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Characterization of the sequence coding for the clathrin coat assembly protein AP17 (σ2) associated with the plasma membrane from *Zea mays* and constitutive expression of its gene

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Abstract

The cDNA and genomic sequences coding for the clathrin coat assembly protein AP17 (σ2) from maize and its corresponding mRNA accumulation have been analyzed. This protein in yeast and mammals has been shown to be part of the associated protein (AP) complex of clathrin in the plasma membrane. The availability of this sequence as well as a previous AP19 in a plant allows one to propose that specific AP complexes exist in plants in the Golgi complex and in the plasma membrane. The AP17 protein is encoded in maize by a single gene, and its mRNA accumulates in all the organs studied. In the immature embryo, it displays a pattern of expression typical of constitutively expressed genes. © 1998 Elsevier Science B.V.

Keywords: Clathrin assembly protein; AP17; Maize; Embryo

1. Introduction

Clathrin-coated vesicles (CCV) are an essential component of the protein transport machinery of the Golgi apparatus, including endocytosis, and in the secretion processes [see Beevers (1996), Mellman (1996) and Robinson (1996) for recent reviews]. In clathrin coats, two complexes may be distinguished: the clathrin complex itself composed of three heavy and three light chains assembled into polyhedral lattices and the assembly of associated protein (AP) complexes. These complexes are located in the cytosolic part of the vesicles, and they are important for the assembly process of clathrin. In mammals and yeasts, at least two AP complexes have been described. One of these complexes, AP-1, is located in the outer trans-Golgi network, whereas the other one, AP-2, is located at the plasma membrane. A third adaptor-like complex, AP-3, has recently been proposed associated to the trans-Golgi network as well as with peripheral membranes (Simpson et al., 1997). These complexes are heterotetramers containing proteins of a high molecular weight, the adaptins, as well as low-molecular-weight proteins that are specific for the two complexes and can be distinguished by their respective molecular weights. AP-1 contains the AP19 protein, whereas AP-2 contains the AP17 protein. These low-molecular-weight proteins have also been termed σ1 and σ2 proteins.

Very little information is available about the components of the secretion machinery in plants. Although CCVs do not seem to be involved in processes such as the transport of storage protein to the vacuole (Hohl et al., 1996), the structure of the complex appears to be similar to the mammalian and yeast systems (Beevers, 1996). Heavy and light clathrin chains have been described in plants (Butler and Beevers, 1993), and homologs of adaptins have been located by using immunological methods (Holstein et al., 1994). It was still not proven whether plants have different adaptin components in the two different complexes, and the AP19 protein has been described in *Camptotheca acuminata* (Maldonado-Mendoza and Nessler, 1996). No information was available on the AP17 component that would confirm the difference between the two AP complexes in plants.

In our laboratory, we are interested in the use of specific patterns of gene expression to analyze the process of early embryogenesis in maize and its modification by specific mutations. To this end, genes having a

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constitutive expression in the plant have been searched in a cDNA library of immature maize embryo. Genes related to the secretion process have been preferentially studied as a number of mutations blocking embryogenesis in *Arabidopsis thaliana*, and these mutations have been shown to occur in genes coding for proteins related to the secretion machinery (Shevell et al., 1994; Lukowitz et al., 1996). In the course of this process, the cDNA and genomic sequences corresponding to the AP17 protein have been cloned in maize, its sequence obtained at cDNA and genomic levels and its expression studied in particular in the immature embryo.

2. Materials and methods

2.1. cDNA and genomic library construction and screening

General recombinant DNA techniques were performed as described by Sambrook et al. (1989). All the genomic and cDNA libraries were made according to the instructions of the manufacturer (Stratagene, La Jolla, CA). The cDNA library from RNA extracted from embryos 12 days after pollination was constructed in λZap vector (Stratagene). The genomic library from genomic DNA extracted from leaves was constructed in EMBL vector (Stratagene). The cDNA library was screened after in-vivo excision using a 12-DAP embryo cDNA probe to obtain clones that were really abundantly expressed in the embryo.

The genomic library was screened using the AP17 probe by the random priming method (Random Primed DNA labeling Kit, Boheringer Mannheim, Germany), following the manufacturer's protocols. Sequencing of the inserts was carried out using automatic fluorescent sequencing (ALF, Pharmacia).

