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ORIGINAL ARTICLE

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COI1 affects myrosinase activity and controls the expression of two flower-specific myrosinase-binding protein homologues in *Arabidopsis*

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Abstract Two cDNA clones homologous to myrosinase-binding proteins (MBPs) were identified by differential display in *Arabidopsis thaliana* (L.) Heynh. The cDNAs (*MBP1* and *MBP2*) correspond to two open-reading frames found in a gene cluster of seven putative *MBP* genes located on chromosome 1. The predicted proteins MBP1 and MBP2 are similar to lectins and plant aggregating factors. In addition, MBP2 contains a region of high content of proline and alanine residues, commonly found in arabinogalactan proteins and hydroxyproline-rich glycoproteins. Transcripts corresponding to *MBP1* and *MBP2* genes are exclusively and abundantly expressed in flowers but are not detected in male-sterile flowers of *coi1* plants, insensitive to jasmonic acid. Northern analysis and in situ hybridization revealed that *MBP* mRNAs are present in higher levels in immature flowers and are localized in several floral organs, including the ovary, ovules, style, anthers and filament. Transcripts of the *Arabidopsis* myrosinase gene *TGG1* show a pattern of expression similar to that observed for the *MBP* genes during flower development; however, they are also abundant in green tissues and are only partially affected by *COI1*. Crude preparations of soluble proteins from leaf and flower extracts of wild-type *Arabidopsis* showed myrosinase activity when sinigrin was used as substrate. In contrast, *coi1* plants showed significantly reduced myrosinase activities in both leaves and flowers. The results show that *COI1* controls *MBP* expression in flowers and significantly affects the expression and activity of myrosinase in *Arabidopsis*.

Keywords *Arabidopsis* (*Coi1* mutant) · Differential display · Flower-specific expression · Mutant (*Coi1*) · Myrosinase-binding proteins

Abbreviations EST: expressed sequence tag · JA: jasmonic acid · MBP: myrosinase-binding protein · MeJA: methyl jasmonate

EMBL accession numbers: *MBP1* (AF054906), *MBP2* (AF222537)

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Introduction

The *coi1* mutant of *Arabidopsis* is insensitive to jasmonic acid (JA) and produces male-sterile flowers (Feys et al. 1994). We have used this mutant to identify JA-responsive genes, and this allowed us to isolate various genes that are normally up-regulated in *Arabidopsis* leaves by JA or wounding. We have noticed that all JA-induced genes we have isolated are constitutively expressed in flowers, but are not found in roots. Examples include the vegetative storage protein gene *VSP* (Benedetti et al. 1995), the coronatine-induced gene *ATHCOR1* (Benedetti et al. 1998) and the oxophytodienoate reductase gene *OPR3* (Costa et al. 2000). In this study, we compared the gene expression profiles of normal and male-sterile flowers of *coi1*, to identify flower-specific genes dependent on JA signal transduction. We report the isolation and characterization of two *Arabidopsis* genes that are specifically expressed in floral organs but are not inducible by JA or wounding in vegetative tissues. The genes identified are homologous to those for myrosinase-binding proteins (MBPs) commonly found in association with myrosinase enzymes in various members of the Brassicaceae.

The myrosinase-glucosinolate system is involved in several aspects of plant development and defense, affecting the behavior of herbivorous insects and

pathogens (reviewed by Rask et al. 2000). In this system, hydrolysis of the glucosinolates by myrosinase enzymes (thioglucosidases) produces substances with a remarkably wide spectrum of biological activities. Some of these compounds are very effective against chewing insects, bacteria and fungi, whereas others can function as signaling molecules or stimulants for insect feeding and oviposition (Rask et al. 2000). Additionally, glucosinolates and their degradation products can provide precursors of plant hormones and serve as metabolic sources of sulfur and nitrogen at particular stages of the plant development (Bones and Rossiter 1996).

The function of MBPs in plants has not been established, and it is not clear yet whether they have a role in the myrosinase-glucosinolate system, since recombinant myrosinases display normal activity in the absence of MBPs or other myrosinase-associated proteins (Chen and Halkier 1999). It has been demonstrated that certain types of myrosinase-associated proteins called epithiospecifiers (ESPs) function as a myrosinase cofactor necessary to modulate the specificity of myrosinases towards the production of particular enzyme products (Bones and Rossiter 1996; Chen and Halkier 1999; Bernardi et al. 2000). However, experimental data supporting a relationship between MBPs and ESPs are still lacking (Bones and Rossiter 1996). Due to their lectin activity, it has also been suggested that MBPs could bind carbohydrates present in insect guts and fungal pathogens, thus enhancing the action of glucosinolates in defense reactions (Rask et al. 2000).

Three myrosinase genes have been identified in *Arabidopsis*, two of them, *TGG1* and *TGG2*, present conserved structures and very similar expression patterns (Xue et al. 1995). We chose myrosinase *TGG1* to compare its transcript levels relative to the *MBP* genes in vegetative tissues and during flower development. Here, we report that myrosinase *TGG1* and *MBP* genes are expressed in flowers in a developmentally similar way, and that *COI1* is also important for *TGG1* expression and myrosinase activity in *Arabidopsis*.

Materials and methods

Plant growth

Seeds of wild-type *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia (Col-0) were germinated in MS medium (Murashige and Skoog 1962), whereas *coi1* seeds from an F_2 population segregating for the Coi phenotype, were first germinated in MS containing 10 μM methyl jasmonate (MeJA) to select homozygous *coi1* plants (Feys et al. 1994). Seedlings were grown for about 10 days in a growth cabinet under white light (70 μmol photons m^{-2} s^{-1}) with a 16-h-day/8-h-night photoperiod at 20 °C, and then transferred to fresh MS medium and grown for 5 days. Seedlings were either transferred to fresh MS or MS-MeJA plates, or moved to soil to grow to maturity.

