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Molecular marker screening of peanut (*Arachis hypogaea* L.) germplasm for *Meloidogyne arenaria* resistance

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A restriction fragment length polymorphism (RFLP) marker linked to a locus for resistance to *Meloidogyne arenaria* (Neal) Chitwood race 1, along with visual evaluation following root staining were used to screen four breeding populations and three lines of peanut (*Arachis hypogaea* L.) in a root-knot nematode infested field. COAN and Florunner peanut cultivars were used as resistant and susceptible controls, respectively. Genomic DNA was isolated from young leaves of plants during the growing season, and Southern blot analysis was conducted using RFLP probe R2430E. Only COAN and the line TP301-1-8 were homozygous for the resistance marker. During evaluation, root masses were counted and the resistance phenotype scored. This field data confirmed the RFLP marker results. Except for COAN and TP301-1-8, all other genotypes displayed high levels of nematode reproduction. The RFLP probe R2430E provided a useful marker for identifying resistance to the peanut root-knot nematode.

Key words: *Arachis hypogaea* L., genetics, host resistance, *Meloidogyne* spp., molecular markers, peanut root-knot nematode.

INTRODUCTION

The worlds leading peanut producing countries include India, China and the United States. In 2013, approximately 432,000 hectares of peanuts were harvested in the United States (NASS, 2014). Root-knot

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Abbreviation: RFLP, Restriction fragment length polymorphism.

nematodes (*Meloidogyne* spp) are the most important peanut nematode pathogens in the US inducing annual yield losses that can exceed 30% (Burrow et al., 2014); thus the monetary loss may reach well over $1 billion (Sasser and Freckman, 1987; Dickson, 1998). The three main species of *Meloidogyne* that cause damage to crop plants are: *Meloidogyne arenaria* (Neal) (Chitwood), *Meloidogyne hapla* (Chitwood), and *Meloidogyne javanica* (Chitwood) (Moens et al., 2009). The predominant pathogenic species in Southern US is *M. arenaria*, and two host races of this pathogen have been identified based on their ability (Race 1) or inability (Race 2) to reproduce on the peanut cultivar Florunner (Sasser, 1954). In the states of Alabama, Georgia, Florida and Texas, as many as 40% of the peanut fields are estimated to be infected with this pathogen (Sturgeon, 1986; Dickson, 1998). The development and deployment of root-knot nematode-resistant peanut cultivars in combination with crop rotation is currently the most effective control method used to reduce root-knot nematode damage (Collange et al., 2011). Resistance to root-knot nematodes from wild *Arachis* species has been introgressed into *Arachis hypogaea* (Choi et al., 1999; Guimarães et al., 2010).

COAN was the first peanut cultivar with a high level of resistance to root-knot nematodes and the resistance in this cultivar was derived from *Arachis cardenasii*, from a backcross introgression pathway involving an interspecific hybrid (TxAG-6) (Simpson and Starr, 1999; Church et al., 2000; Simpson and Starr, 2001). The advent of molecular markers has facilitated monitoring genes that are difficult or time-consuming to select by conventional breeding methods (Botstein et al., 1980; Lander and Botstein, 1989; Holbrook et al., 2013). The resistance in COAN is inherited as a single dominant gene and RFLP markers tightly linked to resistance locus which are easy to score and have been used in breeding programs to identify individuals homozygous for nematode resistance (Choi et al., 1999; Church et al., 2000; Chu et al., 2007; Cason et al., 2010).

There are reports identifying molecular markers linked to genes for resistance to nematodes, but limited data are available to compare the efficiency of marker-assisted selection procedures to other selection techniques (Burow et al., 1996; Burrow et al., 2014). The objective of this study was to evaluate the efficiency of marker-assisted selection using the previously identified RFLP marker for identification of individuals putatively homozygous for resistance.

MATERIALS AND METHODS

Seven University of Florida breeding populations/lines (F1334; F94x30-8-2-2-b3; F94x30-5-2-2-3-b3; F94x30-5-2-3-3-b3; F94x30-8-3-1-b2; F79x4-6; F94x30-8-2-1-b3), and the Texas A&M University breeding line TP301-1-8, were tested in field plots in 2001, at the Plant Science Unit Teaching and Research Center, University of Florida, Gainesville, FL, based on *M. arenaria* (Race 1) reproduction established protocols (Starr et al., 1995). F94x30 lines had the interspecific hybrid TxAG-6 as the parent carrying nematode resistance derived from *A. cardenasii*. Florunner and COAN were included in this test as susceptible and resistant controls, respectively. The area was previously infected and the field plots were two 5.0 m long rows with 90 cm inter rows, with four replications. Ten plants per plot were labeled from each breeding line in each from the 4 replication, total 40 plants per breeding line. After 21 days from emergency, young leaves were collected for DNA extraction from the field plot from those labeled plants. The collected plants were place in plastic bags and then transferred to 10 ml glass tubes, frozen in liquid N_2 and stored at -80°C. To extract the genomic DNA, the frozen samples were ground and DNA was extracted according to the procedure of Rogers and Bendich (1985), with modifications as follows. DNA was extracted from 1.0 g of leaves in 2X CTAB and 10X CTAB extraction buffer at 60°C followed by two chloroform/isoamyl alcohol extractions and precipitation with ice cold isopropanol. DNA precipitates were re-suspended in 100 µl high salt TE buffer and incubated at 65°C for 30 min. The supernatant was then precipitated with two volumes of ice-cold isopropanol and the pellet was washed with 80% ethanol. After drying, the DNA was re-suspended in water followed by RNAse treatment. The DNA pellet was re-suspended in TE buffer and stored at -20°C. Each sample yielded 30 to 50 µg of DNA.

