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Phenotyping and Molecular Marker Analysis for stem-rot Disease Resistance Using F_2 Mapping Population in Groundnut

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Abstract: Sclerotium rolfsii *is one of the dreaded pathogen which causes stem-rot disease by infecting groundnut from seedling to maturity stages and causes potential yield losses. Molecular markers, linked with stem-rot disease resistance gene/QTLs can facilitate the identification of resistant genotypes. In the present study, a stem-rot susceptible genotype (TG-37A) and a stem-rot resistant genotype (NRCG CS85) were crossed and their F_2 population was used for SSR marker analysis. For the phenotypic data, $F_{2:3}$ progenies were screened for stem-rot disease incidence. Parental polymorphism survey was done using 1266 SSR primer pairs so as to identify the polymorphic markers. Among these SSRs, 52 were found to be polymorphic between the parental combination (TG-37A x NRCG CS85).These markers were further utilized for bulked segregant analysis (BSA). Among the polymorphic SSRs, three primers DGR294, DGR470 and DGR510 were able to distinguish both resistant and susceptible bulks and individual plants constituting the bulks. Further genotyping of whole population using identified markers is under way, which may confirm the linkage of putatively linked markers to with the stem-rot resistance.*

Keywords: Arachishypogaea, Sclerotiumrolfsii, *SSR markers, Bulkedsegregant analysis*

INTRODUCTION

Cultivated groundnut or peanut (*A. hypogaea L.*) is anallotetraploid (2n = 4 x = 40) crop grown extensively in nearly 108 countries. Stem-rot caused by *Sclerotium rolfsii* is one of the most devastating fungal diseases, which imposes huge yield-losses. Although, the search for peanut cultivars resistant to *S. rolfsii* originated way back in 1918 [5], but till date a high degree of resistance has not yet been found. Moreover, no marker(s) has been reported to be associated/ linked with any soil-borne pathogens including stem-rot disease resistance in groundnut [7].

Bulked segregant analysis (BSA) is one of the tools which is used to identify the molecular marker(s) linked with any trait of interest in an organism. This measures the variation present in pools of segregants that have been sorted according to phenotype and uses the correlation between these measurements and the pool phenotype to assign a likely map location. This is an improvement over methods that require individual genotyping, and used increasingly for mapping complex traits including those whose genetic control is unknown [4].

Molecular markers and genetic linkage maps are pre-requisites for molecular breeding in any crop including groundnut. Such tools would speed up the process of introgression of beneficial traits into any preferred genotype. Considering the above advantages, the present study was undertaken to rapidly identify the molecular markers linked with the stem-rot disease resistance gene/ QTLs in groundnut through bulked segregant analysis (BSA).

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MATERIALS AND METHODS

Crossing and development of F_2 mapping population

On the basis of our previous screening results, TG-37A (stem-rot susceptible) and NRCG CS85 (stem-rot resistant) were selected as parents [8,9] and were crossed during *kharif*-2013 for the development of F_2 mapping population. Putative F_1's so obtained were first confirmed using polymorphic primer pairs. Further, the confirmed F_1 hybrids were forwarded for the generation of true $F_{2'}$s.

PARENTAL POLYMORPHISM SURVEY

DNA was extracted from fresh leaf tissue of both the parents (TG-37A and NRCG CS85) by CTAB method [2] and parental polymorphism survey was conducted using a total 1266 SSR primer-pairs.

Phenotyping and genotyping of F_2 (TG-37A x NRCG CS85) population

For the stem-rot phenotyping, F_2 seeds were planted in plastic pots (12cm width x11cm long) under the temperature (28±2p C) and humidity (above 70%) controlled P-II glass house conditions. The data for various physiological parameters such as stem-thickness, plant-height, number of nodes, number of branches, hairiness, growth pattern, leaf-color, leaf-shape, leaf-size, inter-nodal distance, stem-pigmentation, flower-size, flower-color, and branching pattern were recorded. Seventy-day old plants were infected with *S. rolfsii* which was multiplied on autoclaved sorghum grains for 15 days @ 2g per pot.

DNA was extracted from the fresh leaf tissues of all the F_2 plants by CTAB method and genotyping is being carried out using the polymorphic SSR markers.

