Isolation and Characterization of AGAMOUS-Like Genes Associated With Double-Flower Morphogenesis in Kerria japonica (Rosaceae)

Jiang Ma1,2†, Xiangling Shen1†, Zhixiong Liu3, Dechun Zhang1, Wen Liu1, Hongwei Liang1, Yubing Wang1, Zhengquan He1 and Faju Chen1*

1 Key Laboratory of Three Gorges Regional Plant Genetics & Germplasm Enhancement (CTGU)/Biotechnology Research Center, China Three Gorges University, Yichang, China, 2 Forestry College, Beijing Forestry University, Beijing, China, 3 College of Horticulture and Gardening, Yangtze University, Jingzhou, China

Double-flower phenotype is more popular and attractive in garden and ornamental plants. There is great interest in exploring the molecular mechanisms underlying the double-flower formation for further breeding and selection. Kerria japonica, a commercial ornamental shrub of the Rosaceae family, is considered an excellent system to determine the mechanisms of morphological alterations, because it naturally has a single-flower form and double-flower variant with homeotic conversion of stamens into petals and carpels into leaf-like carpels. In this study, Sf-KjAG (AGAMOUS homolog of single-flower K. japonica) and Df-KjAG (AGAMOUS homolog of double-flower K. japonica) were isolated and characterized as two AGAMOUS (AG) homologs that occur strictly in single- and double-flower K. japonica, respectively. Our sequence comparison showed that Df-KjAG is derived from ectopic splicing with the insertion of a 2411 bp transposon-like fragment, which might disrupt mRNA accumulation and protein function, into intron 1. Ectopic expression analysis in Arabidopsis revealed that Sf-KjAG is highly conserved in specifying carpel and stamen identities. However, Df-KjAG did not show any putative C-class function in floral development. Moreover, yeast-two-hybrid assays showed that Sf-KjAG can interact with KjAGL2, KjAGL9, and KjAP1, whereas Df-KjAG has lost interactions with these floral identity genes. In addition, loss-of-function of Df-KjAG affected not only its own expression, but also that of other putative floral organ identity genes such as KjAGL2, KjAGL9, KjAP1, KjAP2, KjAP3, and KjPI. In conclusion, our findings suggest that double-flower formation in K. japonica can be attributed to Df-KjAG, which appears to be a mutant produced by the insertion of a transposon-like fragment in the normal AG homolog (Sf-KjAG) of single-flower K. japonica.

Highlights: Sf-KjAG and Df-KjAG are different variations only distinguished by a transposon-like fragment insertion which lead to the evolutionary transformation from single-flower to double-flowers morphogenesis in Kerria japonica.

Keywords: double-flower, AGAMOUS, Kerria japonica, transposon, floral development
INTRODUCTION

Transposon elements (TEs) refer to segments of DNA that can integrate at many different sites along a chromosome (Finnegan, 1985; Kidwell, 2002). In many plants, TEs comprise a major portion of the genome and increase genetic and functional diversity, which might provide more selective potential in plant evolution and domestication (Hirsch and Springer, 2017). In flowering plants, members of the MADS-box transcription factor gene family are known to regulate floral organ development, but few cases of genetic diversity and floral development caused by TE insertion in MADS-box family genes have been reported (Becker and Theissen, 2003; Bennetzen, 2005; El Baidouri et al., 2014).

In Arabidopsis, AGAMOUS (AG), encoding a MADS-box transcription factor, plays crucial functions in floral development, such as regulating stamen and carpel morphogenesis, limiting floral meristem determinacy, and antagonizing A-class genes (Coen and Meyerowitz, 1991; Soltis et al., 2002; Heijmans et al., 2012; Soltis and Soltis, 2014). Over the long term, AG homologous genes in different taxa have undergone sub-functionalization or neo-functionalization, but their functions in regulating the development of reproductive organs are broadly conserved, both in angiosperms and gymnosperms (Winter et al., 1999; Kyozuka and Shimamoto, 2002; Kramer et al., 2004; Zhang et al., 2004, 2015; Arrington, 2006). Mutation or aberrant expression of AG orthologous genes is often involved in double-flower formation (Bowman, 1997; Benedito et al., 2004; Dong et al., 2007; Hsu et al., 2010; Liu et al., 2013; Tanaka et al., 2013; Noor et al., 2014; Sun et al., 2014; Wang et al., 2015; Zhang et al., 2015). For example, loss-of-function of AG in Arabidopsis converts stamens into petals and replaces carpels with another ag mutant flower, known as double-flower (Bowman et al., 1991; Meyerowitz, 1994). In Nigella damascena, simultaneous downregulation of NdAG1 and NdAG2 results in the transformation of stamens into petals and carpels into sepals, as well as the loss of floral determinacy (Wang et al., 2015). Silencing of TAG1 in tomato also causes double-flower formation (Dong et al., 2007). Simultaneous silencing of two C-class MADS-box genes, pMAD3 and FBP6, produces double-flower in Petunia hybrid (Noor et al., 2014). In Camellia japonica, the shift of CjAG expression boundary is associated with double-flower formation (Sun et al., 2014). In Prunus lannesiana, exon skipping of PrsAG results in the complete loss of AG motifs I and II in the C domain, which leads to double-flower formation (Liu et al., 2013). In Magnolia stellata, alternative splicing of MastAG generates three transcripts with divergent functions, which might result in the formation of multiple petals in M. stellata flowers (Zhang et al., 2015).

