkler irrigation was given in the field during evening hours 4 p.m.–6 p.m. regularly to increase the disease pressure. The conidial suspension was prepared and inoculated over the infector rows for LLS development. Disease symptoms were noticed and for an accurate assessment, several plants of each entry were examined for disease severity. All leaves on the main stem were examined and care was taken to eliminate damage due to factors other than LLS. Nine point disease scale (Subrahmanyam et al. 1995) was used to screen the progenies for sources of resistance to LLS. Disease score of 1 for 0 % infection; 2 for 1–5 %; 3 for 6–10 %; 4 for 11–20 %, 5 for 21–30 %; 6 for 31–40 %; 7 for 41–60 %, 8 for 61–80 % and 9 for 81–100 % were recorded. Plants with a disease score of 1–3 and 7–9 were designated as being resistant and susceptible respectively according to Pande and Rao (2001).

Genotyping for late leaf spot disease resistance

SSR analysis

Genomic DNA of the two parents was extracted by CTAB method (Doyle and Doyle, 1987) and the quality was checked by using 0.8 % (w/v) agarose gel electrophoresis. Seventy-seven SSR primer pairs specific to cultivated groundnut were selected from the previous study (Selvaraj et al. 2009) and used. The polymerase chain reaction (PCR) mixtures (10 µl) contained 2 µl template DNA (10 ng), 1 µl of 10× Taq buffer + $MgCl_2$ (15 mM), 1 µl of dNTP (2 mM), 0.5 µl of primers 10 µM (Forward and Reverse), 0.1 µl of *Taq* polymerase (Genei 3 IU/µl) and 4.9 µl of sterile double distilled water. Amplification was performed in 0.2 ml (each tube) thin walled PCR plates (96 wells/plate) in a thermal cycler (Applied Biosystems). The samples were initially incubated at 94.0 °C for 3 min and then subjected to 20 times of the following cycle: 94.0 °C for 30 s (−0.5 °C reduction per cycle), 63.0 °C for 30 s and 72.0 °C for 1 min. This was followed by another 20 cycle of 94.0 °C for 15 s, 55.0 °C for 30 s and 72.0 °C for 1 min. Final Extension was 72.0 °C for 10 min. Amplified products were analyzed using 6 % non denaturing polyacrylamide gel at constant power 350 volts for about 4 h and silver stained (Benbouza et al. 2006).

Single marker analysis

The segregation pattern for SSR markers in the selected F_2 individuals were scored as 1–3 which corresponds to the banding pattern for TMV 2 (P_1), heterozygotes and COG 0437 (P_2) respectively. The molecular and phenotypic data obtained from 120 F_2 individuals were subjected to single marker analysis using one way regression analysis (Sax 1923) using SPSS software. All the marker data and the mean phenotypic traits value of F_3 progenies were used for calculating three marker classes (TMV 2, heterozygotes and COG 0437) and their variances. The significant threshold for association of marker to the trait was set at $P \leq 0.05$ for single marker analysis. The adjusted R^2 (phenotypic variance) value was used as per cent of variance explained by the marker on the particular trait of test and used as a measure of the magnitude of association.

Formation of DNA bulks for BSA

Two bulks of extreme phenotypes (resistance and susceptible) were used for the BSA analysis. Equal quantities of DNA were bulked from susceptible and resistant F_2 plants to give two DNA bulks. Nine plants from resistant and susceptible progenies were pooled for BSA analysis.

Results

Polymorphism of SSR markers

The parents of cross TMV 2 × COG O437 were surveyed with 77 SSR primers to identify polymorphic markers that would discriminate susceptible genotype TMV 2 and the resistant genotype COG 0437. Nine out of 77 primers (11.7 %) were found polymorphic between the parents TMV 2 and COG 0437. The sequence of polymorphic SSR primers is given in Table 1.

