Identification and Characterization of Three Orchid MADS-Box Genes of the AP1/AGL9 Subfamily during Floral Transition[1]

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Gene expressions associated with in vitro floral transition in an orchid hybrid (*Dendrobium* grex Madame Thong-In) were investigated by differential display. One clone, orchid transitional growth related gene 7 (otg7), encoding a new MADS-box gene, was identified to be specifically expressed in the transitional shoot apical meristem (TSAM). Using this clone as a probe, three orchid MADS-box genes, *DOMADS1*, *DOMADS2*, and *DOMADS3*, were subsequently isolated from the TSAM cDNA library. Phylogenetic analyses show that *DOMADS1* and *DOMADS2* are new members of the AGL2 subfamily and SQUA subfamily, respectively. *DOMADS3* contains the signature amino acids as with the members in the independent OSMADS1 subfamily separated from the AGL2 subfamily. All three of the *DOMADS* genes were expressed in the TSAM during floral transition and later in mature flowers. *DOMADS1* RNA was uniformly expressed in both of the inflorescence meristem and the floral primordium and later localized in all of the floral organs. *DOMADS2* showed a novel expression pattern that has not been previously characterized for any other MADS-box genes. *DOMADS2* transcript was expressed early in the 6-week-old vegetative shoot apical meristem in which the obvious morphological change to floral development had yet to occur. It was expressed throughout the process of floral transition and later in the columns of mature flowers. The onset of *DOMADS3* transcription was in the early TSAM at the stage before the differentiation of the first flower primordium. Later, *DOMADS3* transcript was only detectable in the pedicel tissues. Our results suggest that the *DOMADS* genes play important roles in the process of floral transition.

The transition to flowering, the first step in flower development, is triggered by a number of environmental and endogenous signals. In most plant species the transitional phase in the shoot apical meristem (SAM) can be generally divided into three stages: the shift from the vegetative to the inflorescence meristem, the maintaining of the inflorescence meristem, and the differentiation of the floral meristem from the inflorescence meristem (Meyerowitz et al., 1991; Ma, 1994). A variety of genes combined with external and internal cues are involved in the series of biochemical and physiological changes leading to floral induction (McDaniel et al., 1992; Bernier et al., 1993; Levy and Dean, 1998).

Rapid progress is being made in elucidating the molecular mechanisms involved in the floral transition. Particularly, a number of MADS-box genes that function in various steps of the transition from vegetative to reproductive growth have been identified in different plant species. Plant MADS-box genes represent a large family of transcription factors that contain a highly conserved DNA-binding domain (MADS-box) and a second conserved domain (K-box), which is involved in protein-protein interactions (Schwarz-Sommer et al., 1990; Ma et al., 1991). Early acting MADS-box genes during the transition to flowering, such as *SLM4* and *SLM5* from white campion (Hardenack et al., 1994), *AGL8* from Arabidopsis (Mandel and Yanofsky, 1995), *SaMADSA*, *SaMADSB*, and *SaMADSD* from white mustard (Melzer et al., 1996; Bonhomme et al., 1997), and *MdMADS2* from apple (An et al., 1999), are expressed early in the inflorescence meristem. Before the initiation of floral organ primordia, the regulation of floral meristem initiation and development involves many other MADS-box genes, such as *AP1*, *CAL*, *AGL2*, *AGL4*, and *AGL9* from Arabidopsis (Mandel et al., 1992; Bowman et al., 1993; Flanagan and Ma, 1994; Gustafson-Brown et al., 1994; Kempin et al., 1995; Savidge et al., 1995; Mandel and Yanofsky, 1998), *SQUA* from snapdragon (Huijser et al., 1992), *TM5* from tomato (Pnueli et al., 1994), *FBP2* from petunia (Angenent et al., 1994), *OsMADS1* from rice (Chung et al., 1994), and *SaMADSC* from white mustard (Melzer et al., 1996). Phylogenetic analyses of these genes show that almost all of the plant MADS-box genes that are involved in the floral transition belong to the AP1/AGL9 subfamily (Purugganan et al., 1995; Theissen and Saedler, 1996).

Orchids are members of the family Orchidaceae, one of the largest families of flowering plants. Like other flowering plants, the development of orchid flowers begins with the floral transition and continues with the initiation and formation of floral organs. Much information concerning the orchid floral devel-

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opment has been obtained through horticultural and physiological studies (Goh, 1977; Goh and Arditti, 1985). In contrast to a large amount of information concerning the molecular mechanism of floral development in several other flowering plants, very limited molecular studies have been undertaken on orchid floral development (Lu et al., 1993). In recent years in vitro techniques for micropropagation and flowering of orchids have opened new avenues of research into the flowering process (Lakshmanan et al., 1995; Goh, 1996). The shortening of juvenile phase from several years to only a few months and the obvious "landmark" events during floral transition are very helpful in studying the molecular mechanisms involved in the transition from vegetative to reproductive growth in orchids.

In our effort to study the molecular mechanism of floral transition in orchids we have identified eight genes differentially expressed in the transitional shoot apical meristem (TSAM) during the switch from vegetative to reproductive growth by using mRNA differential display method (Liang and Pardee, 1992). One of these genes, otg7 (orchid transitional growth related gene), encodes a new MADS-box transcription factor of the AP1/AGL9 subfamily. Three new members of AP1/AGL9 subfamily were subsequently isolated with the otg7 probe from the cDNA library derived from the TSAM.

The identification and characterization of three new members of the AP1/AGL9 subfamily for the first time provide some detailed information concerning the possible functions of the orchid MADS-box genes in the conversion from vegetative to reproductive growth. Understanding of the function of orchid MADS-box genes and those of orthologs from other plant species would contribute to the elucidation of molecular regulation during floral transition.

RESULTS

Isolation of a MADS-Box Clone Differentially Expressed in the TSAM

In our effort to identify genes in the shoot apical meristem associated with the transition from vegetative to reproductive development, mRNA differential display (Liang and Pardee, 1992) was performed to simultaneously detect genes differentially expressed in the vegetative shoot apical meristems (VSAMs; 6-week-old culture) and TSAM (12-week-old culture). One amplified band (otg7) around 660 bp in size appeared to be present only in the TSAM (Fig. 1). The differential expression pattern of otg7 was further confirmed by DNA dot-blot analysis and northern-blot analysis (data not shown). After sequencing, the gene was subsequently identified as a new member of the MADS-box gene family, most of which play important roles in the regulation of the flowering and flower development. To further study the functions of the MADS-box genes involved in the floral transition in orchids, the otg7 clone was used to screen the cDNA library prepared from the 12-week-old TSAM.

