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ORIGINAL PAPER

# Exon skipping of *AGAMOUS* homolog *PrseAG* in developing double flowers of *Prunus lannesiana* (Rosaceae)

**Zhixiong Liu · Dandan Zhang · Di Liu · Fenglan Li · Hai Lu**

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**Abstract**
***Key message*** **Two transcript isoforms of *AGAMOUS* homologs, from single and double flower *Prunus lannesiana,* respectively, showed different functions.**
*Abstract* The *Arabidopsis* floral homeotic C function gene *AGAMOUS* (*AG*) confers stamen and carpel identity. Loss of *AG* function results in homeotic conversions of stamens into petals and formation of double flowers. In order to present a molecular dissection of a double-flower cultivar in *Prunus lannesiana* (Rosaceae), we isolated and identified a single-copy gene, *AG* homolog from two genetically cognate *P. lannesiana* bearing single and double flowers, respectively. Sequence analysis revealed that the *AG* homolog, *prseag-1*, from double flowers showed a 170-bp exon skipping as compared to *PrseAG* (*Prunus serrulata AGAMOUS*) from the single flowers. Genomic DNA sequence revealed that abnormal splicing resulted in mutant *prseag-1* protein with the C-terminal AG motifs I and II deletions. In addition, protein sequence alignment and phylogenetic analyses revealed that the *PrseAG* was grouped into the euAG lineage. A semi-quantitative PCR analysis showed that the expression of *PrseAG* was restricted to reproductive organs of stamens and carpels in single flowers of *P. lannesiana* 'speciosa', while the *prseag-1* mRNA was highly transcribed throughout the petals, stamens, and carpels in double flowers from 'Albo-rosea'. The transgenic *Arabidopsis* containing 35S::*PrseAG* displayed extremely early flowering, bigger stamens and carpels and homeotic conversion of petals into staminoid organs, but ectopic expression of *prseag-1* could not mimic the phenotypic ectopic expression of *PrseAG* in *Arabidopsis*. In general, this study provides evidences to show that double flower 'Albo-rosea' is a putative C functional *ag* mutant in *P. lannesiana*.

**Keywords** *Prunus lannesiana* · *AGAMOUS* · Exon skipping · Double flower

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## Introduction

The classic ABC-model and expanded ABCE-model explain how a few genes act together to specify the four organ types that make up a perfect flower, stating that (A + E)-functions are needed for the sepal, (A + B + E)-functions are required for the petal, (B + C + E)-functions are necessary for the stamen, and (C + E)-functions are essential for the carpel (Coen and Meyerowitz 1991; Pelaz et al. 2000; Krizek and Fletcher 2005). Mutual repression between the A- and C-functions is integral to the ABCE-model to explain why the A-function expands into the inner two whorls in C-function mutants, causing reproductive organs to homeotically transform into sterile perianth in the inner two whorls (Bowman et al. 1991a; Krizek and Fletcher 2005). Previous studies also revealed that most ABC-genes encode MADS transcriptional

regulators which contain four conserved domains, the MADS-, Intervening-, Keratin-like, and C-terminal (MIKC) domains (Kaufmann et al. 2005; Zahn et al. 2006). The highly conserved MADS domain (approximately 58 amino acids) is responsible for DNA binding, dimerization and accessory-factor interactions (Shore and Sharrocks 1995). The k-domain is important for protein–protein interactions (Yang and Thomas 2004). The region between the MADS- and k-domain is a weakly conserved I domain for dimerization and protein functional specificity (Riechmann et al. 1996; Krizek and Meyerowitz 1996). The C-terminal domain is variable in sequence and length, and is involved in both transcriptional activation and the formation of higher order MADS protein complexes (Egea-Cortlnes et al. 1999; Honma and Goto 2001).

In *Arabidopsis*, the C-class MADS-box gene *AGAMOUS* (*AG*) plays crucial roles in flower development by regulating the organ identity of stamens and carpels, the repression of A-class genes, and floral meristem determinacy (Bowman et al. 1991a; Weigel and Meyerowitz 1994). According to the C-function, *AG* RNA is expressed starting at stage three in the center of the floral meristem that will give rise to the stamens and carpel primordia and becomes limited to the third and forth whorls until late in flower development (Smyth et al. 1990; Drews et al. 1991; Bowman et al. 1991b). In loss-of-function mutants of *AG*, the expression of A-class genes expanding into inner whorls leads to double flowers (flowers with more than the usual number of petals); on the contrary, ectopic expression of *AG* in outer whorls results in flowers with carpelloid sepals and staminoid petals (Mizukami and Ma 1992). Members of *AG* homolog have been identified in all major clades of angiosperms, even in the primitive gymnosperm *Cycas edentata*, indicating that their expression pattern and C-function for controlling reproductive organ (stamen and carpel) identity are conserved (Zhang et al. 2004; Kitahara et al. 2004; Whipple and Schmidt 2006; Teeri et al. 2006; Lv et al. 2007a; Hsu et al. 2010).

*Prunus lannesiana* (Rosaceae) is a popular East Asian original ornamental tree and now widely planted in the world. Many spontaneous double-flower cultivars were kept and propagated for garden landscaping during the early domestication and subsequent breeding process. In order to uncover the possible molecular mechanisms of double flowering cherry flower morphogenesis, we isolated the *AG* homolog from two genetically cognate Japanese flowering cherry cultivars, *P. lannesiana* 'speciosa' bearing single flowers with four normal floral whorls and *P. lannesiana* 'Albo-rosea' bearing double flowers with additional petals, petaloid anthers and leaflike carpels, respectively. Sequence analysis revealed that the *AG* homolog, *prseag-1*, from double flowers, shows a 170-bp exon skipping as compared to *PrseAG* from the single flowers. Genomic DNA sequence and Southern blot analyses revealed that *PrseAG* and *prseag-1* were derived from the consensus pre-mRNA. The abnormally spliced *PrseAG* pre-mRNA was translated to produce a mutant *prseag-1* protein with the C-terminal AG motifs I and II deletions. In this study, we set out to characterize functional differences between the two transcript isoforms, *PrseAG* and *prseag-1*, and to test whether a mutation at this locus results in functional variation. Our results indicate that transgenic *Arabidopsis* containing 35S::*PrseAG* displayed extremely early flowering, bigger stamens and carpels and homeotic conversion of petals into staminoid organs. Abscission of the outer three floral whorls (perianth and stamen) was inhibited during the development of siliques, but ectopic expression of *prseag-1* could not mimic the phenotypic of ectopic expression of *PrseAG* in *Arabidopsis.* In addition, our results also provided evidence to show that the double flower 'Albo-rosea' is a putative C functional *ag* mutant in *P. lannesiana.*

## Materials and methods

### Plant material

The single and double flower buds were collected from Japanese flowering cherry trees growing under natural conditions in Beijing, China. During carpel shape well before anthesis, the juvenile leaves, sepals, petals, stamens and carpels were sampled from two *P. lannesiana* cultivars 'speciosa' and 'Albo-rosea', respectively, and frozen immediately in liquid nitrogen and stored at −80 °C until used.

