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Original Paper

Expression and functional analysis of apple *MdMADS13* on flower and fruit formation

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Abstract Apple *MdMADS13* has a transcription factor with MADS domain. Moreover, it is expressed specifically at petals and carpels. The product forms a dimer with *MdPISTILLATA* (*MdPI*) protein as a class B gene for floral organ formation. Reportedly, in parthenocarpic cultivars of apple (Spencer Seedless, Wellington Bloomless, Wickson and Noblow) the *MdPI* function is lost by genome insertion of retrotransposon, which cultivars show a homeotic mutation of floral organs, petals to sepals and stamens to carpels. Apple fruit is pome from receptacle tissue, and *MdSEPALLATA* (*MdMADS8*/*9*) and *AGAMOUS* homologues *MdMADS15*/*22* involved in the fruit development, the transgenic apple suppressed these gene showed poor fruit development and abnormal flower formation. This article describes that the *MdMADS13* retained expression after blossom and small fruits of parthenocarpic cultivars. Yeast two-hybrid experiment showed specific binding between MdPI and MdMADS13 proteins. Furthermore, transgenic *Arabidopsis* with 35S::MdMADS13 have malformed stamens and carpels. These results suggest strongly that *MdMADS13* is related to flower organ formation as a class B gene with *MdPI*.

Key words: apple, floral organ, MdMADS13, parthenocarpy, retrotransposon.

Introduction

Apple (*Malus*×*domestica* Borkh.) is a widely produced fruit in temperate zones of world. Apple must be pollinated for fruit formation as in other fruit trees. Therefore, stable production of fruits requires normal fertilization and seed development. Natural threats to the pollination are low temperature during blossom time, spring frosts damage to carpels, and global honey bee issues. The merits of parthenocarpy are that it avoids such threats posted by natural conditions. Furthermore, it can save labor requirements for pollination. Reportedly, some apple cultivars show parthenocarpy and homeotic mutation of floral organs. The homeotic mutation replaces petals to sepals and stamens to carpels, known as class B mutation of the floral organ ABC model (Weigel and Meyerowitz 1994). The parthenocarpic cultivars all had an insertion of retrotransposon into the genome of *MdPISTILLATA* (*MdPI*) and lost the expression of *MdPI*, which was an orthologue of *PISTILLATA* of *Arabidopsis* class B gene (Tanaka et al. 2007; Yao et al. 2001). This parthenocarpy and homeotic mutation were co-inherited tightly (Tobutt 1994), but involvement between the parthenocarpy and loss of *MdPI* expression has not been elucidated to date. Our trial undertaken to resolve this enigma was conducted by producing transgenic apples with knockout of *MdPI* expression, as in parthenocarpic apples. Anti-sense *MdPI* expressed transgenic apples had class B homeotic mutation of floral organs as did the parthenocarpic cultivars, but they did not have parthenocarpy as the unexpected result (Tanaka et al. 2016). Anti-sense method enables a decrease in the level of mRNA from a target gene, but it does not provide complete suppression

Abbreviations: MdPI, MdPISTILLATA; SEP, SEPALLATA; AP3, APETALA3; 3AT, 3-amino-1, 2, 4 triazole; X-α-Gal, 5-bromo-4-chloro-3-indolyl-α-D-galactoside; Cl-O, Columbia.

