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 (squamosa promoter binding protein-like) (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).

MicroR172 participates in the photoperiod pathway and is positively regulated by GI (GIANTANEA) 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 (Wa 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’-GCCAAGCTT GTGTTTGCGGGCGTGGCATCATCAAGATTC-3’ and Reverse - 5’-GGAGCTCTTGTCTGCGGATGCAGCATCATCAAGAT-3’.

Sequencing and analysis of *Phalaenopsis* miR172s

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 accession number of the miR172s 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.

Transgenic *Arabidopsis*

The newly identified *PhmiR172* sequences were cloned into the pHV 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 T3 plants. The experiment was carried out with three independent occasions with n = 3/group each time. RNA was extracted with RNAiso Plus (Takara) from T3 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.

| Target gene | Primer sequence (5’ to 3’) | PCR conditions |
|-------------|-----------------------------|----------------|
| **ACTIN**   | Forward: TGGAACCTGCAAGACGC  | 30 s 94 °C, 30 s 55 °C, 30 s 72 °C; 28 cycles |
|             | Reverse: GCACGCCAAGAGTCAAAA |                |
| *PhmiR172*  | Forward: GTTTGCGGGCCTGATCATCAAGATTC | 30 s 94 °C, 30 s 58 °C, 30 s 72 °C; 30 cycles |
|             | Reverse: TTGTCTGCGGATGCAGATCATCAAGAT | |
| *PhAP2*     | Forward: AAGTTCACAGTATAGAGG | 30 s 94 °C, 30 s 55 °C, 30 s 72 °C; 30 cycles |
|             | Reverse: GCATGCGTGCAGGTCGAC | |

Table 1 - Accession numbers of miR172s 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              |

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

Isolation and sequence analysis of *Phalaenopsis* miR172

Since *Phalaenopsis* is monocotyledonous primers were designed based on the sequences of rice miR172s. The stem sequences of rice miR172s 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* miR172s to prevent false clustering with OsmiR172. The two PhmiR172s were closely related to the miR172s of most other species and formed a major highly-related cluster that included most of the miR172s studied. Although the main cluster included miR172s from all of the species, several miR172s from seven species diverged from the main branch, including Oryza 172b/c, Vitis miR172a/b/c and others (Figure 2D).

The levels of miR172s were not identical in the same tissues. For example, only the precursor of PhmiR172-1 was detected in roots and buds, whereas both PhmiR172s were detected at a higher level in the stem and pedicel (Figure 3A).

**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 PhmiR172s and PhAP2 were complementary in some tissues, such as root, stem, leaf and petal, which implied that PhmiR172s 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 PhmiR172s 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 miR172s from other species than did PhmiR172-1. Based
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-pairing 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 (T3). We identified the hygromycin tolerance gene (hpt; hygromycin phosphotransferase) that served as a marker for the vector sequences that remained in transgenic T2 plants along with the 250-bp PhmiR172-2 sequence (Figure 4A). Hpt-positive seeds from T2 and T3 seeds were analysed for gene expression and phenotype. The T3 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 T3 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 miR172s were compared and primers were designed based on the sequences of conserved regions (Figure 1), particularly the conserved nu-

![Figure 4](image-url)

**Figure 4** - Identification and characterization of transgenic and wild-type Arabidopsis. (A) Hpt (hygromycin phosphotransferase marker gene) tests on T2 plants. (B) PhmiR172 transcript detection in controls (CK, transgenic Arabidopsis with empty vector) and experimental groups (positive T1 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, PhmiR172-2-expressing plants and controls. (D) Phenotypes of control (transgenic Arabidopsis with empty vector) and experimental (T1) 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 miR172s was initially identified in Phalaenopsis miR172. PhmiR172-1 diverged

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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.
considerably from the main branch of plant miR172s, 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 miR172s 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 PhmiR172s precursors was negatively correlated with that of the target genes in tissues such as root, stem, leaf and petal. Enhanced expression of miR172s 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 PhmiR172s 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.

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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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