Peanut DNA was quantified by spectrophotometric analysis and 20 µg was digested with *Eco*RI according to the manufacturer's instruction (New England Biolabs, Beverly, MA, USA). Digested DNA was submitted to electrophoresis (34 V) for 16 h on 0.8% agarose gels and transferred to Hybond N+ membrane (Amersham, Arlington Heigts, IL) by capillary blotting (Southern, 1975; Chittenden et al., 1994) and UV cross linked or 3 min at 1600 nm. Dr. Gregory T. Church, from Texas A & M University, provided the R2430E probe, which is 4.2 cM distant from the resistance locus (Choi et al., 1999; Church et al., 2000; Church et al., 2001), which was prepared by PCR amplification. Fifty nanograms of probe DNA was labeled with α-P^{32} dCTP by random primer extension (Feinberg and Vogeltein, 1983). Pre-hybridization and hybridization were performed at 65°C with 7% SDS and denatured salmon sperm DNA (Church and Gilbert, 1984). Samples were washed three times for 20 min each at 65°C with 0.5X SSC and 0.1% SDS. Hybridized blots were auto radiographed using X-ray film (Kodak XAR-5) and two intensifying screens at -70°C for 10 days.

Individuals were scored as homozygous (RR) for resistance if only the band associated with resistance was present; heterozygous (Rr) for resistance if the band associated were present; and susceptible (rr) if the band associated with resistance was absent. The resistance to *M. arenaria* race 1 was measured based on egg masses and galls present on peanut roots and pegs. Nematode reproduction was measured in the same 10 plants previously labeled in each replication and used for the RFLP screening. Two weeks prior to crop maturity, plants were harvested and the soil was washed from the roots with tap water. Roots were then placed into 300 ml beakers containing approximately 900 ml of 0.05% Phloxine B solution for 3 to 5 min (Daykin and Hussey, 1985). To each plant, a root-gall and an egg-mass rating was assigned. Each plant was rated according to the number of egg masses and galls found on roots, pegs and pods. A plant given a rating of 1 (no galls or egg masses on roots, pegs and pods) was considered highly resistant, a plant rated as 2 (1 to 10 egg masses and/or galls on roots and less than 10 egg masses and/or galls on pegs and pods) was considered resistant, a rating of 3 (11 to 100 egg masses and/or galls on roots and between 10 to 50 egg masses and/or galls on pegs and pods) indicated that the plants were susceptible and a plant rated as 4 (> 100 egg masses and/or

Table 1. *Meloidogyne arenaria* reproduction at field assay in peanut breeding population which has the interespecific hybrid TxAG-6 as parent for nematode resistance, the resistant breeding line TP301-1-8, the resistant COAN genotype; and the susceptible Florunner genotype.

Peanut genotypes	Galls and eggs mass index (average ± standard error)	% egg mass on pods and pegs	Classification[1]
F1334	3.40 ± 0.52bc[2]	11-50	S
F94x30-8-2-2-b3	3.50 ± 0.71abc	11-50	S
F94x30-5-2-2-3-b3	3.80 ± 0.42ab	11-50	S
F94x30-5-2-3-3-b3	3.70 ± 0.48 ab	11-50	S
F94x30-8-3-1-b2	3.10 ± 0.32 c	11-50	S
F94x4-6	3.80 ± 0.42 ab	11-50	S
F94x30-8-2-1-b3	3.90 ± 0.31 ab	11-50	S
Florunner	4.00 ± 0.00a	>50	HS
TP301-1-8	1.00 ±0.00 d	0	R
COAN	1.00 ± 0.00 d	0	R

CV=13.51%. [1] HS= High susceptible, R= resistant, S=susceptible. [2] Means within a column followed by the same letter are not statistically different at P=0.05 based on Tukey's multiple range test.

galls on roots and > 50 egg masses and/or galls on pegs and pods) was considered highly susceptible. Nematode count data were subjected to analysis of variance and Tukey test using the SAS statistics program (SAS Institute, Cary, NC, USA).

