Original Paper

The analysis of transgenic apples with down-regulated expression of *MdPISTILLATA*

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Abstract   In parthenocarpic cultivars of apple (*Malus×domestica* Borkh.), *MdPISTILLATA* (*MdPI*) expression has been suppressed by retrotransposon insertion into the *MdPI* genome. In this study, transgenic apple lines were produced that exhibited the same level of *MdPI* depression. The 1P-2 promoter from the *MdPI* genome, which specifies its expression in the petals and stamens, was used for antisense-*MdPI* expression, and rolC:AtFT was included to accelerate flowering. The transgenic apple with rolC:AtFT/1P-2:antisense-*MdPI* showed homeotic changes in the floral organs, whereby petals and stamens were replaced with sepals and pistils, respectively. Line 9–2 of this transgenic apple also showed strong suppression of *MdPI*. Some individuals from this line had deformed floral organs, suggesting that the homeotic changes were incomplete. Other transformants of line 9–2 that had double sepals in the first and second whorls, and many pistils in the third and fourth whorls, as seen in apple cultivars with class B mutations, which demonstrated *MdPI* functioned for floral organs formation same as *Arabidopsis PISTILLATA* gene. The transgenic apples set parthenocarpic fruits (15.7%). However, precocious transgenic apples with rolC:AtFT exhibited more parthenocarpy (14–27%). This indicates that *MdPI* depression cannot explain fruit formation in parthenocarpic cultivars of apple, and so some other as yet unidentified genes must be responsible.

Key words: apple, fruit, *MdPISTILLATA*, parthenocarpy.

Fruit bearing woody plants such as apple (*Malus×domestica* Borkh.) are grown as commercial crops in temperate zones around the world. Most fruit trees require pollination by bees or hand, because normal fertilization and seed development guarantee fruit development. However, pollination is often disrupted by natural conditions, low temperatures, spring frosts and pollinator issues, resulting in unstable fruit production. Consequently, parthenocarpy is considered a favorable trait in many fruit cultivars, allowing fruit production to become stable and saving on labor requirements for pollination. Some apple cultivars are parthenocarpic, and exhibit interesting phenotypes, whereby the flowers lack petals and stamens as a result of homeotic mutations (Yao et al. 2001).

Floral organ identity is specified by class ABC genes (Weigel and Meyerowitz 1994). It has previously been shown that in parthenocarpic cultivars of apple, the class B gene ortholog *MdPISTILLATA* (*MdPI*) has been disrupted by retrotransposon insertion into an *MdPI* genome locus, which caused homeotic changes in the floral organs, and that the parthenocarpy is co-inherited (Tobutt 1994), suggesting that the loss of *MdPI* function is involved in apple parthenocarpy. The ability of *MdPI* as a class B gene has also been demonstrated through complementary experiments with an *Arabidopsis pi-1* class B mutant that had no petals and stamens, with these organs being recovered in the resultant transgenic *pi-1* mutant with 35S:*MdPI*. Furthermore, it has been shown that the 5′ upstream region of the *MdPI* genome (approximately 1.0 kb length) regulates restricted expression in the second and third whorls of flowers (Tanaka et al. 2007).

Examination of *MdPI* expression through the

developmental stages of apple flowers using in situ hybridization method revealed that this gene is expressed from the primordia to the mature organs of the petals and stamens (Wada 2008). It is particularly noteworthy that the receptacle tissue also produced the hybridization signal, indicating that part of this tissue was composed of the basal part of the stamens. This finding supports the appendicular theory for the origin of pome fruits (Pratt 1988; Rohrer et al. 1991), which proposes that the adnate bases of the sepals, petals and stamens encase the true fruit (carpel). By contrast, suppression of the *Arabidopsis* *SEPALLATA* (*SEP*) gene homolog of apple *MdSEP1/2* (*MdMADS8/1/9*) using the antisense method resulted in developmental inhibition of the fruit cortex (Ireland et al. 2013), and in strawberry, the *FaMADS9* of *SEP1/2* homolog of *Arabidopsis* was involved in receptacle development (Seymour et al. 2011), supporting the receptacular theory of rosaceae fruit formation, which argues that the fruit flesh is derived from the receptacle surrounding the carpels.

