Plant Growth Regul (2013) 70:287–296
DOI 10.1007/s10725-013-9800-0

ORIGINAL PAPER

Molecular characterization of loquat *EjAP1* gene in relation to flowering

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Received: 19 November 2012 / Accepted: 22 February 2013 / Published online: 1 March 2013

Abstract *AP1/SQUA*-like gene plays important roles in the development of inflorescences and flowers. In this study, an *APETALA1* (*AP1*) homologue, *EjAP1*, was isolated from loquat (*Eriobotrya japonica* Lindl.), an economically important subtropical fruit of the Rosaceae. *EjAP1* was sequenced and found to be 3,229 bp in length with 6 introns and 7 exons and encoded 239 amino acids. The deduced amino acid sequence contained the typical CaaX-motif of the *AP1* functional proteins and shared high homology with the other *AP1* genes. Phylogenetic analysis at the amino acid level indicated that *EjAP1* belongs to the *AP1/SQUA* subfamily. Transcriptions of *EjAP1* were detected in inflorescence bud, flower bud and flower, but not in vegetative tissues such as vegetative bud. In floral organs, highest expression of *EjAP1* was detected in sepal compared to lower transcription level in petal and stamen, and no transcription in pistil. When ectopic expressed in *ap1-1* mutant of *Arabidopsis*, *EjAP1* can partially complement the mutant mainly in that it can restore the sepal and petal formation of *ap1-1* mutant. These results indicated that *EjAP1* gene may play a similar role as *Arabidopsis AP1* in the floral development of loquat.

Keywords *AP1*-like gene · Flowering · Loquat (*Eriobotrya japonica*)

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Introduction

Flowering, which indicates the switch from vegetative growth to reproductive growth, is an important event in plants. It is especially important for the breeding and production of perennial woody fruit. Early flowering means a shorter breeding cycle for breeders, or early releasing fruits in market which normally follows a higher price. During the last decades, enormous progress has been made in the understanding of how floral meristems are formed and how the proper floral organs emerge from this meristem under the control of a complex gene regulatory network. Most of the molecular information about flowering has been obtained with the model plants such as *Arabidopsis thaliana* and *Antirrhium majus*.

Many floral meristem identity genes, such as *LEAFY (LFY)*, *FRUITFULL* (*FUL*) and *APETALA1* (*AP1*), have been isolated and their functions have been well characterized (Pidkowich et al. 1999; Simpson et al. 1999; Kieffer and Davies 2001; Zik and Irish 2003). Among these genes, a number of MADS-box genes were found functioning in various steps of flowering in different plant species. MADS-box genes belong to transcription factors and contain a highly conserved DNA-binding domain (MADS-box). A major lineage of this gene family is the MIKC-type

proteins which commonly consist of three additional domains besides the highly conserved MADS-box domain: a less-conserved Intervening domain, a moderately conserved Keratin-like domain, and a variable C-terminal region (De Bodt et al. 2003; Becker and Theißen 2003). Studies showed that these domains have different functions (Egea-Cortines et al. 1999; Masiero et al. 2002; van Dijk et al. 2010). AP1/SQUA is a clade member of the MIKC-type proteins and plays important roles in floral meristem identity, floral organs specification and vegetative tissues development. In *Arabidopsis*, *AP1* is required for the establishment of floral meristems and for the specification of floral organ identity, such as that of sepals and petals (Mandel et al. 1992). In floral organs of *Arabidopsis*, expression of *AP1* is only found in sepal and petal. Meanwhile, the sepal and petal were absent in *ap1* mutant. These results suggest that *AP1* could be responsible for the A-function as the ABC model describes (Irish and Sussex 1990; Bowman et al. 1991; Coen and Meyerowitz 1991; Theissen 2001). Mutation of *Antirrhinum AP1* ortholog *SQUA* showed a more complete loss of floral meristem specification (Huijser et al. 1992). It has been reported that environmental factors such as temperature and light affect the flowering time via different pathways (Wellmer and Riechmann 2010; Amasino 2010; Srikanth and Schmid 2011). Many genetic products of various pathways, for example, LFY and FT (FLOWERING LOCUS T), can interact with *AP1* and then initiate its expression (Wagner et al. 1999; Abe et al. 2005; Wigge et al. 2005). On the other hand once AP1 shows its activity, it regulates the expression of other important flowering-related genes, such as the floral repressor *TFL1* (*TERMINAL FLOWER 1*) and *SVP* (*SHORT VEGETATIVE PHASE*) (Wellmer and Riechmann 2010) and those required for early floral organ development (Mandel and Yanofsky 1995; Kaufmann et al. 2010). Several studies reported that overexpression of *AP1* or its homologues can cause an early-flowering phenotype in fruit trees such as apple (Flachowsky et al. 2007), citrus (Peña et al. 2001) and so on. It provides an attractive way for fruit researchers to accelerate the flowering process in woody fruit. Currently, many *AP1* homologues have been isolated and characterized from woody fruit species including apple (Sung et al. 1999; Kotoda et al. 2000), grape (Calonje et al. 2004), citrus (Pillitterri et al. 2004) and peach (Li et al. 2012).

