DOH1, a Class 1 knox Gene, Is Required for Maintenance of the Basic Plant Architecture and Floral Transition in Orchid

Hao Yu, Shu Hua Yang, and Chong Jin Goh

Plant Growth and Development Laboratory, Department of Biological Sciences, Faculty of Science, National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260, Republic of Singapore

We report here the isolation and identification of an orchid homeobox gene, DOH1, from Dendrobium Madame Thong-In. Analyses of its sequence and genomic organization suggest that DOH1 may be the only class 1 knox gene in the genome. DOH1 mRNA accumulates in meristem-rich tissues, and its expression is greatly downregulated during floral transition. In situ hybridization analysis demonstrates that DOH1 is also expressed in the incipient leaf primordia and is later detected in the same region of the inflorescence apex, as in DOMADS1. Overexpression of DOH1 in orchid plants completely suppresses shoot organization and development. Transgenic orchid plants expressing antisense mRNA for DOH1 show multiple shoot apical meristem (SAM) formations and early flowering. In addition, both the sense and antisense transformants exhibit defects in leaf development. These findings suggest that DOH1 plays a key role in maintaining the basic plant architecture of orchid through control of the formation and development of the SAM and shoot structure. Investigations of DOMADS1 expression in the SAM during floral transition reveal that the precocious flowering phenotype exhibited by DOH1 antisense transformants is coupled with the early onset of DOMADS1 expression. This fact, together with the reciprocal expression of DOH1 and DOMADS1 during floral transition, indicates that downregulation of DOH1 in the SAM is required for floral transition in orchid and that DOH1 is a possible upstream regulator of DOMADS1.

INTRODUCTION

Homeobox genes are a universal group of developmentally important transcription factors that have been widely identified in animals, plants, fungi, and yeast (Bürglin, 1994). The evolutionarily conserved homeodomain encoded by these genes consists of three major α-helices that are characterized as a DNA binding domain (Kissingner et al., 1990; Otting et al., 1990). Since the first identification of the homeodomain as a conserved sequence in several Drosophila homeotic genes, animal homeobox genes have been well established as essential regulators involved in developmental decisions that control pattern formation, cell specification, or both (Manak and Scott, 1994).

Plant homeobox genes, by analogy with the regulatory functions of their animal counterparts, have been expected to function in important developmental processes. On the basis of sequence similarities within the homeodomain and flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the flanking -helices that are characterized as a DNA binding domain (Kissingner et al., 1990; Otting et al., 1990). Since the first identification of the homeodomain as a conserved sequence in several Drosophila homeotic genes, animal homeobox genes have been well established as essential regulators involved in developmental decisions that control pattern formation, cell specification, or both (Manak and Scott, 1994).

Plant homeobox genes, by analogy with the regulatory functions of their animal counterparts, have been expected to function in important developmental processes. On the basis of sequence similarities within the homeodomain and flanking regions, plant homeobox genes are categorized into five classes, one of which is the class of the KNOTTED1-like homeobox (knox) genes (Kerstetter et al., 1994; Lu et al., 1996). Characterization of the maize homeobox gene KNOTTED1 (KN1) provided the first evidence that knox genes play an important role in the function of the shoot apical meristem (SAM), which is the source of continuous aboveground organ formation (Vollbrecht et al., 1991; Smith et al., 1992; Jackson et al., 1994). Two subclasses of knox genes have subsequently been distinguished by sequence homology criteria as well as pattern of gene expression (Kerstetter et al., 1994). Thus far, class 1 knox genes have been isolated from widely diverse seed plants, including monocot species (Vollbrecht et al., 1991; Müller et al., 1995; Sentoku et al., 1999), dicot species (Lincoln et al., 1994; Ma et al., 1994; Hareven et al., 1996; Long et al., 1996), and the conifer Picea abies (Sundás-Larsson et al., 1998). The extensive study of class 1 knox genes, especially investigation of the phenotypes of the loss-of-function mutations in KN1, STM, and OSH15, has revealed that this class of genes functions in the maintenance of the SAM, in the development of lateral organs originating from the SAM, or in both (Long et al., 1996; Kerstetter et al., 1997; Sato et al., 1999).

The discovery of recognizable class 1 knox genes involved in plant development has raised one intriguing question: What are the specific networks in which these genes are regulated to activate or repress the function of downstream genes? The answer to this question is beginning to unfold as two aspects related to the study of these genes are gradually being clarified. First, a working model linking the class 1 knox genes and the activities of plant hormones

1 To whom correspondence should be addressed, E-mail dbsgohcj@nus.edu.sg; fax 65-779-5671.
is being established. The change of hormone concentrations in transgenic tobacco plants overexpressing the tobacco NTH15 gene and the rice OSH1 gene has suggested the possible function of homeobox genes in the regulation of hormone metabolism (Tamaoki et al., 1997; Kusaba et al., 1998). The same phenomenon has also been observed in transgenic lettuce plants overexpressing KNAT1 (Frugis et al., 1999). Recently, the findings of increased KNAT1 and STM mRNAs in ipt transgenic plants and the cytokinin-over-producing mutant amp1, together with the previous description of phenotypic similarities between transgenic cytokinin-overproducing plants and plants overexpressing the class 1 knox genes, suggest that plant hormones and class 1 knox genes are linked in the same pathway and that cytokinins may act upstream of KNAT1 and STM (Rupp et al., 1999).

The second important aspect is the possible interaction of homeodomain proteins with other transcription factors, especially the relationship between homeodomain proteins and MADS box proteins. Two instructive examples are the interactions between the yeast proteins MCM1 and MATa2 and the human proteins SRF and Phox1 (Grueneberg et al., 1992). Thus far, studies in plants have yet to demonstrate any relationship between class 1 knox proteins and the MADS box proteins. However, the recently discovered loss-of-function mutants bear a striking similarity to that observed in the maize zgl1 mutants, thus suggesting an interesting possibility of mutual influence between these two kinds of transcription factors (Kerstetter et al., 1997).

Orchids are members of the family Orchidaceae, one of the largest families of flowering plants. Despite numerous studies involving the physiological aspects of this large group, molecular understanding of the developmental control mechanisms in orchids remains lacking. Time-consuming and inefficient orchid transformation systems have limited the investigation of gene function and regulation in vivo. In recent years, in vitro thin-section techniques for micropropagation and flowering of orchids not only have shortened the orchid juvenile phase from several years to only a few months but also have provided more obvious “landmark” events during development (Lakshmanan et al., 1995; Goh, 1996). These advances and improved orchid transformation systems (Yang et al., 1999) facilitate the study of genes involved in orchid growth and development. In evolutionary terms, the orchid family is highly evolved and specialized, with great complexity and a large number of species. Using comparative studies of the functions of genes in orchids and their homologs in other plant species, we may gain important insights into the genes involved in plant development and further elucidate the evolution of related genes and their corresponding developmental processes.

