RESEARCH ARTICLE

Epidermis-Specific Gene Expression in Pachyphytum

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Transcripts of exclusively epidermis-specific expression were obtained by differential screening of a cDNA library prepared from isolated epidermis tissue of a succulent plant, Pachyphytum. Six transcripts were selected and characterized by RNA gel blot hybridization. Five cDNAs represented abundant mRNAs found exclusively in the epidermis. In situ hybridizations with three of these transcripts further substantiated their epidermal location. While two transcripts were present in all cells of the epidermis, one transcript was enriched in subsidiary cells of the stomatal complexes. All of the transcripts were highly expressed in the protoderm of the shoot apical meristem. DNA sequence determination indicated that the mRNAs identified represent novel sequences, encoding yet unknown plant functions. These transcripts, their control elements, and their encoded functions should help to advance our understanding of epidermal cell determination and differentiation.

INTRODUCTION

The epidermis of aerial plant organs performs a number of functions essential for the stability of turgescent tissue, the most important of which is to control water loss. In addition, the epidermis provides mechanical and chemical defenses against pathogens. The ability to perform these functions is accomplished by a number of specialized biochemical pathways. Synthesis of the cuticle, stoma development, and stomatal function, as well as the biogenesis of specialized cell wall components and the synthesis and storage, or external deposition, of pathogen-repelling chemicals are the phenotypic results of this complex biochemistry. The epidermis must therefore be characterized by the expression of specific genes, an aspect of plant molecular biology that has not been investigated in much detail until now. Most of the information that is accumulating concerns organ- or tissue-specific gene expression (Edwards and Coruzzi, 1990) with only few examples of cell specificity of gene expression, such as the homeotic gene AGAMOUS, which is expressed in certain cells during Arabidopsis flower development (Bowman et al., 1991), and tapetal-specific genes that are active during anther development in tobacco (Mariani et al., 1990; Koltunow et al., 1991).

Several groups have identified genes that are expressed in epidermal cells. However, these genes are typically found in more than one cell type; some are environmentally induced and others are expressed only during a limited portion of the plant's life cycle. For example, when epidermal cells respond to UV irradiation or function in the deterrence of pathogen invasion, members of the chalcone synthase and phenylalanine ammonia-lyase gene families are transcriptionally activated in epidermal cells (Schmelzer et al., 1988, 1989); interestingly, in the absence of induction, these genes are expressed in other cell types as well (Schmelzer et al., 1989). Genes determining flower pigmentation are expressed in specialized epidermal cells of snapdragon petals (Jackson et al., 1991; Goodrich et al., 1992), but only for limited periods during ontogeny. Recent reports have identified two genes encoding lipid transfer proteins in maize (Sossountzov et al., 1991) and in carrot (Sterk et al., 1991) that are exclusively epidermis specific during most of the plant's life cycle. One of these genes is expressed in the epidermis of maize leaves; however, its transcript is also found in the vascular bundles of these leaves (Sossountzov et al., 1991). The genes encoding the soybean proline-rich cell wall proteins SbPRP1, SbPRP2, and SbPRP3 show a very complex expression pattern (Wyatt et al., 1992); all are at some time expressed in the epidermis but are expressed at different times in vascular tissues as well. None of these genes are exclusively epidermis specific.

In this study, we capitalized on the fact that the epidermis can be removed easily from the leaves of Pachyphytum (Crassulaceae) to obtain epidermal tissue in amounts large enough to facilitate RNA isolation. We constructed a cDNA library from this isolated epidermal tissue and screened it by differential hybridization to obtain a set of epidermis-specific transcripts. This approach of differential screening excluded genes such as isogenes for general cellular functions from this investigation. Epidermis specificity was demonstrated by RNA gel blot analyses. These findings were investigated further by in situ

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localization of the transcripts. Two of the identified transcripts appeared to be present in all cells of the epidermis. One is abundant in the cells of the stomatal apparatus and less abundant in other cells in the epidermis of mature leaves. Our RNA gel blot analyses indicated that mesophyll cells and cells of the vascular bundles lack these transcripts; these results were confirmed by our in situ experiments. All of the transcripts studied are highly expressed in the protoderm of the shoot apical meristem of Pachyphytum plants, which supports the concept of epidermal origin from the outermost layer of the shoot apical meristem (see, for example, Satina et al., 1940).

The cell-specific transcripts identified here, the genetic elements controlling their epidermis-specific expression, and their encoded functions provide exciting new tools that we can use to obtain information about the specific molecular structure and function of epidermal cells.

RESULTS

Epidermis-Specific cDNAs

The ornamental plant Pachyphytum, which is shown in Figure 1A, is characterized by fleshy leaves from which the epidermis can be removed easily in large segments. An anatomical survey of Pachyphytum leaves of approximately 1 cm in length (Figure 1B) indicates that the epidermis is uniseriate. Ordinary epidermis cells are interspersed by stomatal complexes at regular intervals. Subtending the epidermis is a layer of thick-walled sclerenchyma cells. The transverse section of the leaf is dominated by extremely large mesophyll cells.

The epidermis was peeled from leaves 1 to 3 cm in length. Epidermal peels proved to be reasonably pure, as demonstrated by histological observation. This was mainly due to the presence of the underlying layer of sclerenchyma cells (Figure 1B) which aided in the removal of the epidermis. The peeled material was practically devoid of chlorophyll. Poly(A)$^+$ RNA isolated from the peeled material was used to generate a cDNA library. Differential screening was used to isolate plaques that hybridized only with cDNA specific to the epidermis, and candidate plaques were screened several times. Six cDNAs were selected for further studies. Their tissue-specific expression was monitored by gel blot hybridization, as shown in Figure 2, to RNA from either epidermis peels (lanes E) or mesophyll cell-derived RNA (lanes M).

