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SSR MARKERS ASSOCIATED WITH RESISTANCE TO RUST (*Puccinia arachidis* Speg.) IN GROUNDNUT (*Arachis hypogaea* L.)

T.S.N. VARMA[1], S.L. DWIVEDI[2*], S. PANDE[3], and M.V.C. GOWDA[4]

SUMMARY

Rust (*Puccinia arachidis* Speg.) is one of the important foliar diseases that causes substantial losses to groundnut (*Arachis hypogaea* L.) production and reduces seed quality. Resistance to rust is associated with many undesirable pod and seed characteristics. It is reported to be controlled either by few recessive genes or is inherited as a quantitative trait. DNA marker technology is used to minimize linkage drag and hasten the development of new resistant varieties. This experiment was initiated to study molecular variation among parents and identify SSR markers associated with rust resistance in groundnut. The parents and F_2 populations of the two crosses were evaluated for resistance to rust under greenhouse conditions. None of the SSR primer pairs showed intra-accession variability among parents. Seven and eight primer pairs detected polymorphic variation between ICGV 99003 and TMV 2, and ICGV 99005 and TMV 2, respectively. They either belonged to 'ATT' or 'AG' SSR repeat families. Wilcoxon test was used to test the significance of association between marker and rust disease score. Rust resistance was associated with two SSR alleles ($pPGPseq3A1_{271}$ and $pPGPseq3A1_{390}$) in ICGV 99003xTMV 2 and seven SSR alleles ($pPGPseq5D5_{270}$, $pPGPseq5D5_{295}$, $pPGPseq5D5_{325}$, $pPGPseq16F1_{315}$, $pPGPseq16F1_{424}$, $pPGPseq17F6_{128}$, and $pPGPseq13A7_{292}$) in ICGV 99005xTMV 2. SSR markers associated with rust resistance should facilitate the rapid identification and transfer of chromosomal region(s) into elite breeding lines by using marker-assisted backcross breeding in groundnut.

Key words: DNA polymorphism, peanut, rust, microsatellite markers

Cultivated groundnut (*Arachis hypogaea* L.), also known as peanut, is grown on 26.4 million hectares between latitudes 40° N and 40° S, with a global production of 36.0 million tons and average pod yield of 1.37 t ha^{-1} (FAO, 2004). The vast majority of groundnut is produced under rainfed conditions in Asia and Africa. Several biotic and abiotic stresses contribute to variation in groundnut productivity. For example, rust (*Puccinia arachidis* Speg.) and late leaf spot (LLS; *Phaeoisariopsis personata* Ber. and M A Curtis), the two foliar diseases which occur worldwide, can together cause over 50% loss to groundnut production (Subrahmanyam *et al.*, 1985; Waliyar, 1991), and affect seed

[1] Scientific Officer, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Hyderabad, PO 502 324, India;
[2] Consultant, GT 1, ICRISAT, Patancheru, Hyderabad, PO 502 324, India;
[3] Principal Scientist, ICRISAT, Patancheru, Hyderabad, PO 502 324, India;
[4] Professor, Department of Genetics and Plant Breeding, University of Agricultural Sciences, Dharwad, Karnataka, 580005, India.
* Corresponding author: s.dwivedi@yahoo.com

quality (Dwivedi *et al.*, 1993a). The adoption of resistant cultivars by resource poor farmers is the best option to minimize losses and maintain good product quality. Several sources of resistance to rust and LLS have been identified in groundnut (Singh *et al.*, 1997). However, resistance is associated with many undesirable pod and seed characteristics that breeders have found difficult to eliminate due to linkage drag (Young and Tanksley, 1989). For example, crop duration and pod/seed characteristics of the many newly developed rust resistant breeding lines are still not comparable to the farmers' preferred cultivars.

