The Plant Journal (1998) **15**(2), 185–197

Immunolocalization of a novel annexin-like protein encoded by a stress and abscisic acid responsive gene in alfalfa

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Summary

We report here on the isolation and characterization of a full-length cDNA clone from alfalfa termed *AnnMs2* encoding a 333 amino acid long polypeptide that shows 32–37% sequence identity with both mammalian and plant annexins, and has four tandem repeats. While other plant annexins exhibit a high level of sequence similarity to each other (up to 77% identity at amino acid level), AnnMs2 appears to be a distinct type of plant annexins. All the four endonexin folds contain the conserved eukaryotic motif within this alfalfa protein, but this element is considerably different in the second repeat. The *AnnMs2* gene is expressed in various tissues of alfalfa with elevated mRNA accumulation in root and flower. This gene is activated in cells or tissues exposed to osmotic stress, abscisic acid (ABA) or water deficiency. The recombinant AnnMs2 protein is able to bind to phospholipid in the presence of Ca^{2+}. Indirect immunofluorescence studies using affinity purified rabbit anti-AnnMs2 peptide antibody show mainly nucleolar localization, but the protein sequence lacks the usual nuclear localization signal. The potential role of this novel annexin-like protein in the basic and stress-induced cellular functions is discussed.

Introduction

The annexins are a growing family of at least 13 distinct, but structurally related, proteins that bind to negatively charged phospholipids in a Ca^{2+}-dependent manner (extensively reviewed by Raynal and Pollard, 1994 and Moss, 1997). They are relatively abundant cellular proteins, mainly present in the cytoplasm or intracellular membrane. However, some annexins have also been detected in the nucleus (Mizutani *et al.*, 1992; Raynal *et al.*, 1992; Sun *et al.*, 1992). All annexins have an internally repetitive structure comprising four or eight repeats of a conserved 70–75 amino acid domain termed the annexin repeat. Each repeat contains a conserved 17 amino acid long consensus sequence with its characteristic GXGTDE motif called the endonexin fold. The C-terminal 'core' is thought to be involved in Ca^{2+}-binding, and it is quite distinct from the more common EF hand Ca^{2+}-binding motif (helix-loop-helix) (Kawasaki and Kretsinger, 1994). The N-terminal regions of annexins are highly variable both in length and amino acid composition. These N-terminal domains are almost certainly responsible for the distinct functions of annexins as they contain the major sites for phosphorylation, proteolysis or cellular interactions with other proteins (Raynal and Pollard, 1994).

In mammalian cells, 10 different annexins have been described, several of which have homologues in lower eukaryotes. On the other hand, there are annexins (like *Drosophila* annexin IX and X, *Hydra* annexin XII) that appear to be specific for the individual species (Johnston *et al.*, 1990; Schlaepfer *et al.*, 1992). There are also indications for the existence of unique plant-specific annexins (Seals *et al.*, 1994; Smallwood *et al.*, 1990). However, annexins do not seem entirely ubiquitous as annexin-like coding sequences have not been identified in the *Saccharomyces cerevisiae* genome, and they have not been found in protozoa and prokaryotes.

Mammalian annexins play roles in a number of different intra- and extracellular processes, including the regulation of membrane traffic, transmembrane channel activity, signal transduction, DNA replication, cell–matrix interactions, anti-inflammatory and anti-coagulant activities (reviewed by Raynal and Pollard, 1994), although the exact *in vivo* function of the distinct annexins is still unknown.

Considerably less is known about plant annexins, even though since 1989 on the basis of amino acid sequence similarities, antibody cross-reactivity and functional similarities to mammalian annexins, several annexins have been identified in plants including tomato, maize, tobacco, etc. (Blackbourn *et al.*, 1992; Boustead *et al.*, 1989; Seals *et al.*, 1994). Recently, a partial annexin cDNA sequence has been obtained from *Medicago sativa* (Pirck *et al.*, 1994) and strawberry (Wilkinson *et al.*, 1995), and full-length cDNA sequences from *Capsicum annuum* (Proust *et al.*, 1996), *Zea mays* (Battey *et al.*, 1996) and *Arabidopsis thaliana* (Gidrol *et al.*, 1996). The predicted amino acid

Received 24 June 1997; revised 1 May 1998; accepted 7 May 1998.
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sequences of plant annexins show a high degree of similarity to each other, although they are distinct from the mammalian annexins.

From a functional point of view, plant annexins have been implicated in Ca^{2+}-dependent exocytosis (Battey and Blackbourn, 1993), regulation of callose synthase activity in cotton fibers (Andrawis *et al.*, 1993), Ca^{2+} signal transduction events in the vacuole (Seals *et al.*, 1994), and in the interaction with actin (Calvert *et al.*, 1996). Some of the plant annexins have been shown to possess intrinsic phosphodiesterase activity (Calvert *et al.*, 1996; McClung *et al.*, 1994). Annexins were also suggested to play various roles in secretion and ripening processes (Blackbourn *et al.*, 1992; Clark *et al.*, 1992; Clark and Roux, 1995). Recently, an annexin-like protein has been identified from *Arabidopsis thaliana* which may be involved in the response to oxidative stress (Gidrol *et al.*, 1996).

In this paper we report the isolation and characterization of the cDNA encoding a new annexin-like protein, AnnMs2 from *Medicago sativa*, which is distinct from other plant annexins in several respects. The *AnnMs2* gene has a high mRNA level in flower and root, and it can be activated by stress treatments, such as osmotic stress, drought and abscisic acid (ABA). Our immunolocalization data reveal that the AnnMs2 protein is localized preferentially in the nucleolus, and this leads us to suggest a functional role for this novel plant annexin.

Results

Primary sequence analysis of a new annexin-related protein from Medicago sativa

Searching for stress-activated plant genes, we have isolated and sequenced several cDNAs from a *Medicago sativa* somatic embryo cDNA library. We could identify a full-length cDNA clone which was homologous with known mammalian and a partial *Medicago sativa* annexin cDNA sequence, *AnnMs* (Pirck *et al.*, 1994). The cDNA clone was designated *AnnMs2,* and it is 1533 bp long with a 113 nucleotide long 5′-, a 422 nucleotide long 3′-untranslated region and a poly A tail. Between positions 113 and 1114 it contains an open reading frame (ORF) encoding a 333 amino acid long polypeptide.

Sequence similarity searches using the exhaustive Smith–Waterman search algorithm given in the BLITZ program revealed the highest identity with the human intestine-specific annexin XIIIa (ISA, 35.5%), a slightly lower sequence identity with bovine and rabbit annexin XI (calcyclin-associated annexin 50, CAP-50, Variant I and II, 32.8–32.7%), chicken annexin V (anchorin CII, 32.6%), and 26–32% identity with other types of annexins, especially type I, II, III and V from different sources (rat, mouse, human, bovine, chicken, etc.).

The deduced amino acid sequence of *AnnMs2* is shown in Figure 1 in comparison with the amino acid sequences of *Medicago sativa* (Pirck *et al.*, 1994), *Capsicum annuum* (Proust *et al.*, 1996), *Arabidopsis thaliana* (Gidrol *et al.*, 1996), *Zea mays* p33 and p35 annexins (Battey *et al.*, 1996). AnnMs2 possesses the common primary structure of annexins: a short N-terminal tail and the C-terminal core composed of four repeats of 70–75 amino acids, but it is more difficult to predict the precise position of the repeats than in the case of animal annexins. All four repeats contain the conserved region of annexin repeats termed the endonexin fold with its characteristic KGhGTDExxLIPILApR motif (h: hydrophobic, p: polar, x: variable residue) (Raynal and Pollard, 1994). In contrast to the other plant annexins, the AnnMs2 endonexin folds show considerable similarity to the mammalian motif with the exception of the second repeat (Figure 1). Similarly to other plant annexins, the N-terminal tail is very short (five amino acids). A putative phosphorylation site for protein kinase C, as well as casein kinase II, can be detected near the N-terminus. Between positions 265 and 272 the TLIRVIVTR sequence highly resembles the TLIRIVVTR motif, the actin bundling site of annexin II, that is also present in human annexin XIIIa (Jones *et al.*, 1992; Wice and Gordon, 1992). Further experiments are required to test whether AnnMs2 can bind to actin as was shown for tomato annexins p34 and p35 (Calvert *et al.*, 1996).

