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RFLP tagging of QTLs conditioning specific leaf weight and leaf size in soybean

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Abstract Selection for high specific leaf weight (SLW) in soybean [*Glycine max* (L) Merr.] may increase apparent photosynthetic rate per unit leaf area (AP), which in turn may improve seed yield. In general, the SLW and leaf size are negatively correlated in soybean. To maximize total photosynthetic performance, and perhaps the seed yield, of a soybean cultivar, it would be necessary to establish a large leaf area rapidly while maintaining a high SLW. The objective of the present study was to identify quantitative trait loci (QTLs) conditioning SLW and leaf size in soybean. One hundred and twenty F_4-derived lines from a 'Young' × PI416937 population were evaluated using restriction fragment length polymorphism (RFLP) markers. The genetic map consisted of 155 loci on 33 linkage groups (LGs) covering 973 cM of map distance. The phenotypic data were collected from two different environments – a greenhouse at Athens, Ga. and a field site at Windblow, N.C. The SLW and leaf-size measurements were made on leaves from the 8th and 9th node of soybean plants at the V12 stage of development. Combined over environments, six putative independent RFLP markers were associated with SLW, and four of these loci were consistent across environments. Individually, the six markers each explained between 8 and 18% of the phenotypic variation among lines for SLW. The Young alleles contributed to a greater SLW at four of the six independent marker loci, and transgressive segregation occurred among the progeny for SLW. Three putative independent RFLP markers were associated with leaf size, each explaining between 6 to 11% of the phenotypic variation in the trait, and one of these markers was identified in both environments. There was no correlation between SLW and leaf size in this population. Similarly, none of the six QTLs conditioning SLW were linked to any of the three QTLs for leaf size. In this soybean population, it is possible to select for progeny lines with greater SLW than either parent perhaps without affecting the leaf size. It is feasible to pyramid all of the desirable alleles for greater SLW and large leaf size in a single genetic background.

Key words Soybean · *Glycine max* · QTL · RFLP · SLW · Leaf size

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Introduction

Thompson et al. (1995) observed that increasing apparent photosynthetic rate per unit leaf area (AP) may improve seed yield in soybean, and selection for high specific leaf weight (SLW) may increase AP. Genotypic differences in SLW (Lugg and Sinclair 1979; Nelson and Schweitzer 1988) and AP (Dornhoff and Shibles 1970; Bhagsari et al. 1977; Hesketh et al. 1981; Weibhold et al. 1981) have been demonstrated in soybean. Dornhoff and Shibles (1970) sampled AP and SLW 15 times during the reproductive stage of development and found the two to be correlated ($r = 0.71$**). Buttery et al. (1981) evaluated 12 soybean cultivars and found SLW and AP to be correlated during flowering ($r = 0.76$**), but not during pod fill. Bhagsari et al. (1977) and Hesketh et al. (1981) also reported a positive relationship between SLW and AP in soybean. Pettigrew et al. (1993) found that two cotton, *Gossypium hirsutum*, genotypes (super okra and okra) with a greater SLW and a higher chlorophyll concentration

had a higher AP than the respective isolines with normal leaf types.

Dornhoff and Shibles (1970) and Peet et al. (1977) found AP to be positively correlated with seed yield in soybean. Buttery et al. (1981) reported a strong correlation ($r = 0.78^{**}$) between AP measured at R5 (Fehr and Caviness 1977) and seed yield in 12 cultivars. However, Egli et al. (1970) and Ford et al. (1983) found no such relationship. Apparent canopy photosynthesis rates were found to have a consistent positive relationship to soybean seed yield in a number of studies (Peet and Kramer 1980; Harrison et al. 1981; Boerma and Ashley 1982, 1988). Wells et al. (1986) reported that the quantity of RuBPCase per unit leaf area in soybean was positively correlated with SLW, that the greater SLW of 'Tracy' was translated into more photosynthetic proteins per unit ground area and a higher canopy photosynthesis rate compared to other cultivars with similar leaf-area indices.

