

Research Article

Identification of Expressed Resistance Gene Analogs from Peanut (*Arachis hypogaea* L.) Expressed Sequence Tags

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Available online on 6 February 2013 at www.jipb.net and www.wileyonlinelibrary.com/journal/jipb

doi: 10.1111/jipb.12037

Abstract

Low genetic diversity makes peanut (*Arachis hypogaea* L.) very vulnerable to plant pathogens, causing severe yield loss and reduced seed quality. Several hundred partial genomic DNA sequences as nucleotide-binding-site leucine-rich repeat (NBS-LRR) resistance genes (*R*) have been identified, but a small portion with expressed transcripts has been found. We aimed to identify resistance gene analogs (RGAs) from peanut expressed sequence tags (ESTs) and to develop polymorphic markers. The protein sequences of 54 known *R* genes were used to identify homologs from peanut ESTs from public databases. A total of 1,053 ESTs corresponding to six different classes of known *R* genes were recovered, and assembled 156 contigs and 229 singletons as peanut-expressed RGAs. There were 69 that encoded for NBS-LRR proteins, 191 that encoded for protein kinases, 82 that encoded for LRR-PK/transmembrane proteins, 28 that encoded for Toxin reductases, 11 that encoded for LRR-domain containing proteins and four that encoded for TM-domain containing proteins. Twenty-eight simple sequence repeats (SSRs) were identified from 25 peanut expressed RGAs. One SSR polymorphic marker (RGA121) was identified. Two polymerase chain reaction-based markers (*Ahsw-1* and *Ahsw-2*) developed from RGA013 were homologous to the Tomato Spotted Wilt Virus (TSWV) resistance gene. All three markers were mapped on the same linkage group AhIV. These expressed RGAs are the source for RGA-tagged marker development and identification of peanut resistance genes.

Keywords: *Arachis hypogaea*; expressed sequence tags; resistance gene analogs; Tomato Spotted Wilt Virus.

Liu Z, Feng S, Pandey MK, Chen X, Culbreath AK, Varshney RK, Guo B (2013) Identification of expressed resistance gene analogs from peanut (*Arachis hypogaea* L.) expressed sequence tags. *J. Integr. Plant Biol.* doi: 10.1111/jipb.12037

Introduction

Cultivated peanut, or groundnut (*Arachis hypogaea* L.), is an allotetraploid (AABB; $2n = 4x = 40$) and is one of the most important oilseed crops grown. Peanut is a major source of

edible oil and digestible protein with kernels composed of about 50% oil and 25% protein (Guo et al. 2012). However, very limited genetic variation has been revealed in peanut by using various molecular markers such as restriction fragment length polymorphisms (RFLP) (Kochert et al. 1991;

Paik-Ro et al. 1992), random amplified polymorphic DNA (RAPD) (Subramanian et al. 2000), and simple sequence repeats (SSR) (Liang et al. 2009; Varshney et al. 2009; Qin et al. 2012; Wang et al. 2012). Scarcity of genetic diversity among cultivated peanut accessions is likely derived from the single hybridization event between two ancient diploid species, likely *Arachis duranensis* (A genome) and *A. ipaensis* (B genome) (Seijo et al. 2004, 2007; Favero et al. 2006; Burrow et al. 2009). The low levels of genetic variation make peanut very vulnerable to many plant pathogens (Ratnaparkhe et al. 2011).

Peanut yield and quality are severely constrained by a wide variety of fungal, bacterial, viral, and nematode pathogens. Among the fungal diseases, early leaf spot (*Cercospora arachidicola*) and late leaf spot (*Cercosporidium personatum*) are most prevalent, and occur throughout peanut growing regions. Late leaf spot and rust (*Puccinia arachidis*) diseases often occur simultaneously and can cause 50–70% yield loss in India and some African countries (Khedikar et al. 2010). Spotted wilt disease, caused by Tomato Spotted Wilt Virus (TSWV), has become more prevalent and more severe in the Southeastern United States. For example, TSWV caused approximately 12% peanut yield loss in Georgia in 1997, which represented about a USD \$40 million value (Culbreath and Srinivasan 2011). Therefore, the most promising solution for managing peanut diseases is using resistant cultivars. A high yielding cultivar with improved resistance to multiple pathogens would present tremendous advantages for peanut farmers to combat these diseases.

