Molecular Markers Associated with Water Use Efficiency and Leaf Ash in Soybean

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ABSTRACT

Water use efficiency (WUE) is an important trait that has been associated with drought tolerance of crop plants, and leaf ash (LASH) is generally related to WUE. A restriction fragment length polymorphism (RFLP) map was constructed from a soybean [*Glycine max* (L.) Merr.] population of 120 F_4-derived lines from a cross of 'Young' × PI 416937. The purpose of this research was to identify quantitative trait loci (QTL) associated with WUE and LASH in 36-d-old, greenhouse-grown plants. The experimental design was a randomized complete block with six replications. Significant ($P < 0.01$) phenotypic differences were detected among the lines for both traits. A total of four and six independent RFLP markers were associated with WUE and LASH and if combined each group of markers would explain 38 and 53% of the variability in the respective traits. One marker locus (cr497-1), on USDA Linkage Group J, explained 13.2% of the variation in WUE indicating the presence of a major QTL. The LASH was negatively correlated with WUE ($r = -0.40^{**}$), and two QTL were associated with both WUE and LASH. For each of these QTL, the allele for increased WUE was associated with reduced LASH.

MOST of the soybean hectarage in the USA is grown in areas where erratic and low rainfall often reduces yield. This is particularly evident in the southern USA where the average annual loss has been estimated to be over 1.0 Mg ha^{-1} (Carter, 1989).

A drought tolerant plant introduction (PI 416937) has been identified recently in the USDA soybean germplasm collection and is characterized by tolerance to wilting during severe drought stress (Sloane et al., 1990). The primary drought tolerance mechanism of PI 416937 is a large fibrous root system which is able to extract soil moisture more efficiently than cultivar Forrest. It is also tolerant to aluminum, which gives it an added advantage of root growth in acid soils (Carter and Rufty, 1993). The drought tolerance mechanism of PI 416937 probably results from the maintenance of water supply rather than reduction in the amount of water used. While this is a good strategy for a short duration or intermittent drought, such a strategy may not be as effective under a longer duration drought, during which soil moisture could be completely depleted resulting in earlier plant death. It would be logical to try to combine the soil moisture extracting ability of PI 416937 with improved WUE (total biomass accumulation/total water used). In two greenhouse experiments that included 35 soybean genotypes, we found Young as one of the most efficient genotypes in terms of WUE (unpublished data, 1992-1993).

Masle et al. (1992) reported a negative association between WUE and LASH across a range of C_3 species. Mayland et al. (1993) found that ash concentration (leaf and stem) of crested wheatgrass [*Agropyron desertorum* (Fischer ex Link) Schultes] was negatively associated with WUE. Smith et al. (1982) found a significant negative correlation between WUE and calcium contents in the shoots of ryegrass (*Lolium* sp.). These responses support the concept (Masle et al., 1992) that there is a positive linear relationship between leaf or shoot ash contents and the transpiration ratio (1/WUE). This response would depend on the maintenance of a constant concentration of minerals in the transpiration stream. Environmental conditions could cause variation in the mineral concentration; however, comparison of genotypes under the same environmental conditions should provide repeatable responses. A molecular investigation may provide some insight into the genetic relationships between WUE and LASH.

While WUE can be readily measured in pots in which evaporation can be minimized, it is difficult to control or measure soil evaporation in the field. To avoid the difficulty of measuring WUE of field grown plants, Farquhar et al. (1982) and Farquhar and Richards (1984) proposed the evaluation of the discrimination of ^{13}C by leaves (Δ) during photosynthesis in C_3 plants. The Δ in leaf tissue is correlated with WUE in many crop species (Farquhar and Richards, 1984). Martin et al. (1989) found that 70% of the variation in Δ between *Lycopersicon esculentum* Miller (drought-sensitive cultivated tomato with high Δ) and *L. pennellii* (Cor.) D'Arcy (drought-tolerant wild tomato with low Δ) was associated with three RFLP loci that mapped to three different chromosomes. The results from this indirect mapping of QTL for WUE indicate the possibility of direct mapping of QTL conditioning WUE, provided the phenotypic measurements of WUE can be accomplished.

