RFLP Mapping in Soybean: Association Between Marker Loci and Variation in Quantitative Traits

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Manuscript received March 26, 1990
Accepted for publication July 21, 1990

ABSTRACT

We have constructed a genetic map for soybean and identified associations between genetic markers and quantitative trait loci. One-hundred-fifty restriction fragment length polymorphisms (RFLPs) were used to identify genetic linkages in an F_2 segregating population from an interspecific cross (*Glycine max* × *Glycine soja*). Twenty-six genetic linkage groups containing *ca.* 1200 recombination units are reported. Progeny-testing of F_2-derived families allowed quantitative traits to be evaluated in replicated field trials. Genomic regions, which accounted for a portion of the genetic variation (R^2 = 16 to 24%) in several reproductive and morphological traits, were linked to RFLP markers. Significant associations between RFLP markers and quantitative trait loci were detected for eight of nine traits evaluated. The ability to identify genes within a continuously varying trait has important consequences for plant breeding and for understanding evolutionary processes.

GENETIC linkage of molecular markers to quantitative trait loci (QTLs) has been used to identify individual polygenes and to characterize their genetic action (NIENHUIS *et al.* 1987; OSBORN, ALEXANDER and WILLIAMS 1987; EDWARDS, STUBER and WENDEL 1987; TANKSLEY and HEWITT 1988; BURR, HELENTJARIS and TANKSLEY 1989). As early as 1923, SAX (1923) used a seed-coat color locus to predict variation in a quantitative trait, seed size. Only with a large number of genetic markers, however, can researchers routinely identify QTLs in populations. Morphological markers are too rare for such studies and may vary with the environment. Restriction fragment length polymorphisms (RFLPs) have the potential to "saturate" the genome which will increase the probability of QTL detection.

Soybean [*Glycine max* (L.) Merr.] genetics has developed slowly due to the inherent difficulties in performing crosses, a lack of genetic variation in the germplasm, and a lack of cytogenetic markers. Currently the classical soybean genetic linkage map contains 49 linked markers and covers 530 centimorgans (PALMER and KIANG 1990). In contrast, the maize genetic map contained about 60 linked markers and covered over 700 centimorgans in 1935 (EMERSON, BEADLE and FRAZIER 1935). Early success in maize genetics was largely due to the concentrated effort of a large number of researchers and has been duplicated in only a small number of easily manipulated experimental organisms (*e.g.* Drosophila). In soybean, and many other species, it is doubtful that traditional genetic approaches will generate a detailed genetic map. Molecular markers provide an important genetic tool where traditional genetic studies have been difficult.

If a large number of RFLP markers can be identified in a single segregating population it facilitates establishing genetic linkage among markers and identifying linkage between markers and QTLs. Previous studies in *G. max* have encountered low levels of restriction site polymorphism (DOYLE and BEACHY 1985; APUYA *et al.* 1988; DOYLE 1988), which prevented extensive genetic mapping. A survey of 58 wild and cultivated soybean accessions from the subgenus Soja, however, identified genetically diverse genotypes (KEIM, SHOEMAKER and PALMER 1989).

Glycine soja (Seib. and Zucc.) is a wild relative of the domesticated *G. max* and is considered the progenitor of the domesticated species (HYMOWITZ and SINGH 1987). *G. soja* is interfertile with *G. max* and represents a potential genetic resource for plant breeders. In a crop species with limited genetic variability, such as soybean (DELANNAY, RODGERS and PALMER 1983; SPECHT and WILLIAMS 1984; KEIM, SHOEMAKER and PALMER 1989), wild germplasm becomes valuable as a source of genes not present in domestic germplasm. Unfortunately, *G. soja* also has many undesirable agronomic traits (*e.g.* lodging) to be avoided during introgression of desirable genes. In plant breeding, an understanding of undesirable characters is as important as that of desirable traits.

