Original Paper Plant Biotechnology, **17** (2), 137–144 (2000)

Isolation and Characterization of a MADS-box Gene cDNA, *PnMADS1*, That is Expressed in Both Vegetative and Floral Meristems of *Pharbitis nil*

Michiyuki ONO*[1)], Kimiyo SAGE-ONO**, Wakako MARUYAMA**, Michihiro KAWAKAMI**, Masayasu INOUE* and Hiroshi KAMADA**

**Biotechnology Institute, Akita Prefectural University, Ohgata, Akita, 010-0444 Japan*
***Institute of Biological Sciences, University of Tsukuba, Tsukuba, Ibaraki, 305-8572 Japan*
[1)] *Corresponding author* E–mail address: ono@agri.akita-pu.ac.jp

Received 28 January 2000; accepted 10 February 2000

Abstract

A novel cDNA corresponding to a gene designated *PnMADS1* and encoding a MADS-box protein was isolated from the short-day plant *Pharbitis nil* (Japanese morning glory) cv. Violet. Phylogenetic analysis of the amino acid sequence encoded by *PnMADS1* indicated that PnMADS1 does not belong to any of the major sub-groups of MADS-box proteins. The protein exhibited some similarity to proteins in the so-called 'orphan group' of unclassified MADS-box proteins that are mainly expressed in vegetative organs. The most similar MADS-box genes were *AGL24* and *StMADS16* (53.2% and 52.3% identity at the deduced amino-acid level, respectively). We obtained evidence that *PnMADS1* mRNA accumulates in both vegetative and floral meristems but not in any other vegetative organs. Our results indicate that PnMADS1 is a novel MADS-box protein in terms of both structure and pattern of expression.

Key words: Floral induction - Flower development - MADS box - *Pharbitis nil* - Vegetative development
Footnote: The nucleotide sequence reported in this paper has been submitted to the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under accession number D84512.

1. Introduction

Numerous MADS-box genes have been found in a wide variety of eukaryotes, from yeast to plants and man (Shore and Sharrocks, 1995; Hasebe *et al.*, 1988). Each MADS-box protein contains a DNA-binding domain, known as a MADS box, which consists of 57 strongly conserved amino acids (Schwarz-Sommer *et al.*, 1990; Ma *et al.*, 1991). MADS-box proteins play key roles as transcription factors. In plants, the control of flower development by floral homeotic genes, which include MADS-box genes, has been studied extensively in *Arabidopsis* and *Antirrhinum* (Coen and Meyerowitz, 1991; Yanofsky, 1995). Several MADS-box genes have been isolated from various plant species and characterized, and some of them have been shown to be expressed during vegetative development (Ma *et al.*, 1991; Pnueli *et al.*, 1991; Mandel *et al.*, 1994; Heard and Dunn, 1995; Rounsley *et al.*, 1995; Zachgo *et al.*, 1997).

Pharbitis nil cv. Violet is a short-day plant that has been used as a model plant for studies of photoperiodic induction of flowering (Imamura, 1967; Vince-Prue and Gressel, 1985). Seedlings of *P. nil* cv. Violet grown under continuous light (*i.e.*, under ideal non-inductive conditions) are induced to flower by a single 16-h exposure to continuous darkness. Thus, almost absolute states of vegetative growth and reproductive growth can easily be established (Imamura, 1967; Vince-Prue and Gressel, 1985). We postulated that this feature would be useful for studies of MADS-box genes that are expressed in apical meristems during vegetative growth, as well as during reproductive growth. Analysis of such genes should provide fundamental information about the induction and development of flowers.

In this study, we isolated a novel MADS-box gene from *Pharbitis*, which is expressed in vegetative and reproductive apical meristems. This gene, *PnMADS1*, seems to be unique in terms both of amino acid sequence and pattern of expression.

2. Materials and Methods

2.1 Plant materials

Seeds of *Pharbitis nil* Choisy cv. Violet (obtained from Marutane Co., Kyoto, Japan) were soaked in concentrated sulfuric acid for 30 min, with occasional stirring, and then rinsed in running tap water for 1 h. The seeds were then soaked in a large volume of distilled water for 16 h and sown on wet vermiculite for germination. Germination and growth of seedlings were allowed to proceed at 25 ± 1℃ with continuous illumination from cool-white fluorescent lamps (FLR80H-WA; Matsushita Electronics Co., Tokyo, Japan; 13.6 W m^{-2}) in cultivation chambers (CU-350; Tomy Seiko Co., Tokyo, Japan). When the cotyledons had extended maximally (6 days after treatment with sulfuric acid), photoperiodic treatments were applied (Sage-Ono *et al.*, 1998). The seedlings were divided into two groups and subjected to SD treatment (16 h of continuous darkness, namely, flower-inductive conditions) or to LD treatment (continuous light, namely, non-inductive conditions). Apical buds, including flower buds, young leaves and stems, were excised 2, 3, 4, 5, 7 and 10 days after induction of flowers, frozen in liquid nitrogen and stored at -80℃.