In our effort to determine the molecular mechanisms involved in orchid development, we isolated 12 genes differentially expressed in the vegetative SAM (VSAM) during the in vitro transition from vegetative to reproductive growth, using the mRNA differential display method (Yu and Goh, 2000a). One of these genes, OVG2 (for orchid vegetative growth related 2), encodes a new member of the class 1 knox gene subfamily. The full-length clone, designated DOH1 (for Dendrobium orchid homeobox1), was subsequently isolated with the OVG2 probe from the VSAM cDNA library. In this article, on the basis of our analyses of the phenotypes of sense and antisense DOH1 transgenic orchid plants and studies of the temporal and spatial expressions of DOH1 during the development of in vitro wild-type plants, we report on the function of DOH1 in maintaining the basic plant architecture in orchid. Also, to further our understanding of the important network of interrelated processes involved during floral transition in orchid, we investigated the possible relationship of DOH1 to DOMADS1 of the MADS box family (Yu and Goh, 2000b).

RESULTS

Cloning and Sequence Analysis of a Novel Class 1 knox Gene from Orchid

In previous studies, using the mRNA differential display method, we identified 12 genes in the VSAM associated with the transition from vegetative to reproductive development in orchid (Yu and Goh, 2000a). An amplified band (OVG2) of ~500 bp showed a decreased expression pattern during floral transition (data not shown). Partial sequence analysis revealed that the gene was a new member of the class 1 knox gene subfamily, most of whose members are involved in meristem maintenance or the proper development of lateral organs. To further study the function of the class 1 knox genes involved in the development of orchid plants, we used OVG2, which encoded the well-conserved ELK domain and the homeodomain, to screen the cDNA library derived from 6-week-old VSAMs.

Approximately 400,000 independent plaques were screened under low-stringency conditions. On the basis of our results using restriction mapping and partial sequence analysis, we found that the 26 positive cDNA clones isolated represent only one group of genes. The longest clone, designated DOH1 (GenBank/EMBL/DDBJ accession number AJ276389), was selected for further study. DOH1 is a 1400-bp cDNA clone containing a single open reading frame with the start codon at position 320. The 861-bp coding region of the cDNA encodes a protein with a molecular mass of 32.0 kD and a pI of 4.81. A comparison of DOH1 with a range of homeodomain proteins from other organisms revealed that DOH1 is a novel class 1 knox gene that contains the well-conserved homeodomain, the flanking ELK domain, and the relatively conserved KNOX domain (Kerstetter et al., 1994; Bürglin, 1997). As shown in Figure 1A, the protein encoded by DOH1, especially the three conserved regions, is structurally very similar to maize KN1, Arabidopsis STM, and rice OSH1. To elucidate the evolutionary relationship between the class 1 knox gene in orchid and those in other angiosperm species, we constructed a phylogenetic tree based on analysis of the
Figure 1. Sequence Comparison of Class 1 knox Proteins.

(A) Alignment of the deduced amino acid sequences of DOH1 and its homologs from maize (KN1), Arabidopsis (STM), and rice (OSH1). Black boxes indicate identical amino acids, and dashes indicate gaps introduced to maximize the alignment. The positions of the first amino acid residues are shown on the left.

(B) Phylogenetic analysis of class 1 knox proteins. Orchid class 1 knox protein is indicated by the asterisk. The horizontal branch length is proportional to the estimated number of base substitutions. Bootstrap values (>50%) in 100 replicates are indicated next to the nodes. Genus and species are given in parentheses behind the corresponding protein.
The homeodomain region. Figure 1B shows that DOH1 is placed in an independent branch and is not grouped with its counterparts from any other angiosperm plants.

To investigate the genomic organization of class 1 knox genes in the genome, we prepared an orchid genomic DNA gel blot and hybridized it at low stringency with the complete DOH1 cDNA probe. Figure 2 demonstrates that a single strong band is evident in all of the digests, which not only indicates the presence of DOH1 as a single-copy gene but also suggests the possible absence of other class 1 knox genes in the orchid genome. This assessment was confirmed when we obtained the same result (data not shown) after reprobing the blot at low stringency, using the partial DOH1 probe that encodes the most conserved homeodomain and the flanking ELK domain.

Analysis of DOH1 Gene Expression during Orchid Development

RNA gel blot analysis was performed to determine the expression of DOH1 in various orchid organs. As shown in Figure 3A, DOH1 was strongly expressed in stems (young and old stems) and VSAMs, moderately to weakly expressed in transitional SAMS (TSAMs) and floral buds, but not expressed in roots and leaves (young and old leaves). The size (length) of the single band detected in RNA from orchid tissues was comparable to that of the DOH1 cDNA, confirming that DOH1 is a full-length class 1 knox gene.

RNA gel blot analysis was further undertaken to investigate the different developmental stages at which DOH1 was expressed during in vitro flowering of orchid. Under our tissue culture conditions, thin sections (1 mm thick) of protocorms produced 0.5-cm-long protocorm-like bodies (PLBs) within 1 month; these developed into leafy shoots with the typical VSAM during the next 5 weeks. The typical TSAM was produced in most shoots after another 5 weeks of culture, with the two visible youngest leaves narrowing toward the apex; subsequently, the plantlets entered the reproductive stage. The terminal inflorescence produced as many as seven floral buds, each subtended by a bract. Figure 3B shows that the expression of DOH1 gradually increased in the apical meristem during orchid vegetative development, with the transcript numbers peaking at the end of the vege-
tative stage (8 weeks). *DOH1* expression decreased in the overlapping phase of the vegetative and transitional stages (8 to 9 weeks), then further decreased with the progress of reproductive growth.

**In Situ Localization of *DOH1* Transcripts in Orchid Shoot Apices and Floral Buds**

The spatial and temporal expression patterns of the *DOH1* gene during orchid development were precisely determined by in situ hybridization with digoxigenin-labeled RNA probes. The typical expression of *DOH1* in the SAM at the vegetative stage is shown in Figure 4A. Compared with the control section hybridized with sense RNA probes (data not shown), large amounts of *DOH1* transcripts were detected in both the apical region of the VSAM and the provascular strands of the emerging leaf primordia (Figure 4A). During floral transition, *DOH1* transcripts were distributed throughout the whole zone of the TSAM and in both of the emerging bract primordia, as shown in Figure 4B. At the subsequent stage of inflorescence development, *DOH1* transcripts were present throughout the inflorescence meristem as well as in the developing floral primordia (Figures 4C and 4D). In the inflorescence meristem, the expression of *DOH1* was more concentrated in the outer cell layers than in the central zone of the meristem, whereas its expression was relatively uniform in the developing floral primordia at different stages (Figure 4D).

The pattern of *DOH1* expression was investigated further in developing floral buds (Figures 4E to 4J). In orchid flowers, the stigmas, styles, and stamens are fused in a structure called the gynostemium, or column. As shown in Figures 4E and 4F, *DOH1* signals were stronger in the petal and column primordia than in the sepal primordia, suggesting the distinct expression of *DOH1* in various developing floral organs despite the uniform transcript distribution observed in the floral primordium during floral transition. This variation of gene expression in different floral organs was more obvious in the fully developed floral bud, in which *DOH1* transcripts were most intense in the young petals and column (Figures 4G and 4H). In the mature flower, strong expression of *DOH1* was mainly restricted to the base of the column (Figures 4I and 4J).

