Two *GLOBOSA*-Like Genes are Expressed in Second and Third Whorls of Homochlamydeous Flowers in *Asparagus officinalis* L.

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Garden asparagus (*Asparagus officinalis* L.) has homochlamydeous flowers. Like Liliaceae plants such as lily and tulip, the perianths of asparagus have two whorls of almost identical petaloid organs, called tepals. Floral structures of these homochlamydeous flowers could be explained by a modified ABC model, in which the expression of the class B genes has expanded to whorl 1, so that the organs of whorls 1 and 2 have the same petaloid structure. In this study, we isolated and characterized two *GLOBOSA*-like genes (*AOGLOA* and *AOGLOB*), one of class B gene, from asparagus. Southern blot showed that *AOGLOA* and *AOGLOB* genes are single copy genes. Northern blot analysis indicated that these genes were specifically expressed in male and female flowers. In situ hybridization showed that the expression of *AOGLOA* and *AOGLOB* genes is confined to whorls 2 and 3 (inner tepal and stamen) and not detected in whorl 1 (outer tepal). The other asparagus class B gene, *AODEF*, was also not expressed in outer tepal [Park et al. (2003) *Plant Mol Biol.* 51: 867]. These results indicate that the class B genes are not involved in the outer tepal development in asparagus, not supporting the modified ABC model in asparagus.

Keywords: ABC model — *AOGLOA* — *AOGLOB* — asparagus — *GLOBOSA* — MADS-box gene.

Introduction

In most higher dicotyledonous plants, the floral organs consist of four different whorls, containing sepals, petals, stamens and carpels. The ABC model proposes that three gene functions (A, B and C) act in combination to specify the different organ identities (Coen and Meyerowitz 1991). Based on this model, the A function genes establish sepal identity alone and the combination A- and B-function genes specifies petal formation. The combination B- and C-function genes specifies stamen formation and the C function genes establish carpel identity alone (Weigel and Meyerowitz 1994, Theissen et al.

2000). In *Arabidopsis*, class A genes are represented by *APETALA1* (*AP1*) and *APETALA2* (*AP2*), class B genes comprise *APETALA3* (*AP3*) and *PISTILLATA* (*PI*), and class C gene is *AGAMOUS* (*AG*). The A-, B- and C-function genes, except for *AP2* gene, encode MADS-domain proteins which share a conserved structural organization, the MIKC-type domain structure, including MADS (M), intervening (I), keratin-like (K) and C-terminal (C) domains (Theissen et al. 1996, Theissen et al. 2000). Phylogeny reconstructions demonstrated that MADS-box gene family in different angiosperm species is composed of several defined gene clades (Theissen et al. 1996, Theissen et al. 2000). Most members of each clade share similar functions and expression patterns. For example, B-function genes fall into two closely related clades, namely *DEFICIENS* (*DEF*)- or *GLOBOSA* (*GLO*)-like genes, corresponding genes of *AP3* and *PI*, respectively.

Garden asparagus (*Asparagus officinalis* L.) is a dioecious and an economically important horticultural crop, which has a homochlamydeous flower and the perianth has two whorls of almost identical petaloid organs, called tepals. Like other monocotyledonous plants, such as lily and tulip, asparagus flowers have three outer tepals, three inner tepals, 3+3 stamens, and three carpels (Dahlgren et al. 1985). To explain the flower morphology of tulip, van Tunen et al. (1993) proposed a modified ABC model. In this model, class B genes are expressed in whorl 1 as well as in whorls 2 and 3, so that the organs of whorls 1 and 2 have the same petaloid structure. Recently, two types of class B genes, *TGDEFA*, *TGDEFB* and *TGGLO*, were isolated from tulip, and those genes expressed in whorl 1 as well as whorls 2 and 3 by Northern blot analysis which supports the modified ABC model (Kanno et al. 2003). We also isolated *AODEF* gene, an ortholog of eudicot *DEF*-like gene, from asparagus (Park et al. 2003). *AODEF* was a single copy gene and in situ hybridization showed that this gene was expressed in the inner tepals and stamens (whorls 2 and 3, respectively), but not in the outer tepals (whorl 1). This suggests that *AODEF* gene is not involved in the outer tepal development in asparagus. Then which gene specifies the outer tepal formation in asparagus?

One possibility is that the *GLO*-like gene alone would specify the outer tepal identity in asparagus. In higher eudicots, DEF- and GLO-like proteins cannot homodimerize but make heterodimers, and the B-function is provided by the heterodimers of DEF- and GLO-like proteins (Winter et al.

