# Evidence for Orthologous Seed Weight Genes in Cowpea and Mung Bean Based on RFLP Mapping

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ABSTRACT

A well saturated genomic map is a necessity for a breeding program based on marker assisted selection. To this end, we are developing genomic maps for cowpea (*Vigna unguiculata* 2*N*=22) and mung bean (*Vigna radiata* 2*N*=22) based on restriction fragment length polymorphism (RFLP) markers. Using these maps, we have located major quantitative trait loci (QTLs) for seed weight in both species. Two unlinked genomic regions in cowpea contained QTLs accounting for 52.7% of the variation for seed weight. In mung bean there were four unlinked genomic regions accounting for 49.7% of the variation for seed weight. 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. This suggests that the QTLs in this genomic region have remained conserved through evolution. This inference is supported by the observation that a significant interaction (*i.e.*, epistasis) was detected between the QTL(s) in the conserved region and an unlinked RFLP marker locus in both species.

COWPEA [*Vigna unguiculata* (L.) Walp.] and mung bean [*Vigna radiata* (L.) Wilczek] evolved separately in sub-Sahara Africa and Asia, respectively (SMARTT 1990), and relationships between them, especially at the molecular level, have not been reported. Cowpea belongs to subgenus *Vigna* section *Catiang* while mung bean belongs to subgenus *Ceratotropis*. Crossing experiments between various subgenera and sections of the genus *Vigna* clearly show that mung bean cannot provide immediate germplasm for transfer into cowpea or vice versa due to cross incompatibility (DANA and KARMAKAR 1990). However, because both belong to the same genus, *Vigna* Savi (MARECHAL, MASCHERPA and STAINIER 1978) they share common restriction fragment length polymorphism (RFLP) marker loci (D. I. MENANCIO-HAUTEA, C. A. FATOKUN, L. S. KUMAR, D. DANESH and N. D. YOUNG, in preparation) and, potentially, many orthologous genes. The presence and distribution of such orthologous genes within the genomes of the two species might give an indication of common evolutionary trends.

RFLP analysis has been successfully used to study evolutionary relationships between species. For example, DOEBLEY *et al.* (1990) identified molecular markers linked to morphological traits which differentiate maize from its presumed progenitor, teosinte. Using RFLP analysis HULBERT *et al.* (1990) detected conserved DNA genetic markers in maize and sorghum, both members of the tribe Andropogoneae. BONIERBALE, PLAISTED and TANKSLEY (1988) found a high level of homeology between chromosomes of tomato and potato, with the only detectable chromosomal changes being paracentric inversions at four locations on three chromosomes of potato. By contrast, while tomato and pepper genomes share a similar gene repertoire and conserved nucleotide sequences among homologous genes, extensive chromosome rearrangements were observed between the two genomes (TANKSLEY *et al.* 1988).

In cowpea and mung bean seed weight is one of the three most important yield components and large seeds usually command consumer preference. Hence, breeders have endeavored to develop varieties characterized by larger seeds coupled with other desirable traits. Seed weight is quantitatively inherited with average heritability values of 66.2% and 87.9% in cowpea and mung bean, respectively (FERY 1980). The estimated minimum number of factors influencing seed weight range from 6 to 10 (SENE 1968; ARYEETEY and LAING 1973).

Polygenes controlling important metric traits are usually distributed among several quantitative trait loci (QTLs), which may not be linked to one another. For this reason, incorporating desirable traits governed by unlinked polygenes using conventional breeding methods is not an easy task. Tagging useful agricultural genes, such as those for seed weight, with tightly linked molecular markers should enhance efforts aimed at their improvement. STUBER, EDWARDS and WENDEL (1987) have reported that molecular (isozyme) marker loci can be effective in locating and

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identifying QTLs (or genomic regions) with effects on grain yield in maize.

In this paper we report the detection of genomic regions in cowpea and mung bean that contain QTLs with major effects on seed weight. Our results indicate that most of the variation is controlled by a small number of genes in both species. Moreover, one genomic region with orthologous RFLP markers conserved between cowpea and mung bean contains the QTL with the greatest effect on seed weight in both species.