**Complete Suppression of Shoot Organization and Development in Orchid by Overexpression of *DOH1***

To assess the functional role of *DOH1* in orchid development, we generated both sense and antisense transgenic orchid plants (*3SS::DOH1se* and *3SS::DOH1as* transformants) under the control of the cauliflower mosaic virus (CaMV) 35S promoter by particle bombardment transformation. To avoid obtaining chimeric plants, we used meristematic tissues (PLBs) in the early stages of organization as targets for particle bombardment, and we repeatedly screened calli for kanamycin resistance. Approximately 60% of the shoots obtained after three rounds of isolation and subculture were confirmed as pure transgenic cell lines by polymerase chain reaction (PCR) and DNA gel blot analysis of the genomic DNA from different tissues. After several generations of in vitro propagation, selected transgenic cell lines proved to be stable transformants. Because the phenotypes of independent transgenic lines had high uniformity, whereas the generation of T2 progeny of the Dendrobium orchid by in vitro pollination was unsuccessful, the T1 primary transformants were further analyzed to assess the transgenic effects, as shown in Figures 5 and 6. Transformants carrying a 35S-β-glucuronidase (GUS) construct acted as controls; these were normal in phenotype, like the wild-type plants (data not shown).

A total of 52 independent 35S::*DOH1*se transformants were obtained. Their PLBs showed SAMs of the same size and shape as those in the in vitro wild-type plants. However, all but six transformants exhibited a similar marked phenotypic alteration in the development of the SAM, which continued to grow, forming cylindrical stemlike tissues (SLTs), as shown in Figure 5A. The most intriguing defects exhibited by these transformants were the complete suppression of vegetative organs initiated from SAMs during the elongation of SLTs and the failed subsequent transition to flowering, even after repeated subculture. As shown in Figures 5C and 5D, no new organ primordia emerged in developing transgenic SAMs 250 μm in diameter, a size at which the SAM of a wild-type plant would have initiated several leaf primordia. Histological analysis revealed that the development of the SLT was characterized by the continuous production of morphologically undifferentiated and densely cytoplasmic cells, with the complete absence of any organization of vasculature or normal shoot anatomy (cf. Figures 5E and 5F with 5G). Indeed, these transformants showed complete failure in shoot development. Thus, overexpression of *DOH1* in orchid completely suppressed shoot organization and development.

The other phenotypic change observed in 35S::*DOH1*se transformants was defective leaf development. The term cotyledon-like leaf (CLL) is used here for precise description of the first leaf originating from the same region from which the SAM emerged (Figures 5C, 6E, and 6G). Although the CLL structure has yet to be reported in any orchid literature, its function of sheathing and protection of the developing SAM is comparable to the role of cotyledons in other plant species. In a wild-type orchid protocorm or PLB, the CLL was initiated on the flank of the SAM and developed slowly to only a very small size, whereas the CLL in a typical 35S::*DOH1*se transformant became the only leaf emerging, encircling the SAM, and later developing almost as a normal leaf (cf. Figures 5C and 6E). Aside from fortuitous changes in leaf size, two aberrant patterns in leaf morphology were observed in the typical transgenic CLLs. As shown in Figure...
5B, the split tips of mature transgenic CLLs were the obvious abnormality exhibited, with some variability among independent transformants. The other striking alteration in these leaves was the production of trichome structures, which was not observed in wild-type plants. Trichomes were initiated early on the adaxial surface of the young CLL as well as on the meristematic surface of the callus (cf. Figures 5C and 5D with 6E), and their presence was also obvious on the mature CLL (cf. Figures 5H and 5I).

The presence and integrity of the DOH1 sense construct in 35S::DOH1se transformants were verified by DNA gel blot analysis. An example of the genomic DNA gel blot analysis is shown in Figure 7, in which transgenic lines se1, se7, and se17 appeared to have multiple copies of the DOH1 sense gene, represented by the extra bands in addition to the endogenous DOH1 gene band, the single band detected in wild-type plants. The stronger intensity of some bands, compared with that of the endogenous DOH1 gene band, indicated that several copies of the transgene were represented by each of these bands. Generally, multiple copies of the transgene were present in the genome of each independent 35S::DOH1se transgenic plant. RNA gel blot analysis was also performed to evaluate the levels of DOH1 transcripts in independent transgenic lines. As shown in Figure 8A, high levels of DOH1 expression were detected in mature CLLs of several transgenic lines but not in the CLLs from wild-type plants. Further investigation of all 35S::DOH1se transformants revealed that the expression level of DOH1 transcripts did not correlate with the copy number of the DOH1 sense gene present in individual transgenic lines.

Plant Architecture Altered and Early Flowering Induced in Orchid Expressing the DOH1 Antisense Gene

To further elucidate the function of the DOH1 gene product, we downregulated the expression of DOH1 by using an antisense approach. Of the 41 orchid plants (35S::DOH1as transformants) expressing the DOH1 antisense gene under the control of the CaMV 35S promoter, 34 transformants shared the same phenotypic aberrations in plant architec-

Figure 4. In Situ Localization of DOH1 Transcripts in Shoot Apices and Developing Floral Buds.
(A) A 6-week-old vegetative shoot apex.
(B) A 9-week-old transitional shoot apex.
(C) and (D) A 15-week-old inflorescence apex.
(E) and (F) A 16-week-old young floral bud.
(G) and (H) An 18-week-old developed floral bud.

(I) and (J) Part of a 20-week-old mature flower.
Sections in (A), (B), (D), (F), (H), and (J) were hybridized with the DOH1 antisense RNA probe; those in (C), (E), (G), and (I) were hybridized with the DOH1 sense RNA probe. In (A) through (F), bright-field illumination was used for visualization of hybridization signals; in (G) through (J), dark-field illumination was used.
am, apical meristem; b, bract; c, column; fm, floral meristem; fp1, first floral primordium; fp2, second floral primordium; fp3, third floral primordium; im, inflorescence meristem; lp, leaf primordium; p, petal; s, sepal. Bars = 50 μm.
Figure 5. Phenotypes of Transgenic Orchid Plants Overexpressing the DOH1 Gene.

(A) Shoot development of a typical 35S::DOH1se orchid transformant (top) and a wild-type orchid plant (bottom). Plantlets that had been in culture for 4, 5, 6, and 8 weeks are laid out from left to right. Arrows point to the SLTs.

(B) Leaf from a wild-type plant (left) and the CLLs from 35S::DOH1se orchid transformants (middle and right). The split tips of transgenic CLLs are marked with arrows.

(C) Scanning electron micrograph of a typical SAM of the 35S::DOH1se transformant. The first and only CLL is initiated surrounding the SAM (arrow).

(D) Higher magnification image of (C), showing the mound-shaped SAM and the trichomes (arrows) on the adaxial surface of the CLL.

(E) Median longitudinal section through the young shoot apex of a typical transformant, showing the only surrounding CLL and the emerging SLT (arrow).

(F) Median longitudinal section through the developing shoot apex of a typical transformant. Arrow points to the broadening base of the SLT.

(G) Median longitudinal section through the young shoot apex of a wild-type plant, showing the surrounding CLL and the SAM (arrow) with the emerging leaf primordia (LP).

