Targeting and genotyping RGAs in a mapping population of the AA genome of wild *Arachis*

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**ABSTRACT** - Peanut is an important legume crop. Although it has high levels of morphological diversity, it lacks genetic variability and sources of disease resistance. The transference of resistance genes from wild species is difficult due to the different ploidy level of the wild and cultivated species. Recently, amphidiploids have been produced that can be used as ‘bridges’ to introgress wild genes. Molecular markers are useful to pyramidize desirable genes and track them through generations of backcrossings. Molecular markers based on Resistance Gene Analogs have improved chances to be present in or linked to resistance gene loci. This study describes the development and genotyping of molecular markers based on resistance gene motifs. Specific primers were designed based on unique sequences of an Arachis RGA dataset. The identity of the amplified polymorphic bands was confirmed by sequencing. These markers were genotyped on a F₂ population that segregates for resistance to biotic stress types.

**Key words**: Peanut, molecular markers, resistance gene analogs, marker-assisted selection, *Arachis*.

**INTRODUCTION**

Peanut, *Arachis hypogaea* L. is a legume of great importance, especially in tropical and subtropical areas. It is mainly used for human food, oil and animal fodder. Unlike the morphological diversity of peanut, the genetic diversity is low and the crop lacks effective sources of biotic resistance (Kochert et al. 1996). This could be due to its origin: *A. hypogaea* probably arose from a single event of allotetraploidization of a hybrid of two diploid species. The resulting plant was sexually incompatible with its wild parents and remained isolated, resulting in a genetic bottleneck (Kochert et al. 1991). Wild species, on the other hand, are genetically very diverse and a rich source of resistance to the pathogens that attack cultivated species (Halward et al. 1992, Galgaro et al. 1998).

Biotic stresses represent a major challenge for farmers. The transfer of resistance genes from wild species to crop cultivars is particularly difficult in peanut because of the different ploidy levels that cause a fertility barrier. Resistance against the root-knot nematode, *Meloidogyne arenaria*, has been transferred at least once from wild species: diploid species were intricately crossed, chromosomes duplicated and the resulting hybrid plant was backcrossed with *A. hypogaea* (Simpson 2001). This process is however very time-consuming (C. Simpson, pers. comm).

Recently, the species has been resynthesized from the cross of two wild species, *A. duranensis* and *A. ipaënsis* (Fávero et al. 2005). The chromosome number of the hybrid was duplicated and the plant was crossed...
Design of specific primers and RGA amplification

The translated sequences of 79 Arachis RGAs (Bertioli et al. 2003) were aligned using Clustalx and primers were designed to be as specific as possible. An example of a region used for primer design is shown in Figure 1, and primer details in Table 1. The Primer Melting Temperature (Tm) was calculated using the formula: \( Tm = 4(G+C) + 2(A+T) \).

PCRs were performed as follows: 15 mM MgCl₂, 1U Taq DNA polymerase (Invitrogen), 1X enzyme buffer, 200nM dNTPs, 25 ng genomic DNA, 1 pmol µL⁻¹ of each primer and autoclaved distilled water to 15 µL. Thermocycling was programmed as follows: 5min at 96 °C, 35 cycles of: 96 °C for 1 min, primer-specific annealing temperature for 1 min (Table 1), 72 °C for 1 min, and a final extension step of 72 °C for 5 minutes. When the annealing temperatures of the forward and reverse primers diverged, the lowest temperature was used for amplifications. Reactions were run on a Mastercycler Gradient Thermocycler (Eppendorff), separated on 1% ethidium bromide-stained agarose gel and visualized under UV light.

The reactions were analyzed by polyacrylamide gel electrophoresis as described by Creste et al. (2001). To confirm that the products were RGAs, bands were excised from the gel, soaked in 100µL autoclaved distilled water overnight and maintained at 95 °C for 5 min. A 5 µL aliquot was used as template for a PCR, under the same conditions as the original amplification. PCR products were visualized by agarose gel electrophoresis, purified using a QIAQuick gel extraction kit Qiagen, and quantified by comparing fluorescence in ethidium bromide-stained agarose gel. The sequencing reactions were run on an ABI 3700 (Applied Biosystems), basically as recommended by the manufacturer.

RESULTS

As expected, the NBS region of different Arachis species is conserved (Figure 1). Based on the 79 Arachis RGAs, only six regions with insertions or deletions were therefore considered suitable to design specific primers. Of the six primers designed, S48-2F, S534-R and S5-26-F produced bands from both parents and the hybrid plant. All these three primer pairs were derived from sequences isolated from A. stenosperma. The primers...
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**Figure 1.** Example of an alignment of aminoacid sequences derived from RGAs of *Arachis* spp. using ClustalX. The region used for the design of primer S5_A_378 – fwd is underlined

**Table 1.** Primers designed based in unique contigs of *Arachis* spp. used to amplify RGAs by PCR. Size of PCR product was inferred based on the contig sequence from which primers were derived

