An Effective Method for Cloning a Partial MADS-box Gene in Groundnut

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The key genes in the developmental control of eukaryotes are often members of a very limited number of multigene families which encode transcription factors. Such homeobox genes have been thought to act as homeotic selector genes that are involved in differentiating different body regions from each other, probably by activating or repressing different sets of downstream genes in different parts of the body (Theissen et al. 2000). Recent studies have provided insight that inflorescence and flower development in higher eudicotyledonous flowering plants are determined by a network of regulatory genes which are organized in a hierarchical fashion (Theissen and Saedler 1998). In this, the late- and early-flowering genes are triggered by environmental factors such as day length, light quality and temperature.

The MADS-box is a highly conserved sequence motif found in the family of transcription factors. The conserved domain was recognized after the first four members of the family were identified as MCM1, AGAMOUS, DEFICIENS and SRF (serum response factor). The name MADS was constructed from the "initials" of these four "founder" proteins on which the definition of this gene family is based (Schwarz-Sommer et al. 1990). The MADS-box genes of plants are scattered throughout the entire plant genomes (Theissen et al. 2000) and by now, over a hundred MADS-box sequences have been found in species from all eukaryotics. MADS-domain proteins, like many other eukaryotic transcription factors, have a modular structural organization (Shore and Sharrocks 1995). The family of MADS-domain proteins has been subdivided into several distinct subfamilies. Most MADS-domain factors play important roles in plant developmental processes. Prominently, the MADS-box genes in flowering plants are the "molecular architects" of flower morphogenesis (Coen and Meyerowitz 1991, Angennet et al. 1995).

The MADS domain is by far the most highly conserved region of proteins (Purugganan et al. 1995). Based on the most conserved sequence region of the MADS box, a pair of degenerate primers were designed and used to amplify the genomic DNA of groundnut (*Arachis hypogaea*) in this study. The results indicate that an amplified fragment showed a high homology to the MADS-box protein of *Arabidopsis thaliana*. This study laid the foundation for obtaining the full length of MADS-box gene in groundnut.

Materials and methods

Seeds of groundnut cultivar JL 24 were planted in pots and maintained in the greenhouse. Genomic DNA was isolated from the young leaves by using the method described by Porebski et al. (1997) and Puchooa (2004).

Based on conserved amino acids found in the MADS domain of plant MADS-box genes, degenerate primers were designed to amplify the MADS-box gene homologues. The two forward primers used were MADSF1, 5'-ATGGG(ATCG)(AC)G(ATCG)GG(ATCG)AA(AG)AT (ACG)GA-3' and MADSF2, 5'-(ATCG)TG(CT)GA(CT)GC(ATCG)GA(AG)GA(AG)GT(ATCG)GC-3' and the two reverse primers were MADSR1, 5'-GC(ATCG)AC(CT)TC(ATCG)GC(AG)TC(CT)AA-3' and MADSR2, 5'-GC(ATCG)AC(CT)TC(ATCG)GC(AG)TC(CT)CA(ATCG) AG-3'. A 50-µl PCR (polymerase chain reaction) contained 50 ng genomic DNA, 25 pmol of each degenerate primer, 0.1 mM of each dNTP, and 1X Reaction Buffer (including 2.0 mM MgSO4). Touchdown PCR was used to amplify the expected fragment. The reaction was heated at 94°C for three min, then 1.5 unit of *Taq* DNA polymerase (New England BioLabs) was added to each reaction tube. Ten cycles of

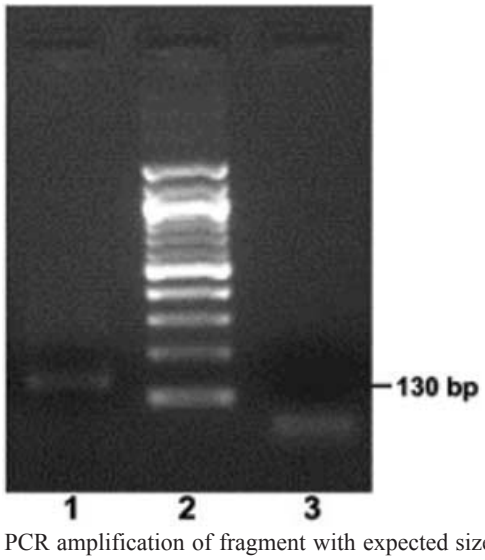

Figure 1. PCR amplification of fragment with expected size – Lane 1: JL 24; Lane 2: 100 bp DNA Ladder; Lane 3: Control (sterile distilled water).

