Sex Plant Reprod (2006) 19: 133–144
DOI 10.1007/s00497-006-0030-8

ORIGINAL ARTICLE

**Akira Kanno · Homare Hienuki · Takuro Ito
Toru Nakamura · Tatsuya Fukuda · Pil-Yong Yun
In-Ja Song · Taichi Kamimura · Toshinori Ochiai
Jun Yokoyama · Masayuki Maki · Toshiaki Kameya**

The structure and expression of *SEPALLATA*-like genes in *Asparagus* species (Asparagaceae)

Received: 2 January 2006 / Accepted: 15 May 2006 / Published online: 14 June 2006

Abstract MADS-box genes encode transcriptional regulators that are critical for a number of developmental processes. In the MADS-box gene family, the *SEPALLATA* (*SEP*) gene subfamily plays an important role in controlling the development of floral organs in flowering plants. To understand the molecular mechanisms of floral development in *Asparagus*, we isolated and characterized several *SEP*-like genes from dioecious *Asparagus officinalis* and hermaphrodite *A. virgatus*: *AOMADS1*, *AOMADS2*, *AOMADS3*, and *AVMADS1*, *AVMADS2*, *AVMADS3*, respectively. Through alignment of the predicted amino acid sequences of various *SEP*-like genes, we defined three characteristic motifs in the C-terminal region of the genes: SEP motif I, SEP/AGL6 motif, and SEP motif II. Of the genes we isolated, AOMADS3 and AVMADS3 had lost the SEP motif II. Phylogenetic analysis revealed that *AOMADS1*, *AOMADS2*, *AVMADS1*, and *AVMADS2* were closely related to *SEP3* from *Arabidopsis*, whereas *AOMADS3* and *AVMADS3* were classified in different clade which is far related to *SEP3* gene. Northern hybridization and RT-PCR showed that three *SEP*-like genes in *A. officinalis* were specifically expressed in the flower buds. In addition, PCR RFLP showed that there was no significant difference in the amount of transcripts of *AOMADS1* and *AOMADS2*. These results suggest that *AOMADS1* and *AOMADS2* may be redundant genes. In contrast, the expression of *AOMADS3* was weaker than that of *AOMADS1* or *AOMADS2*, suggesting that the function of *AOMADS3* may be different than that of *AOMADS1* or *AOMADS2*.

Keywords *Asparagus officinalis* · *Asparagus virgatus* · Gene duplication · Phylogeny · *SEP*-like genes

Communicated by Scott Russell

Akira Kanno and Homare Hienuki contributed equally to this work.

A. Kanno (✉) · H. Hienuki · T. Ito · T. Nakamura
T. Fukuda · I.-J. Song · T. Kamimura · T. Kameya
Graduate School of Life Sciences, Tohoku University,
Katahira 2-1-1, Aoba-ku, Sendai 980-8577, Japan
E-mail: kanno@ige.tohoku.ac.jp
Tel.: +81-22-2175725
Fax: +81-22-2175725

P.-Y. Yun
Institute of Jeju Hi-Tech Industry Development 4-8,
Ara-1 dong, Jeju 690-121, Republic of Korea

T. Ochiai
Graduate School of Science, Hiroshima University,
Kagamiyama 1-4-3, Higashi, Hiroshima 739-8526, Japan

J. Yokoyama · M. Maki
Graduate School of Life Sciences, Tohoku University,
Aoba, Aramaki, Aoba-ku, Sendai 980-8578, Japan

Introduction

Genetic studies of *Arabidopsis* and *Anthirrinum* mutants have led to the ABC model of floral organ determination, in which the combinatorial action of three regulatory functions leads to organ flower formation (Coen and Meyerowitz 1991). Based on single-mutant, double-mutant, and triple-mutant analyses, the ABC model proposes three classes of combinatorial-acting floral organ identity genes, called A, B, and C. A functions specify sepals in the first floral whorl; A plus B specify petals in the second whorl; B plus C specify stamens in the third whorl; and C specifies carpels in the fourth whorl (Coen and Meyerowitz 1991). A few years after the ABC model was proposed, studies on ovule development in petunia led to the isolation and characterization of two MADS-box genes, *Floral Binding Protein 7* (*FBP7*) and *FBP11*, whose expression is turned on just before ovule primordia arise and is maintained in the seed coat after fertilization (Angenent et al. 1995; Colombo et al. 1995; Favaro et al. 2003; Ferrario et al. 2003). Moreover, ectopic expression and co-suppression of these genes in petunia and *Arabidopsis thaliana* indicated that their activity is necessary for determining ovule identity, an activity that has been proposed as a separate floral organ

identity function, the D-function gene (Angenent et al. 1995; Colombo et al. 1995; Favaro et al. 2003). As a result of these studies, the ABC model has become more complex, and now includes a new class of genes referred to as the E-function genes, *SEPALLATA1* (*SEP1*), *SEP2*, and *SEP3*, which are required for B and C floral organ identity functions (Egea-Cortines and Davis 2000; Pelaz et al. 2000; Honma and Goto 2001; Jack 2001).

In *Arabidopsis thaliana*, *SEP1*, *SEP2*, *SEP3*, and *SEP4* are required for specifying the identity of all four whorls of the floral organ, and for floral meristem determinacy (Honma and Goto 2000; Pelaz et al. 2000, 2001; Ditta et al. 2004). *SEP1*, *SEP2*, and *SEP4* are expressed throughout the floral meristem at stage 2, slightly earlier than *SEP3*, which is expressed in a region corresponding to the inner three whorls just before the initiation of floral organ primordia (Flanagan and Ma 1994; Savidge et al. 1995; Mandel and Yanofsky 1998; Ditta et al. 2004). Subsequently, *SEP1* and *SEP2* expressions persist in all floral organ primordial, and *SEP3* is expressed in the inner three whorls. In contrast, *SEP4* becomes more highly expressed in the central dome than in the sepals. Genetic studies of quadruple mutants indicated that the *SEP* genes are functionally redundant in the regulation of floral organ development and have redundant roles in promoting floral meristem determinacy (Pelaz et al. 2000; Ditta et al. 2004). In petunia, which is distantly related to *Arabidopsis*, SEP-like genes function similarly to those in *Arabidopsis*. Vandenbussche et al. (2003b) reported that *FBP2* and *FBP5* in petunia were required for B, C, and D floral organ identity functions, based on the analysis of *fbp2fbp5* double mutants. Moreover, two MADS-box genes in silver birch (*Betula pendula*: Betulaceae), *BpMADS1* and *BpMADS6* (Lemmetyinen et al. 2004), and two genes in apple (*Malus domestica*: Rosaceae), *MdMADS3* and *MdMADS4* (Sung et al. 2000), are phylogenetic and functional homologues to *SEP*-like genes. These results imply that these sets of genes have similar functions among these plants and perhaps function in more distantly related groups as well.

The monocotyledonous plants are a large group, but many of the molecular studies of MADS-box genes in monocots are carried out in the grass species, tulip (Kanno et al. 2003), *Commelina* and *Tradescantia* (Ochiai et al. 2004), *Phalaenopsis* (Tsai et al. 2004), *Agapanthus* (Nakamura et al. 2005), *Muscari* (Nakada et al. 2006), and *Dendrobium* (Skipper et al. 2006). Garden asparagus (*Asparagus officinalis* L.) is a dioecious monocot species belonging to the genus *Asparagus* (Asparagaceae), which is a large genus comprising 100–300 species. Because this genus contains hermaphrodite and dioecious species (Kanno et al. 1997; Ochiai et al. 2002; Fukuda et al. 2005), the genus *Asparagus* is an important and interesting model for studying sex differentiation and floral development. Several MADS-box genes have been isolated and characterized from *A. officinalis* (Caporali et al. 2000; Park et al. 2003, 2004; Losa et al. 2004; Ito et al. 2005) and from the ornamental species, *A. virgatus* (Yun et al. 2004a, b). On the basis of the expression analyses of isolates, Caporali et al. (2000) and Losa et al. (2004) reported the existence of *SEP*-like genes in *A. officinalis* (*AOM1* and *AOM4*), although an additional *SEP*-like gene isolated in this study was not well understood. Here, in order to clarify the function of *SEP*-like genes in the genus *Asparagus*, we isolated and characterized three *SEP*-like genes, *AOMADS1*, *AOMADS2*, and *AOMADS3*, from *A. officinalis*, and three genes, *AVMADS1*, *AVMADS2*, and *AVMADS3* from *A. virgatus*. In addition, we examined the evolutionary histories of these genes in *Asparagus* species.

