Rapid Communication

cDNA Isolation and Gene Expression of the Maize Annexins p33 and p35[1]

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The isolation, cloning, and sequencing of two full-length cDNAs corresponding to the root tip forms of the maize (*Zea mays* L. cv Clipper) annexins p33 and p35 are described. These are the first complete sequences for the widely reported doublet of plant annexins. The predicted sequences can be divided into four repeat domains characteristic of the annexin family, but Ca^{2+} binding by the type-II site typical of annexins would be predicted to occur only in repeats 1 and 4. This reduced number of sites is consistent with previously reported biochemical data indicating a high Ca^{2+} requirement for membrane association. Although the two annexins are very similar (80% amino acid identity), their genes are quite distinct, as demonstrated by their different 3' noncoding regions and Southern blotting. The predicted sequences of the root tip proteins are very similar to regions known from peptide sequencing of the coleoptile proteins. Because a rather small gene family is indicated, the implication is that there may be less functional diversity than in animal cells. Furthermore, the sequence data clearly show that plant annexins form a very distinct group compared with those from other kingdoms.

Annexins are a family of proteins of conserved sequence that associate with phospholipid membranes in the presence of Ca^{2+}. A variety of observations suggest that in animal cells annexins may be regulators of Ca^{2+}-dependent exocytosis (see Creutz, 1992; Raynal and Pollard, 1994). In plants exocytosis is a critical process for cell growth and development, and Ca^{2+} plays an important role in its regulation (Battey and Blackbourn, 1993; Battey et al., 1996). Cell biological and biochemical studies have provided circumstantial evidence that plant annexins may be involved in exocytotic regulation, but knowledge is currently very incomplete (see Clark and Roux, 1995).

The maize (*Zea mays* L.) annexins p33 and p35 bind to and cause aggregation of artificial and native vesicles (Blackbourn et al., 1991; Blackbourn and Battey, 1993), but

the Ca^{2+} concentration required is in the submillimolar range, raising a question about the physiological relevance of this property. Consistent with this high Ca^{2+} requirement, peptide sequence alignments with mammalian annexins indicated that the full complement of Ca^{2+}-binding sites (three to four sites) may be absent from the maize proteins (Blackbourn et al., 1992). Predicted sequences from partial cDNA clones of annexins from other plants are in agreement with this (see Clark and Roux, 1995). Based on experience with animal annexins, in which many distinct family members have been identified, it is also likely that different cell and tissue types contain distinct complements of annexins, and that different annexins are involved in the regulation of distinct cellular processes (see Gruenberg and Emans, 1993; Raynal and Pollard, 1994).

To make progress in understanding these aspects of annexin function, we have isolated, cloned, and sequenced two full-length annexin cDNAs from a maize root tip library. These cDNAs encode proteins with predicted amino acid sequences very similar to those regions of the coleoptile annexins p33 and p35 that are known from peptide sequencing (Blackbourn et al., 1992). Here we report these sequences, some characteristics of the proteins encoded, and the expression pattern of the annexin genes.

MATERIALS AND METHODS

Seeds of *Zea mays* L. cv Clipper were obtained from J.W. Hull (Rainham, Kent, UK). Root tips consisting of the apical 5 mm of root tissue were harvested after seeds had been allowed to imbibe overnight in running water, germinated on moist filter paper, and grown for 1 d at 28°C. The root tips were immediately frozen in liquid nitrogen and stored at −70°C until use.

RNA Isolation, cDNA Library Construction, and Screening

Total RNA was isolated from 2.5 g of root tips by the method of Jepson et al. (1991). Poly(A)$^+$ RNA was purified from total RNA using the Poly ATtract system (Promega). Double-stranded cDNA was synthesized from poly(A)$^+$ RNA, cloned into Uni-Zap XR (Stratagene), and packaged (Gigapack II, Amersham). Library screening was as described in the Uni-Zap XR instruction manual (Stratagene), and in vivo excision was according to the method of Sambrook et al. (1989). Prehybridization was in 5× SSPE, 1% low-fat milk powder, 0.5% SDS, and 200 $\mu g\ mL^{-1}$ sheared salmon sperm DNA; hybridization was in the same buffer

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without salmon sperm DNA. The filters were washed three times in 3× SSC, 0.1% SDS. Hybridization and wash temperatures were 42°C to screen for annexin p35 or 55°C to screen for annexin p33. After secondary and tertiary screening, DNA was prepared from in vivo-excised plasmids using the Wizard or Magic Miniprep kit (Promega).