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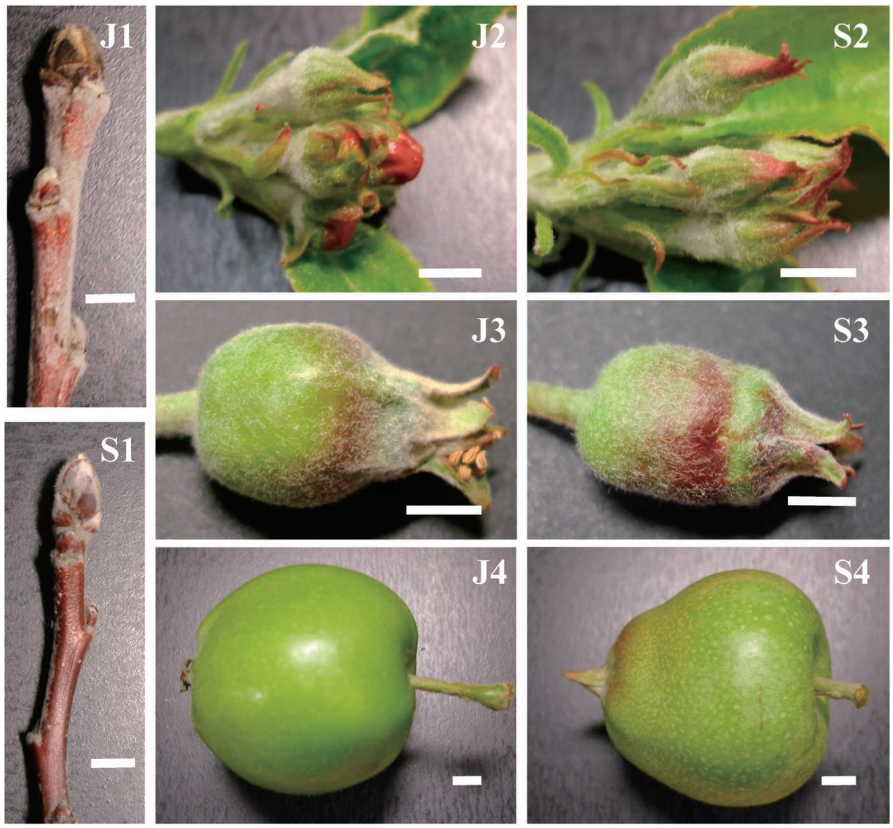

Figure 1. Normal 'Jonathan' and parthenocarpic 'Spencer Seedless' from flower buds to fruits. The J1 to J4 from normal cultivar 'Jonathan' and S1 to S4 from parthenocarpic cultivar 'Spencer Seedless' at each stage. These photographs were taken that J1 and S1 were at April 2, J2 and S2 were at May 1, J3 and S3 were at May 29 and J4 and S4 were at July 2 in 2014 respectively. White bars in photographs represent 0.5 cm.

of *MdPI* expression compared with the parthenocarpic apples. Therefore, it is possible that slight expression of *MdPI* of the transgenic apples interfered with parthenocarpy. The *MdMADS8* and *MdMADS9* were reported as homologues of *Arabidopsis SEPALLATA* (*SEP*) genes (Ireland et al. 2013). A transgenic apple defective with the *MdMADS8/9* by anti-sense method showed suppression of flower and flesh development. *Arabidopsis* SEP were demonstrated to co-work with class B genes, PI and AP3, for floral organ formation (Pelaz et al. 2000). *MdMADS13* is a close homologue of *APETALA3* (*AP3*) (van der Linden et al. 2002). The expression localized at stamens and carpels similarly to *MdPI*. This similar localization suggests that *MdPI* and *MdMADS13* serve as floral organ identity genes like as *PI* and *AP3* of *Arabidopsis*. In this article, the *MdMADS13* was analyzed what role played about the development of apple flower and fruit.

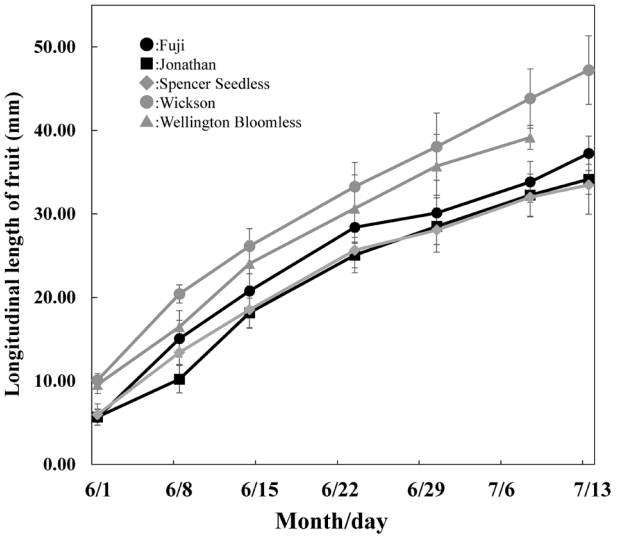

Figure 2. Growth of fruits of normal and parthenocarpic cultivars. Black lines represent normal cultivars 'Fuji' and 'Jonathan.' Grey lines represent parthenocarpic cultivars, 'Wickson,' 'Wellington Bloomless' and 'Spencer Seedless.' Vertical lines represent fruit size (mm) and horizontal line represents date (month/day means June 1 to July 13). Error bars represent standard deviation (n=16–30).