2.2 Construction and screening of a cDNA library

Total RNA was isolated from each sample as described by Ozeki *et al.*, (1990). Poly(A^+) RNA was isolated by chromatography on oligo(dT)-cellulose (Amersham-Pharmacia, Tokyo), as described originally by Aviv and Leder (1972). A cDNA library was constructed from the poly(A^+) RNA of apical buds of *P. nil*, 2, 3, 4, 5, 7 and 10 days after induction of flowering, using commercial kits for synthesis and cloning of cDNA (Amersham-Pharmacia) in accordance with the instructions from the manufacturer. Screening of plaques and preparation of phage DNA were also performed according to the instructions from Amersham-Pharmacia. The molecular probe used for screening the cDNA library was a ^{32}P-labeled fragment of DNA encoding a MADS-box domain. For preparation of the probe, two oligonucleotide primers were synthesized and used for amplification by PCR of the DNA fragment that encoded the MADS-box domain of the *AGAMOUS* (*AG*) gene of *Arabidopsis* (Yanofsky *et al.*, 1990). One was identical to nucleotides (nt) 1 through 27 and the other was complementary to nt 149 through 175 of the published sequence of *AG* cDNA (Yanofsky *et al.*, 1990). A genomic clone of *Arabidopsis* DNA containing the *AG* gene (cloned, sequenced and generously donated by Dr. J. Imamura, Plantech Research Institute, Yokohama, Japan) was used as template for amplification. During the screening of 200,000 recombinant phages in the *P. nil* library, we isolated several positive plaques. The insert DNAs were subcloned into the *Eco*RI site of pBluescript IIKS+ (Stratagene, La Jolla, CA, U.S.A.) and sequenced. One of the clones that we characterized was designated *PnMADS1* (for the gene for MADS1 of ***Pharbitis nil***). Because the cDNA lacked an initiation codon and the 5' non-coding sequence, the full-length cDNA was prepared with a 5'-RACE kit (Clontech Lab. Inc., Tokyo, Japan) using specific primers (Frohman *et al.*, 1998).

2.3 Sequencing and analysis of DNA

The nucleotide sequence of *PnMADS1* cDNA was determined with fluorescent primers and an automated DNA sequencer (model 373A; Applied Biosystems, Chiba, Japan). Nucleotide and amino acid sequences were analyzed with GENETYX-MAC software (Software Development Co. Ltd., Tokyo, Japan). Databases were searched with TFASTA 3.08, TFASTX 3.09 and TBLASTN 2.0.7 (DNA Data Bank of Japan, Mishima, Shizuoka, Japan; Altschul *et al.*, 1997; Pearson and Lipman, 1988). The phylogenetic tree was obtained with the neighbor-joining method (Saitou and Nei, 1987). The amino acid sequences of MADS-box proteins were aligned by using the Clustal X program (Thompson *et al.*, 1997) and then were revised manually. Bootstrap values were also calculated using the same program. The resulting phenogram was drawn with Niplot program (M. Gouy, Universite Claude Bernard-Lyon 1, Lyon, France). Nodes with < 50% bootstrap support were collapsed as described previously by Purugganan *et al.*, (1995).

2.4 Southern hybridization

We prepared a DNA fragment specific for *PnMADS1* as a molecular probe. To avoid cross-hybridization with other MADS-box genes, digestion at the *Pst*I site located 279 bp from the 5' end of *PnMADS1* cDNA was performed to remove the MADS-coding sequence. The rest of the cDNA (the 630-bp *Pst*I fragment) was used for all hybridization experiments. Genomic DNA was isolated from apical buds with small leaves of *P. nil* as described by Rogers and Bendich, (1985). It was digested separately with *Eco*RI, *Bam*HI, *Hin*dIII and *Xho*I. Digested DNA was fractionated by electrophoresis on an agarose gel and bands of DNA were transferred to a nylon membrane filter (Biodyne B; Nihon Pall, Ltd., Tokyo, Japan). The DNA on the

filter was allowed to hybridize with the ^{32}P-labeled 630-bp *Pst*I fragment of *PnMADS1* cDNA in hybridization buffer that contained 6x SSPE (1x SSPE is 0.18 M NaCl, 0.01 M sodium phosphate, and 1 mM Na_2EDTA, pH 7.7), 5x Denhardt's solution (1x Denhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin) plus 0.5% SDS and 150 μg ml^{-1} salmon sperm DNA at 65 ℃ for 16 h. The filter was washed twice with 2x SSPE plus 0.1% SDS for 5 min at room temperature and then twice with 0.1% SSPE plus 0.1% SDS for 30 min at 65 ℃ (high-stringency conditions) (Sage-Ono *et al.*, 1998). For visualization of bands on the filter, we used a bioimaging analyzer with an imaging plate (BAS2000; Fuji Photo Film Co., Ltd., Tokyo, Japan).