Loquat (*Eriobotrya japonica* Lindl.), an evergreen fruit tree native to China, is cultivated mainly in tropical and subtropical regions. Fruits of loquat can be consumed fresh or processed for jam, juice, wine, syrup, or as candied fruits (Lin et al. 1999). Loquat has panicles of small white or yellow flowers and the inflorescence buds are descended from vegetative buds on the shoots. The main process of flowering in loquat is described in Fig. 1. The shoots of loquat can be classified into central branches which are formed primarily and lateral branches which are formed a little later. Meanwhile, the shoots of loquat form in different seasons such as spring, summer and sometimes in autumn. So there is an interesting phenomenon in the flowering of loquat that its inflorescence bud differentiation period lasts for months. For example, in China, inflorescence buds differentiate usually from July (warmer climate) to September (cooler climate) and followed by the blooms from fall to early winter. Furthermore, there are too many flowers exist in the inflorescence. Generally, the main panicle axis bears 5–10 branched secondary axes, with 70–100 flowers, occasionally even more than 100 (Lin et al. 1999). Thus a problem of flower thinning arises in the production of loquat and it is time-consuming and laborious. It is desirable to control the flowers of loquat to an appropriate amount and regulate the flowering time to an appropriate period. Moreover, loquat also has a long juvenile phase as other fruit trees, which impedes both productivity and breeding efficiency.

Although it's very important to clarify the flowering mechanism of loquat, there are few reports about the genetic controlling in it. In this study, we isolated an *AP1*-like gene, *EjAP1*, from loquat and characterized its expression pattern. As a transcription activator belonging to *AP1/SQUA* subfamily, *EjAP1* can partially complement the *ap1-1* mutant of *Arabidopsis*.

Materials and methods

Plant materials

'Zaozhong No.6' loquat, was used as materials in this study, which grew in the germplasm preservation orchard at South China Agricultural University, Guangzhou, China. The developing floral shoot apexes about 1 cm long from the tip were collected from 5 year-old trees from September to December. Flowers and vegetative shoot apexes were also collected from the same trees at appropriate time. All the materials were frozen in liquid nitrogen immediately and stored at −80 °C.

RNA extraction and full-length cDNA cloning

Total RNA was isolated using an improved CTAB method (Asif et al. 2000). The rapid amplification of cDNA ends (RACE) technique was used to isolate the *AP1* homologue in loquat using cDNA of floral buds with 3′ and 5′ Full Race Core Set (TaKaRa, Japan).

In previous study, we have isolated a partial DNA sequence of *AP1* homologue from loquat with the degenerate primers which were designed according to the

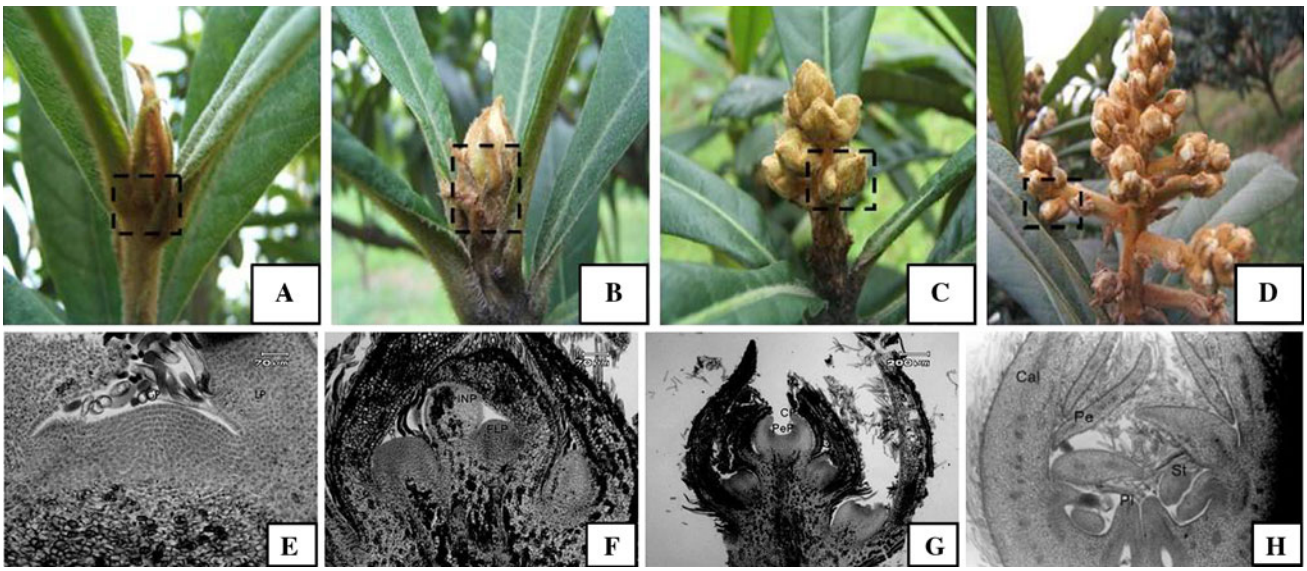

Fig. 1 Flowering process of loquat. **a–d**, four important flowering stages of loquat: **a** vegetative bud; **b** inflorescence bud; **c** forming the inflorescence; **d** inflorescence with developing flowers. **e–h**, tissue slices of the four stages corresponding to **a–d**; **e** the vegetative bud has a flat vegetative cone; **f** the raised inflorescence primordium attached with a flower primordium; **g** the forming of calyx primordium and petal primordium; **h** four flower organs of a flower. *LP* leaf primordium, *INP* inflorescence primordium, *FLP* flower primordium, *CP* calyx primordium, *PeP* petal primordium, *Cal* calyx, *Pe* petal, *St* stamen, *Pi* pistil. Positions of the tissue slices **e–h** are *dotted* corresponding in **a–d**

conserved regions of other reported *AP1* homologues. In this study, 3′ RACE was performed with the 3′-site adaptor primer of the Set and the gene-specific forward-primer FP1 which were designed according to the known sequence. 1 μg total RNA was used to reverse-transcribe the first strand of cDNA with Oligo d(T) primer provided by the Set. Reverse transcription PCR was performed with 1 cycle of 30 °C for 10 min, 55 °C for 25 min, 95 °C for 5 min, 5 °C for 5 min. Totally 20 μl of the first strand cDNA was used for 3′ RACE with 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 2 min.