Materials and methods

Plant material

The roots, shoots, phylloclades, and flower buds of *A. officinalis* L. cv. Mary Washington 500 W and *A. virgatus* Baker were collected from the experimental field at Tohoku University. All materials were frozen in liquid nitrogen immediately after collection and stored at −80°C. For Northern blot analysis, we used 1–2-mm long flower buds, which are representative of a late developmental stage. Vouchers sampled in this study have been deposited in the Herbarium, Graduate School of Science, Tohoku University (TUS).

Isolation of cDNA clones

Partial cDNAs from *A. officinalis* and *A. virgatus* were isolated by the 3′ rapid amplification of cDNA ends (RACE) method (Frohman et al. 1988; Münster et al. 1997). Poly(A)$^+$ RNA prepared from flower buds of *A. officinalis* and *A. virgatus* were used as templates. PCR amplification of the first cDNA strand was done using two MADS-box-specific primers (5′-GATCAAGMGSATC-GAGAA-3′ and 5′-GATGAAGMGSATCGAGAA-3′) and an adapter primer. The PCR products were extracted and purified from agarose gels using a QIAquick Gel Extraction kit (Qiagen), then sub-cloned into the pGEM-T Easy Vector (Promega, USA). Upstream sequences overlapping the 3′ fragments were isolated by 5′ RACE using the 5′/3′ RACE kit (Roche). cDNA clones with complete open reading frames were isolated by PCR, using primers located in the 5′ and 3′ UTR regions, cDNA pools as template. Sequencing was performed using the ABI PRISM DNA Sequencing kit (Perkin-Elmer Applied Biosystems) according to the manufacturer's protocol.

Phylogenetic analysis

We used 124 amino acid sequences from different MADS-domains and K-domains for the phylogenetic analysis. To construct phylogenetic trees for *SEP*-like

genes, the amino acid sequences were aligned using CLUSTAL X (Thompson et al. 1997), based on the BLOSUM protein weight matrix. Phylogenetic relationships were analyzed by the neighbor joining (NJ) method and the maximum-likelihood (ML) method. For NJ analysis, we constructed phylogenetic tree which is drawn by Njplot (Perriére and Gouy 1996). Bootstrap values of this phylogenetic tree were derived from 1,000 replicate runs (Thompson et al. 1994; http://www.ddbj.nig.ac.jp/E-mail/homology.html). For ML analysis, we used the PROML program of the PHYLIP v. 3.6 program package (Felsenstein 2004). We used JTT with constant rates across sites for this analysis (Jones et al. 1992). All indels were counted as missing. We performed ten random sequence addition searches using the J option, and global branch swapping using the G option, in order to identify the ML tree with the best log-likelihood. We then performed a bootstrap analysis (Felsenstein 1985) with 100 replications using the SEQBOOT program of PHYLIP. The GenBank accession numbers of the amino acid sequences used are: *ACaMAGL2* (AY850184: *Acorus americanus*), *AGL6* (M55554: *Arabidopsis thaliana*), *AGLG1* (AY244507: *Triticum monococcum*), *AktSEP3-1* (AY627628: *Akebia trifoliata*), *AMtrAGL2* (AY850179: *Amborella trichopoda*), *AMtrAGL9* (AY850178: *Amborella trichopoda*), *AOM3* (AY383559: *A. officinalis*), *AOM4* (AY383560: *A. officinalis*), *AOMADS1* (DQ344502: *A. officinalis*), *AOMADS2* (DQ344503: *A. officinalis*), *AOMADS3* (DQ344504: *A. officinalis*), *API* (Z16421: *Arabidopsis thaliana*), *ApMADS3* (AB079261: *Agapanthus praecox*), *AVMADS1* (DQ344499: *Asparagus virgatus*), *AVMADS2* (DQ344500: *Asparagus virgatus*), *AVMADS3* (DQ344501: *Asparagus virgatus*), *BoAGL3-a* (AJ508052: *Brassica oleracea*), *BpMADS1* (AJ252070: *Betula pendula*), *CAGL2* (AF135962: *Cucumis sativus*), *CaMADS1* (AF129875: *Capsicum annuum*), *CDM44* (AY173057: *Chrysanthemum morifolium*), *CMB1* (Q39685: *Dianthus caryophyllus*), *CsSEP3* (AY397763: *Chloranthus spicatus*), *DAL1* (X80902: *Picea abies*), *DcMADS5* (AJ271151: *Daucus carota*), *DEFH49* (X95467: *Antirrhinum majus*), *DEFH72* (X95468: *Antirrhinum majus*), *DEFH200* (X95469: *Antirrhinum majus*), *DlMADS7* (AY599760: *Dendrocalamus latiflorus*), *DlMADS17* (AY599754: *Dendrocalamus latiflorus*), *DOMADS3* (AF198176: *Dendrobium grex*), *EGM3* (AF029977: *Eucalyptus grandis*), *FaMADS* (AF484683: *Fragaria ananassa*), *FBP2* (M91666: *Petunia hybrida*), *FBP9* (AF335236: *Petunia hybrida*), *FBP23* (AF335241: *Petunia hybrida*), *FDRMADS1* (AF141966: *Oryza sativa*), *GGM9* (AJ132215: *Gnetum gnemon*), *GGM11* (AJ132217: *Gnetum gnemon*), *GHMADS1* (Accession No. AF538965: *Gossipium hirsutum*), *HcSEP1* (AB089157: *Houttuynia cordata*), *HcSEP2* (AB089158: *Houttuynia cordata*), *HcSEP3* (AB089159: *Houttuynia cordata*), *HvAGL6* (AY541067: *Hordeum vulgare*), *HvAGL9* (AY541068: *Hordeum vulgare*), *LeMADS1* (AY294329: *Lycopersicon esculentum*), *LeMADS5* (AY294330: *Lycopersicon esculentum*), *LpMADS4* (AY198329: *Lolium perenne*), *LpMADS5* (AY198330: *Lolium perenne*), *LpMADS6* (AY198331: *Lolium perenne*), *LpMADS7* (AY198332: *Lolium perenne*), M79 (Y15008: *Oryza sativa*), *MAGL4* (AF185574: *Populus tremuloides*), *MdMADS1* (U78947: *Malus domestica*), *MdMADS3* (U78949: *Malus domestica*), *MdMADS8* (AJ001681: *Malus domestica*), *MdMADS9* (AJ001682: *Malus domestica*), *MdMADS11* (AJ000763: *Malus domestica*), *MpMADS3* (AB050645: *Magnolia praecocissima*), *MpMADS4* (AB050646: *Magnolia praecocissima*), *MpMADS13* (AB050655: *Magnolia praecocissima*), *MTF1* (AJ223318: *Pisum sativum*), *NsMADS3* (AF068722: *Nicotiana sylvestris*), *NtMADS4* (AF068723: *Nicotiana tabacum*), *NUadAGL2* (AY850183: *Nuphar advena*), *OM1* (Q38694: *Aranda deborah*), *OsMADS1* (L34271: *Oryza sativa*) *OsMADS6* (U78782: *Oryza sativa*), *OsMADS7* (U78891: *Oryza sativa*), *OsMADS8* (U78892: *Oryza sativa*), *OsMADS45* (U31994: *Oryza sativa*), *PaMADS1* (AF372840: *Poa annua*), *PapnSEP3* (AY306174: *Papaver nudicaule*), *PatSEP1* (AY306166: *Pachysandra terminalis*), *PdMADS* (AY313211: *Prunus dulcis*), *PhSEP3* (AY306171: *Petunia hybrida*), *PMADS12* (AY370527: *Petunia hybrida*), *PtM6* (AY235222: *Populus tremuloides*), *RbAGL6* (AY306184: *Ranunculus bulbosus*), *RMADS217* (AY551922: *Oryza sativa*), *SaMADSD* (Y08626: *Sinapis alba*), *SEP1* (B39534: *Arabidopsis thaliana*), *SEP2* (AY727621: *Arabidopsis thaliana*), *SEP3* (O22456: *Arabidopsis thaliana*), *SEP4* (P29383: *Arabidopsis thaliana*), *SlSEP1* (AB162019: *Silene latifolia*), *SlSEP3* (AB162020: *Silene latifolia*), *SQUA* (X63701: *Antirrhinum majus*), *SvAGL6* (AY306188: *Syringa vulgaris*), *SvSEP3* (AY306186: *Syringa vulgaris*), *TAGL2* (AY098738: *Lycopersicon esculentum*), *TaMADS* (AF543316: *Triticum aestivum*), *TaMADS12* (AB007505: *Triticum aestivum*), *TDR5* (X60758: *Lycopersicon esculentum*), *VvMADS2* (AF373601: *Vitis vinifera*), *VvMADS3* (AF373602: *Vitis vinifera*), *VvMADS4* (AF373603: *Vitis vinifera*), *ZAG3* (L46397: *Zea mays*), *ZAG5* (L46398: *Zea mays*), *ZMM6* (AJ430692: *Zea mays*), *ZMM7* (Y09302: *Zea mays*), *ZMM24* (AJ430638: *Zea mays*), *ZMM27* (AJ430694: *Zea mays*), *ZMM31* (AJ430640: *Zea mays*).

