Theor Appl Genet (1991) 81:189–198

THEORETICAL AND APPLIED GENETICS

RFLP mapping using near-isogenic lines in the soybean [*Glycine max* (L.) Merr.]*

G. J. Muehlbauer, P.E. Staswick, J.E. Specht***, G.L. Graef, R.C. Shoemaker**** and P. Keim*****

Department of Agronomy, University of Nebraska, Lincoln, NE 68583-0915, USA

Received July 2, 1990; Accepted July 19, 1990
Communicated by A.L. Kahler

Summary. A molecular marker analysis of a near-isogenic line (NIL), its donor parent (DP), and its recurrent parent (RP) can provide information about linkages between molecular markers and a conventional marker introgressed into the NIL. If the DP and RP possess different alleles for a given molecular marker, and if the NIL possesses the same allele as the DP, then it is reasonable to presume a linkage between that molecular marker and the introgressed marker. In this study, we examined the utility of RFLPs as molecular markers for the NIL gene-mapping approach. The allelic status of fifteen RFLP loci was determined in 116 soybean RP/NIL/DP line sets; 66 of the 'Clark' RP type and 50 of the 'Harosoy' RP type. Of the 1740 possible allelic comparisons (116 NILs × 15 RFLP loci), 1638 were tested and 462 (33.9%) of those were informative (i.e., the RP and DP had different RFLP alleles). In 15 (3.2%) of these 462 cases the NIL possessed the DP-derived RFLP allele, leading to a presumption of linkage between the RFLP locus and the introgressed conventional marker locus. Two presumptive linkages, pK-3 – *r* and pK-472 – Lf_1, were subsequently confirmed by cosegregation linkage analysis. Although not yet confirmed, two other associations, pk-7 – *ab* and pK-229 – y_9 seemed to be plausible linkages, primarily because the pk-7 – *ab* association was detected in two independently derived NILs and both markers of the pK-229 – y_9 association were known to be linked to *Pb*. The data obtained in this investigation indicated that RFLP loci were useful molecular markers for the NIL gene-mapping technique.

Key words: *Glycine max* – Near-isogenic lines – Molecular markers – RFLP – Linkage – Genetic map

Introduction

Only a fraction of the soybean (*Glycine max* L. Merr.) conventional markers have been located on the classical genetic linkage map (Palmer and Killen 1987). Kiang (1987), for example, only recently located the position of three of the several known isozyme markers. By comparison, restriction fragment length polymorphisms (RFLPs) provide an extremely efficient means of mapping plant genomes, and RFLP linkage maps have been developed recently in soybean (Apuya et al. 1988; Keim et al. 1989a; R. C. Shoemaker, unpublished data). Because molecular markers in particular are so useful in genetic studies, there is a need to consolidate the RFLP, isozyme, and conventional marker maps into a single linkage map.

Near-isogenic lines (NILs) of soybean can be used to integrate the separate molecular and conventional marker linkage maps (Muehlbauer et al. 1988). Gene mapping with NILs is based on the premise that when a conventional marker is introgressed from a donor parent (DP) into a recurrent parent (RP) through backcrossing (see Fehr 1987), the resultant NIL retains a small number of DP-specific molecular markers in its genome. Most of

* Published as Paper no. 9101, Journal Series, Nebraska Agric. Res. Div. Project no. 12-091. Research partially funded by a grant from the Nebraska Soybean Development, Utilization, and Marketing Board

** Current address: Department of Agronomy and Plant Genetics, University of Minnesota, St. Paul, MN 55108
*** To whom correspondence should be addressed
**** Permanent address: USDA Agricultural Research Service, Cereal and Soybean Improvement Research Unit, Iowa State University, Ames, IA 50011
***** Permanent address: Department of Biological Sciences, Northern Arizona University, Flagstaff, AZ 86011

these retained markers will be linked to the introgressed conventional marker.

For a hypothetical species possessing $n = 20$ chromosomes of 50 cM length, Muehlbauer et al. (1988) calculated that of 100 randomly chosen molecular loci, only four DP-derived alleles would be retained in the BC_5S_1-derived NIL. Of these four markers, two or three would be located on the introgressed marker chromosome, with the others dispersed randomly among the other chromosomes. Therefore, one could examine existing RP/NIL/DP sets to genetically "type" each line for its allelic status at molecular marker loci of interest. If the NIL possessed the DP allele at one or more molecular marker loci, then one could presume linkage between that molecular marker and the introgressed conventional marker. Verification of that linkage would necessitate the generation of appropriate F_2 or F_3 cosegregation data. However, the NIL gene-mapping technique would provide a convenient initial screen , because it would allow one to categorize the molecular and conventional markers into presumptive linkage groups. This information would greatly reduce the number of linkage analyses needed for the integration of the molecular and conventional markers into a single linkage map. Because a large number of soybean NILs have already been created (Bernard 1976), the NIL gene-mapping technique would be particularly effective when used in conjuction with a densely populated RFLP linkage map.

We previously demonstrated the utility of the NIL gene-mapping technique with respect to 12 isozyme loci (Muehlbauer et al. 1989). Data obtained in that study allowed us to position the *Enp* and the *Mpi* isozyme loci next to the *In* and *Dt2* loci, respectively, on the conventional marker linkage map.

In the present study, the effectiveness of the NIL gene-mapping technique was evaluated using RFLP markers. The first step was to determine how much diversity was detectable between the RP and DP at numerous RFLP loci. In this initial screen we used 24 soybean DNA probes and four restriction enzymes to determine the allelic status of RFLP loci in two recurrent parents (RPs), 'Clark' and 'Harosoy', and in 21 donor parents (DPs) that had been used in the development of some of the soybean NILs. Based on this initial data, we determined that 12 of the 24 probes detected at least some degree of DP/RP allelic diversity. We then used these 12 probes to evaluate the allelic status of 15 RFLP loci (2 unlinked loci are detected with each of 3 probes) in 116 soybean RP/NIL/DP sets. The objectives of this study were to use the NIL gene-mapping technique to identify linkages between RFLP markers and introgressed conventional markers, and to evaluate the technique on the basis of data obtained with RFLP loci, with isozyme loci (Muehlbauer et al. 1989), and with hypothetical loci using theoretical calculations (Muehlbauer et al. 1988).