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                   MADS box
AOGLOA    MGRGKIEIKRIENSTNRQVTFSKRRNGIMKKAREISVPCESEISVVVFSSLGKISEFCSP
AOGLOB    MGRGKIEIKRIENSTNRQVTFSKRRNGIIKKAREISVLCEAQVSVLIFSSSGKISDYCSA
LRGLOA    MGRGKIEIKRIENSTNRQVTFSKRRNGIIKKAREISVLCEAQVSVVIFSSSGKMSEYCSP
OSMADS2   MGRGKIEIKRIENSTNRQVTFSKRRSGILKKAREISVLCDAEVGVVIFSSAGKLYDYCSP
PMADS2    MGRGKIEIKRIENSSNRQVTYSKRRNGIIKKAKEITVLCDAKVSLIIFGNSGKMHEYCSP
GLO       MGRGKIEIKRIENSSNRQVTYSKRRNGIMKKAKEISVLCDAHVSVIIFASSGKMHEFCSP
          ************:*****:****.**:***:**:***::.::..::*.. **: ::**.

                                         K domain
          NTTLPKMLEKYQQHSGKKLWDAKHENL SAEIERIKRENDNLQIKLRHLKGEDLNSLNPRE
          NTSFSRILERYQQNCGKKLWDANHESL SAQIDRIKKENDNMQIKLRHLKGEDLNPLTPKE
          STSLPKILERYQVNCGKIWDPKHEHL SAEIDRIKKENDNMQIELRHLKGEDLNSLQPKE
          KTSLSRILEKYQTNSGKILWDEKHKSL SAEIDRIKKENDNMQIELRHLKGEDLNSLQPKE
          STTLPDMLDGYQKTSGRRLWDAKHENL SNEIDRIKKENDNMQVKLRHLKGEDINSLNHKE
          STTLVDMLDHYHKLSGKRLWDPKHEHL DNEINRVKKENDSMQIELRHLKGEDITTLNYKE
          .*:: :*: *: .*: :** :*: *. ,:*:*:*:***.:*::*:*******::.* :*

          LIPIEEALQNGMTNVRAKQMDFLKMLKLNG KMLEEENEKLAYYLHHQELAMERNR-----
          LIPIEDVLQNGLDSVRDKQMNYLKMLKKNE RLLEDEQKRLTYILHHQQLAMEENVR-----
          LIPIEEALENGIRGVREKQNDFLRMLKKNE RILEEDNKRLTYILHHQQLAMDENMR-----
          LIMIEEALDNGIVNVNDKLMDHWERHVRTD KMLEDENKLLAFKLHQQDIALSGSMR-----
          LMVLEEGLTNGLSSISAKQSEILRMVRKND QTLEEEHKQLQYALHQKEMAAMGGNMRMIE
          LMVLEDALENGTSALKNKQMEFVRMMRKHN EMVEEENQSLQFKLRQMHLDPMNDNVMESQ
          *: :*: * ** : * :  .::*:::: * : *:: .:  .

          ----HGCHQGGTDYASE VQMALGVQPMQPNLQLAK
          -DLDLGFHQKNGAFATH MPMAFRVQPIQPNLQENK
          -NLEFAYHHKDGDFSSQ MPMAFRVQPIQPNLHEDK
          -DLELGYHP-DRDFAAQ MPITFRVQPSHPNLQENN
          EVYHQRDRD---YEYQQ MPFALRVQPMQPNLHERM
          AVYDHHHHQNIADYEAQ MPFAFRVQPMQPNLQERF
          :             .:  ::: *** :***:
                             PI Motif
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Fig. 1 Comparison of amino acid sequences among several *GLO*-like genes. The MADS box and K domain, and PI motif are shown in respective boxes. *GLO*-like genes from *A. officinalis* (*AOGLOA* and *AOGLOB*) are highlighted in bold. *OSMADS2* and *LRGLOA* are monocot *GLO*-like genes from *Oryza sativa* and *Lilium regale*, respectively. *PMADS2* and *GLO* are dicot *GLO*-like genes from *Petunia* and *Antirrhinum*. The consensus sequences are indicated by asterisks.

2002b). In some monocotyledonous plants, however, homodimerization of *GLO*-like gene products, LRGLOA and LRGLOB in lily and TGGLO in tulip, has been reported (Winter et al. 2002b, Kanno et al. 2003). Moreover, GGM2, ancestral class B genes from gymnosperms, make homodimers (Winter et al. 2002b) and transgenic *Arabidopsis* in which ectopic expression of this gene showed petaloid sepals as well as staminoid carpels, which is phenotypic features of both 35S::*PI* and 35S::*AP3* plants (Krizek and Meyerowitz 1996, Jack et al. 1994, Winter et al. 2002a). Gel retardation assay showed that GGM2 prefers to homodimerize, rather than to heterodimerize with DEF or GLO (Winter et al. 2002b). These data suggest that the class B gene function would be contributed by the GGM2 homodimers. The function of homodimers of monocot GLO-like proteins is unclear, however, there is a possibility that homodimers of the monocot GLO-like proteins may have a B-function and specify the outer tepal identity in homochlamydeous flowers.

In order to clarify the mechanism of the petaloid tepal development in asparagus, we have isolated and characterized *GLO*-like genes from asparagus and discussed how the class B genes are involved in the flower development in asparagus.

