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RESEARCH ARTICLE

Cloning and characterization of *PhPI9* involved in floral development from *Phalaenopsis* Orchid

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Abstract In the attempt to discover new genes involved in the floral development in monocotyledonousin species, we have cloned and characterized the homologous PISTALLATA-like (*PI*-like) gene from *Phalaenopsis hybrid cultivar* named *PhPI9* (*Ph*alaenopsis PI STILLATA # 9). The cDNA of *PhPI9* has a fragment of 834 bp and has 60% identity with the *PISTILATA* from *Arabidopsis*. The deduced amino acid sequence of *PhPI9* had the typical PI-motif. It also formed a subclade with other monocot PI-type genes in phylogenetic analysis. Southern analysis showed that *PhPI9* was present in the *Phalaenopsis* orchid genome as a single copy. Furthermore, it was expressed only in the lip of the *Phalaenopsis* flower and no expression was detected in vegetative organs. Thus, as a B-function MADS-box gene, *PhPI9* specifies floral organ identity in orchids.

Keywords *Phalaenopsis*, MADS-box gene, floral development, RT-PCR

1 Introduction

The transition from vegetative to reproductive phase is one of the most critical developmental events during the life of a plant. The critical point is the transition from floral primordium to floral organ primordium and the establishment of the floral organ (Bernier, 1988). All these are controlled by several genes besides environmental factors. In Arabidopsis, these genes include *AGAMOUS*(*AG*) (Bowman *et al*., 1990), *APETALA3*(*AP3*) (Jack *et al*., 1990), *PISTILATA*(*P*) (Goto and Meyerowitz 1994), *APETALA1*(*AP1*) (Mandel *et al*., 1992) and *APETALA2*(*AP2*) (Jufuku *et al*., 1994) etc. Most of these genes (except *AP2*) have a conserved domain named MADS-box, which was acquired from four members of the family: *MCM1* (yeast), *AGAMOUS* (*Arabidopsis*), *DEFICIENS* (*Antirrhinum*) and *SRF* (human) (Schwarz-Sommer *et al*., 1990). These genes are named MADS-box and encode transcription factors involved in floral development (Coen 1991; Coen and Meyerowitz 1991; Yanofsky 1995). Furthermore, MADS-box genes are important in the study of the origin of spermatophyte because of their conservation (Chen and Lu, 1997).

At present, most studies have concentrated on the model species *Arabidopsis* and other dicotyledonous species. However, the fact that MADS-box genes also function in monocotyledons arouses great interest for at least two reasons: the structural diversity of their flowers and inflorescences, and the commercial importance of their flowers, seeds, and fruits (Theissen *et al*., 2000). Although the first monocotyledon MADS-box gene is cloned from Orchid (Lu *et al*., 1993), most studies of monocotyledon MADS-box genes are focused in Gramineae (Cacharr *et al*., 1999; Jia *et al*., 2000; Ambrose *et al*., 2000). However, the unique floral morphology has made orchids attractive models for botanists. Unlike dicotyledonous flowers, all orchid flowers have colored sepals. The sepals and petals of orchid are called tepals because of their similar color and shape. The median tepal in the inner whorl generally differs from the rest of tepals; it is named as labellum or lip. Furthermore, the stigma and stamens are always fused to form a highly modified column. *Phalaenopsis* is a member of the *Orchidaceae*, one of the largest flowering plant families (Wu *et al*., 2003). As an ornamental crop, *Phalaenopsis* plays a very important role in the international market. In this paper we have cloned a *PI*-like gene from the cDNA of *Phalaenopsis* named PhPI9 and its GenBank accession number is AY748818.

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2 Material and methods

2.1 Material

Young plants (after germination for one year) of *Phalaenopsis* were grown in a greenhouse under natural light and controlled temperature 15°C/10°C, Day/Night for two weeks, then the temperature was changed to 20°C/15°C (Day/Night), and initiation of the peduncle could be seen. For RT-PCR and Southern analysis, leaves, stems, bulbs and floral organs (sepal, petal, lip and column) were frozen in liquid nitrogen immediately after collection and stored at −80°C.

2.2 Bacterial strains and plasmids

E. coli strain DH5*α* was stored by our lab. Plasmid pGEM(R)-T was bought from Promega.

2.3 Reagent and enzymes

Trizol and Taq plus were the products of Shanghai Biological Engineering Technology Co. Ltd. Super Scriptase TMII Rnase H Reverse Transcriptase were bought from Invitrogen Company, Southern Blot were performed according to the Gene Images Random Prime Labeling Protocol (Amersham Biosciences). PCR products were purified by SiMax™ PCR Products/Agarose Gel Purification Kit (SBS), and the Restriction Endonucleases were bought from Jingmei Biotech Co., Ltd.