5′ RACE was performed after the 3′ end was obtained. A phosphorylated P5 primer designed according to the known 3′ end sequence was used to get the first strand cDNA. The reverse transcription reaction was performed with 1 cycle of 30 °C for 10 min, 50 °C for 50 min, 80 °C for 2 min. The RNA-cDNA hybrid was treated with 1 μl RNase H to remove RNA residues and the single strand cDNA was then looped by T4 RNA Ligase. The looped cDNA was used as template in the following PCR. Two pairs of primers F1/R1 and F2/R2 specialized to the known sequence were designed to perform the PCR. The first PCR with primer F1/R1 was performed with 25 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 2 min. Then the product of 1st PCR was diluted and used as the template for a second PCR. The second PCR was performed with primer F2/R2 with 30 cycles of 94 °C for 1 min, 53 °C for 30 s, 72 °C for 2 min. After the product of the 2nd PCR being cloned and analyzed, we found that we still had not got the full length of loquat, so we designed a new primer R2-2 to perform a third PCR. After cloning of its product, we finally got the full length sequence of the *AP1* homologue from loquat.

According to the sequence got by RACEs, we designed a pair of gene-specific primers (EAP-F, EAP-R) to get the full length clone of this gene which we named *EjAP1*. PCR was performed with 30 cycles of 94 °C for 1 min, 53 °C for 30 s, 72 °C for 2 min.

All the PCR products were cloned in pGEM-T easy vector (Promega, USA) and then sequenced. All the primers mentioned above were listed in Table 1.

Table 1 Primers used in this study

Primer name	Sequence (5′–3′)
FP1	GCAGCAGCTTGATACTACTCTTA
P5	(P)AATGGTTCCAGAGTCAG
F1	CAGGTTCAAGACTGGGAGCAG
R1	ATTTTCCTCCTGTATCGCCTTTC
F2	CAGCCACTTCCATGTCTAAAC
R2	TAAGAGTAGTATCAAGCTGTTGC
R2-2	GCTTGCCTTTGTTGGAGAAG
EAP-F	CCCGGATCCATGGGGGGAGGTAGAGTTCA
EAP-R	GCCGAGCTCTCAAGCAGCAAAGCATCCGA

DNA extraction and cloning of *EjAP1* DNA sequence

Genomic DNA was extracted from young leaves of the same trees. The primers and procedures of PCR were same as those mentioned in the full-length cDNA cloning.

Sequence and phylogenetic analysis

The nucleotide and deduced amino acid sequence of the *EjAP1* were used for BLAST searches in the GenBank databases. Among the BLAST hits, 20 MADS genes were selected for construction of phylogenetic tree. The genes used were *Antirrhinum SQUA*, *AmFUL* (X63701, AY306139); *Arabidopsis AP1*, *CAL*, *FUL* (Z16421, L36925, L36925); Apple *MdMADS2*, *MdMADS5*, *AP1-like* (U78948, AJ000759, Q6YNE5); Pear *PpAP1* (EF423915); *Betula pendula BpMADS3*, *BpMADS4*, *BpMADS5* (X99653, X99654, X99655); Cauliflower *BOAP1* (Z37968); *Corylopsis sinensis CsAP1*, *CsFUL* (AY306146, AY306147); *Heuchera americana HeaAP1*, *HeaFL*, *HeaFUL* (AY306148, AY306149, AY306150) and *Nicotiana tabacum NAP1-1*, *NAP1-2* (AF009126, AF009127). The amino acid sequences were aligned and a tree was generated using the Clustal W method (Higgins et al. 1994). Bootstrap values were derived from 1,000 replicate runs. A phylogenetic tree was constructed using the MEGA 5 software (Tamura et al. 2011).

Expression analysis

Northern blot was used to detect the expression pattern of *EjAP1*. 10 μg total RNA from each sample was electrophoresed with 1.2 % agarose gel and transferred to Hybond N^+ nylon membranes (Roche, Swiss). A gene-specific fragment (498 bp) at the 3′ end of *EjAP1*, which is a specific region in the C-terminal, was used as the probe for hybridization. The specific methods of probe labeling and hybridization are as described in the instruction offered by Roche DIG High Prime DNA Labeling and Detection Starter Kit I (Roche, Swiss).

Function analysis of *EjAP1* with overexpression in *ap1-1* mutant

Construct for *EjAP1* overexpression was made by cloning of the ORF region of *EjAP1* in sense orientation into pBI121 plasmid under the control of CaMV 35S promoter (Fig. 2). *Bam*H I and *Sac* I cloning sites were introduced into the initiation and terminal sites of *EjAP1* sequences by means of PCR. The construct was then introduced into the disarmed strain of *Agrobacterium tumefaciens*, GV3101 and then transformed into *Arabidopsis ap1-1* mutant. Wild-type *Arabidopsis* of Columbia (*col*) was used as a control.