Marker assisted selection by means of RFLP or other molecular markers offers an important alternative for selection of hard to measure traits (Tanksley et al., 1989). The identification of QTL allows the analysis and selection of complex quantitative traits (e.g., drought tolerance) as a set of single-gene traits. A RFLP analysis allows the localization of QTL and the determination of the relative magnitude of their effects on traits of interest (Reiter et al., 1991). Indirect selection for drought tolerant QTL via molecular markers may improve selection efficiency. The objective of this study was to identify

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Abbreviations: WUE, water use efficiency; QTL, quantitative trait loci; LASH, leaf ash; RFLP, restriction fragment length polymorphism; LG, linkage group.

RFLP markers associated with QTL conditioning WUE and LASH.

MATERIALS AND METHODS

Genotypic Assay

A F_4-derived population from a cross between Young × PI 416937 was used to create a RFLP linkage map and for evaluation of WUE and LASH. To obtain DNA for determination of RFLP polymorphism between the parents, seeds of Young and PI 416937 were sown in a greenhouse. Leaves were harvested from seedlings prior to full expansion. DNA was isolated from leaves according to the procedure of Keim et al. (1988) and digested overnight with one of the five restriction enzymes: *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, or *Taq*I. Restricted DNA was then separated by 8 g L^{-1} (w/v) agarose gel electrophoresis for 16 to 20 h at 22 V. Following electrophoresis, a Southern blot was made by transfer to Gene-Screen Plus nylon membranes (DUPONT, Biotechnology Systems, NEN Research Products, 549 Albany Street, Boston, MA). Nylon membranes were placed in 300- by 38-mm glass bottles containing 4 to 10 mL of 0.25 *M* Na_2PO_4 and 70 g L^{-1} (w/v) SDS (sodium dodecyl sulfate), and prehybridized in a rotisserie oven for 4 to 6 h at 65°C. Approximately 25 ng of isolated DNA probe was randomly labeled, and hybridization was conducted overnight in hybridization buffer containing 650 mL of 0.77 *M* Na_2HPO_4 and 350 mL of 200 g L^{-1} (w/v) SDS. A set of about 750 probes from various sources including cDNA and genomic clones of soybean (R.C. Shoemaker, USDA/Iowa State Univ.; K.G. Lark, Univ. of Utah; R.T. Nagao, Univ. of Georgia), *Vigna radiata* (L.) R. Wilczek (N.D. Young, Univ. of Minnesota), *Phaseolus vulgaris* L. (J.M. Tohme, CIAT, Cali, Columbia), *Arachis hypogaea* L. (G.D. Kochert, Univ. of Georgia), and *Medicago sativa* L. (G.D. Kochert) were used to screen for polymorphism between the parental genotypes.

Polymorphic probes were used for mapping a population of 120 F_4-derived lines (each line traces to a different F_2 plant) of Young × PI 416937. DNA was isolated from leaves of eight to 10 plants/line which were grown at the Plant Sciences Farm near Athens, GA, during the 1993 growing season. To supplement this DNA and replace DNA samples of poor yield or low quality, leaves were uniformly collected and pooled from at least 14 additional plants from each line grown in the greenhouse. Nylon membranes containing DNA from each of the 120 lines were screened with polymorphic probes. The linkage map was constructed with marker data with the Kosambi map function of GMendel (Holloway and Knapp, 1993) assuming the data were collected from F_4-derived lines. The LOD minimum of 3.0 and rmax of 0.38 [approximately 50 cM (centimorgan)] was used to construct this map.

Greenhouse Assay

In a greenhouse at 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 to 10 d after planting. Plants were fertilized with 40 mg N, 40 mg P, and 40 mg K per pot. Each pot was then fitted with a lid containing two small holes, one to fit the seedling through and the other one as an opening for watering. A small piece of foam was placed in between the lid and seedling to prevent damage to the stem, and a rubber stopper was placed in the other opening to minimize evaporation of soil moisture. Pots were then watered to bring the soil to field capacity. Water use by each plant was recorded by weighing the pots regularly. When soil moisture dropped to 25% of field capacity, water was added individually to each pot to return soil moisture to field capacity. At 25% of field capacity, plants showed visible signs of mild water stress (e.g., orientation or wilting of leaves and bending of petioles).

