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Cloning and characterisation of a ribosomal RNA gene repeat unit from groundnut

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Abstract Characterisation of a ribosomal RNA gene repeat unit (rDNA) was carried out on groundnut, *Arachis hypogaea* L. The rDNA was cloned, a restriction map constructed and the 5.8S and internal transcribed spacer (ITS1 and ITS2) regions were completely sequenced while the 18S and 25S regions were partially sequenced. The restriction map and the sequences were compared with published maps and sequences from other crops. The cloned groundnut rDNA is about 12 kb in size with approximately 6 kb of intergenic spacer (IGS). The unique *Hin*dIII site in the IGS and two *Eco*RI and four *Bam*HI sites in the transcribed region are features it shares with other legumes, while the presence of four of the five *Xba*I sites in the IGS region is its distinguishing feature.

Keywords *Arachis hypogaea* · Peanut · rDNA

Abbreviations *IGS* Intergenic spacer · *ITS* Internal transcribed spacer · *rDNA* Ribosomal RNA gene repeat unit

Introduction

The cultivated groundnut *A. hypogaea* L. (2*n*=40) is an allotetraploid belonging to section *Arachis*. Its allotetraploid origin was proposed on the basis of stable bivalent pairing with occasional multivalent formation and the presence of two pairs of morphologically distinguishable chromosomes in the genome (Husted 1936). Over 70 *Arachis* species are known, of which only four are tetraploid while all the others are diploid in nature (Gregory et al. 1973). Based on cytological evidence Singh (1988) proposed *A. batizocoi* and *A. duranensis* to be the most likely progenitors. However, observations based on restriction fragment length polymorphism (RFLP) analysis by Kochert et al. (1991) suggested that *A. ipaensis* is likely to be one donor, while either *A. duranensis* or *A. spegazzinni* could be the other. Paik-Ro et al. (1992) indicated that *A. duranensis* is the most closely related species, while there is a lack of homology between *A. hypogaea* and *A. batizocoi*. On the basis of RFLP and cytogenetic evidence Kochert et al. (1996) proposed *A. ipaensis* (B genome) and *A. duranensis* (A genome, female parent) as the progenitor species. Alternatively, Raina and Mukai (1999) proposed *A. villosa* and *A. ipaensis* to be the most likely genome donors of the tetraploid species based on in situ hybridisation at the rDNA and 5S RNA loci and centromere banding pattern. To date, no unequivocal evidence has been obtained with respect to the progenitor species of the cultivated groundnut.

The cultivated groundnut genome has revealed very little variability at the molecular level, although considerable morphological variability exists among its germplasm resources. Isozyme variability is very low in the cultivated germplasm (Grieshammer and Wynne 1990). The RFLP technique revealed very little variation among cultivars (Kochert et al. 1991) and exotic germplasm lines (Halward et al. 1991). Randomly amplified polymorphic DNA (RAPD) analysis revealed a low level of polymorphism (Halward et al. 1992; Bhagwat et al. 1997), while the DNA amplification fingerprinting (DAF) and amplified fragment length polymorphism (AFLP) techniques demonstrated polymorphism (He and Prakash 1997). The general low level of variability in the cultivated groundnut has been attributed to a single and recent polyploidisation event being successively selected and utilised for all further breeding efforts – thus resulting in a highly conserved genome (Young et al. 1996). Conversely, abundant polymorphism has been detected

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among related wild species of *Arachis* using isozymes (Stalker et al. 1990), RFLPs (Kochert et al. 1991) and RAPD markers (Halward et al. 1992).