The *SEP1/2/3/4* genes from *Arabidopsis* specify the sepals, petals, stamens, and carpels (Ditta et al. 2004, Honma and Goto 2001, Pelaz et al. 2000), and the SEP proteins form multimeric complexes with *MADS* class B- and C-gene proteins. Thus, the apple *MdSEP1/2* (*MdMADS8/1/9*) homolog appeared to form complexes with *MdPI* to function as an organ identity gene. Both transgenic apple and strawberry flowers that were suppressed by *SEP* homologs had green or sepaloid petals, indicating that rosaceous flower formation is specified by multimeric complexes with the ABCE genes (Ireland et al. 2013; Seymour et al. 2011). However, the relationship between the loss of *MdPI* function and parthenocarpy remains unclear. Therefore, in this study, we investigated whether transgenic apple with suppressed *MdPI* expression exhibited the same traits as a parthenocarpic cultivar.

Materials and methods

Plant materials

Apple trees of the cultivars 'Fuji' and 'Jonathan,' and the parthenocarpic cultivar 'Spencer Seedless' that were more than twenty years old and growing in the experimental field of the Division of Apple Research at the Institute of Fruit Tree and Tea Science, Morioka, Japan were used in this study. In addition, the apple semi-dwarfing rootstock cultivar 'JM2' was cultured aseptically and shoots were propagated in vitro. The leaflets from the shoots were then cut and used in the transformation experiments, as described previously (Wada et al. 2009).

Vector construction and transformation

The coding and 3′ noncoding region of *MdPI* was amplified from flower cDNA (Yao et al. 2001) using the PCR method. The resulting 850 bp DNA fragment was amplified between the 31 HS and the 51 SC primers (Table 1). The 31 HS primer contains a *Hin*dIII and *Spe*I sites at the 5′ end in this order, and while 51 SC primer contains the *Sac*I site at the 5′ end. The amplified *MdPI* fragment was digested by *Hin*dIII and *Sac*I, and the binary vector pSMAK251 (Yamashita et al. 1995) was also digested by the same restriction enzymes. The restriction enzyme treated *MdPI* fragment was then inserted in the antisense direction into the pSMAK251 vector at the *Hin*dIII and *Sac*I sites.

The *MdPI* genome 5′ upstream region of approximately 1.0 kb (Tanaka et al. 2007) was used as a specific promoter for expression in petals and stamens, and the promoter region was amplified using the PCR method with the PI SH and PI AS primers (Table 1), which contain a *Hin*dIII site and a *Spe*I site, respectively. The pSMAK inserted antisense *MdPI* vector was digested by *Hin*dIII and *Spe*I, and the DNA fragment that was amplified between the PI SH and PI AS primers was treated with the same restriction enzyme. The *MdPI* promoter region was then inserted into the pSMAK antisense *MdPI* vector at the *Hin*dIII and *Spe*I sites, and the resultant product was designated pSMAK 1P-2:antisense-*MdPI*. To accelerate flowering in the transgenic apple, a rolC:AtFT was added to the pSMAK 1P-2:antisense-*MdPI* vector at the *Hin*dIII site (Tanaka et al. 2014). The resultant vector was designated pSMAK rolC:AtFT/1P-2:antisense-*MdPI* (Figure 1), which was intended to cause flowering acceleration and the specific suppression of *MdPI* in transgenic apple. The pSMAK rolC:AtFT/1P-2:antisense-*MdPI* vector was changed the 1P-2 to 35S (cauliflower mosaic virus) promoter, and the reconstructed vector was designated pSMAK rolC:AtFT/35S:antisense-*MdPI*.

The transformation method that was used to generate transgenic apple has been described previously (Wada et al. 2009). Leaflets from the cultured shoots of 'JM2' were infected by *Agrobacterium tumefaciens* strain LBA4404 bearing the pSMAK rolC:AtFT/1P-2:antisense-*MdPI*. These transformed shoots were then planted in vitro on new

Table 1. Primer sets for gene cloning.

