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Anther-specific, developmentally regulated expression of genes encoding a new class of proline-rich proteins in sunflower

Jean-Luc Evrard, Colette Jako, Agnès Saint-Guily, Jacques-Henry Weil and Marcel Kuntz*
Institut de Biologie Moléculaire des Plantes du C.N.R.S., Université Louis Pasteur, Strasbourg, 12 rue du Général Zimmer, 67084 Strasbourg cedex, France (author for correspondence)*

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Abstract

We have used RNA gel blot analysis to demonstrate the anther-specific expression of three genes in sunflower. Expression of these genes was first detected shortly before flower opening, which occurs sequentially on the sunflower inflorescence, and continues during pollination. In contrast, these genes are not expressed (or only weakly expressed) in a male-sterile line in which anther development aborts. *In situ* hybridization experiments showed that these genes are only expressed in the single cell layer of the sunflower anther epidermis. In the case of one of these genes, which codes for an abundant mRNA, we report the peptide sequences deduced from the sequence of two similar but non identical cDNAs. These proteins contain a potential signal peptide and are characterized by the presence of a proline-rich region which reads KPSTPAPPPPPP(PP)K. Our results also suggest that several proline-rich proteins of unknown functions are specifically synthesized during the maturation of anthers in sunflower.

Introduction

Flowering is a complex process which begins with the conversion of a vegetative meristem into a floral meristem [2, 20]. Then, undifferentiated cells divide and differentiate into appropriate floral organs. Various homeotic mutations that affect the development of floral organs have been described in *Arabidopsis* and other plants [4, 13, 16]. Two of these genes have been identified recently by gene tagging [9] or differential screening [26]. We have chosen a further developmental process, namely flower maturation which leads to pollination and fertilization, for studies on the genetic control of flower development. We have chosen sunflower (*Helianthus annuus*) as a model for these studies and, as a first step, we have isolated flower-specific cDNAs from this plant [14].

In sunflower, a member of the Compositae family, the inflorescence head contains numerous fertile flowers (disk flowers), bracts and, at the periphery, a row of sterile flowers (ray flowers). The disk flowers are arranged in arcs and open sequentially from the periphery towards the center of the head (1 to 4 whorls per day). Consequently, a single inflorescence contains a number of flowers at different developmental stages. The flowering process of a disk flower can be divided into several phases characterized by visible morphological changes. First, before flower opening, the filaments sustaining the anther begin to grow. Full

elongation of the filaments leads to the exsertion of the anther tube from the corolla. Maturation of the pollen is completed at this stage which is then followed by the exsertion of the stigma from the anther tube, as a consequence of style elongation followed by shriveling of the filaments. In many sunflower cultivars, flower opening is preceded by pigmentation of the anthers from yellow to brown and by the differentiation of green chloroplasts to yellow chromoplasts in the upper part of the corolla.

Thus, at flowering, sunflower disk flowers exhibit some unique properties and it is likely that these developmental processes involve changes in gene expression. Our approach to unravel the mechanisms regulating the coordinated maturation of the various floral organs is to isolate and study genes which are expressed in a regulated manner during these developmental processes. Of particular interest for such studies will be genes expressed specifically in a single floral organ.

In this paper we report the results of RNA gel blot and *in situ* hybridization experiments demonstrating the anther- and tissue-specific expression of a gene encoding a novel type of proline-rich protein in sunflower. In addition, the expression of this gene during anther development was studied and compared with two other genes specifically expressed in the same anther tissues.

Materials and methods

Plant materials

Sunflower plants (*Helianthus annuus*) were obtained from Semences Cargill (France) and grown under standard greenhouse conditions. Cultivar Ha401B, a fertile imbred line, was used when not otherwise stated. Instead, Ha401A, a male-sterile line which is isogenic to Ha401B, was used.

Labeling of DNA probes and sequencing

The various cDNA-containing plasmids used in this study were cut with *Eco* RI and the cDNAs were isolated from preparative low-melting agarose gels as already described [14]. Labeled cDNA probes were prepared by random priming as described [14, 18] using [α-^{32}P]dCTP (3000 Ci/mmol) for RNA gel blot hybridizations or [α-^{35}S]dATP (800 Ci/mmol) for *in situ* hybridizations. Nucleotide sequences were determined using the 'Sequenase' kit from United States Biochemical Corp. and double-stranded DNA as template [31].