The transcripts represented by the isolated cDNAs were abundant only in isolated leaf epidermis (Figure 2, lanes E). When total RNA from intact leaves was analyzed for the expression of these cDNAs, only weak hybridization signals were observed (data not shown). Directly quantified (by β-scanner) hybridization signals from intact leaves revealed that they represented 5% of that detected in the epidermal tissue. Transcripts represented by five of the isolated cDNAs (EPI2, EPI11, EPI12, EPI13, and EPI15) were relatively abundant in total RNA from epidermis peels (Figure 2); no hybridization of these cDNAs occurred with RNA isolated from leaves from which the epidermis had been removed. Results from gel blot hybridization suggested that the EPI16 transcript constituted a very low-abundance mRNA in the epidermis, but the lack of hybridization to RNA derived from mesophyll cells does not preclude its presence in these cells at even lower levels. Considering the resolution provided by the RNA hybridization technique, it appeared that at least five, and possibly six, of
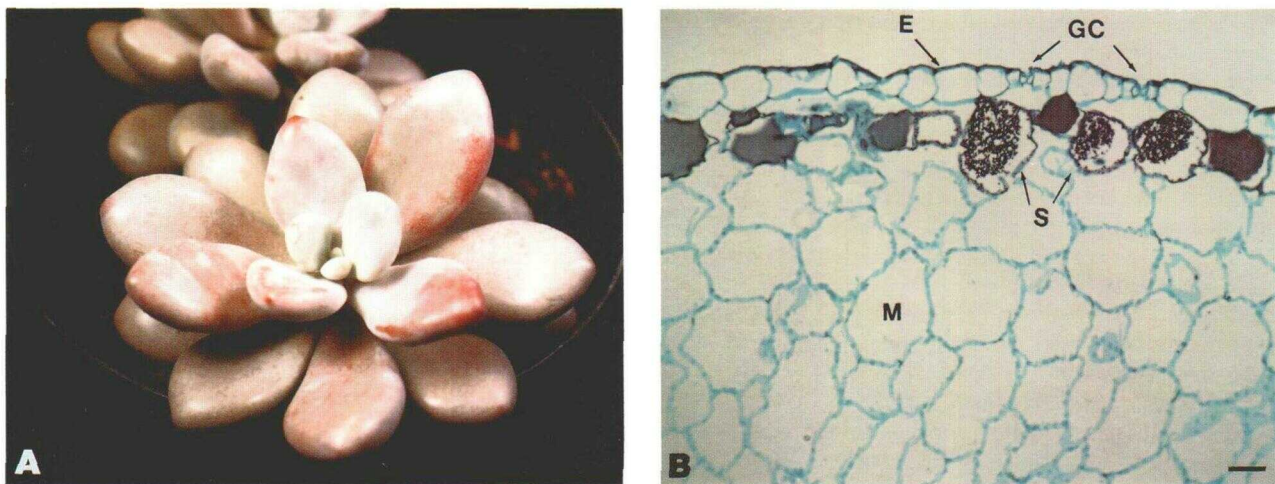

Figure 1. Pachyphytum.

(A) Pachyphytum habitus.
(B) Transverse section through the outer portion of a Pachyphytum leaf and histological staining with safranin O and fast green. E, epidermis cell; GC, guard cells; M, mesophyll cell; S, sclerenchyma cell. Bar = 10 μm.
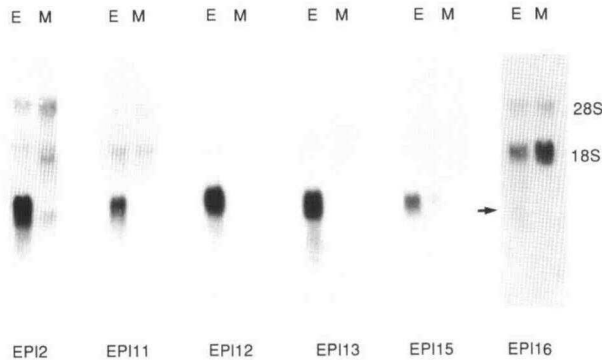
Figure 2. Hybridization of Selected cDNAs to Total RNA from Epidermis Peels and Mesophyll Cells.

Total RNA was isolated from peeled epidermis preparations (E) or from the mesophyll cells (M) after removal of the epidermis. Ten micrograms of RNA was loaded per lane; lanes containing both RNAs were hybridized to the indicated cDNA clone. Exposure times were 12 hr (EPI12, EPI13, and EPI15), 3 days (EPI2 and EPI11), or 7 days (EPI16). The arrow indicates weak hybridization to the low-abundance EPI16 transcript.

the cDNAs selected for this study are expressed exclusively in the epidermis. In situ hybridization experiments with the inserts of cDNAs EPI11, EPI12, and EPI13 support these findings.

Localization by in Situ Hybridization

Leaf sections were hybridized (Cox and Goldberg, 1988) with single-stranded ^{35}S-labeled RNA probes comprising either the antisense or sense strands of the three selected cDNAs (EPI11, EPI12, and EPI13). All three were indicated as exclusively epidermis specific by RNA gel blot hybridization, and they represented relatively abundant transcripts. Because EPI12 and EPI13 show similar patterns of expression, data are only presented here for EPI13. Leaves of different ages were screened initially to find the optimal developmental stage for use in in situ hybridizations. Immature, still-expanding leaves (approximately 1 cm in length) were best suited for our purpose. This is probably due to the high cytoplasm-to-vacuole ratio found in younger leaves that allows unambiguous documentation of the signals at the cellular level.