BULKED SEGREGANT ANALYSIS (BSA)

BSA was performed using polymorphic SSRs. For this, two DNA pools were made of 10 F_2 plants (TG-37A x NRCG CS85) each, exhibiting extreme phenotypes for stem-rot disease (i.e. resistant and suceptible). The bulk was made by mixing equal quantities of DNA from 10 selected individual plants. DNA samples of the two parents and the two bulks in each population were assayed using the selected polymorphic markers. A marker was regarded as putatively linked at this stage if the polymorphic bands recorded in the resistant bulks (RB) and susceptible bulks (SB) were similar to their respective resistant and susceptible parents.

RESULTS AND DISCUSSION

Development of F_2 mapping population

The cross between TG-37A x NRCG CS85 have resulted in 121 putative F_1s, which were confirmed using polymorphic primers. Further, 94 confirmed $F_{1's}$ were forwarded for the generation of $F_{2'}$s and a total of 850 $F_{2'}$s plants were obtained. Since, screening for stem-rot disease is a destructive method, out of 850 F_2 seeds 400 seeds were shown in the field for RILs development, while remaining $F_{2'}$s were used for mapping.

Screening of F_2 population (TG-37A x NRCG CS85) under controlled conditions for stem-rot incidence

For phenotyping, 450 F_2plants were sown in plastic pots under controlled conditions along with their parents during *kharif*-2014. These plants were then artificially infected with *S. rolfsii* after 70 days of sowing. Disease development and wilting (%) were recorded at three days interval after infection. Significant variability was observed in the population for diseases development as well as various other morphological parameters (Fig. 1 and 2). Based on the disease score (as wilting percentage

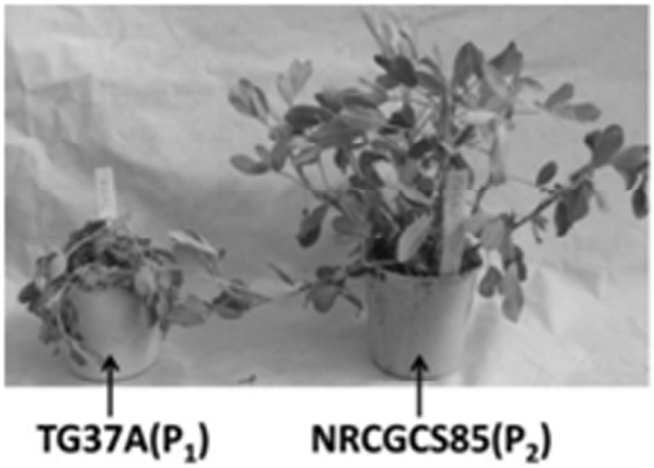

Figure 1: Disease screening of parents of TG37A and NRCG CS85

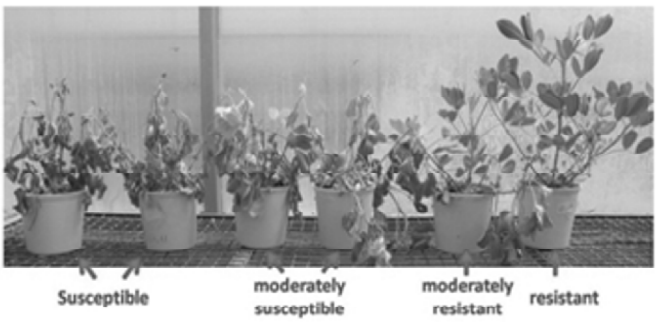

Figure 2: Disease screening of F_2 population (TG37A x NRCG CS85)

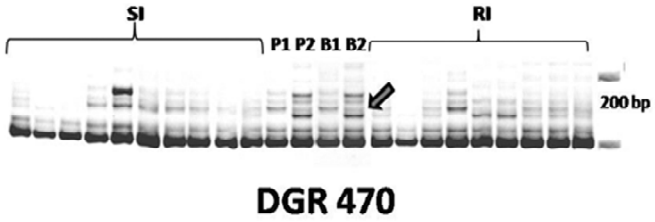

Figure 3: Bulked segregate analysis for F2's derived from the cross TG-37A x NRCG CS85. Where, P1: TG37A, P2: NRCG CS85, B1: Susceptible bulk, B2: Resistant bulk, SI: Susceptible Individuals and RI: Resistant Individuals which constituted the bulk.

on the 12th day of inoculation), all the F_2 plants were grouped in four different categories *viz*. resistant, moderately resistant, moderately susceptible and susceptible, which found to contain 12, 10, 23 and 394 plants respectively.

