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Flowering induced by 5-azacytidine, a DNA demethylating reagent in a short-day plant, *Perilla frutescens* var. *crispa*

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Treatment with 5-azacytidine, a DNA demethylating reagent, induced flowering in *Perilla frutescens* (L.) Britton var. *crispa* (Thunb. ex Murray) Decne. ex L. H. Bailey, an absolute short-day plant under long days. The 5-azacytidine treatment induced slight suppression of vegetative growth but had no obvious effect on any other phenotypes. The Southern hybridization analysis of the genomic DNA isolated from the leaves of 5-azacytidine-treated plants and digested with restriction enzyme, methylation-insensitive *Msp* I or methylation-sensitive *Hpa* II with *P. frutescens* 25S-18S rDNA intergenic spacer probe indicated that the 5-azacytidine treatment caused demethylation of the genomic DNA. The 5-azacytidine-induced flowering was delayed as compared with the short day-induced flowering. Flowers were formed even at the lower nodes which had not been directly treated with 5-azacytidine. The results suggest that DNA demethylation induced flowering by inducing the production of a transmissible flowering stimulus in *P. frutescens*.

Introduction

Flowering is regulated by environmental factors such as night length (photoperiodic flowering), coldness (vernalization), and stresses in many plant species. The recent studies on the regulation mechanism of gene expression by DNA demethylation (Finnegan et al. 1998b) have clarified many aspects of the molecular mechanism of vernalization (Michaels and Amasino 2000, 2001, Sheldon et al. 2000a). In vernalization, the effect of low temperature perceived by the seeds or young seedlings is actualized when the plants reach the adult phase. The once-induced effect of low temperature is transmitted mitotically during the plant's development. This mitotic stability suggests an epigenetic basis for vernalization. DNA methylation/demethylation is involved in the regulation of such epigenetic phenomena (Michaels and Amasino 2000, 2001). In *Arabidopsis thaliana*, the transcript level of floral inhibitor, *FLOWERING LOCUS C* (*FLC*) is downregulated by the cold temperature (vernalization) (Michaels and Amasino 1999, Sheldon et al. 1999). The *FLC* transcript level remains low for the remainder of the plant's life, after the vernalized plant is returned to a normal temperature (Sheldon et al. 2000b). Therefore, DNA demethylation was supposed to be involved in the regulation of *FLC* expression. 5-Azacytidine which prevents the transfer of methyl residue into DNA molecule given to plants replaces cytosine during DNA replication causing demethylation of DNA. The treatment with 5-azacytidine of vernalization-requiring late-flowering mutants *fca* and *fy* of *A. thaliana* induced precocious flowering (Burn et al. 1993). Treatment with 5-azacytidine also induced flowering in the vernalization-requiring *Thlaspi arvense* (Burn et al. 1993). Furthermore, over-expression of

Abbreviations – LD, long days; SD, short days.

antisense-methyltransferase cDNA (*MET1*) induced flowering and downregulation of *FLC* expression in *A. thaliana* (Finnegan et al. 1998a, Sheldon et al. 1999). These results indicate that the flowering genes are upregulated through the decrease in DNA methylation (Michaels and Amasino 2000, 2001). Because *FLC* is a flowering repressor, the gene which is upregulated by DNA demethylation is not *FLC* itself. The gene(s) upstream of *FLC*, such as *VERNALIZATION1* (*VRN1*), *VERNALIZATION2* (*VRN2*) or *VERNALIZATION INSENSITIVE3* (*VIN3*) (Gendall et al. 2001, Levy et al. 2002, Sung and Amasino 2004), may be upregulated by DNA demethylation.