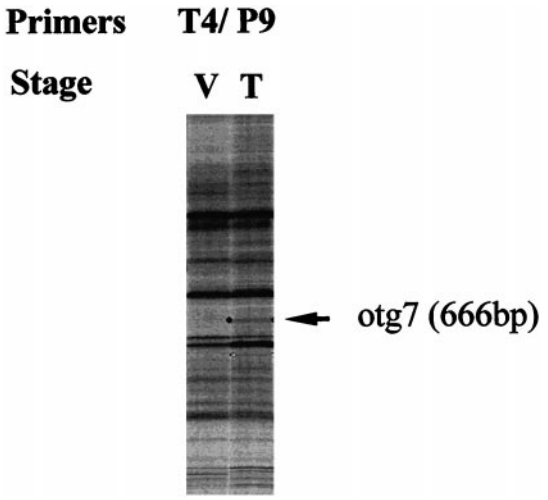

Figure 1. mRNA differential display. Total RNA from VSAM (V) and TSAM (T) were treated with RNase-free DNase I and analyzed by differential display using T4 and P9 primers. Amplified products were separated on 5% (w/v) denaturing polyacrylamide gels under thermostatic conditions. The arrow indicates the gene (otg7) differentially expressed in the TSAM.

Isolation and Sequence Analysis of Three New Members of the AP1/AGL9 Subfamily

A total of 5×10^5 independent plaques were screened under non-stringent conditions. Fifteen positive clones were isolated and sorted into three distinct groups based on their restriction mapping and sequencing results. These results also indicated that clones of each group were coding for the same gene. Thus, the longest clones of each of the groups, designated *DOMADS1* (accession no. AF198174), *DOMADS2* (accession no. AF198175), and *DOMADS3* (accession no. AF198176), were selected for further study. The sequence for *DOMADS1*, *DOMADS2*, and *DOMADS3* each contains a full-length coding region as well as untranslated sequences on both ends. DNA sequence analysis also showed that the otg7 probe is almost identical to *DOMADS1* gene except several base-pair mismatches on both ends of the probe. The deduced amino acid sequences of the three cDNAs are shown in Figure 2. All three of the cDNAs encode proteins that contain 56 conserved amino acid residues of the MADS-box domain at their N-terminal ends and the K-box domain located between residues 91 and 157 (92 and 158 for *DOMADS2*). The MADS-box regions among three genes share above 82% identities, and the sequence conservation in the K-box is between 50% and 65%. The comparison of the predicted protein sequences of *DOMADS* genes with other MADS-box genes in the database showed that *DOMADS1* and *DOMADS3* are most homologous to the *AGL2* and *AGL9* from Arabidopsis (Flanagan and Ma, 1994;

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DOMADS1    MGRGRVEMKRIENKINRQVTFAKRRTGLLKKAYELSVLCDVEVALIIFSNRGKLYEFCSS 60
DOMADS2    .....QL.............S...S.....H.I.....A.....V...K.....YSTD 60
DOMADS3    .....L.................N.............A.....V.....R.F..... 60

DOMADS1    RSMLKTLEKYQKCSDGAPEMTMTSRETQSS-QVEYLKLKSQVEALQRSQRNLLGEDLNPL 119
DOMADS2    S..E.I..R.ERY.YAERALFSNEANP.ADWRL..N...AR..S..K...H.M..Q.DS. 120
DOMADS3    T..T....R.....YN.S.SAVP.KDA.N.-YH...T..AK..Y.....G.......IE. 119

DOMADS1    GGKDLDQLERQLEASLKQIISTRMQYMLDQLGDLQQRELLLFETNKSLGTRV-------- 171
DOMADS2    SI.E.QR..Q...S...F.R.RKT.LI.HSISE..KM.KI.L.Q..T.EKEII.KEKAKA 180
DOMADS3    SS.E.....L...M.....R..KT.L.....C.IKRK.QM.H.A.RA.SMKLKEDGPEIP 179

DOMADS1    ---------------SAL*------------------------------------- 174
DOMADS2    LVQHAPWEKQNQSQYS...PPVISDSVPTPTSRTFQARANEEESPQPQLRVSNTLLPPWM 240
DOMADS3    LELSWPGGETNGSS-ERQQPQ--SDKFFQPLPCS-NPSLQIGYSP*-------------- 220

DOMADS1    -------
DOMADS2    LSHMNGQ* 247
DOMADS3    -------
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Figure 2. Alignment of the deduced amino acid sequences of *DOMADS1*, *DOMADS2*, and *DOMADS3*. Black box, MADS domain; gray box, K-box region. Identical residues to the *DOMADS1* reference sequence are indicated by dots, and gaps introduced to maximize the alignment are shown by dashes. The positions of amino acids are shown on the right.

Mandel and Yanofsky, 1998), *DEFH72* and *DEFH200* from snapdragon (Davies et al., 1996), and *FBP2* from petunia (Angenent et al., 1992). The derived protein sequence of *DOMADS2* shares strong homology with the maize *ZAP1 (*Mena et al., 1995) and the apple *MdMADS2* (Sung et al., 1999).

To determine the evolutionary relationship between *DOMADS* genes and the MADS-box genes from other plant species, a phylogenetic tree based on analysis of the MIK region was constructed (Fig. 3). The tree showed that *DOMADS1* and *DOMADS2* are new members of the AGL2 subfamily and SQUA subfamily (Purugganan et al., 1995; Theissen et al., 1996; Münster et al., 1997), respectively. *DOMADS3* was clustered together with *OSMADS1*, *OSMADS5*, *ZMM3*, and *ZMM8.* This cluster is separated from the AGL2 subfamily for the first time as a separate gene clade, designated as the OSMADS1 subfamily. It is apparent from Figure 4 that the AGL2 and OSMADS1 subfamily can be distinguished from each other with the distinct characteristic amino acids of the second β-pleated sheet in the MADS-domain (Theissen et al., 1996).

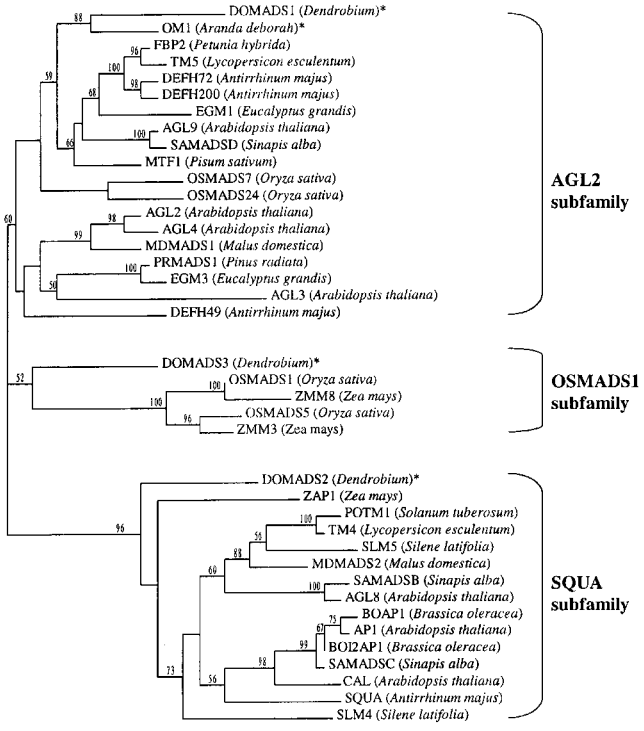

Figure 3. Phylogenetic tree of plant MADS-box genes in the AGL2, OSMADS1, and SQUA subfamilies. Orchid MADS-box proteins are indicated by asterisks. Genus names of respective species are given in the parentheses behind the corresponding protein names. Subfamilies generally representing monophyletic gene clades (Theissen et al., 1996) are indicated by brackets at the right margin. The subfamily of OSMADS1-like genes appears here for the first time as an independent clade. The horizontal branch length is proportional to the estimated number of base substitutions. The numbers next to the nodes indicate bootstrap values (>50%) in 100 replicates.

