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Specific mRNA accumulation of a gene coding for an *O*-methyltransferase in almond (*Prunus amygdalus*, Batsch) flower tissues

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Abstract

A cDNA clone, PAB14B, was isolated from an almond flower cDNA library. The 1245 bp nucleotide sequence contains an open reading frame encoding a polypeptide of 356 amino acids which shows sequence similarity to *O*-methyltransferases (OMTs) from different plant species. The highest identity (49%) was observed with a methyltransferase from pea that catalyses the terminal step in pisatin phytoalexin biosynthesis. Comparison of this almond sequence, with amino acid sequences of described OMTs, identified regions of similarity which probably contribute to the binding site of *S*-adenosyl-L-methionine. Southern blot analysis suggests that PAB14B may be encoded by more than one gene in the almond genome. Flower-specific expression was shown by RNA blot analysis and the OMT transcript was detected in epidermal and vascular floral tissues by in situ hybridization. The available data suggest that this enzyme may be involved in the flavonoid biosynthesis pathway in almond floral tissues.

Keywords: Prunus; *O*-methyltransferase; Flower expression; In situ hybridization

1. Introduction

Plants contain a multitude of secondary products that include one or more methyl groups in their structure, added during their biosynthesis. These methylations are catalysed by a class of enzymes, called methyltransferases (EC 2.1.1.–), many of which use *S*-adenosyl-L-methionine (SAM) as the methyl donor [1]. Other structurally related methyltransferases, which accept SAM as the methyl donor, also methylate DNA, RNA and proteins [2].

Lignin, suberin, flavonoids, anthocyanins and isoflavonoids, are phenylpropanoid compounds that fulfil a variety of key roles in plant growth

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and development, and are involved in the interactions of plants with their environment. There is a wide structural diversity due to a variety of modifications that include specific hydroxylations, glycosylations, acylations, prenylations, sulphations and methylations [3]. The plant methyltransferases that have been most thoroughly studied are the *O*-methyltransferases (OMTs), enzymes that are involved in phenylpropanoid metabolism [1,4].

In studies of plant–pathogen interactions, special attention has been paid to OMTs involved either in the formation of precursors of lignin [5,6], or in the synthesis of the diverse group of antimicrobial secondary metabolites called phytoalexins [7,8]. The enzymatic activity of both classes of OMTs has been shown to increase as a response to infection by pathogens or elicitor treatments and, in several cases, accumulation of the corresponding gene transcript in reacting plant tissues has been reported.

Several plant OMTs involved in the enzymatic methylation of flavonoids have been studied because methylation, in addition to glycosylation, is supposed to play an important role in the transformation of the reactive hydroxyl groups of flavonoids. In most flowers, pigments contain flavonoid derivatives. However, apart from their role in the pigmentation of flowers and fruits, flavonoids display many other functions. They are involved in defence against phytopathogens in plant species, such as French bean [9] and soybean [10], and protection against UV light [11,12]. It has also been proposed that they have a role in the regulation of auxin transport [13] and resistance to insects [14].

The genus *Prunus* includes plant species of interest for different reasons. Almond, peach, apricot, cherry, etc. are among those species of the genus having a high economic interest, and they may be interesting models for molecular studies in tree species as they have a very low DNA content, only twice that of *Arabidopsis thaliana* [15]. A complete genetic map of almond has recently been published including some characterized cDNAs [16,17]. Interesting agronomic characters in these species include those that depend on flower development and this prompted us to analyze genes highly expressed in the floral organs of this plant. In the present paper we report on the isolation of a cDNA coding for a putative OMT of almond (*Prunus amygdalus*) which is the first flower-specific OMT reported so far.

2. Materials and methods

2.1. Plant material

Almond (*Prunus amygdalus*, Batsch) fruit and flower samples from the 'Texas' cultivar were collected at different stages of development from crop fields at the Departament d'Arboricultura Mediterrània, IRTA (Reus, Spain) and were immediately frozen in liquid nitrogen and stored at −80°C until they were used. During almond flower development, three stages have been considered, C, D and F corresponding, respectively to the appearance of calyx, corolla and mature flower with petals completely opened [18].