Although many double-flower phenotypes are noted in natural and domesticated species, the molecular mechanism underlying double-flower formation has only been investigated in few species. In addition, double-flower formation caused by TE insertion in AG orthologous genes was previously observed only in three ornamental plants, including Japanese morning glory (Nitasaka, 2003), Thalictrum thalictroides cultivar ‘Double White’ (Galimba et al., 2012) and Gentiana scabra (Nakatsuka et al., 2015). Kerria japonica, a common ornamental deciduous shrub in Rosaceae, has a single-flower form and a double-flower variant with homeotic conversion of stamens into petals and carpels into leaf-like carpels, and loss of floral meristem determinacy. Based on these observed phenotypes, we identified the formation of double-flower in K. japonica as a candidate for the loss of C-class function. In this study, we isolated two AG-like genes, Sf-KjAG from single-flower K. japonica and Df-KjAG from double-flower K. japonica. Detailed sequencing and expression analyses of Sf-KjAG and Df-KjAG were performed. Further, functional analyses of Sf-KjAG and Df-KjAG under the control of the cauliflower mosaic virus (CaMV) 35S promoter were conducted in the wild-type and homozygous ag-1 mutant Arabidopsis. The results provided strong evidences of high functional conservation of the AG ortholog in K. japonica in specifying carpel and stamen identities and revealed the molecular mechanism of double-flower formation in K. japonica, as well as novel information regarding the involvement of a transposon in double-flower formation and evolution.

MATERIALS AND METHODS

Plant Materials

Flower bud samples of single- or double-flower K. japonica were collected from Shengnongjia forest district and Beijing Forestry University. The samples were then frozen immediately in liquid nitrogen and stored at −80°C until use.

The Arabidopsis ag-1 mutant line (CS3086) in an ecotype Landsberg background was obtained from the Arabidopsis Biological Resource Center, Ohio State University, Columbus, OH, United States.

Rapid Amplification of cDNA Ends

Total RNA was extracted separately from floral buds of the single- and double-flower K. japonica by using the EASYspin plant RNA Extraction kit (Aidlab, China) following manufacturer instructions. The 3′-full rapid amplification of cDNA ends (RACE) Core Set Version 2.0 kit (TaKaRa, Japan) was used to synthesize the 3′ RACE cDNA from 2 μg of DNase I-treated RNA by using 3′ RACE adaptor primer and PrimeScript RTase. The 3′ portion of the AG homolog from single-flower K. japonica was isolated, which yielded a 1038 bp fragment that was amplified from single-flower K. japonica 3′ RACE cDNA (forward primer, 3′AGGSPF1; reverse primer, 3′RACE outer primer). The 5′ RACE cDNA was synthesized from 1 μg of DNase I-treated RNA by using random primer and PrimeScript RTase by using the 5′-Full RACE Kit (TaKaRa, Japan). The 5′ portion of the fragment was then amplified using a pair of specific primers (5′AGGSPRI and the 3′ RACE outer primer). These PCR products were cloned into a pMD18-T vector and then sequenced. The cloning procedure of double-flower K. japonica AG homolog was similar to that of single-flower K. japonica. The accuracy and integrity of AG homologous cDNA sequences from K. japonica were validated by designing primers to amplify...
full-length CDS (primers Sf/Df-KjAGF and Sf-KjAGR for single-flower *K. japonica* AG homolog; primers Sf/Df-KjAGF and Df-KjAGR for double-flower *K. japonica* AG homolog). The products were cloned into pMD18-T vectors and then sequenced. The sequences were deposited in GenBank (accession numbers: KC476500 and KT884658).

**Cloning and Analysis of Sf-KjAG and Df-KjAG Genomic Sequence**

Genomic DNA was extracted from young leaves of single- and double-flower *K. japonica* by using TaKaRa MiniBEST Plant Genomic DNA Extraction kit (Takara, Japan). The Sf-KjAG and Df-KjAG genomic DNA was amplified using Long PCR Enzyme Mix (Fermentas, Lithuania). Forward primer KjAG-GeDNA-F (in identical exon 1 of *K. japonica* and double-flower *K. japonica*) and reverse primer KjAG-GeDNA-R (in exon 8 of *Sf-KjAG*) were used to amplify the full-length genomic DNA sequences of Sf-KjAG and Df-KjAG. The PCR products were cloned into pTOPO-TA vectors (Aidlab, China) and then sequenced. The sequences were deposited in GenBank (accession numbers: KT884659 and KT884660).

**Southern Blotting and Distribution Detection of Sf-KjAG and Df-KjAG Transcripts**

Genomic DNA was extracted from the young leaves of single- and double-flower *K. japonica* using the TaKaRa MiniBEST Plant Genomic DNA Extraction kit (Takara, Japan) following the manufacturer's protocol. Specific hybridization was confirmed by designing probe primers Kj-souF and Kj-souR in the lowest conserved C domain. PCR amplification was performed by separately using single- and double-flower *K. japonica* genomic DNA as template to detect primer specificity, and PCR products were cloned into a pTOPO-TA vector (Aidlab, China) and sequenced. The PCR products (727 bp consensus sequence of Sf-KjAG and Df-KjAG genomic DNA) were amplified using Long PCR Enzyme Mix (Fermentas, Lithuania). Forward primer KjAG-GeDNA-F (in identical exon 1 of Sf-KjAG and Df-KjAG) and reverse primer KjAG-GeDNA-R (in exon 8 of Sf-KjAG) were used to amplify the full-length genomic DNA sequences of Sf-KjAG and Df-KjAG. The PCR products were cloned into pTOPO-TA vectors (Aidlab, China) and then sequenced. The sequences were deposited in GenBank (accession numbers: KT884659 and KT884660).