Genomic Organization of *DOMADS* Genes

DNA gel-blot analysis of orchid genomic DNA digested with several enzymes revealed that a large number of bands were produced with the probe containing the conserved MADS-box region of *DOMADS1* (data not shown). This indicated that a high number of MADS-box genes are present in the orchid genome.

To further investigate the genomic organization of the respective *DOMADS* genes in the genome, the 3′ end of each gene was prepared and used as a probe under high-stringent conditions for the southern blot of orchid genomic DNA digested with *Eco*RI, *Eco*RV, and *Xho*I (Fig. 5). Genomic DNA-blot analysis re-

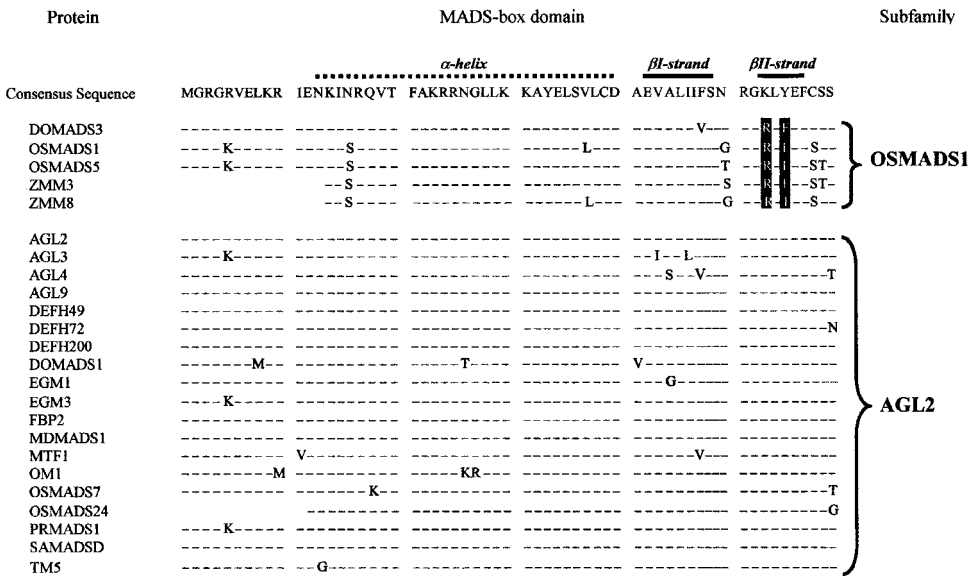

Figure 4. Comparison of MADS-box domains between the genes in the AGL2 and OSMADS1 subfamilies. A consensus sequence (amino acids most frequently encountered at each particular position) is displayed, and only the deviations from consensus are listed in the individual sequence. The identical amino acids with consensus sequence are represented by dashes, and the missing data are indicated by blanks. The α-helix and two β-strands in the MADS-box domain are indicated respectively by the square dots and the lines above the consensus sequence. The characteristic amino acids of the OSMADS1 subfamily, which is distinguished from the AGL2 subfamily, are in black boxes.

vealed that a single, strong band was evident in most of the digests in all three of the blots, indicating the possibilities of the 3′-end regions acting as gene-specific probes. In the *DOMADS2* and *DOMADS3* blots the weakly hybridizing bands in some digests indicated the possible presence of restriction sites within the corresponding orchid genomic DNA.

Northern-Blot Analysis of Differential Expression of *DOMADS* Genes

Under our culture conditions, thin sections (1 mm) of protocorms produce 0.5-cm-long protocorm-like bodies (PLBs) within a month and continue to develop into shoot with the typical VSAM over the next 5 weeks. Most of shoots produce the typical TSAM with narrowing of two visible youngest leaves toward the apex after another 5 weeks in culture. Following this, the growth of the plantlet enters into the reproductive stage. After 15 weeks in culture, the terminal inflorescence has developed to more than 3 mm in length. By 18 weeks, the differentiated floral buds are mostly 2 to 4 mm in length. Northern-blot analysis was undertaken to investigate the different developmental stages at which *DOMADS1*, *DOMADS2*, and *DOMADS3* were expressed during in vitro flowering. To avoid cross-hybridization with other MADS-box genes, the 3′ end of *DOMADS* genes, which are the most divergent regions, were used as probes in all of the northern-blot analyses. As shown in Figure 6, both of the transcripts of *DOMADS1* and *DOMADS3* were first detected in the TSAM of 12-week-old culture. This is the stage in which the first flower primordium just differentiated on the flank of the apical meristem. In the shoot apex (SA) of 15-week-old culture, expression of *DOMADS1* was gradually increased in the inflorescence meristem and later in the floral bud (18-week-old culture). However, the transcript level of *DOMADS3* was decreased somewhat in the inflorescence meristem but appeared higher in the floral bud. The *DOMADS2* transcript was expressed early in the 6-week-old VSAM and continu-

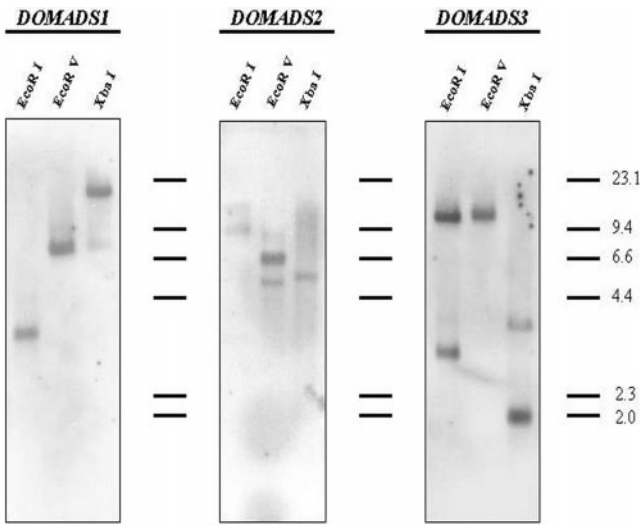

Figure 5. Genomic DNA-blot analysis of *DOMADS* genes. DNA gel blots containing 15 μg of genomic DNA digested with *Eco*RI, *Eco*RV, and *Xba*I were hybridized at high stringency with digoxigenin-labeled probes that were derived from the 3′-specific region of *DOMADS* genes. The size of the DNA markers is given on right in kb.