Materials and methods

Plant materials

This study examined apple trees grown for more than ten years at the Division of Apple Research of the National Institute of Fruit Tree and Tea Science at Morioka in Japan were used for this study. Normal apple cultivars used were 'Fuji' and 'Jonathan.' The parthenocarpic apple cultivars used were 'Spencer Seedless,' 'Wellington Bloomless' and 'Wickson' (Tanaka et al. 2007; Yao et al. 2001). The floral buds to small

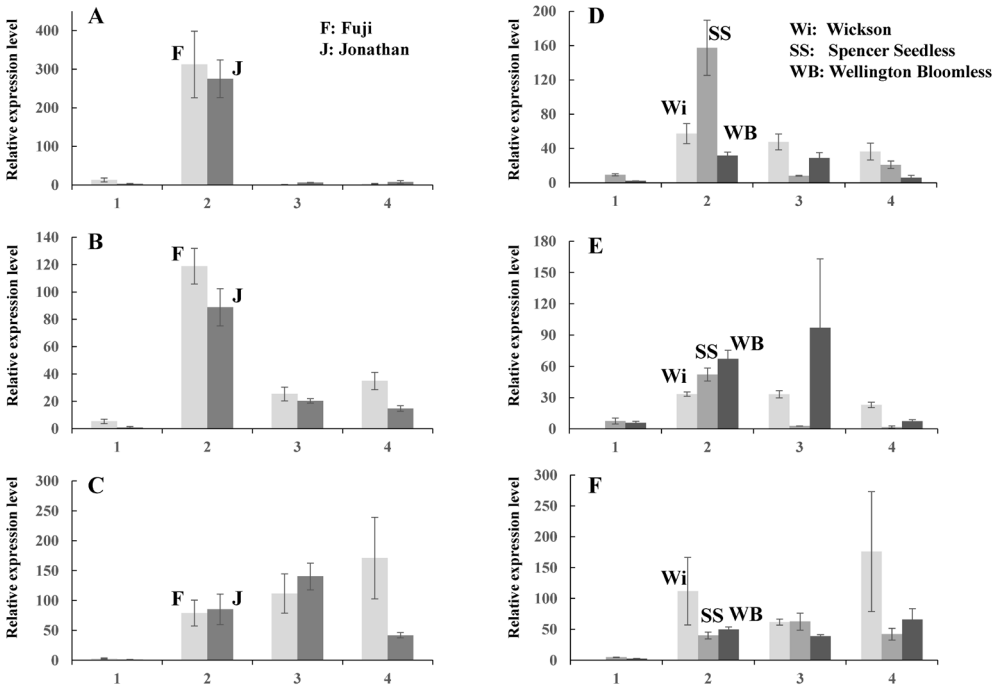

Figure 3. Temporal expression patterns of *MdMADS13* gene in normal and parthenocarpic apples from flower bud to fruit development. In A, B, and C, each gene expression was quantified with normal cultivars, Fuji (F); grey and Jonathan (J); dark grey bar. In D, E, and F, corresponding gene expression was quantified with parthenocarpic cultivars, Wickson (Wi); grey, Spencer Seedless (SS); middle grey and Wellington Bloomless (WB); dark grey. Alphabets above bars indicated each cultivars. The quantity of *MdMADS13* expression shows in A and D, that of *MdMADS15* expression shows in B and E, and that of *MdFT2* shows in C and F. The numbers under figure means sampling date. Normal cultivars (A–C) were collected: 1, April 4; 2, May 4; 3, June 4; and 4, July 4 in 2012. Parthenocarpic cultivars (D–F) were collected: 1, April 12; 2, May 1; 3, June 4; and 4, July 4 in 2012. Relative values were normalized to apple ubiquitin as the internal standard. Error bars represent standard deviation ($n=3$).

fruits from both cultivars were photographed on April 2, May 1, May 29, and July 2 in 2014 (Figure 1). The longitudinal length of developing fruits was measured on June 1, June 8, June 14, June 22, June 30, July 8, and July 13 in 2005 (Figure 2). For quantified RT-PCR, floral buds from normal cultivars were collected on April 4, May 4, June 4, and July 4; parthenocarpic cultivars were collected on April 12, May 1, June 4, and July 4 in 2012 (Figure 3). The harvested samples were frozen immediately with liquid nitrogen and were stored in a −80°C freezer for RNA extraction.

