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Characterization and gene expression of an annexin during fruit development in *Capsicum annuum***

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Abstract Several lines of evidence indicate that annexins, as calcium-dependent phospholipid-binding proteins, are involved in a variety of plant cellular processes. We were interested in determining if annexins are implicated in the highly regulated fruit development of bell pepper. By differential screening of several cDNA libraries, we isolated a full-length cDNA of 1180 bp encoding an annexin. Northern blot analyses show a differential expression pattern of the transcripts during the early stages of development and during ripening. Immunoblots using antiserum raised against p33/p35 from maize reveal that cross-reactive polypeptides of about 30 kDa are present at each stage of fruit development in bell pepper. We partially purified the annexins from seedlings and green fruits. At least one annexin of 32 kDa is present in these plant tissues.

Key words: Calcium-dependent phospholipid-binding protein; Full-length cDNA; Differential pattern of expression; Partial protein purification

1. Introduction

Fruit development in bell pepper is a useful system to study the regulatory mechanisms controlling division, growth and differentiation of plant cells during the early stages of fruit formation and during the ripening process. Different hormonal and physical factors play an important role during the development, but the molecular nature of the signals that control this development is largely unknown. Since calcium is a well-known component of the signal transduction pathway in activating and regulating numerous cellular processes in eukaryotes, we focused our study on calcium and calcium-binding proteins.

Among the calcium-binding proteins, annexins form a family of structurally related proteins that exhibit calcium-dependent binding to phospholipids. In animal tissues, they have been shown to be implicated in multiple aspects of cell biology including regulation of membrane trafficking, transmembrane channel activity, transduction in mitogenic signal and settlement of cell-matrix interactions [1]. However, the precise biological functions of annexins are still unknown, and concerning their occurrence and implication in plant cells, only very liitle information is available [2]: among the hypotheses, it has been proposed that annexins may function in the Golgi-mediated secretion of wall polysaccharides in plant cells [3]. We have isolated the first full-length cDNA encoding a plant annexin. We analyse the expression of bell pepper annexin during fruit development to determine the participation of calcium in this regulated process.

2. Materials and methods

2.1. Plant material

Bell pepper seeds (*Capsicum annuum*, cv. Yolo Wonder) were germinated, and plants were grown under greenhouse conditions. For the partial purification of calcium-dependent phospholipid-binding proteins, maize (*Zea mays* L. cv. DK250) and bell pepper seeds were grown in the dark: 5 days in moist vermiculite for maize [4] and 17 days in moist compost for bell pepper.

2.2. Construction of the cDNA libraries and screening

cDNA libraries were constructed in different vectors, according to the manufacturer's instructions, from bell pepper fruits poly(A)$^+$ mRNA of different stages of development: very young green fruits mRNA in λZap, young green fruits mRNA in λgt11 and red fruits mRNA in λgt10.

A λgt11 library of young green fruit was differentially screened with labelled first-strand cDNA synthesized from poly(A)$^+$ RNA of very young green fruits and of mature green fruits, according to a method [5] used previously for the isolation of fruit-specific cDNAs [6].

An incomplete cDNA (507 bp) was isolated and used as a probe for hybridization screening of the other fruit libraries described above. cDNA isolated by screening of the red and young green fruit libraries were cloned into the *Eco*RI site of pBluescript KS+ (Stratagene). After screening of the very young green fruit library, in vivo excision and phagemide preparation were performed as described in the instruction manual (Stratagene).

2.3. Northern hybridizations

Total RNAs from fruits at the early development stages were extracted according to Arrand [7]. Those from fruits at the late development stages were extracted as described before [8]. RNAs were separated on 1.2% agarose-6% formaldehyde gels, transferred to Hybond-N+ membranes (Amersham). Hybridizations were carried out overnight at 42°C in 5×SSC (1×SSC: 0.15 M NaCl, 0.015 M sodium citrate), 5×Denhardt's solution (1 mg/ml Ficoll, 1 mg/ml polyvinylpyrrolidone, 1 mg/ml BSA), 1% SDS, 20 mg/ml salmon sperm DNA and 50% formamide with the ^{32}P-labelled cDNA. Filters were first washed at 42°C in 2×SSC, 0.1% SDS and then at 65°C in 0.2×SSC, 0.1% SDS before exposure to X-ray film.