The pairwise comparison of the predicted amino acid sequence of *AnnMs2* with the other plant annexin sequences shows clearly (Figure 2) that AnnMs2 is a novel plant annexin-like protein as the identity is only 34–37% (the highest with *Zea mays* p35), slightly different from the identity found between AnnMs2 and animal annexins.

A detailed comparison of the AnnMs2 sequence with 180 individual annexin domains found in the SBASE 5.0 domain sequence library (Murvai *et al.*, 1996) confirmed the presence of all four domains characteristic of annexins, and also showed that the second repeat was more divergent as compared with the other annexins in the database.

Phylogenetic analysis of the four internal repeats of AnnMs2 using the GCG Pileup program (Devereux *et al.*, 1984) as well as pairwise comparison of the four repeats revealed that domains 3 and 4 displayed a higher degree of similarity between each other than with the others, with the second repeat being the most divergent (see results above). The identity among the different internal repeats extends from 20 to 38%.

The AnnMs2 sequence was analysed to identify the predicted type II Ca^{2+}-binding sites within the endonexin folds: the GXGT loop with an acidic E or D residue 42 amino acid downstream of the first Gly. This type of Ca^{2+}-binding site has been defined on the basis of the crystallographic analysis of annexin I and V (Weng *et al.*, 1993).

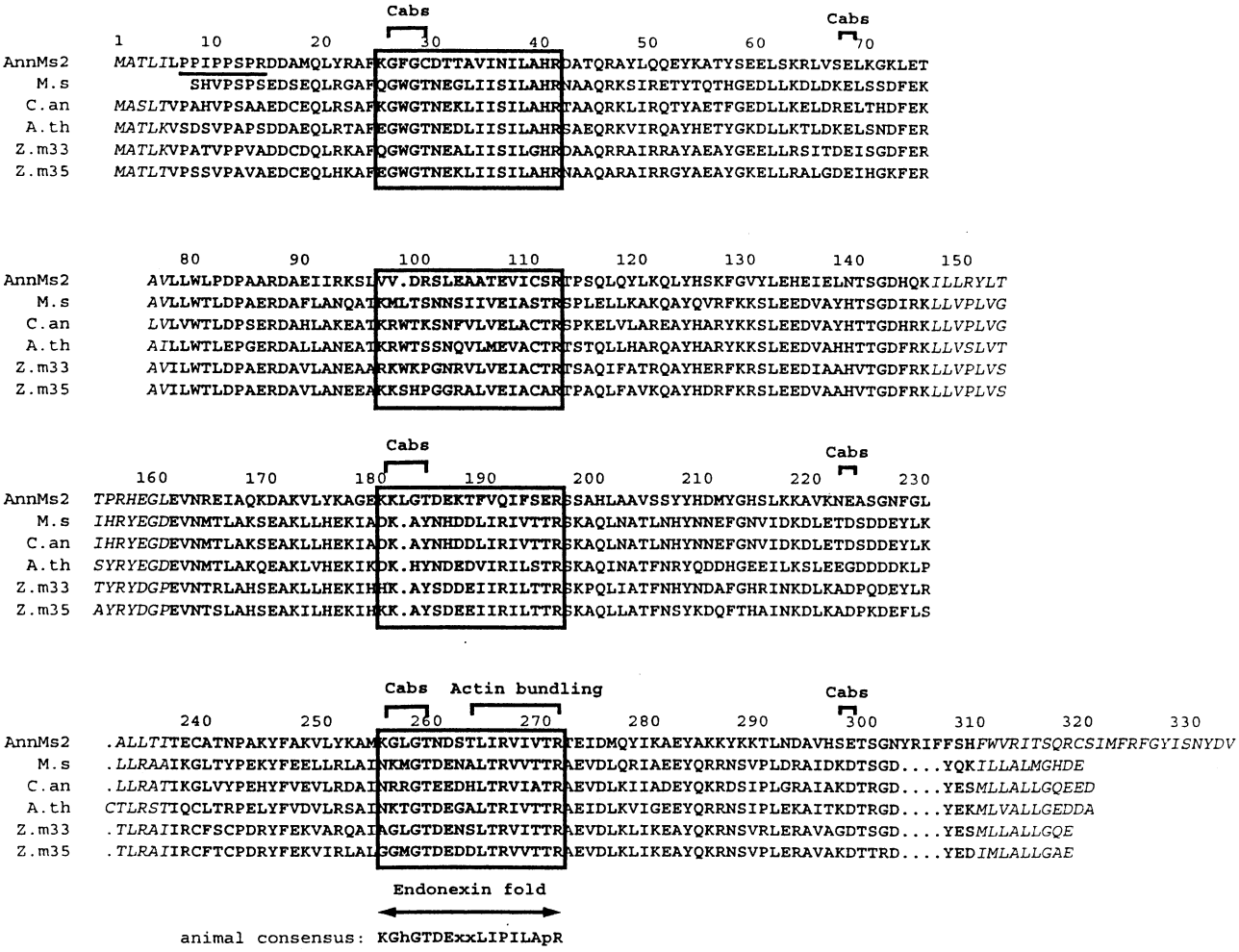

Figure 1. Alignment of the predicted amino acid sequence of *AnnMs2* with those from *Medicago sativa* (M.s; Pirck *et al.*, 1994), *Arabidopsis thaliana* (A.th; Gidrol *et al.*, 1996), *Capsicum annuum* (C.an; Proust *et al.*, 1996) and *Zea mays* (Z.m33 and Z.m35; Battey *et al.*, 1996).
The repeat sequences are bold and residues external to the four repeats are in italic. The boxed area corresponds to the endonexin fold motif. In the consensus sequence h = hydrophobic residue, *P* = polar residue and *x* = variable residue. AnnMs2 possesses three theoretical Ca^{2+}-binding sites type II indicated by Cabs. The Pro-rich region near the N-terminal is underlined.

	AnnMs2	M.s	A.th	C.an	Z.m33
AnnMs2	*	*	*	*	*
M.s	34.2	*	*	*	*
A.th	35.7	66.1	*	*	*
C.an	33.9	77.3	66.1	*	*
Z.m33	36.5	60.7	60.0	62.7	*
Z.m35	37.2	59.9	58.7	59.6	79.6

Figure 2. Comparison of plant annexin sequences.
The percentage identities were calculated pairwise for the amino acid sequences predicted from coding regions of full-length cDNAs from *Medicago sativa* (*AnnMs2*; M.s, Pirck *et al.*, 1994), *Arabidopsis thaliana* (A.th, Gidrol *et al.*, 1996), *Capsicum annuum* (C.an, Proust *et al.*, 1996) and *Zea mays* (Z.m33 and Z.m35, Battey *et al.*, 1996). The identities were calculated using the PROSIS program.

While in other plant sequences only the first repeat contains the GXGT loop and the acidic E in the proper position (except for the *Zea Mays* annexin sequences), in the case of the AnnMs2 sequence the fourth repeat contains the correct type II Ca^{2+}-binding site. In the first and third repeat it is slightly different (GXGC+E and KLGT+E), and this type of Ca^{2+}-binding site cannot be found in the second repeat.