**Sequence Alignments and Phylogenetic Analysis**

Deduced amino acid sequences for Sf-KjAG were subjected to BLAST analysis using sequence information from the GenBank database. During the BLAST searches, multiple C-class proteins from eudicot taxa were selected for alignment. During the construction of phylogenetic trees, we also introduced the A-, B-, D-, and E-class proteins. The GenBank accession numbers of the sequences used are listed in Supplementary Table S2. Full-length amino acid sequences comprising the MADS, I, K, and C domains of these genes were aligned using the ClustalW program under default settings. A phylogenetic tree was constructed using the Maximum likelihood method, including bootstrap analyses with 500 replications, in MEGA6.0 software (Jones et al., 1992; Tamura et al., 2013).

**Semi-Quantitative Reverse Transcription-PCR and Real-Time PCR**

For semi-quantitative reverse transcriptase (RT)-PCR analysis, total RNA was extracted separately from juvenile leaves, sepals, petals, stamens, and carpels of single-flower *K. japonica* and juvenile leaves, sepals, outermost petals, inner petals and leaflike carpels of double-flower *K. japonica* at S9 and D9 stages by using an EASYspin plant RNA Extraction Kit (Aidlab, China). In double-flower *K. japonica*, the outermost 5 petals were considered as the outermost petals, and the remaining petals were considered as inner petals. After treatment with DNase I (TaKaRa, Japan), 1 µg of total RNA was used to synthesize the first-strand cDNA by using the oligo (dT)18 primer and MMLV Reverse Transcriptase. RT-PCR was performed using the following primers: RT-Sf-KjAGF and RT-Sf-KjAGR for Sf-KjAG, RT-Df-KjAGF and RT-Df-KjAGR for Df-KjAG, and RT-KjactinF and RT-KjactinR for ACTIN (reference control). Next, 1 µL of each cDNA sample was submitted to PCR as follows: 94°C for 5 min; thirty cycles of 30 s at 94°C, 30 s at 58°C, 30 s at 72°C; and a 10 min final extension at 72°C. The PCR products from all amplifications were analyzed using electrophoresis on a 1% agarose gel and photographed under ultraviolet light. Further, real-time quantitative PCR was performed to further confirm the expression abundance. The real-time quantitative PCR was performed using SYBR premix Ex Taq (Takara, Japan) and the following primers: qSf/Df-KjAG-F and qSf-KjAG-R for Sf-KjAG, qSf/Df-KjAG-F and qDf-KjAG-R for Df-KjAG, and qKjactin-F and qKjactin-R for ACTIN (reference control). The reaction conditions were as follows: 95°C for 3 min; forty cycles of 95°C for 15 s, and 60°C for 30 s. The abundance of other putative floral organ identity genes such as *KjAP1* (putative A-class), *KjAP2* (putative A-class), *KjAP3* (putative B-class), *KjPI* (putative B-class), *KjAGL2* (putative E-class), and *KjAGL9* (putative E-class) was determined by conducting real-time quantitative PCR expression analyses. The primers used for real-time quantitative PCR are listed in Supplementary Table S1.

Total RNA was extracted from bud samples of single- and double-flower *K. japonica* at different development stages. After treatment with DNase I (TaKaRa, Japan), 1 µg of total RNA...
was used to synthesize first-strand cDNA by using the oligo (dT)$_{18}$ primer and MMLV Reverse Transcriptase. The real-time quantitative PCR of Sf-KjAG and Df-KjAG at different development stages was performed as the protocol mentioned before.

**VECTOR CONSTRUCTION AND TRANSFORMATION**

Full-length coding sequences of Sf-KjAG and Df-KjAG were cloned into the binary vector pBI121 (BD Biosciences Clontech) by separately digesting with XbaI and SacI, and XbaI and Smal, under the control of the CaMV 35S promoter in the sense orientation. The 35S::Sf-KjAG and 35S::Df-KjAG constructs were first transformed into Agrobacterium tumefaciens strain GV3101-90 and then into heterozygous ag-1 Arabidopsis (Landsberg erecta) by using the floral-dip method, as described by Clough and Bent (1998). In addition, pBI121 vector as a negative control was transformed into Arabidopsis.

The putatively transformed Arabidopsis thaliana seeds were germinated on solid 1/2 MS medium containing 35 µg/mL kanamycin. After the seeds were vernalized at 4°C for 2 days, they were transferred to a greenhouse under normal growth conditions (16 h light/8 h dark) at 22°C. After 2 weeks of screening, transformation-positive plants with green true leaves and long roots were transplanted into nutritional soil for cultivation. The transgenic plants were confirmed using quantitative real-time PCR (primers: qRT-Sf-KjAG-F and qRT-Sf-KjAG-R; qRT-Df-KjAG-F and qRT-Df-KjAG-R). The genotypes (wild-type, heterozygous ag-1, or homozygous ag-1) of transformed Arabidopsis were identified using the dCAPS methods (Neff et al., 1998). PCR was performed using a 5 min at 94°C denaturation step; thirty cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 30 s; and a final extension period of 5 min by using the primers ag-f and ag-r. Finally, 20 µL of the total PCR product (25 µL) in each reaction was digested using Afl II (TaKaRa, Japan) for 3 h and then separated on a 4% agarose gel, stained, and visualized under ultraviolet light.

