Biochemical Characterization of Tomato Annexin p35

INDEPENDENCE OF CALCIUM BINDING AND PHOSPHATASE ACTIVITIES*

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Tomato annexin p35 has been cloned and used in a site-directed mutagenesis study to explore the phospholipid binding and catalytic properties of the protein in detail. Analysis of the cDNA sequence of p35 reveals that the annexin has only two typical endonexin folds, corresponding to repeats I and IV. Expression of recombinant p35 in Escherichia coli confirmed both phospholipid binding and a nucleotide phosphatase activity that could be inhibited on interaction of the recombinant annexin with phospholipids. Site-directed mutagenesis in which the acidic residues Glu-68 (repeat I), and Asp-297 (repeat IV) were changed to Asn, generated two mutant forms, E68N and D297N, respectively. Both mutant forms of the annexin continued to express catalytic activity. Changing repeat I had little effect on phospholipid binding, whereas the change to repeat IV abolished this property. These data show that, in this plant annexin, repeat IV plays a more critical role in calcium-dependent phospholipid binding than repeat I, and that the catalytic and phospholipid binding activity of the protein can be separated experimentally.

Since the first annexin cDNA sequence (AnxI) was identified (1), more than 27 distinct annexin genes have been isolated from organisms as diverse as mammals, plants, and primitive eukaryotes such as Giardia and Dictyostelium (reviewed in Ref. 2). Initially, annexins were classified together on the basis of their common property of Ca\(^{2+}\)-dependent phospholipid binding. Subsequent analysis of a number of annexin sequences revealed significant homology predominantly in the core domain that comprises four conserved repeats (or eight repeats in annexin VI). These repeats are approximately 70 amino acids in length. Crystallographic studies have also shown a conserved three-dimensional structure, implying that annexins can be considered as a family of structurally related proteins (3–6). Different members of this protein family have been suggested to function in a range of cellular processes including ion transport, signal transduction, and vesicle trafficking and fusion (4–7).

Plant annexins are similar to animal annexins, in that the proteins bind phospholipids and preferentially negatively charged phospholipids in a Ca\(^{2+}\)-dependent manner. Plant annexins, again similar to their animal counterparts, have been found in many diverse cell and tissue types and are highly abundant in roots and ripening fruits. In early studies, the proteins were implicated in secretory processes (8–11); more recently, plant annexins have been reported to regulate the activity of the cell surface enzyme involved in (1→3)-β-glucan (callose) synthesis (12), as well as exhibiting several catalytic activities of their own, including ATPase and peroxidase activities (13–14).

In studies of tomato annexins, we have recently demonstrated several intriguing properties of the proteins in addition to their Ca\(^{2+}\)-dependent phospholipid binding activity. Both the tomato annexins p34 and p35 were found to bind specifically to F-actin but not to G-actin, at Ca\(^{2+}\) levels of 100–300 μM (15). This Ca\(^{2+}\) requirement is much higher than the Ca\(^{2+}\) level required by human II and VI to bind F-actin (16–17), but is close to that of human annexin I (18). In the same study, we also reported a catalytic activity, related to, but different from, an ATPase activity previously described for maize annexins (13). The tomato annexins catalyzed the hydrolysis of a much wider range of substrates, including nucleotide triphosphates and diphosphates. The annexin nucleotide phosphatase activity was found to constitute ~60% of the total soluble cellular activity and was independent of Mg\(^{2+}\) and Ca\(^{2+}\) (15). Surprisingly, when binding to phospholipids, the catalytic activity of the proteins was inhibited, but was not affected when the annexins were attached to actin filaments (15).

In order to begin to study the structure-function relationships of the tomato annexins in more detail, we have now cloned the genes of p34 and p35. Their sequences show very high homology to those of all other plant annexins that have been sequenced to date. A universal feature of all of the plant sequences is that only repeats I and IV contain the classical endonexin fold, the 17-amino acid consensus motif known to function in the Ca\(^{2+}\) and phospholipid binding activities of annexins from non-plant sources (19). This study describes results of a site-directed mutagenesis strategy to analyze the biochemical properties of p35 and the relative importance of the endonexin folds for phospholipid binding and phosphatase activity.

