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Isolation and characterization of the C-class *MADS-box* gene involved in the formation of double flowers in Japanese gentian

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Abstract

Background: Generally, double-flowered varieties are more attractive than single-flowered varieties in ornamental plants. Japanese gentian is one of the most popular floricultural plants in Japan, and it is desirable to breed elite double-flowered cultivars. In this study, we attempted to characterize a doubled-flower mutant of Japanese gentian. To identify the gene that causes the double-flowered phenotype in Japanese gentian, we isolated and characterized *MADS-box* genes.

Results: Fourteen *MADS-box* genes were isolated, and two of them were C-class *MADS-box* genes (*GsAG1* and *GsAG2*). Both *GsAG1* and *GsAG2* were categorized into the PLE/SHP subgroup, rather than the AG/FAR subgroup. In expression analyses, *GsAG1* transcripts were detected in the second to fourth floral whorls, while *GsAG2* transcripts were detected in only the inner two whorls. Transgenic Arabidopsis expressing *GsAG1* lacked petals and formed carpeloid organs instead of sepals. Compared with a single-flowered gentian cultivar, a double-flowered gentian mutant showed decreased expression of *GsAG1* but unchanged expression of *GsAG2*. An analysis of the genomic structure of *GsAG1* revealed that the gene had nine exons and eight introns, and that a 5,150-bp additional sequence was inserted into the sixth intron of *GsAG1* in the double-flowered mutant. This insert had typical features of a *Ty3/gypsy*-type LTR-retrotransposon, and was designated as *Tgs1*. Virus-induced gene silencing of *GsAG1* by the *Apple latent spherical virus* vector resulted in the conversion of the stamen to petaloid organs in early flowering transgenic gentian plants expressing an Arabidopsis *FT* gene.

Conclusions: These results revealed that *GsAG1* plays a key role as a C-functional gene in stamen organ identity. The identification of the gene responsible for the double-flowered phenotype will be useful in further research on the floral morphogenesis of Japanese gentian.

Keywords: AGAMOUS, *Apple latent spherical virus* vector, Double-flowers, Japanese gentian, LTR-type retrotransposon, *MADS-box* genes

Background

Double-flowered plants are often preferred by consumers because they are larger, more floriferous, and more showy than single flowers [1]. Double-flowered varieties are more common than single-floweredvarieties for several important floricultural plants including carnation (*Dianthus caryophyllus*), rose (*Rosa hybrida*), and chrysanthemum (*Chrysanthemum* × *morifolium*). In other floricultural plants, the development of double-flowered varieties is one of the main breeding aims alongside improvements to floral color, size, scent, vase life, and disease resistance.

Generally, the flowers of dicotyledonous plants are composed of four types of organs; sepals, petals, stamens, and pistils, which are arranged in four whorls. In eudicots, floral organ identities are explained by the ABC model, which has been established from studies on two model plants, *Arabidopsis thaliana* and *Antirrhinum majus* [2].

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The ABC model includes many genes encoding MADS-box transcription factors. According to this model, there are three classes of gene functions. The A-function gene, *APETALA1* (*AP1*, *SQUAMOSA* (*SQUA*) in *A. majus*), is expressed in the first and second whorls. The B-function genes, *APETALA3* (*AP3, DEFICIENCE* (*DEF*) in *A. majus*) and *PISTILLATA* (*PI, GLOBOSA* (*GLO*) in *A. majus*) are expressed in the second and third whorls, and their encoded proteins gain their B-function when they form heterodimers [3]. The C-function genes are expressed in the third and fourth whorls, and play an important role in stamen and pistil formation. Male and female organ identities are specified by a single C-function gene, *AGAMOUS* (*AG*), in Arabidopsis, but by two C-function genes, *PLENA* (*PLE*) and *FARINELLI* (*FAR*), in *A. majus* [4]. The *A. majus ple* mutant was shown to form petal and petaloid organs in place of stamens and carpels, respectively [5], similar to the Arabidopsis *ag-1* mutant. *A. majus PLE* is an ortholog of Arabidopsis *SHATTERPROOF 1/2* (*SHP1/2*), which is involved in the dehiscence of mature fruit [6], but it is not an ortholog of AG. AG/FAR and SHP/PLE are paralogs, but not orthologs derived from a duplication event in a common ancestor [7].

To control floral organ identity, the B- and C-function genes also require *SEPALLATA* (*SEP*), which is defined as an E-function gene [8]. The proposed "quartet model" directly links floral organ identity to the action of four different tetrameric transcription factor complexes composed of MADS-box proteins [9, 10]. Petunia *FBP6* and *FBP11* are expressed in the ovule, and are defined as D-class *MADS-box* genes [11]. Recently, the petunia C- and D-clade genes were shown to have largely overlapping functions specifying ovule identity and floral termination [12]. D-function genes have also been identified in lily (*LMADS2*, [13]), *Eustoma grandiflorum* (*EgMADS2*, [13]), and Arabidopsis (*STK*, [14]).

The deficiency of C-function genes results in the conversion of third-whorl stamens to petals, and fourth-whorl pistils to sepals [15]. This sepal-petal-petal pattern repeats itself many times, resulting in flowers with many petals. In addition to its role in determining floral organ identity, AG also plays a role in terminating flower development [16, 17]. Double-flowered phenotypes result from C-function deficiency in most floricultural plants, including *Ipomoea nil* [18], *Rosa hybrida* [19], *Petunia hybrida* [20], *Cyclamen persicum* [21], and *Cymbidium ensifolium* [22]. Therefore, it is likely that double-flowers of Japanese gentian plants result from lost or impaired C-function gene (s), although this had not been confirmed experimentally.

Japanese gentian (*Gentiana scabra*, *Gentiana triflora,* and their interspecific hybrids) is one of the most popular floricultural plants in Japan, and is used as cut flowers and potted plants [23]. The genus *Gentiana* comprises more than 400 species, and belongs to the family Gentianaceae, which also contains the genera *Eustoma*, *Swertia*, and *Tripterospermum*. The flowers of Japanese gentian have a bell-shaped corolla with five lobes, five stamens partly fused with petals, and one pistil. Organs known as plicae, which are located between the lobes of the corolla, are a typical feature of the *Gentiana* genus. The petals of Japanese gentians are vivid blue, which is conferred by the polyacylated anthocyanin gentiodelphin [24]. The flavonoids of Japanese gentian, the structures of the anthocyanins and flavones, and the biosynthetic structural and regulatory genes associated with these pigments have been well studied [25]. More recently, we determined the structures of flavones that accumulate in the leaves and flowers of *G. triflora* and identified a novel glucosyltransferase gene involved in the formation of flavone-glucosides [26].

However, there have been few studies on the floral morphogenesis in Japanese gentian at the molecular level. Floral homeotic *MADS-box* genes have been isolated and characterized from *E. grandiflorum*, which belongs to the family Gentianaceae [27]. Although Mishiba et al. [28] isolated four *MADS-box* genes from *G. triflora* (*GtMADS1*–*GtMADS4*; Genbank accession numbers AB189429–AB189432), these genes have not been characterized in detail. To date, there have been no systematic characterizations of floral morphological *MADS-box* genes in Japanese gentian.

Here, we attempted to characterize a double-flowered mutant of *G. scabra,* a species closely related to *G. triflora*. We isolated and characterized *MADS-box* genes expressed in gentian flower buds, focusing on C-class *MADS-box* genes. We identified 14 *MADS-box* genes belonging to A, B, C, D, and E classes; these genes are presumably involved in floral development and organ identification. Analyses of a double-flowered mutant revealed that the phenotype was caused by an insertion of a novel retrotransposable element (*Tgs1*) into one of the C-function genes, *GsAG1*. This was confirmed by suppressing *GsAG1* using the *Apple latent spherical virus* (ALSV) vector. To our knowledge, this is the first report of the functional characterization of *MADS-box* genes involved in the floral morphogenesis of Japanese gentian, and the involvement of a retrotransposable element in its double-flowered phenotype.