2.4. Southern hybridization

Genomic DNA was digested with various restriction enzymes (*Bam*HI, *Eco*RI and *Hin*dIII). Fragments were separated on 0.7% agarose gels, denatured, and transferred to Hybond-N+ membranes. Hybridizations were carried out as described above.

2.5. Protein analysis, immunochemical techniques

Total proteins were extracted from different tissues of bell pepper (leaves and roots) and different stages of fruit development (very young green, young green, mature green, orange and red fruits): we use the phenol extraction method (0.1 M LiCl, 1% SDS, 10 mM EDTA and 0.1 M Tris-HCl pH 8 as extracting buffer) [9].

Calcium-dependent phospholipid-binding proteins were isolated from mature green fruits (20 g), dark grown bell pepper seedlings (10 g) and dark grown maize coleoptiles (20 g) as described [4], but omitting the hydroxyapatite purification step and using a soybean

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phospholipid mixture (type IV-S, Sigma) containing 67% phospholipids in weight of the mixture.

30 μg of crude protein samples and 15 μg of purified protein extracts were separated on SDS-PAGE and transferred to nitrocellulose filters for Western blot analyses [10]. Immunodetection was carried out using polyclonal antibodies against annexins p33/p35 from maize raised in rabbit [4] and with peroxidase-conjugated anti-rabbit immunoglobulin G.

3. Results

3.1. Characterization of cDNAs encoding an annexin

By differential screening, we succeeded in isolating a partial cDNA of 507 bp (an open reading frame of 315 bp is followed by an untranslated region of 292 bp). The analysis of the peptide sequences predicted from this cDNA show that one peptide shares an important similarity percentage with the alfalfa annexin. This 507 bp cDNA was used as a probe for hybridization screening of other libraries in order to isolate a full-length cDNA. Libraries from RNA extracted at different stages of fruit development were screened: (i) very young green fruit stage (phase of cellular division and differentiation); (ii) young green fruit stage (phase of cellular expansion); (iii) red fruit stage (phase of the end of ripening). The sequences of all the cDNAs isolated from the different libraries were identical except the length of the 3′ untranslated region, ranging from 170 to 292 bp, indicating that different poly A sites are used.

The cDNA E511 that contains the longest translated region, isolated from the very young green fruit library, contains 1130 bp. Although the absence of upstream STOP codons does not allow the identification of the initiation codon, several lines of evidence indicate that the bell pepper clone is full-length. The first ATG codon of E511 lies within a sequence close to the consensus sequence of Kozak [11], and therefore constitutes likely the start of the open reading frame. All 5′ ends of the different cDNAs isolated from different developmental stages libraries were always located around the same sequence. Two independent *Arabidopsis thaliana* partial sequences, obtained as a part of the systematic sequencing program and appearing in the dbest library under the accession nos. H76460 and T22046, present an identical aaaaatggc sequence without additional ATG codons in the upstream region.

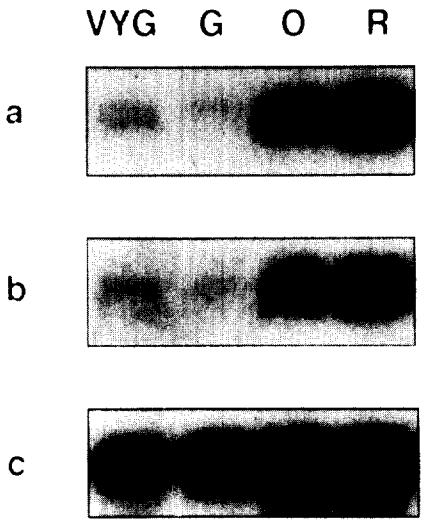

Fig. 2. Northern blot analysis of the annexin transcripts during fruit development. Total RNA samples (10 μg) from very young green fruits (VYG), mature green fruits (G), orange ripening fruits (O) and fully ripe red fruits (R), were tested using the full-length cDNA E511 as probe in (a), the 3′ untranslated region of E511 as probe in (b) and an 18S RNA probe in (c).

