Cloning and Functional Analysis of TCAP3 Gene in *Taxus Chinensis var. mairei*

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***Abstract*—** *Taxus Chinensis var. mairei is a valuable plant species for timber and taxoids isolated from this species are very important compounds that are used for cancer treatment. Although chemical investigation on T. chinensis var. mairei are popular, functional identification of genes isolated from this species is rare. In this investigation, we have isolated TCAP3 gene and analyzed its expression pattern in different tissue and developmental stages through Real time-PCR; then we transformed this gene into Arabidopsis and analyzed its function. Our results demonstrated that its cDNA contains 846 bp bases (coding 197 amino acids) constituted by four typical domains, M, I, K, C with conserved motif, Phylogenetic analysis showed that TCAP3 is more ancient than angiosperm B class genes. Alignment of protein sequence demonstrated the conserved motifs, which illustrated that TCAP3 belongs to gymnosperm Gymno B class MADS-box genes with PI-derived, on C-teminal, which is similar structure to the Gymno B class MADS-box genes that they share the same B class gene specific conserved motif. Expression analysis of TCAP3 in different tissue showed that it only expression in male strobilus, not in leaf, bud and female strobilus at different developmental stages. We divided the stages according to paraffin sections of male strobilus. The results indicated that TCAP3 expresses dynamically along with the male strobilus. Heterologous expression of TCAP3 in Arabidopsis demonstrated that TCAP3 was involved in flower, especially the filaments morphological development.*

***Keywords*— Taxus Chinensis var. Mairei; gene cloning; B-class gene; expression pattern; functional analysis.**

I. INTRODUCTION

Taxus Chinensis var. *mairei* is a typical cretaceous precious tree species scattered distributed in wild and cultivated habitats in eastern, south central, southwest of China. *T. Chinensis* var. *mairei* is a dioecism that has low setting percentage of fruits because of the desynchronized flowering between male and female strobilus. *T. Chinensis* var. *mairei* has low seeds germination rate due to long cycle dormancy. Its natural populations have been endangered or extinct in some original habitats. There are some initial studies on morphology development of male strobilus, but not in molecular level. In this investigation, we have cloned a MADS-box gene that is related to the development of male strobilus from *T. Chinensis* var. *mairei*.

MADS-box genes encode transcription factors that play essential roles in cell signaling and development processes including flowering induction, flower organ identity, and embryo development. The heterodimer formation between B-class MADS-box proteins plays a core role for petal formation through protein-protein interactions in transgenic plants. For example, transgenic gentian ectopically expressing GsPI2 produced an elongated tubular structure that consisted of an elongated petaloid organ in the first whorl and stunted inner floral organs, suggesting that B-class MADS-box proteins might be important for the complete development of petal organs (Nakatsuka, et al. 2016). MADS-box genes in grapevine can be crucial for development of central cell, endosperm (Grimplet, et al. 2016). Hormone-related transcription changes were associated with regulation of MADS-box transcription factor expression in grapevine inflorescence fruit set (Domingos, et al. 2016).

Phylogenetic analysis of MADS-box proteins suggests functional conservation in floral signal integration and meristem determination pathways that may help in devising strategies to improve important traits in apple (Kumar, et al. 2016).

MADS-box transcription factors play central role in peach endodormancy regulation and transitions (Wells, et al. 2015). In sesame (*Sesamum indicum* L.), MADS-box genes were identified from 14 linkage groups of the sesame genome and motif distribution analysis indicated that type II sesame MADS-box genes had more complex structures (Wei, et al. 2015). MADS-box transcription factors play a key role in controlling lateral root development through nitrate signal in Arabidopsis and are positive regulator control lateral and primary root development in rice (Yu, et al. 2015). MADS-box genes are transiently expressed in small numbers of cells in the floral apex in *Nicotiana tabacum* (Mandel, et al. 1994). MADS box genes are involved in proper ovule development in petunia (Angenent, et al. 1995) and regulating floral meristem and floral organ identity (Colombo, et al. 1995). Chromosomal map positions of MADS-box genes were determined in recombinant inbred lines of maize (*Zea mays* ssp. mays). It appears that MADS-box genes are scattered throughout the maize genome (Fischer, et al. 1995).

Studies on distantly related dicot plant species have identified MADS-box genes that specify floral meristem identity and determine the fate of floral organ primordial, providing the basis for further studies into the regulation of floral organ morphogenesis among the grasses (Mena, et al. 1995). In *T. Chinensis* var. *mairei*, investigating the development process of ovulate strobilus and microstrobil for understanding the molecular mechanism of development regulation will not only help us to know sexual reproduction process, but also having important significance in conservation genetics that would help us to generate strategies in the improvement of the seed setting rate and breeding measurements. Meanwhile, It also can provide theoretical support and protection and update of the new way of thinking for the breeding and protection of *Taxus Chinensis* var. *mairei*'s population from the molecular level.

In this investigation, we have isolated TCAP3 and sequenced it; analyzed its expression pattern in different tissue and developmental stages through real time-PCR; then transformed the gene into Arabidopsis mutants to discover its function. This is the first report of cloning and functional analysis of *TCAP3* gene in *Taxus Chinensis var. mairei*.

II. MATERIALS AND METHODS

2.1 Plant Materials

Plants of *Taxus Chinensis* var. *mairei* were grown in campus in Jingzhou, Hubei in China. The stems, leaf, and flower samples were harvested from 8-week-old plants. To examine the spatial expression of TCAP3, the leaves, stems, and flowers of *Taxus Chinensis* var. *mairei* seedlings were collected. All the samples were quickly frozen in liquid nitrogen and preserved at -80 ^{o}C until further analysis.