Whether or not DNA demethylation is involved in photoperiodic flowering remains unknown. The time at which a favorable photoperiodic cue is perceived could not be clearly separated from the start of the formation of flower buds in photoperiodic flowering. Therefore, there has been no way of examining the long-lasting stability of the effect of photoperiodic treatment and epigenetic basis for photoperiodic flowering. In a short-day (SD) plant, *Perilla crispa* (synonym *Perilla frutescens*), however, photoperiodic induction has been characterized as an irreversible and indestructive phenomenon (Zeevaart 1958, 1969). That is, the flowering state did not revert to a vegetative state when the photoinduced plants were exposed to non-inductive light condition. By grafting the photoinduced leaves onto vegetative stocks, the stocks are induced to flower under long-days (LD). A single photoinduced leaf scion could repeatedly induce flowering in the stocks kept under LD. Such longevity of the flowering status in *P. frutescens* is analogous to the stability of cold treatment in vernalization and suggests that the same mechanism is involved in the regulation of photoperiodic flowering in *P. frutescens* and vernalization. DNA demethylation might be involved in the regulation mechanism of photoperiodic flowering in *P. frutescens*.

Here, we report that 5-azacytidine, a DNA demethylating reagent, applied to *P. frutescens* induced flowering under LD, suggesting the involvement of DNA demethylation in the photoperiodic flowering in *P. frutescens*.

Materials and methods

Plant materials and growth condition

Green-leafed and red-leafed varieties of *P. frutescens* (L.) Britton var. *crispa* (Thunb. ex Murray) Decne. ex L. H. Bailey were used as the experimental materials. The nomenclature of *Perilla* is confusing, and the same species has been called by the names of *P. crispa*, *P. frutescens*, *P. ocymoides*, or *P. nankinensis* in the past literature on flowering (Zeevaart 1986). In this paper, we will call it '*Perilla frutescens*'.

The seeds were placed on moist filter paper in a Petri dish 90 mm in diameter and germinated at 25°C under 16-h light and 8-h dark (LD) condition. White light (100–160 $\mu mol \cdot m^{-2} \cdot s^{-1}$) was provided by fluorescent lamps (Toshiba FL40SSW/37). The germinated seeds were planted to soil or vermiculite in a plastic pot 90 mm in diameter and grown under the same condition as before. Once a week, the plants were given a mineral nutrient solution originally reported by Nakayama and Hashimoto (1973) and partly modified as follows: 250 mg l^{-1} NH_4NO_3, 250 mg l^{-1} KNO_3, 250 mg l^{-1} $MgSO_4 \cdot 7H_2O$, 250 mg l^{-1} $Ca(H_2PO_4)_2 \cdot H_2O$, 250 mg l^{-1} KH_2PO_4, 6 mg l^{-1} Fe(III)-EDTA, 2 mg l^{-1} H_3BO_3, 1 mg l^{-1} $MnSO_4 \cdot 4H_2O$, 0.2 mg l^{-1} $ZnSO_4 \cdot 7H_2O$, 0.2 mg l^{-1} $Na_2MoO_4 \cdot 2H_2O$, and 0.1 mg l^{-1} $CuSO_4 \cdot 5H_2O$.

Treatment with 5-azacytidine

The DNA demethylating reagent, 5-azacytidine (Wako Pure Chemical Industries, Ltd., Osaka, Japan), was used at the concentration of 50 or 250 $\mu mol\ l^{-1}$ (Burn et al. 1993). For treatment of the seeds, 5-azacytidine dissolved in water was applied to a cube block of packed vermiculite and fibers (20 mm × 20 mm × 20 mm) (Nisshinbo Industries, Inc., Tokyo, Japan) into which the seeds were inoculated. When shoot apical meristem was treated, the seedlings were treated by dropping 10 µl of a 40% ethanol solution of 5-azacytidine on the plumule of main stem with a microsyringe. The treatment was repeated for 5 or 10 days using a solution freshly prepared every day.

SD treatment

Except otherwise mentioned, plants were grown at 25°C under the LD condition for 4–5 weeks after the seed germination until the fourth leaf pair expanded and then kept in a 8-h light and 16-h dark (SD) condition at 25°C for 5 or 10 days. After the SD treatment, the plants were returned to the LD condition.