A hypothetical three-dimensional model of the AnnMs2 *protein*

As the 3D structure of several animal annexins has already been known from X-ray crystallography and homology modelling studies (Huber *et al.*, 1990a, b; 1992; Weng *et al.*, 1993), it was possible to create a three-dimensional model of the alfalfa annexin protein. The superimposition of the

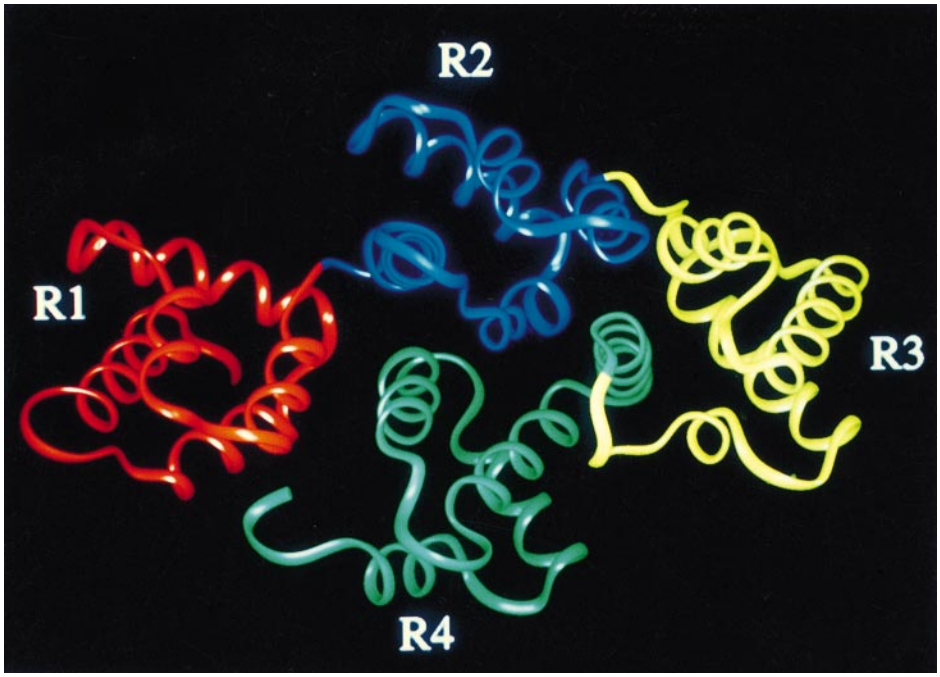

Figure 3. 3D model of the alfalfa annexin constructed by homology modelling.
Repeats 1–4 are coloured red, blue, yellow and green, respectively. The expected structure of the annexin domains (five helices interconnected with loops) is clearly seen in all of the four domains.

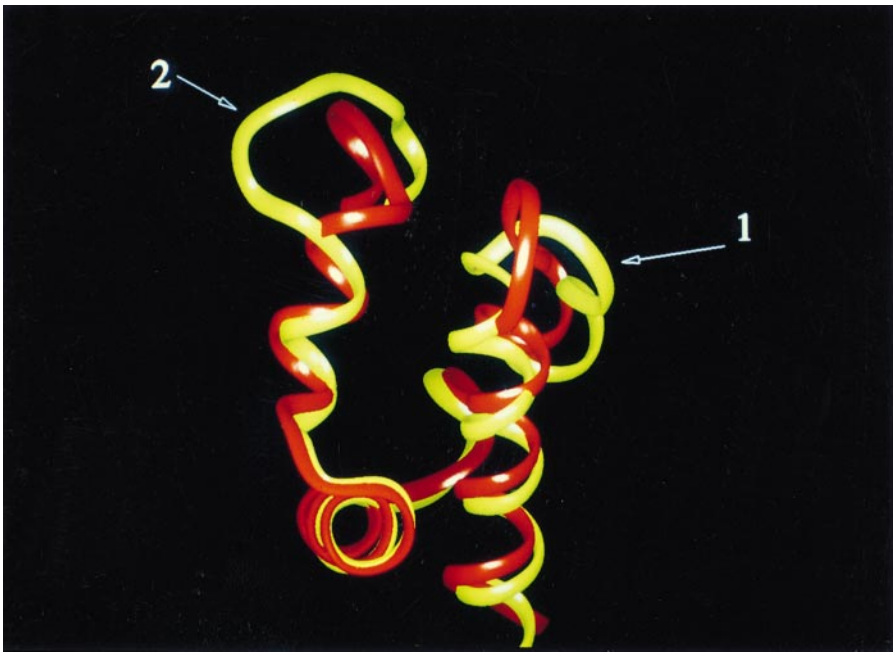

Figure 4. Comparison of the 3D structure of repeat 3 (yellow) with that of repeat 1 (red).
Two regions of structural divergence are highlighted: 1. residues 180–186 (the loop between helix 2 and helix 3) in domains 3 and 2. residues 221–226, the end of helix 5 in domain 3 and the loop leading the domain 4.

structures and the alignment of the structurally conserved regions was used to design a structural template on the basis of which a 3D model was finally constructed through a residue by residue replacement (Figure 3). The reliability of the model was tested by the knowledge-based mean field approach of Sippl as implemented in the PROSA program (Sippl, 1993a; b). The program calculates the Cβ–Cβ pair interaction energy for each residue in the sequence, and correctly folded proteins produce smooth energy plots with negative values. The annexin model gave an energy profile with values corresponding to those of native structures. The energy profile had no positive regions which would indicate misfolded parts in the model (data not shown). The so-called Z-score or normalized energy value was – 3.9, which is within the range of values expected for native proteins of this length. The 3D model of alfalfa annexin clearly shows that the main features of the annexin structure are conserved within this protein.

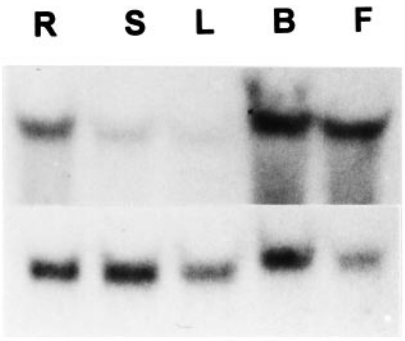

Figure 5. Differential expression pattern of *AnnMs2* in various plant tissues. Total RNAs were extracted from R: root, S: stem, L: leaf, B: bud and F: flower hybridized with the ^{32}P-labelled 5′ *Eco*RI-*Xho*I fragment of the full-length annexin clone. The blot was probed with *Msc27* clone in order to verify the even loading of the RNA samples (lower lane).

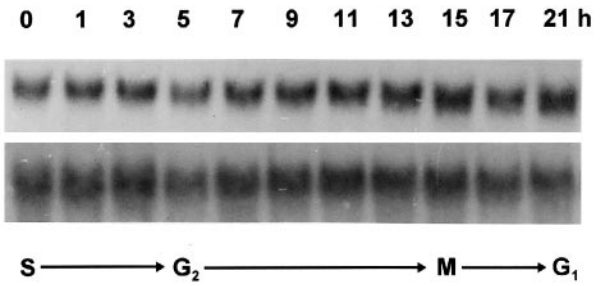

Figure 6. *AnnMs2* is constitutively expressed during cell cycle progression. Alfalfa A2 suspension culture was synchronized with aphidicolin (10 μg ml^{-1}), and samples were taken at indicated time intervals after releasing from the aphidicolin block. The duration of the cell cycle phases (G_1, S, G_2 and M) are indicated by arrows. Internal control: *Cdc2MsC* cDNA clone (lower lane).