2.2 Cloning of the Full-Length cDNA of TCAP3

Total RNA was isolated from *Taxus Chinensis* var. *mairei* seedlings using the CTAB method [40]. The concentration and quality of the RNA were measured using spectrophotometry and agarose gel electrophoresis. The primers TCAP3F1 (5'-GGGAAGATTGAAATAAAAATGATTGAGAAC-3'), TCAP5RP1 (5'- AATATCCAGCGCTTTGCAGGTTC-3'), TCAP5RP2 (5'- CGAAATGTAGGTGGAGGAGTTTGGTC -3'), GADPHF (5'- CGGAGACAGTCGATCAAGC -3'), GADPHR (5'- CCCATCCTCAACCCAATAA -3'), TC-QRT-F1 (5'- GAACACAACCAACAGGCAGGTAAC -3'), and TC-QRT-R1 (5'- GCACTCCTGTATCTCTCCAGAATC -3') were designed and synthesized (Shanghai Sangon, Shanghai, China) according to the gene annotation of *Taxus Chinensis* var. *mairei* in the transcriptome database. One-step RT-PCR was performed, and the DNA fragment was amplified with the one-step RT-PCR kit (Dalian TaKaRa, Dalian, China) under the following conditions: 94 ^{o}C for 5 min; 35 cycles of amplification at 94 ^{o}C for 30 s, 56 ^{o}C for 30 s, and 72 ^{o}C for 1 min; 72 ^{o}C for 7 min for 3'RACE and 94 ^{o}C for 5 min; 35 cycles of amplification at 94 ^{o}C for 30 s, 60 ^{o}C for 30 s, and 72 ^{o}C for 1 min; 72 ^{o}C for 5 min for 5'RACE.

2.3 Bioinformatics Analysis and Molecular Evolution Analyses

The obtained nucleotide sequence and deduced amino acid sequence were compared by a BLAST database search (http://www.ncbi.nlm.nih.gov). The molecular weight of the deduced TCAP3 protein was computed with the Compute tool (http://web.expasy.org). Multiple sequence alignment was performed with the Vector NTI suite 10.0 program (Invitrogen, Paisley, UK). A phylogenetic tree was constructed with CLUSTALX 2.0 (Conway Institute UCD Dublin, Dublin, Ireland, http://www.clustal.org) and MEGA 4.0 (Biodesign Institute, Tempe, AZ, USA, http://www.megasoftware.net). The reliability of the tree was measured by bootstrap analysis with 100 replicates.

2.4 TCAP3 Transcript Analysis by Real-Time PCR

The expression level of TCAP3 was determined by real-time PCR (qRT-PCR). A 1 μg aliquot of the total RNA was used as the template for qRT-PCR. qRT-PCR was performed using a Bio-Rad Mini OpticonTM Real-time PCR Mini Cycler (BioRad, Hercules, CA, USA) with SYBR Premix Ex Taq™ II Kit (Dalian TaKaRa) according to the method of Xu et al. [41]. The primers for TCAP3 [TC-QRT-F1 (5'- GAACACAACCAACAGGCAGGTAAC -3') and TC-QRT-R1 (5'- GCACTCCTGTATCTCTCCAGAATC -3')], and housekeeping gene GAPDH gene [GAPDHF (5'- CGGAGACAGTCGATCAAGC -3') and GAPDHR (5'- CCCATCCTCAACCCAATAA -3')] were designed according to the Sequence Detection System software. Raw data were analyzed with MiniOpticon™ Real-time PCR Detection system.

2.5 Histological observation

For histological studies, the stems, leaf, and flower samples of *Taxus Chinensis* var. *mairei* were fixed in formalin, acetic acid and ethyl alcohol (1:1:18, vol/vol) at room temperature for 48 h, dehydrated through a graded series of ethyl alcohol and tertiary butyl alcohol, and embedded in paraffin (58–60°C). Serial sections of 8 mm thickness were cut with a rotary microtome and sections were stained with a 1% aqueous crystal violet solution.

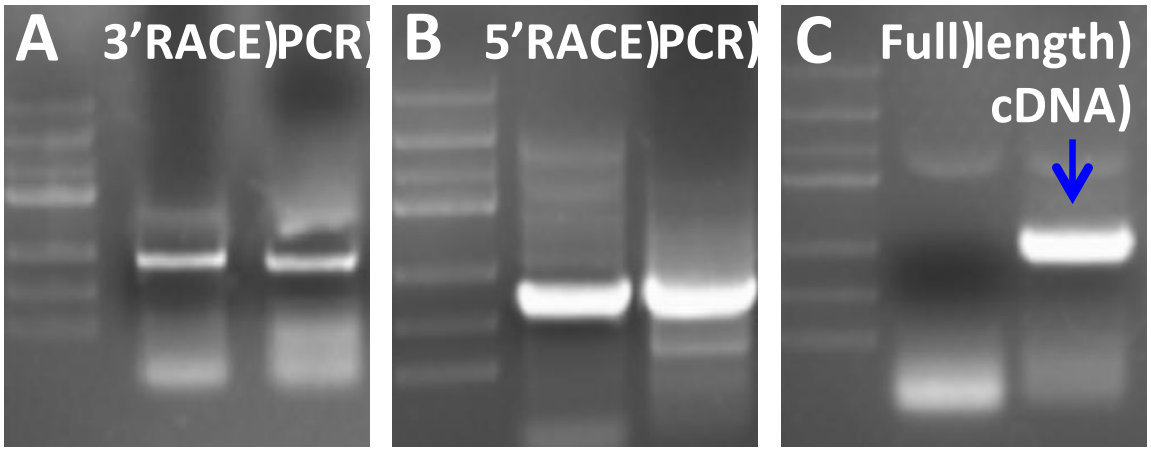
2.6 Transformation of Arabidopsis

Agrobacterium-mediated transformation of Arabidopsis mutants was carried out as described by Tang et al. (2007). *Agrobacterium* strain GV3101-90 carrying the expression vector pBI-TCAP3 was use for infection of Arabidopsis by flower dipping after plants are ready to use. Densities of *Agrobacterium* (OD600=0.3, 0.6, 0.9, 1.2, 1.5, or 1.8.) were tested for their effects on transformation efficiency. Three days after infection, *Agrobacterium* was removed and seeds were used selection in kanamycin-containing plates.

III. RESULTS

3.1 Cloning and Characterization of TCAP3

Using homologous cloning and RACE technique, we isolated a Gymno B class MADS-box gene from *Taxus Chinensis* var. *mairei*. The PCR products (Fig. 1a-c) were sequenced, and results showed that the cDNA sequence of the PCR products was 846 bp (Fig. 1d). The results of BLASTN analysis on NCBI showed that the nucleotide sequence of TCAP3 had a high similarity to those of other TCAP3 genes from *Asparagus officinalis, Muscari armeniacum, Dendrobium crumenatum, Elaeis guineensis, Drimys winteri, Drimys winteri, Drimys winteri, Drimys winteri, Magnolia figo, Michelia alba, Liriodendron chinense, Saruma henryi, Asarum caudigerum, Chimonanthus praecox, Persea Americana, Chloranthus spicatus, Amborella trichopoda, Nymphaea tetragona, Brasenia schreberi, Crocus sativus, Asparagus officinalis, Agapanthus praecox, Wild Malaysian banana, Hypoxis villosa et al*. Phylogenetic analysis (Fig. 2) indicates that the gene we cloned is a member of angiosperm B class MADS-box genes. Therefore, this gene was designated as TCAP3 (GenBank Accession No. KC818630). As shown in Fig. 1, the nucleotide sequence of the CnHMGS gene contained a 846 bp ORF that encodes a predicted protein sequence of 197 amino acid residues.