2.2. Library screening

Polyadenylated RNA, extracted from closed flowers (stage C) was used to construct a cDNA library in the Uni-ZAP XR λ vector (ZAP-cDNA Synthesis Kit; Stratagene). After in vivo excision, 500 clones were hybridized with radiolabelled first-strand cDNA from the same tissue. A number of clones with a high level of expression were sequenced and a 1.2 kb cDNA clone with similarity to published OMTs was among the sequences, although it did not correspond to the full-length cDNA (PAOMTII). PCR was used with a PAOMTII-specific 18-mer oligonucleotide primer and the SK-17 sequence primer (TCTAGAACTAGTGGATC) to amplify PAOMTII sequence from the λZAP-XR library upstream of the PAOMTII primer sequence. PCR amplifications were performed using 10^8 plaque-forming units of the library in 50 ml of 1 × PCR buffer (20 mM Tris–HCl, pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 0.1% Triton X-100) with 200 mM dNTPs, 200 ng of both primers and 2.5 U of Taq DNA polymerase (Amersham). Substrates were heat denatured at 94°C for 10 min before adding the

enzyme, and the DNA was amplified in a Hybaid Omnigene thermal cycler for 35 cycles of 94°C (40 s), 50°C (40 s) and 72°C (1 min). The reaction products were analyzed on a 1.5% agarose gel and subcloned in pBluescript SK II − for sequence analysis.

2.3. Extraction of RNA and RNA blot analysis

Total RNA was extracted from different almond tissues using the methods described by Martin et al. [19] and Haffner et al. [20] depending on the tissue. The tissues analyzed in the present study include seed and pericarp at different times after flowering, flowers from periods C, D and F, petals, sepals, stamen and pistil from the open flower and young leaves and roots. A total of 15 mg of total RNA was separated in a 1.5% agarose-formaldehyde gel and transferred to a nylon membrane (Nytran, Schleicher and Schuell) according to the method originally described by Lehrach et al. [21]. The membranes were hybridized with a 890 bp fragment (*Eco*RI–*Sal*I) of the PAOMTII clone labelled with [^{32}P]dCTP using random oligonucleotides as primers (Boehringer Mannheim). Hybridization and washes were performed according to the protocol described by Church and Gilbert [22].

2.4. DNA preparation and Southern blot analysis

Genomic DNA was extracted from young leaves using the method described by Bernatzky and Tanksley [23]. The DNA was purified by equilibrium centrifugation in a CsCl-ethidium bromide gradient as described by Maniatis et al. [24]. The DNA was digested under standard conditions, electrophoresed on a 0.8% agarose gel and transferred to a nylon membrane (Nytran, Schleicher and Schuell) according to the method originally described by Southern [25]. The membranes were hybridized with a 890 bp fragment (*Eco*RI–*Sal*I) of the PAOMTII clone labelled with [^{32}P]dCTP by random priming (Boehringer Mannheim). Hybridization and washes were performed according to the protocol described by Church and Gilbert [22].

2.5. In situ RNA localisation

PAOMTII mRNA was localized in paraffin-embedded flower sections by the non-isotopic DIG-labelling system (Boehringer Mannheim), basically as described by Langdale [26]. A total of 150–200 nucleotide sense and antisense DIG-labelled riboprobes were generated by alkaline hydrolysis. Almond flowers in the C stage of development were fixed in ethanol/formaldehyde/acetic acid 80:3.5:5 for 1 week at 4°C before being embedded in Paraplast wax. Tissue sections (8 mm thick) were mounted on poly-L-Lys coated glass slides. After deparaffination with xylene, the sections were hydrated and treated for 30 min with proteinase K. Hybridization of the sections was performed at 55°C for 16 h with the riboprobe (2 mg ml^{-1}) in hybridization solution (50% formamide, 6 × SSC, 3% SDS, 100 mg ml^{-1} tRNA, 100 mg ml^{-1} poly (A)). The washes, blocking reaction, antibody incubation and detection reaction were performed according the protocol described by Langdale [26]. The slides were permanently mounted in Histovitrex, examined under a Zeiss model Axiophot FL microscope, and photographed using Kodak Ektachrome 400 film.