2.5 Northern hybridization

Total RNA (20 μg) was fractionated by electrophoresis on a formaldehyde-agarose gel and then bands of RNA were transferred to a nylon membrane filter (Biodyne B). The RNA on the filter was allowed to hybridize with the ^{32}P-labeled 630-bp PstI fragment of *PnMADS1* cDNA. The conditions for hybridization, washing and analysis were as described previously by Sage-Ono *et al.*, (1998).

2.6 Reverse transcription-PCR

Total RNA was prepared from various organs as described above and was then treated with RNase-free DNaseI (Promega) to eliminate contaminating fragments of genomic DNA. Purified total RNA (1 μg) was reverse transcribed with an oligo(dT) primer. The reaction mixture for reverse transcription (total volume 20 μl) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 2.5 μM oligo $(dT)_{36}$ primer, 1 mM dNTPs and 20 U of RNase inhibitor (Takara, Tokyo), and reactions were performed as follows. The mixture was heated at 65 ℃ for 5 min and then at 37 ℃ for 10 min, after which 5 U of AMV reverse transcriptase XL (Life Sciences Inc., St. Petersburg, FL, U.S.A.) were added. The mixture was heated at 42 ℃ for 60 min and at 95 ℃ for 5 min, and then it was stored at -20 ℃ prior to PCR. One or two microliters of this mixture were used as the source of template for PCR in a reaction mixture that contained the *PnMADS1*-specific primers 5'-CAGCAGTTGGAG-AGGTC-3' and 5'-AACTTCATAATAATCTTG-TG-3'. The solution for PCR (total volume 50 μl) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (v/v) gelatin, 0.2 μM *PnMADS1*-specific primers, 200 μM dNTPs, and 1.25 U of AmpliTaq™ DNA polymerase (Perkin Elmer Cetus, Norwalk, CT, U.S.A.). PCR involved 30 to 40 cycles of incubation at 94℃ for 1 min, 54℃ for 1 min, and 72 ℃ for 2 min. Aliquots of duplicate reaction mixtures after PCR were subjected to electrophoresis on a 5% polyacrylamide gel to separate amplified cDNAs. Then cDNAs were blotted onto a nylon membrane filter, allowed to hybridize with a ^{32}P-labeled specific probe and visualized by exposure to a X-ray film. The expected length of the product of PCR was 285 bp and the products corresponded to 410 to 695 bp of *PnMADS1* cDNA. To confirm the quality of the original samples of RNA and to serve as a positive control, the same cDNA templates were used for PCR with primers for the amplification of actin cDNA, namely, 5'-TCCATAATGAAGTGTGATGT-3' and 5'-GGACCTGACTCGTCATACTC-3'. The expected length of the amplified fragment of actin cDNA was 259 bp and the products corresponded to *PnACT1* (accession number in DDBJ, D78204), *PnACT3-1* (D78205), and *PnACT3-3* (D78206). To avoid saturation conditions, we determined the appropriate number of cycles for each PCR.

2.7 In situ hybridization

Apical buds of 5 mm in length were fixed in a solution of 10% formaldehyde, 5% acetic acid and 50% ethanol, embedded in wax (Paraplast plus; Oxford Labware, St. Louis, MO, U.S.A.), and sectioned. The 630-bp *Pst*I fragment of *PnMADS1* cDNA was subcloned into pBluescript IIKS+ to provide templates for T7 polymerase and T3 polymerase for the synthesis of sense and antisense RNA probes. Probes for *in situ* hybridization were labeled with digoxigenin-11-rUTP with a nucleic acid-labeling kit from Boehringer Mannheim Biochemica (Mannheim, Germany). *In situ* hybridization and immunological detection of the hybridized probe were performed as described in the instruction from Boehringer Mannheim Biochemica.

3. Results

3.1 Nucleotide sequence of PnMADS1 cDNA and the deduced amino acid sequence

PnMADS1 cDNA was 906 bp long and contained a 705-bp open reading frame that encoded a putative protein of 234 amino acids, as well as a 34-bp 5' non-coding region and a 167-bp 3' non-coding region. The cDNA appeared to be nearly full-length because it included a region that encoded a complete MADS-box domain, with a conserved initiation codon and a polyadenylated tail. The deduced amino acid sequence was typical of MADS-box proteins from plants. The MADS-box domain was followed by an intermediate domain, a keratin-

like domain, and a carboxy-terminal domain (**Fig. 1**; Ma *et al.*, 1991).