Materials and methods

Plant material

Seed of the 116 RP/NIL/DP sets was obtained from R.L. Bernard (USDA-ARS Soybean Isoline Collection, Department of Agronomy, Urbana, Illinois). Each set consisted of a NIL and its associated RP and DP. Most of the 116 NILs represented BC_5S_1 plant selections from an RP × DP backcross program, but some were derived from intercrosses between previously derived NILs. A few NILs were derived from spontaneous mutations that had occurred in the RPs. The 'Harosoy'-*wm* NIL, for example, was created as a result of a *Wm* to *wm* mutation in 'Harosoy'.

The 116 NILs were constructed from 42 DPs and the two RPs, 'Clark' and 'Harosoy' (Table 1). There were 66 'Clark' NILs and 50 'Harosoy' NILs. In 36 of the 116 sets, gene (s) from a common DP were introgressed into both 'Clark' and 'Harosoy' (i.e., the reason for only 80 data rows in Table 1). In a few cases, a 'Clark' NIL and a 'Harosoy' NIL with the same introgresssed marker (e.g., E_1, Dt_2, dt_1, w_1, Rps_1, etc.) received that marker from different DPs. Finally, in some instances, the introgression involved more than one conventional marker. In total, there were 57 *different* introgressd conventional markers in the 116 NILs listed in Table 1. There also were four NILs with DP cytoplasm; that is, the RP genome had been introgressed into DP cytoplasm (cf. the DPs: PI 101.404A & B, PI 65.388, Medium Green). Consequently, these 116 NILs possess introgressed genes that represent a broad cross-section of the conventional markers reported in the soybean.

DNA isolation and Southern blotting

DNA was isolated from lyophilized young leaves using a procedure described by Saghai-Maroof et al. (1984) with minor modifications suggested by T. Helentjaris (personal communication). Leaf DNA (5 µg) was digested with one of several restriction endonucleases (Table 1) following protocols specified by the supplier. DNA fragments were then electrophoresed overnight in an 0.8% agarose gel containing 0.89 *M* TRIS-borate, 0.089 *M* boric acid, and 0.05 *M* NaEDTA, pH 8.0. After electrophoresis, the DNA was transferred (Southern 1975) to an uncharged nylon membrane obtained from Micron Separations.

Isolation and labeling of soybean probes

Twenty-one soybean probes, pK-2, pK-3, pK-5, pK-7, pK-9 pK-11, pK-14, pK19, pK-21, pK-32, pK-69, pK-80, pK-226b, pK-265, pK-266, pK-272, pK-349, pK-365, pK-411, pK-417, and pK-472, were used in this study, and were derived from a 0.5- to 3.3-kb size-fractionated *Pst*I genomic library (Keim and Shoemaker 1988). Three previously uncharacterized probes (6-39, 6-124, and 5-257) derived from a soybean leaf cDNA library (Staswick 1988) also were used. All 24 probes were used to detect RFLPs in a *G. max* × *G. max* cross (i.e., Minsoy × Noir 1), or in a *G. max* × *G. soja* cross (Keim et al. 1989a).

Plasmid DNA was isolated using a rapid-boiling method adapted from Holmes and Quigley (1981). Soybean DNA inserts were isolated from the plasmids following a procedure developed by Dretzen et al. (1981). Inserts were labeled with ^{32}P using a random primer procedure (Feinberg and Vogelstein 1983).

Hybridization and washes

Membranes were prehybridized overnight in 12 ml of solution (1 ml/12 cm^2 of blot) comprised of 5 × SSC, 1 × Denhardts, 50 m*M* TRIS-Cl, pH 8.0, 0.2% SDS, 10 m*M* NaEDTA, and 100 µg/ml sheared salmon-sperm DNA (T. Helentjaris, personal communi-

cation). Hybridizations were conducted overnight in 8 ml of the same prehybridizing solution at 60–65 °C. Filters were washed twice for 5 min at room temperature in 2 × SSC and 0.5% SDS, followed by two 30-min washes in 0.2 × SSC and 0.1% SDS at 65 °C. Membranes were exposed to X-ray film at −70 °C for 24–48h.

Initial screen for RP/DP allelic diversity

The potential of the 24 probes to detect RFLP diversity with respect to the NIL gene-mapping technique was determined by examining the two RPs, 'Clark' and 'Harosoy', and the following set of DPs: T117, T139, PI 84.987, T145, T175, T176, T201, T203, T204, PI 80.837, T217, PI 91.160, PI 86.024, PI 83.945, T26OH, PI 101.404A, Blackhawk, Chief, CNS, Columbia, and Medium Green. The restriction enzymes used were those listed in Table 1.

Final screen for presumptive linkage

Based on the results obtained in the initial screen for RP/DP allelic diversity, 12 probes were selected for the final evaluation of the allelic status at 15 RFLP loci (2 unlinked loci are detected with each of 3 probes) in the 116 RP/NIL DP trio line sets. In the informative cases where an RP/DP allelic contrast existed for the RFLP locus, a presumptive linkage of the RFLP marker with the introgressed conventional marker was inferred whenever the RFLP locus had a DP/NIL allelic equality and a corresponding RP/NIL allelic contrast.

F_2 and/or F_3 cosegregation data were available for two of the presumptive linkages identified in this study. Population I originated from a cross between two 'Clark' NILs, 'Clark'-$E_1E_1ttP_1P_1RR$ × 'Clark'-$e_1e_1TTp_1p_1rr$. Each F_2 plant was evaluated for allelic status at the RFLP locus pK-3. F_3 lines were scored as true-breeding or segregating for the conventional markers. Population II originated from a cross of a five-gene NIL with its RP, H-29 ('Harosoy'-$Lf_1Lf_1lnlny_9y_9Pd_1Pd_1dt_1dt_1$) × L2 ('Harosoy'). Each F_2 plant was evaluated for allelic status at the pK-472 RFLP locus, and also was scored for leaflet number: homozygous pentafoliolate (Lf_1Lf_1), heterozygous quadrafoliolate (Lf_1lf_1), or homozygous trifoliolate (lf_1lf_1). F_2 plants were scored for other morphological markers segregating in this population. In both populations, the recombination frequency between any two cosegregating loci was calculated using the Linkage-1 computer program acquired from Suiter et al. (1983).