Scoring of flowering response

Flowers of *P. frutescens* are formed as solitary flowers or inflorescences at leaf axils or as a terminal inflorescence at the shoot apex. All the leaf axils on the main stem of the treated plants were dissected under a binocular microscope to determine whether they formed flowers, inflorescences or their primordia. The percentage of plants with at least one flower or inflorescence among

all the plants treated (% flowering) and number of flowers per plant, number of inflorescences per plant, or total number of flowers and inflorescences per plant are presented. The average length of the main stem was presented as an indicator of vegetative growth. Twenty plants were used for each treatment. Each experiment was repeated at least three times.

DNA methylation analysis using genomic Southern hybridization

Referring to the base sequences of *A. thaliana* rDNA, a primer with the sequence of 3′ end of 25S rDNA, 5′-CAGGTTAGTTTTACCCTACTGATGCC-3′ and a primer with the sequence of 5′ end of 18S rDNA, 5′-TTATTGTCACTACCTCCCCGTGTC-3′ were constructed. Genomic DNA was prepared from leaves of red-leafed *P. frutescens*. The polymerase chain reaction was performed with the *P. frutescens* genomic DNA and the primers mentioned above. The cycling condition was 35 cycles of 95°C for 1 min, 50°C for 1 min and 74°C for 5 min. The amplified DNA fragment was cloned into a plasmid vector (pBluescript II SK (-), Stratagene) using TA cloning method. The plasmid was propagated in *Escherichia coli* cells and was purified by the alkaline lysis method. The purified plasmid was digested with restriction enzymes, *Sal* I and *Nco* I, and the products resolved by electrophoresis on an agarose gel obtaining 853-bp 25S rDNA-18S rDNA intergenic spacer region fragment. The fragment obtained was sequenced (DDBJ, Accession No. AB185210). This fragment was labeled with ^{32}P and used as a probe for the Southern hybridization.

The genomic DNA prepared from leaves of red-leafed *P. frutescens* was digested with methylation-sensitive restriction enzyme, *Hpa* II which does not cleave a CCGG site when the inner cytosine is methylated, or methylation-insensitive restriction enzyme, *Msp* I which cleaves this site even when the inner cytosine is methylated. The digested DNA fragments were fractionated on 1.2% agarose gel and transferred to Hybond-N (Amersham) membrane. The fragments were detected by [^{32}P]-25S rDNA -18S rDNA intergenic spacer probe.

Results

Characteristics of flowering behavior

Flowering behavior of *P. frutescens* was first studied. Plants were given 10 SDs after grown under the LD condition for 45 days and then returned to the LD condition. Another lot of plants were continuously grown under the LD condition for 101 days. The SD treatment induced 100% flowering response, whereas the LD-grown plants strictly remained at the vegetative state for more than 3 months (Table 1). The vegetative growth of the flower-induced plants was significantly suppressed, although the duration of the SD treatment was only 10 days during the total growth period of 101 days.

Plants were then induced to flower by treatment with 55 SDs, and the time-depending change in the flowering response was scored after returning to the LD condition (Table 2). The plants continued to produce floral buds even after returning to the non-inductive LD condition. On the other hand, number of vegetative buds was quite small and decreased with time proceeded. Vegetative buds were never formed even after 42 days under LD condition. These results indicate that once-induced flowering state never return to vegetative state.

Table 1. Flowering response and vegetative growth of green-leafed and red-leafed *Perilla frutescens* var. *crispa* under different photoperiodic conditions. Seedlings were grown under a 16-h light and 8-h dark condition for 63 or 64 days after seed sowing and then under an 8-h light and 16-h dark condition for 10 days (SD). The plants were again moved to the 16-h light and 8-h dark condition. The other plants were continuously grown under the 16-h light and 8-h dark condition (LD). Flowering response and vegetative growth were scored 101 or 102 days after the seed sowing.