Results

cDNA cloning of two GLO-like genes from A. officinalis

To isolate MADS-box genes from *A. officinalis*, we used the 3′ rapid amplification of cDNA ends (RACE) method with degenerate primers specific for MADS-box region. The amplified DNA fragment was subcloned and sequenced. A BLAST search using the deduced amino acid sequence of these clones led to the identification of several MADS-box genes. Two of

these clones showed high sequence similarity to maize *ZMM16* (Münster et al. 2001), rice *OSMADS2* (Chung et al. 1995), *Arabidopsis PISTILLATA* (*PI*) and *Antirrhinum GLOBOSA* (*GLO*) genes, which are *GLO*-like genes in higher plants. Two *GLO*-like genes isolated from *A. officinalis* were named *AOGLOA* and *AOGLOB*. The 5′-regions corresponding to the cDNAs were isolated by using the 5′/3′ RACE kit, and cDNA clones comprising the complete coding regions were isolated by PCR. *AOGLOA* cDNA was 900 bp in length and encoded a putative protein of 206 amino acids and *AOGLOB* cDNA was 870 bp and 210 amino acids. The amino acid sequence of *AOGLOA* showed 68% similarity to that of *AOGLOB* (Fig. 1). Multiple sequence alignments with other GLO-like proteins demonstrated that AOGLOA and AOGLOB proteins have a typical MIKC-type domain structure (Fig. 1, Theissen et al. 1996, Theissen et al. 2000, Münster et al. 1997) and the PI motif, MPFx-FRVQPxQPNLQE, which is consensus sequence in the C-domain of PI (GLO) subfamily genes (Kramer et al. 1998).

Phylogeny reconstructions with other known MADS-box genes indicate that *AOGLOA* and *AOGLOB* genes are included in GLO subfamily (Fig. 2). These genes are most closely related to other monocot MADS-box genes and monophyly of monocot *GLO*-like genes are well supported (Fig. 2). In the group of monocot *GLO*-like genes, *AOGLOA* and *AOGLOB* are divided into two small clusters; *AOGLOA* is closely related to *HPI1* (*Hyacinthus*), and *AOGLOB* is related to *OrcPI* (*Orchis*), *LRGLOA* (*Lilium*) and *TGGLO* (*Tulipa*). *MAGLOA* and *MAGLOB*, *GLO*-like genes from *Muscari* (Nakada and Kanno, unpublished data), and *HPI1* fall into each cluster, indicating that two types of *GLO*-like gene might exist in Asparagales, which includes *Hyacinthus*, *Muscari* and *Asparagus*.
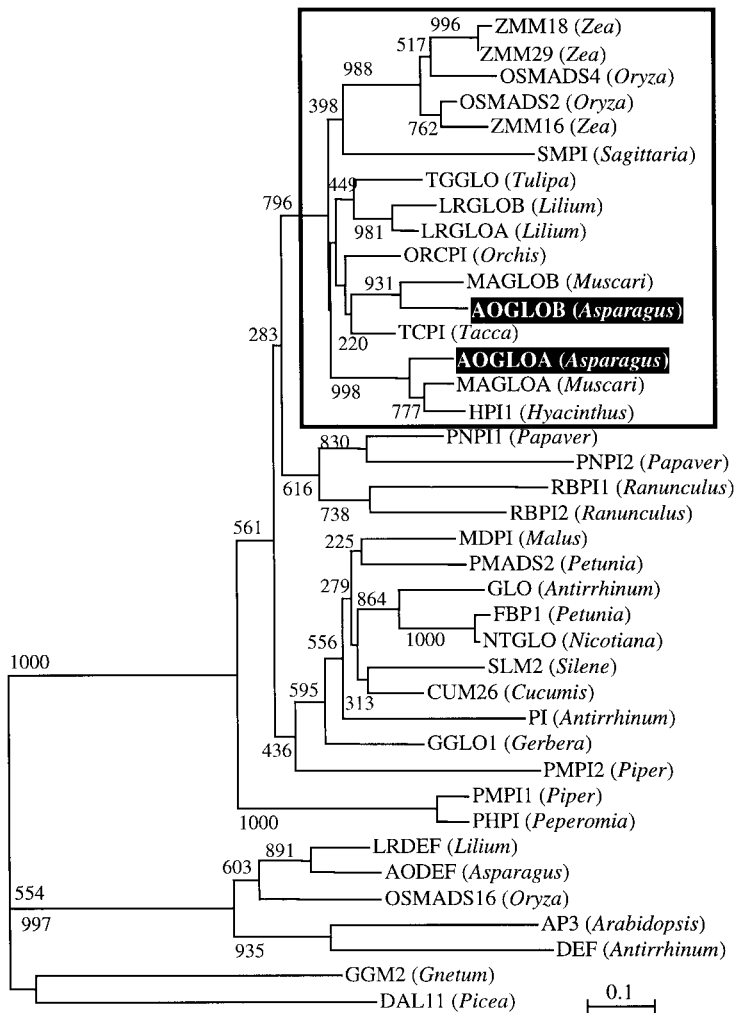
Fig. 2 Phylogeny reconstruction of plant MADS-box genes which belong to the GLO subfamily. This neighbor-joining tree was generated by ClustalW. The numbers next to the nodes give bootstrap values from 1,000 replicates. *AOGLOA* and *AOGLOB* genes are shown by outline letters on solid boxes and the monocot *GLO*-like genes are enclosed by a frame.

