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Molecular cloning of the cDNA coding for the (R)-(+)-mandelonitrile lyase of *Prunus amygdalus*: temporal and spatial expression patterns in flowers and mature seeds

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Abstract. A gene highly expressed in the floral organs of almond (*Prunus amygdalus* Batsch), and coding for the cyanogenic enzyme (R)-(+)-mandelonitrile lyase (EC 4.1.2.10), has been identified and the full-length cDNA sequenced. The temporal expression pattern in maturing seeds and during floral development was analyzed by RNA blot, and the highest mRNA levels were detected in floral tissues. The spatial mRNA accumulation pattern in almond flower buds was also analyzed by in-situ hybridization. The mRNA levels were compared during seed maturation and floral development in fruit and floral samples from cultivars classified as homozygous or heterozygous for the sweet-almond trait or homozygous for the bitter trait. No correlation was found between these characteristics and levels of mandelonitrile lyase mRNA, suggesting that the presence of this protein is not the limiting factor in the production of hydrogen cyanide.

Key words: Cyanogenesis – Flower (gene expression) – Mandelonitrile lyase (in-situ hybridization) – *Prunus* (cyanogenesis)

Introduction

Cyanogenesis, the production of the respiratory poison HCN by biological organisms, was first described in plants almost two centuries ago (see Robinson 1930). The metabolic pathway leading to the production of HCN has been described in various mono- and dicotyledonous species (Seigler 1991). Although low levels of HCN are probably produced by all plants during ethylene biosynthesis (Kende 1989), some species exhibit far greater HCN release due to the catabolism of cyanogenic glycosides or cyanolipids.

The pathway leading to the production of HCN in *Prunus* species has been studied in seed macerates of black cherry (*Prunus serotina*) (Poulton 1993). It has been shown that the initial substrate is (R)-amygdalin [the β-gentibiocide of (R)-mandelonitrile] and that three main enzymes acting successively in the pathway lead to the formation of HCN: amygdalin hydrolase, which produces (R)-prunasin; prunasin hydrolase, which produces (R)-mandelonitrile; and (R)-(+)-mandelonitrile lyase (MDL; EC 4.1.2.10), which yields HCN as one of its products. It has also MDL constitutes approx. 10% of the soluble protein of black-cherry seeds (Zheng and Poulton 1995). Upon crushing mature seeds, amygdalin is rapidly catabolized to HCN and benzaldehyde through the sequential action of the enzymes amygdalin hydrolase, prunasin hydrolase and MDL. Hydrogen cyanide is toxic to most aerobic organisms and its large-scale production occurs only upon tissue damage, so it has been suggested that cyanogenic compounds, in concert with the enzymes that catabolize them, defend these plants against herbivores and pathogens (Nahrstedt 1985). Tissue-level segregation has been demonstrated in sorghum leaves (Kojima et al. 1979) and *Prunus* seeds (Swain et al. 1992a), indicating that compartmentalization of cyanogens and their catabolic enzymes is a way of avoiding damage to the plant tissues. Another hypothesis, which does not exclude the previous one, is that these cyanogenic glycosides and lipids may serve as storage compounds for reduced nitrogen, since they are used for the synthesis of non-cyanogenic compounds during seedling development in *Hevea brasiliensis* and *Ungnadia speciosa* (Selmar et al. 1988).

The bitter flavour of certain almond seeds is due to amygdalin glycoside. The processes involved in its accumulation are not well defined; thus, it is not known whether the glycosides are synthesized de novo in the growing fruit, or whether all, or significant amounts, of

Abbreviations: DAF = days after flowering; MDL = mandelonitrile lyase; PCR = polymerase chain reaction *Gene bank accession*: The cDNA sequence of MDL1 has been submitted to the EMBL data bank under the accession number Y 08211

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the glycosides are translocated from a primary site of biosynthesis in the parent tissue to the storage tissue in the seed (Frehner et al. 1990). In almond, the sweet flavour of the kernel has been of prime importance in the breeding of the different cultivars because the bitter flavour is not in general a desired trait. Through breeding programmes and determination of kernel flavour, it has been proven that the inheritance of kernel flavour is determined by one gene with two alleles, *S* dominant, responsible for a sweet flavour and *s* recessive, responsible for a bitter flavour (Dicenta and García 1993). In spite of the importance of this trait, no molecular studies have been done to explain the difference between sweet and bitter almonds.