Screening of SSR markers for parental polymorphism

Parental polymorphism survey was first done using 550 SSR primers of series like Ah, RM, RN, PM, PMC, S,Seq etc. which were known to have certain level of polymorphism in different groundnut genotypes. However, only 16 (~3%) primers were found polymorphic between parental combination (TG-37A and NRCG CS85). Further, novel EST derived SSR markers developed at our lab [1] were also synthesized and used for the parental polymorphism survey so as to get sufficient number of polymeric markers. A total of 36 new polymorphic SSR markers were identified by screening 716 EST derived SSR markers (5% polymorphism). Thus, as of now, a total of 52 polymorphic primer pairs are available with us for further genotyping of mapping population. Low level of polymorphism is one of the problems faced while working with cultivated groundnuts [3]. For the preparation of saturated linkage map number of polymorphic markers must be more; therefore, more SSRs are being screened.

BULKED SEGREGANT ANALYSIS (BSA)

BSA relies on the informative individuals being grouped so that a particular genomic region is studied against a randomized genetic background of unlinked loci [6]. Presence of low level polymorphism among the selected groundnut genotypes and availability of poorly dense linkage map in groundnut has made the job of linkage mapping more difficult. However, for BSA, linkage maps are not required and it can be effectively 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 [10]. In present study, BSA was performed using polymorphic SSRs (total 52 SSR primer pairs)so as to identify the putative SSR(s) associated with stem-rot disease resistance. Of all polymorphic markers, only three SSRs *viz*. DGR294, DGR470 and DGR510 distinguished the resistant and susceptible bulks (Fig.3). Genotyping of whole population with these three primers is in progress so as to identify the marker(s) linked with stem-rot disease resistance QTLs in groundnut.

GENOTYPING OF F_2 MAPPING POPULATION

Complete genotyping of 443 F_2s (TG-37A x NRCG CS85) have been done using 16 polymorphic SSR primer pairs; while genotyping using remaining polymorphic SSRs is continuing (Fig. 4). Table 1 shows the genotyping data of 16 polymorphic SSRs along with the calculated Chi-square value for each primer for the expected ratio 1:2:1 (co-dominant markers). Chi-square test has been used to reveal the deviation from the expected Mendelian segregation ratio. Expected chi-square ratio was observed for all primers, including IDT 837 and IDT 838 which were of dominant in nature (3:1). Chi-square value of SSR markers smaller than the P value i.e. 5.99 for co-dominant markers (df- 2) and 3.84 for dominant markers (df- 1) at 0.05 probability level indicates that <5% segregation distortion. The

Table 1
Genotyping of F_2 population (TG-37A x NRCG CS85)

S. No.	Primer	Observed genotype in the F_2 population			Total	Expected genotype in the F_2 population			Chi-square value	P value at 0.05 probability level	Position on linkage group
		AB	AA	BB		AB	AA	BB			
1	IDT 734	218	106	116	440	220.0	110.00	110.00	0.49	5.99	_
2	IDT 832	234	111	98	443	221.5	110.75	110.75	2.17	5.99	1
3	IDT 643	217	105	114	436	218.0	109.00	109.00	0.38	5.99	2
4	IDT 736	193	124	117	434	217.0	108.50	108.50	5.53	5.99	1
5	IDT 402	208	101	128	437	218.5	109.25	109.25	4.34	5.99	_
6	IDT 217	204	98	113	415	207.5	103.75	103.75	1.20	5.99	3
7	IDT 149	220	100	110	430	215.0	107.50	107.50	0.69	5.99	_
8	IDT 637	201	106	128	435	217.5	108.75	108.75	4.72	5.99	_
9	IDT 651	205	113	116	434	217.0	108.50	108.50	1.36	5.99	_
10	IDT 837	-	108	332	440	-	110.00	330.00	0.04	3.84	4
11	IDT 838	-	100	329	429	-	107.25	321.75	0.65	3.84	4
12	IDT 45	201	106	132	439	219.5	109.75	109.75	6.19	5.99	2
13	IDT 834	215	91	130	436	218	109	109	7.06	5.99	3
14	IDT 213	192	92	124	408	204	102	102	6.431	5.99	3
15	IDT 631	175	117	133	425	212.5	106.25	106.25	14.440	5.99	_
16	IDT 406	200	102	121	423	211.5	105.75	105.75	2.9574	5.99	_

