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Tissue-Specific Expression of Divergent Actins in Soybean Root

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It has been proposed that the evolution of distinct classes of genes encoding the κ-, λ-, and μ-actins in soybean is the result of an ancient divergence in patterns of actin gene expression. In this study, antisera against a family of synthetic actin peptides from a divergent region within the predicted actin polypeptide sequences have been used to explore the differential expression of plant actins. Antiserum elicited against a 16-residue synthetic λ-actin peptide SAc4:257 reacted with a 46-kilodalton protein in soybean extracts, showed specificity for the λ-peptide over the divergent κ- and μ-actin peptides in enzyme-linked immunosorbent assays, and reacted strongly and preferentially with root protoderm in apical roots and in lateral root primordia. Antiserum elicited against the synthetic κ-actin peptide SAc1:257 reacted with 46-kilodalton protein on protein gel blots, showed partial specificity toward the immunogenic κ-peptide over the divergent λ- and μ-peptides, and reacted strongly with all root tissues with the exception of root cap. These data support the hypothesis that ancient classes of plant actin genes may have been preserved because of their role in developmentally controlled differences in tissue-specific actin expression and/or function. The possibility that other diverse actin classes have unique patterns of regulation is discussed.

INTRODUCTION

Actin is encoded by a small multigene family in most eukaryotes in which it has been examined. In vertebrates, the actin genes and their gene products are divided into two distinct classes, muscle and cytoplasmic, which are distinguished by their mode of expression and by small differences in the protein-coding sequences. However, the sequences used to define muscle or cytoplasmic actins in vertebrates cannot be used reliably to define the subclasses of actin in distant organisms such as plants, protists, and fungi. For this reason, the molecular evolution and divergence of a variety of eukaryotic actin sequences was quantified by comparing the percent replacement nucleotide substitutions (rns) (Shah et al., 1983; Hightower and Meagher, 1986). The divergence of actin genes in animals, fungi, and plants is estimated to occur at the rate of 1% rns/100 million years (±50 million years). Furthermore, plant actin gene sequences have diverged from nonplant sequences at rates similar to those observed for the divergence of animal actins.

The multigene families encoding plant actins are more diverse than those encoding animal actins (Hightower and Meagher, 1985, 1986; Baird and Meagher, 1987). DNA hybridization data were used to divide the six characterized soybean actin genes into three distinct and divergent classes, κ, λ, and μ, with a pair of closely related genes in each class (Hightower and Meagher, 1985). Subsequent analysis of actin sequences from soybean and other plant genomes suggests that additional highly divergent classes of plant actin remain to be characterized (Baird and Meagher, 1987). Sequence analysis of a representative of the κ, λ, and μ classes revealed that they have diverged from each other by 6% to 9% in rns. Muscle and cytoplasmic actin genes within vertebrates differ by no more than 4% to 5% in rns (Hightower and Meagher, 1986). These and other data imply that the three classes of soybean actin diverged from a common ancestor a minimum of 350 million years ago and that their divergence far predated the ascendance of angiosperms. The estimated divergence time for the κ-, λ-, and μ-actin genes corresponds to the emergence of vascular land plants and the concomitant diversity of tissue types and life cycle characteristic of higher plants (Taylor, 1981). Cell types may differ in the use of actin in cytoplasmic streaming, organelle orientation and movement, transport, and cytoskeletal structure (Staiger and Schliwa, 1987). Plant tissues with actively dividing cells will contain high concentrations of actin in the cytokinetic phragmoplast in contrast to tissues expanding by cell elongation. For these reasons, actin genes may have evolved specialized functions and/or patterns of regulation in different cell and tissue types or may

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Table 1. Actin Sequences[a]

Designation	Class	Sequence
SAc1:257	κ	FGCPGVLYQPSMVGME
SAc4:257	λ	FRCPEVLFQPSMIGME
SAc3:257	μ	FRCPKILFQPSMIEME
Chick muscle actin		FRCPETLFQPSFIGME
SAc3:129	μ	CTFNVPAMYVAIQAV

[a] Residues that vary among the homologous sequences of the three divergent classes of plant actin are shown in boldface. Synthetic peptides are named based on their corresponding codon positions (257 to 272 or 129 to 142) within the sequences of the soybean actin genes SAc1, SAc3, and SAc4 (Hightower and Meagher, 1986). The peptide corresponding to amino acids 257 to 272 within the intact chick skeletal muscle actin polypeptide is shown for comparison. A synthetic peptide with this chicken sequence was not made. The SAc3:129 peptide is unrelated to the SAc:257 or chicken sequences and serves as a negative control.

be involved in different developmental processes. Therefore, a two-part hypothesis has been used to explain the existence of ancient divergent classes of plant actin genes: (1) the divergent plant actin genes are differentially expressed with respect to tissue and development and/or (2) they encode actin proteins with divergent functions (Hightower and Meagher, 1986).

The differential levels of expression of the classes of actin in different organs may give some indication as to the general tissue-specific patterns of protein expression to expect. The κ-actins (SAc1, SAc6) and μ-actins (SAc3, SAc7) each express about 0.05% to 1% of the mRNA in the major plant organs, suggesting that these actins may have a generalized pattern of expression in many cell types or, alternatively, may direct the synthesis of moderate quantities of actin in a major tissue type, such as cytoplasmic streaming in vascular tissue. The μ-actin RNAs were found at higher levels in leaves and stems than in roots or hypocotyls. The λ-actin RNAs, SAc2 and SAc4, are expressed at a level approximately 100-fold lower than either the κ- or μ-actin RNAs (Hightower and Meagher, 1985, 1986). The low levels of expression suggest that the λ-actins either may have a highly specialized pattern of expression in a single limited tissue or may direct the synthesis of a small part of the total actin used for cell maintenance.