The ribosomal RNA gene locus (rDNA) has been widely used for phylogenetic and diagnostic purposes. The rDNA in most eukaryotes consists of a series of repeating units containing the 18S, 5.8S and 25S rRNA genes. These units are arranged in a head-to-tail tandem array with internal transcribed spacers (ITS1 and ITS2) separating the rRNA genes within a unit and an intergenic spacer (IGS) separating the adjoining units. The number of these repeat units in plants ranges from 570 to 32,000, and in some crops the repeat units comprise about 7% of the haploid genome (Ingle et al. 1975). Variation is present in the ITS (Nickrent and Doyle 1995; Tucci et al. 1998) and the IGS sequences (King et al. 1993; Liu et al. 1996). ITS regions are more variable than the rRNA genes but are more conserved than the IGS regions. Studies on variability in rDNA show that the coding and IGS regions evolve at different rates. The coding region is highly conserved, whereas the IGS region is variable in sequence and length and shows inter- and intra-specific variation (Rogers and Benedich 1987). This variation provides useful molecular markers for assessing genetic diversity in plants. The IGS, in addition to consisting of transcriptional control elements and the transcription initiation site (TIS), contains repeated DNA sequence families characteristic of a species. Unequal crossing-over within these repetitive families and duplications of the simple repeat sequences (SSRs) are thought to have generated the IGS length variability (Appels and Dvorak 1982) that has been observed in a number of species (Yakura et al. 1984; Polans et al. 1986; Delseny et al. 1990; Maggini et al. 1992; Liu et al. 1996; Borisjuk et al. 1997).

The present investigation was initiated to characterise this gene from the groundnut genome. We report the cloning of a rDNA repeat unit from groundnut, the construction of its restriction map and the partial sequencing of the 18S (3' region) and 25S (5' region) coding regions and complete sequencing of 5.8S coding region and the two internal transcribed spacers. A comparison is made of this restriction map with cloned rDNA maps from leguminous crops like pea, soybean, French bean, broad bean, lens, *Cicer* and yellow lupine. The partial sequence of the 18S and the 25S RNA gene from groundnut is also compared with the corresponding published sequences from other crops.

Materials and methods

Isolation of DNA

Plant DNA was isolated from green leaves of field-grown plants following the method of Dellaporta et al. (1983). Plasmid DNA was isolated by the alkaline lysis method according to Sambrook et al. (1989).

Cloning and subcloning

The *Hin*dIII-digested genomic DNA from groundnut cv. Spanish Improved was separated on 1% LMP agarose (Sigma, USA), and fragments ranging in size from 9 kb to 15 kb were eluted. The *Hin*dIII-digested plasmid vector pUC19 was then ligated to the eluted genomic DNA using T4 DNA ligase (Bangalore Genei, India) at 10°C for 30 min and transformed into *Escherichia coli* DH5α cells (Sambrook et al. 1989). The transformants were selected on Luria agar plates containing ampicillin, X-gal and IPTG. White colonies were picked out, replica-plated and transferred onto nylon membranes (Sambrook et al. 1989). The colony blots were probed with a pea rDNA fragment (see below), and those showing positive signals following autoradiography were analysed further. For subcloning, plasmid pBluescript II SK^+ (Stratagene, USA) was used. The subclones were named pARG 1.1, pARG 1.2, pARG 1.2.1 and pARG 1.3 (see Fig. 2).

Probes

The first probe consisted of the 5.8S coding sequence along with the two flanking ITS sequences amplified from pea (*Pisum sativum*) using the following primers: the upstream primer used was either 5'-GGAAGTAAAAGTCGTAACAAGG-3' or 5'-TCC GTAGGTGAACCTGCGG-3' and the downstream primer was TCCTCCGCTTATTGATATGC. The second probe was the full-length insert from clone pARG-1 (Fig. 2). The probes were labelled using a random primer DNA labelling kit and α-[^{32}P]-dATP (BRIT, BARC, India).

Amplification

Amplification of the 5.8S RNA gene along with both the ITS sequences from groundnut and pea was accomplished using the primers mentioned above in a MJ Programmable Thermal Controller (PTC-100). The polymerase chain reaction (PCR) was carried out in a 25-µl reaction volume using 0.5 U *Taq* DNA polymerase and 1× *Taq* buffer (10 m*M* Tris pH 8.8, 1.5 m*M* $MgCl_2$, 50 m*M* KCl, 0.001% gelatin), 0.2 µ*M* of each primer (synthesised by Bangalore Genei, India), 20 ng of the template DNA and 0.2 m*M* each of the dNTPs (Boehringer Mannheim, Germany). The PCR programme consisted of 25 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and synthesis at 72°C for 2 min. The initial denaturation was at 94°C for 4 min and the final extension was at 72°C for 10 min.