RNA isolation and gel blot analysis

RNA was isolated using the method described by Arrand [1] with some modifications [14]. RNA samples were separated by urea-agarose gel electrophoresis [19] and transferred onto nylon filters (Hybond-N, Amersham). The amount of RNA loaded on the gels were standardized by absorbance measurements at 260 nm and comparison of the ethidium bromide fluorescence of cytosolic rRNAs in gels. These rRNAs can be used as internal standards in most tissues, as already observed [18], with the exception of mature anthers which contain little rRNA. The labeled DNAs were used at a concentration of 3×10^6 cpm/20 ml of hybridization mixture which contained 5 × SSPE (900 mM NaCl, 50 mM sodium phosphate pH 7.7, 0.5 mM EDTA), 50% formamide, 0.5% SDS and 5 × Denhardt's solution. Hybridizations were done at 37 °C, overnight. Before hybridization, filters were incubated in the same conditions, for 2 hours, in the presence of 1 ng/ml of non-homologous and unlabeled single-stranded oligodeoxyribonucleotides. After hybridization, filters were washed in 5 × SSPE, 0.5% SDS at 65 °C and autoradiographed.

In situ hybridizations

Tissues were fixed at 10 °C in 2% paraformaldehyde, 0.2% glutaraldehyde, 0.1% Triton X-100 in 0.1 M sodium phosphate pH 7.0 buffer. 16 μm sections were obtained using a Reichert-Jung 2800N cryomicrotome and treated as described

by Meyerowitz [21]. Hybridizations were performed at 37 °C, overnight, in 5 × SSPE and 50% formamide in the presence of 10 ng of labeled probes (4×10^6 cpm/slide). The sections were then washed 10 min in 5 × SSPE at room temperature and twice in 0.2 × SSPE at 65 °C. The slides were coated with an Ilford K5 nuclear track emulsion to allow simultaneous observation of the sections and the autoradiograms. Alternatively, sections were autoradiographed by Hyperfilm-βmax (Amersham) which were then processed separately.

Results

Organ specificity and temporal expression of a flower-specific cDNA clone

Anthers, corollae and pistils (consisting of the style and the stigma) were dissected from mature fertile flowers (disk flowers) of a sunflower inflorescence. In addition, non-mature fertile flowers were harvested from different developmental stages: developing inflorescence buds (ca. 2 cm) from a 6-week-old plant, closed inflorescences from a 2-month-old plant (just before ray flower elongation and yellowing) and opened inflorescences 2–4 days later (just after ray flower elongation and yellowing). In the latter case, unopened disk flowers were harvested at two different stages: when the corollae were still green at their tops and at a later stage, when the corollae were entirely yellow. These two stages will be referred to as green and yellow unopened flowers, respectively. RNA was extracted from these various floral tissues, as well as from an inflorescence (consisting of disk flowers at various developmental stages), leaves and roots.

We used a flower-specific cDNA, termed SF2, isolated from sunflower [14] as a probe in RNA gel blot analysis. The clone SF2 hybridizes to a 800 nucleotide RNA present in the sunflower inflorescence but undetectable (even after long exposure times) in sunflower leaves and roots (Fig. 1A). As shown in Fig. 1A, this clone hybridizes to RNA extracted from mature anthers,

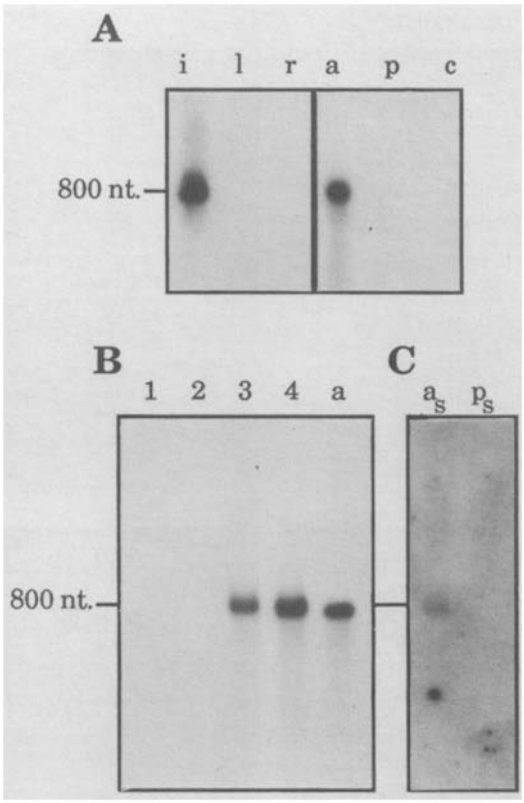


Fig. 1. RNA gel blot analysis of SF2 transcript. A. Poly(A)$^+$ RNAs extracted from inflorescences (i), leaves (l), roots (r) and total RNAs extracted from anthers (a), pistils (p) and corollae (c) were hybridized with labeled SF2 cDNA. B. Total RNAs were extracted from inflorescences at different stages of development and hybridized with SF2 cDNA. The developmental stages were: floral buds (1), closed inflorescence (2), closed green flowers of an opened inflorescence (3), closed yellow flowers of an opened inflorescence (4) and mature anthers (a). Exposure time of the autoradiogram was 4 hours. C. Total RNAs from anthers (a_s) and pistils (p_s) of a male-sterile sunflower were hybridized with SF2 cDNA. A faint hybridization signal is visible for the anther RNAs after overexposure of the autoradiogram (5 days).