Despite the high level of divergence of the plant actins relative to animal actins, the 75% to 80% conservation of overall nucleotide sequence within a plant actin multigene family could complicate in situ RNA hybridization studies for the localization of κ-, λ-, and μ-actin mRNAs due to cross-hybridization of the various sequences (Hightower and Meagher, 1985). Post-transcriptional events affecting protein expression could also result in the misinterpretation of RNA in situ studies even if class-specific hybridization probes were constructed. As an alternative, Bulinski and Gundersen (1986; Bulinski, 1986) have used antisera to divergent peptide sequences to distinguish muscle actins from cytoplasmic actins in vertebrate tissues. Following this approach, antisera to divergent soybean κ-, λ-, and μ-actin peptides were prepared and used to determine whether these actin classes were differentially expressed in various plant tissues. Expression of one class of actin in a particular cell or tissue type would suggest that the divergent plant actin genes have been preserved because (1) they are differentially expressed and/or (2) they encode proteins with divergent functions. In this paper, we present initial data on the tissue-specific expression of the λ-actin-related proteins and the more generalized pattern of expression of κ-actin-related proteins in the roots of soybean seedlings.

RESULTS

Immunogenic Activity of the Actin Peptides

The three synthetic peptides used as immunogens are compared in Table 1. Their amino acid sequences were derived from residues 257 to 272 of representative κ-, λ-, and μ-actins. This is a region of maximum divergence among the three soybean actin classes (Hightower and Meagher, 1986). The chicken skeletal muscle actin polypeptide sequence from the homologous region is also shown in Table 1 for comparison and is more similar to the SAc4:257 λ-actin sequence than the three plant sequences are to each other.

The covalently linked conjugates of keyhole limpet hemocyanin (KLH) with soybean peptides SAc1:257 (κ), SAc4:257 (λ), and SAc3:257 (μ) were used to immunize three rabbits each. Ten days after the second booster injection, titers of the antigenic activity against all three peptides were measured. Titers for all three antisera are shown in Table 2. Samples of antisera from these and the other rabbits taken from 2 weeks to 2 months after the second injection exhibited similar properties in enzyme-linked immunosorbent assays (ELISA). The κ-actin peptide antiserum SAc1:257Ab showed high reactivity with the immunogenic κ-peptide, lower activity with the λ-peptide, and no detectable activity with the μ-peptide. Similarly, the λ-actin peptide antiserum SAc4:257Ab showed high reactivity with the λ-peptide, lower activity with the κ-peptide, and no detectable activity with the μ-peptide. Although the μ-actin peptide antiserum SAc3:257Ab was reactive with all three SAc:257 peptides and chicken actin, it showed no particular specificity toward the immunogenic μ-actin peptide. The antiserum from this particular rabbit showed greater specificity toward the κ-peptide. None of the SAc:257 antisera showed cross-reactivity with BSA or the

Table 2. ELISA Titers of SAc:257 Antisera[a]

Protein or Peptide (0.008 μg/well)	Antipeptide Antisera		
	SAc1:257Ab	SAc4:257Ab	SAc3:257Ab
KLH	1600 (570)	1550 (490)	900 (424)
BSA	0	0	0
Chick muscle actin	200 (0)	0	50 (35)
SAc3:129 (μ) free peptide	*0*	*0*	*0*
Buffer control	0	0	0
SAc1:257 (κ) free peptide	2000 (570)	1600 (0)	500 (141)
SAc4:257 (λ) free peptide	800 (0)	2800 (570)	150 (71)
SAc3:257 (μ) free peptide	0	0	250 (212)

[a] Peptide sequences and nomenclature are described in Table 1 and in the text. Antibodies are named based on the immunogenic peptide (i.e., SAc1:257 immunogen produces SAc1:257Ab). ELISA titers of the IgG fractions are defined as the reciprocal of the dilution required to give half-maximum reaction as described in Tainer et al. (1984). Values for half-maximum reaction are often approximated as intermediate between two dilutions. The titers presented are the average of two replicas, and the standard deviations of these two values from the average are shown in parentheses.

SAc3:129 μ-actin control peptide. Preimmune antisera showed no significant ELISA activity with any protein or peptide assayed.

Antisera to κ- and λ-Actin Peptides React with Actin Proteins

Figure 1 shows the reactivity of the SAc1:257Ab and SAc4:257Ab with soybean proteins of 46 kD, the approximate molecular weight expected for actin on protein gel blots of soybean extracts. The Amersham monoclonal anti-chicken actin, N.350, also reacted with a band of the same molecular weight in soybean extracts. Because of the approximately 100-fold lower levels of λ-actin mRNAs relative to κ- and μ-actin mRNAs (Hightower and Meagher, 1985), the lower reactivity of the SAc4:257Ab relative to the SAc1:257Ab was expected when examining crude protein extracts. All three antisera reacted detectably with 2 μg of purified chicken muscle actin. Of the three antisera, SAc4:257Ab reacted most strongly with chicken actin, consistent with the sequence similarity between SAc4:257 and chicken (Table 1). ELISA data showed that the cross-reactivity of these antipeptide antisera with the nonimmunogenic peptides and with chicken actin increased significantly after the third and fourth booster injections. Because these data suggested a possible decrease in the specificity of the antisera against the immunogenic actin peptide, the antisera from these later bleeds were not used in this study. Although SAc3:257Ab reacted with a 46-kD polypeptide on protein gel blots of soybean extracts (not shown), its complete lack of actin class specificity in ELISAs resulted in its being dropped from further analysis.

