Development and Phenotyping of Recombinant Inbred Line (RIL) Populations for Peanut (*Arachis hypogaea*)

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ABSTRACT

The identification of molecular markers for economically significant traits should greatly improve the speed and efficiency of all peanut (*Arachis hypogaea* L.) breeding programs. Development and phenotypic evaluation of recombinant inbred line (RIL) populations of peanut, along with molecular genotyping, will be essential for association of markers with traits. The primary objectives of this research were to develop 16 structured RIL populations that can be used by the peanut research community, and to begin high-resolution phenotyping of these populations. Crosses were made using a 2 by 8 (common by unique) factorial nested association mapping design. Parents were selected to attempt to maximize genetic diversity while meeting practical breeding objectives. First, two modern runner cultivars (Tifrunner and Florida-07) were selected as common parents because runner cultivars account for about 80% of the production in the U.S. Second, the eight unique parents were selected to supply diversity across market classes and botanical varieties and are donors of favorable alleles for enhancing drought tolerance and resistance to most important disease of peanut in the U.S. The eight unique parents are N08082olJCT (a Bailey derived high oleic breeding line), C76-16, NC 3033, SPT 06-06, SSD 6 (PI 576638), OLin, New Mexico Valencia A, and Florunner. The 16 populations were advanced using summer and winter nurseries. Input from multiple disciplines has resulted in a prioritized list of populations and traits that should be examined, and seed increase has begun to provide the community with material for extensive phenotyping. In-depth phenotyping and genotyping of these populations should result in markers that can be deployed by breeding programs for the development of improved cultivars.

Key Words: Genotyping, Marker assisted selection, peanut, phenotyping, recombinant inbred lines.

Peanut (*Arachis hypogaea* L.) has lagged behind other crop species in the development and use of genetic markers for marker-assisted selection (MAS). Stalker *et al.* (2009) reviewed the early efforts in advancing the science of genomics in peanut and stressed the need for close cooperation between teams of researchers from multiple disciplines to fully utilize the potential of genomics for peanut improvement. They also pointed out the need for development of appropriate populations that could be used for testing markers to establish associations with traits of interest.

Translation of genomics to breeding requires association of molecular markers with phenotypes and the implementation of cost-effective MAS (Collard and Mackill, 2008). Because of sparse genomic information for peanut, researchers have only been able to take advantage of MAS for a limited number of traits, primarily nematode resistance and high oleic acid (Holbrook *et al.*, 2011). Yet in such a narrowly focused project, tremendous (at least 3-fold) gain in the speed of selection was achieved (Chu *et al.*, 2011). These two traits also were exceptional in that nematode resistance was introgressed from a wild species (Simpson and Starr, 2001) where abundant polymorphisms facilitated discovery of markers (Nagy *et al.*, 2010); and for high oleic acid, genes in fatty acid biosynthesis already had been characterized in other oil seed crops enabling straightforward characterization in peanut and discovery of functional mutations (Jung *et al.*, 2000; Lopez *et al.*, 2002). These two traits also are relatively simply inherited in peanut which is in contrast to many other traits for which breeders must select, e.g. disease resistance, drought tolerance, and yield. With the initiation of the Peanut Genome Project (Anonymous, 2008), the acquisition of genome

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Table 1. Attributes for parents of 16 structured recombinant line (RIL) populations.

Parent	Common or unique parent	Market class	Oleic acid[a]	TSWV[bc]	Early leaf spot[bd]	Late leaf spot[be]	White mold[bf]	Sclerotinia[bg]	CBR[bh]
Tifrunner	Common	Runner	L	R	MR	MR	S	U	U
Florida-07	Common	Runner	H	R	S	S	MR	U	U
N08082olJCT	Unique	Virginia	H	MR	MS	U	U	MR	MR
C76-16	Unique	Runner	L	MR	U	U	U	U	U
NC 3033	Unique	Virginia	L	HS	MR	HS	R	U	HR
NM Valencia A	Unique	Valencia	L	S	S	S	HS	HS	U
OLin	Unique	Spanish	H	MS	S	S	U	R	U
SSD 6	Unique	Exotic[i]	L	HR	U	U	U	U	U
SPT 06-06	Unique	Exotic[i]	L	U	HR	HR	U	U	U
Florunner	Unique	Runner	L	HS	S	S	S	S	S

[a]L = Normal oleic, H = High oleic.

[b]R = Resistant, MR = Moderately Resistant, MS = Moderately Susceptible, S = Susceptible, HS = Highly Susceptible, U = Unknown.

[c]TSWV = Tomato Spotted Wilt Virus (genus *Tospovirus*; family *Bunyaviridae*).