whereas no hybridization signal was detected in mature corollae or pistils. As shown in Fig. 1B, the RNA hybridizing with clone SF2 is present in unopened yellow as well as green flowers, but not at earlier developmental stages (e.g. closed inflorescence). Interestingly, the appearance of this transcript precedes the pigmentation of the anther and the elongation of the filament which occur

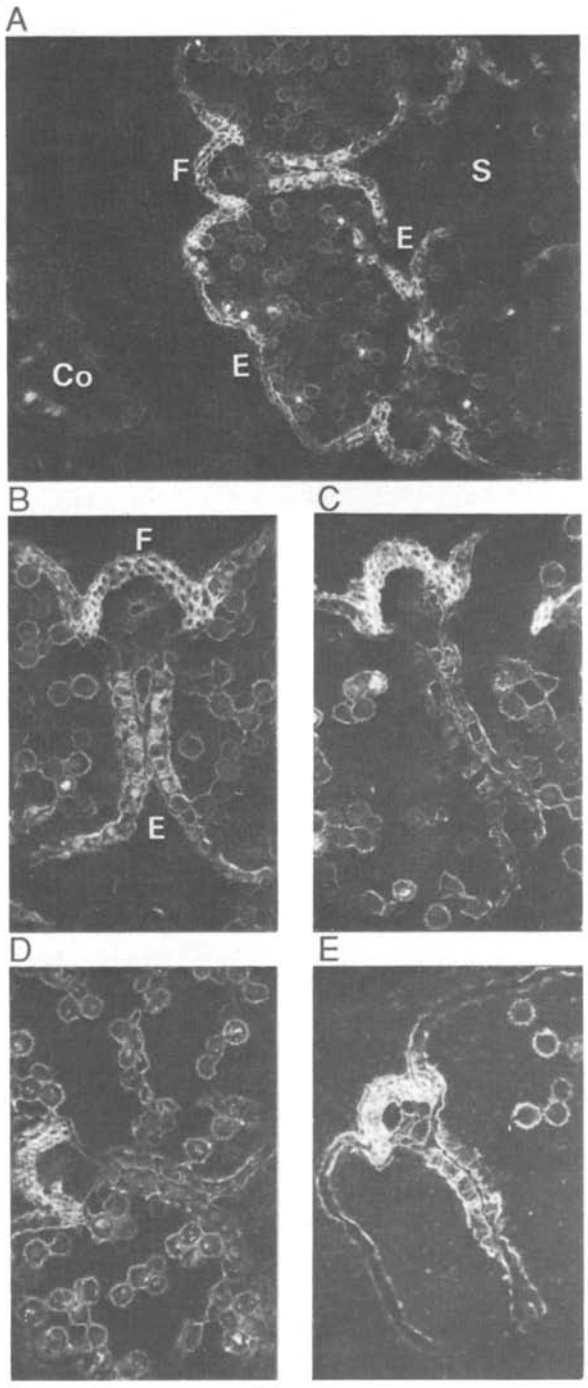


Fig. 2. SF2 mRNA localization in flowers by *in situ* hybridization and dark-field microscopy. A. Low magnification photograph (70 ×) of a transverse section of a mature flower hybridized with labeled SF2 cDNA. Slides were coated with a photographic emulsion (see Materials and methods). A hybridization signal is only detectable in the anther epidermis (E), not in pollen, corolla (Co) or stigma (S). The signal observed in the two-cell layer of the filament (F) is due to light diffraction (compare Fig. 2B and C). B. Higher magnification photograph (100 ×) of a transverse section of a mature anther hybridized with SF2 cDNA. C. Negative control experiment performed with labeled pUC vector. No hybridization signal is detected. The signal detected at the periphery of the cells of the filament is also observed without hybridization and is therefore due to light diffraction (most likely by the cell walls). D. Transverse section through the anther of a closed green flower hybridized with SF2 cDNA. A hybridization signal is detected in the same tissue as in Fig. 2A and B. E. Transverse section through the anther (after exsertion of the pistil) hybridized with SF2 cDNA. Only a very faint hybridization signal is detected in the anther epidermis.

during stamen maturation, immediately before pollen is shed.

