# Isolation and Expression Analysis of *DlAP2* Targeted by *miR*172a in *Dendrocalamus latiflorus*

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## Abstract

Much attention has been paid to the regulation of bamboo flowering, which has become a hot topic to reveal the regulatory mechanisms at the molecular level through methods of molecular biology. Transcription factors of APETALA2 (AP2) sub-family were involved in the growth process of plant development, such as the regulation of floral meristem, ovule and seed development etc. *AP2* gene belonged to class A genes of flower development model, involving in developmental regulation of petals, while its own expression was regulated by *miR*172a, which targeted *AP2* gene with cutting degradation. One *miR*172a-targeted gene named *DlAP2* was isolated from *Dendrocalamus latiflorus* with the methods of RT-PCR and RACE. Sequence analysis showed that the full length cDNA of *DlAP2* was 1 729 bp including 5′ UTR 81 bp, ORF 1 464 bp, 3′ UTR 351 bp and 24 bp polyA. *DlAP2* encoded a putative protein with 487 amino acids, which included two AP2 domains indicating that it belonged to AP2 group of AP2 subfamily in AP2/ERF family. DlAP2 had a high homology with those AP2 from other monocots, among which the highest consistency was 84.6% with that of moso bamboo (*Phyllostacys edulis*) AP2 (AGH68972), and the consistency with transcription factor AP2D23-like (AAW78371) in model plant rice (*Oryza sativa*) and ARGET OF EARLY ACTIVATION TAGGED1 (T NP_001189625) in *Arabidopsis thaliana* were 75.1% and 49.6% respectively. There was one *miR*172a complementary sites (CTGCAGCATCATCAGGATTCT) at 130 bp of the 3′ end in ORF. RLM-5′RACE analysis showed that *DlAP2* was regulated by *miR*172a through cleavage mainly at the site between the 11th and 12th bases. Real-time quantitative PCR results showed that the expression of *miR*172a was a declining trend from buds of 1.0 cm, 1.5 cm, 2.0 cm, top leaves of flowering branches, top leaves of branches without flowering, to that of control in leaves of normal plant, with the highest level in buds of 1.0 cm and the lowest one in leaves of normal plant,

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and the similar level in leaves of flowering branches and that of not flowering branches on the flowering plant. These results demonstrated that the expression of *DlAP2* was showing an opposite trend correspondingly to those of *miR*172, the lowest level was in buds of 1.0 cm and the highest one was in leaves of normal plant, which indicated that *miR*172a had regulatory effect on the expression of *DlAP2*.

## Key Words

*Dendrocalamus latiflorus*, *AP2* gene, *miR*172a, expression analysis

## Introduction

Bamboo, being as a tribe of flowering and evergreen perennial monocots, classified in the subfamily Bambusoideae within the grass family Poaceae that includes rice, maize, wheat and other cereals (Han et al., 2009), is known to the fast-growing characteristics and one of the most important non-timber forest resources in the world (Zhou et al., 2005; Peng et al., 2010). About 2.5 billion people depend on bamboo economically, and the international trade in bamboo amounts to over 2.5 billion US dollars per year (Lobovikov et al., 2007). However, collective death in some bamboo species has occasionally occurred after flowering over a large area, which results in a large loss, even for a devastating blow to bamboo resource base. The reasons for bamboo death after flowering remain unclear. Therefore, much attention had been paid to bamboo flowering, including the study on environmental factors, nutrition factors, the specific pathways and genes involved in bamboo flowering and flower development (Tian et al., 2005; Gao et al., 2007; Lin 2009; Gao et al., 2010; Chen et al., 2013; Peng et al., 2013;Gao et al., 2014). However, few studies have focused on the characterization of microRNA (miRNA) in bamboo (Zhao et al. , 2013; Zhao et al. , 2014; Xu et al., 2014), little is known about how the miRNAs regulate bamboo flowering.