Transcripts Expressed in All Cells of the Epidermis

Sectioned leaves were analyzed for the presence of the EPI13 mRNA. Silver grains indicating the location of hybridization to the antisense strand, as shown in Figure 3A, were found in high density in all epidermal cells of leaves 1 to 3 cm in length, thus confirming the RNA hybridization data. Hybridization signals with the EPI12 and EPI13 antisense probes were concentrated near the outer surface of the epidermal cells

(Figures 3A and 3B). In addition, hybridization signal obtained with the EPI13, but not the EPI12, probe clearly outlined nuclei of the epidermal cells (Figures 3B and 3D). Nuclear identification was confirmed by cytological staining with orange G (data not shown). We did not detect any specific hybridization in any other cells in the leaf (including sclerenchyma, mesophyll, or vascular cells) with the EPI13 antisense probe (Figure 3C).

Signal intensity was inversely proportional to the size (age) of the leaf. Young leaves (Figures 3A and 3B) have epidermal cells in which a larger percentage of the lumen is occupied by cytoplasm than is the case in older cells that are characterized by a prominent central vacuole (Figures 3D and 3E). When the antisense EPI13 probe was hybridized to tissue sections from young leaves, much stronger hybridization signals could be observed. The EPI13 and EPI12 probes showed an even distribution of silver grains in all cells of the epidermis, including the cells of the stomatal apparatus (as shown for EPI13 in Figure 3B). Both transcripts were present in old and young leaves (i.e., leaves that may be more than 6 months old and leaves that had just emerged from the apical meristem), and both transcripts were present in approximately equal abundance.

Transcripts Expressed in Cells of the Stomatal Apparatus

The spatial pattern of silver grain distribution observed following in situ hybridization with the EPI11 antisense probe was unique, showing a high concentration of silver grains restricted to certain areas of the epidermis, as shown in Figure 4A. Unlike the EPI12 and EPI13 mRNAs, the EPI11 probe hybridized predominantly around the stomatal apparatus. Ordinary epidermal cells in leaves 1 to 3 cm long showed a silver grain density that was not dramatically different from that observed with the control sense strand probe (Figure 4B), indicating that the EPI11 transcript might be present only in the subsidiary cells and, perhaps, in guard cells of the stomatal apparatus (Figure 4A). As was the case for the other transcripts, no hybridization was observed in any cells other than epidermal cells.

When localization of the EPI11 transcript was analyzed in paradermal sections suitable for visualizing the leaf surface, additional information about EPI11 expression emerged. Due to the concave shape of Pachyphytum leaves (Figure 1A), this type of glancing sectioning provides visualization of epidermal cells as well as some of the sclerenchyma cells. The latter are shown as dark blue structures in the stained material (Figure 4C). Pachyphytum has an anisocytic stomatal structure (Esau, 1977) in which two guard cells are surrounded by three subsidiary cells, one of which is distinctly smaller than the other two (Popham, 1952). Upon observation of hybridization with antisense probes by dark-field microscopy (Figure 4D), silver grains were observed in all epidermal cells, although with highest density in the subsidiary cells surrounding guard cells. In bright-field observations (Figure 4E), the highest concen-
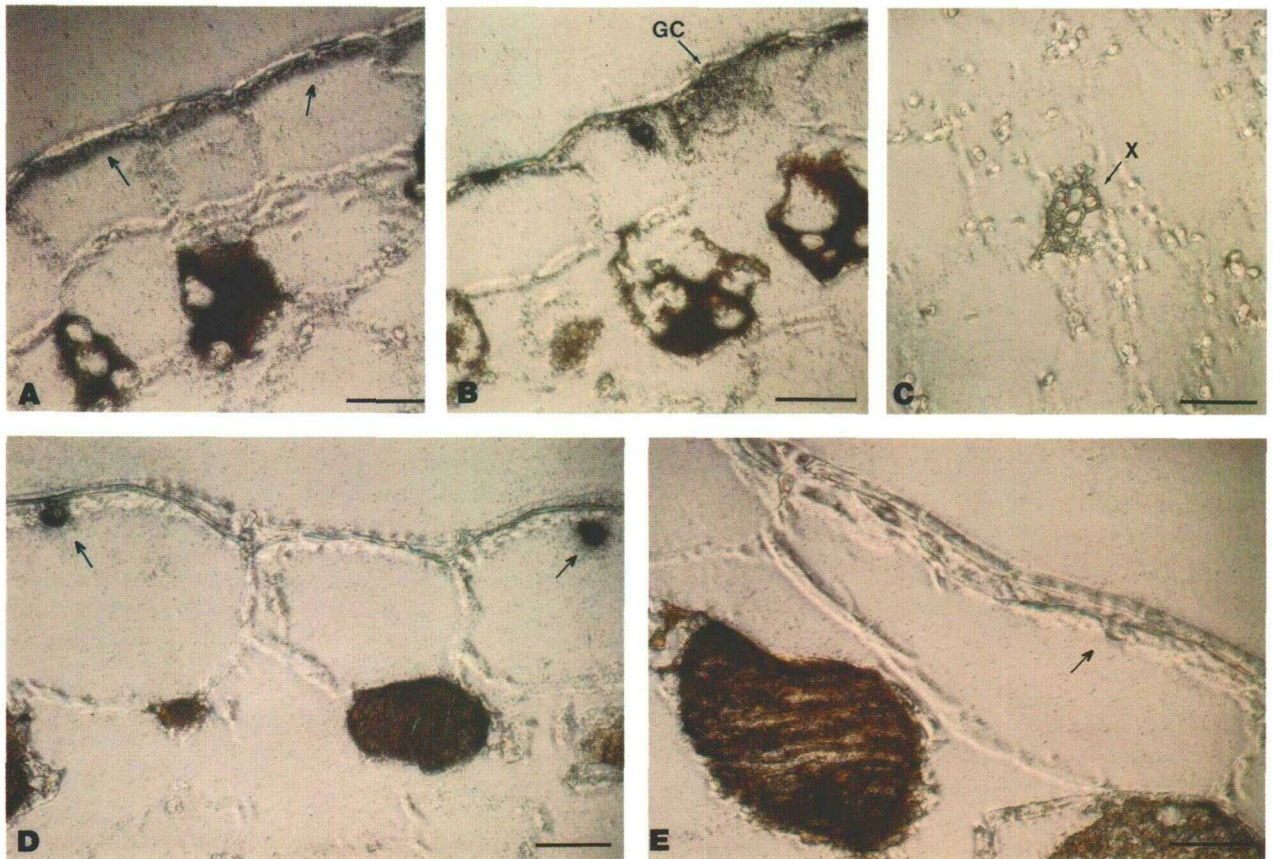
Figure 3. Localization of EPI13 mRNA.