RNA gel blot hybridization of SF2 was also performed with RNA extracted from anthers and pistils and a male-sterile cultivar of sunflower. In this male-sterile line anther pigmentation and filament elongation are strongly reduced and pollen formation is aborted, whereas the other organs of the flower develop normally. As shown in Fig. 1C, only a faint hybridization signal was detected (after overexposure of the autoradiogram) in anthers from the male-sterile line.

Localization of the SF2 transcript to specific tissues

In opened sunflower inflorescence (after ray flower elongation and yellowing), the anthers have a simple structure consisting of a single epidermal cell layer, sustained by the filament, which harbors the maturing pollen grains.

In order to study the tissue-specific expression of the cDNA clone SF2, we used *in situ* hybridization of labeled SF2 DNA to flower cross-sections. Fig. 2A–C show examples of such experiments performed with flowers at the mature anther stage. When flower sections were taken at the lower part of the anther (through the corolla and the stigma), hybridization was visible only in the anther and not in the stigma or corolla (Fig. 2A), as expected from the RNA gel blot analysis. In addition, hybridization was visible only in the

single cell layer of the anther epidermis, not in the filament or the pollen (Fig. 2A). The same observations were made when sections where taken higher in the anther. For instance, when sections were taken above the corolla but through the stigma, hybridization was visible only in the anther epidermis, not in the filament, pollen or style (Fig. 2B). Fig. 2C shows the result of an experiment performed using labeled pUC18 as a negative control.

When these experiments were repeated with flowers at different developmental stages the same pattern of hybridization was observed. A hybridization signal was first detected in the anther epidermal cell layer of unopened green flowers (Fig. 2D), as expected from the RNA gel blot analysis. In addition, these experiments revealed that the level of the SF2 transcript is dramatically reduced in anthers at a later developmental stage, when the pistil is exserted from the internal space of the anther tube (Fig. 2E).

SF2 codes for a novel type of proline-rich protein

The peptide sequence deduced from the nucleotide sequence revealed that the cDNA clone SF2 potentially codes for a 121 amino acid long protein whose NH_2-terminal region contains two polar residues (Asn) at positions 3 and 4, followed by an uninterrupted run of 17 hydrophobic or hydroxylated amino acids (Fig. 3). This NH_2-terminal region therefore resembles a signal peptide, suggesting that the encoded protein is synthesized on membrane-bound polysomes and co-translationally transferred to the endoplasmic reticulum (ER). We have tentatively localized the cleavage site of the signal peptide between Ser_{21} and Glu_{22}. The most striking feature of the proposed 100 amino acid long mature protein (Fig. 3) is the presence of a proline-rich peptide which reads KPSTPAPPPPPPK. The mature protein contains an overall percentage of proline of 11% and, in addition, exhibits a high percentage of lysine (11%) and cysteine (9%). A computer-assisted search for sequence homology did not reveal any significant homology between the

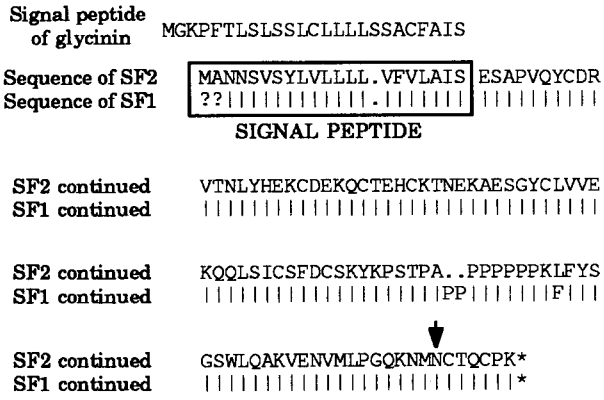


Fig. 3. Peptide sequences of SF2 and SF1. The comparison between SF2 and SF1 reveals only minor differences: one amino acid change and one insertion of two prolines in SF1 (| = no change; ? = unknown; • = deletion or gap). The sequence of the SF2 potential signal peptide is compared with that of glycinin [22]. The arrow indicates the putative site of glycosylation.

mature part of the SF2 protein and other known proteins. Only the potential NH_2-terminal extension of the SF2 protein showed limited homology to known ER-targeting signal peptides (Fig. 3).