Southern blot analysis

DNA gel blot analysis was performed to determine the copy number of *GLO*-like genes in *A. officinalis*. Genomic DNA was digested with *Bam*HI, *Eco*RI, *Hin*dIII and *Sac*I and hybridized with specific probes of *AOGLOA* and *AOGLOB* genes. One or two hybridizing bands were found in each lane, indicating that *AOGLOA* and *AOGLOB* genes are single copy genes (Fig. 3).

Northern blot analysis

To investigate the expression patterns of *AOGLOA* and *AOGLOB* genes, we performed Northern blot analysis. Total RNA was isolated from roots, stems, phylloclades, and flower buds of male and female individuals. To avoid cross hybridization, the 3′-specific cDNA fragments of *AOGLOA* and *AOGLOB* were used as probe. As shown in Fig. 4, *AOGLOA* and *AOGLOB* were specifically expressed in male and female flowers but no signal was detected in vegetative organs, such as roots, stems and phylloclades. Two *GLO*-like genes of *A. officinalis* were strongly expressed in male flowers, but the hybridization signals are weaker in female flowers.
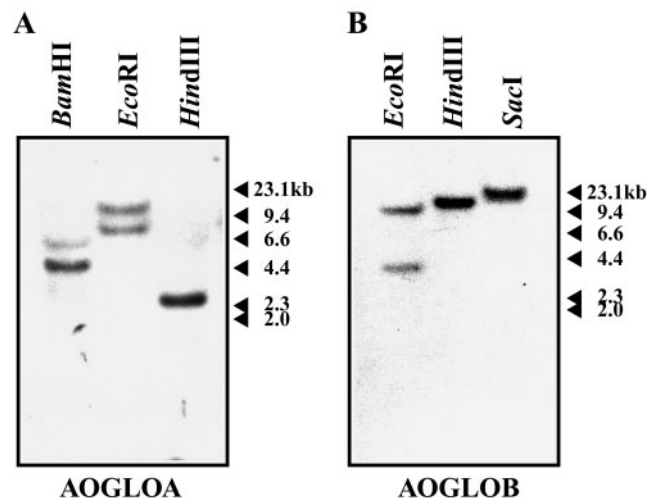

Fig. 3 Southern blot analysis of *AOGLOA* (A) and *AOGLOB* (B) genes in *A. officinalis*. Each lane contains 10 μg of total DNA digested with each of restriction enzymes (*Bam*HI, *Eco*RI, *Hin*dIII, *Sac*I).
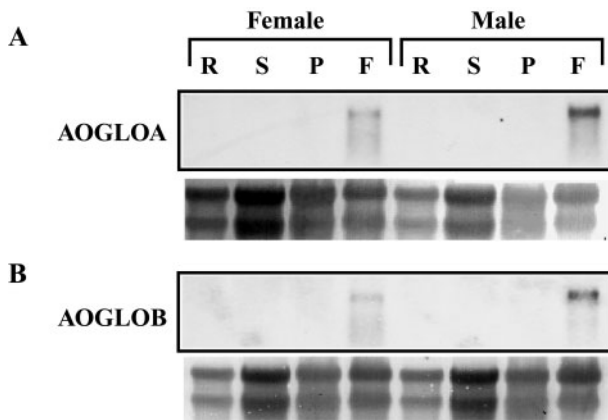
A

	Female	Male

Fig. 4 Northern blot analysis of *AOGLOA* (A) and *AOGLOB* (B) genes in *A. officinalis*. Each lane contains 10 µg of total RNA isolated from roots (R), stems (S), phylloclades (P) and flower buds (F). Probes specific for *AOGLOA* and *AOGLOB* were used for hybridization. The panels below the Northern blots show the ribosomal RNA stained by methylene blue.

In situ hybridization

To investigate the pattern of expression of during floral development, we performed in situ hybridization. Digoxygenin-labeled DNA probes from the specific 3′ end of *AOGLOA* and *AOGLOB* cDNAs were used to identify transcripts of these genes in various developmental stages of male and female flowers. We referred to the stages of *A. officinalis* flower development outlined by Caporali et al. (1994).

As shown in Fig. 5A and 6A, *AOGLOA* and *AOGLOB* genes were expressed in floral primordia of both male and female inflorescences in early hermaphroditic stages, and the expression of *AOGLOA* gene was weaker than that of *AOGLOB* gene in both male and female flowers. *AOGLOA* gene was expressed in central dome of the meristem (Fig. 5B) and the expression was confined to the inner tepal and stamen primordia (Fig. 5C). No expression was observed in the central whorl that would give rise to the carpel (Fig. 5B–D). When tepals and stamens developed from floral primordia, *AOGLOA* transcripts were observed in the inner tepal and stamen but not in the outer tepal (Fig. 5D).