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1    GAAAATTTTCTACTCAGCAACATTCATTTGCATTTGCAAACATTCTTCGTTGAACACAAA

                70         80         90        100        110        120
61   TACGTCTGAAACAATGGGGAGGGGCAAGATTGAAATAAAGAAGATCGAGAACACAACCAA
1                    M  G  R  G  K  I  E  I  K  K  I  E  N  T  T  N

              130        140        150        160        170        180
121  CAGGCAGGTAACATTCTCAAAGAGAAGGGCAGGACTCTTCAAGAAAGCAAGGGAGTTATC
17     R  Q  V  T  F  S  K  R  R  A  G  L  F  K  K  A  R  E  L  S

              190        200        210        220        230        240
181  CATTTTATGTGCAGCAGATGTTGCTGTTATTGTCTTTAACAGCACAGGAAGGCTCTTTGA
37     I  L  C  A  A  D  V  A  V  I  V  F  N  S  T  G  R  L  F  D

              250        260        270        280        290        300
241  CTTTGCAAGCTCCAGCATGAAAAGGATTCTGGAGAGATACAGGAGTGCATGTGGAGGACA
57     F  A  S  S  S  M  K  R  I  L  E  R  Y  R  S  A  C  G  G  H

              310        320        330        340        350        360
301  TGATTGGAACAATGAACATGAGCAAATGTTGTGTCAATTTAGAAACTTGAGGAAAGAAAA
77     D  W  N  N  E  H  E  Q  M  L  C  Q  F  R  N  L  R  K  E  N

              370        380        390        400        410        420
361  TGAGGATCTGCATAGGGAGATAAGGTATGTGATGGGCGAGGATGCAGACTCATTGTCACC
97     E  D  L  H  R  E  I  R  Y  V  M  G  E  D  A  D  S  L  S  P

              430        440        450        460        470        480
421  AAAGCAACTTGATTATCTCGAAGGAAATCTTGAGATTGCAGCGAAGAAAGTTCGAGAAAG
117    K  Q  L  D  Y  L  E  G  N  L  E  I  A  A  K  K  V  R  E  R

              490        500        510        520        530        540
481  AAAGACGGAAGTCTTAAAATATGAACGCCGCAAAACTGAAAGCAAGGTAAATGGGTTGGA
137    K  T  E  V  L  K  Y  E  R  R  K  T  E  S  K  V  N  G  L  E

              550        560        570        580        590        600
541  GCAGAAGTGCATACTTCTTAAGCAATGGCTTGCAACAGCGGAGAACCTTGAGGAATATGA
157    Q  K  C  I  L  L  K  Q  W  L  A  T  A  E  N  L  E  E  Y  D

              610        620        630        640        650        660
601  CCAAACTCCTCCACCTACATTTCGTGTGCAGCCAAGCCAGCCGAACCTGCAAAGCGCTGG
177    Q  T  P  P  P  T  F  R  V  Q  P  S  Q  P  N  L  Q  S  A  G

              670        680        690        700        710        720
661  ATATTAAATAGGGTTTCATGAACACTAAGTATCAATTATATCTCTATCTGTTTCTTTGTG
197    Y

721  TGTGTTAAACTTTAGGCATTTTCCTCCATAGTTGGAAACATATGTTTTCTCATGTATACA
781  AATATATGGTCTTATTTTATTACTGATTGCTAAAAAGAAACAAATTTAGTCTTAAAAAAA
841  AAAAAA
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FIGURE 1. Cloning of *TCAP3* in *Taxus Chinensis* var. mairei by RACE PCR and the cDNA full-length nucleotide acid sequence and deduced amino acid sequence of the *TCAP3* gene. (A) 3' RACE PCR products; (B) 5' RACE PCR products; (C) the cDNA full-length PCR products; and (D) the cDNA full-length nucleotide acid sequence and deduced amino acid sequence of the *TCAP3* gene.

