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Functional Analysis of *MdPI*, the *PISTILLATA* Gene Homologue of Apple, in *Arabidopsis*

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Some cultivars of apple (*Malus*×*domestica* Borkh.) show parthenocarpy. In these cultivars, the *MdPISTILLATA* (*MdPI*) gene, which is homologue of the *PISTILLATA* (*PI*) gene of *Arabidopsis thaliana*, does not function by the insertion of a retrotransposon into *MdPI*. *MdPI* is a class B floral organ identity gene belonging to the MADS-box gene family. When *MdPI* was overexpressed in *Arabidopsis* Columbia ecotype under the control of the cauliflower mosaic virus (CaMV) 35S promoter, the sepals showed petaloid changes. In the case of the *pi-1* mutant of *Arabidopsis* Landsberg *erecta*, apple *MdPI* overexpression led the *pi-1* mutant to recover normal petals and carpeloid stamens. In detailed observations with a scanning electron microscope, we observed that the filaments were also normally recovered. However, the anthers were not. We isolated the *MdPI* promoter from the apple cultivar 'Jonathan', and the *uidA* gene which encodes β-glucuronidase (GUS) was fused as a reporter gene. The *MdPI* promoter fused with GUS was designated p1MDPI.1G. Just petals and stamens of *Arabidopsis* plants transformed with p1MDPI.1G had GUS activity. These results indicated that the *MdPI* gene was related to the development of petals and stamens, and formed petals and filaments. Therefore, the *MdPI* gene had a function equal to *PI*, although the relation between *MdPI* and parthenocarpy in the apple is unknown.

Key Words: *Arabidopsis thaliana*, Homeotic mutation, *Malus*×*domestica*, *MdPISTILLATA*, parthenocarpy.

Introduction

Many plants have four whorls of floral organs: sepals, petals, stamens, and carpels. The identity genes corresponding to these floral organs have been explained in the ABC model for *Arabidopsis* and *Antirrhinum majus* (Weigel and Meyerowitz, 1994). In *Arabidopsis*, the class A genes *APETALA1* (*AP1*) and *APETALA2* (*AP2*) control the formation of sepals; *AP1* and the class B gene *APETALA3* (*AP3*) and *PI* control the formation of petals; *AP3* and *PI* and the class C gene *AGAMOUS* (*AG*) control the formation of stamens; and *AG* controls the formation of carpels (Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994). These genes, except *AP2*, have the MADS-box domain that is a highly conserved DNA-binding region (Riechmann et al., 1996; Shore and Sharrocks, 1995; Theissen and Saedler, 1995). The floral organ identity genes *SEPALLATA1*, *2*, *3*, and 4 (*SEP1*, *2*, *3*, and *4*) in *Arabidopsis* also have B and C functions in petals, stamens, and carpels (Ditta et al., 2004; Pelaz et al., 2000). *AP3* and *PI* form a heterodimer and function in the second and third whorls (Goto and Meyerowitz, 1994; Honma and Goto, 2000; Jack et al., 1992; Lamb and Irish, 2003; McGnigle et al., 1996; Zik and Irish, 2003). In addition, in the quartet model, it was reported that *AP3* and *PI* formed a complex with *AP1* and *SEP* in the second whorl and with *AG* and *SEP* in the third whorl (Theissen, 2001; Theissen and Saedler, 2001). Recently, investigations of genes related to floral bud, floral organ, and fruit formation have been conducted in the apple. The meristem identity genes *AFL1*, *AFL2*, *MdTFL1*, and *MdAP1* (*MdMADS5*) have been isolated and their expression has been investigated in relation to early flowering (Kotoda and Wada, 2005; Kotoda et al., 2000, 2002, 2006; Sung et al., 1999; Wada et al., 2002). In addition, the floral organ identity genes *MdMADS1* to *MdMADS15*, *MdPI*, and *MdTM6* have been isolated (Kitahara et al., 2004; Sung and An, 1997, 1999, 2000; Van der Linden et al., 2002; Yao et al., 1999, 2001).

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A few apple cultivars that have double sepals and many pistils set flowers without petals and stamens. These cultivars show a homeotic mutation, and their flowers are similar to *PI* or *AP3* mutant flowers of *Arabidopsis*. This is because *MdPI* (a *PI* homologue and class B gene) is not expressed because of the insertion of a retrotransposon (Yao et al., 2001). These cultivars have parthenocarpy, and produce fruits without seeds. The relationship between the deficit of the class B gene and parthenocarpy has not yet been clarified in the apple, and has not been observed in *Arabidopsis*. Apple parthenocarpy is inherited by single recessive heredity (Tobutt, 1994). The important issue in the commercial production of fruit such as apples is to ensure stable fruiting. Many fruit tree cultivars are self-incompatible and therefore require pollination. Parthenocarpy will be very useful for fruit tree cultivation, because parthenocarpic cultivars produce fruits without pollination. The use of parthenocarpic cultivars could thus save labor.