## MATERIALS AND METHODS

**Plant materials:** Two cowpea genotypes, IT2246-4 (an improved cultivar) and TVNI 963 (*V. unguiculata* ssp. *dekindtiana* $2N=22$) were crossed at the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria, as were two mung bean genotypes VC3890 (an improved cultivar) and TC1966 (*V. radiata* ssp. *sublobata* $2N=22$) at the Asian Vegetable Research and Development Centre (AVRDC), Shanhua, Taiwan. These two pairs of parents were selected because they differed in several morphological and agronomic attributes.

Seeds obtained from the crosses were sown (at IITA or AVRDC) to produce the $F_1$ generation. Seeds harvested from $F_1$ plants were then planted in the greenhouse at the University of Minnesota, St. Paul. For each cross, 58 $F_2$ plants were grown and used as source of leaves, which were frozen in liquid nitrogen prior to DNA extraction. Plants were allowed to grow back, flower, and set seeds. Data were collected from each plant for mean 100-seedweight.

**DNA extraction, restriction digests, electrophoresis and Southern blotting:** Plant DNA was extracted from frozen leaves using a modification of the technique of DELLAPORTA, WOOD and HICKS (1983). For cowpea, seven restriction enzymes: *Bcl*I, *Bst*NI, *Eco*RI, *Eco*RV, *Hae*III, *Hin*dIII and *Xba*I were used to digest total DNA, while for mung bean, *Bst*NI, *Dra*I, *Eco*RI, *Eco*RV, *Hae*III and *Hin*dIII were used. Digested DNA was electrophoresed on 1% agarose gel, followed by blotting onto a Hybond N+ membrane (Amersham Corporation) using a modification of SOUTHERN (1975), which consisted of blotting in an alkaline solution containing 0.5 M NaOH and 0.5 M NaCl.

**DNA clones:** Three different genomic libraries served as sources of RFLP markers. The first of these consisted of a library of soybean genomic DNA which had been prepared by digestion with the methylation-sensitive restriction enzyme, *Pst*I, and insertion into the bacterial plasmid, pBS+ (Stratagene). This library was generously donated by RANDY SHOEMAKER (Iowa State University, Ames, Iowa). About 90% of the soybean clones showed clear hybridization signals when probed onto cowpea and mung bean DNA. The other genomic libraries were constructed by digesting purified cowpea or mung bean DNA with *Pst*I, which was then separated according to size by sucrose gradient centrifugation, and the fraction between 500 and 3000 base pairs ligated into pUC18 by standard molecular methods (SAMBROOK, FRITSCH and MANIATIS 1988).

**Library screening and DNA hybridizations:** Cloned DNA inserts were labeled using the polymerase chain amplification. Individual bacterial colonies were grown overnight in suspension culture, centrifuged for 5 min at $2000\times g$, rapidly frozen and thawed, and centrifuged at $2000\times g$ for 5 min and used as plasmid templates for the reactions. Two microliters of the supernatant were used as template in a polymerase chain reaction using the M13-forward and -reverse sequence as primers (Promega). Unincorporated nucleotides were removed either by ammonium acetate precipitation or by brief centrifugation through a 1.0-ml column containing Sephadex G50 resin. Forty to 50 ng of each amplified genomic sequence was radiolabeled by the random hexamer reaction of FEINBERG and VOGELSTEIN (1983).

The radiolabeled sequence was then incubated with a blot containing DNA of the parents or $F_2$ for 18–24 hr at 60° in a hybridization solution of 5 × SSC, 0.1 M phosphate buffer, pH 7.5, 1 × Denhardt's solution (2% each of Ficoll, polyvinylpyrrolidone and bovine serum albumin fraction V), 0.1% sodium dodecyl sulfate, and 5% dextran sulfate. After incubation, blots were washed three times for 15 min each at 60° with 2 × SSC, 1 × SSC and 0.5 × SSC, respectively. Each wash solution also contained 0.1% sodium dodecyl sulfate. Following the washes blots were placed against Kodak X-AR film and stored at −80° for 1–5 days to produce autoradiographs.