MATERIALS AND METHODS

Molecular Cloning of Tomato Annexin cDNAs—A tomato root cDNA library constructed in AZAP II (Stratagene) was supplied courtesy of Mike McPherson, Department of Biochemistry and Molecular Biology, University of Leeds, UK. A total of 30,000 recombinant phage were screened in duplicate with radioisotope-labeled alfalfa annexin (23) and bell pepper annexin cDNA (24), following the methods described by Sambrook et al. (20). A number of putative clones were taken to secondary screen. Single colonies corresponding to the signals obtained in the secondary screen were isolated, and plasmids carrying the cDNA inserts were rescued from the AZAP II vector using helper phage VCSM 13 (Stratagene) following the instructions of the manufacturer’s manual. The sequence of these cDNA clones were determined using Sequenase® version 2.0 DNA sequencing kit (U. S. Biochemical Corp.).

Construction of p35 Gene Expression Plasmid—The p35 cDNA was amplified with oligomer 5′-CGGAATTCATGTCTAGTCTTAAA-3′ (5′ primer) and T7 primer (3′ primer), in order to truncate the 5′ polylinker and to obtain the proper reading frame for protein expression.

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The PCR reaction was set up by mixing 10 ng of template DNA, 100 ng of each primer, 1 \( \times \) PCR reaction buffer (Stratagene), 2 mM MgCl\(_2\), 0.2 mM amounts of each deoxynucleoside triphosphate (Pharmacia), and 1 unit of cloned \( Pfu \) DNA polymerase (Stratagene) in a 20-\( \mu l \) reaction. DynaMix (Flowgen) was loaded onto the PCR reaction following the manufacturer’s instructions. The PCR reaction was run on a PTC-200 Peltier Thermal Cycler (MJ Research). OmniGene programmable cycling heat block by one cycle of 5 min of denaturation at 94°C, 2 min of annealing at 55°C, 3 min of extension at 72°C, followed by 35 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 55°C, 2 min of extension at 72°C. The PCR reaction was completed by one cycle of 2 min denaturation at 94°C, 3 min of annealing at 55°C, 3 min of extension at 72°C. The PCR products were electroeluted from 1% (w/v) DNA agarose gel. After extraction with phenol/chloroform, the DNA fragments were digested with EcoRI and XhoI and were subcloned into EcoRI and XhoI sites of pBluescript\(^{\text{KS}}\) vector (Stratagene) using T4 DNA ligase. The cDNA fragment was then excised again using Smal and XhoI. After a fill-in reaction carried out by Klenow fragment, this DNA fragment was subcloned into the Smal site of the GST gene fusion vector pGEX3X (Pharmacia). The final construct that contains the correct orientation of p35 cDNA was named as pGEX35a. All the DNA modifying and restriction enzymes were purchased from New England Biolabs.

### Protein Expression—Recombinant annexin p35 (Rp35) protein was expressed as a fusion protein, containing a GST fusion partner at the N terminus. E. coli strain XL-Blue carrying plasmid DNA pGEX35a was grown at 37°C in 2\% YT media containing 50 \( \mu g/ml \) ampicillin until the \( A_{600 \text{nm}} \) reached 0.5, after which the culture was incubated with 1 \( \text{mM} \) isopropyl-1-thio-\( \beta \)-galactopyranoside for 4 h at 37°C to induce protein expression. To check protein expression, 1 ml of culture was harvested and cracked by boiling for 3 min in 20 \( \mu l \) of 2 \( \times \) SDS loading buffer, and applied to SDS-PAGE following the methods described by Sambrook et al. (20). After separation, the proteins were transferred onto nitrocellulose membrane using Bio-Rad PROTEAN II electrotransfer system at 4°C for 1 h with a voltage of 100 V, in the tris-glycine transfer buffer. The membranes were electroblotted in the conditions described above. The immunoblotting reaction was completed as described previously (15).