2.6. Sequence alignment and phylogeny

The sequence of the almond PAOMTII was aligned with 17 amino acid sequences of *O*-methyltransferases deduced from cDNAs or genes encoding these proteins in plants. The alignment was analyzed by using parsimony and distance-based methods included in PHYLIP (Phylogenetic Inference Package) program version 3.41 [27]. Accession number of the sequences compared: *Capsicum annuum* (CAODMT, U83789), *Chrysosplenium americanum* (CAFLOMT, U16794), *Eucalyptus gunnii* (EGCAOMT, X74814), *Medicago sativa* (MSCAOMT, M63853; MSCHOMT, L10211), *Mesembryanthemum crystallinum* (MCMYOMT, M87340), *Nicotiana tabacum* (NTCAOMTI,

X74452; NTCAOMTII, X74453), *Pisum sativum* (PSHMOMT, U69554), *Populus kitakamiensis* (PKCAOMTA, D49710; PKCAOMTB, D49711), *Populus tremuloides* (PTCAOMTA, U13171; PTCAOMTC, U50522), *Prunus amygdalus* (PACAOMTI, X83217; PAOMTII, AJ223151), *Zea mays* (ZMCAOMT, M73235; ZMZRP4, L14063), *Zinnia elegans* (ZECAOMT, U19911).

3. Results

3.1. Isolation and analysis of almond PAOMTII cDNA clone

In the course of screening an almond (*Prunus amygdalus*, Batsch) flower cDNA library for genes highly expressed in the floral organs of this plant, a clone (PAB14B) containing a sequence homologous to *O*-methyltransferases from different plant species, was isolated. The sequence of the PAB14B clone revealed a 1178 nucleotide insert, excluding a poly (A) tail, thus representing an almost full-length clone of the 1.3 kb gene transcript recognized on Northern blots. In order to obtain the full-length cDNA, an oligonucleotide was synthesized complementary to nucleotides 173–190 of the PAB14B clone, and used in combination with the SK-17 sequence primer to amplify the missing 5′ sequence from the λZAP-XR cDNA library, employing the polymerase chain reaction (PCR). Electrophoretic analysis of the PCR products revealed that they contained cDNA fragments of the expected size. After subcloning and sequencing the PCR products, a new 5′-end fragment of 40 bp was obtained containing a putative ATG start codon. The sequence overlapping the cDNA fragments were identical indicating that they were probably obtained from the same mRNA. The complete sequence (Accession No. AJ223151) contained an open reading frame extending from position 15 to 1085, including the stop codon. The untranslated 3′ region of the sequence contained a putative polyadenylation signal at position 1116 and the encoded translation product corresponded to a 356 amino acid polypeptide.

3.2. Sequence comparison of almond PAOMTII with other plant O-methyltransferases

Searches through the EMBL and SwissProt databanks revealed clear sequence similarities between the deduced amino acid sequence of the PAB14B insert and plant OMTs (30–57% amino acid similarity). In Fig. 1, a comparison is shown of the encoded amino acid sequence with seven plant OMTs representing different putative OMT activities. The highest level of similarity, 49 and 43% amino acid identity, are observed, respectively with HMM, an OMT from pea involved in pisatin phytoalexin synthesis [28], and ZRP4, a putative OMT from maize roots believed to be involved in suberin synthesis [29]. In contrast, when the PAB14B is compared to the reported *P. amygdalus* OMT [30] that was proposed to be involved in lignin biosynthesis, the similarity is lower (33% amino acid identity), thus indicating that these two enzymes may belong to distinct classes of plant OMTs. For this reason, the cDNA has been called PAOMTII. The similarity of the PAOMTII amino acid sequence to all other OMTs is highest in the C-terminal half of the sequence. In particular, the region extending from position 230–360 in Fig. 1 includes sequences conserved between the OMTs involved in lignin [31] and suberin biosynthesis [29], plant OMTs methylating flavonoid compounds [32,33], virus-induced plant OMTs [34] and three animal [35] and one bacterial OMTs [36] with various substrate specificities. These sequences, also present in PAOMTII, are known to be important for *S*-adenosyl-L-Met-dependent binding [2] and are boxed in the figure.