Differential display

Differential display of mRNA was performed based on Liang and Pardee (1992). Total RNA (1 μg) from flowers of wild-type and *coi1* plants was reverse-transcribed and amplified by polymerase chain reaction (PCR) as previously described (Benedetti et al. 1998). A differentially displayed band of about 120 bp (TVGOPP9), detected by the pair of primers OPP9 (OPERON, Alameda, Calif., USA) and T_{12}VG (GIBCO-BRL, Gaithersburg, Md., USA), was purified, cloned and sequenced as described by Benedetti et al. (1998), and used to probe RNA blots.

Screening of the cDNA library

A cDNA library of *Arabidopsis* (Landsberg *erecta*), kindly donated by Dr. Elliot M. Meyerowitz (California Institute of Technology, Pasadena, USA), was screened following the Stratagene protocols using TVGOPP9 as probe. A few independent clones were isolated and sequenced using the dye-terminator reaction kit (Perkin-Elmer, Norwalk, Conn., USA) in an automated ABI DNA sequencer (Applied Biosystems, Warrington, UK). A 1.2-kb *Eco*RI/*Sal*I fragment from clone 1.2 isolated during the first screening was used to re-screen the library. Two full-length clones of approx. 1.6 kb (*MBP1*) and approx. 2.4 kb (*MBP2*) were used in the work described here.

Probes

Specific probes were obtained by digesting *MBP1* and *MBP2* cDNAs with *Eco*RI or *Eco*RI/*Ssp*I, yielding the 286-bp *Eco*RI and 571-bp *Eco*RI/*Ssp*I fragments, corresponding to the 5′ ends of *MBP1* and *MBP2*, respectively. The 286-bp *Eco*RI fragment was subcloned into pBlueScript KS^+ (pMBP1Eco) to generate the sense and antisense RNA probes used in the in situ hybridization. A myrosinase probe was obtained from the expressed sequence tag (EST) number 34862 (clone 185J2T7) corresponding to the myrosinase *TGG1* gene, provided by the *Arabidopsis* Biological Resource Center (Ohio State University). The identity of the EST clone was confirmed by sequencing analysis.

Northern blots

Total RNA from roots, seedlings, leaves, flowers and siliques was extracted according to Verwoerd et al. (1989), whereas total RNA from flowers at different developmental stages was extracted with Trizol reagent (GIBCO-BRL) following the manufacturer's protocol. Aliquots of total RNA (20 μg) were fractionated on formaldehyde-agarose gels (Sambrook et al. 1989), transferred onto nylon membranes Hybond N^+ (Amersham, Little Chalfont, UK) by capillary blotting and fixed by UV cross-linking according to the manufacturer instructions. Blots were hybridized overnight with the ^{32}P-labeled probes described above, at 42 °C, and washed twice for 10 min at 42 °C with 2× SSC (1× SSC is 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7) and 0.1% SDS, and twice with 0.2× SSC and 0.1% SDS, under the same conditions.

Southern blots

Genomic DNA was extracted using the CTAB method described in Ausubel et al. (1989). DNA samples (10 μg) were digested with *Xba*I, *Xho*I or *Eco*RI, electrophoresed in a 1% agarose gel and transferred onto nylon membranes (Hybond N^+). Blots were hybridized for 15 h at 42 °C with the ^{32}P-labeled MBP1- and MBP2-specific probes described above. Membranes were washed once with 2× SSC and 0.1% SDS for 20 min at 42 °C, and twice with 0.2× SSC containing 0.1% SDS for 10 min at 42 °C.

In situ hybridization

RNA probes were prepared from linearized pMBP1Eco using digoxigenin (DIG)-11-rUTP labeling, following the manufacturer's protocol (Boehringer Mannhein, Germany), except that T3 RNA

polymerase was used. Tissues were fixed in FAA (50% ethanol, 10% formaldehyde, 5% acetic acid) overnight at 4 °C, dehydrated through a graded ethanol series followed by a *t*-butanol series, then embedded in Paraplast Plus (Sigma, St. Louis, MO, USA) and sectioned (8 μm). Sections assembled in poly-L-lysine-coated slides were de-paraffinized in xylene, re-hydrated and treated with proteinase K (1 μg/ml) for 30 min. Slides were incubated in 100 mM triethanolamine, 0.1% acetic anhydride for 10 min. Hybridization was carried out at 55 °C in a moist chamber in 50% deionized formamide, 5× SSC, 5% SDS, 100 μg/ml tRNA, 100 μg/ml polyA and the probes at 5 μg/ml. Slides were washed twice in 0.2× SSC, 0.2% SDS at 55 °C for 10 min, and then incubated with RNase A (10 μg/ml) for 20 min. Immunological detection was performed incubating the slides for 1 h in 0.5% blocking reagent, then in diluted anti-DIG-alkaline phosphatase conjugate (1:1,000) in 0.5% BSA for 1 h at room temperature in a moist chamber. The color reaction was developed in 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 50 mM $MgCl_2$, 338 μg/ml nitro blue tetrazolium (NBT), 175 μg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP), and 1 mM levamisole, overnight at room temperature in a dark moist chamber. Sections were dehydrated in ethanol series and mounted with Permount (Fisher Scientific, Pittsburgh, PA, USA) for visualization.