The structural similarities of the four repeats reveal a picture which is different from what could be seen from our sequence comparisons. The repeats were compared by superimposing the conserved regions of the annexin repeats in three dimensions. Initially, repeats 1, 2 or 4 superimpose very well, and the deletion at the end of the first helix in repeat 2 does not lead to any structural difference in this region (data not shown). On the other hand, repeat 3 is quite different from the others as illustrated by the structural comparison in Figure 4. There are two regions of conspicuous differences: (1) residues 180–186 (KKLGTDE), i.e. the loop between helix 2 and helix 3 in repeat 3; and (2) residues 221–226 (KSEASG), i.e. the end of the fifth helix and the loop leading to the first helix of repeat 4, which might suggest a specific role for repeat 3. These residues are located exactly in the regions involved in Ca^{2+}-binding and possibly, in the case of the *Medicago sativa* annexin protein (also in other plant annexins), some repeats are able to bind Ca^{2+} with a modified loop (in vertebrates the GXGT loop is thought to contribute main-chain carboxyl oxygens for Ca^{2+} co-ordination).

Differential expression of the AnnMs2 gene in various organs of alfalfa

Northern analyses of total RNA samples obtained from various plant tissues: roots, stems, leaves, flower buds and flowers, have revealed a differential expression pattern of *AnnMs2* (Figure 5). The *AnnMs2* transcript was most abundant in flowers, flower buds and roots while a smaller quantity was detected in stems and leaves. Hybridization of the same RNA filter with *Msc27* (control cDNA) gave a different expression pattern. Quite strong expression levels were found in most organs, although old leaves and flowers showed reduced levels of transcript. This was also observed by Pirck *et al*. (1993) when they used the same internal control to study the organ-specific expression of PP2aMs.

Considering the observed differences in the *AnnMs2* gene activity in different alfalfa organs, we have tested the cell cycle dependence of RNA accumulation. The rapidly dividing cells of *Medicago sativa* A2 suspension culture were synchronized either by aphidicolin or hydroxyurea. *AnnMs2* is equally expressed in all phases of the cell cycle after aphidicolin synchronization (Figure 6). Similar results were obtained with RNA samples from cells synchronized with hydroxyurea (data not shown).

AnnMs2 gene is activated by different kinds of stress, including drought and abscisic acid (ABA) treatment

Using the 5′-terminal part of the *AnnMs2* gene as a probe, several Northern blot experiments were performed to study gene expression after exposing alfalfa cells or plants to different stress treatments including heat or cold shock, oxidative stress, osmotic shocks generated by increase of the NaCl, mannitol and PEG concentration and dehydration.

Neither heat (37°C) nor cold (4°C) shock had a significant effect on the expression of this gene. In contrast to the *Arabidopsis thaliana* annexin gene (Gidrol *et al*., 1996), a constitutive mRNA level was observed after treatment with salicylic acid (10 and 50 mM SA) and H_2O_2 (1 and 5 mM) for various periods of time (1–24 h) (data not shown). Treatment of alfalfa cell suspension with salt (50 mM or above) or high concentration of mannitol (0.3–0.6 M) significantly increased the level of *AnnMs2* mRNAs (Figure 7a,b). Exposing the cells to PEG we could notice that this gene was rapidly up-regulated (within 1 h) at even 5% PEG treatment. Higher PEG concentration (10 and 15%) did not cause a further increase in the amount of the mRNA (Figure 7c). With increasing periods of time the gene expression did not change significantly and the transcript level was always above that of the control. In accordance with the results of osmotic treatment, water deficiency caused a significant increase of the transcript

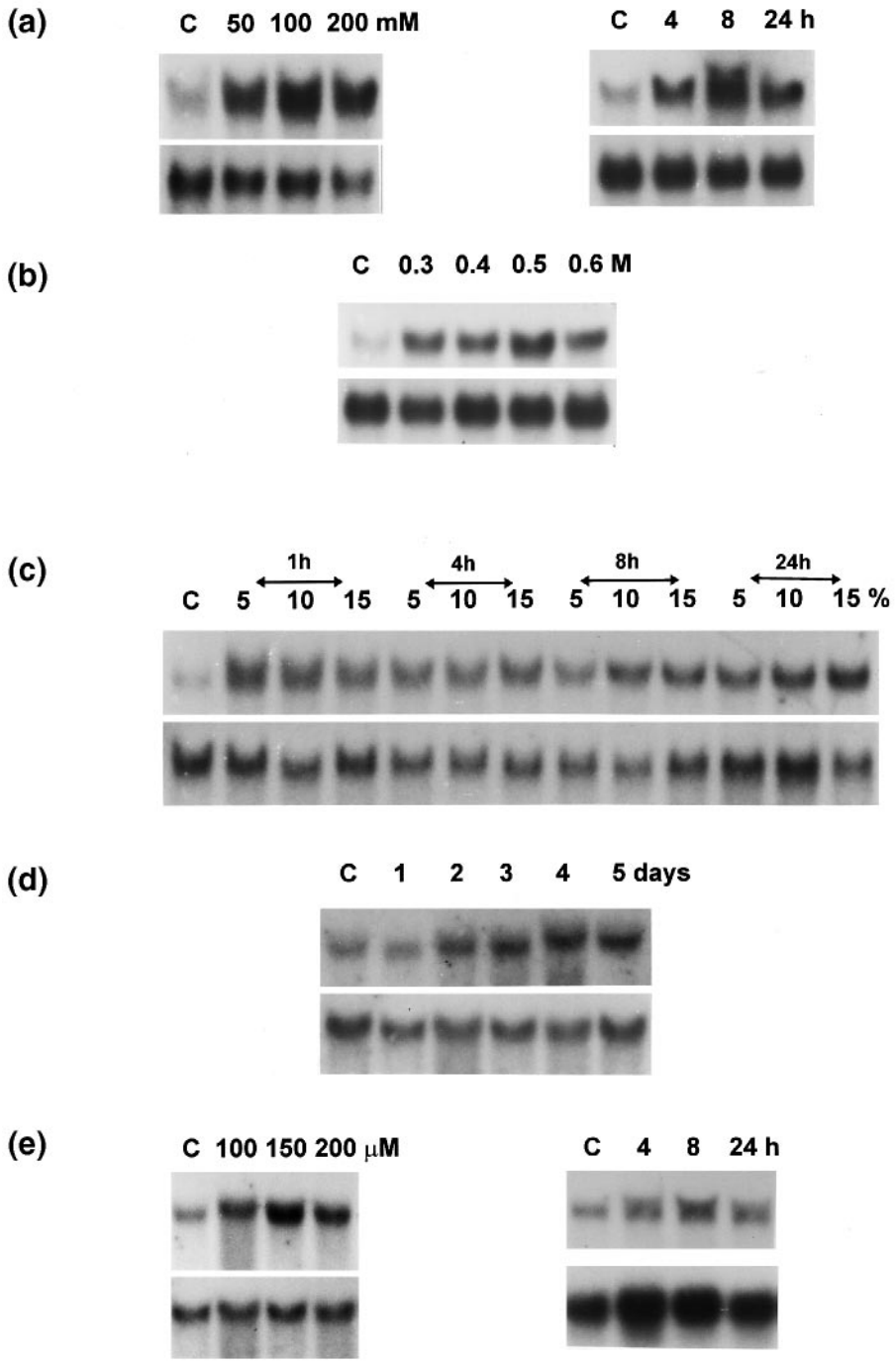

Figure 7. *AnnMs2* is activated by different stress treatments including osmotic shock, dehydration and by the addition of exogenous ABA.
(a) A2 cell suspension was treated with various concentrations of NaCl (50–200 mM) (left) and for different time intervals (4–24 h) using 150 mM NaCl (right).
(b) Treatment with increasing concentration of mannitol (0.3–0.6 M).
(c) The effect of increasing concentration of PEG (5–15%) Samples were taken at different time intervals (1–24 h) as indicated.
(d) Northern blot analysis of the *AnnMs2* transcript during dessication. Samples were taken daily for 5 days.
(e) Treatment with different concentrations of abscisic acid (100–200 μM ABA) (left) and for different time intervals (4–24 h) using 150 μM ABA (right). Control: *Msc27* cDNA probe (lower lane in all cases).

level in alfalfa leaves (Figure 7d). Since the osmotic factors and water deficiency are known to act through the increase of ABA concentration, we have analyzed the effect of ABA on the activity of the *AnnMs2* gene. ABA activation was highest at 150 μM ABA concentration after 8 h of treatment (Figure 7e).

