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Gene introgression from *Arachis glabrata* into *A. hypogaea*, *A. duranensis* and *A. diogoi*

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Summary

Arachis glabrata Benth, variety *glabrata* coll. GK 10596 (PI 276233; ICG 8176) belonging to section *Rhizomatosae* has multiple disease resistance. Fertile hybrids between *A. hypogaea* L. and *A. glabrata*, *A. duranensis* Krapov & W.C. Gregory and *A. glabrata* and *A. diogoi* Hoehne and *A. glabrata* were produced. Introgression of DNA from *A. glabrata* into *A. hypogaea, A. duranensis* and *A. diogoi* was analyzed by isozyme and RAPD analyses. Hybrids were backcrossed and BC_1 seeds were obtained in all the three hybrids. Hybrids were evaluated for the transfer of disease resistance genes from *A. glabrata*, which was confirmed. RAPD analysis with several primers showed that DNA fragment pattern were not simply represented, instead there were new bands and several parental bands were absent in the interspecific derivatives.

Introduction

Arachis hypogaea L., commonly called groundnut, is an important crop of the semi arid tropics. Among important yield constraints are diseases caused by foliar fungal pathogens, viruses and insect pests. At the International Crops Research Institute for Semi Arid Tropics (ICRISAT), India, a rich source of wild germplasm is available that offers an opportunity for improvement of the crop.

Based on morphological and cross-compatibility relationships, Krapovickas & Gregory (1994) grouped the genus *Arachis* into nine sections. Cultivated groundnut (2n=40) belongs to section *Arachis* which includes 24 diploid (2n=20), and one tetraploid wild species. Very few of these diploid species have been successfully used in the improvement of cultivated groundnut (Stalker & Simpson, 1995). There are no reports of production of fertile hybrids between *A. hypogaea* and wild species from other sections, and when attempts were made, the hybrids were sterile (Stalker & Simpson, 1995). Mallikarjuna & Sastri (1985 a & b) and Shen et al. (1995) reported fertile hybrids between *A. hypogaea* and *A. glabrata* Benth, the only tetraploid species known outside section *Arachis*. Interest in the section *Rhizomatosae* species *A. glabrata* exists because it has resistance to diseases caused by fungi, virus, and insect pests (Moss et al., 1988).

This paper reports the first successful attempt to produce fertile hybrids between *A. duranensis* Krapov & W.C. Gregory and *A. diogoi* Hoehne (both of section *Arachis*) with *A. glabrata*. These crosses were possible by overcoming the barriers to hybridization by applying gibberellic acid (GA) to pollinated pistils and rescuing aborting embryos by embryo culture techniques. The hyrid between *A. hypogaea* and *A. glabrata* was also obtained by the use of the above mentioned techniques (Mallikarjuna & Sastri, 1985 a & b). The parents and the hybrids were used for disease screening and isozyme and RAPD analyses.

Materials and methods

Arachis diogoi, *A. duranensis* and *A. glabrata* variety *glabrata* [GK 10596 (PI 276233; ICG 8176)] plants were maintained in a glasshouse. Pollinations were carried out before 10 am. Pollinated pistils were

Table 1. Operon primers used in the study of interspecific groundnut hybrids

Primer code – Sequence	Primer code – Sequence
OPI01-ACCTGGACAC	OPF02-GAGGATCCCT
OPI02-GGAGGAGAGG	OPF04-GGTGATCAGG
OPI03-CAGAAGCCCA	OPF07-CCGATATCCC
OPI18-TGCCCAGCCT	OPF08-GGGATATCGG
OPJ01-CCCGGCATAA	OPF20-GGTCTAGAGG
OPJ06-TCGTTCCGCA	OPF13-GGCTGCAGGA
OPJ13-CCACACTACC	OPH01-GGTCGGAGAA
OPJ17-ACGCCAGTTC	OPH02-TCGGACGTGA
OPJ19-GGACACCACT	OPH03-AGACGTCCAC
OPJ20-AAGCGGCCTC	OPH05-AGTCGTCCCC

