Biochem. J. (1992) 281, 501–505 (Printed in Great Britain)

The pattern of plant annexin gene expression

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Peptide sequence data derived from a plant annexin, P34 [Smallwood, Keen & Bowles (1990) Biochem. J. **270**, 157–161] was used to design amplimers for PCR. A unique fragment of 95 bp, amplified from tomato (*Lycopersicon esculertum*) genomic DNA, was used in Northern analyses and demonstrated a differential pattern of expression in vegetative tissues of tomato, potato (*Solanum tuberosum*) and barley (*Hordeum vulgare*). The tissue-specific abundance of the annexin transcript was found to correlate closely with abundance of annexin protein as revealed by their partial purification and analysis with antisera specific for annexins isolated from tomato suspension-culture cells.

INTRODUCTION

Annexins are a homologous family of proteins, characterized by a Ca^{2+}-dependent affinity for phospholipid [reviewed by Smith & Dedman (1990), Crompton *et al.* (1988), Geisow *et al.* (1987), Glenney (1987) and Geisow & Walker, 1986]. At least eight annexins have been identified to date, including two (annexin 1 and annexin 2) that are of particular interest since they act as substrates for tyrosine kinases and may therefore play key roles in cell-signalling processes (Erikson & Erikson, 1980; Fava & Cohen, 1984; Sawyer & Cohen 1985; Guigni *et al.*, 1985; De *et al.*, 1986; Pepinsky & Sinclair, 1986; Sheets *et al.*, 1987; Karasik *et al.*, 1988; Blay *et al.*, 1989). Recently we have shown that proteins with properties similar to those of annexins can be found in plant cells (Boustead *et al.*, 1989). Detailed analysis of the purified proteins revealed significant sequence similarity to the annexins isolated from other eukaryotes (Smallwood *et al.*, 1990). The two plant proteins, P34 and P35, isolated from tomato suspension-culture cells contained regions nearly identical with the 70-amino-acid repeat domain common to all known annexins. Other regions did not show sequence identity, suggesting some modification to the structure, and possibly the function, of the proteins in plants and animals.

As an efficient entry into the study of annexin gene expression, we have used information from the peptide sequence data to design amplimers for use in the PCR to generate annexin DNA. This has enabled us to determine the pattern of annexin gene expression by Northern analyses of RNA prepared from tissues of tomato and other plant species. We have compared results at the Northern level with the pattern obtained from Western analyses of the same tissues. The results suggest that annexin gene expression is developmentally regulated in plants and the family of annexins is conserved in both dicotyledonous and monocotyledonous plant species.

EXPERIMENTAL

Preparation of plant tissues

Tomato (*Lycopersicon esculentum*, hybrid L2-14, Edinburgh) suspension culture cells were grown, harvested and washed as described by Smallwood *et al.* (1990).

Tomato (*L. esculentum* var. Money Maker) plants were grown from seed on compost in 20 cm × 30 cm (8 in × 12 in) seed trays at a density of approximately 100 plants per tray. Plants were grown under a 16 h photoperiod with light supplied by fluorescent tubes to give a photon lux density of 580 $\mu E \cdot s^{-1} \cdot m^{-2}$ in a temperature regime of 22 °C (light), 18 °C (dark) and a 60% relative humidity as described in Doherty *et al.* (1988). Plants were harvested at 21 days and dissected into peripheral roots, stem, unexpanded apical leaflets and mature leaves.

Barley (*Hordeum vulgare*) grain was soaked in distilled water at 4 °C for 18 h and sown on wet vermiculite. Seedlings were grown under a 16 h photoperiod with light supplied by fluorescent tubes to give a photon lux density of 300 $\mu E \cdot s^{-1} \cdot m^{-2}$ at a constant temperature of 17 °C and 70% relative humidity. Seedlings were harvested at 7 days as described by Clarke *et al.* (1991). The coleoptile was removed and the root tissue was separated; the shoot was divided into a lower region containing the meristematic leaf base and an upper region.