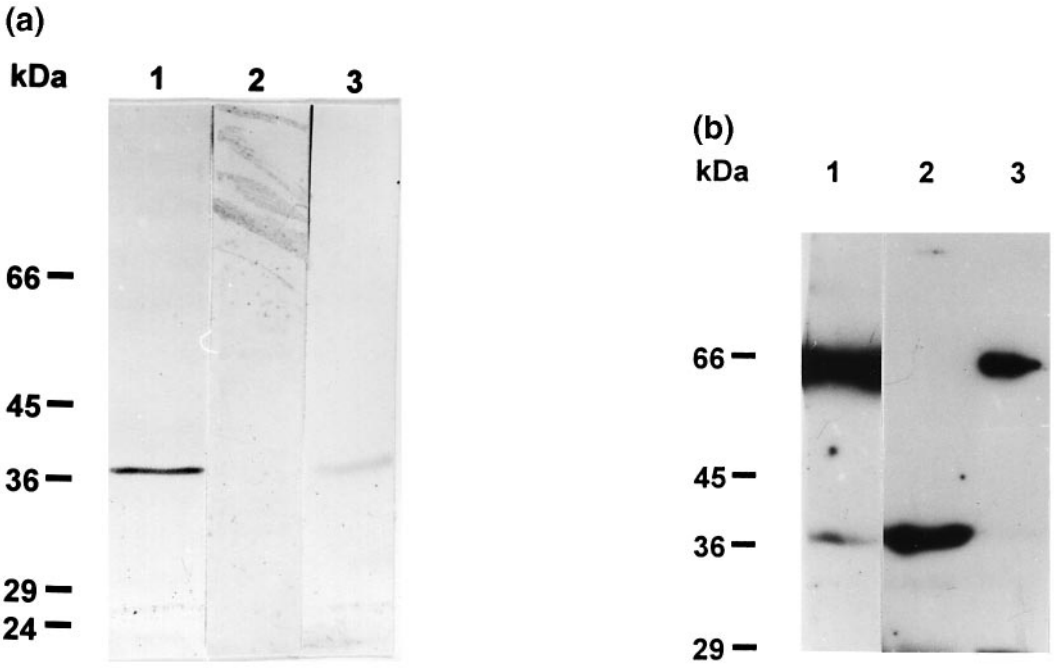

Figure 8. AnnMs2 binds to phospholipids in the presence of Ca^{2+}.
(a) Immunological detection of AnnMs2 in alfalfa cell suspension. Crude protein extract prepared from alfalfa (A2) cells were used for immunoblotting with affinity purified polyclonal anti-C-terminal (CT) AnnMs2 antibody (lane 1). Competition experiment: the anti-CT-AnnMs2 antibody was pre-incubated with the synthetic annexin peptide in 10-fold molar excess before the immunostaining (lane 2). Positive control: bacterially expressed AnnMs2 protein (lane 3). Molecular mass markers are indicated on the left.
(b) Precipitation of the recombinant AnnMs2 protein by exogenous phospholipid extract in the presence of Ca^{2+} and immunodetection of annexin. Crude protein extract from *E. coli* after IPTG induction (lane 1), pellet (lane 2) and supernatant (lane 3) after phospholipid extraction. Equal amounts of proteins (100 μg) were loaded in all lanes. In crude *E. coli* extract both the GST-AnnMs2 and the AnnMs2 protein (proteolytic product) can be detected but only the latter can be precipitated with phospholipid extract.

The recombinant AnnMs2 *protein shows one of the common features of annexins: the phospholipid binding ability in the presence of* Ca^{2+}

In order to analyze the characteristics of the AnnMs2 protein, the cDNA encoding AnnMs2 was inserted into pGEX4T-2 plasmid vector to yield a translation fusion with glutathione-S-transferase, and was expressed in *E. coli*. The pGEX4T-2 vector has been engineered in a way that it contains an oligonucleotide encoding cleavage recognition site of a site-specific protease, thrombin. Therefore, the GST carrier can be cleaved from the expressed fusion protein by thrombin. The recombinant AnnMs2 protein was detected by immunoblotting using affinity purified polyclonal antipeptide antibody. The specificity of the antibody was tested with immunoblot of crude protein extract from alfalfa (A2) cell suspension as well as with competition experiment. Only one specific band with the appropriate molecular mass, i.e. the mass predicted by the cDNA sequence, was recognized by the antibody in crude alfalfa protein extract, and no cross-reacting proteins could be detected (Figure 8a, lane 1). This specific interaction could be competed out by addition of the annexin peptide, even at 10-fold molar excess of the peptide (Figure 8a, lane 2). The size of the specific signal recognized by the antipeptide antibody corresponded to the size of recombinant AnnMs2 protein (Figure 8a, lane 3).

After IPTG induction in crude *E. coli* protein extract we could detect the GST-AnnMs2 fusion protein (≈ 64 kDa) and a faint protein band (≈ 37 kDa) (Figure 8b, lane1). There are data in the literature showing that several GST fusion proteins are able to degrade spontaneously between the GST and the protein (Nakano *et al.*, 1994). This cleavage cannot be avoided by decreasing the incubation temperature, shortening the induction time, or using various protease inhibitors. Most probably, this also happened with the GST-AnnMs2 fusion protein, as by immunoblotting we could detect a band with the appropriate AnnMs2 protein size. Depending on the *E. coli* strain used for expression of the protein, the amount of AnnMs2 showed some variation, but it was faintly detectable even using a protease negative *E. coli* strain, AD202 (as in this case). After phospholipid treatment the AnnMs2 protein was detected in the pellet (lane 2). Although the GST-AnnMs2 fusion protein was highly abundant in crude *E. coli* extract compared with AnnMs2, this protein was not precipitated during phospholipid extraction but was observed entirely in the supernatant (lane 3) (due to adsorption to the pellet, after precipitation the GST-AnnMs2 protein was faintly