Materials and methods

Plant material. Almond (*Prunus amygdalus* Batsch) fruit and flower samples from the Masbovera (*SS*), Primorskyi (*SS*), Garrigues (*Ss*), Texas (*Ss*), and Garrigues amarg (*ss*) cultivars (allelic determination for sweet/bitter trait; Dicenta and García 1993) were collected at different stages of development from crop fields in the Department d'Arboricultura Mediterrània, IRTA (Reus, Catalonia, Spain), and were immediately frozen in liquid nitrogen and stored at −80 °C until they were used. Three stages of almond flower development have been considered C, D, and F corresponding, respectively, to the appearance of calyx, corolla and mature flower with petals completely opened (Felipe 1977).

Library screening. Polyadenylated RNA, extracted from closed flowers 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 the clones with a high level of expression were sequenced and a 1.6-kb cDNA clone with 80% similarity to published MDLs from *Prunus serotina* was among the sequences, although it did not correspond to the full-length cDNA. Polymerase chain reaction (PCR) was used with a PamMDL1-specific 18-mer oligonulceotide primer and the SK-17 sequence primer (TCTAGAACTAGTGGATC) to amplify MDL1 sequences from the λZAP-XR library upstream of the PamMDL1 primer sequence. In order to clone the MDL1 5′-end, the 5′ RACE system (GibcoBRL, Life Technologies) for rapid amplification of cDNA ends was used with 1 μg of total RNA from closed almond flowers, following the manufacturer's procedure.

Blot analysis of RNA and DNA. Whole RNA was extracted from different almond tissues using the methods described by Martin et al. (1993) or Haffner et al. (1978), depending on the tissue. Total RNA samples (20 μg) were electrophoretically separated in a 1.5% agarose-formaldehyde gel. Genomic DNA was extracted from young leaves from different cultivars, using the method described by Bernatzky and Tanksley (1986), and purified by equilibrium centrifugation in a CsCl-ethidium bromide gradient (Maniatis et al. 1982). After electrophoresis on 0.8% agarose gels, both DNA and RNA were blotted onto nylon membranes (Nytran; Schleicher and Schuell) and hybridized with a 400-bp fragment of the PamMDL1 clone labelled with [^{32}P]dCTP by random priming (Boehringer Mannheim). Hybridization and washing were done according to the protocol described by Church and Gilbert (1984).

In-situ RNA hybridization. The MDL1 mRNA was localized in paraffin-embedded flower sections by the non-isotopic digoxigenin-(DIG)-labelling system (Boehringer Mannheim), basically as described by Langdale (1993). Sense and antisense DIG-labelled riboprobes (150–200 nucleotide long) were generated by alkaline hydrolysis. Almond flowers in the C stage of development were fixed in ethanol/formaldehyde/acetic acid (80:3.5:5, by vol) for 1 week at 4 °C before being embedded in Paraplast Plus (Sigma) wax. Hybridization of the sections was done at 55 °C for 16 h with the riboprobe (1 μg ml^{-1}) in hybridization solution (50% formamide, 6 × SSC [1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0], 3% SDS, 100 μg ml^{-1} tRNA, 100 μg ml^{-1} poly A). The slides were permanently mounted in Histovitrex, examined under a Zeiss Axiophot FL microscope, and photographed using Kodak Ektachrome 160T films.