3.2 Similarity to other proteins at the amino acid level

The amino acid sequence deduced from *PnMADS1* cDNA was used in a search for homologous proteins as described in Materials and Methods. Homology scores were relatively low but the results of all searches were consistent (data not shown). Similar genes, listed in descending order of similarity, were *AGL24, StMADS16, StMADS11, AGL17* and *AGL15*. The amino acid sequence of PnMADS1 exhibits 53.2%, 52.3%, 46.4%, 32.5% and 31.4% identical to AGL24, StMADS16, StMADS11, AGL17 and AGL15, respectively. *StMADS11* and *StMADS16* are MADS-box genes for vegetative proteins of potato that show some similarity to *AGL15* and *AGL17* (Carmona *et al.*, 1998). *AGL15* and *AGL17* are members of the so-called 'orphan' group of MADS-box genes that do not belong in the well-characterized major family of MADS-box genes (Purugganan *et al.*, 1995; Rounsley *et al.*, 1995, Perry *et al.*, 1996). Results of a phylogenetic analysis of amino acid sequences are presented in **Fig. 2**. The phylogenetic analysis by the neighbor-joining method indicated that PnMADS1 was distinct from other MADS-box proteins. We aligned the deduced amino acid sequence of PnMADS1 with those of AGL24, StMADS16, and StMADS11. As shown in **Fig. 1**, the MADS-box domain of PnMADS1 was somewhat different from those of the other somewhat similar proteins.

3.3 Results of Southern and Northern analysis

To determine the genomic organization of the *PnMADS1* gene, we performed Southern hybridization using genomic DNA from *P. nil* that had been digested with *Eco*RI, *Bam*HI, *Hin*dIII and *Xho*I. The probe that we used was part of the cDNA, namely, a 630-bp *Pst*I fragment that excluded the coding sequence of the strongly conserved MADS domain. Under stringent conditions, single hybridization bands were obtained (**Fig. 3**). Our results indicated that there was only one copy of the *PnMADS1* gene in the genome of *P. nil* and confirmed that the probe was highly specific to the *PnMADS1* gene. To characterize the temporal and organ-specific expression of the *PnMADS1* gene, we performed

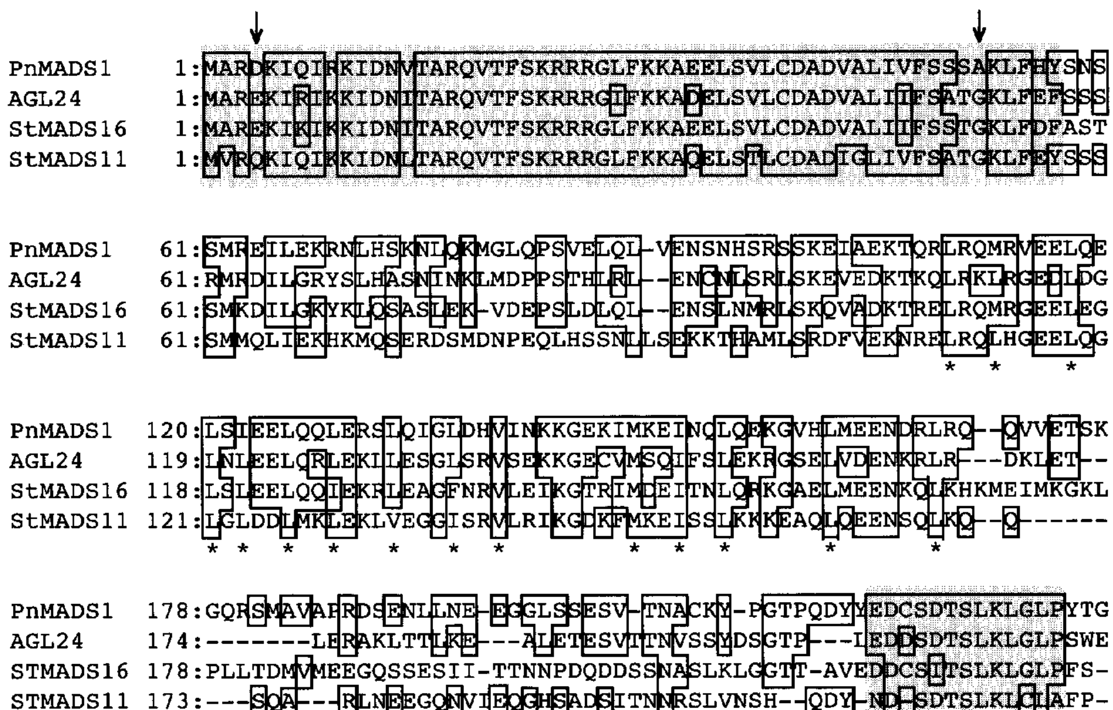

Fig. 1 Alignment of the amino acid sequence deduced from *PnMADS1* cDNA with those of similar proteins. The amino acid sequences are given in the one-letter code and have been aligned, with the introduction of gaps (---), to maximize possible homology. Amino acids that are identical in PnMADS1 and other proteins are boxed. The MADS-box domain (residues 1 to 57) and the carboxy-terminal conserved motif are enclosed in shaded boxes. Asterisks indicate common hydrophobic amino acids (L, I, V, and M) in the region of the keratin-like domain. Asp at position 4 and Ala at position 52 are indicated by vertical arrows.