In this study, we describe the function of *MdPI*. Functional analysis was demonstrated in *Arabidopsis*, because transformation in apple is difficult and it takes several years to set flowers in apples. We examined the phenotype by the introduction of *MdPI* into the wild-type or *pi* mutant of *Arabidopsis*, and determined the expressed tissues by promoter analysis of *MdPI*.

Materials and Methods

A transformation vector of MdPI

The coding sequence of *MdPI* was amplified from *MdPI* cDNA using the PCR method. *MdPI* was amplified using a Takara LA-PCR kit (Takara Biomedicals, Tokyo, Japan) with specific a sense primer, 5'-ATGG-GACGTGGGAAGGTTGA-3', and antisense primer, 5'-CACAAACCGAGTTCATGCAC-3'. The reaction conditions were 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min for 25 cycles. This amplified fragment was linked to the CaMV 35S promoter, and was designated pSMDPI.1+. (Fig. 1A). This vector was inserted into the *Agrobacterium tumefaciens* strain GV3101 (Koncz and Schell, 1986).

Plant materials

Arabidopsis Columbia (Col) and *pi-1* were kindly provided by the Nottingham Arabidopsis Seed Centre, UK. *pi-1* is class B mutant with a Landsberg *erecta* (L*er*) background and shows no petals and stamens. Seeds were kept at 4°C and grown on agar plates containing 1/2 Murashige and Skoog (MS) medium (Wako Pure Chemicals C. Ltd., Tokyo, Japan) in a growth chamber at 22°C under a 16 h light/8 h dark photoperiod.

Transformation of Arabidopsis

The *MdPI* gene was introduced into Col (genotype *PI/PI*) by the floral dip method (Clough and Bent, 1998). The T_0 transgenic seeds were selected on 1/2 MS + 50 mg·L^{-1} kanamycin + 50 mg·L^{-1} cefotaxime medium.

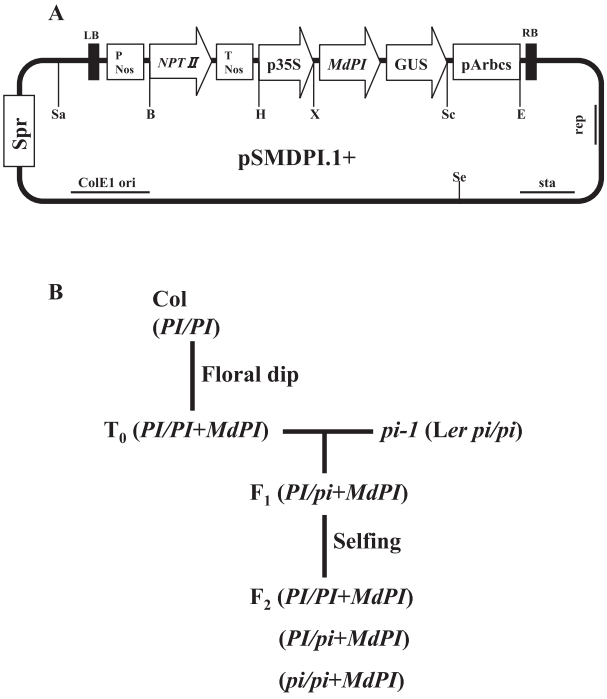

Fig. 1. (**A**) Construct of the vector pSMDPI.1 + for *MdPI* introduction. *MdPI* cDNA was inserted in the sense direction at the *Sma*I site of binary vector pSMAK251. PNos, Nos promoter; Tnos, Nos terminator; p35S, cauliflower mosaic virus (CaMV) 35S promoter; *NPTII*, neophosphotransferase; GUS, *uidA* gene; pArbcs, 3' region of rubisco small subunit; LB, left border; RB, right border; Sa, *Sal*I; B, *Bam*HI; H, *Hind*III; X, *Xba*I; Sc, *Sac*I; E, *Eco*RI; Se, *Spe*I; sta, region involved in plasmid stability; rep, essential region for plasmid maintenance. (**B**) Introduction method of the *MdPI* gene to *Arabidopsis* Col (genotype *PI/PI*). The *MdPI* gene was introduced by the Floral dip method, and T_0 (genotype *PI/PI* + *MdPI*) was obtained. T_0 (genotype *PI/PI* + *MdPI*) was crossed with the *pi-1* mutant (L*er* genotype *pi/pi*), and F_1 plants (genotype *PI/pi* + *MdPI*) were obtained. F_1 plants (genotype *PI/pi* + *MdPI*) were selfed, and F_2 plants (genotypes *PI/PI* + *MdPI*, *PI/pi* + *MdPI*, *pi/pi* + *MdPI*) were obtained.