DNA Sequencing

DNA was sequenced using a Sequenase 2.0 kit (United States Biochemical). Intelligenetics (Mountain View, CA) software (IG-Suite and PCGENE) was used for sequence compilation and analysis; the sequence alignment in Figure 1 was obtained using the Clustal program.

Southern and Northern Blotting

Maize genomic DNA was isolated as described by Larkin et al. (1989). DNA (15 μg) was digested with either EcoRI, HindIII, or BamHI and the restriction fragments were sep-

Figure 1. Amino acid sequences of maize annexins p33 and p35 predicted from their root-tip cDNAs (ZMANNp33, ZMANNp35) and aligned against predicted annexin sequences for alfalfa (MSANN; Pirck et al., 1994), strawberry (FAANN; Wilkinson et al., 1995), and human annexin XIIIa (HSANNXIII; Wice and Gordon, 1992). The numbers 1 to 4 above the top sequence refer to characteristic annexin repeat domains as applied to HSANNXIII (derived from Raynal and Pollard, 1994). Open boxes indicate the critical residues for type-II Ca²⁺-binding sites in animal annexins (Huber et al., 1990; Weng et al., 1993). Amino acid identities (*) and conservative substitutions (.) are indicated.

arated on a 0.9% (w/v) agarose gel. DNA was transferred to a nylon membrane (Hybond N, Amersham) as described by Sambrook et al. (1989). Hybridization was as described for library screening, except that the temperature was 65°C.

Total RNA was isolated from 1-mm segments from the root tip using the method described above. The RNA was fractionated on 1.2% (w/v) formaldehyde agarose gels, and blotted in 20× SSPE onto a nylon membrane. Prehybridization and hybridization were in the same buffer as for library screening, except that 50% (v/v) formamide was included. Ten micrograms of RNA was loaded per lane, quantified by measurement of A_{260}, and confirmed by rRNA staining with ethidium bromide in gels and on blots. Oligonucleotides were end-labeled with [γ-^{32}P]ATP as described by Sambrook et al. (1989); gel-purified PCR products and cDNA fragments were labeled with [α-^{32}P]dCTP by random priming using kits under the conditions recommended by the suppliers (Pharmacia, Stratagene).
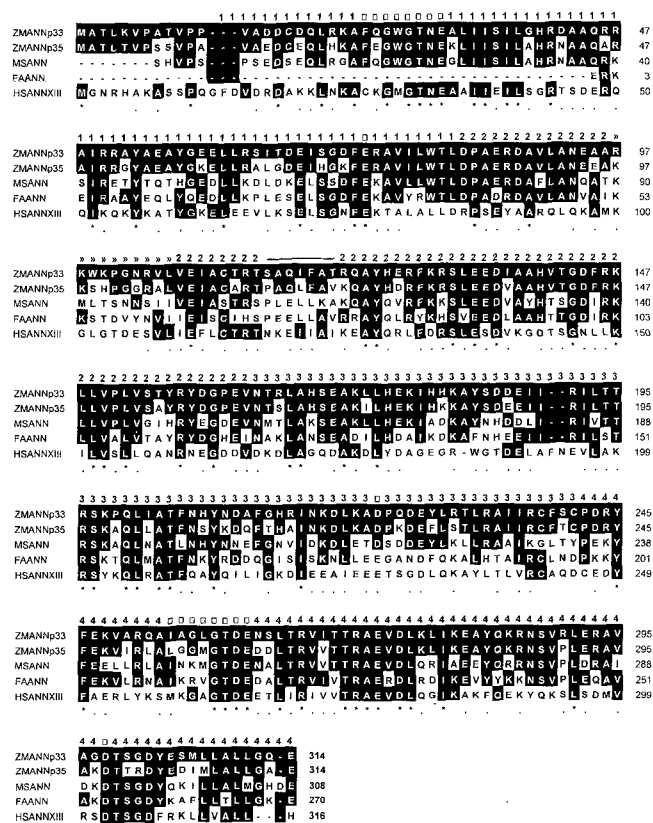
PCR

