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Seed-specific, developmentally regulated genes of peanut

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Abstract Four cDNAs of seed-specific and developmentally regulated peanut (*Arachis hypogaea* L.) genes were identified by differential screening of a peanut-seed cDNA library using cDNA probes constructed from mRNAs isolated from immature and mature stages of the seed. Northern analysis, probed with the four cloned cDNAs, indicated that the genes represented by two cDNAs were expressed abundantly early in seed development, while another two were abundantly expressed later at the cell-expansion stages of seed development. These four genes did not show expression in roots, pegs or leaves. However, one of the early expressed genes was seed coat-specific. One of the clones, Psc11, had significant sequence similarity to subtilisin-like genes in *Arabidopsis* and soybean. Clones Psc32 and Psc33 had significant similarity to the peanut allergen genes Ara h II and Ara h 6, respectively. The sequence of clone Psc12 was unique and did not show significant similarity to any sequence in the databases. One of the four seed-specific clones showed restriction fragment length polymorphism (RFLP) among peanut lines representing the four peanut botanical varieties. These findings indicate that polymorphism exists in peanut seed-storage genes. This contrasts with other genes previously used for genetic mapping of cultivated peanut.

Keywords *Arachis hypogaea* · Gene expression
Seed-specific · Developmental regulation

Introduction

Peanuts (*Arachis hypogaea* L.) show an unusual pattern of fruit development. After fertilization, a new organ called the peg differentiates from the ovary (Smith 1950; Brennan 1969). Little mitotic division occurs in the embryo or endosperm during the active geotropic peg growth until the peg penetrates the soil (Smith 1956). Then, a few days later, rapid embryo cell division begins. This occurs about 10 to 12 days following fertilization (Smith 1956). Seed developmental changes in water content, nucleic acid level, enzyme activities and storage-protein deposition patterns were studied from the earliest stage when kernels can be removed (Pickett 1950; Cherry and Ory 1972; Aldana et al. 1972; Pattee et al. 1974; Basha et al. 1976; Yamada et al. 1980). We know from other studies of genes expressed during seed development that regulation can occur at several levels of control. Many tissue-specific and developmentally expressed genes are regulated at transcription initiation (Falvey and Schibler 1991). Transcription of these genes is initiated primarily by interactions between RNA polymerase II, and in general by specific transacting factors binding to discrete cis-elements (Maniatis et al. 1987).

Embryo-specific seed-storage protein genes, whose mRNA and protein accumulation are correlated with the cell-expansion stage of the embryo (Higgins 1984), have been studied extensively. For example, the cis-element responsible for regulation of the soybean 7S storage protein gene was found in the 5′ promoter region (Chen et al. 1986). Also, DNA binding activity of a soybean lectin gene transacting factor was shown to be developmentally regulated (Jofuku et al. 1987). Evidence suggests that abscisic acid is involved with seed dormancy, expression of seed-storage protein genes and late-embryogenesis abundant genes (Bray and Beachy 1985; Finkelstein et al. 1985; Galau et al. 1986). However, signal transduction mechanisms triggering seed development have yet to be well understood.

Since the seed is the most economically important part of the peanut plant, and concern for peanut being one of the most allergenic foods, understanding seed development at the molecular level is an important research objective. In this paper, we report the identification and characterization of cDNAs of seed-specific and developmentally regulated peanut genes. The clones were iso-

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lated by differential screening of a peanut-seed cDNA library using mRNAs expressed from very immature and mature stages of the seed.