treated with 87.5 mg/L GA to stimulate peg initiation and elongation (Mallikarjuna & Sastri, 1985a). Pods were harvested 30 to 35 days after pollination and embryos were rescued using the technique of *in-ovulo* embryo culture (Mallikarjuna & Sastri, 1985b). Embryos were dissected out of the ovules and cultured on MS (Murashige and Skoog's medium) basal salts with 3% sucrose, 0.7% agar, 0.1 mg/L napthaleneacetic acid (NAA) and 1.0 mg/L benzylamino purine (BAP). Most of the embryos germinated with a healthy shoot but a stunted root system. Many of the shoots formed multiple shoot buds when placed on MS medium with NAA (0.01 mg/L) and BAP (0.1 mg/L). The rooting medium to induce roots on the in vitro grown shoots was made up of 1/10 MS basal medium with 2.0 mg/L indole-3-butyric acid (IBA).

Wild species *A. diogoi* and *A. duranensis* are diploid (2n=20) and belong to section *Arachis*. *Arachis hypogaea*, the cultivated species, is a tetraploid (2n=40) and also belongs to section *Arachis* whereas *A. glabrata* (2n=40) belongs to section *Rhizomatosae*. A hybrid between *A. hypogaea* and *A. glabrata* obtained in another experiment (Mallikarjuna & Sastri, 1985 a & b) was also included in the study.

For isozyme analysis, crude extracts were obtained from immature leaves in the buffer designed for moderately interfering substances as described by Wendel & Weeden (1989). For localizing esterase, isopropanol dehydrogenase and glycerol dehydrogenase isozyme methods described by Manchenko (1994) were followed.

Genomic DNA was extracted from immature leaves of plants grown in a glasshouse. Fresh immature leaves were harvested, lyophilized in liquid nitrogen and stored at –70 °C, and DNA was extracted whenever necessary by the CTAB method (Saghai-Maroof et al., 1984). RAPD-PCR was performed according to the protocols of Williams et al., (1990). Twenty random 10-mer primers (Operon Technologies; Table 1) were used to amplify DNA in Perkin GeneAmp 9600 thermal cycler and PCR products were electrophoresed on 1.5% Agarose gels, stained in ethidium bromide and photographed under UV illumination.

Flower buds of *A. hypogaea* × *A. glabrata*, *A. duranensis* × *A. glabrata and A. diogoi* × *A. glabrata* were fixed in carnoy's II mixture (alcohol: acetic acid: chloroform; 6:3:1). Buds were squashed in 2% acetocarmine and pollen fertility estimates were made on well-stained pollen grains. Five hundred pollen grains were counted from five random flowers and mean percent pollen fertility was calculated.

Parents and the hybrids were tested for their reaction against *Puccinia arachidis* Spegazzini (rust), *Phaeosariopsis personata* van Deighton (late leaf spot; LLS) and *Cercospora arachidicola* Hori (early leaf spot, ELS) diseases by the detached leaf technique described by Subrahmanyam et al. (1982). Diseases were scored on 1–9 scale as resistant (1–2), moderately resistant (3–5), and susceptible (6–9).

The screening method for the three virus diseases viz. PBNV, PMV and PSTV was by mechanical sap inoculation. Plant extracts containing the viruses in an appropriate buffer were applied to the surface of the leaves of healthy-looking hybrid plants. Disease-infected seeds of *A. hypogaea* cv TMV 2 were used as positive controls and healthy uninfected seeds of the same cultivar were used as negative controls. The presence of virus was tested by the ELIZA method as described by Hobbs et al. (1987). Absorbance values at 620 nm were determined with a Titertek Multiscan ELIZA reader. ELISA readings above 1.0 were considered as positive for the presence of virus.