After the transition to the unisexual developmental pathway (stage −1), the expression of *AOGLOA* gene was maintained in second and third whorls of male flowers (Fig. 5E). Additionally, the expression was barely detectable in ovary walls in this stage (Fig. 5F). *AOGLOA* expression of female flower in this stage became weaker in the inner tepal and stamen than that of male flowers (Fig. 5G, H). Also, this transcript was observed in the ovule in female flowers.

The *AOGLOB* gene is strongly expressed in floral primordia of male and female inflorescences (Fig. 6A–C). The gene was expressed in the inner tepal and stamen and no signal was observed in the outer tepal and carpel primordia in both male and female flowers in hermaphroditic stages (Fig. 6C, D).

In unisexual developmental stages, expression of *AOGLOB* gene in inner tepal and stamen of male flower was

clear (Fig. 6E). The expression of this gene was observed in ovary walls in this stage as that of *AOGLOA* gene (Fig. 6F). The transcript of *AOGLOB* was maintained in male flower; however, the expression became weaker in the inner tepal of female flower (Fig. 6G, H). Also, *AOGLOB* gene was expressed in the ovule in female flower (Fig. 6H).

Discussion

In this study, two *GLO*-like MADS-box genes, *AOGLOA* and *AOGLOB*, were isolated from *A. officinalis*. Phylogeny reconstruction showed that *AOGLOA* and *AOGLOB* genes belong to *GLO* subfamily and these genes are closely related to other monocot *GLO*-like genes. Also, C-domain region of *AOGLOA* and *AOGLOB* genes includes a PI-motif, which is a conserved sequence in the *PI* (or *GLO*) family genes (Kramer et al. 1998). These data suggest that *AOGLOA* and *AOGLOB* genes of asparagus are the cognate orthologs of *GLO*. Southern blot analysis indicates that *AOGLOA* and *AOGLOB* genes are single copy genes in asparagus genome. Northern blot analysis showed that these genes are specifically expressed in male and female flowers. Hybridization signals of *AOGLOA* and *AOGLOB* genes were detected in whorls 2 and 3 (inner tepal and stamen), and not in whorl 1 (outer tepal) by in situ hybridization. In our previous report (Park et al. 2003), the other class B gene in asparagus, *AODEF*, has been isolated and this gene was expressed in the inner tepals and stamens (whorls 2 and 3, respectively), but not in the outer tepal (whorl 1). Although the homodimerization of AOGLOA and AOGLOB have not been analyzed yet, our expression data of *AOGLOA*, *AOGLOB* and *AODEF* genes did not support the modified ABC model in asparagus (this study, Park et al. 2003) and also that the asparagus class B genes are not involved in outer tepal development although asparagus has a homochlamydeous flower. This is the first evidence that the B function is not conserved in petaloid tepal identity in monocots. In lower eudicots and basal angiosperms, *DEF*- and *GLO*-like genes are continually expressed during the development of stamens but variably in petals (Kramer and Irish 1999, Kramer and Irish 2000), and it is unclear that what kind of genes would specify petal identity in basal angiosperms. In order to characterize the role of class B genes in asparagus, functional analyses of these genes are needed with transformation into *Arabidopsis* and asparagus. Moreover, *SEP* gene, along with the ABC genes, is required to specify the floral organ identity in *Arabidopsis* (Goto et al. 2001), and SEP protein interacts with PI, AP3 and AP1 proteins in whorl 2 (Goto et al. 2001, Theissen 2001). One *SEP*-like gene, *AOM1*, was isolated from asparagus and this gene was expressed in the floral meristems and the primordia of all floral organs (Caporali et al. 2000). Since the class B genes, *AODEF*, *AOGLOA* and *AOGLOB* genes, are expressed in whorl 2 as well as *AOM1* gene, it is very interesting to analyze the protein–protein interaction between SEP-like protein and class B proteins in asparagus.
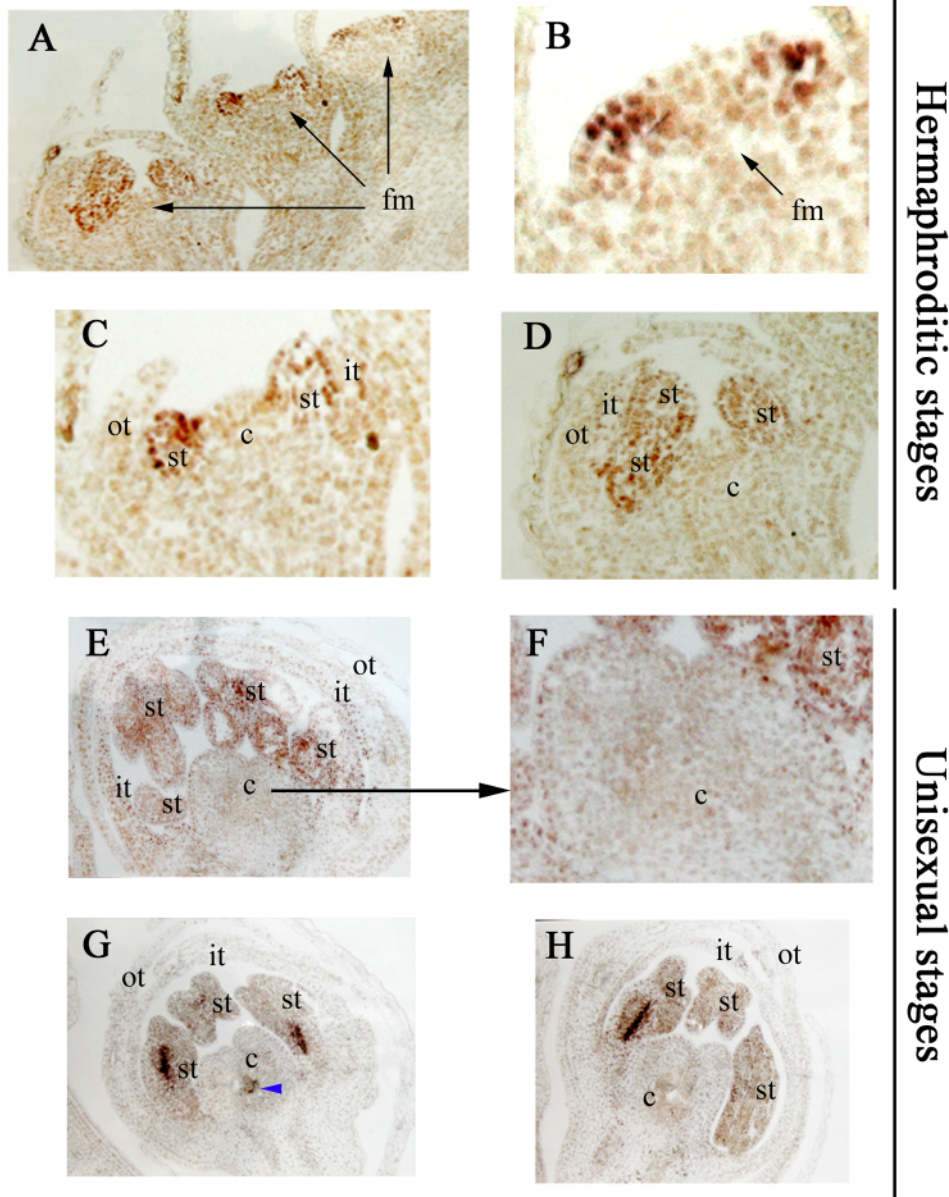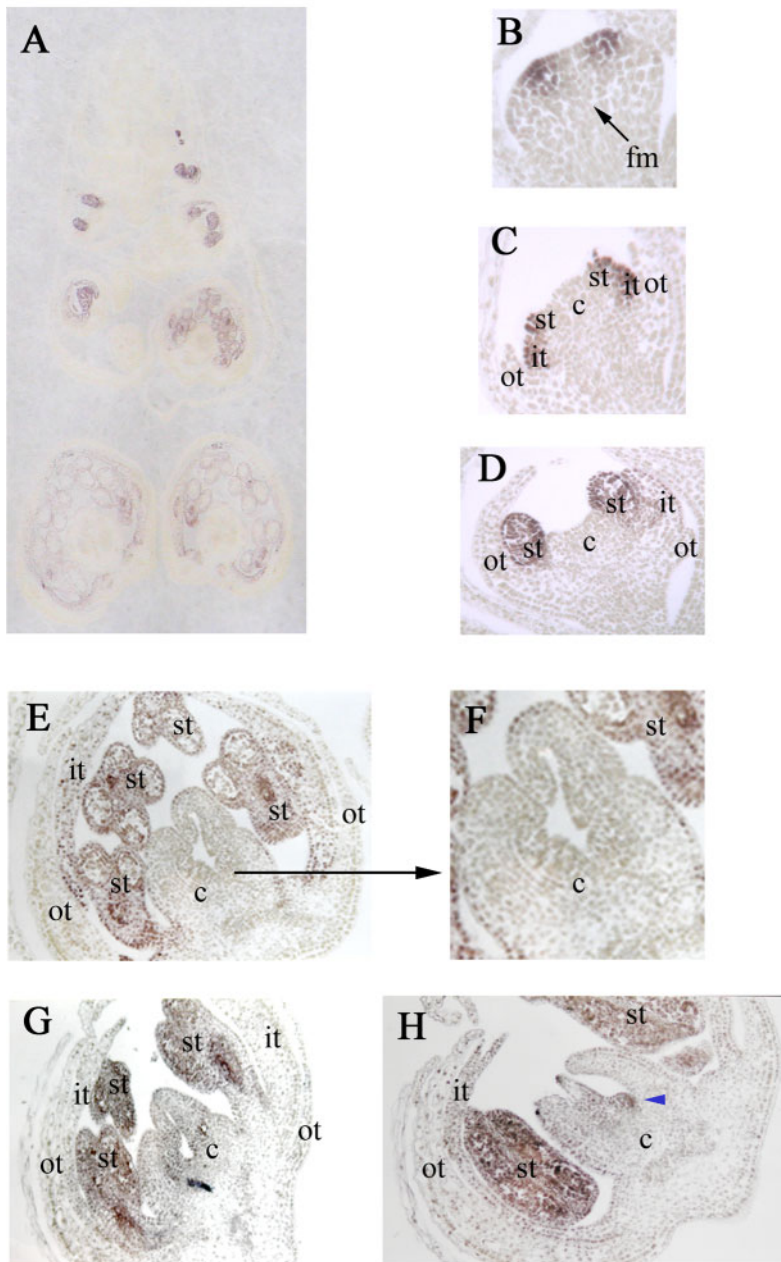
Fig. 5 Accumulation of *AOGLOA* mRNA on longitudinal sections of developing asparagus flowers. (A) Reproductive apex with developing flowers. DNA probe specific for *AOGLOA* transcripts was used. (B, C) Male flowers at hermaphroditic stage (stage –6 to –5). The hybridization signal is visible in the floral meristems, and primordia of inner tepals and stamens (stage –6 to –4). (D) Male flowers at hermaphroditic stage (stage –4 to –3). The hybridization signal is present in inner tepals and stamens. (E) Male flower at unisexual stage (stage –1 to 3). The signal is maintained in inner tepals and stamens. (F) Close-up of the carpel in male flower. The signal is observed in ovary wall. (G, H) Female flowers at unisexual stage (stage –1 to 3). *AOGLOA* is weakly expressed in inner tepals and stamens. Also, the signal is observed in ovule that is indicated by an arrowhead. fm, floral meristem; ot, outer tepal; it, inner tepal; st, stamen; c, carpel.

