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P. J. Maughan · M. A. Saghai Maroof · G. R. Buss

Molecular-marker analysis of seed-weight: genomic locations, gene action, and evidence for orthologous evolution among three legume species

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Abstract The objectives of this study were to use molecular markers to: (1) identify quantitative trait loci (QTL) controlling seed-weight in soybean, (2) characterize the genetic basis of seed-weight expression, and (3) determine whether soybean shares orthologous seed-weight genes with cowpea and/or mung bean. An F_2 population was developed between a large-seeded *Glycine max* breeding line and a small-seeded *G. soja* plant introduction. DNA samples from 150 F_2 individuals were analyzed with 91 polymorphic genetic markers, including RFLPs, RAPDs and SSRs. Seed-weight was analyzed by randomly sampling 100 seeds from each of 150 greenhouse-grown F_2 individuals, and their 150 $F_{2:3}$ lines, from a replicated field trial. Markers associated with seed-weight were identified using the computer program MapMaker-QTL and a one-way analysis of variance. Three and five markers were significantly associated with seed-weight variation ($P<0.01$) in the F_2 and $F_{2:3}$ generations, respectively. Tests for digenic epistasis revealed three significant interactions in both generations. In a combined analysis, these markers and interactions explained 50 and 60% of the phenotypic variation for seed-weight in the F_2 and $F_{2:3}$ generations, respectively. Comparison of our results in soybean (*Glycine*) with those previously reported in cowpea and mung bean (*Vigna*) indicated that soybean and cowpea share an orthologous seed-weight gene. In both species, a genomic region significantly associated with seed-weight spanned the same RFLP markers in the same linkage order. A significant digenic interaction involving this genomic region was conserved in all three species. These results suggest that the exploitation of "comparative QTL mapping" is an invaluable tool for quantitative geneticists working with poorly characterized plant systems.

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P. J. Maughan · M. A. Saghai Maroof (✉) · G. R. Buss
Department of Crop and Soil Environmental Sciences,
Virginia Polytechnic Institute and State University,
Blacksburg, VA 24061, USA

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Introduction

In soybean (*Glycine max*), and other plant species, the majority of economically important agronomic characteristics are controlled in a quantitative fashion. Until recently, plant breeders have relied on phenotypic selection methods to improve specific quantitative traits. Due to the effects of the environment on these traits, such methods can be expensive, time consuming and labor intensive. Recent advances in molecular genetics, in particular the advent of restriction fragment length polymorphism (RFLP) technology, have made possible the genetic dissection of many of these recalcitrant agronomic traits (Stuber 1992). RFLP technology has been successfully used in the study of a wide array of quantitative traits, including fruit characteristics in tomato (Patterson et al. 1991), heterosis in maize (Stuber et al. 1992), and quantitative disease resistance in potato (Kreike et al. 1993) and soybean (Concibido et al. 1994).

Seed-weight, measured as mass per seed, is an important yield component in soybean (Burton 1987). Seed-weight in soybean (*G. max*) is polygenically controlled and can range from 6 to 55 grams per 100 seeds. Soybean cultivars with either very small (<8 g/100 seeds) or very large (>20 g/100 seeds) seed sizes are used directly in the production of many oriental specialty food items, including tofu, natto, and miso. The demand for these "food quality" soybeans in the global market is steadily increasing at a rate of 3–5% per annum (Griffis and Wiedermann 1990). Soybean cultivars with these desired seed sizes are limited in number and generally unadapted to U.S. growing areas. The increasing interest in adapted soybean cultivars that fit into this specific market has made breeding for seed-weight a major objective of some breeding programs. Plant breeders have been successful in manipulating this trait, but the underlying genetic basis for seed-weight inheri-

tance has not been elucidated. Information on the association between genetic markers and seed-weight should help breeders construct beneficial allelic combinations and accelerate the development of specialty cultivars.

