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Genetic Linkage of Randomly Amplified Polymorphic DNA (RAPD) Markers in Sweetpotato

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Abstract. RAPD marker analyses were completed on parents and progeny of two sweetpotato [*Ipomoea batatas* (L.) Lam.] crosses to determine the feasibility of genetic linkage map construction. A total of 100 primers was tested and 96 produced amplified genomic DNA fragments. The average number of polymorphisms per primer was 0.69. A total of 134 polyphorphic markers was observed and 74 (60%) segregated 1 band present : 1 band absent as needed for use in genetic linkage mapping of polyploids. The 60% of RAPD markers that segregated 1:1 shows that genetic linkage mapping of the hexaploid sweetpotato by RAPD marker analysis is feasible. Linkage was determined for all markers that segregated 1:1 and five pairs of linked markers were found. These were the first linked molecular markers found in sweetpotato and they show that construction of a genetic linkage map is feasible. A genetic linkage map will be a valuable tool to assist in genetic improvements.

Information on genetic segregation and linkage in sweetpotato is limited. Reasons for this shortage of information on genetic mechanisms include self-incompatibility and a high percentage of cross-incompatibility (Martin 1965, 1970). The incompatibilities, combined with low seed set in compatible crosses, make seed production for genetic studies difficult. The self-incompatibility also results in outcrossing and increases genetic heterozygosity. In addition, pedigrees are unknown for most cultivars and breeding lines because seeds for cultivar development are produced by open-pollination in polycross blocks. Another reason for limited genetic information is that sweetpotato is a hexaploid with reported 2n chromosome numbers of 90 (Jones, 1965; Magoon et al., 1970; Ting and Kehr, 1955) and 88 to 94 (Shiotani, 1988). Alloploidy (Jones, 1965; Magoon et al., 1970; Ting et al., 1957) and autoploidy (Shiotani, 1988) were hypothesized. Therefore, confusion exists as to interpretation of segregation ratios and methods of analysis for linkage determinations in sweetpotato since expected polyploid segregation ratios and map distances differ depending on chromosome homology.

Using RAPD markers is a molecular approach to circumvent obstacles to genetic analysis, including linkage estimates in sweetpotato. The inheritance of RAPD markers is completely dominant. They should, therefore, segregate 1 band present : 1 band absent in progeny from a simplex x homozygous recessive cross (testcross). RAPD markers that segregate 1:1 satisfy the inheritance requirements of single-dose restriction fragments, which are useful for genetic determinations in polyploids (Wu et al., 1992). They should, therefore, be useful for linkage analysis of the hexaploid sweetpotato. Using RAPD markers for genetic analysis of sweetpotato requires using parents to screen primers for polymorphisms. The progeny from polymorphic parents for a particular primer are then analyzed for marker segregation ratios. Those ratios reveal the presence or absence of heterozygosity for that primer in the dominant (band present) parent and also confirm the 1:1 ratio necessary for genetic linkage analysis.

Methods for RAPD marker genetic mapping in sweetpotato are needed, since no linkages have been reported for the species. The development of a linkage map will expedite production of improved genotypes once linkages between molecular markers and traits such as disease resistance, yield, and quality are located. The purpose of this study was to determine the feasibility of RAPD marker analysis for developing a genetic linkage map in sweetpotato. Objectives to accomplish that purpose were to 1) determine the number of RAPD markers per primer tested, 2) determine the number of markers that segregate 1:1, and 3) identify coupling phase linkages between markers to begin a genetic linkage map.

Materials and Methods

Plant materials. Parents and progeny of the two biparental crosses—MD-708 x 'Vardaman' and 'Vardaman' x 'Regal'—were used for RAPD analysis. MD-708 is a breeding line developed at the Univ. of Maryland and is a polycross seedling of unknown parentage. It was used as a parent because of high stability of yield and quality and moderate nematode resistance. 'Regal' is a cultivar developed at the U.S. Vegetable Laboratory, Charleston, S.C. (Jones et al., 1985), and is a polycross seedling out of breeding line W-99, a polycross seedling out of breeding line South Carolina-1166. 'Regal' was chosen as a parent because of high disease, nematode, and insect resistance. 'Vardaman' is a cultivar developed at Mississippi State Univ. from the controlled cross M97-4 x HM145 (Allison et al., 1981). It was chosen as a parent because it has high early root yield and is drought tolerant.