2.4 Gene clone and sequence analysis

The total RNA from the petal was extracted using Trizol (Sangon, China). Primer B26 5′-GACTCGAGTCGACATC-GAT17-3′ (Jia *et al*., 2000) was used for the first strand cDNA synthesis (RevertAidTM First Strand cDNA Synthesis Kit, Fermentas). PCR amplification was performed under the conditions: 5 min at 94°C for initial denaturation, 30 s at 94°C, 60 s at 55°C, 90s at 72°C for 30 cycles, followed by an extension for 10 min at 72°C using the 5′ primer 5′-ATGGGGG/GAGC/AAAGAGC/AGAGATCAAGA/C-3′ and the 3′ primer5′-ACTCGAGTCGACATCGA-3′ (Jia et al., 2000). PCR products were cloned into the pGEM-T vector (Promega, USA) for identification and sequencing.

We used DNAssist Package to perform sequence analysis for pPI9 and other orthologous MADS-box genes searched from NCBI GenBank.

2.5 Southern blot

Total genomic DNA was isolated from the young leaves of the plant using SDS buffer (Tris-HCl 10 mM pH 8.0, *β*-Mercaptoethanol 2.2% (W/V), NaCl 500 mM, EDTA 20 mM, SDS 1.5%).15 μg of total DNA was digested with *Dra* I or *EcoR* I. The digested DNA was then run on a 0.7% (W/V) agarose gel and transferred to a Hybond-N^+ membrane (Amersham Biosciences). The 3′ UTR sequence of *PhPI*9 was used as the probe and labeled as described by the Gene Images Random Prime Labeling Protocol (Amersham Biosciences). Southern bolt analysis was carried out according to the protocol of Gene Images Random Prime Labeling Module and Detection System (Amersham Biosciences). Hybridization was performed at 60°C and Hybond-N^+ membrane was strictly washed thrice with 1×, 0.5× and 0.1× washing solution respectively. Exposure time for signal detection was reduced to remove the background caused by homologues.

2.6 Phylogenetic analysis

The deduced amino acid sequence of PhPI9, along with other class-B group MADS-box genes, were aligned using the computer program CLUSTAL W. A phylogenetic tree of several known B-class genes was constructed using Neighbor-Joining method provided by the computer program Mega 3. Bootstrap support was indicated on branches. MADS-box genes we searched from GenBank were from *Arabidopsis thaliana*: Ap3 (D21125), PI (D30870); *Amtirrhinum majus*: GLO (X68831), DEF(X52023); *Petunia hybrida*: pMADS2 (X69947), GP(X69946); *Oryza sativa*: OsMADS16 (AF077760); *Zea mays*: ZMM16(AJ292959), ZMM18 (AJ292960), ZMM29(AJ292961), SILKY (AF077760); *Orchis italica*: OrcPI (AB094985); *Phalaenopsis equestris*: pAP3 (AY771993), PeMADS2(AY378149), PeMADS3 (AY378150), PeMADS4(AY378147), PeMADS5 (AY378148); *Hyacinthus orientalis*: HPI1 (AF134114); *Triticum aestivum*: WPI1(AB107991); *Sagittaria montevidensis*: SmPI (AF230712), SmAP3 (AF230705).

2.7 RT-PCR

Total RNA was extracted from the peduncle at various developmental stages, and from bud, root, leaf, sepal, petal, labellum, pistil and stamen of the plant. After determining the OD_{260} of the samples, 1 μg of total RNA was used to synthesize first strand cDNA using the poly (A) tail primer B_{25} using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas). This cDNA, which served as the template in PCR, was carried out as described above using gene-specific primers. The primers of orchid actin-F 5′-TGGAACTGCCAAGACG-3′, and actin-R 5′-GCAGCGAAGATTCAAAA-3′ were used for control amplification. The PCR conditions for actin are the same as those above.