In this study, a segregating population was derived from an interspecific cross between *G. max* and *G.*

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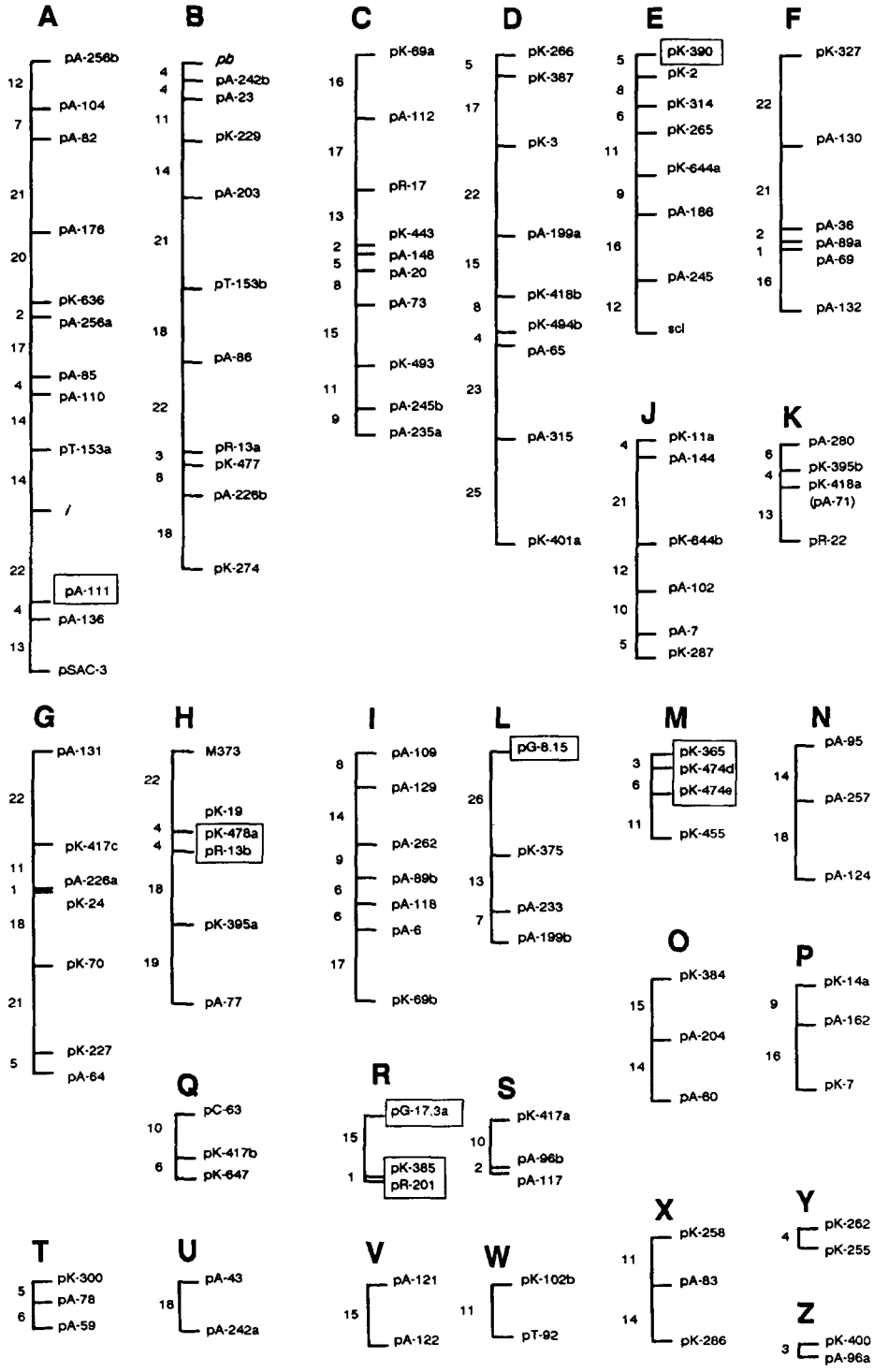

FIGURE 1.—Soybean RFLP genetic linkage map. One thousand two hundred map units are present on 26 linkage groups. Markers predicting significant variation for quantitative traits are boxed (see Table 2).

soja. This population was selected for its diversity at the molecular level (RFLPs) and at the phenotypic level. Our goals were to construct a molecular genetic linkage map and to identify associations between molecular markers and quantitative trait variation.

MATERIALS AND METHODS

Germplasm: The F_2 study population was constructed by W. R. FEHR (Iowa State University) from two parental lines: *G. max* (A81-356022, an Iowa State University breeding line) and a *G. soja* accession (PI 468.916). These parents were chosen for their genotypic diversity (KEIM, SHOEMAKER and PALMER 1989), phenotypic differences (CARPENTER and FEHR 1986; CIANZIO and FEHR 1987; GRAEF, FEHR and CIANZIO 1989) and lack of chromosomal translocations (PALMER *et al.* 1987).