MiRNAs have important functions in different developmental stages and metabolic process by mediating gene silencing at transcriptional and post-transcriptional levels (Llave et al., 2002; Bari et al., 2006). In plants, miRNAs regulate leaf morphogenesis, the development of root and flowers and other key processes, such as response to biotic and abiotic stresses, via interactions with their specific target mRNAs (Palatnik et al., 2003; Chen, 2004; Wang et al.,

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2005; Kidner and Martienssen, 2005; Guo et al., 2005; Jones-Rhoades et al., 2006). MiRNAs have played key roles in the process of plant from flora induction to flora organ specification, while *APETALA2* (*AP2*) gene is one of important factors involved in regulation of flower development. *AP2* expression is regulated at the post-transcriptional level by a miRNA, *miR*172 (Aukerman and Sakai, 2003; Kasschau et al., 2003; Chen, 2004). The discovery of *miR*172 as post-transcriptional negative regulator of AP2 immediately provided a potential means to solve the apparent paradox of AP2 mRNA being ubiquitously expressed.

Ma bamboo (*Dendrocalamus latiflorus*) is an evergreen species locally known as 'tropical giant bamboo', which forms abundant forests in southern China and southeast Asia, and is a valuable natural resource used as food, building material and other human consumption (Lin et al., 2007). To reveal the expression and regulation of *miR*172 and AP2 gene in bamboo plants, flowering *D. latiflorus* was selected as material to carry out the following research. In this paper, the full-length cDNA of *DlAP2* gene was isolated and characterized from *D. latiflorus*, the *miR*172a-mediated cleavage site was investigated using RLM-RACE method, the expression profiles of *miR*172a and *DlAP2* were analyzed in different tissues of flowering *D. latiflorus*. This is the first report of *DlAP2* targeted by *miR*172a in bamboo, which is a primary research of bamboo miRNA and will encourage further design strategies for the directional improvement of bamboo timber and shoots quality by molecular engineering.

## Materials and methods

### Plant materials and strains

The two-year seedlings of bamboo (*Dendrocalamus latiflorus*) from tissue culture were potted in chamber under long-day conditions (16 h light / 8 h dark) at 25°C with the light intensity of 150 μmol·m$^{-2}$·s$^{-1}$. The young branches, buds leaves were collected from the normal seedlings and flowering seedlings respectively, and stored at -80 ºC for further experiment.

*Escherichia coli* strain, DH5α was used as the recipients for routine cloning experiments.

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## Isolation of full length cDNA

Total RNA was isolated from different tissues of *D. latiflorus* with Trizol method according to the protocol recommended by the manufacturer (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized from 500 ng of RNA using the Promega cDNA synthesis system. The 3' cDNA and 5' cDNA were synthesized using the SMART™ RACE kit (Clontech, Mountain View, CA). Primers (DlAP2-F: 5′- ATGGAGCTGGATCTGAACGTG -3′, DlAP2-R: 5′- CGGCTTCTCTACCATTGCAT -3′were designed on the basis of the sequence of the conservative domain of AP2 gene in *Zea may* (EU974370), Gradient PCR amplification was performed with DlAP2-F and DlAP2-R to optimize the annealing temperature.

Specific primers were designed according to the obtained sequence using the procedure described above. The primers used for 5' rapid amplification of cDNA ends (RACE) were DlAP2-5-1 (5-1：5′- GTGCAGGGTGACGCCTCTGTATTTGGA -3′) and DlAP2-5-2 (5′- GCCTGCGGGAGCGGCAAGATTCTGATGT-3′); those used for 3' RACE were DlAP2-3-1 (5'- GATGGGAGGCTCGCATGGGCCAGTTC -3') and DlAP2-3-2 (5'- GCGATCAAATGCAATGGTAGAGAAGCCG -3'). Touch down PCR were performed with DlAP2-5-1, DlAP2-3-1 and a universal primer mix (UPM) as supplied with the SMART™ RACE cDNA amplification kit, and then the PCR amplicons were used as template for a subsequent nested PCR using primer pairs of DlAP2-5-2 or DlAP2-3-2 with the NUP primer supplied in kit. The PCR fragments were cloned into pGEM-T easy vectors (Promega, Madison, WI) using standard protocol and sequenced using an ABI 3730 sequencer (Applied Biosystems, Bedford, MA). The full length cDNA was obtained by the combination of conserved sequence with the 5' and 3' end sequences.

