Identifying a Randomly Amplified Polymorphic DNA (RAPD) Marker Linked to a Gene for Root-knot Nematode Resistance in Sweetpotato

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ABSTRACT. The inheritance of resistance to root-knot nematode race 3 [*Meloidogyne incognita* (Kofoid & White) Chitwood] in sweetpotato [*Ipomoea batatas* (L.) Lam.] was studied in 71 progenies of the F_1 single-cross population produced from the cross of resistant parent 'Regal' and susceptible parent 'Vardaman'. The distribution frequency of the progenies based on log total nematode number (egg + juvenile counts) was a bimodal distribution with a ratio of ≈4 resistant : 1 susceptible. Based on this phenotypic ratio, the proposed genetic model was duplex polysomic inheritance (RRrrrr = resistant parent and rrrrrr = susceptible parent). Bulk segregant analysis in conjunction with the RAPD technique was used to identify a RAPD marker linked to a root-knot-nematode-resistance gene. Of 760 random decamer primers screened, 9 showed polymorphic bands between the two bulk DNA samples. Primer OPI5$_{1500}$ produced a band in the resistant bulk but not in the susceptible bulk, suggesting a linkage in coupling phase. An estimated recombination fraction of 0.2421 ± 0.057 between the marker and the root-knot-nematode-resistance gene indicated linkage.

Root-knot nematodes (*Meloidogyne incognita*) are found in large numbers in areas with warm or hot climates (Jatala, 1991). The greatest production of sweetpotato (*Ipomoea batatas*) is in Asia (Food and Agriculture Organization, 1994) where such climates are common. Nematode injury is a major problem in the southern United States (Clark et al., 1992). Yield and quality of fresh roots are reduced by infection, which results in cracking with accompanying necrosis and protuberances on the fleshy roots (Lawrence et al., 1986).

Struble et al. (1966) examined many combinations of sweetpotato parents and observed different manifestations of nematode resistance among the progenies obtained. One or more genetic factors were postulated to be involved in controlling resistance. Jones and Dukes (1980) reported quantitative inheritance with high heritability (0.75) for resistance as measured by gall index.

In previous reports of inheritance of root-knot-nematode-resistance genes, the physiological race of root-knot nematode used was not identified. Plant resistances to *Meloidogyne incognita* are generally host-race specific (Hartman and Sasser, 1985). More than one race (race 1 to race 4) is capable of infecting sweetpotato (Lawrence et al., 1986; Lawrence and Clark, 1986). Therefore, the exact identity of the nematode population being tested must be known to specify the type of resistance from a given source.

Sweetpotato is a hexaploid with $2n = 6x = 90$ (Jones, 1965; Magoon et al., 1970; Ting and Kehr, 1955). Allopolyploidy (Jones, 1965; Magoon et al., 1970; Ting and Kehr, 1955) and autopolyploidy (Shiotani, 1988) were hypothesized. Ting and Kehr (1955), who gave evidence of allopolyploidy, indicated that, among three genomes of sweetpotato, two are more closely related than the other.

Self-incompatibility and a high percentage of cross-incompatibility were observed in sweetpotato (Martin, 1965, 1970). Due to the cytological complexity and difficulty of producing seed, genetic segregation and linkage evaluations are difficult. Currently, molecular markers are playing an important role in constructing genetic maps and tagging genes of interest (Tanksley, 1983) and should help overcome some of the problems in genetic determinations for sweetpotato. The RAPD technique developed by Williams et al. (1990) is a powerful molecular marker technique useful in genetics and plant breeding (Tingey et al., 1992). RAPD markers were used to tag target genes even in crops with large genomes with highly repetitive DNA such as oats (*Avena sativa* L.) (Penner et al., 1993). In the autotetraploid, alfalfa (*Medicago sativa* L.), cosegregations among 19 RAPD markers in three different gametic series were reported and a RAPD marker associated with embryogenesis was identified (Yu and Pauls, 1993a, 1993b). In sweetpotato, RAPD markers have been used to study genetic segregation and linkage (Hong, 1994) and to construct DNA fingerprints (Connolly et al., 1994).