### Cytomorphological examination and determination of pollen viability

Flower buds at different developmental stages were fixed in FAA (38 % formaldehyde:acetic acid:70 % ethanol = 1:1:18). Then samples were dehydrated in an ethanol series, embedded in paraffin, and serially sectioned at a thickness of 8 μm with a microtome. Subsequently, the sections were stained with safranin-fast green and observed under a Leica DM6000B microscope as published (Johanse 1940). The average number of stamens per single and double flower was counted in 30 samples, and statistical analysis was performed with SPSS13.0 software. Germination of fresh pollen grains from 'speciosa' and 'Albo-rosea' was assessed by hanging drop culture with basic liquid germination medium containing 15 % sucrose at 25 °C for a 50 min incubation (Brewbader and Kwack 1963; Hu 1993). The pollen grain slides were observed under an Olympus BH-2 phase contrast microscope and the

measurements of pollen germination rate are based on 30 readings.

### Isolation of *PrseAG*, *prseag-1* RNA and full-length genomic DNA of *PrseAG*

Total RNA was extracted from the flower buds of *P. lannesiana* 'speciosa' and *P. lannesiana* 'Albo-rosea' with the modified CTAB method according to Chang et al. (1993). The first-stand cDNA was synthesized from 2 μg of the DNase I-treated RNA using oligo(dT)15 adaptor primer and M-MLV reverse transcriptase (TaKaRa, Japan). Isolation of the 3′ end of *PrseAG* was carried out through 3′ RACE using the 3′-full RACE core set Ver.2.0 kit (TaKaRa, Japan) according to the manufacturer's instructions and the gene-specific primer GSPAG (5′-ACAACAA ACCGTCAAGTCAC-3′). The full-length cDNA sequences of *PrseAG* were amplified by PCR using the forward primer PrseAGF (5′-ACCCAAAAGCTTGCAACTATG-3′) and the reverse primer PrseAGR (5′-AAGTGCCTGTAGC TATCATCATCT-3′), respectively. PCR was performed with a 5-min 94 °C denaturation step, followed by 35 cycles of 45 s at 94 °C, 45 s annealing at 57 °C, a 1-min extension at 72 °C, and a final extension period of 10 min. Moreover, the full-length cDNA of *prseag-1* was isolated from double-flower buds of 'Albo-rosea' with the same PCR protocol.

Genomic DNA was extracted from *P. lannesiana* 'speciosa' juvenile leaves using the modified CTAB method according to Doyle and Doyle (1987). The full-length genomic DNA sequence of *PrseAG* was isolated using in-fusion advantage PCR cloning kit (Clontech, USA) according to the manufacturer's protocol. Amplification by PCR was accomplished with *LA Taq* DNA polymerase (TaKaRa, Japan), the forward primer DAGF (5′-GCAGGTCGACTCTAGAGCAACTATGGCCTATG AAAACAAA-3′) and reverse primer DAGR (5′-TCGAG CTCGGTACCCGGGAAGTGCCTGTAGCTATCATCAT CT-3′), as well as 2 μg of RNase-treated genomic DNA. PCR was performed with a 5-min 94 °C denaturation step, followed by 30 cycles of 30 s at 94 °C, 1-min annealing at 60 °C, a 5-min extension at 72 °C, and a final extension period of 10 min. The PCR product was cloned into the pUC19 vector (TaKaRa, Japan) digested with *Sma*I and *Xba*I, and sequenced.

### Sequence alignments and phylogenetic analysis

The deduced amino acid sequences of *PrseAG* were used for BLAST analysis on the Genbank database. During the BLAST searches, multiple AG-like proteins from different lineages were selected for alignment, and four A-class proteins, two B-class proteins and four E-class proteins were also included. Full-length amino acid sequences comprising the MADS, I, K, and C domains of these genes were aligned with ClustalW program under default settings. The Phylogenetic trees were constructed using MEGA4.0 software by the neighbor-joining method (Tamura et al. 2007). The Genbank accession numbers of the sequence data used are as follows: *Prunus lannesiana*, PrseAG (ADK95058), PrseSHP (ADG45819), PrseSTK (ADD91578), CLAP1 (ACT67688); *Prunus persica,* PpMADS4 (AAU29513), PperSHP (ABG75908), PperSTK (ABQ85556); *Prunus mume*, PmAG (ABU41518); *Prunus serotina,* PsAG (ACH 72974); *Malus* × *domestica,* MdMADS15 (CAC80858), MdMADS14 (CAC80857); *Taihangia rupestris,* TrAG (ABB59994), TrSHP (ABB59995); *Rosa rugosa,* MASAKO C1 (BAA90744), MASAKO D1 (BAA90743); *Fragaria* × *ananassa,* STAG1 (AAD45814); *Gossypium hirsutum,* GhMADS3 (AAL92522), GhMADS7 (ABM69045), GhMADS5 (ABM69043); *Theobroma cacao,* TcAG (ABA 39727); *Cucumis sativus,* CUM1 (AAC08528), CUM10 (AAC08529); *Hydrangea macrophylla,* HmAG (BAG 74745); *Antirrhinum majus,* FAR (CAB42988), PLE (AAB25101); *Petunia* × *hybrida,* pMADS3 (CAA51417), FBP6 (CAA48635), FBP7 (CAA57311), FBP11 (CAA 57445); *Solanum lycopersicum*, TAG1 (AAA34197), TAGL1 (AAM33101), TAGL11 (AAM33102); *Torenia fournieri,* TFPLE2A (BAG31394); *Misopates orontium,* MoPLE (CAJ44134); *Arabidopsis thaliana*, AG (NP_ 567569), AGL1/SHP1 (NP_191437), AGL5/SHP2 (NP_ 850377), STK/AGL11 (NP_192734), AP1 (NP_177074), AGL8/euFUL (NP_568929), AGL79/FUL-like (NP_ 189645), AP3 (NP_191002), PI (NP_197524), SEP1 (NP_ 568322), SEP2 (NP_186880), SEP3 (NP_850953), SEP4 (NP_849930).

