EVOLUTION & DEVELOPMENT 9:1, 92–104 (2007)

Two *AGAMOUS*-like MADS-box genes from *Taihangia rupestris* (Rosaceae) reveal independent trajectories in the evolution of class C and class D floral homeotic functions

Shanhua Lü[a,b], Xiaoqiu Du[a], Wenliang Lu[a], Kang Chong[a], and Zheng Meng[a,*]

[a]Laboratory of Photosynthesis and Environmental Molecular Physiology, Institute of Botany, Chinese Academy of Sciences, Xiangshan, Beijing 100093, China
[b]Graduate School, Chinese Academy of Sciences, Beijing 100039, China
*Author for correspondence (email: zhmeng@ibcas.ac.cn)

SUMMARY Duplicate genes may be retained by sub- and/or neofunctionalization through changes in gene expression and/or coding sequence, and therefore have the potential to contribute to the genetic robustness and diversification of an organism. In this study, two MADS-box genes were isolated from *Taihangia rupestris*, a core eudicot species belonging to the Rosaceae. Sequence and phylogenetic analyses revealed that they are clade members of the *euAG* and *PLE* lineages, respectively, and hence the two genes are named *TrAG* (*Taihangia rupestris AGAMOUS*) and *TrSHP* (*Taihangia rupestris SHATTERPROOF*). Southern blot analysis shows that *TrSHP* is a single-copy gene in the *T. rupestris* genome. In situ hybridization analyses show that both *TrAG* and *TrSHP* are mainly expressed in the stamens, carpels, and ovules. When the stamen primordia are firstly observed, *TrAG* is initially expressed in the floral meristem domain that will initiate stamens and carpels. In contrast, no *TrSHP* signal is observed at this developmental stage. At late stages of carpel development, *TrAG* expression is detected in the ovules, ovaries, and developing styles and stigmas, whereas *TrSHP* expression is tightly restricted to the ovules. The transgenic *Arabidopsis* plants containing 35S::*TrAG* and 35S::*TrSHP*, respectively, showed similar phenotypes, including homeotic conversions of sepals into carpelloid structures bearing ovules and petals into staminoid organs, and the fruits shattering prematurely along the dehiscence zone. In addition, the phenotype of the transgenic 35S::*TrSHP Arabidopsis* plants revealed that perianth abscission was inhibited. Yeast two-hybrid assays indicated that TrAG can interact with TrSEP3, whereas TrSHP cannot. The data suggest that the *euAG* and *PLE* paralogs, *TrAG* and *TrSHP*, may have subfunctionalized and/or neofunctionalized through changes in expression patterns and accumulating variations in the coding regions. Taking these findings together with those available expression and functional data from *Arabidopsis* and other species, we conclude that the compensatory ways vary among the eu*AG* and *PLE* lineage pairs in eudicot species.

INTRODUCTION

Gene duplication is believed to be an accelerator for the evolution of organisms (Ohno 1970). Duplicate genes may be retained by sub- and/or neofunctionalization through shifts in gene expression and/or coding sequence, and therefore contribute to innovations of organism morphological features (Force et al. 1999; Gu et al. 2003; Moore et al. 2005). The MADS-box gene family in plants encoding key transcription factors, many of which play an important role in the regulation of the flower development, has undergone extensive gene duplication events (Kramer et al. 1998, 2004a, b; Theissen et al. 2000; Becker and Theissen 2003; Irish 2003; Litt and Irish 2003; Irish and Litt 2005; Zahn et al. 2005). Given their critical roles in shaping flower architecture, functional diversification following gene duplication within the MADS-box gene family is believed to be responsible, to a large extent, for the morphological innovations of the land plants (Becker and Theissen 2003; Irish 2003).

The most well-studied MADS-box genes are the floral organ identity genes, which play a key role in specifying the identities of floral organs. Based on genetic analyses of homeotic mutants primarily in two eudicot species, *Arabidopsis thaliana* and *Antirrhinum majus*, the ABC model was proposed to explain the determination of floral organ identity (Carpenter and Coen 1990; Sommer et al. 1990; Bowman et al. 1991a). According to the model, class A genes are responsible for the identity of sepals, class A and class B genes for petal identity, class B and class C genes for stamen identity, and class C genes for carpel identity. Recently, reverse genetic studies revealed that genes of two additional groups, named class D and class E, are also required to specify the identity of the floral organs. Class D genes are required for ovule development (Colombo et al. 1995; Theissen 2001; Ditta et al. 2004), while class E genes are required for development of proper organ identity in all the whorls of flowers (Pelaz et al. 2000; Honma and Goto 2001). These findings led to the two extensions of the ABC model known as "ABCDE" model and "floral quartet" model, with the latter one predicting the molecular mode of interaction of the different floral homeotic genes at molecular level for the specification of floral organ identities (Theissen 2001; Theissen and Saedler 2001). In the model plant *Arabidopsis*, class A genes are represented by *APETALA1* (*AP1*) and *APETALA2* (*AP2*; Mandel et al. 1992; Jofuku et al. 1994), class B genes by *APETALA3* (*AP3*) and *PISTILLATA* (*PI*; Goto and Meyerowitz 1994; Yang et al. 2003), class C genes by *AGAMOUS* (Yanofsky et al. 1990), class D genes by *SEEDSTICK* (*STK*; formerly *AGL11*) as well as *SHP1* and *SHP2* (Savidge et al. 1995; Pinyopich et al. 2003; Kaufmann et al. 2005), and class E genes by *SEPALLATA1*, *2*, *3*, and *4* (*SEP1*, *2*, *3*, and *4*; previously known as *AGL2*, *4*, *9*, and *3*, respectively; Pelaz et al. 2000, 2001; Honma and Goto 2001; Ditta et al. 2004). Except for *AP2*, all class ABCDE floral homeotic genes represent MIKC^{C}-type MADS-box genes, referring to their conserved structure comprising an M (MADS), I (intervening), K (keratin-like), and C (C-terminal) domain (Sommer et al. 1990; Bowman et al. 1991a, 1993; Becker and Theissen 2003).

The expression patterns of *AG*-like genes from the diverse gymnosperm species are highly restricted to reproductive organs (Tandre et al. 1995, 1998; Rutledge et al. 1998; Winter et al. 1999), strongly suggesting that the ancestral function of the predecessors of the *AG* subfamily was to specify reproductive organs and to distinguish them from nonreproductive organs (Winter et al. 1999). Phylogenetic analyses revealed that two major gene duplication events occurred within the subfamily during the evolutionary history of angiosperms (Becker and Theissen 2003; Kramer et al. 2004a, b; Zahn et al. 2006). The first duplication event occurred before the radiation of extent angiosperms and produced two lineages: (i) the C lineage (the *AG* clade), the members of which are functionally characterized to be involved in specifying stamen and carpel identities and in providing floral meristem determinacy (C function *sensu lato*; Coen and Meyerowitz 1991; Schwarz-Sommer et al. 1992), and (ii) the D lineage (the *AGL11* clade), the members of which are involved in ovule development (D function; Angenent et al. 1995; Colombo et al. 1995, 1997), represented by the class D genes of extant eudicots. Subsequent duplications within the eudicot *AG* lineage occurred close to the base of the core eudicots and led to the paralogous *euAG* and *PLENA* (*PLE*) clades, including the *Arabidopsis* genes *AG* and *SHATTERPROOF1/2* (*SHP1/2*; Becker and Theissen 2003; Kramer et al. 2004a, b; Causier et al. 2005; Zahn et al. 2006).

Functional studies showed that considerable variations in the functions of the *euAG* and *PLE* lineage members exist in different species. *AG* in *Arabidopsis* and its functional equivalent (as discussed in Theissen 2005) in *Antirrhinum*, *PLE*, are paralogs rather than orthologs (Kramer et al. 2004a, b; Causier et al. 2005; Theissen 2005; Zahn et al. 2006), but they have equivalent typical C functions (Bowman et al. 1991a; Drews et al. 1991; Mandel et al. 1992; Mizukami and Ma 1992; Davies et al. 1999). In fact, the ortholog of *AG* in *Antirrhinum*, *FAR*, has a derived function in promoting stamen development (Davies et al. 1999; Kramer et al. 2004a, b; Causier et al. 2005), while the co-orthologs of *PLE* in *Arabidopsis*, *SHP1/2*, have taken new roles in promoting fruit dehiscence (Liljegren et al. 2000), in addition to being partly redundant in specifying the carpel and ovule identity with *AG* (Pinyopich et al. 2003). A number of *AG*-like genes from other core eudicots have been identified (reviewed in Zahn et al. 2006). Among them, however, only two members of the *euAG* lineage, *pMADS3* from *Petunia* and *DUPLICATED* (*DP*) from *Ipomoea nil*, are functionally well characterized (Kapoor et al. 2002; Nitasaka 2003). Till date, little is known about expression patterns and functions of duplicate genes of the *euAG* and *PLE* lineages from the Rosaceae species, which are grouped together with the Brassicaceae (including *Arabidopsis*) into the Rosids (Soltis et al. 1999; APGII. 2003), although the paralogous lineage genes, *MdMADS14* and *MdMADS15* from *Malus* (van der Linden et al. 2002), and *MASAKO C1* and *D1* from *Rosa* (Kitahara and Matsumoto 2000; Kitahara et al. 2004), were reported.