2.2. RNA blot and Southern blot analysis

Tissues were collected from different embryo stages, adult root and leaf of *Zea mays* (W64). All tissues were frozen in liquid nitrogen and kept at $-70^{\circ}C$ until use. Total RNAs were extracted as described by Maniatis et al. (1982). Ten micrograms of total mRNA were denatured and fractionated on a denaturing gel as described by Meinkoth and Wahl (1984). The gel was transferred to a nylon membrane (Nytran; Schleicher and Schuell) using $20\times$ SSC as transfer buffer. Maize genomic DNA was isolated as described by Chen and Dellaporta (1994), and transfered under alkaline conditions on to Nytran (Schleicher and Schuell) following the manufacturer's protocol. Northern and Southern blots were fixed, hybridized and washed as described by Church and Gilbert (1984). Probes were labelled by the random priming method (Random Primed DNA labeling Kit, Boheringer Mannheim, Germany), following the manufacturer's protocols.

2.3. In situ hybridization

Fixation and pretreatment of the tissues were carried out according to (Langdale, 1994). Riboprobes, hybridization, washes, blocking, antibody incubation and detection were performed according to the suppliers' protocols (RNA colour kit for non-radioactive in-situ hybridization, Amersham). Digoxygenin-labelled hybrids were viewed using bright-field microscopy, and photographs were taken using Ektachrome 160 films.

2.4. Sequence aligment and phylogeny

The sequence of the AP17 was aligned with eight amino acid sequences of other AP deduced from cDNAS or genes encoding these proteins. The Multiple Sequence Alignments were analyzed using the ClustalV program (Higgins et al., 1991). The Accession Numbers of the sequences compared are: *Zea mays* AP17 (X96758), *Homo sapiens* AP17 (X97074), *Rattus norvegicus* AP17 (M37194) (Kirchhausen et al., 1991) and AP17B (U75917), *Saccharomyces cerevisiae* AP17 (M37193) (Kirchhausen et al., 1991), *Camptotheca acuminata* AP19 (U53345) (Maldonado-Mendoza and Nessler, 1996), *Mus musculus* AP19 (M62418) (Kirchhausen et al., 1991), *Saccharomyces cerevisiae* AP19 (X70279, Z30314, U17246) (Nakai et al., 1993; Phan et al., 1994).

3. Results

3.1. Sequence of the cDNA and the gene corresponding to the AP17 protein in maize

During the process of examining the patterns of expression of specific groups of genes in the maize immature embryo, genes having a constitutive expression were cloned. This type of expression was defined from the specific mRNA accumulation observed in RNA blots. Genes having a sequence that could be related to specific cellular mechanisms in the cell were chosen, and in particular those related to vesicular transport processes. This is the case of a clone that showed a high homology to the AP17 protein from clathrin coat vesicles already described in yeast and mammals.

The full-length cDNA clone corresponding to the AP17 protein was obtained, and the protein sequence was deduced from its cDNA. The sequence has the X96758 Accession Number in the EMBL Database. In Fig. 1, the comparison of the AP17 protein from maize with the other low-molecular-weight AP protein sequences from other species is shown. The sequences