(A) to **(D)** Bright-field microscopy of sections of Pachyphytum leaf epidermis after in situ hybridization with the EPI13 antisense probe.
(E) Bright-field microscopy of sections of Pachyphytum leaf epidermis after in situ hybridization with the EPI13 sense probe.
Arrows in **(A)** indicate silver grains; arrows in **(D)** and **(E)** point to the nuclei. GC, guard cells; X, xylem. Bars ≈ 10 μm.

tration of silver grains occurred in the three subsidiary cells accompanying the guard cells. We cannot assess the presence of EPI11 transcripts in the guard cells themselves at present due to the extremely small cytoplasmic volume of these cells.

Transcript Expression during Leaf Development

The results presented above illustrate exclusive epidermal specificity of three abundant transcripts (EPI11, EPI12, and EPI13) obtained from a cDNA library constructed from isolated epidermis of Pachyphytum leaves at various developmental stages. To investigate whether, and to what extent, epidermis specificity of these transcripts occurs at the shoot apex, as shown in Figures 5A and 5B, we performed further in situ localization on this tissue. Strong hybridization of the antisense EPI13 probe was observed in the protoderm at the shoot apex (Figure 5C). The epidermis of nascent leaves, P_1, P_2, and P_3

(like those in Figure 5A), contained fewer silver grains (data not shown). This difference in silver grain density may be attributable to the differences in cytoplasm-to-vacuole ratios between these cells, or it may be due to actual differences in transcript abundance. Similar hybridization patterns (with highest grain density in cells at or very close to the shoot apical meristem) were found for the EPI11 (Figure 5E) and EPI12 antisense probes (data not shown).

Analysis of EPI11 expression was extended beyond the shoot apex or leaf primordium to include a developing leaf similar to the one identified as P_2 in Figure 5A. Sections hybridized with the antisense RNA probe showed that the EPI11 mRNA is found within all epidermal cells, even though stomata are already present at this stage of leaf development (Figure 5E). These results suggest that EPI11 expression becomes restricted to stomatal complexes during leaf maturation. Moreover, analyses of small leaves (less than 1 cm long) illustrate a gradient of the EPI11 transcript within the leaf itself. The transcript appears to be uniformly distributed within epidermal cells at

the base of a leaf, but the mRNA becomes limited to stomatal complexes in distal portions of the leaf (data not shown).

Sequence of an Epidermis-Specific Transcript

None of the nucleotide sequences of these six epidermis-specific cDNAs encodes a function that has been described previously in plants (A.M. Clark and C.B. Michalowski, unpublished results). The nucleotide sequence of the EPI11 cDNA is presented in Figure 6 because of the potential relevance of its deduced amino acid sequence to its role in the epidermis, where it is more abundant in cells of the stomatal complex. The 681-nucleotide length of the EPI11 cDNA is consistent with the size of the transcript observed in our RNA gel blot analyses (Figure 2). Although we have not yet determined the transcription start site, it appears that this clone represents

a full-length cDNA; analysis of a region surrounding the translation initiation codon supports this assumption, for the sequence of this region resembles the plant consensus translation start sequence (Lütcke et al., 1987). The deduced amino acid sequence of EPI11 (Figure 6) reveals a single open reading frame of 141 amino acids, corresponding to a molecular mass of 16,148 D.

