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Research Article

An effective homologous cloning method for isolating novel miR172s from *Phalaenopsis hybrida*

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Abstract

MiR172 is an important microRNA that regulates floral development in various plants and downregulates *AP2* family members to relieve the stress on floral determinacy, leading to phase transition from vegetative to reproductive growth. In this work, PCR with primers designed based on the rice *miR172* sequence was used to isolate two *miR172*-like transcripts from *Phalaenopsis hybrida* (*PhmiR172-1* and *PhmiR172-2*) that were very similar to *Oryza miR172d* and *Arabidopsis miR172b*. RT-PCR indicated that the levels of these two transcripts were negatively correlated with the level of the *Phalaenopsis AP2* (*PhAP2*) gene in stem, root, pedicel and sepal, and that both were co-expressed with *PhAP2* in young buds. Overproduction of *PhmiR172-2* in *Arabidopsis* led to early flowering. The homologous cloning method used to isolate the *Phalaenopsis miR172*-like transcripts can be used to isolate miRNAs from other species. These *PhmiR172* transcripts may be used to accelerate the flowering of orchids.

Key words: homologous cloning, *miR172*, miRNA, *Phalaenopsis*, sequence conservation.

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Introduction

MicroRNAs, non-coding RNAs 21-23 bp in size, are critical developmental factors in animals and plants (Bartel, 2004) that were originally thought to transcriptionally down-regulate target genes without reducing the amount of corresponding target RNA (Lee *et al.*, 1993). Later studies showed that miRNAs can also degrade mRNA directly (Llave *et al.*, 2002; Yekta *et al.*, 2004; Allen *et al.*, 2005; Bagga *et al.*, 2005). MicroRNAs are a key factor in maintaining the homeostasis of some transcriptional control pathways and make gene expression more precise (Achard *et al.*, 2004; Chiou *et al.*, 2006).

In plants, many miRNAs are involved in the precise control of flowering time because of its roles in sexual reproduction and maintenance of the species. Many miRNAs are involved in maintaining phase transition, *e.g.*, *miR156* targets *SPLs* (**s**quamosa **p**romoter **b**inding **p**rotein-**l**ike) (Schwab *et al.*, 2005), *miR159* (phytohormone pathway) directs the cleavage of MYB33 transcripts, resulting in the repression of *LEAFY* (Achard *et al.*, 2004), and *miR172* promotes floral transition by repressing the expression of AP2 members (Park *et al.*, 2002; Aukerman and Sakai 2003).

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MicroR172 participates in the photoperiod pathway and is positively regulated by *GI* (*GIGANTEA*) in an age-dependent rather than rhythmic manner (Jung *et al.*, 2007). *MiR156* regulates SPL9 and SPL10 that control the expression of *miR172* by directly promoting the transcription of *miR172b* (Wu *et al.*, 2009). In addition, *miR172* can affect floral organ identity (Zhao *et al.*, 2007; Zhu *et al.*, 2009; Zhu and Helliwell, 2011), possibly through a function of AP2, an A-class gene that specifies perianth organs (Parcy *et al.*, 1998; Wollmann *et al.*, 2010).

The positive effect of *miR172* on the induction of flowering makes it a potential target gene for commercial flowering plants. However, *miR172* has not been isolated from important ornamental plants because precursor sequences are not as conserved as protein coding genes (Griffiths-Jones *et al.*, 2006), although *miR172* transcripts have been identified in many kinds of plants, including tobacco (Kasai *et al.*, 2010), maize (Chuck *et al.*, 2007), apples (Gleave *et al.*, 2010), morning glory (Glazinska *et al.*, 2009) and potato (Martin *et al.*, 2009; Hwang *et al.*, 2011).