Genetic markers: One-hundred-fifty RFLP differences between the *G. max* and *G. soja* parental lines were detected using recombinant DNA clones primarily from a random *Pst*I genomic library (KEIM and SHOEMAKER 1988). Parental DNA digested with *Eco*RI, *Eco*RV, *Dra*I, *Taq*I, *Hin*dIII or *Xba*I were screened by molecular hybridization with each

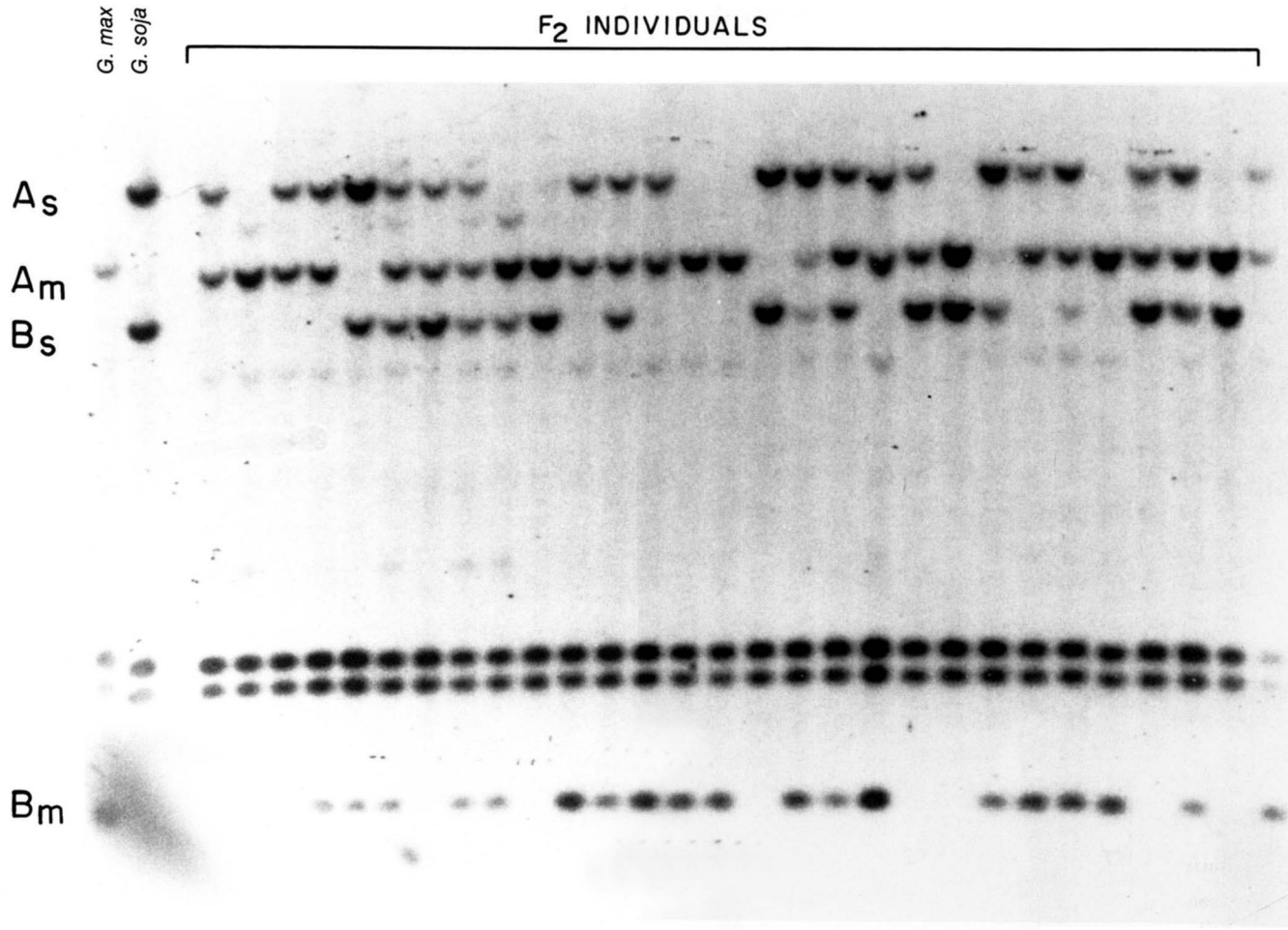

FIGURE 2.—The independent segregation of two loci detected with one probe. DNA from F_2 individuals, digested with the restriction enzyme *Hin*dIII, was probed with pA-199. Allelic combinations of restriction fragments are indicated for each locus (A_s = 4.8 kb, A_m = 2.7 kb, B_s = 1.9 kb, B_m = 0.3 kb).

