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SHORT COMMUNICATION

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The MADS box gene *AOM1* is expressed in reproductive meristems and flowers of the dioecious species *Asparagus officinalis*

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Abstract MADS box genes are implicated in different steps of plant development. Some of them are expressed in vegetative organs. Most of them, however, are expressed in flower tissues and are involved in different phases of flower development. Here we describe the isolation and characterization of an *Asparagus officinalis* MADS box gene, *AOM1*. The deduced AOM1 protein shows the highest degree of similarity with *FBP2* of *Petunia hybrida* and *AGL9* (*SEP3*), *AGL2* (*SEP1*) and *AGL4* (*SEP2*) of *Arabidopsis thaliana*. In situ hybridization analyses, however, show that the expression profile of *AOM1* is different from that of these genes: *AOM1* is expressed not only in flower organs but also in inflorescence and flower meristems. These data indicate a possible function of *AOM1* during flower development as well as in earlier stages of the flowering process. *Asparagus officinalis* is a dioecious species which bears male and female flowers on different individuals. *AOM1*, which is expressed very early during the process of flowering and has a similar expression profile in male and female flowers, does not seems to be involved in asparagus sex differentiation.

Keywords *Asparagus officinalis* · *AOM1* · MADS box genes · Flower development

Introduction

MADS box proteins are a family of transcription factors found in vertebrates, insects, plants and fungi. They are involved in the regulation of different processes (see Shore and Sharroks 1995) and are characterized by the presence of a highly conserved domain of 57 amino acids, the MADS-box (Schwarz-Sommer et al. 1990), which binds DNA. Plant MADS box genes are expressed in different organs and are implicated in both vegetative and reproductive development (Rounsley et al. 1995; Heard et al. 1997; Zhang and Forde 1998). Most plant MADS box genes are expressed in flower tissues and are implicated in the different phases of flower development (for recent reviews see Weigel and Meyerowitz 1994; Yanofsky 1995; Riechmann and Meyerowitz 1997). During the development of unisexual flowers in monoecious and dioecious species, the expression pattern of MADS box genes involved in stamen and pistil differentiation is directly or indirectly controlled by sex determining genes. In most of these species, flowers are hermaphrodite at early stages of development, and sex differentiation results from programmed abortion of the pistil in male flowers and the stamen in female flowers. The moment of transition from hermaphrodite to the unisexual developmental program is different in different species (Dellaporta and Calderon-Urrea 1993). The expression pattern of analyzed MADS box genes in each species is also different (Hardenak et al. 1994; Ainsworth et al. 1995; Mena et al. 1995, 1996).

In the dioecious species *Asparagus officinalis*, the morphological and biochemical events which characterize the transition to the unisexual developmental pathway have been previously defined (Caporali et al. 1994), but nothing is known about the involvement of MADS box genes. As a start toward determining a possible involvement of these genes in asparagus sex differentiation, we isolated the MADS box genes expressed in flowers to compare expression patterns during male and female flower development. We describe the isolation and characterization of an asparagus MADS box gene expressed in reproductive meristems and in developing male and female flowers.

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Materials and methods

Plant material

Male and female plants of *Asparagus officinalis* L. (line UC 127) grown in open fields at a farm near Piacenza (northern Italy) were used in all experiments. Phylloclades (needle-like assimilatory

structures on stems) and flowers were frozen in liquid nitrogen immediately after collection and stored at –80°C. Flowers were divided into different classes according to developmental stage, as described (Caporali et al. 1994). Roots and shoots were collected from 10-day-old seedlings, cultivated in a growth chamber.

Construction and screening of a female-specific cDNA library

Total RNA was extracted from 600 mg of female flower buds 1 mm long, utilizing the GIBCO BRL TRIzol reagent as described by the manufacturer. Starting with this preparation, a λtriplex cDNA library was prepared by CLONTECH Laboratories.