SAc4:257Ab Reacts Strongly and Preferentially with Root Protoderm

Roots are complex organs and their many cell and tissue types have the potential for differential expression of actin. Root tips and root primordia were used for actin localization because they are composed mainly of cytoplasm. More mature portions of the root are predominantly vacuole and cell wall.

The reactivity of SAc4:257Ab and control antiserum with lateral root primordia is shown in Figure 2. A longitudinal section of a soybean root primordium can be seen within the transverse sections of a 5-day-old root (frames a to e). Strong reactivity was detected with one or two cell layers corresponding to the protodermal tissue and the apical portion of the root cap. Weak reactivity was seen in provascular tissue of the vascular cylinder within the root primordia (frames a and b). The general morphology of the tissue was examined in the epifluorescent micrographs of these sections counterstained with the DNA-specific dye 4′,6-diamidino-2-phenylindole (DAPI) (frames c and e). Cell

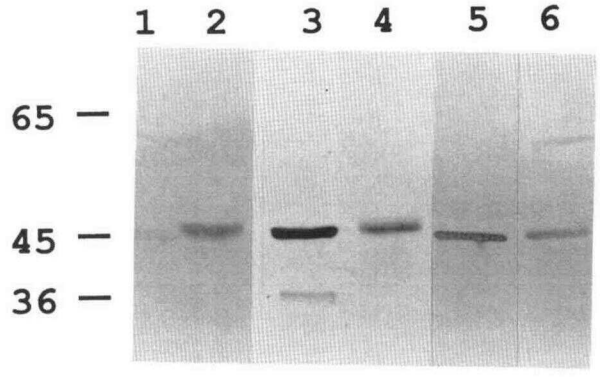

Figure 1. Antisera React with a 46-kD Polypeptide in Crude Extracts from Soybean Seedlings.

Protein gel blots were probed with antipeptide antisera SAc1:257Ab (lanes 1 and 2) and SAc4:257Ab (lanes 3 and 4), and N.350 monoclonal anti-chicken actin antiserum (lanes 5 and 6). Lanes 1, 3, and 5 contain 2 μg of purified chicken actin; lanes 2, 4, and 6 contain 15 μg of soybean seedling crude extract. The antigen-antibody complex was detected using alkaline phosphatase-conjugated secondary antibody. The mobilities of molecular weight standards are indicated on the left.

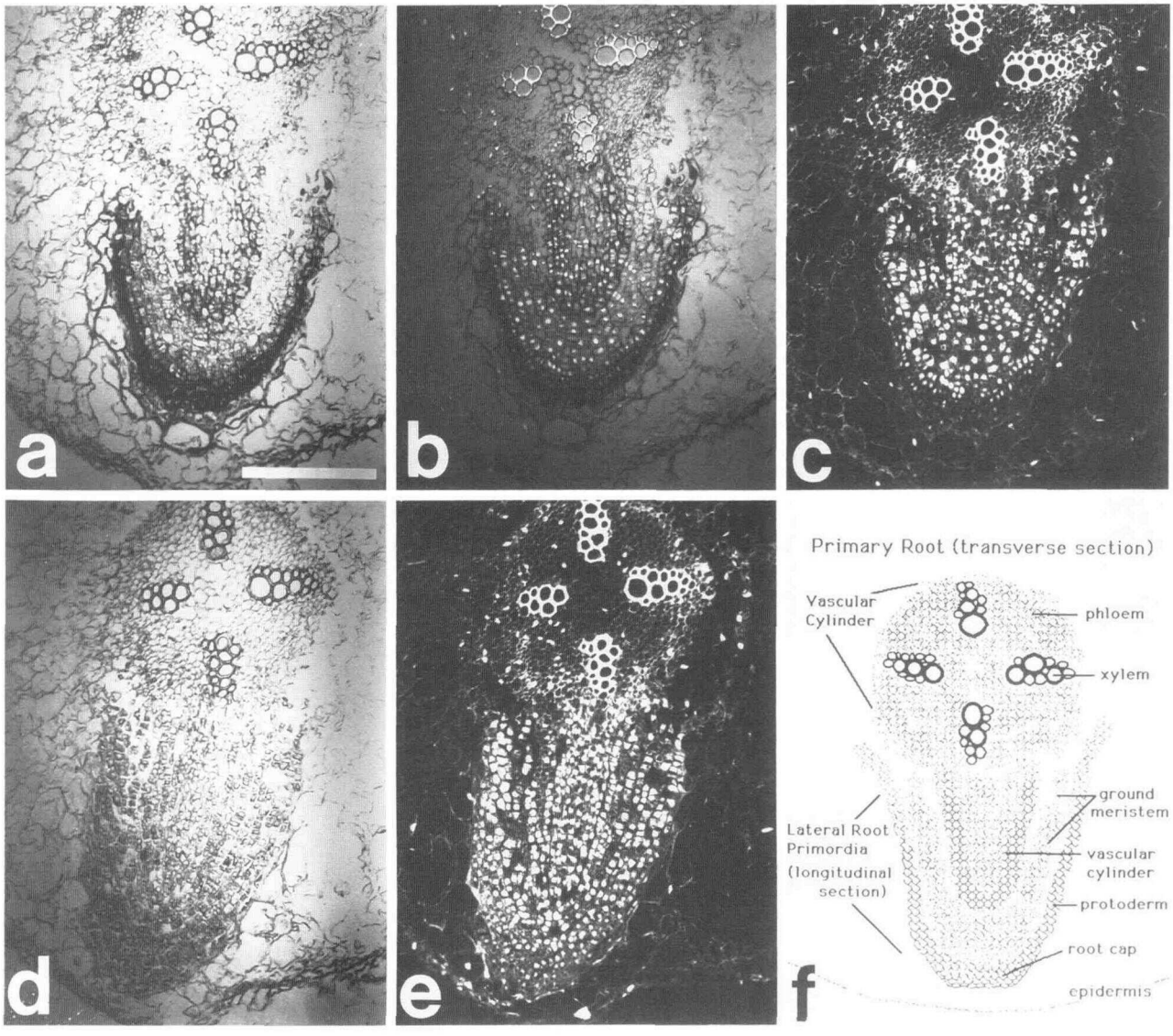

Figure 2. Immunochemical Reactivity of Protoderm in Soybean Lateral Root Primordia with SAc4:257Ab.

