Identification of RAPD Markers Linked to Five Marker Genes (*blu*, *dgs*, *y*, *arg*, and a Flat Pod Mutant) in Common Bean

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ABSTRACT. The development of a complete linkage map, including both classical (visible) and molecular markers, is important to understand the genetic relationships among different traits in common bean (*Phaseolus vulgaris* L.). The objective of this study was to integrate classical marker genes into previously constructed molecular linkage maps in common bean. Bulk segregant analysis was used to identify 10 random amplified polymorphic DNA (RAPD) markers linked to genes for five classical marker traits: *dark green savoy* leaf (*dgs*), *blue* flower (*blu*), silvery [Latin: *argentum*] green pod (*arg*), *yellow* wax pod (*y*) and *flat* pod (a spontaneous mutation from round to flat pod in 'Hialeah' snap bean). The genes for *dark green savoy* leaf (*dgs*) and *blue* flower (*blu*) were located in a previously constructed molecular linkage map. These results indicate that classical marker genes and molecular markers can be integrated to form a more complete and informative genetic linkage map. Most of the RAPD markers were not polymorphic in the two mapping populations used, and molecular markers from those mapping populations were not polymorphic in the F₂ populations used to develop the RAPD markers. Alternative genetic hypotheses for the pod shape mutation in 'Hialeah' are discussed, and the experimental difficulties of pod shape classification are described.

The classical gene linkage map of common bean (*Phaseolus vulgaris*) (2n = 2x = 22) has been rudimentary compared with maize (*Zea mays* L.), barley (*Hordeum vulgare* L.), pea (*Pisum sativum* L.), and tomato (*Lycopersicon esculentum* Mill.) (Bassett, 1991). Although more than 240 genes have been reported (M.J. Bassett, 1996), only about 50 have been placed in any linkage group (Bassett 1991; Gepts et al., 1993; McClean et al., 2002). Several molecular linkage maps have been constructed in common bean (Adam-Blondon et al., 1994; Gepts et al., 1993; Jung et al., 1996, 1997; Nodari et al., 1993; Vallejos et al., 1992); however, these maps include only a few classical marker genes (<http://agronomy.ucdavis.edu/gepts/geptsfab.htm>).

Placing genes controlling classical (visible) marker traits on the genetic linkage map of common bean can be facilitated by tagging the genes of interest using random amplified polymorphic DNA (RAPD) markers and bulked segregant analysis (BSA). Because RAPD marker polymorphisms are abundant in common bean (Skroch and Nienhuis, 1995), the gene of interest can be tagged with RAPD markers and then mapped to an existing linkage map. Therefore, by means of molecular markers, a gene can be placed in a linkage map developed from a mapping population that does not necessarily segregate for that gene.

The objective of this study was to integrate classical marker genes into a previously constructed molecular linkage map in common bean by means described above.

Materials and Methods

PLANT MATERIAL. BSA was used to identify RAPD markers linked to genes for five classical marker traits: *dark green savoy* leaf (*dgs*) (Nagata and Bassett, 1984), *blue* flower (*blu*) (Bassett, 1992), silvery [Latin: *argentum*] green pod (*arg*) (Currence, 1931; Lamprecht, 1947), yellow wax pod (*y*) (Currence, 1931; Lamprecht, 1947), and round pod [German: *elliptische*] (*ea eb*) (Lamprecht, 1932, 1947) or flat pod (no gene symbol given by Ram and Prasad, 1985). Lamprecht (1932) found a 15:1 segregation for flat to round pods, respectively, in one cross and later (Lamprecht, 1947) found a 3:1 segregation with another cross for the same pod shape classes. Ram and Prasad (1985) found a 3:1 segregation for round to flat pods, respectively, which is a reversal of the trait dominance compared with the findings of Lamprecht (1932, 1947). Four segregating populations of 80 F₂ individuals each were evaluated for five morphological traits (Table 1). Two BC₂-F₂ populations (one with *dgs* and the other *blu*) were developed using Florida dry bean breeding line 5-593 as the recurrent parent. The marker genes *dgs* and *blu* are induced mutations developed on Florida dry bean breeding line 7-1404. A BC₃-F₂ population segregating for *y* and *arg* was developed from the Lamprecht source line PI 527858, using 5-593 as the recurrent parent. Finally, a F₂ population segregating for a spontaneous mutation from round pod to flat pod (whether at *ea* or *eb* or another locus is unknown) was developed from the cross 'Hialeah' flat pod mutant x 'Hialeah' round pod. 'Hialeah' is a pure line snap bean variety from Ferry-Morse Co., and Robert Gehin (now with Harris Moran, Sun Prairie, Wis.) supplied the seed of 'Hialeah' and the spontaneous 'Hialeah' flat pod mutant. All of these traits are reported to be inherited as single gene traits (Bassett 1992a; Currence, 1931; Lamprecht, 1947; Nagata and Bassett 1984; Ram and Prasad, 1985), except not always for round pod (Lamprecht, 1932). Our original hypothesis is that the