Protein extraction and myrosinase assay

Rosette leaves and flowers of wild-type and *coi1* plants were homogenized in 0.1 M phosphate buffer (pH 6.0) containing 5 mM benzamidine. The homogenate was maintained in continuous, gentle stirring for 30 min at 4 °C. Insoluble material was removed by centrifugation at 13,000 rpm for 15 min, and the supernatant was filtered through a 0.45-μm filter and precipitated in 70% saturated ammonium sulfate. The pellet was dissolved in 10 mM phosphate buffer, and thoroughly dialyzed against the same buffer. Precipitate formed during dialysis was removed by centrifugation at 4,000 *g* for 10 min. All steps were carried out at 4 °C. Myrosinase activity was determined by measuring the hydrolysis of sinigrin (Sigma) by following the decrease in absorbance at 227 nm (Schwimmer 1961). Assays were performed at 27 °C in 0.75 ml of 33 mM sodium phosphate (pH 6.0), containing 20–50 μg of protein extract and 0.24 mM sinigrin. Assuming that one of the hydrolytic products of sinigrin, allyl isothiocyanate, absorbs light in the UV region, we used the molar extinction coefficient at 227 nm for both the substrate ($\epsilon = 7800$) and product ($\epsilon = 564$) in the calculation of sinigrin concentration at the end of the reaction. Protein was quantified by a Bradford-based method (Bio-Rad, Hercules, Calif., USA).

Results

Isolation of two *MBP* genes from *Arabidopsis* flowers

A small DNA fragment (TVGOPP9) identified by differential display in the wild type but not in male-sterile flowers of *coi1* was cloned and used to probe total RNA from immature and developed flowers of *Arabidopsis*. It was found that TVGOPP9 detected a transcript of approx.1.8 kb expressed only in wild-type flowers (not shown).

A flower cDNA library was screened with TVGOPP9 and a few independent clones were isolated. Sequence analysis of the cDNA ends revealed that two of the clones (1.1 and 1.2) were related and that clone 1.1 contained the entire TVGOPP9 sequence located at its 5′ end. Northern blot analysis showed that, similar to TVGOPP9, clone 1.1 preferentially detected a transcript of approx. 1.8 kb, whereas clone 1.2 detected transcripts of approx. 1.7 kb, approx. 1.8 kb and approx. 2.8 kb, in wild-type flowers (Fig. 1). These transcripts were poorly detected in flowers of *coi1* (Fig. 1).

In order to isolate full-length cDNAs corresponding to the 1.7-, 1.8- and 2.8-kb transcripts, a fragment from clone 1.2 was used to re-screen the library. Twenty-one independent clones were isolated and divided into restriction groups. Clones representative of each group were sequenced, and two related cDNAs homologous to MBPs were identified. One cDNA (*MBP1*) was identical to the previous clone 1.1, whereas a larger one (*MBP2*) represented a full-length cDNA of clone 1.2.

To certify that *MBP2* corresponded to the larger transcript of approx. 2.8 kb observed on Northern blots, a fragment specific to *MBP2* cDNA was used to probe RNA from flowers. Figure 1 shows that the probe derived from *MBP2* preferentially detected the approx. 2.8-kb transcript in wild-type flowers. Although a transcript of approx. 1.7 kb was frequently observed on Northern blots (Fig. 1), a cDNA smaller than *MBP1* was not isolated in our screenings.

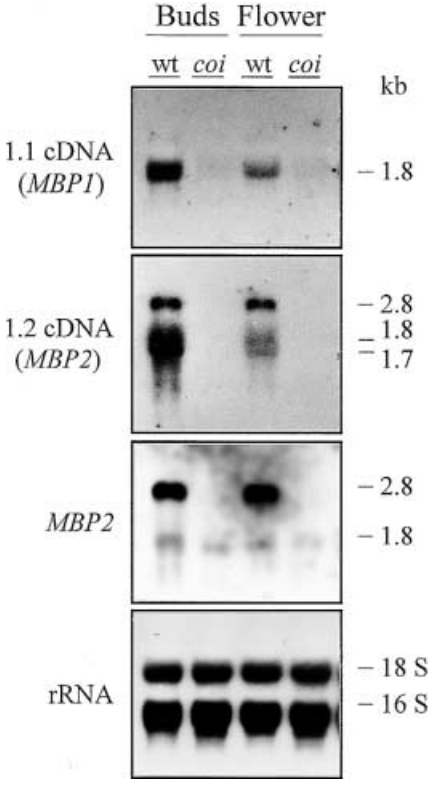

Fig. 1 Expression of *MBP1* and *MBP2* genes in buds and developed flowers of wild-type (*wt*) *Arabidopsis thaliana* and the *coi1* mutant. RNA was probed with cDNA clones 1.1 and 1.2 (*MBP1* and *MBP2*, respectively), and with a fragment specific to *MBP2* cDNA. Transcripts of 1.7, 1.8 and 2.8 kb were detected in the wild type, but not in the male-sterile flowers of *coi1*. Similar amounts of RNA were loaded in each lane as revealed by the hybridization to a 16 S rRNA

Genomic structure and sequence analysis of *Arabidopsis MBP* genes

Sequences from *Arabidopsis* chromosome 1 (AC006216) showed the existence of a cluster of seven related genes homologous to previously described MBPs from *Brassica napus* (Fig. 2A). Sequence alignments between *MBP1* and *MBP2* with BAC F5F19 clearly shows that *MBP1* corresponds to the Col-0 *F5F19.10 MBP* gene. *MBP2* is highly similar to the Col-0 *F5F19.9 MBP* gene (96% identity); however, *MBP2* has additional coding sequences relative to *F5F19.9 MBP* and therefore may represent a different Landsberg allele.