RFLP mapping has previously been used to analyze seed-weight in a variety of crop species. Most recently, the inheritance of kernel weight in two maize-teosinte hybrid populations was investigated by Doebley et al. (1994). In the two populations studied, they detected multiple quantitative trait loci (QTLs) explaining approximately 70% of the phenotypic variance for kernel weight. In soybean, Mansur et al. (1993) utilized 154 genetic markers and detected a single QTL explaining 13% of the phenotypic variation for seed-weight. Fatokun et al. (1992) identified two and four QTLs explaining 53 and 50% of the variation for seed-weight in cowpea and mung bean, respectively. Interestingly, in both cowpea and mung bean, the genomic region with the greatest effect on seed-weight spanned the same RFLP markers in the same linkage order, suggesting that this genomic region has been conserved through the evolution of these two species. Although soybean belongs to a different genus (*Glycine*) than cowpea and mung bean (*Vigna*), all three species are from the tribe Phaseoleae of the family Leguminosae (Smartt 1990). Thus, it is possible that the three species share orthologous seed-weight genes (i.e., genes related by descent from a common ancestor). The purpose of this research was to use genetic markers to: (1) identify quantitative trait loci for seed-weight in soybean, (2) characterize the genetic basis of seed-weight expression in soybean, and (3) to determine whether soybean shares orthologous seed-weight QTLs with cowpea and/or mung bean.

Materials and methods

Genetic materials

An F_2 population with a high level of genetic diversity for seed-weight was developed from an interspecific hybridization between an adapted *G. max* breeding line (V71-370) with large seeds (24 g/100 seeds) and a *G. soja* plant introduction (PI407.162) with extremely small seeds (1.5 g/100 seeds). One-hundred and fifty F_2 plants were grown under normal greenhouse conditions at Virginia Polytechnic Institute and State University. Sixty $F_{2:3}$ seeds from individual F_2 plants and both parents were scarified, divided into two lots, and planted in a randomized complete block design with two replications at the Virginia Crop Improvement Association's seed farm near Mt. Holly, Va. $F_{2:3}$ lines were planted in plots 4 ft long with 7.5 ft between rows. Since plants within $F_{2:3}$ lines were expected to segregate for maturity, and shattering was also expected, $F_{2:3}$ lines were harvested in bulk and dried in large open grain bags when 90% of the plants in the plot reached maturity. After drying, each plot was threshed separately to produce $F_{2:4}$ seed.

RFLP analysis

DNA was isolated from leaf tissue of parental, F_1, and 150 F_2 plants as previously described (Saghai Maroof et al. 1984). RFLP procedures were as described by Zhang et al. (1993). Briefly, 8 μg of plant DNA were digested with one of five restriction enzymes (*BamH*I, *Dra*I, *Eco*RI, *Eco*RV and *Hin*dIII) and electrophoresed on 0.8% agarose gels, followed by standard DNA transfer to nylon membranes via Southern blotting. Southern blots were hybridized overnight with randomly primed ^{32}P-dCTP-labelled probes. Following hybridization, blots were washed four times, twice for 5 min at room temperature with 1×SSC and 0.1% SDS and twice for 10 min at 65°C with 0.1×SSC and 0.1% SDS. After washing, blots were immediately covered with plastic wrap and exposed to X-ray film for 3–5 days.

DNA clones

Two sets of DNA clones derived from three separate *Pst*I genomic DNA libraries were used in this experiment. The first set, consisting of 170 soybean clones, was selected based on the position of the clones in the current USDA-ISU soybean genetic map (Diers et al. 1992) to provide maximum genome coverage. The second or "comparative QTL mapping" set was selected based on previous reports placing them in the vicinity of putative QTLs controlling seed-weight in cowpea and/or mung bean (Fatokun et al. 1992). This set included 11 soybean clones, five mung bean clones, and one cowpea clone. DNA clones were generously provided by R. C. Shoemaker (USDA-ARS, Iowa State University, Ames) and N. D. Young (University of Minnesota, St. Paul, Minn.). A total of 84 clones, out of the 181 tested, were polymorphic between the parents of this cross. Clones derived from soybean, cowpea and mung bean genomic libraries will be designated with the prefix sg, cg, or mg, respectively.