With the technique of bulk segregant analysis (Michelmore et al., 1991), specific genomic regions can be studied against a random genetic background of unlinked loci by observing polymorphisms generated by RAPD markers or restriction fragment length polymorphisms (RFLPs) between two bulk DNA samples derived from a population segregating for a gene of interest. Putative linkages of molecular markers to the target loci can be established with the segregating population. The technique is useful for crops in which nearly isogenic lines are impossible, such as sweetpotato. The procedure can be used with either monogenic or polygenic inheritance with major genes having a large effect on the phenotype (Darvari and Soller, 1994).

In the present study, the inheritance of genes for root-knot nematode (race 3) resistance was evaluated by classical Mendelian procedures based on the distribution of progenies. Bulk segregant analysis was used to identify RAPD markers linked to a root-knot-nematode-resistance gene. The degree of linkage between the resistance gene and a RAPD marker was estimated.

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Materials and Methods

PLANT MATERIALS. Nine sweetpotato cultivars with reported reactions to root-knot nematode (Clark et al., 1992) were tested to determine their reaction to *Meloidogyne incognita* race 3. 'Vardaman' and 'Regal' were selected from those nine cultivars to serve as susceptible and resistant parents, respectively. The 71 segregating progenies from this F_1 single-cross were used to determine the frequency distribution. Polymerase chain reactions (PCRs) were performed with DNA of these two parents and progenies.

SCREENING FOR ROOT-KNOT NEMATODE RESISTANCE. A *Meloidogyne incognita* race 3 population was increased in a greenhouse on tomatoes (*Lycopersicon esculentum* Mill.). Nematode egg inoculum was extracted from tomato roots with 0.5% sodium hypochlorite for 4 min (Hussey and Barker, 1973). Each sweetpotato cutting was planted in a cone-shaped plastic container containing a steamed-pasteurized 1 sand : 1 soil mixture (v/v). The experiment was conducted in the greenhouse in a randomized complete-block design with four blocks. Each cutting was inoculated with 3000 eggs 4 d after planting. Data were collected 45 d after inoculation. Eggs were collected from the entire root system using the same method as egg inoculum preparation. Juveniles (J2) were collected from the soil by gravity screening and centrifugal flotation (Jenkins, 1964). Total nematode number (eggs + juveniles) data were transformed using logarithms to establish variance homogeneity. The log-transformed data were then used to classify resistance according to frequency distribution of progenies.

DNA ISOLATION. A CTAB (hexadecyltrimethyl ammonium bromine) DNA extraction procedure was used to isolate the genomic DNA from 500 mg of young leaves. Leaf samples were ground in polyvinyl-polypyrolidone (pvpp) and liquid nitrogen with a mortar and pestle. CTAB buffer (0.2 M EDTA, 0.1 M Tris pH 8.0, 1.4 M NaCl, 2% CTAB) was mixed with 2-mercaptoethanol (1:4, v/v) just before use and added to the powdered tissue. The mixture was incubated at 65 °C for 1 h. After incubation, an equal volume of a mixture of 24 chloroform : 1 octanal (v/v) was added to the sample. Proteins were separated by centrifugation at 2500 g_n for 15 min at room temperature. The aqueous phase was drawn off and the DNA was precipitated by adding twice the amount of the aqueous phase of cold ethanol and mixing by quick inversion several times. The precipitated DNA was hooked with a glass rod and washed in 0.2 M sodium acetate for 20 min and 0.01 M ammonium acetate for 10 s. The DNA was dissolved in TE buffer (10 mM Tris-HCl pH 8.3, 1 mM EDTA).

BULK SEGREGANT ANALYSIS WITH RAPD MARKERS. The 10 most resistant and 10 most susceptible F_1 single-cross progenies were selected for the respective bulk DNA samples. The two DNA bulks were subjected to PCR. PCR was carried out in a 12.5-μL reaction volume containing 50 ng DNA, 0.2 μM primer, and 100 μM each of 4 dNTPs, 1.4 mM MgCl₂, 10 mM Tris-HCl (pH 8.3), 50 mM HCl, 0.1% Triton X-100, and 1 U of Taq DNA polymerase (Promega Inc., Madison, Wis.). The PCR reactions were overlaid with 15 μL mineral oil and performed in a thermal cycler (GTC-2; Precision Scientific Inc., Chicago). The thermal cycler was programmed for 40 cycles of 94 °C for 1 min, 40 °C for 1 min, 72 °C for 2 min, and 1 cycle of 72 °C for 5 min. DNA products were analyzed by electrophoresis in 1.5% agarose gels at 100 V, stained with ethidium bromide, and photographed under UV light.