Hydrophobicity plots (data not shown) indicate an amino-terminal hydrophobic region (amino acids 5 to 24), followed by a stretch of mostly hydrophilic residues (amino acids 25 to 39). The remaining sequence exhibits a random distribution of charged residues, implying a globular structure of the EPI11 protein. The most abundant amino acids are proline (18%) and lysine (14%); the net charge of the protein (at pH 7) is +11. Several unique repeat motifs are both highly positively charged and proline rich. For example, the motif P/FFHKKD is found three and a half times, the sequence I/VKKHPLPPI
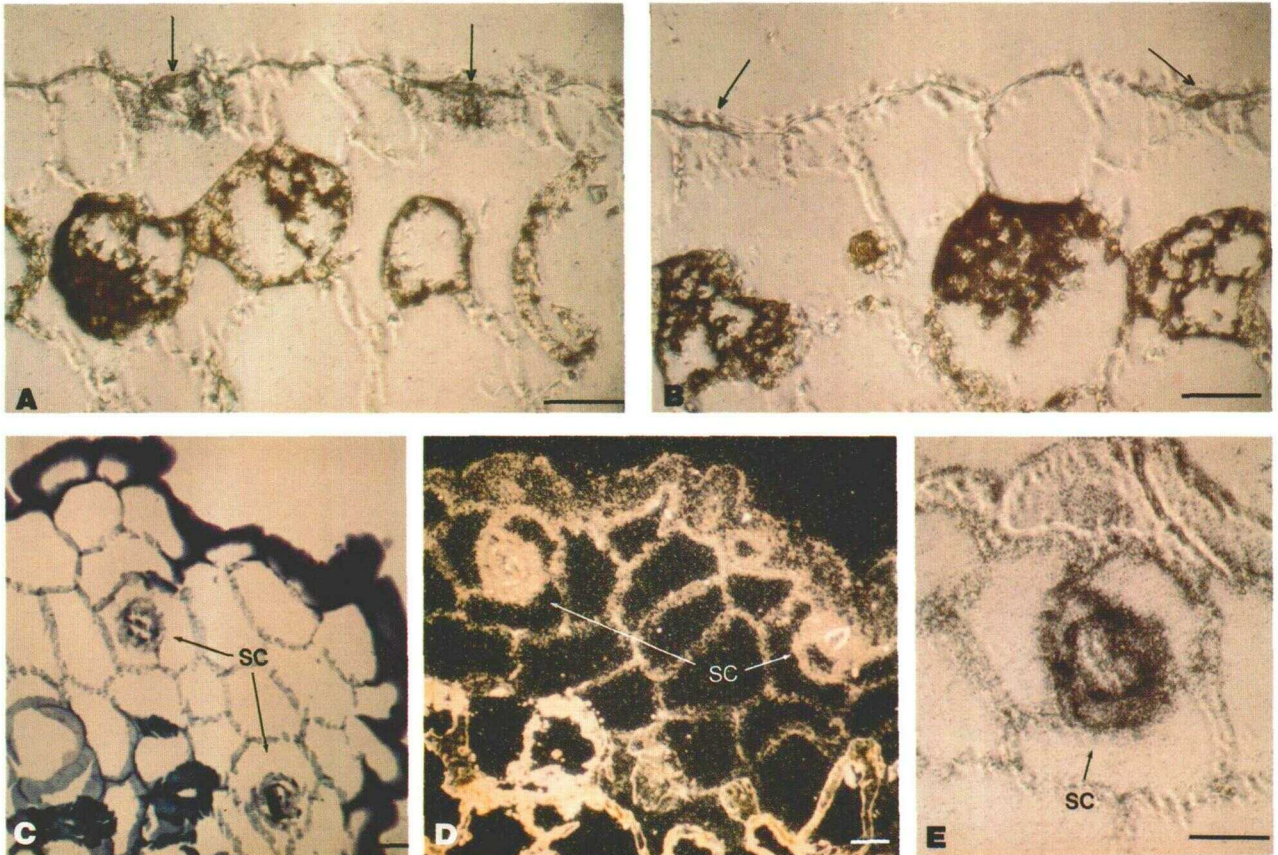

Figure 4. Localization of EPI11 mRNA.

(A) and **(B)** Bright-field microscopy of transverse sections of Pachyphytum leaf epidermis after in situ hybridization of **(A)** antisense or **(B)** sense RNA probes. Arrows point to stomatal complexes.

(C) to **(E)** Paradermal sections of Pachyphytum leaf epidermis. The micrograph in **(C)** shows staining with toluidine blue. The micrograph in **(D)** illustrates dark-field microscopy of a section hybridized with the antisense EPI11 probe, and **(E)** shows bright-field microscopy of a section hybridized with the antisense EPI11 probe. Arrows indicate stomatal complexes (SC).
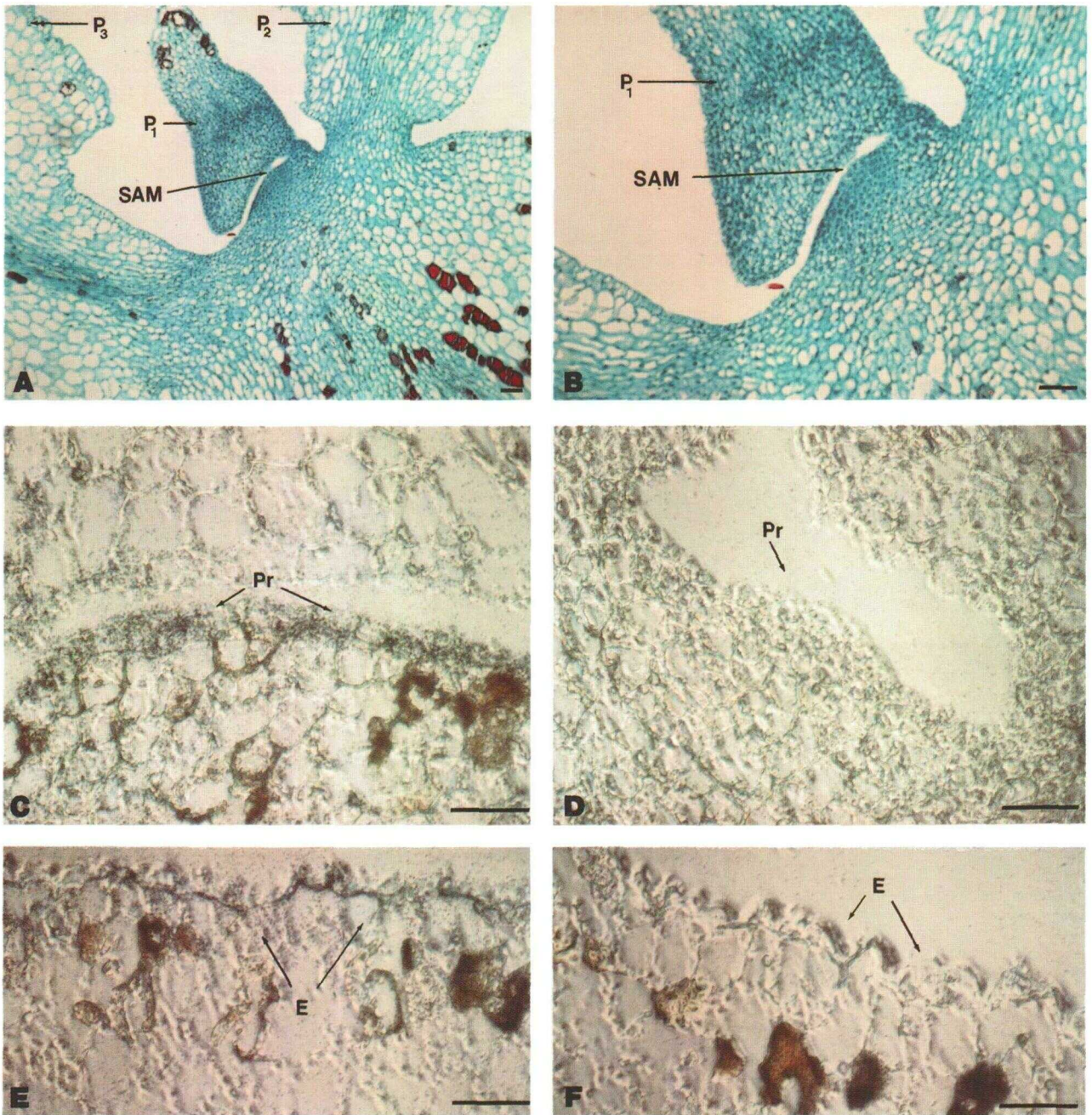
Bars = 10 μm.