Since Southern analysis of genomic DNA (not shown) suggested that at least two genes homologous to the SF2 clone are present in sunflower, we have determined the nucleotide sequence of other flower-specific cDNAs cross-hybridizing with SF2. Figure 3 shows the protein sequence deduced from one of these clones which was previously termed SF1 [14]. This protein contains a similar proline-rich region but in this case with an uninterrupted run of 8 prolines instead of 6 in the SF2 protein. In addition, a single amino acid substitution was found between the SF1 and SF2 protein sequences. It is important to mention that the SF1 and SF2 clones show extensive nucleotide sequence homology (including in the 3′-untranslated region) and are therefore indistinguishable in hybridization experiments.

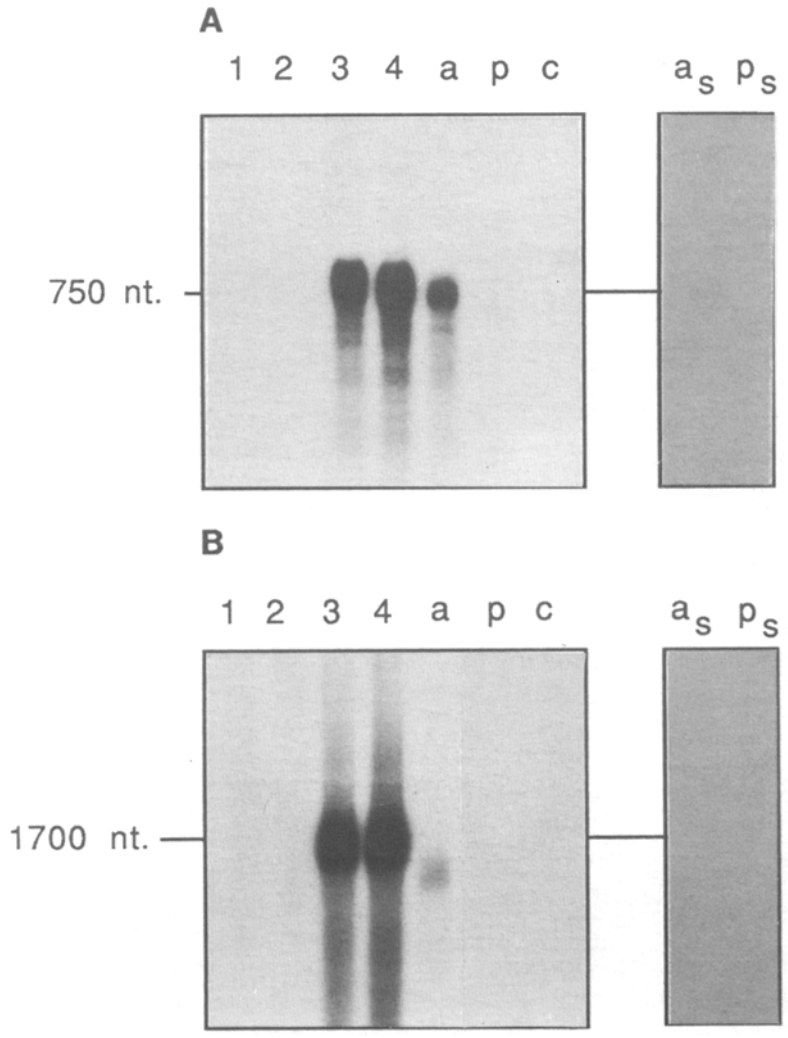
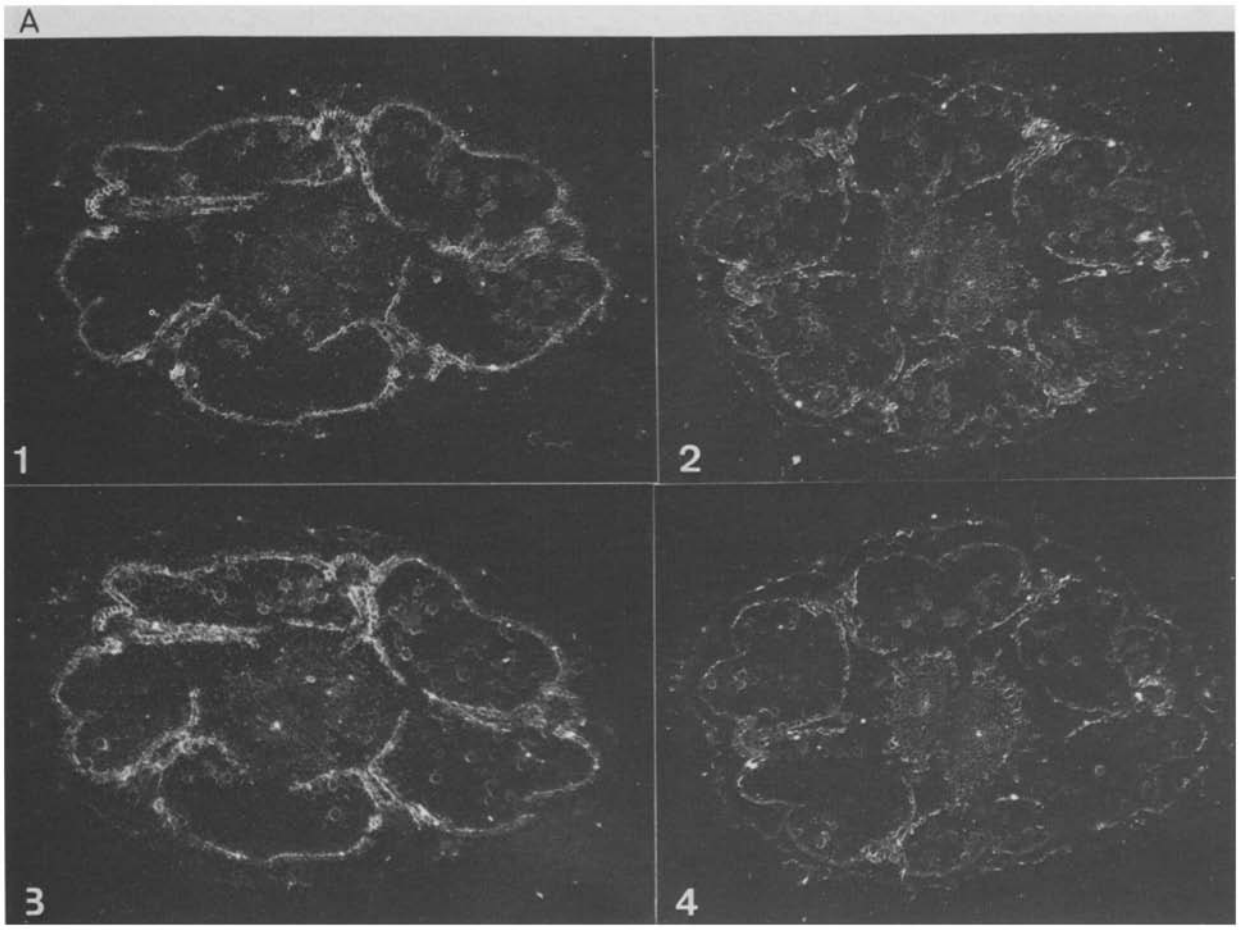