### Southern blot

The genomic DNA from juvenile leaves of 'speciosa' and 'Albo-rosea' were treated with RNase and purified, and then digested with *Eco*RI, *Hin*dIII and *Eco*RV (TaKaRa, Japan), respectively. 20 μg of digested genomic DNA were loaded in each lane and separated on a 1 % agarose gel, and subsequently transferred to a HyBond-$N^+$ nylon membrane (Amersham Biosciences, UK), and then fixed by UV-crosslinking (254 nm UV light for 2 min). A 260-bp consensus sequence of *PrseAG* and *prseag-1*, present in the 3′UTR (3′untranslated region) of *PrseAG* (730–989), was prepared for Southern blot probe A by PCR with the forward primer HAGF1 (5′-TAATGTGCTTGGACCGTCG TC-3′) and the reverse HAGR1 (5′-CACATGAGAGTTG GAGGAAATTG-3′), using the plasmid DNA template of *PrseAG*; A 170-bp missing exon of *prseag-1*, position in C-region of *PrseAG* (559–728), was prepared for Southern blot probe B by PCR with the forward primer HAGF2

(5′-ATAGCTGAGAATGAGAGGAGCCA-3′) and the reverse primer HAGR2 (5′-ACTAATTGAAGGGCCAT CGGATC-3′), using the plasmid DNA template of *PrseAG*. Two probes for Southern blot were labeled with digoxigenin by using DIG high prime DNA labeling and detection starter kit II (Roche, Mannheim, Germany) according to the manufacturer's procedure. Prehybridization, hybridization, stringency washes and immunological detection were performed with DIG high prime DNA labeling and detection starter kit II (Roche, Mannheim, Germany) according to the manufacturer's procedure.

### Semi-quantitative RT-PCR analysis

For semi-quantitative RT-PCR analysis, 2 μg of total RNA extracted from leaves, sepals, petals, stamens and carpels of each cultivar was used to synthesize the first-strand cDNA with an oligo(dT)15 primer as described above. 2 μl of cDNA sample from the RT reaction was used for 30 cycles of PCR as follows: 30 s at 94 °C; 30 s at 60 °C; and 30 s at 72 °C, which were preceded by 5 min at 94 °C and followed by 10 min at 72 °C. 5 μl of the total PCR product (25 μl) in each reaction was analyzed by electrophoresis in 1 % agarose gel and photographed under UV light. RT-PCR was carried out using gene specific primers RTAGF (5′-ACAACAAACCGTCAAGTCAC-3′) and RTAGR (5′-ACGGTCCAAGCACATTAAACTA-3′). As a positive control, 2 μl of the first-strand RT reaction were used for amplification of the *P. lannesiana ACTIN* cDNA using specific primers Psactin-F (5′-TGTGAGTCACACT GTGCCAA-3′) and Psactin-R (5′-GCAGCTTCCATTCC AATGAG-3′).

### Vectors construction and *Arabidopsis* transformation

Full-length *PrseAG* and *prseag-1* cDNA were separately cloned into binary vector pBI121 (BD Biosciences Clontech) using *Xba*I and *Sma*I restriction enzymes under the control of cauliflower mosaic virus 35S promoter in the sense orientation. 35S::*PrseAG* and 35S::*prseag-1* constructs were transformed separately into *A. thaliana* plants (ecotype Columbia) using the floral-dip method according to Clough and Bent (1998) with *Agrobacterium tumefaciens* strain GV3101. The seeds of transgenic *Arabidopsis* plants were selected on solid 0.5 ×MS medium (Murashige and Skoog 1962) containing 50 μg/ml kanamycin at 4 °C for 2 days, and then were transferred to the greenhouse under long-day condition (16 h light/8 h dark) at 22 °C for 10 days. Subsequently, the seedlings were transplanted to soil. The transgenic lines of *A. thaliana* plants were confirmed by Southern blot analysis. The genomic DNA from leaves of transgenic *Arabidopsis* was extracted as previously described (Dellaporta et al. 1983), and then digested with *Eco*RI, as well as *Xba*I and *Sma*I. The 566-bp Southern probe for 35S::*PrseAG Arabidopsis* was prepared by PCR with primers TAGS1 (5′-TGAAAGACCTGAAG AACCTGGAG-3′) and TAGR (5′-TATCCCGGGTCCAA GCACATTAAAC-3′), and the plasmid DNA templates of *PrseAG*. The 342-bp Southern probe for 35S::*prseag-1 Arabidopsis* was prepared by PCR with primers TagS1 (5′-TGAAAGACCTGAAGAACCTGGAG-3′) and TagR (5′-TAACCCGGGAGTGCCTGTAGCTATC-3′), and the plasmid DNA templates of *prseag-1*. These were labeled with digoxigenin by using DIG high prime DNA labeling and detection starter kit II (Roche, Mannheim, Germany) according to the manufacturer's procedure. Southern hybridizations were performed according to the description by Zhang et al. (2009). The genomic DNA from leaves of wild *Arabidopsis* and plasmid DNA of the target genes were served as negative and positive controls, respectively. The stage of flower development in wild-type and transgenic *Arabidopsis* was confirmed according to the description by Smyth et al. (1990).

## Results

### Morphological difference between single and double flowers in *P. lannesiana*

The single flowers from *P. lannesiana* 'speciosa' have four whorls of normal floral organs, including five sepals in whorl 1, five petals in whorl 2, forty-three to fifty-two stamens in whorl 3, and one carpel in whorl 4, respectively (Fig. 1a–c, g). However, the double flowers from *P. lannesiana* 'Albo-rosea' have five normal sepals in whorl 1, thirty to forty petals in whorl 2, seventeen to twenty-seven stamens with petaloid anthers in whorl 3, leaflike carpels instead of normal carpel in whorl 4 (Fig. 1d–f, j), and their stamen numbers were significantly lower ($P < 0.01$) than that of the single flowers from 'speciosa'. Moreover, their petaloid anthers house a large number of sterile pollen grains (Fig. 1k) and some double flowers contain ectopic abortion carpels attached to calyx tube (Fig. 1f, j). In addition, the mean germination rate of fresh grains from 'speciosa' was 72.64 % (Fig. 1i), whereas no fresh pollen germination was observed in 'Albo-rosea' (Fig. 1l).