probe. The random DNA probes pG-17.3, pG-8.15 and M373 were provided by K. G. LARK (University of Utah), and their isolation has been described (APUYA *et al.* 1988). The actin gene probe (pSAC-3) was kindly provided by RICHARD MEAGHER (University of Georgia). Probes designated pT, pR and pC were also from the random *Pst*I library (KEIM and SHOEMAKER 1988), though they were developed and kindly provided by K. G. LARK. The hybridization protocol has already been described (APUYA *et al.* 1988; KEIM, SHOEMAKER and PALMER 1989). Three morphological markers were also evaluated: seed coat color (*i*), pubescent tip (*pb*) (PALMER and KILEN 1987), and seed coat luster (scl). An official gene symbol has yet to be assigned for seed coat luster.

Experimental design: The 60 genotypes used in this study were F_2 plants derived from the *G. max* × *G. soja* cross. DNA from these individuals was extracted according to the procedure of KEIM, OLSON and SHOEMAKER (1988). F_3 progeny derived from each of the 60 F_2 plants were used to measure quantitative traits. F_2 derived lines were grown in plots 1.5 meters long at 33 seeds per meter. Plots were separated by 1.2 meters (within rows) with 1-meter spacing between rows. Entries were arranged in a randomized complete-block design with two replications at each of three unique locations near Ames, Iowa. Because maturity was segregating in this population, planting dates for each location were different: May 1, May 15 and May 29 of 1988.

Quantitative traits: Vegetative and reproductive stages in soybean have been described by FEHR and CAVINESS (1977). Using this study as a guide, we measured the traits on each plot as follows. R1 was the number of days after June 31 when 50% of the individuals in a plot had at least one flower. R8 was the number of days after August 31 when 50% of the individuals in a plot had mature seed-pod color (95% of the pods per plant). Seed-fill length was the number of days between R1 and R8. Six fully expanded leaflets (randomly chosen) per plot were measured at their widest and longest points to determine average leaflet length and width per plot. Stem diameters were measured with calipers midway between the unifoliolate and the first trifoliolate nodes for three mature plants per plot. To determine canopy height, a wooden lath was placed on each plot and used to measure the average height above the ground where the lath was supported. Stem length was defined as the distance from soil surface to the uppermost node with a pod on the main stem. Internode length was defined as the distance between the unifoliolate and the third trifoliolate nodes. Upon maturity, stem and internode lengths were determined on three plants per plot.

Linkage analysis: Genetic relations among markers were determined by maximum likelihood analysis of segregation patterns in the F_2 population. The computer program "Map