Expression analysis

Total RNA was extracted from each parthenocarpic and normal cultivar from each floral bud and small fruits. Extractions were performed using the modified cetyltrimethylammonium bromide method (Wada et al. 2002). The quantified PCR method for *MdMADS13* (Acc. No. AJ251116), *MdMADS15* (Acc. No. AJ251118) and *MdFT2* (Acc. No. AB458504) expression for floral buds to small fruits were performed with 1.0 μg extracted total RNA, which was synthesized to cDNA with oligo(dT) primers. Specific primers for *MdMADS13* designated at the 3′ non-coding region were 313F: TAT TAA GGT CAC TTA TAA CTG C and 313R2: TAA AGC CAA TAC AAG ACA TCC. The amplified DNA fragment was 270 bp. Specific primers for *MdMADS15* were designated in the 3′ non-coding region: the forward primer sequence was 315F: AAT GAT CCA GAT TGC TTG GG; reverse primer sequence was 315R: GTT GGA AGA ACT TGT TAG GG. The amplified DNA fragment was 170 bp. Specific primers for *MdFT2* were used as described in an earlier report (Tanaka et al. 2014). Apple ubiquitin (*MdUBQ*) was quantified as an internal control, as explained in an earlier report of the literature (Takos et al. 2006).

Yeast two-hybrid experiments

The yeast two-hybrid method involved the use of the MATCHMAKER system (Clontech, Palo Alto, CA, USA). Full-length coding regions of *MdPI*, *MdMADS13*, *MdTM6*, *MdMADS10* and *MdMADS15* were generated via PCR amplification with appropriate restriction enzyme sites at both ends and cloned into the binding domain vector pGBKT7 and the activation domain vector pGADT7 provided by the manufacturer. These constructions were confirmed by sequence determination. Yeast strain AH109 was used for transformation by the lithium acetate method (Gietz et al. 1992). The transformants co-transformed with binding domain and activation domain plasmids were selected on selective medium lacking adenine, histidine, leucine and tryptophan (-4) according to the manufacturer's instructions. A 0.5 mM solution of 3-amino-1,2,4 triazole (3AT) was added to the (-4) SD medium. The addition of 3AT clearly reduced the background growth. To verify the interactions between the *MdMADS* genes, we used 20 μg ml^{-1} of 5-bromo-4-chloro-3-indolyl-α-D-galactoside (X-α-Gal) on SD plates to detect yeast

MEL1 activity. The pCL encoded the full-length, wild-type GAL4 protein and provided a positive control for the X-α-Gal assay. The pGKBK T7-53 and the pGADT7-T encoded murine p53 and SV40 large antigen, respectively. The p53 and the large antigen proteins interacted in a yeast two-hybrid assay as another positive control provided by the manufacturer.

Plant transformation

MdMADS13 cDNA coding region was amplified by PCR and was inserted into pSMAK vector for transformation (Yamashita et al. 1995). *Arabidopsis* Columbia (Cl-O) ecotype was used for transgenic experimentation. *Agrobacterium tumefaciens* GV3101 strain harboring binary vector pSMAK 35S::MdMADS13 was infected to two-week-old flower buds of growing *Arabidopsis* Cl-O plants using floral dip method (Clough and Bent 1998). The obtained seeds were sown and selected on 1/2MS medium with kanamycin ($20\,mg\,l^{-1}$). Surviving seedlings were grown as transgenic lines, which were selfing. The resultant seeds were harvested. Then the plants from the seeds were designated as the T1 generation. The T1 flower phenotypes were analyzed under long day conditions (16 h light/8 h dark) and were photographed using a digital CCD camera system (Pixera Pro600ES; Pixera Corp.). The expression of *MdMADS13* in each transgenic *Arabidopsis* was confirmed using RT-PCR with *MdMADS13* specific primers.