To investigate the copy number and relatedness of the *Arabidopsis MBP* genes, two probes derived from the 5′ ends of *MBP1* and *MBP2* were used in Southern blot experiments (Fig. 2B). The two probes detected mainly single strongly hybridizing bands in genomic DNA digested with three different enzymes, under high-stringency conditions (Fig. 2B). Two hybridizing bands were observed in *Xba*I-digested DNA probed with *MBP2*, although no *Xba*I sites are found in either *MBP2* cDNA or genomic *F5F19.9* sequences. We concluded from these experiments that *MBP1* and *MBP2* are represented as single-copy genes in the *Arabidopsis* genome.

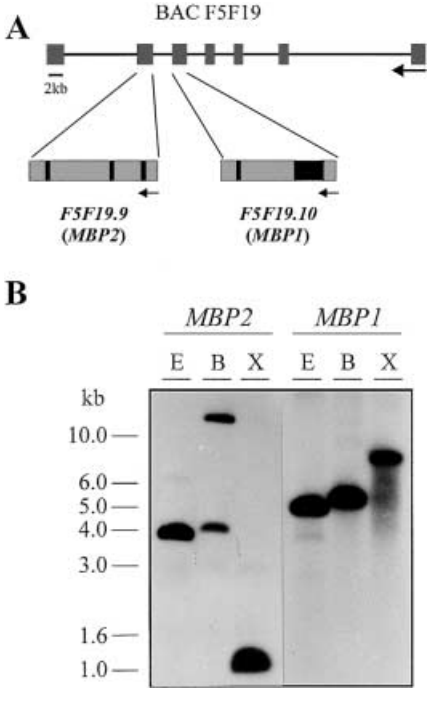

Fig. 2A, B Genomic organization of *Arabidopsis MBP* genes. **A** Schematic representation of the *Arabidopsis* chromosome 1 region (*BAC F5F19*) comprising seven putative *MBP* genes (*boxes*). The genes encoding *MBP1* (*F5F19.10*) and possibly *MBP2* (*F5F19.9*) are shown in detail, with introns (*black*) and exons (*gray*). *Arrows* indicate the orientation of transcription. **B** Southern blot analysis of *Arabidopsis* genomic DNA digested with *Eco*RI (*E*), *Xba*I (*B*) and *Xho*I (*X*), hybridized to *MBP1* (286-bp *Eco*RI fragment) or *MBP2* probe (571-bp *Eco*RI/*Ssp*I fragment)

The *MBP1* and *MBP2* cDNAs possibly encode for proteins of 462 (MBP1) and 664 amino acids (MBP2), which are homologous to MBPs from *Brassica napus* and *Arabidopsis* (Fig. 3). The deduced proteins are composed of three (MBP1) or four (MBP2) repeated domains, a characteristic that is also found in *B. napus* MBPs (Taipalensuu et al. 1997a, c; Geshi and Brandt 1998). In addition, the deduced MBP1 and MBP2 proteins show significant homology to plant agglutination factors and lectins such as jacalin, particularly in their C-terminal regions (Fig. 3).

A special feature of the MBP2 deduced protein is the presence of a 54-amino-acid sequence rich in proline (50%) and alanine (36%) found between the second and third domains (Fig. 3). The proline (P) residues are confined to stretches of PX, where X is frequently alanine (A). The PA-rich region is also found in an MBP from *B. napus* seeds (Taipalensuu et al. 1997a) and in an f-AtMBP recently isolated from *Arabidopsis* flowers (Takechi et al. 1999).

MBP2 is 96% identical to f-AtMBP, and the main difference between the two sequences is the number of PX repeats (Fig. 3). We observed that the deduced polypeptide from *f-AtMBP* (Takechi et al. 1999) almost perfectly matches the protein derived from the genomic sequence *F5F19.9 MBP*, except that it includes four additional amino acid residues in its N-terminal (Fig. 3).

Flower-specific expression of *Arabidopsis MBP* genes

The expression of both *MBP1* and *MBP2* is restricted to floral tissues and requires the *COI1* function (Fig. 4A). Because MBPs have been suggested to be associated with myrosinase enzymes (Bones and Rossiter 1996), we looked at the overall expression of an *Arabidopsis* myrosinase gene *TGG1* (Chadchawan et al. 1993) for comparison. We observed that *TGG1* has a pattern of expression different from that of the *MBP* genes. *TGG1* mRNA was found in green tissues, including leaves and siliques, but, similar to *MBP* transcripts, it is abundant in flowers and absent in roots. Interestingly, *TGG1* expression is only partially affected by the *coi1* mutation (Fig. 4A). Since *TGG1* mRNA was found in flowers and its expression was significantly diminished in *coi1*, we followed its transcript accumulation relative to *MBP1* during stages of flower development. Figure 4B shows that the expression of *TGG1* parallels that of *MBP1* during flower development, and that both transcripts are high in immature flowers, but progressively decrease as flowers begin to open, mature and senesce. Male-sterile flowers of *coi1* do not express *MBP1* in the floral stages examined, but show a low expression of *TGG1* (Fig. 4B).

Since the *MBP* genes and *TGG1* are dependent on *COI1* for flower expression, we tested whether they could be induced by jasmonate in vegetative tissues. Figure 5 shows that *MBP1* and *TGG1* are weakly induced by MeJA in wild-type seedlings after 4 h of jasmonate treatment.