The open reading frame of 541 bp (bp 5 to 946) is followed by a 204 bp untranslated region and a poly A tail. Up to now, this is the first full-length cDNA encoding a plant annexin. In the GenBank and EMBL nucleic acid database, incomplete cDNAs were described: a cDNA from strawberry [12] and the longest incomplete published cDNA from *Medicago sativa* [13].

The nucleotide sequences of the cDNA E511 and the cDNA from *M. sativa* share 64% nucleotide identities. The bell pepper cDNA contains a longer open reading frame (24 additional bp in the 5′ region).

3.2. Predicted amino acid sequence of E511

The amino acid sequence deduced from E511 consists of

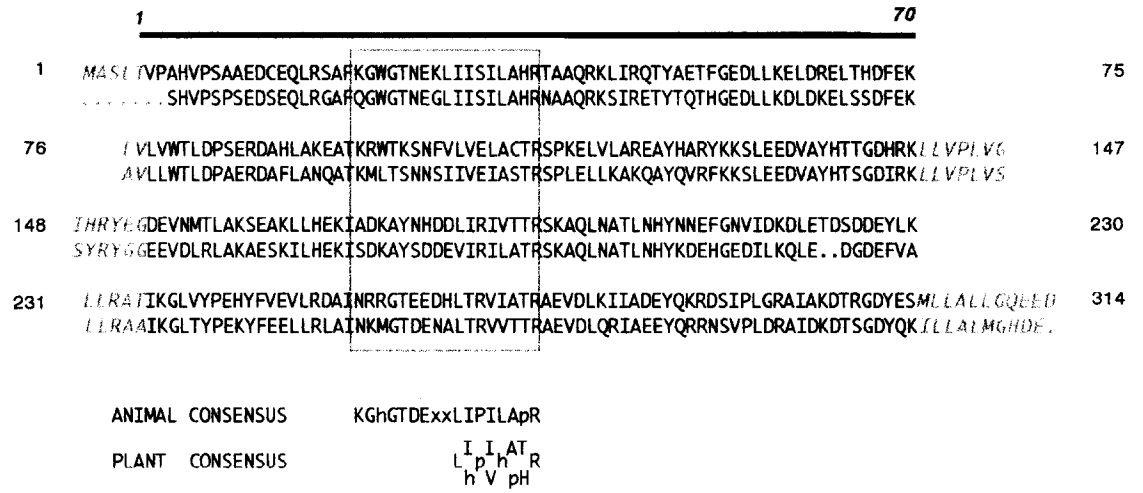

Fig. 1. Alignment of bell pepper (upper line) and alfalfa annexin peptides. The repeat sequences are numbered according to the convention for animal annexins (1–70) [19]. Residues external to the four repeats are represented in gray and italic. The boxed area corresponds to the endonexin-fold domains. In the consensus sequence, h = hydrophobic residue; p = polar residue; s = small residue; a = aliphatic residue according to the Venn diagram [20].

314 residues as shown in Fig. 1. The predicted polypeptide has a molecular weight of 35.8 kDa, a global charge of −10, and an isoelectric point of 5.98. The N-terminal region of the predicted peptide does not have significant similarity with any targeting sequences (for example, signal peptide, nuclear localization sequence or transit peptide) thus suggesting a cytoplasmic localization.

All annexins present similar properties regarding calcium and phospholipids, resulting from a common primary structure. In mammal cells, each annexin is constituted of two different regions, the unique N-terminal domain (the tail), considered to be the regulatory region of the protein, and the C-terminal domain (the core) [1]. The core is composed of 4 repeats of a 70 amino acid sequence containing a highly conserved region called the endonexin-fold with its characteristic KGhGTDExxLIpILApR motif (see Fig. 1).