SSR and RAPD assays

In addition to screening for RFLPs, a set of 40 randomly amplified polymorphic DNA (RAPD) primers (Operon kits B and O; Alameda, Calif.) and eight simple sequence repeat (SSR or microsatellite) primers were screened for polymorphism according to the procedures outlined by Williams et al. (1990) and Maughan et al. (1995), respectively. Of the 40 RAPD primers screened, three detected reproducible polymorphic markers. RAPD markers will be given the prefix R, followed by the Operon kit designation (B or O), followed by the primer number. Of the eight SSR markers screened, four have been described by Akkaya et al. (1992) and four by Yu et al. (1994). Four SSR markers were polymorphic between the parents of this cross. Thus, a total of 91 polymorphic genetic markers, including RFLPs, SSRs and RAPDs, were assayed for linkage relationships with seed-weight in this interspecific soybean population.

Data analysis and QTL mapping

Seed-weights for two generations (F_2 and $F_{2:3}$) were determined by randomly sampling 100 seeds from each F_2 plant and each $F_{2:3}$ family. Trait means, normality, parent-offspring correlations, and analysis of variance F-tests were determined using the computer program SAS (Statistical Analysis Systems, Cary, N. C.). Heritability in standard units was calculated using the correlation coefficient, adjusted for inbreeding (Frey and Horner 1957; Smith and Kinman 1965). Segregation ratios for each marker in the F_2 population were tested for "goodness of fit" to a 1:2:1 or 3:1 ratio using the computer program Linkage-1 (Suiter et al. 1983). The most probable order and map distances among the genetic markers were determined by multiple linkage analysis using the computer program MapMaker 2.0 at LOD = 3.0 (logarithm to the base 10 of the likelihood odds ratio) and with a maximum Haldane distance of 50 centiMorgans (Lander et al. 1987). Molecular marker-QTL associations were analyzed using the computer program MapMaker-QTL 1.1b (Lander and Botstein 1989) and an analysis of variance (see Keim et al. 1990). Only highly significant F-values ($P<0.01$) and/or LOD scores (>2.0) were interpreted to indicate co-segregation of putative QTLs for seed-weight and genetic markers. Two-way analyses of variance were used to test for digenic interactions between markers significantly associated with seed-weight and all other unlinked marker loci. Significant marker loci and interactions were then combined in a multivariate linear regression model to determine their combined effect.

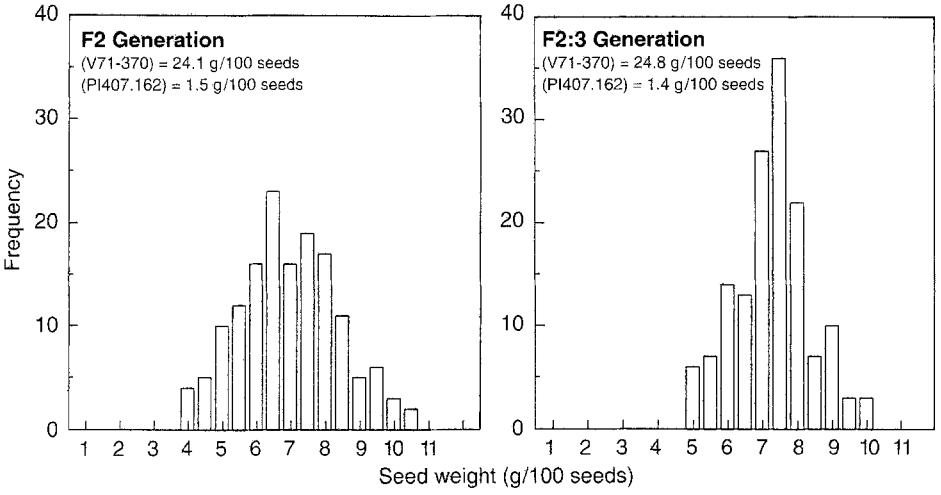

Fig. 1 Frequency distribution of 150 F_2 (*open bars*) and $F_{2:3}$ (*filled bars*) plants for 100 seed-weight. Both generations exhibited approximately normal distributions. Mean seed-weight and expected midparental values for the F_2 and $F_{2:3}$ generations were 6.7 and 7.1 g/100 seeds and 12.8 and 13.1 g/100 seeds, respectively. Mean seed-weight of the parents (V71-370 and PI407.162), the F_2 population and the $F_{2:3}$ population are shown in parentheses

Since multiple one-way ANOVAs were performed on the same data set, there is a large risk of committing a type-I error (P=0.60). Lowering the P-value required for exclusion, however, creates the possibility of missing a weak linkage to an important QTL. In this study, only highly significant F-values (P<0.01) are interpreted to indicate the significant association of a molecular marker locus and a seed-weight QTL. We attempt to confirm our QTL mapping results by using data sets generated from two generations (F_2 and $F_{2:3}$) of the same population grown in two different environments (greenhouse vs field).