	Photoperiodic condition	% flowering	Inflorescences/ plant	Main stem length (mm)
Green-leafed	SD	100	13 ± 0.88	76 ± 6.1
	LD	0	0 ± 0	301 ± 13.3
Red-leafed	SD	100	10 ± 1.7	147 ± 7.13
	LD	0	0 ± 0	283 ± 19.0

Table 2. Changes in flowering response and vegetative growth of red-leafed *Perilla frutescens* var. *crispa* after flower-inducing short-day treatment. Seedlings were grown under a 16-h light and 8-h dark condition for 34 days after seed sowing until four pairs of leaves were fully expanded and then given SD (16-h dark a day) treatment for 55 days. The plants were then moved to the 16-h light and 8-h dark condition. Flowering response and vegetative growth were scored immediately, 21 or 42 days after the end of the flower-inducing SD treatment. Total number of the solitary flowers on the main stem and flowers on inflorescences was scored (Flower buds/plant). Number of the vegetative buds was also scored.

Days after SD treatment	% flowering	Flower buds /plant	Vegetative buds /plant	Main stem length (mm)
0	100	130 ± 17	0.10 ± 0.069	99 ± 11
21	100	340 ± 44	0.048 ± 0.048	100 ± 10
42	100	460 ± 39	0 ± 0	98 ± 8.1

Table 3. Flowering response of green-leafed and red-leafed *Perilla frutescens* var. *crispa* to 5-azacytidine. Seeds treated with 5-azacytidine at the concentrations indicated for 5 days at 25°C under a 16-h light and 8-h dark condition were planted in soil and grown under the same conditions for 73 days. Number of the solitary flowers and that of inflorescences were scored.

	5-azacytidine (μmol l^{-1})	% flowering	Solitary flowers/plant	Inflorescences/plant	Main stem length (mm)
Green-leafed	0	0	0 ± 0	0 ± 0	53 ± 2.5
	50	0	0 ± 0	0 ± 0	53 ± 4.1
	250	50	1.5 ± 1.5	0 ± 0	65 ± 9.0
Red-leafed	0	0	0 ± 0	0 ± 0	83 ± 7.1
	50	0	0 ± 0	0 ± 0	84 ± 17
	250	60	4.4 ± 1.8	0 ± 0	48 ± 7.2

Induction of flowering by 5-azacytidine

Seeds of red-leafed and green-leafed *P. frutescens* were treated with 5-azacytidine at 50 or 250 μmol l^{-1} and grown under continuous LD condition. Both varieties were induced to flower by 5-azacytidine at 250 μmol l^{-1} (Table 3), and the plants formed only solitary flowers. Although treatment with 5-azacytidine suppressed the vegetative growth of the red-leafed variety slightly, no obvious effects on the other phenotypes were found.

The effect of 5-azacytidine was next examined by the application to the shoot apical meristem using the red-leafed variety which showed a higher response to the treatment of seeds. Because the 5-azacytidine treatment of seeds was effective at 250 μmol l^{-1}, this concentration was used hereafter. 5-Azacytidine was applied to the shoot apical meristem of the seedlings grown under LD condition until the fourth leaf pair expanded (Table 4). The plants thus treated were grown under a continuous LD condition, and the flowering response was scored. The treatment of 5-azacytidine to the shoot apical meristem also induced flowering. Solitary flowers were not formed, and only inflorescences were formed by this treatment.

Detection of demethylation of genomic DNA induced by 5-azacytidine

Genomic DNA was extracted from leaves of the 5-azacytidine-treated and non-treated red-leafed *P. frutescens*, and each DNA sample was digested with *Msp* I *and Hpa* II. Southern hybridization was performed with the 25S-18S rDNA intergenic spacer probe. The signal pattern in the DNA fragments digested with *Msp* I was almost identical in the 5-azacytidine-treated sample and control one (Fig. 1). In both 5-azacytidine-treated and control samples, the digestion with *Msp* I gave less signals in the high molecular weight region than did *Hpa* II. These results indicate that the *P. frutescens* rDNA intergenic spacer region is highly methylated. When the DNA samples were digested with *Hpa* II, the 5-azacytidine-treated sample gave signals in the lower molecular weight region at which no signals were detected in the control sample. This means that the 5-azacytidine treatment promoted demethylation of the rDNA intergenic spacer region.