The experimental design was a randomized complete block with six replicates. Because of the large size of the experiment and limitation in greenhouse space, the replicates were grown sequentially in time with 3 to 4 wk between successive replicates. The experiment was started in mid June of 1994 and was completed by early October of the same year. High-pressure sodium lighting (575 μmol m^{-2} s^{-1} of photosynthetically active radiation 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. Temperature ranged from 28 to 37°C during the day and from 19 to 23°C during the night. Daytime relative humidity ranged between 35 and 65%. Plants were spaced at about 20 by 25 cm 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 two sides (along the length of the bench). The pots were regularly rotated to minimize border and locational effects within the test.

At 36 d after planting, the leaves and the stem of each plant were harvested separately, and the roots were carefully separated from the soil by washing them with a gentle flow of water from a hose onto a wire mesh. Roots severed during washing were retrieved from the mesh. Plant parts were dried in an oven at 68°C for 96 h and the leaf, stem, and root dry weights were recorded in grams. Total dry weight (TDW = leaf dry weight + stem dry weight + root dry weight) was calculated. The WUE was then determined as follows: WUE = TDW / amount of H_2O used in liters. The LASH was determined according to the procedure described by Masle et al. (1992) and was expressed as milligrams per gram leaf dry weight.

Data Analysis

The WUE and LASH data were compared with the RFLP marker data. Each marker locus was evaluated for linkage to QTL affecting each 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 locus (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 ($P < 0.01$) with an *F*-test from the type III means squares obtained from GLM procedure of SAS (SAS Institute, Cary, NC). Two-factor analysis of variance was used to detect epistatic interactions between all possible pairs of independent significant RFLP markers.

RESULTS AND DISCUSSION

Genetic Map

A genetic map was constructed with 155 polymorphic RFLP markers (Fig. 1). Of these 155 markers, 126 were co-dominant and 29 were dominant markers. The letter