## Sequence analysis

Sequence analysis was carried out with the DNASTAR software package, and the cDNA sequences were subjected to a similarity search against the NCBI database (http://www.ncbi.nlm.nih.gov) using the Blastx algorithm with default parameters. The target sites of miRNA were analyzed using the online software

(http://plantgrn.noble.org/psRNATarget/). On the basis of this assembled sequences, the putative amino acid were analyzed using the software of Motif Scan (Sigrist et al., 2010), ProtScale (Gasteiger et al., 2005) and SOMPA (Geourjon and Deléage, 1995) to predict the physicochemical properties, structure and functional domains. Three-dimensional model analysis was carried out by using AP2 template of Arabidopsis. A neighbor joining (NJ) tree was constructed using the MEGA4.0 software package and the CLUSTAL algorithm in conjunction with the homologue sequences of AP2.

## *MiR*172a-mediated cleavage site analysis of the target gene

Primers for 5′RACE were designed on the basis of prediction for target miRNA sites, outer primer 172F1 (5′- CCAATCCACTACAAGAAACCACCCCGG -3′) and Inner primer 172F2 (5′- CTTCCCGGCAAAT TTACAGTGTGGC -3′) were synthesized respectively. The cDNA for RLM-RACE was synthesized using Full RACE Kit with TAP (Code No.6107) followed the instruction manual of the kit. Outer PCR was carried out using outer primer and 172F1 with final volume of 20 μl containing 10×GC Buffer Ⅱ 2 μL, 1×cDNA Dilution Buffer Ⅱ 8 μL, cDNA template 2 μL, 172F1 (10 μM) 1 μL, 5′RACE Outer Primer(10 μM) 1 μL, *LA Taq*（5 U·μL$^{-1}$）0.25 μL and ddH$_2$O 5.75 μL. The PCR procedure involved in an initial denaturation period at 94°C for 3 min, followed by 30 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72°C for 30 s, then with a final extension period at 72°C for 10 min.

After that, inner PCR was performed inner primer and 172F2 with final volume of 20 μl containing 5×PrimerSTAR Buffer (Mg$^{2+}$ plus) 4 μL, dNTP mix (2.5 mM each) 1.6 μL, outer PCR reaction product 1 μL, 172F2（10 μM） 1 μL, 5′RACE Inter Primer（10 μM）1 μL, PrimerSTAR HS DNA Polymerase（2.5 U·μL$^{-1}$）0.2 μL and ddH$_2$O 11.2 μL. The inner PCR procedure involved in an initial denaturation period at 98°C for 3 min, followed by 30 cycles at 98 °C for 30 s, 55 °C for 30 s, and 72°C for 30 s, then with a final extension period at 72°C for 10 min. The PCR fragments were cloned into pGEM-T easy vectors and sequenced.

## Real time-PCR analysis

The stem-loop primer (5′- CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCT GCAGCA-3′) for cDNA synthesis from miRNA was designed using stem-loop primer method, and the quantitative analysis of expression *miR*172 was carried out with specific

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primer (miR172a- F：5′- AGCAGCATATATAGAATCCTGATG -3′) and universal primer (miR172a- R：5′- CTCAACTGGTGTCGTGGA GTC -3′). Primers were designed from non-conserved region of the isolated *DlAP2* using ABI Primer express 3.0 (F1: 5'-TTACTGAAGTTGGTGCTGAAGGT -3'; R1: 5'- TTACTGAAGTTGGTGCTGAAGGT -3').

The cDNA templates were synthesized using the total RNA isolated from buds of 1.0 cm, 1.5 cm, 2.0 cm, top leaves of flowering branches, top leaves of branches without flowering and the leaves of normal plant, respectively. Real time fluorescent quantitative PCR amplification and analysis were carried out using Light Cycler® 480 Real-Time PCR System with LightCycler® 480 SYBR Green I Master kit (Roche Applied Science). The final volume was 10 μl containing 2×SYBR Premix Ex Taq 5 μl, 0.4 μl of each primer (5 μM), 1 μl of cDNA and 3.2 μl of nuclease-free water. Amplification was performed according to the procedure of an initial denaturation period at 95°C for 2 min, followed by 45 cycles at 95 °C for 30 s and 58 °C for 60 s. Bamboo *NTB* gene (Fan et al., 2013) was selected as a reference gene for *DlAP2*, as well as U6 snRNA for miRNA (Zhao et al., 2013).