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spontaneous revertant mutation from round pod to flat pod (the wild phenotype) occurred at a single locus, but we do not know the locus involved.

MORPHOLOGICAL DATA. The four segregating populations were evaluated by growing each individual in a 8-cm pot, using standard cultural practices in a greenhouse at Madison, Wis. The populations segregating for *blu*, *dgs*, *y* and *arg* were grown during Summer 1996. The population segregating for the round pod trait was grown during Summer 1997. The five traits were genetically classified based on phenotype and the known genetics.

DNA EXTRACTIONS AND RAPD REACTIONS. DNA was extracted from fresh immature trifoliate leaves of every plant in each of the four segregating populations. The DNA isolation procedure of Johns et al. (1997) was used. RAPD reaction mixtures were formulated as described by Skroch and Nienhuis (1995). RAPD reactions were performed in 96-well plates in a thermal cycler (PTC-100; MJ Research, Watertown, Mass.). The cycling temperature settings were the same as used by Johns et al. (1997). Reaction products were separated by electrophoresis on 1.5% agarose gels, stained with ethidium bromide, and photographed over a ultraviolet light transilluminator with Polaroid 667 film.

BULKED SEGREGANT ANALYSIS. Two equimolar bulks were constructed per population. One bulk consisted of the DNA template from eight individuals displaying the phenotype of interest for each homozygous recessive (*dgs/dgs*, *blu/blu*, *arg/arg*, *y/y* and flat pod). The other bulk consisted of the DNA template from eight individuals displaying the contrasting dominant phenotype (*Dgs* __, *Blu* __, *Arg* __, *Y* __ and round pod). The contrasting bulks were screened, using 10-base primers from Operon and BC. A total of 260 RAPD primers were used: 240 from Operon kits A to Z and AA to AW, and 20 from BC 200 to 640. All data were scored as 1 = the presence or 0 = the absence of the DNA fragment for each polymorphic band. RAPD markers were named with the original number of the primer, either from Operon Technologies (Alameda, Calif.) or the University of British Columbia (BC), and their approximate size. The approximate length in base pairs of the RAPD markers was determined by comparing each RAPD marker with size markers from a 100-base pair ladder (Gibco BRL) and rounding to the nearest 50 base pairs. Primers showing polymorphic bands between the contrasting bulks were amplified for each individual making up the contrasting bulks. If the polymorphic band persisted, the primer was used to perform linkage analysis.

LINKAGE ANALYSIS. The putatively linked RAPD markers identified using BSA were assayed for cosegregation among the characterized individual genotypes from the respective population. The linkage analysis was performed using MAPMAKER Macintosh version 2.0 (Lander et al., 1987). A logarithm of odds (LOD) score

of 3.0 and a maximum recombination fraction of 0.3 were used as linkage threshold criteria. Map distances were estimated using the Kosambi function (Kosambi, 1944). Segregation ratios for the five morphological traits and 10 putatively linked RAPD marker loci were tested for deviation from the expected 3:1 segregation ratio by chi-square goodness-of-fit analysis.