Results

Phenotypic analysis

Analysis of seed-weight distribution in the F_2 and $F_{2:3}$ generations showed continuous variation (Fig. 1). Seed-weight ranged from 3.6 to 10.4 g/100 seeds in the F_2 generation and from 4.6 to 10.0 g/100 seeds in the $F_{2:3}$ generation. Seed-weights equal to that of the parents were not observed in either generation. The means of the F_2 and $F_{2:3}$ generations were 6.7 and 7.1 g/100 seeds, respectively. The expected midparental values for the F_2 and $F_{2:3}$ generations were 12.8 and 13.1 g/100 seeds, respectively. In both generations the population means were closer to the weight of the small-seeded parent (Fig. 1). A Shapiro-Wilks test for normality, as required by MapMaker-QTL and ANOVA, indicated that seed-weight was normally distributed in both generations (P>0.10). Standard unit heritability, calculated from the parent-offspring correlation and adjusted for inbreeding, was 0.54 and is in the range of previous heritability estimates for seed-weight in soybean (Burton 1987). Seed-weights from the F_2 and $F_{2:3}$ generations were highly correlated (r=0.81).

QTL analysis and epistasis

The low-resolution map produced from the genetic data consisted of 77 markers, forming 21 linkage groups spanning 780 centiMorgans (map not presented). An additional 14 markers segregated independently of any other genetic marker. Deviations from the expected 1:2:1 genotypic ratios were significant for three (3.3%) of the 91 markers scored (sgA487, P=0.02; sgK227, P=0.01; sgA955, P=0.003). These three loci were located on separate linkage groups, showed excess *G. max* alleles, and were not significantly associated with seed-weight variation.

Searches for seed-weight QTLs in the F_2 generation using MapMaker-QTL and a one-way ANOVA identified three highly significant (P<0.01) marker loci (Table 1). Individually these markers, sgA118, sgA816 and sgK385, explained 21.0, 7.8 and 10.5% of the total variation for seed-weight in the F_2 generation (Table 1). Two-way ANOVA tests for epistasis between these significant

Table 1 Location, gene action and effect of markers significantly associated with seed size

Marker	Gen[a]	LG[b]	MM[c]	MS	SS	%Var	$P>F$	Mode[d]
sgA118	F_2	H	7.7	6.6	5.8	21.1	0.001	
	$F_{2:3}$		7.5	7.1	6.4	14.2	0.001	AR
sgA816	F_2	M	7.3	6.5	6.4	7.8	0.004	
	$F_{2:3}$		7.6	6.9	6.7	9.8	0.001	DA
sgA023	F_2	L	7.1	6.8	6.3	4.3	0.049	
	$F_{2:3}$		7.5	7.1	6.6	8.7	0.002	AR
sgK384	F_2	J	7.1	6.8	6.2	5.7	0.017	
	$F_{2:3}$		7.5	7.2	6.5	10.5	0.001	AR
sgT153	F_2	A	7.1	6.8	6.2	4.9	0.032	
	$F_{2:3}$		7.5	7.1	6.6	7.1	0.007	ARD
sgK385	F_2	L	7.5	6.6	6.2	10.5	0.001	
	$F_{2:3}$		7.2	7.0	6.9	0.9	0.535	AD

[a] The generation in which the QTL was detected (F_2 or $F_{2:3}$)
[b] Linkage group as designated in the current USDA-ISU map
[c] MM-homozygous *G. max*; MS-heterogous; SS-homozygous *G. soja*; measured as g/100 seeds
[d] The possible pure modes of gene action (Mode) for each QTL as detected in the $F_{2:3}$ generation (A-additive; D-dominant; R-recessive). The most likely mode is listed first

Table 2 Significant interactions between genetic markers as determined using two-way ANOVA