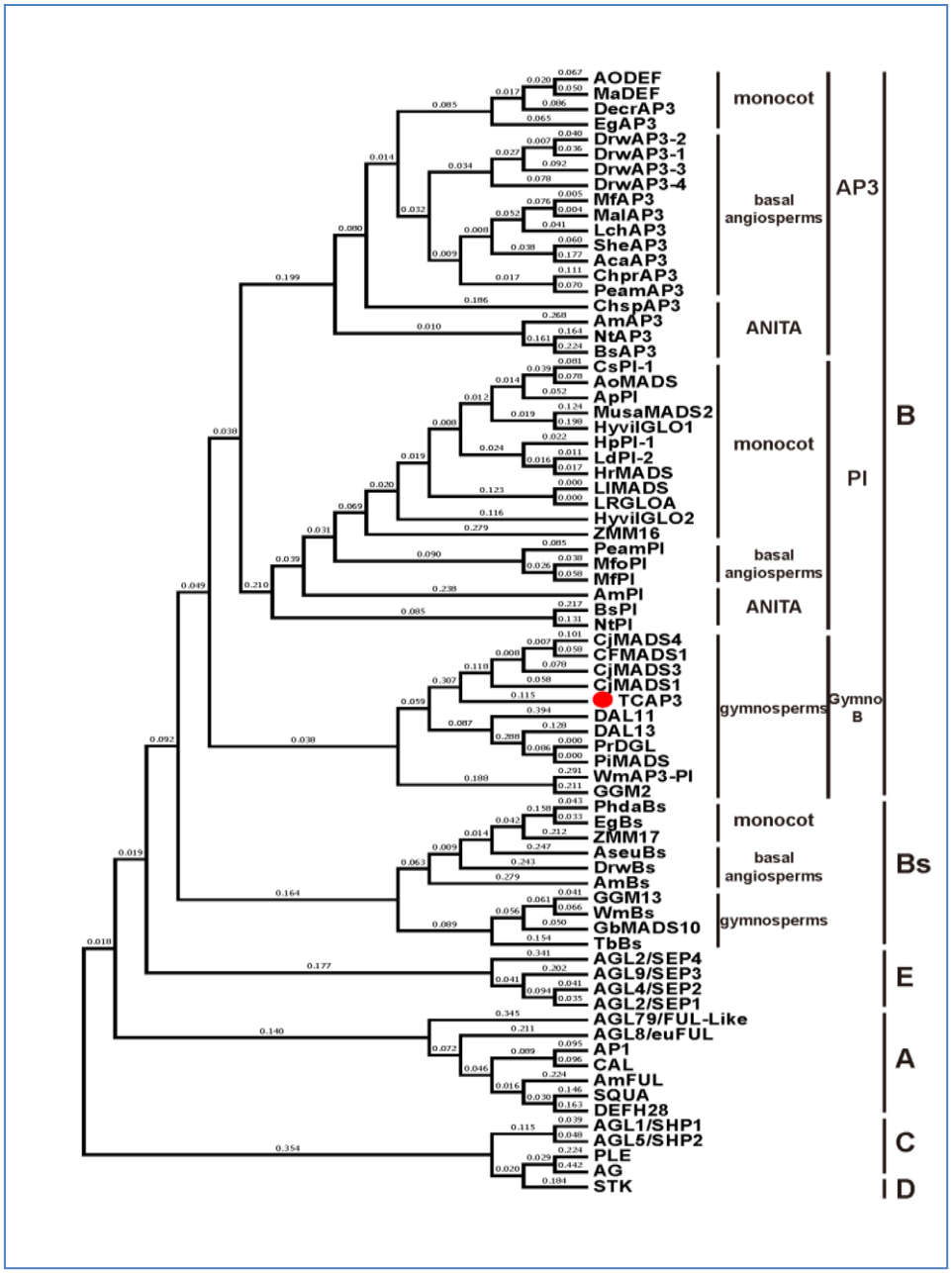


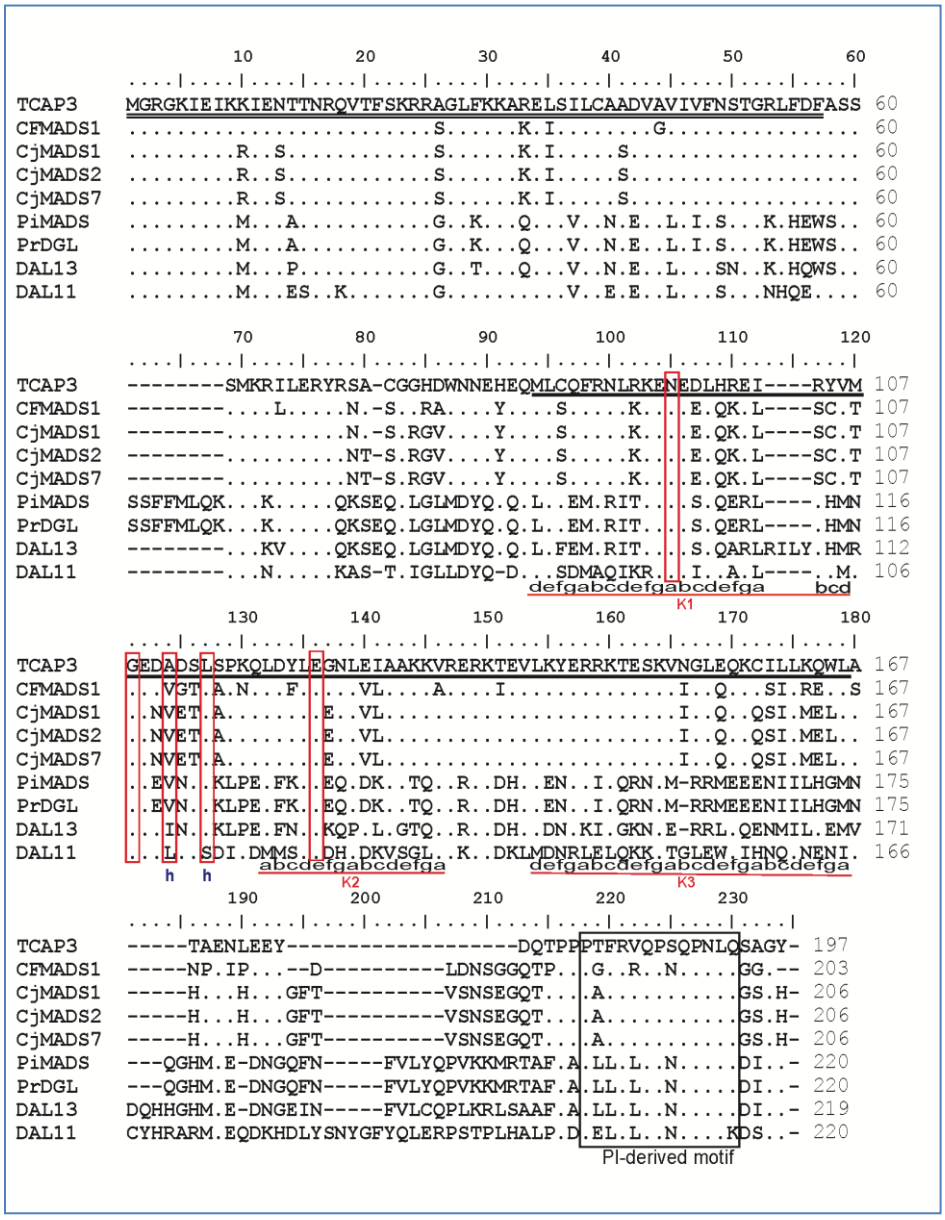
FIGURE 2. THE PHYLOGENETIC TREE ANALYSIS OF TCAP3.