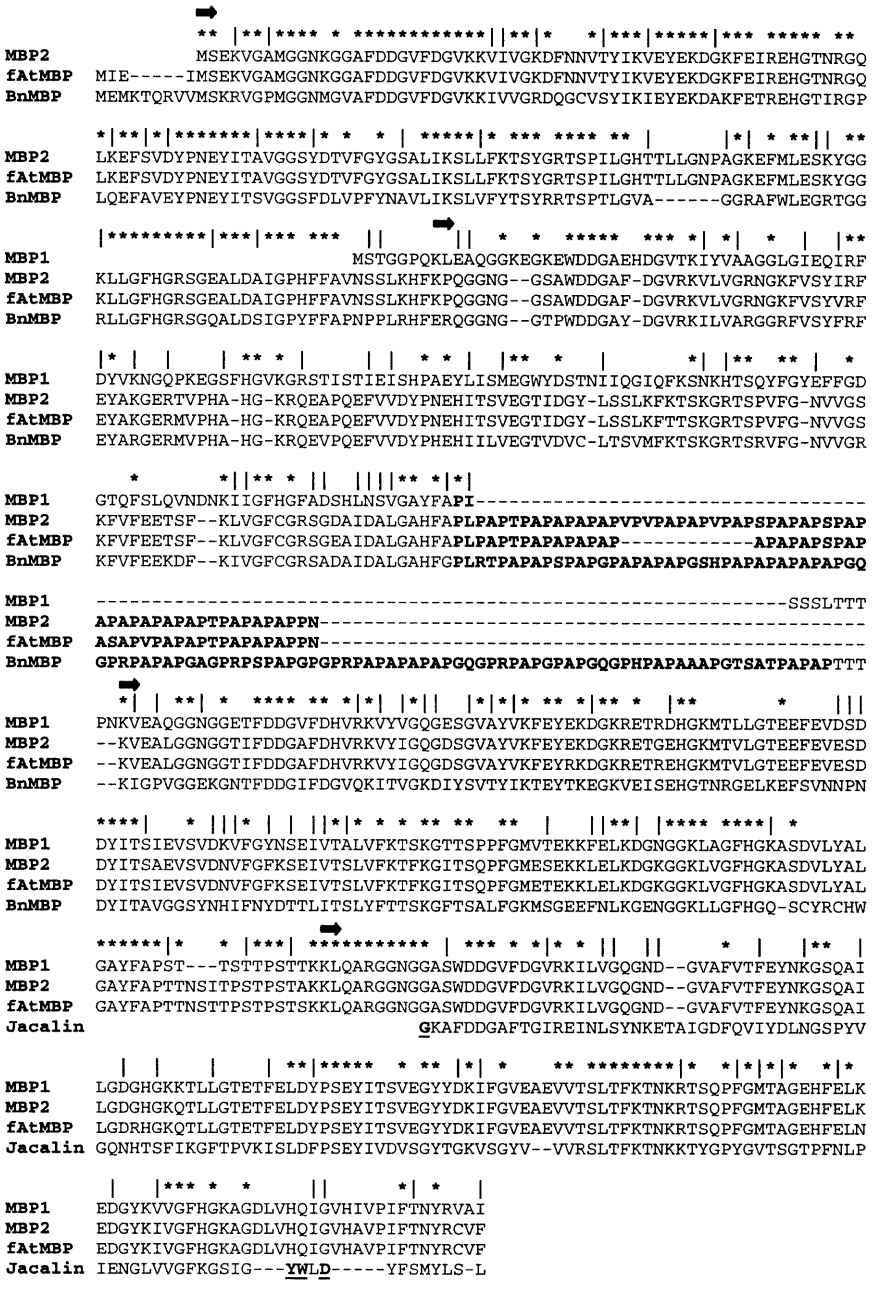

Fig. 3 Protein alignment of *Arabidopsis* MBPs 1 and 2 and related sequences. Comparison of the predicted MBP1 (AF054906) and MBP2 (AF222537) proteins with *Arabidopsis* fAtMBP (AB027252), *Brassica napus* BnMBP (U59446) and lectin jacalin α-chain from *Artocarpus integrifolia* (L03795). *Asterisks* and *lines* indicate identical and similar residues, respectively. *Arrows* separate different MBP domains and the proline-rich region is in *bold*. Residues that are important for carbohydrate binding in jacalin protein are *bold-underlined*. The last 18 amino acid residues of the C-terminal of BnMBP were removed to optimize the alignment

In situ localization of the *Arabidopsis MBP1* transcript

To further examine the expression of *MBP1*, a probe derived from a 286-bp *Eco*RI fragment of *MBP1*, previously shown to hybridize specifically to the 1.8-kb *MBP1* transcript (Fig. 4B), was used in in situ hybridization experiments. *MBP1* mRNA was detected in male and female organs, petals and pedicels (Fig. 6A, D–F). Hybridization signals (purple-blue) were stronger in immature flowers (Fig. 6A, D) but less intense in developed and in *coi1* flowers (Fig. 6E, F and B, respectively). As observed in Fig. 6D and E, most of the female structures, particularly the ovary, ovules and style were labeled, relative to the sense (brownish) hybridization (Fig. 6G). The hybridization signal was also observed in stamens, with intense labeling of the tapetal cells (Fig. 6A, D, E). However, less intense staining of the tapetal cells was detected in wild-type flowers hybridized with the sense probe (Fig. 6C) and in *coi1* flowers hybridized with the antisense probe (Fig. 6B).

