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Full Length Research Paper

Validation of SSR markers associated with QTLs for pod and kernel traits in groundnut (*Arachis hypogaea L.*)

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A set of 64 groundnut genotypes were used to validate reported molecular markers associated with QTLs of pod and kernel traits viz., PM36, PGS19D09 and IPAHM103 for oil content (percent); PM375 for 100-pod weight (g), pod length (mm) and kernel length (mm); PM137, PGS19D09; PM384 for 100-kernel weight (g) and PM45 for days to maturity. Genotype data were generated using 64 genotypes and the seven markers associated with various phenotypic traits. Phenotyping of the studied material was accomplished during post rainy season, 2011-12. Single marker analysis was performed to ascertain the relationship between the marker and traits. The results indicated that markers PM36, IPAHM103 and PGS19D09 for oil content; PM375 for 100-pod weight, pod length and kernel length; PM137 and PM384 for 100-kernel weight explained more than 10 percent of phenotypic variation. In addition, these markers also showed association with other traits. Hence these markers could be considered as a potential tool for marker assisted breeding in groundnut.

Key words: Groundnut, pod and kernel traits, QTLs, single marker analysis, phenotypes.

INTRODUCTION

Tailoring the genetic architecture of crop plants to suit the growing needs of human beings in terms of increased yield and improved quality has imposed significant dividend in case of cereals. However, plant breeding efforts are yet to make such an impact in case of groundnut (*Arachis hypogaea L.*), an important oilseed crop. This crop is globally grown in an area of 23.0 m ha with the production of 50 million metric tons with an average yield 1.6 tons per ha (FAOSTAT, 2010). Groundnut is an important crop for both its versatility and value. The high protein and energy contents make groundnut valuable as a subsistence crop in some countries, and the demand for its oil allows groundnut to be sold as a cash crop.

Molecular markers and genetic linkage maps are pre-requisites for molecular breeding in any crop; such tools would speed up the process of introgression for benefi-cial traits into preferred varieties. A large number of studies in various crop species have used molecular markers as a tool to identify major genes/QTLs to introduce a new character into elite germplasm. The knowledge of the location of these genes and specific alleles offers the possibility to apply marker assisted selection (MAS). Groundnut molecular breeding has been hindered by a shortage of polymorphic genetic markers due to a very narrow genetic base (Halward et al., 1991; Kochert et al., 1991).

Microsatellites (SSRs) are markers of choice in groundnut because they are co-dominant. SSRs can be used to separate cultivars (Moretzsohn et al., 2005) and this marker system holds great potential for developing useful markers to improve selection efficiency for traits in groundnut breeding. In spite of substantial effort over the last few years by a number of research groups, the number of SSRs that are polymorphic for *A. hypogaea* is still limiting for routine application, creating the demand for the discovery of more polymorphic markers within cultivated germplasm. New efforts for the development of SSR genomic markers are important in order to increase the availability of this class of markers for genetic studies of the *Arachis* species. Many hundreds of SSR makers have been developed during recent years (Jayashree et al., 2005; Luo et al., 2005; Ma et al., 2007), with less than 30% being polymorphic among *A. hypogaea* lines. However, many mapped markers identified in preliminary genetic studies are not suitable for direct use in MAS. Validation of markers is required before use in MAS, since it tests their linkage to, and association with QTL and their effectiveness in postu-

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Table 1. List of 64 groundnut genotypes used in this study.

Genotypes	Habit	Special features	Genotypes	Habit	Special features
CO6	SSP	Released variety	ICGV07368	SSP	Low oil%
ICGV07222	SSP	High pod yield	ICGV06139	B	High pod yield
ICGV07165	B	High pod yield	ICGV07018	SSP	High pod yield
ICGV07108	B	High pod yield	ICGV04093	B	High pod yield
VRI6	SSP	Released variety	ICGV06146	B	High pod yield
ICGV07038	SSP	High pod yield	ICGV98432	B	Low oil%
GG-20	B	Released variety	ICGV06050	B	High pod yield
TG-37	B	Released variety	ICGV05097	B	High pod yield
ICGV07166	B	High oil%	ICGV03128	SSP	High oil%
ICGV07229	B	High oil%	ICGV06321	B	High oil%
ICGV03042	B	High oil yield	ICGV06420	SSP	High oil%
ICGV07023	B	High oil%	ICGV05141	SSP	High oil%
ICGV07148	B	High oil%	ICGV06100	SSP	High oil%
ICGV07019	B	High oil%	ICGV05100	B	High oil%
ICGV07145	SSP	High oil%	ICGV06138	SSP	High oil%
ICGV02411	B	High oil yield	ICGV05032	B	High oil%
TAG24	B	Early maturity	ICGV06216	B	High oil yield
GIG31	B	High pod yield	ICGV06814	SSP	High oil yield
K6	B	Released variety	ICGV06423	SSP	High oil yield
TMV2	B	Drought tolerant	ICGV06424	B	High oil yield
JL24	B	Released variety	ICGV06099	SSP	High oil yield
ABHAYA	B	Released variety	ICGV06046	SSP	High oil yield
GPBD4	B	Released variety	ICGV06049	B	High oil yield
TCGS1043	B	High pod yield	ICGV06051	SSP	High oil yield
ICGV07359	SSP	Low oil%	ICGV05155	B	High oil yield
ICGV06188	B	Low oil%	ICGV06041	SSP	High oil yield
ICGV06110	SSP	Low Oil%	ICGV04061	SSP	High oil yield
ICGV07014	B	High oil yield	ICGV06039	SSP	High oil yield
ICGV07041	SSP	High oil yield	ICGV03057	SSP	High oil yield
ICGV06236	B	Low Oil%	ICGV03043	SSP	High oil yield
ICGV05191	B	Low Oil%	ICGV03109	SSP	High oil yield
ICGV00440	B	Low Oil%	ICGV06042	SSP	High oil yield