Electrophoresis and blotting

PCR-amplified products were separated on 1.5% agarose gels in Tris borate buffer (0.1 *M* Tris, 0.083 *M* boric acid, 1 m*M* EDTA, pH 8.4) with a *Hae*III digest of ϕX174 as the molecular-weight marker. The groundnut genomic DNA and plasmid DNA from the clones were digested with restriction enzymes according to the manufacturer's instructions (Bangalore Genei, India). The restricted DNA was resolved on 0.8% agarose gels and then blotted onto nylon membranes (Boehringer Mannheim, Germany), which were subsequently baked at 120°C for 30 min in order to fix the DNA. The *Hin*dIII-digested genomic DNA from groundnut was resolved on 1% LMP agarose, and DNA fragments ranging in size from 9 kb to 23 kb were eluted from the gel according to Sambrook et al. (1989).

Hybridisation and autoradiography

The labelled probes were hybridised to Southern-blotted DNA from the clones following the procedure of Church and Gilbert (1984). Briefly, prehybridisation was performed in Gilbert's solution containing 7% SDS at 65°C for 4 h. The labelled probe was denatured, then added to the same solution, and hybridisation was carried out overnight at 65°C. After two stringent washes in 0.1× SSC with 0.2% SDS at 65°C for 30 min the blot was exposed to X-ray film (Indu, India).

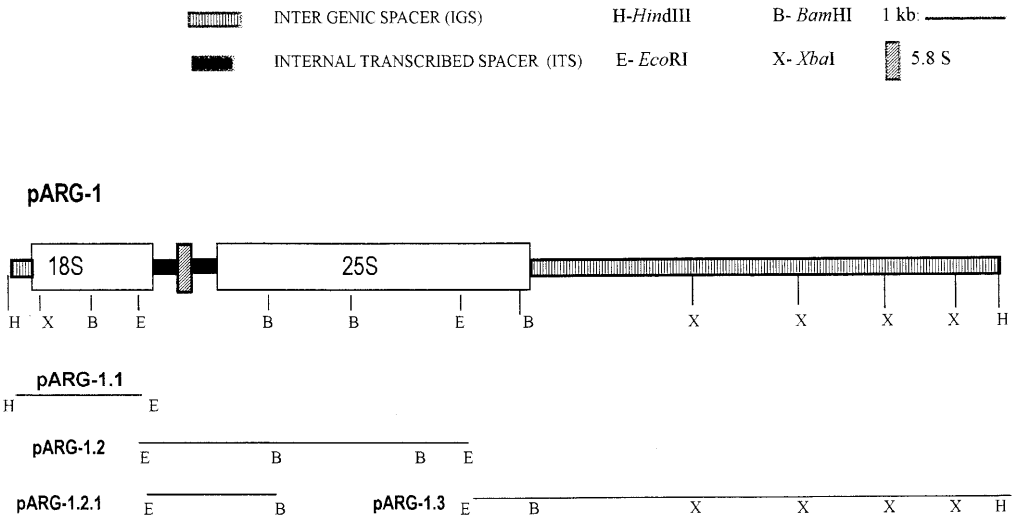

Fig. 1 Restriction map of the cloned rDNA repeat (pARG-1) of groundnut. Depicted are the clone pARG-1 and its subclones (pARG-1.1, pARG-1.2, pARG-1.2.1 and pARG-1.3) showing restriction cleavage sites

Sequencing

Sequencing reactions were carried out by Bangalore Genei (Bangalore, India) following Sanger's method (Sanger et al. 1977) in an automated sequencer (ABI Prism) using the ABI fluorescence dye terminator kit (PE Applied Biosystems). The sequencing of pARG 1.2.1 from the *Eco*RI site downstream was accomplished using the T7 primer (5'-AATACGACTCACTATAG-3'), which gave about 600 bases of readable data. The sequencing of pARG-1.2.1 from the *Bam*HI site upstream was done using the T3 primer (5'-ATTAACCCTCACTAAAG-3'), which gave 513 bases of readable sequence. From this 3' sequence a primer was synthesised (5'-CCTCGTGGTGCGACAGG-3') that was subsequently used to read a further 670 bases upstream.