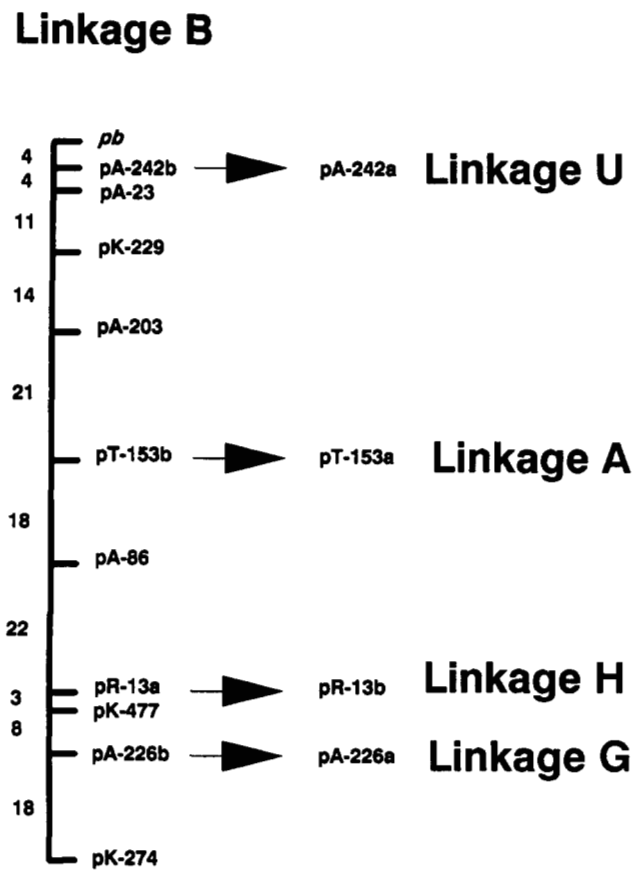

FIGURE 3.—Duplicate loci on linkage group B. Linked loci in linkage group B do not have linked duplicates in other linkage groups.

Maker" (LANDER *et al.* 1987) was used to analyze these data and establish the "best" order of loci. The statistical evidence in favor of the "best" map was summarized as a lod score ($\log_{10}$ of the odds ratio). A minimum lod score of 3.0 (except of markers pA-203 and pT-153b where lod = 2.8) was used for the pairwise linkage analysis. Lod scores for the combined markers of different linkage groups ranged from 3 to 20.

Statistical analysis of quantitative data: The statistical program "General Linear Models (PC-SAS)" was used for quantitative data analysis. Initially, quantitative data was analyzed using standard analysis of variance (ANOVA) methods for a randomized-complete-block design experiment. Variance was partitioned according to experimental factors including location, replication within locations, genotypes (means of the F_2-derived families), and genotype-by-location interactions. Both locations and replications were considered random effects. All traits had significant genotypic effects and were further analyzed on an individual marker basis. Marker-genotypes classes (homozygous *G. soja*, heterozygous, homozygous *G. max*) were used to sort the F_2 -derived lines so that one-way ANOVAs could be performed on the quantitative data. The *F* statistic was used to determine if the means of different marker-genotype classes were significantly different ($P < 0.01$). Variation explained by markers was described by using the R^2 value which is the proportion of the total variance among the 60 lines' means explained by the marker-genotype classes. The 9 traits were sorted and analyzed for 150 markers.

Because 150 one-way ANOVAs were performed for each trait, a great probability for type I errors exists. Therefore, only those associations significant at $P < 0.01$ are reported here. Even when a significance level of $P < 0.01$ is used, however, there is still a risk of concluding a marker is linked to a QTL although it is not. Whereas, when a lower significance level is used important loci may be overlooked. Assuming a genome size of 1.2 Morgans and 150 markers, we have calculated using the procedures of LANDER and BOTSEIN (1989) that for any trait, the probability of a false QTL association is near 1.0 at the significance level of $P < 0.05$. The probability decreases to 0.78, 0.14 and 0.01 for $P < 0.01$, 0.001 and 0.0001, respectively. This study constitutes only the initial identification of QTLs, and markers significant near the $P < 0.01$ level will require further confirmation.

RESULTS

The frequency of polymorphisms observed in this population was greater than that observed in previous studies of soybean RFLP markers. Over 500 random genomic clones were used as probes to screen restriction digested parental DNAs (five different restriction enzymes). Some 400 of these proved to be low copy DNA sequences (KEIM and SHOEMAKER 1988) of which *ca.* 40% detected polymorphisms between the *G. soja* accession (PI468.916) and the *G. max* line (A81-356022). This is a twofold increase over the frequency observed between two *G. max* Plant Introductions used previously (APUYA *et al.* 1988). Seemingly, a large number of RFLP markers identified in this study resulted from DNA rearrangements since about half of the variable probes detected polymorphisms with two or more restriction enzymes. DNA rearrangements have been found to be the most plausible mechanism causing a polymorphism to be detected by multiple enzymes and this has been substantiated by restriction mapping of polymorphic regions (APUYA *et al.* 1988). RFLP markers where the heterozygote and one of the homozygotes cannot be distinguished (similar to dominance in classical markers) account for 10% of the polymorphisms between these two lines. These "dominant" RFLP markers could have been caused by sequence deletions in one parent, and thus, also by DNA rearrangements.