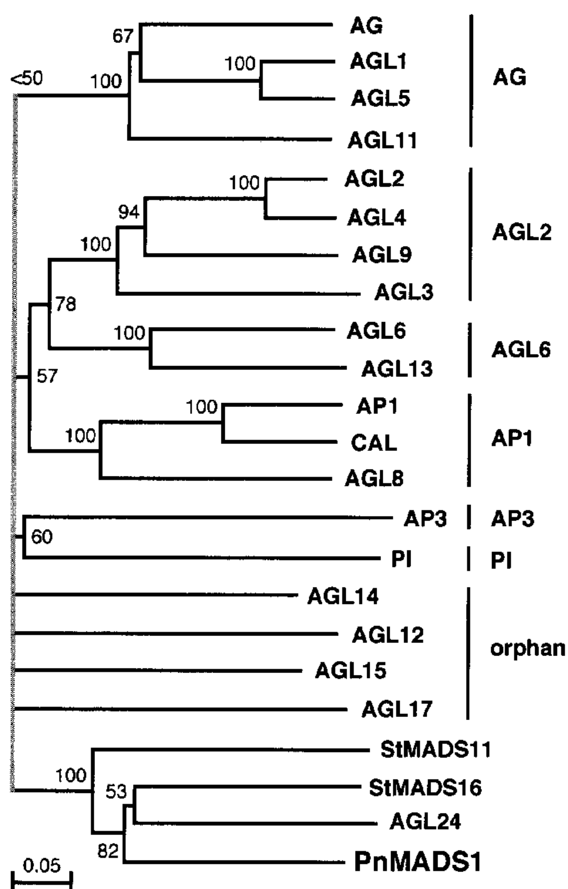

Fig. 2 A phylogenetic tree of plant MADS-box proteins based on the neighbor-joining method (Saito and Nei, 1987). PnMADS1, StMADS11, StMADS16 and characterized MADS-box proteins from *Arabidopsis* were presented. The horizontal branch length is proportional to the estimated number of amino acids substitutions per residue. (Bar=0.05 aa substitution per residue). Numbers at the branch points represent the bootstrap values for percentage of 1,000 replicate trees. This is an unrooted tree. Nodes with < 50% bootstrap support are collapsed. Sources of the amino acid sequences are as follows: AG (Genbank accession number P17839), AGL1 (P29381), AGL5 (P29385), AGL11 (AAC49080), AGL2 (P29382), AGL4 (P29384), AGL9 (O22456), AGL3 (P29383), AGL6 (P29386), AGL13 (AAC49081), AP1 (P35631), CAL (AAA64789), AGL8 (Q38876), AP3 (P35632), PI (P48007), AGL14 (CAB44326), AGL12 (AAC49085), AGL15 (Q38847), AGL17 (AAD15571), StMADS11 (AF008652), StMADS16 (AF008651) and AGL24 (AAC63139). The different gene groups are indicated on the right as described (Purugganan *et al.*, 1995, Winter *et al.*, 1999).

Northern blotting using total RNA extracted from apical buds and several vegetative organs of *P. nil*. Most samples of RNA did not yield any positive

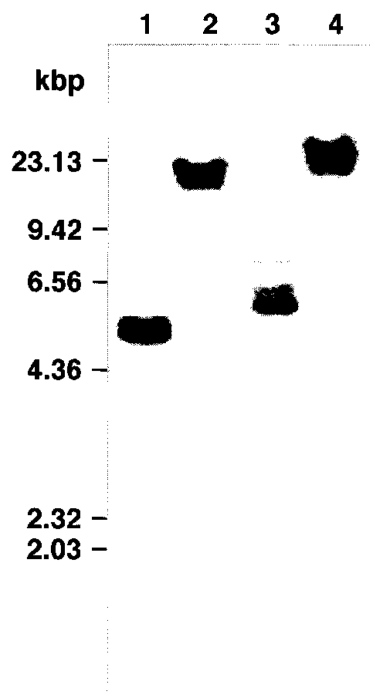

Fig. 3 Southern hybridization. Genomic DNA was prepared from *Pharbitis nil* cv. Violet. It was digested with *Eco*RI (lane 1), *Bam*HI (lane 2), *Hin*dIII (lane 3) and *Xho*I (lane 4) and subjected to Southern hybridization. Results after washing under high-stringency conditions are shown. The probe used was the ^{32}P-labeled 630-bp *Pst*I fragment of *PnMADS1* cDNA. The mobilities of marker fragments are shown on the left in kilobase pairs (kbp).

signals (data not shown). However, weak signals were detected in the case of apical buds harvested 7 days and 10 days after short-day treatment (data not shown). The mRNAs were about 1.0 kb long, as estimated from the mobilities of rRNAs, which were used as size markers.