In bell pepper annexin, the C-terminal domain is composed of 4 repeats of 70 residues, but it is more difficult to predict their precise position. We determined the 17 residues constituting the endonexin-fold of the first repetition (KGWGTNEKLIISILAHR), which is very close to the consensus sequence. The properties of several residues (for example, R in position 36) strictly conserved allow the identification of the 3 other repeats. A decreasing similarity percentage for the different endonexin-fold in the four repeats appears: the first, then the fourth, the second and lastly the third. A bell pepper characteristic motif, shorter but strictly conserved, can be deduced from the comparison of the four repeats: LI/hpI/VhA/pT/HR. The endonexin-fold of the first repetition seems to be well conserved in bell pepper annexins. A bell pepper p35 annexin was isolated [14] and its partial amino acid sequence, consisting of 22 residues in this endonexin-fold, shares 86% identities with the amino acid sequence de-

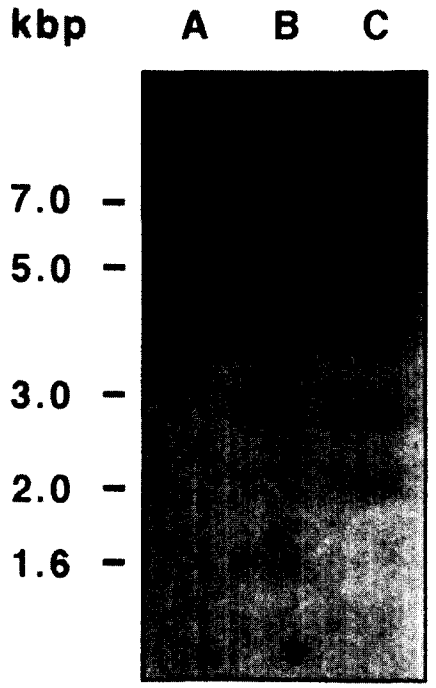

Fig. 3. Southern hybridization of 10 μg genomic DNA restricted by *Bam*HI (lane A), *Eco*RI (lane B) and *Hin*dIII (lane C). The blot was probed with ^{32}P-labelled full-length E511 cDNA.

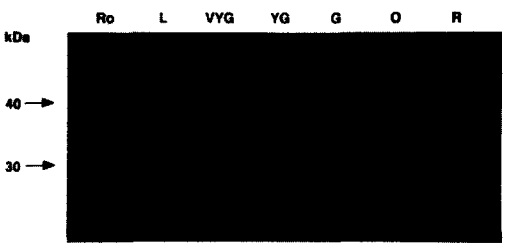

Fig. 4. Immunological analysis of samples from roots (Ro), leaves (L), very young green fruits (VYG), young green fruits (YG), mature green fruits (G), orange ripening fruits (O) and fully ripe red fruits (R). In each case 30 μg of proteins were subjected to SDS-PAGE, followed by immunoblotting using antiserum against the 33–35 kDa annexin doublet from maize.

rived from E511. The second important region of annexin is the tail. The bell pepper one, with only five residues, is very short in comparison with the length found in animals.

In Fig. 1, alignment of bell pepper and alfalfa annexins (alfalfa annexin is the only other plant predicted peptide containing almost the four complete repeats) is shown. The amino acid sequences derived from the two cDNAs share 83% similarities and 67% identities. Whereas the four bell pepper endonexin-folds are largely divergent from the conserved mammal motif (except in the first repeat), plant endonexin-folds are much more similar.

3.3. Developmental regulation of annexin gene expression

Total RNAs were extracted from fruits at different development stages and analyzed on Northern blots. The full-length cDNA E511 probe hybridizes to a transcript of 1.2 kb (Fig. 2a). The transcripts accumulate transiently in very young green fruits and the transcript level remains low in the following step of mature green fruits. During ripening, annexin mRNAs accumulate strongly. The overall accumulation in the fully ripe red fruit can be estimated as a 3.5-fold increase. In Fig. 2c, the blot was hybridized with an 18S RNA probe, indicating that the differences in expression of the E511 transcripts were not due to variation in sample loading or integrity.