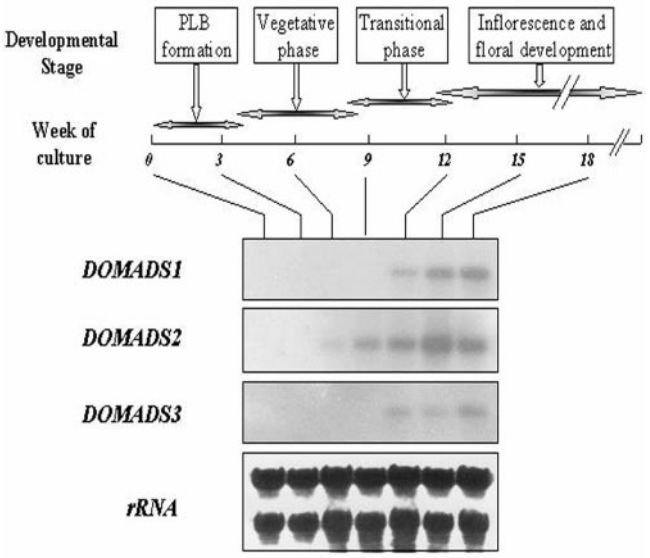

Figure 6. Expression of *DOMADS* genes during the development of the orchid. The temporal scheme of main events during the orchid development is outlined above the northern results. The horizontal double arrows above the temporal scheme indicate the different developmental phases of the orchid. From left to right, total RNA (30 μg per lane) was successively prepared from thin sections of protocorms (0-week length = 1 mm), PLBs (3-week length = 4–5 mm), VSAMs including the youngest leaf primordium (6-week length = 1.5 mm), TSAMs including bracts and the youngest leaf primoridium (9- and 12-week length = 2 mm), inflorescence meristems including bracts and the youngest leaf primordium (15-week length = 3 mm), and floral buds (18-week length = 2–4 mm). Blots were hybridized with the specific digoxigenin-labeled probes described in "Genomic DNA-Blot Analysis." The ribosomal RNAs stained by methylene blue indicate the amount of total RNA loaded in each lane.

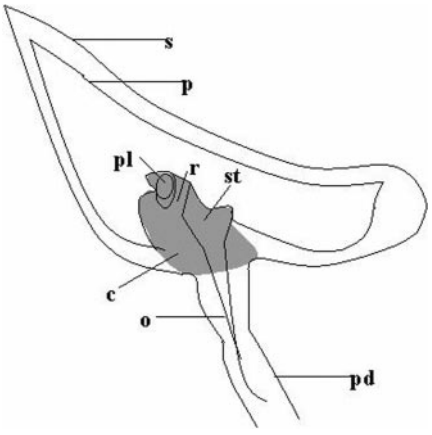

Figure 7. A schematic median vertical section of the orchid flower bud showing the different floral parts. c, Column (fused structure of stigmas, styles, and stamens indicated by shaded region); o, ovary; p, petal; pd, pedicel; pl, pollinarium; r, rostellum; s, sepal; st, stigma.

ously expressed during the floral transition, and the transcript level reached a peak in the inflorescence meristem and remained high in the young floral buds (Fig. 6).

Total RNA isolated from different tissues and different floral organs (Fig. 7) was used for further analysis of the expression of *DOMADS* genes. Northern analysis showed that all three of the genes were exclusively expressed in flowers, and no signals were detected in vegetative tissues (PLB, root, stem, and leaf) except for a very weak signal detected in stems when hybridized with the *DOMADS1* probe (Fig. 8A).

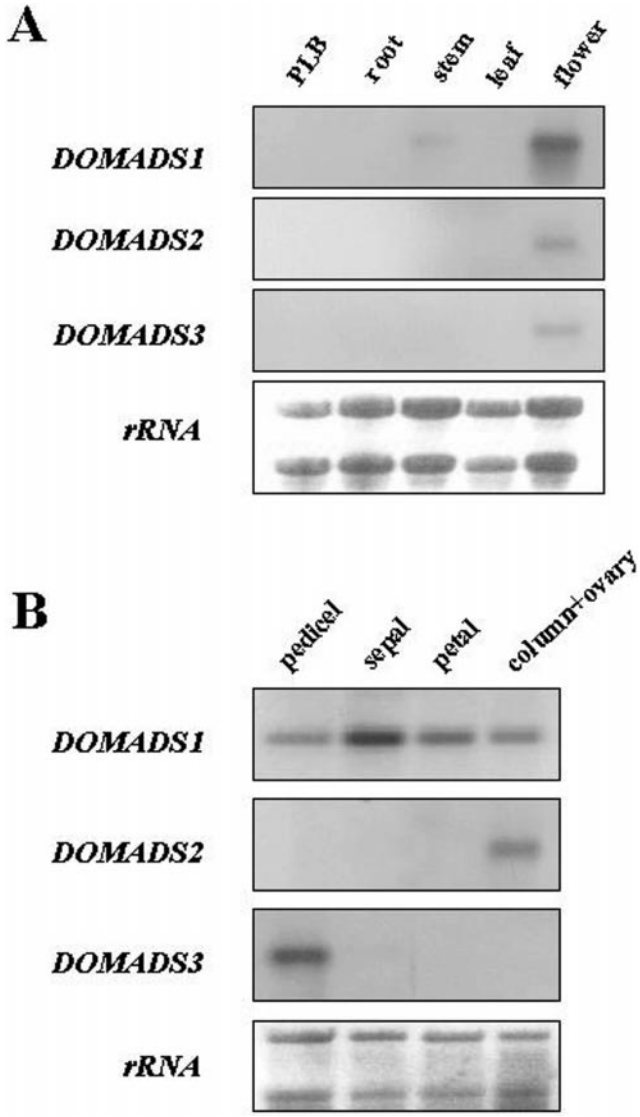

Figure 8. Northern analysis of *DOMADS* genes in different orchid tissues (A) and in different floral organs (B). All of the blots were hybridized with the specific digoxigenin-labeled probes described in "Genomic DNA-Blot Analysis." The ribosomal RNAs stained by methylene blue indicate the amount of total RNA loaded in each lane. A, The blots contain 25 μg of total RNA extracted from different tissues in each lane. B, The blots contain 15 μg of total RNA extracted from different mature floral organs in each lane.