Taihangia rupestris Yü et Li, a rare and endangered species, which only distributes at the south margin of the Taihang Mountain, China, belongs to the Dryadeae, Rosoideae, and Rosaceae. Compared with other genera of this tribe, such as *Dryas*, *Geum*, *coluria*, and *waldsteinia*, *T. rupestris* has advanced features as well as primitive ones. It has bisexual and/or unisexual flowers and always herbaceous habit. The temperature during the initiation of floral primordia is key factor for developing uni- or bisexual flower. We observed that the stamen or pistil aborts irreversibly when the temperature is higher than 18°C or lower than 3°C, respectively (unpublished data). The most suitable temperature scope for bisexual flowers is 6–16°C (Lu 1996). The plant has determinate inflorescences, which unusually consist of two or three florets born in the axils of each leaf. Five sepals and five petals, as well as numerous stamens and carpels, are visible from the outer whorl to the inner whorl when the floret develops to maturation in the bisexual flower (Lu et al. 1983; Lu 1996). Sepals and petals arrange alternately (M. Tang et al. unpublished). Finally, aggregated achenes form and partly dehisce along ventral suture (Yü and Li 1980, 1983; Lu et al. 1983; Lu 1996). The bisexual flower and fruits of *T. rupestris* are shown in Fig. 1.

To parse the various degrees of functions of the duplicate genes in different species, it will be a prerequisite to assess gene structures, expression patterns, and functions by

Fig. 1. Bisexual flower and fruits of *Taihangia rupestris* are shown in A and B, respectively.

comparing studies of large numbers of species (Irish 2003). Here, two MADS-box genes were isolated and analyzed from *T. rupestris*, a core eudicot species belonging to the Rosaceae. Sequence and phylogenetic analyses revealed that they fall into the clades of *euAG* and *PLE* lineages, respectively, and hence the two genes are named *TrAG* and *TrSHP*. Their expression patterns, functions, and biochemical interaction patterns were analyzed using RNA in situ hybridization, constitutive expression in *Arabidopsis* and yeast two-hybrid assays. Our data suggest that the *euAG* and *PLE* paralogs in *T. rupestris* have sub- and neofunctionalized at various degrees compared with the counterparts in the other species. The functional dynamic shifts and diversification between the two genes and their closest counterparts in other plant species are discussed. This comparative study among orthologs and paralogs suggests that functional redundancy, sub- and/or neofunctionalization following gene duplication, occur by various compensatory ways in expression patterns, coding regions, and biochemical pathways in different species (Kramer et al. 2004a, b; Zahn et al. 2006; this work).

MATERIALS AND METHODS

Plant material

T. rupestris was collected from the Taihang Mountain, Northern China, and was cultivated in the Botanical Garden, Institute of Botany, Chinese Academy of Sciences, Beijing, China. The bisexual flowers were obtained under the condition of 10–12°C.

Isolation of *TrAG* and *TrSHP*

Total RNA was extracted using Plant RNA Purity Reagent (Invitrogen, Carlsbad, CA, USA) from the inflorescences of *T. rupestris* across a range of developmental stages. Poly (A) mRNA was purified from total RNA using Oligotex mRNA Mini Kit (Qiagen, Hilden, Germany), and single-stranded cDNA was synthesized by priming with the oligonucleotide PTA using SuperscriptIII Reverse Transcriptase (Invitrogen). Next, 3′ rapid amplification of cDNA ends (3′ RACE) was used for double-stranded cDNA synthesis by combining primer AP with degenerate primer B2 (for *TrAG*) or L2 (for *TrSHP*). PCR products of about 1.0 kb were cloned into the pGEM-T vector (Promega, Madison, WI, USA). To obtain the 5′ partial cDNA ends of *TrAG* and *TrSHP*, 5′ rapid amplification of cDNA ends (5′ RACE) was used. Internal gene-specific primers P3D6, 69-1 and 69 were designed for *TrAG*, and primers 86-1-1 and 86-1-2 were used for *TrSHP*. The full-length cDNA of *TrAG* was obtained by 3′ RACE with primers 3N69 and AP. A *Hin*dIII recognition site lies in overlay of 3′ partial cDNA sequence and 5′ partial cDNA sequence, and so the full-length cDNA of *TrSHP* was recombined into the pGEM-T vector using the bridge vector pBluescript SK+(Stratagene, La Jolla, CA, USA) with *Hin*dIII digestion. Three independent cDNA clones were sequenced with the ABI PRISM dye terminator kit (PE Applied Biosystems, Foster City, CA, USA). The sequences of primers mentioned above are shown in Table S1.

Genomic DNA extraction and southern blot

Genomic DNA was extracted using DNeasy Plant Mini Kit (Qiagen) from *T. rupestris* young leaves according to the manufacturer's protocol, and digested with *Hin*dIII, *Eco*RI, or *Bam*HI. Eight micrograms of digested genomic DNA was loaded in each lane and separated on a 0.8% agarose gel, and subsequently transferred to a HyBond-N+nylon membrane (Amersham Biosciences, Little Chalfont, UK). A 3′ end fragment comprising the entire C-region of *TrSHP* (506–945) was labeled with [a-^{32}P] dCTP using Prime-a-Gene® label as a probe. Prehybridization and hybridization were performed at 65°C in the solution containing 6 × SSC, 5 × Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), and 100 µg/ml denatured salmon sperm DNA, and then washed to a final stringency of 0.1 × SSC, 0.1% SDS at 65°C for 15 min.

Phylogenetic analysis

Previously published amino acid sequences of MADS-box genes were all obtained from the GenBank database, and aligned with

CLUSTALX1.81 under default settings and refined by hand. The neighbor-joining (NJ) trees were constructed by alignment of full-length amino acid sequences comprising the MADS, I, K, and C domains using the MEGA3 (Molecular Evolutionary Genetic Analyses, version1.1, Pennsylvania State University) package. Bootstrap values were based on 1000 replicates. Gaps were encoded as missing data.

Vectors construction and *Arabidopsis* transformation

Full-length *TrAG* and *TrSHP* cDNA were separately cloned into binary vector pBI121 (BD Biosciences Clontech) under the control of cauliflower mosaic virus 35S promoter in the sense orientation. 35S::*TrAG* and 35S::*TrSHP* constructs were transformed separately into *A. thaliana* plants (ecotype Columbia) using the floral-dip method according to Clough and Bent (Clough and Bent 1998) with *A. tumefaciens* strain C58C1/pMP90 (Koncz et al. 1984). The seeds of transgenic *Arabidopsis* plants were selected on solid 0.5 × MS medium (Murashige and Skoog, 1962) containing 50 mg/l 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.

Scanning electron microscopy

Transgenic materials were fixed and treated, then dried in liquid CO_2, coated with gold and observed as published (Xu et al. 2005).

RNA in situ hybridization

Tissue fixation and hybridization were performed as described (Ferrándiz et al. 2000; Li et al. 2005) with the following modifications. Digoxigenin-labeled RNA probes were synthesized from *TrAG* (542–971) and *TrSHP* (506–945) gene-specific 3′ ends subcloned into pGEM-T vector (Promega). The anti-sense and sense probes were generated using the DIG Northern Starter Kit (Roche, Mannheim, Germany) according to the instructions. Final wash was performed in 0.1 × SSC at 58°C for 45 min.

Northern hybridization

Total RNA was extracted using TRIZOL reagent (Invitrogen) from *Arabidopsis* leaves following the manufacturer's instructions. Twenty-five micrograms of total RNA were loaded and separated on a 1.0% formaldehyde agarose gel in each lane, and subsequently transferred to a HyBond-N+nylon membrane (Amersham Biosciences). Gene-specific 3′ end fragment of *TrAG* or *TrSHP* were labeled with [a-^{32}P] dCTP using Prime-a-Gene® label. Prehybridization and hybridization were performed at 65°C in the solution containing 6 × SSC, 5 × Denhardt's solution, 0.5% SDS and 100 μg/ml denatured salmon sperm DNA, and then washed to a final stringency of 0.1 × SSC, 0.1% SDS at 65°C for 30 min.

Yeast two-hybrid assays

Yeast two-hybrid assays were performed using the GAL4-based MATCHMAKER Two-Hybrid System (Clontech, Palo Alto, CA, USA). *Saccharomyces cerevisiae* strain AH109, GAL4 activation domain (AD) cloning vector pACT2, and GAL4 DNA-binding domain (DNA-BD) cloning vector pAS2 were used. The method of constructing the fusion protein plasmids is as follows. Full-length cDNA of *TrFUL*, *TrAP1*, *TrPI*, *TrAG*, *TrSHP*, and *TrSEP3* were amplified by PCR with *Nco*I restriction enzymes digest site at the 5′ end at the start codon and *Bam*HI at the 3′ end, as well as *Nco*I and *Eco*RI sites were introduced for *TrTM6*, and then digested using restriction enzymes for cloning into pACT2 and pAS2, respectively. All constructs were verified by sequencing. Yeast transformation was performed using the lithium acetate method. The transformants cotransformed with BD and AD fusion plasmids were confirmed by growth on dropout medium lacking leucine (Leu) and Tryptophan (Trp) and by PCR and restriction enzymes analyses. Interactions were analyzed on the selective dropout medium lacking Leu, Trp, Adenine (Ade) and histidine (His) supplemented with 20 mM 3′-aminotriazole (3-AT) and by β-Galactosidase activity (LacZ) assay. Growth of yeast on selective media was scored after 3 days of incubation at 30°C. The LacZ activity was measured by the colony-lift filter assay and the colonies that turned blue in < 7 h were selected. All these assays were performed according to the manufacturer's protocols. The transformants cotransformed fusion plasmids of AP3 and PI in absence of MADS domain from *Arabidopsis* were used as a positive control and the transformant transformed fusion plasmid pAS2 was used as a negative control.