The total photosynthetic performance of a soybean plant depends on the magnitude of the carbon dioxide exchange rate, the duration of carbon dioxide exchange rate, and photosynthetic leaf area (Ford et al. 1983). To maximize total photosynthetic performance, and perhaps the yield, of a soybean cultivar, it is important that the cultivar be able to establish a large leaf area rapidly while maintaining a high SLW. However, the relationship between SLW and leaf photosynthesis often does not carry over into plant weight or yield (Nelson 1988), because plants with a low SLW may have a higher growth rate (VanArendonk and Pooter 1994). The relatively greater leaf area expansion of plants with a low SLW probably accounts for their faster growth rates and resulting higher weights, as has been reported for several species (Potter and Jones 1977; Nelson 1988). Wiebold and Kenworthy (1985) concluded that since single leaflet expansion and total leaf area expansion rates are negatively correlated to SLW and AP, it may be difficult to identify cultivars that combine a fast rate of leaf expansion with a fast rate of AP.

The total leaf area of a soybean plant is a function of the size of individual leaves multiplied by the total number of leaves. PI416937, a plant introduction from Japan, has a much larger leaf size than 'Young' (unpublished data, Mian and Ashley 1994). We also observed that the two genotypes had the same number of leaves on their main stems.

In general, individual leaf size and SLW are negatively correlated and are quantitative in nature. Thus, it may be difficult to increase the magnitude of one trait without decreasing the magnitude of the other trait through conventional breeding procedures (Wiebold and Kenworthy 1985). The use of molecular markers to identify QTLs for SLW and leaf size would provide information on linkage or pleiotropy as causes of the negative association between the two traits. The use of molecular markers for the simultaneous improvement of SLW and leaf size in soybean may be more efficient than direct selection based on the phenotype because of the potential application of markers in breaking the negative association between the two traits. Molecular tags can yield information regarding the linkage, or the lack of it, between the QTLs conditioning SLW and leaf size, making it possible to identify and pyramid the independent QTLs conditioning the two traits.

Mansur et al. (1993) identified two QTLs for leaf size in soybean on LG 2 and 16 on the genetic map of a 'Minsoy' × 'Noir 1' population. The QTL on LG 2 explained 20%, and the QTL on LG 16 explained 25%, of the variation in leaf area. Using a recombinant inbred population of Minsoy × Noir 1, Mansur et al. (1996) detected two QTLs for leaf size on LG 10a and 11 of the genetic map. Apart from these reports we do not know of any other published data on the molecular tagging of QTLs for leaf area or SLW in soybean. The specific objectives of the present study were to identify RFLP loci associated with QTLs conditioning SLW and leaf size in a F_4-derived soybean population, and to determine the association between the SLW and leaf-size QTLs.

Materials and methods

Genotypic assay

A soybean population derived from a cross of Young × PI416937 was used to construct a genetic linkage map and to evaluate phenotypic traits. Young is a highly productive Maturity Group VI cultivar. PI416937, a Maturity Group VI plant introduction from Japan, is characterized as having larger and thicker leaves than the leaves of the commercially grown soybean cultivars of southern USA (Sloane et al. 1990).

The Young × PI416937 population consisted of 120 lines which were created by single-seed-descent with each line originating from a different F_2 plant. DNA isolation, Southern blotting, and hybridization procedures have been described previously (Lee et al. 1996; Mian et al. 1996). In short, RFLPs were surveyed from DNA isolated from lyophilized young leaves of parents grown in the greenhouse. The DNA was isolated from leaves according to the procedure of Keim et al. (1988), and digested overnight with each one of five restriction enzymes (*Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, or *Taq*I). Following electrophoresis of DNA fragments, a Southern blot was made by transfer to an uncharged nylon membrane. Nylon membranes were placed in 300 × 38-mm glass bottles containing 4–10 ml of 0.25 M Na_2PO_4 and 7% SDS, and pre-hybridized in a rotisserie oven for 4–6 h at 65°C. About 25 ng of isolated DNA probe were labeled with ^{32}P using a random primer procedure, and hybridization was conducted overnight. Approximately 750 probes from various sources, including cDNA and/or genomic clones of soybean (R. C. Shoemaker, USDA/Iowa State University; K. G. Lark, University of Utah; R. T. Nagao, University of Georgia), *Vigna radiata* (N. D. Young, University of Minnesota), *Phaseolus vulgaris* (J. M. Tohme, CIAT), *Arachis hypogaea* (G. D. Kochert, University of Georgia), and *Medicago sativa* (G. D. Kochert), were used to screen for polymorphisms between Young and PI416937.