Crosses were made by covering flower buds 1 d before opening and hand transferring pollen at anthesis. Self-pollination was prevented by restricted pollen movement from the flower covers and by the high level of self-incompatibility in sweetpotato (Martin, 1965, 1970).

Plants used for RAPD analysis were grown in a double-layered polyethylene greenhouse. Stem cuttings 24 cm long were placed in 8-L polyethylene pots. The potting medium used was 1 ground pine bark (≤60 mm) : 1 river sand : 2 sphagnum peat (by volume)

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amended with 5 kg dolomitic limestone/m^3 and 4.5 Kg micromax/m^3 (Grace Sierra Co., Foglesville, Pa.). Plants were fertilized bimonthly with 20 g of Sta-Green 12N–2.6P–5K controlled-release fertilizer (Persell, Sylacauga, Ala.). The daily temperature varied from 21 to 32 °C depending on outdoor ambient temperature. Plants received natural lighting.

Genomic DNA was extracted from young leaves 10 to 20 mm long. CTAB-DNA extraction was used on individual plants of parents and progeny. Five grams of leaf tissue was ground with 1 g polyvinylpyrrolidone in liquid nitrogen. Before nitrogen evaporation, 5 mL of 2% CTAB extraction buffer [0.2 M EDTA, 0.1 M tris-HCl pH 8.0, 1.4 M NaCl, 2% CTAB (cetyltrimethylammonium bromide) plus 0.4% 2-mercaptoethanol added just before use] was added and the tissue homogenized. The homogenate was incubated in a water bath at 65 °C for 1 h, then cooled to room temperature. An equal volume of 24 chloroform : 1 octanol was added and mixed with the homogenate until the solution became homogeneous. Solutions were then microcentrifuged at 3400× *g* for 15 min. The clear supernatant was transferred to a new 12×75-mm tube and 2 to 3 times the volume of 20 °C ethanol was mixed with the supernatant. The tubes were inverted gently until DNA precipitated. The DNA was spooled out and washed in 76% ethanol with 0.2 M sodium acetate for 20 min, then in 76% ethanol with 0.01 M ammonium acetate for 10 s. Finally, the DNA was dissolved in 400 µL of TE buffer (10 mM tris-HCl pH 7.4, 1 mM EDTA pH 8.0).

Standard RAPD reaction conditions (Williams et al., 1990) were optimized for use with sweetpotato. Reaction mixture volumes of 12.5, 25, and 50 µL were compared in 3 replications using 3 parents and 20 progenies. Gels were indistinguishable among reaction volumes. Since the needed amount of TAQ DNA polymerase depends on reaction volume, the 12.5-µL volume was used to reduce costs. Therefore, conditions as modified consisted of a 12.5-µL reaction mixture containing 50 mg DNA, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM tris-HCl pH 9.0, 0.1% Triton X-100, 100 µM each of dATP, dCTP, dGTP, and dTTP (Promega, Madison, Wis.), 1 unit *Taq* DNA polymerase (Promega), and 0.2 µM random primer. The reaction solution was overlaid with 1 drop of mineral oil (Sigma, St. Louis). Random primers were from the Univ. of British Columbia Biotechnology Laboratory (Vancouver, BC) (Set 6) and Operon Technologies (Alameda, Calif.) (Sets J, K, Q, R, T). Polymerase chain reaction (PCR) was completed in a Precision GTC-2 genetic thermocycler programmed for 40 cycles of 1 min at 94 °C, 1 min at 40 °C, 2 min at 72 °C, followed by 5 min at 72 °C for final extension. Amplified products were separated by electrophoresis in 1.5% agarose gels and stained with ethidium bromide.