3 Results

3.1 Cloning and sequence analysis

Because the MADS domain is highly conserved among the different members of this gene family, it is possible to isolate

additional MADS-box genes by RT-PCR. Degenerate oligonucleotides were designed to this region and PCR was carried out. The DNA sequence analysis showed that one of the obtained cDNA sequences, named *PhPI9* (834 bp), contains a putative ORF (210 aa), a 3′ untranslated region and a poly (A) tail. The sequence was deposited in GenBank under the accession number ***AY748818***. *PhPI9* shows 60% amino acid sequence identity with *PISTILLATA* from *Arabidopsis*, and 68% amino acid sequence identity with the MADS-box containing protein *PI* from *Chloranthus spicatus*. The deduced amino acid sequence alignment with other MADS-box proteins demonstrated that *PhPI9* has the typical MIKC-type domain and within the C terminal region, there is the highly conserved PI sequence motif (MPFxFRVQPxQPNLQE) (Yang *et al*., 2003) (Fig. 1). The conserved amino acid residue E-97 and N-98 in other PI-like genes (Yang *et al*., 2003) also founded in PhPI9 is further confirmation that the gene we cloned belong to a PI-like MADS-box gene family.

The identical amino acids were indicated in black boxes, and the similar amino acids were presented in the gray boxes. M-box, K-box and PI-motif were underlined, and the gene pPI9 is highlighted in italic.