Sixteen transgenic plants were obtained and grown in a growth chamber at 22°C under a 16 h light/8 h dark photoperiod. The pollen of one individual of the T_0 transgenic plants (genotype *PI/PI* + *MdPI*) was crossed with the *pi-1* mutant (L*er*; genotype *pi/pi*) in order to perform a complementary experiment in which *MdPI* was introduced. The approximately thirty F_1 transgenic plants (genotype *PI/pi* + *MdPI*) that were obtained were self-fertilized, and some of their F_2 seeds were selected using kanamycin. In the F_2 plants (genotype *PI/PI* + *MdPI*, *PI/pi* + *MdPI*, and *pi/pi* + *MdPI*), the flower phenotype expressing *MdPI* was analyzed. A flow chart showing the route to the transgenic plants is shown in Figure 1B.

Genotype analysis of F_2 plants

The genomic DNA was isolated from the rosette leaves of some F_2 plants by the modified CTAB method (Kotoda et al., 2000). The introduction of *MdPI* was confirmed by determining whether the *MdPI* was

amplified from these DNAs by PCR using specific primers, the sense primer 5'-CAGCCAAATCTCCAG-GAGAG-3', and the antisense primer 5'-CACAAAC-CGAGTTCATGCAC-3'. The reaction conditions were 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min for 25 cycles. The Col genotype represents *PI/PI*, and the *pi-1* genotype from L*er* represents *pi/pi*. The Col *PI* gene differs from the L*er pi* gene only in terms of a few bases. Specific primers, the sense primer 5'-GTTATCTG-GCAAGAAACTATGGG-3', and the antisense primer 5'-AGAATAATGACCTGAGCTCC-3', were designed to partially amplify both *PI* and *pi* genomic DNA. The amplified bands from several genomic DNAs were the same length (1.1 kb) and were digested using the restriction enzyme *Bsr*I (NEW ENGLAND Bio Labs, UK). The L*er* band was digested by the site and separated into 570 bp and 530 bp bands, while the Col band was not. The F_2 plants showed three genotypes, *PI/PI* + *MdPI*, *PI/pi* + *MdPI*, and *pi/pi* + *MdPI* (Fig. 1B), and therefore, the amplified bands digested by *Bsr*I were used to determine the genotypes. This rescue experiment was confirmed by observing the phenotype of the *Arabidopsis* flowers, which expressed 35S::MdPI with a *pi/pi* background. The *PI/PI* + *MdPI* plants had a 1.1 kb band. The *PI/pi* + *MdPI* plants had three bands (1.1 kb, 570 bp, and 530 bp). The *pi/pi* + *MdPI* plants had only the shorter bands (570 bp and 530 bp).

Scanning electron microscopy

The F_2 plants (*pi/pi* + *MdPI*) were observed with a light microscope and a scanning electron microscope (SEM). SEM observations were performed as follows according to the method of Kubono and Ito (2002). Transgenic plants were fixed in 5% glutaraldehyde for 24 h at 4°C, then in 0.1% osmium tetroxide for 90 min at 4°C. They were then dehydrated through a graded ethanol series and dried with a Hitachi Critical Point Dryer (Hitachi, Ibaraki, Japan). The plants were coated with gold using an IB-3 Ion Coater (Eiko, Ibaraki, Japan), and observed with a JSM-5310 LV SEM (JEOL, Tokyo, Japan) operating at 5 kV.

MdPI promoter cloning

Genomic DNA was isolated from the leaves of the apple cultivar 'Jonathan' using the modified CTAB method (Kotoda et al., 2000). After partial digestion with *Sau*3AI, the genomic library (9–20 kb) was inserted at the *Bam*HI site of phage lambda EMBL3 (Stratagene, La Jolla, CA, USA). The library was screened using a probe from *MdPI* cDNA. One million plaques were screened using the *MdPI* probe. Screening revealed three positive clones of *MdPI*. Phage DNAs were purified using the QIAGEN Lambda Mini Kit (QIAGEN K.K., Tokyo, Japan). The phage DNA digested with *Sal*I was subcloned into pBluescript II SK + (*Sal*I site) using a DNA Ligation kit (Takara Biomedicals). The plasmid was extracted using a QIAprep Spin Miniprep Kit (QIAGEN K.K.). The nucleotide sequence of the upstream region of *MdPI* (about 4.3 kb) was determined using an SQ 5500 S automatic sequencer (Hitachi). The nucleotide sequence was analyzed using GENETYX-MAC software (Fig. 2A) (Software Development Co., Tokyo, Japan). Sequence homology was searched for with a DDBJ BLAST search. A 1.0 kb upstream sequence including the *MdPI* start site was amplified using a Takara LA-PCR kit (Takara Biomedicals) with the specific sense primer 5'-TAAATTTGGAGTCCCCT-TCC-3', and the antisense primer 5'-ATCTCTCAGT-ATTCTTGTTCTCTCTATTTTCTCC-3'. The reaction