Primers were first tested for polymorphisms using parental cultivars and then with progeny and parents for genetic determinations. Markers were scored present (+) or absent (–). Only well-resolved, clearly defined bands were included for analysis. Segregating markers were tested for goodness of fit to a 1A : 1a phenotypic segregation ratio by chi-square analysis. Linkage analyses were completed for markers fitting a 1:1 segregation ratio. Recombination fractions were determined using the maximum likelihood estimator for coupling, $r_c = (b + c)/(a+b+c+d)$, where a, b, c, and d = four classes of progeny AB, Ab, aB, and ab (Wu et al., 1992).

Results

Photographs of RAPD gels differing only in season of DNA extraction were compared throughout this study and were consistently indistinguishable. Those results showed that RAPD markers were not influenced by the environmental variables present in this experiment. Using the parents MD-708, 'Regal', and 'Vardaman', 100 primers were screened and 96 produced amplified DNA fragments. Numbers of polymorphic bands per primer tested were similar between the crosses MD-708 x 'Vardaman' and 'Vardaman' x 'Regal' and averaged 0.69 over both crosses (Table 1). The similar number of polymorphisms in the two separate crosses did not indicate that one cross was more genetically variable than the other.

In the two crosses, 124 RAPD markers were observed and 74 (60%) segregated 1:1 and were useful for linkage determinations. Photographs of parents and samples of segregating progenies are presented in Figs. 1 and 2. The 60% of markers in the test-cross situation from noninbred parents appears promising for genome mapping. This percentage was comparable to results on conifers by Carlson et al. (1991) who reported that 7 of 12 (58%) polymorphic loci segregated 1:1. They were also working with highly heterozygous parents and stated that this percentage of 1:1 segregations verified feasibility of genetic mapping.

Since self-incompatibility was essential to ensure biparental crosses in this study, RAPD marker segregation ratios were observed for indications of self-pollination and none were found. Progeny from homozygous recessive (band absent) female parents x dominant (band present) male parents consistently included phenotypically dominant individuals and no segregation ratio was skewed toward excessive numbers of homozygous recessives. Those results indicated that self-incompatibility effectively prevented self-fertilization in the parents used in this study.

A prerequisite to linkage analysis in hexaploids is identifying polymorphic markers in coupling phase because excessive progeny numbers are necessary to estimate repulsion linkages (Wu et al., 1992). To determine phase of linkage between RAPD markers, segregation ratios were compared to those expected for linked and nonlinked markers (Table 2). Coupling linkages would result in increased numbers of AB and ab progenies relative to Ab and aB progenies, while repulsion would give greater numbers of Ab and ab relative to AB and ab (Table 2). These comparisons are true for disomic, tetrasomic, and hexasomic inheritance and are, therefore, useful for linkage phase determination in sweetpotato.

All markers that segregated 1:1 were included in two-point linkage analyses and five pairs of linked markers were revealed (Table 3). All linked marker pairs appeared to be in coupling phase since distortion from 1 AB : 1 Ab : 1aB : 1ab were all decreases in aB and Ab with relatively equal increases in AB and ab (Table 3). Higher numbers of Ab and aB in relation to AB and ab as expected for repulsion linkage were not observed. One pair of linked

Table 1. Number of primers used, number of polymorphisms, and number of RAPD markers segregating 1:1 in two sweetpotato crosses.