3.4 Temporal and spatial expression of PnMADS1 mRNA

Northern hybridization yielded only weak signals. Therefore, we applied a more sensitive method, namely, RT-PCR and detected *PnMADS1* mRNA in apical buds at all stages of development (**Fig. 4**). The RNA isolated from other vegetative organs, namely, roots, stems, petioles and cotyledons, did not yield any signals. By contrast, positive controls with actin-specific primers consistently gave positive results. We also examined the spatial expression of *PnMADS1* mRNA within apical buds at different developmental stages by *in situ* hybridization (**Fig. 5**). At the early stage of development of flower buds (3 days after floral induction), *PnMADS1* was expressed in meristematic domains (**Fig. 5A**). This pattern of expression was indis-

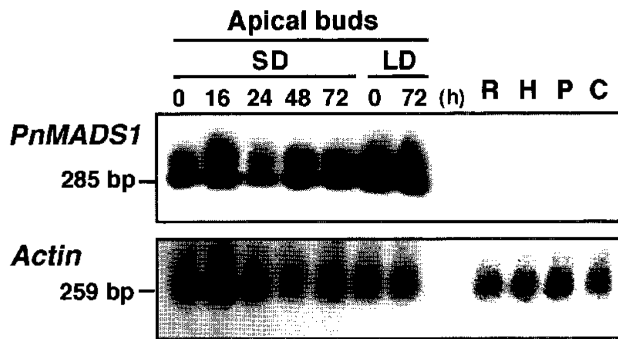

Fig. 4 Detection of *PnMADS1* mRNA by RT-PCR. Total RNA isolated from apical buds, roots (R), hypocotyls (H), petioles (P) and cotyledons (C) was analyzed for the presence of *PnMADS1* mRNA by RT-PCR. Apical buds were harvested at the times indicated. Primers for RT-PCR were specific for *PnMADS1* and a gene for actin (as an internal control). Products of RT-PCR were separated on a polyacrylamide gel, blotted onto a nylon membrane filter, allowed to hybridize with ^{32}P-labeled specific probes, and exposed to X-ray film for visualization. The lengths of products of PCR specific for *PnMADS1* and actin were 285 bp and 259 bp, respectively.

tinguishable from that in vegetative meristems (data not shown). In the floral meristems collected 7 days and 9 days after floral induction, we observed the preferential accumulation of *PnMADS1* mRNA in petals, stamens and ovule primordia but not in sepal primordia (Figs. 5C and 5E). However, we detected no hybridization signals in floral buds collected 14 days after floral induction (**Fig. 5F**). No significant signal can be detected with the sense RNA (**Fig. 5B** and **5D**).

4. Discussion

We isolated the cDNA for a MADS-box gene, *PnMADS1*, from a cDNA library constructed from apical buds of *Pharbitis nil* Choisy, cv. Violet after the induction of flowering. The amino acid sequence deduced from *PnMADS1* did not correspond to that of any of the major and well-characterized MADS-box proteins. The most similar MADS-box gene was *AGL24*, which exhibited 53.2% identity at the amino acid level. Such homology was lower than that between most MADS-box genes in any specific sub-family, which is usually more than 60% or 70%. MADS-box proteins consist of several domains, namely, a MADS-box domain, an intermediate domain, a keratin-like domain and a carboxy-terminal domain (Ma *et al.*, 1991, Purugganan *et al.*, 1995). These domains in PnMADS1 were distinct, as shown in **Fig. 1**. The

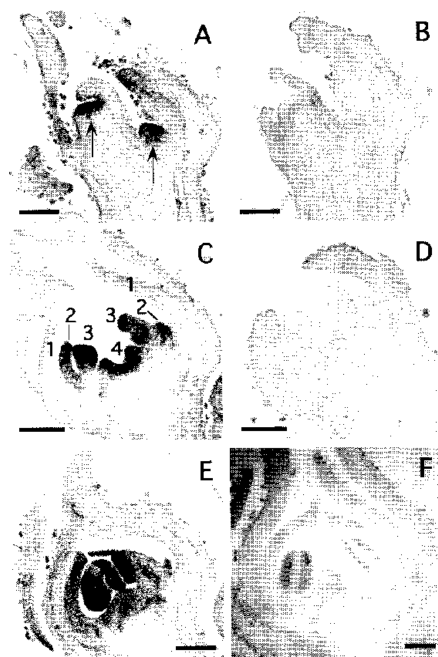

Fig. 5 *In situ* hybridization of *PnMADS1* mRNA. Longitudinal sections of apical buds of *Pharbitis* were allowed to hybridize with digoxigenin-labeled antisense RNA (A, C, E, F) and sense RNA (B, D) derived from a *PnMADS1*-specific fragment of cDNA that excluded the coding sequence of the MADS-box domain. Apical buds were collected 3 days (A, B), 7 days (C, D), 9 days (E) and 14 days (F) after the short-day induction of flowering. Bars indicate 50 μm. Arrows in panel A indicate the meristematic domains. Numerals in panel C refer to the whorls that developed into sepals (1), petals (2), stamens (3) and carpels (4).