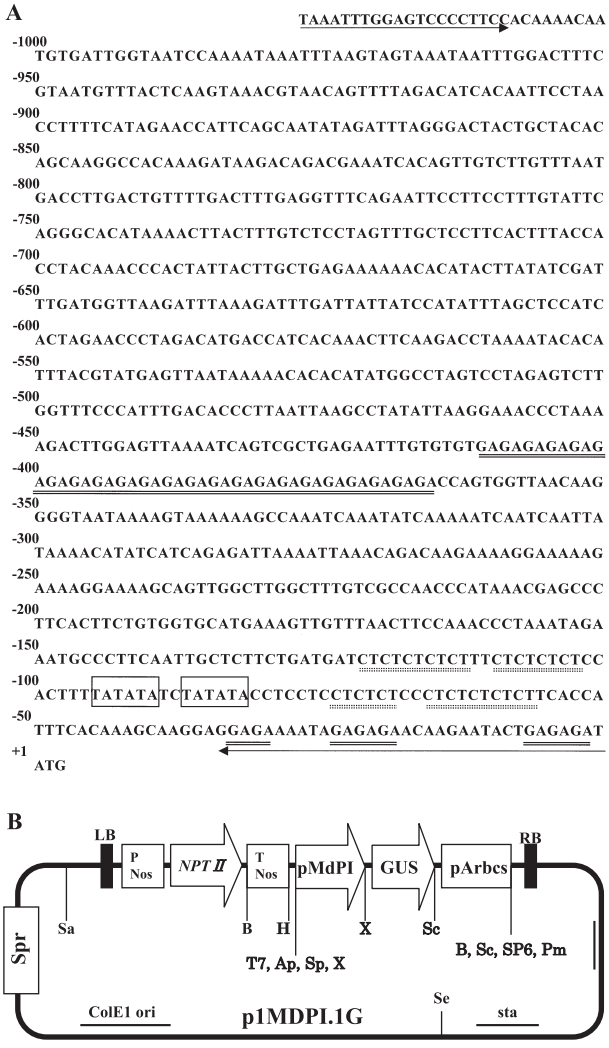

Fig. 2. (**A**) DNA sequence of the *MdPI* upstream region. A putative TATA box is indicated by the square. The primers are indicated by arrows. GA and CT repeats are indicated by double under lines and double dotted under lines, respectively. (**B**) Construction of the vector p1MDPI.1G with the *MdPI* promoter, which was inserted in the sense direction into binary vector pSMAK312 Blue. pNos, Nos promoter; Tnos, Nos terminator; *NPTII*, neophosphotransferase; GUS, *uidA* gene; LB, left border; RB, right border; Sa, *Sal*I; B, *Bam*HI; H, *Hind*III; T7, T7 promoter; Ap, *Apa*I; Sp, *Spe*I; X, *Xba*I; Sc, *Sac*I; SP6, SP6 promoter; Pm, *Pme*I. sta, region involved in plasmid stability; rep, essential region for plasmid maintenance.

conditions were 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min for 25 cycles. The 3' end of the PCR product was linked to the β-glucuronidase (GUS) gene in the frame and was designated p1MDPI.1G (Fig. 2B).

GUS assay

The transformation vector p1MDPI.1G was introduced into Col by the floral dip method (Clough and Bent, 1998). The obtained T_0 transgenic plants comprised nine lines. The flowers and inflorescences of the transgenic *Arabidopsis* plants were soaked in staining buffer (10 μM $NaPO_4$ (pH 7.4), 500 μg·mL^{-1} X-glucuronidase (5-Bromo-4-chloro-3-indolyl-β-D-glucuronide cyclohexylammonium salt), 500 μM potassium ferricyanide, 500 μM potassium ferrocyanide, and 0.1% Triton X-100) and deaerated for 10 min. They were then incubated at 37°C overnight. The solution was then exchanged for a 70% ethanol solution, which bleached the flowers overnight. The stained flowers were observed and photographed under a stereoscopic microscope (OLYMPUS, Tokyo, Japan).

Results and Discussion

Effects of 35S::MdPI in Arabidopsis

We chose the *Arabidopsis* transformation system because the generation period is much shorter than that of the apple and the transformation is easier. We introduced 35S::MdPI into *Arabidopsis* (Col) by the floral dip method, and obtained sixteen T_0 transgenic plants (Fig. 3C) that survived on the selection medium with 50 mg·L^{-1} kanamycin. In the flowers of all T_0 transgenic lines, the margin of sepals changed to white petaloid organs. This result was the same as the results reported for *Arabidopsis* into which 35S::PI was introduced (Lamb and Irish, 2003). The other floral organs were unchanged. This finding suggested that *MdPI* had the same function as *PI* and was affected by *AP3* expressed slightly in the sepals. *AP3* is expressed at the base of the first whorl organs and throughout the second and third whorl organs (Krizek and Meyerowitz, 1996).