To prepare large quantities of recombinant proteins, 500 ml of culture were set up in the conditions described above. The cells were harvested by centrifugation at 5,000 \( \times g \) for 5 min and were resuspended in 5 ml of ice-cold 1\% phosphate-buffered saline. The suspended cells were sonicated using a Vibra Cell Ultrasonic processor (SONICS & MATERIALS) on ice in short bursts until the solution turned clear. Cell debris was removed by centrifugation at 40,000 \( \times g \) for 5 min. The protein in a supernatant fraction was collected by adding 500 \( \mu l \) of 50\% glutathione-coupled Sepharose gel (Pharmacia, supplied as an approximately 75\% slurry), and recovered in elution buffer (20 \( \mu l \) reduced-form glutathione, 100 \( \mu l \) Tris-\( \text{HCl, pH 8.0,} \) 120 \( \mu l \) NaCl), according to the manufacturer’s instructions. The purified recombinant proteins were dialyzed against a dialysis buffer (20 \( \mu l \) Tris-\( \text{HCl, pH 6.5,} \) 0.02\% (w/v) Na\(_2\text{SO}_4\)) for 4 h overnight. The proteins were collected and stored at 4°C for further experiments.

### Phospholipid Binding Assay—Phospholipid vesicles were prepared using sucrose solution method (21). A 100-\( \mu l \) bed volume of phospholipids (Sigma) was homogenized in distilled H\(_2\)O, followed by a centrifugation for 5 min at 12,000 \( \times g \). The pellet was resuspended in 240 \( \mu l \) sucrose and was incubated at room temperature for 2 h. The liposomes were harvested by adding two volumes of 1\% binding buffer (100 mM KCl, 2 mM MgCl\(_2\), 20 mM HEPES/KOH, pH 7.0) and centrifuging for 10 min at 12,000 \( \times g \). The liposomes were resuspended in 100 \( \mu l \) of 1\% binding buffer. An aliquot of 10 \( \mu l \) of liposomes was used for the phospholipid binding assay.

GST protein or the GST-Rp35 fusion protein was incubated for 15 min at room temperature with liposomes in the presence of 1\% binding buffer and the appropriate concentration of Ca\(^{2+}\) or EGTA. Supernatant fractions and pellets were separated by sedimentation for 10 min at 12,000 \( \times g \). The supernatant fractions were collected and acetone-precipitated. The pellets were washed once with the same buffer used in the phospholipid binding assay. The proteins that bound to phospholipids were eluted with 100 \( \mu l \) EGTA and acetone-precipitated. All the samples were mixed in 20 \( \mu l \) of SDS-PAGE loading buffer, boiled for 3 min, and loaded onto a 10\% (w/v) SDS-polyacrylamide gel.

### Measurement of Nucleotide Phosphatase Activity—Phosphatase ac-

\(^{2}\) The abbreviations used are: PCR, polymerase chain reaction; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; BE, bovine brain extract type VII.

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Expression and Characterization of Tomato Annexin p35

The results showed that the phospholipid binding properties of GST-Rp35 were similar to those of the native annexin. The fusion protein was stable in the presence of Ca\(^{2+}\) and EGTA, and was not affected by the concentration of these ions. The ATPase activity of GST-Rp35 was also measured, and it was found to be similar to that of the native annexin. The results indicate that the GST-Rp35 fusion protein is a potential target for the characterization of tomato annexins.
phatidylserine and phosphatidylcholine, or were made from BE. As expected, when the liposomes only consisted of phos-
phatidylcholine, the fusion protein did not bind and was recov-
ered in the supernatant. The control, using GST alone, was
unable to bind to liposomes. The interaction of the fusion pro-
tein with liposomes could be inhibited by the inclusion of 1 mM
EGTA in the assay buffer, leading to recovery of GST-Rp35 in
the supernatant. Thus, the presence of the GST did not inhibit
nor alter the expected phospholipid binding activity of the
recombinant annexin.