Comparison of flowering response induced by 5-azacytidine with that induced by SD

Red-leafed *P. frutescens* grown under LD condition until the fourth leaf pair expanded were then treated with 250 μmol l^{-1} 5-azacytidine to the shoot apical meristem or kept under SD for 10 days. The flowering response induced by 5-azacytidine was weaker than that induced by SD (Fig. 2A). The time to flowering from the treatment was longer in the treatment with 5-azacytidine than in that with SD.

In another experiment, the plants were grown as above, but all the leaves except the youngest (the fourth leaf pair) were removed just before starting the 5-azacytidine or SD treatment, and the period of the treatment was reduced to 5 days. Again, the flowering

Table 4. Flowering response of red-leafed *Perilla frutescens* var. *crispa* to 5-azacytidine applied to shoot apical meristem. The seedlings were grown at 25°C under a 16-h light and 8-h dark condition for 45 days (until the fourth leaf pair expanded). Then 10 μl of a 40% ethanol solution with or without 250 μmol l^{-1} 5-azacytidine was applied to the shoot apical meristem once a day for 5 days. The plants were then grown under the same conditions for 81 days. Number of the solitary flowers and that of inflorescences were scored.

5-azacytidine (μmol l^{-1})	% flowering	Solitary flowers/plant	Inflorescences/plant	Main stem length (mm)
0	0	0 ± 0	0 ± 0	370 ± 58
250	67	0 ± 0	3.0 ± 1.2	270 ± 42

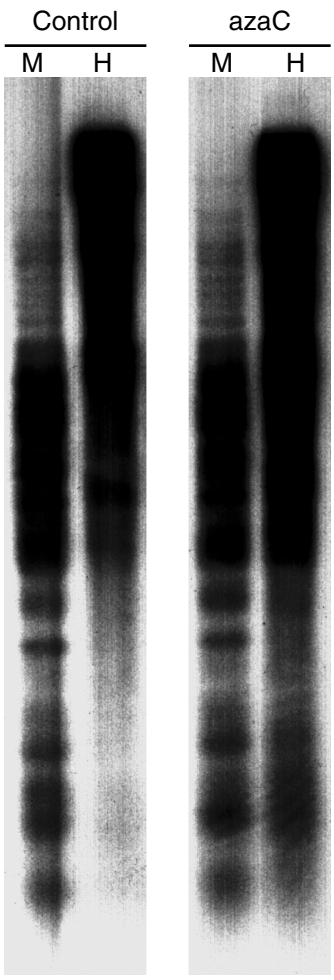

Fig. 1. Southern hybridization of genomic DNA of red-leafed *Perilla frutescens* var. *crispa*. Genomic DNA was extracted from 5-azacytidine-treated (azaC) and non-treated control (Control) plants grown under a 16-h light and 8-h dark condition, digested with *Msp* I (M) and *Hpa* II (H). The digested DNA fragments were resolved by electrophoresis and hybridized with [^{32}P]-25S-18S rDNA intergenic spacer probe.

response by 5-azacytidine was weaker and later than that by SD (Fig. 2B).

Fig. 3 shows the nodes at which flowers or inflorescences were formed in the plants treated with 5-azacytidine for 5 days (Fig. 2B). The flower or inflorescence was first formed at the fourth or nearest node and continued to be formed at both the higher and lower nodes as time proceeded. The flowers and/or inflorescences were formed at successive nodes. Within the plants which were induced to flower, the lowest node position at which inflorescence was formed was 3.3 ± 0.67 on day 25 after the end of the treatment, and was lowered to 1.8 ± 0.48 on day 35.