A genetic map was constructed from the 150 segregating markers. One hundred thirty markers (20 markers remain unlinked) were genetically linked to form 26 linkage groups (Figure 1). Redundant linkage groups must exist in our map because the haploid chromosome number of *G. max* is known to be 20 (HYMOWITZ and SINGH 1987). Because of this, the RFLP linkage group letter designations are tentative and will change with further study. Currently, the classical genetic map consists of 17 linkage groups (PALMER and KIANG 1990), the majority of which are two-point linkages. Many of these two-point linkages may be present on the same chromosome, but have not been connected either because the appropriate populations have not been analyzed, or because large

distances separate them. The RFLP map reported here consists of *ca.* 1200 centimorgans, in contrast to the *ca.* 530 centimorgans present in the previous map (PALMER and KILEN 1987). In conjunction with RFLP markers, several segregating morphological markers were evaluated in the F_2 population. In two instances, these markers were present in both classical and RFLP genetic maps. The pubescence marker *pb* occurred in the RFLP linkage group "B," and in the classical linkage group 14. The seed coat marker *i* was in RFLP linkage group "A" and the classical linkage group 7. Integration of the remaining genetic linkage groups will require segregation analysis in other populations, the use of aneuploid lines (HELENTJARIS, WEBER and WRIGHT 1986), or the use of near-isogenic lines (MUEHLBAUER *et al.* 1988).

Some DNA probes detected two RFLP loci segregating independently in the F_2 population. Segregation of two loci detected with probe pA-199 is illustrated in Figure 2. Duplicate loci detected with probe pG-17.3 (previously described) were attributed to an ancestral tetraploidy state for soybean (APUYA *et al.* 1988). Almost all probes used in this study detected multiple fragments, but multiple polymorphic fragments existed in only 23 of the 104 probes (127 linked RFLP markers). An example of nonpolymorphic fragments can also be observed in Figure 2. Duplicate markers are indicated in Figure 1 by a suffix letter designation (*e.g.*, pT-153*a*). In most instances (with the exception of pA-256a and b, and pK474e and d), the duplicate RFLP markers occur in independent linkage groups. In maize studies, similar observations have been attributed to the existence of ancient homoeologous chromosomes (HELENTJARIS, WEBER and WRIGHT 1988). In soybean, however, we have found that duplicate loci linked in one group do not occur again in another single linkage group. Figure 3 illustrates an instance in which none of the duplicate loci from linkage group "B" formed another homoeologous group. Six other examples of linked duplicate loci exist in the RFLP genetic map, and none of these seem to define a second homoeologous group (Figure 1).

NIENHUIS *et al.* (1987) reported a skewed distribution of segregating alleles that favored the wild parent in an interspecific cross of tomato (*Lycopersicon hirsutum* × *Lycopersicon esculentum*). This result has been attributed to gametic selection which favored the wild accession over the cultivated line (ZAMIR, TANKSLEY and JONES 1982). In this study, only 20 out of 150 markers deviated significantly (chi-square test, $P < 0.05$, data not shown) from the expected ratios, and these deviations were in both the *G. max* and the *G. soja* directions. Thus, the genetic distortions noted in other studies (NIENHUIS *et al.* 1987) do not exist in this soybean F_2 population (Figure 4).

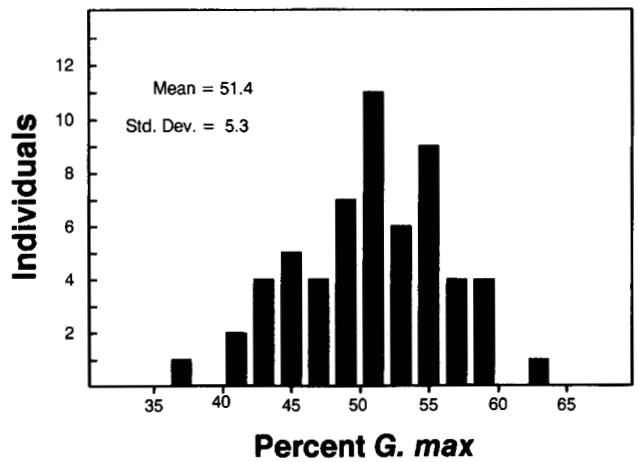

FIGURE 4.—Genotypic composition of F_2 individuals. The genotype composition of an individual was determined by averaging all the markers for each individual.