Nucleotide Phosphatase Activity of GST-Rp35—Tomato an-
nexins have previously been shown to hydrolyze a range of
nucleotide phosphates (15). To confirm the catalytic activity
was indeed a property of the gene product and not a contami-
nant in the purified native protein preparation, enzyme assays
were carried out with the recombinant GST-Rp35 fusion pro-
tein. The results are summarized in Table I. GST-Rp35 re-
leased Pi from a wide range of compounds including ATP, CTP,
GTP, TTP, and UTP at similar levels of hydrolytic activity.
ADP was also hydrolyzed but at a lower rate when compared
with the activities toward nucleotide triphosphates. In con-
trast, a much lower hydrolytic activity was observed toward

![Fig. 1. Amino acid sequence alignments of the tomato annexins. A, the partial amino acid sequences obtained from micro-sequence analysis (25) confirm the identity of the two annexin cDNA clones. X in the partial sequences indicates unidentified residue. B, putative en-
donexin folds of plant annexines were aligned to each other, and to the repeats from human annexins I and V. Asterisks indicate the residues that form hydrogen bonds to further stabilize the type II Ca²⁺-binding structure in each animal annexin repeat (28). The putative P-actin
binding motif is highlighted in bold.](image)

![Fig. 2. Expression and purification of GST-Rp35. Proteins extracted from E. coli expressing annexin p35 were either analyzed di-
rectly or after purification, using 10% (w/v) SDS-polyacrylamide gel
electrophoresis. Protein was visualized by Coomassie staining, or after
Western blotting with antiserum raised against native tomato annexins
(15). M, molecular weight markers; lane 1, total protein (10 µg); lane 2,
GST-Rp35 (10 µg) eluted from glutathione-Sepharose; lane 3, as in lane
2, but probed with antisera to annexin p35.](image)
AMP, fructose 1-phosphate, UDP-glucose, and inorganic pyrophosphate. When GST alone was assayed as an internal control, negligible activity was detected. Michaelis-Menten kinetics of GST-Rp35 phosphatase activity suggested GTP and ATP as the optimal substrates (Table I).

The effect of phospholipids on the catalytic activity of GST-Rp35 was analyzed in the presence of BE liposomes and 1 mM Ca\(^{2+}\), or 1 mM EGTA. The assays shown in Table II all contained liposomes. In the presence of EGTA, i.e. non-binding conditions, the enzyme activity of GST-Rp35 was near identical to that shown in Table I, implying that the presence of liposomes alone had no effect on catalytic activity. However, in the presence of phospholipids and 1 mM Ca\(^{2+}\), i.e. under conditions in which the GST-Rp35 was bound to the phospholipid vesicles, the enzyme activity was inhibited >67.5%.

**Site-directed Mutagenesis Studies**—To gain further insight into the Ca\(^{2+}\)-dependent phospholipid binding activity of Rp35, as well as the relationship of phospholipid binding and nucleotide phosphatase activity, two point mutants were constructed. The two conserved acidic residues (Glu-68 and Asp-297) that locate 40 residues downstream of the endonexin fold in repeats I and IV (Fig. 1B) of the plant annexin were changed to Asn. In studies on animal annexins, these residues have been shown to be involved in Ca\(^{2+}\)-dependent phospholipid binding activity (26).

The mutant proteins were purified with the same yield in *E. coli* as GST-Rp35 (data not shown), indicating that the point mutations did not affect expression, protein synthesis, or protein stability. Binding experiments with BE vesicles were carried out over a range of Ca\(^{2+}\) concentrations, to compare GST-Rp35 with E68N and D297N mutants. As shown in Fig. 4, GST-Rp35 only started to bind to phospholipids at 0.5 mM Ca\(^{2+}\). A Ca\(^{2+}\) concentration of 1 mM was required for E68N mutant to bind to phospholipids. In stark contrast, D297N was not recovered in the pellet, irrespective of Ca\(^{2+}\) concentration.

The three recombinant proteins, GST-Rp35, E68N, and D297N, were also analyzed for their catalytic activity. As shown in Table III, each of the three proteins were active, with near identical specific activity when assayed against 3 mM ATP in the presence of 1 mM EGTA and BE liposomes. This shows that the point mutations do not affect the phosphatase activity of Rp35. When Ca\(^{2+}\) replaced EGTA in the assay mix, the activity of GST-Rp35 was inhibited as expected. The presence of 1 mM Ca\(^{2+}\) also partially inhibited the activity of E68N, and this inhibition was increased by increasing the Ca\(^{2+}\) concentration to 5 mM, most probably reflecting the increased binding of the mutant to phospholipids at the higher Ca\(^{2+}\) concentration. In contrast, the presence of Ca\(^{2+}\) had no effect on the enzyme activity of D297N, presumably since the mutant protein was unable to bind to phospholipids.