Potato (*Solanum tuberosum*, spp. *tuberosum*, var. Maris Piper) plants were grown as described by Hammond-Kosack *et al.* (1991) and harvested at 14 days. Material was divided into root, stem and leaf.

Generation of plant annexin DNA

High-molecular-mass genomic DNA was prepared (Jofuku & Goldberg, 1989) from tomato plants. Plant DNA (500 ng) was combined in a final reaction volume of 50 μl containing Amplitaq buffer (Cetus), 200 μM-dNTP mix (Pharmacia) and 100 pmol of each primer, N347 and C345 (Fig. 1). The reaction mixture was denatured at 95 °C for 5 min, and 2 units of *Taq* polymerase (Cetus) added. The reaction mix was overlaid with 50 μl of mineral oil and incubated for 40 cycles of 95 °C for 1 min and 72 °C for 1 min in an LRP mark III programmable heater. PCR products were ethanol-precipitated and electrophoresed through a 2% Nusieve GTG LMP agarose gel (FMC Bioproducts, Rockland, ME, U.S.A.).

The band was excised and microcentrifuged (13000 *g* for 5 min) through Spin-X filters (Costar) to recover the DNA for sequencing using the Pharmacia T7 polymerase system or to hexaprime label (Feinburg & Vogelstein, 1984) with [^{32}P]dCTP. PCR products from five different amplifications were combined and sequence data derived, in both directions, on three separate gels.

Abbreviations used: PBS, phosphate-buffered saline (145 mM-NaCl/10 mM-sodium phosphate, pH 7.4).

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Northern-blot analysis

RNA (10 μg/track) was electrophoresed through 1.4% agarose/0.6 M-formaldehyde gels containing ethidium bromide (0.5 μg/ml) (Davis *et al.*, 1986). The gels were blotted on to Hybond-N membrane (Amersham), prehybridized, then hybridized to labelled hexaprimer fragment at 42 °C in 50% (v/v) formamide (Gurr & McPherson, 1991). After hybridization, the membranes were washed to a final concentration of 0.1 × SSC (1 × SCC is 0.15 M-NaCl/0.015 M-sodium citrate) + 0.1 SDS at 42 °C before exposure to X-ray film at −70 °C.

Purification and analysis of annexin proteins

Annexins were purified from 3.5 g wet weight of tissue as described previously (Boustead *et al.*, 1989) with the following modifications. Tissue was homogenized in 12 ml of buffer A [50 mM-Hepes/NaOH (pH 7.4)/0.15 M-NaCl] using a Polytron instrument (setting 4; 3 × 10 s bursts) and filtered through one layer of Miracloth. Before centrifugation, 0.2 M-EGTA was added to give a final concentration of 10 mM and the filtrate was incubated on ice for 15 min. Annexins were selectively precipitated using Ca^{2+} and exogenous phospholipid as described previously (Boustead *et al.* 1989) and re-extracted using buffer A, containing 15 mM-EGTA, into a final volume of 5 ml.

Protein present in 1 ml of extract (derived from 0.7 g wet weight starting material) was precipitated using 12% trichloroacetic acid and centrifuged for 10 min at 10000 *g*. The pellets were washed three times in ice-cold acetone and resuspended in 40 μl of SDS/PAGE sample buffer (Laemmli, 1970). After being shaken for 45 min on a mixer, samples were boiled for 4 min, centrifuged for 3 min at 10000 *g* and the supernatants were transferred to clean Eppendorf tubes. Aliquots (10 μl) were applied to 0.5 mm-thick SDS/10%-PAGE gels (LKB midget system) (Laemmli, 1970).

Proteins separated by SDS/PAGE were electrophoretically transferred on to nitrocellulose as described by Towbin *et al.* (1979) or stained in the gel with 0.1% Brilliant Blue in acetic acid/methanol/distilled water, 1:4:5 (by vol.) and destained in isopropan-2-ol/acetic acid/distilled water (5:4:31, by vol.).