RAPD markers putatively linked to each of the five genes (three for *dgs*, one for *blu*, two for *arg*, one for *y*, and three for flat pod) were assayed for polymorphism among the parents of two mapping populations: BAC-6 x HT-7719 (Jung et al., 1996) and PC-50 x XAN-159 (Jung et al., 1997). If the RAPD marker was polymorphic between the parents, then the marker was analyzed in the two F_2 recombinant inbred line (RIL) populations of 70 individuals each. These RAPD reactions were performed to generate linkage analysis data between the five morphological traits and these two previously constructed molecular linkage maps (Jung et al., 1996, 1997).

Results and Discussion

MORPHOLOGICAL DATA AND SEGREGATION RATIOS. Aberrant F_2 ratios deviating from the expected 3:1 were detected at the *arg* locus and the putative flat pod locus, which were the result of an excess of homozygous recessive individuals. This deviation can contribute to the experimental error associated with the linkage estimates computed, leading to inconclusive linkage estimates. Because the inheritance of flat pod is uncertain, the observed aberrant ratio can be attributed to one or more of several causes: incomplete dominance of the round pod allele, disturbed segregation at the locus (Bassett, 1995; Bassett et al., 1990, 1999), epistatic gene interaction, or environmental effects.

Among the five morphological traits included in this study, the pod shape trait (pod cross-section) was the most difficult to classify. This was due to the presence of seemingly intermediate shapes between clearly round pod and clearly flat (fully elliptical) pod that were observed under the conditions of this study. The intermediate pod shapes were classified as flat. When 30 progeny tests (12 individuals in each) were carried out to check for misclassification in the population segregating for pod shape, three corrections were made (data not shown); however, the deviation persisted. Although flat pod vs. round pod was reported to be inherited as a single gene trait (Lamprecht, 1947; Ram and Prasad, 1985), the results of this study suggest the occurrence of epistasis in this population. Lamprecht (1932) also reported a 15:1 segregation ratio between flat (elliptical) and round pods, respectively, for which he created the two-gene model *ea eb*. The observed segregation for 45 round to 35 flat pods (Table 1) gives a perfect fit to a 9:7 ratio. We speculate that duplicate genes can each give the intermediate pod shape; and the double

Table 1. Segregation in F_2 populations for five marker gene traits (*blu*, *dgs*, *y*, *arg*, and flat pod) in common bean observed in four crosses given below.

Cross ^z	Phenotype/genotype	Phenotypic class		χ^2 3: 1	<i>P</i>
		Normal	Mutant		
<i>blu</i> BC ₁ 5-593 x <i>Blu</i> 5-593	Purple (<i>Blu</i> ⁺)/blue (<i>blu blu</i>) flowers	60	20	0.00	1.00
<i>dgs</i> BC ₁ 5-593 x <i>Dgs</i> 5-593	Normal (<i>Dgs</i> ⁺)/dark green savoy (<i>dgs dgs</i>) leaves	63	17	0.60	0.44
<i>y arg</i> BC ₂ 5-593 x <i>Y Arg</i> 5-593 ^y	Green (<i>Y</i> ⁺)/yellow (<i>y y</i>) pods	60	20	0.00	1.00
	Green (<i>Arg</i> ⁺)/silver (<i>arg arg</i>) pods	52	28	4.27	0.04
Hialeah flat pod mutant x Hialeah (round pod) ^x	Round (?)/flat (?) pods ^w	45	35	15.00	<0.001

^zThe first two crosses involve induced mutations made in Florida breeding line 7-1404, which were backcrossed into the recurrent parent, Florida breeding line 5-593. The third cross involves two natural marker traits for pod color. Hialeah is a snap bean with round pods; spontaneous mutations for flat pods occur at rates high enough to be a commercial problem for pure seed production.

^yThe phenotype of *y arg* is white pod.

^xFor the data 45, 35, the χ^2 (9:7) = 0.00, *P* = 1.00.

^wThe identity of the gene for pod shape is unknown.

Table 2. Segregation in F_2 populations for 10 RAPD markers linked to classical marker gene traits (*blu*, *dgs*, *y*, *arg*, and flat pod) in common bean.