The library was screened with the MADS box domain sequence from the *PLENA* gene of *Antirrhinum majus* (kindly provided by Dr. Zsuzsanne Schwarz-Sommer, Max Planck Institute for Plant Breeding, Cologne, Germany) and the MADS box homologues clones were hybridized with the 3' specific end of *PLENA*. Radioactive probes were prepared utilizing the random primed DNA labeling kit (Roche Molecular Biochemicals). Hybridizations were performed following the procedure suggested by CLONTECH.

DNA sequencing

The *AOM1* cDNA clone was sequenced at the ENEA Gene Lab (Rome, Italy). Sequence analyses were performed using the programs GCG Bestfit, ExPASy Blast N, ExPASy Blast P, and GCG Profile Scan.

RTPCR

Total RNA was extracted from male and female flowers (stages 2 and 5), roots and shoots of young seedlings, and phylloclades of adult plants, with the TRIPURE isolation reagent (Roche) as recommended by the manufacturer. For first strand cDNA synthesis, 5 µg of total RNA was used and cDNA synthesis was primed by using the polyT primer O RACE T (Gibco) and the reverse transcriptase Gibco Supertranscript II RT, following the directions of the manufacturer. To detect the *AOM1* mRNA, primer P1 (AGAGGGAGAGTTGAGCTGAAGA) located inside the MADS box of *AOM1* (nucleotides 71–92) or primer P2 (TTCTGCAGCAGCTCTAGCATG) located immediately outside the MADS box (nucleotides 233–253) was used for PCR, along with primer P3 (CAGGCATGTAATTGCTCACGC) located 10 bp upstream of the translational termination codon (nucleotides 770–750). PCR was performed with Perkin Elmer Taq polymerase in the thermal cycler Perkin Elmer gene amp PCR system 9600, set for 5 min at 92°C and 35 cycles of 1 min at 92°C, 1 min at 55°C, and 1 min at 72 °C, followed by a final extension of 7 min at 72°C.

In situ hybridization

Shoot apexes of flowering branches or single flowers at different stages of development were fixed and embedded in paraffin according to Canas et al. (1993). Digoxigenin-labelled antisense RNA probes, derived from the sequence downstream of the MADS box and the K box of *AOM1* cDNA, were generated by in vitro transcription according to the instruction provided with the in vitro transcription kit (Roche). Hybridization and immunological detection were performed as described by Canas et al. (1993).

Results

An asparagus MADS-box gene, designated *AOM1* (*Asparagus officinalis* MADS-box 1), was isolated from the cDNA library constructed from young female asparagus flowers using a probe specific for *PLENA*, a stamen and carpel identity gene of *Antirrhinum majus*. The *AOM1* cDNA (total length 1036 bp) contains a single open reading frame, 5' and 3' untranslated regions and a poly(A)+ tail. *AOM1* has the highly conserved MADS domain (nucleotides 67–234) and the K domain (nucleotides 397–510). Nucleotide number 64 is the first base of the translation initiation codon (Fig. 1). The deduced AOM1 protein shows the highest degree of similarity with FBP2 of *Petunia hybrida* (83%), and AGL9 (79%), AGL2 (72%) and AGL4 (72%) of *Arabidopsis thaliana*. The MADS domains of the first three proteins and that of AOM1 are identical, while in the MADS domain of AGL4 there are two substitutions. The degree of similarity of AOM1 with PLENA is lower, 82% within the MADS box and 60% downstream of the MADS box.