lation and selection in the target phenotype in independent populations and different genetic backgrounds. In terms of converting SSRs into informative markers for groundnut genetics and breeding, validation of SSR markers would provide useful markers for MAS. The previously identified markers linked with various QTLs need to be validated before utilizing in the marker assisted breeding programme. Hence in the present study, attempts were made to validate groundnut SSR markers associated with various pod and kernel traits of groundnut which have been reported by various authors. These results further confirm their potential involvement in improvement of various yield contributing traits.

MATERIAL AND METHODS

Plant Material

In this present investigation, 64 genotypes of groundnut (Advanced Breeding lines and Check varieties selected based on the 3 years study from a set of 180 lines) (Table 1) were used for validation. They were raised in a randomized block design with three replications at the Oilseeds farm, Centre for Plant Breeding and Genetics, Tamil Nadu Agricultural University, Coimbatore during post rainy season, 2011-12. Normal agronomic practices were followed under irrigated condition. The data were recorded for 11 pod and kernel traits viz., 100-pod weight

Table 2. List of primers used for validation.

Primer	Traits associated	PVE %	Population used	Authors
PM36	Oil content (%)	6.98	$F_{9:10}$ generations of 146 recombinant inbred lines (RILs) of TG26 x GPBD 4	Sarvamangala et al. (2011)
IPAHM103	Oil content (%)	5.72	$F_{9:10}$ generations of 146 recombinant inbred lines (RILs) of TG26 x GPBD 4	Sarvamangala et al. (2011)
PM375	100-pod weight (g) Kernel length (mm) Pod length (mm)	10.17, 12.45, 9.19	$F_{2:6}$ recombinant individuals in two DNA pools (high and low) resulting from a cultivated cross between a runner (Tamrun OL01) and a Spanish (BSS 56) peanut	Selvaraj et al. (2009)
PM137	100-kernel weight (g)	6.9	F_5 RIL population of TMV 2 x COG 0437	Priyadharshini et al. (2012)
PGS19D09	100-kernel weight (g) Oil content (%)	5.1,	F_5 RIL population of TMV 2 x COG 0437	Priyadharshini et al. (2012)
PM384	100-kernel weight (%)	-2.5* (b value)	F_2 population (TMV 2 X COG 0437)	Shoba et al. (2010)
PM45	Days to maturity	17.69	$F_{2:6}$ recombinant individuals in two DNA pools (high and low) resulting from a cultivated cross between a runner (Tamrun OL01) and a Spanish (BSS 56) peanut	Selvaraj et al. (2009)

Table 3. Variability for different characters among 64 groundnut genotypes.

Characters	Maximum	Minimum	Mean	CV (%)
100-pod weight (g)	182.6	62.4	95.9	24.4
100- kernel weight(g)	46.4	14.4	28.4	27.5
Pod length (mm)	35.6	21.4	26.9	11.3
Pod width (mm)	19.5	10.5	12.9	11.4
Kernel length (mm)	17.6	9.8	12.5	11.3
Kernel width (mm)	8.7	4.8	6.6	11.1
Shell thickness (mm)	1.1	0.6	0.9	12.6
Shell weight (g)	4.5	1.1	2.0	32.9
Shelling (%)	78.3	38.9	59.2	13.9
Oil content (%)	62.2	29.2	48.3	13.9
Days to maturity	129	110	119.1	6.4

(g), pod length (mm), pod width (mm), 100-kernel weight (g), kernel length (mm), kernel width (mm), shell weight (g), shell thickness (mm), shelling %, days to maturity and oil content (%). Phenotyping was done with the mean value of five plants.

SSR Markers and QTLs

A total of 7 SSR markers (Table 2) which were published by various workers with high level of association between QTLs and various pod and kernel traits of groundnut were chosen for validation in this present study.