Results and discussion

Fruit formation of normal and parthenocarpic cultivars

Apple flower initiation started the prior summer, developing floral organs gradually under their scales. The floral buds remained in a dormant state in winter time. After sufficient chilling, they broke to bud with increasing temperature during spring time. Photographs taken at the beginning of April showed hard and small floral buds from both cultivars, but the floral organs had already matured in their buds (Figure 1-J1, S1). One month later, at the beginning of May, the flower buds from the base were about to blossom. Normal 'Jonathan' showed pink petals (Figure 1-J2). Parthenocarpic 'Spencer Seedless' had pinkish tips of sepals (Figure 1-S2). One month later, the blossoms had already fallen. Both cultivars had developed small fruits of about 1.0–2.0 cm diameter by the end of May (Figure 1-J3, S3). 'Jonathan' still had anthers under sepals (Figure 1-J3). Both cultivars showed similar size and shape. However, the fruits of 'Spencer Seedless' were not pollinated. One month later, at the beginning of July, both fruits were growing well. The fruits size had increased to four times the longitudinal and vertical length of fruits measured at the beginning of June (Figure 1-J4, S4). However, the fruit shape of 'Spencer Seedless' differed from that of the normal cultivar 'Jonathan.' The bottom side with sepals grew less than the upper side with peduncles, seemingly dependent on the double ovaries of the parthenocarpic cultivars (Yao et al. 2001). The growth patterns of both cultivars were described by measuring the longitudinal lengths of both fruits (Figure 2) during June 1–July 13 at one week intervals. The growth rates of normal and parthenocarpic cultivars were almost equal, as the figure shows. Furthermore, the horizontal length patterns of the fruits were similar. These results suggest that parthenocarpic cultivars were able to initiate and develop flowers to fruits without pollination and the trait was involved in the loss of *MdPI* expression. However, direct suppression of *MdPI* by antisense method in transgenic apple had not led to parthenocarpy (Tanaka et al. 2016). To clarify the genes influenced by *MdPI*, next we also examined the relation between *MdMADS13*, a possible another class B gene, and fruit formation, as described below.

Temporal expression of floral genes

For normal apple 'Jonathan' and parthenocarpic 'Spencer Seedless,' fruit development was similar temporally, but 'Jonathan' was pollinated and 'Spencer Seedless' was not. Actually with respect to fruit development, temporal expressions of the *MdMADS13* differed between normal and parthenocarpic cultivars (Figure 3A, D). The *MdMADS13* of normal cultivars was regulated to express strictly at matured flowers (Figure 3A) and was localized at petals and stamens similar to *MdPISTILLATA* of apple class B gene (Kitahara et al. 2004; Tanaka et al. 2007, 2016; van der Linden et al. 2002). Little expression was found in April, June, and July. However, *MdMADS13* in parthenocarpic cultivars maintained low expression in June and July (Figure 3D). *MdMADS15* was found to be a homologue of the *AGAMOUS* (Lu et al. 2007; Yanofsky et al. 1990) gene from *Arabidopsis*, which contributes to formation of stamens and carpels in apple (Klocko et al. 2016). Normal cultivars expressed *MdMADS15* from matured flowers at the beginning of May and decreased expression in June and July (Figure 3B). These expression patterns supported our inference that *MdMADS15* was involved in floral organ formation and in fruit developments, the expression of *MdMADS15* of parthenocarpic cultivars started in May and decreased gradually in June–July (Figure 3E), which represented similar patterns to that of normal cultivars. The homologue of *FT* gene of *Arabidopsis* (Kobayashi et al. 1999), *MdFT2* from apple showed constant expression during May–July (Figure 3C, F) (Kotoda et al. 2010). *FT* gene is known as a flowering regulator of *Arabidopsis* (Kobayashi et al. 1999). In the apple case, *FT* homologue *MdFT* gene also plays a key role in flowering, but additional expression was detected in fruit. The expression of *FT* homologue in fruit was reported in citrus too (Nishikawa et al. 2007), which suggested that the *FT* homologue played some role in the fruit

development of apple and citrus. Three parthenocarpic cultivars showed expression of *MdMADS13* after blossoming. In fact, a notable trait was their faint expression in April. Two normal cultivars expressed *MdMADS13* in May, but it was difficult to detect after May. In normal flowers, petals and stamens died after blossoming. Then it was natural that *MdMADS13* expression vanished in fruit development because of the class B gene. The ectopic expression of *MdMADS13* in flowers and fruits of parthenocarpic cultivars suggested that *MdPI* regulated expression of *MdMADS13*. The *MdMADS15* expression appeared to have no affection with parthenocarpic cultivars, which suggested a close connection between *MdPI* and *MdMADS13*.