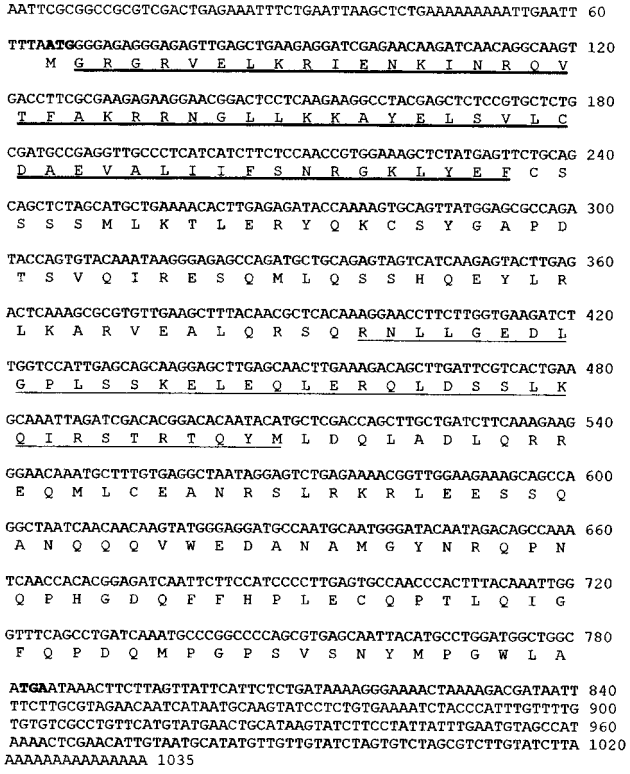


Fig. 1 Nucleotide and deduced amino acid sequence of the *AOM1* cDNA clone. Position of the MADS box (nucleotides 67–234) and the K box (nucleotides 397–510) are underlined

Analysis of the genomic DNA of asparagus through Southern blot hybridization, utilizing as a probe the *AOM1* region downstream of the MADS box, indicates that only one copy of *AOM1* is present in the asparagus genome (not shown).

RNA analysis

As a start toward determining the expression pattern of *AOMI*, RNA from roots and shoots of young seedlings, phylloclades and flowers was analyzed with the RTPCR, utilizing two pairs of primers of 22 nucleotides designed

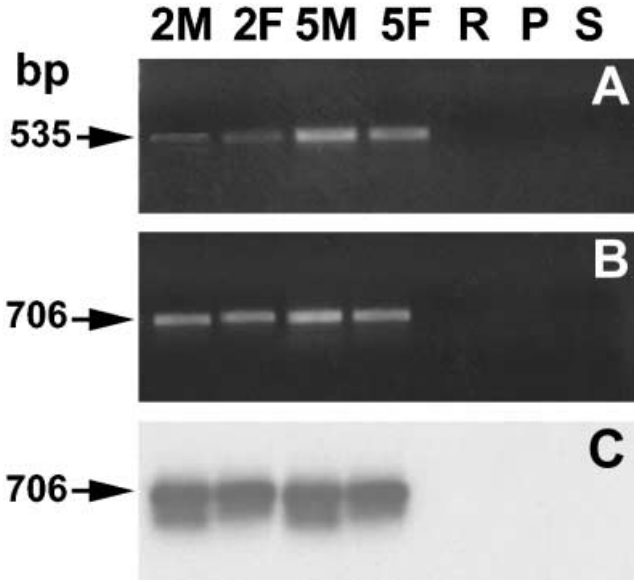


Fig. 2A–C RTPCR analysis on mRNA prepared from flowers, roots, shoots, and phylloclades. **A** Amplification products obtained with primers P2 and P3 designed to obtain the region downstream of the MADS box. **B** Amplification products obtained with primers P1 and P3 designed to obtain the complete cDNA. **C** Southern blot analysis of the amplification products shown in 3B. *2M* and *5M* Male flowers at stages 2 and 5; *2F* and *5F* female flowers at stages 2 and 5; *R* root; *P* phylloclades; *S* shoot

to obtain amplification products which include the complete cDNA (primers P1 and P3, see Materials and methods) or the region downstream the MADS box (primers P2 and P3). In both assays an amplification product was present only in flower samples (Fig. 2A,B). The specificity of the product and its absence in root, shoot and phylloclade were confirmed by Southern blot analysis of the amplification products (Fig. 2C). These results indicate that *AOM1* is expressed in flower but not in vegetative tissues.