Identification of SSR markers linked to late leaf spot resistance through BSA

The F_1s along with their parents for the cross TMV 2 × COG 0437 were raised for studying F_2 performance during Dec–Mar, 2008–2009. A total of 120 $F_{2:3}$ progenies of the cross TMV 2 × COG 0437 were evaluated during Jun–Sep, 2009. Nine plants each of susceptible and resistant progenies for LLS were selected and pooled separately to form disease susceptible bulk and disease resistant bulk. Nine polymorphic SSR primers were used to survey the bulk. Among the polymorphic SSR markers, five SSR primers viz., PM 210, PM 375, PM 384, PGP05D05 and Lec-1 were able to distinguish the bulks. Among the five primers, only three primers viz., PM 375_{162}, pPGPseq5D5$_{220}$ and PM 384_{100} were confirmed with individual plants (Fig. 1a–c). Differences between the bulked extremes and the respective individuals were very clear in all the figures.

Single marker analysis

Disease scoring for LLS was taken from the 120 $F_{2:3}$ individuals to determine the association of marker to the respective phenotype. The markers were subjected to single factor regression analysis by using the marker and the respective phenotype. The regression values (*b*) of the SSR primers for LLS score are furnished in Table 2. The regression value b was significantly different from zero indicating the particular marker was linked to QTL. Among the nine SSR markers, primers viz., PM 375, PM 384, pPGPseq5D5, PM 137, PM 3, PMc 588 and Ah 4-26 were linked with LLS severity score. The phenotypic variation explained by these markers ranged from 32 to 59 %.

Validation of markers

The markers PM 375, pPGPseq5D5 and PM 384 were also used to survey five resistant and six susceptible genotypes of groundnut. The primer PM 384 was able to distinguish the resistant (100 bp allele) and susceptible genotypes (120 bp allele) except for one genotype in each category. The other two primers were unable to distinguish the resistant and susceptible genotypes clearly.

Discussion

Identification of resistant breeding lines for LLS disease is a major challenge to groundnut breeders. So the present study was undertaken to develop mapping population derived from resistant and susceptible parents followed by screening with SSR

Table 1 The sequence of polymorphic SSR primers

SSR primers	Forward sequence	Reverse sequence
PM 384	GGCGTGCCAATAGAGGTTTA	TGAAAACCAACAAGTTTAGTCTCTCT
pPGPseq5D5	AAAAGAAAGACCTTCCCCGA	GCAGGTAATCTGCCGTGATT
PM 137	AACCAATTCAACAAACCCAGT	GAAGATGGATGAAAACGATG
PM 3	GAAAGAAATTATACACTCCAATTATCG	CGGCATGACAGTCCTATGTT
PMc 588	CCATTTTGGACCCCTCAAAT	TGAGCAATAGTGACCTTGCATT
PM 343	AGAAACGAGGAGCTCGACAA	GCTCATTTTGATGGAATGAGAG
PM 377	ACGCTCACATGTTTGCTTTG	GCTCGATTTGATTTGGGTGA
PM 375	CGGCAACAGTTTTGATGGTT	GAAAAATATGCCGCCGTTG
Ah4-26	TGGAATCTATTGCTCATCGGCTCTG	CTCACCCATCATCATCGTCACATT