We assessed whether *MdPI* recovers the phenotype of the *pi-1* mutant (L*er*) by crossing *pi-1* with the pollen of one individual T_0 transgenic plant in which 35S::MdPI

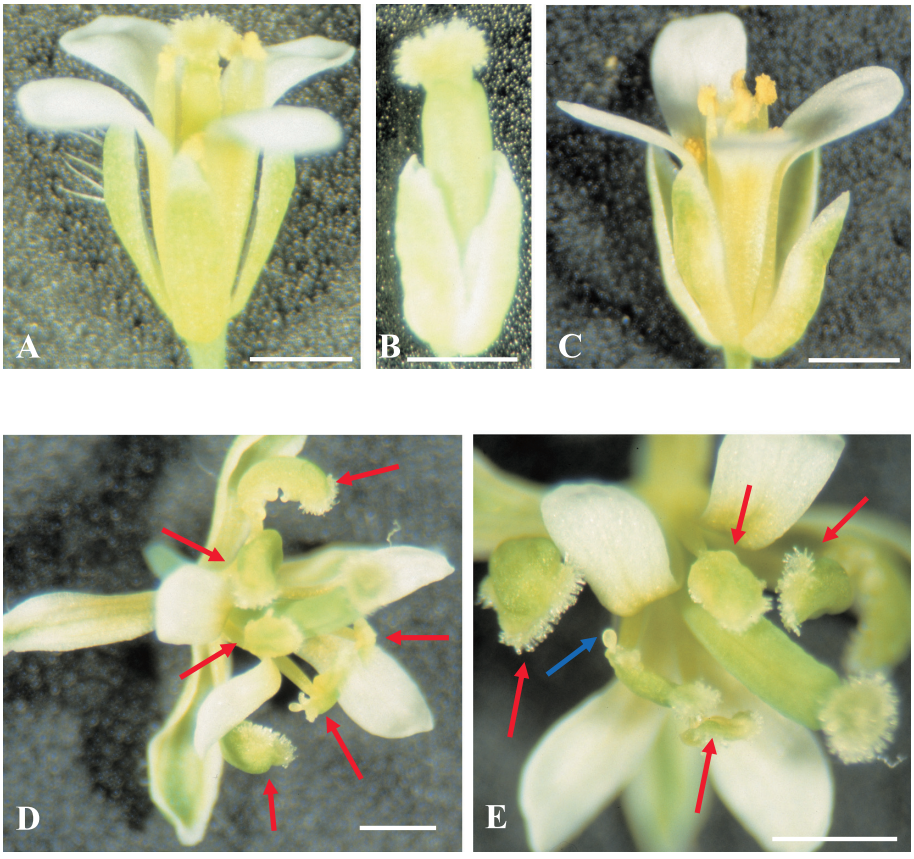

Fig. 3. Effect of 35S::MdPI introduction in *Arabidopsis*. Light micrographs of *Arabidopsis* flowers are shown. (**A**) Normal flower (Columbia). (**B**) *pi-1* mutant flower: has a single big carpel and double sepals (Landsberg *erecta*). (**C**) 35S::MdPI transformed Columbia flower (T_0), the margin of sepals showed white-like petals. (**D**) F_2 plants (*pi/pi* + *MdPI*) recovered four normal petals completely, but had carpel-like stamens (red arrows). The sepals also showed a white margin the same as petals of C. The carpel appeared to show no effects. (**E**) Magnification of D. Some carpeloid stamens were unclosed with an ovule-like sphere at the base of the carpeloid stamens. (blue arrow). Bars = 500 μm.

was introduced (Fig. 1B). After the self-pollination of F_1, some F_2 seeds were selected on a 1/2 MS agar plate with kanamycin. Phenotype recovery brought about by the introduced *MdPI* was confirmed in the F_2 generation. The introduction of *MdPI* into some F_2 transgenic lines (lines 1 to 6) was confirmed by PCR using the genomic DNA (Fig. 4A). In this study, the introduction of *MdPI* was able to be confirmed in all investigated lines. The *PI* or *pi* partial fragment was amplified from the same genomic DNA of antecedent transgenic plants by PCR (Fig. 4B). The PCR products were digested by the restriction enzyme *Bsr*I. The F_2 transgenic plants with *MdPI* with the *pi/pi* and *PI/pi* backgrounds had the recognition site of *Bsr*I, but the *PI/PI* background plants did not. The F_2 transgenic lines 1, 3, and 5 were considered to have the *pi/pi* background because PCR products were cut by *Bsr*I (Fig. 4C). The other lines (lines 2 and 4) with normal floral organs were not cut by *Bsr*I. This suggested that lines 2 and 4 had a *PI/PI* background. However, line 6 with normal floral organs was confirmed to have two bands. It was suggested that line 6 had a hetero genotype with a *PI/pi* background. The margin of sepals of F_2 transgenic lines 1, 3, and 5 had changed to petaloid organs as in *MdPI*-introduced