Phalaenopsis is an important horticultural plant with a long vegetative period of at least 15 months. These plants flower only under strict temperature, humidity and photoperiod conditions, which makes them more expensive. In an attempt to shorten the flowering time of *Phalaenopsis*, two novel *miR172* transcripts of *Phalaenopsis hybrida* were isolated by RT-PCR and characterized. These *miR172*

transcripts should be useful in genetic engineering of the phase transition in *Phalaenopsis* species. The homologous cloning method described here can also be used to isolate other pre-miRNAs from non-model organisms.

Materials and Methods

Plant material

Phalaenopsis hybrida (~20 months old) was grown in a greenhouse under standard conditions (16/8 h light/dark cycle at 25-28 °C).

Amplification of *Phalaenopsis miR172* sequences

Genomic DNA extracted from leaf tissue according to the method of Dellaporta *et al.* (1983) was used as a template for PCR amplification under the following conditions: 5 min at 94 °C for initial denaturation, followed by 30 cycles of 30 s at 94 °C, 60 s at 55 °C and 90 s at 72 °C with a 10 min extension at 72 °C. The primers used for PCR were designed against conserved *miR172* sequences (enzyme restriction sites and protective bases are underlined): Forward - 5'-GCCAAGCTTGTGTTTGCGGGCGTGGCATCATCAAGATTC-3' and Reverse - 5'-GCGAGCTCTTGTCTGCGGATGCAGCATCATCAAGAT-3'.

Sequencing and analysis of *Phalaenopsis miR172*s

The PCR products were cloned into pGEM-T vectors (Promega, USA) for identification and sequencing. The secondary structures of identified miRNA precursors were predicted with the software RNA fold. The *miR172* sequences from *Aegilops tauschii, Arabidopsis thaliana, Brachypodium distachyon, Elaeis guineensis, Glycine max, Manihot esculenta, Oryza sativa,* Phalaenopsis hybrida, Populus trichocarpa, Solanum tuberosum, Sorghum bicolor and *Vitis vinifera* were aligned using CLUSTAL X software (Thompson *et al.*, 1994). Table 1 shows the name and GenBank number of the *miR172*s from *O. sativa (OsmiR172)* and *A. thaliana.* Phylogenetic analysis was done with Mega5.0 software.

Semi-quantitative RT-PCR

Total RNA was extracted from root, stem, leaf, pedicel, bud, sepal, petal, labellum and pistil of *P. hybrida* using RNAiso Plus (Takara). After treatment with DNase I, 1 μg of total RNA was used to synthesize first strand cDNA using a PrimerScript reverse transcriptase kit (Takara). Sequence alignment was used to design a pair of primers to amplify the *Phalaenopsis AP2* gene. The primer sequences and PCR conditions used are listed in Table 2. All of the reactions were initiated with a 5 min denaturation at 94 °C.

Table 1 - Accession numbers of *miR172*s from *A. thaliana* (*AtmiR172*), *O. sativa* (*OsmiR172*), and *P. hybrida*.

Species	GenBank accession no.
Arabidopsis thaliana	
AtmiR172a	FM163881.1
AtmiR172b	EU549208.1
AtmiR172c	EU549230.1
AtmiR172d	EU549247.1
AtmiR172e	EU549268.1
Oryza sativa	
OsmiR172a	HM139602.1
OsmiR172b	HM139609.1
OsmiR172c	HM139615.1
OsmiR172d	HM139626.1
Phalaenopsis hybrida	
PhmiR172-1	JN122376
PhmiR172-2	JN122377

Transgenic *Arabidopsis*

The newly identified *PhmiR172* sequences were cloned into the pHB vector (Ren *et al.*, 2005) for constitutive expression, and new blossomed flowers of *Arabidopsis* were infected with *Agrobacterium* strain EHA105-35S::*PhmiR172*. The seeds of transgenic plants were screened in hygromycin (0.5 mg/mL). Vegetative days and rosette leaf number before flowering were counted in wild-type and T_3 plants. The experiment was carried out with three independent occasions with n = 3/group each time. RNA was extracted with RNAiso Plus (Takara) from T_3 plants, followed by treatment with DNase I and cDNA synthesis. The primers and PCR conditions for the *Phalaenopsis* genes and the internal control are described in Table 1.