A phylogenetic analysis (Fig. 2) of eighteen different plant OMT sequences, including those shown in Fig. 1, indicates that most of them are included in two separate groups: one group comprises a distinct branch of caffeic acid OMTs (CAOMT) involved in the formation of lignin precursors and the second one includes different OMTs involved in the biosynthesis of phytoalexins, suberin and flavonoid derivatives. The PAOMTII amino acid sequence is grouped in this second heterogeneous group.

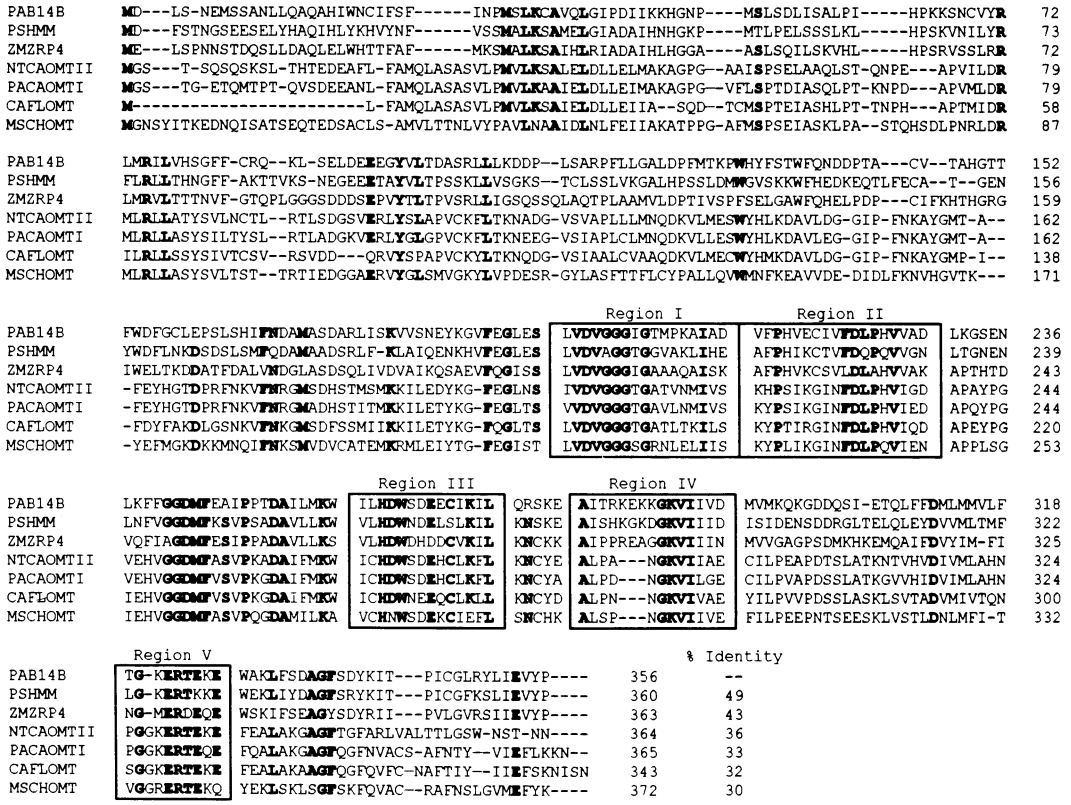

Fig. 1. Alignment of the predicted amino acid sequence from PAOMTII with plant OMTs representing different putative activities: PSHMM, pea 6a-hydroxymaackiain 3-OMT; ZMZRP4, putative maize OMT expressed in roots; NTCAOMTII, pathogen- induced tobacco caffeic acid OMT; PACAOMTI, almond caffeic acid OMT; CAFLOMT, 3′/5′ flavonol OMT from *Chrysosplenium americanum*; MSCHOMT, alfalfa isoliquiritigenin 2′-OMT. Gaps (–) are introduced for maximum alignment. Percentages of amino acid sequence identity of the various OMTs with PAOMTII are indicated at the end of the alignment. Boxed areas correspond to the putative SAM-binding site regions proposed for other plant OMTs and the bold areas indicate highly conserved sequences.