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>Contig</th>
<th>Species</th>
<th>Access</th>
<th>Sequence 5'-3'</th>
<th>PCR product size</th>
<th>Tm</th>
</tr>
</thead>
<tbody>
<tr>
<td>S48-2F</td>
<td>S4_164</td>
<td><em>A. stenosperma</em></td>
<td>V10309</td>
<td>Fwd: CAG.GAG.AAT.GTG.ATT.GGT.TTA</td>
<td>215 bp</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Rev: TT.GAG.CAT.ATA.TTG.CTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C8V11R.abi</td>
<td>incomplete</td>
<td><em>A. cardenasi</em></td>
<td>GKP10017</td>
<td>Fwd: TCG.CTT.GTT.ACG.TAC.AAA.GCA</td>
<td>260 bp</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Rev: TTT.AGG.TGA.CAC.TAT.AGA.ATA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S534-R</td>
<td>S5_A_384</td>
<td><em>A. stenosperma</em></td>
<td>S5</td>
<td>Fwd: CCC.AAT.TTG.AAG.AAC.ACT.GCA</td>
<td>314 bp</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Rev: AAA.CAG.TGT.TAC.TCC.ATG.TGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S5-26-F</td>
<td>S5_A_378</td>
<td><em>A. stenosperma</em></td>
<td>S5</td>
<td>Fwd: GGC.ATT.GTA.AAG.AAC.ACT.GCA</td>
<td>418 bp</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Rev: TGA.AAC.TGA.GCA.ATT.CTA.GAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SMP7_3-F</td>
<td>SmP6_A_56P</td>
<td><em>A. simpsonii</em></td>
<td>V13710</td>
<td>Fwd: TCC.ATA.TAT.TGT.GTA.ATG.GAT.TT</td>
<td>222 bp</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Rev: CAT.CAT.CAA.GAA.CAA.CAA.GAA.CAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D6-6-3-F</td>
<td>incomplete</td>
<td><em>A. duranensis</em></td>
<td>V14167</td>
<td>Fwd: ATG.AGG.ATC.CTG.ATA.GAT.GGG</td>
<td>240 bp</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Rev: GGA.CAA.TTT.CCT.TGC.CGA.TCA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

D6-6-3-F, C8V11R.abi and SMP7_3-F however produced no bands in the PCRs.

The amplification products from the parental accession and hybrid on agarose gel showed a single band. The same PCR products presented multiple bands when resolved by PAGE, most of which were monomorphic, though all primers presented a few strong polymorphic bands (Figure 2).

These polymorphic bands were isolated, reamplified and sequenced to confirm the identity. Most traces indicated the presence of a single sequence within amplified bands (eg. top and bottom traces, Figure 3), while
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Figure 2. Polyacrylamide gel with products of S534-R and S248-2F primers with parentals and hybrid. Reactions were performed in duplicates as follows: K = *A. duranensis* K7988, H = hybrid, V = *A. stenosperma* V10307. Polymorphic bands are indicated by arrows.

other traces suggested that bands are mixtures of sequences (middle trace, Figure 3). The sequence of one polymorphic band (S526-F1, not shown) did not show homology with any sequence of the *Arabidopsis* or *Arachis* databank, whereas sequence analysis of the other six polymorphic bands (S48-2-1, S48-2-2, S48-2-3, S534-1, S534-2, and S534-3) confirmed that they were highly homologous RGAs to the original clone based on which they were designed (not shown). The bands S48-1, S48-2, S534-1 and S534-2 were V10309-derived, whereas S48-3 and S534-3 were K7988-derived (Figure 2). Each set of bands amplified by the same primer pair are variants of the same RGA, with size or mobility difference and a few SNPs (sequence nucleotide polymorphisms). An example of a SNP in the sequences amplified by primer pair S534-R is shown in Figure 3. The strongest bands, S534-1 and S48-2-3, were genotyped on the complete mapping population, which consists of 93 F2 plants derived from the hybrid of a cross of *A. stenosperma* (accession V10309) and *A. duranensis* (K7988). These species both have the AA genome. An example of genotyping gel is given in Figure 4.

**DISCUSSION**

This study is part of a project that aims to use resistance genes from wild relatives of peanut in breeding programs of cultivated peanut. Resistance Gene Analogs are often clustered in the genome and are frequently part of resistance genes (Zhang et al. 2002). However, since RGAs belong to a complex gene family, their use as molecular markers is complicated as it requires the use of techniques that are sensitive enough to distinguish similar sequence regions, such as PCR followed by PAGE.