Table 2 - PCR primers and conditions used in this work.

Target gene	Primer sequence (5' to 3')	PCR conditions
ACTIN	Forward: TGGAACTGCCAAGACG Reverse: GCAGCGAAGATTCAAAA	30 s 94 °C, 30 s 55 °C, 30 s 72 °C; 28 cycles
PhmiR172	Forward: GTGTTTGCGGGCGTGGC ATCATCAAGATTC Reverse: TTGTCTGCGGATGCAGC ATCATCAAGAT	30 s 94 °C, 30 s 58 °C, 30 s 72 °C; 30 cycles
PhAP2	Forward: AAGTTCACAGTATAGAGG Reverse: GCATGCCTGCAGGTCGAC	30 s 94 °C, 30 s 55 °C, 30 s 72 °C; 30 cycles

Results

Isolation and sequence analysis of *Phalaenopsis* miR172

Since *Phalaenopsis* is monocotyledonous primers were designed based on the sequences of rice *miR172*s. The stem sequences of rice *miR172*s were aligned (Figure 1) and the core regions of miRNA and miRNA* were found to be conserved, which facilitated the PCR cloning.

Two novel *Phalaenopsis miR172*-like (*PhmiR172*) sequences of different lengths were isolated by PCR using genomic DNA as the template (Figure 2A). Random amplification resulted in fragments of different lengths: the 100-bp fragment was referred to as *PhmiR172-1* and the 250-bp fragment as *PhmiR172-2*. Sequence analysis was undertaken before characterizing the function of these fragments by expression analysis and in transgenic plants. The predicted secondary structures of the two *PhmiR172* precursors had a unique stem-loop region (Figure 2C) and shared a number of identical nucleotides, in addition to the region matching the primers (Figure 2B).

The stem loop sequences of *miR172* from 12 species, along with *PhmiR172-1* and *PhmiR172-2,* were subjected to phylogenetic analysis (Figure 2D). The regions matching the primer sequences were removed from the *Phalaenopsis miR172*s to prevent false clustering with *OsmiR172*. The two *PhmiR172*s were closely related to the *miR172*s of most other species and formed a major highly-related cluster that included most of the *miR172*s studied. Although the main cluster included *miR172s* from all of the species, several *miR172*s from seven species diverged from the main branch, including *Oryza 172b/c*, *Vitis miR172a/b/c* and others (Figure 2D).

Expression of *PhmiR172* and *Phalaenopsis AP2*

Semi-quantitative RT-PCR was used to examine the expression pattern of *miR172* and its potential target, the *AP2* family gene, in *P. hybrida* tissues. The same primers were used to amplify *PhmiR172-1* and *PhmiR172-2* because of the short length of *PhmiR172-1*. RT-PCR indicated that the transcription levels of *PhmiR172*s and *PhAP2* were complementary in some tissues, such as root, stem, leaf and petal, which implied that *PhmiR172*s might affect the transcription of AP2 (Figure 3A). Both versions of *miR172* were mainly expressed in root, stem, pedicel and bud, while *PhAP2* transcripts were limited to leaf, pedicel, bud and petal. *MiR172* was expressed at a relatively high level in root and stem, and no *AP2* mRNA was detected in these tissues. *AP2* mRNA was expressed in leaf and petal, where no *miR172* was detected. The detected transcript of *PhAP2* was cloned and sequenced, but was only recovered as a partial mRNA (107 bp; too short to be deposited in GenBank). Phylogenetic analysis was done to confirm its identity (Figure 3B).