Results and discussion

Initial screen

The initial screen of 21 DPs and two RPs ('Clark' and 'Harosoy') with 24 soybean DNA probes allowed us to assess the extent of RFLP diversity between the DPs and RPs used in the construction of numerous lines in the NIL collection. Fifteen RFLP loci, detected by 12 of the 24 probes, were polymorphic for two or more paired combinations of the 21 DPs and the two RPs. The 15 RFLP loci detected by these 12 probes were: pK-2, pK-3, pK-7, pK-9, pK-11a, pK-11b, pK-14a, pK-14b, pK-69a, pK-69b, pK-80 pK-229, pK-417b, pK-472, and 6-39, where a and b indicate two unlinked loci detected by the same probe. Another probe, pK-411, detected an allelic contrast between CNS (a DP) and the two RPs, but it was not used in the final screen. The 11 remaining probes were monomorphic with respect to these two RPs and 21 DPs (T203, T204, and T260H were not tested with pK-365).

Final screen

In the final 12-probe screen, 1740 RP/NIL/DP comparisons (116 NIL sets × 15 soybean RFLP loci) were possible, and all but 102 of these were eventually evaluated (Table 1). Of the 1,638 tested cases, 1,361 were evaluated in a two-step manner by first testing the RP and DP for allelic differences at each RFLP locus, an *a priori* approach recommended by Muehlbauer et al. (1988). Polymorphisms (i.e., allelic contrasts) were found for the RP and DP in 462 of the 1,361 cases (i.e., the "+" and "#" cells in Table 1), indicating that these 462 cases would be "informative" when the allelic status of the appropriate NILs was determined in the second step. In contrast, an RP/DP allelic equality was found for 899 of the 1,361 cases (i.e., the "−" cells in Table 1), indicating that the two parents of the NIL had identical alleles with respect to the RFLP locus in question. This obviated the need to examine the corresponding NIL.

Approximately 34% (100 × 462/1,361) of the tested RP/DP cases were of the informative type in this evaluation of 15 RFLP loci and 116 trio line sets (Table 1). By comparison, in a previous study of 12 isozyme loci and 63 trio line sets, we found that 34% of the RP/DP cases (100 × 256/756) were informative (Muehlbauer et al. 1989). Aside from the fact that this amount of RP/DP diversity (i.e., one-third of the tested cases) allows for a reasonable successful application of the NIL gene-mapping technique (Muehlbauer et al. 1988), the identical percentages suggest that RP/DP diversity is equivalent for both isozyme and RFLP loci.

In 194 of the 1,638 tested cases, only the NIL/RP comparison was evaluated; the corresponding DP was not tested (i.e., the "R" cells in Table 1). In all 194 cases, the RP and NIL exhibited an allelic equality. Restricting the evaluation to just the RP/NIL components of each set is generally the most efficient approach when searching for linkage. However, in those instances where several NILs have been derived from one RP × DP cross, it might be more efficient to first determine RP/DP allelic status (i.e., contrast or equality) to discern if an evaluation of the NILs is even necessary.

Three 'Clark' NILs (*i*, k_1, k_2) and three 'Harosoy' NILs (*i*, k_2, *wm*) originated as a spontaneous mutation of the corresponding wild-type conventional marker in the RPs (i.e., the "M" cells in Table 1). No allelic differences in any RFLP locus were observed between the RP and its NIL in any of the 83 cases involving these six NILs. Apparently, these spontaneous mutations did not coordinately result in a polymorphism for any of the RFLP loci detected by the DNA probes and restriction enzymes used in this study.

Table 1. Allelic status of various RFLP loci in a set of near-isogenic lines (NILs) and corresponding donor parents (DPs) and recurrent parents (RPs). RFLP loci are listed by probe and restriction enzyme