### Cloning and sequence analyses of *PrseAG* and *prseag-1*

A full-length cDNA of *PrseAG* from 'speciosa', and *prseag-1* from 'Albo-rosea' were obtained, respectively, by using homology-based cloning and RACE techniques following the procedure described earlier. The *PrseAG* was 1,063 bp and contained a 732-bp ORF encoding for 243 amino acids, as well as a 331-bp 3′ UTR including poly-A

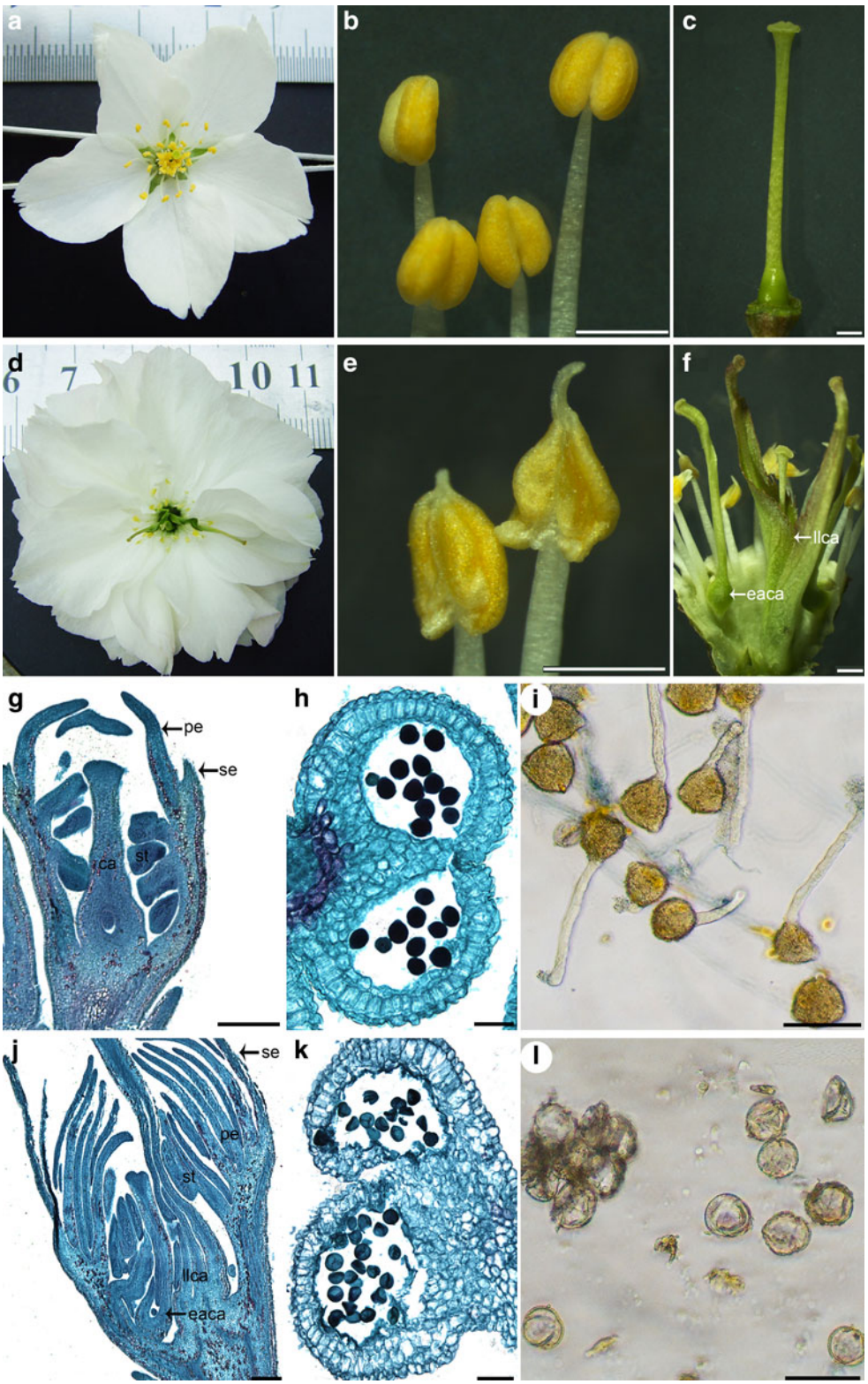


**Fig. 1** The morphological and cytological observation on single and double flowers of *P. lannesiana*. **a** The single flower from *P. lannesiana* 'speciosa'. **b** The stamens from 'speciosa'. **c** The carpel from 'speciosa'. **d** The double flower from *P. lannesiana* 'Albo-rosea'. **e** The petaloid anthers from 'Albo-rosea'. **f** The leaflike carpels and ectopic abortion carpels attached to calyx tube from 'Albo-rosea'. **g** The flower bud of *P. lannesiana* 'speciosa'. **h** Mature pollens of 'speciosa', **i** Pollen germination of 'speciosa', **j** The flower bud of *P. lannesiana* 'Albo-rosea', **k** Mature pollens of 'Albo-rosea'. **l** Pollen germination of 'Albo-rosea'. *Bars* 1000 μm (**a**–**f**), 200 μm (**g**, **j**), 50 μm (**h**, **i**, **k**, **l**). *llca* leaf-like carpel, *eaca* ectopic abortion carpels, *le* juvenile leaf, *se* sepal, *pe* petal, *st* stamen, *ca* carpel

tail (Genbank accession number: GU377079); while the ORF of the *prseag-1* was only 627 bp and encoded for 208 amino acids (Genbank accession number: GU377080). Sequence and phylogenetic analyses revealed that the *PrseAG* (*Prunus serrulata AGAMOUS*) is grouped into the *euAG* lineage (Fig. 2). Moreover, cDNA sequence comparisons showed that *prseag-1* is a mutation of *PrseAG* with a 170-bp coding region omission. To ascertain whether they were derived from the consensus pre-mRNA, genomic DNA of *PrseAG* and *prseag-1* were sequenced. The results showed that the complete omission of the seventh exon (170 bp) from the genomic sequence of *PrseAG* (Genbank accession number: HM036349) produced the *prseag-1*. Moreover, the 170-bp exon skipping results in the completely lesion of the *prseag-1* C-terminal AG motifs I and II (Fig. 3) (Kramer et al. 2004), a region conserved in plant AG-like proteins and proposed to function in transcriptional activation, mediating protein–protein interactions, the formation of higher order MADS protein complexes and functional specificity (Egea-Cortlnes et al. 1999; Honma and Goto 2001; Tzeng et al. 2004; Lamb and Irish 2003). Conceptual translation reveals that PrseAG and *prseag-1* share a consensus predicted protein sequence throughout the M, I, and K domain, but have a different C-terminal region. To further ascertain whether *PrseAG* is duplicated in the *P. lannesiana* genome, Southern blot analyses were performed using two different specific probes of *PrseAG* (A 260-bp consensus sequence of *PrseAG* and *prseag-1*, present in 3′UTR, as probe A; the 170-bp omission exon of *prseag-1* as probe B). One hybridization band is observed, indicating that *PrseAG* is a single-copy gene in both *P. lannesiana* 'speciosa' and 'Albo-rosea' genomes (Fig. 4). Furthermore, Southern blot analysis with probe B also revealed that the complete 170-bp exon is located in genomic DNA of the double flower cultivar 'Albo-rosea'. Moreover, Southern blot analyses with two different probes (A and B) showed the same banding pattern, and genomic DNA sequences further confirmed that there are no recognition sites of *Eco*RI, *Hin*dIII and *Eco*RV between/in probe regions. These results indicated that the gene locus was not mutated in double flower cultivar 'Albo-rosea', and *PrseAG* and *prseag-1* were derived from the consensus pre-mRNA.