Interestingly, the same expression pattern was obtained using the full-length cDNA probe or the specific probe from the 3′ untranslated region (Fig. 2b). This result indicates that the transcript level measured reflects the variation of one transcript rather than the resultant of several different transcripts. The detection of one mRNA can be correlated with the characterization of only one cDNA type at all the fruit development stages.

3.4. Genomic analyses

To ascertain that the differential pattern of the transcript expression is due to the variation of one transcript, we have analyzed genomic DNA to determine the number of related genes. *Bam*HI, *Eco*RI and *Hin*dIII cleaved genomic DNA was analyzed by Southern blot hybridization using the complete cDNA as probe. As shown in Fig. 3, hybridization reveals a unique strong signal in all three lanes. One additional minor signal is observed with the *Eco*RI and *Bam*HI digestions, and three with the *Hin*dIII digestion. These results confirm the presence of a single gene copy, but a small number of related genes exist in the bell pepper genome.

3.5. Determination of the presence of annexin in different bell pepper tissues by Western blot analysis

We have performed Western blot analyses using antiserum raised against the 33–35-kDa annexin doublet from maize, kindly provided by Dr. Battey. We have cloned the E511 cDNA in the expression vector pBluescript to determine whether these antibodies cross-react with bell pepper annexin. The in vivo produced peptide fused with the β-galactosidase is recognized by the maize antibodies (data not shown).

Total proteins were extracted from several tissues (roots and leaves) and from all the fruit development stages. The antiserum strongly detects only one bell pepper polypeptide of about 30 kDa (see Fig. 4) in all these crude extracts (in leaves and green mature fruits, the peptide of 20 kDa revealed by the antibodies represents likely a degradation product). The detection of one polypeptide at all the fruit development stages correlates well with the characterization of only one cDNA type.

Moreover, the strong accumulation of the transcript level during ripening can be compared with the increasing amount of the protein.

3.6. Isolation of calcium-dependent phospholipid-binding proteins from bell pepper and maize

Whereas the antiserum recognizes two polypeptides in maize, it detects only one annexin in bell pepper fruits. This result prompted us to perform partial purification of the annexin proteins in order to overcome the possible low abundance of other calcium-dependent phospholipid-binding proteins and the divergence of their amino acid sequences. The purification procedure was used in parallel on fruit tissues and etiolated bell pepper seedlings and on maize coleoptiles as control [4].

Staining (Fig. 5a) and immunoblotting (Fig. 5b) revealed the doublet of M_r 33–35 kDa with purified maize extracts. However, the pattern is different with bell pepper green fruits in which three polypeptides of 32, 35 and 38 kDa respectively are detected by staining, but only the polypeptide of 32 kDa is recognized by the antiserum. The results of this Western blot allow us to assume that at least the 32 kDa peptide corresponds to an annexin.

To determine if the detection of only one annexin was restricted to fruit, we decided to isolate calcium-dependent phospholipid-binding proteins from bell pepper seedlings. Staining of purified extracts revealed proteins of 32 and 38 kDa and the antiserum reacted only with that of 32 kDa. Thus, seedlings contain also an annexin of 32 kDa. However, to ascertain that the same protein is present in fruits and seedlings, it will be necessary to isolate the seedling cDNA.

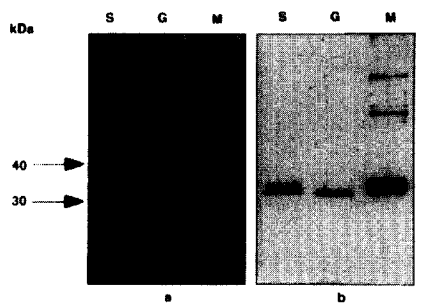

Fig. 5. Purification of Ca^{2+}-dependent phospholipid-binding proteins. 15 μg of purified proteins extracts were resolved in SDS-PAGE, followed by staining in (a) and by immunoblotting using antiserum against p33/p35 from maize in (b). Protein samples from dark grown bell pepper seedlings (S), mature green fruits (G) and dark grown maize coleoptiles (M) were analyzed.