Materials and methods

Plant materials

Field-grown peanuts of the high oleic acid peanut line, FL435, were harvested at 50 and 80 days after flowering. Because seed maturity depends upon the position of the peg on the plant and the position of the kernel within the pod (Smith 1956), morphological characteristics of pericarp (pod), seed coat, and seed were used to classify the kernels into four arbitrary seed-maturity stages (Table 1). Kernels were immediately frozen in liquid N_2 after being removed from the shell, and stored at –70°C until needed for RNA isolation. Peanut kernels, including the pericarp and embryo, were left intact and are referred to as 'seed' in this publication. Immature leaves, developing pegs and young roots were also harvested from line FL435, frozen, and stored (as described above) for RNA isolation. Immature leaves of peanut lines F1035, 44–314 and 89–509, and FL435, representing runner, virginia, valencia and spanish botanical varieties, respectively, were frozen and stored for DNA isolation.

Nucleic acid isolation

DNA was isolated from peanut leaf by first using a nuclei separation step, as described previously (Paik-Ro et al. 1992). RNA isolation from peanut tissues was based on a phenol/SDS method (Ausubel et al. 1990). Seven grams of tissue were ground in liquid nitrogen with a mortar and pestle. The powder was immediately mixed with 75 ml of extraction buffer (0.2 M Tris, 0.1 M LiCl, 5 mM EDTA, 1% SDS pH 8.2), transferred to a blender containing 25 ml of equilibrated phenol (pH 8.2), and mixed at top speed for 2 min. Chloroform (25 ml) was added and blended for an additional 30 s at low speed. The mixture was transferred to a 250-ml polypropylene bottle and centrifuged for 20 min at 10,000 rpm. The supernatant was transferred to a new bottle and extracted twice with phenol and chloroform, and one last time with chloroform. RNA was precipitated with 2 M LiCl, dissolved in sterile water, and stored in 2 vol of absolute ethanol at –20°C. The quantity of RNA was estimated spectrophotometrically at OD_{260} nm. RNA quality was examined on northern gels by the appearance of distinct rRNA bands.

Poly A^+ RNA was isolated using an oligo-dT column (Collaborative Res. Inc.) prepared by washing with sterile water and loading buffer (0.5 M LiCl, 10 mM Tris.Cl, 1 mM EDTA, 0.1% SDS). The poly A^+ RNA was isolated from 2 mg of total RNA in 0.5 M LiCl which was passed through the column with loading buffer. Ribosomal RNA was washed out of the column with middle wash buffer (0.15 M LiCl, 10 mM Tris.Cl, 1 mM EDTA, 0.1% SDS), and the poly A^+ RNA was eluted with elution buffer (2 mM EDTA, 0.1% SDS) (Ausubel et al. 1990). The quantity of mRNA was measured spectrophotometrically at OD_{260} nm.

Northern and Southern analyses

Northern blots were made by denaturing and electrophoresing 10 µg of total RNA per lane along with an RNA-marker ladder (Bethesda Research Laboratories) for 4 h at 80 V on a 1.2% formaldehyde denaturing gel (2.2 M formaldehyde) with 1×MOP buffer (Ausubel et al. 1990). Following electrophoresis, RNA was transferred by capillary blotting to a Hybond-N membrane (Amersham Corporation, Arlington Heights, Ill.) using 20×SSC (1×SSC=0.15 M NaCl, 0.015 M sodium citrate, pH 7.5).

Southern blots were made by electrophoresing *Eco*RI, *Eco*RV, *Hin*dIII and *Xba*I endonuclease-digested total DNA and *Hin*dIII-digested lambda/ *Hae*III-digested Phi-X174 molecular markers (Bethesda Research Laboratories) at 25 V for 15 h in a 0.8% agarose gel/Tris-phosphate buffer system. The DNA gel was treated with 0.25 M HCl for 10 min, denatured with 1.5 M NaCl/0.5 M NaOH, neutralized with 1.5 M NaCl/0.5 M Tris.Cl/0.001 M EDTA (pH 7.2), and transferred by capillary blotting overnight (Southern 1975) to a Hybond-N membrane as for northern blotting.