Resistance to rust in *A. hypogaea* is conferred either by a few recessive genes (Knauft, 1987; Kalekar *et al.*, 1984; Paramasivam *et al.*, 1990) or predominantly controlled by additive, dominance, additive x additive, and additive x dominance genetic effects (Reddy *et al.*, 1987; Varman *et al.*, 1991). In addition, partial dominance has been reported in some diploid species (Singh *et al.*, 1984).

Extensive variation for morphological and physiological traits has been observed in both wild *Arachis* species and cultivated groundnut. Abundant polymorphism in wild *Arachis* species has been observed whereas little variation has been reported in cultivated groundnut (Kochert *et al.*, 1991; Halward *et al.*, 1991; Paik-Ro *et al.*, 1992; Stalker *et al.*, 1994; He and Prakash, 1997; Hopkins *et al.*, 1999). The studies at ICRISAT revealed, in contrast, sufficient random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and simple sequence repeats (SSR) variability among cultivated germplasm that may be exploited for genetic enhancement in groundnut (Subramanian *et al.*, 2000; Dwivedi *et al.*, 2001, 2002a,b; Dwivedi and Gurtu, 2002; Dwivedi *et al.*, 2003c). Both RAPD and restriction fragment length polymorphism (RFLP) markers have been used to monitor introgression of wild *Arachis* chromosome segments into cultivated groundnut (Garcia *et al.*, 1995), and few RAPD (RKN 229, RKN 410, and RKN 440) and RFLP (R2430E, R2545E, and S1137E) markers linked with root-knot nematode resistance have been reported (Burow *et al.*, 1996; Choi *et al.*, 1999).

The present study was initiated to study intra- and inter-accession polymorphic variation among rust resistant and susceptible parents and to identify SSR markers associated with resistance to rust in two crosses in groundnut.

MATERIALS AND METHODS

Plantm materials and disease screening

ICGV 99003, ICGV 99005, and TMV 2 were selected for the study. ICGV 99003 and ICGV 99005 are phenotypically stable tetraploid rust resistant interspecific derivatives (Dwivedi *et al.*, 2002b). The former originates from the cross *A. hypogaea* x (*A. duranensis* x *A. stenosperma)* and the latter from TMV 2 x (*A. hypogaea* x (*A. batizocoii* x *A. duranensis*). TMV 2 belongs to subspecies *fastigiata* var *vulgaris*. It is highly susceptible to rust but is a widely grown cultivar in southern India. Phenotypically, both ICGV 99003 and ICGV 99005 looked like subsp. *hypogaea* var. *hypogaea*. They were crossed with TMV 2 to produce F_1 and F_2 generations. The F_2 population of 107 individuals from the cross ICGV 99003xTMV 2 and 101 individuals from ICGV 99005xTMV 2 and about 10 plants of the parents were grown under greenhouse conditions in 15-cm diameter plastic pots containing autoclaved alfisol and farmyard manure (v/v 4:1 ratio). High disease pressure under greenhouse conditions was created (Dwivedi *et al.*, 2002b). The rust inocula were produced and maintained on incubated and inoculated detached leaves of the susceptible cultivar, TMV 2 in a Percival Plant Growth Chamber using a temperature of 23°C and 12 h

photoperiod. The 35-day old plants were inoculated uniformly in the evening with rust inoculum containing 20,000 uredospores ml^{-1}, with an automizer, and immediately transferred into dew chambers at 23°C to ensure wetness of the leaf surface during the night. The pots were removed from the dew chambers on the following morning and returned to the greenhouse to maintain a dry period during the day. This alternate wet (16 h) and dry (8 h) period treatments were repeated for 10 days. The pots were then kept permanently in the greenhouse till the completion of the experiment. Observation on rust disease was recorded on individual plants at 45 days after inoculation using a 1 to 9 scale (Subrahmanyam *et al.*, 1995).