Donor parent	Marker(s) introgressed into the NIL[b]	RP[c]	RFLP loci and restriction enzymes[a]														
			pK02	pK03	pK07	pK09	pK11a	pK11b	pK14a	pK14b	pK16a	pK16b	pK18	pK22	pK41 7b	pK44	pK63
			RV	RV	HIII	RI	RI	RI	RI	RI	RI	RI	DI	RI	HIII	RV	RI
			Tabular data code[a]														
T 31	p_2Ir*	C, H	R, R	R, R	R, R	−, N	R, R	R, R	R, N	R, N	R, N	R, N	R, R	R, R	R, R	R, R	R, R
T 85	Fli*	C,	+	+	+	−	−	−	−	+	+	+	R	+	−	−	−
T 117	Dt_2	C, H	−, +	−, +	+, +	−, −	−, −	−, +	−, −	−, +	−, −	−, −	−, −	+, −	−, −	+, −	−, +
T 125	ir^m	C,	−	#	+	−	−	+	−	−	−	−	R	+	−	−	−
T 135	y_9*	C, H	−, R	−, R	−, R	−, −	−, −	+, −	−, −	+, −	−, −	−, −	R, R	−, #	−, R	+, R	−, −
T 138	y_7y_8	C, H	+, R	−, R	−, −	−, −	−, −	−, +	−, −	−, +	−, −	−, −	R, R	−, +	−, R	+, R	+, −
T139	y_3	C, H	−, N	−, N	−, −	−, −	−, −	+, −	−, −	N, −	−, −	−, −	−, −	−, N	−, −	+, −	−, N
T 139	w_1	C,	−	−	−	−	−	+	−	+	−	−	−	−	−	+	−
PI 84.987	dt_1	C,	−	R	+	−	−	−	−	N	−	−	−	+	−	−	−
PI 84.987	e_3	C,	−	R	+	−	−	−	−	N	−	−	−	+	−	−	−
PI 84.987	pc	C, H	−, +	R, R	+, +	−, −	−, −	−, +	−, −	N, −	−, −	−, −	−, −	+, −	−, −	−, +	−, +
T 145	r	C,	R	#	+	−	−	+	−	+	−	−	−	−	−	+	−
T 145	p_1	C, H	R, +	+, −	+, +	−, −	−, −	+, −	−, −	+, −	−, −	−, −	−, −	−, +	−, −	+, −	−, +
T 175	E_1	C,	+	+	+	−	−	+	−	−	−	−	+	+	−	−	+
T 176	lw_1	C, H	−, +	−, +	+, +	−, −	−, −	+, −	−, −	−, N	−, N	−, N	−, −	−, −	−, −	+, −	−, +
T 176	R	, H	+	+	+	−	−	−	−	N	N	N	−	−	−	−	+
T 201	rj_1	C, H	−, +	−, +	−, −	−, −	−, −	−, +	−, −	−, +	−, −	−, −	+, +	−, +	−, −	+, −	+, −
T 201	I	C,	−	−	−	−	−	−	−	−	−	−	+	−	−	+	+
T 203	fc	C, H	−, +	+, −	+, +	−, −	−, −	+, −	−, −	−, N	−, −	−, −	−, −	+, −	−, −	−, +	+, −
T 204	ln	C, H	+, −	+, −	+, +	−, −	−, −	−, +	−, −	−, N	−, −	−, −	−, −	+, −	−, −	−, +	−, +
T 204	lo	C, H	+, −	+, −	+, +	−, −	−, −	−, +	−, −	−, N	−, −	−, −	−, −	+, −	−, −	−, +	−, +
T 204	e_3	, H	−	−	+	−	−	+	−	N	−	−	−	−	−	+	+
PI 80.837	Pd_1	C, H	+, −	+, −	+, +	−, −	+, +	+, +	−, −	−, +	+, +	−, −	−, −	+, −	−, −	+, −	−, +
PI 80.837	Dt_2	, H	−	−	+	−	+	+	−	+	+	−	−	−	−	−	+
T 217	E_1	, H	+	−	+	N	−	−	−	N	−	−	−	−	−	+	+
T 217	lb_1	C,	−	+	+	+	−	+	−	−	−	−	−	+	−	−	−
T 219 H	$Y_{11}y_{11}$	C,	R	R	R	−	−	+	−	−	−	−	R	−	R	R	+
T 235	Wm*	C, H	R, R	R, R	R, N	−, −	−, −	+, −	−, −	+, −	−, −	−, −	R, N	+, −	R, N	R, R	+, −
PI 91.160	Ps	C, H	+, −	+, −	+, +	−, −	−, −	+, −	−, −	+, N	−, −	−, −	+, +	−, +	−, −	−, +	+, −
PI 91.160	w_1	, H	−	−	+	−	−	−	−	N	−	−	+	+	−	+	−
PI 91.160	td	C,	+	+	+	−	−	+	−	+	−	−	+	−	−	−	+
PI 86.024	dt_1	C, H	+, −	+, −	+, N	−, −	−, −	−, N	−, −	N, −	−, −	−, −	−, −	+, −	+, N	−, N	−, N
PI 86.024	e_2	C,	+	+	+	−	−	−	−	N	−	−	−	+	+	−	−
PI 86.024	Lf_1	C, H	+, −	+, −	+, +	−, −	−, −	−, +	−, −	N, −	−, −	−, −	−, −	+, −	+, +	−, #	−, +