For each condition, the qRT-PCR experiments were performed as biological triplicates and expression levels were normalized according to that of the internal control. The relative value of the gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Statistical tests were performed on the qRT-PCR data using SPSS (Statistical Product and Service Solutions) 18.0 software. Error bars representing the standard deviation were derived from the three experiments in triplicate.

## Results

### Gene isolation and sequence analysis

*AP2* homologue genes have high similarities among monocotyledon plants which facilitate the design of primers for RT-PCR to clone DlAP2. As a result of the RT-PCR with DlAP2-F and DlAP2-R, a 990 bp nucleotide fragment was obtained, which had high identities with those in *Oryza sativa*, *Zea mays* and *Triticum aestivum*. Subsequently, a 387 bp fragment from the 5'-end region and a 774 bp fragment from 3'-end region were obtained by 5'- and 3'-RACE, respectively. After analysis of the obtained sequences, a 1729 bp full length cDNA

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was identified containing a 1464 bp ORF, a 81 bp 5′ untranslated region (UTR) and a 184 bp 3′ UTR. There was a high degree of matching target site for *miR*172a in the coding frame near the 3 'end of the 130 bp. The ORF encoded a putative peptide of 487 amino acids including two conserved domains of AP2/ERF which indicated it belonged to the AP2 subfamily of AP2/EREBP family. The theoretic isoelectric point (pI) and calculated molecular mass of DlAP2 were 6.859 and 52.75 kDa, respectively. The GenBank accession numbers of *DlAP2* is KM267641 (Figure 1).