[d]Early Leaf Spot [*Cercospora arachidicola* (S. Hori)].

[e]Late Leaf Spot [*Cercosporidium personatum* (Berk. & M.A. Curtis)].

[f]White Mold (*Sclerotium rolfsii* Sacc.).

[g]Sclerotinia [*Sclerotinia minor* Jagger (Kohn, 1979)].

[h]CBR = Cylindrocladium black rot [*Cylindrocladium parasiticum* Crous, Wingfield, and Alfenas, syn *C. crotalariae* (Loos) Bell and Sobers].

[i]SSD 6 is PI 576638, an *Arachis hypogaea* var. *hirsuta* accession. SPT 06-06 is an interspecific breeding line with *A. cardenasii* in its pedigree.

sequence and potential for discovery of markers will outpace the ability to associate these markers with complex traits that have significant genotype by environment effects, i.e. the bottleneck becomes phenotyping because of the need to collect multiple years of data in multiple locations. Anticipating this need, the peanut breeding community initiated the development of several recombinant inbred line (RIL) populations.

A large group of peanut breeders and molecular geneticists met in the spring of 2008 to develop a plan for identifying molecular markers for MAS. Much of the meeting was devoted to the selection of the most appropriate parents to use for the development of RIL populations that will be needed for mapping and marker development. The group agreed to develop 16 structured RIL populations using a 2 by 8 (common by unique) factorial nested mating association mapping design. Parents were selected to attempt to maximize genetic diversity while meeting practical breeding objectives (Table 1). First, two modern runner cultivars [Tifrunner (Holbrook and Culbreath, 2007), and Florida-07 (Gorbet and Tillman, 2009)] were selected as common parents because runner cultivars account for about 80% of the production in the U.S. Tifrunner was also chosen since according to the Peanut Genome Strategic Plan 2012–2016, it was selected as the candidate genotype for reference genome sequencing and baseline expression profiling by RNA-seq technology. As of January 2013, a rough draft of the peanut genome sequence from Tifrunner has been generated, although considerable refinement and quality control of the assembly must be completed before the sequence is released. Second, the eight unique parents were selected to supply diversity across market classes and botanical varieties and are donors of favorable alleles for enhancing drought tolerance and resistance to most important diseases of peanut in the U.S. The eight unique parents are N08082olJCT (a Bailey derived high oleic breeding line), C76-16, NC 3033 (Beute *et al.*, 1976), SPT 06-06, SSD 6 (PI 576638), OLin (Simpson *et al.*, 2003), New Mexico Valencia A (Hsi and Finker, 1972), and Florunner (Norden *et al.*, 1969).

Eight of these cross combinations (Tifrunner × N08082olJCT; Tifrunner × SPT 06-06; Tifrunner × C76-16; Tifrunnner × NC 3033; Florida-07 × N08082olJCT; Florida-07 × SPT 06-06; Florida-07 × C76-16; and Florida-07 × NC 3033) were designated as Set A (Table 2) and were completed in time to send to the 2008-2009 winter nursery. Leaf tissue was sampled from these F_1 plants and frozen for DNA extraction and marker analysis to test for hybridity. F_2 populations were grown in 2009 in Tifton, GA and Raleigh, NC. Four-hundred F_2 plants were harvested from each of the eight populations, and these populations have been advanced using summer and winter nurseries. Generation advance was achieved using small plots

Table 2. Timeline for the development of 16 recombinant inbred line (RIL) populations.

Year	Set A[a] (location[c])	Set B[b] (location[c])
2008	Produce hybrid seed (T & R)	
	F_1 plants (PR)	
2009	F_2 population (T & R)	Produce hybrid seed (T & M)
	F_3 population (PR)	F_1 plants (PR)
2010	F_4 population (T & R)	F_2 population (T & M)
	F_5 population (PR)	
2011	F_6 population (T & R)	F_3 population (M)
	Individual Plant Harvest	F_4 population (PR)
2012	F_7 line increase plots (T & R)	F_5 population (M)
	Begin phenotyping and genotyping	
2013	Continue seed increase as needed	F_6 population (M)
	Continue phenotyping	Individual Plant Harvest
2014	Continue seed increase as needed	F_7 line increase (M & T)
	Continue phenotyping as needed	Begin phenotyping and genotyping
2015		Continue seed increase as needed
		Continue phenotyping
2016		Continue seed increase as needed
		Continue phenotyping as needed

[a]Set A = Tifrunner × N08082olJCT; Tifrunner × SPT 06-06; Tifrunner × C76-16; Tifrunner × NC 3033; Florida-07 × N08082olJCT; Florida-07 × SPT 06-06; Florida-07 × C76-16; and Florida-07 × NC 3033.