Yeast two-hybrid analysis between apple MADS genes

The PI protein of *A. thaliana* interacts with AP3 protein and combines a structure that functions as a transcription regulator for petal and stamen identity (Honma and Goto 2001). We investigated the interaction between MdPI and MdMADS13 protein with yeast two-hybrid experiments (Figure 4). Other apple MADS genes, *MdTM6*, *MdMADS10* and *MdMADS15* products, were also investigated with regard to protein–protein interactions with MdPI or MdMADS13. Phylogenetic analysis indicated that *MdTM6* was located in the class B gene group (Kitahara et al. 2004) and *MdMADS10* and *MdMADS15* were in the class C group. As expected, MdPI and MdMADS13 formed heterodimers with each combination of the DNA binding domain vector and the activation domain vector (Figure 4d, j). On the other hand, homodimer formation of MdPI or MdMADS13 was not detected (Figure 4e, k). The putative class B gene *MdTM6* product showed no interaction with MdPI or MdMADS13 (Figure 4g, m). Nevertheless, the similarity in amino acids sequences between MdMADS13 and MdTM6 was high (88%) and K-box between MdMADS13 and MdTM6 were almost identical. The interaction of two putative class C genes, *MdMADS10* and *MdMASD15* products, with both MdPI and MdMADS13 was also assessed. MdMADS15 showed weak interaction with MdPI and MdMADS13, respectively (Figure 4h, n). But MdMADS10 had no binding abilities (Figure 4i, o). These results suggested that *MdPI* and *MdMADS13* play the role of class B genes as floral organ identity genes together, like the PI and AP3 proteins of *Arabidopsis*. Apple *MADS* genes, *MdMADS8/9* (*SEPALLATA1/2*) and *MdMADS10/15, AGAMOUS*-like genes are reportedly involved in fruit flesh formation (Ireland et al. 2013; Klocko et al. 2016). Tomato mutant *pistillate* (a possible orthologue of *PISTILLATA*) exhibited floral organ mutation and parthenocarpic traits (Olimpieri and Mazzucato 2008). Quartet models for flower organ formation were required