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   1   ACGCGGGGGTCTGGTTGTTTGGGTTTGAGGGGTGTTGATTGGGAGTTGGGATTGATCTTGGTTGAG
  67   GAGGTGGAGGAGGAGATGGAGCTGGATCTGAACGTGGCGGACGGGGCGCCGGAGAAGCCGGAAGCG
                     M   E   L   D   L   N   V   A   D   G   A   P   E   K   P   E   A
 133   ATGGCGCGGAGCGACTCCGGCACGTCGGACTCGCCGGTGCTGAACGCGGAGGCGTCCGGCGGCGGA
         M   A   R   S   D   S   G   T   S   D   S   P   V   L   N   A   E   A   S   G   G   G
 199   GGCGCCGCGGGTGCGCCCGCGGAGGAGGGCTCCAGCTCGACGCCCCCGCCGCTGGCGGTGCTCGAG
         G   A   A   G   A   P   A   E   E   G   S   S   S   T   P   P   P   L   A   V   L   E
 265   TTCAGCATCATGAGGAGCTCGGCGTCGGCCGAGGGCGAGAAAGACGTGGGCGTTGCCGATGACGAG
         F   S   I   M   R   S   S   A   S   A   E   G   E   K   D   V   G   V   A   D   D   E
 331   GAGGAGGCCACGCCGTCGCCTCTGCGGCGGCAGCAGCTCGTCACCCAGCAGCTCTTCCCGGTCGAC
         E   E   A   T   P   S   P   L   R   R   Q   Q   L   V   T   Q   Q   L   F   P   V   D
 397   GCCGGCCCGCCGCGGCCCGTGCCGCAGCCTGGGGCCGAGCTCGGGTTCTTACGCCCCGAGCCGCCG
         A   G   P   P   R   P   V   P   Q   P   G   A   E   L   G   F   L   R   P   E   P   P
 463   GGCCCGCAGCCGGACATCAGAATCTTGCCGCTCCCGCAGGCGCACGCGCCGCCGGCGCAGCCGCAG
         G   P   Q   P   D   I   R   I   L   P   L   P   Q   A   H   A   P   P   A   Q   P   Q
 529   GCTACCAAGAAGAGCCGCCGCGGCCCGCGCTCCCGCAGCTCGCAGTACCGCGGCGTCACCTTCTAC
         A   T   K   K   S   R   R   G   P   R   S   R   S   S   Q   Y   R   G   V   T   F   Y
 595   CGCCGCACCGGCCGCTGGGAATCCCATATTTGGGATTGCGGCAAGCAAGTGTACTTAGGTGGATTT
         R   R   T   G   R   W   E   S   H   I   W   D   C   G   K   Q   V   Y   L   G   G   F
 661   GACACTGCTCATGCTGCTGCAAGGGCGTATGATCGAGCGGCGATCAAGTTCCGCGGCATCGACACG
         D   T   A   H   A   A   A   R   A   Y   D   R   A   A   I   K   F   R   G   I   D   T
 727   GACATAAACTTCGATCTTAGTGACTACGAGGACGACATGAAGCAGGTGAAGAGCCTATCCAAGGAG
         D   I   N   F   D   L   S   D   Y   E   D   D   M   K   Q   V   K   S   L   S   K   E
 793   GAGTTCGTGCACGTCCTGCGACGGCAGAGTACTGGCTTCTCGCGAGGCAGCTCCAAATACAGAGGC
         E   F   V   H   V   L   R   R   Q   S   T   G   F   S   R   G   S   S   K   Y   R   G
 859   GTCACCCTGCACAAGTGCGGCCGATGGGAGGCTCGCATGGGCCAGTTCCTCGGCAAGAAGTACATA
         V   T   L   H   K   C   G   R   W   E   A   R   M   G   Q   F   L   G   K   K   Y   I
 925   TATCTTGGGCTATTCGACAGCGAAGTAGAGGCTGCAAGGGCTTATGATAAGGCTGCGATCAAATGC
         Y   L   G   L   F   D   S   E   V   E   A   A   R   A   Y   D   K   A   A   I   K   C
 991   AATGGTAGAGAAGCCGTGACGAACTTCGAGCCTAGCACATATGATGGGGAGATGCTTACTGAAGTT
         N   G   R   E   A   V   T   N   F   E   P   S   T   Y   D   G   E   M   L   T   E   V
1057   GGTGCTGAAGGTGCAGATGTCGATCTGAACTTGAGCATATCTCAACCAGCTTTGCAGAGCCCCCAA
         G   A   E   G   A   D   V   D   L   N   L   S   I   S   Q   P   A   L   Q   S   P   Q
1123   AGGGATAAGAACTCCCTTGGTCTGCAGCTGCACCATGGATTATTTGATGGCTCTGAAGTGAAAAGA
         R   D   K   N   S   L   G   L   Q   L   H   H   G   L   F   D   G   S   E   V   K   R
1189   GCTAAGATTGATGCTCCCTCTGAACTGGCTGGCCGCCCTCATCGGTTCCCTCTTCTGACCAAGCAT
         A   K   I   D   A   P   S   E   L   A   G   R   P   H   R   F   P   L   L   T   K   H
1255   CCACCAGTCTGGCCTGCCCAATCTCACCCCATATTTTCAAATAATGAGGATGCATCTAGAGATCAT