Nucleic acids were fixed onto the membranes by U.V. crosslinking on a transilluminator for 2 min at 300 nm. Probe DNA was labeled by the random-priming extension method (Feinberg and Vogelstein 1983). Blots were prehybridized for 4 h in 30 ml of 7% sodium dodecyl sulfate (SDS) buffer (7% SDS, 0.5 M $NaHPO_4$, 1 mM EDTA, 1% BSA) at 65°C with denatured salmon sperm DNA added. Hybridization was done overnight in 10 ml of 7% SDS buffer at 65°C with denatured labeled probe and salmon sperm DNA added (Ausubel et al. 1990). Washing was done for 10 min each at 65°C with 2×SSC, then 1×SSC, and finally with 0.5×SSC (all washes included 0.1% SDS). Hybridized blots were autoradiographed on x-ray film (Kodak XAR-5) for three to seven days at –70°C.

Table 1 Characteristics of pericarp (pod), kernel and seed coat used to determine the maturity stage in this report. The Seed index, pericarp and kernel characteristics are from Pattee et al. (1974)

Maturity stage	Days after pollination	Seed index	Pericarp characteristics (pod)	Kernel size	Kernel characteristics
1	30	4	Very watery, soft and spongy, nearly full size	<150 mg	Completely white, mostly seed coat, seed very small
2	40	5	Soft, not as watery, endo-carp fleshy, no cracks		Flat, white starting to turn pink at embryonic axis end
	45	6	Endocarp beginning to show cracks	300 mg	Torpedo shaped, embryonic axis-end pink
3	55	7	Endocarp beginning cottony appearance		Torpedo to round, non-embryonic axis-end to pink
	65	8	Endocarp beginning to dry out, cracks more numerous	600 mg	Round pink all over
	75	9	Endocarp beginning to show brown blotches		Dark pink at embryonic axis end, light pink elsewhere
4	85	10	Endocarp has many dark blotches	600 mg	Large, dark pink all over, seed coat drying out
	95	11	Endocarp almost completely brown	600 mg	Dark pink, seed coat drying out

Transcript level was quantified either by measuring the radioactivity on the hybridized blots with the PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.), or by 2-D densitometer readings of autoradiographs. Measurements derived from the selection of different backgrounds were averaged.

Differential screening of peanut cDNA library

An *A. hypogaea* seed cDNA library constructed in lambda gt11 using RNA from the spanish-type high oleic acid peanut line, FL435, (courtesy by Dr. A. G. Abbott, Clemson University) was differentially screened. About 1,000 plaque-forming units (pfu) of lambda clones were plated with Y1090 cells on 8.5 cm×8.5 cm lambda agar plates (Ausubel et al. 1990). Plates were incubated for 5 h at 37°C until the plaques just began to appear and then were stored at 4°C overnight.

Plaques from each plate were blotted onto four Hybond-N membranes for duplicate hybridizations with two cDNA probes. Membranes were further denatured and neutralized on 3 MM paper with plaque side up, and U.V. cross-linked for 2 min at 300 nm. Complimentary DNA probes were made and labeled from mRNA isolated from stage 1 and stage 3 seed. Each mRNA (2 µg) was reacted with 1 µg of oligo-dT primer (BRL), 1 mM dATP, 1 mM dGTP, 1 mM dTTP, 6 µM dCTP, 100 µCi $dCTP^{\alpha 32}$, and 200 U reverse transcriptase (BRL) for 1 h at 37°C to a specific activity of 10^8–10^9 cpm/µg. RNA templates were hydrolyzed and neutralized as described by Sambrook et al. (1989).