[The species, protein names, and GenBank accession number are as following: *Asparagus officinalis:* AoDEF (BAC75969); *Muscari armeniacum:* MaDEF (BAE48147); *Dendrobium crumenatum:* DecrAP3 (AAZ95249); *Elaeis guineensis:* ElguAP3 (AAW66883); *Drimys winteri:* DrwAP3-2 (AAR87684); *Drimys winteri:* DrwAP3-1 (AAR87683); *Drimys winteri:* DrwAP3-3 (AAR87685); *Drimys winteri;* DrwAP3-4 (AAR87686); *Magnolia figo*: MfAP3 (AAC42592); *Michelia alba:* MalAP3 (AFN68916); *Liriodendron chinense:* LchAP3 (AFN68933); *Saruma henryi:* SheAP3 (AAR87676); *Asarum caudigerum:* AcaAP3 (AGO59776); *Chimonanthus praecox:* ChprAP3 (ABK34952); *Persea americana:* PeamAP3 (AAR06682); *Chloranthus spicatus*: ChspAp3 (AAR06664); *Amborella trichopoda:* AmAP3 (BAD42444); *Nymphaea tetragona:* NtAP3 (BAD42348); *Brasenia schreberi*: BsAP3 (BAD42352); *Crocus sativus:* CsPI-1 (ABB22777); *Asparagus officinalis:* AoMADS (BAD13496); *Agapanthus praecox:* ApPI (BAC66962); *Wild Malaysian banana:* MusaMADS2 (XP_009404965); *Hypoxis villosa:* HyvilGLO1 (ACR16042); *Habenaria petelotii:* HpPI-1 (ACD85108); *Ludisia discolor:* LdPI-2 (ACD85108); *Habenaria radiate:* HrMADS (BAH03321); *lilium longiflorum:* LiMADS (BAB91551); *Lilium regale:* LRGLOA (ABD92703); *Hypoxis villosa:* HyvilGLO2 (ACR16043); *Zea mays:* ZMM16 (NP_001105136); *Persea Americana:* PeamPI (AAR06672); *Magnolia fordiana:* MfoPI (AFN68737); *Magnolia fordiana:* MfPI (AFN68737); *Amborella trichopoda*: AmPI (XP_006847167); *Brasenia schreberi:* BsPI (BAD42353); *Nymphaea tetragona:* NtPI (BAD42349); *Cryptomeria japonica:* CjMADS4 (AAL05441); *Calocedrus formosana:* CfMADS1 (AFI98666); *Cryptomeria japonica:* CjMADS3 (BAG48503); *Cryptomeria japonica:* CjMADS1 (AAL05440); *Taxus chinensis var mairei:* TCAP3 (KC818630); *Picea abies:* DAL11 (AAF18373); *Picea abies:* DAL13 (AAF18377); *Pinus radiate:* PrDGL (AAF28863); *Welwitschia mirabilis:* WmAP3/PI (AGV28071); *Gnetum gnemon:* GGM2 (CAB44448); *Phoenix dactylifera:* PhdaBs (XP_008807981); *Elaeis guineensis:* EgBs (XP_010914210); *Zea mays:* ZMM17 (NP_001105130); *Asarum europaeum:* AseuBs (Q9LLA7); *Drimys winteri:* DrwBs (AAR87687); *Amborella trichopoda*: AmBs (XP_006829168); *Gnetum gnemon:*

GGM13 (Q9XGJ4); *Welwitschia mirabilis:* WmBs (AGV28074); *Ginkgo biloba:* GbMADS10 (BAD93174); *Taxus baccata:* TbBs (AGK89797); *Arabidopis thaliana:* AGL2/SEP1 (NP_568322); *Arabidopis thaliana:* AGL9/SEP3 (NP_001185081); *Arabidopis thaliana:* AGL4/SEP2 (NP_186880); *Arabidopis thaliana:* AGL3/SEP4 (NP_178466); *Arabidopis thaliana:* AGL79/FUL-Like (NP_189645); *Arabidopis thaliana:* AGL8/euFUL (AAA97403); *Arabidopis thaliana:* AP1 (NP_177074); *Arabidopis thaliana:* CAL (D7KQR8); *Antirrhinum majus:* AmFUL (AAP83363); *Antirrhinum majus:* SQUA (CAA45228); *Antirrhinum majus:* DEFH28 (AAK72467); *Arabidopis thaliana:* AGL1/SHP1 (NP_191437); *Arabidopis thaliana:* AGL5/SHP2 (NP_850377); *Antirrhinum majus:* PLE (AAB25101); *Arabidopis thaliana:* AG (NP_001190766); *Arabidopis thaliana:* STK (NP_001190696); The bars represent evolutionary distance. The reliability of the tree is measured by bootstrap analysis with 100 replicates.]

3.2 Characterization of the Deduced TCAP3 Protein

The deduced TCAP3 protein contained 197 amino acids. Computer Tool was used to calculate the molecular weight of the deduced TCAP3 protein, which were predicted to be 21.6 kDa. Alignment of protein sequence demonstrated that the TCAP3 belonged to gymnosperm Gymno B clade (Figs. 3 and 4). TCAP3 protein has 197 amino acids that shares the conserved motifs of gymnosperm Gymno B clade including four typical domains, M, I, K, C (Fig. 3). TCAP3 protein has the conserved motif and PI-derived fragment (Fig. 4) on C-terminal that is similar structures of the angiosperm B class genes, indicating that TCAP3 shares the same B class gene specific conserved motif and is a member of angiosperm B class genes.



Dots indicate gaps inserted for alignment optimization. Double underline represents the MADS domain. Single underline represents the K domain that consists of K1, K2 and K3 amphipathic helixs, repeated arrangement as abcdefg model. In this domain, there are lots of conservative pots. The region between MADS domain and K domain is I domain. The understream of K domain is C-terminal domain, containing a PI-derived motif. The species, protein names and GenBank accession numbers are as following: *Taxus chinensis var mairei:* TCAP3 (KC818630); *Calocedrus formosana:* CfMADS1 (AFI98666); *Cryptomeria japonica:* CjMADS1 (AAL05440); *Pinus radiate:* PrDGL (AAF28863); *Picea abies:* DAL11 (AAF18373); *Picea abies:* DAL13 (AAF18377);*Gnetum gnemon:* GGM2 (CAB44448).

FIGURE 3. COMPARISON OF DEDUCED AMINO ACID SEQUENCES ENCODED BY TCAP3 WITH RELATED GYMNO B HOMOLOGENES.