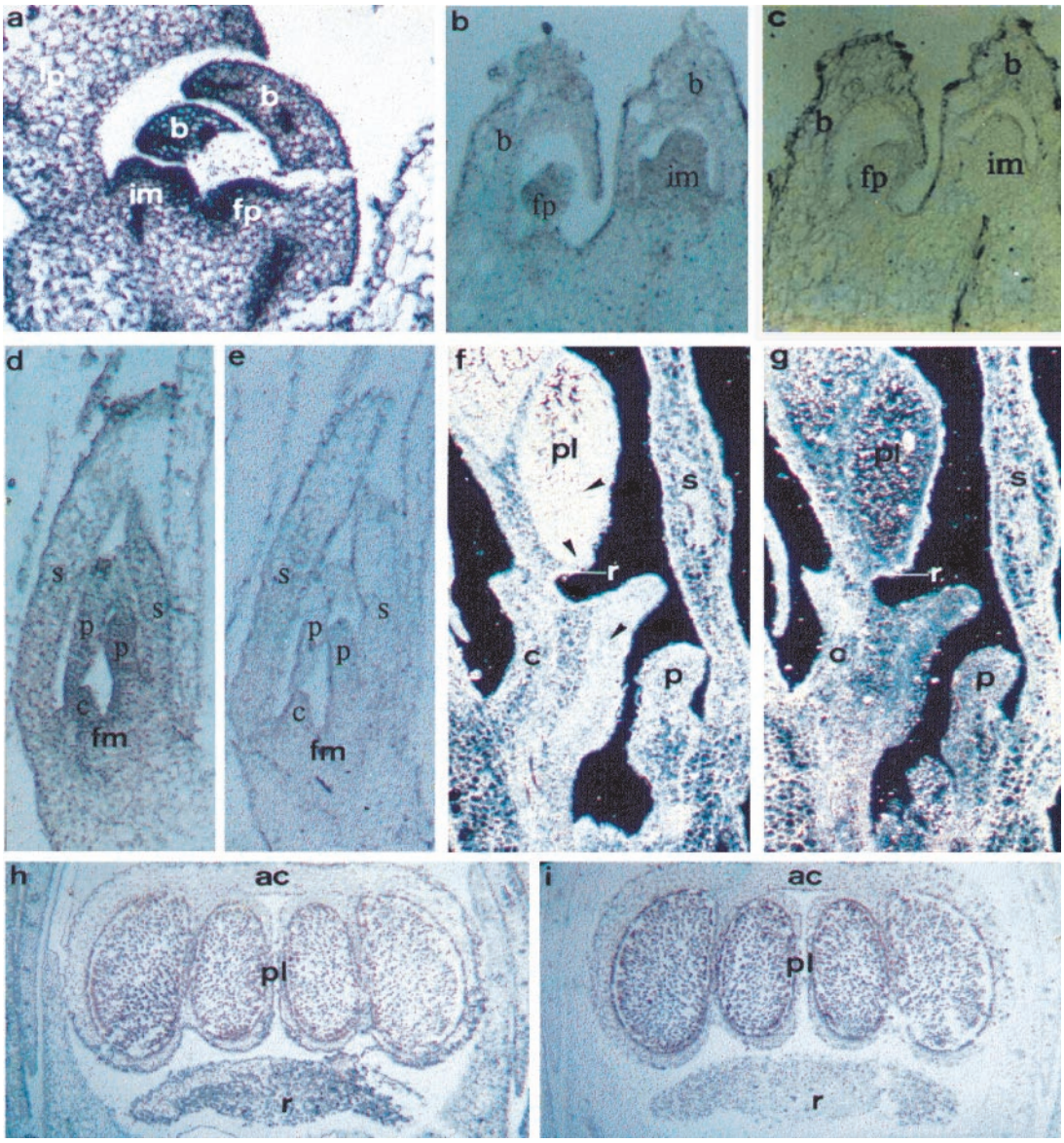

Figure 9. In situ localization of *DOMADS1* expression in longitudinal sections of SA and developing floral buds. Sections hybridized with the *DOMADS1*-specific antisense RNA probe (a, b, d, f, and h) or the *DOMADS1* sense RNA probe (c, e, g, and i) are shown. Hybridization signals were visualized using a blue filter in bright-field illumination (a–e, h, and i) or dark-field illumination (f and g). Expression of *DOMADS1* in: a, the SA of 12-week-old culture in the apical region of the inflorescence meristem and the first floral primordium (magnification, ×100). b and c, The SA of 15-week-old culture in the inflorescence meristem and the developing floral primordium (magnification, ×80). d and e, The young developing floral bud of 17-week-old culture in all of the floral organ primordia and the basal floral meristem (magnification, ×60). f and g, The floral bud of 19-week-old culture mainly in the maturing pollinarium, the rostellum, and the column (arrowheads) (magnification, ×50). h and i, The mature flower of 23-week-old culture in the rostellum located below the pollinarium (magnification, ×20). ac, Anther cap; am, apical meristem; b, bract; c, column; fm, floral meristem; fp, floral primordium; im, inflorescence meristem; lp, leaf primordium; p, petal; pl, pollinarium; r, rostellum; s, sepal.

In mature flowers the expression of the three *DOMADS* genes produced different patterns (Fig. 8B). *DOMADS1* was expressed in all of the floral organs, including pedicels, sepals, petals, column (gynostemium, a fused structure of stigmas, styles, and stamens), and ovaries. The transcript of *DOMADS2* was only detectable in the column and ovary and that of *DOMADS3* was only detected in the pedicel.

In Situ Localization of *DOMADS* Genes in Shoot Apical Meristems and Floral Buds

The detailed spatial and temporal expression patterns of *DOMADS* genes during floral transition was assessed by RNA in situ hybridization. *DOMADS1* transcript was first detected in the 12-week-old TSAM, in which the first floral primordium emerged