**YEAST TWO HYBRID ASSAY**

Further evidence for Df-KjAG loss-of-function was provided by conducting yeast two hybrid (Y2H) assays by using Sf-KjAG, Df-KjAG, KjAGL2, KjAGL9, KjAP1, KjAP3, and KjPI, with HIS3, AUR1-C, LacZ, and ADE2 serving as reporter genes by using the Matchmaker Gold Yeast Two-Hybrid System (Clontech, United States). If the co-transformed yeast grew and turned blue on the selective medium (lacking histidine and adenine), which consist of five sepals, five petals, numerous stamens, and five carpels (Figure 1A). Conversely, in double-flower K. japonica, whorl 3 is replaced with numerous petals (Figure 1B), and whorl 4 is replaced with 6 to 10 leaf-like carpels (Figures 1G–J). Additional phenotypes can be observed within these two whors: the petals of double-flowers dwindle down centripetally (Figure 1D); some petals in the center still retain filament-like and anther-like structures (Figures 1D–F); and partial leaf-like carpels are observed to be fused with the petals (Figures 1B,H,J, 7B).

**DF-KJAG OF DOUBLE-FLOWER K. JAPONICA IS A TRUNCATED MUTANT OF SF-KJAG OF SINGLE-FLOWER K. JAPONICA**

Based on morphological observation and previous study findings, we speculated that the double-flower formation of K. japonica might be caused by the loss-of-function of C-class genes. Therefore, we separately isolated AG homologous genes from single- and double-flower K. japonica. Phylogenetic analyses revealed that the AG homolog cloned from single-flower K. japonica is nested in the euAG clade (Supplementary Figure S3). Therefore, the AG homolog isolated from single-flower K. japonica is referred to as Sf-KjAG (AGAMOUS homolog of single-flower K. japonica), and that isolated from double-flower K. japonica is referred to as Df-KjAG (AGAMOUS homolog of double-flower K. japonica). The cDNA alignment revealed that exon 1 of Df-KjAG was identical to that from Sf-KjAG, but lacked the I, K, and C domains. The Sf-KjAG transcript contains a 741 bp open reading frame encoding 246 amino acids (aa), which contain a 57 aa MADS domain, a 32 aa I domain, an 82 aa K domain, and a 57 aa C-terminal domain (Supplementary Figure S4). An 18 aa N-terminal extension, which is usually observed in euAG clade members (Kramer et al., 2004), was also found in Sf-KjAG (Supplementary Figure S4). Moreover, the Sf-KjAG protein contains two specific and highly conserved motifs of AG homologous proteins, AG motifs I and II. In contrast, the Df-KjAG transcript only contains a 303 bp open reading frame encoding a 100 aa protein, which only contains the MADS domain. Genomic sequence alignment revealed that Sf-KjAG and Df-KjAG share identical sequences except for an extra 2411 bp fragment inserted into intron 1 of Df-KjAG genomic DNA. Moreover,
the inserted segment caused premature transcription termination and provided multiple alternative splice acceptor sites; therefore, exons 2–6 of Df-KjAG mRNA are from the inserted segment. According to the unified classification system for eukaryotic transposable elements (Wicker et al., 2009), further identification revealed that the inserted fragment contains two target repeats (ATTTATAT) and two inverted repeats (TAGGGTGAGGC), suggesting a similarity to transposable elements. However, the inserted segment is neither a DNA transposon since it lacked sequences encoding transposase, nor a retro-transposon for the presence of introns, but the lack of long terminal repeats (Figure 2B). In addition, BLAST analysis of the inserted sequences revealed that a 108 bp sequence (from 1014 to 1122 bp of the inserted segment) has nearly 95% similarity with P. mume DNA-directed RNA polymerase V subunit 1 (LOC103330828). Another 118 bp fragment (from 1509 to 1627 bp of the inserted segment) was found to share nearly 93% similarity with M. × domestica DNA-directed RNA polymerase V subunit 1-like (LOC103438702). However, the DNA-directed RNA polymerase encoded by the sequences is truncated, and an obvious frame-shift mutation was noted at the 1232 bp loci of the inserted segment.

**Southern Blotting and Distribution Detection of Sf-KjAG and Df-KjAG Transcripts**

The result of Southern blotting with an identical sequence of Sf-KjAG and Df-KjAG as a probe revealed that both Sf-KjAG and Df-KjAG are a single copy (Figure 2C). Multiplex PCR showed that the Df-KjAG transcript can be detected only in double-flower samples and Sf-KjAG transcript only in single-flower K. japonica samples (Figure 3B).

**Expression Patterns of Sf-KjAG and Df-Kjag in K. japonica**

Semi-quantitative RT-PCR indicated that the Sf-KjAG transcript is strictly expressed in stamens and carpels and absent in leaves, sepals, and petals in single-flower K. japonica (Figure 3A). Similar to Sf-KjAG, Df-Kjag is expressed in the inner two whorls
Before real-time quantitative PCR on buds at different developmental stages, the stages of buds were determined using the traditional paraffin section method (Supplementary Figure S1). Real-time quantitative PCR analyses revealed that the expression level of *Sf-KjAG* increased gradually from the anther primordia stage (S1) to the primary sporogenous cell stage (S2) and reached a peak at the secondary sporogenous cell stage (S3). Further, its expression decreased and remained steady at the pollen mother cell stage (S4), late uninucleate stage (S5), and double nucleus stage (S6). Subsequently, the expression decreased gradually at the double nucleus stage (S7) and pollen maturation stage (S8) and remained lower until flowering (S9; Figure 3C). Compared with that of *Sf-KjAG*, the expression level of *Df-KjAG* was considerably lower in all development stages; in particular, during early flower development, the expression level of *Df-KjAG* was reduced by nearly four hundred times (Figure 3C).