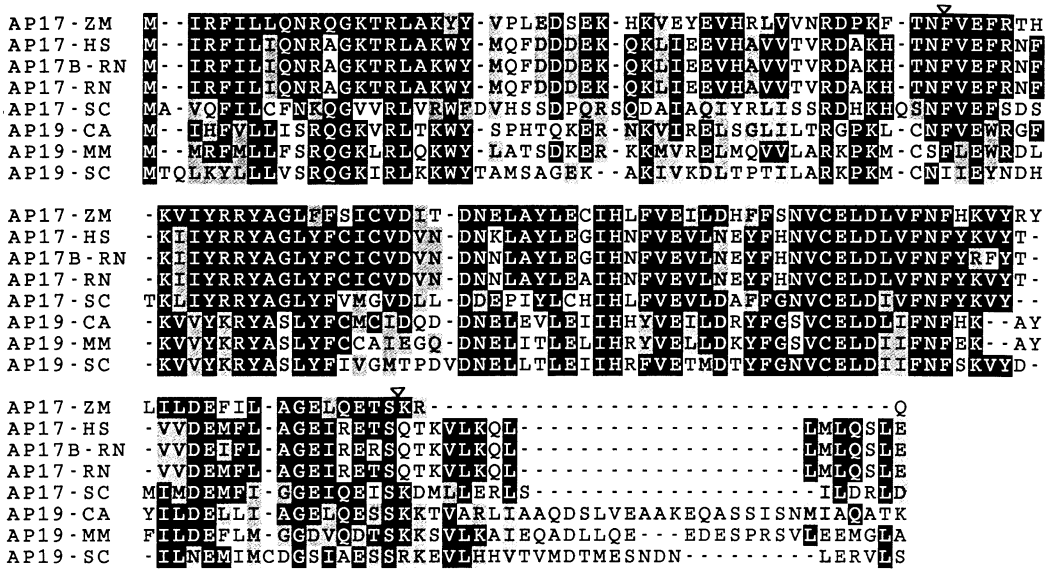

Fig. 1. Alignment of the AP17 protein from *Zea mays* (lane 1) with the other low-molecular-weight AP protein sequences from other species using Clustal V. Amino-acid residues identical to the AP17 sequence of *Zea mays* are shown in black boxes. Amino-acid residues similar to the AP17 sequence of *Zea mays* are shown in grey boxes. Zm-AP17, *Zea mays* (AP17); Hs-AP17, *Homo sapiens* (AP17); Rn-AP17, Rn-AP17B, *Rattus norvegicus* (AP17); Sc-AP17, *Saccharomyces cerevisiae* (AP17); Ca-AP19, *Camptotheca acuminata* (AP19); Mm-AP19, *Mus musculus* (AP19); Sc-AP19, *Saccharomyces cerevisiae* (AP19). Intron positions of the genomic clone of Zm-AP17 are shown by triangles pointing down.

are mainly from mammals and yeasts but a sequence from a plant (*Camptotheca acuminata)* corresponding to the AP19 protein is also available (Maldonado-Mendoza and Nessler, 1996). Partial cDNA sequences (ESTs) from rice and *Arabidopsis thaliana* are also available in the databases, but as they code for incomplete protein sequences, they have not been considered in this analysis. The similarity of the protein from maize is 65% with the human and rat sequences of the AP17 group, and 58% and 51% with the human and mouse sequences, respectively, of the AP19 group. When compared with the yeast sequences, the similarity is also higher with the AP17 protein: the maize sequence has a 52% similarity with AP17 and 45% similarity with AP19. The protein deduced from the maize cDNA has a molecular weight of 16.9 kDa. This fact and the similarity of the sequence indicate that the maize sequence cloned belongs to the AP17 group. Interestingly, when the similarity of the maize AP17 protein with the other plant sequence avaliable, the AP19 from *Camptotheca acuminata*, is calculated, a value of 58% is obtained, a value that is higher than those obtained after comparing the yeast AP17. This could indicate a degree of convergence among plant sequences, a point that will be discussed below. This classification of the maize sequence in the AP17 group of proteins is confirmed when a phylogenetic tree analysis including all the AP sequences available is carried out. Different programs of sequence classification were used (result not shown), and the maize AP17 never clustered with the AP19 sequences that always form a group in a branch of the tree, including the one from *Camptotheca acuminata*. For this reason, this clone is attributed to the AP assembly complex from the plasma membrane.

3.2. Genomic structure and mRNA accumulation corresponding to the maize AP17 protein

The AP17 protein is encoded in the genome of maize by a single sequence. This is shown in Fig. 2, where a single band appears in a Southern blot with different restriction enzymes. The genomic sequence of the maize AP17 protein has also been identified and partially sequenced. The location of introns in the sequence is shown in Fig. 1 as triangles. Only data from fission yeast sequences are available, and the position of the introns does not coincide with that found in maize.

The AP17 gene has been cloned in a cDNA library from the maize immature embryo as a gene having a similar level of expression in different embryo stages. To confirm this fact, RNA blot experiments were carried out on RNAs extracted from different embryo and endosperm stages and different parts of the plant. The experiment is shown in Fig. 3. It appears that in all organs studied from both the seed and the adult plant, the mRNA corresponding to AP17 protein is present. The overall level of mRNA accumulation may vary in the different organs of the plant. Among those studied, the lowest mRNA level has been observed in endosperm 20 days after pollination, a period of seed development in maize when transcriptional activity is limited to the accumulation of storage materials.