Until now, about 70 *R* genes, which confer resistance to various diseases have been cloned from different plant species by either map-based cloning or transposon tagging methods (Johal and Briggs 1992; Whitham et al. 1994; Dixon et al. 1996; Sanseverino et al. 2009). The majority of *R* genes share a few highly conserved domains such as a nucleotide binding site (NBS), leucine-rich repeats (LRR), protein kinases (PK), transmembranes (TM), and toll and interleukin-1 receptor (TIR) domains (Ali and Yan 2012). These conserved domains facilitate the isolation of *R* genes or resistance gene analogs (RGAs) from the same or from other plant species. Based on the presence of specific conserved domains, plant *R* genes can be grouped into at least four classes (Xiao et al. 2006; Sanseverino et al. 2009). *R* genes containing NBS-LRR domains form the largest class of *R* genes (Meyers et al. 1999, 2003; Xiao et al. 2006). This class can be further divided into two sub-classes, TIR-NBS-LRR and non-TIR-NBS-LRR, based on the presence of a TIR domain on the N-terminus (Meyers et al. 1999, 2003). For example, the *N* gene of tobacco, which confers resistance to Tobacco mosaic virus, possesses an N-terminal NBS and C-terminal LRR domains, plus a TIR domain upstream of NBS (Whitham et al. 1994), while the *Rps2* of *Arabidopsis*, which confers resistance to

the bacterium *Pseudomonas syringae*, contains an N-terminal NBS and at least 14 imperfect C-terminal LRR domains with a coiled-coil (CC) domain upstream of NBS (Bent et al. 1994). The second class of *R* genes consists of those with LRR and PK domains, such as *Xa21*, found in rice, with an intracellular serine/threonine kinase domain and 23 extracytoplasmic LRRs. *Xa21* confers resistance to rice leaf blight, caused by the bacterium *Xanthomonas oryzae* pv. *oryzae* (Song et al. 1995). The third class is those which contain a serine/threonine kinase (protein kinase) domain. An example of this class is the tomato *Pto* gene with 27 serine and 13 threonine residues, which confers resistance to bacterial speck caused by *Pseudomonas syringae* pv. *tomato* (Martin et al. 1993). The fourth class is those of the *R* genes containing large extracellular LRR domains, like the tomato *Cf-2* gene. The *Cf-2* gene, which provides resistance to the leaf mold pathogen *Cladosporium fulvum*, contains 37 LRRs (Dixon et al. 1996). The fifth class includes all other *R* genes, which confer resistance to pathogens by different mechanisms, such as the *Hm1* gene of maize. *Hm1* confers resistance to the leaf spot fungus *Cochliobolus carbonum*. *Hm1* was the first *R* gene to be cloned, and encodes for a reductase enzyme that detoxifies the *C. carbonum* HC-toxin (Johal and Briggs 1992).