Degenerate primers were designed based on the annexin peptide sequence for p33 from maize (see Blackbourn et al., 1992) and were used in conjunction with an oligo-dT primer to amplify an annexin sequence from cDNA synthesized from root tip poly(A)⁺ RNA (see above). For the synthesis of 3′ noncoding region probes to p35 and p33, 3′- and 5′-specific primers were used. In all cases reactions were carried out using 1.3-unit Taq polymerase PCR buffer (Perkin-Elmer) and 0.25 mM ultrapure dNTPs (Pharmacia). Between 21 and 35 cycles were run at annealing temperatures of 42 to 60°C, according to the primer, using a Techne PHC-1 (Scotlab, Strathclyde, UK). PCR products for labeling were electrophoresed in 1.5 to 2% agarose gels, and the DNA was eluted from the gel using a kit (QIAEX, Qiagen, Dorking, UK). When necessary, the products were cloned into the PCR vector kit using a cloning kit (TA kit, Invitrogen, Abingdon, Oxon, UK).

RESULTS

Isolation of Annexin cDNA Clones

Degenerate oligonucleotides were designed based on amino acid sequences of known annexin peptides from maize coleoptiles (Blackbourn et al., 1992). Direct screening of the maize root tip cDNA library with these oligonucleotides as probes was unsuccessful. However, PCR employing an oligonucleotide (AAGGCICAG/AATITTC/TG-CIAC) based on the coleoptile p33 peptide sequence (Blackbourn et al., 1992) (the equivalent region predicted by the cloned root tip p33 cDNA is overlined in Fig. 1) and the oligo-dT primer amplified an approximately 600-bp product that sequencing and database searches revealed to be similar to the annexin family of proteins. When the library was screened using this fragment as a probe, approximately 100 positives were obtained from 240,000 plaque-forming units, of which 10 were excised in vivo and sequenced at the 5′ and 3′ ends. The partial sequences revealed seven identical, annexin-like cDNAs of differing lengths. The longest cDNA clone was sequenced and its

	ZMANN33	ZMANN35	MSANN	FAANN	CAANN	HSANNXIII
ZMANN33						
ZMANN35	80	—				
MSANN	60	60	—			
FAANN	56	56	56	—		
CAANN	63	60	77	53	—	
HSANNXIII	35	34	35	36	35	—

Figure 2. Comparison of annexin identities. The percentage identities were calculated pairwise for the amino acid sequences predicted from coding regions of full-length annexin cDNAs from maize (ZMANN33, ZMANN35), bell pepper (CAANN; Proust et al., 1996), and humans (HSANNXIII; Wice and Gordon, 1992), and partial cDNAs from alfalfa (MSANN; Pirck et al., 1994) and strawberry (FAANN; Wilkinson et al., 1995). The identities were calculated using the PALIGN program in PCGENE (Intelligenetics).

predicted amino acid sequence is shown in Figure 1 (top sequence row); in the 43-amino-acid region corresponding to the coleoptile p33 peptide sequenced by Blackbourn et al. (1992) there are seven differences. The open reading frame encodes a protein of 35.6 kD, which has a high degree of identity with partial annexin cDNAs from *Medicago sativa* (Pirck et al., 1994) and *Fragaria ananassa* (Wilkinson et al., 1995). The most similar nonplant annexin is the "intestine-specific" form of human annexin I, now classed as annexin XIIIa (35% identity; see Figs. 1 and 2) (Wice and Gordon, 1992; Fiedler et al., 1995). Expression in *Escherichia coli* yields a 34-kD protein on SDS-PAGE, which strongly cross-reacts with antibodies to coleoptile p33/p35 (data not shown).