The results obtained by RNA blot do not inform about the distribution of specific mRNA among cell

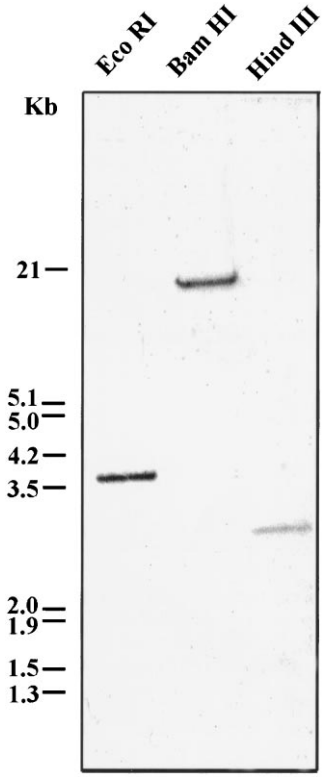

Fig. 2. Southern blot analysis of the AP17 gene. Each lane contains 10 μg of genomic DNA restricted with *Eco*RI, *Bam*HI, *Hin*dIII. The filters were hybridized with a probe corresponding to the full-length AP17 cDNA.

types in a given organ. Maize immature embryo sections offer the possibility of analyzing an organ containing cells having different stages of development and evolving towards distinct cellular functions. Advantage has been taken of this fact in the analysis of other maize genes such as HRGP (Ruiz-Avila et al., 1992) or LTP (Sossountzov et al., 1991). For this reason, immature embryo sections were hybridized in situ with the AP17 probe. The results are shown in Fig. 4. In the young embryo (8 DAP), the AP17 probe hybridized with the upper part of the embryo that becomes the embryo proper. Hybridization is also observed in the endosperm transfer cells. At later stages of embryogenesis the AP17

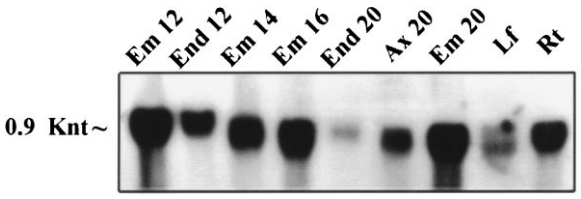

Fig. 3. RNA blot analysis of the AP17 gene. RNA was isolated from different embryo and endosperm stages and different parts of the plant using the full-length fragment as a probe. Em 12, Embryo 12 DAP (days after pollination); End 12, endosperm 12 DAP; Em 14, embryo 14 DAP; Em 16, embryo 16 DAP; End 20, endosperm 20 DAP; Ax 20, axis 20 DAP; Em 20, embryo 20 DAP; Lf, leaf; Rt, root.