Blots were prehybridized for 4 h in 25 ml 7% SDS solution at 65°C with denatured salmon sperm DNA added. Duplicate blots from each plate were hybridized with stage 1- and stage 3- specific probes, respectively. Hybridization was done overnight in 10 ml of 7% SDS solution at 65°C. Blots were washed under low (0.5% BSA, 1 mM Na_2EDTA, 40 mM $NaHPO_4$, 5% SDS) and high (1 mM Na_2EDTA, 40 mM $NaHPO_4$, 1% SDS) stringency conditions (Ausubel et al. 1990). Washed blots were autoradiographed at –70°C on χ-ray film for 1 to 24 h. Hybridization patterns between the two sources of probes were compared on autoradiographs. Differentially hybridized plaques were located and isolated, re-plated, and differentially hybridized with both probes twice more. Final plaques were resuspended in SM buffer and phage plate lysates were prepared in 5 ml of SM (Ausubel et al. 1990). Plate lysate (1 µl) was used for PCR amplification of the peanut inserts by the polymerase chain reaction (Herman et al. 1990).

Subcloning

Peanut inserts and plasmid vectors, pT7/T3 alpha-19 (Bethesda Research Laboratories) or pGEM 11Zf(–) (Promega), were digested with *Eco*RI or *Not*I and the vectors dephosphorylated with 0.1 U of calf intestine phosphatase (Promega) for 30 min. Inserts and vector DNAs were phenol/chloroform extracted, ethanol-precipitated, and dissolved in TE to a final concentration of 100 ng/µl. A 1:3 molar ratio, peanut insert to vector (100 ng), were used in the ligation reaction.

DH5-alpha competent cells (BRL) were transformed with a ligation product (1 to 10 ng DNA) and subjected to blue and white screening on ampicillin and X-gal plates. Plasmids were isolated from white colonies following the modified miniprep alkaline-extraction procedure (Birnboim and Doly 1979).

Sequencing and analyses

Subcloned recombinant plasmids were sequenced by the University of Florida's Interdisciplinary Center for Biotechnology Research sequencing core laboratory by the florescent dideoxynucleotide method using a thermocycling reaction and an ABI 373a DNA sequencer (Foster City, Calif.). Partial or complete sequencing of the cDNA clones was done from each end using T3, T7 or SP6 vector primers. Sequence searching was performed at the National Center for Biotechnology Information using the BLAST network service.

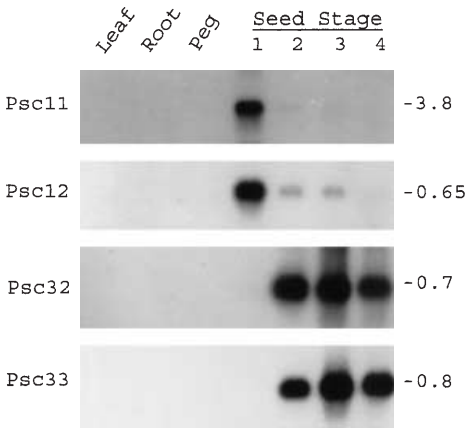

Fig. 1 Northern analyses of the transcripts of the four seed-specific, developmentally regulated clones. The membrane contained total RNA from leaf, root, peg and four seed-maturity stages. Sizes of the transcripts are listed on the right in kb

Results and discussion

Differential screening of 2,000 plaques from the peanut seed lambda cDNA library produced five clones representing seed-specific and developmentally regulated genes. Those cDNA clones were isolated based on the distinct hybridization pattern differences between the very immature seed (stage 1) mRNA-derived cDNA probe and the more mature seed (stage 3) mRNA-derived probe. Four of those clones are reported here. Clones designated Psc11 (GenBank AF366558) and Psc12 (GenBank AF366559) displayed hybridization signals only with the stage 1 mRNA-derived cDNA probe. Clones Psc32 (GenBank AF366560) and Psc33 (GenBank AF366561) showed hybridization signals only with the stage 3 mRNA-derived cDNA probe.