**DISCUSSION**

This study describes the cloning, heterologous expression, and biochemical analysis of the recombinant p35 annexin of tomato plants. In an earlier report, we showed that the native annexins purified from tomato cells exhibited a catalytic activity that was inhibited when the proteins were bound to phospholipid vesicles (15). Using recombinant protein, and a site-directed mutagenesis approach, we have now explored these properties and their relatedness in more detail.

In contrast to annexins from animal sources, which typically comprise variable N termini followed by four repeats each containing the characteristic endonexin fold (reviewed in Refs. 3–6 and 27), all the plant annexins identified to date are highly homologous throughout their sequences. In each case, their N terminus is short, and the proteins contain only two endonexin folds (L/M-K-G-X-G-T-X\(_{10–12}\)D/E) in repeats I and IV (Fig. 1B). Given the sequence of p35 also shows these features, it is possible that the relatively high Ca\(^{2+}\) required by the tomato annexin for phospholipid binding reflected that either repeats II and III contain a low affinity Ca\(^{2+}\)-binding structure, or repeats I and IV are the only sites that interact with Ca\(^{2+}\) and
Ca$^{2+}$ was found to play a much more critical role than repeat I in the mutants were compared with wild-type, interestingly repeat IV phospholipid interaction domains (26). When the properties of these revealed a ranking of Ca$^{2+}$ importance for the activation of enzymes such as myosin light ATPase (31). Similar results have also been found for animal proteins were incubated with liposomes reconstituted from BE, at a range of Ca$^{2+}$ concentrations from 0.1 mM to 10 mM. The pellet (P) and supernatant (S) fractions separated by centrifugation were analyzed by 10% (w/v) SDS-polyacrylamide gel electrophoresis and visualized by Coomassie staining.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Ca$^{2+}$/chelator</th>
<th>Specific activity</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>GST-Rp35</td>
<td>EGTA</td>
<td>28.9 ± 0.7</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Ca$^{2+}$</td>
<td>9.9 ± 1.1</td>
<td>65.7</td>
</tr>
<tr>
<td>E68N</td>
<td>EGTA</td>
<td>26.5 ± 0.6</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Ca$^{2+}$</td>
<td>20.1 ± 0.4</td>
<td>24.1</td>
</tr>
<tr>
<td></td>
<td>Cr$^{3+}$ (5 mM)</td>
<td>14.6 ± 1.2</td>
<td>44.9</td>
</tr>
<tr>
<td>D297N</td>
<td>EGTA</td>
<td>26.8 ± 0.9</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Ca$^{2+}$</td>
<td>26.7 ± 0.4</td>
<td>6.6</td>
</tr>
</tbody>
</table>

Phospholipids.

To investigate the role of repeats I and IV in this process, we generated two mutants in which the charged residue located 40 residues downstream of the endonexin fold in either repeat I or repeat IV was removed. These acidic residues located in the DE loop of the repeats help to stabilize the type II Ca$^{2+}$-binding site of annexins (28). Thus, in this way, the Ca$^{2+}$ binding ability of the protein can be abolished via elimination of the Ca$^{2+}$ co-ordination, without affecting the structure of the phospholipid interaction domains (26). When the properties of these mutants were compared with wild-type, interestingly repeat IV was found to play a much more critical role than repeat I in the Ca$^{2+}$-dependent phospholipid binding activity of tomato annexin p35.

While annexins bind Ca$^{2+}$ using a different mechanism from that of calmodulin and other EF Ca$^{2+}$-binding proteins (29–30), a common feature is that all the proteins have more than one Ca$^{2+}$-binding site. Multiple mutations in calmodulin have revealed a ranking of Ca$^{2+}$-binding structures in order of importance for the activation of enzymes such as myosin light chain kinase, adenyl cyclase, and plasma membrane Ca$^{2+}$-ATPase (31). Similar results have also been found for animal annexins, in which, for example, increasing the number of mutated repeats of annexin IV caused a progressive elevation in the Ca$^{2+}$ requirement for membrane binding and aggregation (26). Our data on the tomato annexin p35 now reveal a different pattern. Rather than a quantitative decrease in its Ca$^{2+}$-dependence of phospholipid binding, the mutation in repeat IV completely abolished the activity of the annexin, whereas a mutation in repeat I caused very little difference to its properties.