Primer	Oligonucleotides (5′ to 3′)
Gene Cloning	
31 HS	ATAAGCTTACTAGTCACAAACCGAGTTCATGCAC
51 SC	ATGAGCTCATGGGACGTGGGAAGGTTGAG
PI SH	ATAAGCTTTAAATTTGGAGTCCCCTTCC
PI AS	ATACTAGTATCTCTCAGTATTCTTGTTC
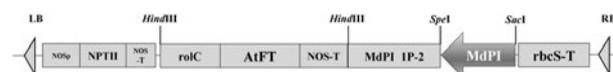
Figure 1. Schematic representation of the transformation vector pSMAK rolC:AtFT/1P-2:antisense-*MdPI*.

NOSp, nopaline synthase promoter; NPTII, neomycin phosphotransferase II; NOS-T, terminator of nopaline synthase; rolC, *Agrobacterium rhizogenes rolC* promoter; AtFT, *Arabidopsis FLOWERING LOCUS T*; MdPI 1P-2, *MdPISTILLATA* promoter region (approximate 1.0 kb); rbcS-T, *Arabidopsis* Rubisco-S-2B terminator; LB, left border; RB, right border. *MdPI* cDNA is described by the left-pointing arrow to show the antisense direction for transcription. Each restriction enzyme site is indicated by bold letters.

medium supplemented with 50 mgl⁻¹ kanamycin and 50 mgl⁻¹ meropenem (Dainippon Sumitomo Pharma Co., Osaka, Japan) each month. After several months, antibiotic resistant shoots were selected and cultured on root-induction medium. The rooted shoots were then transplanted into sterile soil in a plastic pot. Following a 3–4 week acclimatization period, the resultant transgenic apples were grown in closed greenhouses under natural light conditions.

Analysis of transgenic apple flowers

Three to five flowers were collected from each line of rolC:AtFT and rolC:AtFT/1P-2:antisense-*MdPI* transgenic apples in the culture room and used for RNA extraction with the CTAB method (Wada et al. 2002). Wild type flowers were also collected from 'Fuji' and 'Jonathan' apple trees growing in the orchard of the apple research station at the time of blooming in May 2008, and RNA was extracted using the same method. For each sample, 1.0 µg RNA was electrophoresed with 0.8% agarose gel, which was blotted with a positively charged nylon membrane (Roche Diagnostics GmbH, Mannheim, Germany). DIG-labeled RNA of the antisense *MdPI* coding region was synthesized using a labeling kit and T7 RNA polymerase, according to the manufacturer's instructions (Roche). Hybridization was performed in a DIG Easy Hyb™ (Roche) at 68°C for 16 h followed by two rinses in 2× saline sodium citrate (SSC) containing 1% (w/v) sodium dodecyl sulphate (SDS) at room temperature for 10 min and two washes in 0.1× SSC containing 1% (w/v) SDS at 68°C for 15 min. Chemiluminescent signals were visualized using an ImageQuant LAS4000 (GE healthcare, Japan). The hybridization signals were quantified with image analyzing software of LAS4000 (GE healthcare, Japan).

Microscopy

Transgenic apple lines were observed under a stereoscopic microscope (Leica DMR HC RB-5, Germany) and photographed with a Pixera Pro600ES digital CCD camera system (Pixera Corporation, Kanagawa, Japan). To visualize the surface structure of the transgenic apple flower organs at a microscale, non-fixed flowers were observed under a scanning electron microscope (SEM) (TM-1000 Miniscope, Hitachi, Ibaraki, Japan).