most significant differences were an Asp residue at position 4 and an Ala residue at position 52 in the MADS-box domain. MADS-box domains in the databases rarely contain these residues at these positions. The sequences of MADS-box domains within a single sub-family are usually much more homologous to each other. It appears that *PnMADS1* might be related to *AGL24*, *StMADS16*, and *StMADS11* but it is clearly not a member of the same sub-family. This conclusion is supported by the results of studies of gene expression. However, there was a common motif in the carboxy-terminal sequence (**Fig. 1**). This motif is weakly conserved among members of the 'orphan group', namely, *AGL15* and *AGL17* (Carmona *et al.*, 1998). The *PnMADS1* gene appears to be the gene for a novel MADS-box protein that is similar to members of the orphan group of MADS-domain proteins (Purugganan *et al.*, 1995).

We studied the temporal and spatial patterns of expression of the transcript of *PnMADS1*. Since only faint signals corresponding to *PnMADS1*

mRNA were obtained by Northern hybridization, we used RT-PCR and *in situ* hybridization to detect transcripts. Expression of *PnMADS1* was restricted to apical meristems during both vegetative and reproductive growth. Expression of *PnMADS1* was detected in meristematic domains fated to form the inner three whorls, namely, petals, stamens, and ovary, of the flower **(Fig. 5)**. The pattern of expression of *PnMADS1* at the late stage of flower development is similar to that of *TM5*, a MADS-box gene in tomato (Pnueli *et al.*, 1991) and to that of *FBP2* of petunia (Angenent *et al.*, 1992), in spite of the very low degree of homology at the amino acid level. However, *TM5* and *FBP2* are not expressed in indeterminate vegetative shoot meristems (Pnueli *et al.*, 1991; Angenent *et al.*, 1992). By contrast, expression of *TobMADS1*, a ubiquitously expressed MADS-box gene in tobacco is detectable in both floral and vegetative organs of the tobacco plant (Mandel *et al.*, 1994). Similar results have been reported for *AGL3* of *Arabidopsis* (Huang *et al.*, 1995) and *TM3* of tomato (Pnueli *et al.*, 1991). However, unlike *PnMADS1*, *TobMADS1*, *AGL3* and *TM3* mRNAs are detectable in all organs of the respective plants. *StMADS16* and *StMADS11* are structurally similar to *PnMADS1* but their patterns of expression are completely different. *StMADS16* and *StMADS11* are expressed in all vegetative organs of potato plants, but not in floral tissues. There are no reported data on the expression of *AGL24*, to our knowledge. Thus, *PnMADS1* might be a novel MADS-box gene that is expressed specifically in shoot meristems and that contributes to the developmental program in both vegetative and reproductive organogenesis.

Acknowledgements

The authors thank Dr. J. Imamura for the gift of a genomic clone of *AG*, Dr. M. Hasebe for initial phylogenetic analysis and Dr. K. Nakahara for the phylogenetic analysis. This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas (no. 07281101; Genetic Dissection of Sexual Differentiation and Pollination Processes in Higher Plants) from the Ministry of Education, Science, Culture and Sports, Japan, and by a Grant-in-Aid for the "Research for the Future" Program (JSPS-RFTF97L00601) from the Japan Society for the Promotion of Science.

References

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res., **25:** 3389-3402.

Angenent, G.C., Busscher, M., Franken, J., Mol, J.N.M., Van Tunen, A.J., 1992. Differential expression of two MADS-box genes in wild-type and mutant petunia flowers. Plant Cell., **4:** 983-993.

Aviv, H., Leder, P., 1972. Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid cellulose. Proc. Natl. Acad. Sci. USA, **69:** 1408-1412.

Carmona, M.J., Ortega, N., Garcia-Maroto, F., 1998. Isolation and molecular characterization of a new vegetative MADS-box gene from *Solanum tuberosum* L. Planta, **207:** 181-188.

Coen, E.S., Meyerowitz, E.M., 1991. The war of the whorls: Genetic interactions controlling flower development. Nature., **353:** 31-37.

Frohman, M.A., Duch, M.K., Matin, G.R., 1998. Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. Proc. Natl. Acad. Sci. USA, **85:** 8998-9002.