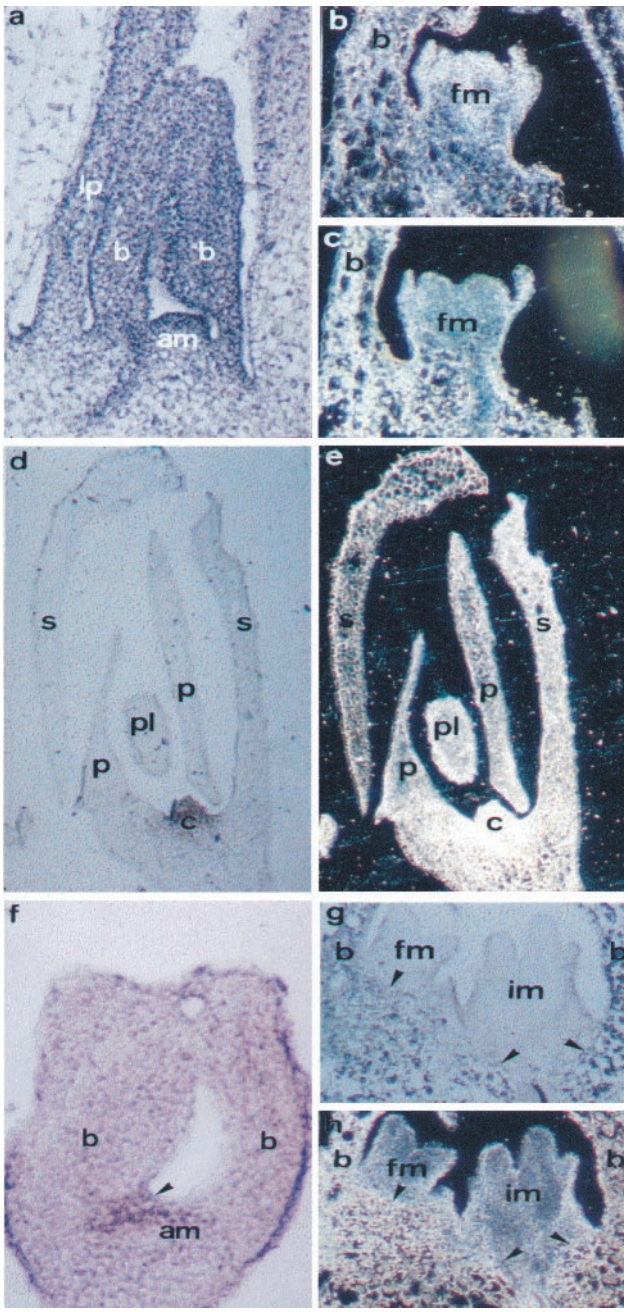

Figure 10. In situ localization of *DOMADS2* and *DOMADS3* transcripts in longitudinal sections of SA and developing floral buds. Sections hybridized with the *DOMADS2*-specific antisense RNA probe (a, b, d, and e) and the *DOMADS3*-specific antisense RNA probe (f–h) or the *DOMADS2* sense RNA probe (c) are shown. Hybridization signals were visualized using a blue filter in bright-field illumination (a, d, f, and g) or dark-field illumination (b, c, e, and h). Accumulation of *DOMADS2* transcripts in: a, the SA of 11-week-old culture in the TSAM, both of the bract primordia and the last leaf primordium (magnification, ×100). b and c, The first floral primordium of 16-week-old culture in the central zone of the floral meristem (magnification, ×80). d and e, The floral bud of 18-week-old culture in the column primordium located at the bottom of the floral bud (magnification, ×30). Accumulation of *DOMADS3* transcripts in: f, the SA of 11-week-old culture in the central zone of the TSAM and the region where the floral primordium would initiate on one flank of the apical meristem (Fig. 9a). The expression of *DOMADS1* was more concentrated in the apical region of the TSAM and the floral primordium than in the bract primordia. At a later stage, the *DOMADS1* transcript was present throughout the inflorescence meristem and the floral primordium (Fig. 9, b and c). In the young, developing floral buds, *DOMADS1* was almost uniformly expressed in all of the floral organs, including sepals, petals, column, and also the basal floral meristem (Fig. 9, d and e). At the later stage of the development of floral buds, *DOMADS1* was expressed in all of the floral organs (Fig. 9, f and g). Relatively strong signals were detected in the maturing pollinarium (pollinium apparatus), rostellum (a platform bearing the pollinarium), and the column (Fig. 9, f and g). In the column of mature flower the strong expression level of *DOMADS1* was only detectable in the rostellum located below the pollinarium as compared with the control hybridized with the *DOMADS1*-sense RNA probe (Fig. 9, h and i). No significant signals could be observed in the anther cap and the mature pollinarium (Fig. 9, h and i).

In situ hybridization showed that the *DOMADS2* transcript was detectable in the early 11-week-old TSAM at the stage when both of the bract primordia were well defined but before the first floral primordium was formed (Fig. 10a). The hybridization signals could be detected at a high level in the apical meristem, both of the bract primordia, and the last leaf primordium (Fig. 10a). Similar to the distribution of *DOMADS1* transcript in the 12-week-old TSAM (Fig. 9a), *DOMADS2* was also expressed in both of the apical meristem and the emerging floral primordium (data not shown). In the longitudinal section of the flower meristem, the accumulation of *DOMADS2* transcript was mainly detected in the central zone of the meristem where the column primordium was developing (Fig. 9, b and c). In flower development, *DOMADS2* was mainly expressed in the column primordium located at the bottom part of the floral bud (Fig. 10, d and e).

The *DOMADS3* transcript was also first detected in the 11-week-old TSAM at the stage when the first flower primordium had not emerged (Fig. 10f). The signal was strong in the central zone of the TSAM and the flanking region where the floral primordium would initiate, weak signals were also detectable in both of the bract primordia (Fig. 10f). In the progressively more developed inflorescence axis, *DOMADS3* accumulated uniformly in the region below the floral meristem and the area flanking the procambium tissues under the inflorescence meristem. No expres-

(arrowhead; magnification, ×110). g and h, The SA of 16-week-old culture in the region below the floral meristem and the area flanking the procambium tissues below the inflorescence meristem (arrowheads; magnification, ×80). am, Apical meristem; b, bract; c, column; fm, floral meristem; im, inflorescence meristem; lp, leaf primordium; p, petal; pl, pollinarium; s, sepal.

sion was observed in the floral meristem or the inflorescence meristem (Fig. 10, g and h).

DISCUSSION

In this study we have isolated and characterized three orchid MADS-box genes that are successively activated in the shoot apical meristem of *Dendrobium* Madame Thong-In during in vitro floral transition. Sequence comparisons of *DOMADS1* and *DOMADS2* with other MADS-box proteins have revealed that they are highly similar to the various members in the AGL2 and SQUA subfamilies (Theissen et al., 1996; Münster et al., 1997), respectively. Sequence analyses also indicate that *DOMADS3* is a new member of the new cluster, OSMADS1 subfamily, separated from the AGL2 subfamily, as was suggested earlier (Theissen et al., 1996). In the MADS-box domain, the apparent difference between the AGL2 and OSMADS1 subfamily occurs in the second β-pleated sheet, which is the important component of the DNA-binding domain and oligomerization domain. Therefore, the sequence difference in the β-sheet indicates that the AGL2 and OSMADS1 subfamilies may have distinct DNA-binding specificity and the affinity for promoter elements (Huang et al., 1996; Liu et al., 1999). It is also interesting to note that, so far, all of the members in the OSMADS1 subfamily were isolated from monocotyledonous plants, indicating the possible functional diversification between the monocot and the dicot during the evolutionary development of flowering.

Most of the MADS-box genes are involved in the regulation of the various steps of flower development including the transition from vegetative to reproductive growth. So far, in situ localization of the MADS-box genes involved in the floral transition has shown three different expression patterns in apical meristems. The first type of genes, which includes *AGL8/FRUITFULL* from Arabidopsis (Mandel and Yanofsky, 1995; Gu et al., 1998) and *SaMADSA*, *SaMADSB*, and *SaMADSD* from white mustard (Melzer et al., 1996; Bonhomme et al., 1997), is expressed early in inflorescence meristems, but not in nascent floral meristems. Another type of genes, including the white campion *SLM5* (Hardenack et al., 1994), the apple *MdMADS2* (Sung et al., 1999), and the orchid *DOMADS1* in this study, is expressed early in both of the inflorescence meristem and the emerging floral primordia. Unlike white campion and apple, orchid has an indeterminate inflorescence meristem from which floral meristems initiate on the flanks. It has been suggested that the expression of *SLM5* and *MdMADS2* in both inflorescence meristems and floral meristems may reflect the structural difference between the determinate and indeterminate inflorescence (Hardenack et al., 1994; Sung et al., 1999). However, our results show that the expression of the MADS-box gene (*DOMADS1*) in both meristems is also present in plants with indeterminate inflorescence. Thus, the structural difference of inflorescences may not be the main reason leading to this expression pattern. The third type of MADS-box genes involved in the floral transition expresses in nascent floral primordia but not in inflorescence meristems. These genes include *AP1* and *CAL* from Arabidopsis (Mandel et al., 1992; Kempin et al., 1995), *SQUA* from snapdragon (Huijser et al., 1992), *OsMADS1* from rice (Chung et al., 1994), and *SaMADSC* from white mustard (Melzer et al., 1996).