Results

Isolation and characterization of an almond MDL1 cDNA clone. In the course of screening an almond (*Prunus amygdalus*) flower cDNA library for genes highly expressed in the floral organs of this plant, a clone, designated D12D, containing a sequence homologous to MDL was isolated. The full-length cDNA was cloned and the levels of mRNA accumulation in different parts of the plant analyzed. As the sequence corresponded to one of the possible MDLs from almond, this clone was named MDL1.

The D12D cDNA clone contained an insert of 1667 bp but its 5′-end was incomplete compared to the size of the transcript assessed by RNA blot (2100 nucleotides). To obtain the full-length clone, an oligonucleotide was synthesized complementary to nucleotides 107–124 of the D12D clone. This was used in combination with the SK-17 sequence primer to amplify the 5′ MDL1 sequence from the λZAP-XR cDNA library, employing PCR. Electrophoretic analysis of the PCR products revealed that they contained a cDNA of the expected size, but not a full-length clone. The 5′ RACE system for rapid amplification of cDNA ends was used to clone the MDL1 5′-end. An MDL1 20-mer oligonucleotide (positions 73–92) was used to synthesize the first-strand cDNA from total RNA from closed flowers, and a second 20-mer oligonucleotide (positions 64–83) was used for PCR to amplify the MDL1-tailed cDNA. After subcloning and sequencing a PCR product of approx. 500 bp, the full-length almond MDL1 cDNA sequence with the new 5′ fragment of 49 bp was obtained.

The alignment of the *P. amygdalus* MDL protein sequence and two *P. serotina* (Cheng and Poulton 1993; Zheng and Poulton 1995) sequences, deduced from the respective cDNAs, is shown in Fig. 1. The three proteins have a high similarity of 81.6% (PamMDL1/PseMDL1), 82.3% (PamMDL1/PseMDL3) and 84.8% (PseMDL1/PseMDL3). The almond MDL1 protein sequence has a putative signal peptide of 27 residues which has been shown to be processed in black cherry, because it is not found in the mature PseMDL1 protein (Cheng and Poulton 1993). The three MDL proteins share four putative N-glycosylation sites, consistent with the known glycoprotein character of *P. serotina* MDLs (Wu and Poulton 1991).

The almond MDL mRNA shows the highest accumulation levels during flower development. The level of mRNA accumulation corresponding to the almond MDL was examined by RNA blot analysis using total RNA from

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                                  ↓
PamMDL1   MEKSTMSVILFVLHLLVLHLQYSEVHSLANTSAHDFSYLKFVYNATDTSLEGSYDYIVIGGGTSGCPLAATLSEK   75
PseMDL1   MEKSTMSAILLVLHLFVLLLQYSEVHSLATTSNHDFSYLRFAYDATDLELEGSYDYVIVGGGTSGCPLAATLSEK   75
PseMDL3   MVKSTMSAVLLVLHIFVLHLQYSEVQSLANTSSHDFSYLSFVYDATDPELEGSYDYIIVGGGTAGCPLAATLSAN   75
          * ***** .*.***..** ******.*** ** ****** * *.***  *******...****.********* .

YKVLLLERGTIATEYPNTLTADGFAYNLQQQDDGKTPVERFVSEDGIDNVRARILGGTTIINAGVYARANISFYSQTGIEWDLD  159
YKVLVLERGSLPTAYPNVLTADGFVYNLQQEDDGKTPVERFVSEDGIDNVRGRVLGGTSMINAGVYARANTSIYSASGVDWDMD  159
YSVLVLERGSLPTEYPNLLISDGFVYNLQQEDDGKTPVERFVSEDGIDNVRGRVLGGTSMINAGVYVRANTSFFNQTGIEWDMD  159
* **.****...* *** * .*** *****.********************.*.****..****** *** *... .*..**.*

LVNKTYEWVEDAIVVKPNNQSWQSVIGEGFLEAGILPDNGFSLDHEAGTRLTGSTFDNNGTRHAADELLNKGDPNNLLVAVQAS  243
LVNKTYEWVEDTIVFKPNYQPWQSVTGTAFLEAGVDPNHGFSLDHEAGTRITGSTFDNKGTRHAADELLNKGNSNNLRVGVHAS  243
LVNQTYEWVEDTIVFEPDSQTWQTVIGTAYLEAGILPNNGFSVDHLAGTRLTGSTFDNNGTRHASDELLNKGDPNNLRVAVQAA  243
****.******.**  *. * **.* * ..****. *..***.** ****.*******.*****.*******..*** *.*.*.