DNA extraction

Leaves were collected from 64 genotypes in two leaf stages and genotyping was done with the pool of five plant DNA and DNA extraction was performed according to the cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1990). The extracted DNA content was

Table 4. Number of alleles and polymorphism information content (PIC) of the primers.

S.No.	Primer	No. of alleles	PIC value
1	PM36	15	0.90
2	IPAHM103	9	0.84
3	PM375	6	0.79
4	PM137	9	0.88
5	PGS19D09	8	0.80
6	PM384	6	0.80
7	PM45	3	0.10
	Mean	**8**	**0.73**

measured using DNA standards in agarose gel (0.8 % w/v). The 10 µl PCR cocktail contained 20 ng of 2 µl DNA, 1 µl of 10XTaq buffer, 0.2 µl of 25 mM Mgcl2, 1.0 µl of 0.2 mM of dNTP, 0.5 µl of 0.5 uM of each forward and reverse primer, 4.5 µl of sterile water and 0.3 µl of 0.03 IU Taq DNA polymerase. DNA amplification was performed in a Veriti® 96-Well Fast Thermal Cycler (Applied Biosystems Inc., Foster city, CA). DNA samples were denatured initially at 94 °C for 3 min, then subjected to the following 20 cycles: 94 °C for 30 s, 63 °C for 30 s with a decrement of 0.5 °C per cycle, and 70 °C for 1 min. This was followed by another 20 cycles of 94 °C for 15 s, 55 °C for 30 s, and 70°C for 1 min. A 10 min extension was performed at 72 °C as the last step. Amplified products were analyzed using 6% Polyacrylamide gel electrophoresis at 150 volts DC for 4 hrs and silver stained in accordance with the protocol described by (Benbouza et al., 2006).

Data Scoring and Data Analysis

Clear and unambiguous bands were scored for their presence or absence with the score 1 indicating their presence and 0 indicating their absence. The data matrix of binary codes thus obtained was subjected to further analysis. Phenotypic values of all the 64 genotypes were subjected to associate with corresponding marker score for its significance by using simple regression in SPSS software (version.16).

Simple linear regression method (Haley and knott, 1992) was used to identify significant maker trait association. The linear equation formed was as follows:

$Y = \mu + f(\text{marker}) + \text{error}$

where, Y = phenotypic trait value; µ = population mean and f (marker) = function of the molecular marker.

The potential relationship between the marker and trait was established considering the significance of the regression coefficient at 5 and 1 per cent probability. The phenotypic variance explained was expressed in terms of adjusted R2 values.

RESULTS AND DISCUSSION

Phenotype

The phenotypic variation observed among 64 groundnut genotypes is summarized in the Table 3. The traits 100-pod weight, 100-kernel weight and shell weight had high coefficient of variation (>20 %). Whereas pod length, pod width, kernel length, kernel width, shell thickness, shelling % and oil content recorded medium level coefficient of variation (10-20%). The trait days to maturity had the least coefficient of variation (<10%).

Genotype

Polymorphic Information Content of the Markers

The number of alleles generated by the primer varied from 3 to 15. The PIC value ranged from 0.10 to 0.90 for the seven primers used (Table 4). Among all the primers used, five as viz., PM36 (0.90), PM137 (0.88), IPAHM103 (0.84), PM384 (0.80), PGS19D09 (0.80) and PM375 (0.79) exhibited the high PIC value. The higher PIC value indicated the informativeness of the primer and hence these markers may be considered as informative primers.

Single Marker Analysis

Simple linear regression was calculated using phenotypic traits and genotype of each marker. The results indicated that each marker had association with many traits and a single trait related to many markers (Table 5). The marker which is having a strongest relationship can be judged from its adjusted R2 value which will give the percentage of variability explained (PVE) by the particular trait.

Single marker analysis among the 11 traits revealed the association of marker with various traits as follows: a total of seven markers for 100-pod weight (5.4 to 12.3% PVE), six markers for 100-kernel weight (7.2 to 12.3% PVE),

Table 5. Single marker analysis of 64 genotypes for various traits.