In contrast to the three groups of MADS-box genes mentioned above, *DOMADS2* and *DOMADS3* appear to be novel MADS-box genes. The expression of *DOMADS2* is strong in the last leaf primordium flanking around the TSAM (Fig. 10a), and the transcript situation at this point indicates the onset of transcription of this MADS-box gene may have started even in the VSAM. The result of RNA-blot analysis (Fig. 6) also shows weak expression of *DOMADS2* in the typical VSAM (6-week-old culture). The acquisition of reproductive competence in plants is often marked by phase change (Poethig, 1990; Lawson and Poethig, 1995). In our orchid system the onset of transcription of *DOMADS2* and its successive expression during early floral transition are accompanied by an obvious morphological change in the vegetative structures, which is the narrowing of two visible youngest leaves toward the SA. This observation enhances the inference that some genes identified as important in controlling the floral transition may also be involved in vegetative phase change (Levy and Dean, 1998). The expression pattern of *DOMADS2,* from early in VSAM and increases in later stages of flower development, suggests that *DOMADS2* is one of the earliest regulatory genes during the transition to flowering. This pattern has not been previously demonstrated for any other MADS-box genes.

The interesting aspect of *DOMADS3* arises from the analysis of the change of its expression in the TSAM. Although the early expression of *DOMADS3* is detectable in the central zone of the 11-week-old TSAM where the first floral primordium has yet to emerge (Fig. 10f), its transcript later disappears in both of the developing inflorescence meristem and the floral meristem (Fig. 10, g and h). Its expression is mainly in the region immediately below the floral meristem, where the tissues may later develop into pedicels (Fig. 10, g and h). Combined with the analysis of its expression pattern in mature flowers (Fig. 8b), our results suggest that *DOMADS3* is a novel MADS-box gene that may function as a regulatory factor not only in the process of the early floral transition but also in the development of pedicel tissues.

Our analysis of the different expression patterns of members in the AP1/AGL9 subfamily during floral transition is in agreement with the suggestion that

the sorting of MADS-box genes into different families by sequence comparison may reflect the distinct functional roles these genes play in flower development (Purugganan et al., 1995; Theissen and Saedler, 1996; Theissen et al., 1996). Although the members in the AP1/AGL9 subfamily show diverse expression patterns during flower development, almost all of the MADS-box genes characterized so far during floral transition belong to the AP1/AGL9 subfamily. This indicates that members of AP1/AGL9 subfamily could act at the top of the proposed regulatory hierarchy of MADS-box genes controlling flower development (Mandel and Yanofsky, 1995; Rounsley et al., 1995). Furthermore, the distinct expression patterns in the SAM demonstrated by the early acting members of AP1/AGL9 subfamily during floral transition also suggest that a more subtle regulatory hierarchy of MADS-box genes may be operating for controlling the successive events in the shift from vegetative to inflorescence meristems, maintenance of inflorescence meristems, and the differentiation of floral meristems from inflorescence meristems.

Our attempt to search for the possible correspondence between orchid MADS-box genes and the orthologs from other plant species suggests that there is no conservation in MADS-box genes function during floral transition in all of the flowering plants. In Arabidopsis, a number of early-acting MADS-box genes are organized to successively function in the restricted apical meristem region as floral transition proceeds. During the switch from vegetative to reproductive development, *AGL8* RNA accumulates early to high levels only in the inflorescence apical meristem (Mandel and Yanofsky, 1995), whereas *AP1* and *CAL* RNAs accumulate early only in the stage-1 flower primordia (Mandel et al., 1992; Kempin et al., 1995). This is followed by the onset expression of *AGL2*, *AGL4*, and *AGL9* in stage-2 flower primordia (Flanagan and Ma, 1994; Savidge et al., 1995; Mandel and Yanofsky, 1998). Shortly thereafter, floral organ identity genes, such as *AP3*, *PI*, and *AG*, become active in the regulatory networks to specify identities of floral organs (Rounsley et al., 1995; Mandel and Yanofsky, 1998). Compared with the activities of Arabidopsis orthologs in floral transition, the early-acting orchid MADS-box genes show two different patterns. First, the onset expression of *DOMADS2* and *DOMADS3* (especially for *DOMADS2*) is much earlier than *AGL8*, *AP1*, and *CAL*. This result shows that the earliest activity of MADS-box genes involving in the flower development can be traced back to the typical VSAM where the signals for floral transition may have been accumulated but the morphological change has yet to be developed. Second, the transcripts of *DOMADS1* and *DOMADS2* are accumulated in both of the inflorescence meristem and the floral meristem, whereas in Arabidopsis, the expression of *AGL8* is limited in the former region and *AP1* and *CAL* in the latter region. The distinct expression patterns in two different plant species suggest that through evolution, different plant species may have set up their own regulatory systems to control the activities of MADS-box genes involved in the floral transition.

The orchid flower is highly evolved with a column (parts of whorl 3 and whorl 4 of "normal" flowers) and an elaborate petal, the labelum. Anther development proceeds rapidly so that the pollen grains (in pollinia) are mature and functional at anthesis, whereas ovule development only starts after successful pollination. The *DOMADS1* transcript is found in all of the floral organs and their primordia. The dramatically changed expression pattern of *DOMADS1* in the pollinarium during the development of the floral bud could suggest the function of *DOMADS1* as an early regulator in the formation of pollen mother cells. Our studies also show the specific expression of *DOMADS2* and *DOMADS3* in columns and pedicels, respectively, indicating their possible functions in the regulation of floral organ identity. It is interesting to note that almost all of the MADS-box genes involved in the regulation of floral transition also function in the later stages of flower development (Mandel and Yanofsky, 1995; Melzer et al., 1996; Bonhomme et al., 1997; Sung et al., 1999).

The identification of *DOMADS1*, *DOMADS2*, and *DOMADS3* as early-acting genes during floral transition is the first step toward the elucidation of the molecular mechanisms of floral transition in orchids. A fundamental question is how the early-acting MADS-box genes are triggered by environmental and/or endogenous signals to mediate or activate the subsequent series of activities leading to flower development. The present results contribute to the understanding of the molecular events regulating the switch from vegetative to reproductive growth in flowering plants. Combined with our previous horticultural and physiological studies (Goh, 1977; Goh and Arditti, 1985), the present study also provides a better understanding of the development of orchid flowers.

MATERIALS AND METHODS

Plant Material

The source of all of the plant materials was the self-pollinated F_1 progenies of orchid (*Dendrobium* Madame Thong-In), a hybrid of *Dendrobium* Somsak × *Dendrobium* Suzie Wong. Thin-section explants, PLBs, and vegetative and transitional shoots were all maintained in modified liquid Knudson C medium (Knudson, 1946) supplemented with 2% (w/v) Suc, 15% (v/v) coconut water, and 5 μM benzyladenine, and grown at 24°C under a 16-h photoperiod of 35 μmol m^{-2} s^{-1} from daylight fluorescent lamps on rotary shakers at 120 rpm. Different tissues were dissected, frozen in liquid nitrogen, and stored at −80°C.