Probes polymorphic with respect to the parents were used for creating the genetic map. The DNA was isolated from young unfolded trifoliate leaves of 8–10 plants/line which were grown in a field near Athens, Ga., in 1993. Multiple sets of nylon membranes containing DNA from each of the 120 lines were screened with polymorphic probes. The nomenclature for RFLP loci consisted of a probe

designation, followed by the restriction endonuclease designation [*Dra*I (D); *Eco*RI (E); *Eco*RV (V); *Hin*dIII (H); *Taq*I (T)], and a dashed-number suffix for an anchored probe or a letter (lower case) suffix for a non-anchored probe when more than one locus was detected by that probe. A RFLP locus was accepted as an "anchor" when it had the same probe/enzyme combination and an identical banding pattern with the corresponding RFLP locus on the USDA/ARS-ISU map. The linkage map was constructed with marker data using the Kosambi map function of GMendel (Holloway and Knapp 1993) assuming the data were collected from F_4-derived lines. For grouping markers, linkage thresholds of 3.0 for a minimum LOD score and an rmax of 0.38 (approximately 50 cM) were used to construct the map.

Phenotypic assay

Greenhouse evaluation

In a greenhouse in Athens, Ga., 120 F_4-derived lines and the two parents were grown in plastic pots containing 3.5 kg of methyl bromide-fumigated Pacolet sandy loam soil (a member of the clayey, Kaolinitic, thermic family of Typic Hapludults) amended with sand to a texture of 800 g kg^{-1} sand, 120 g kg^{-1} silt, and 80 g kg^{-1} clay. Four seeds were planted in each pot and seedlings were thinned to one per pot 8–10 days after planting. Plants were fertilized with 40 mg N, 40 mg P, and 40 mg K per pot at the time of thinning.

The experimental design was a randomized complete block with six replicates. Because of the large size of the experiment and limitations in greenhouse space, the replicates were grown sequentially in time with 2–3 weeks between successive replicates. The experiment was started in mid April of 1996 and was completed by early July of the same year. High-pressure sodium lighting (575 umol m^{-2} s^{-1} 20 cm above the pot soil level) was used after dusk to maintain a 15-h light and 9-h dark cycle throughout the experiment. The same lights were also used during days with complete cloud cover. The temperature ranged from 28 to 37°C during the day and from 19 to 23°C during the night. The day time relative humidity ranged between 50 to 80%. Plants were spaced at about 30×30 cm^2 to avoid the shading effect from neighboring plants. One column of border pots was included at the two ends of the experiment (along the width of the bench), but no border pots were provided on the other two sides (along the length of the bench). The pots were regularly rotated to minimize border and locational effects within each replicate.

At the V12 stage of development or about 45 days after planting, the leaves from the 8th and 9th node of each plant were harvested between 08:00 to 10:00 h following a sunny day. The leaflets were separated from the petiolules. The leaf area was measured immediately by using a portable LI 3000 leaf-area meter (LI-COR Inc., Lincoln, Neb.). The leaves from each plants were then put in a paper bag, placed in an oven for drying at 68°C for 96 h, and the dry weight of the leaves from each plant were recorded.

Field evaluation

The parents and 120 lines were grown a field in 1994 at Windblow (Sandhills Research Station), N.C. The individual plots were 2.88 m wide and 3.05 m long with three rows. The soil type was a sandy, salicious, thermic Arenic Paleudult. The experimental design was a randomized complete block with three replications. At the V12 stage of development, the leaves from the 8th and 9th node from a single plant in each plot were collected, packaged in a polyethylene ziplock bag, and placed under ice in an ice box. The leaves were collected following a sunny day between 08:00 to 10:00 h. The leaf samples were then transported to the laboratory where the leaf area and leaf dry weight was determined as described earlier.