These observations led us to the conclusion that we had isolated the root tip isoform of the maize coleoptile annexin p33. To isolate cDNAs encoding p35, we synthesized an oligonucleotide to the region most dissimilar in the p33 predicted sequence and the known p35 peptide sequence (double arrowheads in Fig. 1). This was used, in conjunction with a restriction fragment of p33 from the *Eco*RI cloning site at the 5' end to a *Sca*I site at base 555, to make a differential screen for p35. The p33 restriction fragment was used as a general probe to identify clones with similarity to annexins in the primary screen; 36 good positives were picked and taken to a secondary screen, this time using the restriction fragment and oligonucleotide in hybridizations on duplicate lifts. One colony from each filter that hybridized to the p33 restriction fragment but showed no hybridization to the oligonucleotide was selected for in vivo excision. Of the 12 clones selected in this way, 8 proved to contain inserts that appeared on sequencing to be cDNAs of different lengths derived from a common annexin mRNA very similar to coleoptile p35. The complete predicted amino acid sequence is shown in Figure 1

(second sequence row). In the 113-amino-acid region corresponding to coleoptile p35 peptides sequenced by Blackbourn et al. (1992) there are 13 differences. The open reading frame encodes a protein of 35.3 kD, and alignment with the predicted sequence for p33 reveals a high degree of identity (80%), with the expected divergence in the region used to design the oligonucleotide (Fig. 1). The four characteristic repeat domains of approximately 70 amino acids are each numbered above the sequences in Figure 1 and are based on the alignments for mammalian annexins described by Raynal and Pollard (1994). The key residues responsible for the type-II Ca^{2+} binding site in mammalian annexins (see Weng et al., 1993, for terminology) are also indicated above the sequence rows (open boxes). p33 and p35 would both be expected to bind Ca^{2+} in the first and fourth repeats but not in the second and third. However, the acidic "cap" residue essential for Ca^{2+} binding (Huber et al., 1990) is conserved in domain III.

Very recently a full-length cDNA was reported for an annexin from bell pepper (Proust et al., 1996), which is most similar to the amino acid sequence predicted from the alfalfa partial cDNA, and slightly closer to the maize p33 sequence than to p35 (Fig. 2).

Accumulation of p33 and p35 Transcripts in Root and Coleoptile

Northern blots of RNA isolated from the three most apical 1-mm segments from the root tip and from coleoptiles were probed with the PCR-amplified 3' noncoding regions of p33 and p35, and with a coding sequence probe that consisted of the whole p33 cDNA. A single hybridizing band was present in blots with all three probes, and there was less transcript in the apical 0- to 1-mm root tip segment than in the more proximal 1- to 2-mm and 2- to 3-mm segments (Fig. 3). Coleoptile expression was slightly
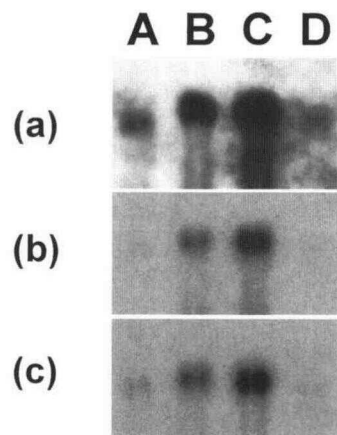
Figure 3. Accumulation of annexin p33 and p35 transcripts in maize root tips and coleoptiles. Northern blots contained 10 μg of total RNA (quantified by measurement of A_{260} and confirmed by rRNA staining with ethidium bromide) from the following tissues: lanes A through C, 0 to 1, 1 to 2, and 2 to 3 mm from the root tip, respectively; lane D, coleoptiles. Northern blots were probed with full-length p33 cDNA (a) and the 3' noncoding regions of p33 cDNA (b) and p35 cDNA (c).

weaker than in these root segments using the coding sequence probe, but was almost undetectable with the probes based on the 3' untranslated regions of p33 and p35. However, a more comprehensive analysis is necessary to determine whether the p33 and p35 cDNAs isolated from the root tip are specific to this tissue.