Results

Isolation of *MADS-box* genes from Japanese gentian

The fragments of Japanese gentian *MADS-box* genes were amplified using degenerate primers designed from the conserved domain of AGAMOUS proteins, as described by Kramer et al. [29, 30]. After subcloning, 96 clones were sequenced, and 14 independent clones were identified. Using 5′-RACE technology, we obtained eight

independent clones of complete full-length cDNA sequences, whereas the 5′-upstream fragments corresponding to the other six clones were not obtained. In a phylogenetic analysis based on the deduced amino acid sequences, these Japanese gentian *MADS-box* genes clustered into four functional clades (Fig. 1, Additional file 1: Figures S1 and S2).

There were two gentian A-clade *MADS-box* genes; *GsAP1* (Genbank accession number LC022772) and *GsFUL* (LC022780). Core eudicot species have two types of A-class MADS-box lineage genes, *euAP1* and *euFUL* [31]. *GsAP1* and *GsFUL* were categorized into *euAP1* and *euFUL*, respectively (Additional file 1: Figure S1). The deduced amino acid sequence of *GsAP1* showed 63.9 % identity with that of *GsFUL*.

We also identified another six *MADS-box* genes, which were categorized as B-class genes (Additional file 1: Figure S2). The B-class *MADS-box* genes form three subgroups, *euAP3/DEF*, *TM6* (paleoAP3), and *PI/GLO* [32]. *GsAP3a* (LC022769) and *GsAP3b* (LC022774) were categorized into the *AP3/DEF* subgroup, while *GsPI1* (LC022770), *GsPI2* (LC022771), and *GsPI3* (LC022773) were categorized into the *PI/GLO* subgroup. *GsTM6* (LC022767) belonged to the TM6 subgroup derived from the *AP3/DEF* subgroup. The deduced amino acid sequence of *GsAP3a* exhibited 78.0 % and 59.8 % identities with those of *GsAP3b* and *GsTM6*, respectively. The deduced amino acid sequence of GsAP3b showed 60.1 % identity with that of GsTM6. GsAP3a exhibited 60.3 %, 77.1 %, and 72.4 % identities, while GsAP3b exhibited 56.7 %, 71.5% and 73.1 % with Arabidopsis AP3, *Antirrhinum* DEF, and petunia GP, respectively. GsTM6 exhibited 58.8 %, 57.3 %, and 52.4 % identities with tomato TDR6, petunia TM6, and rose MADSKO B3, respectively. GsPI1 exhibited 93.7 % and 86.3 % identity with GsPI2 and GsPI3, respectively, while GsPI2 showed 80.2 % identity with GsPI3. The GsPIs exhibited 55.7 %–58.9 %, 58.1 %–64.2 %, 68.1 %–70.8 %, and 59.9 %–67.3 % identities with Arabidopsis PI, *Antirrhinum* GLO, petunia pMADS2, and petunia GLO1, respectively.

The C-clade *MADS-box* genes can be separated into two subgroups, *AG/FAR* and *SHP/PLE* [7]. We isolated two Arabidopsis *AG*/*SHP* orthologs, *GsAG1* (LC022775) and *GsAG2* (LC022779), from Japanese gentian floral buds, and both belonged to the *SHP/PLE* subgroup (Fig. 1). No clones in the AG/FAR subgroup were obtained by degenerate PCR or by searching the gentian flower normalized library described by Nakatsuka et al. [33]. The deduced amino acid sequence of *GsAG1* showed 63.9 % identity with that of *GsAG2*. GsAG1 showed 68.8 %, 66.8 %, and 65.2 % amino acid sequence identity with petunia FBP6 [34], *A. majus* PLENA [5], and *I. nil* PEONY [18], respectively, whereas GsAG2 showed 68.4 %, 63.5 %, and 66.4 % identity, respectively.

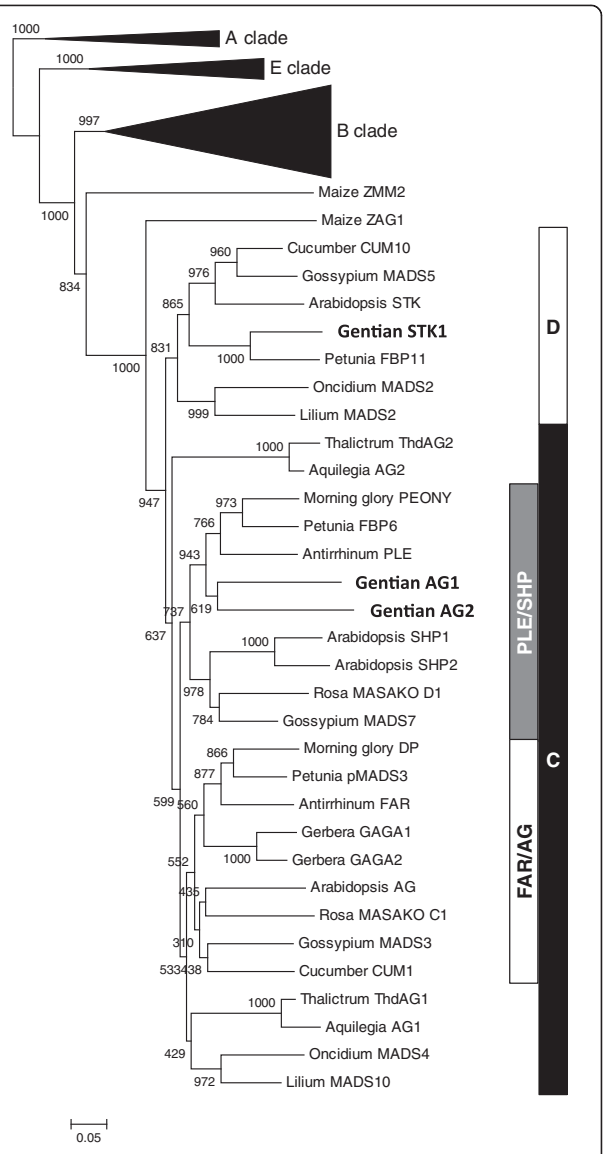

Fig. 1 Phylogenetic tree of C/D-class MADS-box proteins. Phylogenetic tree was constructed by the neighbor-joining method using ClustalW and visualized using MEGA6. Genbank accession numbers of amino acid sequences used in phylogenetic analysis are as follows: *Arabidopsis thaliana* AG (NP_567569), SHP1 (NP_001190130), SHP2 (NP_850377) and STK (NP_192734); *Antirrhinum majus* FAR (CAB42988) and PLE (AAB25101); *Aquilegia alpina AG1*(AAS45699) and AG2 (AAS45698); *Cucumis sativus* CUM1 (AAC08528) and CUM10 (AAC08529); *Gentiana scabra* GsAG1 and GsAG2 (this study); *Gerbera hybrida* GAGA1 (CAA08800) and GAGA2 (CAA08801); *Gossypium hirsutum* MADS3 (AAL92522), MADS5 (ABM69043) and MADS7 (ABM69045); *Ipomoea nil* DP (BAC97837) and PEONY (BAC97838); *Lilium longiflorum* MADS2 (AAS01766) and MADS10 (AIJ29174); *Oncidium hybrida* MADS2 (AIJ29175) and MADS4 (AIJ29176); *Petunia hybrida* FBP6 (CAA48635), FBP11 (CAA57445), PFG (AAF19721) and pMADS3 (Q40885); *Rosa rugosa* MASAKO C1 (BAA90744) and MADSKO D1 (BAA90743); *Thalictrum dioicum* ThdAG1 (AAS45683) and ThdAG2 (AAS45682); *Zea mays* ZAG1 (AAA02933) and ZMM2 (NP_001104926). Numerals beside branches indicate bootstrap values from 1,000 replicates. Scale bar indicates 0.05 amino acid substitutions per site

GsSTK1 (LC022768) showed high sequence similarity to STK (AGL11), which is encoded by a D-class *MADS-box* gene in Arabidopsis and regulates ovule development [35]. The deduced amino acid sequence of GsSTK1 showed 85.1 %, 80.9 %, and 64.9 % identity with that of *Eustoma grandiflorum* MADS1 [13], petunia FBP7 [36] and Arabidopsis STK [14], respectively. We also isolated three *SEP* orthologs, designated as *GsSEP1* (LC022776), *GsSEP2* (LC022777), and *GsSEP3* (LC022778), all of which were E-function *MADS-box* genes (Additional file 1: Figure S1).