VEKILFSSNTSNLSAIGVIYTDSDGNSHQAFVRGNGEVIVSAGTIGTPQLLLLSGVGPESYLSSLNITVVQPNPYVGQFLYNNP  327
VEKIIFS-NAPGLTATGVIYRDSNGTPHRAFVRSKGEVIVSAGTIGTPQLLLLSGVGPESYLSSLNIPVVLSHPYVGQFLHDNP  326
VEKIIFSSNTSGVTAIGVIYTDSNGTTHQAFVRGEGEVILSAGPIGSPQLLLLSGVGPESYLTSLNISVVASHPYVGQYIYDNP  327
****.** *.. ..* **** **.*  *.****. ****.*** **.***************.**** ** ..*****.. .**

RNFINNFPPNPIEASVVTVLGIRSDYYQVSLSSLPFSTPPFSLFPTTSYPLPNSTFAHIVSQVPGPLSHGSVTLNSSSDVRIAP  411
RNFINILPPNPIEPTIVTVLGISNDFYQCSFSSLPFTTPPFSFFPSTSYPLPNSTFAHFASKVAGPLSYGSLTLKSSSNVRVSP  410
RNFINILPPNPIEASTVTVLGITSDFYQCSISSLPFDTPPFSFFPTTSYPLPNQTFAHIVNKVPGPLSHGTVTLNSSSDVRVGP  411
***** .******.. ****** .*.** *.***** *****.**.******* ****... *.**** *..**.***.**..*

NIKFNYYSNSTDLANCVSGMKKLGDLLRTKALEPYKARDVLGIDGFNYLGVPLPENQTDDASFETFCLDNVASYWHYHGGSLVG  495
NVKFNYYSNPTDLSHCVSGMKKIGELLSTDALKPYKVEDLPGIEGFNILGIPLPKDQTDDAAFETFCRESVASYWHYHGGCLVG  494
NVKFNYYSNLTDLSHCVSGMKKLGEVLSTDALEPYKVEDLPGIDGFNILGIPLPENQTDDAAFETFCRESVASYWHYHGGCLVG  495
 *.**********..*******.*..* * ** ***  *. **.*** **.***.*****.***** . .********** ***

KVLDDSFRVMGIKALRVVDASTFPYEPNSHPQGFYLMLGRYVGLQILQERSIRLEAIHNI--QESM-----------         559
KVLDGDFRVTGIDALRVVDGSTFPYTPASHPQGFYLMLGRYVGIKILQERSASD---------LKILDSLKSAASLVL         563
KVLDDGFRVTGINALRVVDGSTFPSTPASHPQGFYLMLGRYMGIQILQERSASEDAIRNLGFQENILDSPKSTSSFAF         573
****. *** ** ******.****  * *************.*..******          .
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Fig. 1. Alignment of three MDL proteins: PamMDL1 (*Prunus amygdalus*, Y08211), PseMDL1 (*Prunus serotina*, U78814) and PseMDL3 (*P. serotina*, U51562). *Arrow*, putative signal peptide cleavage site; *asterisks*, identical amino acids in all three sequences; *dots* conservative amino acid replacements; *bold letters*, putative N-glycosylation sites conserved in the three sequences. Gaps (-) have been introduced for maximum alignment