Efforts have been made to isolate RGAs from peanut. Bertoli et al. (2003) identified 78 RGAs from the peanut cultivar 'Tatu' and four wild diploid species by using degenerate primers targeting the P-loop, GLPL, and RNBS-D motifs of the NBS domains. Subsequently, they mapped 34 candidate RGAs to a linkage map by using a combination of methods of amplified fragment length polymorphism (AFLP), NBS profiling, RGA-AFLP, and sequence characterized amplified region (SCAR) markers (Leal-Bertioli et al. 2009). Yuksel et al. (2005) isolated 234 RGAs from the peanut cultivar 'Florunner UF-439-16-1003-2' using degenerate primers based on the NBS-LRR and LRR-TM conserved domains. Ratnaparkhe et al. (2011) reported six RGAs from a BAC clone library. More recently, Wang et al. (2012) reported 3 784 BAC clones containing RGAs and two RGA-SSR markers, which were mapped to a linkage map. In summary, there are currently 355 partial genomic DNA sequences for NBS-LRR encoding RGAs (Bertioli et al. 2003; Yuksel et al. 2005). All these RGAs were derived from genomic DNA sequences and not from expressed sequences. Dilbirligi and Gill (2003) concluded that the majority of genomic *R*-gene-like sequences from six crop plants did not have detectable transcripts, indicating that most of them could be non-functional. The peanut expressed sequence tag (EST) project has made significant progress (Guo et al. 2009; Feng et al. 2012b), and this resource has proven to be valuable for various genome-scale experiments (Guo et al. 2008b; Luo et al. 2010; Pandey et al. 2012b). Currently, there are 252,832 peanut ESTs available in the National Center for Biotechnology Information

(NCBI) GenBank database (<http://www.ncbi.nlm.nih.gov/>) (10 August, 2012), including 178,490 ESTs from cultivated peanut, 35,291 ESTs from *A. duranensis* (AA genome), 6,264 ESTs from *A. stenosperma* (AA genome), and 32,787 ESTs from *A. ipaensis* (BB genome). Therefore, the objectives of this study were to identify expressed RGAs from these peanut ESTs and to develop potential polymorphic markers for a genetic mapping population.

Results

Identification of RGAs from peanut ESTs

We identified 385 putative RGAs from the publicly available peanut ESTs at NCBI. The tBLASTn algorithm was used to identify expressed peanut RGAs homologous to (parts of) the full length protein sequences of 54 known plant *R* genes (Table 1). As a result, a total of 1,053 *R*-gene-like ESTs homologous to the different classes of known *R* genes were recovered with $E \leq e^{-10}$. After alignment and assembly,

385 unigenes were identified as peanut expressed RGAs, consisting of 156 contigs and 229 singletons (Table S1). The average length of the RGAs was 736 bp, ranging from 313 bp to 2,647 bp. A BLASTX search against GenBank indicated that the expressed RGAs could be classified as (i) NBS-LRR containing proteins (69 total); (ii) protein kinases (PK) (191 total); (iii) LRR-PK/TM (82 total); (iv) Toxin reductase (28 total); (v) LRR-domain containing proteins (11 total); and (vi) TM-domain containing proteins (four total).

Of the 1,053 ESTs, 649 were from cultivated peanut (11 genotypes) and 404 were from wild peanut (three genotypes) (Table 2). Among the 14 peanut genotypes, wild diploid peanut 'DUR25' contributed the largest number of *R*-gene-like ESTs, followed by cultivated peanut '06-4104' and 'Tifrunner'. The percentage of *R*-gene-like ESTs in the wild peanut 'DUR25' is 0.62% (219/35,291), and was higher than that of others except for the cultivars 850 (1.21%, 9/745) and Yueyou 523 (1.30%, 5/385). The 1,053 *R*-gene-like ESTs were mainly derived from tissue obtained from seeds (468), followed by roots (333), leaves (208), and gynospores (44) (Table 2).