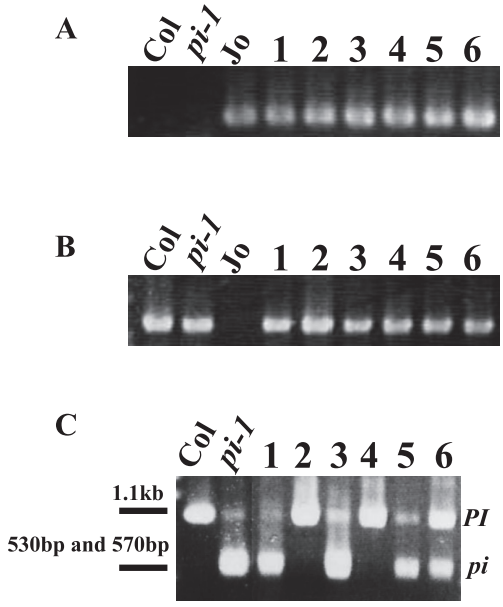

Fig. 4. (**A**) *MdPI* was amplified from F_2 transgenic lines (lines 1 to 6) and 'Jonathan' by PCR using the genomic DNA. The amplification of bands was confirmed in all investigated individuals with *MdPI*. (**B**) The F_2 transgenic lines (lines 1 to 6) and controls were amplified partially for both *PI* and *pi* genomic DNA. The band was amplified in all individuals except for 'Jonathan'. (**C**) Investigation of the genotype of 35S::MdPI-transformed F_2 transgenic lines. The genomic PCR product of Col (*PI/PI* background) was not digested by *Bsr*I, but *pi-1* (Landsberg *erecta*) (*pi/pi* background) was digested. From the left, Columbia, *pi-1*, F_2 transgenic lines 1 to 6. Lines 1, 3, and 5 (*pi/pi* background) were digested by *Bsr*I. Lines 2 and 4 (*PI/PI* background) were not digested by *Bsr*I. Line 6 had the hetero genotype (*PI/pi* background). Col: Columbia; Jo: 'Jonathan'.

Col (T_0) and their petals were completely recovered; however, normal stamens were not (Fig. 3D, E). The distal parts of the stamens were changed to stigmanoid organs corresponding to the position of the anthers. The carpeloid stamens had unclosed carpels, and the ovules were exposed to the outside (Fig. 3E). For this F_2 phenotype, no stamens were observed in all individuals. The petals of the F_2 plants (*pi/pi* + *MdPI*) were normally recovered when *MdPI* was overexpressed under the control of the 35S promoter. Even though the 35S promoter should be overexpressed in all organs, the F_2 plants (*pi/pi* + *MdPI*) could not recover their stamens fully and no change was observed in the carpels. This result suggested that *MdPI* has a weak interaction with *AP3*, *AG*, and *SEPALLATA* (*SEP1*, *2*, *3*, and *4*) in the stamens, because *PI* in *Arabidopsis* interacts with *AP3*, *AG*, and *SEPALLATA* (Ditta et al., 2004; Theissen, 2001; Theissen and Saedler, 2001). However, we found that the F_2 plants (*pi/pi* + *MdPI*) formed filamentous stamens when the temperature of the culture room was lower than that of the growth chamber. In the growth chamber at 22°C, the distal half of the recovered stamens was carpeloid. This suggested that temperature is an important factor in the effectiveness of *MdPI* on floral organ formation in *Arabidopsis*. The temperature would affect the subunit formation of *MdPI* and other MADS products.

The fine structures of the complemented plants with the *pi/pi* background were observed by SEM (Fig. 5). Although a number of stamens appeared to have normal filaments, the carpeloid form was observed in some stamens (Fig. 5C). The distal half of the carpeloid stamens had a carpeloid structure, and showed carpel cells with papillae and stomata (Fig. 5F–H), while the proximal half remained filamentous, and had equal oblong cells (Fig. 5E) like normal filaments of the wild-type (Fig. 5D). The stamens showed either carpeloid or filamentous forms (Fig. 5F, I). The distal half of the apparently normal filaments also had carpel cells with stomata (Fig. 5I). The filamentous stamen showed a boundary between the filamentous and carpeloid tissues (allow). Therefore, the distal half of the carpeloid stamens and filamentous tissues seems to have carpel tissues. Ovules were formed on the bases of the carpeloid stamens, not on the filaments (Fig. 5C, F arrow), and a micropyle and suspensor structure was observed (Fig. 5J). *MdPI* functions in the formation of petals and stamens and belongs to a homologue of the class B floral identity gene *PI* in *Arabidopsis* (Yao et al., 2001). However, in the present study, the overexpression of 35S::MdPI with the *pi/pi* background showed that the anthers were not recovered and carpeloid organs were observed. This result indicates that *MdPI* also functions in the formation of petals and the filaments of stamens.