Results and discussion

The percentage of pods formed was very low in the crosses *A. diogoi* × *A. glabrata* and *A. duranensis* × *A. glabrata* (Table 2), but in the cross *A. diogoi* × *A. glabrata* all the pods had embryos large enough (3.0 to 4.5 mm) to be directly cultured in vitro. In the cross *A. duranensis* × *A. glabrata*, 36% of the embryos (3.0 to 4.5 mm) were large enough to be directly cultured. In *A. hypogaea* × *A. glabrata* none of the aborting

Table 2. Results of interspecific hybridization between *A. glabrata* and wild species from section *Arachis*

Cross	No. pollinations	No. pegs (%)	No. pods (%)	Pollen fertility (%)
A. dura. × *A. glab.*	890	73 (8.2)	11 (1.2)	31
A. diog. × *A. glab.*	266	29 (11)	3 (0.9)	30
A. hypo. × *A. glab.*	433	61 (14)	39 (9)	26

seeds had embryos sufficiently large to be directly cultured (Mallikarjuna & Sastri, 1985b). In the cross *A. diogoi* × *A. glabrata* and *A duranensis* × *A. glabrata*, although few seeds were obtained, embryos were normal in appearance with two well developed cotyledons to be directly cultured on the medium. Although it took more than 120 days for the embryos to develop into plants, the plants grew normally and flowered profusely. Pollen fertility in both the crosses ranged from 15 to 31%. Although the hybrids were normal in the cross *A. diogoi* × *A. glabrata*, the leaves exhibited virus-like symptoms. These plants were tested for viruses by ELISA and were found to be healthy and devoid of virus. Smartt (1964) observed similar symptoms in *A. diogoi* (synonym = *A. chacoense*) crosses.

These results lead to the conclusion that the genomes of *A. duranensis* and *A. diogoi* (A genome; Smartt et al., 1978) and *A. glabrata* (RR genome; Smartt & Stalker, 1982) may be closely related. This hypothesis is further substantiated because in the cross *A. diogoi* × *A. glabrata* and *A. duranensis* × *A. glabrata*, embryos were big enough with well developed cotyledons to be directly cultured on the medium to obtain plants. Similarly hybrid plants were obtained between *A. hypogaea* (A and B genome; Smartt et al., 1978) and *A. glabrata*.

Amongst the three isozymes used in the study, esterase distinguished *A. diogoi* and *A. duranensis* from *A. glabrata* and was also able to confirm the hybridity of the F_1 plants. The two hybrids of the present study and the hybrid between *A. hypogaea* and *A. glabrata* (Mallikarjuna and Sastri, 1985 a & b) had one unique band not present in any of the parents (Figure 1). This unique band may be the result of recombination between A genome of the female parents and the R genome of *A. glabrata*, the common pollen donor. Although glycerol dehydrogenase and isopropanol dehydrogenase were able to distinguish the parents, the hybrids resembled their maternal parent in their isozyme profile.

Table 3. Summary of the RAPD data for the presence (+) or absence (–) of polymorphic amplification products specific to *A. glabrata*

Primer	*A. hypo.* × *A. glab.*	*A. dura.* × *A. glab.*	*A. diog.* × *A. glab.*
OPJ01	–	+	–
OPF04	+	+	–
OPH03	+	–	+
OPJ17	+	+	–
OPF02	+	–	–
OPF04	+	+	–
OPH01	+	–	–
OPH02	+	+	–
OPI03	+	–	–
OPJ06	+	+	–
OPF20	+	+	+

Out of twenty primers used to distinguish *A. hypogaea* × *A. glabrata* from its parents, nine primers showed bands specific to *A. glabrata*, the pollen donor. A greater number of bands were specific to *A. glabrata* in the hybrid than those to *A. hypogaea*. Six primers showed bands specific to *A. glabrata* in the hybrid *A. duranensis* × *A. glabrata* and two primers showed bands specific to *A. glabrata* in the hybrid *A. diogoi* × *A. glabrata* (Table 3). In *A. diogoi* × *A. glabrata*, the number of bands specific to *A. diogoi* were greater than those specific to *A. glabrata*. With primer OPH02, the DNA fragment pattern of the parents *A. diogoi* and *A. glabrata* and the hybrid *A. diogoi* × *A. glabrata* revealed that parental patterns were just not represented, instead some parental bands were absent and few new bands were present (Figure 2). Similarly with primer OPF20, *A. duranensis* × *A. glabrata* showed unique bands not present in either parents (Figure 3). These results confirm that during the synthesis of hybrids, parental band patterns are not simply represented in the hybrids, The absence of some parental bands as well as the presence of new bands