Number of Annexin Genes

Figure 4 shows the pattern of bands when digests of genomic DNA are hybridized with the p33 and p35 cDNAs. Hybridization of these probes to the cDNA inserts of p33 and p35 established that under our experimental conditions there is little or no cross-hybridization (data not shown), and this is borne out by the distinct banding patterns in Figure 4, a and b. There are no restriction sites for EcoRI, BamHI, or HindIII in the p33 cDNA, whereas the p35 cDNA contains a single site for EcoRI and a single site for HindIII. Therefore, the relatively simple pattern of hybridization suggests only small gene families for these annexins.

DISCUSSION

Previous research has suggested that much of the specificity of function of individual members of the annexin family results from unique features of the N-terminal sequence (see Raynal and Pollard, 1994). The start codons for p33 and p35 have been identified based on the conserved nucleotide sequences identified by Kozak (1984) and the predicted and, for p33, expressed, protein molecular weights. However, there are no in-frame stop codons in the sequenced regions upstream of these start codons. The full-length sequences of maize p33 and p35 show that in both cases the N-terminal region is relatively short, with the predicted beginning of the first of the four repeats only

Figure 4. Analysis of the complexity of the p33 and p35 genes in maize. Southern hybridization of 15 μg of maize genomic DNA digested with HindIII (lanes A), EcoRI (lanes B), and BamHI (lanes C). a, Probed with p33 cDNA; b, probed with p35 cDNA.

12 amino acids downstream from the start Met residue (Fig. 1). In this respect the maize annexins are most similar to annexins IV and V. A search using the PROSITE program in PCGENE (Intelligenetics) revealed a potential phosphorylation site at the most N-terminal Thr (residue 3 in Fig. 1) in p33; this was not a potential site in p35. Such differences may account for the small but consistent difference in the apparent molecular masses of p33 and p35, in spite of their identical length (314 amino acids; see Fig. 1). However, there is no clear identity with the N-terminal sequences of other members of the annexin family.

The predicted amino acid sequences for the maize annexins have been divided into four repeat domains based on the alignment with annexin XIIIa (Fig. 1). The absence of the type-II Ca^{2+}-binding sites from repeats 2 and 3 makes this less convincing than for animal annexins; the maize annexin repeats show only about 20% identity, whereas their identity to Medicago and Fragaria annexins is much greater (55–60%). This has been noted for the partial Medicago cDNA sequence in relation to plant peptide sequences (Pirck et al., 1994). Partial cDNAs for plant annexins indicate that all have the type-II Ca^{2+}-binding site in the first repeat (Clark and Roux, 1995), and the same appears to be true for the sequence predicted from the full-length bell pepper cDNA (Proust et al., 1996). The maize annexins have this site not only in repeat 1, but also in repeat 4, which could have important functional implications for the maize proteins. There may also be Ca^{2+} binding in a modified, lower affinity site in repeat III, where the acidic residue is present at position 223 (Fig. 1; see Fiedler and Simons, 1995).

Outside the plant kingdom, the maize annexins are most similar to annexin XIIIa, originally described as an intestine-specific form of human annexin I (Wice and Gordon, 1992). The association of this annexin with the apical membrane of secretory cells of the intestinal epithelium suggests a role in the secretory pathway, a finding that is made more compelling by the recent discovery of annexin XIIIb (Fiedler et al., 1995). This annexin appears to regulate secretion in the NSF/SNAP/SNARE-independent apical pathway of canine kidney cells (Ikonen et al., 1995). However, the identity of the maize annexins to annexin XIIIa is relatively low (35%) and does not extend to the N terminus, where annexin XIIIb differs most radically from XIIIa. Therefore, although the similarity of maize annexins to annexin XIII is noteworthy, there is nevertheless considerable divergence from nonplant annexins, and the sequence data so far available indicate that plant annexins form a very distinct group. However, it seems likely that the celery annexin-like protein with high Ca^{2+} sensitivity (Seals et al., 1994) may be a novel type.