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           P   P   V   W   P   A   Q   S   H   P   I   F   S   N   N   E   D   A   S   R   D   H
    1321   AACAGGAGGCCAGAGGGGAGCACCGGGGGTGTTCCCAGCTGGGCATGGAAAGTGAGCCACCCTCCA
           N   R   R   P   E   G   S   T   G   G   V   P   S   W   A   W   K   V   S   H   P   P
    1387   CCCACACTACCATTGCCGCTGTTCTCGTCGTTGTCATCGTCATCCGCTGCAGCATCATCAGGATTC
           P   T   L   P   L   P   L   F   S   S   L   S   S   S   S   A   A   A   S   S   G   F
    1453   TCCAGAACCGTCAAGATAGCTATCTCCACCACCCCATCGACCTCCCTCCAGTTCGACCCGATGGCG
           S   R   T   V   K   I   A   I   S   T   T   P   S   T   S   L   Q   F   D   P   M   A
    1519   CCATCATCGTCGAACCACCACCGCTGAATAGAAGCCACACTGTAAATTTGCCGGGAAGCCGGCATC
           P   S   S   S   N   H   H   R
    1585   TTTTTTCCCTCCCGACGTTTCAACGTCTTCGGTTTTGCGCCGGGGTGGTTTCTTGTAGTGGATTGG
    1651   ATTCATGACTGTATTTGCATGCTGCCCAATTGAAAATGTTCCTATTTGCACGCACAAAAAAAAAAA
    1717   AAAAAAAAAAAAA
```

Fig.1 Nucleotide sequence and deduced amino acid sequence of *DlAP2*. Single underline: AP2/ERF domains; Square frame: *miR*172a binding site.

## Protein properties analysis and structure prediction

The analysis result showed that there contained 56 strongly basic amino acids, 59strongly acidic amino acids, 146hydrophobic amino acids, 119 polar amino acids in the protein encoded by *DlAP2*. Protein hydrophilicity / hydrophobicity analysis indicated DlAP2 had strong hydrophilicity.

The prediction of protein structure demonstrated that there also contained a proline-rich region (103-148) and a serine-rich region (444-483) besides the two AP2/ERF domains. There had one amidation site, one N-terminal glycosylation site, one cAMP- and cGMP-dependent on protein kinase phosphorylation site, six casein kinase     phosphorylation sites, 8 N- myristoylation sites and 5 protein kinase C phosphorylation sites. The secondary structure analysis indicated DlAP2 protein contained four kinds of structure such as no rules of curling, β fold, extension chain and α helix, in which the no rules of curling was the most one with 315 aa covering 64.68% of DlAP2, followed by α helix with 97 aa (19.92%), next extension chain with 53 aa (10.88%) and β fold with 22 aa (4.52%). Homology modeling was used for the AP2 domains in DlAP2. Using the Arabidopsis AtERF1 the GBD (GCC-box binding domain) protein three-dimensional configuration as a template (1gccA), the three-dimensional configuration of AP2 domains in DlAP2 was predicted. They all had typical structure of AP2 with 3 α helix and 3 β folds, which had similarity to the template with 45.16% and 32.79%, respectively (Figure 2).
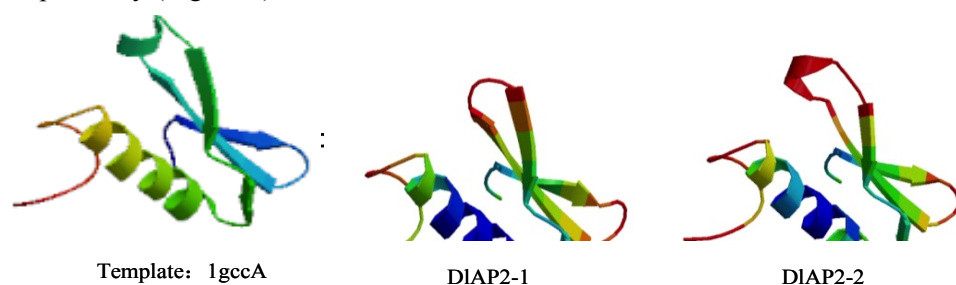


Template：1gccA          DlAP2-1          DlAP2-2

Fig.2 Three-dimensional configuration prediction of AP2 in DlAP2

## Phylogenetic Analysis of DlAP2

The deduced amino acid sequence of DlAP2 was analyzed with AP2 from other plant species using Blastp in NCBI database. The results showed that there were 91 protein sequences with more than 50% homologous to DlAP2, among which AP2 (AGH68972) of *Phyllostachys edulis* had the highest identities (84.6%), and those of transcription factor AP2D23-like (AAW78371) in *Oryza sativa* and ARGET OF EARLY ACTIVATION TAGGED1 (NP_001189625) in Arabidopsis thaliana were 75.1% and 49.6% respectively.