Results and discussion

Analysis of transgenic apple

In total, 1120 'JM2' leaflets were transformed by rolC:AtFT/1P-2:antisense-*MdPI* and 116 kanamycin resistant shoots were obtained after three months in vitro. Of these, 43 shoots set flowers in vitro within 6 months. Eight lines grew vigorously and were planted in pots following rooting, all of these lines bloomed within a year. Homeotic changes in the floral organs were observed in three lines, with apparent differences in the homeotic replacements between these lines

(Figure 2). Line 9–2 of the rolC:AtFT/1P-2:antisense-*MdPI* transgenic apple had double sepals and excess pistils without petals and stamens, as occurs in the parthenocarpic cultivar 'Spencer Seedless' (Figures 2C, E). But the transgenic apple hardly showed fertility compared with 'Spencer Seedless.' By contrast, line 213 of the rolC:AtFT/1P-2:antisense-*MdPI* transgenic apple had pinkish sepaloid petals, stamens, and fewer pistils (Figure 2F), while line 284 flowers (Figure 2D) appeared to have the same phenotype as line 66 rolC:AtFT transgenic apple flowers (Figure 2B), which had accelerated flowering (Tanaka et al. 2014).

Northern hybridization indicated that the expression of *MdPI* in line 9–2 was approximately ten times weaker than in the other transgenic lines and the normal cultivars (Figure 3). This demonstrated that the homeotic changes in the petals and stamens of the transgenic apple corresponded with a reduction in the *MdPI* expression level. This clearly demonstrated that the *MdPI* played a role of floral identity gene of class B same as *Arabidopsis PISTILLATA*. In this study, we used the rolC:AtFT/1P-2:antisense-*MdPI* vector (Figure 1) to generate homeotic changes, but these were observed only in three lines, despite 43 lines exhibiting precocious flowering. This suggested that the antisense method was less effective for suppressing the target gene than rolC:AtFT was for inducing flowering. It has previously been shown that *MdTFL1* suppression using the antisense method causes flowering acceleration in transgenic apples (Flachowsky et al. 2012; Kotoda et al. 2006; Sasaki et al. 2011), indicating that gene reduction methods are sufficient to induce flowering by decreasing *MdTFL1* expression. Therefore, the *MdPI* suppression by antisense method would be expected to produce more transgenic plants which had homeotic flowers like as Class B mutation. The reason was unclear why enough numbers of transgenic apples with the apetalous phenotype were not obtained.