Data analysis

The SLW was calculated as: leaf dry weight/leaf area. The data on SLW and leaf size from each environment, as well as combined over environments, were analyzed using the GLM procedure of SAS (SAS Institute, Cary, N.C.). Replications, locations, and lines were considered as random effects in the combined analysis over environments. For each of the two traits, a single-factor analysis of variance (ANOVA) was conducted to evaluate each marker locus for linkage to a presumed QTL affecting the trait by contrasting the mean performance of the two homozygous RFLP classes. Due to the limited number of heterozygous lines available for a given marker loci (expected number of 15 = 12.5% of 120 lines), the heterozygous class was excluded from the analysis. For each of the marker loci, the RFLP class-means were compared for the determination of significant differences ($P \leqslant 0.01$) using an F-test from the type-III means squares obtained from GLM procedure of SAS. The proportion of the total phenotypic variance among lines that could be explained by a marker was estimated by R^2 = (sum of squares for the markers)/(sum of squares among lines). A two-factor analysis of variance was used to detect epistatic interactions ($P \leqslant 0.01$) between all possible pairs of independent RFLP loci.

Results and discussion

Genetic map

The details on the genetic map of this population were reported in Lee et al. (1996) and Mian et al. (1996). In short, the genetic map was constructed with 155 polymorphic RFLP markers. Of these 155 markers, 126 were co-dominant and 29 were dominant. One hundred and forty markers were mapped to 33 linkage groups covering more than 973 cM. Fifteen markers remained unlinked. Twenty six of the thirty three linkage groups were identified with the USDA/ARS-ISU soybean genetic map (Shoemaker and Specht, 1995).

RFLP markers associated with SLW

There was no genotype × environment interaction for SLW. In order to demonstrate the consistency of QTLs across environments, the results of the combined analysis, as well as the results from each environment, are reported. Combined over environments, the two parents did not differ in SLW (Table 1). The progeny differed significantly ($P \leqslant 0.01$) in their SLW with the highest progeny having a 31% greater SLW than the lowest progeny. The SLW of the highest progeny was also greater than that of either parent.

Combined across the environments, ten RFLP markers on five LGs were associated with SLW (Fig. 1). Six of these markers represent putative independent QTLs (greater than 50 cM from other independent marker loci for the trait, and the marker acted in an additive manner, i.e., no epistasis with other independent markers, in explaining the total variation for the trait) (Table 2). Four of the independent marker loci (Blt043H, A122D-1, A381D-1, and Gc409E-b) were

consistent across environments. The remaining two marker loci on LGs L(1) and L(2) had significant association with SLW only in the greenhouse, but not in the Windblow field environment. Individually, the six independent markers explained between 8 and 18% of variation in SLW in the combined analysis over environments. Marker Blt043H on LG B1 explained the highest amount of variation in this trait ($R^2 = 18\%$).

No epistatic interactions were detected among the six putative independent markers. Thus, these marker loci were additive to one another and if combined would explain 72% of total variation in SLW. The heritability of this trait based on the means of nine replicates was 59%. Thus, all of the genetic variation in SLW in this population could be explained by the six putative independent QTLs.

Young alleles contributed to greater SLW at four (A122D-1, A381D-1, A489V, and Gc409E-b) of the six independent loci (explaining 46% of the phenotypic variation among line means), while the PI416937 allele was responsible for greater SLW at the Blt043H and EV2E-1 marker loci (explaining 26% of the phenotypic variation) (Table 2). This ability of both parents to contribute towards the greater SLW of the progeny explains the transgressive segregation observed among

Table 1 Mean specific leaf weight (SLW) and leaf size for parents and the extreme progeny lines combined over environments