Table 1. Known *R* genes from plants used in this study

Plant	<i>R</i> genes	Protein GB ID	Structure	Plant	<i>R</i> genes	Protein GB ID	Structure
Apple	<i>Vf1</i>	CAC40825	LRR	Potato	<i>R1</i>	AAU95638	CC-NBS-LRR
<i>Arabidopsis</i>	<i>Fls2</i>	BAB11088	LRR-PK		<i>Rgc1</i>	AAF76163	CC-NBS-LRR
	<i>Pbs1</i>	ABR46085	PK		<i>Rx</i>	CAB50786	CC-NBS-LRR
	<i>Rpm1</i>	CAA61131	CC-NBS-LRR	Rice	<i>Pib</i>	BAA76282	CC-NBS-LRR
	<i>Rpp1</i>	AEE77906	TIR-NBS-LRR		<i>Pi-ta</i>	BAF91352	CC-NBS-LRR
	<i>Rpp4</i>	AAE83818	TIR-NBS-LRR		<i>Rpr1</i>	BAA75812	CC-NBS-LRR
	<i>Rpp5</i>	AAE83827	TIR-NBS-LRR		<i>Xa1</i>	BAA25068	CC-NBS-LRR
	<i>Rpp8</i>	AAC78631	CC-NBS-LRR		<i>Xa21</i>	AAC80225	LRR-PK
	<i>Rpp13</i>	AAF42832	CC-NBS-LRR		<i>Xa26</i>	ABD36512	LRR-PK
	<i>Rps2</i>	AAM90883	CC-NBS-LRR	Sugarbeet	<i>Hs1</i>	AAW03319	LRR-TM
	<i>Rps4</i>	CAB50708	TIR-NBS-LRR	Tobacco	<i>N</i>	AAA50763	TIR-NBS-LRR
	<i>Rps5</i>	AAC26126	CC-NBS-LRR	Tomato	<i>Cf-2</i>	AAC15779	LRR-TM
	<i>Rpw8.1</i>	ACJ05900	TM		<i>Cf-4</i>	CAA05268	LRR-TM
	<i>Rpw8.2</i>	AAP45326	TM		<i>Cf-5</i>	AAC78591	LRR-TM
		<i>Ssi4</i>	AAN86124	TIR-NBS-LRR		<i>Cf-9</i>	CAA05274
Barley	<i>Rcy1</i>	BAC67706	CC-NBS-LRR		<i>Hero</i>	CAD29728	CC-NBS-LRR
	<i>Rpg1</i>	ABK51312	PK		<i>I2</i>	AAD27815	CC-NBS-LRR
	<i>Mla1</i>	AAG37354	CC-NBS-LRR		<i>Mi-1</i>	AAC97933	CC-NBS-LRR
	<i>Mla6</i>	CAC29242	CC-NBS-LRR		<i>Prf</i>	AAC49408	CC-NBS-LRR
Flax	<i>Mlo</i>	CAB06083	TM		<i>Pto</i>	AAB47421	PK
	<i>L6</i>	AAA91022	TIR-NBS-LRR		<i>Sw-5</i>	AAG31013	CC-NBS-LRR
	<i>M</i>	AAB47618	TIR-NBS-LRR		<i>Cre3</i>	AAC05834	CC-NBS-LRR
Maize	<i>P2</i>	AAK28806	TIR-NBS-LRR	Wheat	<i>Vrga1</i>	AAF19148	CC-NBS-LRR
	<i>Hm1</i>	AAC04333	Toxin reductase		<i>Yr10</i>	AAG42168	LZ-NBS-LRR
	<i>Hm2</i>	ABY68564	Toxin reductase		<i>Lr1</i>	ABS29034	CC-NBS-LRR
Lettuce	<i>Rp1-d</i>	AAD47197	CC-NBS-LRR		<i>Lr10</i>	AAQ01784	CC-NBS-LRR
	<i>Dm3</i>	AAD03156	CC-NBS-LRR	Pepper	<i>Bs2</i>	AAF09256	CC-NBS-LRR