**Functional Analysis of Sf-KjAG and Df-KjAG by Transforming Arabidopsis**

In order to gain further insight into the function of *Sf-KjAG* and *Df-KjAG*, we constructed ectopic expression vectors with the genes under the control of the CaMV 35S promoter and transformed them into heterozygous *ag-1* mutant *Arabidopsis* plants by using Agrobacterium-mediated transformation. The transgenic plant genotypes were screened using the dCAPs method (Supplementary Figure S2).

We obtained 56 35S::*Sf-KjAG* transgenic *Arabidopsis* in the wild-type background. Among these transgenic plants, 17 (30.36%) could be phenotypically distinguished from untransformed wild-type plants. From these 17 plants, 9 (16.07%) showed homeotic transformation from sepals into carpelloid structures bearing stigmatic papillae and ovules (Figures 4B–F), and petals into filament-like structures (Figures 4C,G,H). The remaining 8 (14.29%) plants showed flower phenotypes consisting of shortened petals converted to filament-like structures, and shrunken and revolute sepals (Figures 4G,H). In addition, the abscissions of the outer three floral whorls (sepals, petals, and stamens) were inhibited; thus, these flower tissues remained persistent until silique development (Figure 4I).

We also obtained thirty-seven 35S::*Sf-KjAG* plants in the homozygous *ag-1* mutant background. Compared with that in the homozygous *ag-1* mutant *Arabidopsis*, 13 (35.14%) plants exhibited obvious floral organ phenotype changes. In these lines, the missing stamens and carpels of the *Arabidopsis* *ag-1* mutants were rescued (Figures 5B–H). The number of recovered carpels varied from one to three per flower, and the number of recovered stamens ranged from zero to fourteen (Figures 5B–H). These
stamens and carpels recovered in homozygous ag-1 mutant prevented the formation of constantly nested sepals and petals in the flower center (Figures 5B–H). Moreover, the number of petals decreased (Figures 5B–H). In addition, two (7.41%) plants exhibited splaying and white-edge sepals (Figure 5H). Forty-three 35S::Df-KjAG transgenic Arabidopsis plants in the wild-type background were obtained. None of these transgenic plants showed common phenotype alterations of AG homolog ectopic expression, such as the formation of carpelloid sepals and stamenoid petals. Instead, 10 (23.26%) plants showed phenotypic alterations involving stamen number reduction and runtish anthers (Figure 4I). Four (9.30%) plants maintained five stamens (Figures 4J–L), and other 6 (26.09%) plants had four stamens (Figures 4M,N). There were twenty-two 35S::Df-KjAG transgenic plants in the homozygous ag-1 mutant background that showed no phenotype rescue compared with those in the non-transgenic homozygous ag-1 mutant.

Transgenic plants and relative expression levels were confirmed by quantitative RT-PCR. The results showed that Sf-KjAG expression levels were higher in transgenic plants with

**FIGURE 3 |** Expression pattern analysis of Sf-KjAG and Df-KjAG. (A) Tissue-specific expression pattern analysis of Sf-KjAG and Df-KjAG by semi-quantitative RT-PCR. (Aii) Expression pattern analysis of Sf-KjAG and Df-KjAG in different floral tissues by real-time quantitative PCR. le, leaves; se, sepals; pe, petals; st, stamens; ca, carpels; o-pe, outermost petals; i-pe, inner petals; le-ca, leaf-like carpels. (B) Distribution detection of Sf-KjAG and Df-KjAG in single- and double-flower K. japonica by multiplex PCR. Lane 1 is the PCR with plasmid which carries the Sf-KjAG full-length CDS as a template. Lanes 2 is the PCR with plasmid which carries the Df-KjAG full-length CDS as a template. Lanes 3–14 are the PCR with the cDNA of single-flower K. japonica as templates. Lanes 15–24 are the PCR with the cDNA of double-flower K. japonica as templates. (C) Expression analysis of Sf-KjAG and Df-KjAG at different development stages by real-time quantitative PCR. Stars indicated $p < 0.05$ by student's t-test.
obvious transformation of sepals into carpellodium structures and petals into stamenoid petals than in those with no phenotypic alterations in the wild-type background (Figure 6A). In the homozygous ag-1 mutant Arabidopsis, Sf-KjAG expression levels were higher in the transfectants with obvious phenotypic rescue of stamens and carpels than in those with no phenotypic alterations (Figure 6A). The Df-KjAG expression levels were higher in the transgenic plants with obvious phenotypic alterations of stamen number reduction and runtish anthers than in those with no phenotypic alterations in the wild-type background (Figure 6B). Further, although expression levels of Df-KjAG were also high in the homozygous ag-1 mutant Arabidopsis, no phenotype alteration was noted in the transformants (Figure 6B).

**Interaction Pattern Comparison of Sf-KjAG and Df-KjAG**

In previous studies, AG homologs in organ-specific programs required the formation of protein–protein complexes with the putative floral organ identity transcription factors (Coen and Meyerowitz, 1991; Theißen and Saudler, 2001; Liu et al., 2010).