Transverse sections in the region of lateral root formation were reacted with λ-actin peptide antiserum SAc4:257Ab [**(a)** to **(c)**] and preimmune antiserum [**(d)** and **(e)**].
(a) and **(d)** Bright-field images.
(b) Combined bright-field and epifluorescent image.
(c) and **(e)** Epifluorescent images of DAPI-stained nuclei and autofluorescent cell walls.
(f) Interpretive diagram.
The antigen-antibody complex was detected using immunogold enhanced with silver. Bar = 200 μm.

walls autofluoresce at this excitation wavelength, giving the outline of individual cells. Figure 2f shows interpretation and nomenclature (Esau, 1977). A comparison of Figure 2c with 2e shows that the DAPI signal was quenched in protodermal and root cap cells because of a high density of silver grains. No significant reactivity was detected with mature root epidermal tissue, cells of the mature vascular cylinder, or in sections of emerging leaves (data not shown). Little nonspecific reactivity was detected in these tissue sections when a high concentration of preimmune antiserum was used (Figures 2d and 2e).

Figures 3a and 3c show the reactivity of SAc4:257Ab with apical root sections. The antiserum reacted specifically with soybean root protoderm and the statocytes in

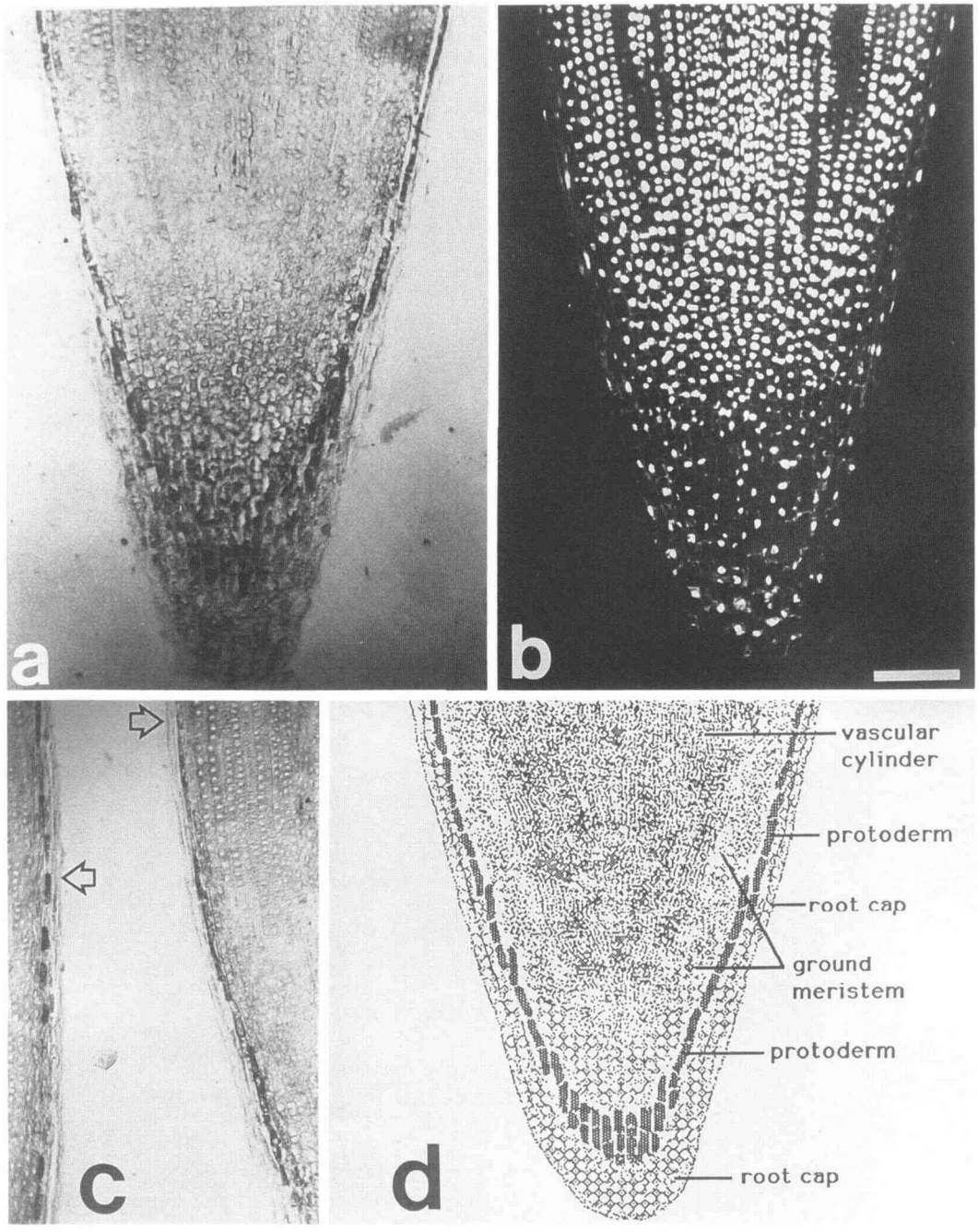

Figure 3. Immunochemical Reactivity of Protoderm in Soybean Apical Root with SAc4:257Ab.