In some dioecious plants, the relationship between floral organ development and sexual differentiation has been investigated by analyzing the expression of floral homeotic genes. In the dioecious plant white campion, A-, B- and C-functional genes are isolated and characterized (Hardenack et al. 1994). In male flowers of white campion, the fourth whorl development is altered in early stage. The expression of *SLM2* and *SLM3*, class B genes of white campion, is different in male and female flowers. This result suggests that the white campion gynoecium-suppressing genes act upon the factors that determine the boundaries of the whorls and *SLM2* and *SLM3* transcription is affected as a consequence. But definite relationship between sex determination and the expression of *SLM2* and *SLM3* is not yet known. *A. officinalis* flowers possess stamen and carpel in early flower development stages. In late developmental stages, however, the stamen in female flower is degen-

Hermaphroditic stages

Unisexual stages

Fig. 6 Accumulation of *AOGLOB* mRNA on longitudinal sections of developing asparagus flowers. (A) Reproductive apex with developing flowers. DNA probe specific for *AOGLOB* transcripts was used. (B, C) Male flowers at hermaphroditic stage (stage –6 to –5). The hybridization signal is clearly visible in the floral meristems, and primordia of inner tepals and stamens (stage –6 to –4). (D) Male flowers at hermaphroditic stage (stage –4 to –3). *AOGLOB* is expressed in inner tepals and stamens. (E) Male flower at unisexual stage (stage −1 to 3). It is equally expressed in hermaphroditic and unisexual stages. (F) Close-up of the carpel in male flower. The signal is observed in ovary wall as well as *AOGLOA*. (G, H) Female flowers at unisexual stage (stage −1 to 3). When the signal is compared with male flowers, *AOGLOB* is weakly expressed in inner tepals and stamens of female flowers. Also, the signal is observed in ovule that is indicated by an arrowhead.

erated and the development of carpel in male flower is arrested. The class B genes, *AOGLOA* and *AOGLOB*, show different expression pattern in late developmental stages of flowers in both sexes. The expression of *AOGLOA* and *AOGLOB* is reduced and disappeared in whorls 2 and 3 in unisexual stages of female flowers; however, the expression is maintained in this stage of male flowers. Based on these results, we suggest that the expression of *AOGLOA* and *AOGLOB* may be affected by the degeneration of stamen in the female flower. Further experiments will be needed to clarify the relationship between the expression of class B genes and flower organ degeneration.