Southern blot analysis

Total DNA was extracted from phylloclades of *A. officinalis* as described by Honda and Hirai (1990). Purified total DNA was digested with three restriction enzymes (*Eco*RI, *Hin*dIII, and *Bam*HI) and separated on a 0.6% agarose gel. Hybridization was performed at 42°C in a solution containing 50% formamide, ×5 SSC, 0.1% *N*-lauroylsarcosine, 0.02% SDS, and 2% blocking reagent (Roche). Gene-specific probes were labeled with the PCR DIG Probe Synthesis Kit (Roche). Washes were performed twice in ×2 SSC, 0.1% SDS at room temperature for 5 min, and twice in ×0.5 SSC, 0.1% SDS at 65°C for 10 min.

Northern blot analysis

Total RNA was isolated from roots, stems, phylloclades, and flower buds of *A. officinalis* by the method of Chomczynski and Sacchi (1987). Total RNA from each sample (10 μg each) was separated by electrophoresis on a 1.2% agarose gel containing ×1 MOPS solution and 5% formaldehyde, then transferred to positively charged nylon membranes (Roche). Hybridization was carried out at 65°C overnight in a solution of ×5 SSC, 50% formamide, 0.02% SDS, 0.1% *N*-lauroylsarcosine, and 2% (w/v) blocking reagent (Roche) using the same DIG-labeled gene-specific probes that were used for Southern hybridization.

Gene-specific RT-PCR

Expression of the *AOMADS3* in each floral organ of *A. officinalis* was characterized by PCR analysis of cDNA isolated from dissected tissues. Gene-specific primers were designed based on known sequences from *AOMADS3*. The following thermocycling conditions were employed: (94°C, 2 min) × 1 cycle; (94°C, 30 s; 54°C, 30 s; 72°C, 60 s) × 35 cycles; (72°C, 10 min) × 1 cycle. Amplified products were run on a 1% agarose gel and digitally photographed.

RT-PCR RFLP analysis

RT-PCR RFLP analyses of *AOMADS1* and *AOMADS2* genes were conducted to analyze the relative expression levels of *AOMADS1* and *AOMADS2*. There are nucleotide substitutions present in *AOMADS1* and *AOMADS2* that alter their susceptibility to certain restriction enzymes. We used *Pst*I (CTGCAG) to discriminate between the two genes, because the *AOMADS1* cDNA contains a *Pst*I site in I-region, whereas the *AOMADS2* cDNA does not (Fig. 7A).

cDNA prepared from flower buds of *A. officinalis* was used as a template. The following thermocycling conditions were employed: (94°C, 2 min) × 1 cycle; (94°C, 30 s; 54°C, 30 s; 72°C, 60 s) × 35 cycles; (72°C, 10 min) × 1 cycle. Amplified products were digested with *Pst*I, separated on a 1% agarose gel, and digitally photographed.

Results

Sequencing and phylogenetic analyses of the *SEP*-like genes in *A. officinalis* and *A. virgatus*

We isolated several cDNA clones of MADS-box genes from *A. officinalis* using the RACE method. BLAST searches revealed that three of these clones had high sequence similarity to the *AOM1* gene (Caporali et al. 2000). This suggested that the three cDNA clones represented *SEP*-like genes, and we named them *AOMADS1*, *AOMADS2*, and *AOMADS3*. Using 5′ RACE, the 5′ region of each of the cDNAs was obtained, and full-length cDNA clones were isolated by PCR using gene-specific primers. At least two independent clones were sequenced for each gene. Using a similar procedure, we isolated three *SEP*-like genes from *A. virgatus*, designated *AVMADS1*, *AVMADS2*, and *AVMADS3*.

The predicted amino acid sequence of *AOMADS1* and *AOMADS2* were almost identical to those of *AOM1* and *AOM4*, respectively, so that these genes are likely to be the same gene (Caporali et al. 2000; Losa et al. 2004). Amino acid sequence alignment of several *SEP*-like genes revealed that the *SEP*-like genes from *A. officinalis* and *A. virgatus* have well-conserved MADS-domains and K-domains. Moreover, we identified three regions that followed the K-domain sequence that was also conserved among previously published sequences (Fig. 1). We termed these regions "SEP motif I," "SEP/AGL6 motif," and "SEP motif II." Of these motifs, the SEP/AGL6 motif is especially well conserved, not only in *SEP*-like genes, but also in *AGL6*-like genes, which are the outgroup lineages. Vandenbussche et al. (2003a) and Zahn et al. (2005) have also suggested that this region is a typical motif in *SEP*-like genes. Both SEP motif I and SEP motif II were conserved in the SEP-like lineage, but not in the outgroup lineages, although there were some exceptions (Fig. 1).

We conducted phylogenetic analyses of *SEP*-like genes to determine the phylogenetic positions of the *SEP*-like genes in *A. officinalis* and *A. virgatus* that we isolated in this study. We used as our data set most of the published genes in the MADS-box gene family. To construct the phylogenetic tree, we used the amino acid sequences of the well conserved MADS-domains and K-domains. Figure 2 shows the result of the NJ analysis and Fig. 3 shows the result of the ML analysis. *AGL6*-like genes, *AP1* and *SQUA*, were used as outgroups in both phylogenetic analyses. In both analyses, all *SEP*-like genes comprise a monophyletic group with high support value, and this group is the sister to the monophyletic group of *AGL6*-like genes (Figs. 2, 3). In the monophyletic group of *SEP*-like genes, each group of genes from *A. officinalis* and *A. virgatus*: *AOMADS1*/*AOM1* and *AVMADS1*, *AOMADS2*/*AOM4* and *AVMADS2*, and *AOMADS3* and *AVMADS3*, respectively, is also monophyly. Among the *SEP*-like genes in *A. officinalis* and *A. virgatus*, *AOMADS1*/*AOM1* and *AVMADS1* are more closely related to *AOMADS2*/*AOM4* and *AVMADS2* than to *AOMADS3* and *AVMADS3*.

Genomic organization and expression analyses of *SEP*-like genes from *A. officinalis*

To determine the copy number of *AOMADS1*/*AOM1*, *AOMADS2*/*AOM4*, and *AOMADS3* in the *A. officinalis* genome, we performed genomic Southern blot hybridization. Total DNA was digested with *Eco*RI, *Hin*dIII, or *Bam*HI and hybridized with the C-terminal region of