Preparation of antisera to plant annexins

Annexins were purified from tomato suspension-culture cells as described by Boustead *et al.* (1989) and separated by SDS/PAGE. The P34 band was excised and injected intramuscularly into rats, together with Freund's adjuvant, at fortnightly intervals. At 10 days after the final injection of antigen, antiserum was collected. The antiserum was not cross-reactive with P35, the higher-molecular-mass annexin isolated from tomato suspension-culture cells, nor with proteins remaining in solution after the selective precipitation of annexins with Ca^{2+} and phospholipid (results not shown). Preimmune serum showed no reaction with annexins (results not shown). Antiserum raised in sheep against celelectrin, an annexin derived from *Torpedo marmorata* (electric eel) was used to detect P35 (the antiserum was kindly given by Dr. J. H. Walker, Department of Biochemistry and Molecular Biology, University of Leeds, Leeds, U.K.).

Western-blot analysis

Partially purified annexins separated by SDS/PAGE were transferred on to nitrocellulose as described by Towbin *et al.* (1979) and incubated in phosphate-buffered saline (PBS) containing 2 mg of BSA for 1 h to block unoccupied sites. The blots were then incubated in 1:200 dilution of antiserum in PBS/BSA for 24 h with continuous agitation, followed by 10 min washes in: (i) PBS, (ii) PBS/0.5% Tween 20 and (iii) PBS/0.5% Tween 20/0.5 M-NaCl. Horseradish peroxidase-conjugated anti-rat IgG or anti-sheep IgG antibody (Sigma) was used to reveal cross-reactive proteins.

Peptide mapping

Polypeptides were partially digested by *Staphylococcus aureus* V-8 proteinase using the 'in-gel' method of Cleveland (1983). Products of proteolysis, separated on 1.5 mm-diameter SDS/15%-PAGE gels (LKB midget system), were silver-stained as described by Merril (1990).

RESULTS

Generation of a plant annexin DNA probe

A previous study (Smallwood *et al.*, 1990) provided sequence data from peptides derived from *Staphylococcus aureus* V8 proteinase digestion of annexin polypeptides. Information in one sequence taken from a 16 kDa peptide of annexin P34 was used to generate oligonucleotide primers for PCR. The primers (N347 and C345), designed to incorporate inosine residues at positions of multiple redundancy (see Fig. 1), could potentially hybridize respectively to 2048 and 8192 complementary sequences. Despite this high redundancy, a unique 95 bp fragment was amplified from tomato genomic DNA as shown in Fig. 1. Under the conditions described, no 'artefactual' bands were observed in ten independent amplification experiments. Fig. 2 shows the nucleic acid sequence from the 95 b.p. fragment generated from genomic DNA of tomato. Comparison of the predicted amino acid sequence of the amplified PCR product from tomato with the known sequence of the authentic P34 peptide fragment indicates a difference in four out of the 32 amino acid residues (positions 13, 18, 19 and 23 in Fig. 2).

Developmental regulation of plant annexin gene expression

The PCR-amplified product from tomato genomic DNA was used as a probe in Northern analyses of total RNA prepared from the tissues of tomato, potato and barley. The results are shown in Fig. 3. The DNA probe hybridized to a transcript of 1.1 kb that corresponds in size to that expected for mRNA coding the annexin polypeptide. A differential pattern of expression was observed in analyses of roots, stems and leaves. In 21-days-old tomato plants, the transcript was most abundant in roots and less in stem tissue. When leaves were analysed, only the youngest, non-expanded, apical leaflets expressed annexin RNA. No transcript was detectable in more mature leaves. When the blots were stripped and rehybridized with a cDNA probe encoding the small subunit of ribulose-bisphosphate carboxylase (Eckes *et al.*, 1985), identical levels of expression in both samples of leaf tissue were observed (Fig. 3*b*). A similar pattern of annexin gene expression was also observed when the tomato DNA probe was used in Northern analysis of RNA extracted from 14-day-old potato plants (Fig. 3). Similarly, in analyses of RNA extracted from barley seedlings, the heterologous probe also detected a transcript of 1.1 kb, which showed the highest level in the roots. The abundance was lower in the leaf base, and no transcripts could be detected in the upper part of the leaf.