**Expression Pattern Comparison of A-, B-, and E-Class Genes in the Single- and Double-Flower K. japonica**

To investigate whether the loss-of-function of Sf-KjAG could affect the expression patterns of other related putative floral organ identity genes, we performed real-time quantitative PCR to compare the expression patterns of KjAGL2, KjAGL9, KjAP1, KjAP2, KjAP3, and KjPI in both single- and double-flower K. japonica. In single-flower K. japonica, KjAGL2 and KjAGL9 were found to be expressed in all floral organs; KjAP1 was specifically expressed highly in sepals and petals and little in stamens and carpels; KjAP2 was expressed in all the whorls, whereas its level in stamen was relatively less; and KjAP3 and KjPI expression levels were high in petals and stamens and little in sepals and carpels (Figure 7C). In double-flower K. japonica, the KjAGL2 expression was decreased in inner petals and leaf-like carpels; the KjAGL9 expression was increased in inner petals and leaf-like carpels; KjAP1 was ectopically expressed in inner petals and leaf-like carpels; KjAP2 expression was increased in the inner petals and decreased in leaf-like carpels; and both KjAP3 and
Although many double-flower phenotypes are noted in Double-flower varieties are more popular and attractive for single- and double-flower plants. Notably, the expression levels of these A-, B-, and E-class genes in K. japonica were expressed ectopically in leaf-like carpels (Ma et al., Mechanism of Double-Flowers Formation in Kerria japonica, 2018). A recent whole-genome duplication event might have occurred in Rosaceae 35.4–66.5 million years ago, and this was before pear (Maloideae, Rosaceae) and apple (Maloideae, Rosaceae) divergence, but not noted in strawberry (Rosoideae, Rosaceae; Wu et al., 2013). According to apple and strawberry genomes deposited in NCBI database, their AG orthologs are duplicated and single, respectively. Like Sf-KjAG and Df-KjAG (Figure 2C), both AG homologs in P. lannesiana 'speciosa' and 'Albo-rosea' (Rosaceae, Prunoideae) are a single copy (Liu et al., 2013). K. japonica and strawberry are grouped into Rosoideae, which might not have undergone the recent whole-genome duplication event that occurred before Maloideae divergence, but after Rosaceae divergence.

Sequence alignments revealed a transposon-like fragment insertion within intron one of Df-KjAG genomic DNA (Figure 2). However, partial structural elements related to transposition were lacking and two truncated coding sequences similar to polymerase genes were present in the transposon. Transposable elements often undergo rearrangement and truncations, such as abortive transposition events that lead to local rearrangements and deletions of internal sequences or nested insertions within other elements (Sanmiguel et al., 1996; Gorbunova and Levy, 1997; Yang et al., 2004; Krishnaswamy et al., 2010; Hirsch and Springer, 2017). Therefore, the transposition might be followed by transposable element rearrangements and truncations. Insertions of TEs within coding regions are generally mutagenic and result in strong loss-of-function, but insertions within introns or untranslated region are often tolerated and have minimal impact on transcription and splicing (West et al., 2014; Hirsch and Springer, 2017). In Gentiana scabra, an LTR-retrotransposon insertion within the intron 6 of GsAG1 (C-class gene) did not generate novel transcripts (Nakatsuka et al., 2015). In 'Rae Ime' and 'Spencer Seedless' (apatulous and parthenocarpic apple variants), the splicing of MdPI (B-class gene) separately inserted by transposons within the fourth intron and the sixth intron was also not affected (Yao and Dong, 2001). However, in double-flower K. japonica, the transposon insertion within the intron of AG homolog caused premature transcription termination and generated novel transcript Df-KjAG, whose exons 2–6 were spliced from the inserted segment, which might be because the inserted transposon provided alternative splice-acceptor sites. In T. thalictroides cultivar 'Double White,' novel

**DISCUSSION**

Double-flower varieties are more popular and attractive for higher ornamental values and more commercial interest. Although many double-flower phenotypes are noted in natural and domesticated species, the molecular mechanisms of double-flower formation in most varieties are not well understood. The gradually reduced petals centripetally and remnant filament-like and anther-like structures are the typical characteristics of extra petals transmuted from stamens in double-flower (Reynolds et al., 1984). Considering the morphology (Figures 1G–J), we speculated that the extra petals of double-flower K. japonica might be transmuted from stamens, and leaf-like structures are from carpels. Similarity of mutant phenotypes between K. japonica and other double-flower varieties caused by abnormal function or expression of AG homologous genes, such as P. lannesiana 'speciosa' and 'Albo-rosea,' T. thalictroides 'Double White' and C. japonica, the K. japonica double-flower formation might be related to the loss-of-function or aberrant expression of AG homologs.