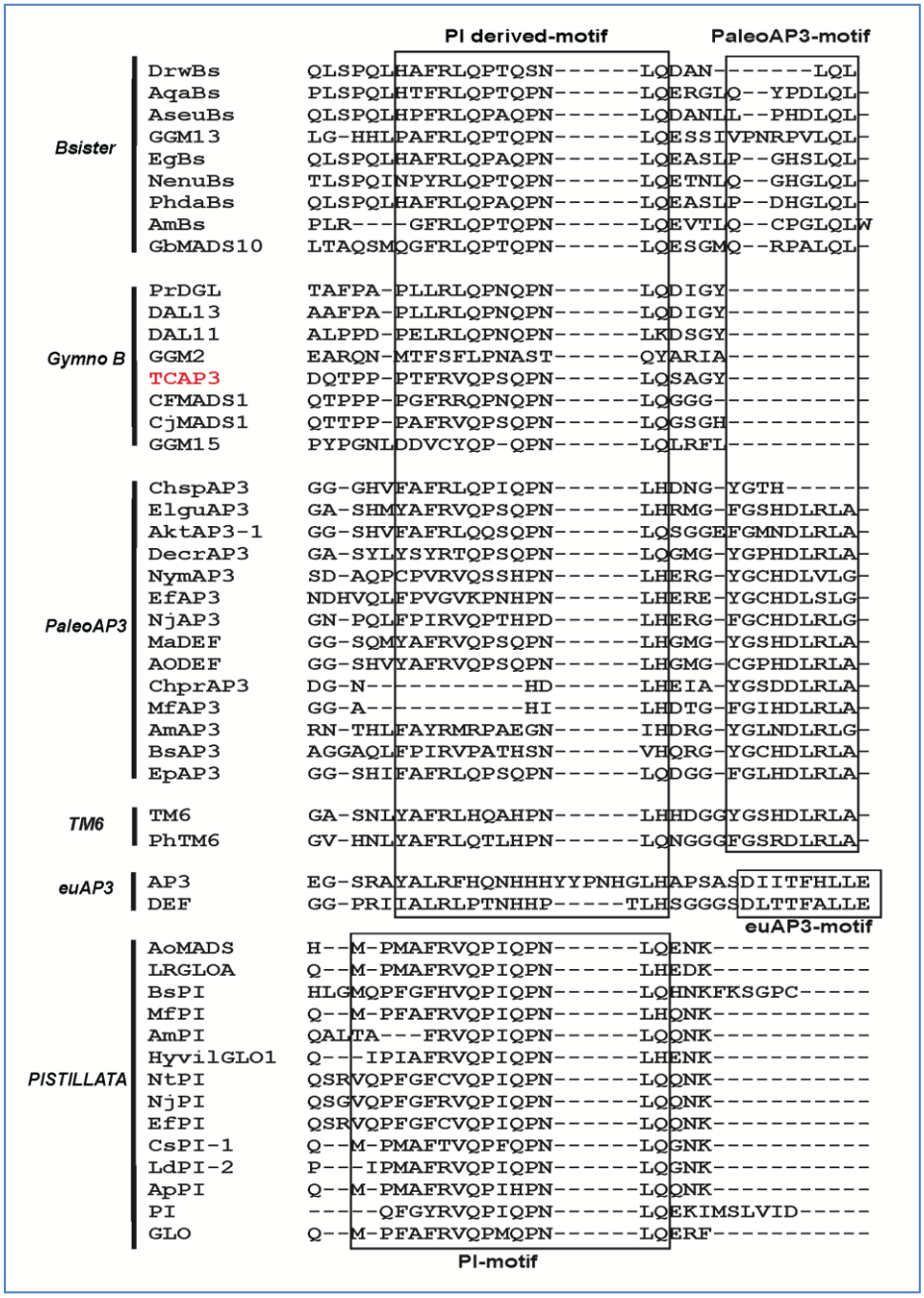


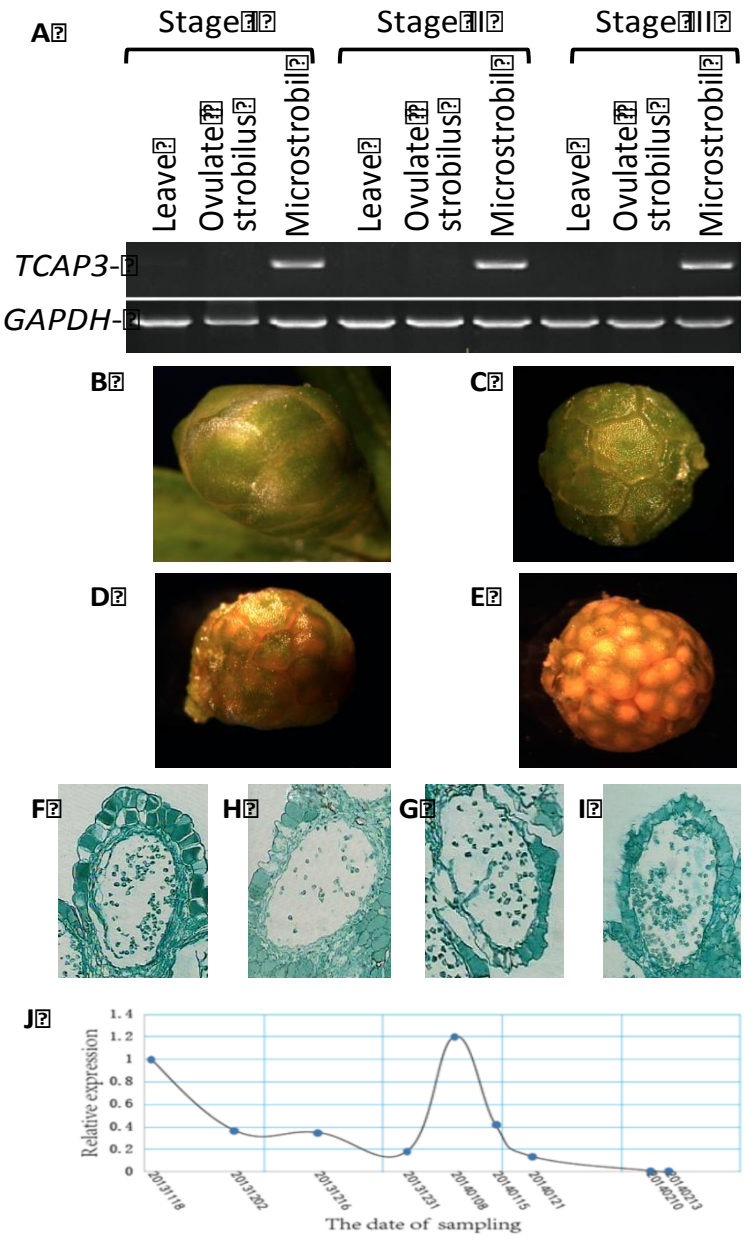
FIGURE 4. MOTIF ANALYSIS OF B-CLASS GENES AND B SISTER GENES.