Characters	Primers/allele	PVE (R^2) %	Reference
100-pod weight (g)	PM36	12.3	
	IPAHM103	12.0	
	PM375	11.4	
	PM45	11.2	
	PM137	10.5	
	PM384	9.8	
	PGS19D09	5.4	
100-kernel weight (g)	PM137	12.3	Priyadharsini et al. (2012)
	PM45	10.4	
	PM384	10.3	Shoba et al. (2010)
	IPAHM103	9.2	
	PM36	7.5	
	PGS19D09	7.2	Priyadharsini et al. (2012)
Pod length (mm)	PM375	16.1	Selvaraj et al. (2009)
	PM36	8.9	
	PM45	5.5	
Pod width (mm)	PM375	8.5	
	PM137	8.5	
	IPAHM103	7.1	
	PM36	5.8	
Kernel length (mm)	PM36	20.1	
	PM375	14.3	Selvaraj et al. (2009)
	PGS19D09	7.6	
	IPAHM103	6.2	
Kernel width (mm)	PM36	13.0	
	PGS19D09	5.8	
	IPAHM103	5.5	
Shell thickness (mm)	IPAHM103	8.0	
	PM36	7.7	
Shell weight (g)	PM375	12.7	
	PM36	8.5	
	PGS19D09	7.6	
Shelling (%)	PGS19D09	6.6	
	IPAHM103	4.9	
Oil content (%)	IPAHM103	17.6	Sarvamangala et al. (2011)
	PM36	16.7	Selvaraj et al. (2009)
			Sarvamangala et al. (2011)
	PGS19D09	10.1	Priyadharsini et al.(2012)
Days to maturity	PM36	22.6	
	PGS19D09	16.8	
	PM384	9.4	
	IPAHM103	8.9	
	PM375	7.4	
	PM45	4.5	Selvaraj et al. (2009)

three markers for pod length (5.5 to 16.1 % PVE), four markers for pod width (5.8 to 8.5 % PVE), four markers for kernel length (6.2 to 20.1 % PVE), three makers for kernel width (5.5 to 13.0 % PVE), two markers for shell thickness (7.7 to 8.0 % PVE), three markers for shell weight (7.6 to 12.7 % PVE), two markers for shelling percentage (4.9 to 6.6 % PVE), three markers for oil content (10.1 to 17.6 % PVE) and six markers for maturity (4.5 to 22.6 % PVE).

Validation of Markers Associated with Pod Traits

Among the seven SSR markers linked to 100-pod weight, markers PM36, PM375, IPAHM103, PM137 and PM45 were considered as important as they can account for more than 10 percent PVE of the trait. Among the three markers associated to pod length, marker PM375 had 16.1 % PVE. This marker was reported for pod length by (Selvaraj et al., 2009). For pod width, out of the four markers, PM375 and PM137 had higher PVE (8.5%).

Validation of Markers Associated with Kernel Traits

In case of 100-kernel weight, though six SSR markers were found to be linked, the marker PM137 had the highest PVE (12.3%). This marker had been reported by (Priyadharsini et al., 2012). Apart from this, PM384 (10.3% PVE) and PGS19D09 (7.2% PVE) also contributed significantly to the 100-kernel trait. These two markers were reported by (Shoba et al., 2010 and Priyadharsini et al., 2012) respectively. Four markers were found to be associated with kernel length and among the markers, PM36 and PM375 recorded higher PVE of 20.1 and 14.3 % respectively. Among these markers, PM 375 was reported to be associated with kernel length (Selvaraj et al., 2009). Three markers were found to be relevant in the case of kernel width, of which marker PM36 had more than 10% PVE and two markers were found related to the shell thickness with 7.7 and 8.0 % PVE. Three markers PM36, PGS19D09 and PM375 were found to be related with the shell weight. Among these markers, PM375 had the highest contribution of 12.7 % PVE. The trait shelling percentage had association with two markers viz., PGS19D09 and IPAHM103 with 6.6% and 4.9% PVE respectively.

In case of oil content, three markers viz., PM36, IPAHM103 and PGS19D09 were found to be linked, and all the three markers have shown the highest PVE of more than 10%. These markers were reported by (Selvaraj et al., 2009; Sarvamangala et al., 2011 and Priyadharsini et al., 2012). Four days to maturity, six markers were found to be linked, of which PM36 and PGS19D09 had the highest PVE (22.6 and 16.8%) and PM45 marker with 4.5% PVE was already reported by (Selvaraj et al., 2009).

In the present study, most of the markers were found to be related to more than one trait. The marker PM36 was found to be associated with all ten traits except shelling %. Similarly marker IPAHM103 was related to all traits except pod length and shell weight. Also markers PM375 and PGS19D09 were linked to more than five traits. Markers PM137 and PM384 was associated with traits viz, 100-pod weight and 100-kernel weight. This indicates that probably the same set of genes is controlling the expression of these characters. Moreover, phenotypically these characters have more association with each other. Hence these markers may be useful for yield improvement programme.

Molecular markers linked with QTL/major genes for traits of interest are being routinely developed in several crops using materials derived from planned crosses such as F2, RIL, DH populations, etc. Many times the marker identified in a specific population may not be useful in other populations. Hence validation in other mapping population is highly important before using these markers. Validation of the identified markers/QTLs in a set of genotypes is one of the best methods because the results can be generalised than the results obtained from biparental mating populations. Hence we believe that the markers which have been validated have a potential use in MAS programme for pod and kernel traits in groundnut.

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