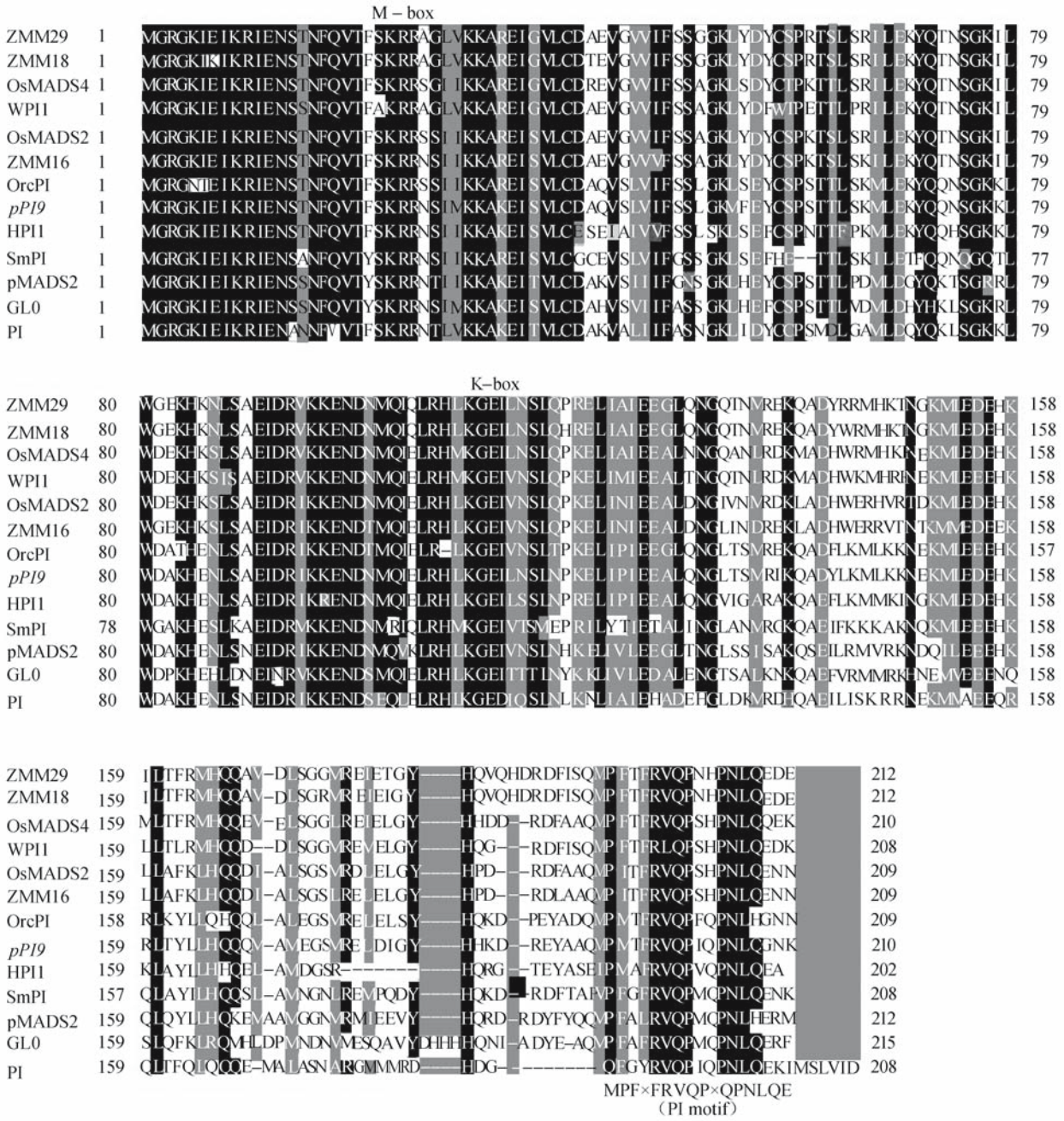

Fig. 1 Alignment of the deduced amino acid sequences of *pPI9* with other PI-type MADS-box proteins

3.2 Southern blot

Southern blot analysis was carried out to assess the copy number of *PhPI9* in the *Phalaenopsis* genome. To avoid cross hybridization, the probe used was the 3′ region of *PhPI9*, which is not conserved amongst PI-type genes. The genomic DNA was digested with *Dra* I and *EcoR* I. The results found that *PhPI15* is present as a single copy in the *Phalaenopsis* genome (Fig. 2) (Because there is a D*ra* I site in the sequence of the probe, we can see two signals in the lane of *Dra* I).

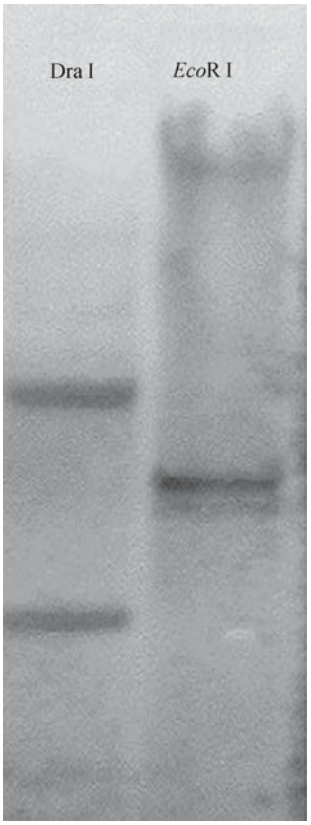

Fig. 2 Southern analysis for *PhPI9*
DNA was digested with *Dra* I or *EcoR*II, fractionated on a 0.7% agarose gel. Southern bolt was probed with the 75bp 3′UTR fragment of DNA (excluding the polyA tail).

3.3 Phylogenetic analysis

To determine the phylogenetic relationships between *PhPI9* and other B-class MADS-box genes, a phylogenetic tree was constructed using full-length amino acid sequences. The tree showed that the class-B gene family can be divided into PI-type and AP3-type clades, and both clades could be separated into dicot and monocot sub-clades. The topology of the phylogenic tree showed that the *Phalaenopsis PhPI15* gene was classified into the subclade of monocot PI-type genes (Fig. 3), and also formed a monophyletic subclade with the other B-class genes from monocots.

3.4 RT-PCR

Semi-quantitative RT-PCR analyses were used to examine the specific expression of *PhPI9* in different plant tissues, specifically roots, leaves, buds, and the tip of the peduncle at different developmental stages and different floral organs

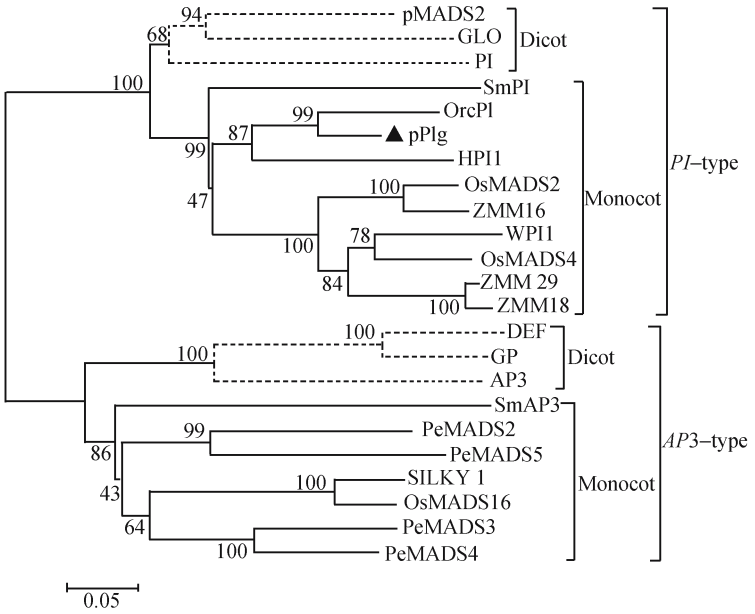

Fig. 3 Phylogenetic analysis of *PhPI9* with other B group MADS-box genes
The amino acid sequences were aligned using the computer program CLUSTAL W, and the dendrogram was then obtained using the neighbor-joining method. *PhPI9* is highlighted with a triangle ▲.