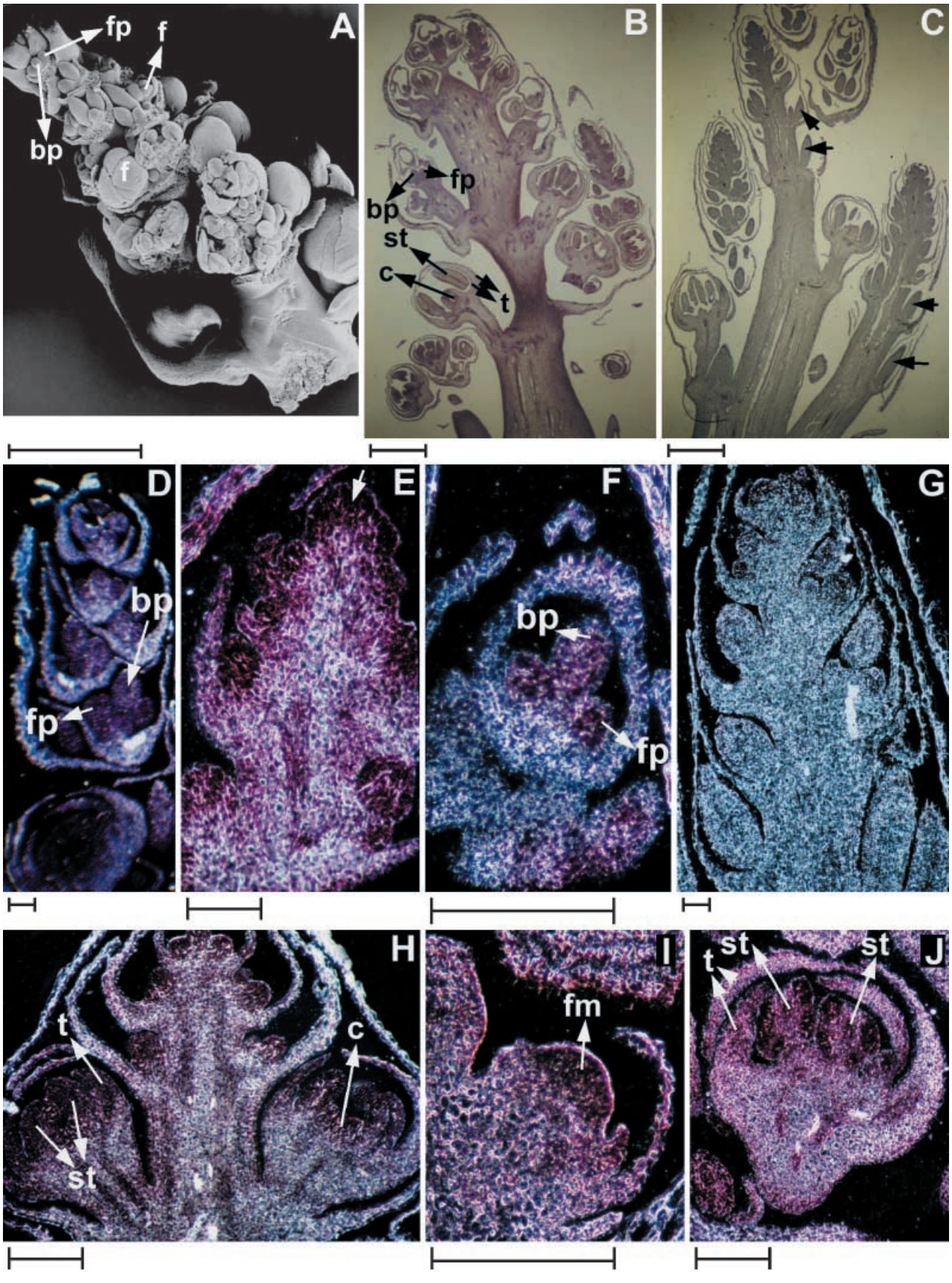
To determine the temporal and spatial pattern of *AOM1* expression in male and female flowers, the accumulation of *AOM1* mRNA was analyzed by in situ hybridization.