Interaction	Gen[a]	$P>F$	%Var[b]
Soybean[c]			
sgA118*sgA426	F_2	0.007	8.2
	$F_{2:3}$	0.005	9.4
sgA816*mgM185b[d]	F_2	0.002	11.5
	$F_{2:3}$	0.004	9.5
sgA118*sgA112	F_2	0.008	8.1
	$F_{2:3}$	0.141	4.5
sgA118*sgA023	F_2	0.027	6.1
	$F_{2:3}$	0.007	8.5
Cowpea[e]			
sgA816*cgO103	F_2	0.006	–
Mung bean[e]			
sgA816*mg182	F_2	0.001	–

[a] The generation in which the interaction was detected (F_2 or $F_{2:3}$)
[b] Percent phenotypic variation explained by individual interactions calculated as a partial R^2
[c] Soybean epistatic data as determined in this study
[d] Marker locus mgM185b is linked to cgO103 in cowpea and is located near significant seed-weight QTLs in both cowpea and mung bean
[e] Cowpea and Mungbean epistatic data taken from Fatokun et al. (1992)

marker loci and all other unlinked marker loci revealed three significant digenic interactions involving three other molecular marker loci (Table 2). A factorial analysis (6 loci; 2 alleles per locus) indicated that the three significant marker loci and three interacting loci explained 50% of the variation for seed-weight in the F_2 generation.

In the $F_{2:3}$ generation, five genomic regions associated with seed-weight were identified by MapMaker-QTL and one-way ANOVA (Table 1). Individually, these genomic intervals explained from 7.1 to 14.2% of the total variation for seed-weight (Table 1). Of the five significant marker loci identified in the $F_{2:3}$ generation, only two (sgA118 and sgA816) were significant ($P<0.01$) in the F_2 generation (Table 1). However, the remaining three marker loci (sgA023, sgK384 and sgT153) were significant at $P<0.05$ in the F_2 generation. Two-way ANOVA tests identified three highly significant digenic interactions, two of which were detected in the F_2 generation (sgA118*sgA426 and sgA816*mgM185b; Table 2). A factorial analysis with eight loci and two alleles per locus explained 60% of the total phenotypic variation for seed-weight. The significant marker loci identified in this study are located on five separate linkage groups (Table 1).

Gene action

In both generations, all *G. max* alleles at all marker loci with significant effects on seed-weight were associated with greater seed-weight. These results were not unexpected since the *G. max* parent had significantly larger seeds than the *G. soja* parent. Gene action at individual marker loci was evaluated by comparing the fit of individual QTLs to three Mendelian models (i.e., either dominant, recessive, or additive) using MapMaker-QTL 1.1b. A one-LOD reduction in likelihood indicates that a given type of gene action is unlikely. The modes of inheritance for the individual QTLs, presented in Table 1, could not be rejected as unlikely. The gene action of all six marker loci affecting seed-weight was consistent with an additive model (Table 1). Three of the six markers (sgA118, sgA023 and sgK384) also conformed to a dominance model (of the *G. soja* factor), while a recessive model was deemed unlikely for two of the markers (sgA816 and sgK385). At the remaining marker (sgT153) neither dominance nor recessiveness could be deemed unlikely (Table 1).

Discussion

Analysis of seed-weight in the F_2 and $F_{2:3}$ generations of this cross showed a continuous distribution, indicative of a quantitative trait. The mean values of both generations were closer to the value of the small-seeded parent, suggesting partial dominance of the alleles for small seed-weight (Fig. 1). This observation is consistent with previous reports in several plant species (Drabo et al. 1984; Vallejos and Chase 1991; Doebley et al. 1994). Nienhuis et al. (1987) reported a similarly skewed distribution that favored the alleles for the wild parent of an interspecific cross in tomato, which they attributed to gametic selection. In the present study, only three marker loci deviated from their expected 1:2:1 genotypic ratios (sgA487, sgK227 and sgA955). These three loci showed excess *G. max* alleles, and were not significantly associated with seed-weight variation. Furthermore, the genotypic composition of this soybean population appears to be normally distributed ($P>0.10$) with a mean *G. max* fraction of 0.48, close to the expected 0.50 fraction, suggesting that dominance of small seed-weight, and not gametic (or zygotic) selection, was responsible for the smaller than expected population means.