The A-function genes included AP1-like *MADS-box* genes, and also AP2-like genes harboring two continuous AP2 domains. We isolated a *GsAP2* ortholog (LC022781) from the gentian petal normalized library described by Nakatsuka et al. [33]. The *GsAP2* cDNA was 1,813-bp long, and encoded a protein of 456 amino acid residues (Additional file 1: Figure S3). The *miR172*-target nucleotide sequences of *AP2* were conserved within the *GsAP2* coding regions.

Spatial expression analysis of *MADS-box* genes in different floral organ, leaves, and stems

The spatial expression patterns of isolated *MADS-box* genes were analyzed by semi-quantitative RT-PCR in wild-type Japanese gentian (Fig. 2). Among the A-clade *MADS-box* genes, *GsAP1* expression was restricted to the first and second whorls and stem tissues, while *GsFUL* transcripts were detected in all of the tissues tested. *GsFUL* was strongly expressed in the first and second floral whorls and also in stem tissues.

The expressions of *GsAP3a, GsAP3b,* and *GsTM6*, belonging to the AP3/DEF subfamily, were detected in all four whorls of the floral organs. There were high transcript levels of *GsAP3a* and *GsAP3b* in the petal and stamen, and high transcript levels of *GsTM6* in the pistil organs in addition to whorls 2 and 3. Transcripts of *GsAP3a*, *GsAP3b,* and *GsTM6* were detected in stem organs, but barely detected in leaves. In contrast to the AP3/DEF subfamily, the PI/GLO subfamily genes *GsPI1*, *GsPI2* and *GsPI3* were expressed only in the petal and stamen organs (Fig. 2). The transcript levels of *GsPI2* and *GsPI3* were approximately equal in the petal and stamen organs, whereas there were higher transcript levels of *GsPI1* in the petal than in the stamen. The three *GsPI* genes were expressed at undetectable levels in vegetative organs. Thus, the expression profiles of the *GsPI* genes belonging to PI/GLO subgroup differed from those of the genes in the AP3/DEF and TM6 subgroups.

The two C-class *MADS-box* genes, *GsAG1* and *GsAG2*, were strongly expressed in the third (stamen) and fourth whorls (pistil). Transcripts of *GsAG1* were also present in petals. Transcripts of both *GsAG1* and *GsAG2* were at very low levels or undetected in

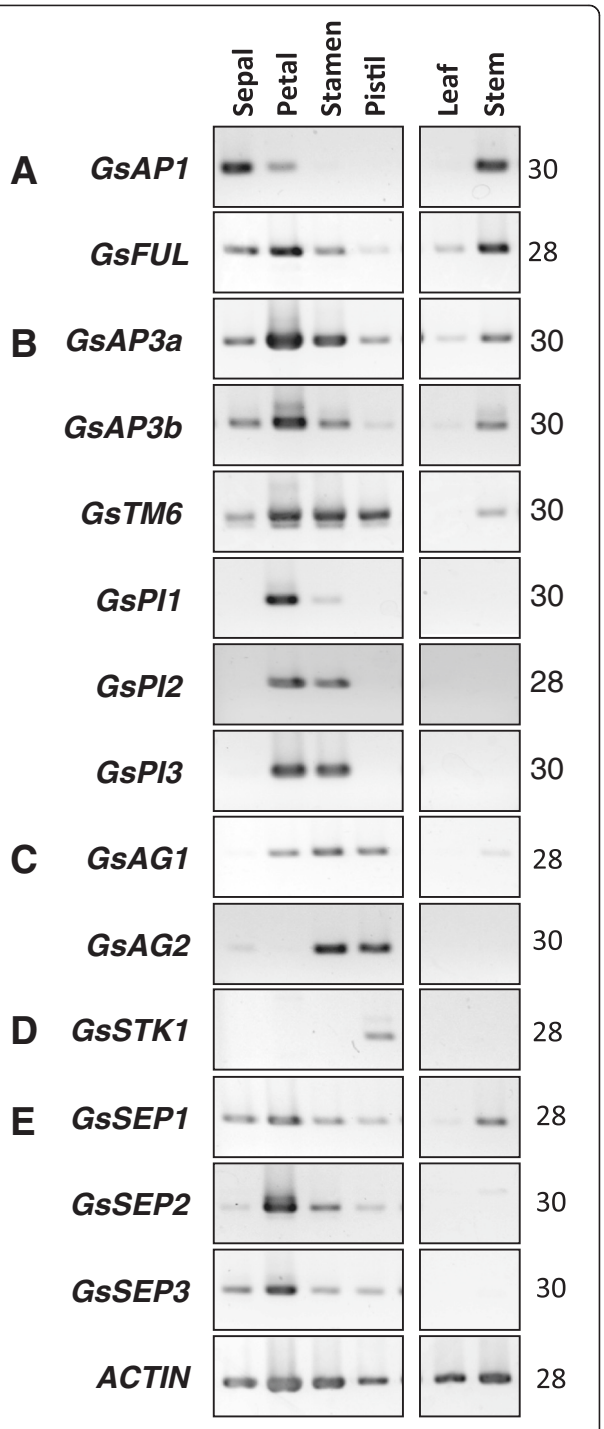

Fig. 2 Spatial expression profiles of *MADS-box* genes in floral organs of Japanese gentian. Semi-quantitative RT-PCR analysis was performed using total RNAs isolated from sepals, petals, stamens, and pistils of floral buds, and from leaves and stems. Expression profiles of 14 MADS-box genes were investigated: A-clade (*GsAP1* and *GsFUL*), B-clade (*GsAP3a*, *GsAP3b*, *GsTM6*, *GsPI1*, *GsPI2* and *GsPI3*), C-clade (*GsAG1* and *GsAG2*), D-clade (GsSTK1), and E-clade (*GsSEP1*, *GsSEP2* and *GsSEP3*) genes. *Actin* served as the reference gene. Gene names and cycle numbers are indicated at the left and right of panel, respectively

vegetative tissues (leaves and stems). Transcripts of *GsSTK1* were detected only in pistils, and not in other whorls, leaves, or stems. The three E-class *MADS-box* genes, *GsSEP1*, *GsSEP2,* and *GsSEP3*, showed similar expression profiles in floral organs. Transcripts of *GsSEP*2 and *GsSEP3* were detected all floral whorls but not in leaves or stems, whereas *GsSPE1* transcripts were detected in all floral whorls and in stems.

Heterologous expressions of *GsAG1* and *GsAG2* in Arabidopsis

To investigate the functions of *GsAG1* and *GsAG2*, we produced four and six lines of T_2 transgenic Arabidopsis plants overexpressing *GsAG1* or *GsAG2*, respectively. Ectopic expressions of C-class *MADS-box* genes in Arabidopsis and tobacco have been used to evaluate the function of AG orthologs from several plants [37, 38]. Ectopic expressions of *AG* genes have been shown to induce the *ap2* mutant phenotype; that is, pistil-stamen-stamen-pistil [39] Of the four *GsAG1*-overexpressing Arabidopsis lines, three formed carpeloid organs instead of sepals, and showed partial disappearance of petals (Fig. 3b–d). No morphological changes were observed in all six *GsAG2*-overexpressing Arabidopsis lines (Fig. 3e–f). These results revealed that the biological functional ortholog of Arabidopsis *AG* was *GsAG1,* not *GsAG2*.

Expression analysis of *MADS-box* genes in a double-flowered mutant

Next, we attempted to identify the cause of double-flowers in a gentian mutant. The double-flowered mutant had petaloid organs instead of stamens in the third whorl (Fig. 4a). The petaloid organ consisted of a petal structure fused to a sterile stamen. Some individuals of the double-flowered mutant also formed a slightly abnormal pistil that contained another incomplete pistil.