3.3. Southern hybridization

Southern analysis was performed on genomic DNA from almond leaves (Fig. 3). Almond DNA (5 mg), cleaved separately with *Bam*HI, *Eco*RI and *Hin*dIII restriction enzymes, which do not cut the PAOMTII insert, was separated by agarose gel electrophoresis and blotted onto nylon filters. The blot was probed with a PAOMTII fragment of 850 bp (*Eco*RI–*Sal*I) which hybridized to one or more bands, suggesting that the almond PAOMTII may be encoded by more than one gene, although probably no more than two or three.

3.4. Accumulation of almond PAOMTII mRNA in plant organs

Accumulation of the gene transcript corresponding to the insert of PAOMTII was studied by RNA blot hybridization of total RNA isolated from various almond organs in different stages of development (Fig. 4A). The analysis revealed that the PAOMTII mRNA, of $\approx$1.3 kb, specifically accumulated in flowers. During floral development, the PAOMTII gene transcript starts to accumulate in low but detectable levels at the C floral stage, increasing rapidly during the D floral stage, which corresponds to a completely devel-

oped but closed flower. Following this stage, PAOMTII mRNA levels decrease with maturation of the whole flower. The analysis of total RNA extracted from different organs of the mature flower, shown in Fig. 4B, revealed significant differences in the levels of PAOMTII mRNA. While stamen and sepals appear to contain similar high levels of the PaOMTII transcript, lower mRNA levels are detected in petals and, particularly, in pistils.

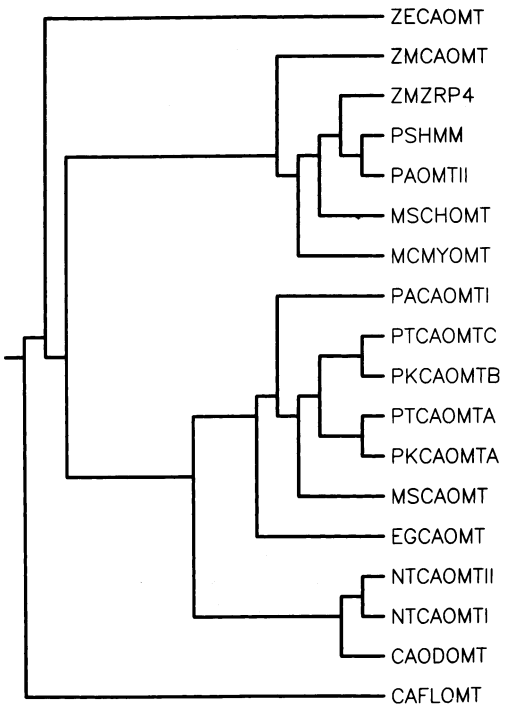

Fig. 2. Phylogenetic analysis of plant OMT amino acid sequences using the parsimony-based method in the PHYLIP program package. Sources for OMT sequences: CAODMT, *O*-diphenol-OMT from *Capsicum annuum*; CAFLOMT, 3′/5′ flavonol OMT from *Chrysosplenium americanum*; EGCAOMT, caffeic acid OMT from *Eucaliptus gunnii*; MSCAOMT, alfalfa caffeic acid OMT; MSCHOMT, alfalfa isoquiritigenin 2′-OMT; MCMYOMT salt stress-induced myo-inositol-OMT from *Mesembryanthemum crystallinum;* NTCAOMTI and NTCAOMTII, tobacco pathogen-induced caffeic acid OMTs; PSHMM, 6a-hydroxymaackiain 3-OMT from *Pisum sativum*; PKCAOMTA and PKCAOMTB, caffeic acid OMTs from *Populus kitakamiensis;* PTCAOMTA and PTCAOMTC, caffeic acid OMTs from *Populus tremuloides;* PACAOMTI, almond caffeic acid OMT; PAOMTII, sequence described in this work; ZMCAOMT, maize caffeic acid OMT; ZMZRP4, putative maize OMT expressed in roots; ZECAOMT, caffeic acid OMT from *Zinnia elegans*.