RESULTS

Sequence analyses of the *TrAG* and *TrSHP* genes from *T. rupestris*

cDNAs of two MADS-box genes were cloned. Sequence and phylogenetic analyses revealed that they are grouped into the *euAG* and *PLE* lineages, respectively (Fig. 2A). Therefore, the two genes are named *TrAG* (*<u>T</u>aihangia <u>r</u>upestris <u>AG</u>AMOUS*) and *TrSHP* (*<u>T</u>aihangia <u>r</u>upestris <u>SH</u>ATTER<u>P</u>ROOF*). Conceptual translation reveals that TrAG has 232 amino acids (aa) and TrSHP 229 aa. TrAG has a 57-aa MADS domain, 33-aa I domain, 67-aa K domain, and 75-aa C domain (Fig. S1A). TrSHP has a 57-aa MADS domain, 32-aa I domain, 67-aa K domain, and 73-aa C domain (Fig. S1B). Multiple alignments of the aa sequences of TrAG and TrSHP with those of *AG*-related proteins from *Arabidopsis*, *Antirrhinum*, *Petunia*, and from Rosaceae species indicate that they share high similarities to the predicted protein sequence throughout the M, I, and K domain (Fig. S1, A and B; Table S2). Three regions of the heptad $(abcdefg)_n$ repeats previously identified in the K-domain, namely K1 (87–108), K2 (121–135), and K3 (143–168) subdomains, were also found in the two proteins (Fig. S1, A and B) (Yang et al. 2003). Like the case found in all MIKC-type MADS proteins except for a few FLC-like proteins (GenBank accessory no. AF312665; AF312666; AF312667; Alvarez-Buylla et al. 2000; Ratcliffe et al. 2001; Scortecci et al. 2001; Yang et al. 2003), a conserved glycine also occurs at position 110 in the K domain of TrSHP (Fig. S1B). But it is notable that alanine, not glycine, is present at position 110 in the K domain of TrAG (Fig. S1A). The same was also found in several other MADS-box proteins within

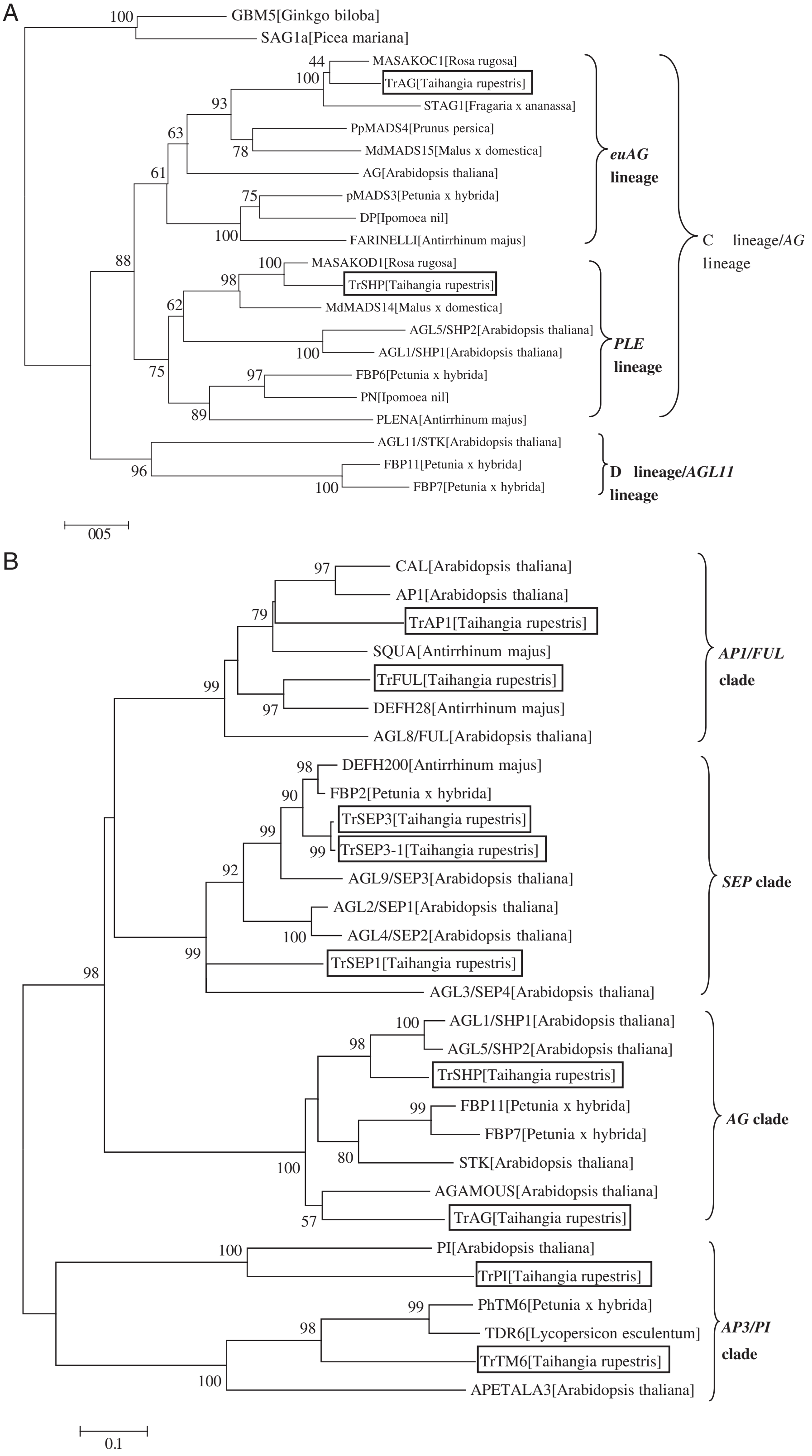

Fig. 2. In (A) phylogenic analyses of represented *AG*-related MADS-box genes. TrAG and TrSHP from *Taihangia rupestris* are boxed. The values on the nodes are percentages of bootstrap confidence levels from 1000 replicates. Gymnosperm *AG*-like genes, GBM5, and SAG1a, were used to root the trees and lineages were defined as published Kramer et al. (2004a, b) and Zahn et al. (2006). The accession numbers of the sequence data used are as follows: MASAKOC1 (BAA90744), MASAKOD1 (BAA90743), PpMADS4 (AAU29513), pMADS3 (CAA51417), AG (NP_567569), FBP6 (CAA48635), AGL5/SHP2 (NP_850377), STK (NP_849351), FBP11 (CAA57445), FBP7 (CAA57311), AGL1/SHP1 (NP_191437), FARINELLI (CAB42988), PLENA (AAB25101), DP(AB006182), PN(AB006183), MdMADS14(CAC80857), MdMADS15 (CAC80858), STAG1 (AF168468), GBM5 (AAM76208), SAG1-a (AAC97157). Names of lineage are labeled by brackets at the right margin. The species designations listed below are indicated in brackets in the figure. In (B) phylogenic analyses of MADS-box genes from *T. rupestris* and other represented MADS-box genes from *Arabidopsis*, *Antirrhinum* and *petunia*. TrAP1, TrFUL, TrTM6, TrPI, TrAG, TrSHP, TrSEP1, TrSEP3, and TrSEP3-1 from *T. rupestris* are boxed. Unroot tree was generated. The accession numbers of the sequence data used are as follows: AP1 (CAA78909), CAL (NP_564243), SQUA (CAA45228), DEFH28 (AAK72467), AGL8/FUL (NP_568929), PhTM6 (AAS46017), APETALA3 (BAA04665), TDR6 (AAM33100), PI (AAD51988), DEFH200 (CAA64743), SEP3 (NP_850953), FBP2 (M91666), AGL3 (U81369), AGL2 (AAU82024), AGL4 (M55552), AG (NP_567569), AGL5/SHP2 (NP_850377), AGL1/SHP1 (NP_191437), STK (NP_849351), FBP11 (CAA57445), FBP7 (CAA57311). The species designations listed below are shown in brackets in the figure. The values on the nodes are percentages of bootstrap confidence levels from 1000 replicates and less than 50% are cutoff.

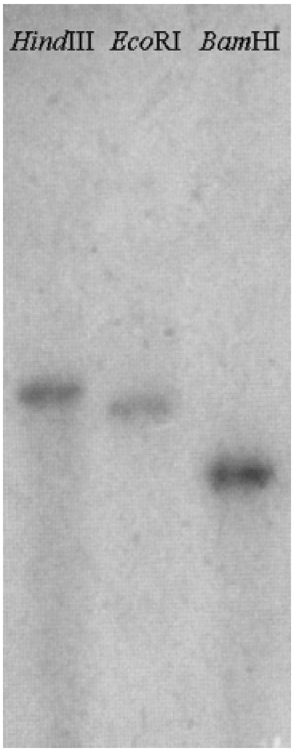

Fig. 3. Southern blot analysis of *TrSHP*. The genomic DNA extracted from *Taihangia rupestris* was digested with *Hin*dIII, *Eco*RI, or *Bam*HI. Each lane contains 8 μg DNA.

the *euAG* lineage from the Rosaceae (Fig. S1A). Obviously, the C-terminal domains of the predicted proteins of TrAG and TrSHP are less well conserved (Table S2). Nevertheless, they also have diagnostic aa motifs (Fig. S1, A and B), denoted as AG motifs I and II (Kramer et al. 2004a, b).

To ascertain whether *TrSHP* is duplicated in the *T. rupestris* genome or not, Southern blot analysis was performed using the specific probe of *TrSHP*. Only a hybridization band is observed, indicating that *TrSHP* is a single-copy gene in the *T. rupestris* genome (Fig. 3).