(H) and (I) Scanning electron micrograph of the adaxial surface of the mature CLL from a typical transformant showing trichomes (H) and the mature CLL from a wild-type plant showing no trichomes (I).

Bars in (C) to (I) = 100 μm.

ture. Figure 6A shows a typical 35S::DOH1as transformant developed to form two independent shoots from the same meristematic region. As shown in Figure 6D, five independent 35S::DOH1as transformants exhibited a more severe phenotype, characterized by the formation of three or more shoots from the same meristem. Wild-type Dendrobium orchids develop sympodially, with the new shoots being initiated from axillary buds at the base of the older plants and later detaching into independent plantlets. The growth of typical transgenic plants with the multiple-shoot phenotype differed markedly from that of wild-type plants, displaying a unique developmental pattern. In these transformants, multiple shoots emerged and developed simultaneously from the fasciated SAMs that had been separated and independent from each other on the same meristem region (cf. Figures 6E and 6G and Figures 6F and 6H).

Despite the initial split appearance, the fasciated SAMs developed as integral parts of the whole: the CLL was produced to cover the whole SAM region, just as in a wild-type plant (Figures 6E and 6G); some leaves were initiated that
Figure 6. Phenotypes of DOH1 Antisense Transgenic Orchid Plants.

(A) Shoot development of a typical 35S::DOH1as transformant (top) and a wild-type plant (bottom). Peripheral leaves of the transformant were removed to facilitate viewing of the multiple-shoot phenotype. Plantlets that had been in culture for 3, 4, 5, 6, and 8 weeks are laid out from left to right.

(B) Leaf phenotypes of a wild-type plant (far left) and several 35S::DOH1as transformants.

(C) Top view of a 35S::DOH1as transformant. Arrows point to both of the shoots that originated simultaneously from the same meristem region. Leaves initiated surrounding both of the shoots are marked with asterisks.

(D) Side view of a 35S::DOH1as transformant, showing the simultaneous growth of three separate young shoots from the same meristem region. Peripheral leaves encircling the shoots were dissected for viewing the multiple-shoot phenotype. Arrows indicate the initiated roots.

(E) Scanning electron micrograph of formation of the SAM of a wild-type plant. The new emerging SAM sheathed by the CLL is indicated by an arrow.

(F) Scanning electron micrograph of the shoot tip of a young wild-type plant.

(G) Scanning electron micrograph of the SAM formation of a typical 35S::DOH1as transformant. Split SAMs sheathed by the CLL are marked by arrows.

(H) Scanning electron micrograph of the young plantlet of a typical 35S::DOH1as transformant. Arrows indicate the young shoots simultaneously developing from the same meristem region.

(I) Median longitudinal section through the young shoot apex of a typical transformant. Two new SAMs flanked by developing leaf primordia (LP) are marked with arrows.

(J) Median longitudinal section through the developing shoot apex of a typical transformant. Positions at which new leaf primordia (LP) will initiate from the respective SAMs (S) are marked with asterisks.

Bars in (E) to (J) = 100 μm.
surrounded all of the developing shoots (Figures 6C and 6I); and roots formed at the common basal structure (Figure 6D). However, compared with wild-type plants, fasciated meristems in a typical transformant retained the function of the SAM for themselves. They initiated leaf primordia independently on their own SAMs (Figures 6H and 6J) and later produced inflorescences from their own TSAMs (data not shown).

In most of the orchid 35S::DOH1as transformants, all of the transgenic lines exhibited different degrees of dwarfing, as illustrated in Figure 6A. In addition, leaf phenotypic aberrations were also observed (Figure 6B), although the degree of deviation from wild-type plants differed among independent lines.

The constitutive expression of DOH1 in the antisense orientation in orchid also resulted in early flowering. Of the 41 35S::DOH1as transformants, 25 plants produced typical TSAMs with the narrowing youngest leaves and later entered the reproductive stage. The ratio of flowering in transgenic plants was almost the same as that in wild-type plants. However, the average time for vegetative development before transition in these transformants was only 3.5 weeks, 10 days shorter than that required in wild-type plants, whereas the time for other developmental stages remained unchanged (Figure 9B). The multiple shoots in individual transformants developed their inflorescences simultaneously with the same temporal scheme seen from PLB formation to flower development—indicating once again that the multiple shoots are integral parts of the whole. Other than some abnormal floral organs produced in several lines, no overall phenotypic defects were observed in transgenic flowers.

Genomic DNA gel blot analysis of 35S::DOH1as transformants, as shown in Figure 7, revealed that multiple copies of the DOH1 antisense gene were integrated into the orchid chromosomes. To determine whether the aberrant phenotype was correlated with the downregulation of endogenous...
DOH1 transcripts, we analyzed the amounts of DOH1 sense mRNA and antisense mRNA in 6-week-old VSAMs from wild-type plants and antisense transformants. As shown in Figure 8B, the expression of the DOH1 antisense gene was detected in the VSAMs from all analyzed transgenic lines, and the decrease of the corresponding DOH1 sense mRNAs was obvious, albeit with some degree of variability. These results indicated that the presence of DOH1 antisense mRNA degraded the DOH1 mRNA—probably an important factor in the phenotypic effects exhibited by 35S::DOH1as transformants.

Analysis of DOMADS1 Expression in Orchid 35S::DOH1as Transgenic Plants

Our previous study identified DOMADS1, a new MADS box gene of the AP1/AGL9 subfamily, as a marker gene differentially expressed in the TSAM during floral transition (Yu and Goh, 2000b). The expression of DOMADS1 was first detected in the 12-week-old TSAM; during the subsequent increase in DOMADS1, DOH1 expression was greatly diminished (Figure 3B). This reciprocal expression pattern of DOH1 and DOMADS1 in the TSAM during floral transition in wild-type plants and the early flowering exhibited by 35S::DOH1as transformants should indicate a certain relationship between these two genes during the transition to flowering. Therefore, we investigated the molecular basis of their possible interaction by analyzing the expression of DOMADS1 mRNA in 35S::DOH1as transformants. As shown in Figure 9A, the expression of DOMADS1 was apparent in 10-week-old TSAMs from all of the selected early-flowering transformants, whereas appreciable DOMADS1 expression was not detected in wild-type plants, suggesting that the early expression of DOMADS1 was concomitant with advancing the floral transition and flower development in these transformants. Examination of DOMADS1 expression in a plant of the transgenic line as31, which showed the earliest flowering of all of the transformants, further demonstrated that the shift of onset of DOMADS1 expression corresponded to the early transition to flowering (Figure 9B).

We isolated a 3.6-kb DOMADS1 promoter fragment by using genomic DNA walking strategy. Deletion analysis of this fragment indicated that it is sufficient for normal DOMADS1 expression (H. Yu and C.J. Goh, unpublished results). To study in greater detail the regulation of DOMADS1 expression during floral transition, we fused the 3.6-kb DOMADS1 promoter to the GUS reporter gene and searched for GUS activity in wild-type and transgenic line as31 plants carrying the DOMADS1::GUS construct. At least eight independent GUS-positive transgenic lines from the two different backgrounds were studied. The patterns of GUS staining among independent lines with the same background were indistinguishable. In wild-type plants, GUS staining was observed early in the 12-week-old TSAM and also later in the inflorescence meristem and developing flo-

**Figure 9.** RNA Gel Blot Analysis of the DOMADS1 Gene in Orchid 35S::DOH1as Transformants.

(A) Expression of DOMADS1 in TSAMs from wild-type and 35S::DOH1as transgenic plants. RNA was isolated from 10-week-old TSAMs (length, 2 mm) from wild-type plants (wt) and independent transformants (lines as4, as10, as15, as23, as27, as31, and as38). The gel blot, containing 20 μg of total RNA in each lane, was hybridized with the digoxigenin-labeled RNA probe derived from the 3′-specific region of the DOMADS1 gene.