Figure 5. Localization of the EPI11 and EPI13 Transcripts in Apical Meristems and Young Leaves.

(A) and **(B)** Longitudinal sections of the shoot apical meristem region stained with toluidine blue.

(C) and **(D)** Antisense and sense, respectively, EPI13 probes hybridized to shoot apical meristem regions that are comparable to those shown in **(A)** and **(B)**.

(E) and **(F)** Antisense and sense, respectively, EPI11 probes hybridized to sections from young leaves.

E, epidermis; P_1, P_2, and P_3, nascent leaves; Pr, protoderm; SAM, shoot apical meristem. Bars = 10 μm.

is present two and a half times, and the sequence YVHPGIP is present twice (Figure 6).

There is limited sequence similarity of the EPI11 amino acid sequence to that of several SbPRPs (Hong et al., 1987, 1990) and soybean ENOD2 (Franssen et al., 1989). A 25% identity between the entire deduced EPI11 amino acid sequence (141 amino acids) and the amino-terminal portion of ENOD2 (145 amino acids of a total of 309 residues) was indicated. Identities with the SbPRPs are approximately 35%, but do not extend over the entire EPI11 sequence. Alignments of amino acid sequences start at residues 50 or 62 for the EPI11 amino acid sequence and amino acids 175 and 156 for the SbPRP1 and SbPRP2 proteins, respectively. These alignments extend to the carboxyl termini of the three amino acid sequences. Sequence length is 256 amino acids for SbPRP1 and 230 amino acids for SbPRP2. Similarity with the 90–amino acid long SbPRP3 begins at residue 32 and residue 90 of the EPI11 amino acid sequence extending throughout the remaining portions of both sequences.

The deduced EPI11 amino acid sequence, like that of the ENOD2 and SbPRPs, contains a putative amino terminal signal sequence, as shown in Figure 7. Significantly, this sequence is 88% similar (16 of 18 residues) to a signal peptide identified in β-1,3-glucanase from tobacco (Payne et al., 1990). Although cleavage of the signal peptide has not been demonstrated for any of these deduced sequences, signal peptide cleavage has been demonstrated for other glucanases (Shinshi et al., 1988).

Figure 6. Nucleotide and Deduced Amino Acid Sequences of the EPI11 cDNA.

The deduced amino acid sequence encoded by 187 codons is characterized by a putative amino-terminal transmembrane region (underlined) and by several repeat units. The deduced amino acid sequences of these repeat units are P/FFHKKD (double underlined sequences numbered 1 to 4), I/VKKHPLPPI (arrowheads a to c), and YVHPGIP (dashed lines designated α and β). The start codon is underlined; the putative polyadenylation sites are indicated by asterisks.
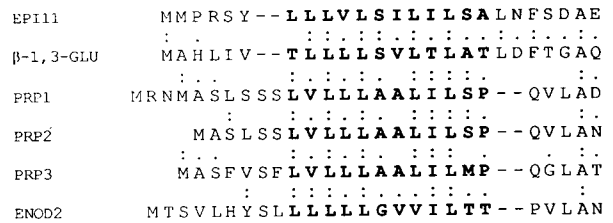

Figure 7. Homology of the EPI11 Amino-Terminal Region with Signal Peptides of Other Plant Proteins.

The amino-terminal region of the deduced amino acid sequence of EPI11 is compared to the deduced amino-terminal regions of β-1,3-glucanase (B-1,3-GLU) and to corresponding regions of three proline-rich cell wall proteins (PRP1, PRP2, and PRP3) as well as to a deduced nodulin amino acid sequence (ENOD2). Boldface letters indicate a region of high homology.