To determine organ specificity and verify developmental regulation, Northern analyses with the four cDNA clones as probes were conducted on total RNA extracted from leaves, roots, pegs, and seeds of maturity stages 1, 2, 3 and 4 (Fig. 1). Northern analyses showed that none of the four peanut clones hybridized to transcripts from leaves, roots or pegs. In seed, Psc11 and Psc12 showed abundant hybridization at stage 1 to transcripts of 3.8- and 0.65-kb, respectively, with decreased signals at stages 2 and 3 and a further reduction at stage 4 (Fig. 1). Densitometer measurements indicated that stage 2 transcript levels of Psc11 and Psc12 were reduced to 7% and 8% of the stage 1 level, respectively, were not changed from stage 2 to 3, but then were decreased further at stage 4 to 2% and to 1% of the stage 1 level. Psc11 also hybridized to a smaller seed-specific, non-developmentally regulated transcript (not shown in Fig. 1.).

Northern analyses of Psc32 and Psc33 indicated that their transcript sizes were 0.7- and 0.8-kb, respectively. Their transcript levels increased significantly from a very low level at stage 1 to a high level at stage 2; then the levels increased slightly at stage 3 and started de-

creasing at stage 4. (Fig. 1). Densitometer measurements showed that the transcript levels increased 83-fold in Psc32 and 75-fold in Psc33 from stage 1 to stage 2. From maturity stage 2 to stage 3, the levels increased 10% and 20%, respectively in Psc32 and Psc33, then decreased to 70% of stage 3 at stage 4 for both transcripts. The cell-division stage of the embryo occurs early and apparently shifts to the cell-expansion stage between stage 1 and stage 2; hence the dramatic change in gene expression between those two stages.

Sequence and transcript size differences of those clones indicate that they represent different genes. Sequence searches using BLAST revealed that Psc12 is unique with no sequences in GenBank and other databases having a significant similarity to it. Psc11 was found to have a nucleotide similarity of about 63% to the *Arabidopsis thaliana* subtilisin-like serine protease (EC 3.4.21.14) gene, AIR3 (Neuteboom et al. 1999), and about 76% similarity to a 94 nucleotide portion of the soybean (*Glycine max*) "expressed sequence tag" (EST) clone Gm-c1004 (GenBank Accession AI441432), also believed to represent a subtilisin-like protease (SLP). Those sequence similarities suggest that peanut Psc11 is also a SLP. Proteolysis by SLPs is believed to play a key role in protein processing and to be associated with a variety of important functions such as development, stress response, defense mechanisms and adaptation to environmental change. Molecular mechanisms regulating the specific aspects of SLPs in protein-processing are poorly understood. Although there are a large group of SLPs that include over 200 members, only a few SLPs have been cloned and characterized in plants and associated with a variety of functions. The AIR3 SLP gene, to which Psc11 has sequence homology, was found to be associated with lateral root initiation following auxin treatment (Neuteboom et al. 1999), so it does not appear to be functionally related to the peanut Psc11 which is expressed only in the very immature peanut seed. Another *Arabidopsis* SLP cDNA clone (*ara12*), presumed to have more function similarity because of high expression in the immature siliques (Ribeiro et al. 1995), does not have significant sequence similarity to Psc11. Other isolated and characterized plant SLPs further demonstrate the diversity of function. An *Alnus glutinosa* actinorhizal root-nodule SLP cDNA (*ag12*) was found to be expressed specifically in the nodule, especially in the early initiation stage (Ribeiro et al. 1995). At least two SLPs of the P69 family (P69B and P69C) isolated from tomato were found to be induced by viroid infection (Tornero et al. 1997) and are believed to be involved in the defense system.

Both Psc32 and Psc33 have significant similarities to the major peanut seed-protein genes *Ara h II* (Burks et al. 1992; Stanley et al. 1997) and *Ara h 6* (Kleber-Janke et al. 1999). The Psc32 translated polypeptide has about 98% similarity to that of *Ara h II* and about 71% similarity to that of the *Ara h 6* gene. The Psc33 translated peptide has about 75% similarity to that of *Ara h II* and about 94% similarity to that of *Ara h 6*.