Analyses of the flowers in the later stages of development, when tapetal cells had degenerated, revealed that the overall staining of female organs and petals is maintained, while staining of male parts is reduced and limited to the filament (Fig. 6F). No hybridization signal was detected in pollen grains or sepals.

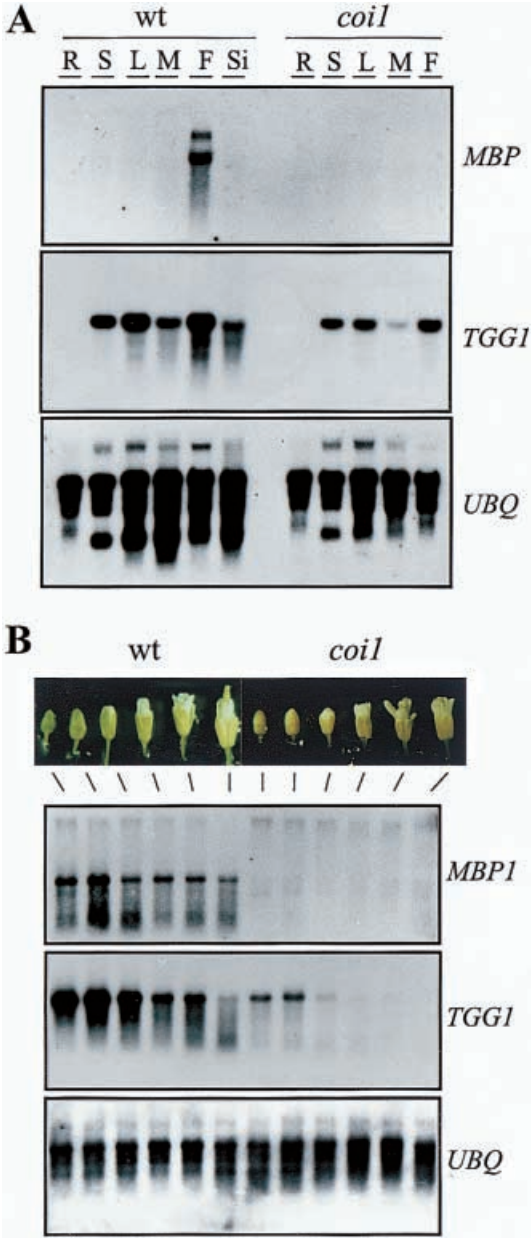

Fig. 4A, B Flower specific expression of the *Arabidopsis MBP* genes, and their dependency on *COI1*. **A** *MBP1* and *MBP2* transcripts are expressed exclusively in flowers (*F*) of wild-type plants. Transcripts of a myrosinase gene (*TGG1*) are nevertheless present in seedlings (*S*), young (*L*) and mature (*M*) leaves, flowers (*F*) and siliques (*Si*), but not in roots (*R*) of both wild-type and *coi1* plants. However, the *coi1* mutation also affected *TGG1* expression. **B** The accumulation and disappearance of *MBP1* and *TGG1* transcripts occurs in parallel during the stages of flower development, but expression of *TGG1* is only partially affected by *COI1*. Total RNA hybridized to a ubiquitin gene is shown (*UBQ*)

Myrosinase activity is increased in flowers and is affected by *COI1*

MBPs are thought to play a role in the myrosinase-glucosinolate system, and because both *MBP* and *TGG1* transcript levels are relatively lower in *coi1* tissues, we measured myrosinase activity from leaf and flower extracts using sinigrin as substrate (Fig. 7). We observed that the myrosinase activity toward sinigrin is higher in flowers than in leaves of *Arabidopsis* and that this activity is significantly reduced in these organs in the *coi1* mutant. On average, the myrosinase activity was 60% and 90% lower in *coi1* flowers and leaves, respectively (Fig. 7).

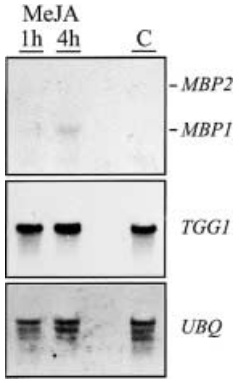

Fig. 5 Transcript accumulation of *MBP1*, *MBP2* and *TGG1* in wild-type *Arabidopsis* seedlings in response to MeJA. Northern analysis of seedlings treated with MeJA (10 μM) for 1 and 4 h reveals a very weak *MBP1* and *TGG1* response to jasmonate, compared to untreated controls (*C*). *MBP2* transcripts could not be detected. The ubiquitin (*UBQ*) control for RNA loading is shown

Discussion

This work presents the isolation and characterization of two flower-specific *MBP* genes, which are absent in male-sterile flowers of the *Arabidopsis coi1* mutant. Transcripts related to *MBP* genes have been identified in *Arabidopsis* flowers (Utsugi et al. 1996), and a gene (*f-AtMBP*) encoding a flower-specific MBP similar to MBP2 has been described (Takechi et al. 1999). Indeed, the sequencing of the *Arabidopsis* genome has revealed the existence of a gene cluster in chromosome 1 harboring seven open-reading frames homologous to MBPs (AC006216). The cDNAs corresponding to the two major transcripts observed in developing flowers are represented in this gene cluster. *MBP1* is identical to the Col-0 *F5F19.10* gene, except for a single amino acid change. However, the sequence of *MBP2* differs slightly from its putative corresponding Col-0 allele in chromosome 1, particularly in the PX repeated region, which is longer in MBP2 relative to F5F19.9 MBP (same as f-AtMBP). A few amino acid changes between MBP2 and F5F19.9 MBP may reflect an ecotype variation.