Fig. 4. RNA gel blot analysis of SF18 and SF19 transcripts. Total RNAs were extracted from inflorescences at different stages of development and hybridized with labeled SF18 cDNA (A) or SF19 cDNA (B). The developmental stages were: floral buds (1), closed inflorescence (2), closed green flowers of an opened inflorescence (3), closed yellow flowers of an opened inflorescence (4) and mature anthers (a), pistils (p) and corollae (c). RNA gel blot analysis of total RNAs from anthers (a_s) and pistils (p_s) of a male-sterile sunflower revealed only a faint hybridization signal in anthers. Exposure times of the autoradiograms were 3 days for each experiment.

Fig. 5. Localization of SF18 and SF19 mRNAs in flowers by *in situ* hybridization. A. Visualization of the transcripts in flower sections by dark-field microscopy (51 ×). Experiments were performed as described in Materials and methods and Fig. 2. 1) and 3) Transverse sections of a yellow unopened flower hybridized with labeled SF18 and SF19 cDNAs, respectively. 2) and 4) Negative control experiments performed with labeled pUC vector (see legend to Fig. 2C). These experiments show the background signals which are mainly due to light diffraction by cells. B. Bright-field micrographs of transverse flower sections compared with the corresponding autoradiographs. 1) and 4) Bright-field micrographs (28 ×). 2) and 5) Autoradiography (Hyperfilm-βmax, Amersham) of 1) and 2) after hybridization with labeled SF18 and SF19 cDNAs, respectively. 3) and 6) Negative controls.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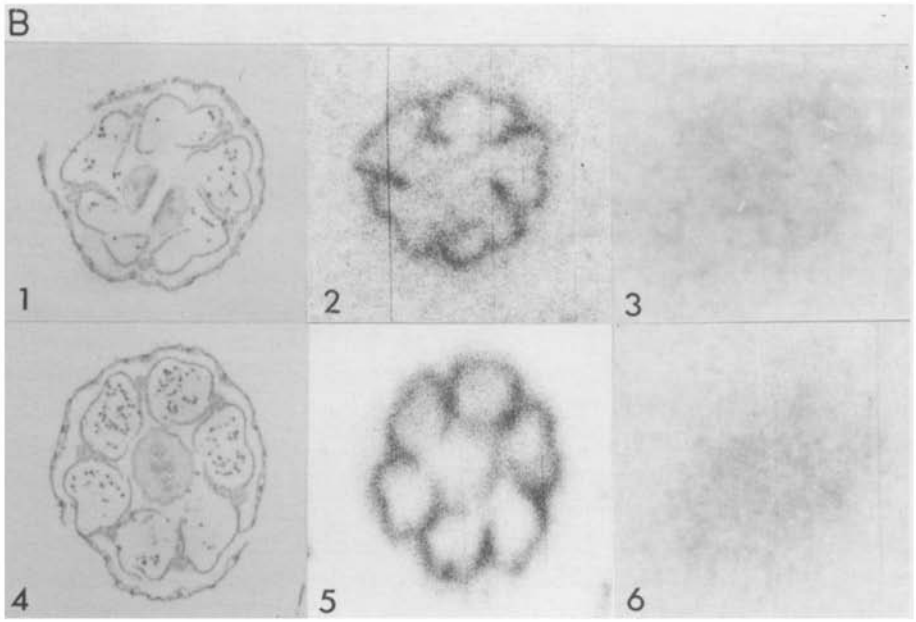
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Other developmentally regulated cDNAs coding for proline-rich proteins are present in anthers

In order to determine if transcripts encoding other proline-rich proteins are also specifically present in sunflower anthers, similar hybridization experiments were performed on additional flower-specific clones [14].

Two cDNA clones (termed SF18 and SF19, respectively) which showed anther specificity were further characterized (Fig. 4). These clones exhibit a pattern of hybridization during development similar to that of SF2/SF1. Furthermore, like SF2/SF1, SF18 and SF19 correspond to genes which show a maximum level of expression in yellow unopened flowers. Like SF2/SF1, the SF18 and SF19 transcript levels are dramatically reduced in anthers from male-sterile sunflower plants. The main difference between the hybridization patterns of these three transcripts is that the SF19 transcript, unlike the SF2/SF1 and SF18 transcripts, appears to be present in considerably lower amounts in mature anthers than in immature anthers.

In order to study the tissue-specific expression of the cDNA clones SF18 and SF19, we also used *in situ* hybridization experiments. Since the SF18 and SF19 transcripts are present in significantly lower amounts than the SF2/SF1 transcripts, these experiments (Fig. 5) were performed using flowers at the yellow unopened stage (i.e. when these transcripts are most abundant). In addition, to allow unambiguous visualization of these transcripts, the results of two different methods of autoradiography (see Materials and methods) are presented in Fig. 5A and B. Both types of experiments revealed that, like the SF2/SF1 transcripts, the SF18 and SF19 transcripts are present specifically in the anther epidermis.

In the case of SF19 (which is not a full-length clone) the deduced peptide sequence revealed that the encoded protein consists (at least partially) of repeated $P^{V}_{L}PP^{L}_{I}P$ motives. Interestingly, the protein sequence deduced from the nucleotide sequences of the cDNA clones SF18 [8] also shows a high percentage of proline but is different from that of SF2 and SF19, suggesting that several proline-rich proteins are specifically synthesized during the late stages of anther maturation in sunflower.

Discussion

We have analyzed RNA gel blots with flower-specific cDNA probes from sunflower. Three strictly anther-specific cDNAs have been characterized. The corresponding genes are expressed at a late developmental stage of anthers, namely before and during the pigmentation and elongation process which precedes pollen shedding in sunflower. The corresponding genes are also expressed in mature anthers, although these organs contain little ribosomal RNAs and therefore must have a limited translational capacity. These genes were found by *in situ* hybridization to be present exclusively in the anther epidermal cell layer.

The peptide sequence deduced from one of these cDNA clones (SF2), which is a full-length clone and which corresponds to an abundant transcript, is reported here. This peptide sequence and especially the NH_2-terminal part suggests that this protein is translocated into the lumen of the ER. The SF2 protein also contains a potential glycosylation site near its COOH-terminus (Fig. 3). However, whether the SF2 protein passes then through the Golgi apparatus and whether it is subsequently transported to the vacuole or the extracellular space remains to be established.

