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Inheritance and AFLP Tagging of Leaflet Mutants in Mungbean (*Vigna radiata* (L.) Wilczek)

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ABSTRACT

Leaflet type is a canopy characteristic related to light interception, thus modification of canopy structure can alter seed yield. Two multiple leaflet mutants were obtained from gamma-rays irradiation and used in studying the mode of inheritance and tagging with AFLP marker. The cross between large-heptafoliate leaflet with small-pentafoliate leaflet mutants gave all F_1 plants with normal trifoliate leaflets. The F_2 plants segregated in a 9:3:3:1 ratio of large-trifoliate: large-heptafoliate: small-pentafoliate: small-heptafoliate plants, suggesting that the genes controlling leaflet size and leaflet number were independent loci. The gene symbols N_1,n_1 and N_2,n_2 were proposed to control leaflet number. Since there was no plant found with large-pentafoliate leaflets, it was hypothesized that the N_2 allele expressed pleiotropic effect on both leaflet number and leaflet size. Thus the genotypes of the above-mentioned F_2 could be assigned as $N_1\_N_2\_$, $n_1n_1N_2\_$, $N_1\_n_2n_2$, and $n_1n_1n_2n_2$, respectively. Another possibility was that there was another locus with *S* and *s* alleles controlling the leaflet size and tightly linked with N_2 and n_2, respectively. There were 3 AFLP markers linked to number of leaflets per leaf and all of them corresponded to the N_1 allele of the small-pentafoliate parent.

Key words: *Vigna radiata*, mungbean, inheritance, leaflet mutant, AFLP marker

INTRODUCTION

Mungbean (*Vigna radiata* (L.) Wilczek) is a widely-grown, short-duration grain legume crop in South and Southeast Asia. It is an important source of inexpensive protein in most Asian diets and a significant component of various cropping systems. However, the average yields in the farmers' fields are still low, ranging between 500 to 800 kg/ha. One reason is due to the use of traditional cultivars and low management inputs by most farmers. However, there is a rather limited genetic variation in the existing mungbean germplasm to boost up mungbean yield by plant breeder. An alternative is to create genetic variation throught mutagenesis.

Several types of multifoliate leaflet mutant have been found in legume crops and express a potential in altering grain yield. Dwivedi and Singh (1985) reported that narrow leaf

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character in mungbean appears to be governed by two recessive genes symbolized by nl_1 and nl_2. Bhadra (1991) reported that a nine-foliate leaflet character was monogenic recessive to normal trifoliate leaf. He proposed the symbols *tf* and *Tf* for the genes regulating these two characters.

Molecular markers can be used to tag genes controlling traits of interest and to form into a partial linkage group. This is particularly useful as a starting point in constructing a more informative molecular linkage group for mungbean crop that molecular marker technology is at the beginning stage. The AFLP marker was chosen in this study because of its excellent reproducibility, which was essential if screening protocols were to be established (Jones *et al*., 1998; Matthes *et al*., 1998). AFLP can screen a large number of loci for polymorphism and simultaneously detects a greater number of DNA markers than any other polymerase chain reaction based detection system (Vos *et al*., 1995). Linkage map has recently been developed in some crops including genus *Vigna* (Tomooka *et al*., 2002; Somta *et al*., 2006).

The objectives of this experiment were: 1) to study the inheritance of multifoliate leaflet mutants in mungbean, and 2) to identify AFLP markers associated with the multifoliate leaflet character.

MATERIALS AND METHODS

Inheritance of multifoliate leaflets

Plant materials

A cross was made between two parental lines, one with large-heptafoliate leaflets (L-7) and the other with small-pentafoliate leaflets (S-5) during early rainy season 2002 at Kasetsart University, Kamphaeng Saen Campus. The L-7 parent was a BC_9 progeny having the most popular Thai cultivar 'Kamphaeng Saen 1' as the recurrent parent and the large-heptafoliate leaflet mutant (V5926) from AVRDC - the World Vegetable Center, Taiwan as the donor parent (Kowsurat *et al*., 1999). The S-5 parent was a new mutant line obtained from gamma-rays irradiation of F_2 seed from a cross between the cultivated 'Chai Nat 36' with the wild mungbean 'TC 1966' (Srinives *et al*., 2000). The leaflet size of this mutant was only about 1/5 of the normal one (Fig. 1). The S-5 was used as the paternal plant since it had purple hypocotyl which was a dominant character for identifying the true F_1 hybrid from crossing with the green hypocotyl L-7, used as the maternal