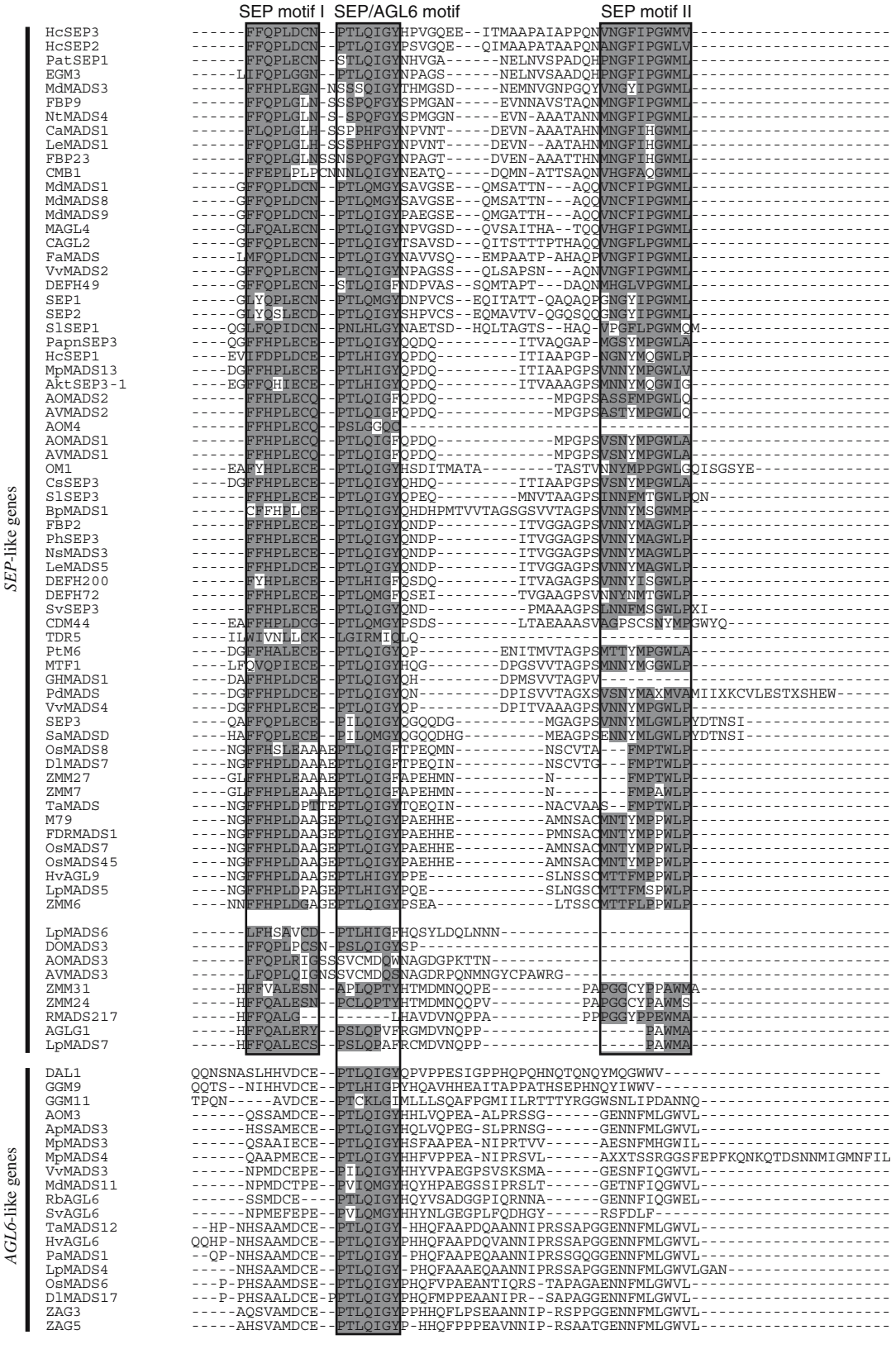

Fig. 1 Alignment of the C-terminal regions of the predicted amino acid sequences for select representatives of *SEP*-like genes, and their outgroup lineage, *AGL6*-like genes. Three highly conserved regions, *SEP*-motif I, *SEP*/*AGL6*-motif, and *SEP*-motif II, are indicated with *boxes*. Residues in these regions that are highly conserved with respect to the *SEP1* consensus sequences are *shaded*

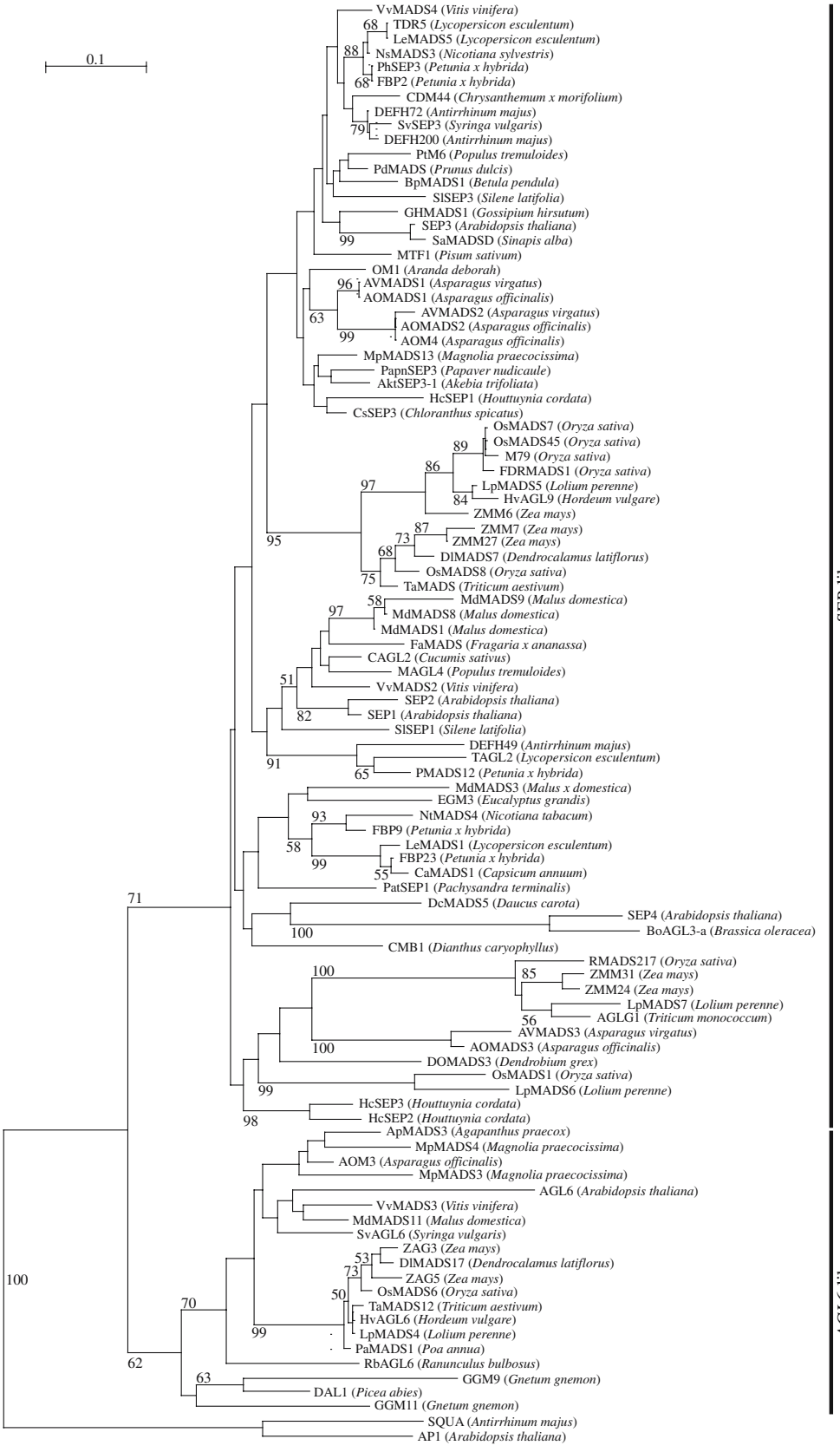

Fig. 2 Phylogenetic trees of *SEP*-like genes generated by the *NJ* method. Numbers represent bootstrap values of 50% or more support