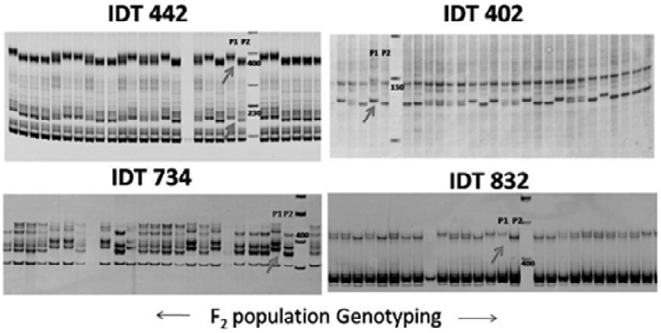

Figure 4: Genotyping result of F_2 population (TG-37A x NRCG CS85)

markers showing more than 5% segregation distortion is to be confirmed again. Linkage map was prepared using the data of 16 SSR primers listed in Table 1. Nine out of total 16 primers used were assembled in 4 linkage groups, while remaining has not shown linkage with any of the primers used (Fig.5). Further genotyping of whole population with remaining polymorphic SSRs would help to prepare denser genetic linkage maps and help in the identification of markers linked to stem-rot disease resistance.

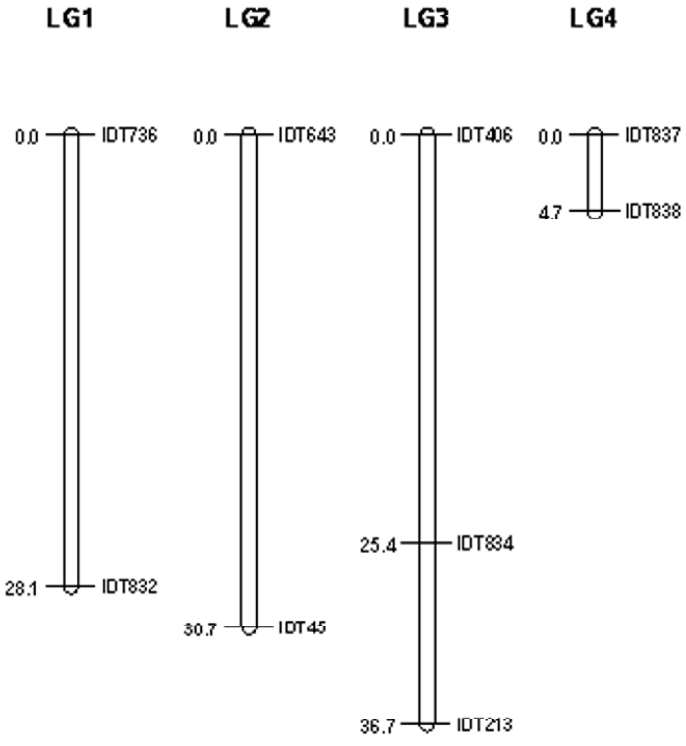

Figure 5: Linkage map prepared using 16 polymorphic SSRs primers

CONCLUSION

In this study, a total of 52 polymorphic SSR markers were identified, of which three (DGR294, DGR470 and DGR510) also distinguished the bulks in BSA, which could be putatively linked with the stem-rot disease resistance. Further, genotyping of complete mapping population with polymorphic markers identified would not only confirm the linkage of markers to stem-rot disease resistance, but also result in a linkage map. After intensive validation of the identified markers linked with the stem-rot disease resistance, it may be used for the MAS for incorporating the stem-rot disease resistance in various groundnut genotypes. Further, the newly identified marker can be added into the already existing groundnut linkage map.

Acknowledgments

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