(B) Time course of DOMADS1 expression in a plant of the 35S::DOH1as transgenic line as31. The temporal schemes of main events during the development of wild-type and DOH1 antisense transgenic plants are illustrated above the RNA gel blot results. The horizontal bars in the graph indicate the different developmental phases of orchids. The average time of each phase was defined by calculating these values in 100 wild-type plants and in all 25 independent 35S::DOH1as transformants that could produce flowers. The time of onset of expression of DOMADS1 in wild-type and transgenic line as31 plants is labeled with an asterisk and an ×, respectively. Total RNA (30 μg per lane) was extracted from thin sections of protocorms (8 weeks old; length, 1 mm), VSAMs (6 weeks old; length, 1.5 mm), TSAMs (8 and 10 weeks old; length, 2 mm), and inflorescence meristems (12, 14, and 16 weeks old; length, 3 to 5 mm) from a transgenic line as31 plant. Hybridization was performed as in (A).

The amount of total RNA loaded in each lane was determined by comparing the staining of the rRNAs with methylene blue.
Sections of the Apical Meristem from Orchid Plants Harboring Histochemical Analysis of GUS Activity in Longitudinal Diagrams of the Apical Meristem

Figure 10. Histochemical Analysis of GUS Activity in Longitudinal Sections of the Apical Meristem from Orchid Plants Harboring DOMADS1::GUS Constructs.

(A) A 9-week-old transitional shoot apex from a transgenic line as31 plant carrying DOMADS1::GUS constructs.
(B) A 9-week-old transitional shoot apex from a wild-type plant carrying DOMADS1::GUS constructs.
(C) An 11-week-old inflorescence apex from the same plant as in (A).
(D) A 13-week-old floral bud from the same plant as in (A).

Blue staining in (A), (C), and (D) indicates GUS activity. am, apical meristem; b, bract; c, column; fm, floral meristem; fp, floral primordium; im, inflorescence meristem; lp, leaf primordium; p, petal; s, sepal. Bars = 300 μm.

DISCUSSION

DOH1, a New Member of the Class 1 knox Gene Family

In this study, we described the isolation and characterization of an orchid homeobox gene, DOH1, encoding a member belonging to the class 1 knox gene family. Sequence analysis revealed that DOH1 possesses all of the features that characterize class 1 knox genes, including the highly conserved homeodomain with an invariant third helix, the ELK domain with repeating hydrophobic residues, and the relatively conserved KNOX domain just upstream of the ELK domain (Kerstetter et al., 1994; Lincoln et al., 1994; Bürglin, 1997). The homeodomain among DOH1, KN1, STM, and OSH1 shares >79% identity, whereas the overall identity of the coding regions of these genes is <47% (Matsuoka et al., 1993; Kerstetter et al., 1994; Long et al., 1996).

Characterization of the spruce class 1 knox gene, HBK1, indicates that this gene class was present before the split between the conifer and angiosperm lineages and that its function has been conserved throughout the evolution of seed plants (Sundás-Larsson et al., 1998). This is perhaps not surprising in view of its important regulatory function in the SAM and in shoot organization and development. Phylogenetic analysis of the homeodomain sequences from previously reported angiosperm class 1 knox genes demonstrates that despite the evolutionary distances between some genes, dicot and monocot genes occur in this family without very clear demarcations by subfamilies, indicating the maintenance of a high degree of sequence conservation throughout the evolution of angiosperm plants (Figure 1B). Nevertheless, the clustering of some dicot and monocot genes into separate subgroups, such as the KNA1 group (KNA1, NTH120, KNA1, KN1, and TK1), the STH1 group (LET6, NTH15, SBH1, and STM), and the KN1 group (OSH1, HVH21, HVKNOX3, and KN1), may also suggest the different evolutionary processes in dicots and monocots.

Studies in some plant species, such as maize (Kerstetter et al., 1994), rice (Sentoku et al., 1999), and tobacco (Nishimura et al., 1999), indicate the presence of a large family of class 1 knox genes in these plant genomes. Although homeodomain proteins with sequence similarities in animals and yeast tend to function redundantly in a similar developmental event (Sharkey et al., 1997), most class 1 knox genes in a given plant species show distinct spatial and temporal expression patterns during developmental processes, indicating different and unique biological functions of these genes in plant growth (Kerstetter et al., 1994; Nishimura et al., 1999; Sentoku et al., 1999). Our present study suggests the possibility that only one class 1 knox gene is present in the genome of Dendrobium Madame Thong-In, as was also observed in another orchid hybrid, Dendrobium Sonia (S.H. Yang and C.J. Goh, unpublished results). This is in great contrast to the presence of two or more homologs reported in all other plants. It is interesting to speculate on the evolutionary
implication of such differences. If the molecular function of class 1 knox genes is well conserved in seed plants, the single class 1 knox gene in orchid may indicate the pleiotropic effects of DOH1 in orchid plant development and the regulation of this gene by complex regulatory networks.

Expression of DOH1 during Orchid Development

In agreement with the expression of most class 1 knox genes in other plant species, DOH1 transcripts were detectable in meristem-rich tissues such as protocorms, SAMs, and stems but not in roots and leaves. Three important features of DOH1 expression have been revealed in orchid. First, expression of DOH1 was detected in incipient leaf primordia, which is quite different from the expression patterns exhibited by its homologs in other plant species with simple leaves. Although the observation of the expression of tomato class 1 knox genes, TKN1 and TKN2, in the leaf primordia partly supported the premise that this gene class is required for the formation of compound leaves (Hareven et al., 1996; Chen et al., 1997; Parnis et al., 1997), our results suggest that the structural difference between simple and compound leaves may not result solely from the expression of class 1 knox genes in leaf primordia. In orchids, despite the fact that the expression of DOH1 is undetectable in young and old leaves, the defects in leaf development in DOH1 sense and antisense transformants strongly suggest the importance of DOH1 expression in leaf primordia in mediating subsequent leaf development. As suggested by Taylor (1997), the morphological plasticity of leaves in different plant species may result partly from mutual interactions between knox proteins and other regulatory elements in leaf development. Thus, other important regulators aside from the class 1 knox genes could be involved in the determination of simple or compound leaf development. Second, DOH1 transcripts in the SAM were more concentrated in but not limited to the outermost cell layer (Figures 4A and 4B), which is different from the reported class 1 knox genes from other monocots. Because the cell activities in the outer layers are directly involved in the proper organization of lateral organ primordia (Taylor, 1997; Reiser et al., 2000), the unique expression of DOH1 in SAM may indicate its function in lateral organ initiation.