TABLE 1

Trait variation due to experimental factors

Traits	R^2 (%)		
	Genotype	Location	G*L
R1	56**	30**	7*
R8	83**	4**	8**
Seed-fill (R1 to R8)	53**	20**	12*
Stem diameter	54**	2**	21*
Stem length	25**	NS	3**
Canopy height	64**	2**	NS
Leaf width	79**	5*	7**
Leaf length	58**	22**	10**

NS = not sigificant, (*) $P < 0.05$, (**) $P < 0.01$. Replications within location were significantly only for leaf width, and leaf length.

Significant genotypic variation was present in all traits measured. Twenty-five to 83% of the variation (R^2) in our study was explained by genotypic effects (Table 1). Location and genotype-by-location interactions were significant for most traits but accounted for a lesser amount of the variation than did the genotypic effect. Segregation within F_2-derived lines could hinder the accurate evaluation of a trait. The lack of significant replication effects (except for leaf length and width, where less than the full plot was used for evaluation, see MATERIALS AND METHODS) suggests that an each plot contained enough individuals to avoid errors caused by within-family segregation.

Genomic regions important in determining quantitative variation were identified in 8 of 9 traits. Markers in these regions were significantly ($P < 0.01$, Table 2) associated with a portion of the phenotypic variation. No significant associations were detected for internode length. Table 2 lists the 20 different RFLP marker associations with traits that were significant. The variation explained by each marker was greater than 16% (R^2). In several instances the markers asso-

TABLE 2

Markers that detect significant phenotypic variation

Trait	Marker	R^2	$P <$	Linkage group
Leaf width	pA-111	0.24	0.0009	A
	pK-390	0.17	0.006	E
	pK-411	0.16	0.008	Unk.
Leaf length	pR-13b	0.18	0.005	H
	pK-478a	0.19	0.003	H
Stem diameter	pG-17.3a	0.24	0.0006	R
	pK-385	0.17	0.006	R
	pR-201	0.17	0.006	R
Canopy height	pK-390	0.16	0.009	E
	pR-13b	0.20	0.002	H
Stem length	pK-18	0.19	0.004	Unk.
First flower (R1)	pK-365	0.21	0.001	M
	pK-474a	0.23	0.0008	M
	pK-474b	0.21	0.002	M
Seed pod maturity (R8)	pK-472	0.18	0.004	Unk.
	pR-13b	0.18	0.004	H
	pK-365	0.17	0.006	M
	pK-474a	0.18	0.004	M
	pK-474b	0.21	0.002	M
Seed-fill (R1 to R8)	pG-8.15	0.18	0.006	L

R^2 = The amount of variation explained. Unk. = unknown.

ciated with a trait were genetically linked (see Table 2) and, therefore, may have been detecting the same QTL or a cluster of QTLs. Linkage group "R" contains at least one stem diameter QTL which was significantly associated with all three markers in that linkage group. Likewise, linkage groups "M" and "H" contained several markers significant for R1, R8, and leaf length (Table 2).

Several markers were significantly associated with more than one trait. Marker pK-390 was associated with two vegetative traits (leaf width and canopy height); whereas marker pR-13b was associated with leaf length, canopy height (vegetative traits), and reproductive stage R8. Linkage group "M" contains gene(s) for both the R1 and R8 reproductive stages (Table 2). None of the markers associated with R1 or R8 proved significant predictors of the seed-filling period. A marker on linkage group "L," however, predicted 18% of the variation in the seed-fill period (Table 2).

DISCUSSION

RFLPs have been used in this study to mark regions of the genome and then to partition the variation of quantitatively inherited traits. Because of the relatively small population size (60 genotypes), only genomic regions responsible for large portions of the variation could be detected. In eight out of nine traits analyzed, however, there were genomic regions accounting for more than 16% of the variation (Table 2). Markers that detect large portions of the variation within the continuous phenotypic variation seems to be generally true of the traits studied. Mutations in a few major genes have been postulated as being important for the domestication of crop species (SIMMONDS 1976). QTLs observed in our population would be a reflection of the parental cross where a domesticated soybean line (*G. max*) was mated with its wild progenitor (*G. soja*). Genetic mapping of genes selected during soybean domestication will facilitate the use of *G. soja* as a genetic resource.