However, *PhmiR172* and *PhAP2* transcripts were detected in early flower structures, such as pedicels and buds (Figure 3A), which indicated a mode of dynamic regulation between *PhmiR172* and its target during early flowering. The levels of *PhmiR172-1* and *PhmiR172-2* were not identical in the same tissues. For example, only the precursor of *PhmiR172-1* was detected in roots and buds, whereas both *PhmiR172*s were detected at a higher level in the stem and pedicel (Figure 3A).

Overexpression of *PhmiR172* in *Arabidopsis* promotes flowering

PhmiR172-2 showed greater sequence similarity with *miR172*s from other species than did *PhmiR172-1*. Based

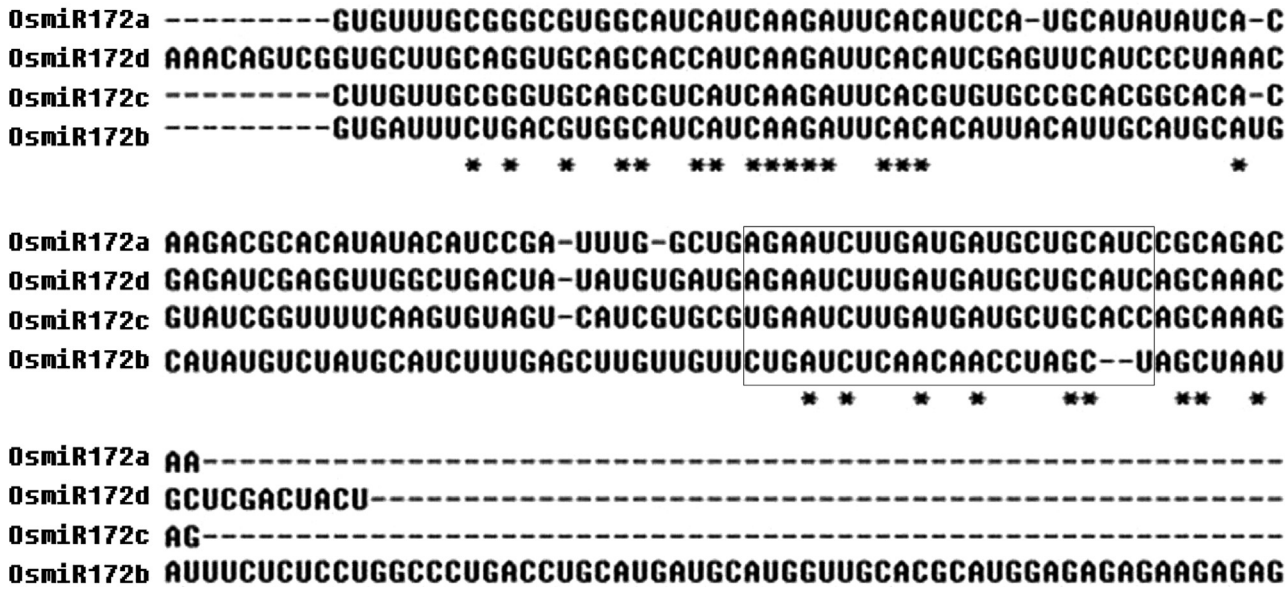


Figure 1 - Sequence alignment of rice *miR172*s. *Conserved nucleotides. The core region is framed.