Longitudinal sections of root apex were reacted with λ-actin peptide antiserum, SAc4:257Ab [**(a)** to **(c)**].
(a) Bright-field image.
(b) Epifluorescent image of DAPI-stained nuclei and autofluorescent cell walls of section shown in **(a)**.
(c) Immunostaining further up from the root apex. Arrow indicates 1 mm from tip of the root cap.
(d) Interpretive diagram.
The antigen-antibody complex was detected using immunogold enhanced with silver. Bar = 100 μm.

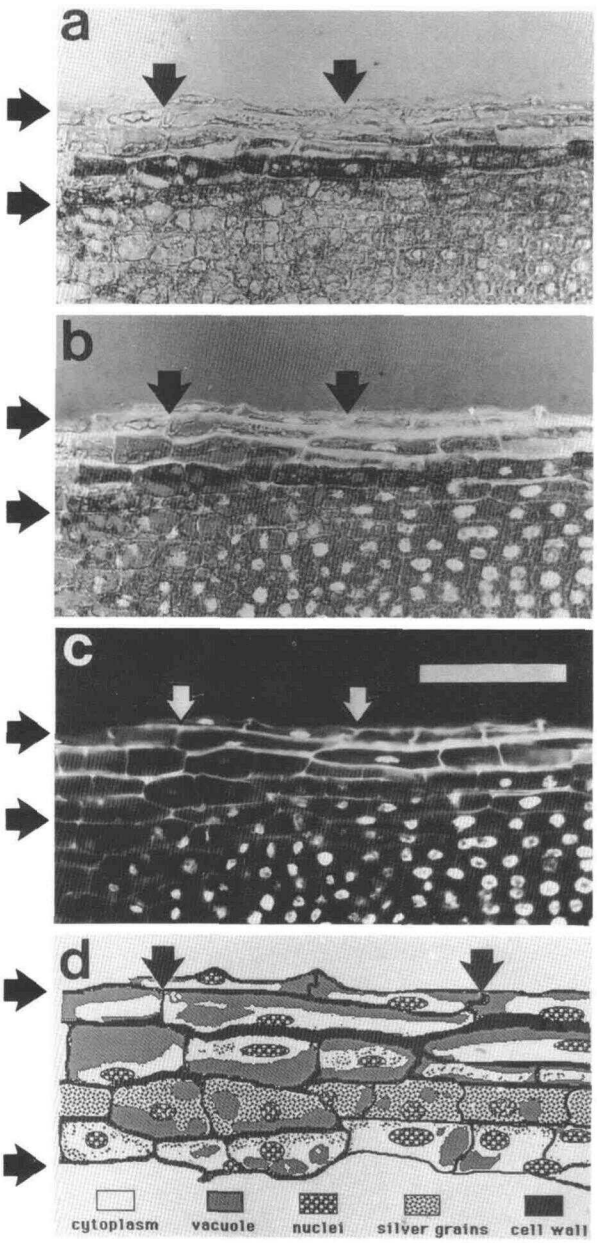

Figure 4. *Immunochemical* Reactivity of Soybean Apical Root Protodermal Cells with SAc4:257Ab.

Longitudinal sections of a protodermal region were reacted with λ-actin peptide antiserum, SAc4:257Ab [**(a)** to **(c)**].
(a) Bright-field image.
(b) Combined bright-field and epifluorescent image.
(c) Epifluorescent image of DAPI-stained nuclei and autofluorescent cell walls.
(d) Interpretive diagram of the portion bracketed by arrows in **(a)** to **(c)**.
This image was taken approximately 0.5 mm from the apical root tip. The antigen-antibody complex was detected using immunogold enhanced with silver. Bar = 80 μm.

the interior portion of the root cap. The epifluorescent micrograph of DAPI-stained cells (Figure 3b) and interpretive diagram (Figure 3d) are presented to identify cell and tissue types. The exterior portion of the root cap did not react significantly, despite the high concentrations of actin that must be present for cytoplasmic streaming in this tissue. Staining of the protoderm began to diminish 1 mm to 1.5 mm from the tip of the root apex (Figure 3c) and ceased before the protoderm escaped from the root cap and became epidermal tissue. It was typical to see alternate staining of protodermal cells, as is shown in the left side of Figure 3c. Relative to protoderm, undetectable or low levels of reactivity were observed with adjacent cortical or provascular tissues. At the same antiserum concentrations and levels of silver enhancement, no tissue reacted with preimmune antiserum (data not shown). Control experiments using 100 mM galactose or 100 mM sucrose in primary antibody incubations showed that signals are not due to nonspecific binding of the antibodies to common plant polysaccharides. No reactivity of SAc4:257Ab was detected in mature epidermal tissue approximately 10 mm from the root tip or at several other points examined along the hypocotyl.

Immunochemical staining of the protodermal tissue with a SAc4:257Ab, shown in Figure 3, was examined at higher magnification in Figure 4. Because of the high density of silver grains, reactivity of the cytoplasm in the protodermal cell layer was obvious in the bright-field image and in the combined bright-field and epifluorescent image (Figures 4a and 4b, respectively). An interpretive diagram is presented in Figure 4d. The quenching of DAPI-stained nuclei due to the presence of silver grains was apparent in the protoderm (Figure 4c). Areas of the cytoplasm, but not vacuolar areas of the single protodermal cell layer, had a high density of silver grains (Figures 4b and 4d). The two to three outer layers of root cap cells did not react significantly with antiserum. The cytoplasm of cortical cells immediately adjacent to protoderm appeared to have a lower density of silver grains, whereas the reaction of central cortical cells was not detectable.