Levels of annexin protein in different plant tissues

Owing to the low affinity of available antisera and the low abundance of annexins in some vegetative tissues of plants, it proved necessary to purify the proteins partially before Western analyses. The purification utilized the annexin characteristic of Ca^{2+}-dependent interaction with phospholipid to precipitate the proteins selectively from an EGTA extract of plant tissues (see the Experimental section for details). This procedure was applied

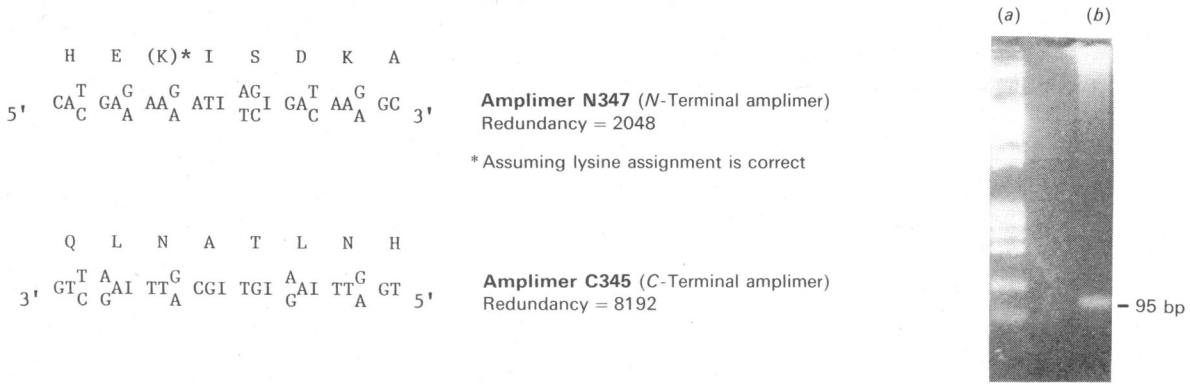

Fig. 1. Amplification of the 95-bp probe to tomato annexin P34

Primers were designed based based on the tomato P34 sequence (Smallwood *et al.*, 1990) and hybridized to tomato genomic DNA in the PCR. (*a*) Markers; (*b*) products of PCR.

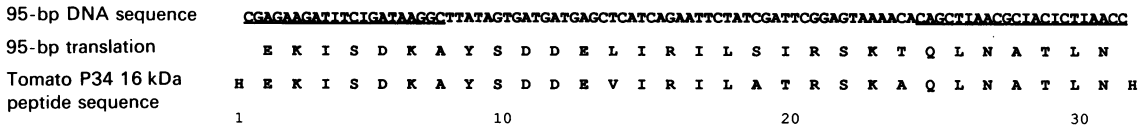

Fig. 2. Sequence of the PCR product aligned with the P34 amino acid sequence on which amplimers were based

Primer sites are underlined. The first two 5′ and the last 3′ base of the 95 bp product were not determined.

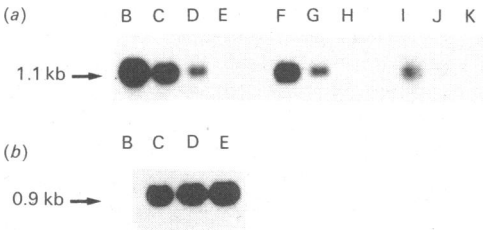

Fig. 3. Northern blots of total RNA: (*a*) probed with 95-bp PCR-amplified fragment; (*b*) probed with small subunit of ribulose-bisphosphate carboxylase

Lane B, tomato roots; C, tomato stems; D, young tomato leaves; E, mature tomato leaves; F, potato roots; G, potato stems; H, potato leaves; I, barley roots; J, barley leaf bases; K, barley leaves.