Discussion

Flowering by 5-azacytidine

5-Azacytidine, a DNA demethylating reagent induced flowering under a non-inductive LD condition in the SD plant, *P. frutescens* (Tables 3 and 4). Because *P. frutescens* is an absolute SD plant (Table 1), it is certain that 5-azacytidine brought about the flower-inducing effect although the flowering response was not always 100%. The rDNA intergenic spacer region is known to be rich in CG sequences and therefore highly methylated. The Southern hybridization analysis revealed that 5-azacytidine promoted demethylation of this region (Fig. 1) indicating that 5-azacytidine apparently induced DNA demethylation. This suggests that the genes involved in the regulation of flowering were also demethylated by the 5-azacytidine treatment.

The induction of flowering by DNA demethylation was first reported for the low-temperature requiring ecotypes and mutants of *A. thaliana* and *T. arvense* (Burn et al. 1993). Then the same effects were reported in winter wheat (Brock and Davidson 1994, Yong et al. 2003) and *Cichorium intybus* (Demeulemeester et al. 1999). All these plants had to be exposed to a low temperature to be induced to flower, and DNA demethylation was considered to be involved in the regulation mechanism of vernalization. However, we report here for the first time the flower-induction by DNA demethylation in a plant whose flowering is photoperiodically induced. Like the effect of coldness in vernalization, the effect of SD in the photoperiodic flowering in *P. frutescens* is persistent (Table 2). Thus, it is reasonable to consider these effects to be epigentically regulated by DNA demethylation. In many other plant species, such as *Pharbitis nil*, *Lemna gibba* and *Lemna paucicostata*, the photoperiodic induction is reversible (Thomas and Vince-Prue 1997). It is necessary to examine the effect of 5-azacytidine in other species which have members showing reversible flowering and irreversible flowering.

The flowering induced by 5-azacytidine treatment was accompanied by only slight suppression of vegetative growth with no obvious changes in the other phenotypes (Table 3). The suppression of vegetative growth was also observed in the plants induced to flower by SD treatment (Table 1). Flowering is originally the change of growth mode from vegetative to reproductive. Therefore, the suppression of vegetative growth observed may be the result of transition to flowering. This means that the 5-azacytidine treatment may cause flowering per se. *A. thaliana* transformed with antisense-methyltransferase cDNA (*MET1*) showed many