Expression patterns of *TrAG* and *TrSHP* in *T. rupestris*

To compare the temporal and spatial expression patterns, the transcript accumulations of *TrAG* and *TrSHP* were analyzed by in situ hybridization at a series of floral developmental stages. Both *TrAG* and *TrSHP* signals are not detected in the flower primordium at very early stages (Fig. 4, B and F). *TrAG* is initially expressed in the stamen primordia and floral meristem region that will give rise to the stamen and carpel primordia (Fig. 4C). In contrast, no *TrSHP* signal occurs until the stamen primordia are differentiated (Fig. 4, F and G). Subsequently, both genes are expressed in the carpel primordia and the region will produce the carpel primordia, and in developing stamens and carpels (Fig. 4, D, H, and I). At late stages of carpel development, the *TrAG* expression is broadly detected in the ovules, ovaries, developing styles, and stigmas (Fig. 4E), while the accumulation of the *TrSHP* mRNA is tightly confined to the ovules (Fig. 4J). The results suggest that the functions of *TrAG* and *TrSHP* may be overlapping, but divergent.

Ectopic expression of *TrAG* and *TrSHP* in *Arabidopsis*

The coding regions of *TrAG* and *TrSHP* accumulate changes in the sequence level, suggesting that their activities are likely to be altered. To gain further insight into the function of *TrAG* and *TrSHP*, we transformed *Arabidopsis* plants with the genes under the control of the cauliflower mosaic virus 35S promoter. Among 62 35S::*TrAG* independent transgenic plants, 53 displayed severe phenotypes and nine weak phenotypes. Among thirty-six 35S::*TrSHP* independent transgenic plants, 29 lines showed severe phenotypes and seven showed weak phenotypes. Ectopic expressions of *TrAG* and *TrSHP* in *Arabidopsis* were confirmed by Northern blot analysis from five independently transformed plants with each of the constructs. Both *TrAG* and *TrSHP* are not expressed in the leaves of wild-type plants but are expressed in the leaves of the transgenic plants (Fig. 5, A and B). Transgenic lines 69-7 and 69-11 show higher RNA expression of *TrAG* with stronger phenotypes compared with lines 69-4, 69-1, and 69-3 with moderate phenotypes (Fig. 5A). The accumulation levels of *TrSHP* transcripts in different lines are also consistent with phenotypic alterations (Fig. 5B). The *35S::TrAG* transgenic lines 69-7 and 69-11, and the *35S::TrSHP* lines 86-12 and 86-8 with stronger phenotypes were selected for further analyses. Both the 35S::*TrAG* and 35S::*TrSHP* transgenic *Arabidopsis* plants are extremely early flowering (even before the fourth rosette leaf appeared; Figs. 6B and 7A). The height of all the transgenic plants is reduced (data no shown). The rosette leaves and cauline leaves curl upwardly (Figs. 6B and 7A) and become smaller (Fig. 7C). The flower buds are prematurely open before stage 13 (Figs. 6C and 7E). The sepals are converted homeotically into carpel-like structures with stigmatic papillae and ovules (Figs. 6, E and F and 7, G, L, and O), and the bases of the petals transformed homeotically into filament-like structures (Figs. 6D and 7F). Stigmatic papillae and ovules form on the cauline leaves (Figs. 6, J and K and 7L). The ovaries are bent (Figs. 6, L–N and 7, F and J–K). Both the 35S::*TrAG* and 35S::*TrSHP* fruits shatter prematurely along the dehiscence zone before seed maturity, and ovules are exposed (Figs. 6, G–K and 7H). Terminal flowers form in both the 35S::*TrAG* and 35S::*TrSHP* transgenic plants (data not shown). Moreover, the flowers of the 35S::*TrSHP* transgenic plants terminated occasionally producing only four flowers (data not shown). These observations suggest that the

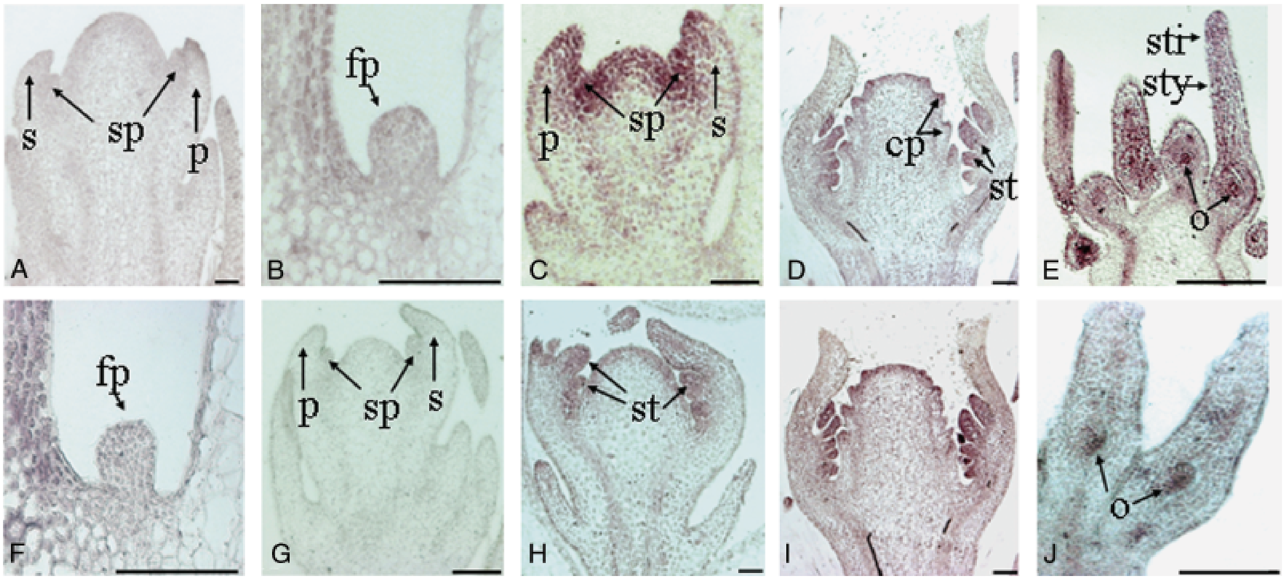

Fig. 4. Expression patterns analyses of *TrAG* and *TrSHP* in *Taihangia rupestris*. (A) Hybridized with sense *TrAG* RNA probe. (B–E) Hybridized with anti-sense *TrAG* RNA probe. (F–J) Probed with the anti-sense *TrSHP* RNA probe. fp, floral primordium; s, sepal; p, petal; cp, carpel primordia; sp, stamen primordia; st, developing stamen; o, ovule; sty, developing style; sti, developing stigma. Scale bar = 50 μm.

functional potential of the encoded proteins remained largely conserved despite the sequence changes that accompanied the evolution in paralogous subclades of the *AG*-like genes.

However, there are some differences observed between 35S::*TrAG* and 35S::*TrSHP* plants. Flower buds of 35S::*TrAG* are dark green in color (Fig. 6C), while those of 35S::*TrSHP* are yellow green (Fig. 7E). It is notable that perianth abscission is inhibited and sterility increased with shorter siliques in transgenic 35S::*TrSHP Arabidopsis* plants (Fig. 7, J–K). The results indicate that *TrSHP* besides its general conservation has also neofunctionalized via changes in the coding regions.

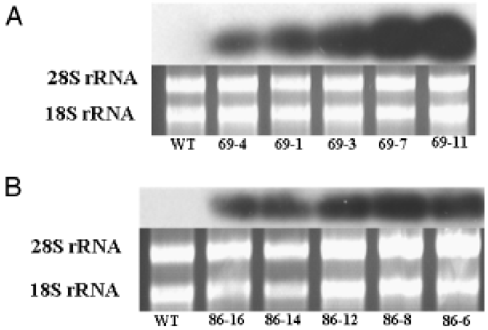

Fig. 5. Northern blot analyses of constitutive expression of *TrAG* (A) and *TrSHP* (B) in *Arabidopsis* leaves. WT, wild-type *Arabidopsis* leaves. 69-4, 69-1, 69-3, 69-7, 69-11, 35S: *TrAG* transgenic *Arabidopsis* lines; 86-16, 86-14, 86-12, 86-8, 86-6, 35S: *TrSHP* transgenic *Arabidopsis* lines.

Interaction patterns of TrAG and TrSHP in *T. rupestris*

The comparison of interaction patterns can provide additional evidence to functional similarities or differences among related proteins (Immink et al. 2003). Therefore, we analyzed the interaction partners of TrAG and TrSHP, respectively, among other MADS-box genes isolated from *T. rupestris*. A GAL4-based yeast two-hybrid experiment was used to assay protein–protein interactions in vivo (in yeast cells). *TrFUL*, *TrAP1*, *TrTM6, TrPI*, and *TrSEP3* belonging to the members of *SQUA*/*AP1*, *SQUA*/*AP1*, *DEF*/*AP3*, *GLO*/*PI*, and *AGL2* subfamily, respectively, were isolated from *T. rupestris* (Fig. 2B; our unpublished data). The Ade, His, and LacZ reporter genes in the yeast cannot be automatically activated by any proteins above (data not shown), indicating that these proteins can be used for further yeast two-hybrid assays. In this assay, different combinations of TrAG or TrSHP with proteins mentioned above were tested. The consensus results were obtained by swapping the DNA-BD and AD fusion vectors. Yeast cells only cotransformed the fusion proteins of TrAG and TrSEP3 can grow on the selective media lacking Leu, Trp, Ade, and His and activate the expression of the LacZ reporter gene (Fig. 8). The results show that TrAG interacts only with TrSEP3 (Fig. 8), but not with TrFUL, TrAP1, TrTM6, TrPI, or TrSHP, whereas TrSHP did not interact with any of the proteins mentioned above. Additionally, neither TrAG nor TrSHP formed homodimers under the conditions tested (data not