Although not indicated by the computer search, the signal sequences of the six amino acid sequences discussed here share significant similarity in the hydrophobic core (Figure 7). Also conserved is an alanine residue preceding the hypothetical cleavage site. The putative signal sequence of EPI11 conforms to rules suggested by von Heijne (1988), including the nature of the residues surrounding the processing site. The presence of such a signal peptide suggests that the EPI11 protein might be secreted to the cell wall or to the extracellular space, although only further experiments can show whether this sequence actually serves as a transport signal.

DISCUSSION

The epidermis of plants performs a multiplicity of specialized functions that can be characterized by the expression of specific genes. Epidermal features including color and cell structure are often used as easily scored genetic markers. For example, trichomes serve as epidermal structural markers in Arabidopsis (Marks and Feldmann, 1989) and in tomato (Goffreda et al., 1990), whereas composition and deposition of cuticular waxes in maize determine the appearance of the kernel epidermis (Coe et al., 1988). Epidermal mutants of maize (Knotted) (Hake and Freeling, 1986) and Arabidopsis (keule) (Mayer et al., 1991) have been used to follow the fate of individual cells in developing organs. The analysis of cuticular wax layer composition has long been a domain of phytochemical and biochemical plant analyses (Kolattukudy, 1987). Anatomical investigations of the epidermis are many (Popham, 1952; Esau, 1977), and many studies have been directed toward understanding the physiology of stomatal complexes (Zeiger, 1983). However, surprisingly little is known about the metabolism, development, or molecular and biochemical mechanisms of the plant epidermis.

The lack of molecular information about epidermis function

can be explained in part by the difficulties encountered in obtaining noncontaminated preparations of epidermal cells. We have overcome this constraint by studying Pachyphytum. The basic anatomy of the Pachyphytum leaf (Figure 1B) facilitates the removal of the epidermis; located underneath the uniseriate epidermis, sclerenchyma cells provide thick cell walls along which the epidermis can be removed without including any of the underlying cells. The purity of our epidermis preparation and the epidermis specificity of our cDNA transcripts are demonstrated by RNA gel blot hybridizations (Figure 2). Gene probes isolated from the cDNA library derived from epidermal peels do indeed hybridize in a cell-specific manner. Four of the six transcripts isolated are abundant in the epidermis-derived RNA; they are virtually absent from mesophyll RNA and are present in total leaf RNA equivalent to low-abundance mRNA.

Using in situ hybridization, we further examined the distribution of EPI transcripts within the Pachyphytum leaf. Interestingly, two patterns of distribution were observed. The EPI12 and EPI13 transcripts are distributed evenly within the epidermis. The hybridization pattern is affected by the stage of leaf development, as illustrated by the localization of the EPI13 transcript (Figure 3). While this transcript is found throughout the epidermal cytoplasm in a young leaf, hybridization of this transcript becomes limited to the region around the nuclei of epidermal cells in older leaves. During leaf maturation, most of the cell volume is occupied by a vacuole, leaving only a rather small volume of cytoplasm, like that around nuclei, available for hybridization (Figure 4E). Because neither EPI11 nor EPI12 was found to be concentrated around nuclei in mature epidermal cells, factors other than age must be responsible for the intriguing nuclear or perinuclear localization of the EPI13 transcript.

The second pattern of transcript distribution observed is represented by the EPI11 isolate. In older leaves, EPI11 mRNA is found predominantly in the subsidiary cells of the stomatal complex and possibly also in the guard cells themselves. A lower level of expression is found in ordinary epidermal cells when paradermal (rather than transverse) sections are examined (Figures 4F and 4G). This may be due to the relationship between the cytoplasm content and the plane of the section examined (compare Figures 1B and 4E). EPI11 expression may be regulated developmentally. It is difficult at this point to state unequivocally that the EPI11 gene is transcriptionally regulated in a manner such that the early message is ubiquitous in "young" epidermis, while in mature leaves it becomes expressed specifically in subsidiary cells. Additional insights might be obtained through analysis of elements controlling EPI11 expression and, especially, characterization of its gene product.

The abaxial and adaxial leaf surfaces of Pachyphytum show no differences in terms of hybridization; both exhibit comparable amounts of hybridization for all transcripts tested. Asymmetrical expression in the adaxial (upper) epidermis of mature leaves has been shown for SbPRP3, a member of a proline-rich cell wall protein gene family (Wyatt et al., 1992).

Like the EPI11, EPI12, and EPI13 transcripts, the SbPRP3 transcript is expressed uniquely in the epidermis of mature leaves. While the SbPRP3 transcript is localized in the adaxial epidermis of mature leaves, it is not expressed in the epidermis of the growing hypocotyl. Unlike the SbPRP3 mRNA, the three EPI transcripts identified here show early expression because they are present in Pachyphytum shoot apices (Figure 5). Moreover, their expression seems to be localized to a single layer of cells at the shoot apex, which is consistent with the one cell–layer theory for origin of epidermal tissue (Sussex, 1989). Early expression of EPI mRNAs suggests their active participation in Pachyphytum leaf epidermis ontogeny.

Two transcripts that are expressed specifically in the shoot apex have been identified previously. For example, Kelly et al. (1990) describe a transcript, A3, that is expressed specifically in the vegetative shoot apex of tobacco. This transcript is also present during apical transition to flowering. The A3 mRNA is assumed to be active in meristematic tissue only because its presence in any of the mature leaves could not be demonstrated. The transcript of another gene, extracellular protein 2 (EP2), which encodes a nonspecific lipid transfer protein in carrot, has been localized in the epidermis of the shoot meristem and leaf primordia (Sterk et al., 1991). EP2 is absent, however, from mature plant organs. In contrast to A3 and EP2, the EPI clones analyzed here continue to be transcribed in mature leaves, albeit at different steady state levels.