Plant DNA isolation and formation of DNA pools

Young leaves from two-week-old greenhouse grown plants of the parents and F_2s were bulked-harvested from individual plants and immediately placed in liquid nitrogen for DNA extraction. DNA was extracted by CTAB method (Saghai-Maroof *et al.*, 1984). The quantity and quality of DNA were assessed by a spectrophotometer and electrophoresis in 0.8% agarose gel with known concentrations of lambda DNA. DNA pools were formed following Michelmore *et al.* (1991) to identify markers associated with resistance to rust. The F_2 segregants with extreme phenotypes, either highly resistant or susceptible to rust, were selected to form the resistant and susceptible bulks. The five resistant and four susceptible plants from TMV 2 x ICGV 9905 and four resistant and six susceptible plants from TMV 2 x ICGV 99003 were selected to constitute resistant and susceptible F_2 bulks. Similarly, five plants from each parent were bulked to form resistant parent and susceptible parent bulks. DNA (0.5 ng l^{-1}) from individually selected resistant and susceptible plants were pooled to form the resistant parent, susceptible parent, resistant F_2, and susceptible F_2 bulks.

SSR analysis

Forty-six SSR primer pairs specific to cultivated groundnut were selected for the study (Ferguson *et al.*, 2004). The polymerase chain reaction (PCR) mixtures (20l) contained 1.0 l (5 ng) of genomic DNA, 2.0 l of 10X PCR buffer, 4.0 l of 10 mM $MgCl_2$, 1.5 l of 2mM dNTPs, 2.5 l of 4p moles SSR primer (both forward and reverse), 8.2 l of double distilled water, and 0.8 l of 1 unit *Taq* DNA polymerase (Gibco BRL, Life Technologies, USA). Amplification was performed in 0.2 ml thin-walled tubes placed in a Thermal Cycler (DYAD Engine Peltier Thermal cycler, MJO2451, USA). The samples were initially incubated at 94°C for 2 min, and then subjected to 35 repeats of the following cycle: 94°C for 45 sec, 60°C for 1 min, 72°C for 1.5 min. Five l of orange dye [1 g of Methyl orange dye (4-dimethylaminoazobenzene-4-sulfonic acid, sodium salt, Sigma-Aldrich, USA) powder was added into 100 ml solution containing 10 ml of 0.5 M EDTA (pH 8.0) + 1 ml of 5 M Nacl + 50 ml glycerol + 39 ml distilled water] was added into PCR products prior to electrophoresis. Amplified products were analyzed using 6% non-denaturing polyacrylamide gel (15 ml of acrylamide/bisacrylamide 29:1 (W/W) + 7.5 ml of 10 X TBE + 53 ml of distilled water + 90 l of TEMED + 350 l of Ammoniumpersulphate). Electrophoresis was performed at constant power 500 volts for about 4 h and silver stained (Bassam *et al.*, 1991).

Statistical analysis

Single-marker analysis was used to detect association of a marker with rust disease score. Wilcoxon test was used to test the significance of association between markers and rust disease score (Siegel, 1956). This test detects whether the phenotypic distributions of the two genotypic classes of a marker differ significantly with respect to the marker.

RESULTS

Intra- and inter-accession polymorphism among parents

None of the 46 SSR primer pairs showed intra-accession variability among ICGV 99003, ICGV 99005, and TMV 2 (data not presented). Primer pairs pPGPseq3A1, pPGPseq3A8, pPGPseq1B9, pPGPseq2D12B, pPGPseq5D5, pPGPseq2G3, and pPGPseq2G4 in ICGV 99003 and TMV 2 and pPGPseq3A8, pPGPseq13A7, pPGPseq1B9, pPGPseq5D5, pPGPseq16F1, pPGPseq17F6, pPGPseq2G3, and pPGPseq2G4 in ICGV 99005 and TMV 2 (Table 1) showed inter-accession polymorphic variation (Table 2). Eighty percent of the polymorphic markers were trinucleotide repeats whereas only 20% were dinucleotide repeats. The trinucleotide primer pairs belong to the $(ATT)_n$ and the dinucleotide to the $(AG)_n$ SSR repeat motifs. The most common SSR repeat motif reported in plants are $(AT)_n$ followed by $(A)_n$, $(CT)_n$, $(AAT)_n$, and $(AAC)_n$ (Wang *et al.*, 1994).