The third important feature exhibited by the DOH1 gene is its decreased expression in the SAM during floral transition. Downregulation of class 1 knox genes during floral transition has been reported in two dicots, Arabidopsis (Lincoln et al., 1994) and tobacco (Nishimura et al., 1999). In contrast to the high expression of Arabidopsis KNAT1 and tobacco NTH20 in the peripheral region of the VSAM, the respective transcripts were obviously excluded in the inflorescence meristems. This demonstrates an interesting and possibly important feature of these plants during the transition from vegetative to reproductive growth (Lincoln et al., 1994; Nishimura et al., 1999). Downregulation of DOH1 transcripts in the SAM also occurs during orchid floral transition. However, the decreased expression of DOH1 in this process is quite different from that of KNAT1 in Arabidopsis and NTH20 in tobacco: DOH1 transcripts were still present in the outer cell layers of the inflorescence meristem and throughout the floral primordia. It is interesting that the expression patterns exhibited by DOH1 and the previously identified DOMADS1 gene of the MADS box family are reciprocal during floral transition. Furthermore, during floral development, at the position at which DOH1 transcripts continue to be expressed, their expression decreases precisely at the position at which the DOMADS1 gene is strongly expressed. Therefore, the possible regulatory interactions between DOH1 and DOMADS1 might be an essential part of the developmental program that leads to normal floral transition and flower development in orchids.

DOH1 Function in Maintaining the Basic Plant Architecture

The suggestion that class 1 knox genes serve to maintain cells in a state of indeterminacy is based on the study of their expression in the wild-type context and the observation of phenotypes exhibited by dominant and recessive mutations as well as gain-of-function transgenic plants (Taylor, 1997). The most compelling evidence indicating the involvement of this gene class in SAM formation or maintenance comes from the characterization of the loss-of-function alleles of STM in Arabidopsis (Long et al., 1996) and KN1 in maize (Kerstetter et al., 1997). To evaluate the function of DOH1 in comparison with that of other class 1 knox genes, we constructed transgenic orchid plants expressing DOH1 sense and antisense mRNAs under the control of the CaMV 35S promoter. We successfully identified antisense transgenic plants for a class 1 knox gene, possibly through the absence of complementary functions conferred by other redundant factors in the orchid genome. Analyses of the phenotypic properties of sense and antisense transgenic plants revealed that DOH1 functions to maintain the basic plant architecture mainly by regulating the formation and development of the SAM (Figure 11). In a typical flowering plant, the SAM is responsible for establishing the entire aboveground plant body. The SAM in a typical 35S::DOH1se transformant showed the same size and shape as that in a wild-type plant, whereas the disorganized SAM in a typical 35S::DOH1as transformant became a fasciated SAM. Many mutants showing meristem fasciation at VSAMs or inflorescence meristems have been identified in nature, but the molecular mechanisms underlying these phenomena remain to be investigated (Kerstetter and Hake, 1997). The occurrence of meristem fasciation in the transgenic orchid subjected to the slight reduction of DOH1 transcripts (Figure 8B) suggests that the initiation of the SAM is sensitive to the downregulation of DOH1 and that a certain amount of DOH1 expression is an essential factor required
Compared with wild-type plants, as Arabidopsis and orchid.

SAM seems to be shared by distantly related species, such as knox genes, the cooperative feature of the functionality of class 1

The exact regulatory pattern may be distinct in different spe-

The present investigation of the biological roles of DOH1 in orchid development positions DOH1 in the central role in the developmental programs that lead to the construction of normal plant architecture and provides new insights into the pivotal function of class 1 knox genes in the formation and maintenance of the SAM as well as in the initiation and development of lateral organs.

**DOH1 as a Possible Upstream Regulator of DOMADS1**

In a wild-type orchid, the onset of DOMADS1 expression in the SAM during floral transition is accompanied by a marked turnover of DOH1 transcripts, and both kinds of transcripts demonstrate their pivotal role in regulating lateral organ initiation as well as converting the SAM from indeterminate to determinate. Despite hypotheses that downregulation of class 1 knox genes may be required for leaf initiation (Hake et al., 1995; Clark, 1997; Kerstetter and Hake, 1997), previous investiga-

One possible explanation for this difference is that DOH1 may be the only class 1 knox gene in orchid crucial to leaf initiation, and its overexpression undoubtedly causes marked defects in this process. In other plants, a group of class 1 knox genes with different functions may be organized to re-

Our studies demonstrate that the normal expression of DOH1 in wild-type plants and the downregulation of DOH1 in antisense transgenic plants do not prevent progress in the development of lateral organs and in the transition from vegetative to reproductive growth, whereas the upreg-

A possible relationship between the CLL and the SAM is also interesting. When the orchid SAM is initiated from cal-

Figure 11: Scheme of the Orchid Plant Architecture of Wild-Type Plants and 35S::DOH1se and 35S::DOH1as Transformants.

Compared with wild-type plants, 35S::DOH1se transformants grow without initiation of lateral organs from the SAM and do not enter the reproductive stage, whereas 35S::DOH1as transformants exhibit the simultaneous growth of multiple shoots with their own lateral organs and inflorescences. Leaves in the graph are not labeled. C, CLL; I, inflorescence; S, SAM.

for the normal formation of the SAM. However, because the SAMs, either normal or abnormal, are formed in wild-type plants as well as in sense and antisense transformants with different amounts of DOH1 expression, we believe that the establishment of the SAM in orchid is not dictated solely by DOH1. The initiation of the SAM in Arabidopsis has been characterized as a gradual and dynamic process that is di-

Figure 11: Scheme of the Orchid Plant Architecture of Wild-Type Plants and 35S::DOH1se and 35S::DOH1as Transformants.

Compared with wild-type plants, 35S::DOH1se transformants grow without initiation of lateral organs from the SAM and do not enter the reproductive stage, whereas 35S::DOH1as transformants exhibit the simultaneous growth of multiple shoots with their own lateral organs and inflorescences. Leaves in the graph are not labeled. C, CLL; I, inflorescence; S, SAM.

The normal function of the SAM is accomplished by main-

The normal function of the SAM is accomplished by main-

The normal function of the SAM is accomplished by main-

for the normal formation of the SAM. However, because the SAMs, either normal or abnormal, are formed in wild-type plants as well as in sense and antisense transformants with different amounts of DOH1 expression, we believe that the establishment of the SAM in orchid is not dictated solely by DOH1. The initiation of the SAM in Arabidopsis has been characterized as a gradual and dynamic process that is di-

The normal function of the SAM is accomplished by main-

The normal function of the SAM is accomplished by main-

for the normal formation of the SAM. However, because the SAMs, either normal or abnormal, are formed in wild-type plants as well as in sense and antisense transformants with different amounts of DOH1 expression, we believe that the establishment of the SAM in orchid is not dictated solely by DOH1. The initiation of the SAM in Arabidopsis has been characterized as a gradual and dynamic process that is di-

Figure 11: Scheme of the Orchid Plant Architecture of Wild-Type Plants and 35S::DOH1se and 35S::DOH1as Transformants.