PI 83.945	f	C, H	+, −	−, +	+, +	−, −	+, +	+, −	−, −	+, −	−, −	−, −	−, −	−, +	−, −	−, +	+, −	
T 255	lf_2	C,	R	R	R	−	−	+	−	+	−	−	R	−	R	R	−	
T 260 H	Ms_1ms_1	C,	+	−	+	−	−	+	−	+	−	−	−	+	−	−	−	
PI 101.404 A	Rps_1	C,	R	R	+	+	−	−	+	#	+	#	R	+	+	R	−	
PI 101.404 A	wild cyt[e]	C,	R	R	+	N	−	−	+	#	+	#	R	+	+	R	−	
PI 101.404 B	wild cyt[e]	C,	R	R	+	N	−	−	+	#	+	#	R	+	+	R	−	
P.I. 65.388	Grac-cyt[e]	C,	R	R	R	N	R	R	N	N	R	R	R	R	R	R	R	
Black Eyebrow	i[k]*	C,	R	R	R	−	−	+	−	−	−	−	R	+	R	R	+	
Blackhawk	Rps_1	C, H	−, +	−, +	+, +	−, −	+, +	+, −	−, −	+, −	−, −	−, −	+, +	−, +	−, −	+, −	+, −	
Chief	Np	C, H	−, +	−, +	−, −	−, −	−, −	+, −	−, −	−, N	−, −	−, −	−, −	−, +	−, −	+, −	+, −	
Chief	S[t]Np	C,	−	−	−	−	−	+	−	−	−	−	−	−	−	+	+	
CNS	rxp	C, H	+, −	−, +	+, +	−, −	−, −	+, −	+, N	+, N	+, N	−, N	−, −	+, −	−, −	−, +	−, +	
CNS	Rps_2	, H	−	+	+	−	−	−	N	N	N	N	−	−	−	+	+	
Columbia	d_1d_2	C, H	+, −	−, +	+, +	−, −	+, +	+, −	−, −	−, +	+, +	−, −	−, −	−, +	−, −	+, −	+, −	
Columbia	d_1	, H	−	+	+	−	+	−	−	+	+	−	−	+	−	−	−	
Columbia	d_2	C, H	+, −	−, +	+, +	−, −	+, +	+, −	−, −	−, +	+, +	−, −	−, −	−, +	−, −	+, −	+, −	
Columbia	Gd_1d_2	C, H	+, −	−, +	+, +	−, −	+, +	+, −	−, −	−, +	+, +	−, −	−, −	−, +	−, −	+, −	+, −	
Hawkeye	ImIr*	C,	R	R	R	N	R	R	N	N	N	N	R	R	R	R	R	
Hawkeye	Imr*	C,	R	R	R	N	R	R	N	N	N	N	R	R	R	R	R	
Higan	dt_1	, H	−	−	+	N	−	−	−	N	−	−	−	−	−	+	+	
Higan	S	C, H	+, −	+, −	#, +	+, N	−, −	+, −	−, −	−, N	−, −	−, −	−, −	+, −	−, −	−, +	−, +	
Higan	pa_1	, H	−	−	+	N	−	−	−	N	−	−	−	−	−	+	+	
Higan	pa_2	C, H	+, −	+, −	#, +	+, N	−, −	+, −	−, −	−, N	−, −	−, −	−, −	+, −	−, −	−, +	−, +	
Higan	pa_1pa_2	C, H	+, −	+, −	#, +	+, N	−, −	+, −	−, −	−, N	−, −	−, −	−, −	+, −	−, −	−, +	−, +	
Higan	Ps^s	C, H	+, −	+, −	+, +	+, N	−, −	+, −	−, −	−, N	−, −	−, −	−, −	+, −	−, −	−, +	−, +	
Higan	l_2	C, H	+, −	+, −	+, +	+, N	−, −	+, −	−, −	−, N	−, −	−, −	−, −	+, −	−, −	−, +	−, +	
Higan	t	C,	+	+	+	+	−	+	−	−	−	−	−	+	−	−	−	
Kanrich	Rpm*	C,	R	R	R	−	−	+	−	+	−	−	R	+	R	R	+	
Kingwa	ab*	C, H	R, R	R, R	#, #	−, −	−, −	−, +	+, +	+, +	−, −	+, +	R, R	−, +	R, R	R, R	−, +	
Kingwa	Pb*	C, H	R, R	R, R	+, R	−, −	−, −	−, +	N, +	N, +	−, −	N, +	R, R	−, +	R, R	R, R	−, +	
Laredo	W_3w_4	C, H	R, R	R, R	R, R	−, −	+, +	−, +	−, −	+, +	+, +	+, +	R, R	−, +	R, R	R, R	+, −	
Laredo	w_4	C, H	R, R	R, R	R, R	−, −	+, +	−, +	−, −	+, +	+, +	+, +	R, R	−, +	R, R	R, R	+, −	
Medium Green	cyt-G[e]	C, H	−, +	+, −	−, −	−, −	−, −	+, −	−, −	−, +	−, −	−, −	+, +	+, −	−, −	+, −	−, +	
Ogemaw	ior*	C,	R	+	R	−	−	+	−	−	−	−	R	+	R	R	R	
Seneca	L_1*	C, H	R, N	R, N	+, N	−, −	−, −	+, −	−, −	+, −	−, −	−, −	R, N	+, −	R, N	R, N	+, −	
Sooty	td*	C,	N	−	N	−	−	−	−	N	N	N	N	−	N	N	N	
Sooty	B_1i	C,	R	−	R	−	−	−	−	+	+	+	R	−	R	+	+	

Table 1. (Continued)

Donor parent	Marker(s) introgressed into the NIL[b]	RP[c]	RFLP loci and restriction enzymes[a]														
			pK02 RV	pK03 RV	pK07 HIII	pK09 RI	pK11a RI	pK11b RI	pK14a RI	pK14b RI	pK16a RI	pK16b RI	pK18 DI	pK22 RI	pK41b7 HIII	pK47b2 RV	pK63|39 RI
Soysota	n*	C, H	R, R	R, R	R, R	−, −	−, −	+, −	+, +	+, −	−, −	−, −	R, R	+, −	R, R	R, R	+, −
Clark	i	C,	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
Clark	k_1	C,	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
Clark	k_2	C,	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
Clark	i^j	, H	+	+	−	−	−	+	−	+	−	−	−	+	−	+	+
Clark	T	, H	+	+	−	−	−	+	−	N	−	−	−	+	−	+	+
Harosoy	i	, H	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
Harosoy	k_2	, H	N	N	M	M	M	M	M	M	M	M	N	M	M	N	M
Harosoy	wm	, H	M	M	N	M	M	M	M	M	M	M	N	M	N	M	M

[a] Restriction enzyme codes: DI, DraI; RI, EcoRI; RV, EcoRV; HIII, HindIII

[b] The introgressed conventional marker symbols and the phenotypes these markers condition are described by Palmer and Kilen (1987). The asterisk that follows some of the gene symbols indicates that the NIL also possesses the *Rps*$_1$ and *rxp* genes for disease resistance

[c] RP, Recurrent parent (C, 'Clark'; H, 'Harosoy')

[d] Tabular data codes:
minus (−) = RP/DP allelic equality; NIL noninformative and thus not evaluated
plus (+) = RP/DP allelic contrast; RP/NIL allelic cquality; presumptive independence
pound (#) = RP/DP allelic contrast; RP/NIL allelic contrast; presumptive linkage
letter (R) = RP/NIL allelic equality; DP was not evaluated
letter (M) = RP/NIL allelic equality; NIL is a spontaneous mutant of the RP
letter (N) = Not tested.

[e] These NILs were derived by backcrossing the RP genome into DP cytoplasm

RP/DP allelic diversity for RFLP loci

A substantial number of RFLP allelic contrasts must exist between the RP and DP if the NIL gene-mapping technique is to be an effective tool (Muehlbauer et al. 1988). The degree of RP/DP diversity detected for each probe is shown in the individual data columns in Table 1. The calculated percentages of RP/DP alllelic contrasts detected at each RFLP locus (relative to all that were tested) for the 'Clark' and 'Harosoy' NILs, respectively, were as follows: pK-2: 35,28; pK-3: 35,32; pK-7: 60,67; pK-9: 13,0; pK-11a: 12,20; pK-11b: 56,33; pK-14a: 10,6; pK-14b: 43,52; pK-69a: 19,18; pK-69b: 13,9; pK-80: 11,11; pK-229: 50,39; pK-417b: 9,2; pK-472: 29,45; 6-39: 35,54. The overall averages for the 'Clark' and 'Harosoy' NIL groups were 35.7% ($100 \times 275/771$) and 31.7% ($100 \times 187/590$), respectively, indicating that RP/DP allelic contrasts were only slightly more frequent for 'Clark' compared to 'Harosoy'.