different organs of the plant, as well as from different developmental stages. As Fig. 2A illustrates, MDL mRNA was not detectable during phase I [0–62 d after flowering (DAF)] of seed development when the fruit exhibits a dramatic increase both in size and in fresh and dry weights. However, the MDL transcript was detectable (after 100 DAF) in the middle of phase II (63–145 DAF) when cotyledons develop, occupying most of the seed volume and accumulating storage proteins. It increased to reach a maximum at approx. 130 DAF. Subsequently, MDL transcript levels declined in phase III (146–180 DAF) during fruit maturity. In contrast, the almond MDL mRNA was only detected during phase I of pericarp development, although the abundance of mRNA was low. Surprisingly, the highest expression levels detected were in flowers, being similar in the three stages of development C (flower bud), D (closed flower) and F (mature flower). Expression was low in roots and considerably higher in leaves. Important differences in mRNA MDL levels were observed in the floral organs from mature flowers (Fig. 2B). Stamens and sepals appeared to contain similar high levels of MDL mRNAs. In petals, lower levels were detected whereas pistils seemed to accumulate very low levels of MDL transcript. To test for possible differences in MDL mRNA accumulation levels in almond cultivars classified as homozygous (cv. Masbovera) or heterozygous (cv. Texas) for the sweet trait or homozygous (cv. Garrigues amarg) for the bitter one, we compared the MDL mRNA levels during floral and seed development of these cultivars. However, no appreciable differences could be observed between the three almond cultivars from stages C to F of floral development (Fig. 2C) or during fruit maturation (data not shown).

Southern blot analysis. To study MDL gene organization, genomic DNA from almond cultivars Masbovera,

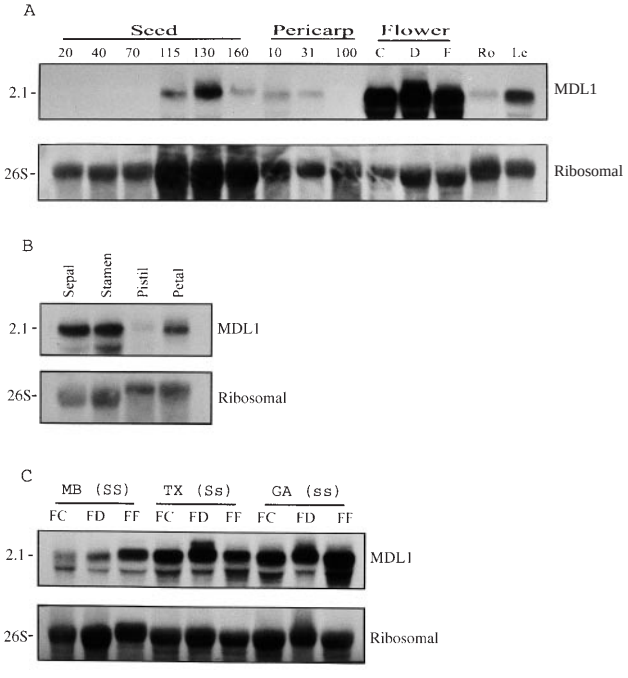


Fig. 2A–C. Northern blot analysis of *Prunus amygdalus* MDL1 using a fragment of 400 bp of the almond MDL1 cDNA. **A** Each lane contains 20 μg of total RNA from different tissues of the Texas (Ss) cultivar: seeds and pericarp at different stages of development, flowers from early development (stage C) to completely mature (stage F), roots (*Ro*) and leaves (*Le*). *Numbers* indicate days after flowering. **B** The lanes contain 15 μg of total RNA from sepals, pistils, stamens and petals from mature flowers of the Texas cultivar. **C** The lanes contain 20 μg of total RNA from flowers at an early developmental stage (stage C) to completely mature flower (stage F) of three almond cultivars: Masbovera (*MB;SS*), Texas (*TX;Ss*) and Garrigues amarg (*GA;ss*). The cDNA probe for 26S ribosomal from *Zea mays* was used as a control. The transcript size, in knt, (2100 nucleotides) is indicated on the left of each μDL1 blot