Genotype	Trait	
	SLW mg/cm^2	Leaf size cm^2/leaf
Young	2.99	152
PI416937	2.91	223
High progeny	3.49	248
Low progeny	2.66	146
$LSD_{0.05}$	0.34	34
Heritability (%)	59	61

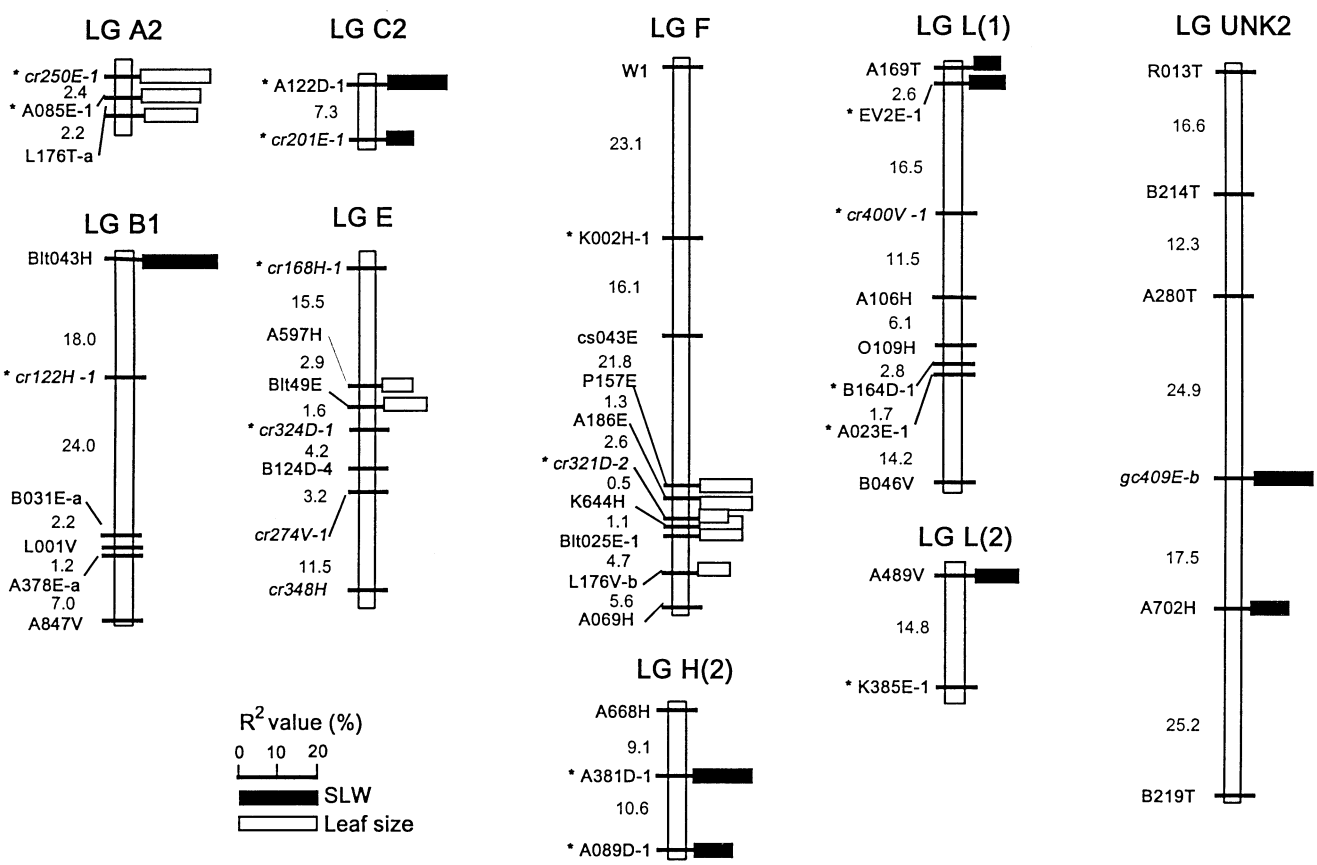

Fig. 1 RFLP linkage groups A2, B1, C2, E, F, H, L(1), L(2), and Unk2 of the Young × PI416937 soybean population showing marker positions and estimated map distances (cM) on the left-hand side, and USDA/ARS-ISU linkage group designation (Shoemaker and Specht 1995) on the top of each linkage group. The numbers in *parenthesis* with the linkage group designation indicate that two or more separate linkage groups in this population were identified within one USDA linkage group. The length of *horizontal bars* indicate R^2 values for the loci associated with the trait. * indicates an anchored probe which had the same probe/enzyme combination and an identical banding pattern with its SoyBase (1995) image. A marker locus is identified with a probe designation, followed by the restriction endonuclease designation, and a *dashed-number suffix* for an anchored probe or a *letter* (*lower-case*) *suffix* for a non-anchored probe, when more than one locus was detected by that probe

Table 2 Putative independent marker loci associated with variation in specific leaf weight of F_4-derived lines from the cross of Young × PI416937 based on single-factor analysis of variance

RFLP locus	Linkage group[a]	Combined		Allelic mean (mg/cm^2)		Environments			
						Greenhouse		Windblow	
		P	R^2 (%)	Young	PI416937	*P*	R^2 (%)	*P*	R^2 (%)
Blt043H	B1	0.0001	18	2.97	3.14	0.0001	16	0.005	8
A122D-1	C2	0.0004	12	3.13	3.00	0.006	7	0.003	9
A381D-1	H(2)	0.0004	12	3.14	3.01	0.001	10	0.01	6
EV2E-1	L(1)	0.004	8	3.02	3.13	0.002	9	–	–
A489V	L(2)	0.0016	9	3.11	3.00	0.0008	10	–	–
Gc409E-b	Unk2	0.0002	13	3.14	3.00	0.0007	11	0.009	6

[a] Shoemaker and Specht (1995)

Table 3 Putative independent marker loci associated with variation in leaf size of F_4-derived lines from the cross of Young × PI416937 based on single-factor analysis of variance