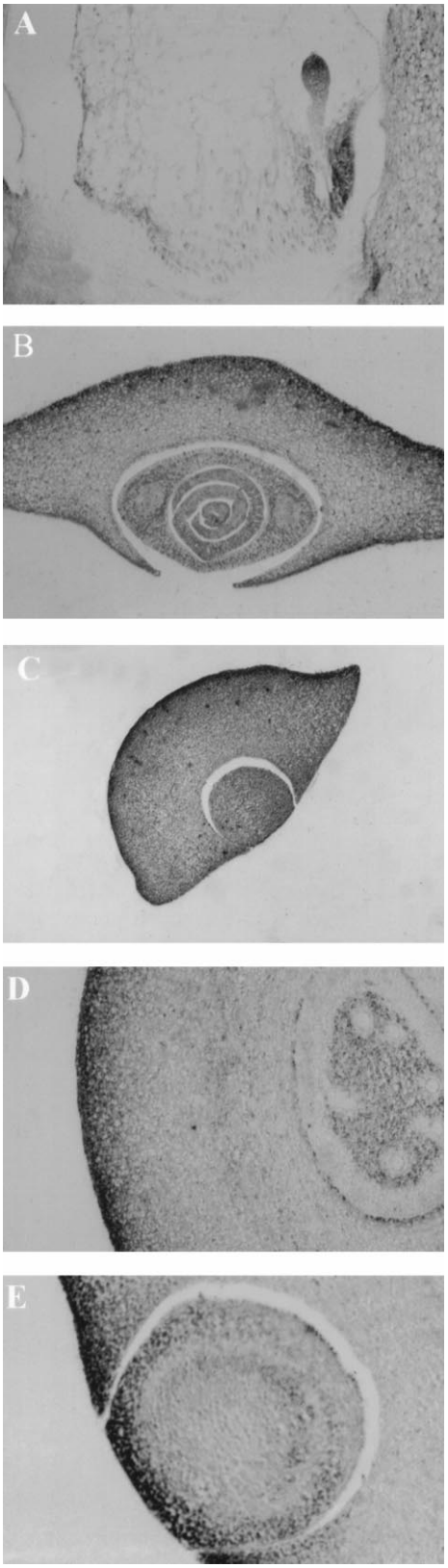

Fig. 4. In-situ hibridization of AP17 mRNA in embryo sections. (**A**) longitudinal section of an 8-DAP embryo. (**B**) Transverse section of a 20-DAP embryo at the shoot level. (**C**) Transverse section of a 20-DAP embryo at the coleorhiza level. (**D**) Transverse section of a 20-DAP embryo at the primary root level. (**E**) Transverse section of a 20-DAP embryo at the coleorhiza level.

mRNA is observed as a background hybridizing to all the embryo sections. Both in the embryo and scutellum, a gradient of mRNA is observed from the subepidermal layer towards the inner layers of the scutellum. In this organ, these cells and the provascular cells of the axis are those accumulating the higher levels of RNA (result not shown). The pattern observed for AP17 is the same one that can be observed for genes related to general metabolic mechanisms such as GAPDH (V. Stiefel, pers. commun.), and it may be related to genes having a constitutive expression such as AP-17.

4. Discussion

Vesicular transport is among the essential functions of the plant cell. The Golgi apparatus occupies a central position in this function, being the location of carbohydrate and glycoprotein biosynthesis that will be followed by transport towards the cell wall. It can be then understood that mutations in genes involved in secretion and endocytosis processes have been the first ones detected in the analysis of mutations that block embryo development in *Arabidopsis thaliana* (Shevell et al., 1994; Lukowitz et al., 1996). In the search of genes having a constitutive expression in the immature embryo of maize, a clone having a homology with a component of the assembly protein (AP) complex of the clathrin vesicles was cloned, and the full-length cDNA and a genomic fragment were sequenced. Analysis of the deduced sequence reveals that it belongs to the AP17 class of proteins, a component of the assembly complex of the clathrin coat vesicles involved in the secretion to the plasma membrane (Kirchhausen et al., 1991).

The identification of the protein encoded by the cDNA is deduced mainly from the similarity with proteins reported from mammals and yeast but also from the predicted molecular weight of the protein. The alignment of the sequence can be done with a reasonable degree of identity with the reported sequences corresponding to the AP17 class of proteins, and when using the phylogenetic tree analysis, it clusters with these proteins instead of the AP19 group of sequences. It is interesting to note that using total similarity analysis, the values obtained comparing to the only other plant protein sequence reported, the AP19 protein from *Camptotheca acuminata* (Maldonado-Mendoza and Nessler, 1996), demonstrate a higher percentage of identity between these two proteins than that calculated comparing the maize sequence with the AP17 protein from yeast. This fact may reflect the need of the two AP17 and AP19 proteins to interact with the same adaptin polypetides to form the assembly protein complex while taking part in complexes that are specific to the subcellular targeting.

The AP17 is encoded in the genome of maize by a single gene that contains two introns. This gene appears to have a constitutive type of expression. This is in accordance with the function of the protein that is important for the transport mechanisms of the cell, and therefore it takes part in an essential cellular function. This fact is confirmed by the accumulation of mRNA in all the organs studied with a lower level in maturating endosperm, a moment when transcriptional activity is concentrating in the biosynthesis of storage material. This is in accordance with the fact that storage product deposition appears not to involve clathrin coated vesicles (Hohl et al., 1996).

In the embryo, the AP17 mRNA shows a background of accumulation in the whole sections studied. It also shows a gradient in both the embryo and scutellum from the subepidermal layers towards the inner parts of the organ. This pattern corresponds to the general distribution of RNA accumulation except in the meristematic regions where genes related to proliferation are highly accumulated. This pattern has been observed in the genes that are important for basic metabolic and cellular mechanisms. A gene taking part in the vesicular transport machinery is clearly expected to show such behaviour.

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