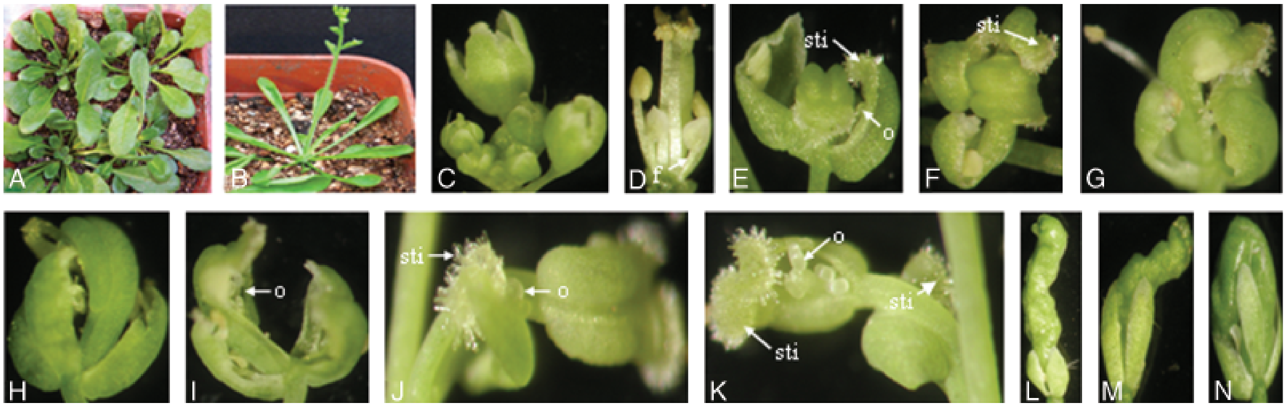

Fig. 6. Constitutive expression of 35S::*TrAG* in *Arabidopsis* plants. (A) Wild-type *Arabiposis* plants and (B–N) 35S::*TrAG* plants. (A) Wild-type (Col ecotype) plants grown for 22 days under long-day condition. (B) Curled rosette leaves and cauline leaves, and early flowering in comparison with (A). (C) Prematurely open flower buds and dark green in color. (D) Sepals are removed and the base of the petal is transformed into filament-like (f) structures. (E and F) Sepals are transformed into carpel-like structures including the stigmatic papillae (sti) and ovules (o). (G–K) Valves are ripped before stage 19 along the dehiscence zone and ovules are exposed. (J) Carpelloid–cauline leave with stigmatic papillae (sti) and ovules (o). (L–N) Ovary bent.

shown). In this assay, except for cotransformants of TrAG and TrSEP3, yeast cells of the negative control and other combinations cannot grow on the dropout medium lacking Leu, Trp, Ade, and His in spite of prolonged incubation of 2 days at 30°C.

DISCUSSION

So far expression patterns of the *euAG* and *PLE* paralogs from Rosaceae species are not known in detail (Kitahara and Matsumoto 2000; van der Linden et al. 2002). Here, in situ hybridization analyses reveal that the onset of *TrAG* expression is earlier than that of *TrSHP* during flower development. *TrAG* is initially expressed in the floral meristem region where the stamen and carpel primordia will initiate, whereas no *TrSHP* signal could be seen until the stamens were differentiated. After stamen primordia had emerged, the *TrSHP* signal was firstly detected in the floral center region where the carpel primordia will initiate. Then, both genes were expressed in the developing stamens and carpels. At late stages of flower development, *TrAG* was broadly expressed in the carpel, whereas the expression of *TrSHP* was highly restricted to ovules. These results suggest that *TrAG* may be required for determination of the floral meristem and for the specification of stamen and carpel identity, and hence may function as a class C floral organ identity gene; *TrSHP* may share redundant roles in promoting stamen development and specifying carpel identity with *TrAG*. Considering their critical roles in producing reproductive organs, the overlapping expressions of both genes in developing stamens and carpels might be an advantage conferred by genetic buffering in *T. rupestris*.

Previous studies have showed that the expression patterns of the *euAG* and *PLE* lineage genes are overlapping, but also variable in different species. In *Arabidopsis*, the *AG* transcripts are initially detected in the meristem domain that initiate the stamen and carpel primordia when the sepal primordia emerge at stage 3, as defined in the literature (Smyth et al. 1990), and continue to exist in stamens and carpels, and later in the developing fruit (Bowman et al. 1991b; Drews et al. 1991; Savidge et al. 1995), while the *SHP1/2* transcripts are firstly detected throughout the carpel primordia at stage 7, after the base of the stamens becomes stalked and petal primordia become hemispherical, and then get restricted to the ovules and develop pistil and fruit (Savidge et al. 1995; Catherine et al. 1996; Flanagan et al. 1996). The expression patterns of the paralogous genes are consistent with their functions: *AG* plays a key role in floral meristem determinacy and reproductive organ identity, whereas *SHP1* and *SHP2* are redundantly involved in regulating the shattering of the fruit when the seeds mature, in addition to their functions in promoting carpel and ovule development (Liljegren et al. 2000; Pinyopich et al. 2003). In *Antirrhinum*, the expression patterns of *FAR* and *PLE* are very similar with only slight differences, in expression levels in different floral organs during flower development (Davies et al. 1999). Loss-of-function analyses suggest that *PLE* in *Antirrhinum*, an *AG* paralog, functions as primary C-function gene, while the *AG* ortholog, *FAR*, functions in promoting stamen development. This demonstrates that functional evolution of paralogous *AG*-like genes has taken different routes in different species (Davies et al. 1999; Kramer et al. 2004a, b; Causier et al. 2005; Irish and Litt 2005; Theissen 2005). Similar expression patterns of the *euAG* and *PLE* paralogous genes, *pMADS3* and *FBP6*, are

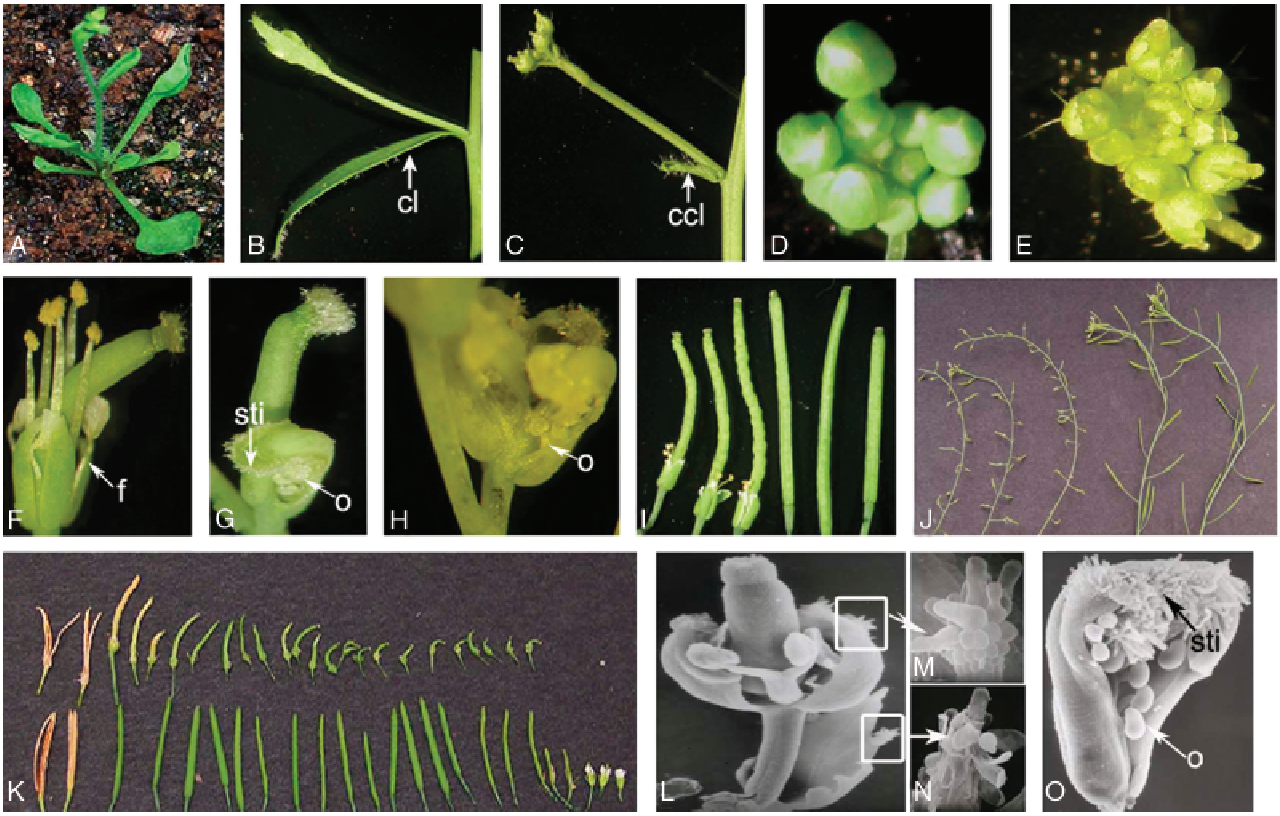

Fig. 7. Constitutive expression of *TrSHP* in *Arabidopsis* plants. (B and D) Wild-type *Arabiposis* plants, and (A, C, E–O) 35S::*TrSHP* plants. (L–O) was taken by scanning electron microscopy. (A) Curled rosette leaves and cauline leaves, and early flowering. (B) Wild-type cauline leaves (cl). (C) Cauline leaves becoming smaller and curled (ccl) in comparison with (B). (D) Inflorescence of the wild type, and sepals enclose the flower buds before stage 13. (E) Prematurely open flower buds and yellow green in color in comparison with (D). (F) Base of the petal is converted into filament-like (f) and ovary bends. (G) Carpelloid-sepal bearing stigmatic papillae (sti) and ovules (o). (H) Valves are ripped along dehiscence zone and ovules are observed. (I) Three 35S::*TrSHP* fruits are shown on the left and three wild-type fruits are on the right; perianth abscission inhibited. (J) Three 35S::*TrSHP* inflorescences are shown on the left and two wild-type inflorescences are on the right; sterility increased and ovary bent. (K) 35S::*TrSHP* fruits are shown on the top row and wild-type fruits are on the bottom; perianth abscission inhibited, sterility increased, and ovary bent. (L) Stigmatic papillae form on the top of the sepals and margin of the cauline leaf; two filaments are fused together at the base. (M and N) Close-up of (L) marked in the box (upper and bottom, respectively); stigmatic papillae form O, carpelloid-sepal with stigmatic papillae and ovules.

observed in *Petunia* (Tsuchimoto et al. 1993; Kater et al. 1998; Kapoor et al. 2002). In line with their expression patterns *pMADS3* and *FBP6* have redundant but distinct functions in both conferring floral determinacy and specifying floral organ identity (Kapoor et al. 2002). Taken together, these data demonstrate that different shifts in expression patterns following gene duplication occurred among the duplicated genes of the eudicots *AG* lineage in different species (Kramer et al. 2004a, b; Duarte et al. 2005; Zahn et al. 2006), suggesting that the paralog pairs have undergone various degrees of sub- and/or neofunctionalization.