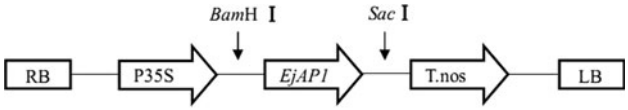

Fig. 2 Vector construction. ORF of *EjAP1* were inserted in sense orientation between the *BamH*Iand *Sac*I sites of the binary vector pBI121. *RB* right border; *P.35S* cauliflower mosaic virus 35S promoter; *T.nos* nos terminator; *LB* left border

Seed stocks of *ap1-1* and *col* were obtained from the Arabidopsis Biological Resource Center (ABRC) at Ohio State University, Columbus, USA. Transformation was performed by a floral-dip method (Clough and Bent 1998). Transformants were selected on medium containing MS salts and vitamins supplemented with 50 mg/L kanamycin. Then, the resistant plants were transferred to potting mixture and grown under long-day condition. RNA from inflorescences of the transformants was used to synthesize cDNA and RT-PCR was employed to detect whether *EjAP1* is expressed in *ap1-1* mutants with primers of EAP-F and EAP-R.

Results

Isolation and sequence analysis of *EjAP1*

A combination of PCR techniques were used to isolate the *AP1*-like gene from loquat. By 3′ RACE, a 568 bp length sequence with poly-A tail was obtained. To obtain the transcription initiation site, twice 5′ RACE procedures were performed. With the first 5′ RACE, the sequence extended 247 bp forward based on the known 3′ region, but its transcription initiation site was not found. After the second 5′ RACE was performed, a new 141 bp sequence which containing the initiation codon was obtained. Pair of gene-specific primers was designed according to the sequences got by RACEs and used in new PCR procedures for the isolation of the *EjAP1*, and finally the ORF of *EjAP1* was obtained (Fig. 3).

The full cDNA sequence of *EjAP1* is 938 bp in length (GenBank accession number AY880261) and contained a 720 bp ORF with a 3′ untranslated region of 217 bp and a 5′ untranslated region of 1 bp. *EjAP1* encoded a predicted polypeptide with 239 amino acids. The g*EjAP1* sequence was obtained by the amplification using genomic DNA as a template. The sequencing result showed that g*EjAP1* (GenBank accession number AY880262) was 3,229 bp in length which indicated that there should be intron(s) in the *EjAP1* gene. Alignment of the cDNA and genomic DNA sequences of this gene showed that there are six introns in the genomic DNA of *EjAP1*. The first intron is 1,562 bp and the last intron is 453 bp, while the others are very short

Fig. 3 ORF sequence of *EjAP1*. The primers used in cloning are *underlined* and the names of the primers were superscript. The sequence with *italic letters* was got by the 1st 5′RACE and the sequence with *boldface letters* was got by the 2nd 5′RACE, other sequence was got by 3′RACE. The initiation codon and the termination codon were *boxed*

ATGGGGGGAGGTAGAGTTCAGCTTAAGCGCATAGAGAACAAGATCAACAGGCA
GGTGACTTTTTCCAAAAGAAGAACTGGGCTGCTGAAGAAGGCTCATGAGATCTCTGT
CTTGTGTGATGCTCAAGTTGCTGTGGTTGT*CTTCTCCAACAAAGGCAAGC*$^{R2\text{-}2}$*TCTTTGAGT*
ACGCCACAGATTCATGCATGGAGCAAATCCTCGAACGCTATGAAAGATACTCTTACGCCGAAAGA
CAGCTAGTCGAACCAGAATTTGAGTCGCAGGGTAACTGGACCTTTGAATATTGCAGACTAAAGGC
TAAGGTTGAGGTGTTGCAAAGAAACCATAGGCACTATTTGGGAGAAGACCTGGATTCCTTGACTC
*TCAAGGAAATCCAAAATTTGGA*GCAACAGCTTGACACTGCTCTTA^{R2}AACAAATTCGATTAAG
AAAAAACCAACTCATGCATGAATCCATCTCTGAGCTTCAGAGAAAGAGAAAGGCGATACA
GGAGGAAAAT^{R1}AACTTGCTAGCAAAGAAGATAAAGGAGAAGGAGAAGGCTGCAGCTCAG
CCCCAGGTTCAAGACTGGGAGCAG^{F1}CAGAACATTGGCCTTGACCTGCTTCCACAGCCACT
TCCATGTCTAAAC^{F2}AATGGGGGGACGCAGCAAGATGAATTCCTTCAAGTGAGGAGGAACC
AGCTGGACCTGACTCTGGAACCATT^{P5}ATATTCATGCCATCTCGGATGCTTTGCTGCTTGA

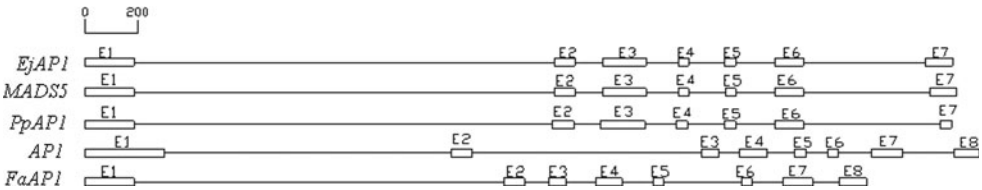

Fig. 4 Schematic diagram of the exons (*boxes*) and introns (*lines*) of *EjAP1* and other *AP1* homologues from apple (*MADS5*), pear (*PpAP1*), *Arabidopsis* (*AP1*) and strawberry (*FaAP1*)

with not more than 200 bp in length (101, 117, 131 and 145 bp in length, respectively). The intron distribution of *EjAP1* gene coincides with the *MADS5* gene of apple and the *PpAP1* gene of pear which also has six introns, but unlike some other *AP1* homologues such as *AP1* and *FaAP1* which generally have seven introns (Fig. 4).