Nucleic Acid Isolation

Total RNA was isolated from various plant tissues according to the method of Murray and Thompson (1980) with some modifications. Frozen materials were ground in liquid nitrogen and extracted with the buffer containing 2% (v/v) β-mercaptoethanol, 2% (w/v) hexadecyltrimethylammonium bromide, 100 mM Tris [tris(hydroxymethyl)-aminomethane]-HCl (pH 7.5), 20 mM EDTA, 2 M NaCl, and 1% (w/v) polyvinylpyrrolidone. The homogenate was incubated in a water bath at 65°C for 15 min with occasional shaking. After centrifugation, the aqueous phase was extracted at least twice with an equal volume of chloroform: isoamyl alcohol (24:1, v/v). Total RNA was precipitated by adding 0.25 volume of 10 M lithium chloride and kept overnight on ice. The pellet was washed twice with 70% (v/v) ethanol, dried, and dissolved in diethylpyrocarbonate-treated water. RNA purity and concentration were determined spectrophotometrically. The integrity of RNA was evaluated by separation on a glyoxal-agarose gel. Genomic DNA was isolated from leaves by the method described by Carlson et al. (1991).

Differential Display Analysis

Differential display analysis (Liang and Pardee, 1992) was performed using the Delta Differential Display Kit (CLONTECH Laboratories, Palo Alto, CA) according to the manufacturer's recommendation. Total RNA samples from VSAMs (6-week-old culture) and TSAMs (12-week-old culture) were treated with RNase-free DNase I (CLONTECH Laboratories) to remove residual DNA. The treated total RNA (2 μg each) was used for the first-strand cDNA synthesis. PCR amplification of cDNA products was performed according to the manufacturer's instructions in the presence of [α-^{35}S]dATP by using 90 combinations of upstream "P" primers and downstream "T" primers supplied in the kit (CLONTECH Laboratories). Amplified PCR products were separated on a 5% (w/v) denaturing polyacrylamide gel in thermostatic conditions. Gels were dried under vacuum at 75°C for 40 min and exposed to x-ray films overnight. Differential displayed bands were excised, eluted in 50 μL of sterile water at 100°C for 5 min, and re-amplified by PCR. The products were analyzed on agarose gels. Bands of the expected size were purified by using the QIAEXII Gel Extraction Kit (Qiagen, Valencia, CA) and cloned into pGEM-T Easy Vector (Promega, Madison, WI). Heterogeneous inserts for each clone with the same size were distinguished from each other by arbitrary double-digestion with *Rsa*I and *Sau*3A. cDNA clones containing different inserts of the expected size were further selected by DNA dot-blot analysis according to the method of Corton and Gustafsson (1997). The identified clones were checked by northern-blot analysis of their expression patterns in VSAM and TSAM. One differentially expressed clone, otg7, was sequenced and used as a probe to screen the TSAM cDNA library.

cDNA Library Construction and Screening

Poly(A^+) RNA was isolated by oligo(dT) column chromatography from total RNA extracted from the TSAM (12-week-old culture) at 2 mm in length. A cDNA library was constructed from the purified mRNA using the ZAP-cDNA/GigapackIII Gold Cloning Kit (Stratagene, La Jolla, CA). The library was subsequently amplified and stored in a 7% (v/v) dimethyl sulfoxide solution at −80°C. The amplified cDNA library containing approximately 500,000 plaques was screened under low-stringency conditions with the digoxigenin-labeled MADS-box domain sequence (otg7 probe). Plaque lifts were performed on duplicate nylon membranes (positively charged, Boehringer Mannheim, Basel) as suggested by the supplier. The plaque hybridization was performed in DIG Easy Hyb (Boehringer Mannheim) at 42°C for 2 h. Low-stringency washes were done twice at room temperature for 15 min with 2× SSC and 0.1% (w/v) SDS, once at 42°C for 15 min with 0.5 × SSC and 0.1% (w/v) SDS. Immunological detection of the hybridization signals was carried out using the DIG Detection Kit (Boehringer Mannheim).

Sequencing and Sequence Analysis

Isolated cDNA clones were sequenced using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Applied Biosystems, Foster City, CA) and an ABI PRISM 377 DNA sequencer (Perkin-Elmer Applied Biosystems). Sequence data were compared with all of the known sequences in the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) database with the BLAST search program (Altschul et al., 1997).

Alignment of deduced amino acid sequences was made by using the Clustal W multiple sequence alignment program (version 1.7, June 1997). The sequences used for phylogenetic analyses included the MADS-box domain plus the 110 amino acids downstream of the MADS-box domain (Purugganan et al., 1995). Phylogenetic trees were constructed with the neighbor-joining algorithm by using the NEIGHBOR program in the PHYLIP program (Phylogeny Inference Package, version 3.57c, Department of Genetics, University of Washington, Seattle). The evolutionary distances were calculated by the PHYLIP program PROTDIS under the Dayhoff and PAM matrix. The statistical significance of trees was tested by bootstrap analysis using the SEQBOOT and CONSENSUS programs in the PHYLIP program.

Southern-Blot Analysis

Ten micrograms of genomic DNA was digested with different restriction enzymes, resolved on 0.7% (w/v) agarose gels, and then blotted onto nylon membranes (positively charged, Boehringer Mannheim). Blots were hybridized overnight with the specific digoxigenin-labeled DNA probes described below at 42°C in DIG Easy Hyb buffer (Boehringer Mannheim). These blots were washed twice with 2× SSC and 0.1% (w/v) SDS for 5 min at room temperature, once with 0.5× SSC and 0.1% (w/v) SDS for 15 min at room temperature, and finally once with 0.1×

SSC and 0.1% (w/v) SDS for 15 min at 65°C. Chemiluminescent detections were performed with CDP-star (Boehringer Mannheim) according to the manufacturer's instruction, and the blots were then exposed against x-ray films for 5 to 30 min before development. The specific DNA probes were synthesized from 3′ end of MADS-box clones using the DIG-High Prime Labeling Kit (Boehringer Mannheim).

Northern-Blot Analysis

Total RNA was separated on glyoxal-agarose gels and transferred onto nylon membranes (positively charged, Boehringer Mannheim) by capillary blotting. The RNA blots were hybridized overnight at 50°C in DIG Easy Hyb buffer (Boehringer Mannheim) with the specific digoxigenin-labeled DNA probes used in DNA-blot analysis. The washing and detection of blots were performed as described in DNA-blot analysis.

In Situ Hybridization

For synthesis of antisense and sense probes, the 3′ ends of three cDNA clones were introduced into the pGEM-T Easy vector (Promega). The single-stranded antisense and sense RNA probes were transcribed in vitro with T7 or SP6 polymerase using the DIG RNA Labeling Kit (Boehringer Mannheim). The labeled probes were partially hydrolyzed to an average length of 150 bases (Angerer and Angerer, 1992).

Tissues from shoot apical meristems or floral buds at different developmental stages were fixed in 3% (v/v) formaldehyde, 5% (v/v) acetic acid, and 60% (v/v) ethanol. The fixed materials were dehydrated and embedded in paraffin using standard methods. Longitudinal sections (10 μm) were transferred onto poly-D-Lys coated slides. In situ hybridization was performed essentially according to the method of Sung et al. (1999). For colorimetric detection of hybridization signals, the slides were developed overnight with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate solutions (Boehringer Mannheim) at room temperature. Sections were photographed on a microscope (TMS-F, Nikon, Tokyo) using bright or dark field optics.

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