Among the protein sequences sharing some similarities with the deduced EPI11 amino acid sequence are three soybean cell wall proteins (Hong et al., 1987, 1990) and a soybean early nodulin protein ENOD2 (Franssen et al. 1990). While the soybean cell wall protein transcripts are detected in the leaf epidermis, though not exclusively, the ENOD2 mRNA is found in nodule parenchyma (van de Wiel et al., 1990). Based on transcript localization and similarities to proline-rich cell wall proteins, it has been implied that ENOD2 contributes to the special morphology of the nodule parenchyma. This specialized morphology might provide an oxygen diffusion barrier to protect an oxygen-sensitive nitrogenase. Because one of the functions of the epidermis is control of gas exchange, it remains a possibility that EPI11, like ENOD2, might also contribute to the unique morphology of ordinary epidermal cells or to the stomatal complexes only.

The results discussed here represent initial characterization of the molecular nature of epidermal cells in the leaves and shoot apical meristem of Pachyphytum. It is not unexpected that the cDNAs described here do not share significant similarities with sequences present in gene banks. Our analysis of isolated epidermal tissue greatly amplified the epidermis-specific signal. Based on our estimations from β scanning of our RNA gel blots, epidermal-specific signals, even the most abundant ones, represent only 5% of total leaf mRNA. This may explain the difficulties encountered to date in obtaining epidermis-specific sequences in heterologous RNA gel blot experiments. A more successful approach may be to use the Pachyphytum probes described here to screen either genomic or cDNA libraries. To date, one of the EPI counterparts, that

of EPI11, has been detected through a screen of an Arabidopsis genomic library. The analysis of this Arabidopsis clone is in progress. It is anticipated that this, as well as further studies of these Pachyphytum clones, including a detailed analysis of their controlling elements, will provide us with information on the specific molecular structure and function of epidermal cells.

METHODS

Plant Materials

Pachyphytum sp plants were purchased from local nurseries and grown in a greenhouse at the University of Arizona. Epidermal peels were collected from mature leaves (ranging in length from 1 to 3 cm). After an incision was made at the leaf tip, the adaxial and abaxial epidermis layers were removed with a forceps in one movement. Peels were immediately frozen in liquid nitrogen and stored at −70°C. Whole leaves and leaves from which the epidermis had been removed were also frozen.

RNA Isolation

Total RNA from either epidermal peels or mesophyll cells was extracted using the method of Hughes and Galau (1988). The procedure yielded approximately 10 μg of total epidermal RNA and 20 μg of total mesophyll RNA per g of tissue. Poly(A)$^+$ RNA was isolated from total RNA using oligo(dT)-cellulose chromatography. Approximately 10 μg of epidermis-specific mRNA was converted to double-stranded cDNA (Bethesda Research Laboratories cDNA synthesis kit) and was ligated to EcoRI/NotI adapters (Invitrogen, San Diego, CA) and subsequently ligated to the λ ZAPII vector arms (Stratagene). The epidermis-specific cDNA library contained approximately 3×10^5 recombinant plaques. The library was plated at a density of approximately 4000 plaques per 15-cm-diameter plate that were lifted onto nitrocellulose filters in duplicate. The filters were screened by differential hybridization with epidermis-specific or mesophyll-specific radioactively labeled first-strand cDNA. Autoradiograms were screened for hybridization associated with the epidermis-specific cDNA probe only. Inserts of selected plaques were converted into plasmid vectors according to established protocols (Stratagene).

RNA Hybridization

Total RNA isolated from either epidermis layers or mesophyll tissues was separated on denaturing (2.2 M formaldehyde) 1.2% agarose gels, and the RNA was transferred to nitrocellulose filters (Schleicher & Schüll, BA86). Hybridization with ^{32}P-labeled inserts of the cDNA clones was carried out in 50% formamide, $5 \times$ SSC ($1 \times$ SSC is 0.15 M NaCl, 0.015 M sodium citrate), $1 \times$ Denhardt's solution (0.02% Ficoll, 0.02% PVP, 0.02% BSA), 1% SDS, and 100 μg/mL of denatured salmon sperm DNA for 16 hr at 42°C. After hybridization, the filters were washed twice with $2 \times$ SSC (room temperature) and twice with $0.1 \times$ SSC (42°C), dried, and exposed to x-ray film (X-Omat; Kodak) for various lengths of time.

Tissue Preparation and in Situ Hybridization

Small segments of Pachyphytum leaves were fixed with 2% glutaraldehyde in 0.05 M sodium cacodylate buffer, dehydrated in an ethanol series, and subsequently infiltrated in paraffin (melting point, 59°C). Paraffin-embedded tissue was cut into 9- or 10-μm thick sections that were stained for histological observation or were used for in situ hybridization.

In situ hybridizations were carried out essentially as described by Cox and Goldberg (1988). The ^{35}S-labeled, single-stranded RNA probes were synthesized from linearized EPI cDNA clones using either T3 or T7 RNA polymerases. The in vitro synthesized RNA was hydrolyzed to a molecular mass equivalent of approximately 200 nucleotides. Hybridizations were performed for 16 hr at 42°C with 2 to 5 $\times$ 10^4 cpm/μL probe per slide. After hybridization, the slides were exposed to NTB-2 photographic emulsion (Kodak) at 4°C for 3 weeks.

DNA Sequencing

Sequencing of both strands of cDNA inserts was done according to the Sequenase protocol (U.S. Biochemicals). Sequence data were analyzed using the departmental VAX with sequence comparison and analysis programs as described previously (Cushman et al., 1989; Michalowski et al., 1989). The nucleotide sequence of the EPI11 cDNA has been submitted to GenBank as accession number M95528.

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