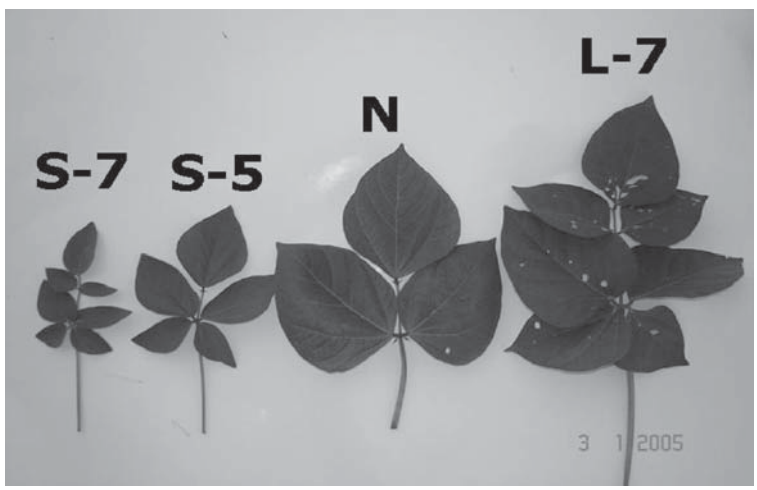

Figure 1 Leaflet types of mungbean progenies derived from the cross between L-7 and S-5.

plant. The F_1 seeds were sown and harvested individually and four F_1 plants with the highest number of F_2 seeds were grown in the field to form F_2 families. Field management of the trials followed the optimum recommended practices advocated by Park (1978). The number of F_2 plants were recorded according to leaflet number (3, 5, and 7), and leaflet size (large and small).

Genetic data analysis

The number of F_2 plants was tested againt a 3:1 ratio for segregation in a single locus and 9:3:3:1 for 2 independent loci using the Chi-square (χ^2) goodness-of-fit test suggested by Mather (1951). The heterogeneity among the 4 F_2 families were also tested accordingly.

Tagging of multifoliate leaflet genes using AFLP markers

Extraction of recombinant inbred lines

From F_2 and on, the normal trifoliate leaflet plants were individually harvested each time until F_5 where four families each with four phenotypes (normal-trifoliate, large-heptafoliate, small-pentafoliate, and small-heptafoliate) were finally obtained. The 16 mungbean lines were considered isogenic lines in regard to leaflet number, but uniform in the genetic background (93.75 % the same in each family).

AFLP marker analysis

Young expanded leaves from 3 plants each of the 16 isogenic mungbean lines and their parents were collected for DNA extraction using the modified CTAB method of Doyle and Doyle (1987). Two hundred nanograms of genomic DNA from each line was digested and ligated simultaneously in a total volume of 30 μl at 37 °C over night. The genomic DNA was digested with 10U *Eco*RI and 10U *Mse*I (Fermentaz INC., Maryland, USA), while ligation required adapters of 5 ρmol of EcoRI and 50 ρmol of MseI.

Preamplification (PCR I) was performed in a total volume of 10 μl containing 1 μl of the 10-fold dilution ligated DNA fragments, 0.5 μl each of *Eco*RI and *Mse*I primers with one selective nucleotide (5 μM), 1 μl of 10x buffer, 0.6 μl of $MgCl_2$ (25 mM), 2 μl of dNTP ((1 mM) and 0.2 μl of *Taq* DNA polymerase (Fermentaz INC., Maryland, USA) (5U/ μl). The PCR procedure followed initial denaturation step at 94 °C for 2 min, 20 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s and extension at 72 °C for 60 s, then incubated at 72 °C for 5 min as the final extension. The PCR I product was diluted 10-fold and used as the template for selective amplification (PCR II). The PCR II procedure began with denaturation step at 94 °C for 2 min, 12 cycles of denaturation at 94 °C for 30 s, annealing at 65 °C for 30 s (less 0.7 °C per cycle after the first cycle), extension at 72 °C for 60 s, denaturation for 24 cycles at 94 °C for 30 s, annealing at 56 °C for 30 s, extension at 72 °C for60 s, followed by the final extension at 72 °C for 2 min.

The PCR II products were loaded on 4.5 % denaturing polyacrylamide gel with 1x TBE at 60 W for 80 min. DNA fragments were detected by silver staining method as described by Promega Corp., USA. Different DNA fragments amplified with each primer were treated as discrete characters and numbered sequentially. Genotypes were scored for the presence (1) or absence (0) of each fragment. A single factor analysis of variance was carried out to identify the association between leaflet types and AFLP markers using Proc ANOVA (SAS Inst., 1999).