![Quantitative analysis of Sf-KjAG and Df-KjAG expression in transgenic Arabidopsis. (A)](image) The expression of Sf-KjAG in the wild-type and homozygous ag-1 mutant transgenic Arabidopsis. (B) The expression of Df-KjAG in the wild-type and homozygous ag-1 mutant transgenic Arabidopsis. WT are the transgenic plants with the pBI121 vector only (negative control) in the wild-type background; ho are the transgenic plants with the pBI121 vector only (negative control) in the homozygous ag-1 mutant background. Sf-KjAG/W-28 and Sf-KjAG/ho-37 are the transgenic plants with no phenotype alteration in the wild-type and homozygous ag-1 mutant background, respectively. Sf-KjAG/W-7(4g)/2(4d)/8(4e)/21(4c)/23(4b) and Sf-KjAG/ho-9(5b)/7(5d)/17(5e)/13(5f)/42(5h) are the 3SS:Sf-KjAG transgenic plants with obvious phenotype alterations in the wild-type and homozygous ag-1 mutant background, respectively. Df-KjAG/W-15/1 are the 3SS:Df-KjAG transgenic plants with no phenotype alteration in the wild-type background. Df-KjAG/W-14(d)/2(4d)/39(4m)/11(4n) are the 3SS:Df-KjAG transgenic plants with phenotypic alterations of stamen number reduction and runtish anthers in the wild-type background. Df-KjAG/ho-28/29/12/6/11 are the transgenic plants with phenotypic alterations of stamen number reduction and runtish anthers in the wild-type background. Df-KjAG/W-15/1 are the transgenic plants with obvious phenotype alterations in the wild-type and homozygous ag-1 mutant background. Numbers and letters in brackets are corresponding to those in Figures 4, 5.
FIGURE 7 | Interaction and expression analysis of floral organ identity genes in K. japonica. (A) The ligation schematic of Sf-KjAG, Df-KjAG and Sf-KjAGΔ (MADS domain of Sf-KjAG was deleted) to pGBKTK7 vector with the GAL4-binding domain (BD). (Aii) Interaction analysis of Sf-KjAG, Df-KjAG, and Sf-KjAGΔ with KjAGL2, KjAGL9, KjAP1, KjAP3, KjPI, Sf-KjAG, and Df-KjAG by Y2H. PC, positive control; NC, negative control; Sf-KjAGΔ, MADS domain of Sf-KjAG is deleted; SD(-Leu/-Trp), synthetically defined medium lacking tryptophan and leucine; SD(-Leu/-Trp/-His/-Ade/+AbA/+X-a-Gal): synthetically defined medium lacking tryptophan, leucine, histidine and adenine and supplemented with Aureobasidin A and X-a-GAL. (B) Partial leaf-like carpels are adnate with petals in double-flower K. japonica. (C) Expression pattern analysis of KjAGL2, KjAGL9, KjAP1, KjAP2, KjAP3, and KjPI in single- and double-flower K. japonica. se, sepal; pe, petal; o-pe, outermost petal; st, stamen; i-pe, inner petal; ca, carpel; le-ca, leaf-like carpel. Stars indicated $p < 0.05$ by student’s $t$-test.
splicing also occurred at the alternative splice-acceptor sites provided by an transposon insertion within the exon 4 of ThtAG1 (Galimba et al., 2012).

In Arabidopsis, the C-class gene AG plays key roles in determining the identity of stamens and carpels, limiting floral meristem determinacy and repressing A-class genes (Coen and Meyerowitz, 1991; Soltis et al., 2002; Heijmans et al., 2012). In the case of AG transgenic expression, homeotic transformation of sepals into carpels or carpelloid structures and petals into stamen-like structures in the wild-type Arabidopsis, and rescue of the stamens and carpels in the ag mutant Arabidopsis are typical phenotype alterations (Mizukami and Ma, 1992; Zhang et al., 2004; Lü et al., 2007). Such phenotype alterations have also been found in the Sf-KjAG transgenic Arabidopsis, which might suggest that Sf-KjAG plays the conserved function in specifying stamen and carpel identities, similar to those of AG in Arabidopsis. The I, K, and C domains are important to AG homologous genes, especially the C domain is essential for AG function (Egeacortines et al., 1999; Honma and Goto, 2001; Lamb and Irish, 2003; Tseng et al., 2004). As expected, Df-KjAG did not show any putative AG homologous function; instead, its expression in the wild-type Arabidopsis reduced stamen number and affected anther development (Figures 4J–N), which indicated that the truncated Df-KjAG might inhibit normal AG function. Inhibition of normal AG function was also observed in transformants with truncated AG homologous genes transformed into Arabidopsis (Mizukami and Ma, 1992; Zhang et al., 2015). The Df-KjAG only contains the MADS domain, which is mainly responsible for target DNA binding (Shore and Sharrocks, 1995; Riechmann and Meyerowitz, 1997; Yang and Jack, 2004; Liu et al., 2010). Therefore, we speculated that the reduced stamen number and runtish stamens might be caused by competitive inhibition against the endogenous AG of Arabidopsis at downstream MADS domain target DNA in Df-KjAG transformants. These results suggested that Df-KjAG has lost the AG homologous function of reproductive organ determinacy. Loss-of-function or aberrant expression of AG orthologous genes often promotes double-flower formation, such as in Arabidopsis, C. japonica, T. thalictroides, Japanese morning glory, N. damascena, and tomato (Bowman, 1997; Benedito et al., 2004; Dong et al., 2007; Liu et al., 2013; Tanaka et al., 2013; Noor et al., 2014; Sun et al., 2014; Nakatsuka et al., 2015; Wang et al., 2015). Therefore, we speculated that the loss-of-function of Df-KjAG induces double-flower formation in K. japonica.