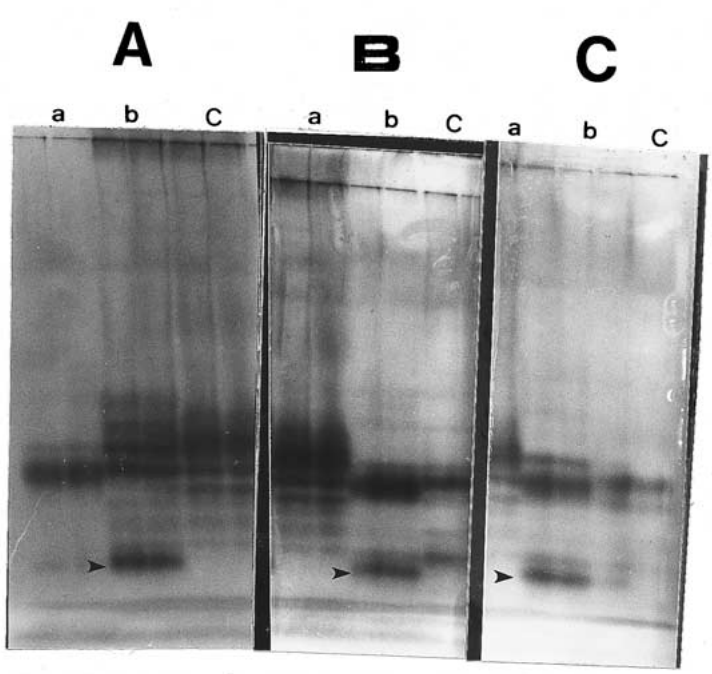

Fig. 1. Isozyme profile of hybrids RIMG, RIMD and RIMC and their parents.

Figure 1. Esterase isozyme profile. A. Esterase isozyme profile of *A. hypogaea* × *A. glabrata* and its parents: a. Isozyme profile of *A. hypogaea* (two lanes); b. Isozyme profile of *A. hypogaea* × *A. glabrata* (two lanes); c. Isozyme profile of *A. glabrata* (two lanes). B. Esterase isozyme profile of *A. duranensis* × *A. glabrata* and its parents: a. Isozyme profile of *A. glabrata* (two lanes); b. Isozyme profile of *A. duranensis* × *A. glabrata* (two lanes); c. Isozyme profile of *A. duranensis* (two lanes). C. Esterase isozyme profile of *A. diogoi* × *A. glabrata* and its parents: a. Isozyme profile of *A. glabrata* (two lanes); b. Isozyme profile of *A. diogoi* × *A. glabrata* (three lanes); c. Isozyme profile of *A. diogoi* (one lane).

suggest the elimination and/or rearrangements of the genetic material occuring after or during the fusion of the genomes. For example, oligonucleotide fingerprinting of re-synthesized *Brassica napus* showed presence of new bands not observed in the parents (Poulsen et al., 1993).

Screening the parents and the hybrids for foliar diseases showed that the hybrid *A. hypogaea* × *A. glabrata* had acquired multiple foliar disease (rust, LLS and ELS) resistance from *A. glabrata* because *A. hypogaea*, the female parent in the cross, was susceptible to all the three diseases. Hybrid plant *A. duranensis* × *A. glabrata* was resistant to rust and ELS but not to LLS. Rust resistance is a trait inherited from *A. glabrata* because *A. duranensis*, the other parent in the cross, is moderately resistant to rust. An important observation was the susceptibility to LLS. Both *A. duranensis* and *A. glabrata* showed resistant reaction to LLS (Table 4). Hybrid *A. diogoi* × *A. glabrata* was resistant to rust, a character inherited from *A. glabrata* because *A. diogoi* is moderately resistant to rust but the hybrid was susceptible to LLS, unlike either of the parents.