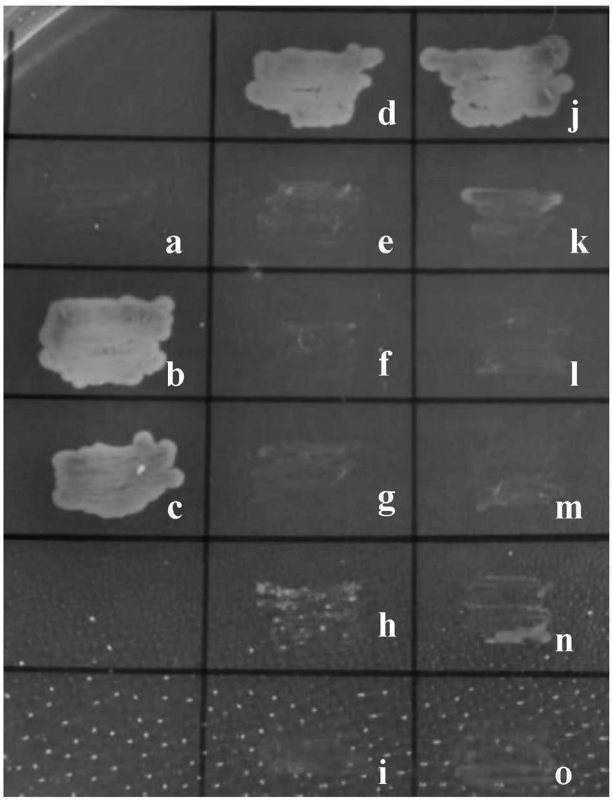

Figure 4. Interactions between MdPI and MdMADS13, or other MdMADS proteins detected by yeast two-hybrid system. The yeast strain AH109 was co-transformed with both binding and activation vectors containing different MdMADS combinations. Transformed yeasts were streaked on the SD medium without adenine, histidine, leucine and tryptophan, with 0.5 mM 3AT and 20 μg ml^{-1} X-gal. a; pGBKT7+pGADT7, b; pGBKT7+pCL1, c; pGBKT7-53+pGAD T7-T, d; pGBKT7-MdPI+pGADT7-MdMADS13, e; pGBKT7-MdPI+pGADT7-MdPI, f; pGBKT7-MdPI+pGADT7, g; pGBKT7-MdPI+pGADT7-MdTM6, h; pGBKT7-MdPI+pGADT7-MdMADS15, i; pGBKT7-MdPI+pGADT7-MdMADS10, j; pGBKT7-MdMADS13+pGADT7-MdPI, k; pGBKT7-MdMADS13+pGADT7-MdMADS13, l; pGBKT7-MdMADS13+pGADT7, m; pGBKT7-MdMADS13+pGADT7-MdTM6, n; pGBKT7-MdMADS13+pGADT7-MdMADS15, o; pGBKT7-MdMADS13+pGADT7-MdMADS10.

of the combination for *MADS* gene products of four kinds (Honma and Goto 2001). Based on results of yeast two-hybrid experiments, the MdPI and MdMADS13 heterodimer were inferred as combining with other MADS gene products such as apple *SEP*-like genes and another *AG*-like genes.

Transgenic Arabidopsis by 35S::MdMADS13

Transgenic *Arabidopsis* with kanamycin resistance were selfed. The resultant T1 generations were sowed in pots to analyze their flower phenotypes. Nine individuals of T1 generation line 3 were obtained, all of which were transgenic plants with malformed carpels or stamens (Table 1, Figure 5). Line 3 plants often had unclosed

Table 1. Numbers of floral organs in transgenic *Arabidopsis* with 35S::MdMADS13.

Line	Rosette leaves	Cauline leaves	Sepal	Petal	Stamen	Carpel	Malformed stamen	Malformed carpel	*n*
Cont.	5.6±0.5	2.5±0.52	4.0±0	4.0±0	5.8±0.42	1.0±0	0	0	10
3-5	5.2±0.7	3.3±1.06	4.0±0	3.9±0.27	6.0±0.95	1.4±0.77	3	12	13
3-7	5.8±0.35	3.8±0.64	4.0±0	4.0±0	6.0±0.81	1.5±0.84	1	9	10
3-8	5.4±0.54	4.8±0.44	4.0±0	4.0±0	5.5±0.52	1.1±0.33	1	7	9
3-9	5.6±0.54	4.0±0	4.0±0	4.0±0	5.3±0.3	1.3±0.67	0	9	11
5-4	5.0±0	3.8±0.44	4.0±0	4.0±0	5.8±0.44	1.0±0	0	0	5
5-5	6.5±0.57	3.0±0	4.0±0	4.0±0	6.5±0.57	1.0±0	0	0	8
6-2	5.3±0.67	4.1±1.1	4.0±0	4.0±0	5.85±0.36	1.0±0	0	0	14
6-3	7.2±0.83	3.4±0.52	4.0±0	4.0±0	5.6±0.69	1.0±0	0	0	10

Values are mean±standard error.