Gene	RAPD markers	Observed frequency (presence:absence) ^z	χ^2 3:1	P
<i>blu</i>	BC412 ₁₃₅₀	63:17	0.60	0.44
<i>dgs</i>	AE18 ₉₀₀	64:16	1.07	0.30
	P7 ₁₂₅₀	61:19	0.07	0.80
	I16 ₅₀₀	68:12	4.27	0.04
<i>y</i>	BC412 ₁₇₀₀	58:22	0.27	0.61
<i>arg</i>	C16 ₁₂₅₀	56:22	0.43	0.51
	AQ15 ₆₅₀	56:22	0.43	0.51
Flat pod ^y	T15 ₅₅₀	53:27	3.27	0.07
	A14 ₈₀₀	54:26	2.40	0.12
	G8 ₁₁₅₀	51:27	3.85	0.05

^zThe band is linked to the dominant gene for each trait: presence = band is visible, absence = no band observed. The associated classical marker trait segregation is given in Table 1.

^yThe inheritance of the spontaneous flat pod mutation has not been resolved.

Fig. 1. RAPD markers linked to five genes (*blu*, *arg*, *dgs*, *y*, and the flat pod locus) in common bean. Marker names are given on the right and map distances (cM) are given on the left of each linkage group. Linkage analysis was performed using MAPMAKER v. 2.0 with the following parameters or function: LOD score of 3.0, maximum recombination fraction of 0.3 and the Kosambi function.

recessive gives the true flat pod, where variability (within the two elliptical pod shape classes) makes unambiguous classification in the field impossible. Pod shape is affected by genes for two distinct aspects of pod development: pod fleshiness and pod fiber (J. Myers, personal communication). Future investigations of mutations from round to flat pods may need to disassemble pod shape into its components, pod wall thickness and pod fiber. Also, pod shape may change with developmental stages: elliptical in immature stage, round at harvest maturity, and creaseback at physiological maturity.

An alternative hypothesis for the inheritance of the flat pod mutation assumes incomplete dominance of the round pod allele (no quantitative data were recorded for F_1 versus the parents) in combination with disturbed segregation at a single locus. The heterozygote expresses the intermediate pod shape described above. In common bean, disturbed segregation at a single locus that fit a 9:7 ratio in F_2 and was confirmed in F_3 progeny tests was previously reported (Bassett, 1993; Bassett et al., 1999). Further research is needed to determine which of the above two hypotheses is correct.

IDENTIFICATION OF RAPD MARKERS. Thirteen RAPD markers were identified by BSA in the four populations used in this study.

However, when individual genotypes were characterized, only 10 RAPD markers were linked (Table 2). One marker (BC412₁₃₅₀) was associated with *blu* at 4.3 cM, and three (P7₁₂₅₀, AE18₉₀₀, and I16₅₀₀) were associated with *dgs* at 4.1, 4.2, and 12.6 cM, respectively (Fig. 1). BC412₁₇₀₀ was associated with *y* at 7.3 cM, and AQ15₆₅₀ and C16₁₂₅₀ were associated with *arg* at 10.6 and 12.1 cM, respectively (Fig. 1). Three markers (T15₅₅₀, A14₈₀₀ and, G8₁₁₅₀) were associated with pod shape at 20.9, 27.2, and 35.7 cM, respectively (Fig. 1). All the identified linkages are in coupling phase with the dominant allele for each marker trait, as expected, due to the design of the experiment in this study, i.e., the use of F_2 segregating populations and BSA for dominant traits (Michelmore et al., 1991). Segregation ratios consistent with dominant monogenic inheritance were observed for all of the RAPD markers with the exception of I16₅₀₀ (Table 2). The consistently large chi-square values for the three markers (two nearly significant) for pod shape are probably not due to chance. They may be due to misclassification of heterozygotes as recessive phenotypes (single-gene hypothesis) or the confounding effects of epistasis (two-gene hypothesis). The RAPD marker I16₅₀₀, associated with *dgs*, displayed an excess of DNA fragments present, which might be attributed to chance.