The function of these *MBP* genes in flowers of *Arabidopsis* is currently unknown. In *B. napus*, MBPs have been suggested to participate in the myrosinase-glucosinolate system that is implicated in defense against herbivores and pathogens (reviewed by Bones and Rossiter 1996; Rask et al. 2000). MBPs are able to form complexes with myrosinases, as evidenced by

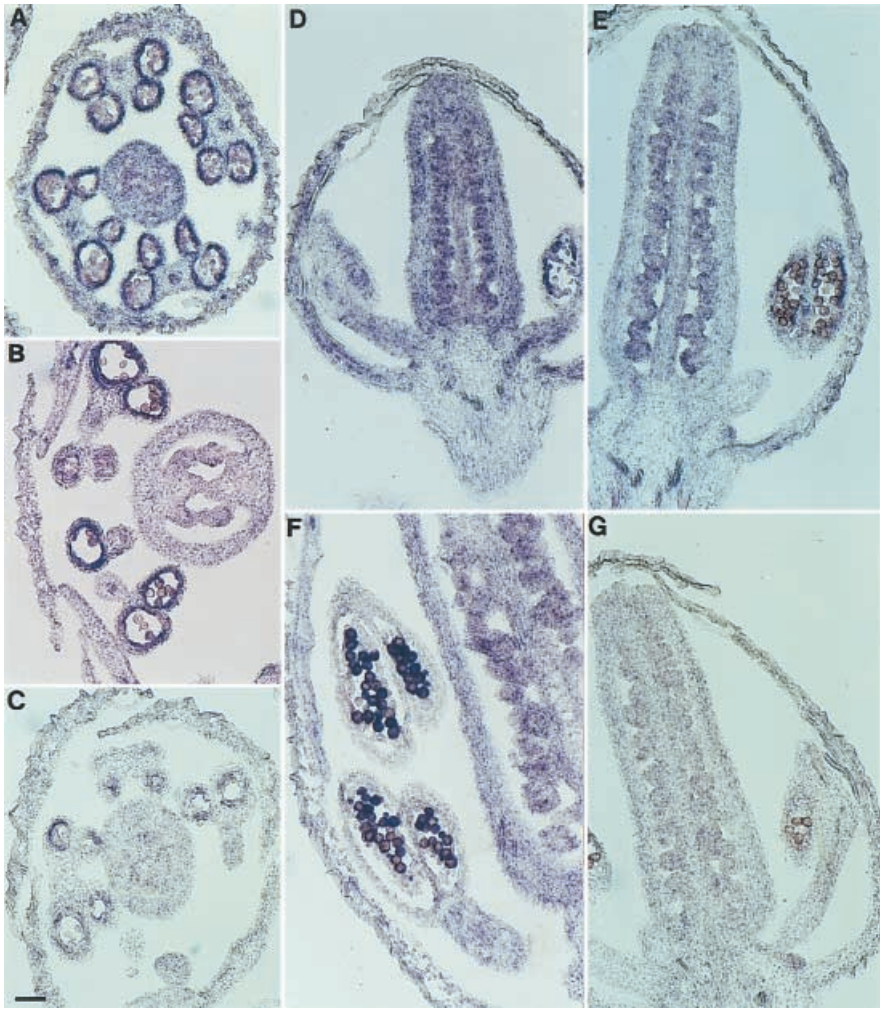

Fig. 6A–G In situ localization of *MBP1* in flowers of wild-type *Arabidopsis*. **A–C** Transverse sections of the wild type (**A**) and the *coi1* mutant (**B**) hybridized with the antisense probe are compared with the wild type probed with sense *MBP1* (**C**). **D–G** Longitudinal sections of immature (**D**) and developed (**E, F**) wild-type flowers hybridized with antisense *MBP1* or sense control (**G**). Hybridization signal (*blue*) is found in male and female organs more intensely in developing flowers (**A, D**) relative to wild-type controls (**C, G**) and *coi1* (**B**). Stronger signals are observed in the ovary, ovules, filament and tapetal cells. Bar = 30 μm

immunoaffinity experiments (Falk et al. 1995; Geshi and Brandt 1998; Geshi et al. 1998). In addition, purified MBPs from *B. napus* seeds were shown to possess lectin activity, suggesting that they could specifically bind to glycosylated myrosinases (Taipalensuu et al. 1997c). In this respect, the C-termini of the *Arabidopsis* MBPs are very similar to those of a maize aggregating factor (Esen and Blanchard 2000) and lectins such as jacalin from *Artocarpus integrifolia* and agglutinin from *Maclura pomifera* (Young et al. 1991). Jacalins constitute a distinct group of lectins, known to bind human IgA_1 specifically through the galactose-terminal oligosaccharides (Skea et al. 1988). Although the amino acid residues of jacalin important for the methyl-galactose binding are not fully conserved in the *Arabidopsis* and *B. napus* MBPs, *B. napus* MBP also binds IgA in vitro through its jacalin homologous region (Geshi and Brandt 1998).