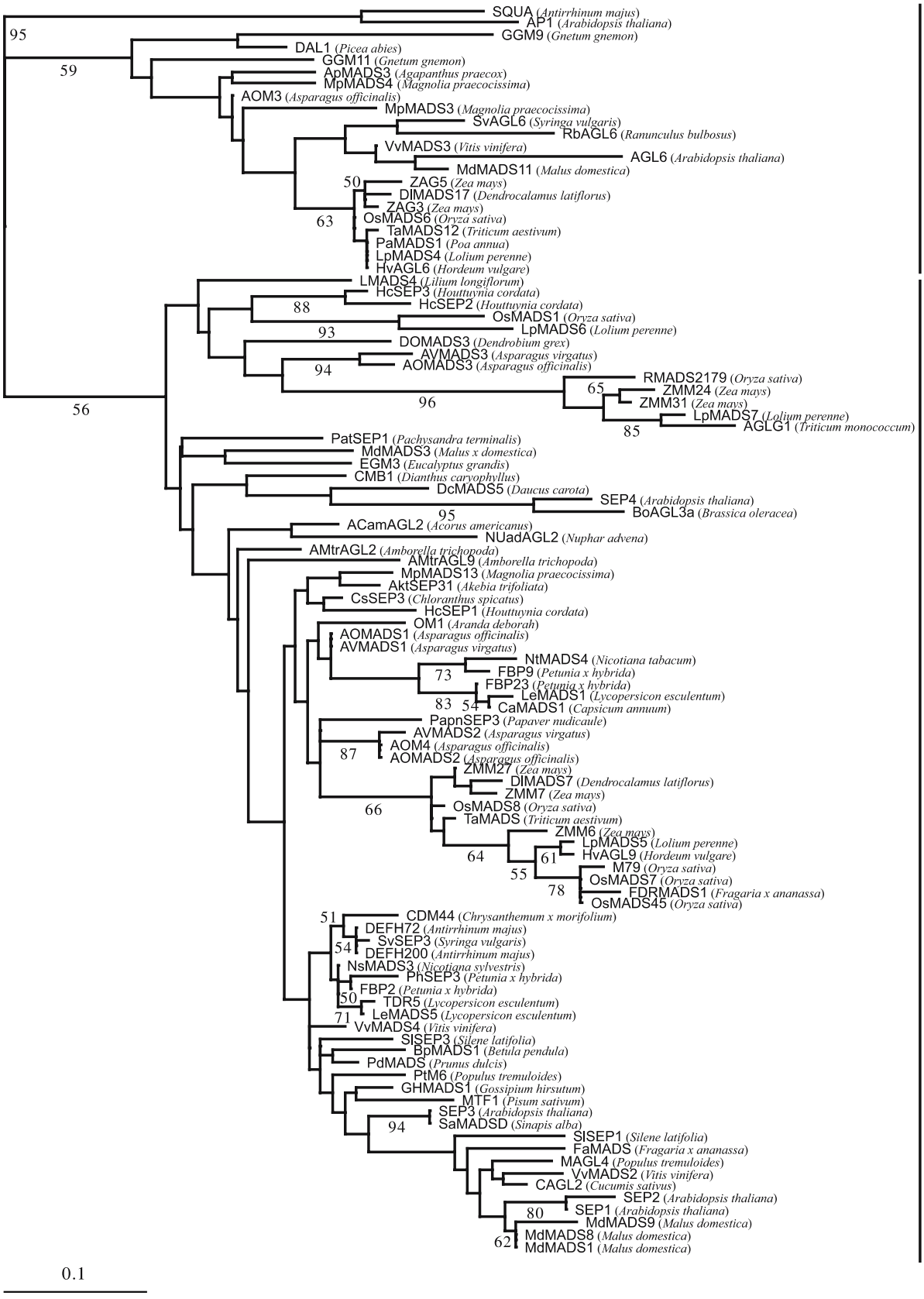

Fig. 3 Phylogenetic tree for *SEP*-like genes obtained using the *ML* method. The log-likelihood of the best *ML* tree is -4,577.43. The numbers below the branches are the bootstrap support values ≥50%. *SEP*-like genes in *Asparagus* species are indicated in *bold*

each cDNA as a gene-specific probe. When probed with an *AOMADS1*-specific probe, a single hybridization band was observed in the samples digested with *Eco*RI and *Hin*dIII, whereas two bands were detected in the samples digested with *Bam*HI, indicating that the *AOMADS1* gene occurs as a single copy in the *A. officinalis* genome (Fig. 4). Using an *AOMADS2*-specific probe, we observed several bands, which seemed to be the result of cross-hybridization with *AOMADS1* gene. These results of *AOMADS1* and *AOMADS2* were consistent with previous report of *AOM1* and *AOM4*, respectively (Caporali et al. 2000; Losa et al. 2004). Several bands were also observed for *AOMADS3* gene, indicating that it is present as a low-copy gene (Fig. 4).

The expression patterns of the *SEP*-like genes in *A. officinalis* were analyzed by Northern hybridization analysis using total RNA isolated from roots, stems, phylloclades, and flower buds. We observed that *AOMADS1*/*AOM1* and *AOMADS2*/*AOM4* were expressed specifically in flower buds (Fig. 5). No transcripts for *AOMADS1*/*AOM1* and *AOMADS2*/*AOM4* were detected in vegetative tissues such as roots, stems, and phylloclades. It should be noted, however, that as the sequence homology between *AOMADS1*/*AOM1* and *AOMADS2*/*AOM4* is very high, it is difficult to distinguish between the expression of one or the other of the two genes. Expression of *AOMADS3* could not be detected in any organs by Northern hybridization analysis, so we performed RT-PCR analysis (Fig. 6). RT-PCR analysis revealed that *AOMADS3* was also expressed specifically in flower buds (Fig. 6). These results indicated that expression of *AOMADS3* is lower than that of *AOMADS1*/*AOM1* and *AOMADS2*/*AOM4* in flower buds (Figs. 5, 6).

To analyze the relative amounts of *AOMADS1*/*AOM1* and *AOMADS2*/*AOM4* transcripts, RT-PCR RFLP analyses were performed using cDNA pools from male and female flower buds from *A. officinalis*. The results showed that there was no significant difference in the amounts of *AOMADS1*/*AOM1* and *AOMADS2*/*AOM4* transcripts in male and female flower buds (Fig. 7).

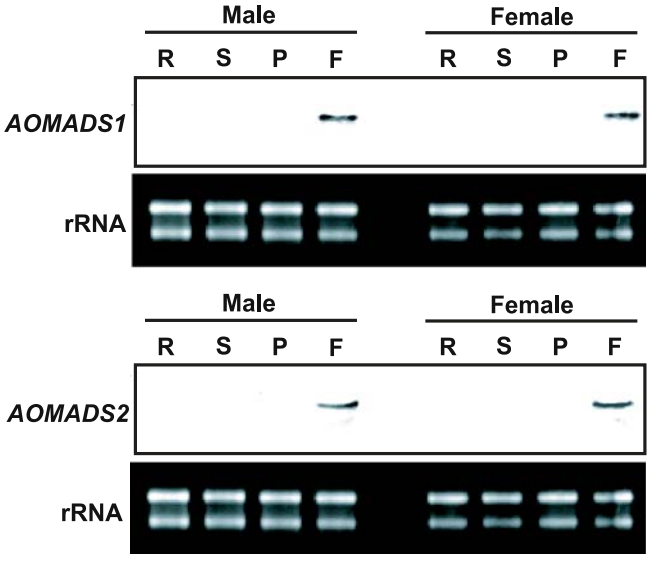

Fig. 5 Northern blot analyses of *AOMADS1* and *AOMADS2* in *A. officinalis*. Each *lane* contains 10 μg of total *RNA* from roots (*R*), stems (*S*), phylloclades (*P*), and flower buds (*F*). Ethidium bromide-staining of *rRNA* is shown below the panel of Northern blot hybridization

Discussion

SEP-like genes have highly conserved C-terminal motifs

Recent observations of a wide range of MADS-box genes have the potential to illuminate many of the

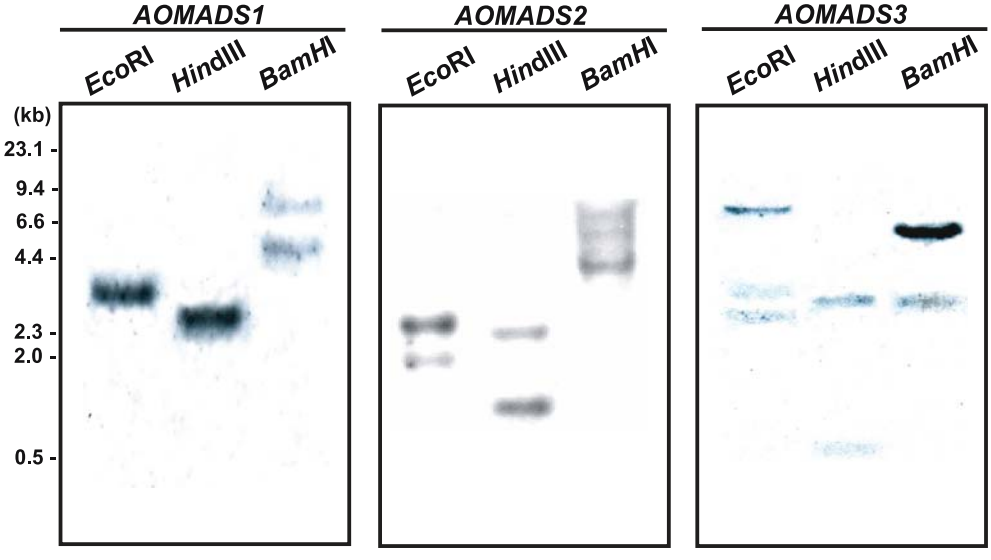

Fig. 4 Southern blot analyses of *AOMADS1*, *AOMADS2*, and *AOMADS3* in *A. officinalis*. Each *lane* contains 20 μg of total DNA digested with *Bam*HI (*B*), *Eco*RI (*E*), or *Hin*dIII (*H*), as indicated above the lanes