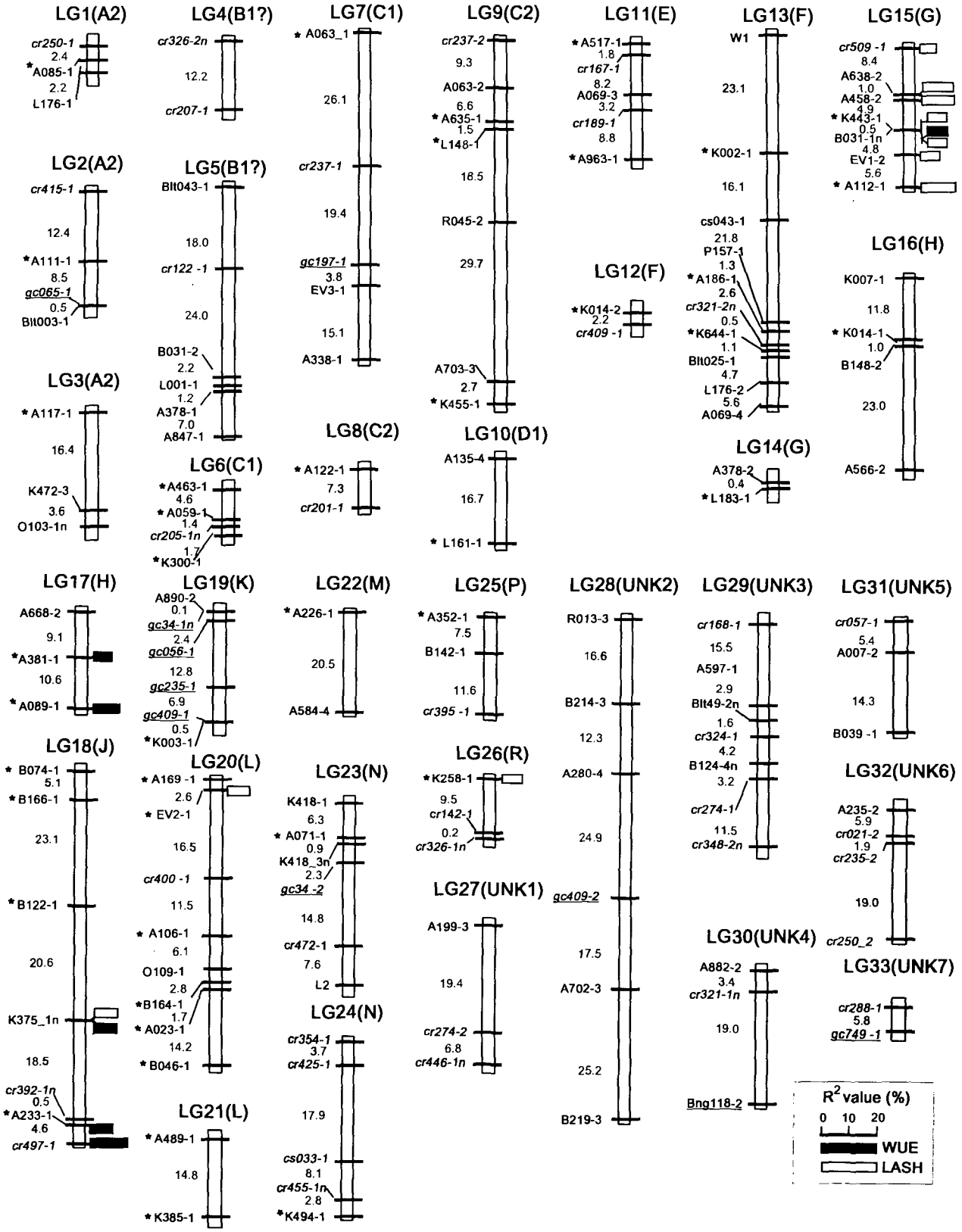

Fig. 1. Soybean RFLP genetic linkage map showing marker positions and estimated map distance (cM) on the left-hand side, and USDA linkage group (Shoemaker and Specht, 1995) in parentheses on the top of each linkage group. Length of bars indicates R^2 values for the loci associated with WUE and LASH of 36-d-old greenhouse plants. * represents an anchored probe which had an identical banding pattern with the image in SoyBase (1995). Markers from *G. max* are are depicted in regular boldface type, those from *P. vulgaris* in underlined boldface type, those from *A. hypogaea* in italicized boldface type, and those from *M. sativa* are underlined in italicized boldface type. A marker locus is identified by a probe designation and a dashed number suffix, where the latter identifies the specific locus of the two or more loci detected by that probe.

"n," for null, was used as a suffix with these dominant RFLP markers. A dashed number suffix was attached to each locus name so that a specific locus would be identifiable from any other locus detected by the same probe. It would also facilitate the comparison of any marker locus on this map with those on the USDA soybean genetic map (Shoemaker and Specht, 1995). One hundred-forty markers were mapped to 33 linkage groups covering more than 973 cM. Fifteen markers remained unlinked. The RFLP loci on this map were compared with the available images of SoyBase (1995) to determine the correspondence of a given locus to the linkage groups of the USDA soybean genetic map (Shoemaker and Specht, 1995). The common loci were also compared with another soybean genetic map constructed from a F_2-derived population of PI97100 × 'Coker 237' (unpublished data, 1995). Each linkage group was designated according to its corresponding linkage group on the USDA map on the basis of RFLP anchor probes (Shoemaker and Specht, 1995). Each of the anchor probes had the same restriction enzyme and an identical banding pattern with the USDA map.

Of the 33 linkage groups in our map, seven could not be identified with the USDA map. Twenty-six linkage groups corresponded to the USDA map, two of which were indirect and a "?" was attached to their designations. Specifically, cr207 on linkage group (LG) 4 was linked to A588-1 with 7.5 cM distance on the map of PI97100 × Coker 237, and A588-1 belongs to LG B1 of the USDA map. LG 5 probably corresponds to LG B1, because cr122-1 is on LG B1 on the PI97100 × Coker237 map. Further mapping with anchor markers of unidentified linkage groups from our population will be needed to confirm the corresponding linkage groups on the USDA map.

The total length of this map was 973 cM, as opposed to that of 1600 cM when Lee et al. (1996) constructed the map using Mapmaker (Lander et al., 1987) as if the same data were collected from F_2-derived lines (Lee et al., 1996). Another notable difference of this map from the Lee et al. (1996) map is that the two segments of LG J of the previous map are joined to form LG 18 in this map (Fig. 1).