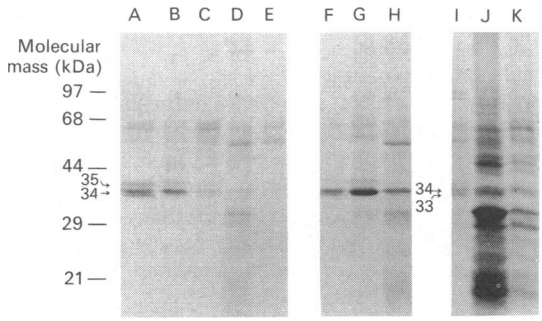

Fig. 4. SDS/PAGE analysis of an extract in annexins

Lane A, tomato suspension-culture cells. Lanes B–K are as in Fig. 3.

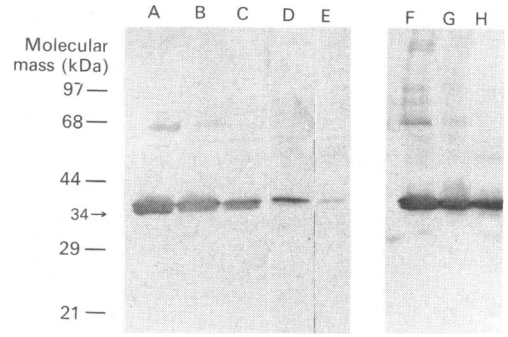

Fig. 5. Western blot of an extract enriched in annexins, stained with anti-P34 antiserum

A nitrocellulose blot of a gel identical with that shown in Fig. 4 was probed with antiserum raised against P34 derived from tomato suspension-culture cells. The lanes are as in Fig. 4.

to the same plant tissues that had been analysed in Northern blots and described above. Whether losses during purification procedure are equivalent from all tissues studied is not known.

After separation of the polypeptides by SDS/PAGE, the products were either revealed by staining with Coomassie Blue (Fig. 4) or blotted on to nitrocellulose and probed with antisera specific for P34 (Fig. 5) or P35 (Fig. 6). Track A of Figs. 4 shows the two polypeptides which can be extracted from tomato suspension-culture cells using this method and have been referred to previously as 'P34' and 'P35' (Smallwood *et al.*, 1990). Tracks B–E correspond to the polypeptides which are extracted when an identical procedure is applied respectively to roots, stems and leaves of 21-day-old tomato plants. The doublet of polypeptides is clearly visible in root and stem tissue, but additional polypeptides are also present, particularly in leaf tissue. Tracks F–H show equivalent samples, but which were prepared from potato plant tissues. Again a doublet of characteristic molecular mass is present, as well as additional polypeptides. When the procedure was applied to barley seedling tissues, a much more complex pattern of polypeptides was revealed (tracks I–K), although

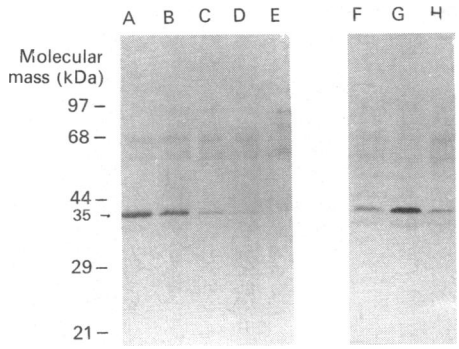

Fig. 6. Western blot of an extract enriched in annexins, stained with antiserum specific for P35

The lanes are as in Fig. 4.