**Data analysis:** All $F_2$ plants were scored for RFLP markers, as well as for seed weight, and then analysed using the Mapmaker (LANDER *et al.* 1987), Mapmaker-QTL (LANDER and BOTSTEIN 1989) and Statview-II computer programs. Linkage analysis was carried out with the Mapmaker program, while QTL analysis was with Statview-II and Mapmaker-QTL. Putative QTLs were inferred for loci showing a significance of $P < 0.01$ in one way ANOVA using Statview-II. With Mapmaker-QTL putative QTLs were inferred whenever the LOD score exceeded 2.0. A LOD score is the $\log_{10}$ of the ratio between the odds of one hypothesis (the presence of a QTL in this case) *vs.* an alternative hypothesis (no QTL). Statview-II was also used to carry out two-factor analysis of variance between RFLP loci in order to determine if there were any significant interaction among loci. Again, a significant level of $P < 0.01$ was used to uncover potentially significant interactions.

## RESULTS AND DISCUSSION

**Frequency distribution of seed weight:** Seed weight among $F_2$ plants showed continuous variation, suggesting polygenic inheritance in both crops (Figure 1). None of the $F_2$ plants had seeds as heavy as the heavier seed-producing parents, IT2246-4 (cowpea) with 13.12 g/100 seeds and VC3890 (mung bean) with 6.04 g/100 seeds. In this study, the fact that no $F_2$ individual had seeds as heavy as the heavier seed producing parents could be attributed to the few number of plants representing these populations. The greenhouse environment in which they were sown would also have contributed to this observation. The mean values of both $F_2$ populations were nearer those of the parents with lower seed weight, suggesting partial dominance of small seed weight. This observation is consistent with reports from earlier studies by DRABO *et al.* (1984), LELEJI (1975) and SEN and MURTY (1960).

**Detection of QTLs:** QTLs for seed weight were investigated by linear correlation between genotypic classes for each RFLP marker (104 in mung bean and 84 in cowpea) and seed weight. It should be recognized that linear correlation only gives an accurate