Construction of phylogenetic tree showed that AP2 homologous proteins from monocots and dicots were clustered into two large branches, DlAP2 and AP2 of *P. edulis* clustered together nearby the homologous proteins from monocots such as *Hordeum vulgare*, *Triticum aestivum*, *Brachypodium distachyon*, *Setaria italica*, *Zea mays* and *Sorghum bicolor*, while farther away from those of dicots (Figure 3).
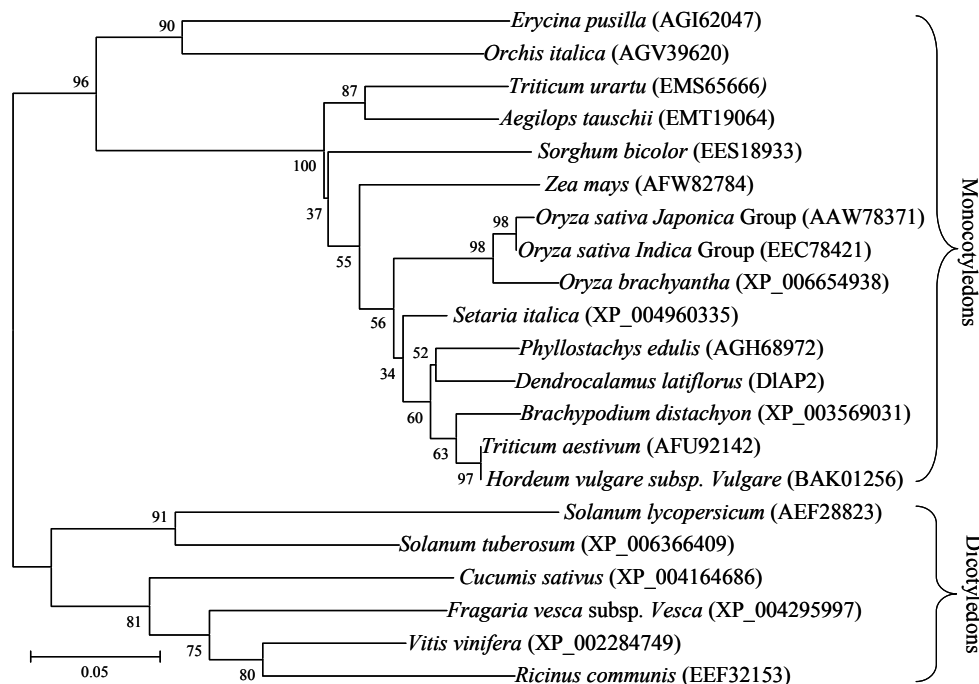


Fig.3 Phylogenetic tree analysis based on AP2 homologue proteins. Numbers on major

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branches indicated bootstrap estimates for 1000 replicate analyses.

## *MiR*172a-mediated cleavage site identification in *DlAP2*

RLM-5′RACE and sequencing methods were used to confirm the *miR*172a-mediated cleavage site in *DlAP2*. The result of sequencing showed that there were nine samples with cleavage sites between the $11^{th}$-$12^{th}$ bases, and only one sample with a cleavage site between the $12^{th}$-$13^{th}$ bases in the ten sequenced samples (Figure 4). This result was consistent with that of AP2 mediated by *miR*172a in Arabidopsis, which cleavage sites were mainly between the $11^{th}$-$12^{th}$ bases. We conclude that *miR*172a regulates its target gene *DlAP2* in *D. latiflorus* by a translational mechanism.

<div align="center">
9/10     1/10

miR172 target sequence in *DlAP2*:  C T G C A G C A T C A T C A G G A T T C T

| | | | | | | | | | | | | | | | | | | | |

*miR*172a:  G A C G U C G U A G U A G U C C U A A G A
</div>

Fig.4 Target sites for *miR*172 in three AP2-like transcripts. Cleavage sites are indicated by arrows. The numbers of 5′ RACE clones sequenced that correspond to each cleavage product are indicated.

## Expression analysis of *miR*172a and *DlAP2* in different tissues

The expression patterns of miRNAs could provide clues to their functions (Yao et al., 2007). Real time PCR was employed to detect the expression level of both *miR*172a and *DlAP2* in different tissues of flowering bamboo (*D. latiflorus*).
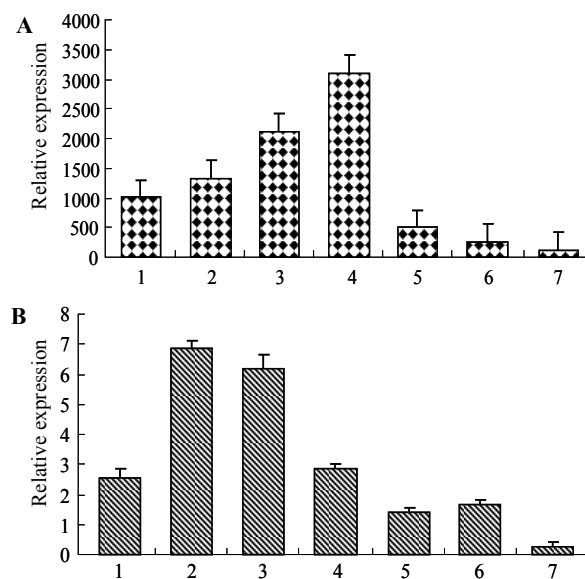


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Fig.5 *miR*172a (A) and *DlAP2* (B) expression in different tissues. 1: Young branch of flowering plant; 2: 1.0 cm bud; 3: 1.5 cm bud; 4: 2.0 cm bud; 5: Top leaf on the flowering branch; 6: Top leaf on the unflowering branch of flowering plant; 7: leaf of unflowering plant.

The result demonstrated that the expression of *miR*172 was a declining trend from buds of 1.0 cm, 1.5 cm, 2.0 cm, top leaves of flowering branches, top leaves of branches without flowering, to that of control in leaves of normal plant, with the highest level in buds of 1.0 cm and the lowest one in leaves of normal plant, and the similar level in leaves of flowering branches and that of not flowering branches on the flowering plant (Figure 5A). Meanwhile the expression of *DlAP2* was showing an opposite trend correspondingly to those of *miR*172, the lowest level was in buds of 1.0 cm and the highest one was in leaves of normal plant (Figure 5B), which indicated that *miR*172 had regulatory effect on the expression of *DlAP2*.