A reporter gene analysis in *Arabidopsis* has previously demonstrated that the 1P-2 promoter in the rolC:AtFT/1P-2:antisense-*MdPI* vector regulates specific expression in the petals and stamens (Tanaka et al. 2007), and in situ hybridization analysis revealed that the expression of *MdPI* is localized in the petals and stamens during apple flower development (Wada 2008). Therefore, we constructed a rolC:AtFT/35S:antisense-*MdPI* vector and used this to develop transgenic apple from 840 leaflets of 'JM2.' This resulted in 58 kanamycin resistant shoots being obtained, 11 of which exhibited flowering in vitro. Four of these 11 precocious apple plants showed homeotic changes in the floral organs, with petals and stamens being replaced by sepals and pistils, respectively, in vitro (Figure 2G). However, these transgenic apple plants were unable to maintain their growth and eventually all died in vitro, suggesting that the over expression of antisense *MdPI*
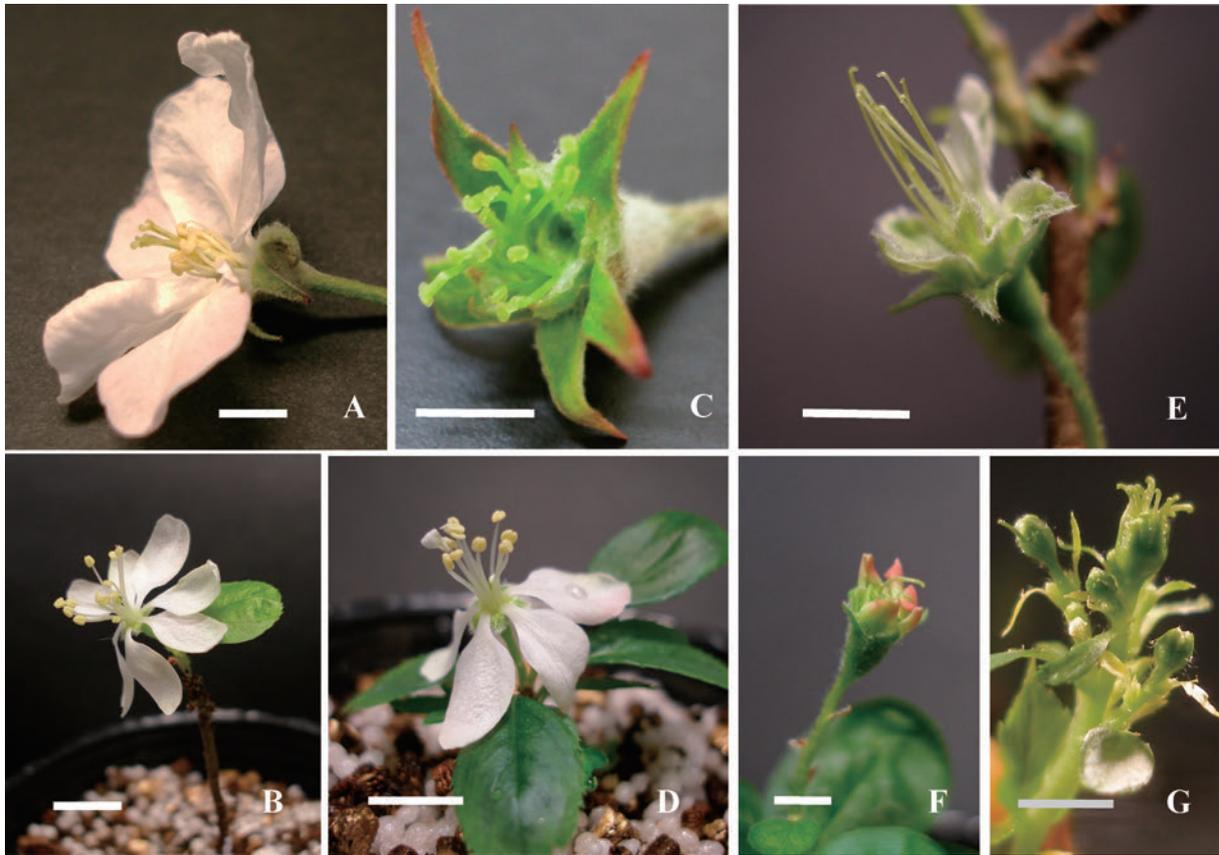
Figure 2. Various flowers of wild type, parthenocarpic, and transgenic apples.
(A) A wild type flower of cultivar 'Fuji.' (B) A transgenic apple flower of rolC:AtFT line 66. (C) A flower of the parthenocarpic apple cultivar 'Spencer Seedless' exhibiting homeotic changes. (D), (E), and (F) Flowers of various rolC AtFT/1P-2:antisense-*MdPI* transgenic apple, lines: line 284, which had a similar phenotype to B (D); line 9–2, which had homeotic floral organs like C (E); and line 213, which had sepaloid petals, no stamens, and fewer carpels (F). White scale bar:1.0 cm. (G) A transgenic apple of rolC:AtFT/35S:antisense-*MdPI*, which had sepaloid petals, no stamens and fewer carpels. These flowers were very small (grey scale bar: 3.0 mm).
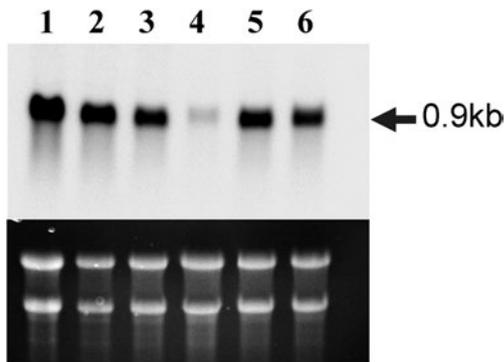
Figure 3. Northern analysis of *MdPI* expression in wild type and transgenic apples.
Lanes 1–2: Flowers from the non-transformed cultivars 'Jonathan' (1) and 'Fuji' (2). Lanes 3–4: Flowers from the rolC AtFT/1P-2:antisense-*MdPI* transgenic apple lines 284 (3) and 9–2 (4). Lanes 5–6: Flowers from the rolC:AtFT transgenic apple lines 66 (5) and 421 (6). The lower image shows the RNA gel electrophoresis, which was stained with ethidium bromide before blotting. The hybridization probe was DIG-labelled antisense *MdPI*. The number on the right indicates RNA size; kb: kilobase.