Results and discussion

The aim of the investigation reported here was to characterise the types of ribosomal RNA genes present in the groundnut genome and to clone them. Our analysis of groundnut DNA digested with different restriction endonucleases when hybridised to the pea probe showed the presence of a single *Hin*dIII site in the 12-kb repeat. Consequently, this restriction site was used for constructing a genomic library of groundnut in the size range of 9–12 kb. When the library was screened with the pea probe, two clones hybridised; these were labelled pARG-1 and pARG-7. Both contained the same-sized insert, and restriction analysis revealed that they differed in the orientation of the insert. Further studies were then restricted to pARG-1. The length of the repeat unit from groundnut is similar to that found in *Cicer* (Patil et al. 1995). The length of the IGS after subtraction of the predicted length of the coding sequence would be approximately 6 kb, as in *Cicer* (Fig. 1).

*Eco*RI-digested pARG-1 DNA, when hybridised with the full-length insert, produced three hybridising fragments of 8.9 kb (containing the 2,686-bp vector sequence in addition to the IGS), 4 kb and 1.6 kb, respectively (Fig. 2). Of these three fragments, the 4.1-kb fragment hybridised to the pea probe. *Bam*HI digestion revealed

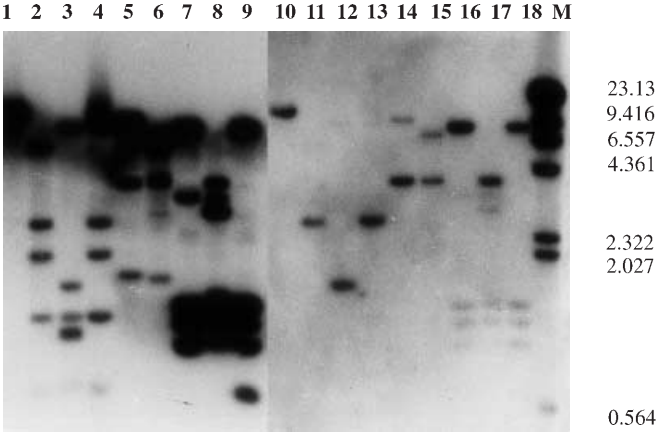

Fig. 2 Hybridisation profile obtained from groundnut. *Lanes 1–9* Clone (pARG-1) DNA digested with *Hin*dIII, *Hin*dIII and *Bam*HI, *Bam*HI and *Eco*RI, *Bam*HI, *Eco*RI, *Hin*dII and *Eco*RI, *Xba*I, *Xba*I and *Eco*RI, *Xba*I and *Hin*dIII, respectively. The full-length insert from pARG-1 was used as the probe. *Lanes 10–18* The profile obtained from the same blot re-probed with the amplified product containing the 5.8S gene along with the ITS sequences. *Lane 19* *Hin*dIII-digested lambda DNA used as a size marker

five bands – 8.5, 2.6, 2.1, 1.1 and 0.6 kb – of which the 2.1-kb band hybridised to the pea probe. Digestion with *Xba*I resulted in a five-band hybridisation pattern, with bands of 8.6, 3.6, 1.3, 1.1 and 0.9 kb; of these, the 8.6-kb band hybridised to the pea probe. The positions of the *Xba*I sites subsequent to the first *Xba*I site in the IGS were tentatively placed with respect to each other. By comparing the hybridisation profiles obtained from the restrictions with *Bam*HI-*Eco*RI, *Xba*I-*Eco*RI, *Bam*HI-*Hin*dIII, *Eco*RI-*Hin*dIII, and *Xba*I-*Hin*dIII (Fig. 2), we located the restriction site positions with respect to each other (Fig. 1). Groundnut genomic DNA restricted with the same enzymes when hybridized to pARG-1 insert revealed a similar profile (data not shown).