Table 2. Distribution of peanut *R*-gene-like expressed sequence tags (ESTs)

cDNA-ESTs	Leaves	Roots	Seeds	Gynospores	Total
GT-C20	27 (0.32%) [†]		18 (0.21%)		45 (0.26%)
Tifrunner	65 (0.35%)		129 (0.39%)		194 (0.37%)
Florunner	1 (0.40%)				1 (0.40%)
850	9 (1.21%)				9 (1.21%)
A13	3 (0.19%)		2 (0.40%)		5 (0.24%)
Luhua 14			31 (0.36%)		31 (0.36%)
Minhua 6			4 (0.52%)		4 (0.52%)
Shanyou 523			13 (0.59%)		13 (0.59%)
Yueyou 523			5 (1.30%)		5 (1.30%)
06-4104	33 (0.15%)	95 (0.46%)	72 (0.35%)		200 (0.32%)
Chiba-Handachi	70 (4.60%)	28 (0.42%)		44 (0.77%)	142 (0.52%)
<i>Arachis ipaensis</i> PI468321		73 (0.46%)	83 (0.49%)		156 (0.48%)
<i>Arachis stenosperma</i> V10309	0 (0.00%)	29 (0.48%)			29 (0.46%)
<i>Arachis duranensis</i> DUR25		108 (0.74%)	111 (0.54%)		219 (0.62%)
Total	208 (0.53%)	333 (0.52%)	468 (0.42%)	44 (0.77%)	1053 (0.42%)

[†]The percentage of *R*-gene-like ESTs in the total ESTs from corresponding tissues.

Development of SSR markers

The MISA program (<http://pgrc.ipk-gatersleben.de/misa>) was used to identify SSRs from peanut RGAs (Thiel et al. 2003). A total of 28 SSRs were identified from 25 peanut RGAs with three RGAs containing two SSRs (Table 3). AAG/CTT (eight RGAs) and AAT/ATT (seven RGAs) were the most abundant motifs. Twenty-six SSR primer pairs were designed and used to screen for polymorphisms between the parental lines of two mapping populations (Table S2). All the primer pairs yielded amplification products using a DNA template from either one of the four parental lines. RGA121-SSR was polymorphic between the parental lines, GT-C20 and Tifrunner, and SunOleic 97R and NC94022 (Figure S1).

Sequence analysis and mapping of RGA121

RGA121 was 1,021 nucleotides long and had an uninterrupted open reading frame (ORF), which translated into a fragment of 339 amino acid residues (Figure S2). Within this fragment, two domains, a leucine-rich repeat N-terminal domain (A₃₅ to D₆₉) and a protein kinase domain (T₈₇ to Y₃₂₄), were revealed by searching the Pfam database (<http://pfam.sanger.ac.uk/>) (Punta et al. 2012), which indicated that RGA121 belongs to the LRR-PK gene family. The program BLASTP was used to

search for RGA121 homologs in GenBank, and the soybean receptor protein kinase TMK1-like showed the highest similarity with $E \leq 2e-171$.

In *Arabidopsis*, at least four LRR-PKs are involved in pathogen resistance. *FLAGELLIN-SENSITIVE 2* (*FLS2*) contributes to the perception of the bacterial elicitor flagellin in *Arabidopsis* (Gomez-Gomez and Boller 2000); *BIR1* and *SOBIR1* are involved in the regulation of cell death and innate immunity (Gao et al. 2009); *ERECTA*, a putative RLK with an extracellular LRR domain and an intracellular kinase domain, functions in both plant development and pathogen defense responses (Godiard et al. 2003). Sequence comparison showed RGA121 had 34%, 38%, 41%, and 38% sequence identities with *FLS2*, *BIR1*, *SOBIR1*, and *ERECTA*, respectively.

In order to locate markers linked to RGA121 on the peanut genomic map, SSR analysis was conducted using 165 RILs of the T population and 363 RILs of the S population (Figure S3). In the T population, 88 and 72 RILs contained alleles from the parental line GT-C20 and Tifrunner, respectively. In the S population, the numbers of alleles from the parental line NC94022 and SunOleic 97R were 161 and 157, respectively (Table 3). A χ^2 test indicated the segregation ratio was found to be the expected 1:1 in both mapping populations ($P < 0.05$). Based on the linkage map developed by the total 363 RILs from the S populations (Pandey et al.