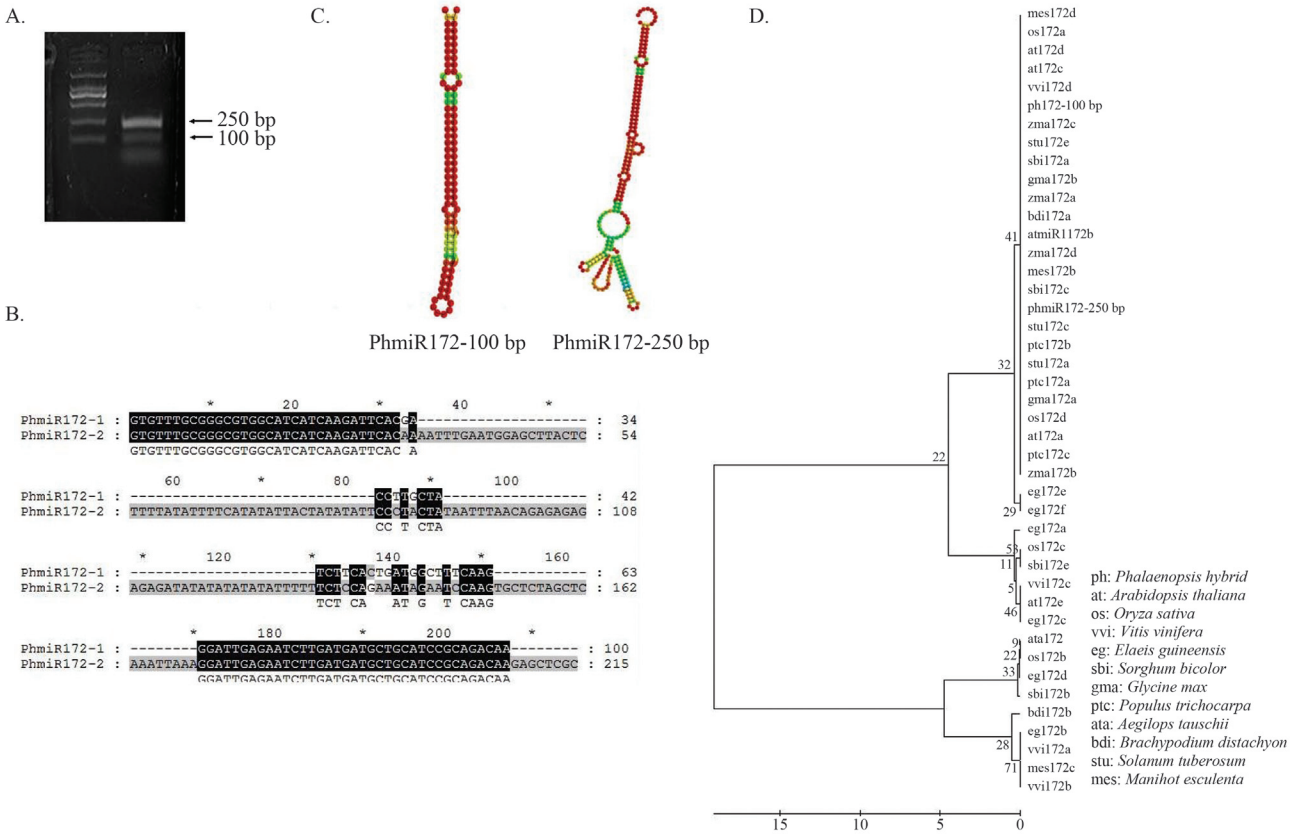


Figure 2 - Cloning and sequence analysis of Phalaenopsis miR172s. (A) PCR amplification of novel PhmiR172 sequences. (B) Sequence alignment of PhmiR172-1 and PhmiR172-2. Dark regions represent identical nucleotides. The primer sequences are indicated under the alignment. *Conserved nucleotides. (C) Secondary structures of precursor PhmiR172s, both with unique stem-loop structures. The red color indicated the higher possibility of base-pairing.When the color is inclined to be red, the possibility of base-paring is becoming higher. (D) Phylogenetic tree of *miR172* precursors from *Arabidopsis*, *Oryza* and *Phalaenopsis* constructed using the maximum evolution method. The horizontal scale at the bottom was the scale for substitution rate (%). The numbers at the nodes refer to the bootstrap value (maximum is 100), which implied the reliability of existing clades in the tree. The number in each clade represented the percentage of success for constructing the existing clade. The system has performed 1000 replicates to construct the phylogeny.