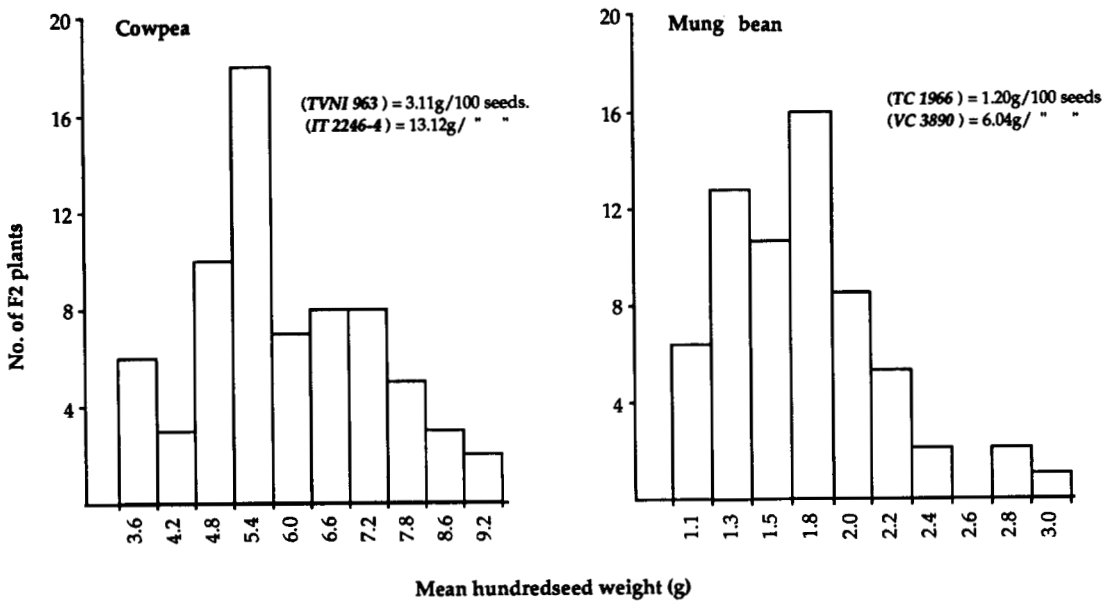


FIGURE 1.—Frequency distributions of cowpea and mung bean $F_2$ plants for 100-seedweight. In both populations, phenotypes of the heavier seed producing parents were not recovered. The cowpea and mung bean genotypes crossed are in parenthesis followed by their mean 100-seedweight.

estimate of effects of QTLs that fall exactly at a marker locus. This means that the effects of QTLs located further away from markers are underestimated. By contrast, Mapmaker-QTL is based on interval mapping, which seeks to eliminate this potential underestimation (LANDER and BOTSTEIN 1989).

**Seed weight QTLs in cowpea:** Both Statview-II and Mapmaker-QTL identified the same two genomic regions around *pO103* on linkage group II and *pA816* on linkage group VI as containing major QTLs for seed weight (Table 1; Figure 2). The interval with the greatest effect on seed weight, as revealed by Mapmaker-QTL analysis, was between markers *pO103* and *pA509* on linkage group II. The genomic region comprising a 10:1 likelihood support interval for the position of a putative QTL was estimated to span 14 cM (Figure 2). This interval represents the range within which the likelihood falls by a LOD of 1.0 or less (PATERSON *et al.* 1988). The QTL(s) in this interval on linkage group II accounted for 36.5% of seed weight variation and the second region on linkage group VI accounted for 32.9% based on Mapmaker-QTL analysis. Together QTLs in these two intervals explained 52.7% of the variation for seed weight. The amount of variation in seed weight explained by these two regions is quite substantial in view of the quantitative nature of this trait.

**Seed weight QTLs in mung bean:** Mapmaker-QTL program identified four unlinked regions as having major QTLs for seed weight (Figure 2). Together, these four unlinked regions of the mung bean genome explained 49.7% of variation for seed weight. Based on Mapmaker-QTL, the interval *pM182-pA124* on linkage group II contained a major QTL(s) accounting for 32.5% of the variation for this trait. The 10:1 likelihood support interval for the presence of a putative QTL in this region spanned 31 cM. Using Statview-II, three of the same regions were identified

TABLE 1

**RFLP markers and linkage groups associated with seed weight QTLs in mung bean and cowpea**

| | Linkage group | | Mung bean | | Cowpea | |
|---|---|---|---|---|---|---|
| Markers | Mung bean | Cowpea | *P* | LOD[a] | *P* | LOD |
| *pM228* | II | — | 0.0006 | 3.581 | — | — |
| *pA124* | II | — | 0.0001 | 4.370 | — | — |
| *pM182* | II | II | 0.0004 | 4.781 | 0.0072 | 2.189 |
| *pM185b* | — | II | — | — | 0.0006 | 3.331 |
| *pA487* | II | II | 0.0026 | 3.157 | 0.0083 | 2.845 |
| *pO103* | — | II | — | — | 0.0001 | 4.498 |
| *pA509* | II | II | 0.0020 | 2.986 | 0.0006 | 3.290 |
| *pK286* | II | — | 0.0038 | 3.403 | — | — |
| *pK443* | — | II | — | — | 0.0100 | 2.027 |
| *pM254* | — | II | — | — | 0.0017 | 3.402 |
| *pA110* | — | II | — | — | 0.0019 | 2.976 |
| *pM185a* | I | — | 0.0030 | 1.928* | — | — |
| *pA60* | I | — | 0.0193** | 2.598 | — | — |
| *pK472a* | VI | — | 0.0148** | 2.055 | — | — |
| *pA816* | — | VI | — | — | 0.0008 | 2.991 |
| *pA226* | — | VI | — | — | 0.0069 | 2.654 |
| *pA955* | III | — | 0.0315** | 2.26 | — | — |

*P* = 0.01 (ANOVA). (—) = Clone not mapped (*i.e.*, not polymorphic) or had no significant relationship with seed weight.