RFLP locus	Linkage group[a]	Combined		Allelic mean (mg/cm^2)		Environments			
						Greenhouse		Windblow	
		P	R^2 (%)	Young	PI416937	*P*	R^2 (%)	*P*	R^2 (%)
A085E-1	A2	0.0006	11	183	197	0.002	9	0.004	8
Blt049	E	0.009	6	185	196	–	–	0.002	9
K644H	F	0.009	6	194	183	–	–	0.0005	11
A635T-1	C2	0.02[b]	5	192	184	0.05	4	0.05	4

[a] Shoemaker and Specht (1995)
[b] Although this marker does not meet the $P \leqslant 0.01$ criterion, it was consistent across the two environments

the progeny in this population. This also implies that considerable improvement in SLW over either parent is possible by selecting for the favorable alleles at all, or most, of these independent loci.

RFLP markers associated with leaf size

There was no significant genotype × environment interaction for leaf size, and combined over environments the leaf size of the parents as well as the progeny lines differed significantly ($P \leqslant 0.01$) (Table 1). PI416937 averaged a 46% greater leaf size than Young. The progeny did not exhibit significant transgressive segregation for this trait. The highest F_4-derived line had a 69% greater leaf size than the lowest line.

A total of 11 markers were associated with leaf size (Fig. 1). Three of the markers were putative independent QTLs and if combined would explain 23% of the variation in leaf size (Table 3). Locus A085E-1 on LG A2 explained the largest variation (11%) of all the markers, and the PI416937 allele at this locus was responsible for a larger leaf size of the progeny. At two of the three independent marker loci, PI416937 alleles were associated with a larger leaf size of the progenies while the Young allele did so at the remaining locus (Table 3). The locus (A085E-1) on LG A2 was consistent across environments, whereas the other two loci were detected only in the field environment at Windblow, but not in the greenhouse.

