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-
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 N₂, 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 (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.

**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 1xMOP 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 EcoRI, EcoRV, HincII and XbaI endonuclease-digested total DNA and HindIII-digested lambda/ HaeIII-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. cross-linking 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 NaHPO₄, 1 mM EDTA, 0.1% 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)

<table>
<thead>
<tr>
<th>Maturity stage</th>
<th>Days after pollination</th>
<th>Seed index</th>
<th>Pericarp characteristics (pod)</th>
<th>Kernel size</th>
<th>Kernel characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30</td>
<td>4</td>
<td>Very watery, soft and spongy, nearly full size</td>
<td>&lt;150 mg</td>
<td>Completely white, mostly seed coat, seed very small</td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>5</td>
<td>Soft, not as watery, endo-carp fleshy, no cracks</td>
<td>300 mg</td>
<td>Flat, white starting to turn pink at embryonic axis end</td>
</tr>
<tr>
<td>3</td>
<td>55</td>
<td>7</td>
<td>Endocarp beginning to show cracks</td>
<td>600 mg</td>
<td>Torpedo shaped, embryonic axis-end pink</td>
</tr>
<tr>
<td>4</td>
<td>85</td>
<td>10</td>
<td>Endocarp has many dark blotches</td>
<td>600 mg</td>
<td>Torpedo to round, non-embryonic axis-end to pink</td>
</tr>
<tr>
<td>95</td>
<td>11</td>
<td>Endocarp almost completely brown</td>
<td>600 mg</td>
<td>Round pink all over</td>
<td></td>
</tr>
</tbody>
</table>

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 OD₂₆₀ nm.