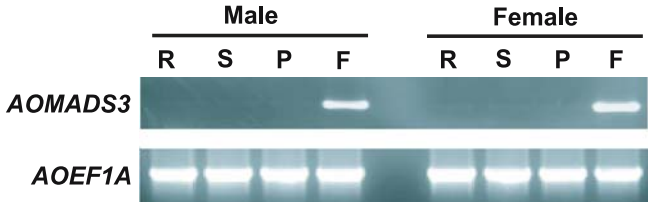

Fig. 6 Gene-specific *RT-PCR* of *AOMADS3* using *cDNA* from dissected organs of *A. officinalis*. *AOEF1A*, encoding elongation factor 1A gene, is used as a positive control. *R* roots; *S* stems; *P* phylloclades; *F* flower buds

developmental processes that underlie plant organ development (Alvarez-Buylla et al. 2000; Theissen et al. 2000). Moreover, alignment studies of various angiosperm MADS-box genes have revealed that some lineages of the MADS-box gene families contain highly conserved motifs (Kramer et al. 1998, 2004; Vandenbussche et al. 2003a; Yun et al. 2004b). For instance, Kramer et al. (1998) demonstrated that the *GLO*-like and *DEF*-like genes (B-class genes) encode the characteristic amino acid motifs of each lineage: the paleoAP3 motif, euAP3 motif, PI motif-derived, and PI motif. Moreover, Kramer et al. (2004) defined two characteristic amino acid motifs in the C-terminal regions of *AGAMOUS* (*AG*)-like genes, AG motifs I and II. In *AG*-like genes, Yun et al. (2004b) reported that nine amino acid residues following AG motif II were conserved among previous published sequences of various monocots, and termed it the MD motif. Furthermore, from the alignment of the C-terminal regions of many MIKC-type MADS-box genes from a wide range of angiosperms, representing all major gene families, Vandenbussche et al. (2003a) reported that most lineages of MADS-box gene family contained several conserved motifs in their C-terminal regions. These results indicate that members of the same MADS-box gene subfamily usually contain highly conserved C-terminal motifs.

Conserved C-terminal motifs in the *SEP*-like genes have been reported as the "AGL2 motif," "ZMM3 motif," and "ZMM7 motif" (Vandenbussche et al. 2003a). Of these three motifs, ZMM3 and ZMM7 motifs are conserved in four grass species: *Hordeum vulgare*, *Oryza sativa*, *Triticum aestivum*, and *Zea mays* (Vandenbussche et al. 2003a). Recently, Zahn et al. (2005) have also reported the conserved C-terminal motifs in *SEP*-like genes, SEP I motif and SEP II motif. We compared the previously published sequences of *SEP*-like and *AGL6*-like genes from a large range of seed plants, including *A. officinalis* and *A. virgatus* (Fig. 1). The result is that amino acid residues in the C-terminal region are highly conserved among *SEP*-like genes in seed plants. It is very interesting to note that a part of the SEP I motif (Zahn et al. 2005) is well conserved among the *AGL6*-like genes as well (Fig. 1), so that we termed this region as the "SEP/AGL6 motif." The eight amino acid residues before the sequence of the SEP/AGL6 motif, which is a part of SEP I motif (Zahn et al. 2005), was conserved among previously published sequences of *SEP*-like genes in seed plants, therefore, we termed this region as "SEP motif I." As these motifs are conserved among more distantly related species, they may play important roles in determining partner specificity in higher-order complex formation or that they contain activation domains. However, the SEP/AGL6 motif is conserved not only in *SEP*-like genes, but also in *AGL6*-like genes, so the specialized functions of *SEP*-like genes are not characterized solely by the SEP/AGL6

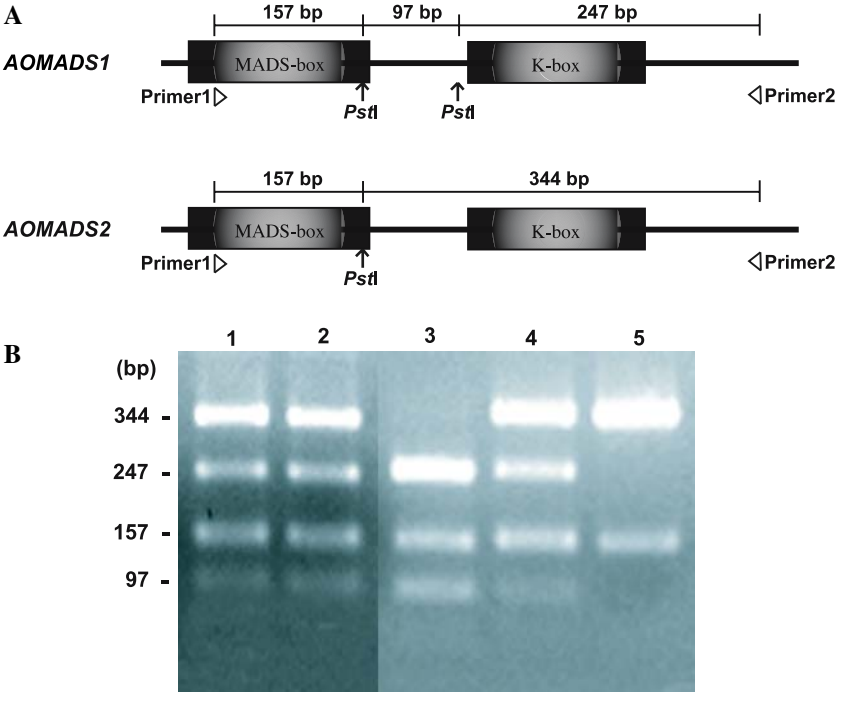

Fig. 7 *RT-PCR RFLP* analysis of *AOMADS1* and *AOMADS2*. **a** The schema of expected restriction sites for distinguishing *AOMADS1* and *AOMADS2* by *RT-PCR RFLP*. **b** *RT-PCR RFLP* profiles of *AOMADS1* and *AOMADS2* in *A. officinalis*. *Lane 1* male flower buds; *lane 2* female flower buds; *lane 3 cDNA* of *AOMADS1*, *lane 4 cDNA* mixture of *AOMADS1* and *AOMADS2* (1:1); *lane 5 cDNA* of *AOMADS2*

motif. We found another conserved motif, termed as "SEP motif II," downstream of the SEP/AGL6 motif, which was previously called SEP II motif (Zahn et al. 2005). The SEP motif I is more highly conserved than the SEP motif II, suggesting that the SEP motif II may have additional functions and that the SEP motif I involves in the original functions of the *SEP*-like genes.

There are a variety of roles for the predicted C-terminal sequence motifs in plants. For example, the first half of the C-terminal regions of DEFICIENS and GLOBOSA in *Antirrhinum majus* appears to be essential for in vitro ternary complex formation with the SQUAMOSA protein (Egea-Cortines et al. 1999). In addition, Lamb and Irish (2003) reported that the PI motif in the C-terminal of the PISTILLATA in *Arabidopsis* is necessary for the specification of organ identity, although the PI motif was positioned outside the functional MADS-domains and K-domains. In the HOX ortholog proteins, which are the most important transcriptional factors involved in determining the body plan in animals, the gain or loss of the QA motif in the C-terminal region of Ultrabithorax (Ubx) contributes to the evolution of hexapod body patterns in animals (Galant and Carroll 2002; Ronshaugen et al. 2002). Thus, specific protein sequences outside the MADS-domain of MADS-box genes and the homeodomain of HOX ortholog genes are linked to the generation of diversity in the plant and the insect body plans (Galant and Carroll 2002; Levine 2002; Ronshaugen et al. 2002; Lamb and Irish 2003). In the view of these previous results, the well-conserved C-terminal SEP-motif I, SEP/AGL6-motif, and SEP-motif II identified in our study are particularly intriguing, as they may play a role in generating floral diversity. Moreover, the conservation of sequence specificity in SEP motif I, SEP/AGL6 motif, and SEP motif II may reflect a similar conservation of biochemical and/or functional activity. Additional studies using sequences that lack C-terminal SEP motif I, the SEP/AGL6 motif, and SEP motif II are needed in order to examine the role of these motifs in complex formation.

Perspectives on the functional evolution of *SEP*-like genes in *Asparagus*

In plants, gene duplication and divergence of the MADS-box genes has likely played a fundamental role in the elaboration of the plant body plan (Theissen et al. 2000). Moreover, the MADS-box genes have undergone a significant amount of gene duplication, and it is the increased number of MADS-box genes, as well as their recruitment to new roles, that has likely contributed to the evolution of new plant morphologies (Theissen et al. 2000). *SEP*-like genes are a separate subfamily of the MADS-box gene family (Becker and Theissen 2003). Ermolaeva et al. (2003) indicated that *SEP1* and *SEP2* have recently undergone duplication in the evolution of flowering plants, but their relationships to *SEP3* and *SEP4* were not clear. Some studies have suggested that *SEP1* and *SEP2* were more closely related to *SEP4* than to *SEP3* (Yu and Goh 2000; Lemmetyinen et al. 2004; Zahn et al. 2005), whereas others suggested that *SEP3* was the closest relative of *SEP1* and *SEP2* (Purugganan 1998; Lawton-Rauh et al. 2000; Sung et al. 2000; Becker and Theissen 2003; Parenicova et al. 2003; Vandenbussche et al. 2003b). Our phylogenetic analysis by the NJ method supports the latter hypothesis, showing that *SEP3* is more closely related to *SEP1* and *SEP2* (Fig. 2), whereas the other analysis by the ML method showed that the phylogenetic relationship of these four genes are not clear (Fig. 3).