Keim et al. (1989b) noted that molecular locus diversity, when *measured as the number of alleles present at a given RFLP locus*, was low in the soybean (i.e., typically no more than two alleles per locus, one of which usually predominated in frequency). This conclusion is based on an evaluation of the allelic status at 17 RFLP loci in 58 diverse accessions from the genus *Glycine*, subgenus *Soja*. Molecular locus diversity, when *measured as the fraction of the RP/DP × RFLP loci combinations that exhibit an allelic contrast for a given locus*, as it was in the present study, tended to be much greater (i.e., one-third of the tested cases). The obvious inference is that the DPs as a group are quite different from both RPs. Indeed, Muehlbauer et al. (1989) noted that the DPs tended to be strains with odd phenotypes that had been identified from divergent sources (e.g., germ plasm collections) by soybean geneticists and breeders. Not surprisingly, RP/DP allelic diversity at the molecular marker loci seems to mirror the RP/DP allelic diversity noted by breeders at the conventional marker loci.

An NIL collection based on two quite different RPs (as is the case for soybean) has an advantage over a collection based on just a single RP. For example, the ancestral pedigree for 'Clark' is ('Mandarin' × 'Manchu') × 'Richland', whereas that for 'Harosoy' is ('Mandarin', 'Ottawa' × 'A.K. Harrow'). The data in Table 1 indicate a greater potential for RP/DP allelic contrasts when NILs were derived from both 'Clark' and 'Harosoy'. Notably, these two RPs differed from each other (i.e., had an allelic contrast) at 7 of the 15 RFLP loci (i.e., pK-2, pK-3, pK-11b, pK-14b, pK-226b, pK-472, and 6-39). For these 7 loci, a noninformative 'Clark'/DP comparison was often counterbalanced by an informative 'Harosoy'/DP comparison, and vice-versa (Table 1). Because the same DP often was used as an introgressed marker source for the two sets of NILs, probes detecting an RFLP *between* 'Clark' and 'Harosoy' would guarantee at least one RP/DP allelic differential in those comparisons where two NILS were available for each conventional marker.

Presumptive linkages

Of the 462 cases with a RP/DP banding difference, analysis of the corresponding NILs indicated that the RFLP banding pattern of the NIL was identical to its RP in all but 15 cases. Recovery of the RP-derived RFLP allele in the NIL of the remaining 447 cases (i.e., the "+" cells in Table 1) suggested that the RFLP loci involved in these 447 cases were not on the chromosome containing the introgressed conventional marker. Muehlbauer et al. (1988) calculated that there is a 2.5% probability of error when making such a presumption for any specific RFLP marker in a single RP/NIL/DP trio set (assuming BC_5S_1-derived NILs; 20 chromosomes, each of 50 cM genetic map length). The 2.5% probability is squared (i.e., 0.0625%) when the presumption is based on two independently derived NILs involving the same introgressed gene (e.g., 'Clark' and 'Harosoy' NILs).

There were 15 RP/NIL/DP sets in which the RFLP allele in the NIL was identical to that in the DP, but different from that in the RP (i.e., the "#" cells in Table 1). Of these 15 cases, 12 had 'Clark' and 3 had 'Harosoy' as an RP. Eight of the 42 DPs in Table 1 were implicated, T125, T135, T145, PI 86.024, PI 101.404A, PI 101.404B, 'Higan', and 'Kingwa'. These presumptive linkages included the RFLP loci pK-3, pK-7, pK-14b, pK-69b, pK-229, and pK-472, and the conventional marker loci $R/r^m/r$, Y_9/y_9, Lf_1/lf_1, Rps_1/rps_1, $S/s^t/s$, Pa_2/pa_2, and Ab/ab (Table 1). The detection of 15 presumptive linkages among 462 informative RP/NIL/DP cases computes to 3.2%, which is comparable with the 4.1% predicted in calculations made by Muehlbauer et al. (1988).

DNA banding patterns obtained for the RP/NIL/DP set involving the RFLP marker pK-3 and the conventional marker *r* are depicted in the leftmost three lanes of Fig. 1. This type of banding pattern, and analogous ones in the other 14 cases, was the basis for presuming linkage between the following pairs of conventional and RFLP marker loci: pK-3 with both *r* and r^m; pK-7 with *ab, S, pa_2;* pK-14b with rps_1; pK-69b with rps_1; pK-229 with y_9; pK-472 with lf_1 (Table 1). For pK-14b and pK-69b, and RP/NIL difference also was detected for NILs in which the RP genome (rather than a conventional marker) had been introgressed into DP cytoplasm. These two anomalous cases will be discussed later.