RESULTS AND DISCUSSION

In the field tests to confirm resistant genotypes based on RFLP analysis, no evidence of segregation was found in the breeding materials for the *M. arenaria* resistance locus. The gall and egg mass index for the resistant cultivar COAN was 1, whereas the mean gall and egg mass index on roots and pods of the susceptible cultivar Florunner was 4.00 (Table 1). Reproduction of *M. arenaria* on each of the lines was measured by the number of galls and egg masses on roots, ranged from 0 to greater than 100 and, as measured in percentages, ranged from 0 to > 50% galls and egg masses on pods and pegs. The breeding populations had a gall and egg mass index ranging from 1.00 to 3.90 with greater than 11% egg masses on pods and pegs, which was more than the reproduction found on Florunner ($P<0.001$) (Table 1). TP301-1-8 had no nematode parasitism with a gall and egg mass index equal to 1.0 on roots and pods, which was not significantly different ($P>0.05$) from the reproduction on COAN (Table 1). TP301-1-8 has a single gene for resistance with an *RR* genotype (Simpson and Starr, 2001). In previous report, Church et al. (2000) identified 29% of the TP301-1-8 breeding line as homozygous for resistance, using the RFLP probe R2430E. In a study to identify RFLP markers linked to resistance to *M. arenaria* in six BC_5F_2 peanut breeding populations derived from the interespecific hybrid TxAG-7, Choi et al. (1999) estimated that resistance was conditioned by a single dominant gene.

The RFLP probe R2430E used for screening was linked to high levels of nematode resistance. The resistant and susceptible alleles were quite distinct and easy to score (Figure 1). All populations with a susceptible RFLP genotype (Figure 1) had a susceptible phenotype based on nematode reproduction (Table 1). Previously, R2430E was shown to be 4.2 centiMorgans (cM) from the nematode resistance locus (Church et al., 2001).

In the present investigation, the RFLP probe R2430E was effective in identifying homozygous individuals resistant to *M. arenaria* Race 1, with a high level of confidence. An advantage of using this marker is the opportunity to screen peanut genotypes without relying on inoculation tests with the pathogen, which is cumbersome and time-consuming. Marker-assisted selection also allowed an evaluation of resistance to be performed three months prior to the measurement of nematode reproduction. In addition to saving time, the use of marker-assisted selection for nematode resistance will reduce the cost associated with planting and maintenance, by reducing the number of field plantings to those plants actually carrying the resistance gene in subsequent field trials.

Conflict of Interests

The author(s) have not declared any conflict of interests.

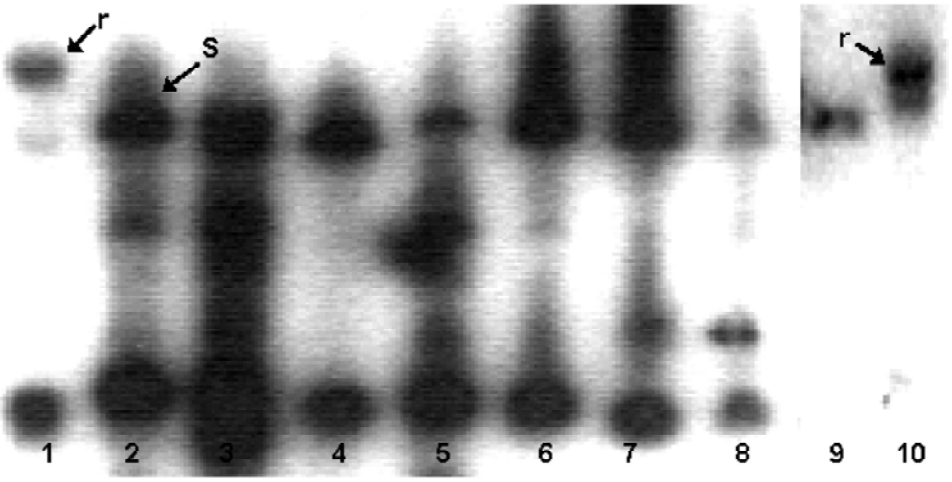

Figure 1. RFLP locus R2430E linked to resistance to *Meloidogyne arenaria* in peanut breeding lines. r = resistant and s = susceptible alleles. Lane 1 is the control resistant COAN genotype; lane 2 is the control susceptible Florunner genotype; lanes 3 to 9 are the breeding lines F1334; F94x4-6; F9430-8-2-1-b3; F94x30-8-3-1-b2; F94x30-8-2-2-b3; F94x30-5-2-2-3-b3, F94x30-5-2-3-3-b3, respectively; line 10 is the resistant breeding line TP301-1-8.

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