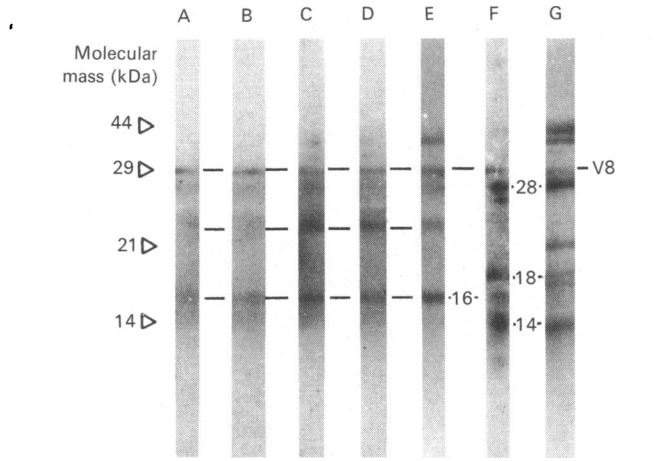

Fig. 7. Cleveland maps of tomato, potato and barley annexins

Lane A, potato P34 (lower band); B, tomato P34 (lower band); C, potato P34 (upper band); D, tomato P34 (upper band); E, authentic P34 from tomato suspension-culture cells; F, barley P33; G, authentic P35 from tomato suspension-culture cells. V8, *Staphylococcus* V8 proteinase; 14, 16, 18 and 28, molecular masses of peptide fragments.

again products of molecular mass 34 and 33 kDa were visible in extracts from root and stem tissue.

Samples were also probed with antisera specific for authentic P34 or P35 from tomato suspension-culture cells. The results in Fig. 5 arise from probing the blot with antisera raised against tomato P34 and reveal a closely apposed dimer of molecular mass 34 kDa in both potato and tomato tissues. As yet it is not known whether the dimer arises from 'artefactual' proteolysis during protein extraction, alternative post-translational modifications or represents a separate gene product; all these explanations have precedents from studies of animal annexins (Glenney, 1986; Roy Choudhury *et al.*, 1988; Ando *et al.*, 1989*a*). The two members of the dimer are, however, related to P34 rather than P35, as shown by their cross-reaction with antisera specific for P34 (Fig. 5), the absence of cross-reaction with anti-calelectrin antiserum (Fig. 6) and the similarity of their peptide maps (Fig. 7). No material cross-reactive with anti-P34 antiserum could be detected in blots of barley extracts (results not shown). This indicates that epitopes have not been conserved between the distantly related monocot species and members of the Solanaceae.

Since antisera raised against tomato P35 also cross-reacted with both P34 bands (results not shown), an antiserum raised against calelectrin derived from *Torpedo marmorata* (previously characterized; Boustead *et al.*, 1989; Smallwood *et al.*, 1990), was used to detect specific polypeptides antigenically related to P35. The results are shown in Fig. 6 and demonstrate a pattern of cross-reactive products very similar to that shown for P34 in Fig. 5.

Comparison of peptide maps of plant annexin polypeptides

In order to confirm the similarity in proteins extracted from the different plant tissues, polypeptides in the region of 34 kDa were excised from gels after SDS/PAGE and subjected to digestion with *S. aureus* V8 proteinase as described by Smallwood *et al.* (1990). The results are shown in Fig. 7 and compare polypeptides from tomato, potato and barley with authentic P34 and P35 isolated from tomato suspension-culture cells. The results confirm the pattern is near-identical for the proteins from tomato and potato tissues. The 34 kDa polypeptide derived from barley appears to share some peptides in common with annexins derived from members of the Solanaceae.

DISCUSSION

Previous work from our laboratory provided good evidence for the existence of annexins in higher plants (Boustead *et al.*, 1989; Smallwood *et al.*, 1990). The two polypeptides, P34 and P35, purified from tomato suspension-culture cells, contained sequences similar to those of other eukaryotic annexins and interacted with phospholipid in a Ca^{2+}-dependent manner. The aim of the present work was to investigate the distribution of annexins in differentiated plant tissues and to broaden the studies into a range of other plant species.

PCR amplification of tomato genomic DNA, using highly redundant primers based on tomato P34 amino-acid-sequence data, yielded a unique 95-bp fragment (Fig. 1). The tomato DNA sequence data were of sufficient quality to demonstrate that the fragment corresponded to a plant annexin sequence. All other eukaryotic annexins contain multiple repeats of the P34 sequence on which the amplimers were based (Pepinsky *et al.*, 1988). Translation of the PCR-amplified tomato DNA sequence revealed four amino acid residues which differ from the peptide sequence derived from P34. It is possible that an alternative repeat from P34 or P35 has been amplified, accounting for the anomalies between DNA and protein sequence data.