* = values included because of significant *P* or ** significant LOD.

[a] LOD is the $\log_{10}$ of the odds ratio that supports evidence for the presence of a QTL in the region.

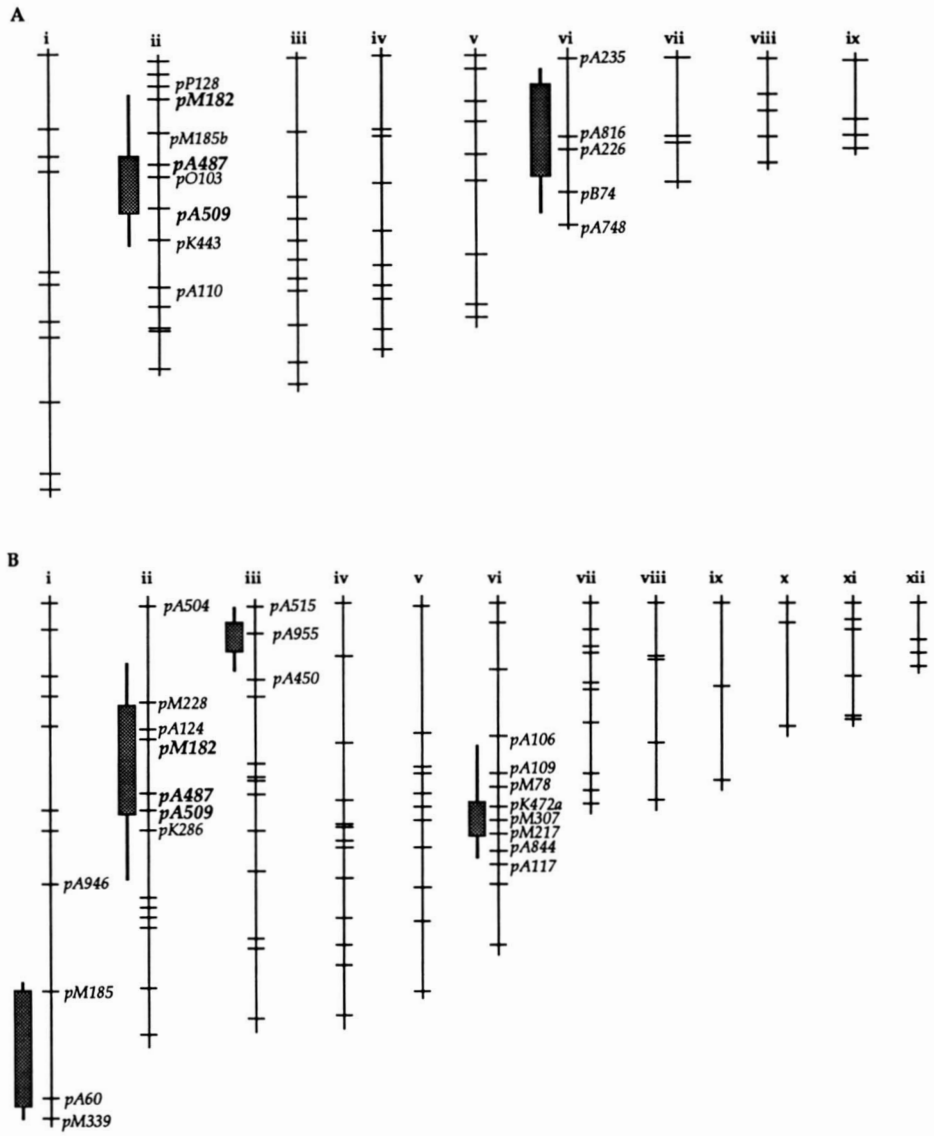


FIGURE 2.—RFLP maps of cowpea (A) and mung bean (B) showing locations of putative QTLs for seed weight. Linkage group II of both species have QTLs with the highest effects on seed weight and are spanned by identical markers. Both species have 11 pairs of chromosomes each, while the current RFLP maps span 9 linkage groups in cowpea and 12 in mung bean. The short cross lines on the chromosomes indicate the positions of markers while the broad and intermediate bars adjacent to chromosomes are the intervals over which LOD scores are within 1.0 and 2.0 log units (see Table 1) respectively as indicated by Mapmaker-QTL program.