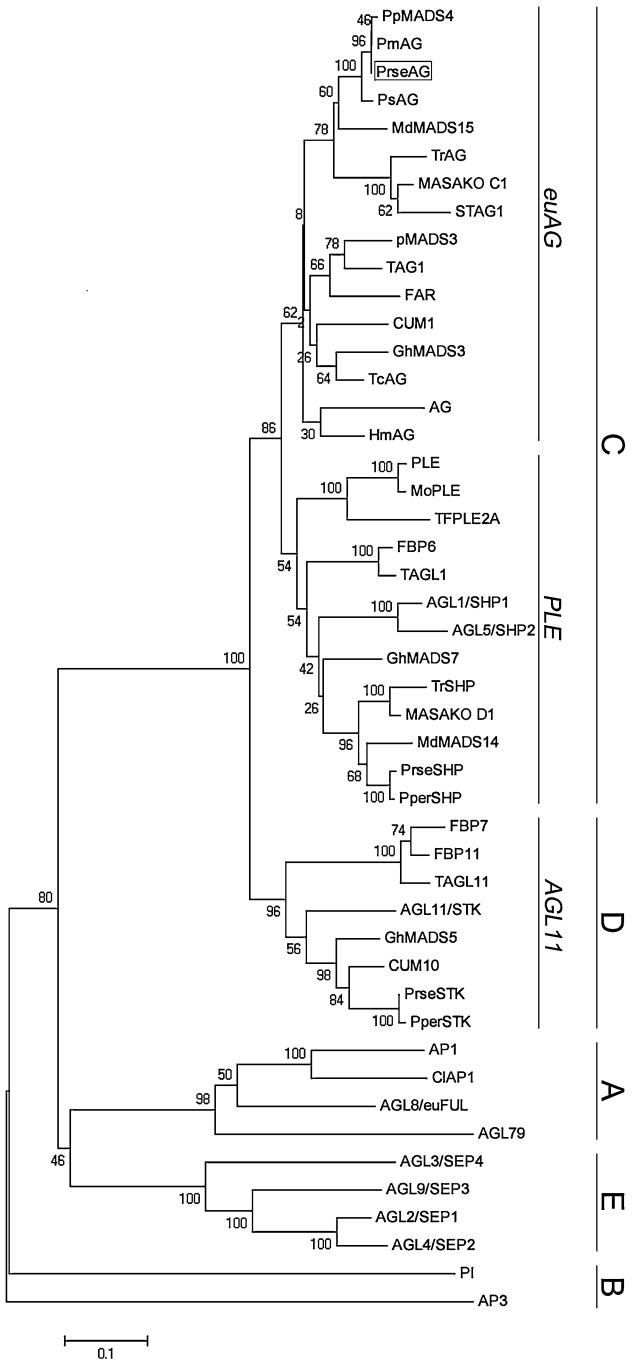


**Fig. 2** Phylogenetic analysis of AG-like MADS-box proteins. PrseAG from *P. lannesiana* is *boxed*

Expression analysis of *PrseAG* and *prseag-1* in single and double flowers of *P. lannesiana*, respectively

Semi-quantitative RT-PCR analysis, performed using gene specific primers, showed that *PrseAG* was transcribed in the stamens and carpels of the flower buds from *P. lannesiana* 'Speciosa'. The expression of *PrseAG* was restricted to reproductive structures and excluded from outer whorls of the perianth and leaves, which displayed a similar spatial expression pattern as the *AG* in *Arabidopsis.* Whereas the *prseag-1* mRNA was highly transcribed throughout petal, stamen and carpel in double flower from 'Albo-rosea' (Fig. 5).