Some markers were significantly associated with variation in more than one trait. This phenomenon was observed between reproductive stages (pK-365), between vegetative traits (pK-390), and also between vegetative and reproductive traits (pR-13b). A developmental sequence in soybean may interrelate both vegetative and reproductive traits. For example, vegetative growth precedes reproduction, and when flowering commences, vegetative growth is curtailed. A single QTL could influence both vegetative and reproductive traits simply by controlling flowering time (R1). It is also possible that a regulatory gene (*e.g.*, phytochrome) could control different traits in a more direct fashion. In contrast to these pleiotropic examples, genomic regions may contain multiple QTLs that affect different traits.

Evaluation of quantitative traits in molecular marker studies has involved two strategies. One method has been to measure the traits on the same plants that have been evaluated with markers. Typically, thousands of individuals are used in order to estimate accurately the effect of QTLs upon the traits (EDWARDS, STUBER and WENDEL 1987; STUBER, EDWARDS and WENDEL 1987). A second strategy has involved the use of progeny testing (COWEN 1988; LANDER and BOTSEIN 1989) in which fewer individuals are genotyped, and traits are measured on their progeny (from either self-fertilization or crossing to a tester line) in replicated trials. We have demonstrated that even with a relatively small population, progeny testing can be used to identify major quantitative genes. Often, the expense of genotyping thousands of individuals will prohibit QTL studies. If the goal, however, is to identify the genes having a major effect on a trait, smaller and less expensive studies can still be fruitful.

Caution must be used in extrapolating the results from this study to cultivated soybean. First, it has been observed that recombination distances between genetic markers are less in *G. max* × *G. soja* than in *G. max* × *G. soja* populations (GRIFFIN and PALMER 1987). If this is generally true, the recombinational genome size could be much greater in cultivated soybean. What appears to be a "saturated" map in one population may not be in another, though the ar-

rangement of markers should be the same. Secondly, the importance of particular QTLs will change in different populations (TANKSLEY and HEWITT 1988). A marker significantly associated with trait variation in this study need not be significant in adapted germplasm. The advantages of using a diverse cross for RFLP are many, but in some instances the results may only apply to this population.

Duplicate loci have been cited as evidence of a tetraploid state in a diploid organism's evolutionary past (HELENTJARIS, WEBER and WRIGHT 1988; WENDEL *et al.* 1989). Many of soybean's qualitative traits segregate as if controlled by a single locus, but there are some examples of traits controlled by two loci (as expected from a tetraploid, PALMER and KILEN 1987). Unlike maize (HELENTJARIS, WEBER and WRIGHT 1988), duplicate soybean RFLP loci fall into a pattern inconsistent with a tetraploidy origin (Figure 3). This is evidence that the duplications we observe may originate by mechanisms other than polyploidy. For example, one set of duplicate RFLP loci reside on the same linkage group (Figure 2, pK-474d,e: linkage "M"). This would be consistent with a "gene cluster" such as that observed in mammalian globin genes (MANIATIS *et al.* 1980). Low-copy mobile elements (*e.g.*, *Tgm*, RHODES and VODKIN 1988) are another example of duplicated sequences such as those observed in this study. Mobile elements generate RFLPs and, therefore, could have been preferentially selected during this study. Alternatively, these duplications could have been generated by chromosome doubling followed by chromosome "scrambling" via translocations. We have noted that relatively few probes detect duplicate polymorphic loci, but essentially all probes hybridize to multiple DNA fragments. These nonpolymorphic fragments could represent homeologous loci, but obtaining more information about them is difficult. Reconciling our results with those expected in a tetraploid will require more examples of duplicate markers.

As in other systems (*e.g.*, maize and tomato), molecular markers have provided a tool for greatly enhancing genetic studies. We have described a soybean molecular genetic map encompassing more markers and more map units than has been described using classical markers. The probes used in this study are available to both public and private institutions. With the construction of this map, a research tool is available for efficiently carrying out genetic studies such as those for the identification of QTLs.

We wish to thank KARL G. LARK, RICHARD MEAGHER and WALTER FEHR for providing probes or germplasm for this study. This is Journal Paper No. J-13956 of the Iowa Agriculture and Home Economics Experiment Station, Ames Iowa. Project No. 2763. The mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture or Iowa State University and does not imply its approval to the exclusion of other products that may be suitable. This work was supported, in part, by Iowa State Biotechnology grant No. 480-46-09.

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Communicating editor: A. H. D. BROWN