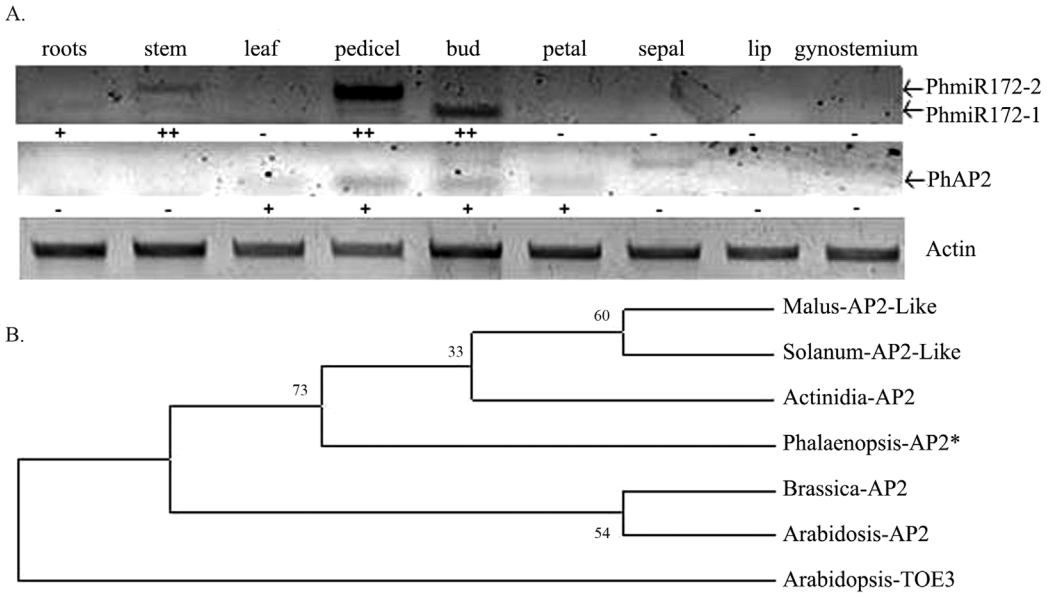


Figure 3 - Semi-quantitative RT-PCR analyses of *PhmiR172s* and its target *PhAP2* gene in different tissues of *P. hybrida*. (A) Semi-quantitative RT-PCR of *PhmiR172s* (+: expression of *PhmiR172-1*; ++: expression of *PhmiR172-1* and *PhmiR172-2*; -: no expression) and *PhAP2* (+: expression; -: no expression). (B) Phylogenetic analysis of PhAP2 and AP2 sequences from other species.

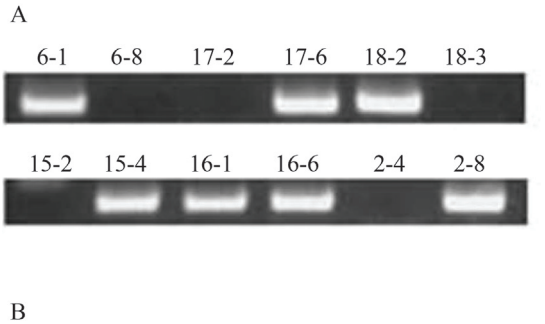
on this finding, we inserted the *PhmiR172*-2 transcript into *A. thaliana* plants to obtain homozygous plants overexpressing *PhmiR172-2* (T_3). We identified the hygromycin tolerance gene (hpt; hygromycin phosphotransferase) that served as a marker for the vector sequences that remained in transgenic T_2 plants along with the 250-bp *PhmiR172-2* sequence (Figure 4A). Hpt-positive seeds from T_2 and T_3 seeds were analysed for gene expression and phenotype. The T_3 plants expressed transcripts that were absent in control wild-type (WT) plants, although the sizes of these transcripts were not uniform (Figure 4B).