Interestingly, the MBP2 predicted protein presents a repetitive proline-rich region (PX). Proline-rich motifs $(Hyp\text{-}X)_n$ are widespread in arabinogalactan proteins (AGPs) belonging to the family of hydroxyproline (Hyp)-rich glycoproteins (HGRPs). In these proteins, Pro residues are usually post-translationally hydroxylated to form Hyp, and X is usually alanine, threonine or serine. Non-contiguous Hyp residues are sites of arabinogalactan polysaccharide attachment (Shpak et al. 1999). AGPs are found in a variety of organs and tissues,

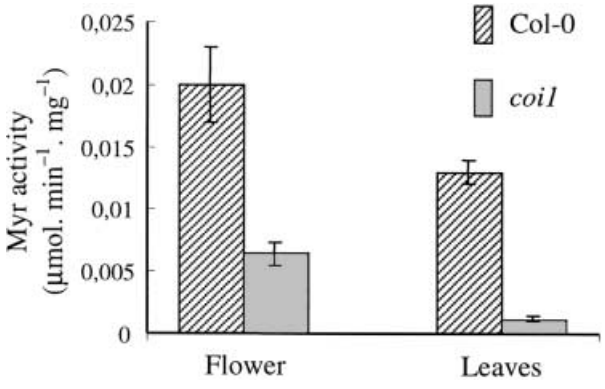

Fig. 7 Specific myrosinase activity in flowers and leaves of *Arabidopsis*. Protein extracts (20–50 μg) from the wild type (*Col-0*) and the *coi1* mutant were assayed for myrosinase activity toward sinigrin in sodium phosphate buffer pH 6.0, at 27 °C, as described in *Materials and methods*. Decay in absorbance due to sinigrin breakdown was measured at 227 nm. Values are means ± SD of four measurements

such as in mature pollen grains of alfalfa (Qiu et al. 1997), and intercellular spaces between stylar transmitting tissue cells (Gao et al. 1999). AGPs and other pistil-specific proline-rich proteins (Goldman et al. 1992; Cheung et al. 1993) possibly mediate cell-cell recognition and interactions in pollination and fertilization. Chimerical proteins containing a lectin domain fused to an HRGP motif with a possible role in fertilization processes are also found in tobacco flowers (Wu et al. 1993). It is possible, therefore, that these MBPs have a role in male fertility since *coi1* flowers do not express them.

The expression of *Arabidopsis* MBPs restricted to floral organs is distinct from the expression profiles of *B. napus* MBPs and MyAPs (myrosinase-associated proteins) described previously. *MBP* and *MyAP* from *B. napus* possess seed-specific transcripts that show no induction upon MeJA or wounding (Falk et al. 1995; Taipalensuu et al. 1997a, b), and transcripts that are vegetatively expressed, and wound- and MeJA-inducible (Taipalensuu et al. 1997b, c; Geshi and Brandt 1998). We observed that *Arabidopsis* seedlings treated with MeJA show a small induction of *MBP1* and *TGG1*, but not *MBP2*. Similarly, *f-AtMBP* did not respond to wounding, dehydration stress, MeJA, salicylic acid, or abscisic acid treatments. However, transcripts related to *MBP* genes were induced by such treatments in *Arabidopsis* vegetative tissues (Takechi et al. 1999), suggesting that *Arabidopsis* also has non-inducible organ-specific and vegetative inducible *MBP* genes. More recently, an EST homologous to a JA-induced *MBP* was identified in wounded leaves of *Arabidopsis* and shown to depend on *COI1* for expression (Reymond et al. 2000). This shows that *Arabidopsis* has two classes of *COI1*-dependent *MBP* genes which are differentially regulated in different tissues.

In situ hybridization showed that *MBP1* was expressed in male and female organs, petals and pedicels more intensely in immature flowers. Accordingly, *MBP1* transcripts detected on Northern blots are increased during the initial stages of flower development, but are maintained after anthesis, which may correspond to the hybridization signals observed in female organs and petals at later stages. Takechi et al. (1999) were able to detect a similar pattern of mRNA expression during flower development in wild-type plants using an antisense probe specific to *f-AtMBP* (similar to *MBP2*). Transcripts of *f-AtMBP* were detected mainly in ovules and styles of immature flowers, but also in pistils, styles, stamens, petals and embryos (Takechi et al. 1999).

Transcripts of myrosinases *TGG1* and *TGG2* have been found in sepals, petals and female organs of *Arabidopsis* flowers, but not in pollen grains or stamens (Xue et al. 1995). Thus, the presence of *MBP1* and *f-AtMBP* (Takechi et al. 1999) transcripts in stamens of *Arabidopsis* flowers could indicate an alternative function of MBPs not related to the myrosinase-glucosinolate system. Except in *B. napus*, where both myrosinase and MBPs were co-localized to myrosin grains found in myrosin cells of cotyledons (Geshi et al. 1998), a similar correlation between MBPs and myrosinases has not been established in other plants. Here we show that *TGG1* and *MBP1* mRNAs are expressed in flowers in a developmentally similar way. However, *TGG1* mRNA is present in tissues where *MBP* transcripts are absent, a strong indication that *MBP1* and *MBP2* are not required for *TGG1* function, particularly in leaves. Accordingly, myrosinase activities toward sinigrin determined in *coi1* are lower in leaves than in flowers, relative to the wild type. These observations do not exclude the possibility of MBPs being functional as components of a myrosinase-glucosinolate system in *Arabidopsis*, but alternatively, suggest that they could play a role, in principle as lectins, during flower development, or in defense against insects (Rask et al. 2000).

The fact that *coi1* plants have significantly lower levels of myrosinase activities in leaves and flowers is of particular relevance since these plants are more susceptible to attack by insects and fungi (McConn et al. 1997; Staswick et al. 1998; Vijayan et al. 1998). Further studies will be required to determine whether the reduced myrosinase activity in *coi1* plants is directly linked to the increased susceptibility of this mutant to insect herbivory and fungal infection.

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