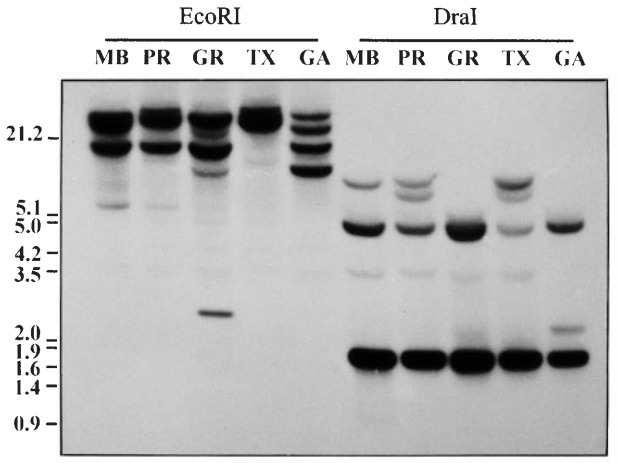


Fig. 3. Southern blot analysis of *Prunus amygdalus* DNA. Each lane contains 10 μg of genomic DNA from the following almond cultivars: Masbovera (*MB*; *SS*), Primorskyi (*PR*; *SS*), Garrigues (*GR*; *Ss*), Texas (*TX*; *Ss*) and Garrigues amarg (*GA*; *ss*), digested with *Eco*RI and *Dra*I. The blot was hybridized with a probe corresponding to a fragment of 400 bp of the almond MDL1 cDNA. The size markers (kb), corresponding to lambda-phage DNA digested with *Eco*RI and *Hind*III, are shown on the left

Primorskyi, Garrigues, Texas and Garrigues amarg was digested with *Eco*RI (for which there is no restriction site in the cDNA sequence) and DraI (for which there are two internal restriction sites, liberating a 1869-bp fragment). Southern blot analysis using a 403-bp *Xho*I-*Xho*I fragment of MDL1 cDNA as a probe, suggests (Fig. 3) that there are probably two or three MDL-related genes in the almond genome. This result indicates that the MDL1 probe used predominantly detects one band but that other genes with high similarity to MDL1 also hybridize with this probe. For this reason the mRNA levels observed both by RNA blot and in-situ hybridization probably correspond to the expression of all these genes together.

In-situ localization of MDL1 mRNA in flower buds. To elucidate the spatial distribution pattern of MDL mRNA in flowers, we used in-situ hybridization on tissue sections from almond flower buds. There was a high level of expression in parenchymal cells, both in petals (Fig. 4D,E) and in the style of the carpel, where the expression was specifically localized in two opposite stylar zones (Fig. 4B), although no signal was detected in the ovary (Fig. 4A). In sepals, the MDL mRNA was detected in small, meristematic cell groups localized along the internal side of the sepal (Fig. 4H,I). No signal was detected in petals, styles and sepals after hybridization with MDL1 sense mRNA (Fig. 4F,C and G, respectively), the color in Fig. 4C being that of the background. As can be seen in Fig. 4J, no signal was observed in stamens, although the MDL transcript was detected in stamens from mature flowers, by Northern blot. To eliminate the possibility that the stamens were not in good condition, we used in-situ hybridization of PamL TPI cDNA (Suelves and Puigdomènech 1997) as a control and, as expected, a signal was observed in the epidermis of the anthers (Fig. 4K). This suggests that there is no MDL expression at this stage of development or that it is too low to be detected.