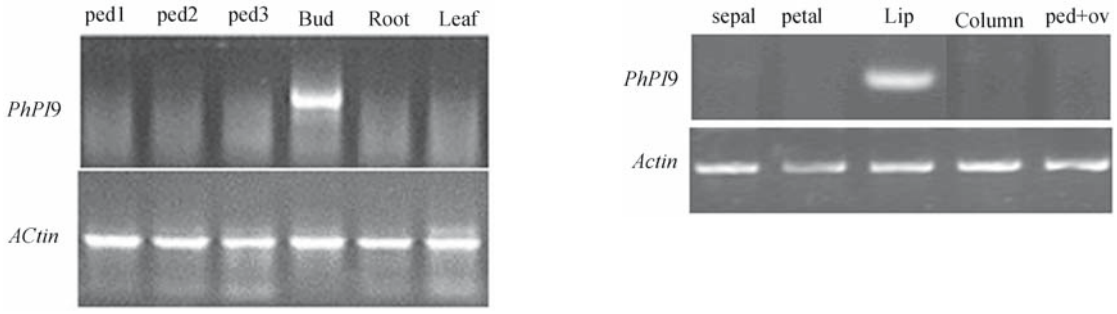

Fig. 4 Detection of the *PhPI9* expression
We used actin as the control agent. Ped1: peduncle with two nodes; Ped2: peduncle with three nodes; Ped3: peduncle with four nodes; sep: sepal; pet: petal; Maker Dl2000 indicated the molecular weight from the top down: 2000 bp, 1000 bp, 750 bp, 500 bp, 200 bp, 100 bp.

in a mature flower. The result showed that this gene was not expressed in the vegetative phases. It was expressed in the reproductive phase and pressed in the lip of the *Phalaenopsis* flower (Fig. 4).

4 Discussion

Genes of the MADS box family encoding transcription factors are found in a diverse range of eukaryotic organisms, including yeasts, plants, insects, amphibians and mammals (Prakash *et al*., 2003). The MADS-box gene family plays a key role in plant development, especially the specification of the floral organs. Moreover, they are involved in promoting resistance against adversity (Lozano *et al*., 1998). The structure of plant MADS-box genes are diverse at the genome level. *PhPI9* cloned from *Phalaenopsis* Orchid had 60% identity with the *PI* from *Arabidopsis* at the amino acid level. Its DNA sequence contained the integrated ORF region and a poly (A) tail. The deduced amino acid sequence has the typical MIKC structure, including M domain (58AA), K domain (107AA). There was a PI-motif at the C terminal of PhPI9 which is observed in other *PI*-like genes (Kramer *et al*., 1998) (Fig. 1). These indicated that *PhPI9* may be another *PI*-like gene in orchids.

The results of semi-quantitative RT-PCR illustrated that *PhPI9* was only expressed in reproductive organs and not in vegetative tissues of the plant (Fig. 4). The expression pattern was similar with the typical ABC model (Goto *et al*., 1994). But unlike other PI-like genes in dicotyledonous species, which are only expressed in the second, third and fourth whorls of developing flowers (Goto and Meyerowitz, 1994), *PhPI9* only expressed in the lip and not in other petal and stamen, suggesting that this MADS-box gene may only be involved in lip formation. A further study is being carried out to investigate the function of *PhPI9*.

Orchidaceae is one of the largest angiosperm families among more than 20,000 species. It came into being one hundred and twenty million years ago. The highly evolved orchid flower is unique to the well-known monocots, such as maize and rice; their highly reduced flowers make them unsuitable for the study of flower development. All expected flower whorls are present in orchids, and the sophisticated flower organization offers an opportunity to discover new variant genes and different levels of complexity within morphogenetic networks. A study of the flower development of orchids not only can test the validity of the classical ABC model in monocots, but also investigate MADS-box genes involved in this highly structured flower and help understanding of the evolution of orchid species.

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