To identify the candidate gene responsible for the formation of double flowers, we compared the spatial expression profiles of C-class *MADS-box* genes between the double-flowered mutant and the typical single-flowered gentian cv. Alta (Fig. 4b). The transcript levels of *GsAG1* in the third and fourth whorls were significantly lower in the double-flowered mutant than in the single-flowered cultivar. In contrast, the abundance of *GsAG2* transcripts was not significantly different between the wild-type cultivar and the double-flowered mutant. The transcript levels of *GsAP2* in the inner two whorls were higher in the double-flowered mutant than in the wild-type plants (Fig. 4b). There were also differences between the wild-type cultivar and the double-flowered mutant in the transcription profiles of other A-class *GsAP1* and *GsFUL* genes in the second and third whorls. Slight differences in the expression patterns of some genes might be because of the different genetic backgrounds of the single-flowered cultivar and the double-flowered mutant. However, these results suggested that *GsAG1*, a C-class *MADS-box* gene, was the most likely candidate gene responsible for the double-flowered phenotype.

Genomic structures of *GsAG1* and *GsAG2* in Japanese gentian

In spatial expression analyses of Japanese gentian *MADS-box* genes, reduced *GsAG1* transcript levels were detected in male and female organs of the double-flowered mutant

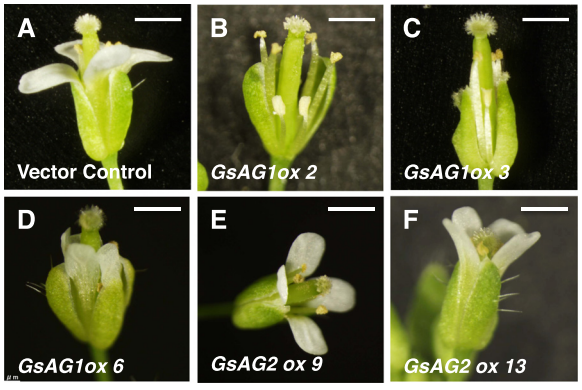

Fig. 3 Typical floral phenotypes of *GsAG1*- and *GsAG2*-expressing transgenic Arabidopsis plants. **a** Vector-control flower with normal sepal and petal organs. **b**–**d** Flowers of *GsAG1*-overexpressing transgenic lines nos. 2, 3, and 6 with sepals and petals converted into pistiloid and stamenoid organs, respectively. **e**–**f** Flowers of *GsAG2*-overexpressing transgenic lines nos. 9 and 13 with normal floral phenotypes. Expression of transgene in each T_2 transgenic plant is illustrated in Additional file 1: Figure S4. Bar = 10 mm

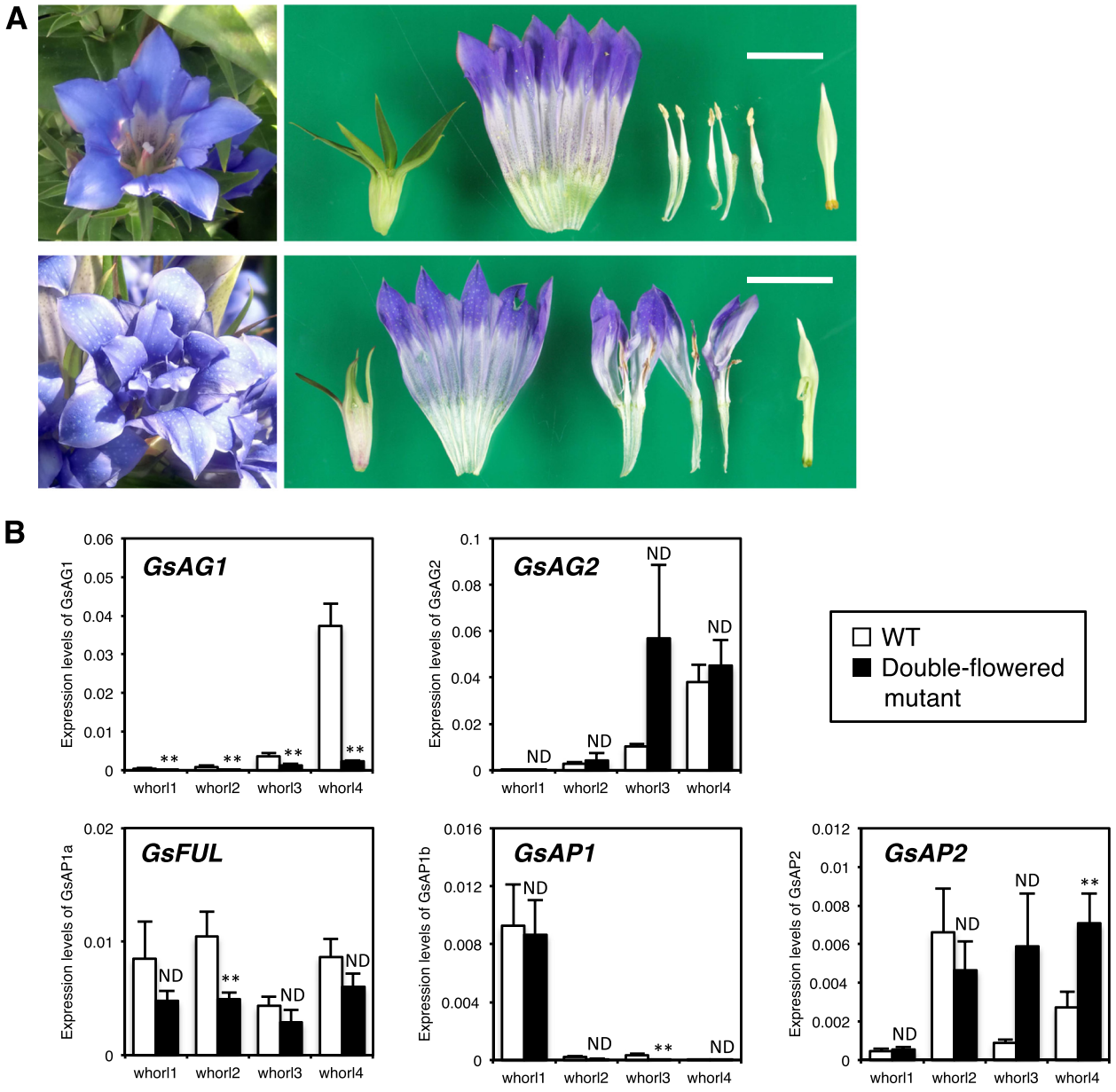

Fig. 4 Phenotype of double-flowered gentian mutant and spatial expression analysis of *MADS-box* genes. **a** Typical floral phenotypes of single flower cv. Alta (upper panels) and double-flowered mutant (lower panels). Bar = 2 cm. **b** qRT-PCR analysis of floral *MADS-box* genes in single-flowered cultivar (WT) and double-flowered mutant. Total RNAs were isolated from each whorl organ of floral buds at flower developmental stage 3 as defined by Nakatsuka et al. [58]. Values are the average of four biological replicates ± standard deviation. White bar indicates single-flowered gentian cv. Alta. Black bar indicates double-flowered mutant. ** and ND indicate significant difference ($P < 0.01$) and no significant difference, respectively (Student's *t*-test)

(Fig. 4b). Therefore, we determined the genomic sequences of *GsAG1* and *GsAG2* in the double-flowered mutant and control plants.

The genome sequence corresponding to *GsAG1* cDNA was 15.3-kb long, and consisted of nine exons and eight introns (Fig. 5a). The number and position of introns were conserved between Arabidopsis *AG* and *GsAG1*. The second and third introns of *GsAG1* (4.3 kb and 6.7 kb, respectively) were considerably longer than those of the corresponding introns in *AG* genes in other plants (2,998 bp and 102 bp, respectively, in Arabidopsis). The genomic sequence of *GsAG2* was 9.5-kb long and consisted of nine exons and eight introns, like *GsAG1* (Fig. 5b). The second intron of *GsAG2* was 6.6-kb long, but the third intron was shorter than that of *GsAG1*. The second intron of Arabidopsis *AG* contains transcriptional