was harmful to their development. By contrast, the rolC:AtFT/1P-2:antisense-*MdPI* transgenic apple plants grew vigorously both in vitro and in a greenhouse. These results indicated that the specific expression of antisense-*MdPI* by the *MdPI* promoter 1P-2 caused

the same changes to the floral organs as are seen in parthenocarpic apple cultivars. The two vectors that we used contained different promoters (1P-2 or 35S) for the expression of antisense-*MdPI*. The cauliflower mosaic virus 35S promoter is able to enhance gene expression in every tissue of the plant (Benfey and Chua 1990), which resulted in a much greater increase in expression. However, there was a little difference between the number of transgenic apple plants with altered floral organs between the two promoters, indicating that the reduction in *MdPI* expression was not affected by the intensity of the promoter. The specific promoter 1P-2 led the success of production for the transgenic apple with expected phenotype.

Changes in the floral organs

The floral organs of the rolC:AtFT/1P-2:antisense-*MdPI* transformed apple plants exhibited a variety of homeotic changes. Line 213 had pinkish sepaloid petals in the second whorl (Figure 2F), while line 9–2 clearly had sepals in place of petals in the second whorl, in which the trichomes on the surface were the same as are seen in native sepals (Figure 2E). Thus, we concluded that the flowers produced by line 9–2 showed a homeotic change

Figure 4.   Phenotypes of various pistils from rolC:AtFT/1P-2:antisense-MdPI transgenic apple.
(A) Longitudinal section of a transgenic apple flower from line 9–2, with the sepals removed; ovules (arrow head) were detected in the ovary. (B) and (C) Isolated pistils without the ovary from line 9–2 flowers, showing various shapes of the basal part; one pistil had a twisted style. (D) Isolated pistil with two anther-like swellings on the base. (E) A line 9–2 flower from which the pinkish sepaloid petals had been removed and all pistiloids had two anther-like swellings on the base, as seen in (D). White scale bar: 1.0 mm.
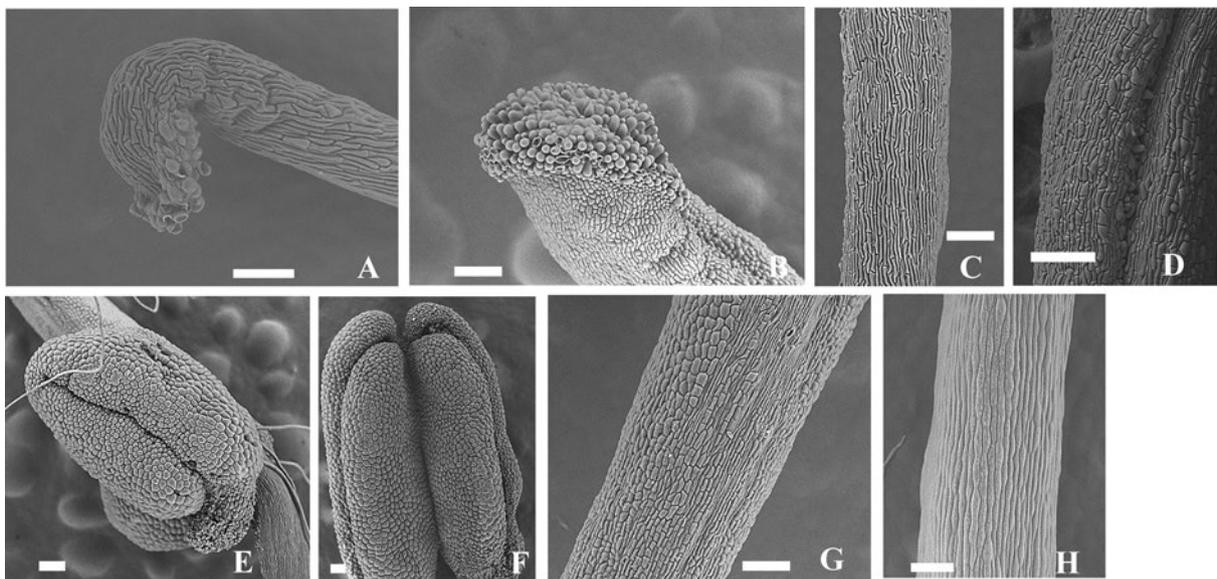
Figure 5.   Comparison of the fine structures of the floral organs from line 9–2 transgenic apple and normal cultivar.
(A) and (B) Close-up pictures of the papillae on the stigma in line 9–2 (A) and cultivar 'Fuji' (B). (C) and (D) Surface cell shapes on the style of line 9–2 (C) and 'Fuji' (D). (E) Anther-like organs on the basal part of the pistiloids in line 9–2. (F) Anther of 'Fuji.' (G) Surface cells on the stem-like basal part of the pistiloid in line 9–2 (magnified basal part of Fig. 4D; arrow head). (H) Surface cells of the filament in 'Fuji.' White scale bar: 100 μm.