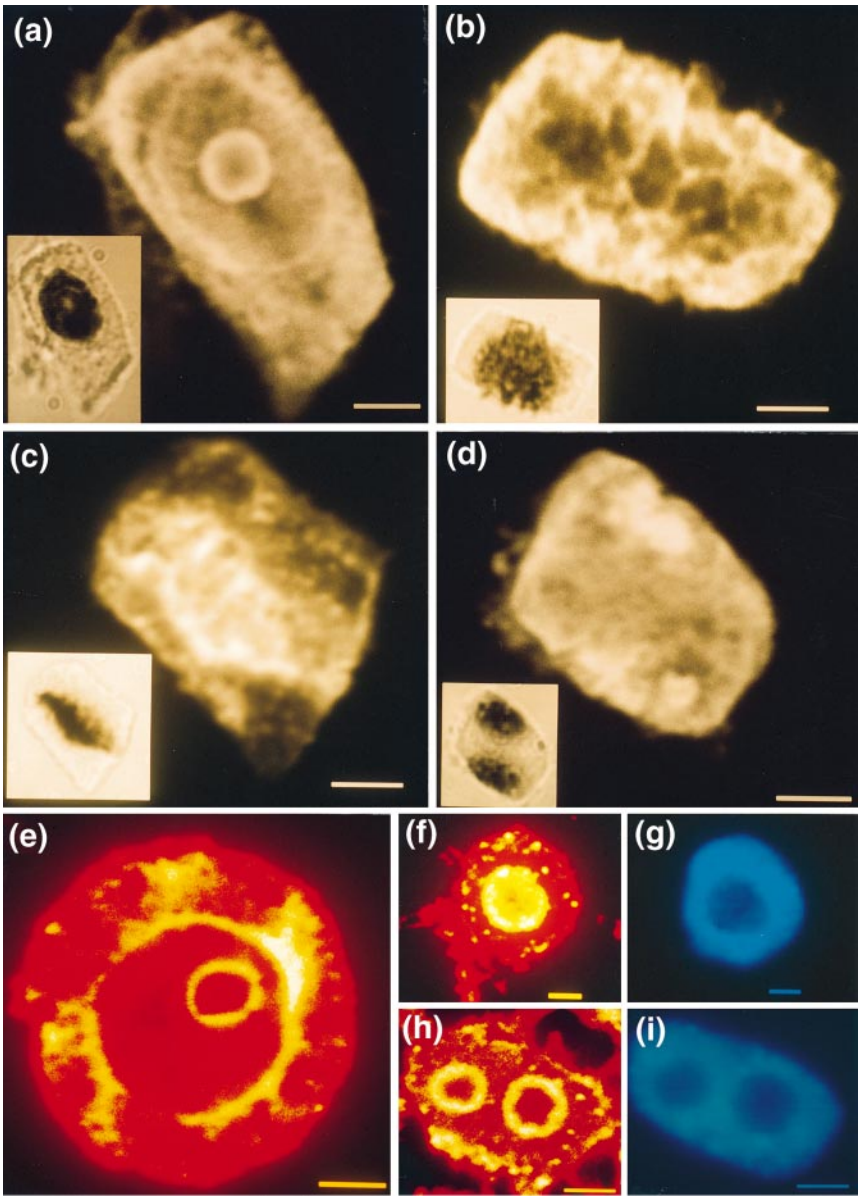

Figure 9. Immunolocalization of annexin in root tip cells and in isolated protoplasts or intact nuclei.
(a) Interphase cell with nucleolar, perinuclear and cytoplasmic signal. (b) Prophase labelling showing enrichment of the signal around chromosomes.
(c) Perichromosomal labelling around metaphase chromosomes.
(d) Reappearing of nucleolar labelling at late telophase. Insets show superposition of DAPI images onto bright field pictures after image subtraction and gamma correction. Bar represents 2.5μ.
(e) Interphase with nucleolar, perinuclear and cytoplasmic labelling.
(f,h) Spotty nucleoplasmic labelling together with nucleolocortical signal.
(g,i) Corresponding nuclear stainings with DAPI. Bar represents 2.5 μ.

detectable in the pellet but after washing the pellet with buffer containing 3 mM $CaCl_2$ it was only present in the supernatant). Most probably, the GST motif (26 kDa) can modify the conformation of the AnnMs2 protein and therefore the fusion protein is not able to bind to phospholipids. In all lanes, equal amounts of proteins were loaded to clearly demonstrate the phospholipid binding ability of the recombinant AnnMs2 protein.

Nucleolar and cytoplasmic localization of the novel annexin-related protein

Indirect immunofluorescence microscopy using affinity purified annexin antibody to label annexin in cells of root tip squash preparations from *Medicago sativa* showed a strong and specific staining of nucleolar cortex, perinucleus and cytoplasm in interphase cells (Figure 9a). In addition, patchy nucleoplasmic signals were also detected. The simultaneous staining with DAPI indicated the localization and morphology of nuclei and nucleoli in the analyzed cells.

In mitotic cells at the beginning of prophase dissolution of nucleolar and perinuclear labelling was followed by enrichment of the signal around the chromosomes (Figure 9b). During metaphase and anaphase, strong labelling was recorded around mitotic chromosomes (Figure 9c). We observed nucleolar labelling in late telophase (Figure 9d) and early interphase. Perinuclear labelling was recorded after nuclear membrane formation. No specific reaction was observed in the control experi-

ments when the antibody was pre-incubated with the specific peptide.

Using protoplast preparations we could confirm the nucleolar, perinuclear and cytoplasmic labelling (Figure 9e) in interphase and perichromosomal labelling in mitotic cells. Further investigation of the nucleoplasmic signal, under very gentle fixation conditions (2% formaldehyde, 15min), revealed granules between nucleolar cortex and perinucleus (Figure 9f–i). This suboptimal fixation often led to burst protoplasts and release of intact nuclei.

In summary, using different preparation procedures we could clearly detect a specific pattern in annexin distribution in the cells at different phases of the cell cycle.

Discussion

After screening a *Medicago sativa* somatic embryo cDNA library we could identify a full-length cDNA (*AnnMs2*) encoding a 333 amino acid long polypeptide which is homologous with known mammalian and plant annexins. It has the conserved primary structure of the eukaryotic annexins with four tandem repeats and the typical 17 bp long conservative motif termed the endonexin fold. Although AnnMs2 shows similarity to animal annexins, several differences can be recognized, as in the case of other plant annexins (Clark and Roux, 1995). The AnnMs2 protein is unique in that it has equally low homology with plant and animal annexins (30–37%), whereas plant annexins have high sequence identity with each other (up to 77% identity at amino acid level). Despite the relatively low homology of this alfalfa protein with other annexins, our data strongly suggest that the AnnMs2 protein belongs to the multigene annexin family although it is a distinct type of plant annexins. This relationship is supported by the unambiguous four domain annexin structure, the domain homology search with known mammalian annexins, the 3D-modelling based on known X-ray crystallography and homology modelling data, and furthermore by the similarities observed with other plant annexins. Recently, we have discovered evidence which strongly supports the idea that despite the low sequence homology AnnMs2 belongs to the annexin multigen family. According to our observations the AnnMs2 protein shows one of the common features of annexins, namely the Ca^{2+}-dependent phospholipid binding ability, as from a crude protein extract of *Escherichia coli* the recombinant protein can be precipitated with exogenous phospholipid in the presence of Ca^{2+}.

Northern analyses revealed that the *AnnMs2* gene was highly expressed in growing tissues. It is an interesting new finding that the mRNA level of the gene can be significantly elevated by various environmental stresses including NaCl, mannitol and PEG treatments. The common feature of all these factors is that they can change the osmotic status of the cells, as also happens under severe water deficiency.

On the basis of homology modelling studies, the 3D structure of AnnMs2 and the sequence homology observed in certain animal annexins, especially type I, V, VI and VII which all exhibit *in vitro* voltage-gated Ca^{2+}-channel activity (Pollard *et al.*, 1992), we propose that the AnnMs2 protein can function as an ion channel. Both ion and water channels are likely to be important in regulating water flux and the relevance of these channels to drought stress has been supported by the isolation of channel protein genes expressed in response to water deficit (Ingram and Bartels, 1996). Considering our data mentioned above we might suggest a potential role of AnnMs2 in osmotic adjustment of plant cells.

There is increasing evidence that the plant phytohormone abscisic acid (ABA) plays an important role in the response of plants to drought stress. It appears that drought stress increases the level of endogenous ABA which, in turn, induces several genes, the so-called 'drought-induced' genes (Bray, 1993; Skriver and Mundy, 1990). Many of these genes are also induced by the application of exogenous ABA, but not all drought-induced genes appear to be ABA-dependent. It is generally accepted that ABA-dependent and ABA-independent signal transduction cascades operate between the initial signal of drought stress and the expression of specific genes (Bray, 1993; Shinozaki and Yamaguchi-Shinozaki, 1996; Skriver and Mundy, 1990). The addition of exogenous ABA increased the level of *AnnMs2* transcript (Figure 7e), which suggests that *AnnMs2* might belong to the drought-induced gene family, and its regulation is linked to the ABA signalling pathway, although further investigations are required to support this theory.