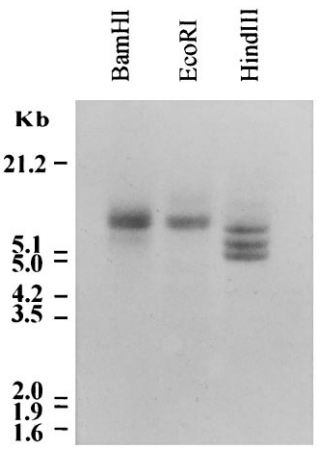

Fig. 3. Southern analysis of *Prunus amygdalus* DNA. Each lane contains 5 mg of genomic DNA restricted with *Bam*HI, *Eco*RI and *Hin*dIII. The blot was hybridized with a probe corresponding to a fragment of 890 bp of the PAB14B clone. The size markers, corresponding to λ phage DNA digested with *Eco*RI and *Hin*dIII, are shown on the left.

To elucidate the spatial expression pattern of the PAOMTII gene in flowers, we used in situ hybridization of the PAOMTII mRNAs on tissue sections from almond flower buds. We used this floral stage because, although the PAOMTII mRNA detected levels were low, it was not possible to obtain good enough sections from more developed flowers because of their larger size. Analysis of transversal sections of almond flowers indicates that, at this developmental stage, the PAOMTII transcripts are present in epidermal cells both in anthers (Fig. 5E) and styles (Fig. 5A) and in a small group of central cells that belong to the transmitting style tissue. In petals, the PAOMTII mRNA is detected in epidermal and vascular cells (Fig. 5C). In sepals, the expression seems to be restricted to the vascular tissue located on the surface (Fig. 5H,I). No signal was detected after hybridization with PAOMTII sense mRNA in styles, petals, anthers or sepals (Fig. 5B,D,FG, respectively).

4. Discussion

Here we report the isolation and characterization of an almond cDNA sequence that displays

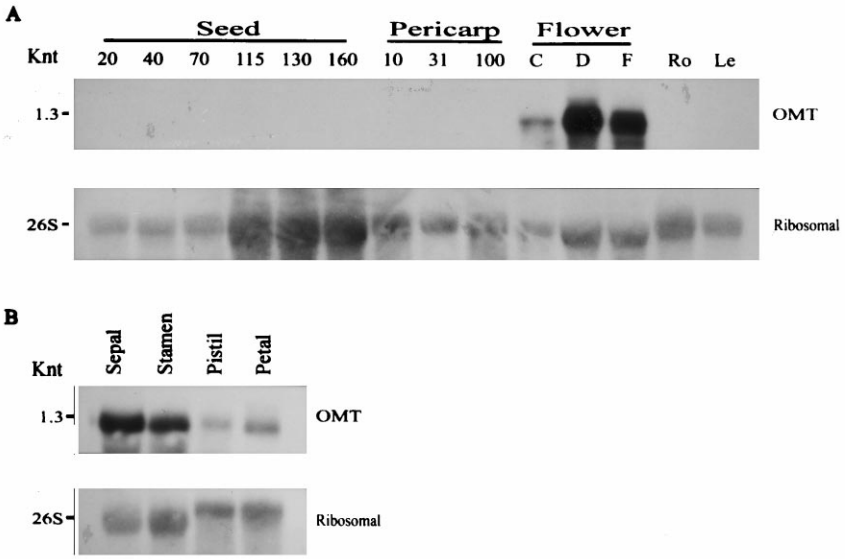

Fig. 4. RNA blot analysis of *Prunus amygdalus* PAOMTII using a fragment of 890 bp of the almond PAB14B cDNA. (A) Each lane contains 15 mg of total RNA from different tissues of the 'Texas' cultivar: seed and pericarp in different stages of development, flower from early development (stage C) to completely mature flower (stage F), root (ro) and leaf (le). Numbers indicate days after flowering. (B) The lanes contain 15 mg of total RNA from sepal, stamen, pistil and petal of mature flowers. The cDNA probe for 26S ribosomal RNA from *Zea mays* was used as a control.