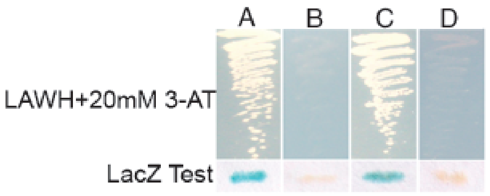

Fig. 8. Interaction analyses of TrAG and TrSHP, respectively, with TrSEP3 MADS-box proteins isolated from *Taihangia rupestris*. LWAH+20 mM 3-AT, selective dropout medium lacking Leu, Trp, His, and Ade supplemented with 20 mM 3-aminotriazole; LacZ test, β-galactosidase reporter gene assay. A, positive control; B, negative control; C, cotransformations of TrAG and TrSEP3; D, cotransformations of TrSHP and TrSEP3.

Obviously, partial functional redundancy has been maintained among the *euAG* and *PLE* paralogous genes (Kramer et al. 2004a, b), reflecting the conserved activity provided by the protein coding regions (Pinyopich et al. 2003; Zahn et al. 2006). In *Arabidopsis*, ectopic expression of *AG*, *SHP1*, or *SHP2* resulted in similar alterations in morphogenetic features: the homeotic transformations of sepals into carpel-like structures and petals into stamen-like organs in transgenic plants (Mandel et al. 1992; Mizukami and Ma 1992; Liljegren et al. 2000; Favaro et al. 2003; Pinyopich et al. 2003). Furthermore, overexpression of the *SHP2* gene is sufficient to largely rescue the development of wild-type third and fourth whorl organs in *ag* mutants (Pinyopich et al. 2003). These findings indicate that both *AG* and *SHP2* maintained similar protein activity in specifying stamen and carpel identity. In this study, phenotypes of both the 35S::*TrAG* and 35S::*TrSHP Arabidopsis* plants resemble those caused by the ectopic expression of *AG*. The results strongly suggest that both *TrAG* and *TrSHP* maintained ancestral activities in the coding regions, even though expression divergence occurred probably due to changes in regulatory regions. Ectopic expressions of many genes within the *euAG* or *PLE* lineage, such as *CaMADS1* from *Hazelnut* (Rigola et al. 2001), *CUM1 from Cucumber* (Kater et al. 1998; Perl-Treves et al. 1998), *TAG1* from *Lycoperscion* (Pnueli et al. 1994; Busi et al. 2003), *Vvmads1* from *Vitis vinifera* (Boss et al. 2001), and *NAG1* from *Nicotiana* (Kempin et al. 1993), resulted in phenotypic alterations mimicking those of ectopic expression of *AG* in *Arabidopsis* (Mizukami and Ma 1992). These results corroborate the view that many members of the *euAG* and *PLE* paralogous lineages, which originated from a relatively recent gene duplication (Becker and Theissen 2003; Pinyopich et al. 2003; Kramer et al. 2004a, b; Theissen 2005), maintained protein sequences that can still provide the assumed ancestral function, even though they have undergone sub- and/or neofunctionalization events through their shifts in expression patterns (Pinyopich et al. 2003; Ferrario et al. 2004; Kramer et al. 2004a, b; Zahn et al. 2006). However, functional studies also revealed that sub- and/or neofunctionalization also occurred among the members of the eudicot *AG* lineage through changes at the protein level (Kater et al. 1998; Causier et al. 2005). Both the *35S::FAR Antirrhinum* and *Arabidopsis* plants displayed homeotic changes of petals into stamenoid organs but sepals showed almost no obvious phenotype. In contrast, 35S::*PLE Antirrhinum* and *Arabidopsis* plants showed a conversion of sepals into carpelloid organs but less apparent transformation of petals into male organs (Causier et al. 2005). This demonstrates that *SHP* in *Arabidopsis* and *FAR* in *Antirrhinum* underwent independent and different subfunctionalization (Causier et al. 2005).

As discussed above, in *Arabidopsi*s plants, constitutive expression of *AG*, *SHP1*, or *SHP2* genes result in ectopic ovule formation, and further genetic analyses indicated that *AG*, *SHP*, and *STK* genes in *Arabidopsi*s play a redundant role in promoting ovule identity (Mandel et al. 1992; Mizukami and Ma 1992; Favaro et al. 2003; Pinyopich et al. 2003), suggesting that members of C-lineage have partially retained D function. In our study, ectopic expression of either *TrAG* or *TrSHP* in *Arabidopsis* was sufficient to induce the formation of ectopic ovules, demonstrating that each of the genes is sufficient to promote ovule development. These results appear that C-lineage members have independently acquired the D function, which is not strictly retained by the D-lineage members in the evolution.

T. rupestris has a single copy of *TrSHP*, like the most core eudicot species except the members of the Brassicaceae family, such as *Arabidopsis* (Becker and Theissen 2003; Kramer et al. 2004a, b; Moore et al. 2005; Zahn et al. 2006). In *Arabidopsis*, *SHP1* and *SHP2* were produced by a very recent gene duplication event within the *PLE* lineage (Moore et al. 2005), which have subfunctionalized (Liljegren et al. 2000).

Our data suggest that *TrAG* and *TrSHP* have retained the conserved ancestral function at the protein levels. However, yeast two-hybrid assays showed that the TrAG protein was capable of interacting with TrSEP3, whereas TrSHP was not, indicating that the paralogs have diverged in interaction behaviors in *T.rupestris*. The interaction patterns are very similar to those of their counterparts in *Antirrhinum*. FAR can interact with a *SEPALLATA3*-like protein, DEFH72, whereas no interaction between PLE and DEFH72 was observed (Davies et al. 1996b). This is different from the interaction behaviors of their counterparts in *Arabidopsis* and *Petunia*. In *Arabidopsis*, however, both AG and SHP can interact with SEP3 (Fan et al. 1997; Favaro et al. 2003; de Folter et al. 2005). In *Petunia*, both pMADS3 and FBP6 can also interact with the SEPALLATA3-like protein, FBP2 (Kapoor et al. 2002; Immink et al. 2003). These results, combined with previous discussions on the expression divergence, suggest that paralogous pairs within the *euAG* and *PLE* lineage have sub- and/or neofunctionalized in various ways in different species by combinatorial alterations in the regulatory region, coding region, or protein–protein interaction pattern. This functional diversification may have contributed to survival of the organismic lineages in evolution.

Altogether, the members of the *AG* subfamily exhibit functional dynamic shifts and diversification in evolution. However, no matter how sub- and neofunctionalization occurred among the paralogous genes in the *AG* subfamily, specifically, in the *euAG* and *PLE* lineage, different species have representative genes that function in reproductive organs (including stamens, carpels, and ovules). They very likely collectively fulfill the ancestral functions in specifying male and female reproductive organs. Maintenance of multiple copies providing this ancestral function might be buffering to contribute to the genetic robustness of an organism in the evolution.

Acknowledgments

We thank Huiyu Tian, Suzhen Zhao, Yongqiang Wang, Guilan Yang, and Hongyan Shan for lab assistance. We are grateful to Prof. Dr. Günter Theissen for critical reading of the manuscript. We also thank anonymous reviewers for helpful comments on the manuscript. This work was supported by National Nature Science Foundation of China (Grants 30170093, 30121003, and 30530090), and partially supported by National Basic Research Program of China (2006CB100202).

GENBANK ACCESSION NUMBERS

TrAG (DQ248948)
TrSHP (DQ248949)
TrFUL (DQ248945)
TrAP1 (DQ248944)
TrTM6 (DQ248946)
TrPI (DQ248947)
TrSEP3 (DQ372071)

REFERENCES

Alvarez-Buylla, E. R., et al. 2000. MADS-box gene evolution beyond flowers: expression in pollen, endosperm, guard cell, and trichomes. *Plant J.* 24: 1–11.

Angenent, G. C., et al. 1995. A novel class of MADS box genes is involved in ovule development in petunia. *Plant Cell* 7: 1569–1582.

APGII. 2003. (The Angiosperm Phylogeny Group) An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG II. *Bot. J. Linn. Soc.* 141: 399–436.

Becker, A., and Theissen, G. 2003. The major clades of MADS-box genes and their role in the development and evolution of flowering plants. *Mol. Phylogenet. Evol.* 29: 464–489.

Boss, P. K., Vivier, M., Matsumoto, S., Dry, I. B., and Thomas, M. R. 2001. A cDNA from grapevine (Vitis vinifera L.), which shows homology to AGAMOUS and SHATTERPROOF, is not only expressed in flowers but also throughout berry development. *Plant Mol. Biol.* 45: 541–553.