RESULTS AND DISCUSSION

All the F_1 from L-7 x S-5 were trifoliate leaflet plants, suggested that there were at least 2 loci of gene controlling the leaflet number. Assuming that the L-7 and S-5 carried the genotype $n_1n_1N_2N_2$ and $N_1N_1n_2n_2$ respectively, the F_1 should have the genotype $N_1n_1N_2n_2$. There was

no large-pentafoliate plant (L-5) found among the plants segregating from F_2 to F_5. Instead, there were only 4 classes of leaflet number and size, viz. large-trifoliate, large-heptafoliate, small-pentafoliate, and small-heptafoliate. The numbers of plants in different leaflet classes from each F_2 family were tested against a 9:3:3:1 ratio of the respective phenotypes $N_{1\_}N_{2\_}$, $n_1n_1N_{2\_}$, $N_{1\_}n_2n_2$, and $n_1n_1n_2n_2$ (Table 1). The χ^2-test results supported the hypothesis that there were 2 loci of gene controlling number of leaflet. The combined data did not deviate significantly from the 9:3:3:1 ratio. Heterogeneity among the families were not significant, revealing that the segregation of this traits among the F_2 families agreed well with each other. With this model of gene action, the N_2 allele should have a pleiotropic effect on leaflet size so that the plants with $N_{2\_}$ and n_2n_2 phenotypes always have large and small leaflets regardless of leaflet number. The χ^2-test for the goodness-of-fit of 3 large: 1 small leaflet plants as supposedly controlled by the n_2 locus is given in Table 2. The test results supported that the n_2 locus also conditioned leaflet size in all of the tested families. The N_1 allele dictated normal-trifoliate at the present of N_2 but showed pentafoliate in $N_{1\_}n_2n_2$, whereas n_1n_1 genotypes expressed heptafoliate regardless of the genotypes in n_2 locus (Table 1). Another possibility with less likely was that the n_2 locus was tightly linked with the third locus (say *s*) controlling the leaflet size. With the latter hypothesis the *S* allele attached to the N_2 allele so

Table 1 Chi square test for independence (9:3:3:1 ratio) between the n_1 and n_2 alleles controlling leaflet number in 4 F_2 mungbean families from the cross between L-7 and S-5 parents.

Family	No. of plants					$\chi^2_{(3)}$	Prob
	N	L-7	S-5	S-7	Total		
	$N_{1\_}N_{2\_}$	$n_1n_1N_{2\_}$	$N_{1\_}n_2n_2$	$n_1n_1n_2n_2$			
1	117	44	42	23	226	6.448	0.10 - 0.05
2	64	21	27	12	124	3.627	0.50 - 0.30
3	172	47	41	17	277	4.460	0.30 - 0.20
4	82	24	20	8	134	1.695	0.80 - 0.70
Total	435	136	130	60	761	4.806	0.30 - 0.20
Heterogeneity (9 df)						11.424	0.30 - 0.20

Table 2 Chi-square test for goodness-of-fit against a 3:1 ratio for leaflet size (large vs small) as supposedly controlled by the n_2 locus in 4 F_2 mungbean families from the cross between L-7 and S-5 parents.

Family	No. of plants[1/]		$\chi^2_{(1)}$	Prob
	$N_{2\_}$	n_2n_2		
1	161	65	1.705	0.20 - 0.10
2	85	39	2.753	0.10 - 0.05
3	219	58	2.437	0.20 - 0.10
4	106	28	1.204	0.30 - 0.20
Total	571	190	0.000	< 0.99
Heterogeneity (3 df)		8.099	0.05 - 0.01	

[1/] No. of plants with large leaflets ($N_{2\_}$) was obtained from N and L-7; those with small leaflets (n_2n_2) were from S-5 and S-7.

tight that they always co-segregated so that the respective genotypes for the large-trifoliate, large-heptafoliate, small-pentafoliate, and small-heptafoliate should be $N_1\_N_2\_S\_$, $n_1n_1N_2\_S\_$, $N_1\_n_2n_2ss$, and $n_1n_1n_2n_2ss$, respectively. The theoretical genotype $N_1\_n_2n_2S\_$ (supposedly showing large-pentafoliate leaflet) was not found in this study, due to no crossing over occurred between N_2 and S.

The F_2 population segregated into a 9:3:3:1 ratio in leaflet size and number, indicating that each character was controlled by a separate locus of genes. A gene action with epistatic expression was proposed for alleles controlling leaflet number. $N_1\_$ gave trifoliate leaf upon the presence of $N_2\_$ genotype, but gave pentafoliate leaflet at the presence of n_2n_2. Whereas n_1n_1 expressed heptafoliate regardless the presence of $N_2\_$ or n_2n_2. The previous study reported by Sripisut and Srinives (1986) indicated that lobed and trifoliate leaflets were dominant over normal and multiple leaflets. Each trait was governed by a single locus of gene on different chromosomes. Chhabra (1990) observed that trifoliate (normal) trait was monogenically dominant over pentafoliate in mungbean. Thus it was clear that the small heptafoliate (with the proposed genetic symbol $n_1n_1n_2n_2$) mutant allele in this study was not the same as those previously reported.