of petals to sepals. By contrast, the stamens produced by line 9–2 took various forms (Figure 4), with some changing to pistiloid organs (Figures 4A, B, C). One of these pistiloids had distinct ovaries with ovules (Figure 4A), while others were often twisted, and had no ovaries or swelled tissues at the bases (Figures 4B, C). In other flowers, the pistils exhibited chimeric shapes, in which the proximal half appeared to be staminoid and the distal half was pistiloid (Figures 4D, E). Those floral organs were observed under SEM to confirm their fine

structures (Figure 5). The pistiloids of line 9–2 had papillae on the stigma, as seen in normal pistils (Figures 5A, B). Furthermore, although the pistiloids of line 9–2 were thinner than normal styles, the surface cell shape was identical to the rectangular shape seen in typical styles (Figures 5C, D). These results indicated that the homeotic change of stamen to pistil was caused by the suppression of antisense-*MdPI*.

Some enigmatic transformants were also observed, with mingled pistils and stamens, which looked like a

Table 2.   Floral organ number in transgenic apples.

Line/Cultivar	Sepal	Petal	Stamen	Pistil	n
Fuji	5.0±0.2	5.0±0	19.8±0.2	5.0±0	20
Jonathan	5.0±0.3	5.0±0	20.0±0.2	5.0±0	20
rolC:AtFT					
66	5.0±0.4	7.7±2.1	12.7±3.6	0	13
421	5.9±1.1	7.6±1.8	13.2±3.4	0.4±1.3	20
rolC:AtFT/1P-2:antisense-*MdPI*					
9–2	5.8±1.8	7.1±1.8[a]	0	11.4±3.3[b]	19
284	5.3±0.9	7.0±2.1	16.0±4.0	0.6±1.1	15

[a] inner sepals at second whorl. b: pistils with ovaries.

type of chimeric organ (Figures 4D, E). The distal part of this organ looked like pistil, and SEM observations revealed that the organs had papillae on the top and the same cell shape as is seen on the surface of the style. By contrast, the basal part contained yellowish double swellings that looked like anthers (Figures 4D, E). The cell shapes on the surface of these anther-like tissues were round, similar to that seen in wild anthers, and they also had dehiscent zones (Figures 5E, F). However, these did not undergo dehiscence or produce mature pollen. According to the proposed ABC model of floral organ development, the class B/C genes specify stamen formation and the class C gene specifies carpel formation. Thus, it appeared that each part of these chimeric organs was controlled by different identity genes.