As expected, the T_3 plants had an early flowering phenotype ($p = 0.02$), flowering on average three days earlier than the controls (Figure 4C), *i.e.*, the 23rd day of growth (counting from seed germination, Figure 4D 2) compared to the 26th day for WT plants(Figure 4D 7). There was no difference in leaf number between WT and transgenic plants when they blossomed ($p = 0.4$) (Figure 4C). The phenotypes of WT and transgenic plants are shown in Figure 4D.

Discussion

MiR172 is an miRNA that regulates flower development by targeting the *TOE1* and *AP2* family genes (Aukerman and Sakai, 2003). These genes belong to the A family in the ABC flowering model proposed by Bowman *et al.* (1991) and control early floral whorls. Overexpression of *miR172* induced early flowering and changed the floral organ identity. In *Arabidopsis*, pAP2::AP2m3 transgenic lines, which escape repression by *miR172*, have a dramatic phenotype involving indeterminate floral tissues (Chen, 2004), which suggests the importance of *miR172* in regulating the floral meristem via targeting of *AP2*. *Arabidopsis miR172* defines the boundary of B family gene expression (Chen, 2004) and restricts AP2 expression to the stamen to prevent stamen-petal transformation (normally associated with AP2 overexpression) (Wollmann *et al.*, 2010).

Phalaenopsis is an economically important flower with a long flowering period that leads to high prices. To accelerate the flowering period of *Phalaenopsis* by genetic engineering, we isolated two forms of *miR172* from *P. hybrida*. We had previously failed to isolate *Phalaenopsis miR172* using the rapid identification of 5' and 3' ends of cDNA. However, since this miRNA is conserved among various species and forms a stem-loop structure we deduced that miRNA* should also show some degree of conservation. For this, rice *miR172*s were compared and primers were designed based on the sequences of conserved regions (Figure 1), particularly the conserved nu-



C

	Controls	Transgenetic T3 lines
FD	23 ± 0.3	26 ± 1
N_{RL}	10	10 ± 0.2

FD = Flowering Day; N_{RL} = Number of Rosette Leaf

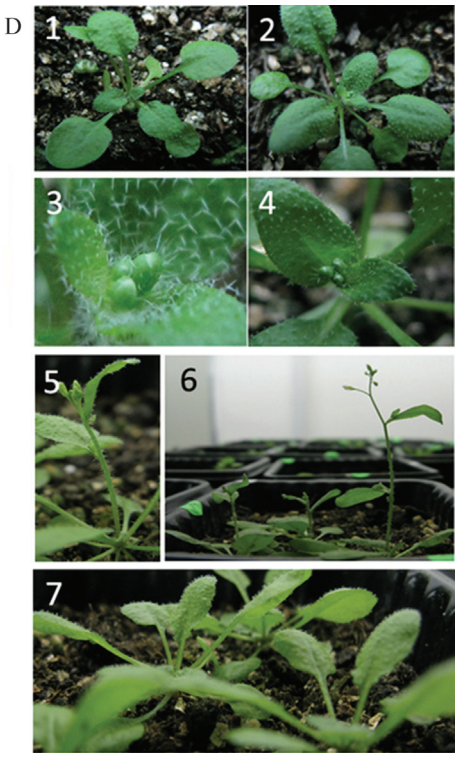


Figure 4 - Identification and characterization of transgenic and wild-type *Arabidopsis*. (A) Hpt (hygromycin phosphotransferase marker gene) tests on T_2 plants. (B) *PhmiR172* transcript detection in controls (CK, transgenic *Arabidopsis* with empty vector) and experimental groups (positive T_3 transgenic plants with *PhmiR172*). (C) Statistics of flowering days (FD, start from seeding, $p = 0.02$) and number of rosette leaves (NRL) when blossoming ($p = 0.4$) of T_3 *PhmiR172-2*-expressing plants and controls. (D) Phenotypes of control (transgenic *Arabidopsis* with empty vector) and experimental (T_3) plants. (1) Controls on the 23rd day of growth in which there was no flowering, (2) Transgenic plants starting to flower on day 23, (3) Transgenic buds on day 23, (4) Transgenic buds on day 26, (5) Transgenic plant on day 26, (6) Transgenic plants on day 29, and (7) Control plant starting to flower on day 26.

cleotides at the 3’ ends of both primers. RT-PCR using genomic DNA as the template yielded two *Phalaenopsis miR172* precursors. This method can be used to isolate other homologous miRNAs because the stem-loop sequences of most miRNAs are conserved between *Arabidopsis* and rice miRNAs* (Figure 5).