In *A. officinalis*, Caporali et al. (2000) and Losa et al. (2004) suggested that the expression profiles of *AOM1*, *AOM3*, and *AOM4* were similar to those of the *SEP* genes in *Arabidopsis thaliana*. Moreover, *AOM1* and *AOM4* could not be distinguished based on in situ hybridization analyses (Caporali et al. 2000; Losa et al. 2004). We isolated *AOMADS1* and *AOMADS2*, which seem to be the same as *AOM1* and *AOM4*, respectively. The results of our expression analysis of *AOMADS1*/*AOM1* and *AOMADS2*/*AOM4* indicated that these genes were expressed in the flower buds (Fig. 5), and there was no significant difference in the amounts of *AOMADS1*/*AOM1* and *AOMADS2*/*AOM4* transcripts (Fig. 7b). As the expression patterns and the amount of the transcripts of these genes were very similar, these genes could be redundant. Our phylogenetic analysis showed that *AOMADS1*/*AOM1* and *AOMADS2*/*AOM4* were related to *Arabidopsis SEPALLATA* genes (Figs. 2, 3), suggesting that *AOMADS1*/*AOM1*/*AVMADS1* and *AOMADS2*/*AOM4*/*AVMADS2* may be the functional homologues to *Arabidopsis SEPALLATA* genes. In addition to *AOMADS1*/*AOM1*/*AVMADS1* and *AOMADS2*/*AOM4*/*AVMADS2*, we isolated two additional *SEP*-like genes, *AOMADS3* from *A. officinalis* and *AVMADS3* from *A. virgatus*. Although *AOMADS1*/*AOM1*/*AVMADS1* and *AOMADS2*/*AOM4*/*AVMADS2* are closely related to *SEP3* gene, which were also described in the previous studies (Zahn et al. 2005; Malcomber and Kellogg 2005), *AOMADS3* and *AVMADS3* were classified in different clade which is far related to *SEP3* gene (Figs. 2, 3). The alignment of the amino acid sequences of *SEP*-like genes showed that *AOMADS3* and *AVMADS3* genes lacked the SEP-motif II in their C-terminal sequences (Fig. 1). It is interesting to note that in the group of *SEP*-like genes that included *AOMADS3*, *AVMADS3*, and sister groups, all had either lost or had a poorly conserved SEP-motif II in their C-terminus (Fig. 1). Our expression analyses of *SEP*-like genes in *A. officinalis* revealed that *AOMADS1*/*AOM1* and *AOMADS2*/*AOM4* were expressed at equal levels in the flower buds (Figs. 5, 7) and that the amount of *AOMADS3* transcript was lower than that of *AOMADS1*/*AOM1* and *AOMADS2*/*AOM4* (Figs. 5, 6). Therefore, it seems that *AOMADS3* may have undergone a functional shift from the original function of *AOMADS1*/*AOM1* and *AOMADS2*/*AOM4*. *AOMADS3* would have any functions

for floral development in asparagus because this gene specifically expressed in flower buds, although the expression level is very weak.

Expression analyses of *AOMADS1*/*AOM1* and *AOMADS2*/*AOM4* revealed that there was no difference in the level of expression of each gene (Fig. 7; Caporali et al. 2000; Losa et al. 2004). As these two genes appear to be the products of a recent duplication event (Figs. 2, 3), each gene may not have accumulated enough mutational changes to result in a change in their functions. However, a recent study on *Arabidopsis thaliana* indicated the presence of some nucleotide sites in the MADS-domains and K-domains that had high probabilities of positive Darwinian selection. Their results indicated that these sites play important roles in the acquisition of novel functions (Martinez-Castilla and Alvarez-Buylla 2003). Therefore, it is likely that functional changes in *AOMADS1*/*AOM1* and *AOMADS2*/*AOM4* of *A. officinalis* occur as a result of changes at a few sites in each gene and may be a major source of innovation in floral development. Further comparative analyses using transgenic plants will illuminate the functional evolutionary histories of not only *AOMADS1*/*AOM1* and *AOMADS2*/*AOM4* but also *AOMADS3* of *A. officinalis*.

Acknowledgments We thank Mr. H. Tokairin for collaboration in culturing the plants. We are additionally grateful to all the members of Kanno's lab for providing help and advice. This work was supported, in part, by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

References

Alvarez-Buylla ER, Liljegren SJ, Pelaz S, Gold SE, Burgeff C, Ditta GS, Vergata-Silva F, Yanofsky MF (2000) MADS-box gene evolution beyond flowers: expression in pollen, endosperm, guard cells, roots, and trichomes. Plant J 24:457–466

Angenent GC, Franken J, Busscher M, van Dijken A, van Went JL, Dons HJM, van Tunen AJ (1995) A novel class of MADS box genes is involved in ovule development in petunia. Plant Cell 7:1569–1582

Becker A, Theissen G (2003) The major clade of MADS-box genes and their role in the development and evolution of flowering plants. Mol Phylogenet Evol 29:464–484

Caporali E, Spada A, Losa A, Marziani G (2000) The MADS box gene *AOM1* is expressed in reproductive meristems and flowers of the dioecious species *Asparagus officinalis*. Sex Plant Reprod 13:151–156

Chomczynski P, Sacci N (1987) Single-step method of RNA isolation by acid guanidium thiocyanate–phenol–chloroform extraction. Anal Biochem 162:156–159

Coen ES, Meyerowitz EM (1991) The war of the whorls: genetic interactions controlling flower development. Nature 353:31–37

Colombo L, Franken J, Koetje E, van Went J, Dons HJ, Angenent GC, van Tunen AJ (1995) The *Petunia* MADS-box gene *FBP11* determines ovule identity. Plant Cell 7:1859–1868

Ditta GA, Pinyopich A, Robles P, Pelaz S, Yanofsky MF (2004) The *SEP4* gene of *Arabidopsis thaliana* functions in floral organ and meristem identity. Curr Biol 14:1935–194

Egea-Cortines M, Davis B (2000) Beyond the ABCs: ternary complex formation in the control of floral organ identity. Trends Plant Sci 5:471–476

Egea-Cortines M, Saedler H, Sommer H (1999) Ternary complex formation between the MADS-box proteins *SQUAMOSA*, *DEFICIENS* and *GLOBOSA* is involved in the control of floral architecture in *Antirrhinum majus*. EMBO J 18:5370–5379

Ermolaeva MD, Wu M, Eisen JA, Salzberg SL (2003) The age of the *Arabidopsis thaliana* genome duplication. Plant Mol Biol 51:859–866

Favaro R, Pinyopich A, Battaglia R, Kooiker M, Borghi L, Ditta G, Yanofsky MF, Kater MM, Colombo L (2003) MADS-box protein complexes control carpel and ovule development in *Arabidopsis*. Plant Cell 15:2603–2611

Felsenstein J (1985) Confidence limits on phylogenetics: an approach using the bootstrap. Evolution 39:783–7913

Felsenstein J (2004) Inferring phylogenies. Sinauer, Sunderland

Ferrario S, Immink RG, Shchennikova A, Busscher-Lange J, Angenent GC (2003) The MADS box gene *FBP2* is required for *SEPALLATA* function in petunia. Plant Cell 15:914–925

Flanagan CA, Ma H (1994) Spatially and temporally regulated expression of the MADS-box gene *AGL2* in wild-type and mutant *Arabidopsis* flowers. Plant Mol Biol 26:581–595

Frohman MA, Dush MK, Martin GR (1988) Rapid production of full-length cDNA from rare transcripts: amplification using a single gene-specific oligonucleotide primer. Proc Natl Acad Sci USA 85:8998–9002

Fukuda T, Ashizawa H, Suzuki R, Nakamura T, Ochiai T, Kanno A, Kameya T, Yokoyama J (2005) Molecular phylogeny of the genus *Asparagus* (Asparagaceae) inferred from plastid *petB* intron and *petD-rpoA* intergenic spacer sequences. Plant Species Biol 20:123–134