Employing both MapMaker-QTL and one-way ANOVA, and a $P<0.01$ criterion, we identified three and five putative QTLs affecting seed-weight in the F_2 and $F_{2:3}$ generations of this cross, respectively (Table 1). Two of the five QTLs identified in the $F_{2:3}$ generation were detected ($P<0.01$) in the F_2 generation. The remaining three QTLs (detected by markers sgA023, sgK384, and sgT153) were significant in the F_2 generation, but at a lower significance criterion ($P<0.05$; Table 1). One marker locus (sgK385) which was highly significant in the F_2 generation, was not significant in the $F_{2:3}$ generation ($P=0.53$). It may be that the effect of this putative QTL was dependent upon the environment (greenhouse versus field) in which it was tested. Changes in the importance of specific QTLs among different environments have been reported in tomato for fruit characteristics (Paterson et al. 1991). Alter-

natively, it may be that this marker has been erroneously declared significant in the F_2 generation when in reality it is not (Type-I error). These results confirm the importance of mapping quantitative trait loci in multiple environments.

The low level of variation (7.1–21.1%) explained by individual markers in these generations confirms the quantitative nature of seed-weight inheritance (Table 1). For all of the QTLs identified, at least two of the three types of gene action (additive, dominant, or recessive) could not be rejected. The inability to reject two modes of inheritance may be the result of the limited size of the population (150 entries), but more likely suggests that these QTLs exhibit partial dominance or recessiveness (Paterson et al. 1991). In addition to detecting major seed-weight QTLs, we also detected several highly significant digenic interactions (Table 2). Previously, epistasis has been detected in mapping studies of soybean for hard seededness (Keim et al. 1990) and seed protein and oil content (Lark et al. 1994), and in cowpea and mung bean for seed-weight (Fatokun et al. 1992). The importance of these interactions is evidenced by their significant partial R^2 values (Table 2). The importance of epistasis in phenotype expression is supported by a large body of research in quantitative genetics (Allard 1988). In a combined analysis, the significant markers and digenic interactions accounted for 50 and 60% of the variation for seed-weight in the F_2 and $F_{2:3}$ generations, respectively. The magnitude of seed-weight variation explained by these markers is substantial in view of the quantitative nature of the trait. The unexplained variation in this experiment may be attributed to the environment, to weak linkage relationships between molecular markers and seed-weight QTLs, other significant seed-weight QTLs not detected in this study as a result of the incomplete genome coverage, or due to the individual effects or interactions among seed-weight QTLs which are too small to be detected relative to experimental error.

To determine whether soybean shares orthologous seed-weight QTLs (loci related by descent) with cowpea and/or mung bean, a set of 17 "comparative QTL mapping" probes (see Material and methods) which detected loci linked to seed-weight genes in cowpea and mung bean were specifically chosen to be included in the genetic analysis of seed-weight in the present study. Three of these RFLP markers (sgA816, sgK024 and sgA226) were linked on soybean linkage group M and were signficantly ($P<0.01$) associated with seed-weight variation in both the F_2 and $F_{2:3}$ generations (Fig. 2). These same RFLP markers detected a highly significant seed-weight QTL in cowpea, but not in mungbean. However, the linear order of these marker loci is conserved in all three species (Fig. 2). Tests for digenic interactions between markers in this genomic region and other unlinked marker loci revealed a significant interaction between marker loci sgA816 and mgM185b (Table 2). In this study, when markers sgA816 and mgM185b were homozygous for the *G. soja* alleles they interacted in a synergistic manner to produce greater seed-weight (Fig. 3). Similarly, Fatokun et al. (1992) showed that the same marker locus, sgA816 (or a nearby QTL), significantly influenced the expression of seed-weight by interacting with marker