F_1 heterozygosity as detected by SSRs

Twenty-five plants of the cross ICGV 99003 x TMV 2 and 15 of ICGV 99005 x TMV 2 were assessed for F_1 heterozygosity using 10 polymorphic primer pairs. Twenty-two F_1 plants of the cross ICGV 99003 x TMV 2 and 14 F_1 plants of the cross ICGV 99005 x TMV 2 had alleles from both the mapping parents and, therefore, were identified as true F_1 hybrids. The remaining plants had alleles only from the female parent and were, therefore, identified as selfed and discarded before harvest. The seeds obtained from genuine F_1 hybrid plants were used to raise the F_2 population.

SSR markers associated with resistance to rust

Bulk DNA samples of the resistant and susceptible parents and F_2 bulks were screened for polymorphic loci using seven primer pairs (pPGPseq3A1, pPGPseq3A8, pPGPseq1B9, pPGPseq2D12B, pPGPseq5D5, pPGPseq2G3, and pPGPseq2G4) in ICGV 99003 x TMV 2 and eight primer pairs (pPGPseq3A8, pPGPseq13A7, pPGPseq1B9, pPGPseq5D5, pPGPseq16F1, pPGPseq17F6, pPGPseq2G3, and pPGPseq2G4) in ICGV 99005 x TMV 2. Amplified fragments that were present in the resistant bulk and the resistant parent but absent in the susceptible bulk and susceptible parent were revealed by the primer pair pPGPseq3A1 in ICGV 99003 x TMV 2 and by the primer pairs pPGPseq13A7, pPGPseq5D5, pPGPseq16F1, and pPGPseq17F6 in ICGV 99005 x TMV 2 (Figure 1). The parents and F_2 plants involved in resistant and susceptible bulks were also individually analyzed by using the same primer pairs that were used in the bulked segregant analysis (Figure 2 and Table 4 for the cross ICGV 99005 x TMV2 and Figure 3 and Table 3 for the cross ICGV 99003 x TMV2). Wilcoxon test detected significant differences between these markers and disease score in ICGV 99003 x TMV 2 (P=0.002) and ICGV 99005 x TMV 2 (P=0.003). Both bulked and individual plant analysis revealed that the SSR alleles $pPGPseq3A1_{271}$ and $pPGPseq3A1_{390}$ in ICGV 99003 x TMV 2 and $pPGPseq13A7_{292}$, $pPGPseq5D5_{270}$, $pPGPseq5D5_{295}$, $pPGPseq5D5_{325}$, $pPGPseq16F1_{315}$, $pPGPseq16F1_{424}$, and $pPGPseq17F6_{128}$ in ICGV 99005 x TMV 2 were associated with resistance to rust. Susceptibility to rust was associated with SSR alleles $pPGPseq3A1_{289}$ and $pPGPseq3A1_{409}$

Table 1. Sequence information of the selected polymorphic SSR primer pairs used.

Primer	Forward primer	Reverse primer	Annealing temperature (°C)	
			Forward	Reverse
pPGPseq3A1	ATCATTGTGCTGAGGGAAGG	CACCATTTTTCTTTTTCACCG	60	59
pPGPseq3A8	ATACGTGACTTGGGCCAGAC	AGTGAAAAATACACCCAACGAA	60	58
pPGPseq13A7	AATCCGACGCAATGATAAAAA	TCCCCTTATTGTTCCAGCAG	59	60
pPGPseq1B9	CGTTCTTTGCCGTTGATTCT	AGCACGCTCGTTCTCTCATT	60	60
pPGPseq2D12B	AAGCTGAACGAACTCAAGGC	TGCAATGGGTACAATGCTAGA	59	59
pPGPseq5D5	AAAAGAAAGACCTTCCCCGA	GCAGGTAATCTGCCGTGATT	60	60
pPGPseq16F1	TGGAGGGAAAAACATTTTGG	CCTGGAGGGGTGAGAGGT	60	60
pPGPseq17F6	CGTCGGATTTATCTGCCAGT	AGTAGGGGCAAGGGTTGATG	60	61
pPGPseq2G3	ATTCACAAGGGGACAGTTGC	ATTCAAGCCTGGGAAACAGA	60	60
pPGPseq2G4	TTCTTGGTTCCTTTGGCTTC	TGCTCAAGTGTCCTTATTGGTG	59	60