The *Eco*RI site located in the 3' end of 18S is highly conserved even in such diverse genera as wheat, rye

GAATTCCTAG TAAGCGCGAG TCATCAGCTC GCGTTGACTA CGTCCCTGCC CTTTGTACAC
ACCGCCCGTC GCTCCTACCG ATTGAATGGT CCGGTGAAGT GTTCGGATCG CGGCGACGTG
GGCGGTTCGC TGCCGGCGAC GTTGTGAGAA GTCCACTGAA CCTTATCATT **TAGAGGAAGG**
AGAAGTCGTA ACAAGGTT**TC CGTAGGTGAA CCTGCGG**AAG GATCATTGTC GATGCCGCAC
AAACCAGGAT TGACGCGCGA ACGAGTCCAC AAACACCCGA GGCGGGGAAGGGCCGGCCGT
GCGCGGCCGG CGCCCCGTCT CAAACAAGAA CAAAACCCCG GCGCGGAAAG CGCCAAGGAA
GCCAAACGTT TCTGCTCTCC CCGCCGGCTT CCGGAGACGG CATCCGGTCG GGCGAGGAGT
GACCACAAGA GTTAAAGAAC GACTCTCGGC AACGGATATC TCGCTCTTGC ATCGATGAAG
AACGTAGCGA AATGCGATAC TTGGTGTGAA TTGCAAGAAT CCCGTGAACC ATCGAGTCTT
TGAACGCAAG TTGCGCCCGA AGCCCTTAGG CTGGAGGGCA CGCCTGCCTG GGTGTCAACC
AAAAGGCGCC CCCCCGTCTC GCCCGTCCCA GGGCACGGGG GAGGGGGCGA ACGTTGGCCT
CCCGGGAGCC CCTGGCTCGC GGTTGGTTCA AAGAGACGGG CTCTTGGTGG GGAGCGGCAC
CGCGGCAGAT GGTGGTCGAG AACAACCCTC GTGGCCAGTC GCGCGCGCCT CTCCCCCGGT
TCAAGGCACG GCGACCCGCG GGCGACGTGG ATCGTCCCGA GCGCGACCTC AGGTCAGGCG
GGGCTACCCG CTGAGTTTAA **GCATATCAAT AAGCGGAGGA** AAAGAAACTA ACGAGGATTC
CCCTAGTAAC GGCGAGCGAA CCGGGAAGAG CCCAGCATGA GAATCGGTCG CCCCTGGCGT
CTGAATTGTA GTCTGGAGAA GCGTCCTCAG TGGCGGACCG GGCCGAAGTC CCCTGGAAGG
TGGCGCCAGA GAGGGTGAGA GCCCCGTTGT GCCCGGACCC TGTCGCACCA CGAGGCGCTG
TTTGCGAGTC GGTTGTTTGG GAATGCAAGC CCTAATCGGG CGGTAAATTC CGTTCCAAGG
CTAAATACTG GCGTGAGACC GATAGCGAAA AGTACCGCGA GGGAAAGAAG AAAGGACTT
TGAAAAGAGA GTCAAAGAGT GCTTGAAATT GTCGGGAGGG AAGCGGATGG GGCCGGCGA
TGCGCCCCGG TCGGATGTGG AACGGCGACG CTGGTCCGCC AATCGACTCG GGGCGTCGAC
CGACGCGGAT TGCAACGGTG GCCCAAGCCC GGGCCGTCGA TAGGCCCGCT GGATACGTCA
TCGTTGCGAT TGTGGAAGGC AGCGCGCGCC CGCTGGCGTG CTTCGGCACC TGCGCGCTCC
GGGCGTCGGC CTGTGGGCTC CCCATTCGGC CCGTCTTGAA ACACGGACCA AGGAGTCTGA
CATGTGTGCG AGTCAACGGG TGAATAAACC CGCGGGGCGC AAGTAAGCTA ATTGGCGGGA
TCC//

Fig. 3 Sequence of pARG-1.2.1 (Genbank accession no. AF156675) containing the 3' end of the 18S ribosomal RNA gene (bases 1–228), ITS1 (bases 229–463), the complete sequence of the 5.8S region (bases 464–597), ITS2 (bases 598–642) and the 25S ribosomal RNA gene, 5' sequence (bases 643–1,563). Sequences in *bold* indicate sites for the one downstream and two upstream primers used

grass, marine angiosperm *Posidonia oceanica* and all legumes. The additional *Eco*RI site in the 25S region has been reported to be present in most legumes (Rafalski et al. 1983; Doyle and Beachy 1985; Jorgensen et al. 1987). The four *Bam*HI sites present, one in the 18S and three in the 25S region, are a feature groundnut shares with other legumes such as pea, yellow lupine, broad bean, *Lens* sp. and *Cicer* sp.; the exception is soybean, which has only three sites. The presence of the single *Hin*dIII site in the rDNA has also been reported for pea, broadbean and *Cicer* sp.