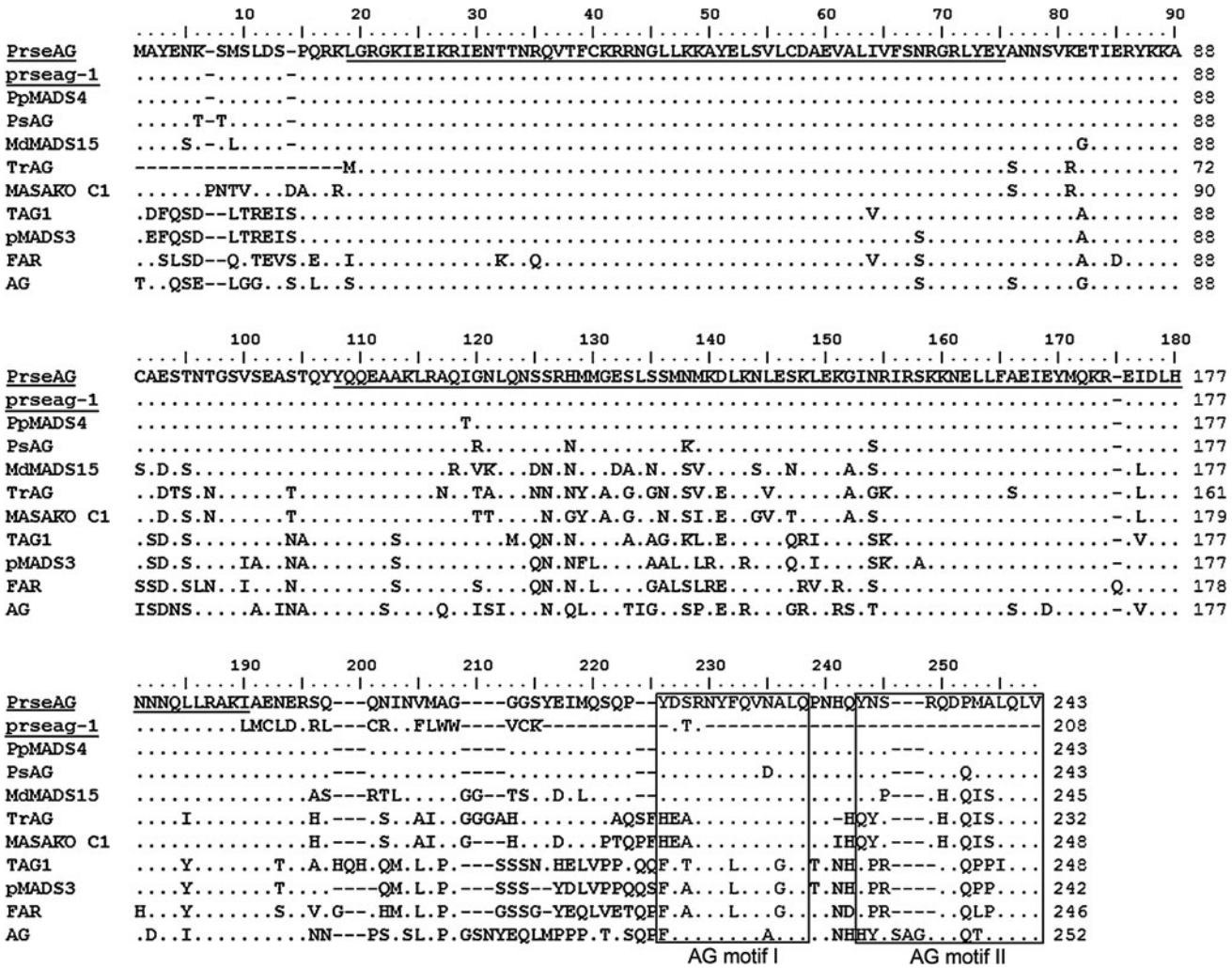


**Fig. 3** Sequence alignments of PrseAG, prseag-1 and the other AG-related MADS domain proteins. The first and second *underlined* regions represent the MADS domain and the K domain, respectively. The AG motifs I and II in the C-terminal region are *boxed.* The two motifs are highly conserved for AG-like proteins. Amino acid residues identical to PrseAG are indicated as *dots*. To improve the alignment, *dashes* were introduced into the sequence

## Ectopic expression of *PrseAG* and *prseag-1* in *Arabidopsis*

In order to gain further insight into the function of *PrseAG* and *prseag-1*, functional analyses were carried out by over expressing *PrseAG* and *prseag-1* in *Arabidopsis*, respectively. The two different transcript isoforms under the control of the cauliflower mosaic virus 35S promoter were introduced into *Arabidopsis* via *Agrobacterium* transformation. Transgenic plants were confirmed by Southern blot (Fig. 6), and their phenotypic alterations were analyzed.

Among 41 35S::*PrseAG* independent transgenic plants, 28 displayed strong phenotypes, six weak phenotypes and seven showed no obviously phenotypic alterations. The 35S::*PrseAG* transgenic lines with strong phenotypes flowered very early (even before the sixth rosette leaf appeared) and displayed reduced height (Fig. 7Ia). The rosette leaves and cauline leaves curled upward and were smaller (Fig. 7Ib). The flower buds opened prematurely, usually before stage 10 (Fig. 7Ic, Id), while the flower buds of 35S::*prseag-1* transgenic and wild *Arabidopsis* opened after stage 12 (Fig. 7IIe, IIf, IIIe, IIIf). At stage 12, the sepals and petals of 35S::*PrseAG* transgenic plants lengthened slowly, and there was a large gynoecium out of them (Fig. 7Ie). In contrast, the perianth of 35S::*prseag-1* transgenic and wild *Arabidopsis* lengthened relatively rapidly (Fig. 7IIe, IIIe). From stage 15 to 16, the petal bases of 35S::*PrseAG* transgenic *Arabidopsis* transformed homeotically into filament-like structures (Figs. 7Ig, 8), whereas the stamen and carpel were larger (Fig. 7If–Ih). Moreover, abscission of outer three floral whorls (perianth and stamen) was inhibited during silique development, and the siliques were bumpy (Fig. 7Ii, Ij). The seed set rate of the bumpy siliques was lower than that

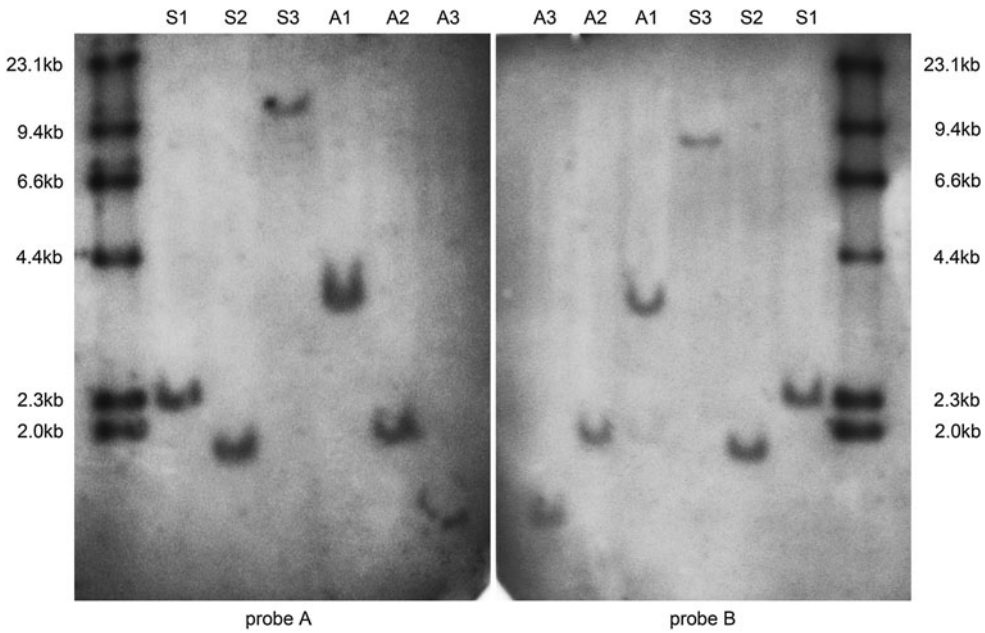


**Fig. 4** Southern blot analyses of *PrseAG* in *P. lannesiana* 'speciosa' and 'Albo-rosea'. The genomic DNA from *P. lannesiana* 'speciosa' and 'Albo-rosea' digested with *Eco*RI (S1, A1), *Hin*dIII (S2, A2) *Eco*RV (S3, A3), respectively. The *PrseAG*-specific probe A was a 260-bp consensus sequence of *PrseAG* and *prseag-1*. The *PrseAG*-specific probe B was the 170-bp omission exon of *prseag-1*. (S1–S3: 'speciosa', A1–A3: 'Albo-rosea')

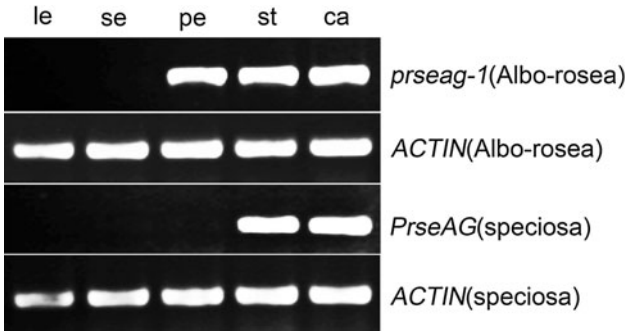


**Fig. 5** Expression analyses of *PrseAG* and *prseag-1* by semi-quantitative RT-PCR with *ACTIN* as control. *le* juvenile leaf, *se* sepal, *pe* petal, *st* stamen, *ca* carpel

of normal siliques in wild and 35S::*prseag-1* transgenic plants. However, 27 35S::*prseag-1* independent transgenic plants showed normal vegetative growth with no pronounced floral homeotic modification (Fig. 7IIa–IIj).