PhmiR172-2 showed higher sequence identity with *miR172* from *Arabidopsis* and rice than did *PhmiR172-1*. Phylogenetic analysis indicated that *PhmiR172-2* formed a cluster with rice *miR172d* and *Arabidopsis miR172b*. The shared conserved sequence among *miR172*s was initially identified in *Phalaenopsis miR172*. *PhmiR172-1* diverged

Figure 5 - Sequence alignment of *Arabidopsis* and *Oryza* miRNAs. *Conserved nucleotides. The core sequences of the miRNAs are underlined. At: *Arabidopsis thaliana*; Os: *Oryza sativa*.

Figure 5 (cont.) - Sequence alignment of *Arabidopsis* and *Oryza* miRNAs. *Conserved nucleotides. The core sequences of the miRNAs are underlined. At: *Arabidopsis thaliana*; Os: *Oryza sativa*.

considerably from the main branch of plant *miR172*s, which suggested that it may be specific to *P. hybrida*. The predicted secondary mRNA structure of the *Phalaenopsis miR172* precursor included a unique stem-loop that probably contributed to the relative stability of this mRNA in cells and facilitated its recognition for subsequent splicing.

The expression of *Phalaenopsis miR172*s and their target, *PhAP2*, was investigated. The primers for *PhAP2* were designed based on conserved sequences such that the overall level of *PhAP2* expression should represent or include that of several members of the AP2 family. As expected, semi-quantitative RT-PCR indicated that the level of *PhmiR172*s precursors was negatively correlated with that of the target genes in tissues such as root, stem, leaf and petal. Enhanced expression of *miR172*s may suppress the levels of *Phalaenopsis* AP2 family members, suggesting an antagonistic relationship between *miR172* and the AP2 family in *Phalaenopsis* species. In this regard 5' RACE PCR could be useful for demonstrating target cleavage. Real-time PCR would provide better quantification of the expression, but for *PhmiR172-1* and *PhmiR172-2* combined.

Intriguingly, the expression levels of the two *PhmiR172*s were not identical. The 250-bp transcript was detected mainly in stem and pedicel, while the 100-bp transcript was detected mainly in bud and root. This divergent expression suggested different functions and regulatory mechanisms for the two miRNAs, a conclusion consistent with the sequence divergence between these two members, as indicated by the phylogenetic analysis.

The over-expression of *PhmiR172* in *Arabidopsis* can lead to early flowering and provides evidence for the conserved function of *miR172* among plants. Further exogenous expression of *PhmiR172* should be done using *Phalaenopsis* as the host. In previous work, the over-expression of *miR172* led to altered floral organ identity in *Arabidopsis* and rice (Zhao *et al.*, 2007; Zhu *et al.*, 2009); this phenomenon was not observed here.

In conclusion, the cloning and functional verification of *PhmiR172* will provide a better understanding of the control of flowering time in *Phalaenopsis*. In addition, the newly identified *Phalaenopsis miR172*s can be used in genetic engineering to accelerate the flowering time of this orchid. This homologous cloning method can be applied to miRNAs from a wide variety of plant species.

Acknowledgments

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Internet Resources

RNA structure analysis software, RNA fold, http://bibiserv.techfak.uni-bielefeld.de/rnafold/submission.html (December 16, 2012).

Mega5.0 software, http://www.megasoftware.net/faq.php (January 7, 2012).

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