[The species, protein names, and GenBank accession number are as following: *Drimys winteri:* DrwBs (AAR87687); *Aquilegia alpina:* AqaBs (AAR87674); *Asarum europaeum:* AseuBs (Q9LLA7); *Gnetum gnemon:* GGM13 (Q9XGJ4); *Elaeis guineensis:* EgBs (XP_010914210); *Nelumbo nucifera*: NenuBs (XP_010258808); Phoenix dactylifera: PhdaBs (XP_008807981); *Amborella trichopoda*: AmBs (XP_006829168); *Ginkgo biloba:* GbMADS10 (BAD93174); *Pinus radiate:* PrDGL (AAF28863); *Picea abies:* DAL11 (AAF18373); *Picea abies:* DAL13 (AAF18377); *Gnetum gnemon:* GGM2 (CAB44448); *Taxus chinensis var mairei*: TCAP3 (KC818630); *Calocedrus formosana:* CfMADS1 (AFI98666); *Cryptomeria japonica:* CjMADS1 (AAL05440); *Gnetum gnemon:* GGM15 (CAC13991); *Chloranthus spicatus*: ChspAp3 (AAR06664); *Elaeis guineensis*: ElguAP3 (AAW66883); *Akebia trifoliate*: AktAP3-1 (AAT46097); *Dendrobium crumenatum*: DecrAP3 (AAZ95249); *Nymphaea tetragona:* NymAP3 (BAD42348); *Euryale ferox:* EfAP3 (BAD42346); *Nuphar japonica:* NjAP3 (BAD42354); *Muscari armeniacum:* MaDEF (BAE48147); *Asparagus officinalis:* AoDEF (BAC75969); *Chimonanthus praecox:* ChprAP3 (ABK34952); *Magnolia figo*: MfAP3 (AAC42592); *Amborella trichopoda:* AmAP3 (BAD42444); *Brasenia schreberi*: BsAP3 (BAD42352); Euptelea pleiosperma: EpAP3 (ADC79696); *Vitis vinifera:* TM6 (NP_001267937); *Petunia x hybrid:* PhTM6 (AAS46017); *Arabidopis thaliana:* AP3 (NP_191002); *Antirrhinum majus:* DEF (P23706); *Asparagus officinalis:* AoMADS (BAD13496); *Lilium regale:* LRGLOA (BAB91551); *Brasenia schreberi:* BsPI (BAD42353); *Magnolia fordiana:* MfPI (AFN68737); *Amborella trichopoda*: AmPI (XP_006847167); *Hypoxis villosa:* HyvilGLO1 (ACR16043); *Nymphaea tetragona:* NtPI (BAD42349); *Nuphar japonica:* NjPI (BAD42356); *Euryale ferox:* EfPI (BAD42347); *Crocus sativus:* CsPI-1

(ABB22777); *Ludisia discolor:* LdPI-2 (ACD85108); *Agapanthus praecox:* ApPI (BAC66962); *Arabidopis thaliana:* PI (P48007); *Antirrhinum majus:* GLO (Q03378).]

3.3 Expression analysis of TCAP3

Expression of TCAP3 in different tissues (leave, ovulate strobilus, and microstrobil) and at different developmental stages has been examined by semi-quantitative PCR analysis. Our results showed that TCAP3 only expressed in male strobilus, not in leaf bud and female strobilus (Fig. 5a). Different developmental stages were determined based on the paraffin sections of male strobilus (Fig. 5b-e and f-i). TCAP3 expresses dynamically along with the male strobilus. The first stage, middle of November, the male strobilus was in microsporocyte formation stage and TCAP3 expression started with high level but declined along with the end of the formation; the second stage, December, development of male strobilus was very slow and even in dormancy, and expression of TCAP3 was at low level; the third stage, starting from the end of December, along with meiosis of microsporocyte, male strobilus began to grow rapidly and so did TCAP3 with its expression peak in beginning of January; the fourth stage, when meiosis finished and microspore were freed, TCAP3 expression went down along with the end of differentiation and development of male strobilus and declining of temperature; the fifth stage, the end of January, when the temperature went up and free microspores expanded rapidly, along with microspores were mature and dispersed, TCAP3 expression decreased till extremely low (Fig. 5j). Therefore, the expression of TCAP3 was fit and played important role in the development of male strobilus.



a. Semi-quantitative PCR analysis showing *TCAP3* expression in the different tissues of *Taxus chinensis var mairei*, including leave, ovulate strobilus, and microstrobil, at different developmental stages (Stage I, stage II, and stage III). *GADPH* was used as control. b-e Micrographs of the misrosporogenesis including Microspore, Microspore mother cell, Meiosis period, and Vacuolated period of free microspores. f-i Histology of the misrosporogenesis including Tetrad stage, Vacuolated period of free microspores, Vacuolated period of free microspores, and Mature poll. j The real-time quantity PCR analysis of *TCAP3* in different developmental stages of microstrobil.

FIGURE 5. EXPRESSION ANALYSIS OF TCAP3 IN TAXUS CHINENSIS VAR MAIREI.

3.4 Function analysis of TCAP3

The constructed overexpression vector with CaMV35S promoter and TCAP3 gene was transferred into *Arabidopsis* mutants, *ap3-3* and *pi-1*, through *Agrobacterium*-mediated transformation. Transgenic plants were screened, identified and analyzed (Fig. 6a and b). In transgenic plants, the tips of sepals were petalized, and some petals changed its cross distribution into triangular with two petals overlap to each other; meanwhile, filaments were shorten and stretched to outside and some even between perianths and sepals, which looked like less filaments (Fig. 6c and d). These phenotypes demonstrated that TCAP3 was involved in flower, filaments morphological development and was conserved in its functions.