Table 2. SSR repeat motif and allele size (bp) in ICGV 99003, ICGV 99005, and TMV 2 in groundnut.

	ICGV 99003 x TMV 2				ICGV 99005 x TMV 2		
	Allele size (bp)				Allele size (bp)		
Primer	ICGV 99003	TMV 2	SSR repeat motif	Primer	ICGV 99005	TMV 2	SSR repeat motif
pPGPseq3A1	271	289	$(ATT)_6$				
	390	409	$(ATT)_7$				
pPGPseq3A8	186	171	$(ATT)_5$	pPGPseq3A8	183	170	$(ATT)_5$
pPGPseq1B9	275	287	$(AG)_6$	pPGPseq1B9	276	285	$(AG)_5$
pPGPseq2D12B	260	240	$(ATT)_7$				
pPGPseq5D5	287	275	$(AG)_6$	pPGPseq5D5	270	260	$(AG)_5$
	314	300	$(AG)_7$		295	288	$(AG)_4$
	343	329	$(AG)_7$		325	312	$(AG)_6$
pPGPseq2G3	272	278	$(ATT)_2$	pPGPseq2G3	272	278	$(ATT)_2$
pPGPseq2G4	267	254	$(ATT)_5$	pPGPseq2G4	273	257	$(ATT)_6$
				pPGPseq16F1	315	300	$(ATT)_5$
				pPGPseq17F6	128	135	$(ATT)_3$
				pPGPseq13A7	292	304	$(ATT)_4$

Table 3. SSR allele of the primer pair pPGPseq3A1 associated either with resistance or susceptibility to rust in the F_2 population of the cross ICGV 99003 x TMV 2 in groundnut.

Mapping parent and F_2 plant	Sample identity	Rust disease severity score	pPGPseq3A1 allele (bp) size
ICGV 99003	1400	3	271, 390
TMV 2	1401	8	289, 409
F_2	1445	3	271, 390
F_2	1454	3	271, 390
F_2	1419	3	271, 390
F_2	1462	3	271, 390
F_2	1398	8	289, 409
F_2	1477	8	289, 409
F_2	1529	8	289, 409
F_2	1602	8	289, 409
F_2	1611	8	289, 409
F_2	1517	8	289, 409

Rust disease score was recorded on 1 to 9 scale where 1 = no disease, 2 = 5% foliage damaged, 3 = 6-10% foliage damaged, 4 = 11-20% foliage damaged, 5 = 21-30% foliage damaged, 6 = 31-40% foliage damaged, 7 = 41-60% foliage damaged, 8 = 61-80% foliage damaged, and 9 = 81-100% foliage damaged (Subrahmanyam *et al.* 1995).

in ICVB 99003 x TMV 2 and pPGPseq13A7$_{304}$, pPGPseq5D5$_{260}$, pPGPseq5D5$_{288}$, pPGPseq5D5$_{312}$, pPGPseq16F1$_{300}$, pPGPseq16F1$_{412}$, and pPGPseq17F6$_{135}$ in ICGV 99005 x TMV 2.