SAc1:257Ab Reacts with All Soybean Root Tissues Examined with the Exception of Root Cap

A strong reactivity of the SAc1:257Ab with most root tissues is shown in Figure 5. The antibody had a much higher reactivity with the cytoplasm of all cells in the internal portion of the root than did the SAc4:257Ab. The absolute level of staining with the SAc1:257Ab in any one cell appears to be less than for those tissues stained with SAc4:257Ab, although immunochemical results are difficult to compare quantitatively. However, it is clear that the quenching of DAPI fluorescence due to high densities of silver grains, as was observed in the protoderm for SAc4:257Ab, did not occur for the SAc1:257Ab without

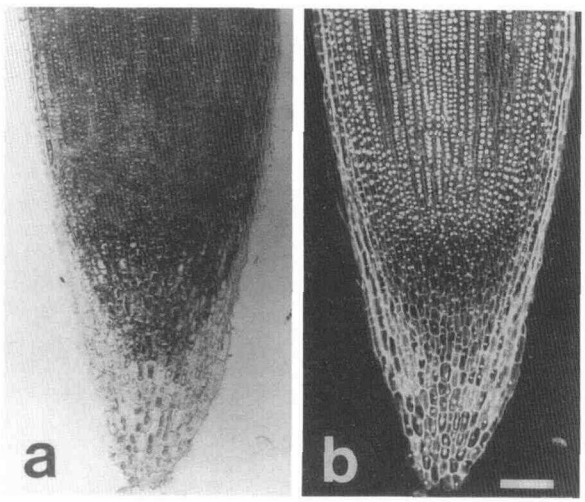

Figure 5. Immunochemical Reactivity of Soybean Apical Root with SAc1:257Ab.

A longitudinal section of the root apex was reacted with κ-actin peptide antisera, SAc1:257Ab.
(a) Bright-field image.
(b) Epifluorescent image of DAPI-stained nuclei and autofluorescent cell walls.
The antigen-antibody complex was detected with immunogold enhanced with silver. Bar = 100 μm.

much longer time for silver grain development. The SAc1:257Ab showed negligible reactivity toward most of the root cap and weak reactivity toward a few interior root cap cells. The Amersham anti-chicken actin monoclonal antiserum, N.350, showed a similar, although weaker, reactivity with most tissues in soybean root sections (not shown) even when relatively large amounts of antiserum were used. Similar to both the SAc4:257Ab and SAc1:257Ab, the monoclonal antiserum failed to react with actin in soybean root cap. Considering the high levels of cytoplasmic streaming that occurs in root cap cells and the large amount of actin involved in this process, soybean root cap actins must differ significantly in their amino acid sequences from the SAc1, SAc4, and chicken actin sequences used as immunogens.

DISCUSSION

Immunogenic versus Antigenic Activity of Actin Peptides

The use of a synthetic peptide as an immunogen to generate antibodies against a denatured or native polypeptide is a relatively new technique. Coupled peptides are potentially more immunogenic than peptide sequences within the intact parent polypeptide because they are both accessible and highly mobile (Tainer et al., 1984; Fieser et al., 1987). Our ELISA results (Table 2) show that peptide coupled to KLH is an effective immunogen for generating high-titer actin peptide antibodies. Antisera from the six other rabbits examined but not included in Table 2 show considerable antipeptide titers after the second booster injection.

Based on ELISA data, the SAc4:257Ab used in this study and two other independent SAc4:257 antisera (not shown) reacted less strongly with the SAc1:257 peptide, which differs by 4 residues, and did not react detectably with SAc3:257 peptide, which differs by 3 of the 16 amino acid residues; the SAc1:257Ab used in this study and other independent SAc1:257 antisera (not shown) reacted moderately with SAc4:257 peptide, which differs by 4 amino acids, but did not react detectably with SAc3:257 peptide, which differs by 6 amino acids. On protein gel blots, both SAc4:257Ab and SAc1:257Ab reacted with purified chicken actin. In the corresponding amino acid sequence, the chicken actin differed by 2 and 6 residues from the immunogenic peptides. This cross-reactivity with nonimmunogenic sequences suggests that the carboxy-terminal sequence shared by λ- and κ-actin and chicken muscle actin contributes significantly to the immunogenicity of the λ- and κ-peptides. The SAc3:257 μ-peptide, which failed to react with either SAc4:257Ab or SAc1:257Ab, differed in the carboxy-terminal sequence by the nonconservative substitution of a glutamic acid (E) for a glycine (G) present in the other three sequences (Table 1). The subtle differences in antigenic reactivity of peptides as a result of single amino acid changes is discussed in depth in Fieser et al. (1987). It should also be noted that peptide ELISA data may not reflect either the level of antigenicity or degree of specificity of an antiserum toward the intact parent protein (Tainer et al., 1984). Unfortunately, purified κ-, λ-, and μ-actin proteins are not yet available and, thus, could not be used to examine the specificity of the various antisera toward the intact proteins. Nonetheless, the partial specificity of SAc1:257Ab toward κ-actin peptide and of SAc4:257Ab toward λ-actin peptide demonstrated that these antisera could be used to explore the localization of λ-actin- and κ-actin-related proteins in tissue sections.