The pattern of annexin gene expression was studied using the PCR-amplified tomato annexin fragment as a homologous and heterologous probe in Northern analyses. In tomato and potato plants, highest transcript levels were found in root tissue, less in stem and least in young expanding leaves, with no hybridization signal detectable in mature tomato leaves. A similar distribution of annexin transcripts was also found in the unrelated monocot, barley. Since P34 and P35 contain near-identical sequence over the region covered by the 95-bp probe (Smallwood *et al.*, 1990), it is possible that it will hybridize to transcripts encoding one or both of the polypeptides. Only one hybridizing band is present in the Northern analyses, but the transcripts encoding P34 and P35 are predicted to be of a very similar length and hence would not be resolved on the gel system used.

Since transcript abundance is not necessarily reflected at the protein level, tissues identical with those used in Northern analyses were subjected to an annexin-enrichment protocol and the results were analysed by SDS/PAGE and immunoblotting. Figs. 4 and 5 demonstrate that the distribution of P34 protein between tomato and potato plant tissues correlates well with the pattern determined by Northern analyses. The spatial pattern of P35 expression was essentially identical with that found for P34 in tomato plants (Fig. 6). The potato data indicate greater

abundance in stem of the 37 kDa polypeptide that cross-reacts with anti-calelectrin antiserum.

It may be noted that a more complex mixture of polypeptides was enriched from tomato and potato leaf tissue. Mammalian annexins are encoded by a multi-gene family, and the expression of different members of the family is developmentally regulated (Braslau *et al.*, 1984; Gould *et al.*, 1984; Smith & Dedman, 1986; Celeste Carter *et al.*, 1986; Glenney *et al.*, 1987; Saihto & Miret, 1987; Lozano *et al.*, 1989; McKanna & Cohen, 1989). The additional polypeptides in leaf tissue may thus represent related, but distinct, annexins, possibly substituting functionally for those which have been characterized. More likely these polypeptides share features with annexins which result in their co-purification during the enrichment protocol. This is also implied by their lack of cross-reactivity with antisera and the absence of hybridizing transcripts in mature tomato leaves. In this context, a 60 kDa polypeptide cross-reactive with antiserum raised against tomato P34 was enriched from tomato suspension-culture cells and root tissues from tomato and potato. It is not yet clear whether this polypeptide represents a separate gene product or if it is a result of P34 polymerization, for example by a transglutamination reaction, for which there are precedents in mammalian systems (Pepinsky *et al.*, 1989; Ando *et al.*, 1989*b*).

A much more complicated pattern of polypeptides was enriched from barley seedling tissues. Although none of the barley polypeptides interacted with either of the antisera used in the present study, peptide maps of the putative barley annexins did indicate a similarity with tomato annexins. It remains to be seen whether either of these two barley proteins is encoded by the mRNA hybridizing to the tomato 95-bp probe.

The biological implications of the plant annexins distribution established in the present study must remain speculative, since their function has yet to be determined. The data eliminate leaf-specific functions for the two plant annexins, P34 and P35. The high abundance of the proteins in root and stem tissue could be correlated with specialized functions of these tissues; however, the highest abundance of annexin protein was found in undifferentiated suspension-culture cells, which might suggest a more generalized role in plant cell function. This function could, for example, be related to the secretory activity of these cells, which are known to export large quantities of pectin into the extracellular matrix (Smallwood *et al.*, 1990) At least one mammalian annexin has been shown to be involved in secretion (Ali *et al.*, 1989).

It has been demonstrated in other eukaryotic systems that the expression of some annexins is up-regulated in proliferating as compared with quiescent cells (Schlaepfer & Haigler, 1990; Keutzer & Hirschorn, 1990). Such a correlation would also be suggested in the tomato plant system, since neither message nor protein could be detected in mature leaves, the only organ system assayed in which cell division had ceased.

Antiserum to calelectrin was kindly provided by Dr. J. H. Walker (University of Leeds). M.S. acknowledges support of a Science and Engineering Research Council studentship..

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Received 5 April 1991/6 June 1991; accepted 13 June 1991