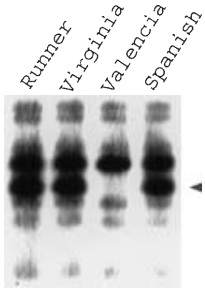

Fig. 2 Southern analyses of Psc 11 on DNA from runner, virginia, valencia and spanish peanut leaves. The *arrow head* indicates the polymorphic DNA fragment

The *Ara h II* and *Ara h 6* genes are related to each other with a 77% nucleotide similarity, both are major peanut seed protein allergens and both are classified as conglutins. These relationships suggest that *Ara h II*, *Ara h 6* and Psc32 and Psc33 represent genes belonging to the same family of peanut allergens, and that Psc32 and Psc33 is also conglutins. Psc32 and Psc33 were isolated from the same peanut line indicating that both versions of this allergenic conglutin are present in the peanut seed.

Peanuts have been identified as one of our most-allergenic foods with hypersensivity reactions occurring in about 1% of children. This food allergy is a lifelong health problem and represents the most common cause of fatal and near fatal food-related allergic reactions. The problem is complicated by the fact that peanuts often are a hidden additive in various foods (Dean 1989) and even some peanut oils are allergenic (Hoffman et al. 1994) which makes avoiding contact difficult for sensitized people. Peanuts are complex, containing over 30 proteins, of which half are estimated to bind serum IgE of sensitized individuals (Dean 1989). In addition to Ara h II and Ara h 6, five other peanut allergens have been identified, their cDNAs isolated and characterized (*Ara h I*, Burks et al. 1991; *Ara h 3*, Burks et al. 1998; *Ara 4* through *7*, Kleber-Janke et al. 1999). Because of the extent and seriousness of these hypersensitivity reactions, these allergens and their associated genes are being intensely studied to identify specific epitopes, develop methods of detecting them in foods and develop strategies to reduce peanut hypersensitivity (Roy et al. 1999). Research to reduce peanut allergenicity by engineering peanut proteins is also being initiated.

Southern analyses using the four cDNA clones as probes on *Eco*RI-digested genomic DNA of peanut lines F1035, 44–314, 89–509 and F435 representing the four botanical varieties of cultivated peanut, runner, virginia, valencia and spanish, respectively, confirmed that the cloned cDNAs represent different genes. The Psc11 used as a probe detected restriction fragment length polymorphism between the valencia and other botanical varieties with the 4.51-kb fragment found in other peanut varieties being missing in the valencia type (Fig. 2).

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References

Aldana AB, Fites RC, Pattee HE (1972) Changes in nucleic acids, protein and ribonuclease activity during maturation of peanut seeds. Plant Cell Physiol 13:515–521

Ausubel AM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (1990) Current protocols in molecular biology. Greene Publishing Associates and Wiley-Interscience, New York

Basha SMM, Cherry JP (1976) Composition, solubility, and gel electrophoretic properties of protein isolated from Florunner (*Arachis hypogaea* L.) peanut seeds. J Agric Food Chem 24: 359–365

Birnboim HC, Doly J (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res 7:1513–1523

Bray EA, Beachy RN (1985) Regulation by ABA of beta-conglycine expression in cultured developing soybean cotyledons. Plant Physiol 70:746–750

Brennan JR (1969) The peanut gynophore. The Biologist 51:71 –82

Burks AW, Williams LW, Helm RM, Connaughton C, Cochrell G, O'Brian T (1991) Identification of a major peanut allergen, Ara h I, in patients with atopic dermatitis and positive peanut challenges. J Allergy Clin Immunol 88:172–179

Burks AW, Williams LW, Connaughton C, Cockrell G, O'Brian T, Helm RM. (1992) Identification and characterization of a second major peanut allergen, Ara h II, with use of a sera of patients with atopic dermatitis and positive peanut challenge. J Allergy Clin Immunol 90:962–969

Burks AW, Sampson HA, Bannon GA (1998) Peanut allergens. Allergy 53:725–730

Chen ZL, Schuler MA, Beachy RN (1986) Functional analysis of regulatory elements in a plant embryo-specific gene. Proc Natl Acad Sci USA 83:8560–8564