[b]Set B = Tifrunner × SSD 6; Tifrunner × OLin; Tifrunner × New Mexico Valencia A; Tifrunner × Florunner; Florida-07 × SSD 6; Florida-07 × OLin; Florida-07 × New Mexico Valencia A; Florida-07 × Florunner.

[c]Location: T = Tifton; R = Raleigh; M = Marianna; PR = Winter nursery (Puerto Rico).

of bulked seed to minimize attrition. A random individual plant was harvested from each F_6 to provide seed for line increases. Seed increase to provide the community with material for extensive phenotyping of the Set A RIL populations was begun in 2012. F_1 seed for the other eight combinations (Tifrunner × SSD 6; Tifrunner × OLin; Tifrunner × New Mexico Valencia A; Tifrunner × Florunner; Florida-07 × OLin; Florida-07 × SSD 6; Florida-07 × New Mexico Valencia A; and Florida-07 × Florunner) designated as Set B, were first sent to the 2009-2010 winter nursery. An update on the current status of these populations is presented in Table 2. Our goal was 400 RILs per population, however, a few populations are slightly smaller than 400 RILs.

The parents for these populations were selected to sample phenotypic diversity as broadly as possible across elite and exotic gene pools and supply materials for identifying loci underlying the maximum number of traits possible. The segregating populations from these crosses will supply a wealth of material for cultivar development and introgression of novel favorable alleles from elite and exotic sources into modern cultivars. Moreover, segregation of common alleles across populations will increase statistical power for estimating genetic effects necessary for applying MAS in peanut.

It is not feasible to phenotype all populations for all economically significant traits. Input from peanut breeders and molecular geneticists has resulted in a list of populations and traits that should be examined first. Phenotyping projects either underway or proposed with these RIL populations are summarized in Table 3.

Seed increase for most of the RIL populations in set A was begun in the summer of 2012; however, based on expected large variation for resistance to late leaf spot [*Cercosporidium personatum* (Berk. & M.A. Curtis)], seed increase for the Florida-07 × SPT 06-06 populations was begun in the 2011-2012 winter nursery. This allowed for a late leaf spot phenoytping trial in the field in 2012, and a typical quantitative inheritance was observed in this population. Seventy-nine polymorphic simple sequence repeat (SSR) markers were used to genotype the parents and the 195 RILs included in the late leaf spot field trial, and a low-density map will be constructed in 2013. This population also has potential to be useful for genetic mapping of additional traits such as pod and kernel characteristics and resistance to early leaf spot [*Cercospora arachidicola* (S. Hori)]. Phenotyping for early leaf spot disease in North Carolina using the genotyped population is planned to begin in 2013.

The ranges of late leaf spot responses in field studies in RIL populations developed from crosses of Tifrunner × GTC-20 and NC 94022 × SunOleic 97R (Qin *et al.*, 2012), and Gregory × Tifguard (Gill *et al.*, 2012) have been large and work is in

Table 3. Recombinant (RIL) phenotyping in progress and proposed.

RIL population	Trait	Principal investigators
Florida-07 × SPT-06-06	Resistance to late leaf spot[a]	P. Ozias-Akins, C. Holbrook, A. Culbreath, S. Jackson
	Resistance to early leaf spot[b]	T. Isleib
	Resistance to TSWV	Culbreath
Tifrunner × NC 3033	Pod fill	R. Hovav, P. Ozias-Akins, S. Jackson
	Drought tolerance	T. Sinclair
	Resistance to late leaf spot[a]	A. Culbreath, P. Ozias-Akins, C. Holbrook
	Resistance to white mold[c]	T. Brenneman
	Resistance to TSWV[d]	Culbreath
	Resistance to CBR[e]	T. Brenneman
Florida-07 × NC 3033	Resistance to CBR[e]	T. Brenneman
Florida-07 × C76-16	Preharvest aflatoxin contamination	P. Ozias-Akins, C. Holbrook, S. Jackson
Tifrunner × C76-16	Drought tolerance	C. Chen

[a]Late Leaf Spot [*Cercosporidium personatum* (Berk. & M.A. Curtis)].
[b]Early Leaf Spot [*Cercospora arachidicola* (S. Hori)].
[c]White Mold (*Sclerotium rolfsii* Sacc.).
[d]TSWV = Tomato Spotted Wilt Virus (genus *Tospovirus*; family *Bunyaviridae*).
[e]CBR – Cylindrocladium black rot [*Cylindrocladium parasiticum* Crous, Wingfield, and Aflenas, syn *C. crotalariae* (Loos) Bell and Sobers].