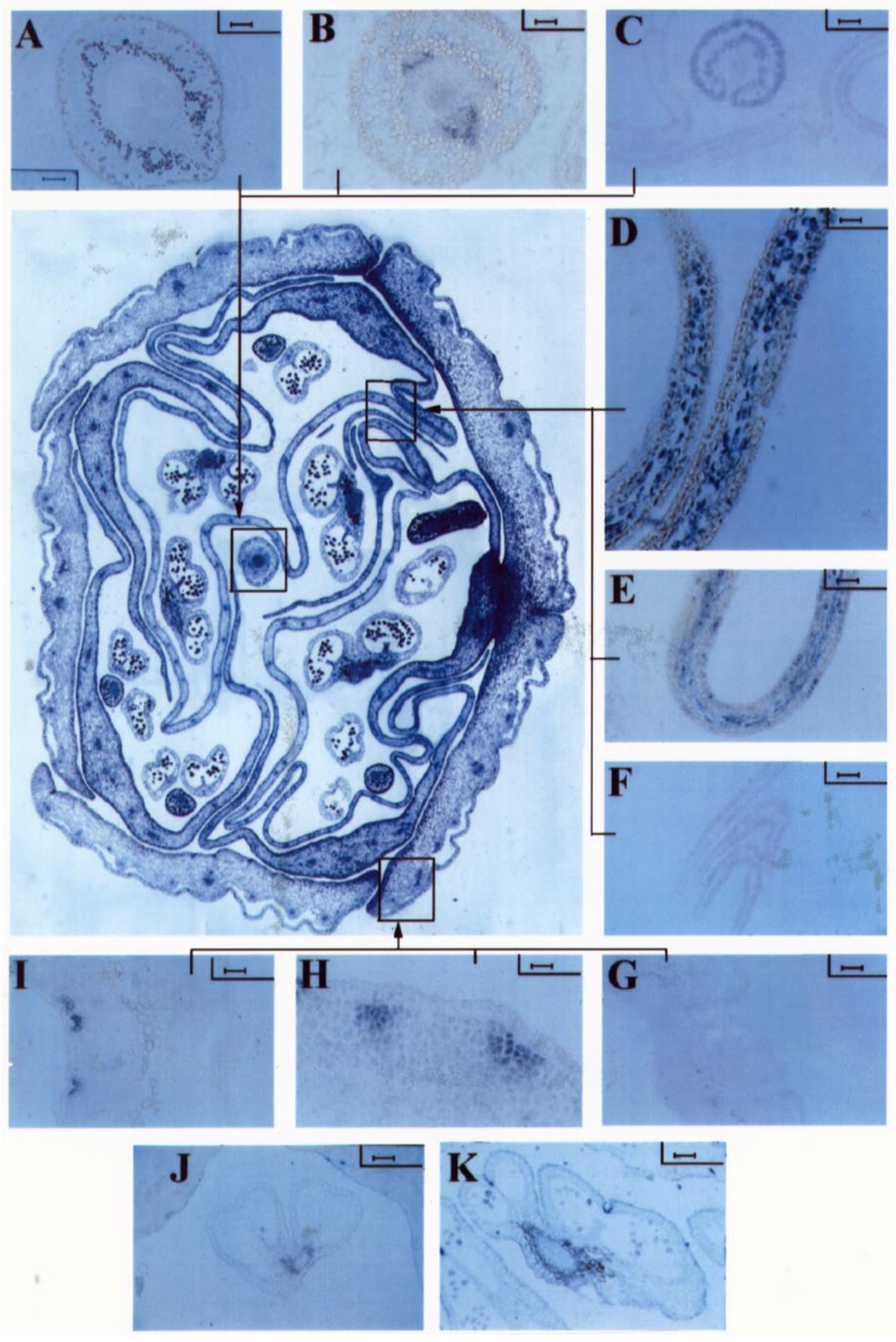
Fig. 4A–K. Localization of MDL1 in almond flower buds by in-situ hybridization. The hybridization was observed as dark-blue areas. The approximate location of transverse sections is indicated by *boxed areas* in the main panel of the figure. **A, B, D, I** Transverse sections of ovary, style, petal and sepal, respectively, hybridized with the antisense probe. **E, H** A detail of a transverse section of petal and sepal, respectively, hybridized with the antisense probe. **C, F, G** Transverse sections of style, petal and sepal, respectively, hybridized with the sense probe. **G** Section of the stamen; **K** Control of stamen hybridized with an LTP1 probe. Bars = 100 μm (**A, C, D, F, G, I, J, K**); 50 μm (**B, D, E**); 25 μm (**H**)