As previously described (Caporali et al. 1994), the shoot apex of asparagus is a complex structure which, during the flowering season, produces the primordia of leaves, branches and flowers. Leaf primordia develop as scales and, inside each scale, in axillary position, a row of three primordia arises. The two lateral primordia develop as flowers, while the central primordium gives rise to a lateral branch which will produce other flowers and branches. At the end of the flowering season the lateral primordia of the internal row develop as phylloclades. The picture obtained by morphological analysis indicates that the asparagus shoot apex behaves as a reproductive apex, producing flowers and lateral inflorescences during the flowering season, and as a vegetative apex, producing phylloclades and branches after the flowering season. The structure of the reproductive shoot apex as it appears at SEM and at light microscope in longitudinal section is shown in Fig. 3A,B. In both pictures flower and branch primordia and several developing flowers are visible. A light microscope picture of a longitudinal section of an apex taken at the end of the flowering season is shown in Fig. 3C. Two developing flowers are still present, but the primordia which arise inside the scales are elongating and will develop as phylloclades. An asparagus flower has six tepals arranged in two whorls and, during the hermaphrodite stages of development, six stamens and a tricarpellate pistil. After the transition to unisexual developmental stages, female flower stamens collapse. The first target of stamen degeneration is the tapetum. Tapetum cells are very short-lived and their death is followed by degeneration of the sporogenous tissue and the external tissues. In male flowers the style does not develop and the ovary stops growing, but remains viable. Development of ovules is arrested at macrosporogenesis (Caporali et al. 1994).

In situ hybridization analysis has been performed on longitudinal sections of shoot apexes collected in the middle and at the end of the flowering season (reproductive and vegetative apexes, respectively), and on isolated flowers. In the reproductive shoot apex, *AOM1* mRNA is detected in the apical dome, in the primordia of flowers and branches and in developing flowers. In the apical dome and in the two types of primordia, *AOM1* is uniformly expressed. In the developing lateral branches, the hybridization signal follows the same pattern (Fig. 3D–F). When the apex switches from reproductive to vegetative phase, the hybridization signal disappears from both the apical meristem and the primordia at the axils of leaves, which at this moment will develop as phylloclades or lateral branches (Fig. 3G). During the first stages of flower development the expression pattern of *AOM1* is identical in male and female flowers. *AOM1* is uniformly expressed in the floral meristem and throughout the primordia of all organs (Fig. 3H–J). Later, the hybridization signal disappears from tepals, while in stamens and pistils it becomes restricted to specific cell types: sporogenous tissue and tapetum in anthers, and placenta and developing ovules in the ovary (Fig. 4A–C). After the transition to the unisexual developmental pathway, in male flowers *AOM1* is expressed in tapetum cells and also in ovules, even after the arrest of ovary development (Fig. 4D). In female flowers *AOM1* is expressed at a high level throughout the developing ovule, while the hybridization signal rapidly disappears from the degenerating anthers. In more mature flowers, when macrosporogenesis occurs the hybridization signal disappears from ovules and ovary in both male and female flowers.

Conclusions

We report the isolation and characterization of a MADS box gene from *Asparagus officinalis*, *AOM1*. Like other plant members of this family of genes, *AOM1* encodes a putative transcription factor with a DNA binding domain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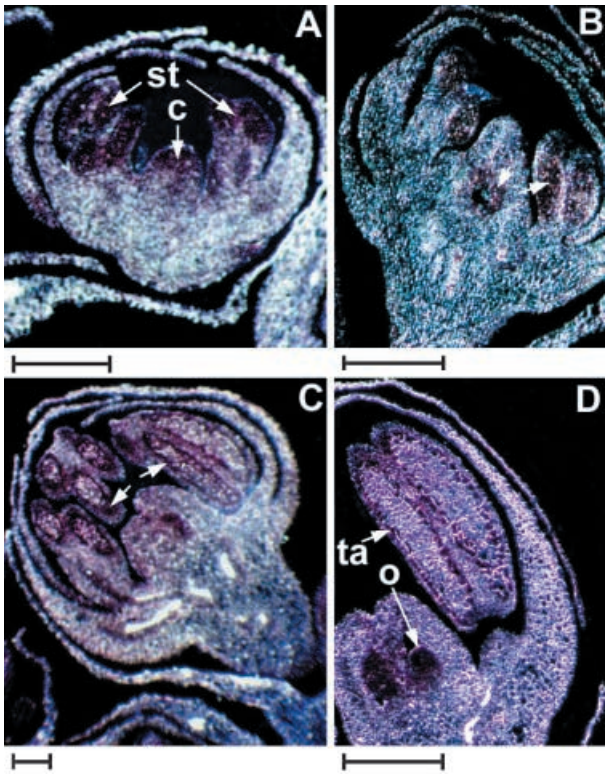