Bowman, J. L., Alvarez, J., Weigel, D., Meyerowitz, E. M., and Smyth, D. R. 1993. Control of flower development in *Arabidopsis thaliana* by APETALA1 and interacting genes. *Development* 119: 721–743.

Bowman, J. L., Drews, G. N., and Meyerowitz, E. M. 1991a. Expression of the Arabidopsis floral homeotic gene AGAMOUS is restricted to specific cell types late in flower development. *Plant Cell* 3: 749–758.

Bowman, J. L., Smyth, D. R., and Meyerowitz, E. M. 1991b. Genetic interactions among floral homeotic genes of Arabidopsis. *Development* 112: 1–20.

Busi, M. V., et al. 2003. MADS-box genes expressed during tomato seed and fruit development. *Plant Mol. Biol.* 52: 801–815.

Carpenter, R., and Coen, E. S. 1990. Floral homeotic mutations produced by transposon-mutagenesis in *Antirrhinum majus*. *Genes Dev.* 4: 1483–1493.

Catherine, A., Flanagan, C. A., Hu, Y., and Ma, H. 1996. Specific expression of the AGL1 MADS-box gene suggests regulatory functions in *Arabidopsis gynoecium* and ovule development. *Plant J.* 10: 343–353.

Causier, B., et al. 2005. Evolution in action: following function in duplicated floral homeotic genes. *Curr. Biol.* 15: 1508–1512.

Clough, S. J., and Bent, A. F. 1998. Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana. Plant J.* 16: 735–743.

Coen, E. S., and Meyerowitz, E. M. 1991. The war of the whorls: genetic interactions controlling flower development. *Nature* 353: 31–37.

Colombo, L., Franken, J., VanderKrol, A. R., Wittich, P. E., Dons, H. J. M., and Angenent, G. C. 1997. Downregulation of ovule-specific MADS box genes from petunia results in maternally controlled defects in seed development. *Plant Cell* 9: 703–715.

Colombo, L., et al. 1995. The petunia MADS box gene FBP11 determines ovule identity. *Plant Cell* 7: 1859–1868.

Davies, B., Egea-Cortines, M., de Andrade Silva, E., Saedler, H., and Sommer, H. 1996b. Multiple interaction amongst floral homeotic proteins. *EMBO J.* 15: 4330–4343.

Davies, B., Motte, P., Keck, E., Saedler, H., Sommer, H., and Schwarz-Sommer, Z. 1999. PLENA and FARINELLI: redundancy and regulatory interactions between two Antirrhinum MADS-box factors controlling flower development. *EMBO J.* 18: 4023–4034.

de Folter, S., et al. 2005. Comprehensive interaction map of the Arabidopsis MADS box transcription factors. *Plant Cell* 17: 1424–1433.

Ditta, G., Pinyopich, A., Robles, P., Pelaz, S., and Yanofsky, M. F. 2004. The SEP4 gene of *Arabidopsis thaliana* functions in floral organ and meristem identity. *Curr. Biol.* 14: 1935–1940.

Drews, G. N., Bowman, J. L., and Meyerowitz, E. M. 1991. Negative regulation of the Arabidopsis homeotic gene AGAMOUS by the APETALA2 product. *Cell* 65: 991–1002.

Duarte, J. M., et al. 2005. Expression pattern shifts following duplication indicative of subfunctionalization and neofunctionalization in regulatory genes of Arabidopsis. *Mol. Biol. Evol.* 1–29.

Fan, H. Y., Hu, Y., Tudor, M., and Ma, H. 1997. Specific interactions between the K domains of AG and AGLs, members of the MADS domain family of DNA binding proteins. *Plant J.* 12: 999–1010.

Favaro, R., et al. 2003. MADS-box protein complexes control carpel and ovule development in Arabidopsis. *Plant Cell* 15: 2603–2611.

Ferrándiz, C., Gu, Q., Martienssen, R., and Yanofsky, M. F. 2000. Redundant regulation of meristem identity and plant architecture by FRUITFULL, APETALA1 and CAULIFLOWER. *Development* 127: 725–734.

Ferrario, S., Immink, R. G., and Angenent, G. C. 2004. Conservation and diversity in flower land. *Curr. Opin. Plant Biol.* 7: 84–91.

Flanagan, C. A., Hu, Y., and Ma, H. 1996. Specific expression of the AGL1 MADS-box gene suggests regulatory functions in *Arabidopsis gynoecium* and ovule development. *Plant J.* 10: 343–353.

Force, A., Lynch, M., Pickett, F. B., Amores, A., Yan, Y. L., and Postlethwait, J. 1999. Preservation of duplicate genes by complementary, degenerative mutations. *Genetics* 151: 1531–1545.

Goto, K., and Meyerowitz, E. M. 1994. Function and regulation of the Arabidopsis floral homeotic gene PISTILLATA. *Genes Dev.* 8: 1548–1560.

Gu, Z., Steinmetz, L. M., Gu, X., Scharfe, C., Davis, R. W., and Li, W. H. 2003. Role of duplicate genes in genetic robustness against null mutations. *Nature* 421: 63–66.

Honma, T., and Goto, K. 2001. Complexes of MADS-box proteins are sufficient to convert leaves into floral organs. *Nature* 409: 525–529.

Immink, R. G. H., Ferrario, S., Busscher-Lange, J., Kooiker, M., Busscher, M., and Angenent, G. C. 2003. Analysis of the petunia MADS-box transcription factor family. *Mol. Gen. Genom.* 268: 598–606.

Irish, V. F. 2003. The evolution of floral homeotic gene function. *BioEssays* 25: 637–646.

Irish, V. F., and Litt, A. 2005. Flower development and evolution: gene duplication, diversification and redeployment. *Curr. Opin. Genet. Dev.* 15: 454–460.

Jofuku, K. D., den BoerI, B. G. W., Van MontaguI, M., and Okamuroa, J. K. 1994. Control of Arabidopsis flower and seed development by the homeotic gene APETALA2. *Plant Cell* 6: 1211–1225.

Kapoor, M., et al. 2002. Role of petunia pMADS3 in determination of floral organ and meristem identity, as revealed by its loss of function. *Plant J.* 32: 115–127.

Kater, M. M., et al. 1998. Multiple AGAMOUS homologs from cucumber and petunia differ in their ability to induce reproductive organ fate. *Plant Cell* 10: 171–182.

Kaufmann, K., Melzer, R., and Theissen, G. 2005. MIKC-type MADS-domain proteins: structural modularity, protein interactions and network evolution in land plants. *Genes* 347: 183–198.

Kempin, S. A., Mandel, M. A., and Yanofsky, M. F. 1993. Conversion of perianth into reproductive-organs by ectopic expression of the tobacco floral homeotic gene NAG1. *Plant Physiol.* 103: 1041–1046.

Kitahara, K., Hibino, Y., Aida, R., and Matsumoto, S. 2004. Ectopic expression of the rose AGAMOUS-like MADS-box genes 'MASAKO C1 and D1' causes similar homeotic transformation of sepal and petal in Arabidopsis and sepal in Torenia. *Plant Sci.* 166: 1245–1252.

Kitahara, K., and Matsumoto, S. 2000. Rose MADS-box genes 'MASAKO C1 and D1' homologous to class C floral identity genes. *Plant Sci.* 151: 121–134.

Koncz, C., Kreuzaler, F., Kalman, Z., and Schell, J. 1984. A simple method to transfer, integrate and study expression of foreign genes, such as chichen ovalbumin and alpha-actin in plant tumors. *EMBO J.* 3: 1029–1037.

Kramer, E. M., Dorit, R. L., and Irish, V. F. 1998. Molecular evolution of genes controlling petal and stamen development: duplication and divergence within the APETALA3 and PISTILLATA MADS-box gene lineages. *Genetics* 149: 765–783.

Kramer, E. M., Jaramillo, M. A., and Di Stilio, V. S. 2004a. Patterns of gene duplication and functional evolution during the diversification of the AGAMOUS subfamily of MADS box genes in angiosperms. *Genetics* 166: 1011–1023.

Kramer, E. M., Jaramillo, M. A., and Di Stilio, V. S. 2004b. Patterns of gene duplication and functional evolution during the diversification of the AGAMOUS subfamily of MADS box genes in angiosperms. *Genetics* 166: 1011–1023.

Li, G. S., Meng, Z., Kong, H. Z., Chen, Z. D., Theissen, G., and Lu, A. M. 2005. Characterization of candidate class A, B and E floral homeotic genes from the perianthless basal angiosperm *Chloranthus spicatus* (Chloranthaceae). *Dev. Genes Evol.* 215: 1–13.

Liljegren, S. J., Ditta, G. S., Eshed, Y., Savidge, B., Bowman, J. L., and Yanofsky, M. F. 2000. SHATTERPROOF MADS-box genes control seed dispersal in Arabidopsis. *Nature* 404: 766–770.

Litt, A., and Irish, V. F. 2003. Duplication and diversification in the APETALA1/FRUITFULL floral homeotic gene lineage: implications for the evolution of floral development. *Genetics* 165: 821–833.

Lu, W. L. 1996. Development of sexual organs in *Taihangia rupestris*-different temperature requirements for both sexual organ development in a bisexual flower. *Acta Bot. Sinica* 38: 174–179.

Lu, W. L., Shen, S. H., and Wang, F. H. 1983. Studies on reproductive biology of *Taihangia rupestris* II investigation and study of sexual and asexual reproduction. *Chin. Biodivers.* 3: 8–14.

Ma, H., Yanofsky, M. F., and Meyerowitz, E. M. 1991. *Agll-Ag16*, an Arabidopsis gene family with similarity to floral homeotic and transcription factor genes. *Genes Dev.* 5: 484–495.