(Table 1) as containing QTL(s) for seed weight. The QTL spanned by markers *pA515, pA955* and *pA450* on linkage group III as revealed by Mapmaker-QTL was not detected by Statview-II.

**Evidence for orthologous genes for seed weight:** The genomic regions in cowpea and mung bean that have the most effect on seed weight, span the same RFLP markers in both species (Figure 3). These markers, listed in Table 1, are colinear in arrangement on homologous linkage groups in both crops (linkage groups II in cowpea and mung bean). Moreover, the genomic regions of cowpea and mung bean with the highest supportive evidence for the presence of seed weight QTLs coincided (Figure 3).

**Evidence for epistasis between QTLs:** The proportion of total variation for seed weight accounted for by the identified QTLs was about 50% in both crop species. The model adopted in Mapmaker-QTL analysis tends to underestimate total genetic variance since only additive, dominance and recessive genetic components of variation are included in the model (PATERSON *et al.* 1991). Consequently, we cannot explain all likely causes of variation by the detected major QTLs. It should be noted that the $F_2$ plants used in this study were sown in pots placed on benches in a greenhouse. Normally, both cowpea and mung bean are sown in the field. Additional sources of variation could be environment, measurement error, interaction with environment, presence of genes influencing other traits that affect seed weight indirectly, QTLs with effects too slight to detect, and interaction between QTLs (PATERSON *et al.* 1991).

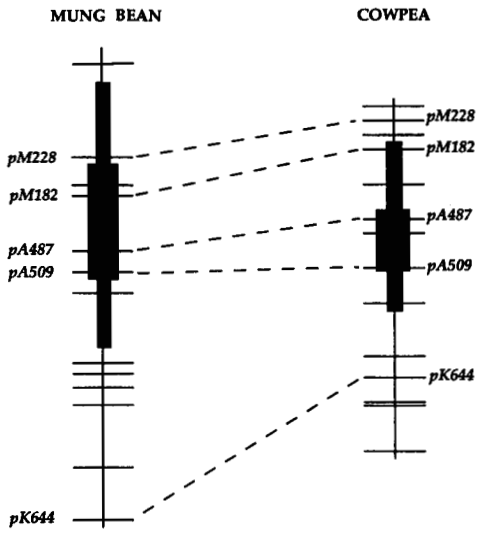


FIGURE 3.—Conserved regions of mung bean and cowpea genomes having the same RFLP markers arranged in similar order (joined by broken lines). The broad and intermediate shaded areas are the intervals over which LOD scores are within 1.0 and 2.0 log units respectively as indicated by Mapmaker-QTL program.

A two-factor ANOVA was carried out to determine the significance of inter-loci interactions (Table 2) among the detected QTLs for seed weight. This analysis revealed that in cowpea the QTL at *pO103* on linkage group II and a second locus located near *pA816* on linkage group VI, influenced one another significantly. The results suggest that the allele of *V. dekindtiana* at *pA816* diminished the effects of *V. unguiculata* allele on seed weight at *pO103*. Likewise in mung bean a QTL located near marker *pA816* significantly influenced the QTL near *pM182* (Table 2) although on its own, *pA816* showed no significant effect on seed weight. When *pA816* was homozygous for *V. radiata* ssp. *sublobata* allele, it diminished the effect of *pM182* when homozygous for *V. radiata* allele. The two markers *pO103* and *pM182* span the same regions of cowpea and mung bean genomes (linkage group II) containing putative QTLs accounting for the highest effect on seed weight. While the strength of evidence for interaction between these loci is only moderate (primarily due to the small population size, 58 $F_2$ plants, used in the analysis), it is noteworthy that a second unlinked locus appears to interact significantly with the major seed weight QTL(s) on linkage group II in both species. In quantitative genetic studies earlier reported (ALLARD 1988; SPICKETT and THODAY 1966), interloci interactions between QTL alleles had significant effects on phenotype. However, QTL mapping studies carried out on tomato (PATERSON *et al.* 1991) and maize (EDWARDS, STUBER and WENDEL 1987) did not show strong evidence for the presence of interaction between detected QTLs.