4. Discussion

Bell pepper fruits have a particularly interesting potential as a biological system to understand problems related to plant development, for two reasons: (i) fruit is a uniquely valuable plant organ to study how cell division, growth and differentiation are controlled [15]; (ii) bell pepper is classified as a non-climacteric fruit [16], and might present a particular behavior in response to hormonal factors during ripening. Early development and ripening are highly programmed processes; their understanding depends on the dissection of the transduction pathway involving the particularly signals taking place during this fruit development. Therefore, we have concentrated our efforts on calcium-binding proteins, and more precisely on annexins, calcium-dependent phospholipid-binding proteins.

We have isolated the first full-length cDNA encoding a plant annexin and it allows us to predict the primary structure of bell pepper annexin. While the structural features are well conserved in mammals (the core is the conserved part of the molecule and strictly defines the annexin family), it seems that, in plants, several variations are found. Indeed, the 17 amino acid endonexin-fold sequence in bell pepper is largely divergent from the characteristic motif in mammals. Comparison of bell pepper and alfalfa annexin endonexin-folds leads to the conclusion that plant C-terminal domain is well conserved but divergent from the mammalian counterpart. Nevertheless, antibodies raised to the 33–35-kDa doublet from maize recognize p68 (annexin VI) present in chicken-gizzard annexin preparations [4] indicating homology between plant Ca^{2+}-dependent phospholipid-binding proteins and animal annexins.

In mammals, the tail is extremely variable and its length varies from a few amino acids to more than 160. It contains the major sites for phosphorylation, proteolysis or interactions with other proteins. The N-terminal domain of the bell pepper annexin contains only 5 amino acids and therefore seems too short to be a regulatory domain. However, since no other N-terminal part of a plant annexin has been described before, it is not possible to establish whether the 5 residues are sufficient to constitute a regulatory domain and if the short N-terminal region is a characteristic of plant annexin. The length of the tail and the primary structure of the core are not conserved in annexin from plants and mammals. This poses two fundamental questions: what are the exact biological functions of the annexin, and are they conserved in mammals and plants?

Annexins are ubiquitous (they have been described in molds, mammals, and more recently in plants), and different mammal cells or organs show a typical distribution pattern of the various annexins. In plant cells, Western blots show that at least two annexins can be detected in maize coleoptile, tomato suspension-culture cells and tissues, potato and barley tissues [17].

However, several lines of evidence from our present work indicate that bell pepper fruits contain only one annexin. We have characterized only one type of cDNA by screening libraries constructed at different fruit development stages, the

differential pattern of annexin mRNA reflects the variation of one transcript and the isolated cDNA corresponds to a single gene copy. Moreover, the antiserum raised against p33/p35 from maize detects only a 32 kDa polypeptide in crude protein samples and in partially purified extracts from bell pepper fruits. However, a small number of related genes exist in the bell pepper genome and we could identify 2 other calcium-dependent phospholipid-binding proteins in green mature fruits. These 2 proteins might be encoded by divergent mRNAs, not detected by the E511 cDNA probe. The 2 other proteins are not recognized by the maize antiserum, but the use of antibodies from mammals can provide an alternative assay to detect other annexins (immunoblottings were carried out using polyclonal antibodies raised against anchorin CII from chicken to identify annexins in the fern rhizoids) [18].

The characterization of the E511 gene will be a useful tool in future studies. The determination of the promoter structure and DNA binding factors will allow an understanding of the molecular nature of the signals that involve calcium and calcium-binding proteins during the highly controlled fruit development and ripening.

To determine the precise biological functions of annexins, the key issue will be to study specific annexin properties in vivo (many properties have been described in vitro only) [1]. Towards this end, one approach could be the stable transformation of bell pepper or more easily transformed tomato plants with constructs directing up- and down-regulated expression of annexins.

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