Amino acid sequence alignment and phylogenetic analysis

Alignment of the amino acid sequence encoded by *EjAP1* and other *AP1*-like genes showed that they share highly conserved regions with each other, mostly in the MADS-box region, then the K-box region, while the C-terminus is the least conserved portion of the *AP1* homologues (Fig. 5). Just as many other AP1-like proteins, the putative protein product of *EjAP1* contained the conserved CFAA motif at the C terminus, which was a typical CaaX box (where *C* is Cys, *a* is an aliphatic amino acid, and *X* is Cys, Met, Ser, Ala or Glu) recognition motif for farnesyltransferase (FTase) (Yalovsky et al. 2000).

A phylogenetic tree was calculated using the full length amino acid sequences of EjAP1 protein and the selected AP1-like proteins with Neighbor-Joining Method (Fig. 6). The EjAP1 proteins fall in a group with PpAP1 of pear and the two AP1-like proteins in apple, MdMADS5 and MdAP1-like. All the MADS-box proteins selected were classified into two groups, AP1/SQUA clades and FUL/FUL-like clades. EjAP1 was grouped into AP1/SQUA clades, indicating its close relationship to AP1 of *Arabidopsis* and SQUA of *Antirrhinum*.

Expression analysis

To determine the involvement of *EjAP1* in floral development of loquat, we studied its temporal and spatial patterns of expression. According to the developing phases, a series of materials were collected in turns for the transcriptional detection of *EjAP1*. The materials were generally divided into four groups: vegetative buds, inflorescence buds, inflorescences with the forming flowers and inflorescences with blooming flowers (Fig. 7a).

In the northern blotting of *EjAP1*, transcription was barely detected in the vegetative buds (Fig. 7a: lane 1–2). During the swelling period of the buds when the inflorescence meristems are being initiated, the transcript of *EjAP1* was detected although in a low level (Fig. 7a: lane 3–5). In the third group materials, transcription of *EjAP1* increased significantly when the inflorescence meristems are dividing to generate new branch meristems and flower meristems (Fig. 7a: lane 6–10). When floral organs are forming and the flowers are beginning to blooming, *EjAP1* could also be detected in a high transcription level, although it decreased gradually following the development process (Fig. 7a: lane 11–14). With the ending of flowering, little transcription was found (Fig. 7a: lane 15). There was also no transcription of *EjAP1* was detected in the some other vegetative materials such as leaf (data not shown).

It was reported that as an A-function gene of ABC model, *AP1* of *Arabidopsis* participates in the construction of the outer two whorls floral organs. To examine whether the *EjAP1* plays the same role during flowering, we detected its transcription in the floral organs of loquat.

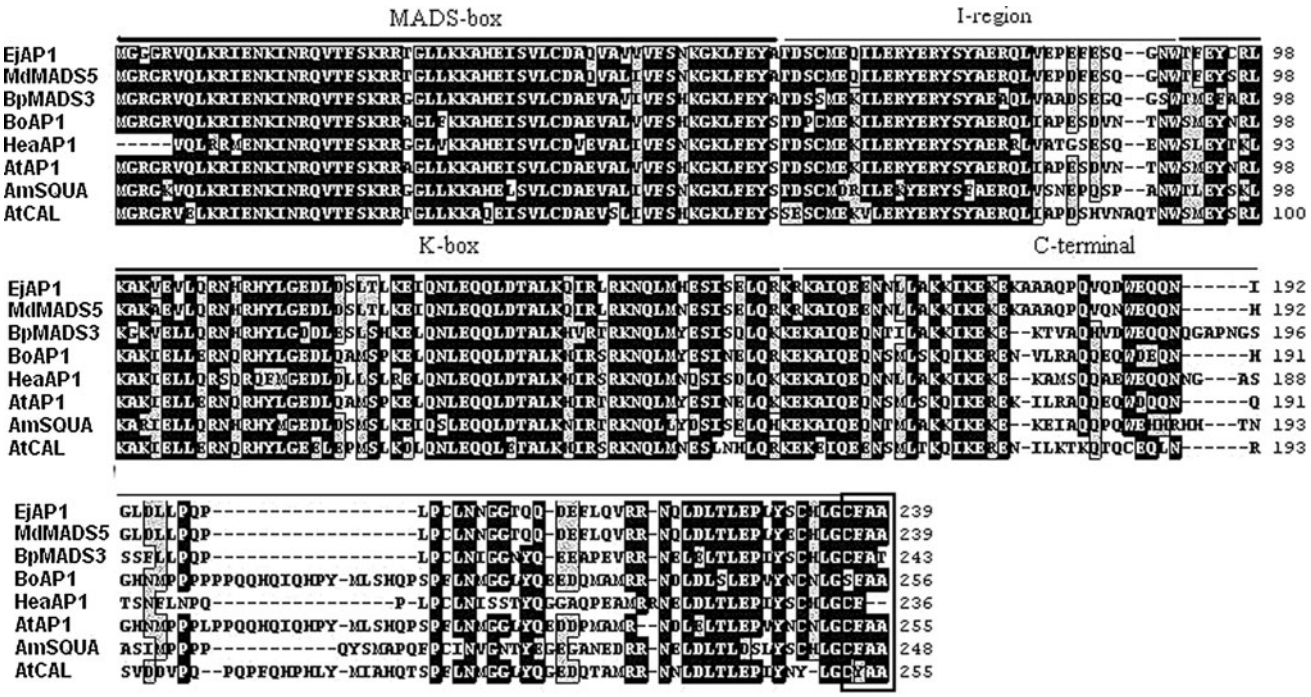

Fig. 5 Alignment of the predicted amino acid sequences of *EjAP1* and other AP1-like proteins from different species. Identical amino *acids* in all proteins are in white letters highlighted by black background, while identical amino acids in more than 75 % of the sequences are highlighted by *grey* background. *Dashes* indicate gaps to maximize alignment. The MADS-box, I, K, and C-domains are shown by *lines* on the *bottom of the alignment*. CFAA motif at the end of C-terminus is boxed

Northern blotting result showed that *EjAP1* was also expressed in the petal and stamen of loquat with a lower level besides the high transcription level in sepal (Fig. 7b).