DISCUSSION

Resistance to rust is quantitative in nature in crosses involving wild *Arachis* species (Singh *et al.*, 1984). None of the five common SSRs polymorphic between resistant and susceptible parents were found associated with rust resistance in the two crosses studied, *i.e.* marker(s) associated with rust resistance in ICGV 99005 x TMV 2 were different from those that detected such association in ICGV 99003 x TMV 2. It is possible that ICGV 99003 and ICGV 99005 may have different resistant gene(s). While both ICGV 99003 and ICGV 99005 have one common wild species (*A. duranensis*) in their pedigree's, the other wild species differ: *A. stenosperma* in ICGV 99003 and *A. batizocoii* in ICGV 99005. It is possible that these two species are contributing different genes conferring resistance to rust that need to be further investigated by crossing the two resistant parents to demonstrate the independent segregation of these markers.

Groundnut is the most important oilseeds crop in India. There are several biotic and abiotic stresses that adversely affect groundnut production at the farm level. Among them, rust is one of the major foliar diseases that not only reduce pod yield but also adversely influence fodder and seed quality (Dwivedi *et al.*, 2003a). Several sources of resistance to rust have been reported in cultivated and wild *Arachis* germplasm.

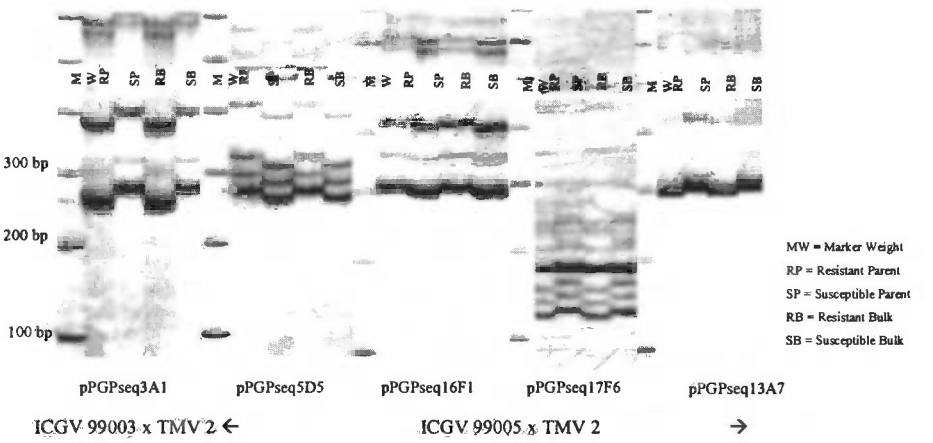

Figure 1. Bulked segregant analysis of the resistant and susceptible F_2 bulks along with the parents in two crosses in groundnut.

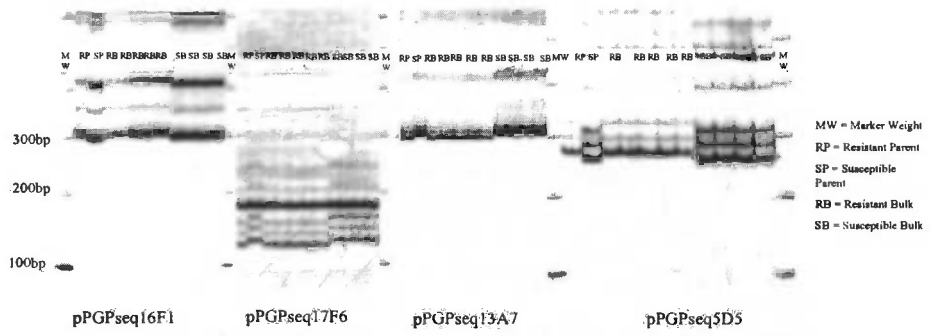

Figure 2. SSR markers pPGPseq16F1, pPGPseq17F6, pPGPseq13A7, and pPGPseq5D5 associated with resistance to rust in the F_2 population of the cross ICGV 99005 x TMV 2 in groundnut.

Table 4. SSR allele of the primer pairs pPGPseq5D5, pPGPseq16F1, pPGPseq17F6, and pPGPseq13A7 associated either with resistance or susceptibility to rust in the F_2 population of the cross ICGV 99005 x TMV 2 in groundnut.