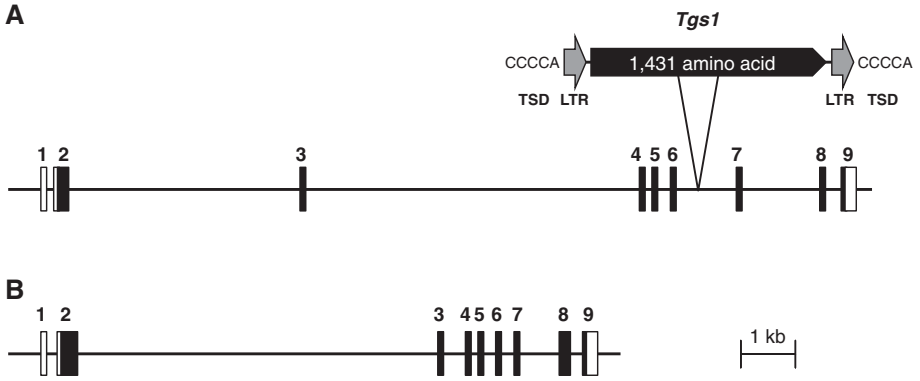

Fig. 5 Genomic structures of *GsAG1* and *GsAG2*. **a** Genomic structure of *GsAG1* in Japanese gentian. Open boxes show untranslated regions, filled boxes show translated regions with exons. Numerals above boxes indicate exon number. Scale bar indicates 1 kb. Open arrow indicates insertion position of transposable element *Tgs1* in *gsag1* in double-flowered mutant. TSD, target site duplication; LTR, long terminal repeat. *Tgs1* is 5,150-bp long with an ORF encoding 1,431 amino acid sequences of a gag-pol polyprotein, 334 bp of LTRs, and 5 bp of TSDs. **b** Genomic structure of *GsAG2* in Japanese gentian cv. Alta

regulation regions [7, 40]. The second intron region of both *GsAG1* and *GsAG2* had several *cis*-elements; a CArG box (CW_8G), a LFY binding site (CCANTG) and a 70-bp region (CCAATCA repeat) (data not shown).

Genomic structures of *GsAG1* and *GsAG2* in the double-flowered mutant

Next, we compared the genomic structures of *GsAG1* and *GsAG2* between the wild-type cultivar and the double-flowered mutant. Genomic PCR analyses targeting the sixth intron region of *GsAG1* amplified a fragment from wild type, but not from the double-flowered mutant (data not shown). Therefore, we sequenced the sixth intron of *GsAG1* in the double-flowered mutant using genome walking technology. The sixth intron of *GsAG1* in the double-flowered mutant had a 5,150-bp insertion that was not present in wild type. This inserted sequence had typical features of an LTR-retrotransposon, including a 5-bp target site duplication (TSD, CCCCA) and a 334-bp perfectly matching long terminal repeat (LTR) at both ends (Fig. 5a). The insert was designated as *Tgs1* (transposable element of *Gentiana scabra* 1). *Tgs1* encoded a 1,431-amino acid sequence of a *gag-pol* polyprotein belonging to the *Ty3*/*gypsy*-type retrotransposon group. There was no difference in the genomic structure of *GsAG2* between the double-flowered mutant and the wild-type cultivar (data not shown).

Suppression of *GsAG1* by virus-induced gene silencing

To confirm whether the deficiency of the *GsAG1* gene contributed to the double-flowered phenotype in Japanese gentian, we attempted to suppress the expression of *GsAG1* using VIGS. We used *Apple latent spherical virus* (ALSV) vectors because they have been used for reliable and effective VIGS in a broad range of plants [41, 42].

Gold particles coated with pEALSR1 and pEALSR2L5R5 were bombarded into *in vitro*-grown plants of transgenic Japanese gentian overexpressing *AtFT* [43]. One month after the bombardment, the proliferation of ALSV in inoculated plants was confirmed by RT-PCR analysis. The proliferation of ALSV was detected in almost all plantlets (data not shown), confirming that the direct bombardment of plasmid vectors was suitable to inoculate ALSV into gentian.

Twenty-two and 20 *AtFT*-overexpressing gentian plants were inoculated with either an empty ALSV vector (pEALSR1/pEALSR2L5R5) or the ALSV-GsAG1 vector (pEALSR1/pEALSR2-GsAG1), respectively. RT-PCR analysis confirmed that the biolistic inoculation of ALSV vectors resulted in a 90 % inoculation frequency (data not shown). The gentian plants inoculated with ALSV vectors were acclimated in a closed greenhouse, and set flowers after 1–3 months of acclimation. There was no significant difference in flower phenotype between wild type and plants inoculated with an empty ALSV vector (Fig. 6a). Six out of 14 surviving plants inoculated with ALSV-GsAG1 formed petals in place of stamens (Fig. 6b). The qRT-PCR analysis showed that plants showing the conversion phenotype by infection with ALSV-GsAG1 had significantly suppressed *GsAG1* transcript levels, compared with those in plants inoculated with the empty vector (Fig. 6c). The transcript levels of *GsAG2* were not affected by ALSV-GsAG1 infection. There was no significant morphological change in the pistils of ALSV-GsAG1-inoculated plants.

Discussion

In this study, we isolated 14 *MADS-box* genes expressed in floral buds of *G. scabra*: two A-class genes (*GsAP1* and *GsFUL*), six B-class genes (*GsAP1a*, *GsAP1b*, *GsTM6*,

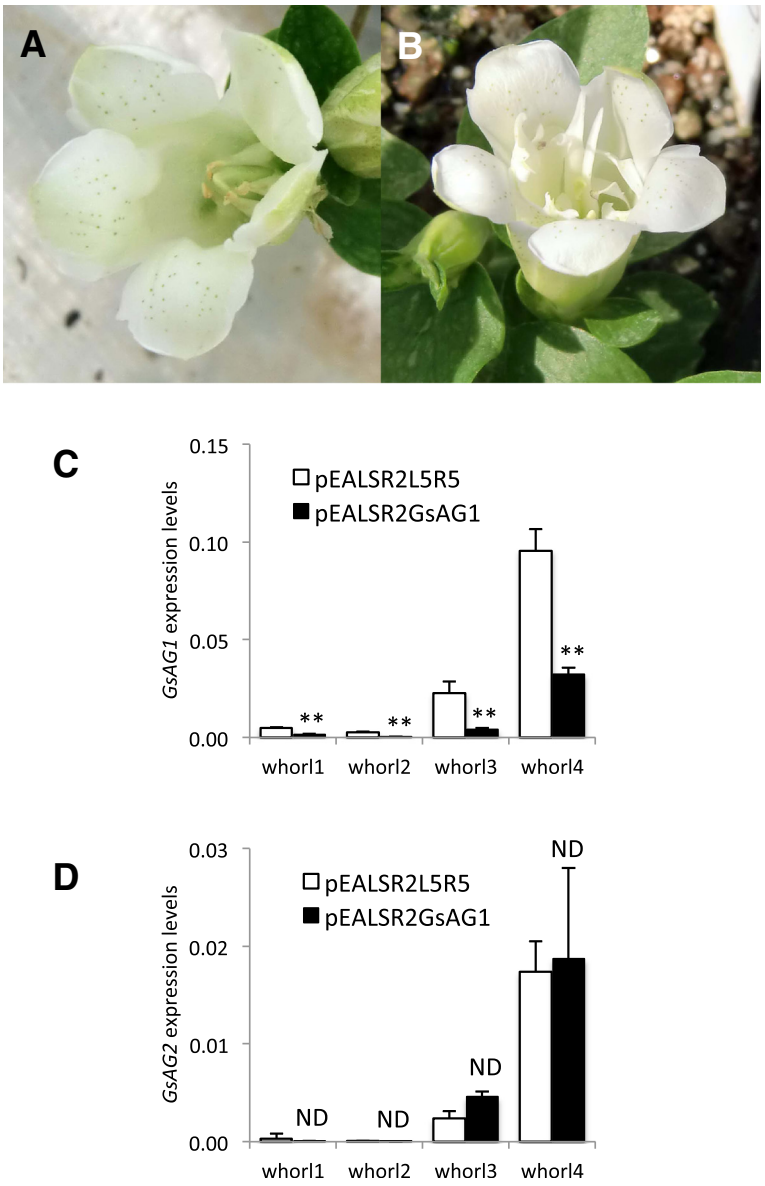

Fig. 6 Effects of *GsAG1* suppression by VIGS. Typical flower phenotypes of control ALSV-empty (pEALSR1/pEALSR2L5R5, **a**) and ALSV-AG1 (pEALSR1/pEALSR2-GsAG1)-infected plants (**b**). Spatial expression patterns of *GsAG1* (**c**) and *GsAG2* (**d**) in ALSV-empty and ALSV-GsAG1-infected plants. Flowers from three independent plants were examined for each treatment. Values are mean ± standard deviation ($n = 3$). ** and ND indicate significant difference ($P < 0.01$) and no significant difference, respectively (Student's *t*-test)

GsPI1, *GsPI2* and *GsPI3*), two C-class genes (*GsAG1* and *GsAG2*), one D-class genes (*GsSTK1*), and three E-class genes (*GsSEP1, GsSEP2,* and *GsSEP3*) (Fig. 1, Additional file 1: Figure S1 and Figure S2). Mishiba et al. [28] cloned four *MADS-box* genes, *GtMADS1–GsMADS4,* from *G. triflora*, a closely related species of *G. scabra*. Our analyses confirmed that GtMADS1–GtMADS4 are orthologs of *GsFUL*, *GsAG2*, *GsAG1,* and *GsSEP1*, respectively.