RFLP Markers Associated with WUE

There were significant ($P < 0.01$) differences among the progeny lines for WUE (Fig. 2). Young was 16.5% more efficient at producing dry matter with a unit of water than PI 416937. The highest progeny produced 17.2% more dry matter with a unit of water than the lowest progeny. The WUE of the 120 F_4-derived lines showed continuous variation, typical of a quantitative trait (Fig. 2). The progeny lines did not exhibit transgressive segregation for this trait, and the WUE of the most efficient F_4 line was almost the same as that of Young.

Six RFLP markers were associated with WUE; one on LG 15(G), two on LG 17(H), three on LG 18(J) (Fig. 1). The marker cr497-1 (LG 18) explained the largest amount of variation (13.2%) of all markers, and

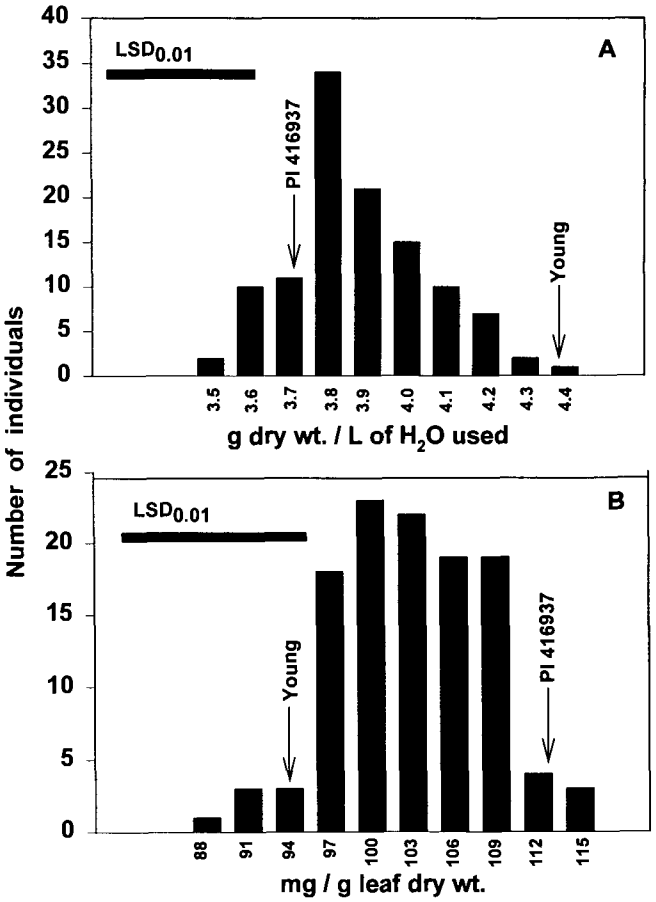

Fig. 2. (A) Frequency distribution of the 120 F_4-derived lines for water use efficiency (WUE), and (B) frequency distribution of the 120 F_4-derived lines for leaf ash contents (LASH).

the Young allele at this locus contributed to greater WUE of the progeny lines. Four of the six markers represent putative independent QTL (greater than 25 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 total variation for the trait). The loci cr497-1 and K375-1n on LG 18 (J) were both considered as independent loci even though they were 24 cM away from each other (Fig. 1). This is because of the amount of variation explained by markers A233-1 and cr392-1n that lies between the two supposedly independent QTL. The effect of the QTL associated with marker locus cr497-1 diminishes rapidly (to about $R^2 = 5\%$, $P = 0.03$, at marker locus cr392-1n). Following this trend, it is not possible for the same QTL to have the effect detected at locus K375-1n ($R^2 = 7.5\%$, $P = 0.003$).

No epistatic interactions were detected among the four putative independent markers. Thus, these four marker loci were additive to one another and if combined together would explain 38% of total variation in WUE. The heritability of this trait based on means of six replicates was 50%. Thus, most of the genetic variation was explained by the four putative independent QTL.

Young alleles contributed to greater WUE at three of the four independent loci (explaining 30% of the variation) while the PI 416937 allele was responsible for

Table 1. Putative independent RFLP loci associated with variation in WUE and LASH.