Materials and Methods

Plant material

Plants of *A. officinalis* L. cv. Mary Washington 500W were used in this work. The plants were grown in the field at Tohoku University, Japan. For Southern and Northern blot analyses, flower buds, roots, stems and phylloclades, which are needle-like branchlets, were frozen in liquid nitrogen immediately after collection and stored at –80°C.

cDNA cloning

Partial cDNAs were isolated using the 3′ RACE method (Frohman et al. 1988, Münster et al. 1997) using 5′/3′-RACE kit (Roche Diagnostics Co., U.S.A.). As template, poly (A)+ RNA pre-

pared from male flower buds of *A. officinalis* was used. Three MADS-box degenerate primers (5′-GACARGTCACKTTYTCKAAGC-3′, 5′-GATCAAGMGSATCGAGAA-3′, 5′-GATGAAGMGSATCGAGAA-3′) were used for the 3′ RACE procedures. To identify full-length cDNA sequence, we performed 5′ RACE using 5′/3′-RACE kit (Roche). The sequences of the primer used in 5′ RACE procedures were SP1 (5′-GCCATTCTGCAAGGCTTCTT-3′), SP2 (5′-CGTTCAATCTCCGC-ACTCAA-3′) and SP3 (5′-GGGCTACAGAACTCGCTAAT-3′) for *AOGLOA*, and SP1 (5′-GGTC AATCTGGGCACTTAGA-3′), SP2 (5′-CCTTGAGAACGAGGTGTTAG-3′) and SP3 (5′-GCCTTCTTGATG-ATCCCGTT-3′) for *AOGLOB*. PCR products were cloned into pGEM-T vector (Promega, U.S.A.). Sequencing of cDNAs was performed with the ABI PRISM dye terminator kit (PE Applied Biosystems, U.S.A.) according to the manufacturer's protocol.

Phylogenetic analysis

Predicted amino acid sequences were aligned using ClustalW program (Thompson et al. 1994). The continuous region from MADS to K domains including the less conserved I region was used for phylogenetic analysis. Phylogenetic tree was constructed by the neighbor joining method. Bootstrap values were derived from 1,000 replicate runs (Thompson et al. 1994). Neighbor-joining trees were illustrated with NJplot (Perrière and Gouy 1996). The GenBank accession numbers of the amino acid sequences used are: *AODEF* (AB094964), *AP3* (M86357), *CUM26* (AF043255), *DAL11–1* (AF158539), *DEF* (X52023), *FBP1* (M91190), *GGLO1* (AJ009726), *GGM2* (AJ132208), *GLO* (X68831), *HPI1* (AF134114), *LRDEF* (AB071378), *LRGLOA* (AB071379), *LRGLOB* (AB071380), *MDPI* (AJ291490), *NTGLO* (X67959), *ORCPI* (AB094985), *OSMADS16* (AF077760), *OSMADS2* (L37526), *OSMADS4* (L37527), *PHPI* (AF052865), *PI* (D30807), *PMADS2* (X69947), *PMPI1* (AF052866), *PMPI2* (AF052867), *PNPI1* (AF052855), *PNPI2* (AF052856), *RBPI1* (AF052859), *RBPI2* (AF052860), *SLM2* (X80489), *SMPI* (AF230712), *TCPI* (AF230713), *TGGLO* (AB094967), *ZMM16* (AJ292959), *ZMM18* (AJ292960), *ZMM29* (AJ292961).

Southern blot analysis

Total DNA was isolated from phylloclades by the CTAB method (Murray and Thompson 1980). Ten μg of total DNA was digested with each of restriction enzymes (*Bam*HI, *Eco*RI, *Hin*dIII, *Sac*I), electrophoresed on 0.6% agarose gel and blotted onto positively charged membrane (Roche). Hybridization was performed with DIG Luminescent Detection Kit (Roche) as recommended by the supplier under strict conditions. The respective C domains and 3′-UTR region of *AOGLOA* and *AOGLOB* cDNA fragments were labeled with DIG-High Prime kit (Roche) as probe.

Northern blot analysis

Total RNA was isolated from flower buds (>3 mm), phylloclades, stems, roots by SDS-phenol method (Kisaka et al. 1996). Ten μg of total RNA were separated by electrophoresis on 1.2% agarose gels containing 5% formaldehyde and 1× MOPS. The gel was blotted onto positively charged nylon membrane (Roche) using standard blotting techniques overnight (Sambrook and Russell 2001). The respective C domains and 3′-UTR region of *AOGLOA* and *AOGLOB* cDNA fragments were used as probe and labeled with DIG-High Prime Kit (Roche). Hybridization was performed with DIG Luminescent Detection Kit (Roche).

In situ hybridization

Male and female flowers at various developmental stages were collected from *A. officinalis*. Samples were fixed in FAA (50% ethanol, 5% acetic acid and 3.7% formaldehyde), embedded in Paraplast

Plus (Sigma-Aldrich). The paraffin-embedded tissues were sliced into 12 μm sections with a rotary microtome and then attached to microscopic slides. The sections were treated with 1 μg ml⁻¹ proteinase K for 30 min at 37°C. Tissue sections were prehybridized for 2 h at 42°C with a solution containing 50% formamide, 0.5× blocking reagent, 4× SSC and 0.15 mg ml⁻¹ tRNA. The downstream region of K domain in each *AOGLOA* and *AOGLOB* cDNA fragments was labeled with PCR DIG labeling mix (Roche) as probe. The labeled DNA probes were hybridized to the tissue sections. After hybridization, the tissues were washed with 50% formamide, 4× SSC for 20 min at 42°C.

References

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