Discussion

The main physiological role of MDL is the catalysis of the dissociation of (R)-mandelonitrile to HCN and an aldehyde or ketone (Yemm and Poulton 1986). We have cloned the first MDL cDNA of *P. amygdalus*, from an almond closed-flower cDNA library. This cDNA codes for a polypeptide of 559 amino acids, including a 27-amino-acid leader peptide. The sequence similarity to MDL1 and 3 from *P. serotina* is almost 85% and, together with the conservation of four putative N-glycosylation sites, suggests that the almond MDL1 is functionally related to MDLs previously described. Most MDLs exist as multiple forms but the comparison of the three available *Prunus* MDL sequences shows that they are very similar except in the last 27 amino acids which correspond to a highly hydrophobic region.

The almond MDL1 protein, like other MDLs, has a putative signal sequence suggesting that the protein is transported into the ER. This fact is in agreement with the already-reported localization of MDL in protein bodies in the seed of *P. serotina* (Poulton 1993), although the signal for retention or getting to another compartment in the cell is not evident from the protein sequence. Genomic Southern blot analysis using an MDL1 cDNA fragment as a probe indicates that, in the almond genome, two or three MDL-related genes exist, which may encode different isoenzymes showing variations in primary structure and in glycosylation pattern, as occurs in MDL proteins from *P. serotina* (Yemm and Poulton 1986). According to this result the mRNA accumulation levels observed here probably correspond to the expression of genes having a high degree of similarity to that coding for MDL1.

We have analyzed the MDL expression pattern in different tissues of the plant and during seed maturation and floral development. Our results indicate that the pattern of almond MDL expression during seed development is similar to that described in developing black-cherry seeds (Zheng and Poulton 1995). The black-cherry pericarp has been considered acyanogenic (Swain et al. 1992b) because, although it accumulates cyanogenic glycosides, it lacks catabolic enzymes. In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contrast, in almond pericarp from young seeds (until 60 DAF) we have detected MDL expression, but the pericarp may still be acyanogenic as the onset of significant cyanogenic glycoside accumulation in bitter almond is around 50 DAF (Frehner et al. 1990).

The highest MDL expression levels were detected during floral development. Analysis of the expression levels in different floral organs from mature flowers gave very high levels of MDL mRNA in sepals and stamens, whereas in petals the levels were lower and hardly detectable in pistils. In-situ hybridization in flower buds showed that MDL expression was restricted to the parenchymal cells of petals and styles, and groups of meristematic cells in sepals. No signal was detected in the stamen by in-situ hybridization, in contrast to Northern blot analyses, where a high level of expression was observed. A possible explanation for this apparent discrepancy is that the Northern blot analyses used stamens from fully mature flowers, whereas in the in-situ hybridization, immature stamens from closed flower buds were used.

This study describes for the first time, the MDL expression pattern in floral tissues and the high accumulation of MDL mRNA during flower development. This raises the question of what biological role MDL plays in flowers. In *P. serotina* seeds, MDL constitutes approx. 10% of the soluble protein which is mainly located in protein bodies of the cotyledon parenchymal cells, and is believed to be multifunctional, serving both as a storage protein and in cyanogenesis (Zheng and Poulton 1995). However, even though storage-protein accumulation in vacuoles (VSP) of vegetative tissues such as leaves has been described (Wittenbach 1982), it seems improbable that MDL1 protein may be a storage compound in floral tissues, which have a very fast development. It is proposed that the (R)-(+)-mandelonitrile lyase coded by the MDL1 gene has a protective function during floral development, contributing to the defence mechanisms of the flower.

We followed the changes in the amount of MDL mRNA during seed maturation and floral development in three almond cultivars classified as homozygous or heterozygous for the sweet trait or homozygous for the bitter one. Our results indicate that there are no significant differences in MDL expression level either during the maturation of the seed or through floral development. This suggests that the MDL is not the limiting step producing the difference between sweet and bitter almonds.

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