Confirmation of two of the presumptive linkages

Populations in which appropriate markers were cosegregating were available for a linkage analysis of two of the foregoing presumptive linkages. In population I, pK-3

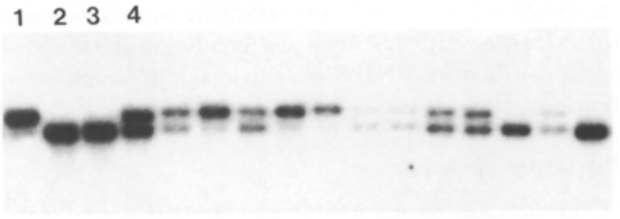

Fig. 1. DNA banding patterns of three members of an RP/NIL/DP set, probed with pK-3, is presented in *lanes 1–3* of this gel, where *lane 1* is the RP 'Clark' (*-RR*), *lane 2* is the DP T145-(*rr*), *lane 3* is the NIL 'Clark'-*rr*. The banding pattern supports a presumption of linkage between the pK-3 and the *r* loci. The same gel also depicts the banding patterns detected for the F_1 (*lane 4*) and the F_2 progeny (*remaining lanes*) of a cross segregating for the RFLP locus pK-3 and the conventional locus *R/r*. The parental cross was 'Clark'-$E_1E_1ttP_1P_1RR \times$ *'Clark'*-$e_1e_1TTp_1p_1rr$

Table 2. Chi-square (χ^2) analyses of the cosegregation data obtained for two presumptive linkages, pK-3 – *r* and pK-472 – Lf_1

Population	Loci pair	Data type[a]	n[b]	χ^2	*df*	P[c]
1	P_1–*r*	$F_{2:3}$	34	39.23	4	0.0000001
	r–pK-3	$F_{2:3}$	34	25.35	4	0.00004
	P_1–pK-3	$F_{2:3}$	34	20.81	4	0.0003
	t–E_1	$F_{2:3}$	34	26.64	4	0.0002
	P_1–E_1	$F_{2:3}$	34	7.88	4	0.10
	P_1–*t*	$F_{2:3}$	34	5.69	4	0.22
	E_1–pK-3	$F_{2:3}$	34	5.35	4	0.25
	E_1–*r*	$F_{2:3}$	34	5.33	4	0.26
	r–*t*	$F_{2:3}$	34	3.91	4	0.42
	t–pK-3	$F_{2:3}$	34	2.13	4	0.71
2	Lf_1–pK-472	$F_{2:3}$	31	24.45	4	0.00007
	Lf_1–Pd_1	F_2	31	6.07	2	0.05
	Pd_1–pK-472	F_2	31	6.02	2	0.05
	ln–Pd_1	F_2	31	2.58	1	0.11
	y_9–Pd_1	F_2	31	2.30	1	0.13
	ln–y_9	F_2	31	1.58	1	0.21
	Lf_1–*ln*	F_2	31	2.25	2	0.32
	y_9–pK-472	F_2	31	1.72	2	0.42
	ln–pK-472	F_2	31	1.15	2	0.56
	Lf_1–y_9	F_2	31	0.00	2	0.99

[a] Genotypes for the RFLP markers were classified in the F_2 generation. Morphological marker genotypes were scored in the $F_{2:3}$ generation for population I, and in the F_2 generation for population II, except for the Lf_1/lf_1 locus, where specification of the F_2 genotypic segregation was possible by identification of the incompletely dominant, heterozygous Lf_1/lf_1 phenotype

[b] Number (n) of F_2 or $F_{2:3}$ progeny

[c] Probability (P) of a greater value of χ^2; linkage-1 program (Suiter 1983)

cosegregated with the *r* and P_1 markers. The *r* and P_1 markers are knwon to be linked by about 21 cM (Palmer and Kilen 1987). In population II, the pK-472 marker cosegregated with the lf_1 marker.

Linkage analysis of population I confirmed a nonindependent assortment of pK-3, *r* and P_1 (Table 2). Some of the banding patterns abtained in the linkage analysis of population I are reproduced in Fig. 1. The recombination distances were estimated to be 14.3 ± 4.6 cM between pK-3 and *r*, 16.2 ± 5.0 cM between pK-3 and p_1, and 10.9 ± 4.0 cM between p_1 and *r* (Table 3). The latter recombination value was significantly lower than the 20.9 ± 2.4 cM value previously reported by Weiss (1970) for the $p_1 - r$ linkage, but this difference may be due to our small sample size of 34 F_2 plants. Although these data confirmed the presumptive linkage of pK-3 to *r*, the assignment of chromosomal order is still equivocal (P_1–*r*–pK-3 likely, but P_1–pK-3–*r* possible). However, these data do position the pK-3 marker in Conventional Linkage Group 2 (p_1 and *r*). The pK-3 marker has been positioned in Linkage Group G of the current RFLP linkage map (R.C. Shoemaker, unpublished), which includes the RFLP loci pK-266, pK-387, pK-3, pA-199a, pK-418b, pK-494b, pA-65, pK-315, and pK-401a. Because the r^m allele of the $R/r^m/r$ locus has been suggested to be a mutatable allele that may have a transposable element associated with it (Chandlee and Vodkin 1988), it would be of particular interest to position this locus relative to the other RFLP loci in Linkage Group E.

Linkage analysis of population II confirmed the presumptive linkage of pK-472 with lf_1 (Table 2). The recombination distance estimated for these two loci was 14.10 +/– 4.82 cM (Table 3). The pK-472 marker has been placed in Linkage Group E of the current RFLP map (R.C. Shoemaker, unpublished), which includes pK-472, DIAP, pa-63 pA-426b, pA-426a pK-255, pK-262, and pA-338. The lf_1 marker has not yet been placed on the conventional marker map. The other four markers, y_9, *ln*, dt_1, and pd_1, segregating in population II were inherited independently of lf_1, pK-472, and each other (Table 2).

Consideration of the other presumptive linkages

F_2 cosegregation data were not available to permit confirmation of the remaining presumptive linkages. However, the data in Table 1 and in some literature reports do provide support for two of the other presumed linkages.