Our interest in annexins has centered on their possible role in exocytosis (see Battey and Blackbourn, 1993). Therefore, we isolated and sequenced cDNAs from a root tip library that included hypersecretory cells of the root cap. Northern blotting provides no evidence for enhanced expression of the annexins in the root cap, compared with the more proximal regions of the root; definite conclusions about a role for the maize annexins in exocytosis from hypersecretory cells will require more detailed analysis using in situ hybridization. Immunolocalization studies on
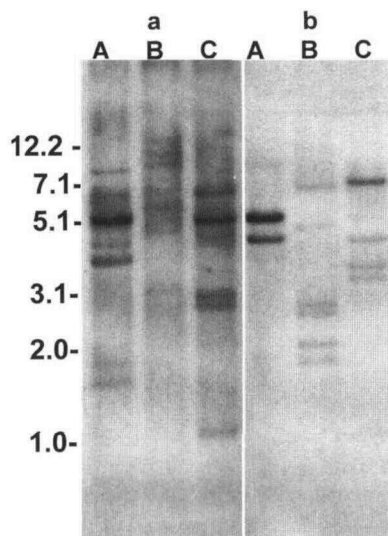
pea root caps (Clark et al., 1992) suggest that this would be a profitable approach. The weaker expression in coleoptiles may indicate the existence of tissue-specific annexin isoforms in maize, and this would be consistent with the differences between the coleoptile p33/p35 peptide sequences and the predicted root tip sequence (Fig. 1). However, Southern blotting indicates that there may be only a small number of copies of the p33 and p35 genes in the maize genome. Only sequencing of annexin cDNAs derived from coleoptile RNA will establish conclusively whether maize coleoptiles and root tips contain distinct forms of the annexins p33 and p35.

p33 and p35 are remarkably alike (80% amino acid identity), yet they are clearly distinct and encoded by different genes. This suggests that the presence of the two annexins is important for their function. Consistent with this, we have observed differences in the relative amounts of the two proteins in extracts from different sources. Furthermore, the Ca^{2+} requirements for phospholipid binding are quite different for p33 and p35: p33 has a requirement for 120 μM free Ca^{2+} for one-half maximal binding to liposomes, whereas p35 requires 370 μM (Blackbourn et al., 1991). This difference is also found for binding to native membranes (A.D. McClung and N.H. Battey, unpublished data). The primary structure is very similar in the type-II Ca^{2+}-binding domains of the two annexins (see above), so this may mean that other, lower-affinity sites are important in these annexins; this has proved to be the case for annexin XII (Luecke et al., 1995). An in vivo association of p33 and p35 is also a real possibility; we found no evidence in any of our library screens for a cDNA representing the p68 annexin present in coleoptiles (Blackbourn et al., 1991; McClung et al., 1994) and in root tips (data not shown). The overexpression of the maize annexins is therefore an important goal made more accessible by the work described here because it should allow structure determinations and resolution of questions about Ca^{2+} binding and a p33/p35 association.

The full-length sequences for maize p33/p35 do little to clarify the basis for the novel ATPase activity in maize annexins described by McClung et al. (1994) and subsequently confirmed for tomato by Calvert et al. (1996). There are no Walker-type nucleotide binding sites (Walker et al., 1982) in the predicted amino acid sequence, but not all nucleotide binding/hydrolyzing proteins have these (Schaap et al., 1994). The effects of the Lys-rich regions that replace the Ca^{2+}-binding loops in repeats 2 and 3 on the tertiary structure need to be resolved, as do the functional implications of these changes. An additional goal is to determine whether recombinant p33 and p35 possess, together or individually, ATPase activity.

ACKNOWLEDGMENTS

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