Screening tests for the presence of peanut bud necrosis virus (PBNV) by aphid inoculation did not show disease symptoms on *A. glabrata* or the hybrid *A. hypogaea* × *A. glabrata*, although insect feeding spots were observed. This meant that although initially the insect tried to feed on the leaves it was not successful in transmitting the virus, and hence the disease. Similarly, hybrid *A. hypogaea* × *A. glabrata* inoculated with peanut stripe virus (PSTV) showed no disease symptoms. ELISA tests showed readings for the presence of PBNV and PSTV of 0.07 and 0.08, the same that of negative control. Wild species *A. duranensis* and the hybrid *A. duranensis* × *A. glabrata* showed disease symptoms and the presence of virus in ELISA tests for PSTV. The hybrid *A. hypogaea* × *A. glabrata* showed peanut mottle virus (PMV) mottling symptoms in younger leaves. ELISA tests for the presence

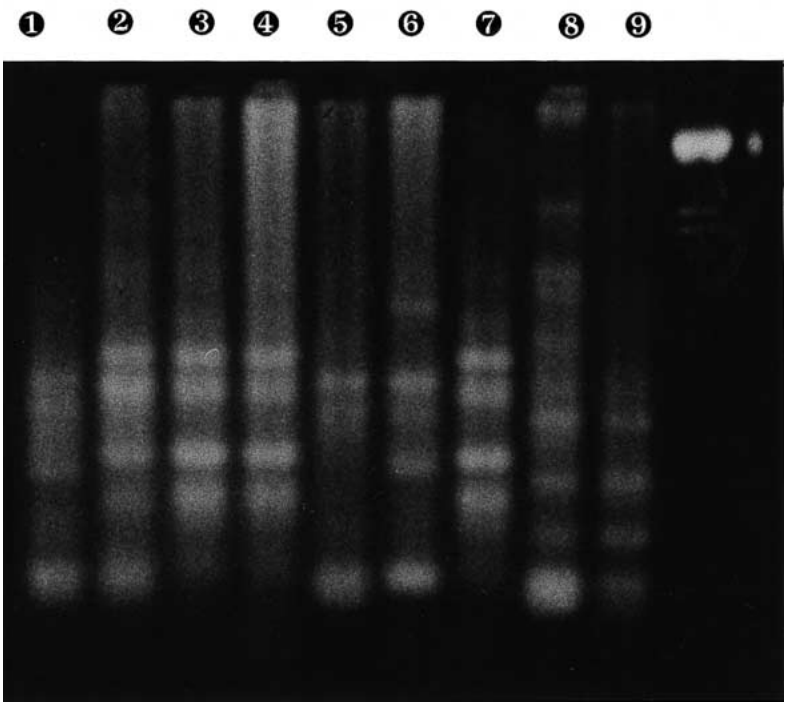

Figure 2. RAPD profile with primer OPH02. Lane 1. RAPD profile of *A. hypogaea*. Lane 2. RAPD profile of hybrid *A. hypogaea* × *A. glabrata*. Lane 3. RAPD profile of *A. glabrata*. Lane 4. RAPD profile of *A. glabrata*. Lane 5. RAPD profile of hybrid *A. duranensis* × *A. glabrata*. Lane 6. RAPD profile of *A. duranensis*. Lane 7. RAPD profile of *A. glabrata*. Lane 8. RAPD profile of *A. diogoi* × *A. glabrata*. Lane 9. RAPD profile of *A. diogoi*.