Cross	Progeny no.	No. of primers	No. of polymorphic primers	No. of polymorphic bands (RAPD markers)	RAPD markers per primer	No. of markers segregating 1:1
MD-708 x Vardaman	170	80	24	57	0.71	34
Vardaman x Regal	76	100	37	67	0.67	40

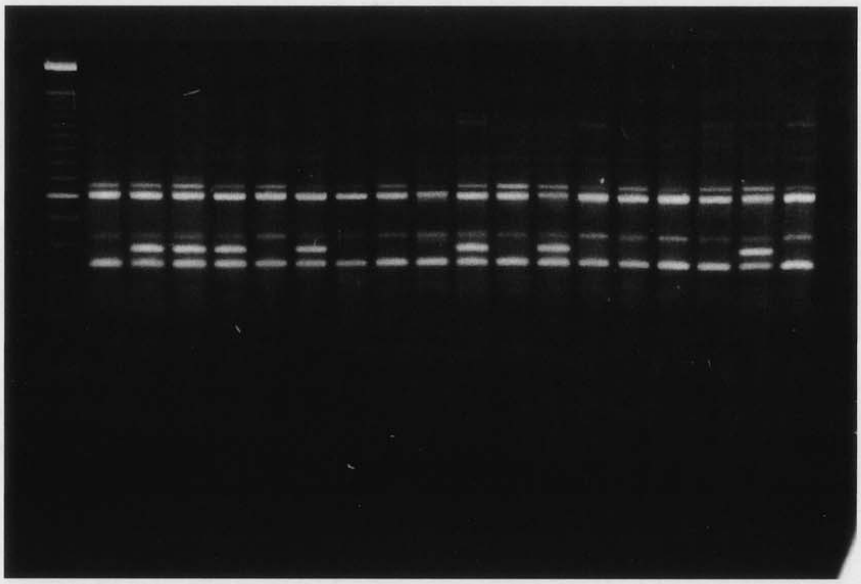

Fig. 1. RAPD profiles of parents and progenies of the sweetpotato cross MD-708 x 'Vardaman'. Primer = OPT17; lane 2 = MD-708, lane 3 = 'Vardaman', lanes 4–20 = progenies, lane 1 = 100-bp DNA ladder; brightest band near center of lane is 600 bp. Polymorphic band at 390 bp.

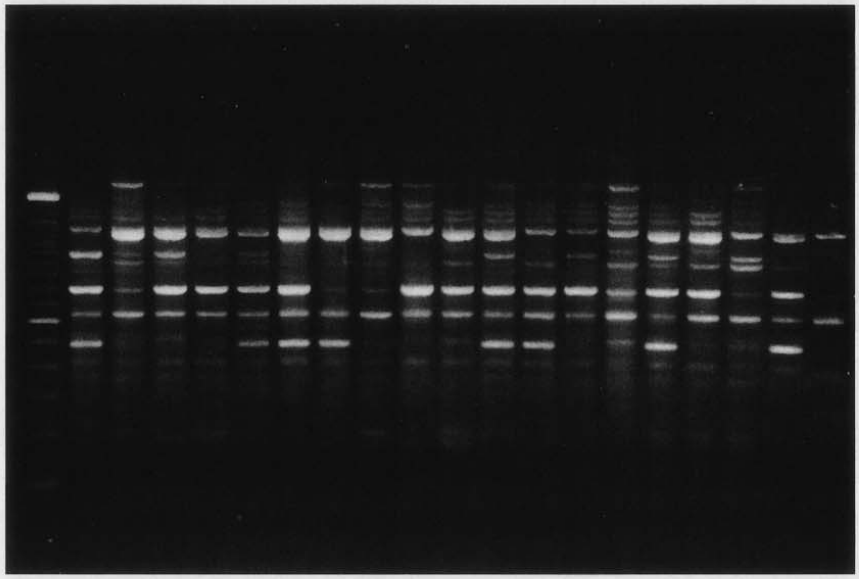

Fig. 2. RAPD profiles of parents and progenies of the sweetpotato cross 'Vardaman' x 'Regal'. Primer = OPK6; lane 2 = 'Vardaman', lane 3 = 'Regal', lanes 4–20 = progenies, lane 1 = 100 bp ladder. Polymorphic band at 490 bp.

markers was observed in cross MD-708 x 'Vardaman' and four pairs were observed in 'Vardaman' x 'Regal' (Table 3). Each marker pair probably represented a separate linkage group since there was no linked marker in common between pairs.