Seven hundred and sixty random decamer primers (Operon Technologies, Inc., Alameda, Calif., and Univ. of British Columbia, Vancouver, Canada) were screened for polymorphisms between the resistant bulk and the susceptible bulk. The polymorphic

primers were then tested for linkage to the root-knot resistance gene in the individual F_1 single-cross progenies of the entire population.

DATA ANALYSIS. The inheritance of resistance was determined by plotting the frequency distribution of F_1 single-cross progenies using log-transformed data. Expected ratios for a proposed genetic model and test of linkage were confirmed using a χ^2 goodness-of-fit test. The recombination fraction (p) was calculated by the maximum likelihood method (Mather, 1964).

Results

INHERITANCE OF ROOT-KNOT NEMATODE RESISTANCE. To distinguish between qualitative and quantitative inheritance, the frequency distribution of the segregating F_1 single-cross progenies for log total nematode number was examined. The bimodal distribution of progenies based on the log of total nematode number suggested qualitative inheritance (Fig. 1). Resistant and susceptible groups of progenies were separated at the point where decreasing log total nematode numbers changed to increasing numbers (log total nematode number = 2.0), with 53 progenies classified resistant and 18 progenies classified susceptible. Since, the occurrence of primarily bivalent pairing with only limited quadrivalent formation at meiosis allows the assumption of no double reduction (Jones, 1967), expected genetic segregation ratios were based on the assumption of random chromosome segregation. The ratio of resistant to susceptible progenies fit a 4:1 ratio ($\chi^2 = 1.26$, p = 0.262). Consequently, duplex hexasomic inheritance was proposed with genotype of resistant and susceptible parents represented as RRrrrr and rrrrrr, respectively. The 4:1 ratio was confirmed in another experiment (data not shown) using 50 F_1 single-cross progenies from the same population ($\chi^2 = 0.25$, p = 0.635).

BULK SEGREGANT ANALYSIS FOR SCREENING PRIMERS. According to the proposed genetic model, bulk segregant analysis was used for screening primers. If a marker (M) segregating in a simplex pattern (1:1) was completely linked to the resistance (R) gene in coupling, the expected phenotypic ratio of progenies would be 5 RM : 3 Rm : 0 rM : 2 rm (Table 1). DNA pooled from susceptible plants was not expected to have a marker band, whereas DNA pooled from resistant plants would have a marker band with the probability of 5/8. Therefore, only primers that generated a band in
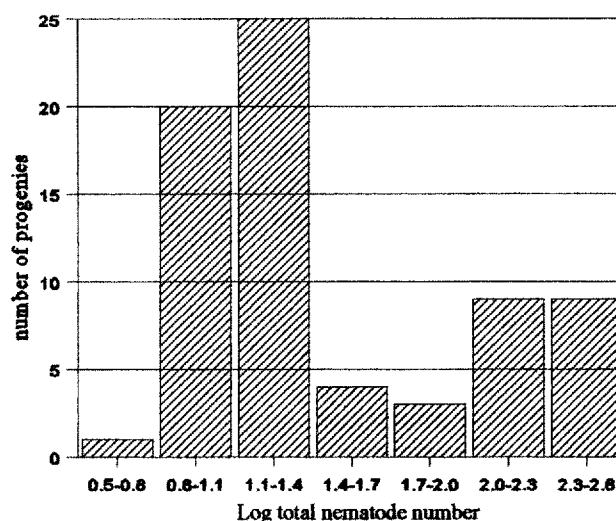

Fig 1. Frequency distribution of F_1 single-cross progenies for log total nematode number.

the resistant bulk, but not in the susceptible bulk, would be useful for detecting a linkage between a marker and the resistance gene. On the other hand, if a marker was completely linked to the resistant gene in repulsion, the phenotypic ratio of progenies would be 7 RM : 9 Rm : 3 rM : 1 rm. In this case, bulk segregant analysis could not detect a polymorphism between two bulks because the resistant and susceptible bulks could have a marker band. Therefore, only linkages in coupling could be identified.