In Arabidopsis, AP1 and FUL function independently; the former controls sepal and petal identities, and the latter controls fruit development and determinacy [31]. In other core eudicots, plants with defective *AP1* genes formed leaf-like sepals, but their petal identity was unaffected [44]. Therefore, *euFUL* genes play an early role in promoting the transition to reproductive meristems and a late role in fruit development. In Japanese gentian, *GsAP1* expression was restricted to the first and second whorls of the floral bud and stem, and it was expressed strongly in the sepals and stems (Fig. 2). Conversely, *GsFUL* was expressed in all tested organs, and was strongly expressed in petals and stems (Fig. 2). As well as *GsAP1* and *FUL*, *GsAP2* might also act as an A-

function gene (Additional file 1: Figure S1). *GsAP2* was strongly expressed in the second whorl (Fig. 4b). In Arabidopsis, the expression of *AP2* is regulated by miR172 through translational inhibition [45]. The nucleotide sequence of *GsAP2* contained a conserved *miR172* target sequence (data not shown). Therefore, the whorl-specific expression of *GsAP2* might be controlled by miR172 in gentian, like in other plants.

In Arabidopsis and *A. majus*, B-function, which specifies petal and stamen identities, is determined by a heterodimer consisting of one AP3/DEF protein and one PI/GLO protein [46, 47]. *AP3*/*DEF* lineages can be categorized into two subgroups; *euAP3* and *paleoAP3* [29]. *euAP3* is widely distributed in higher eudicots, whereas *paleoAP3* is distributed in lower eudicots, magnoliid dicots, monocots, and basal angiosperms [48]. In addition, a number of higher eudicot species contain both *euAP3* and *paleoAP3* (designated as *TM6*). *AP3/DEF* genes belonging to the euAP3 (*GsAP3a* and *GsAP3b*) and TM6 (*GsTM6*) groups were isolated from Japanese gentian (Additional file 1: Figure S2). Three *euAP3*, one *TM6*, and two *PI/GLO* genes were also identified from *Eustoma grandiflorum* in the family Gentianaceae [27]. Therefore, it seems that a *TM6* gene encoding a B-class MADS-box protein is present in the family Gentianaceae, but not in the Solanaceae [35] or Asteraceae [49]. Geuten and Irish [50] reported that the PI/GLO lineage was duplicated and separated into GLO1 and GLO2 lineages in the Solanaceae. Their results also implied that the GLO1 lineage has been lost from the Gentianales and the GLO2 lineage lost from the Lamiales. The results of the present study indicated GsPI1 to GsPI3 in Japanese gentian are in the GLO2 lineage (Additional file 1: Figure S2). *GsAP3a*, *GsAP3b*, and *GsTM6* were expressed in all floral whorls (Fig. 2). High transcript levels of *GsAP3a* and *GsAP3b* were detected in the second (petal) and third whorls (stamen), and GsTM6 was expressed at high levels in whorls 2–4. On the other hand, the expressions of the three *GsPIs* were clearly restricted to the second and third whorls (Fig. 2). These differences in expression profiles among *euAP3*, *TM6*, and *PI/GLO* were also reported in petunia [48]. In petunia, *PhTM6* is mainly expressed in third and fourth whorls and is involved in stamen development but not petal development, while *PhDEF* is involved in both petal and stamen development [51, 48].

Both *GsAG1* and *GsAG2* were categorized into the SHP/PLE subgroup but not the AG/FAR subfamily (Fig. 1). In this study, we could not find any paralogous genes belonging to the *AG/FAR* subgroup by degenerate PCR technology. In *E. grandiflorum*, which also belongs to the family Gentianaceae, three *SHP*/*PLE* subgroup genes (*EgPLE1* to *EgPLE3*) were identified, but no *AG*/*FAR* subgroup genes [27]. The *AG/FAR* subgroup of C-class *MADS-box* genes is responsible for male and female organ identity in several plant species. This subgroup of genes includes Arabidopsis *AG* [52], petunia *pMADS3* [53], and *I. nil DUPLICATED* (*DP*, [18]). Members of the SHP/PLE subgroup also play a major role in floral organ identity in *A. majus* [5]. Therefore, *AG/FAR* subgroup genes might have disappeared from some species in the Gentianaceae, leaving *SHP/PLE* subgroup genes to function as C-class genes, although further analysis such as whole-genome sequencing should be conducted to confirm this hypothesis.

There is only one C-class *MADS-box* gene, a single copy of *AG*, in Arabidopsis. However, there are two *AG* paralogs in some plant species, including *A. majus* (PLE/FAR, [4]), petunia (pMADS3/FBP6, [34]), cucumber (CUM1/CUM10, [34]), maize (ZAG1/ZMM2, [54]), *I. nil* (DP/IN, [18]), and cyclamen (CpAG1/CpAG2, [21]). In maize, *ZAG1* transcripts accumulate in developing ears rather than in tassels, whereas *ZMM2* transcripts are more abundant in tassels [54]. In the *ple* single mutant of *A. majus*, the fourth whorl develops as two sepaloid/carpeloid/petaloid organs. The fourth whorl organs of *ple*/*far* double mutants develop as four or five well-formed petals [4]. Thus, *PLE* and *FAR* appear to contribute unequally to the specification of male and female organs.

GsAG1 transcripts were detected in the inner three whorls, whereas *GsAG2* transcripts were restricted to the third and fourth whorls (Fig. 2). *GsAG1* transcripts were detected in petal organs (whorl 2) in the RT-PCR analysis (Fig. 2) but not in the qRT-PCR analysis (Fig. 4). The RT-PCR and qRT-PCR analyses were performed using floral buds at different floral development stages, S1 (immature bud) and S3 (just before anthesis), respectively. In general, *AG* is expressed in either the third or fourth whorls [15]. Therefore, *GsAG1* expression in the second whorl in Japanese gentian appears to be a unique phenomenon. This may be because the petals and stamens of Japanese gentians are fused at their lower halves. Therefore, at an early floral developmental stage, young petal organs might contain stamen primordia. As shown in the qRT-PCR analysis (Fig. 4), no *GsAG1* transcripts were detected in the second whorl because both petal and stamen organs were completely distinguishable at the later stage of floral development.

The heterologous expression of *GsAG1* in transgenic Arabidopsis caused the conversion of sepals into carpeloid organs, indicating its *AG* function (Fig. 3b). In contrast, *GsAG2*-expressing Arabidopsis showed no significant changes in morphogenesis compared with the empty vector control (Fig. 3c). Ectopic expressions of Arabidopsis *AG* or *Antirrhinum PLE* specified homeotic conversion of the first and second whorl organs, causing sepals to develop as carpels and petals to develop as stamens [37, 7]. The ectopic expression of *Antirrhinum* FAR converted petals to stamens, but did not alter sepal

identity [7]. Thus, heterologous expression analyses in Arabidopsis do not always correctly evaluate the function of C-class *MADS-box* genes from other plant species.