Ectopic expression of *EjAP1* in *ap1-1* mutant of *Arabidopsis*

Sequence homology and expression analysis of *EjAP1* indicates that it may be involved in the construction of loquat floral organs. To confirm it, we overexpressed *EjAP1* in *Arabidopsis ap1-1* mutant plants. Compared to the wild-type flowers (Fig. 8b), flowers of *ap1-1* mutant lack petals and the sepals always turn to bract-like organs (Fig. 8a: Bowman et al. 1993). The *35S*::*EjAP1 ap1-1* plants frequently carried flowers where the petal whorl was partially restored and the first whorl of flowers in the transgenic plants seems more like to sepal (Fig. 8c). Molecular analysis performed by RT-PCR confirmed that the *EjAP1* gene was expressed in restored plants (Fig. 9). These results indicated that *EjAP1* could partially complement the *ap1-1* mutant of *Arabidopsis*.

Discussion

Loquat is now commercially produced in many countries of East Asia and Mediterranean. Although the crop improvement of loquat is being carried out both by breeding programs and selection accessions from germplasm resources, the varieties grown mostly come from selections (Badenes et al. 2009). One possible reason for the slow breeding program in loquat is its long juvenile phase that lasts several years. Identification of genes involved in flowering provides a deeper insight of its flowering mechanism and a possible way to shorten its juvenile phase by shifting these genes within the fruit trees. Actually, there are several reports about the early-flowering phenotype in fruit trees caused by introduction of the exogenous flowering related genes. It is most successful in apple, for example, the ectopic expression of *MdFT1* (an ortholog of *FT* in apple, Tränkner et al. 2010), *BpMADS4* (an *FUL* ortholog of silver birch, Flachowsky et al. 2007) or the silencing of the flowering repressor *MdTFL1* (an ortholog of *TFL1* in apple, Kotoda et al. 2006) can shorten the juvenile phase in apple respectively. The molecular mechanism of flowering in loquat is poorly understood and few flowering related genes have been isolated. In this study, we isolated *EjAP1*, an *AP1*-like MADS-box gene, from loquat and preliminary estimated its characterization in the progress of flowering.

Seven published genes which belong to the *APETALA1* family of proteins and showed high homology to the *EjAP1* were selected for the multiple alignment process. The amino acid alignment of *EjAP1* with other homologues

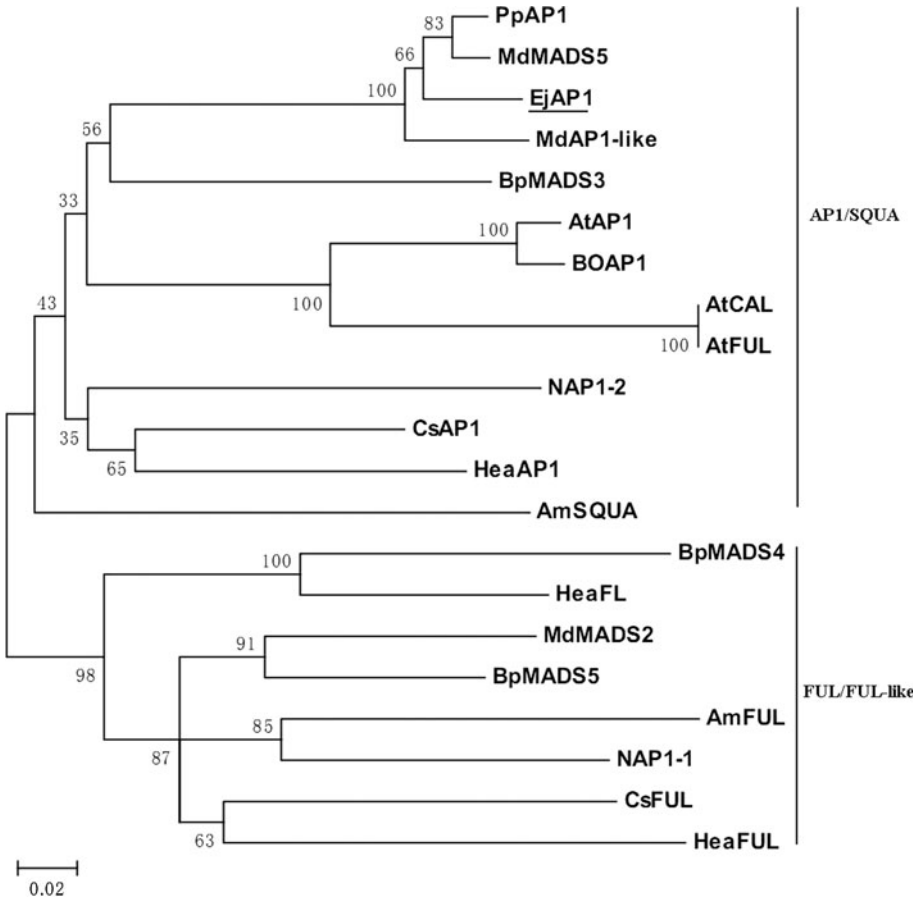

Fig. 6 Phylogenetic tree of the predicted *AP1* homologues proteins from different species