From studies on annexin V, a conformational change in the protein was found at high Ca$^{2+}$ concentrations. The Trp residue (Trp-187) in the AB loop of repeat III changed from a buried to an exposed state, eventually leading to the formation of a new phospholipid-binding site (32–35). This type of Ca$^{2+}$-dependent conformational change has also been reported in other annexins (36–38). Interestingly in this context, a Trp residue (Trp-27) is present in repeat I of p35 and all other plant annexin sequences so far identified. Crystallographic analyses of p35, currently under way, will demonstrate whether the Trp residue is buried in the plant annexin structure. If so, it is possible that binding of Ca$^{2+}$ to repeat IV of the plant protein may induce a comparable conformational change leading to a favored state of repeat I for Ca$^{2+}$ and phospholipid binding.

The recombinant p35 exhibited catalytic activity, confirming our earlier data using native annexins purified from tomato cells (15). While a number of controls in that study strongly suggested the catalytic activity resided in the annexins and not a contaminant that co-purified, it was nevertheless important to confirm categorically the activity to be a property of the tomato annexin. The catalytic specificity of p35 is very broad, with a wide range of nucleotide triphosphates hydrolyzed. There is also significant hydrolytic activity against other phosphate compounds. Interactions with nucleotide phosphates have also been reported in studies of animal annexins (39–42). For example, annexin VI binds to ATP-agarose (39) and annexin I binds to ATP and to cAMP (40), although neither has been reported to have hydrolytic activity against the compounds. Studies on annexin VII have shown the protein can act as a hydrolase, but only against GTP (41). As yet, it is not known whether the proteins act as enzymes in vivo. However, if phospholipid binding in vitro reflects membrane binding within the cell, the data on GST-Rp35 suggest that at any given time, its enzyme activity will be regulated by its intracellular distribution, i.e. whether it is on or off the cytoplasmic surface of a membrane. It will be of interest to determine whether the ATPase activity of the maize annexins (13), and the GTPase activity of annexin VII (41) are similarly inhibited when the proteins are bound to phospholipids.

From the site-directed mutagenesis analysis, it is highly probable that the catalytic site of p35 is distinct from the phospholipid-binding site, given that D297N does not bind phospholipids yet exhibits catalytic activity. These data imply that the two properties of p35, Ca$^{2+}$-dependent phospholipid binding and phosphatase activity, are independent. In turn, it is likely that the inhibition of enzyme activity that is displayed when the protein is bound to phospholipid vesicles arises from steric hindrance.

In the earlier study on tomato annexins, we also demonstrated binding of the proteins to F-actin. At present, we have not detected this property using GST-Rp35, either when the recombinant protein was assayed as a fusion protein with GST or following cleavage of GST (data not shown). It may be significant that, previously, a protein of 70 kDa was eluted from the F-actin affinity matrix, which on SDS-PAGE revealed a single band of ~35 kDa, implying an annexin dimer interacted with the actin filaments. The resolution of the gel was insufficient to clarify whether the band was composed solely of p35 or contained both p35 and p34. Dimerization has been reported for several annexins from both plant and animal
sourced such as synexin, calelectin, and the annexin from green pepper (43–45). Certainly, a conserved amino acid motif IRI can be detected in repeat III of p35 and within the sequences of other plant annexins (Fig. 1B). This motif, in particular the Ile-1 residue, has been reported to correspond to the F-actin-binding site of myosin (46–47). A synthetic nonapeptide corresponding to residues 286–294 of annexin II, containing the motif, has been shown to inhibit the F-actin binding activity of the annexin (48). Similarly, the presence of the motif can also be correlated with the F-actin binding activities of annexin I and VI (17, 49). Further studies will be needed on Rp35, with respect to its dimerization in vitro, its ability to dimerize with p34, and the different properties of annexin homo- and heterodimers.

The studies described in this report provide some insight into the biochemical properties of the tomato annexin p35. Solving the three-dimensional structure of the protein is now required to further investigate the relatedness of these properties in parallel to determining the function of the protein in the plant.

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