## Discussion

In ornamental plants a double flower is one of the major ornamental traits that have been subjected to artificial selection pressure during the early domestication and subsequent breeding process, and is retained for its showy aspect in many horticulture crops. However, the molecular

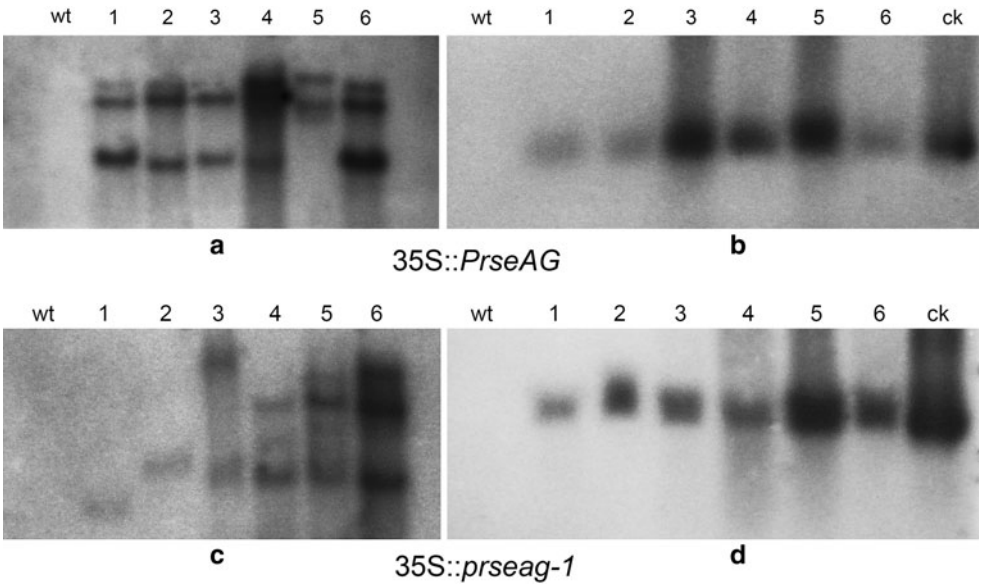


**Fig. 6** The 35S::*PrseAG* and 35S::*prseag-1* transgenic *Arabidopsis* were confirmed by Southern blot. **a** The genomic DNA from *PrseAG* transgenic *Arabidopsis* plants digested with *Eco*RI. **b** The genomic DNA from *PrseAG* transgenic *Arabidopsis* plants digested with *Xba*I and *Sma*I. **c** The genomic DNA from *prseag-1* transgenic *Arabidopsis* plants digested with *Eco*RI. **d** The genomic DNA from *PrseAG* transgenic *Arabidopsis* plants digested with *Xba*I and *Sma*I. CK positive control, 1–6: transgenic plants, *Wt* Wild-type plant