LOCATION OF RAPD MARKERS ON A MOLECULAR LINKAGE MAP. Two RAPD markers, BC412₁₃₅₀ and I16₅₀₀, associated with *blu* and *dgs*, respectively, were found to be polymorphic between the parents of the mapping population PC-50 x XAN-159 (Jung et al., 1997). Therefore, the 70 RI lines from the cross PC-50 x XAN-159 were analyzed and scored to determine the location of BC412₁₃₅₀ and I16₅₀₀ in this mapping population. The RAPD marker BC412₁₃₅₀, associated with *blu*, was located in linkage group 1 of the PC-50 x XAN-159 map. BC412₁₃₅₀ was flanked by C7₉₀₀ at 4 cM and on the other side by X14₅₅₀ at 13 cM (Fig. 2). The RAPD marker I16₅₀₀, associated with *dgs*, was putatively located in linkage group 3 of the PC-50 x XAN-159 map (Fig. 2). I16₅₀₀ was flanked by Z19₅₅₀ at 17 cM and on the other side by *Pu-a*, the gene for abaxial leaf pubescence, at 20 cM (Jung et al., 1998).

Although a putative linkage was reported by Bassett (1992) between *blu* and *Fin* (the gene for indeterminate plant habit located in classical linkage group IV), the location of *blu* is still considered to be inconclusive because of the disturbed within-locus segregation (M.J. Bassett, personal communication), and therefore, has not been included in later revisions of the classical linkage map (Gepts et al., 1993). In this study, *blu* was located in molecular linkage group 1 on the PC-50 x XAN-159 map, which also contains a portion of the classical linkage group I. This classical linkage group contains the complex *C* locus (Bassett, 1991). If this is true, an important hypothesis, derived from the supposed linkage between *blu* and *Fin* (Bassett, 1992), can be proposed: classical linkage groups IV and I constitute parts of the same linkage group. To test this hypothesis, a second marker located in the molecular linkage group 1 should also be linked to *blu*, or vice versa. Therefore, several RAPD markers known to be located in molecular linkage group 1 were tested, including C7₉₀₀ and X14₅₅₀, which flank the marker BC412₁₃₅₀ (Fig. 2) and, hence, were more likely to be linked to *blu*. However, none of them showed polymorphism in the population segregating for *blu*. Only the marker BC412₁₃₅₀ was linked to *blu*, so that no other markers could be tested in the mapping population PC-50 x XAN-159. As a result, the location of *blu* in molecular linkage group 1, and consequently in the classical linkage group I, remains speculative until this hypothesis can be tested further.

According to Bassett (1991), *dgs* is located in the classical

Fig. 2. Putative location of *blu* and *dgs* genes in linkage groups 1 and 3 of the previously constructed PC-50 x XAN-159 molecular linkage map in common bean (Jung et al., 1997). A and C are linkage groups from Fig. 1. Marker names are given on the right and map distances (cM) are given on the left of each linkage group. *Pu-a* and *C* are genes for abaxial leaf pubescence and seed coat color, respectively. Linkage analysis was performed using MAPMAKER v. 2.0 with the following parameters or function: LOD score of 3.0, maximum recombination fraction of 0.3 and the Kosambi function.

linkage group VI, which includes other gene markers, such as *y* (Awuma and Bassett, 1988). In our present study, *dgs* is putatively located in the linkage group 3 of the PC-50 x XAN-159 map. If this hypothesis is true, molecular linkage group 3 would correspond with classical linkage group VI. Following the same reasoning used to test the hypothetical location of *blu*, several RAPD markers known to be located on molecular linkage map 3 were tested, including Z19₅₅₀ and G3₁₁₅₀ (Fig. 2), for polymorphism in the population segregating for *dgs*. In the same way, the other two RAPD markers found to be linked to *dgs* (P7₁₂₅₀ and AE18₉₀₀) were tested for polymorphism in the mapping population PC-50 x XAN-159. However, none were polymorphic in either population, leaving the hypothetical location of *dgs* in the molecular linkage group 3 unproven. Consequently, the equivalence between this linkage group and the classical linkage group VI can only be regarded as inconclusive.

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