Hasebe, M., Wen, C.-K., Kato, M., Banks, J.A., 1998. Characterization of MADS homeotic genes in the fern *Ceratopteris richardii*. Proc. Natl. Acad. Sci. USA, **98:** 6222-6227.

Heard, J., Dunn, K., 1995. Symbiotic induction of a MADS-box gene during development of alfalfa root nodules. Proc. Natl. Acad. Sci. USA, **92:** 5273-5277.

Huang, H., Tudor, M., Weiss, C.A., Hu, Y., Ma, H., 1995. The *Arabidopsis* MADS-box gene *AGL3* is widely expressed and encodes a sequence-specific DNA-binding protein. Plant Mol. Biol., **28:** 549-567.

Imamura, S., 1967. Photoperiodic induction and the floral stimulus. In: Imamura, S. (Ed.): Physiology of Flowering in *Pharbitis nil*, 15-28. Japanese Society of Plant Physiologists, Tokyo.

Ma, H., Yanofsky, M.F., Meyerowitz, E.M., 1991. *AGL1-AGL6*, an *Arabidopsis* gene family with similarity to floral homeotic and transcription factor genes. Genes and Dev., **5:** 484-495.

Mandel, T., Lutziger, I., Kuhlemeier, C., 1994. A ubiquitously expressed MADS-box gene from *Nicotiana tabacum*. Plant Mol. Biol., **25:** 319-321.

Ozeki, Y., Matsui, K., Sakuta, M., Ohashi, Y., Kano-Murakami, Y., Yamamoto, N., Tanaka, Y., 1990. Differential regulation of phenylalanine ammonia-lyase genes during anthocyanin synthesis and by transfer effect in carrot cell suspension cultures. Physiol. Planta., **80:** 379-387.

Pearson, W.R., Lipman, D.J., 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA, **85:** 2444-2448.

Perry, S.E., Nichols, K.W., Fernandez, D.E., 1996. The MADS domain protein AGL15 localizes to the nucleus during early stages of seed development. Plant Cell., **8:** 1977-1989.

Pnueli, L., Abu-Abeid, M., Zamir, D., Nacken, W., Schwarz-Sommer, Z., Lifschitz, E., 1991. The MADS-box gene family in tomato: Temporal expression

during floral development, conserved secondary structures and homology with homeotic genes from *Antirrhinum* and *Arabidopsis*. Plant J., **1:** 255-266.

Purugganan, M.D., Rounsley, S.D., Schmidt, R.J., Yanofsky, M.F., 1995. Molecular evolution of flower development: Diversification of the plant MADS-box regulatory gene family. Genetics., **140:** 345-356.

Rogers, S.O., Bendich, A.J., 1985. Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. Plant Mol. Biol., **5:** 69-76.

Rounsley, S., Ditta, G.S., Yanofsky, M.F., 1995. Diverse roles for MADS-box genes in *Arabidopsis* development. Plant Cell., **7:** 1259-1269.

Sage-Ono, K., Ono, M., Harada, H., Kamada, H., 1998. Accumulation of a clock-regulated transcript during flower-inductive darkness in *Pharbitis nil*. Plant Physiol., **116:** 1479-1485.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol., **4:** 406-425.

Schwarz-Sommer, Z., Huijser, P., Nacken, W., Saedler, H., Sommer, H., 1990. Genetic control of flower development by homeotic genes in *Antirrhinum majus*. Science., **250:** 931-936.

Shore, P., Sharrocks, A.D., 1995. The MADS-box family of transcription factors. Eur. J. Biochem., **229:** 1-13.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The Clustal X windows interface: flexible strategies for multiple sequence alignment aides by quality analysis tools. Nucleic Acids Res., **24:** 4876-4882.

Vince-Prue, D., Gressel, J., 1985. *Pharbitis nil*. In: Halevy, A. (Ed.): Handbook of Flowering, 47-81. CRC Press, Boca Raton.

Winter, K.-U., Becker, A. Munster, T. Kim, J.T., Saedler, H., Theissen, G., 1999. MADS-box genes reveal that gnetophytes are more closely related to conifers than to flowering plants. Proc. Natl. Acad. Sci. USA, **96:** 7342-7347.

Yanofsky, M.F., 1995. Floral meristems to floral organs: Genes and controlling early events in *Arabidopsis* flower development. Annu. Rev. Plant Physiol. Plant Mol. Biol., **46:** 167-188.

Yanofsky, M.F., Ma, H., Bowman, J.L., Drews, G.N., Feldmann, K.A., Meyerowitz, E.M., 1990. The protein encoded by the *Arabidopsis* homeotic gene *agamous* resembles transcription factors. Nature., **346:** 35-39.

Zachgo, S., Saedler, H., Schwarz-Sommer, Z., 1997. Pollen-specific expression of *DEFH125*, a MADS-box transcription factor in *Antirrhinum* with unusual features. Plant Cell., **11:** 1043-1050.