Cherry JP, Ory RL (1972) Electrophoretic characterization of six selected enzymes of peanut cultivars. Phytochemistry 12:283–289

Dean TP (1989) Immunological responses in peanut allergy. Clin Exp Allergy 28:7–9

Falvey E, Schibler U (1991) How are the regulators regulated? FASEB 5:309–314

Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132:6–13

Finkelstein RR, Tenbarge KM, Shumway JE, Crouchy ML (1985) Role of ABA in maturation of rapeseed embryos. Plant Physiol 78:630–636

Galau GA, Hughes DW, Dure III L (1986) Abscisic acid induction of cloned cotton late embryogenesis-abundant (Lea) mRNAs. Plant Mol Biol 7:155–170

Herman J, Lee P, Saya H, Nakajima M (1990) Application of polymerase chain reaction for rapid subcloning of cDNA inserts from lambda gt11 clones. Biotechniques 8:376–379

Hoffman DR, Collins-Williams C (1994) Cold pressed peanut oils may contain peanut allergen. J Allergy Clin Immunol 93:801

Higgins TJV (1984) Synthesis and regulation of major proteins in seeds. Annu Rev Plant Physiol 35:191–221

Jofuku KD, Okamuro JK, Goldberg RB (1987) Interaction of an embryo DNA-binding protein with a soybean lectin gene upstream region. Nature 328:734–737

Kleber-Janke T, Crameri R, Appenseller U, Schlaak M, Wolf-Meinhard B (1999) Selective cloning of peanut allergens, including profilin and 2S albumins, by phage display technology. Int Arch Allergy Immunol 119:265–274

Maniatis T, Goodbourn S, Fischer JA (1987) Regulation of inducible and tissue-specific gene expression. Science 236:1237–1244

Neuteboom LW, Ng JMY, Kyper M, Clijdesdale OR, Hooykaas PJJ (1999) Isolation and characterization of cDNA clones corresponding to mRNAs that accumulate during auxin-induced lateral root formation. Plant Mol Biol 39:273–278

Paik-Ro OG, Smith RL, Knauft DA (1992) Restriction fragment length polymorphism evaluation of six *Arachis* section species. Theor Appl Genet 84:201–208

Pattee HE, Elizabeth BJ, Singleton JA, Sanders TH (1974) Composition changes of peanut fruit parts during maturation. Peanut Sci 1:57–62

Pickett TA (1950) Composition of developing peanut seed. Plant Physiol 25:210–223

Ribeiro A, Akkermans AD, Van Kammen AB, Bisseling T, Pawlowski K (1995) A nodule-specific gene encoding a subtilisin-like protease is expressed in early stages of actinorhizal nodule development. Plant Cell 7:785–794

Roy K, Mao H, Huang S, Leing KW (1999) Oral gene delivery with chitosan-DNA nanoparticles generates immunologic protection in a murine model of peanut allergy. Nature Med 5:387–391

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Smith BW (1950) *Arachis hypogaea*. Aerial flower and subterranean fruit. Am J Bot 37:802–815

Smith BW (1956) *Arachis hypogaea*. Embryogeny and the effect of peg elongation upon embryo and endosperm growth. Am J Bot 43:233–240

Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98: 503–517

Stanley SJ, King N, Burks AW, Huang SK, Sampson H, Cockrell G, Helm RM, West CM, Bannon GA (1997) Identification and mutational analysis of the immunodominant IgE binding epitopes of the major peanut allergen *Ara h 2*. Arch Biochem Biophys 342:244–253

Tornero P, Conejero V, Vera P (1997) Identification of a new pathogen-induced member of the subtilisin-like processing protease family from plants. J Biol Chem 272:14412–14419

Yamada T, Aibara S, Morita Y (1980) Accumulation pattern of arachin and its subunits in maturation of groundnut seeds. Plant Cell Physiol 21:1217–1226