The presented indirect immunofluorescence studies revealed a mostly nucleolar localization of this novel alfalfa annexin-like protein. Although the annexins are localized mainly in the cytoplasm or in the intracellular membrane, the nuclear localization is not unique. Similar studies indicated that a human annexin, type V was located within the cytoplasm and nucleus of cultured human cells, and further examination revealed that annexin V was associated with the nucleolus within the nucleus (Sun *et al.*, 1992). It was also reported that a 50 kDa annexin (CAP-50, calcyclin-binding protein-50) type XI was localized in the nuclei (but not nucleoli) of 3YI fibroblasts in a calcium-dependent manner (Mizutani *et al.*, 1992).

Since the primary function of the nucleolus is the synthesis of ribosomal RNA and the assembly of ribosomes, AnnMs2 may have a role in ribosome synthesis, assembly, transport and function. The presence of specific labelling in the cytoplasm of interphase cells rules out the possibility that it may have a primary role in the establishment or maintenance of nucleolar structure. Using laser scanning

confocal microscopy it was observed that the nucleolar signal was concentrated at the cortical region which is known to contain ribosomal precursor particles in advanced stages of maturation (Diaz de la Espina *et al.*, 1992). Spotty signals in the nucleoplasmic region may represent forms of nucleocytoplasmic translocation of these precursors to the ribosomal subunits. However, signals detected in the cytoplasm and in the perinucleus may have connections with the presence of the antigen in the vicinity of assembled, mature ribosomes either free in the cytoplasm or bound to endoplasmic reticulum and nuclear membrane.

Further studies on mitotic cells revealed that antibody appears to be enriched on the surfaces of the chromosomes which is a typical pattern for some nucleolar and ribosomal proteins (Hernandez-Verdun and Gautier, 1994). During telophase and early interphase, immediate and strong accumulation of annexin labelling over the area in which the future nucleoli will be positioned further supports the hypothesis that there is a close connection between AnnMs2 and formation of intact nucleoli which will immediately start ribosomal biogenesis.

In the AnnMs2 sequence we could not identify nuclear localization signal (NLS) such as the NLS of the SV40 large T antigen (a stretch of basic amino acids: PKKKRKV) (Kalderon *et al.*, 1984) or a bipartite NLS consisting of two clusters of basic amino acids separated by 10–12 amino acids spacer (Robbins *et al.*, 1991). A long N-terminal tail (as in the case of annexin XI), which is necessary and sufficient for its nuclear localization (Mizutani *et al.*, 1995), is also missing. Near the N-terminal of AnnMs2 there is a Pro-rich region which may be important for nuclear targeting lacking the typical NLS.

Concerning the cellular function of the described alfalfa annexin-like protein, presently we have experimental data from two different approaches. On the one hand there are the Northern hybridization experiments that suggest an active state of this gene in growing tissues and considerable mRNA accumulation upon stress treatments, and on the other hand there are the immunocytological data mentioned above. We think that these two lines of evidence can focus the attention onto the role of AnnMs2 as either a structural or functional element in cells with increased need for synthesis of new proteins, especially under stress conditions. The direct functional characterization of this novel protein requires the production of transgenic plants ectopically expressing the *AnnMs2* gene either sense or antisense orientation.

Experimental procedures

Plant material

In most experiments a highly homogenous, fast growing cell suspension culture of *Medicago sativa* ssp. varia (genotype A2) was used. The suspension culture was derived from callus tissue originated from stem segments, and was grown in MS medium (Murashige and Skoog, 1962) supplemented with 0.2 mg l^{-1} kinetin and 1 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) according to Bögre *et al.* (1988). Alfalfa plants were grown in soil under greenhouse conditions. Plant materials were immediately frozen in liquid nitrogen after harvesting, and stored at –70°C.

Stress treatments

Alfalfa A2 suspension culture was treated with various concentrations of NaCl (50–200 mM), mannitol (0.3–0.6 M), PEG (5–15%) and ABA (100–200 μM), and for different time intervals: 4, 8, 24 h at 150 mM NaCl and 150 μM ABA, and for 1, 4, 8, 24 h in the case of the PEG-treatments.

Alfalfa plants grown under greenhouse conditions were subjected to gradually developing drought stress because of water deficiency. Control plants were watered regularly during the experiment. Samples were taken daily for 5 days, and total RNA was extracted from leaf and stem tissues.

Synchronization of the cell cycle, flow cytometric analysis and determination of the mitotic index

To synchronize the cells the alfalfa cell suspension (A2) was treated either with hydroxyurea (Sigma Co.) at a final concentration of 10 or 15 mM or aphidicolin (Sigma Co.) at a final concentration of 10 μg ml^{-1} for 36 h. The cells were then washed three times with fresh MS medium and cultured for synchronous growth (Magyar *et al.*, 1993). Nuclei were isolated and flow cytometric analysis was performed according to Savouré *et al.* (1995). The mitotic index was determined microscopically on ethanol:acetic acid (3:1) fixed cells after carbol fuchsin staining. The determination of cell cycle phase boundaries was according to Magyar *et al.* (1997) on the basis of the flow cytometric data.

RNA isolation and Northern analysis

Total RNA was extracted according to the phenol extraction method of Maes and Messens (1992) with slight modification as described by Magyar *et al.* (1997). The amount of RNA was quantified both spectroscopically (A_{260}) and by gel electrophoresis. Equal amounts of total RNA (20 μg) were loaded on 1.2% formaldehyde-agarose gels containing 0.01% ethidium bromide, and were blotted to Hybond N membranes (Amersham). In all cases the filters were examined under UV light to verify the efficiency of the transfer and to test the quality and quantity of loaded RNA samples. After hybridization in Rapid-hyb buffer (Amersham) at 65°C the filters were washed twice with 2×SSC, 0.1% SDS and once with 1×SSC, 0.1% SDS. The 5′ part of the cDNA clone, a 591 bp long *Eco*RI-*Xho*I fragment, was used as a hybridization probe which was labelled with [α-^{32}P]dCTP using the Megaprime ^{TM}DNA labelling system (Amersham). As an internal control in most cases we used a cDNA clone *Msc27* (Accession no. X98618) which encodes the alfalfa homologue of the human translationally controlled tumor protein (Páy *et al.*, 1992). To study the cell cycle dependent transcription of the *AnnMs2* gene the RNA filter was hybridized with another probe, *Cdc2MsC*, which was shown to be constitutively expressed during cell cycle (Magyar *et al.*, 1997). Each individual Northern experiment was repeated at least three times.

Cloning and DNA sequencing

For the cDNA cloning we used a *Medicago sativa* RA3 somatic embryo cDNA library which was constructed using the λZAPII Kit from Stratagene, and kindly provided by H. Hirt (Institute of Microbiology and Genetics, University of Vienna, Vienna, Austria). *In vivo* excision and phagemide preparations were performed as described in the instruction manual supplied with the 'Lambda ZAP II/*Eco*RI Cloning Kit' by Stratagene. Manual sequencing was performed on the double-stranded DNA in both directions according to the dideoxy chain termination method (Sanger *et al*., 1977) using the T7 sequencing kit from Pharmacia LKB. Oligonucleotide sequencing primers were synthesized by standard cyanoethyl-phosphoramidite method with Expedite synthesizer (Model 8909, PerSeptive Biosystems).