Table 3. Genotyping data of RGA121-SSR in T and S populations

RIL population	No. allele from parent 1	No. allele from parent 2	Heterozygous	Missed band	Total
T population	88	72	5	0	165
S population	161	157	16	29	363

2012a), RGA121 was mapped on the linkage group AhIV (Figure 1).

Mapping of the putative *Ahsw* gene

Previously, a cDNA clone (GO324202) from peanut with 37% amino acid identity to tomato *Sw-α*, a gene providing resistance to TSWV (Brommonschenkel and Tanksley 1997), was revealed and named as *Ahsw* (Chen et al. 2008; Feng et al. 2012a). Two putative genes, *Ahsw-1* and *Ahsw-2*, were identified by using 5' and 3' rapid amplification of cDNA ends (RACE) and electronic polymerase chain reaction (PCR) methods. In the present study, GO324202, JK158518 and JR554938, were integrated into RGA013. The length of RGA013 was 1,758 bp. RGA013 contained an uninterrupted ORF, which translated into a fragment of 561 amino acids. BLASTP indicated that RGA013 contained a NB-ARC domain from K172 to Q466 (Figure S4) and was similar to the soybean putative disease resistance protein At1g50180-like (LOC100814688) (79% identity). Three gene-specific markers were developed

in the 3' variant region for each *Ahsw* gene (Table S2). Primers *Ahsw1-c* and *Ahsw2-b* detected a polymorphism between the two parental lines SunOleic 97R and NC94022, and were further used to genotype the S population. *Ahsw-1* and *Ahsw-2* were mapped onto the linkage group AhIV, along with RGA121 (Figure 1).

Discussion

Peanut resistance to plant pathogens has been identified for a variety of peanut cultivars and wild relatives, which indicates that peanuts do have *R* genes (Bertioli et al. 2003; Qin et al. 2012). Knowledge of expressed *R* genes or RGAs will significantly enhance our ability to better understand the host-pathogen genetic interaction and will facilitate the development of breeding new resistance peanut cultivars. Identifying and mapping *R* genes from cultivated peanuts is very difficult due to the large size of the peanut genome (~2,800 Mb) and the relatively low marker density of linkage maps (Guo et al. 2008a; Varshney et al. 2009; Hong et al. 2010; Qin et al. 2012; Wang et al. 2012). In contrast, there are 252,832 *Arachis* ESTs available in GenBank, which we show to be useful for RGA discovery. In the present study, 385 putative RGAs were successfully identified from the peanut EST database. These RGAs provide a large set of sequence data for RGA-tagged marker development.

Identification of RGAs by data mining

Generally, there are two methods that are used in RGA identification: the PCR-based method and data mining. The PCR-based method uses degenerate primers based on the conserved domains of known *R* genes, and has been successfully used to identify RGAs from a number of plant species (Kanazin et al. 1996; Gowda et al. 2002; Hunger et al. 2003; Gao et al. 2010). In peanut, a total of 355 genomic RGAs have been isolated using the PCR-based method (Bertioli et al. 2003; Yuksel et al. 2005; Nagy et al. 2010; Ratnaparkhe et al. 2011). We compared these genomic RGAs with *Arachis* ESTs available in GenBank (252, 832 ESTs), and found only 61 genomic RGAs that matched 18 *Arachis* ESTs ($E \leq e-10$). This indicates that only a small fraction of RGAs from genomic DNA sequences are expressed.