Since the SF2 protein exhibits a relatively high proline content, we have compared its sequence to those of well-characterized cell wall proteins, such as extensins or other proteins called PRPs [6, 7, 15], which also contain an unusually high proportion of proline or hydroxyproline. However, the SF2 protein does not show any primary sequence similarity to these cell wall (hydroxy)-proline-rich proteins. In addition, whereas the proline residues are found in characteristic repeated motifs in the PRPs or extensins, most of the proline residues are clustered in a particular region of the SF2 protein. However, like exten-

sins, the SF2 protein is also rich in lysine residues which, as discussed by Cassab and Varner [5], could link proteins to polysaccharides such as pectins. Alternatively, lysine residues, upon action of peroxidases (which are also found in cell walls), may be involved in protein cross-linking [5]. In spite of its high lysine content, the SF2 protein is only slightly basic, whereas extensins are strongly basic proteins due to their high lysine content and their relatively low content in acidic amino acids.

Another noteworthy feature of the SF2 protein is that it is cysteine-rich, which distinguishes it from extensins or PRPs, but which is also a characteristic of thionins, namely cell wall proteins involved in the defense mechanism of plants [3], and of lectins (for a review, see [24]). However, the SF2 protein does not show any sequence similarity to either thionins (which are not proline-rich), lectins or other cell wall hydroxyproline-rich proteins such as arabinogalactan proteins (for a review, see [24]). In addition, the comparison of the SF2 protein with proline-rich proteins not located in the cell wall, such as the prolamine storage proteins (for a review, see [23]), or with proteins whose cellular location is unclear, such as the soybean nodulin-75 [10], did not reveal any sequence similarity. The same observations were made for the protein sequence deduced from another anther-specific cDNA, which was called SF18 and which also has a high proline content [8]. In addition, the protein sequence deduced from the partial sequence of a third anther-specific cDNA (SF19) also revealed a high proline content. Therefore, our results suggest that these proline-rich proteins are specifically synthesized in the anther epidermis before and during pollen shedding. It also appears that these proteins are members of a new class of proline-rich proteins and it is one of our goals to determine the biological function(s) of these proteins. It should be noted that RNA gel blot analysis of the SF2, SF18 and SF19 cDNAs did not reveal any hybridizing signal when using anther RNA from another plant, namely tobacco, suggesting either that these transcripts are not present in tobacco anthers or that the sequences of these genes are poorly conserved from one plant to another.

A second cDNA clone, termed SF1, homologous to SF2 was also identified and found to potentially encode an almost identical protein which differs by one amino acid substitution and the insertion of two proline residues in the proline-rich motif of SF2. Since the nucleotide sequences of SF1 and SF2 are quite similar, it was not possible to discriminate between the SF1 and SF2 transcripts in hybridization experiments. However, since differential expression of individual members of a multigene family has been reported [17] it will be of interest to determine if this is also the case here. In particular, it is possible that the transcription of individual members of this SF1/SF2 gene family accounts for different percentages of the overall transcript levels.

Several cDNAs corresponding to genes expressed preferentially in anthers have been described in both monocotyledonous and dicotyledonous plants, many of them showing preferential or exclusive expression in the male gametophyte [12, 27, 28, 29]. In particular, cDNA clones corresponding to transcripts which reach maximum levels in mature pollen grains, but which are also present in the epidermal and endothecial cell layer of the anther wall, have been isolated from tomato [29]. On the other hand, cDNA clones corresponding to transcripts which accumulate early during anther development and decay prior to anther maturation, have been isolated from tobacco and shown to be specific for the tapetum [11]. Tapetum-specific cDNAs have also been characterized in tomato [25]. In addition, cDNAs corresponding to transcripts which persist throughout anther development have also been characterized in tobacco and one of these transcripts was shown to be present in various anther tissues as well as in pistils [11]. Studies on the chalcone flavonone isomerase genes have revealed the specific expression in immature anthers of one of these genes, whereas a second gene is expressed in anthers at a later stage as well as in corolla and tube tissue [30].

Together, these results and the data presented here demonstrate that specific genes are activated in a precise temporal and spatial framework during anther maturation. The expression pattern of

many of these genes appears complex and further work is required before the molecular basis of anther development is better understood. However, these genes represent useful tools for such studies. In particular, the genes studied here are of interest since they are specifically expressed in a single cell type (the epidermis) of sunflower anthers. Furthermore, the expression of these genes is regulated in a coordinated manner during the last stage of anther maturation, namely before and as pollen is shed. Preliminary results have suggested that the regulation of the expression of these genes occurs at the transcriptional level (unpublished data). Work is now in progress to identify the regulatory elements involved in the expression of these genes.

Acknowledgements

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