In the preliminary screening for constructing the RAPD linkage map (data not shown), DNA from the two parents was used to identify polymorphic primers. Of 760 primers, 372 showed polymorphisms; 728 polymorphic bands were scored. When DNA from the 2 bulks was used to screen the 372 polymorphic primers, 9 primers were polymorphic between the 2 bulks. One primer, OPI5, produced a band in the resistant bulk but not in the susceptible bulk, suggesting a putative linkage in coupling phase. Therefore, this primer was used for detecting linkage. The presence and absence of the polymorphic band at 1500 bp in the resistant and susceptible bulk, respectively, and examples of banding patterns of the susceptible and resistant parents and progenies are presented in Fig. 2. The susceptible parent 'Vardaman' did not produce a band showing a homozygous recessive genotype for the band and for the resistance gene (Fig. 2a). 'Regal' was the resistant parent and yielded a band. It was therefore dominant for the band and the resistance gene. In Fig. 2b, susceptible progenies that produce bands are visible and, as expected, result from recombination to produce rM (susceptible with a marker band) progenies. Banding patterns of resistant progenies in Fig. 2c include resistant progenies with no marker in lanes 5, 12, and 13 as expected according to the genetic model.

LINKAGE DETECTION AND ESTIMATION OF RECOMBINATION FRACTION. Phenotypes and expected ratios in the F_1 single-cross progenies produced from nematode resistant 'Regal' (RRrrrr) × susceptible 'Vardaman' (rrrrrr) in combination with a simplex RAPD (Mmmmmm) marker that is linked or not linked are presented in Table 1. The χ^2 test of independence between the resistance gene and OPI5$_{1500}$ marker ($\chi^2 = 8.48$, p = 0.037) indicated that the OPI5$_{1500}$ marker was linked to the resistance gene. The recombination fraction (p) was also determined by the maximum likelihood equation for the asymmetrical duplex-simplex case with random chromosome segregation by using the general gamete proportion and the observed numbers in Table 1.

The likelihood equation can be shown as follows (Mather, 1964):

$$\theta = (n!/a_1!a_2!.....a_j!) \times (m_1)^{a1}(m_2)^{a2}...(m_j)^{aj}$$

where θ = likelihood function, a = observed number, m_j = expected number, and n = $a_1 + a_2 +...+a_j$.

The maximum value of θ is obtained by differentiating and equating to 0. For ease of differentiation, the likelihood function is transformed to log likelihood (L):

$$L = log(71!/28!25!4!14!) + 28log(0.5 - 0.2p) + 25log(0.3 + 0.2p) + 4log(0.2p) + 14log(0.2 - 0.2p)$$

$$dL/dp = (-28/0.5 - 0.2p) + (25/0.3 + 0.2p) + (4/0.2p) - (14/0.2 - 0.2p) = 0$$

A rearrangement of this equation gives

$$28.4p^3 - 38.2p^2 - 17.2p + 6.0 = 0$$

The value of p was obtained by successive trials (Hutchinson, 1929) and the associated standard error was estimated by second derivative of log likelihood giving p = 0.2421 ± 0.097.

Discussion

To identify DNA markers linked with the root-knot nematode-resistance gene, selection of a characteristic for determining the level of resistance is essential. Total nematode number (eggs + juveniles) was chosen for this study because nematode population development was able to differentiate the influence of *M. incognita* on resistant and susceptible plants (Lawrence et al., 1986).

A bimodal distribution of progeny was obtained indicating qualitative inheritance of the resistance gene. The effect of dominant allele (R) over recessive allele (r) was large enough to separate the susceptible group from the resistant group using the frequency distribution of progenies even though quantitative data of total nematode number were influenced by environmental or experimental error. The broad range of total nematode numbers in the resistant progenies may be caused by dosage levels of the resistant allele. For example, total nematode number would be greater with a simplex plant than with a duplex plant, but genotype × environment interaction prevented a clear separation between the resistance level.