In addition to these three marker loci associated with leaf size at the $P \leqslant 0.01$ level of significance, RFLP marker A635T-1 on LG C2 was significant at $P \leqslant 0.05$ for the combined data and at each individual environment (Table 3). The Young allele for this locus was associated with large leaf size. This RFLP marker was 75 cM from the A122D-1 marker for SLW on the USDA/ARS-ISU map and unliked in our map. We could not confirm that any of the leaf-size QTLs identified in this population were in common with any of the QTLs reported by Mansur et al. (1993, 1996) due to lack of correspondence of the LGs of their maps with those of the USDA/ARS-ISU genetic map.

The heritability of leaf size was 61% based on a selection unit of the mean of nine replicates. No epistatic interactions were found among these independent markers. Thus, only a part of the genetic variation in the trait could be explained by the detected QTLs. This means that there are leaf-size QTLs that remained undetected in this study.

Relationship between SLW, leaf size, and other traits

Based on line means from the combined analysis, leaf size was not correlated with SLW. This finding contradicts a number of previous reports (Potter and Jones 1977; Wiebold and Kenworthy 1985; VanArendonk and Pooter 1994) documenting a negative relationship between the two traits. The lack of correlation between the two traits in this population was also supported by the fact that none of the three leaf-size QTLs were linked to any of the six SLW QTLs identified. This unusual relationship between SLW and leaf size in this population may have resulted from the use of PI416937 as a parent in the cross. Even though PI416937 had a 46% larger leaf size than Young, its SLW was similar to that of Young. Thus, in this population, it may be feasible to select progeny with all of the alleles for greater SLW and larger leaf size. As has been mentioned earlier, progeny with a significantly greater SLW than either parent can be selected from this population. The SLW of the progeny with the largest leaf size was 3.24 mg/cm^2, which was only 9% lower than the progeny with the largest SLW. Also, the leaf size of the progeny with the greatest SLW was 222 cm^2/leaf, which was nearly equal to that of PI416937 and statistically similar to the progeny with the largest leaf size (Table 1).

Marker locus A381D-1 on LG H(2) was also linked to a water-use efficiency (WUE) QTL (Mian et al., 1996). The Young allele at this locus was responsible for the greater WUE of the progeny lines. The Young allele at marker locus A381D-1 also contributed to the greater SLW of the progeny. With a tight linkage between the two QTLs for SLW and WUE or with pleiotropy, such a result would be expected. A positive relationship between specific leaf weight (SLW) and water use efficiency (WUE) has been reported in peanut (*Arachis hypogaea*) (Wright 1993, 1994; Brown and Byrd 1996). Nelson (1988) stated that the basis for the relationship between SLW and WUE is not clear, but the often-observed positive correlation between SLW and leaf photosynthesis may contribute to the relationship. If growth is limited by leaf photosynthesis, a greater SLW may result in a greater WUE.

Marker locus Blt043H, the marker associated with the largest SLW QTL, was also associated with maturity and plant height in this population. PI416937 contributed the alleles for taller plants and later maturity at this locus. The PI416937 allele at this locus was also responsible for a greater SLW. It is not clear to us how a QTL for plant height or maturity would affect the SLW of a soybean plant at such an early stage of development (V12). Rather, it is possible that the greater SLW at the early stages of development, may result in a larger and probably a taller plant.

Marker EV2-1 on LG L for SLW also conditioned aluminum tolerance in this soybean population. The Young allele at this locus is associated with increased aluminum tolerance, while for SLW the PI416937 allele contributes to a greater SLW. Consequently, it would be necessary to break the linkage between these two QTLs before selecting for the favorable allele for one trait without adversly affecting the other.

In summary, the transgressive segregation of the progeny for SLW, the lack of a negative relationship between SLW and leaf size, and the absence of linkage between the SLW QTLs and the leaf-size QTLs in this soybean population are important findings of this study. In this soybean population, it is possible to select for progeny lines with a greater SLW than either parent, perhaps without affecting leaf size. It is feasible to combine all of the desirable alleles for a greater SLW and large leaf size in a single genetic background. Pyramiding the favorable alleles for both SLW and large leaf size in a soybean genotype may translate to its faster vegetative growth, and perhaps higher seed yield, through the maximization of its total photosynthetic performance.

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