Tissue-Specific/Developmental Expression of the κ- and λ-Actins

The SAc4:257Ab reacted with protein in the cytoplasm of protodermal cells (Figure 4) but subcellular localization was not apparent. The specific expression of this or any class of actin proteins in the cytoplasms of protodermal tissue and provascular tissue was not anticipated from our knowledge of actin functions in plants. Several explanations are

possible for the observed expression of actin in protoderm. First, the most apical portion of this region of the root is loosely defined as the common initials of a number of cell types in the root. Soybean, like many dicots, has open apical root development in which a common set of initials gives rise to protodermal, epidermal, ground meristem, and root cap tissues, and a separate set of initials gives rise to the vascular cylinder. Possibly, the SAc4-related actin is active during development of the common initials. Second, protodermal tissue is involved in transport processes encompassing both the excretion of proteins and the uptake of nutrients from the soil. Special cytoskeletal requirements might be placed on the cell because of their transport functions. Electron microscope examination of soybean roots revealed numerous actin-like filaments associated with high concentrations of golgi in protoderm (K. Vaughn, personal communication). Alternatively, unique demands may be placed on the cytoplasm of protodermal cells by the tremendous physical pressures generated in an apical root and in lateral root primordia and could be reflected in the cytoarchitecture of the cell and the expression of novel actin proteins.

The low level of SAc4 mRNA expression (Hightower and Meagher, 1985) suggests that SAc4 might be expressed in a unique, defined tissue or cell type. Likely candidates for SAc4 expression would have been root cap cells or the cytokinetic phragmoplast of dividing cells, which are known to contain high levels of actin. However, no reactivity of SAc4:257Ab was seen within the phragmoplast of dividing cells in the apical meristem or with the exterior cells of the root cap (Figure 3). Although not shown in detail, the SAc4:257Ab and SAc1:257Ab antisera did react with the central statocytes of the apical root cap, implying that both antisera may detect actins involved in graviperception (Hensel, 1986; Staiger and Schliwa, 1987).

From our data, the possibility that a low level of λ-actin expression occurs in all root, hypocotyl, stem, and leaf tissues cannot be ruled out. SAc4:257 antiserum from a different rabbit from the one examined herein showed stronger reactivity with all tissues (data not shown) in addition to its strong reactivity with protoderm. When compared with the SAc4:257Ab examined in detail, this antiserum was presumed to have less specificity toward λ-actin proteins. No internal soybean actin protein standards are available for distinguishing class-specific actin expression from weak cross-reactivity of antisera with other classes of actin.

The SAc1:257Ab (κ-actin peptide antiserum) showed reactivity with all root tissues with the exception of root cap. Because κ-actins express high levels of RNA in leaves, hypocotyls, and roots, SAc1:257Ab might be reacting specifically with the κ class of widely expressed plant actins whose distribution includes protodermal tissue. Based on the antigenic specificity determined for this antiserum from ELISAs, it seems equally possible that SAc1:257 reacts with at least two classes, the κ- and λ-actins, but not with all actin classes. It does not react with the SAc3:257 μ-actin peptide in ELISAs or with root cap actin in tissue sections. It is possible that SAc1:257Ab could react with both κ and λ classes of actin in protoderm if both are expressed. In either case, these data on the SAc1:257Ab, and the high degree of specificity of the SAc4:257Ab with protoderm, are consistent with higher levels of λ-class actins in protoderm.

The inability to detect significant levels of λ-actin in protoderm of emerging leaves (data not shown) is surprising, considering that such strong levels of λ-actin expression were detected in protoderm of apical root and lateral root primordia and that SAc4 RNA was detected in leaves, albeit at very low levels. Protoderm in leaves and roots have some developmental and morphological properties in common and might be expected to have common patterns of gene expression. The first two true leaves emerging at 3 and 5 days after germination were examined in this study. The λ-actin may be expressed at earlier or later stages of development than those examined or protein levels for the λ-actin may not directly correlate with mRNA expression in all tissues. The lack of detectable protein expression in leaves is consistent with the proposal that λ-actin is involved in transport in root protoderm.

Plant Actin Divergence Predicts Differential Expression

The differential expression of λ-actin- and κ-actin-related antigens in soybean root tissues and the lack of detection of either antigen in most of the root cap demonstrates that tissue-specific and developmental control may be exerted on the highly divergent plant actins. These results suggest that some of the ancient classes of plant actin genes have been maintained as separate gene lineages over hundreds of millions of years because (1) they are differentially expressed and/or (2) they may encode actin proteins with divergent functions required in these tissues but not in others (Hightower and Meagher, 1986). The extreme diversity that has been observed for actin sequences within a single plant species and the high degree of complexity of some plant actin gene families (Hightower and Meagher, 1986; Baird and Meagher, 1987) may reflect the diversity of roles played by actin in plant cells.

METHODS

Production and Characterization of Antisera to Actin Peptides

The amino acid sequences for residues 257 to 272, predicted from the soybean actin gene sequence for SAc1 (κ class), SAc4 (λ class), and SAc3 (μ class) actins are shown in Table 1 along with other relevant sequences used as controls. SAc3:129 peptide

is from an unrelated region of a μ-actin sequence. These peptides were synthesized on an Applied BioSystems 430A instrument (Merrifield and Barany, 1980) and purified by reverse-phase chromatography on a Brownlee C8 resin (RP-300) elutriated with 0.1% trifluoroacetic acid and a gradient of increasing acetonitrile (0% to 80%). Coupling of the cysteine residues in the peptide to the lysine residues in the carrier protein KLH was performed as described in Green et al. (1982). The coupling reaction for this peptide was monitored by observing the loss of active sulfhydryls in coupled and uncoupled peptides with 5,5′-dithio-bis(2-nitrobenzoic acid) (Ellman, 1959). The estimation of >90% coupling for most reactions suggests 10 to 20 peptide molecules coupled to each KLH molecule. Rabbits were injected once with sufficient peptide-KLH conjugate to contain 100 μg of coupled peptide mixed with Freund's complete adjuvant and again 14 days and 28 days later mixed with Freund's incomplete adjuvant. Ten days after the third injection, rabbits were bled and the IgG fraction was purified by protein A affinity chromatography. After chromatography, the IgG fraction prepared from 1 mL of serum was in a final volume of approximately 6 mL. A monoclonal antiserum to chick actin, No. 350, (Amersham Corp.) was used in several control experiments. The manufacturer states that this antiserum reacts with actin proteins from all eukaryotes tested.