Only one marker linked with the nematode resistance gene out of 728 polymorphic bands was obtained in this study. Two reasons may explain this low percentage of success in identifying molecular markers. First, sweetpotato has a very large chromosome complement—90 chromosomes. The resistance gene most likely occupies a very small region of the genome and, therefore, the probability of tagging this specific region is quite small compared to tagging a specific region in diploid plants with smaller chromosome complements. Secondly, bulk segregant analysis can only detect linkage in the coupling phase for this genetic model. Since repulsion-phase linkages could not be detected, a low percentage of linkage between markers and the resistance gene was expected

Table 1. Expected phenotypic segregation proportions and general gametic proportions in the F_1 single-cross population for linkage or independence between a root-knot nematode resistance gene and a simplex marker in asymmetrical duplex-simplex coupling based on random chromosome segregation.

Progeny phenotype[z]	General phenotypic proportion	Phenotypic proportion		Observed no.	Observed proportion
		Linked	Nonlinked		
RM	0.5 – 0.2p[y]	0.5	0.4	28	0.70
Rm	0.3 + 0.2p	0.3	0.4	25	0.36
rM	0.2p	0	0.1	4	0.1
rm	0.2 – 0.2p	0.2	0.1	14	0.35

[z]R = resistant, r = susceptible, M = band present, m = band absent.
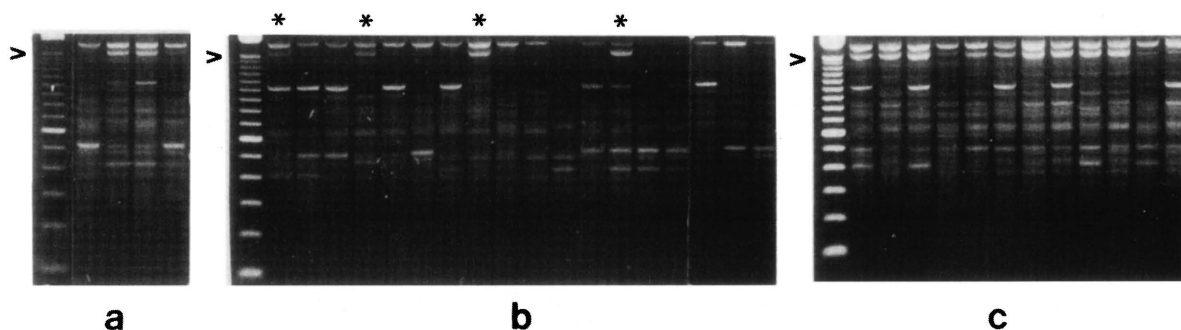[y]p = Recombination fraction.

Fig 2. Bulk segregant analysis of OPI5₁₅₀₀. (a) Lane 2 = susceptible parent, lane 3 = resistant parent, lane 4 = resistant bulk, lane 5 = susceptible bulk. (b) All susceptible progenies (* = recombinant progenies, susceptible with marker). (c) Example of resistant progenies. Arrows indicate the polymorphic band at 1500 bp. The first lane of each picture shows 100-bp molecular mass marker.

compared to diploid plants, in which bulk segregant analysis can detect coupling and repulsion phase or 100% of linkages. Regardless of the low percentage of linkage detected, bulk segregant analysis helped identify a molecular marker linked to root-knot nematode resistance in sweetpotato, which has no near-isogenic lines.

The identification of a RAPD marker linked with the nematode resistance gene proved that genetic linkages between molecular markers and other traits of interest are probable in sweetpotato. This linkage should be beneficial by providing an initial step for screening root-knot nematode resistance in sweetpotato. More molecular markers with closer distances need to be identified so that combinations of these markers can be used to screen seedling progenies for root-knot nematode resistance more effectively than the single marker identified here. In addition, a higher-density map is necessary to identify molecular linkages with other important qualitative and quantitative traits. The identification of such linkages will provide a valuable breeding tool for the genetic improvement of sweetpotato.

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