shows that the MADS-box regions of those homologues are highly conserved and K-box are somewhat less conserved regions, while the C-terminus is the least conserved portion (Fig. 5). This is consistent with other reports (Becker and Theißen 2003; Kaufmann et al. 2005). Litt and Irish (2003) identified and defined several subclades of *AP1*-like genes, specifically *euAP1*, *euFUL* and *FUL-like* in core eudicots. To determine the evolution relationships between EjAP1 and other core eudicots MADS-box proteins, a phylogenetic tree was constructed (Fig. 6). This analysis grouped EjAP1 within the AP1-like clades. EjAP1 falls into the small clade with the MdMADS5 and MdAP1-like, two AP1-like proteins of apple (*Malus* × *domestica* Borkh.), which is agree with the traditional taxonomy for both loquat and apple are species of the Rosaceae.

Although the protein structure of EjAP1 showed a high similarity with other AP1-like proteins, its structure in genome range which only exists six introns is different from most of other reported *AP1*-like genes which always have been shown with an eight exons/seven introns distribution (Shan et al. 2007; Chi et al. 2011). But it is not unique for the genome structure of *EjAP1*, similar exon/intron distribution also can be found in the *AP1* homologues of apple (*MdMADS5*, Kotoda et al. 2002) and pear (*PpAP1*, unpublished). They consist with each other not only in the number of introns but also the similar lengths of introns (Fig. 4), although lengths of their last introns show a little difference. This is not surprising, because all these three kinds of fruit trees belong to the *Maloideae* subfamily of Rosaceae. Differences between the exon/intron distributions of them and other *AP1*-like genes may come from a genomic mutational event during the evolution.

Northern blotting results revealed that *EjAP1* started to express following the transition from vegetative bud to inflorescence bud. With the development of the inflorescence and forming of the attached single flower buds on it, transcription level of *EjAP1* increased rapidly. The highest transcription level was detected in the buds ready to flowering. When the flowering began, its transcription level decreased gradually and was hardly detected in the end of flowering. This expression pattern coincides with the theory that *AP1*/*SQUA*-like genes play key roles in the formation of inflorescence and floral meristems (Mandel et al. 1992; Shan et al. 2007). Although most of *AP1*/*SQUA*-like genes can be expressed in reproductive tissues, there are also many reports which declare that their transcriptions