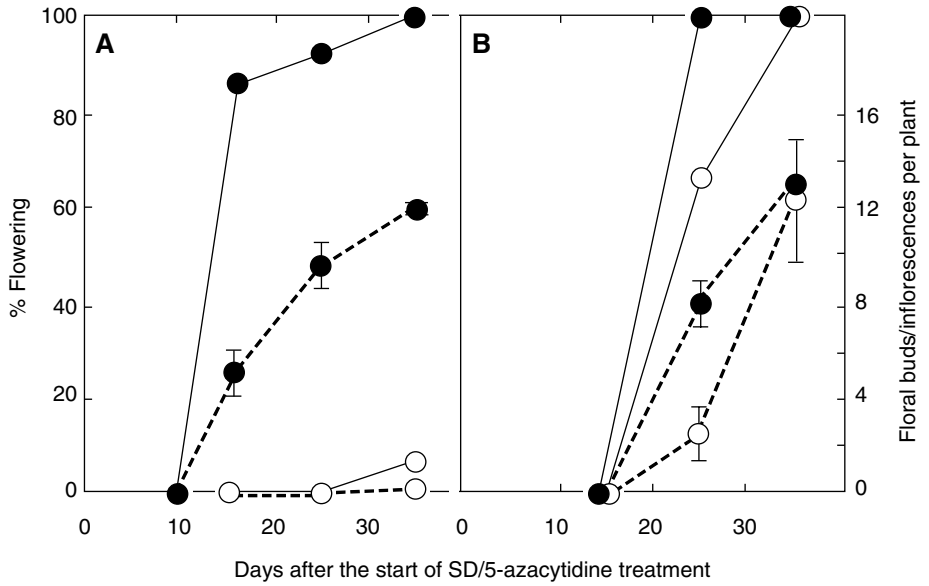

Fig. 2. Flowering responses induced by 5-azacytidine treatment and by SD treatment in red-leafed *Perilla frutescens* var. *crispa*. Seedlings grown at 25°C under a 16-h light and 8-h dark condition for 45 days (until the fourth leaf pair expanded) were given 10 μl of 40% ethanol solution containing 5-azacytidine of 250 μmol l^{-1} once a day (○), or exposed to an 8-h light and 16-h dark condition (●). The treatments were repeated for 10 (A) or 5 (B) days. For the 5-day treatments, all leaves excepting the youngest ones at the fourth node were removed. The plants were then grown under the same conditions as before the treatment, and the percentage flowering (solid lines) and total number of flowers and inflorescences per plant (broken lines) were determined on day 10–35 after the start of the treatments.

abnormalities such as reduced apical dominance, smaller plant size, altered leaf size and shape, and decreased fertility (Finnegan et al. 1996). The genes whose expression is upregulated in *P. frutescens* may be less than in *A. thaliana*, or the 5-azacytidine treatment may have a weaker DNA demethylating effect than the overexpression of antisense-*MET1*. In any case, the present result that the 5-azacytidine treatment induced flowering almost specifically in *P. frutescens* suggests that the expression of flowering-related genes is likely to be almost specifically upregulated. This means that the present experimental system may be useful to study the regulation mechanism of the flowering-related genes in *P. frutescens*.

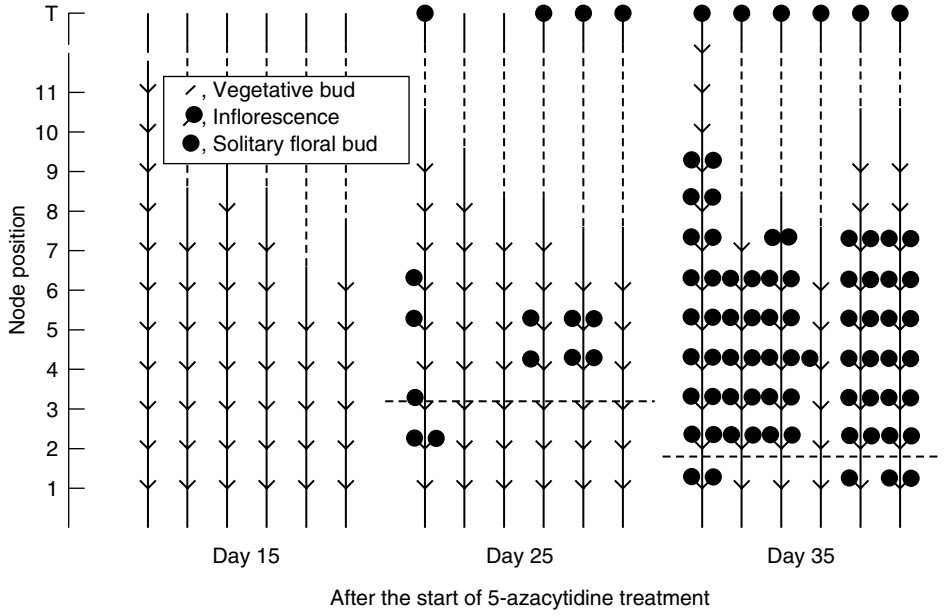

Fig. 3. The node positions at which flowers or inflorescences were formed in each of the 5-azacytidine-treated plants in the experiment shown in Fig 2B. The node position on the main shoot was counted from the bottom: the cotyledonal node was 0. T means the terminal of main shoot. The vertical broken lines indicate the average position of the lowest floral node.