amplification were carried out, with denaturation at 94°C for 50 sec, annealing at 61°C to 50°C for 50 sec and extension at 72°C for 60 sec. The annealing temperature of the reaction was decreased 2°C every second cycle from 61°C to a "touchdown" at 50°C. Subsequently, all the reaction tubes underwent thirty cycles with the annealing temperature at 50°C, followed by a final extension at 72°C for 10 min. PCR products of the expected size were excised from agarose gels and cloned into pGEM-T easy vector (Promega). The clones were confirmed by PCR and restriction enzyme digestion prior to sequencing. The sequences were aligned with nucleotide sequences in GeneBank by using MegaBlast search program (http://www.ncbi.nlm.nih.gov).

Results and discussion

A 130 bp of the expected fragment named ApMADS1 was obtained by PCR amplification by using the primers MADSF1 and MADSR2 (Fig. 1). ApMADS1 was recovered from agarose gel and cloned into pGEM-T easy vector. Positive clones were confirmed by colony PCR and *Eco*RI digestion (Fig. 2) and sequenced. Figure 3 shows the nucleotide sequences and deduced amino-acid sequences. When alignment was carried out using the GeneBank nucleotide database and Megablast search program, ApMADS1 was 79% homologous to MADS-box transcription factor HAM137 from sunflower (*Helianthus annuus*). When discontiguous MegaBlast and Refseq_RNA were chosen, ApMADS1 showed identity ranging from 79% to 84% with 26 MADS-box mRNA. It can be concluded that ApMADS1 is a kind of MADS-box gene in groundnut.

Homology cloning method is effective and efficient, and used widely for gene cloning as confirmed by the result in this study. Compared with common homology cloning method, genomic DNA was used as the template for PCR amplification in this study. Therefore, this method is more simple and cheaper. Based on the sequence of ApMADS1, specific primers were designed and used for the amplification of 5' and 3' RACE. Some expected fragments from 5' and 3' RACE were obtained. Further work on the characterization of putative genes is currently ongoing. Our results indicated that the homology cloning method based on genomic DNA is feasible for MADS-box gene cloning in groundnut. The

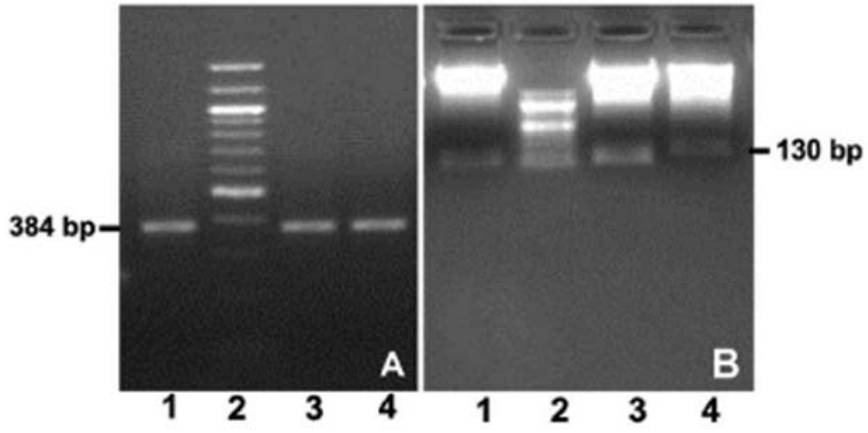

Figure 2. Clones confirmation by PCR amplification (A) using forward and reverse primer of M13 and *Eco*RI digestion (B) – Lanes 1, 3, 4: Clone containing expected fragment; Lane 2: 100 bp DNA Ladder.

ATGGGGCGTGGGAAGATAGAGATCAAGAGGATTGAGAACACTACAAATCGCCAAGTAACC
M G R G K I E I K R I E N T T N R Q V T
TTTTGCAAGCGCGGAAATGGTCTTCTCAAGAAAGCTTATGAGTTATCTGTGCTGTGTGACG
F C K R G N G L L K K A Y E L S V L C D
CCGAGGTGG
A E V

Figure 3. Nucleotide sequences and deduced amino acid sequences (shaded regions indicate the position of forward and reverse primers).

availability of such genes can be used to study the control of floral patterning thus providing an ideal genetic tool kit to study the diversification of flower architecture and its possible alteration through genetic engineering (Pavan Prakash and Kumar 2002).

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