The introduction of *MdPI* indicated that *MdPI* is effective primarily in the first whorl, as the sepals edges turned white and developed a smooth surface without

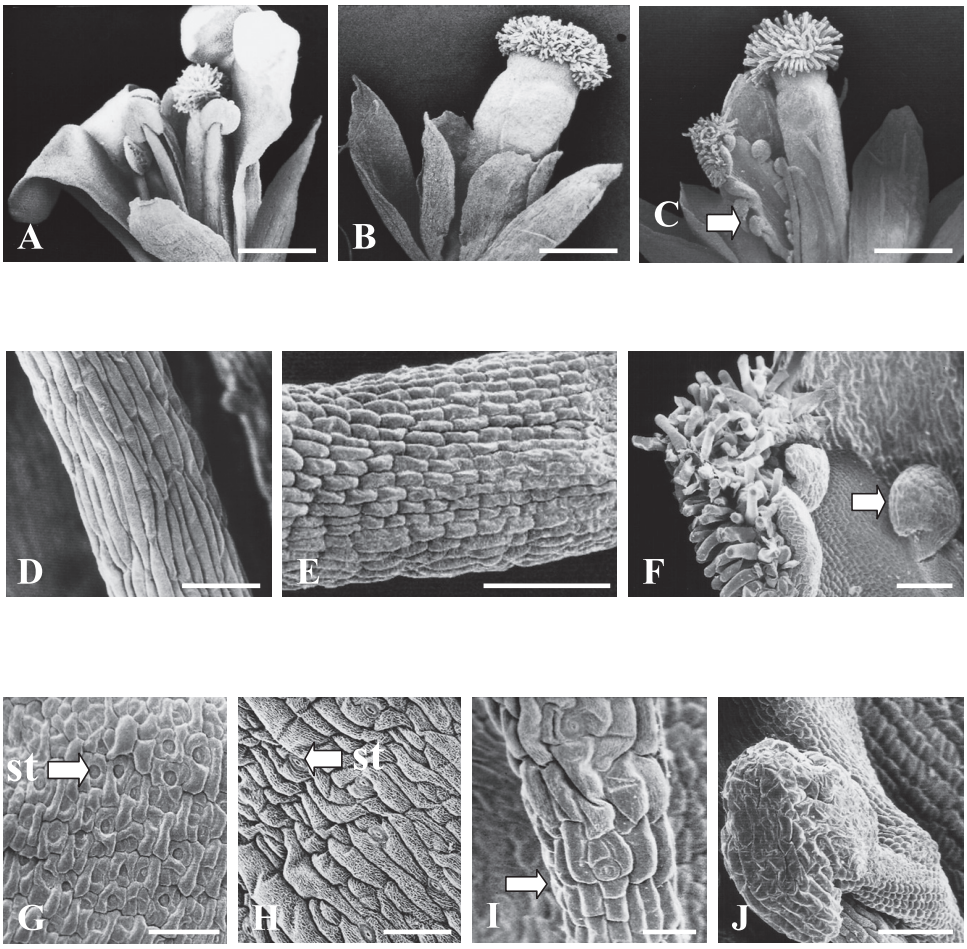

Fig. 5. Observations of the F_2 plants (*pi/pi + MdPI*) by scanning electron microscopy. The F_2 plants (*pi/pi + MdPI*) are C, E, F, H, I, and J. (**A**) Normal flower *Arabidopsis thaliana* (Columbia). (**B**) *pi-1* (Landsberg *erecta*) flower. (**C**) The flower of F_2 plants (*pi/pi + MdPI*). The tops of the recovered stamens were carpeloid. The arrow shows an ovule. (**D**) Filament cells of the wild-type (Col). (**E**) Filament cells of F_2 plants (*pi/pi + MdPI*) in recovered stamens. The filaments had normal cells the same as the wild-type. (**F**) Carpeloid stamen of the F_2 plants (*pi/pi + MdPI*). Their tips had normal carpel cells. The arrow shows an ovule. (**G**) Carpel cells of the wild-type. st: stomata. (**H**) Cells of the F_2 plants' (*pi/pi + MdPI*) carpeloid stamen. (**I**) Boundary of filament and carpeloid tissues in the filamentous stamen (arrow). (**J**) The arrow of C was expanded. Ovule exposed outside. An ovule was formed in the carpeloid stamens and had a micropyle and suspensor. A, B, and C, bar = 500 μm; D, E, G, H, and J, bar = 50 μm; F, bar = 100 μm; I, bar = 20 μm.

trichomes such as petals. This change in the sepals has been considered to be caused by the simultaneous expression of both *MdPI* and *AP3* in the first whorl of Col. Lamb and Irish (2003) reported that the introduction of 35S::PI and 35S::AP3 into *pi-1* changed the first and second whorls to petals and the third whorl to a mix of normal stamens, carpeloid stamens, and filaments. In the case of *pi-2*, it replaced Ser_{75} with Phe (Goto and Meyerowitz, 1994). Flowers of *pi-2* have sepals in the second whorl and carpeloid organs, filaments, or an absence of organs in the third whorl (Bowman et al., 1991). In the case of *pi-2* whose homeotic mutation is not more severe than that of *pi-1*, the phenotype was fully recovered by the introduction of 35S::PI and 35S::AP3. This is because the development of the stamens is based on the level of *PI* protein (Krizek and Meyerowitz, 1996). Normal stamens might be recovered by the introduction of *MdPI* in *pi-2*. In addition, in order to analyze the floral organ identity genes, not only *PI* but also *MdMADS13* or *MdTM6* (Kitahara et al., 2004; Yao et al., 1999), the *AP3* homologue in apple, should be introduced together.