It is possible that *MdPI* may play a greater role in the development of second whorl than the third whorl. This hypothesis was supported by a complementary experiment in which a *pi* mutant from *Arabidopsis* was transformed by *MdPI* overexpression, resulting in complete recovery of petal formation, but imperfect rescue of the stamens (Tanaka et al. 2007). The proximal stems of the chimeric pistiloid that attached to the receptacles were greenish and thicker than the filaments of wild stamens, in which the surface cells were extremely slender (Figure 4D, Figures 5E-H). Furthermore, the surface cells on the basal side of the pistiloid were not homologous with wild type filament cells (Figures 5G, H), indicating that they were derived from a different tissue. Since *MdPI* expression was partly detected in the vascular bundles of the stamens in the receptacles of wild apple using in situ hybridization (Wada 2008), it is possible that the proximal stems of these chimeric pistiloids may be derived from the receptacles.

Comparison with parthenocarpic cultivar
Apple *MdPI* functions as a class B floral organ identity gene in the ABC model (Tanaka et al. 2007; Wada 2008; Weigel and Meyerowitz 1994; Yao et al. 2001). The loss of class B function leads to the petals changing to sepals and the stamens changing to pistils. The same

Table 3.   Parthenocarpy in *MdPI*-suppressed transgenic apple.

Line	Parthenocarpy (%)	Fruit length×width (mm)
rolC:AtFT		
66	6/22 (27)	3–11×7–13
421	5/35 (14)	7–20×10–25
rolC:AtFT/1P-2:antisense-*MdPI*		
9–2	3/19 (15.7)	9–21×12–21
284	2/15 (13.3)	11×15–17

homeotic changes are observed in the apetalous flowers of the apple cultivar 'Spencer Seedless' (Figure 2C), and it has previously been demonstrated that this mutation caused by a transposon insertion into the *MdPI* genome (Yao et al. 2001). This cultivar also sets parthenocarpic fruits and it appears that there is recessive co-inheritance of these traits (Tobutt 1994). Therefore, it is widely believed that the suppression of *MdPI* is closely related to parthenocarpy.

To demonstrate the relationship between *MdPI* expression and parthenocarpy in apple, we attempted to make a precocious *MdPI* suppressed transgenic apple. Flowers of the line 9–2 rolC:AtFT/1P-2:antisense-*MdPI* transgenic apple had a similar phenotype to class B mutation flowers, with homeotic changes of petals to sepals and stamens to pistils (Figure 2E, Table 2). Some line 9–2 flowers did not exhibit these homeotic changes (Figures 2, 4, 5), while others completely changed to appear like those of the parthenocarpic cultivar 'Spencer Seedless' (Figure 2) (Yao et al. 2001). Thus, we successfully produced apple flowers with a class B mutation. We then examined parthenocarpy in the homeotic flowers from line 9–2. Table 3 shows the percentage of fruit sets that occurred without pollination and the size of the resultant fruits. Lines 66 and 421, which contained rolC:AtFT, occasionally exhibited 14–27% parthenocarpy, and similarly line 9–2 with homeotic changes and early flowering exhibited 13–15% parthenocarpy. Furthermore, there was no difference in fruit size between rolC:AtFT and rolC:AtFT/1P-2:antisense-*MdPI* transgenic apple lines. These results suggested that the suppression of *MdPI* is not involved in parthenocarpy. The average fruit size of pollinated

'JM2' apples was 3.0 cm long by 3.0 cm wide. 'JM2' never set fruit without pollination. The parthenocarpic fruits from transgenic apple plants were smaller than found in normal cultivars, parthenocarpic fruits such as 'Spencer Seedless' have a similar fruit size to normal, pollinated cultivars (Tanaka et al. 2007; Yao et al. 2001). Therefore, these findings further suggest that parthenocarpy is not directly caused by *MdPI* suppression as a result of transposon insertion.

Rather, the insertion may have influenced another gene adjacent to the *MdPI* locus. Therefore, studies will need to analyze mRNA expression around the transposon insertional site on *MdPI* gene for further research. And *MADS* genes for floral organ identities of apple should be analyzed about relationship to *MdPI* and expression in parthenocarpic cultivars.

Acknowledgements

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