Fig. 4A–D Dark-field micrograph of longitudinal sections from developing flowers showing in situ hybridization with *AOM1*-specific antisense mRNA probe. **A,B** Female flowers at hemaphroditic stage; **C,D** male flowers after the developmental switch. During flower development the hybridization signal disppears from tepals (**A**), and becomes restricted to sporogenous tissue in stamens and to placenta and ovule in the ovary (**B**, *arrows*). In male flowers, at microsporogenesis the hybridization signal becomes restricted to tapetum and ovule (**C,D**). *st* Stamen; *c* carpel; *o* ovule; *ta* tapetum. *Bars* 200 µm

and a K domain, which is thought to be involved in protein-protein interactions (Ma et al. 1991). *AOM1* shows a high degree of similarity with *FBP2* of *Petunia hybrida*, and *AGL9*, *AGL2* and *AGL4* of *Arabidopsis thaliana*.

These four genes are expressed in the inner three whorls of the developing flower (Angenent et al. 1992; Mandel and Yanofsky 1998; Flanagan and Ma 1994; Savidge et al. 1995). The expression of *AGL2*, *AGL4* and *AGL9*, which were recently renamed *SEP1* (*SEPALLATA*), *SEP2* and *SEP3*, is required for B and C floral organ identity function (Pelaz et al. 2000). The expression pattern of *AOM1* during flower development is very similar to that of *FBP2* and the three *SEP* genes; this finding and the sequence similarity indicate that *AOM1* could be an orthologue of these genes. *AOM1*, however, is also expressed in the early flower meristem and in the apical meristem of reproductive shoots and branches. This indicates a possible function of *AOM1* not only in flower development but also in earlier stages of the flowering process. The function analysis of the rice MADS box gene *OsMADS5*, which is related to the *AGL2* family and is expressed in anther and carpel, indicates that it could also be involved in controlling flowering time (Kang and An 1997). *AOM1* could have a similar function.

AOM1 does not seem to be involved in asparagus sex differentiation. It is expressed very early during the process of flowering, and it is equally expressed in male and female flowers during the hermaphrodite developmental stages. After the switch to the unisexual stages, in female flowers it desappears from anthers following their degeneration, but in male flowers it continues to be expressed in ovules, including after the arrest of ovary growth.

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◀ **Fig. 3A–J** *AOM1* expression in asparagus shoot apex and flowers. **A** SEM micrograph of a reproductive apex with flower and branch primordia and developing flowers. **B** Bright-field photograph of a longitudinal section of a reproductive apex with developing branches and flowers. **C** Bright-field photograph of a longitudinal section of a vegetative apex with elongated primordia developing as phylloclades (*arrows*). **D–J** Dark-field micrograph of longitudinal sections of apexes showing in situ hybridization with *AOM1*-specific antisense mRNA probe. **D,E,F** Details of a reproductive apex. The hybridization signal is present in the apical dome (**E**, *arrow*), and in the primordia of flower and branches (**D**, **E**). **G** Detail of a vegetative apex. The hybridization signal is not detectable. **H** Lateral branch and two flowers derived from the axillary primordia. **I** Detail of a flower meristem. The hybridization signal is visible throughout the flower meristem and the primordia of all flower organs. **J** A more mature flower. The hybridization signal is desappearing from tepals. *fp* Flower primordium; *bp* branch primordium: *f* flower; *t* tepal; *st* stamen; *c* carpel; *fm* flower meristem. *Bars*: **A–C**=1 mm; **D–J**=200 µm

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