The case for the linkage to pK-7 to *ab* is strengthened because RP/NIL allelic contrasts were detected in both the 'Clark' and 'Harosoy' NILs (Table 1). Muehlbauer et al. (1988) have shown that if a DP-derived allele at a molecular marker locus is detected in two independently derived NILs, then the probability of error associated with a presumption of linkage would be greatly decreased (equal to the square of the probability for one NIL). Muehlbauer et al. (1989) later demonstrated that in two cases where a presumption of linkage between an isozyme and conventional marker was based on two

Table 3. Observed and expected F_2 genotypic segregation data for the linkage of pK-3 with p_1 and r in population I and the linkage of pK-472 with Lf_1 in population II

Loci pair	Genotypic classes[a]									Linkage parameter[b]		
	e	g	j	f	hi	k	l	m	n	*n*	*p*	s_p
pk-3–P$_1$												
Obs:	3	2	0	4	14	2	0	2	7	34	16.20	4.95
Exp:	2.125	4.25	2.125	4.25	8.5	4.25	2.125	4.25	2.125	34	50.00	–
pK-3–r												
Obs:	0	2	3	4	13	3	9	0	0	34	14.27	4.63
Exp:	2.125	4.25	2.125	4.25	8.5	4.25	2.125	4.25	2.125	34	50.00	–
P$_1$*–r*												
Obs:	0	1	6	5	13	0	8	1	0	34	10.85	4.02
Exp:	2.125	4.25	2.125	4.25	8.5	4.25	2.125	4.25	2.125	34	50.00	–
pK-472–Lf$_1$												
Obs:	5	3	0	2	14	3	0	0	4	31	14.10	4.82
Exp:	1.9375	3.875	1.9375	3.875	7.75	3.875	1.9375	3.875	1.9375	31	50.00	–

[a] Class designations as per Allard (1956)

[b] *n*, Number of progeny; *p*, recombination value as calculated by the Linkage-1 computer program (Suiter 1983); s_p, standard error of the *p* estimate

NILs, those linkages were eventually confirmed with F_2 linkage data. Therefore, the presumed linkage of pK-7 and *ab* has a high probability of eventual confirmation. If so, this would place the *ab* locus in Linkage Group F of the current RFLP, map, which is comprised of the pK-14a, pK-162, pK-7, and pA-570 (R.C. Shoemaker, unpublished). The fact that a presumptive linkage was not detected between the pK-14a and the *ab* loci, even though there was a RP/DP allelic contrast (Table 1), does not invalidate a presumption of linkage, since the map distance between pK-7 and pK-14a is 25 cM (R.C. Shoemaker, unpublished). Muehlbauer et al. (1988) calculated that the average DP-derived is centered ± 12.5 cM around the introgressed conventional marker. If the marker order is pK-14a – pK-7 – *ab*, then retention of pK-14a in the *ab* NIL would do not have been probable given the large recombination distance.

The evidence for linkage between pK-229 and y_9 is supported by two observations. First, the y_9 and *pb* loci have been placed in Conventional Linkage Group 14, and recombine at a frequency of 27.3 ± 1.1 cM (Thorson et al. 1989). Second, the *pb* locus is in Linkage Group A of the RFLP map, which is currently comprised of pSAC-7 pA-242b, *pb*, pA-23, pK-229, pA-454, pA-374, pA-203, pT-153b, pA-86, pA-458, pA-386, pR-13a, pK-477, pA-226b, pA-427c, and pK-274 (R.C. Shoemaker, unpublished). Both observations strongly support a linkage of pK-229 to y_9.

The remaining presumptive linkages were somewhat anomalous. These include the presumed linkages of the pK-14b and pK-69b RFLP loci with the Rps_1 marker (Linkage Group 10 of the conventional map), and with some DP-derived DNA that was apparently retained in the two NILs created by the introgression of the RP genome into the DP cytoplasm of PI 101.404A and PI 101.404B (Table 1). The pK-69b locus has been placed in Linkage Group J of the RFLP map. The pK-14b locus is not linked to the pK-69b locus and is located in Linkage Group D of the current RFLP map (R.C. Showmaker, unpublished). No presumptive linkages of the pK-14b and pK-69b loci with Rps_1 were detected in other informative NIL cases (See footnote b in Table 1). The residual amount of PI 101.404A (or B) genome retained in these three NILs included DNA segments that encompass the RPs_1, pK-14b, and pK-69b loci. The probability of retaining, by chance alone, three presumably unlinked DP-derived alleles in three independently derived NILs is extremely low. PI 101.404A and PI 101.404B are known to be homozygous for a chromosome interchange (Palmer and Kilen 1987). Perhaps the interchange involves two chromosomes that contain these three loci, and was somehow responsible for the retention of three normally unlinked alleles in the three NILs. Further study will be required for a resolution of this anomaly.

The presumptive linkages of pK-7 with *S* and pa_2 were detected in the 'Clark' NILs, but not in the 'Harosoy' NILs, even though informative DP/RP contrasts existed for both. Moreover, the *S* and pa_2 loci are not known to be linked with each other, or with the *ab* locus (which in this paper exhibited presumed linkage to pK-7). Consequently, the presumptive linkages of pK-7 to *S*, and pK-7 to pa_2, are probably false and will likely be refuted when a conventional linkage analysis of F_2 data is performed.

Implications of this investigation

The data presented in this paper indicated that RFLP loci are useful markers for the NIL gene-mapping technique as described by Muehlbauer et al. (1988). Several presumptive linkages were detected between RFLP and conventional marker loci in the NILs. Two of these linkages were confirmed with F_2 cosegregation data (i.e., pK-3 with P_1 and *r*; pK-472 with lf_1). The evidence for two other presumptive linkages, while not conclusive, is strong enough to suggest that these also will prove to be authentic linkages. F_2 segregation data will, of course, be needed to confirm these and the remaining presumptive linkages.

Two approaches are available for workers contemplating the use of the soybean NIL stocks for the integration of molecular and conventional marker linkage maps. The first apprach would be one similar to that recently employed by Young et al. (1988). A specific RP/NIL set would be challenged with as many RFLP probes as possible in order to identify a large number of linkages of RFLP loci to some *given* introgressed marker of particular interest. Saturating the chromosomal segment containing the conventional marker with linked RFLP loci would be the primary objective. The second approach would be to challenge to a large number of DP/NIL/RP sets with a battery of systematically-spaced RFLP probes (viz-a-viz their position on a saturated RFLP map). Detection of at least one presumptive linkage of an RFLP locus to *each* conventional marker locus would be the primary objective. Such information would greatly accelerate the integration of the molecular and conventional marker linkage maps. A consolidated linkage map would be quite useful, particularly for those interested in eventually cloning conventional markers that control morphological, physiological, and ontological traits of scientific or commercial interest.

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