In single-flower K. japonica, Sf-KjAG was only expressed in stamens and carpels, which was consistent with the tissue expression patterns of AG and other AG homologous genes in species such as T. rupestris and P. lannesiana (Lü et al., 2007; Liu et al., 2013). In double-flower K. japonica, although Df-KjAG has lost the C-class function, its expression was still limited to inner whorls, which might be attributed to the repressors of AG homologs. In Arabidopsis, the inner whorl-specific activation of AG was achieved by the repressors of AG, such as BELLRINGER (BLR), LEUNIG (LUG), and SEUSS (SEU). BLR and SEU/LUG complex can be recruited to AG cis-regulatory region (such as the second intron of AG) and prevents the ectopic AG expression in the two outer whorls of the flower (Franks et al., 2002; Bao et al., 2004; Sridhar et al., 2006). However, the Df-KjAG expression level was significantly lower than that of Sf-KjAG in the inner two whorls (Figure 3A) and at all development stages (Figure 3C). Similar expression patterns were also found in T. thalictroides ‘Double White’, Cyclamen persicum, and double-flower Arabidopsis (de Folter et al., 2005; Gómezmena et al., 2005; Tanaka et al., 2013). In Arabidopsis, AG can form protein heterodimers with SEP3 to act in a positive auto-regulatory loop that maintains and amplifies AG expression (Honma and Goto, 2001; de Folter et al., 2005; Gómezmena et al., 2005; Sridhar et al., 2006; Alvarez-Buylla et al., 2010). In our Y2H analyses, Df-KjAG protein could not form protein complexes with KjAGL9, an SEP3 orthologous protein (Figure 7A). Therefore, the reduced Df-KjAG levels might be attributed to the fact that Df-KjAG protein cannot form a protein complex to maintain and amplify Df-KjAG expression. Loss-of-function of Df-KjAG also changed the expression patterns of KjAGL2 and KjAGL9 (putative E-class genes), KjAP1 and KjAP2 (putative A-class genes), and KjAP3 and KjPI (putative B-class genes) in whorls three (inner petals) and four (leaf-like carpels) of double-flower K. japonica. Although E-class genes may be the upstream genes of AG homologs, expression pattern modifications of E-class genes (AGL2 in Arabidopsis, and DEFH200 and DEFH72 in Antirrhinum majus) have been reported previously in the Arabidopsis ag mutant and Antirrhinum majus plena (C-class gene) mutant (Flanagan and Ma, 1994; Davies et al., 1996). These findings might suggest that the expression regulation among floral organ identity genes is not strictly hierarchical, and the expression of upstream genes can also be affected by the latter genes. The expression pattern changes of A-, B-, and E-class genes are broadly consistent with the mutant phenotypes observed in double-flower K. japonica. According to the ABCE model, A- and E-class genes are responsible for sepal development; A-, B-, and E-class for petal development; B-, C-, and E-class for stamen development; and C- and E-class for carpel development (Coen and Meyerowitz, 1991; Soltis et al., 2002; Liu et al., 2010; Heijmans et al., 2012; Soltis and Soltis, 2014). In double-flower K. japonica, transformation of stamens into petals (Figure 1) might lead to the similar expression of A-, B-, and E-class genes between inner petals and normal petals (Figure 7C); ectopic expression of B-class genes as well as expression of A- and E-class genes might be responsible for the adnation of petals with leaf-like carpels in whorl four (Figure 7C). In double-flower Japanese gentian, expression patterns of its A-class genes (GsAP1 and GsAP2) were also modified in whorls 3 and 4 (Nakatsuka et al., 2015). These findings might suggest that the expression patterns of floral organ identity genes in different whorls are maintained by mutual regulation, and aberrant expression of certain genes might lead to boundary shift or expression modification of other floral organ identity genes. The correct function of AG homologs in organ-specific programs requires the formation of protein–protein complexes with the putative floral organ identity transcription factors (Coen and Meyerowitz, 1991; Theiflen and Saedler, 2001; Liu et al., 2010). The protein–protein interaction patterns of Sf-KjAG are
consistent with those of AG in Arabidopsis and AG orthologous genes in other species such as Petunia hybrida and Antirrhinum majus (Davies et al., 1996; Favaro et al., 2003; Ferrario et al., 2003; Leseberg et al., 2008; Liu et al., 2010), which might suggest that Sf-KjAG plays the analogous function in floral organ development, similar to that of other AG homologous genes. The I domain is shown to be responsible for the selective formation of DNA-binding dimers; the K domain, for protein–protein interactions; and C domain, for the formation of higher-order complexes and functional specificity (Shore and Sharrocks, 1995; Riechmann et al., 1996; Riechmann and Meyerowitz, 1997; Honma and Goto, 2001; Yang and Jack, 2004; Kaufmann et al., 2005; Liu et al., 2010). Thus, the lack of I, K, and C domains of Df-KjAG may well explain the loss of normal interaction capability and function in specifying stamen and carpel determinacy. In addition, SF-KjAGA (MADS domain of SF-KjAG was deleted) can still interact with KjAP1, KjAGL2, and KjAGL9, which might indicate that the MADS domain of SF-KjAG seems to be dispensable in the dimerization with KjAP1, KjAGL2, and KjAGL9, and their dimerization mainly depends on I, K, and C domains (Figure 7A). Once the MADS domain of SF-KjAG protein was deleted, the remaining domains could generate strong interactions with KjPI, which cannot interact with SF-KjAG (Figure 7A). This might suggest that the MADS domain of SF-KjAG also plays a role in specific- or selective-dimerization, besides the main function in binding to target DNA.

CONCLUSION

Although we did not verify the double-flower formation directly in K. japonica, considering that woody plants have lengthy generation periods and are not amenable to the standard techniques of functional genomics, multiple evidences have been provided to show that the double-flower formation of K. japonica is likely to result from the loss-of-function of AG homologous gene by a transposon insertion into an intron of the AG homologous genomic DNA. The transposon insertion caused premature transcription termination and generated normal transcript by aberrant splicing at alternative splice-acceptor sites, and subsequently resulted in prominent loss in protein–protein interaction and function. Further, the loss-of-function of Df-KjAG would affect the expression patterns of itself and other related floral organ identity genes. Furthermore, we showed that a transposon within introns is caused by flower homeotic mutation, which provides further evidences supporting the potential roles of these elements in plant domestication and evolution.

AUTHOR CONTRIBUTIONS

FC conceived and directed this study. JM and XS performed the experiments, analyzed the data, and wrote the manuscript. ZL, DZ, WL, HL, YW, and ZH provided suggestions and revised the manuscript.

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SUPPLEMENTARY MATERIAL

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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