homology to *O*-methyltransferases from other plant species. The initial clone PAB14B was isolated after screening a flower cDNA library carried out in order to obtain genes highly expressed in almond flower organs. The complete cDNA sequence was obtained from a flower cDNA library by PCR, using a specific oligonucleotide primer in combination with the SK-17 sequence primer. The complete deduced amino acid sequence is significantly similar to reported OMTs, particularly in the carboxy terminal half of the protein containing three regions (I, III and IV) previously shown to be conserved in proteins requiring SAM as a substrate [2]. The sequence identity between the protein sequence and previously described caffeic acid OMTs is notably low (not exceeding 35%). Using phylogenetic analysis, the PAB14B protein is not grouped with caffeic acid OMTs, and, in particular, its sequence clearly differs from the first *P. amygdalus* OMT isolated which has sequence similarity to caffeic acid OMTs [30]. For this reason, the cDNA has been called PAOMTII. This result may indicate that these enzymes belong to distinct classes of plant OMTs, suggesting that PAOMTII protein is probably not involved in the methylation of caffeic and hydroxyfeluric acids in lignin biosynthesis.

The PAOMTII sequence is more closely related to the HMM sequence from pea. The HMM enzyme catalyzes the terminal step in the pisatin (the major phytoalexin produced by pea upon microbial infection) biosynthetic pathway [28]. However, the patterns of expression are quite distinct; while the PAOMTII transcript is specifically accumulated in floral tissues, the HMM mRNA is accumulated in pea seedlings treated with $CuCl_2$, which is used to elicit pisatin biosynthesis [28].

This is the first description of a flower specific OMT, although the accumulation of caffeic acid OMT mRNA in flowers has been published in maize [31], alfalfa [37] and *Zinnia elegans* [38]. However, the mRNA levels observed in these cases are higher in lignified tissues such as roots, stems and petioles. Although the mRNA accumulation of PAOMTII in the mature tissues of the adult plant has not been studied, its mRNA is not detectable in the leaf and the young root where