Most double-flowered phenotypes result from a deficiency of C-function genes [2]. The qRT-PCR analysis showed that *GsAG1* transcripts were markedly decreased in the third and fourth whorls of double-flowered Japanese gentian, compared with those in single-flowered wild-type Japanese gentian (Fig. 4b). No *GsAG1* transcripts were detected in the doubled-flower mutant by RT-PCR using several primer combinations (data not shown), and no truncated *GsAG1* transcripts were detected by 3′-RACE. A sequencing analysis revealed that the double-flowered mutant had an insertion of a 5,150-bp putative retrotransposable element in the sixth intron of *GsAG1* (Fig. 5a). This transposable element, *Tgs1*, had the typical features of *Ty3*/*gypsy*-type retrotransposable elements (Fig. 5a). In the *duplicated* (*dp*) mutant of *I. nil*, the mutation was due to the rearrangement of genomic structure by the *Em/Spm* transposable element [18]. The *Antirrhinum ple* mutant was shown to have an insertion of the *Tam3* transposable element in the second intron of *PLE*, and *ple* mRNA was hardly detected in the floral organs of the mutant [5]. It was also reported that a double-flowered ranunculid mutant was associated with the insertion of a solo LTR retrotransposon into the fourth exon of *ThAG1* [55]. Thus, it is likely that the expression of *GsAG1* would be interrupted by the insertion of the long transposable element in the sixth intron.

VIGS is a useful tool for the functional analysis of genes in horticultural plants that are recalcitrant to other means of genetic transformation [56]. Petunia plants in which both *pMADS3* and *FBP6* were silenced by VIGS formed petaloid organs in place of carpels, depending on the cultivar [57]. Most viral vectors are excluded from meristematic tissue, and therefore, gene silencing in the meristem is not possible in most instances [56]. In this study, we used VIGS to silence *GsAG1* and observed that stamens were converted into petaloid organs (Fig. 6b). These results strongly suggested that the deficiency of *GsAG1* was responsible for the double-flowered phenotype of this mutant. Enhanced transcript levels of *GsAP2* were detected in the third and fourth whorls of the double-flowered mutant (Fig. 4b). In contrast, the spatial expression profiles of *GsAP1* and *GsFUL* were similar between the single-flowered cultivar and double-flowered plants. Mizukami and Ma [39] reported that AG antagonizes the function of AP2. Therefore, we speculated that *GsAG1* controls the whorl-specific expression of *GsAP2*.

In the double-flowered gentian mutant, the fourth-whorl pistil was not converted into petals, possibly because of the function of GsAG2. Compared with single-flowered gentian, the double-flowered mutant showed increased expression of *GsAG2* in the third whorl (Fig. 4b). There were also increased transcript levels of *GsAG2* in double-flowered transgenic gentians in which *GsAG1* was suppressed by VIGS (Fig. 6b). In *Antirrhinum*, *PLE* is required for full expression of *FAR*, whereas *FAR* negatively regulates the expression of *PLE* [4]. It is possible that *GsAG1* negatively regulates the expression of *GsAG2* in the third whorl of Japanese gentian. Unfortunately, there are no *GsAG2*-deficient mutants in nature; therefore, to show the function of the GsAG2, the suppression of *GsAG2* by VIGS should be attempted in future studies. In cyclamen, *CpAG1* is involved in stamen formation, and the deficiency of *CpAG1* caused the homeotic conversion of stamens into petals, resulting in double-petal phenotypes [21]. Overexpression of CpAG2-SRDX (a chimeric repressor) in the cyclamen *cpag1* mutant resulted in a multiple-petal phenotype, and the conversion of pistils into petals [21]. Thus, two C-class MADS orthologs contribute to male and female organ identity. Noor et al. [57] demonstrated that VIGS suppression of both *MADS3* and *FBP6* resulted in the conversion of the stamen/carpel into petal/petaloid organs, resulting in double flowers.

The current hypothesis is that GsAG1 plays an important role in male organ identify, while GsAG2 plays important roles in female organ identity and in terminating flowering. To confirm this hypothesis, *GsAG2*- and *GsAG1/GsAG2*- knockdown or knockout lines of Japanese gentian should be generated and analyzed in further studies.

Conclusions

We investigated the causal factor (s) of a double-flowered mutant in Japanese gentian. We isolated and characterized 14 *MADS-box* genes and revealed that a novel retrotransposable element (*Tgs1*) inserted into the sixth intron of *GsAG1* gene is responsible for the mutant flower phenotype. This was confirmed by ALSV-based VIGS system in combination with Arabidopsis *FT*-expressing early flowering transgenic gentian plants. Further investigations will be required to fully understand the developmental regulation of floral morphogenesis in Japanese gentian. As variation in floral shape is currently limited in Japanese gentians, we believe that this information will be helpful for breeding gentian cultivars with variation in floral shape in the future.

Methods

Plant materials

Japanese gentian (*Gentiana scabra*) cv. Alta was grown in a field at the Iwate Agricultural Research Center (Kitakami, Iwate, Japan). The double-flowered mutant was purchased from Iwasaki-Engai Co. (Kitahiroshima,

Hokaido, Japan) and grown as potted plants in the greenhouse of Iwate Biotechnology Research Center. Floral bud samples were collected at developmental stage 1, as defined by Nakatsuka et al. [58], and then stored at −80 °C until RNA extraction.

Isolation of MADS-box genes from gentian flower buds

Total RNAs were isolated from the floral buds of Japanese gentian and purified using RNAiso Plus and Fruit-mate kits (Takara-bio, Otsu, Shiga, Japan). The cDNAs were synthesized by an RNA PCR kit (AMV) Ver. 3 (Takara-bio). The candidate gentian *MADS-box* genes were isolated using degenerate primers as described by Kramer et al. [29, 30]. The amplified fragments were subcloned into the pCR4TOPO TA cloning vector (Invitrogen, Carlsbad, CA, USA) and sequenced using a BigDye terminator ver. 1.1 cycle sequencing kit and an ABI PRISM 3130xl DNA sequencer (Applied Biosystems, Foster City, CA, USA). To obtain the full-length cDNA of each gentian *MADS-box* gene, 5′-rapid amplification of cDNA ends (5′-RACE) was performed using a GeneRacer kit with SuperScript III RT (Invitrogen). The amplified fragments were subcloned and sequenced as described above. Nucleotide sequences were translated into deduced amino acid sequences using CLC Sequence Viewer 7 (CLC bio, Aarhus, Denmark) and compared using the BLAST network service at the National Center for Biotechnology Information. The phylogenetic tree was constructed using ClustalW with neighbor-joining algorithm and visualized using MEGA ver. 6 software [59].

The *GtAP2* ortholog, which is not categorized as a MADS-box protein, was found by BLAST searches of in-house gentian petal cDNA library data [33], and then the full-length cDNA was obtained using RACE technology as described above.

Gene expression analysis

To investigate the spatial expression profiles of gentian *MADS-box* genes, we performed semi-quantitative reverse transcription-PCR (RT-PCR) and quantitative RT-PCR (qRT-PCR) analyses. Total RNAs (1 μg) were isolated from each organ as described above, and then genomic DNA was eliminated and cDNAs were synthesized using gDNA Eraser and PrimeScript RT, respectively (Takara-bio).

For the RT-PCR analyses, the reaction mixture (50 μL) consisted of 1 × *Ex Taq* buffer, 0.2 mM dNTPs, 0.4 μM each primer, 2.5 U *Ex Taq* polymerase (Takara-bio), and 1 μL template cDNA. The PCR cycling conditions were as follows: 2 min at 94 °C, 26–34 cycles of 20 s at 95 °C, 40 s at 55 °C, and 1 min at 72 °C, and final extension for 10 min at 72 °C. The PCR products were electrophoresed on a 1.5 % agarose gel in TAE buffer and then stained with ethidium bromide.

The qRT-PCR analyses were performed with the StepOne Plus system (Applied Biosystems) using SYBR GreenER qPCR Super Mix (Invitrogen) as described previously [33]. Briefly, the reaction mixture (10 μL) consisted of 1 × Master Mix, 0.2 μM each primer, and 1 μL template cDNA. The cycling conditions were as follows: 95 °C for 20 s, followed by 40 cycles of 95 °C for 1 s and 60 °C for 20 s. The specificity of each amplification reaction was checked by a melting curve analysis. Fluorescence was measured at the end of each annealing step. The data were analyzed using StepOne Plus Software Version 2.2.2. The transcript level each gene was calculated relative to that of the reference gene *GtUBQ*. qRT-PCR analyses were performed using four biological replicates, and data were statistically analyzed by Student's *t*-test. The sequences of all primers used in this study are listed in Additional file 1: Table S1.