progress to combine genetic characterization of those populations with disease response data to determine if useful markers for resistance can be developed. Field results from 2012 with the Florida-07 × SPT 06-06 population likewise were promising for late leaf spot, but additional characterization is needed for that population. In addition to field evaluations, Gill *et al.* (2012) evaluated the Gregory × Tifguard population for components of late leaf spot resistance using lateral stem and detached leaf assays. Studies of this type are time and labor intensive, and are not feasible for large RIL populations. However, component characterization of select lines identified from field studies to represent a range of leaf spot responses should provide valuable information for reference to the genetic profile as well as field disease scores.

The RIL populations with the unique parent NC 3033 crossed with either Florida-07 or Tifrunner are also targeted as high priority populations for phenotyping. The former is expected to segregate for cylindrocladium black rot [*Cylindrocladium parasiticum* Crous, Wingfield, and Alfenas, syn *C. crotalariae* (Loos) Bell and Sobers] (CBR) resistance as well as tomato spotted wilt virus (genus *Tospovirus*; family *Bunyaviridae*) (TSWV) resistance. The latter should segregate for TSWV, late leaf spot, and white mold (*Sclerotium rolfsii* Sacc.) resistance, and both should segregate for pod filling percentage.

Research has documented that the parents of the population derived from Tifrunner and NC 3033 diverged substantially for two physiological mechanisms that contribute to drought tolerance (Shekoofa, personal communication). Low hydraulic conductance in the plants appears to be the basis for expression of both traits, and hydraulic conductance is related to the activity of membrane proteins mediating water transport, i.e. aquaporins. The activity of aquaporins can be tested by treating plants with silver, an aquaporin inhibitor (Sadok and Sinclair, 2010). The silver inhibitor test has been effectively applied to a RIL marked population of soybean resulting in the identification of four QTLs (Carpentieri-Pipolo *et al.*, 2012). Phenotyping of the Tifrunner × NC 3033 RIL population using the silver test is expected to lead to QTL markers in peanut.

Previous studies have documented that C76-16 is a unique drought tolerant source for peanut breeding programs (Holbrook *et al.*, 2007; Dang *et al.*, 2012). A pilot study on maturity demonstrated that C76-16 matures significantly earlier than Tifrunner. Phenotyping of the RIL population derived from the cross of Tifrunner × C76-16 should be useful in identifying QTLs underlying drought tolerance and maturity in peanut.

High resolution phenotyping will be essential for associating genetic markers with traits of interest. We have developed an electronic library consisting of more than 40 references on methods to phenotype peanut for economically significant traits. These references are available from the senior author, and should help to standardize the phenotypic data from various locations and projects.

All four populations on which phenotyping is underway will be genotyped with 80–120 SSRs in

order to generate a framework map onto which other marker types will be placed. Reduced representation sequencing using a version of RAD-Seq will be carried out initially on parents of all four populations. Bioinformatic analysis of these data will require the development of an analysis pipeline that will distinguish homeologous SNPs from true allelic SNPs since most pipelines already published use relatively short sequences or require a reference genome for alignment, which is not yet available for peanut. Initial analysis of parents, some of which is already underway, along with communication with other testing variations of genotype by sequencing (GBS) for peanut, will provide data for identifying the most cost-effective method for GBS, which we anticipate will be more economical than array-based genotyping. SNP genotyping will be conducted on up to 200 individuals from each population. This will allow the development of a breeder-centric database with many of the basic elements found in Gramene (Ware *et al.*, 2002), Soybase (Grant *et al.*, 2010), and Solanaceae Genomics Network (Mueller *et al.*, 2005), and will be a valuable resource for breeders, molecular biologists, and others engaged in translational genomics and marker-assisted breeding.

Bernardo (2008) developed a thorough and thought provoking perspective article on the lessons learned from 20 yrs of research on QTLs and MAS for complex traits in plants. A major conclusion was that finding QTLs for complex traits is easy and common, but exploiting these QTLs in applied plant breeding programs is much more difficult, and much less common. To increase the probability that discovered QTLs will be used in selection programs, he suggested the active involvement of plant breeders at the beginning of the research project. The selection of parents for the 16 RIL populations for peanut was a multidisciplinary attempt to balance the maximization of genetic diversity while maintaining utility for cultivar development. The continued active involvement of plant breeders, molecular geneticists, pathologists, physiologists, and other disciplines should result in the identification and use of molecular markers for routine development of improved peanut cultivars.

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