Modelling

All modelling procedures including energy minimisation and molecular dynamics were performed using the INSIGHT II/DISCOVER software (Biosym Technologies Inc., San Diego, CA, USA) implemented on a Silicon Graphics Indigo workstation. Energy calculations were carried out using the CVFF force field (Dauber-Osguthorpe *et al*., 1988).

The structure of alfalfa annexin was modelled according to similarity with known eukaryotic annexin structures. The atomic co-ordinates for all proteins were taken from the Brookhaven Protein Data Bank (Bernstein *et al*., 1977). The modelling was carried out essentially as described previously (Chagolla *et al*., 1994), and the model was evaluated using the Prosall program (Sippl, 1993a,b) using Cβ–Cβ pair interaction potentials and surface potentials as provided by the author.

Antibody production, immunoblotting

Polyclonal antibody was raised in rabbit using a synthetic peptide CSIMFRFGYISNYD (which corresponds to the C-terminal 14 amino acids of AnnMs2) coupled to KLH (Keyhole limpet hemocyanin) through the Cys residue at the N-terminus. Crude IgG fractions were separated from the whole sera by ammonium sulphate fractionation, then further purified on peptide-coupled Pharmacia EAH Sepharose 4B affinity column (Harlow and Lane, 1988).

Protein extracts from alfalfa (A2) cells were prepared for Western analysis, as described by Magyar *et al*. (1997). The SDS-polyacrylamide gels were transferred onto polyvinylidene difluoride (Millipore, Bedford, MA, USA) membranes in 50 mM Tris base-50 mM boric acid buffer at constant current (20 mA) overnight. The filters were blocked in 5% milk powder-0.05% Tween 20 in TBS (25 mM Tris–HCl pH 8.0, 150 mM NaCl) buffer for 2 h at room temperature, reacted with the first antibody (using affinity purified antiannexin antibody) at a concentration of 1 μg of IgG per ml of blocking buffer for 2 h at room temperature, washed three times with TBST (0.2% Tween 20 in TBS, pH 8.0), and reacted with alkaline-phosphatase conjugated goat anti-rabbit IgG according to the manufacturer's instructions (Sigma). Signals were developed by the standard nitro-blue-tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate staining method. We also set up a more sensitive assay for immunodetection: peroxidase-conjugated anti-rabbit IgG was used as a second antibody and the proteins were visualized by chemiluminescence (ECL, Amersham) according to the manufacturer's recommendation.

The specificity of the affinity purified antibody was controlled by competition experiment. The purified antibody was pre-incubated with 10-fold and 100-fold molar excess of the synthetic peptide for 2 h on ice before the immunoreaction.

Bacterial expression of M. sativa *annexin-GST fusion protein*

For the expression of the annexin-like protein the Glutathione-S-Transferase (GST) Gene Fusion System (Pharmacia) was used. The *AnnMs2* cDNA construct (in pBluescript SK +) coding for the annexin-like protein was digested with *Eco*RI and *Kpn*I, the fragment was purified after agarose gel electrophoresis, and ligated with the *Eco*RI-*Kpn*I digested pBluescribe(+) vector (Stratagene). The *Eco*RI-*Sal*I insert was cut out of this construct, and after purification it was ligated with the *Eco*RI-*Sal*I digested pGEX 4T-2 vector to yield a translational fusion with the glutathione-S-transferase. The expression of the fusion protein was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to 0.5 mM final concentration, and purified on glutathione-sepharose according to Smith and Johnson (1988).

Precipitation of the recombinant AnnMs2 protein with exogenous phospholipid extract

Overnight culture of the *E. coli* transformed with the recombinant pGEX plasmid was diluted 1:10 with fresh 2YT medium containing 100 mM glucose and grown at 25°C to OD_{600} 0.5 before adding IPTG to 0.5 mM. The bacterial suspension was grown for a further 3 h at 25°C then the cells were harvested by centrifugation and resuspended in PBS buffer containing 1 mM DTT and 2 mM PMSF. Cells were lysed on ice by mild sonication and after adding Triton X-100–1%, were subjected to centrifugation at 8000 ***g*** for 15 min at 4°C. 2 M EGTA was added to the supernatant (final concentration 10 mM) and after centrifugation at 15000 rev min^{-1} (27000 ***g***) for 30 min at 4°C in a Sorvall SS34 rotor, it was further treated with bovine phospholipid extract (type VII, Sigma) in the presence of Ca^{2+} (final concentration 15 mM) according to Smallwood *et al*. (1990). After the phospholipid extraction the supernatant and the pellet were analysed by immunoblotting together with the crude *E. coli* protein extract after IPTG induction.

Immunolocalization studies

Root tips of 2-day-old *Medicago sativa* seedlings (2 mm long) were fixed with 4% formaldehyde, the cell walls were partially digested with 2% (w/v) cellulase Onozuka R-10, and after gentle squashing the cells were spotted onto Poly L-Lysine (Sigma) coated slides to air-dry (Goodbody and Lloyd, 1994). The slides were post-digested with a strong enzyme mixture (4% Cellulase Onozuka RS, 1% Macerozyme R-10, 1% Driselase, 2% Pectinase, 0.2% Pectolyase in 80 mM $CaCl_2$, 0.5% MES pH 6.5) for 1 h then incubated with rabbit anti-annexin antibody (1 μg μl^{-1}) at 1–100 dilution for 12 h at 4°C in MTSB (50 mM PIPES pH 6.9, 5 mM $MgSO_4$, 5 mM EGTA) and 2% BSA. For the control experiment the antibody was incubated with the peptide in 10 times higher molar concentration for 1 h at 4°C. TRITC (Tetramethyl rhodamino-5-isothiocyanate) conjugated goat antirabbit secondary antibody (Southern Biotech Assoc.) was applied for 1 h at 23°C in the same buffer at 1–70 dilution. The same buffer without BSA was used for both antibody washing steps. Before the last wash 100 μl DAPI (4′,6-diamidino-2-phenylindole HCl) was used (1 μg μl^{-1}) for nuclear staining. The cells were covered with Fluoromount-G mounting solution (Southern Biotech. Assoc.) and analyzed with

either CCD Camera (ZVS-47 DEC, Carl Zeiss Inc.) connected to Zeiss Axiovert 135 M microscope (for UV excitation of DAPI) or Confocal Laser Scanning Microscope (Zeiss LSM 410) (pinhole of 15, eight averaging of 2 sec scans using Plan NeoFluar 100×/1.30 pol objective was used in combination with TRITC filter set for antibody detection).

Suspension cells of *Medicago sativa* were used for the labelling of protoplasts 1 day after refreshing the medium. A dense drop of cells was totally digested with the enzyme mixture used for the root tip preparation (except that 0.3 M sorbitol was also added to the buffer) at 26°C for 4 h on an orbital shaker. After washing the protoplasts with the enzyme buffer, they were layered onto coated slides. Following settling they were fixed on the slide using either 4% fresh formaldehyde in the same buffer for a period of 30 min or 2% for 15 min. Upon gentle washing of the fixative (3 × 20 min) they were air-dried completely to permeabilize the plasma membranes. For antibody labelling, detection and control experiments the same parameters were used as before.

Acknowledgements

The authors are grateful to János Györgyey for his help in the computer analysis, to Zsuzsa Keczán for preparation of the manuscript, and to Béla Dusha and Etelka Mitrov for photographic work. We would also like to acknowledge Heribert Hirt for the alfalfa cDNA library, Sotir Zakharier for the synthetic annexin peptide to affinity purify the polyclonal antibody and our colleagues, especially Attila Fehér and Csaba Seregélyes for their helpful comments on the manuscript.

This work was supported by grants from the Körber Foundation (Hamburg, Germany), and ICGEB (Trieste, Italy) (Grant No. CRP/HUN94–01).

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EMBL database accession number Y11348.