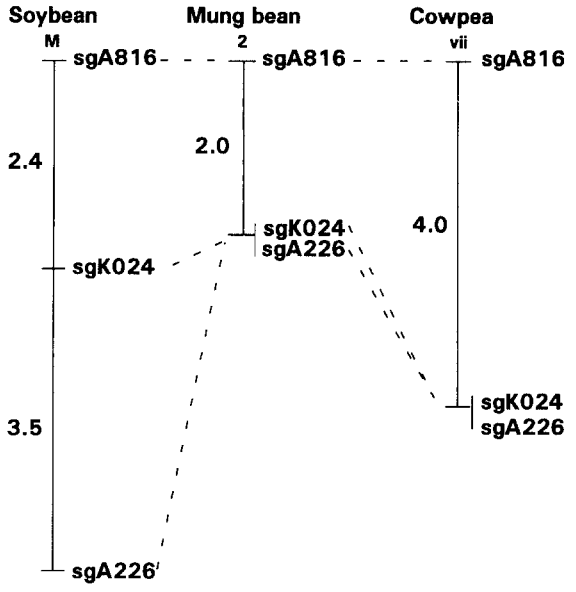

Fig. 2 Orthologous genomic regions influencing seed-weight in soybean, cowpea and mung bean. RFLP markers in this region are significantly associated with seed-weight in soybean and cowpea. Epistatic interaction between marker sgA816 and unlinked markers are significant in all three genomes (Table 2 and Fig. 3). Colinearity of RFLP markers is evident in all three genomes

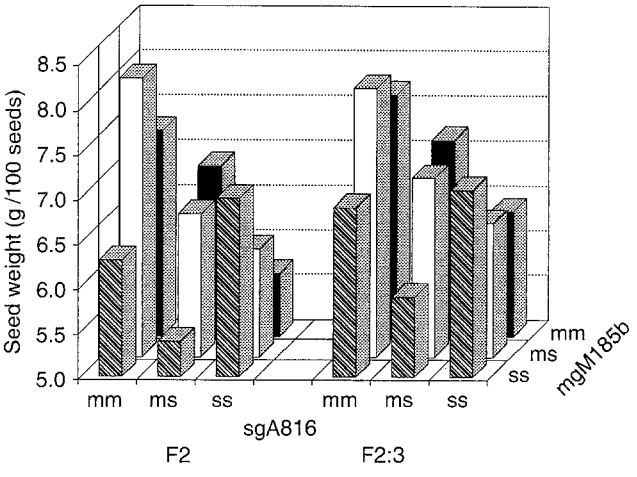

Fig. 3 Epistatic interaction between marker loci sgA816 and mgM185b in the F_2 and $F_{2:3}$ generations of a cross between *G. max* and *G. soja*. Mean seed-weights are plotted relative to their genotypic class at markers sgA816 and mgM185b. When markers sgA816 and mgM185b were homozygous for the *G. soja* allele they interacted in a synergistic manner to produce larger seed-weight

cgO103 in cowpea and with marker mgM182 in mung bean. Interestingly, marker locus mgM185b is linked to cgO103 in cowpea and is located near a significant seed-weight QTL in both cowpea and mung bean.

Fatokun et al. (1992) also reported the conservation of a different seed-weight QTL on cowpea linkage group ii and mung bean linkage group 1 (Menancio-Hautea et al. 1993). In soybean the linear order and linkage relationships of the same molecular markers is conserved on link-

age group A (data not presented), and there is modest evidence (P=0.04) for the presence of a seed-weight QTL at this genomic region of linkage group A in soybean. The lack of highly significant evidence for a seed-weight QTL at this region in the soybean genome may indicate that: (1) no QTL for seed-weight is present in this genomic region of soybean, (2) the importance of this region has been diluted during the evolution of soybean, (3) this genomic region is not polymorphic in this cross (or perhaps in the species) and, therefore, is not detectable through standard RFLP mapping techniques, or (4) the QTL is not detectable in the available 150 genotypic combinations studied. It should be noted that a highly significant seed-weight QTL (linked to marker sgT153) was identified on soybean linkage group A in this study, but it was well separated from the comparative molecular markers associated with seed-weight variation in cowpea and mung bean.

In the present study we identified several seed-weight QTLs in soybean. These QTLs have been described by genomic location, magnitude of effect, gene action (dominance and additive effects), and digenic interactions. Furthermore, we report evidence for the conservation of at least one orthologous seed-weight QTL between soybean and cowpea. The conservation of genetic linkage between molecular markers and important agronomic genes has important implications for intrepreting genetic information among related species.

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