GUS expression analysis using the MdPI upstream region

We isolated the apple *MdPI* upstream region from the genomic library (Fig. 2A). Three positive clones were obtained; the resultant clones were identical. The length of the possible cloned *MdPI*, including the upstream region of *MdPI*, was approximately 4 kb. We could not find a clear homology of this upstream region with the *PI* or *PI* homologue promoter regions (Honma and Goto, 2000; Lännenpää et al., 2005). The *PI* promoter of *Arabidopsis* does not have the CArG box, which is present in the *AP3* promoter (Hill et al., 1998; Honma and Goto, 2000; Tilly et al., 1998). The *MdPI* promoter

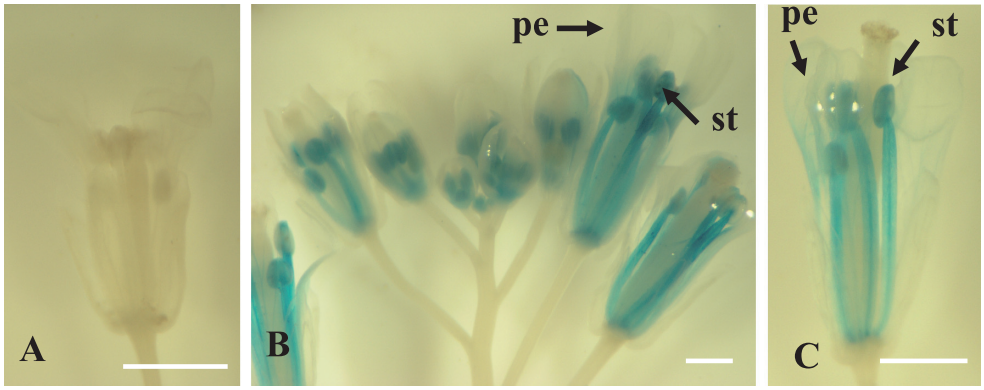

Fig. 6. GUS expression patterns conferred by the *MdPI* promoter. Inflorescence and flowers of *Arabidopsis* with the 1.0 kb pMdPI::GUS. The transgenic plants showed GUS activity only in petals and stamens. (**A**) Non-transgenic flower (Columbia). (**B**) pMdPI::GUS-transformed *Arabidopsis* infloresence. (**C**) pMdPI::GUS-transformed *Arabidopsis* flower. pe: petal, st: stamen (arrows). Bars = 500 μm.

in the apple similarly does not have the CArG box. The *MdPI* promoter has a TATA box at −90 bp in the non-coding upstream region (Fig. 2A). Repeated sequences of GA (from −2 bp to −33 bp and −366 bp to −411 bp) and CT (from −57 bp to −74 bp and −103 bp to −122 bp) were found flanking the TATA box. These repeated sequences are similar to those of the *PI* promoter of *Arabidopsis*, and are assumed to make a stem-loop structure. This stem-loop structure may have activated the combination with an RNA polymerase. In addition, there was an almost complete GA repeated sequence 46 bp long from −366 bp to −411 bp. This sequence may be important in the transcriptional regulation of *MdPI*, because the promoter region of *PI* has similar repeated sequences and was considered to relate to the transcriptional regulations (Honma and Goto, 2000). The coding region of *MdPI* of 'Jonathan' has 98% homology with the *MdPI* sequence of 'Granny Smith' (Yao et al., 2001). These finding suggest that the upstream region is the promoter region of *MdPI*. The slight difference in homology between 'Jonathan' and 'Granny Smith' is thought to depend on the allele.

This 1.0 kb upstream region fused to GUS was introduced into *Arabidopsis*. Nine independent transgenic lines were selected. The specific GUS expression patterns were observed only in petals and stamens (Fig. 6B, C). This indicated that *MdPI* is a class B gene whose expression is limited in petals and stamens, as found in the analysis with *PI* and *AP3* promoter (Honma and Goto, 2000; Tilly et al., 1998). However, the GUS activity in petals was slight. This suggested that the cell layer of the petals is thin and the substrate is difficult to maintain.

These results indicated that the *MdPI* gene was related to the development of petals and stamens, and formed petals and filaments. Therefore, the *MdPI* gene had a function equal to *PI*, although the relation between *MdPI* and parthenocarpy in apple is unknown.

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