Production of transgenic Arabidopsis plants

GsAG1 and *GsAG2* ORFs under the control of the CaMV35S promoter were each inserted into a binary vector harboring the kanamycin resistance (*NPTII*) gene to produce the plasmids pSkan-35S:: GsAG1 and pSkan-35S:: GsAG2, respectively. Each binary vector was transformed into *Agrobacterium tumefaciens* EHA101 by electroporation (MicroPulser: Bio-Rad, Tokyo, Japan). *A. thaliana* ecotype col-1 was transformed by the floral dip method as described by Clough and Bent [60]. Positive transformants were selected on germination medium containing 50 mg L^{-1} kanamycin. *GsAG1*- and *GsAG2*-expressing T_2 transgenic plants (homozygous) were obtained by self-pollination. The floral morphogenetic phenotypes were observed in four *GsAG1*-expressing lines and in six *GsAG2*-expressing lines.

Determination of genomic structures of *GsAG1* and *GsAG2*

The genomic nucleotide sequences of *GsAG1* and *GsAG2* were obtained using genome walking technology with a GenomeWalker Kit (Clontech, Takara-bio). Genomic DNA was isolated from young leaves of *G. scabra* 'Alta' and the double-flowered mutant using Nucleon PhytoPure (GE Healthcare Ltd., Buckinghamshire, UK). Amplified fragments were subcloned and then sequenced as described above.

Virus-induced gene silencing of *GsAG1* in gentian plants

To investigate the function of *GsAG1*, we conducted virus-induced gene silencing (VIGS) using the *Apple latent spherical virus* (ALSV) vector [41]. The trigger fragment was amplified using primers harboring *Xho*I or *Bam*HI sites (Additional file 1: Table S2), and then subcloned into the pGEM-T Easy vector (Promega, Madison, MI, USA). The fragment was excised by double-digestion

with *Xho*I and *Bam*HI, and then ligated into pEALSR 2L5R5 [61] digested with the same enzymes. Large-scale plasmid purification was conducted using a NuleoBond Xtra Midi plus kit (Macherey-Nagel, Takara-bio).

The ALSV vector was inoculated into *G. hybrida* 'Polarno White' plants overexpressing the Arabidopsis *FLOWERING LOCUS T* (*AtFT*) gene. The *AtFT*-expressing gentian plants flower earlier than wild-type gentian [43]; therefore, they are useful for studies on floral morphogenesis. Gentian plants expressing *AtFT* were grown *in vitro* and inoculated with ALSV vectors by the PDS-1000/He particle Delivery system (Bio-Rad Laboratory). A 0.5-mg aliquot of gold particles (1.0 μm diameter; Bio-Rad Laboratories, Hercules, CA, USA) was mixed with 100 μL plasmid solution, which contained 5 μg pEALSR1 and 5 μg pEALSR2L5R5 derivatives, 10 μL 10 M ammonium acetate, and 220 μL isopropanol. The mixture was kept at −20°C for at least 1 h. Gold particles coated with plasmid DNA were washed three times with 1 mL ethanol and re-suspended in 10 μL ethanol. Particles were bombarded with 1,100 psi pressure at a distance of 10 cm from the microcarrier holder. After bombardment, virus-infected plants were acclimated and then grown in a closed greenhouse until flowering.

Availability of supporting data

The GenBank/EMBL accession numbers of genes identified in this study are: *GsAP1* (LC022772), *GsFUL* (LC022780), *GsAG1* (LC022775), *GsAG2* (LC022779), *GsSTK1* (LC022768), *GsSEP1* (LC022776), *GsSEP2* (LC022777), *GsSEP3* (LC022778) and *GsAP2* (LC022781).

Phylogenetic data have been deposited in TreeBASE respository and is available under the URL http://purl.org/phylo/treebase/phylows/study/TB2:S17877.

Additional file

Additional file 1: Table S1. Sequences of primers used for RT-PCR and qRT-PCR analyses. **Table S2.** Sequences of primers used for ALSV vector construction. **Figure S1.** Phylogenetic tree of A- and E-class MADS-box proteins. *Arabidopsis thaliana*, AP1 (NP_177074), CAL (NP_564243), FUL (NP_568929), SEP1 (NP_568322), SEP2 (AAU82009), SEP3 (NP_564214), SEP4 (NP_178466); *Antirrhinum majus* SQUA (CAA45228); *Gentiana scabra* GsAP1 and GsFUL (this study); *Petunia hybrida* FBP2 (Q03489) and FBP5 (AAK21248). Phylogenetic tree was constructed using the neighbor-joining method with ClustalW and visualized using MEGA6. Numerals beside branches indicate bootstrap values from 1,000 replicates. Scale bar indicates 0.05 amino acid substitutions per site. **Figure S2.** Phylogenetic tree of B-class MADS-box proteins. Phylogenetic tree was constructed by the neighbor-joining method using ClustalW and visualized using MEGA6. Genbank accession numbers of amino acid sequences used in phylogenetic analysis are as follows: *Antirrhinum majus* DEF (P23706), GLO (Q03378); *Arabidopsis thaliana*, AP3 (AAD51903), PI (AAD51984); *Chrysanthemum* × *morifolium* CDM86 (AA022986), CDM115 (AA022985); *Gentiana scabra* AP3a, AP3b, PI1, PI2, PI3, TM6 (this study); *Gerbera hybrida* GDEF1 (Q9ZS28), GDEF2 (Q9ZS27), GDEF3 (ACV53813), GGLO1 (Q9ZS26); *Onchidium hybrida* MADS3 (AA045824), MADS5 (ADJ67234), MADS8 (ADJ67236), MADS9 (ADJ67235); *Oryza sativa* MADS2 (AAB52709), MADS16 (Q944S9); *Petunia* × *hybrida* GLO1 (AAS46018), GP (CAA49567), pMADS2 (QO7474); *Phalaenopsis equestris* MADS2 (AAR26628), MADS3 (AAR26629), MADS4 (AAR26626), MADS5 (AAR26627), MADS6 (AAV83997); *Rosa rugosa* MASAKO B3 (BAB63261), MASAKO BP (BAB11939), MASAKO euB3 (BAC79180); *Solanum lycopersicum* TDR6 (XP_004232453); *Torenia fournieri* DEF (BAG24492), GLO (BAG24493). Numerals beside branches indicate bootstrap values from 1,000 replicates. Scale bar indicates 0.05 amino acid substitutions per site. **Figure S3.** Phylogenic tree constructed using deduced amino acid sequences of GsAP2 and other AP2 orthologs. Phylogenetic tree was constructed by the neighbor-joining method using ClustalW and visualized using MEGA6. Numerals beside branches indicate bootstrap values from 1,000 replicates. Amino acid sequences of AP2 orthologs were used as data sets as described by Karlova et al. [62]. Scale bar indicates 0.05 amino acid substitutions per site. **Figure S4.** Expression of transgenes in GsAG1-expressing and GsAG2-expressing T_2 Arabidopsis plants. Four *GsAG1*-expressing lines (nos. 2, 3, 5 and 6) and six *GsAG2*-expressing lines (nos. 1, 3, 6, 7, 9 and 13) of transgenic plants were obtained. VC, vector control plants (harboring binary vector pIG121Hm). Semi-quantitative RT-PCR analysis was performed using total RNAs isolated from leaves. Analyses targeted *GsAG1*, *GsAG2,* and *Actin2* as a reference gene. Gene names and cycle numbers are indicated at the left and right of panel, respectively.

Abbreviations

AG: AGAMOUS; ALSV: *Apple latent spherical virus*; AP1: APETALA1; AP2: APETALA2; FAR: FARINELLI; GLO: GLOBOSA; LTR: Long terminal repeat; PLE: PLENA; qRT-PCR: quantitative reverse transcription-PCR; RT-PCR: semi-quantitative reverse transcription-PCR; SEP: SEPALLATA; SHP: SHATTERPROOF; STK: SEEDSTICK; TSD: Target site duplication; VIGS: Virus-induced gene silencing.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TN and MN designed the research, TN, MS, EY, KF, N. Yamagishi, and MN performed the research, N. Yoshikawa helped to design ALSV, and TN and MN wrote the paper. All authors read and approved the final manuscript.

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