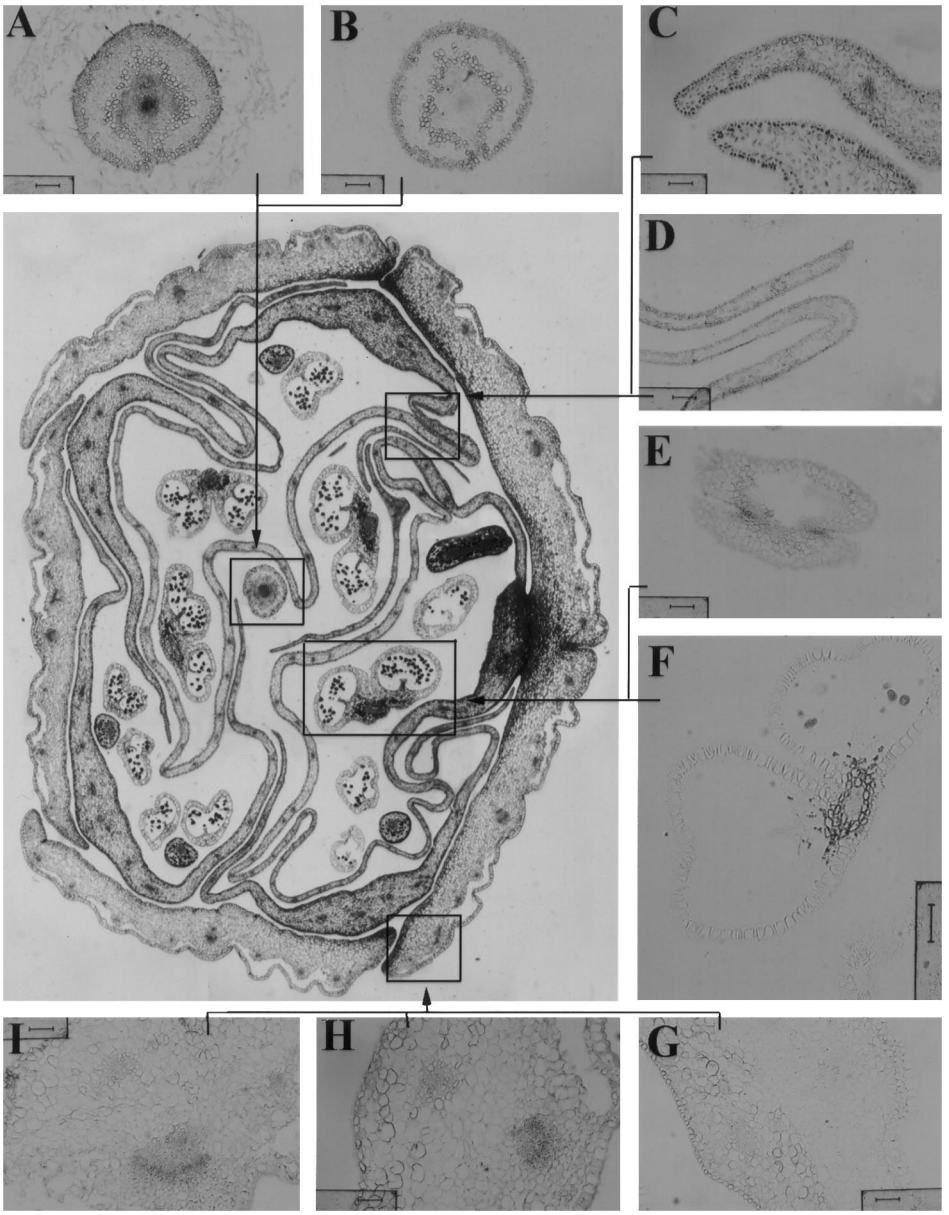

Fig. 5. Localisation of PAOMTII mRNA in almond flower buds by in situ hybridization. The hybridization was observed as darker areas. Bars = 100 μm in A, B, D, E, F, G and I; bars = 50 μm in C and H. The approximate location of transverse sections is indicated by squares on the left side of the figure. A, C, E, H and I: Transverse sections of style, petal, anther and sepal, respectively, hybridized with the antisense probe. B, D, F and G: Transverse sections of style, petal, anther and sepal, respectively, hybridized with the sense probe.

the previously described almond OMT cDNA [30] detected mRNA accumulation. By RNA blot analysis it can be concluded that the almond PAOMTII mRNA is accumulated in the four floral organs, although the expression levels are higher in sepals and stamens. In anthers and styles, the PaOMTII transcript has been located, by in situ hybridization, in epidermal cells, while

in sepals and petals it is mainly located in the vascular tissues. In spite of the divergence of the amino acid sequences of the different OMTs, they share a cellular expression pattern restricted to the epidermal and vascular tissues of distinct plant organs [29,33]. From the Southern blot results of plant genomic DNA, the PAOMTII appears to be encoded by more than one gene in the almond genome, as has been described for the aspen caffeic acid OMT [39], and the alfalfa caffeic acid OMT [37]. In the case of the HMM gene from *Pisum sativum*, two cDNA sequences have been described showing that more than one gene coding for this protein exists in this genome [28].

Sequence analysis and patterns of expression indicate that the almond PAOMTII clone may be representative of a new class of enzymes having an *O*-methyltransferase activity. The high expression levels detected during floral development and the similarity to pea HMM may reflect the involvement of this enzyme in the methylation of flavonoid derivatives accumulated in flower tissues. Its presence in the floral organs may also indicate a function in the pigmentation of the flower or defence mechanisms in the floral tissues.

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