The characterization of a few wild *Arachis* accessions for resistance to several pests showed that all accessions tested were more resistant than cultivated peanut (Nelson et al. 1989, Leal-Bertioli et al. 2000, Leal-Bertioli et al. 2003, Fávero 2005). This consistent high resistance level is very relevant for RGA isolation in these species. A large-scale RGA analysis was performed (Bertioli et al. 2003) but the correlation of RGAs with resistance genes could only be established by converting these into markers and placing them within a genetic map. This map would have to be based on a cross of contrasting parents for disease resistance. A microsatellite-based map for the AA genome was created (Moretszohn et al. 2005) and several attempts were made to use RGAs as molecular markers. The first strategy was Southern Blot, which had been used successfully in other crops (Collins et al. 2001, Madsen et al. 2003). Twelve representative RGAs from different clades were used as probes, with a high polymorphism degree between parents (Guimarães et al. 2005). These markers are now being placed on the *Arachis* genetic map. It is an established technique, although rather time-consuming, and requires large amounts of high quality DNA, which is not always possible for seasonal plants like *Arachis*. Alternative strategies are therefore required, which would make the screening of a larger number of RGAs and their placement on a map possible.

The placement of RGAs on genetic maps has been accomplished for several crops using Southern Blot, PCR using specific or degenerate primers and AFLP using adaptors designed for RGA motifs (Donald et al. 2002, De Giovanni et al. 2004, Xu et al. 2005, Yan et al. 2005). In this study, specific primers were designed for
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**Figure 3.** Example of a SNP (sequence nucleotide polymorphism) found in the sequences of polymorphic bands reamplified from polyacrylamide gels of amplified products using primer S534-R (vertical bar).

**Figure 4.** Example of segregation of a polymorphic band S48-2-3 (RGA) produced by PCR with the specific primers S48-2F For and Rev (indicated by black arrow). These primers were designed based on the sequence of the RGA clone S4_A_164 derived from accession V10309 of *A. stenosperma*. Patterns of the individuals J10 to J54 are shown. “K” is the parental *A. duranensis* K7988, “H” is the F1 hybrid plant and “V” is parental *A. stenosperma* V10309.

For some regions that were unique in the RGA alignment, it was not possible to design primers because (a) the primer pairs were autocomplementary (b) Tm of forward or reverse primers were not compatible (c) the region between primer pairs was too short for PCR. Six pairs were designed in the suitable regions for primer design, so the principle of specific amplification could be tested. All primers amplified the original species from which they were sequenced, but only three amplified DNAs of the accessions that were used for the creation of the segregating population. The other primers did not show any product from these accessions, so their sequence was not homologous to any RGA from V10309 and K7988. Three primers S48-2F, S534-R and S5-26-F showed a single amplification product when resolved on agarose gel. However, when separated on polyacrylamide, several bands of different molecular
weights appeared, some of which were polymorphic. This can be explained by the fact that although the primers were designed for single sequences in the alignment, this did not rule out the possibility of an amplification of other homologous sequences in the genome, as they are part of a multigene family. Of the seven polymorphic bands produced when using these three primers, only one was not significantly homologous to the RGAs (S526-F1). The fact that the primers were designed for an RGA region did not exclude the possibility that they would bind to another, unrelated region of the genome. Therefore, the reaplification and sequencing of the bands generated is mandatory to certify the identity of the genetic marker.

The markers developed here were genotyped on a F2 population that segregates for resistance to biotic stresses. In parallel to this study, bioassays are being performed with this F2 population in order to associate resistances to molecular markers. Introgression lines are being created by the tetraploidization of wild hybrids and their hybridization with peanut (Favero et al. 2005). These lines are back-crossed with peanut, and their progeny is phenotyped for fungal diseases. The identification of molecular markers tightly linked to genes of interest, in this case, of pathogen resistance, would allow the implementation of a Marker-Assisted Selection program (MAS), which can increase the effectiveness of the back-crosses, overcome linkage drag, and reduce the time for selection of resistant lines. In such cases, the resistance mechanism may be unknown, but the resistance-related loci can be selected through generations of selection by simple PCR (Hinchliffe et al. 2005). This would greatly speed up and optimize the development of improved peanut varieties with incorporation of new wild disease-resistance genes.

Desenvolvimento e genotipagem de marcadores RGAs em população de mapeamento genoma AA de Arachis silvestre

RESUMO - O amendoim é uma leguminosa de grande importância mundial. Embora tenha alta variabilidade morfológica, tem baixa variabilidade genética e poucas fontes de resistências. A transferência de genes a partir de espécies silvestres é difícil devido à diferença de ploidia entre estas e a cultivada. Recentemente, foram produzidos anfidiplóides que podem ser utilizados como ‘pontes’ para a introgressão de genes silvestres. Marcadores moleculares são úteis para piramidar estes genes e monitorá-lo através das gerações de retrocruzamentos. Marcadores moleculares baseados em regiões análogas a genes de resistência (RGAs) têm maiores chances de estar ligados a locos de resistência. Este trabalho descreve o desenvolvimento e genotipagem de marcadores moleculares baseados em motivos de genes de resistência. Primers específicos foram construídos para seqüências únicas de RGAs de Arachis. A identidade das bandas polimórficas amplificadas foi confirmada por sequenciamento. Estes marcadores foram genotipados em uma população F2 segregante para resistência a estresses bióticos.

Palavras-chave: Amendoim, marcadores moleculares, análogos a genes de resistência, seleção assistida por marcadores, Arachis.

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