## Discussion

Unlike other MADs-box gene family, AP2 had unique AP2 domains and were important factors in the regulation of floral development in plants, which were divided into three subfamilies including AP2, EREBP and RAV1/RAV2 (Lin et al., 2007).There is a *miR*172 binding site in *AP2* gene of Arabidopsis using large-scale sequencing, and it was confirmed that the expression of *AP2* was regulated by *miR*172 using the transgenics of *miR*172 and the *apetala2* mutant (Jones-Rhoades et al., 2006; Zhao et al., 2007). There was one *miR*172a complementary sites (CTGCAGCATCATCAGGATTCT) at 130 bp of the 3′ end in ORF of *DlAP2*. RLM-5′RACE analysis showed that *DlAP2* was regulated by *miR*172a through cutting mainly at the site between the 11$^{th}$ and 12$^{th}$ bases.

The expression of *miR*172a in flowering tissues of *D. latiflorus* was significantly higher than that of unflowering leaves, which is similar to *miR*172 over-expression can lead to early flowering tomato (Martin et al., 2009). With the elongation of the flower bud, the expression of *miR*172a was up-regulated, while that of *DlAP2* gene was down-regulated, which may be explained by the finish tend of the development of flower buds reached 2.0 cm (the differentiation is complete in each part of its floral organ). Little expression changes of *miR*172a and *DlAP2* were found in young branches, top leaves of flowering branches and top leaves of branches without flowering, this may be that they are in a state of equilibrium in vegetative organs. This was

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confirmed in Arabidopsis, in which any member of the AP2 family, including TOE2, SNZ and SMZ meditated by *miR*172, expressed constitutively would result in delayed flowering during the transition from vegetative growth to reproductive growth (Jung et al., 2007).

It was reported that *miR*172 exercised regulatory functions together with other miRNAs besides regulating target gene only by itself. For example, *miR*172 was not only involved in developmental regulation infancy, but also in the regulation of maize inflorescence meristem under an indirect effect *miR*156 (Chuck et al., 2007; Chuck et al., 2008). Transcript cleavage and translational inhibition both play a role in AP2 regulation by *miR*172, although assessing the relative importance of the two processes is confounded by a negative feedback loop in which AP2 represses its own transcription (Schwab et al., 2005; Mlotshwa et al., 2006). Recent report indicated that 84 conserved miRNAs belonging to 17 families, and 81 novel miRNAs were identified in *D. latiflorus*, and one hundred and sixty-two potential targets were predicted for the 81 novel bamboo miRNAs. Several targets for the novel miRNAs are transcription factors that play important roles in plant development (Zhao et al., 2013). Therefore, *miR*172a regulation of growth and development for *D. latiflorus* will be a complex regulatory network, further study on the mutual adjustment mechanism of other miRNAs and their target genes in *D. latiflorus* should be carried out, in order to make a comprehensive analysis of its growth and development.

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