Compared with wild-type plants, 35S::DOH1se transformants grow without initiation of lateral organs from the SAM and do not enter the reproductive stage, whereas 35S::DOH1as transformants exhibit the simultaneous growth of multiple shoots with their own lateral organs and inflorescences. Leaves in the graph are not labeled. C, CLL; I, inflorescence; S, SAM.

The normal function of the SAM is accomplished by main-

The normal function of the SAM is accomplished by main-

for the normal formation of the SAM. However, because the SAMs, either normal or abnormal, are formed in wild-type plants as well as in sense and antisense transformants with different amounts of DOH1 expression, we believe that the establishment of the SAM in orchid is not dictated solely by DOH1. The initiation of the SAM in Arabidopsis has been characterized as a gradual and dynamic process that is di-

Figure 11: Scheme of the Orchid Plant Architecture of Wild-Type Plants and 35S::DOH1se and 35S::DOH1as Transformants.

Compared with wild-type plants, 35S::DOH1se transformants grow without initiation of lateral organs from the SAM and do not enter the reproductive stage, whereas 35S::DOH1as transformants exhibit the simultaneous growth of multiple shoots with their own lateral organs and inflorescences. Leaves in the graph are not labeled. C, CLL; I, inflorescence; S, SAM.

The normal function of the SAM is accomplished by main-

The normal function of the SAM is accomplished by main-

The normal function of the SAM is accomplished by main-

The normal function of the SAM is accomplished by main-

The normal function of the SAM is accomplished by main-

for the normal formation of the SAM. However, because the SAMs, either normal or abnormal, are formed in wild-type plants as well as in sense and antisense transformants with different amounts of DOH1 expression, we believe that the establishment of the SAM in orchid is not dictated solely by DOH1. The initiation of the SAM in Arabidopsis has been characterized as a gradual and dynamic process that is di-

Figure 11: Scheme of the Orchid Plant Architecture of Wild-Type Plants and 35S::DOH1se and 35S::DOH1as Transformants.

Compared with wild-type plants, 35S::DOH1se transformants grow without initiation of lateral organs from the SAM and do not enter the reproductive stage, whereas 35S::DOH1as transformants exhibit the simultaneous growth of multiple shoots with their own lateral organs and inflorescences. Leaves in the graph are not labeled. C, CLL; I, inflorescence; S, SAM.
are later located in the inflorescence meristem and the developing floral primordia. Thus, one can reasonably deduce a possible relationship between these two genes during the process of floral transition and development. Because the gradual downregulation of DOH1 occurs just before the onset of DOMADS1 expression, we hypothesize that temporally, DOH1 may act negatively as one of the upstream regulators of DOMADS1. Further investigation showed the early flowering in 3SS::DOMADS1 transfectants to be coupled with the early onset of DOMADS1 expression. Also, in a transgenic plant in the 3SS::DOH1 context, the upstream cis element required for normal DOMADS1 expression drove GUS expression in the same temporal sequence in which DOMADS1 was expressed in this transfectant. These results not only reinforce the suggested function of DOMADS1 as a marker gene during floral transition (Yu and Goh, 2000b) but also support our hypothesis that the downregulation of DOH1 is important for triggering DOMADS1 expression. Sequence analysis of the 3.6-kb DOMADS1 promoter region has shown several TGAC core sequences with the preferred neighboring base pairs for possible DNA binding sites for class 1 knox genes (Krusell et al., 1997; H. Yu and C.J. Goh, unpublished results). This finding suggests the potential identity of DOH1 as a direct upstream regulator of DOMADS1.

In most flowering plants, floral transition is controlled by several environmental and endogenous signals. Because the decreased expression of DOH1 is also observed in other lateral organs, downregulation of DOH1 is an essential factor but not the exclusive factor controlling the expression of specific target genes that trigger the transition to flowering in orchids. Extensive study of class 1 knox genes in other plants has provided a working scheme to link these genes with the biological activities of plant hormones (Tamaoki et al., 1997; Kusaba et al., 1998; Frugis et al., 1999; Rupp et al., 2000). Therefore, further elucidation of the relationships among developmental genes, such as class 1 knox genes and MADS box genes, and the establishment of possible links between these genes and the activities of hormones will help unravel the complex mechanisms involved in floral transition.

**Differential Display Analysis**

Differential display analysis was performed with the Delta Differential Display Kit (Clontech, Palo Alto, CA) according to the manufacturer’s recommendations. Total RNA samples from vegetative shoot apical meristems (VSAMs; 6-week-old culture) and transitional shoot apical meristems (TSAMs; 12-week-old culture) were used for differential display analysis. The cloning of differential display bands was performed as described previously (Yu and Goh, 2000a). One differentially expressed clone (OVG2) was sequenced and used as a probe to further screen the 6-week-old VSAM cDNA library.

**cDNA Library Construction and Screening**

Poly(A)+ RNA was isolated by oligo(dT) column chromatography from total RNA extracted from the VSAM (1.5 mm long after 6 weeks of culture). A cDNA library was constructed subsequently from the purified mRNA by using the ZAP-cDNA Gigapack III Gold Cloning Kit (Stratagene, La Jolla, CA). The amplified cDNA library (~400,000 plaques) was screened under low-stringency conditions with the digoxigenin-labeled OVG2 probe by using a digoxigenin labeling and detection kit (Boehringer Mannheim).

**Sequence and Sequence Analysis**

Representative cDNA clones were sequenced on both strands by the dideoxynucleotide chain-termination method by using an ABI PRISM 377 DNA sequencer according to the manufacturer’s protocol (Perkin-Elmer, Foster City, CA). Alignment of deduced amino acid sequences was performed with the CLUSTAL W program (Human Genome Center, Baylor College of Medicine, Houston, TX). Sixty-four amino acids of the homeodomains were used for phylogenetic analysis. Phylogenetic trees were constructed with the neighboring algorithm using the NEIGHBOR program in the Phylogeny Inference Package (PHYLIP; Department of Genetics, University of Washington, Seattle). Evolutionary distances were estimated by the PHYLIB-P program PROTDIST under the Dayhoff and PAM matrices. The statistical significance of trees was tested by bootstrap analysis with the SEQBOOT and CONSENSUS programs in PHYLIP (Felsenstein, 1993).

**DNA Gel Blot Analysis**

Ten micrograms of genomic DNA (per lane) was digested with different restriction enzymes, separated on 0.8% agarose gels, and then blotted onto positively charged nylon membranes (Boehringer Mannheim). Blots were hybridized overnight with the digoxigenin-labeled DNA probes at 42°C in DIG Easy Hyb buffer (Boehringer Mannheim). Chemiluminescent detection was performed with CDP-star (Boehringer Mannheim) according to the manufacturer’s instructions. The DNA probes were labeled using the DIG-High Prime Labeling Kit (Boehringer Mannheim).

**RNA Gel Blot Analysis**

Total RNA was separated on 1% agarose gels and transferred onto positively charged nylon membranes (Boehringer Mannheim) by capillary blotting. RNA gel blots were hybridized overnight in DIG

**METHODS**

**Plant Materials**

All plant materials came from self-pollinated F1 progeny of Dendrobium Madame Thong-In, a hybrid of Dendrobium Somsak×Dendrobium Suzie Wong. The culture of plant materials was described in a previous study (Yu and Goh, 2000b).