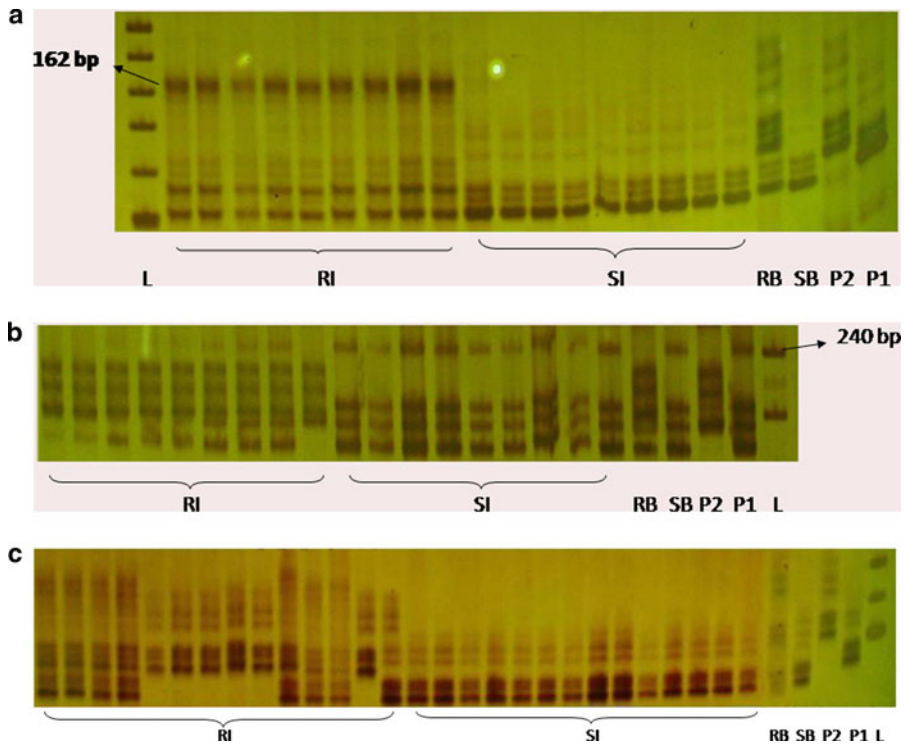

Fig. 1 BSA analysis for the LLS susceptible and resistant progenies using the SSR Marker **a** PM 375, **b** pPGPseq5D5 and **c** PM384. *P1* TMV 2, *P2* COG 0437, *SB* susceptible bulk, *RB* resistant bulk, *SI* susceptible individuals, *RI* resistant individuals, *L* 20 bp Ladder

Table 2 Single marker analysis for SSR primers linked to LLS in the cross TMV 2 × COG 0437

SSR markers	*b* value	R^2
PM 375	−2.90**	48.0
PM 384	−2.28**	59.0
pPGPseq5D5	−3.77**	59.0
PM 137	−1.92**	43.0
PM 3	−1.74**	43.0
PMc 588	−1.57**	32.0
PM 343	0.04	–
PM 377	0.02	–
Ah 4-26	−2.16	30.0

** Significant at 1 % level

markers towards their linkage with resistance. The advent of molecular markers has enabled to dissect quantitative traits into their single genetic components (Dudley 1993). It also assists in the selection and pyramiding of the beneficial QTL alleles through marker-assisted breeding (Ribaut et al. 2002). In the usual method to map QTLs, each plant of a large mapping population (normally in multiples of hundred) should be genotyped with numerous molecular markers and it is a time consuming process and labour intensive one. The difficulty of genotyping all the plants in a mapping population can be reduced through selective genotyping through BSA. BSA involves selection of extremely resistant and susceptible lines and pooling their DNA into two bulks viz., resistant and susceptible bulks (Michelmore et al. 1991). The efficiency of this strategy relies mainly on allele differences, the larger the difference the more efficient the pooling strategy. Possibility of using DNA pooling strategies for mapping QTL in F_2, back cross, recombinant inbred and double haploid populations have been discussed by Wang and Paterson (1994). Hence, BSA renders an easier way to locate markers linked to disease resistance traits.

In the present study, a total of 120 F_2s were developed from a cross between TMV 2, a LLS susceptible cultivar with a LLS resistant genotype COG 0437. The DNA of the parents was surveyed and 11.69 % polymorphism was observed. Tang et al. (2007) reported that the high polymorphic per cent for the markers PM 375 and PM 384 (the markers were used in the present study) viz., 100 and 87.5 % respectively for *Arachis hypogaea* var. *hypogeae* accessions. Low level of polymorphism showed by

SSRs in groundnut was reported by many authors (Varshney et al. 2007; Selvaraj et al. 2009). Low level of genetic polymorphism in cultivated groundnut was attributed to its origin from a single polyploidization event that occurred relatively recently on an evolutionary time scale (Young et al. 1996). Varshney et al. (2007) suggested that the low level of polymorphism was due to the marker techniques used. They also emphasized the importance of development of SSR markers from longer SSR enriched libraries, BAC-end sequences and SNP (single nucleotide polymorphism) markers.