AFLP marker associated with leaflet characters

A total of 180 primer combinations were evaluated for detection of polymorphism between L-7 and S-5 parental genotypes. Amplification was observed and 94 primer pairs showed polymorphism between them. From 94 primer combinations, 47 of them showed clear and sharp bands and thus used for amplifying the fragments of the 16 isogenic lines. Twenty primer pairs could distinguish between the parents and between the isogenic lines and produced 56 polymorphic DNA bands.

The results of single factor analysis of variance showed that a total of 15 AFLP markers significantly associated with leaflet size and leaflet number (Table 3). The size of detected fragments range from 82-413 bp. There were 12 markers associated with leaflet size, 10 of them were contributed from P_1 (L-7) alleles, the other 2 markers, viz. ACT_AGC and GCC_ACA1 were from P_2 (S-5) alleles. For the number of leaflets per leaf, 3 markers were contributed from P_2 alleles.

Three markers, AAA_CTT3, ACG_CAC1, and GCC_ACT3, showed association with the genes controlling leaflet number. Marker AAA_CTT3 was from P_2 allele, while ACG_CAC1 and GCC_ACT3 were from P_1 alleles. The marker AAA_CTT3 was likely linked with N_1 allele, while the markers ACG_CAC1 and GCC_ACT3 were linked with n_1. However, the markers did not correspond to leaflet size (Table 3), revealing that the genes controlling leaflet size and n_1 locus were located on different chromosomes or probably on the same chromosome but far in distance (> 50 cM). Although a set of AFLP markers has been identified to link with leaflet number and leaflet size, more investigation on their map distance is still needed to be further explored.

CONCLUSION

Crossing between 7 large leaflet (L-7) and 5 small leaflet (S-5) mungbean mutants resulted in the normal-trifoliate (N) F_1. The F_2 could be classified into number of leaflets per leaf and leaflet size with large-trifoliate ($N_1\_N_2\_$), small-pentafoliate ($N_1\_n_2n_2$), large-heptafoliate ($n_1n_1N_2\_$), and small-heptafoliate ($n_1n_1n_2n_2$) at the dihybrid ratio of 9:3:3:1. The finding was thus evident that leaflet number character was controlled by n_1 and n_2 loci of genes. However, all 3 AFLP markers associated with leaflet number in this study corresponded to n_1 locus only. The n_2 locus could have a pleiotropic effect upon the

Table 3 A single factor analysis of variance showing association of AFLP markers with leaflet size and leaflet number.

Line no.	Marker	Marker size (bp)	Leaflet size (N, L-7 vs S-5, S-7) Allele mean P_1	Allele mean P_2	Prob	Leaflet number (N, S-5 vs L-7, S-7) Allele mean P_1	Allele mean P_2	Prob
1	AAA_CAG2	200-249	0.70	0.17	0.04	-	-	-
2	AAA_CAG3	151-200	0.70	0.17	0.04	-	-	-
3	AAA_CTA1	200-249	0.80	0.00	< 0.01	-	-	-
4	AAA_CTT2	200-249	0.88	0.13	< 0.01	-	-	-
5	AAA_CTT3	82-100	-	-	-	0.17	0.7	0.04
6	ACG_CAC1	200-249	-	-	-	0.22	0.86	0.01
7	ACG_CAG4	311-413	1.00	0.18	< 0.01	-	-	-
8	ACG_CAG1	151-200	1.00	0.10	< 0.01	-	-	-
9	ACG_CAG2	151-200	1.00	0.30	< 0.01	-	-	-
10	ACT_ACG	151-200	0.86	0.27	0.01	-	-	-
11	ACT_AGC	200	0.33	0.83	0.05	-	-	-
12	CAG_ACG3	100-118	0.73	0.17	0.03	-	-	-
13	CT_AAT	100-118	0.86	0.27	0.01	-	-	-
14	GCC_ACA1	151-200	0.30	1.00	< 0.01	-	-	-
15	GCC_ACT3	200-249	-	-	-	0.27	0.86	0.01

leaflet size such that the N_2 allele controlled large leaflet size as well. Another hypothesis was that the n_2 locus might be closely linked with the *s* locus so that there was no progenies with large pentafoliate leaflet (hypothetically carrying $N_1\_n_2n_2S\_$ genotype).

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