Mandel, M. A., Bowman, J. L., Kempin, S. A., Ma, H., Meyerowitz, E. M., and Yanofsky, M. F. 1992. Manipulation of flower structure in transgenic tobacco. *Cell* 71: 133–143.

Mizukami, Y., and Ma, H. 1992. Ectopic expression of the floral homeotic gene AGAMOUS in transgenic Arabidopsis plants alters floral organ identity. *Cell* 71: 119–131.

Moore, R. C., Grant, S. R., and Purugganan, M. D. 2005. Molecular population genetics of redundant floral-regulatory genes in *Arabidopsis thaliana*. *Mol. Biol. Evol.* 22: 1–103.

Murashige, T., and Skoog, F. 1962. A revied media for rapid growth and bioassay with tobacco cultures. *Physiologia Plantarum* 15: 473–497.

Nitasaka, E. 2003. Insertion of an En/Spm-related transposable element into a floral homeotic gene DUPLICATED causes a double flower phenotype in the Japanese morning glory. *Plant J.* 36: 522–531.

Ohno, S. 1970. *Evolution by Gene Duplication*. Springer-Verlag, New York.

Pelaz, S., Ditta, G. S., Baumann, E., Wisman, E., and Yanofsky, M. F. 2000. B and C floral organ identity functions require *SEPALLATA* MADS-box genes. *Nature* 405: 200–203.

Pelaz, S., Tapia-Lopez, R., Alvarez-Buylla, E. R., and Yanofsky, M. F. 2001. Conversion of leaves into petals in Arabidopsis. *Curr. Biol.* 11: 182–184.

Perl-Treves, R., Kahana, A., Rosenman, N., Xiang, Y., and Silberstein, L. 1998. Expression of multiple AGAMOUS-like genes in male and female flowers of cucumber Cucumis sativus L. *Plant Cell Physiol.* 39: 701–710.

Pinyopich, A., et al. 2003. Assessing the redundancy of MADS-box genes during carpel and ovule development. *Nature* 424: 85–88.

Pnueli, L., Hareven, D., Rounsley, S. D., Yanofsky, M. F., and Lifschitz, E. 1994. Isolation of the tomato AGAMOUS gene TAG1 and analysis of its homeotic role in transgenic plants. *Plant Cell* 6: 163–173.

Ratcliffe, O. J., Nadzan, G. C., Reuber, T. L., and Riechmann, J. L. 2001. Regulation of flowering in Arabidopsis by an FLC homologue. *Plant Physiol.* 126: 122–132.

Rigola, D., Pe, M. E., Mizzi, L., Ciampolini, F., and Sari-Gorla, M. 2001. CaMADS1, an AGAMOUS homologue from hazelnut, produces floral homeotic conversion when expressed in Arabidopsis. *Sex Plant Reprod.* 13: 185–191.

Rutledge, R., et al. 1998. Characterization of an AGAMOUS homologue from the conifer black spruce (*Picea mariana*) that produces floral homeotic conversions when expressed in Arabidopsis. *Plant J.* 15: 625–634.

Savidge, B., Rounsley, S. D., and Yanofsky, M. F. 1995. Temporal relationship between the transcription of two Arabidopsis MADS box genes and the floral organ identity genes. *Plant Cell* 7: 721–733.

Schwarz-Sommer, Z., et al. 1992. Characterization of the Antirrhinum floral homeotic MADS-box gene deficiens: evidence for DNA binding and autoregulation of its persistent expression throughout flower development. *EMBO J.* 11: 251–263.

Scortecci, K. C., Micheals, S. D., and Amasino, R. M. 2001. Identification of a MADS-box gene, FLOWERING LOCUS M, that represses flowering. *Plant J.* 26: 229–236.

Smyth, D. R., Bowman, J. L., and Meyerowitz, E. M. 1990. Early flower development in Arabidopsis. *Plant Cell* 2: 755–767.

Soltis, P. S., Soltis, D. E., and Chase, M. W. 1999. Angiosperm phylogeny inferred from multiple genes as a tool for comparative biology. *Nature* 402: 402–404.

Sommer, H., et al. 1990. Deficiens, a homeotic gene involved in the control of flower morphogenesis in *Antirrhinum majus*: the protein shows homology to transcription factors. *EMBO J.* 9: 605–613.

Tandre, K., Albert, V. A., Sundas, A., and Engstrom, P. 1995. Conifer homologues to genes that control floral development in angiosperms. *Plant Mol. Biol.* 27: 69–78.

Tandre, K., Svenson, M., Svensson, M. E., and Engstrom, P. 1998. Conservation of gene structure and activity in the regulation of reproductive organ development of conifers and angiosperms. *Plant J.* 15: 615–623.

Theissen, G. 2005. Birth, life and death of developmental control genes: new challenges for the homology concept. *Theory Biosci.* 124: 199–212.

Theissen, G. 2001. Development of floral organ identity: stories from the MADS house. *Curr. Opin. Plant Biol.* 4: 75–85.

Theissen, G., and Saedler, H. 2001. Plant biology. Floral quartets. *Nature* 409: 469–471.

Theissen, G., et al. 2000. A short history of MADS-box genes in plants. *Plant Mol. Biol.* 42: 115–149.

Tsuchimoto, S., van der Krol, A. R., and Chua, N. H. 1993. Ectopic expression of pMADS3 in transgenic petunia phenocopies the petunia blind mutant. *Plant Cell* 5: 843–853.

van der Linden, C. G., Vosman, B., and Smulders, M. J. M. 2002. Cloning and characterization of four apple MADS box genes isolated from vegetative tissue. *J. Exp. Bot.* 53: 1025–1036.

Winter, K. U., Becker, A., Munster, T., Kim, J. T., Saedler, H., and Theissen, G. 1999. MADS-box genes reveal that gnetophytes are more closely related to conifers than to flowering plants. *Proc. Natl. Acad. Sci. USA* 96: 7342–7347.

Xu, Y. Y., et al. 2005. Activation of the WUS gene induces ectopic initiation of floral meristems on mature stem surface in *Arabidopsis thaliana*. *Plant Mol. Biol.* 57: 773–784.

Yü, T. T., and Li, C. L. 1983. The systematic position of genus Taihangia in rosaceae. *Acta Phytotaxon. Sinica* 21: 229–235.

Yü, T. T., and Li, C. L. 1980. Taihangia Yü et Li-A new genus of rosaceae from China. *Acta Phytotaxon. Sinica* 18: 469–472.

Yang, Y., Fanning, L., and Jack, T. 2003. The K domain mediates heterodimerization of the Arabidopsis floral organ identity proteins, APETALA3 and PISTILLATA. *Plant J.* 33: 47–59.

Yanofsky, M. F., Ma, H., Bowman, J. L., Drews, G. N., Feldmann, K. A., and Meyerowitz, E. M. 1990. The protein encoded by the Arabidopsis homeotic gene agamous resembles transcription factors. *Nature* 346: 35–39.

Zahn, L. M., et al. 2006. Conservation and divergence in the AGAMOUS subfamily of MADS-Box genes: evidence of independent sub- and neofunctionalization events. *Evol. Dev.* 8: 30–45.

Zahn, L. M., et al. 2005. The evolution of the SEPALLATA subfamily of MADS-box genes: a pre-angiosperm origin with multiple duplications throughout angiosperm history. *Genetics* 169: 2209–2223.

SUPPLEMENTARY MATERIALS

The following supplementary material is available (on request) for this article online: http://www.blackwellpublishing.

com/products/journals/suppmat/EDE/EDE05042/ EDE05042sm.htm Figure S1, Table S1, Table S2.

Fig. S1. (A and B) The alignments of the predicted amino acid sequences of TrAG (in A) and TrSHP (in B) from *T. rupestris* with representatives of other *AG*-related proteins. Gaps (-) are introduced to maximize alignment and the dot (.) indicate identical amino acid to AG (in A) and SHP1 (in B), respectively. MADS, I, K and C domains are underlined and defined by single, double, dash line and arrow respectively, according to Ma et al. (1991). Heptad (abcdefg)$_n$ repeats identified by Yang et al. (2003) in the K domain are represented by K1, K2, and K3 subdomians. Hydrophobic amino acids occupy the positions 113 and 116 like the same observed in most MIKC-type MADS-box proteins and highlighted in blue box. Two highly conserved regions, AG motif I and AG motif II are highlighted in green box. Conserved Gly-110 (in TrSHP and representatives of other MADS-box proteins) are marked in bold and red, and the same position is occupied by the Ala in TrAG, MASAKOC1 and STAG1. The proteins isolated from Rosaceae within the *euAG* lineage (in A) always contain Asn at position 47, and Asn-47 is highlighted in black box. His-158 (in A) and Gln-158 (in B) are boxed. Representatives of other MADS-box proteins: STAG1 (AF168468) from *Fragaria* × *ananassa*, MASAKOC1 (BAA90744) and MASAKOD1 (BAA90743) are from *Rosa rugosa* Thunb. ex Murray, PpMADS4 (AAU29513) from *Prunus persica*, MdMADS15 (CAC80858) and MdMADS14 (CAC80857) are from *Malus* × *domestica*; AG (NP_567569), SHP1 (NP_191437) and SHP2 (NP_850377) from *Arabidopsis thaliana*; FARINELLI (CAB42988) and PLENA (AAB25101) from *Antirrhinum*; pMADS3 (CAA51417) and FBP6 (CAA48635) from *petunia*.

Table S1. Primers used for isolating *TrAG* and *TrSHP*.

Table S2. Comparison of the aa identity of M, I, K, C, and MIKC domains between TrAG and TrSHP, respectively, with other closely related proteins from *Arabidopsis*, *Antirrhinum* and *Petunia*.