Recently, data mining has been successful in RGA discovery from wheat, sugarcane, maize, and common bean (Dilbirligi and Gill 2003; Rossi et al. 2003; Xiao et al. 2006; Liu et al. 2012). Dilbirligi and Gill (2003) compared four different data-mining approaches, namely domain search, individual and multiple motif searches, consensus sequence search, and individual full-length search, to identify *R*-gene-like sequences from wheat ESTs, and concluded that the individual full-length

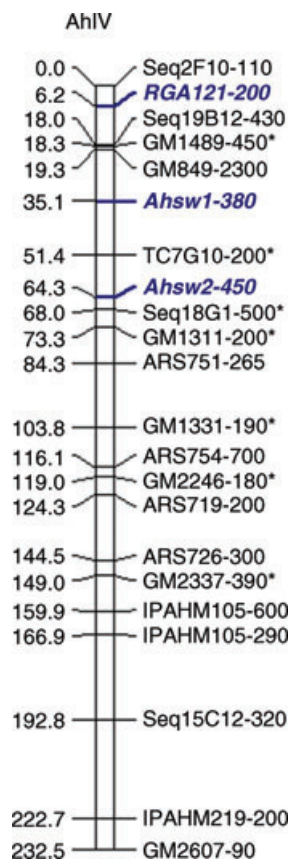


Figure 1. Linkage mapping of RGA121 and *Ahsw* putative genes on peanut map.

search was the most efficient method (Dilbirligi and Gill 2003). Xiao et al. (2006) adopted three methods, namely modified RACE, AFLP and data mining (individual full-length search) to identify RGAs from maize, and showed that the data mining method revealed the largest number of RGAs. In this study, we used the individual full-length search method and identified 385 peanut expressed RGAs. Nagy et al. (2010) found three *R*-gene homologs (RGC144b, RGC154 and RGC240) linked to *Rma*, a dominant root-knot nematode resistance gene. In the present study, RGA106 is identical to the reported RGC154, but the RGA106 is much longer in the 3' end than RGC154.

RGA121 and *Ahsw* were mapped on the same linkage group AhIV

Mapping of *R* genes or RGAs is an important step toward understanding the relationship between specific chromosomal regions and disease resistance. In the present study, RGA121, *Ahsw-1* and *Ahsw-2* were mapped onto the peanut linkage group AhIV (Figure 1), along with a QTL LLS_TF11E6 in control of late leaf spot resistance, and a QTL TSWV_DW10E1 resistant to TSWV (Pandey et al. 2012a).

Despite the importance of disease resistance in peanut, relatively little is known about the chromosomal regions that carry the genes required for disease resistance. This has hampered the use of markers in the development of resistant cultivars. During the last 5 years, there have been attempts to map peanut disease resistance. Leal-Bertioli et al. (2009) mapped 34 candidate RGAs and five QTLs associated with enhanced resistance to late leaf spot on the genetic map of the *A*-genome of *Arachis*. A putative QTL, cp4.1 resistant to late leaf spot, was mapped on the linkage group A4 near the marker TC7G10. The two markers most near to cp4.1, p25M46-2 and As26A, are RGAs (Leal-Bertioli et al. 2009). Linkage group A4 and AhIV shared a common marker, TC7G10. Moreover, TC7G10 is located between *Ahsw-1* and *Ahsw-2* in the present study (Figure 1). Sujay et al. (2012) identified 28 QTLs for late leaf spot resistance and 15 QTLs for rust resistance by using two RIL populations. Among them, two QTLs, QTL_{R4-LLS}13 and QTL_{R5-LLS}14 conferring resistance to late leaf spot, and one QTL, QTL_{R4-Rust}06 in control of rust infection, were located on the linkage group AhVII near the markers GM1311 and GM2246. These two markers were also mapped on the upper regions of linkage group AhIV in our study. Furthermore, GM2246 was conditioned within the QTL TSWV_DW10E1. Therefore, RGA clusters may exist in the upper regions of the linkage group AhIV. Numerous RGAs have been shown to co-localize with resistance genes/QTLs (Pflieger et al. 2001; Wisser et al. 2005; Xiao et al. 2007), and such RGAs in peanut would be highly suitable candidates for use as markers for breeding. Therefore, further studies will be needed to determine

the function of RGA121 and *Ahsw* or RGA013. Recently, Shirasawa et al. (2012) reported a high-density linkage map for cultivated peanut with 1,114 loci. Linkage group LG04.1 shared two markers, TC7G10 and Seq15C12, with linkage group AhIV in the present study. Such high-saturation peanut linkage will hence be needed for the fine mapping of RGA121 and *Ahsw* and for map-based cloning.