Presence of low level polymorphism among cultivated groundnut parents promotes lacking of linkage map development from cultivated x cultivated groundnut crosses. However, for BSA, linkage map requirement is not needed and it can be used where insufficient polymorphism exists for a map. One more advantage is that the approach relies on the dramatic reduction in the number of marker assays when compared to building a genetic map for the purpose of identifying markers associated with a phenotype (Wenzl et al. 2007). In this study, we have used the BSA technique to identify SSR marker(s) associated with LLS resistance in cultivated groundnut. Among the nine polymorphic SSR markers, five SSR primers viz., PM 210, PM 375, PM 384, pPGPseq5D5 and Lec-1 were able to distinguish the bulks. The primer PM 375 was linked to seed length, pod length, and 100-seed weight in the recombinant inbred population of the cross Tamrun OL01 and BSS 56 (Selvaraj et al. 2009), indicating that the chromosomal regions identified by the above marker may contain linked genes or a gene with pleiotropic effects on multiple traits. Among the five primers, only three primers viz., PM 375_{162}, pPGPseq5D5$_{220}$ and PM 384_{100} were confirmed with individual plants. Among these markers, pPGPseq5D5 was reported for the rust resistance in ICGV 99005 × TMV2 mapping population (Varma et al. 2005). Hence QTLs for both LLS and rust may be located in the same chromosome region. Though the parent TMV 2 is the common parent in both studies, the resistant donor is different. Few reports are there for linking of LLS and rust resistant genes in cultivated groundnut. Modified BSA was used for rust resistance in F_2 mapping population derived from the cross VG 9514 and TAG 24 as reported by Mondal et al. (2007). From their study, the primer J7(5′-CCTCTCCGACA-3′) could produce a single coupling phase marker ($J7_{1350}$) and the repulsion phase marker ($J7_{1300}$) linked to rust resistance. Mace et al. (2006) identified the loci associated with LLS and rust. From their studies, 5 SSR loci have been identified with significant association to rust resistance genes (pPGPseq-17 F6, pPGPseq-2F05, pPGPseq-8E12, pPGPseq-13A10 and pPGPseq-16C6); 3 SSR loci have been identified with significant association to LLS resistance genes (pPGPseq-2B10, pPGPseq-2F05 and Ppgp13A7. Among these loci,

Table 3 Details of the genotypes for their LLS resistance and presence of PM 384 allele

Genotype	Disease reaction to LLS	LLS score	PM 384 allele		Pedigree
			100 bp	120 bp	
ALR 2	Resistant	2	Present	–	Selection ICGV 86011 from the cross (Dh.3-20XUsa-20) × NCAC 2232
ALR 3	Resistant	2	–	Present	(R 33-1 × ICG (FDRS) 68) × (NcAc 17090 × ALR 1)
GPBD 4	Resistant	2	Present	–	–
TMV 1	Resistant	2	Present	–	Virginia runner. Mass selection from West-African variety "Saloum" culture AH-25
VRIGN 6	Resistant	2	Present	–	ALR 2 × VG 9513
CO 2	Susceptible	9	–	Present	A bunch mutant from POL-1 by treatment with Ethyl-Methane Sulphonate at 20 %
CO 3	Susceptible	9	–	Present	VRI 3 (VG 55) × JL 24.
COGn 4	Susceptible	9	–	Present	TMV 10 × ICGS 82.
ICGV 00351	Susceptible	8	Present	–	ICGV 87290 × ICGV 87846
TMV 7	Susceptible	9	–	Present	Spanish bunch selection from "Tennesse white"
VRI 2	Susceptible	9	–	Present	JL 24 × CO 2

pPGPseq-2F05, being associated with both LLS and rust resistant genes.

In single marker analysis, the markers viz., PM 375, PM 384, pPGPseq5D5, PM 137, PM 3, PMc 588 and Ah 4-26 had shown higher R^2 values. According to Collard et al. (2005), QTLs accounting for more than 10 % of phenotypic variation (R^2) are major QTLs. Several researchers used this approach to establish marker phenotype association where the phenotypes possessed continuous distribution. The markers identified through BSA were also confirmed with single marker analysis. Hence these markers could be utilized in the marker assisted breeding programme.

Validation of markers for LLS resistance

The markers PM 375, pPGPseq5D5 and PM 384 were validated for their association with resistance to LLS over a set of resistant and susceptible genotypes (Table 3). The validated primer on various genotypes will be much more useful in marker assisted breeding because of applicability over wide range of background than on a single background. Among the three primers confirmed in BSA and SMA analysis, the primer PM 384 alone was able to distinguish the resistant (100 bp allele) and susceptible genotypes (120 bp allele) except for one genotype in each category. Hence the primer PM 384 is a potential marker for marker assisted breeding for LLS over a wide range of genotypes.

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