ELISA Titers

ELISA assays on the IgG fractions follow the procedure of Tainer et al. (1984) with the following exceptions. Nonspecific binding was blocked with 5% normal goat antisera diluted in PBS. Horseradish peroxidase-conjugated goat anti-rabbit IgG (Calbiochem) was used to detect the antibody-antigen complex. Titers are expressed as the reciprocal of the antibody dilution, which gives 50% of maximum absorbance of light at 490-nm wavelength. This titer value is also referred to as the half-maximum reaction in the text.

Protein Gel Blots

The reactivity of antisera was assayed on protein gel blots containing 2 μg of purified chicken muscle actin (Sigma) or 15 μg of soybean extract. Soybean crude extract was prepared by grinding with a Polytron 1 g of 5-day-old soybean seedlings (containing emerging leaves, hypocotyls, stems, and roots) in 2 mL of Laemmli buffer (Laemmli, 1970), immediately boiling the mixture for 5 min, and centrifuging to remove debris. Based on unpublished two-dimensional gel data, actin is 0.1% to 0.2% of the total protein, and 15 μg of crude extract should contain only 15 ng to 30 ng of actin. Each divergent actin class should represent an even smaller portion of this sample.

Polyacrylamide gels were electroblotted to Immobilon (Millipore Corp.) (Towbin et al., 1979). Filters were blocked with 3% BSA, 5% goat antisera in Tris-buffered saline (50 mM Tris, 200 mM NaCl, pH 7.4) for 1 hr at room temperature. Primary and secondary antibodies were diluted in blocking solution. Filters were incubated with either 100 μg of anti-peptide IgG or a 1:2000 dilution of an actin monoclonal antibody (Amersham N.350) at 4°C overnight. The antibody-antigen complexes were detected using alkaline phosphatase-conjugated goat anti-rabbit IgG or goat anti-mouse IgM (Sigma) and the substrates (5-bromo-4-chloro-3-indolyl phosphate (165 μm/mL) and nitro blue tetrazolium (330 μm/mL).

Preparation of Soybean Tissues for Light Microscopy

Soybean seedlings were germinated in a pipet washer with constant water circulation overnight at 28°C. Germinated seedlings then were wrapped loosely in cheesecloth and were given a 1-min spray of water every 3 hr for the next 3 days to 5 days. Four regions of the seedlings were sampled: 5-mm-long apical root tips, 3 × 3-mm sections of root containing emerging root primordia, lateral roots 50 mm up from the apical root, and leaves. Tissues were fixed overnight at 4°C in a solution of 4% paraformaldehyde, 0.5% sucrose neutralized with NaOH. Tissues were passed from fixative through PBS, ethanol, and xylene to paraffin over a 10-hr period. Sections 8 μm thick were deparaffinized immediately before the first day of the immunogold procedure.

Immunogold Localization

The 2-day immunogold localization procedure follows the recommendations of Janssen Life Sciences Products with more extreme blocking conditions and the addition of extra washes. After rinsing in BSA-Tris (2.5% BSA in PBS, 0.13 M NaCl, 0.01 M sodium phosphate, pH 7.2), sections were incubated 6 hr at 4°C in 100 μL to 200 μL 100% goat serum. Excess goat serum was then removed but not washed from the slide. For primary antibody incubations, sections were incubated with either 5 μg or 10 μg of anti-peptide IgG or a 1:200 dilution of actin monoclonal antibody in 200 mL of 5% goat antiserum in PBS and placed in a humid chamber at 4°C overnight. Following primary antibody incubation, slides were washed 4 × 10 min with PBS. Secondary antibody (Janssen AuroProbe LM goat anti-rabbit IgG or goat anti-mouse IgM colloidal gold) was diluted 1:80 in 5% goat antiserum in PBS and incubated 1 hr at room temperature. Slides were rinsed 3 × 10 min and 2 × 3 min in PBS and 3 × 3 min in distilled water.

For the silver enhancement of the antigen-antibody complex, silver grains were allowed to develop in enhancer-initiator for 3 min to 5 min and then sections were rinsed briefly with distilled water. The enhancement was repeated with a fresh mixture of enhancer and initiator and this reaction was monitored using the 10× objective under bright-field. When silver grains were sufficiently developed (4 min to 6 min), the reaction was stopped by quickly rinsing off enhancer-initiator, followed by 2 × 5 min washes in glass-distilled water. Recent versions of the Janssen AuroProbe Kit require as long as 20 min for silver grain development.

Slide Preparation

Semi-permanent slides were prepared by applying a DAPI-glycerol mixture (20 μm/mL DAPI, 0.1 × PBS, 10 mM Na azide, 90% glycerol) to a coverslip before placing the coverslip on the slide. Slides can be kept 6 months when stored in a closed container at 4°C.

Bright-field and epifluorescent photomicrographs were taken of silver-enhanced immunogold-stained sections using a 10× or 40× Zeiss Neofluor lens, Kodak Technical Pan Film 2415, and Kodak HC110 developer diluted 1:36. Exposures for bright-field and epifluorescence photomicrography were approximately 1/30 sec and 4 sec to 8 sec, respectively.

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