Comparison of flowering induced by 5-azacytidine and that induced by SD

5-Azacytidine effectively induced flowering even when applied to the shoot apical meristem of *P. frutescens* seedlings (Table 4) as compared with the previous works in which 5-azacytidine was treated to seeds (Sano et al. 1990, Burn et al. 1993, Brock and Davidson 1994, Finnegan et al. 1998a, Demeulemeester et al. 1999). As reported by Zeevaart (1958), *P. frutescens* seedlings respond to SD treatment only after the fourth leaf pair expanded. Therefore, we could compare the effect of 5-azacytidine and that of SD by applying these treatments to the plants at the same developmental stage: when the fourth leaf pair expanded. The flowering response induced by 5-azacytidine was weaker than that induced by SD (Fig. 2). Because 5-azacytidine was given to the shoot apical meristem, it would not affect the DNA of the leaves already developed at the time of treatment. The leaves kept under the LD condition are known to inhibit the flowering response of the whole plant (Raghavan and Jacobs 1961, Purse 1984). This may have caused the weaker flowering response. To eliminate such an inhibitory influence, we removed these leaves at the time of the treatment with 5-azacytidine (Fig. 2B). The flowering rate reached 100%, although the total number of flowers and inflorescences per plant was smaller than those on the plant exposed to SD. Thus, the LD leaves appeared to inhibit both photoperiodic flowering and 5-azacytidine-induced flowering. The sensitivity of *P. frutescens* plants to SD does not depend on plant age (Zeevaart 1958). Similarly, 5-azacytidine induced flowering when applied to seeds (Table 3) and seedlings (Table 4) indicating that the sensitivity to 5-azacytidine does not depend on plant age. Such similarities in the characteristics between flowering induced by 5-azacytidine and that by SD suggest that 5-azacytidine treatment mimicked SD. Therefore, DNA demethylation may be involved in the regulation mechanism of photoperiodic flowering in *P. frutescens*.

The process of flowering induced by 5-azacytidine

Photoperiodic flowering consists of two major processes, that is, flower induction in which the flowering stimulus is generated in leaves, and flower evocation in which the shoot apical meristem responds to the flowering stimulus transported from the leaves. It is important to determine which process 5-azacytidine affects. If the 5-azacytidine treatment induced flower evocation, this process would be directly induced skipping the preceding flower induction process. If this were true, the time from the 5-azacytidine application to the flower formation would be shortened resulting in earlier flowering than in the SD-induced flowering. However, the flowering induced by 5-azacytidine was delayed as compared with that induced by SD (Fig. 2). We supposed that this was because the 5-azacytidine treatment was effective only in the leaves formed after the treatment, while the SD treatment would affect even the developed leaves. To avoid this difference, we removed all the leaves except the youngest ones as discussed above. Even then, the flowering by 5-azacytidine was delayed (Fig. 2B). This suggests that the process induced by 5-azacytidine was not flower evocation. In the plants treated with 5-azacytidine when the fourth leaf pair expanded, a flower or inflorescence was first formed at around the fourth node, and then additional flowers and/or inflorescences were formed at the higher and lower nodes (Fig. 3). The flowers and/or inflorescences formed at the higher nodes had originated from the cells generated by the shoot apical meristem which had been treated with 5-azacytidine. On the other hand, the flowers and/or inflorescences formed at the lower nodes had originated from the cells generated by the axilary meristem not treated with 5-azacytidine. Therefore, the flowering occurring at the lower nodes is not the result of a direct effect of 5-azacytidine treatment, but the influence of the treatment of the upper part of the plant with 5-azacytidine. The possibility that 5-azacytidine itself moved could be eliminated, because 5-azacytidine is very unstable as we needed to use freshly prepared solution every day during the 5 or 10 days treatment. This suggests that a transmissible flowering stimulus was generated by the 5-azacytidine treatment.

The transmissible flowering stimulus has been hypothetically called 'florigen', and the identification of it is one of the major goals of the flowering study. Recently, mRNA or protein of the floral pathway integrator *FLOWERING LOCUS T* (*FT*) was reported to be a transmissible flowering stimulus in *A. thaliana* (Abe et al. 2001, Huang et al. 2005, Wigge et al. 2005). We do not have currently any informations to argue whether *P. frutescens* has the same regulating mechanism as does *A. thaliana*. The genes involved in the regulation of flowering in *P. frutescens* should be cloned, and their expression should be studied in the connection with photoperiodic condition.

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