Table 4. Results of foliar and viral disease screening of the parents and hybrids

Identity	Rust	LLS	ELS	PMV	PSTV	PBNV
A. glabrata	R	R	R	R	R	R
A. hypogaea	S	S	S	S	S	S
A. duranensis	MR	R	MR	S	S	S
A. diogoi	MR	R	R	R	S	R
A. hypo. × *A. glab.*	R	R	R	S	R	R
A. dura. × *A. glab.*	R	S	R	NT	S	NT
A. diog. × *A. glab.*	R	S	NT	R	NT	NT

Abbreviations: R = resistant, MR = moderately resistant, S = susceptible, NT = not tested, LLS = late leaf spot, ELS = early leaf spot, PMV = Peanut mottle virus, PSTV = peanut stripe virus, PBNV = peanut bud necrosis virus.

Table 5. Pod formation in interspecific hybrids crossed with *A. hypogaea*

Cross	No. pollinations	No. pegs (%)	No. pods (%)
(*A. hypo.* × *A. glab.*) × *A. hypo.*	881	32 (3.6)	10 (1.1)
(*A. diog.* × *A. glab.*) × *A. hypo.*	573	49 (8.5)	3 (0.5)
(*A. dura.* × *A. glab.*) × *A. hypo.*	487	17 (3.4)	7 (1.4)

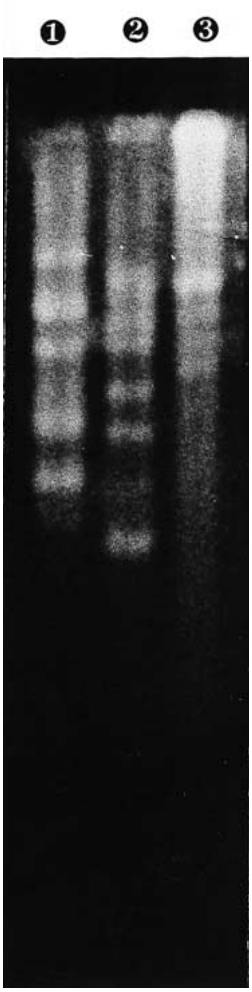

Figure 3. RAPD profile with primer OPH20. Lane 1. RAPD profile of *A. glabrata*. Lane 2. RAPD profile of hybrid *A. diogoi* × *A. glabrata*. Lane 3. RAPD profile of *A. diogoi*.

of PMV showed the presence of virus. Hybrid *A. diogoi* × *A. glabrata* did not show the PMV mottling symptoms nor was the virus detected in ELISA tests. The results of the screening tests for the virus diseases PMV, PSTV and PBNV are tabulated in Table 4.

Leaf morphology of the hybrids was intermediate between their parents. Growth habit of *A. hypogaea* × *A. glabrata* resembled *A. glabrata* whereas *A. duranensis* × *A. glabrata* and *A. diogoi* × *A. glabrata* had an upright growth habit unlike *A. glabrata* or their female parent.

Hybrid plants *A. hypogaea* × *A. glabrata*, *A. duranensis* × *A. glabrata* and *A. diogoi* × *A. glabrata* were used as female parent and back-crossed with *A. hypogaea* cv. ICGS 44. Bold seeds were obtained, but percent pod set was low (2%) compared to the number of pegs formed (3–9%; Table 5). Ovule and embryo culture was essential to obtain hybrids, but BC1 seeds were bold, and plants were obtained without the intervention of in vitro techniques. These results further confirm the fact that A genome of section *Arachis* is closely related to R genome of *A. glabrata*. Upon stabilization of the genomes in the hybrids a large number of bold and mature seeds may be obtained.

According to Smartt & Stalker (1992), tetraploid species of the section *Rhizomatosae* are more recently evolved than the diploid species of the section. The present paper confirms the assumption that one of the genomes of the section *Arachis* played a major role in the evolution of tetraploid members of the section *Rhizomatosae*. The species in the two sections are not distantly related and can be used for the improvement of cultivated groundnut. By setting mature BC1 seeds in the hybrids between *A. glabrata* and members from the section Arachis, not only do these crosses provide resistance to important diseases caused by foliar pathogens and viral diseases, but they bring in much desired variability.

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