The *Xba*I site at the 5' end of the 18S region has also been observed in pea, soybean, broadbean, *Lens* sp., *Cicer* sp. (Patil et al. 1995) and French bean (Falquet et al. 1997). However, the four *Xba*I sites in the IGS region are perhaps a unique feature of the *Arachis hypogaea* cloned repeat. One *Xba*I site has been reported in the IGS of French bean (Falquet et al. 1997) and the monocot *Posidonia oceanica* (L.), a marine angiosperm (Tucci et al. 1998). The length heterogeneity in the IGS is known to result from duplications of the simple repeated DNA elements that are accumulated there (Hembleben et al. 1988). In mungbean a 450-bp *Eco*RI fragment has been found to be duplicated in the upstream region of the 18S gene, and these were arranged adjacent to each other. The number of these subrepeats varied to give altered IGS lengths (Gerstner et al. 1988). In *Phaseolus coccineus*, the region upstream of 18S contains four islands, each including one *Sau*3AI and two *Hpa*II restriction sites, and this region shows length heterogeneity (Magginni et al. 1992). The five *Xba*I sites present at the corresponding position in the groundnut rDNA may also be due to the presence of duplicated repeat sequences, which could be characteristic of the species.

Three subclones were obtained from pARG-1 (Fig. 1) for further analysis. Subclone pARG-1.1 contained the 1.4-kb *Hin*dIII-*Eco*RI fragment containing most of the 18S coding region. The 4.1-kb *Eco*RI-*Eco*RI fragment was cloned to give pARG-1.2, which contains the 3' region of 18S, the 5.8S coding region, the two ITS sequences and most of the 25S coding region. Subclone pARG-1.3 was obtained by cloning the 6.4-kb *Eco*RI-*Hin*dIII fragment containing the 3' region of the 25S gene and most of the IGS.

The *Eco*RI-*Bam*HI fragment of pARG-1.2 was further subcloned to give pARG-1.2.1. This fragment was completely sequenced to read the 1,563 bases (Fig. 3). The 513-base sequence upstream of the *Bam*HI site coding for the 5' region of the 25S RNA gene was compared with corresponding sequences available in GenBank. Of the corresponding 25S RNA gene sequences available only four sequences were from plants belonging to the order Fabales. The analysis revealed that when the common sequence length available (244 bp) was compared, the *A. hypogaea* 25S RNA gene matched best with that of the legumes, soybean and broadbean, both giving 93% (227/244) identity. In soybean three gaps were inserted, while in broadbean four gaps had to be inserted to obtain the alignment. Pea showed 91.4% identity with three gaps, French bean gave 88.9% identity with three gaps, while *Arabidopsis thaliana* also gave a significant match with 92.2% identity.

A similar comparison with 171 bases of the sequence from the 3' end of the 18S RNA gene gave the best matches with both broadbean and pea, which showed 98.2% identity with two gaps. Other significant matches were with soybean (97.6% identity and three gaps), mungbean (96.5% identity and two gaps) and *A. thaliana* (95.9% identity and only one gap).

Cultivated groundnut is an allotetraploid, while nearly all *Arachis* species are diploid. Studies based on DNA sequence analysis have indicated that the two diploid progenitors could be *A. duranensis* (A genome, female) and *A. ipaensis* (B genome). Since only one genomic *Hin*dIII band was detected by the rDNA probe, the implication is that both contributing diploids have similar-sized repeat units. To confirm this we hybridised pARG-1 to the *Hin*dIII-digested genomic DNA from *A. ipaensis* and *A. duranensis* and found that a single band of approximately 12 kb hybridised (data not shown). Detailed RFLP analysis using pARG-1 as the probe has been initiated to establish the phylogenetic relationships among related species of *Arachis.*

In conclusion, the cloned groundnut rDNA repeat has four *Xba*I sites in the IGS region, a characteristic feature only observed in this species so far. The 25S and 18S rRNA gene partial sequences show the best homology

with broadbean and soybean among the leguminous plants. Analysis of the Indian groundnut germplasm for investigating the variability in the IGS region is underway in our laboratory.

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