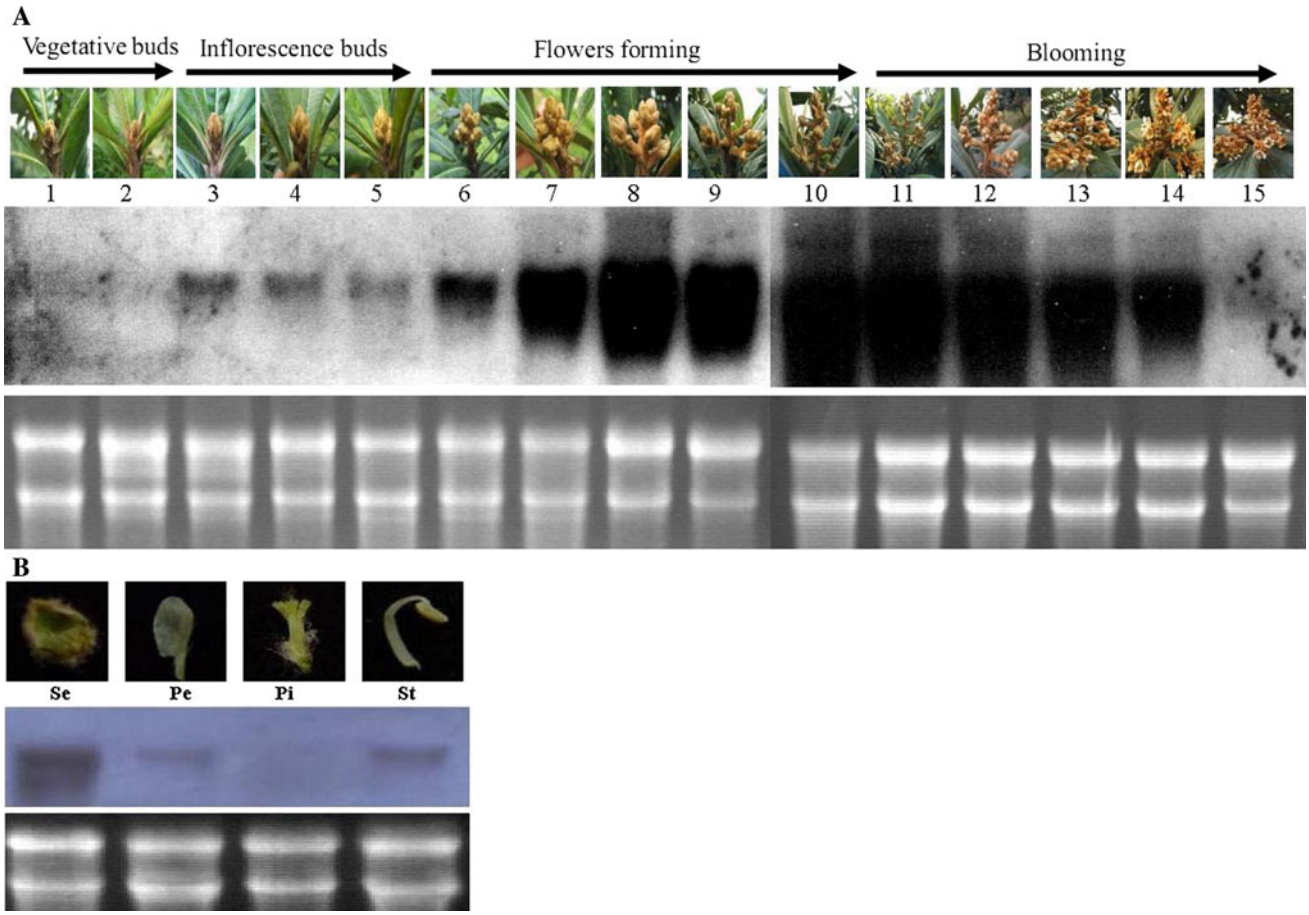

Fig. 7 Expression of *EjAP1*: A, during the flowering of loquat. Materials were collected following its flowering development process. For each lane, 20 μg of total RNA was loaded, blotted, and hybridized with an *EjAP1* probe. rRNA stained with ethidium bromide showing the equivalence of RNA loading between the samples; B in floral organs of loquat; *Se* sepal; *Pe* petal; *Pi* pistil; *St* stamen

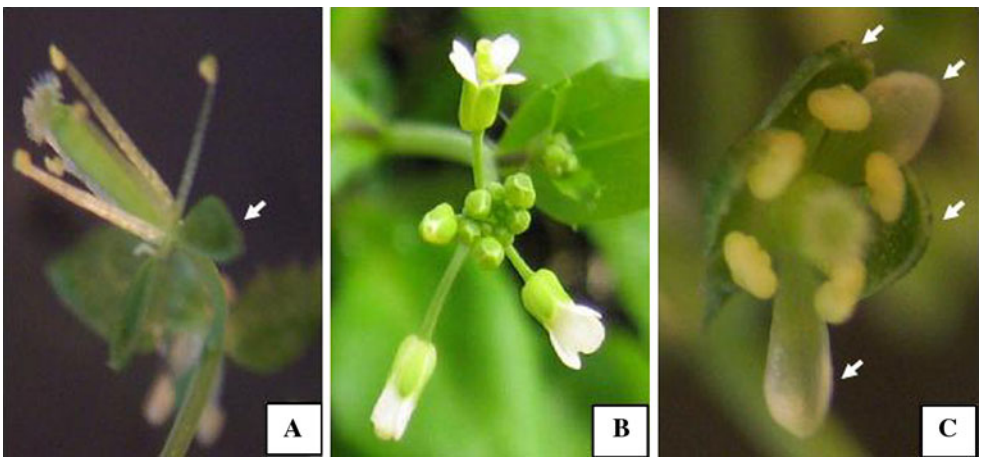

Fig. 8 Overexpression of *EjAP1* in *ap1-1* mutant. **a** flower of *ap1-1* mutant lacks petal and sepal turns to more like a bract (*white arrowhead*); **b** wild-type Columbia flowers; **c** flower of a *35S::EjAP1 ap1-1* plant, the petals and sepals are partially restored (*white arrowhead*)

are not restricted to the reproductive tissues and also can be found in vegetative tissues. For example, the rice homologue *OsMADS15* can be detected in vegetative tissues such as root, node and mature leaves (Lu et al. 2012). Different expression patterns imply the multiple functions of *AP1*-like genes though their sequences are very similar

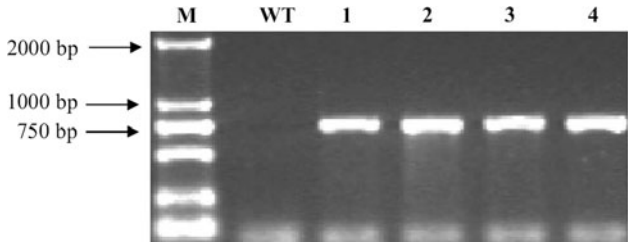

Fig. 9 RT-PCR detection of *EjAP1* expression in transformants. *M* Marker; *WT* Wild-type Columbia plants; *1–4* Transformants

to each other. In our study, *EjAP1* do not show transcription in vegetative tissues, such as vegetative bud (Fig. 7a: lane 1–2) and leaf (detected by RT-PCR, not shown).

Besides the roles in the forming of inflorescence and floral meristems, *AP1* of *Arabidopsis* participates in the construction of the outer two whorls floral organs. But few reports coincide with that. The expression pattern in the floral organs of *AP1/SQUA*-like genes varies in different species. For example, the *AP1* homologues from crocus (Tsaftaris et al. 2004) and citrus (Pillitterri et al. 2004) express in all four floral whorls. *MdMADS5*, the closely relative homologue of *EjAP1* in apple, shows its transcription only in sepal (Kotoda et al. 2000). Expression detection of *EjAP1* showed that it was express not only in sepal and petal, but also in stamen, suggesting its possible function in the construction of floral organs. Results of ectopic expression of *EjAP1* in the *Arabidopsis ap1-1* mutant further confirm this possibility: it can partly restore the floral organs which were absent in the *ap1-1* mutant plants.

The identification of *EjAP1* as an early-action gene during floral transition provides the basis for further investigations to elucidate the molecular mechanisms of floral transition in loquat as well as broadening the tools available for floral manipulation and juvenile phase reduction.

Acknowledgments This study was financially supported by grants of Key Laboratory of Innovation and Utilization for Germplasm Resources in Horticultural Crops in South China of Guangdong Higher Education Institutes, South China Agricultural University (no. KBL11008).

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