Discussion

RAPD marker analyses were completed on F_1 progeny of two crosses between heterozygous sweetpotato parents. Of the primers tested, 90% produced amplified DNA segments and 62 RAPD markers per cross were observed. Of those 62 markers, 37 (60%) segregated in a 1:1 ratio. Five pairs of markers were linked in coupling and were located in five separate linkage groups. This low number of linkages is probably due to the large chromosome number giving many linkage groups and a lower probability of linkage among markers than in most other species with lower chromosome numbers. The percentage of linked markers should increase with each additional primer tested due to the higher probability of linkage as marker density increases.

Continuation of the mapping procedure used here will result in separate maps for the parents MD-708, 'Regal', and 'Vardaman.' Separate maps for the two parents of a cross can be combined by using RAPD markers that are heterozygous in both parents or by using codominant markers. Linkage determinations between markers will provide map distances for combining the markers into a single map. In this study, 29% of markers segregated 3:1 (heterozygous in both parents) and will be useful for combining maps. Therefore, using markers heterozygous in both parents seems to be a good method for combining maps after additional linkages become available.

The dominant inheritance pattern of RAPD markers was not a

Table 2. Expected phenotypic segregation ratios of marker pairs in testcrosses with disomic and polysomic inheritance.

Parental genotype[z]	Progeny phenotype	Linked				Not linked			
				Hexasomic				Hexasomic	
		Disomic	Tetra.	Chsome[y]	Chtid[x]	Disomic	Tetra.	Chsome	Chtid
Bisimplex coupling	AB	1	1	1	0.8	1	1	1	0.7
$AB(ab)_5$	Ab	0	0	0	0	1	1	1	0.8
	aB	0	0	0	0	1	1	1	0.8
	ab	1	1	1	1.0	1	1	1	1.0
Bisimplex repulsion	AB	0	1	1.0	0.6	1	1	1	0.7
$AbaB(ab)_4$	Ab	1	2	1.5	1.1	1	1	1	0.8
	aB	1	2	1.5	1.1	1	1	1	0.8
	ab	0	1	1.0	1.0	1	1	1	1.0

[z]Genotype of the band present (heterozygous) parent crossed to band absent (homozygous recessive parent).
[y]Random chromosome assortment.
[x]Random chromatid assortment.

Table 3. Linked RAPD markers in sweetpotato.

Cross	Marker pair	Phenotype (progeny no.) AB	Ab	aB	ab	χ^2	Recombination
MD708 x Vardaman	OQ10-420/OK9-740	58	21	23	68	33.1*	0.2588
Vardamen x Regal	OK2-430/OJ9-1050	31	7	14	24	17.8*	0.2763
Vardaman x Regal	OK9-400/OR13-430	34	7	10	25	25.6*	0.2237
Vardaman x Regal	OK14-600/OT16-780	31	3	13	29	28.2*	0.2105
Vardaman x Regal	OQ8-1200/OR5-800	29	4	8	35	36.9*	0.1579

*Significant for 1:1:1:1 $P \leq 0.001$.

serious disadvantage for linkage analysis in this study. Since only markers segregating 1:1 were used for linkage determination, the required cross was a heterozygous x homozygous recessive parents and only heterozygous and homozygous recessive progeny are possible from that cross. Consequently, no ambiguous genotypes resulted. Only crosses between two heterozygous parents result in ambiguous genotypes and such crosses were not used to determine linkage in this study. This observation agrees with results on apple by Hemmat et al. (1994), who stated that the hindrance of RAPD dominance found in many annual crops seems to be less of a problem in highly heterozygous species that are longer lived.

These results show that constructing a genetic linkage map for sweetpotato is feasible and confirms the hypothesis that RAPD marker analyses should overcome the problems of self-incompatibility, genetic heterozygosity, polyploidy, and high chromosome numbers for genetic analysis of sweetpotato. The five pairs of markers linked in coupling phase are the beginning of a genetic linkage map and the map should progress rapidly with the testing of additional primers.

A genetic linkage map will be a valuable tool for increased genetic improvement of sweetpotato.

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