**Evolutionary significance:** It is noteworthy that a major QTL for seed weight, which may be common to both cowpea and mung bean, has been retained by both species. In maize (*Zea mays*) and sorghum (*Sorghum bicolor*) clusters of genes with similar linear order have been reported (HULBERT *et al.* 1990), although traits governed by these genes were not stated. In the present study the QTLs located near identical, colinear DNA markers in both species accounted for the greatest amount of variation in seed weight. In the process of evolution, frequencies of some alleles, particularly those concerned with plant survival and fitness, tend to increase, as do frequencies of alleles of associated marker loci (ALLARD 1988). As the evolution of crop plants to their present day forms involves both natural and artificial selection, alleles that are strongly associated with agriculturally important characters such as yield and its components are, therefore, likely to become more frequent in today's crop cultivars.

TABLE 2

**Two-way ANOVA for interacting RFLP marker loci**

| Plant | Source | *F* test | *P* |
|---|---|---|---|
| Cowpea | *pA816* (A) | 12.63 | 0.0009 |
| | *pO103* (B) | 7.04 | 0.0109 |
| | (A × B) | 4.67 | 0.0063 |
| Mung bean | *pA816* (A) | 4.19 | 0.0218 |
| | *pM182* (B) | 8.75 | 0.0006 |
| | (A × B) | 5.87 | 0.0007 |

The RFLP markers *pA816* and *pO103* interacted in cowpea as *pA816* and *pM182* in mung bean with significant effects on seed weight in both species. Markers *pO103* and *pM182* span the same region (linkage group II in both species) containing putative QTLs accounting for the greatest effects on seed weight.

Cowpea and mung bean are distantly related, showing only 70% nucleotide homology based on RFLP analysis (C. A. FATOKUN, D. DANESH, N. D. YOUNG and E. L. STEWART, submitted). A comparison of the genomes of the two species also shows that extensive chromosome rearrangements have taken place in one or both genomes (D. I. MENANCIO-HAUTEA, C. A. FATOKUN, L. S. KUMAR, D. DANESH, in preparation). Cowpea and mung bean also differ significantly from one another in their general appearance, centers of diversity, and are completely sexually incompatible (DANA and KARMAKAR 1990).

**Implications for crop improvement:** The usefulness of RFLP maps in crop improvement depends on identifying RFLP markers that are tightly linked to desirable genes. In the present study we identified RFLP markers which are linked with seed weight in cowpea and mung bean. Moreover, the fact that we did not recover the heavier seed producing parental types among $F_2$ plants did not preclude identification of individuals with potential for producing progenies that are capable of giving high seed weight in subsequent generations. Using marker-assisted selection in

combination with the graphical genotype concept (YOUNG and TANKSLEY 1988), it should be possible to select plants in the $F_2$ generation that are most likely to contain desired genes. Moreover, the gene action at some of these loci was mainly additive, based on the dominance to additive (d/a) ratio (results not shown), which implies that plants selected in early generations, such as the $F_2$, should produce progeny individuals with the desired trait.

In cowpea and mung bean, seed weight is governed by several genes, the major ones were detected in the present study. With more saturated DNA marker maps, an ultimate aim of our studies, it should be possible to identify additional QTLs (genes) for this and other desirable traits. An ensemble of the different QTLs contributing to increased seed weight can then be more readily established in standard cultivars of these crops.

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