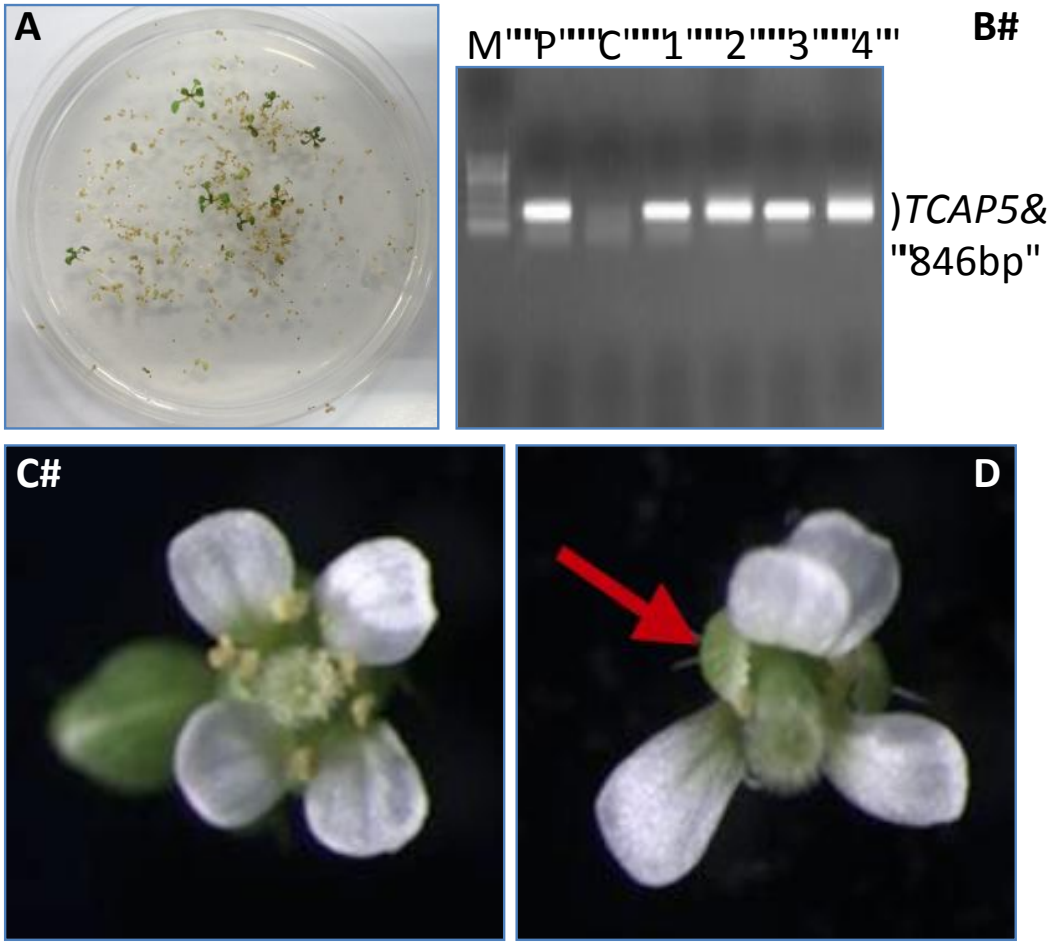


FIGURE 6. TRANSGENIC ARABIDOPSIS THALIANA ACQUIRED BY AGROBACTERIUM MEDIATED METHOD.

[a Genotype identification of 35S::*TCAP3* transgenic Arabidopsis ap3-3 mutants. b confirmation of transgenic plants by PCR, lines M DNA ladder, P plasmid positive control, C negative control, 1-4 35S::*TCAP3* transgenic Arabidopsis lines. c-d The floral phonotype of the wild-type Arabidopsis (c) and the 35S::*TCAP3* transgenic Arabidopsis.]

IV. DISCUSSION

In recent years, there has been a remarkable progress in the understanding of the molecular regulation of floral signal integration and meristem determination in plant. MADS-box proteins, a well-conserved family of transcription factors in eukaryotic organisms, regulate primary metabolism, cell cycle, and cell identity (Gramzow, et al. 2014; Huang, et al. 2014; Lee, et al. 2014; Sun, et al. 2014). MADS-box transcription factors are involved in *S. sclerotiorum* growth and virulence (Qu, et al. 2014). In *B. rapa*, MADS-box genes are involved in stress resistance in addition to their growth and developmental functions, providing the basis for functional characterization and exploitation of the candidate genes for genetic engineering (Saha, et al. 2015). In *Pinus tabuliformis*, MADS-box genes are related to the molecular regulation of cone development and the origin of hermaphroditism (Niu, et al. 2016). In Arabidopsis mutants, flower development is remarkably associated with differential co-occupancy of binding motifs of MADS box transcription factors (Wang, et al. 2016). Currently, functional identification of MADS-box genes in *T. Chinensis var. mairei* has not been reported.

Expression profiling of MADS-box genes has been reported in different species. In *Sapium sebiferum* (Linn.) Roxb, 61 MADS box genes that are involved in flower development have been identified, providing functional genomic information for the genetic engineering to shorten the juvenile period and improve yield by regulating flower development (Yang, et al. 2015). In peach, 79 MADS-box genes distributed across all eight peach chromosomes and frequently located in clusters of two or more genes (Wells, et al. 2015). In sesame (Sesamum indicum L.), gene structure analysis revealed from 1 to 22 exons of sesame MADS-box genes and expression profiles of MADS-box genes in seven sesame transcriptomes indicated that MADS-box genes played significant roles in sesame flower and seed development (Wei, et al. 2015). In grape, expression profiling of MADS-box genes from six cultivars suggests their function in ovule development and may represent potential ovule identity genes involved in parthenocarpy that may be useful in seedlessness-related molecular breeding programs (Wang, et al. 2015).

MADS-box genes play an important role in regulating rice floral meristem and organs identity that are crucial process for reproductive success and rice yield. In rice, mis-splicing of OsMADS32 transcripts in the cfo1-3 mutant resulted in an extra eight amino acids in the K-domain of OsMADS32 protein, indicating that MADS-box genes regulate rice lodicule and stamen identity by interacting with two PI-like proteins via its K domain (Wang, et al. 2015). The MADS-box gene family has diverse developmental roles in flower pattern formation, gametophyte cell division and fruit differentiation. In apple (*Malus domestica*), 146 MADS-box genes were identified and were phylogenetically clustered into six subgroups with the MADS-box genes from Arabidopsis and rice. Expression profiling of all of the apple MADS-box genes indicates that the MADS-box genes are involved in various aspects of the physiological and developmental processes of the apple (Tian, et al. 2015).

To understand the function of MADS-box genes in flower development and growth in *T. Chinensis var. mairei*, we have cloned the TCAP3 gene in *T. Chinensis var. mairei*, analyzed its functional domains, and produced transgenic plants to figure out its function on stamen development. Our results indicate that TCAP3 is very conservative on its structure, very close to male strobilus development, and very conservative on regulating flower and stamen development.

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AUTHOR CONTRIBUTIONS

Y.F., Z.L., R.Q., and W.T. designed the experiments and analyzed the data. W.T. drafted the manuscript. Y.F., Z.L., and R.Q. performed the experiments and the gene sequence data analysis. Z.L. and R.Q. performed qRT-PCR. Y.F., Z.L., and R.Q. contributed the functional complementation. All authors read and approved the manuscript.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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