Trait	RFLP locus	Linkage group	P	R^2	Allelic means	
					Young	PI 416937
				%	g dry wt./L H_2O	
WUE	B031-1n	15(G)	0.0100	8.5	3.98	4.06
	A089-1	17(H)	0.0033	8.7	4.03	3.94
	cr497-1	18(J)	0.0002	13.2	4.03	3.92
	K375-1n	18(J)	0.0029	7.5	4.02	3.93
				%	mg/g leaf dry wt.	
LASH	A112-1	15(G)	0.0001	13.8	104	100
	A458-2	15(G)	0.0003	12.7	104	99
	K375-1n	18(J)	0.0036	7.2	100	103
	EV2-1	20(L)	0.0150	6.2	103	100
	K258-1	26(R)	0.0098	6.3	101	103
	cr107-1	unlinked	0.0083	6.6	100	103

greater WUE at the remaining independent marker locus (explaining 8% variation) (Table 1). Based on these findings, both parents can potentially contribute to greater WUE of the progenies. With a larger number of lines from this cross it should be possible to identify progeny with greater WUE than Young.

RFLP Markers Associated with LASH

The LASH of the progeny lines differed significantly ($P < 0.01$) and showed a continuous distribution indicating the quantitative nature of the trait (Fig. 2). PI 416937 averaged a 20% greater LASH than Young. The progenies did not exhibit significant transgressive segregation for this trait. The highest F_4-derived line had 32% higher LASH than the lowest line.

A total of 11 markers were associated with LASH (Fig. 1). Seven of these markers were on LG 15(G), one on LG 18(J), one on LG 20(L), one on LG 26(R), and one was unlinked. Six of the markers were putative independent QTL and if combined would explain 53% of the variation in LASH (Table 1). The independent markers A112-1 and A458-2 on LG 15 represent an exception to our previously described guideline for determination of putative independent QTL. Even though the two markers are only 16 cM away from each other, the amount of variability explained by the markers that are between these two markers suggests the presence of two different QTL (Fig. 1). We also expect that the QTL associated with marker locus $A112^{-1}$ is located beyond this marker (opposite to PEV1). However, this is speculation and there might instead be a single QTL.

The heritability of LASH was 74% based on a selection unit of the mean of six replicates. No epistatic interactions were found among these independent markers. Marker locus A112-1 on LG 15 explained the largest variation (13.8%) of all markers, and the Young allele at this locus was responsible for higher LASH of the progenies. At three of the six independent marker loci, PI 416937 alleles were associated with higher LASH of the progenies while the Young allele did so at the remaining three marker loci (Table 1).

Relationship between WUE and LASH

Based on genotypic means, LASH had a negative correlation ($r = -0.40^{**}$) with WUE. This finding is in agreement with previous studies (Mayland et al., 1993; Masle et al., 1992). At two genomic regions, one on LG 15(G) and one on LG 18 (J), QTL were associated with both WUE and LASH, and together explained 16 and 21% of the variation in WUE and LASH respectively (Table 1 and Fig. 1). The contribution of each parental allele at these two loci supports the negative association between WUE and LASH, and these loci may provide some genetic basis for the correlation between the two traits. At the locus on LG 15, the PI 416937 allele was associated with greater WUE and lower LASH of the progeny while at marker locus K375-1n (LG J), the Young allele contributed to greater WUE and lower LASH of the progeny (Table 1). In this study, however, the overall relationship between WUE and LASH was not strong enough to justify using LASH as an indirect selection criterion for improving WUE.

We do not know of any previous report on identification of RFLP markers related to WUE and LASH of soybean. This study demonstrates the utility of using molecular markers to study quantitative traits. Traditional heritability estimates provide information on the ratio of genotypic to phenotypic variation. The use of molecular markers provides additional information on the identification of the number of QTL affecting the trait, genomic location of the QTL, and which parent possesses the positive allele at each QTL. These results can be used to determine the specific parents for hybridization to obtain maximum improvement of the trait and to provide guidance in determining the number of progeny required to effectively select an individual with the maximum expression of the trait.

There are appreciable differences among soybean lines in their WUE, and we believe that with finer mapping of these QTL significant gain could be made by using marker assisted selection to improve this trait which is otherwise difficult to evaluate experimentally.

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