Galant R, Carroll SB (2002) Evolution of a transcriptional repression domain in an insect HOX protein. Nature 415:910–913

Honda H, Hirai A (1990) A simple and efficient method for identification of hybrids using nonradioactive rDNA as probe. Jpn J Breed 40:339–348

Honma T, Goto K (2000) The *Arabidopsis* floral homeotic gene *PISTILLATA* is regulated by discrete *cis*-elements responsive to induction and maintenance signals. Development 127:2021–2030

Honma T, Goto K (2001) Complexes of MADS box proteins are sufficient to convert leaves into floral organs. Nature 409:525–529

Ito T, Suzuki G, Ochiai T, Nakada M, Kameya T, Kanno A (2005) Genomic organization of the *AODEF* gene in *Asparagus officinalis* L. Genes Genet Syst 80:95–103

Jack T (2001) Relearning our ABCs: new twists on an old model. Trends Plant Sci 6:310–316

Jones DT, Taylor WR, Thornton JM (1992) The rapid generation of mutation data matrices from protein sequences. Comput Appl Biosci 8:275–282

Kanno A, Lee YO, Kameya T (1997) The structure of the chloroplast genome in members of the genus *Asparagus*. Theor Appl Genet 95:1196–1202

Kanno A, Saeki H, Kameya T, Saedler H, Theissen G (2003) Heterotopic expression of class B floral homeotic genes supports a modified ABC model for tulip (*Tulipa gesneriana*). Plant Mol Biol 52:831–841

Kramer EM, Dorit RL, Irish VF (1998) Molecular evolution of gene controlling petal and stamen development: duplicate and divergence within the *APETALA3* and *PISTILLATA* MADS-Box gene lineages. Genetics 149:765–783

Kramer EM, Alejandra-Jaramillo M, DiStilio VS (2004) Pattern of gene duplication and functional evolution during the diversification of the *AGAMOUS* subfamily of MADS box genes in angiosperms. Genetics 166:1011–1023

Lamb RS, Irish VF (2003) Functional divergence within the *APETALA3*/*PISTILLATA* floral homeotic gene lineages. Proc Natl Acad Sci USA 100:6558–6563

Lawton-Rauh AL, Albarez-Buylla ER, Purugganan MD (2000) Molecular evolution of flowering plants. Trends Ecol Evol 15:144–149

Lemmetyinen J, Hassinen M, Elo A, Porali I, Keinonen K, Makela H, Sopanen T (2004) Functional characterization of *SEPAL-*

LATA and *AGAMOUS* orthologues in silver birch. Physiol Plant 121:149–162

Levine M (2002) How insects lose their limbs. Nature 415:848–849

Losa A, Caporali E, Spada A, Marziani S, Marziani G (2004) *AOM3* and *AOM4*: two MADS box genes expressed in reproductive structures of *Asparagus officinalis*. Sex Plant Reprod 16:215–221

Malcomber ST, Kellogg EA (2005) *SEPALLATA* gene diversification: brave new whorls. Trends Plant Sci 10:427–435

Mandel MA, Yanofsky MF (1998) The *Arabidopsis AGL9* MADS box gene is expressed in young flower primordia. Sex Plant Reprod 11:22–28

Martinez-Castilla PM, Alvarez-Buylla ER (2003) Adaptive evolution in the *Arabidopsis* MADS-box gene family inferred from its complete resolved phylogeny. Proc Natl Acad Sci USA 100:13407–13412

Münster T, Pahnke J, DiRosa A, Kim JT, Saedler H, Theissen G (1997) Floral homeotic genes were recruited from homologous MADS-box genes preexisting in the common ancestor of ferns and seed plants. Proc Natl Acad Sci USA 94:2415–2420

Nakada M, Komatsu M, Ochiai T, Ohtsu K, Nakazono M, Nishizawa NK, Nitta K, Nishiyama R, Kameya T, Kanno A (2006) Isolation of *MaDEF* from *Muscari armeniacum* and analysis of its expression using laser microdissection. Plant Sci 170:143–150

Nakamura T, Fukuda T, Nakano M, Hasebe M, Kameya T, Kanno A (2005) The modified ABC model explains the homochlamydeous flower morphology of *Agapanthus praecox* ssp. *orientalis* (Agapanthaceae). Plant Mol Biol 58:435–445

Ochiai T, Sonoda T, Kanno A, Kameya T (2002) Interspecific hybrids between *Asparagus schoberioides* Kunth and *A. officinalis* L. Acta Hort 589:225–229

Ochiai T, Nakamura T, Mashiko Y, Fukuda T, Yokoyama J, Kanno A, Kameya T (2004) The differentiation of sepal and petal morphologies in Commelinaceae. Gene 343:253–262

Park JH, Ishikawa Y, Yoshida R, Kanno A, Kameya T (2003) Expression of *AODEF*, a B-functional MADS-box gene, in stamens and inner tepals of dioecious species *Asparagus officinalis* L. Plant Mol Biol 51:867–875

Park JH, Ishikawa Y, Ochiai T, Kanno A, Kameya T (2004) Two *GLOBOSA*-like genes are expressed in second and third whorls of homochlamydeous flowers in *Asparagus officinalis* L. Plant Cell Physiol 45:325–332

Pelaz S, Ditta GS, Baumann E, Wisman E, Yanofsky MF (2000) B and C floral organ identity functions require *SEPALLATA* MADS box genes. Nature 405:200–203

Pelaz S, Tapia-López R, Alvarez-Buylla ER, Yanofsky MF (2001) Conversion of leaves into petals in Arabidopsis. Curr Biol 11:182–184

Purugganan MD (1998) The molecular evolution of development. BioEssays 20:700–711

Ronshaugen M, Mcginnis N, McGinnis W (2002) Hox protein mutation and macroevolution of the insect body plan. Nature 415:914–917

Savidge B, Rounsley SD, Yanofsky MF (1995) Temporal relationship between the transcription of two *Arabidopsis* MADS box genes and the floral organ identity genes. Plant Cell 7:721–733

Skipper M, Johansen LB, Pedersen KB, Frederiksen S, Johansen BB (2006) Cloning and transcription analysis of an *AGAMOUS*- and *SEEDSTICK* ortholog in the orchid *Dendrobium thyrsiflorum* (Reichb. f.). Gene (in press)

Sung SK, Yu GH, Nam J, Jeong DH, An G (2000) Developmentally regulated expression of two MADS-bpx genes, *MdMADS3* and *MdMADS4*, in the morphogenesis of flower buds and fruits in apple. Planta 210:519–528

Theissen G, Becker A, Di Rosa A, Kanno A, Kim JT, Münster T, Winter KU, Saedler H (2000) A short history of MADS-box genes in plants. Plant Mol Biol 42:115–149

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 24:4876–4882

Tsai WC, Kuoh CS, Chuang MH, Chen WH, Chen HH (2004) Four *DEF*-like MADS box genes displayed distinct floral morphogenetic roles in *Phalaenopsis* orchid. Plant Cell Physiol 45:831–844

Vandenbussche M, Theissen G, Van de Peer Y, Gerats T (2003a) Structural diversification and neo-functionalization during floral MADS-box gene evolution by C-terminal frameshift mutations. Nucleic Acids Res 31:4401–4409

Vandenbussche M, Zethof J, Souer E, Koes R, Tornielli GB, Pezzotti M, Ferrario S, Angenent GC, Gerats T (2003b) Toward the analysis of the petunia MADS box gene family by reverse and forward transposon insertion mutagenesis approaches: B, C, and D floral organ identity functions require *SEPALLATA*-like MADS box genes in petunia. Plant Cell 15:2680–2693

Yu H, Goh CJ (2000) Identification and characterization of three orchid MADS-box genes of the AP1/AP9 subfamily during floral transition. Plant Physiol 123:1325–1336

Yun PY, Ito T, Kim SY, Kanno A, Kameya T (2004a) *AVAG1* gene is involved in the development of reproductive organs in ornamental asparagus, *Asparagus virgatus*. Sex Plant Reprod 17:1–8

Yun PY, Kim SY, Ochiai T, Fukuda T, Ito T, Kanno A, Kameya T (2004b) *AVAG2* is a putative D-class gene from an ornamental asparagus. Sex Plant Reprod 17:107–116

Zahn LM, Kong H, Leebens-Mack JH, Kim S, Soltis PS, Landherr LL, Soltis DE, dePamphilis CW, Ma H (2005) The evolution of the *SEPALLATA* subfamily of MADS-box genes: a preangiosperm origin with multiple duplications throughout angiosperm history. Genetics 169:2209–2223