Mapping parent and F_2 plant	**Sample identity**	**Rust disease score[+]**	**SSR allele size (bp)**			
			pPGPseq5D5	pPGPseq16F1	pPGPseq17F6	pPGPseq13A7
ICGV 99005	1001	3	270, 295, 325	315, 424	128	292
TMV 2	1002	8	260, 288, 312	300, 412	135	304
F_2	1288	2	270, 295, 325	315, 424	128	292
F_2	1359	2	270, 295, 325	315, 424	128	292
F_2	1157	3	270, 295, 325	315, 424	128	292
F_2	1296	3	270, 295, 325	315, 424	128	292
F_2	1357	3	270, 295, 325	315, 424	128	292
F_2	1095	8	260, 288, 312	300, 412	135	304
F_2	1112	8	260, 288, 312	300, 412	135	304
F_2	1121	8	260, 288, 312	300, 412	135	304
F_2	1127	7	260, 288, 312	300, 412	135	304
F_2	1134	8	260, 288, 312	300, 412	135	304

Rust disease score was recorded on a 1 to 9 scale where 1 = no disease, 2 = 5% foliage damaged, 3 = 6-10% foliage damaged, 4 = 11-20% foliage damaged, 5 = 21-30% foliage damaged, 6 = 31-40% foliage damaged, 7 = 41-60% foliage damaged, 8 = 61-80% foliage damaged, and 9 = 81-100% foliage damaged (Subrahmanyam *et al.*, 1995).

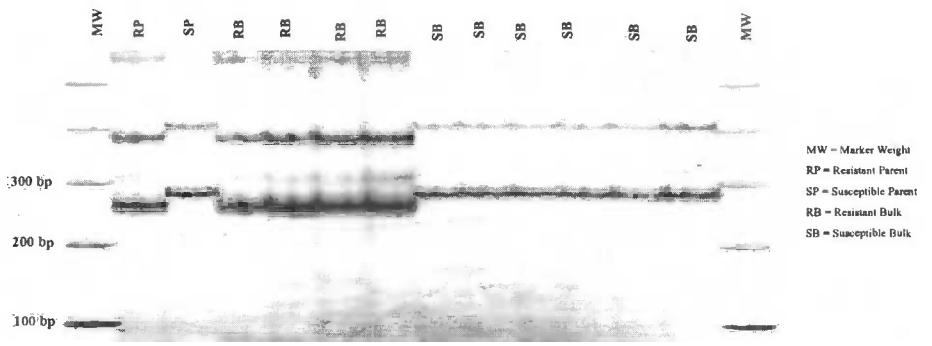

Figure 3. SSR marker pPGPseq3A1 associated with resistance to rust in the F_2 population of the cross ICGV 99003 x TMV2 in groundnut.

However, they are not suitable for commercial cultivation mainly because of the undesirable pod/seed characteristics (Dwivedi *et al.*, 2003b). The discovery of DNA markers has revolutionized genetic analysis and opened up new avenues in crop improvement that can achieve results in a much shorter period than may be expected through conventional breeding techniques. The utility of RFLP- and RAPD-based assays has been demonstrated for monitoring gene introgression (Garcia *et al.*, 1995) and for identifying markers linked with resistance to nematodes in groundnut (Burow *et al.*, 1996; Choi *et al.*, 1999). Using AFLP assay, Herselman *et al.* (2004) reported AFLP markers linked with resistance to the aphid-vector of the groundnut rosette disease that causes havoc to groundnut production in disease epidemic years in Africa (Subrahmanyam *et al.*, 1977, 1991). This is the first report that describes SSR markers' association with rust resistance in cultivated groundnut. This should facilitate the rapid identification and transfer of chromosomal region(s) associated with resistance to rust into elite breeding lines by using marker-assisted backcross breeding (MABB). It is expected that MABB should minimize the linkage drag, reduce the need for field-testing of breeding populations for disease reaction, and shorten the time required for the development of new cultivars.

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