**Nucleic Acid Isolation**

Total RNA was isolated from various plant tissues by the method described previously (Yu and Goh, 2000b). Genomic DNA was isolated from leaves according to the method of Carlson et al. (1991).
Easy Hyb buffer either at 50°C with the digoxigenin-labeled DNA probes or at 68°C with the digoxigenin-labeled RNA probes. The detection of blots and synthesis of DNA probes were performed as described above. The single-stranded RNA probes were synthesized as described below.

**In Situ Hybridization**

The digoxigenin-labeled antisense and sense DOH1 RNA probes were transcribed in vitro from the entire coding region using the DIG RNA Labeling Kit (Boehringer Mannheim). For synthesis of the antisense and sense DOMADS1 RNA probes, the 3′ end was introduced into the pGEM-T Easy vector (Promega) and transcribed in vitro as described above. For in situ hybridization, labeled probes were partially hydrolyzed to an average length of 150 bases.

Plant materials were fixed in a solution of formaldehyde/acetate acid/ethanol (3:5:60 [v/v]). The fixed materials were dehydrated and embedded in paraffin by standard methods. Microtome sections (10 μm thick) were mounted on poly-L-lysine-coated slides. In situ hybridization and immunological detection were performed according to the method of Sung et al. (1999), and sections were photographed through a microscope (model TMS-F; Nikon, Tokyo, Japan).

**Construction of Recombinant DNA**

The entire DOH1 cDNA with blunt ends was obtained by polymerase chain reaction (PCR) amplification with Vent DNA Polymerase (New England Biolabs, Beverly, MA). The primers used for PCR were the forward primer (5′-CTGTGCCACCCACCGAGTC-3′) and the reverse primer (5′-AATTAACCCTCACTAAAGGG-3′). For construction of the DOH1 sense vector, the PCR products were cut with SacI to produce the 5′ cohesive end and cloned into the Smal and SalI sites of the PBI121 binary vector (Clontech) downstream of the cauliflower mosaic virus (CaMV) 35S promoter and instead of the PBI121 binary vector (Clontech) downstream of the cauliflower mosaic virus (CaMV) 35S promoter in the PBI121 binary vector, where the SacI cohesive end was cloned into the XbaI and SacI sites downstream of the CaMV 35S promoter in the PBI121 binary vector, where the SacI cohesive end was introduced by PCR.

The sequences of all constructs were analyzed to eliminate mutations introduced by PCR.

**Orchid Transformation by Particle Bombardment**

Before bombardment, thin-section explants (1 mm thick) from protocorm-like bodies (PLBs) were cultured for 3 days in liquid-modified Knudson C medium containing 2% sucrose [w/v], 15% coconut water [v/v], and 5 μM benzyladenine (Yu and Goh, 2000a) and then placed on a 2-cm-diameter central core on solid modified KC medium in a 90-mm-diameter Petri dish. Plasmid DNA of sense and antisense constructs was coated on gold particles 1.1 mm in diameter by co-precipitation, as described by Klein et al. (1987). The precultured thin-section explants were bombarded with a Biolistic PDS-1000/He particle gun (Bio-Rad) under the following conditions: helium gas pressure, 1350 p.s.i.; partial vacuum, 700 mm Hg; and distance from the stopping plate to the samples, 9 cm. Each bombardment delivered 0.8 μg of plasmid DNA attached to 0.5 mg of gold particles. The bombarded thin sections were cultured for 4 days on solid modified KC medium and subsequently transferred to the same solid medium supplemented with 200 mg/L kanamycin for selection of drug-resistant calli. To avoid obtaining chimeric plants, the selected calli were cut into small pieces and cultured on solid modified KC medium supplemented with 250 mg/L kanamycin for screening of new calli. After this process was repeated for a third time, the selected calli were subcultured every 14 days. The PLBs that proliferated from calli were further screened by PCR and DNA gel blot analysis and maintained by culturing on solid modified KC medium containing 300 mg/L kanamycin. To observe the different developmental processes, wild-type and selected transgenic plants were cloned propagated and cultured on liquid or solid modified KC medium, as described previously (Yu and Goh, 2000a).

**Histological Analysis**

Tissues for histological analysis were fixed with 5% formaldehyde (v/v) and 5% acetic acid (v/v) in 70% ethanol solution. After dehydration in ethanol and infiltration with butanol, the tissues were embedded in paraffin. Microtome sections (10 μm thick) were stained with 0.1% toluidine blue.

**Scanning Electron Microscopy**

Tissues were fixed at 4°C for 2 hr in 0.1 M phosphate buffer (pH 7.2) containing 10% formaldehyde (v/v), 5% acetic acid (v/v), 45% ethanol (v/v), and 1% Triton X-100 (v/v). After three washes in 0.1 M phosphate buffer (pH 7.2), samples were dehydrated through a graded ethanol series and subjected to critical-point drying in liquid CO2. Mounted specimens were coated with ~50 nm of gold and viewed in a JSM-T220A scanning electron microscope (JEOL, Tokyo, Japan) with an accelerating voltage of 15 kV.

**Histochemical Staining of GUS Activity**

Tissues for GUS staining were incubated at 37°C overnight in staining solution containing 50 mM NaPO4, pH 7.0, 2 mM 5-bromo-4-chloro-3-indolyl-β-glucuronic acid, 1 mM K3Fe(CN)6, and 1 mM K4Fe(CN)6. After staining, the tissues were cleared of chlorophyll, fixed, and dehydrated according to the method of Topping and Lindsey (1997). For histochemical localization of GUS activity, the dehydrated tissues were infiltrated with butanol, embedded in paraffin, and cut into 15-μm-thick sections. After a brief incubation in xylene, sections were photographed through a Nikon TMS-F microscope with use of bright-field illumination.

**ACKNOWLEDGMENTS**

We are grateful to our colleagues in the Department of Biological Sciences for their collaboration and assistance in this research. We
thank Dr. Venkatesan Sundaresan and Dr. Prakash Kumar for critical reading of the manuscript. We also thank Choii Lan Lee for help in plant tissue culture. H.Y. and S.H.Y. were supported by postgraduate scholarships from the National University of Singapore. This work was supported by a research grant (No. R-154-000-095-112) from the National University of Singapore.

Received May 31, 2000; accepted August 27, 2000.

REFERENCES


DOHI, a Class 1 knox Gene, Is Required for Maintenance of the Basic Plant Architecture and Floral Transition in Orchid
Hao Yu, Shu Hua Yang and Chong Jin Goh
Plant Cell 2000;12;2143-2159
DOI 10.1105/tpc.12.11.2143

This information is current as of June 30, 2017

Permissions

eTOCs
Sign up for eTOCs at:
http://www.plantcell.org/cgi/alerts/ctmain

CiteTrack Alerts
Sign up for CiteTrack Alerts at:
http://www.plantcell.org/cgi/alerts/ctmain

Subscription Information
Subscription Information for The Plant Cell and Plant Physiology is available at:
http://www.aspb.org/publications/subscriptions.cfm

© American Society of Plant Biologists
ADVANCING THE SCIENCE OF PLANT BIOLOGY