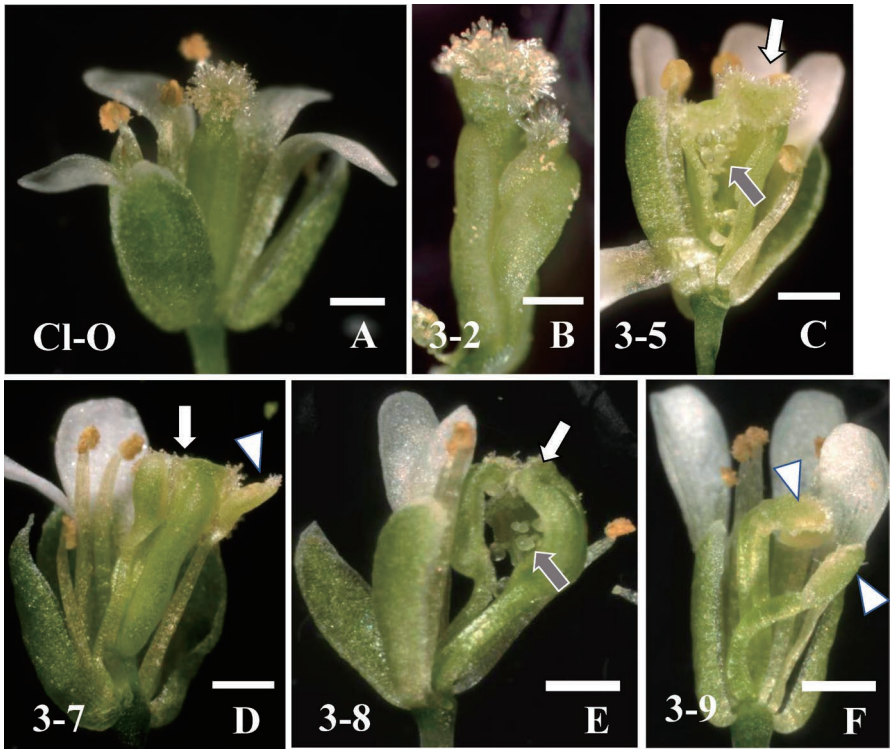

Figure 5. Transgenic *Arabidopsis* flowers carrying with 35S::MdMADS13. Transgenic *Arabidopsis* with 35S::MdMADS13, line 3 T1 generations had malformed stamens and pistils: A, normal flower of Cl-O; B, line 3-2 had doubled carpels removed sepals and petals; C, line 3-5 had unfused carpels (white arrow) and naked ovules (grey arrow); D, line 3-7 had an unfused carpel (white arrow) and stigma on the top of filaments (white arrowhead); E, line 3-8 had unfused carpel (white arrow) with naked ovules (grey arrow); F, stigma on the top of filaments (white arrowhead). White bars represent 0.5 cm.

carpels and naked ovules (Figure 5C, E). Moreover, stamens at whorl 3 changed to papillae-like tissues from anthers (Figure 5D, F). From line 5 and line 6, two individuals each were of identical normal form, the same as wild type (Table 1), but all three lines showed expression of induced *MdMADS13* by RT-PCR, there was no difference of the expression amount between the three lines. It was difficult to explain their different phenotypes between the transgenic *Arabidopsis* plants. Actually, it might depend on the difference of mRNA level or stability, or translation of the induced gene. Numbers of floral organs from line 3 showed that sepals, petals, and stamens were identical to wild type, but the number of carpels was greater than that of wild type (Table 1). Overexpression of *MdMADS13* strongly affected carpel formation and caused slight homeotic change of anthers to papillae. These effects were not the same as *Arabidopsis* with overexpressed *APETALA3* (Jack et al. 1994). The 35S::AP3 in *Arabidopsis* had a change of carpels to stamens, which resulted from the ectopic expression of *AP3* in whorl 4 of the flower. For another class B gene, *PISTILLATA* overexpression changed sepals to petaloid mosaic organs in whorl 1 (Krizek and Meyerowitz 1996). Sequence homology and phylogenetic analysis indicated *MdMADS13* as a closer orthologue of *AP3* compared with *Arabidopsis* (van der Linden et

al. 2002). Therefore, the product of *MdMADS13* might interfere between pairing of AP3 and AG proteins. MdMADS13 was presumed to work as a partner of MdPISTILLATA, as in *Arabidopsis* AP3 and PI. Apple parthenocarpic cultivars reportedly lost *MdPI* expression by retrotransposon (Yao et al. 2001). The loss of *MdPI* involved in the ectopic expression of *MdMADS13* if the *MdPI* regulated *MdMADS13* expression. We tried to produce a transgenic apple with 35S::MdMADS13 to analyze how *MdMADS13* contributed to developments of flowers and fruits. Many kanamycin-resistant apples were obtained, but the plants did not grow and died in vitro. *MdMADS13* was a kind of transcription factor that affected the expression of many genes. Normal growth of the transgenic apple was likely to have been disturbed by the overexpression of *MdMADS13*. It is necessary to consider a choice of promoter to resolve this difficulty that hinders research. For example, one might use a fruit-specific promoter for additional research. As another approach, a specific suppression of *MdMADS13* by genome editing might reveal a new aspect of flower and fruit formation of apple.

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