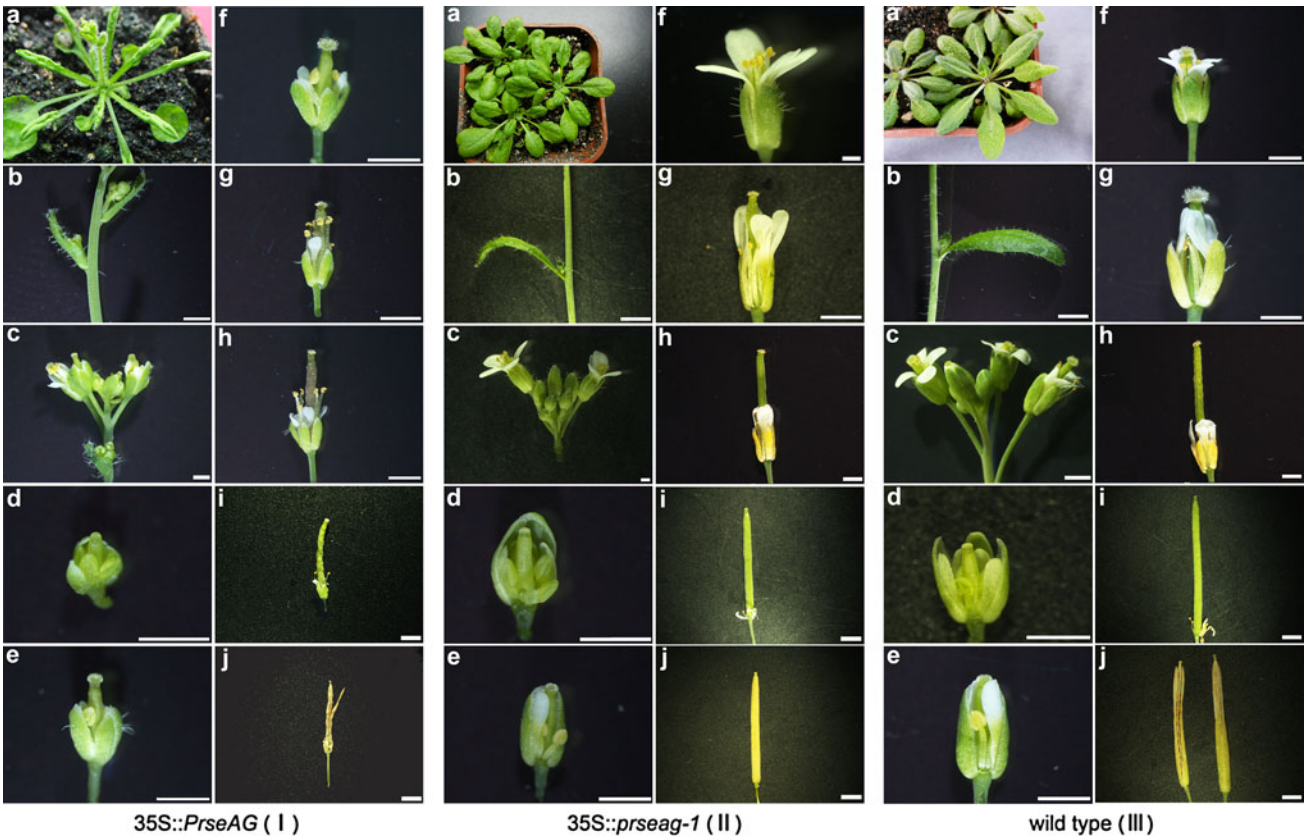


**Fig. 7** Comparison of the phenotypes of wild type, 35S::*PrseAG* and 35S::*prseag-1* transgenic *Arabidopsis*. **Ia**–**Ij** Constitutive expression of 35S::*PrseAG* transgenic *Arabidopsis* plants. **IIa**–**IIj** Constitutive expression of 35S::*prseag-1*. **IIIa**–**IIIj** Wild type *Arabidopsis* plants. **Ia** 35S::*PrseAG* transgenic *Arabidopsis* plants, early flowering in comparison with **IIa** and **IIIa**. **Ib** Curled rosette leaves and cauline leaves of 35S::*PrseAG* transgenic *Arabidopsis*. **IIb**, **IIIb** Cauline leaves of 35S::*prseag-1* transgenic and wild type *Arabidopsis,* respectively. **Ic** Inflorescence of 35S::*PrseAG* transgenic *Arabidopsis* plants in comparison with **IIc** and **IIIc**. **Id** Flower of 35S::*PrseAG* transgenic *Arabidopsis* at development stage 10, Flower buds were prematurely open. **IId**, **IIId** Flower from 35S::*prseag-1* transgenic and wild type *Arabidopsis,* respectively at the same development stage with **Id**. **Ie** Flower of 35S::*PrseAG* transgenic *Arabidopsis* at development stage 12 with large gynoecium out of perianth. **IIe**, **IIIe** Flower from 35S::*prseag-1* transgenic and wild type *Arabidopsis* respectively at the same development stage with **Ie**. **If** Flower at development stage 15. **IIf**, **IIIf** Flower from 35S::*prseag-1* transgenic and wild type *Arabidopsis* respectively at the same development stage with **If**. **Ig**–**Ih** Flower bud at development stage 16 with petal bases transformed homeotically into filament-like structures (**Ig**), whereas the stamen and carpel developed bigger (**Ig**, **Ih**). **IIg**–**h**, **IIIg**–**h** Flower of 35S::*prseag-1* transgenic and wild type *Arabidopsis* respectively at development stage 16 with petals and sepals withering. **Ii** Flower at development 17 with bumpy siliques, perianth and stamens abscission inhibited. **IIi**, **IIIi** Flower of 35S::*prseag-1* transgenic and wild type *Arabidopsis,* respectively at development 17 with all organs fall from green siliques. **Ij** Flower at development at stage 18 with withering perianth and stamens abscission inhibited. **IIj**, **IIIj** Flower of 35S::*prseag-1* transgenic and wild type *Arabidopsis,* respectively at stage 18 with siliques turn yellow

mechanisms controlling the development and floral organ morphogenesis of double flowers are not well understood although they are of great commercial interest.

In *Arabidopsis*, the C-function gene *AGAMOUS* plays a key role in specifying sexual organ identity. *AG* loss-of-function results in a shift of the boundaries of the A-class gene toward center of the flower which transforms stamens into petals and carpels into another *ag* flower and generates double flowers (Bowman et al. 1991a; Weigel and Meyerowitz 1994).

To investigate the C-function floral organ identity genes in the regulation of single and double flower development of *P. lannesiana*, we have identified and characterized a C-class gene, *PrseAG,* from the single flower *P. lannesiana* 'Speciosa'. Based on a sequence alignment, two highly conserved motifs specific to C proteins, AG motifs I and II (Kramer et al. 2004), were found in the C-terminal regions of the *PrseAG*. Phylogenetic tree analysis revealed that *PrseAG* belongs to the clade of *euAG* lineages. However, *prseag-1,* a transcript isoform resulting from a complete exon skipping of *PrseAG* gene, was found in the double flower *P. lannesiana* 'Albo-rosea'. Moreover, the 170-bp exon skipping results in the complete lesion of the prseag-1 C-terminal AG motifs I and II (Kramer et al. 2004), a region conserved in plant AG-like proteins and proposed to function in transcriptional activation, mediating

**Fig. 8** The petal of 35S::*PrseAG* transgenic *Arabidopsis* is converted into filament-like structure

protein–protein interactions, the formation of higher order MADS protein complexes and functional specificity (Egea-Cortlnes et al. 1999; Honma and Goto 2001; Tzeng et al. 2004; Lamb and Irish 2003). Therefore, the C-function of *prseag-1* for specifying the stamen and carpel identity may be lost partially or completely.

Expression analysis revealed that *PrseAG* mRNA is flower specific and strongly detected only in the stamens and carpels of 'Speciosa'. This spatial expression pattern matches well with that of *AG* from *Arabidopsis* and *AG* orthologs from other species in Rosaceae, such as *TrAG* of *Taihangia rupestris* and *PsAG* of *Prunus serotina* (Zahn et al. 2006; Lv et al. 2007b; liu et al. 2010). However, the *prseag-1* mRNA is highly transcribed throughout petals, stamens and carpels in the double flowers of 'Albo-rosea', which suggests that it is the result of different *PrseAG* activities. In the double flower 'Albo-rosea', the decreased stamens are homeotic into additional petals so the high expression of mutant *prseag-1* gene in petals of *P. lannesiana* 'Albo-rosea' was probably persistent in its ancestral expression region after function loss. Similar results reported in a double-flower ranunculid mutant with petaloid sepals throughout, an alternative splicing isoform of C-function lost expressed in petaloid sepals (Galimba et al. 2012).

To ascertain further the function of the two transcript isoforms, *PrseAG* and *prseag-1*, we transformed *Arabidopsis* with the coding region of both transcript isoforms under the control of the cauliflower mosaic virus 35S promoter in the sense orientation considering that woody plants have large, uncharacterized genomes and lengthy generation periods and are not amenable to the standard techniques of functional genomics. Our data showed that *PrseAG* gene was able to promote early flowering, as well as the development of stamens and carpels, and convert the petals into stamenoid structures in *Arabidopsis,* resembling those caused by the overexpression of *AG* (Mizukami and Ma 1992). The results strongly suggest that *PrseAG* maintained ancestral activities in the coding regions for specifying the organ identity of stamens and carpels as well as floral meristem determination. Furthermore, previous studies have revealed that ectopic expression of *AG* orthologs from other plants in the Rosaceae, such as *TrAG* from *Taihangia rupestris* (Lv et al. 2007a) and *MASAKO C1* from *Rosa rugosa* (Kitahara et al. 2004), can also mimic the phenotypic of ectopic expression of *AG* in *Arabidopsis* (Mizukami and Ma 1992). These results further indicated that the *AG* orthologs from Rosaceae maintained protein sequences that can still provide the assumed ancestral function. However, the phenotypes of 35S::*prseag-1* transgenic plants exhibit no floral homeotic modification except flowering earlier than wild-type plants. The results indicated that *prseag-1* could not mimic the phenotypic ectopic expression of *PrseAG* in *Arabidopsis*, and the C-function of *prseag-1* was completely lost. This functional loss may have affected the development of stamens and carpels, as well as transformed the stamens into petaloid organs and carpels into leaflike structures, which contributed to generate double flowers in *P. lannesiana* 'Albo-rosea'.

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