Materials and Methods

Data mining of peanut RGAs

The protein sequences of 54 known *R* genes corresponding to different *R* gene classes were used to search for (tBLASTn) peanut homologs in the GenBank EST database (est_others; Organism: *Arachis*; <http://www.ncbi.nlm.nih.gov>; Altschul et al. 1997). The 54 *R* genes that were used for the data mining study are listed in Table 1 along with their original plant species, putative structures and GenBank accession numbers. Those sequences with $E \leq e^{-10}$ were clustered to develop unigenes with parameters set at 100 bp for overlap length and 95% for nucleotide identity, and all the unigenes were considered as putative peanut RGAs (Xiao et al. 2006; Liu et al. 2012). The resulting unigenes were in turn used to search the GenBank databases by BLASTX to confirm their putative annotations.

Development and mapping of peanut RGA markers

The MISA program (<http://pgrc.ipk-gatersleben.de/misa>) was used for identification of SSRs in peanut RGAs (Thiel et al. 2003). SSRs were considered to be those sequences containing motifs with a size of two to six nucleotides and di-nucleotide, tri-nucleotide, tetra-nucleotide, penta-nucleotide, and hexa-nucleotide motifs with minimum repeat of 6, 5, 5, 5, and 5, respectively. The BatchPrimer3 v1.0 (<http://probes.pw.usda.gov/batchprimer3/>) was used to design SSR primers for the RGAs containing SSRs with default parameters (You et al. 2008). Two gene-specific markers, *Ahsw-1* and *Ahsw-2*, were also developed based on the gene sequences (Brommonschenkel and Tanksley 1997; Chen et al. 2008; Feng et al. 2012a). These SSRs and gene-specific markers were used for screening the four parental lines of two recombinant inbred line (RIL) mapping populations, the T population derived from Tifrunner and GT-C20, and the S population developed from SunOleic 97R and NC94022 (Qin et al. 2012). Those with polymorphisms were used to genotype individuals of the T and S populations. Markers were tested for segregation distortion by χ^2 test for goodness of fit to a 1:1 ratio. Linkage analysis was performed by using JoinMap 4.0 (Van Ooijen, 2011).

DNA extraction and PCR

Genomic DNA was isolated from young leaflets of the four parents, 165 RILs of the T population and 363 RILs of the S population (Qin et al. 2012). A Nano-Drop 1000 spectrophotometer (Nano Drop Technologies, USA) was used to evaluate the quality and concentration of all DNA. All of the DNA samples were diluted to 20 ng/μL, and PCR reactions were carried out in 10 μL as described by Qin et al. (2012). Polymerase chain reaction products were analyzed on 6% or 9% non-denaturing polyacrylamide gel (PAGE) and visualized by silver staining as described by Fountain et al. (2011).

Acknowledgements

We thank Billy Wilson and Sara Beth Pelham for technical assistance in the field and the laboratory. This research was partially supported by funds provided by the Georgia Agricultural Commodity Commission for Peanuts, the National Peanut Board and the Peanut Foundation. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture (USDA). USDA is an equal opportunity provider and employer.

Received 15 Aug. 2012 Accepted 24 Jan. 2013

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(Co-Editor: Merijn R. Kant)

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Sequences of 385 putative expressed RGAs.

Table S2. Primers for 28 peanut RGA-SSRs and two putative *Ahsw* genes.

Figure S1. PCR amplification of twelve selected RGA-SSRs.

Figure S2. Sequence analysis of RGA121.

Figure S3. Example for genotyping of T and S populations by RGA121-SSR.

Figure S4. Sequence analysis of RGA013.