Temperature-dependent Flower Malformation in Carnations (*Dianthus caryophyllus* L.)

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Temperature regimes that cause malformed flowers were examined and histological observation was carried out at the developmental stage of flowers by using mutants of the potted carnation (*Dianthus caryophyllus* L.) ‘Cherry’ producing malformed flowers according to cultivation season. Plants of normal (WT) and malformed (*mlf*) lines were grown under several temperature regimes. All WT plants produced normal flowers, whereas *mlf* lines showed a variety of malformed floral phenotypes, including phyllody-like proliferated sepaloids, proliferated petaloids, proliferated pistillodes with or without petals, secondary flower formation, and a flattened receptacle. In Experiment 1, *mlf* plants produced no malformed flowers when grown under constant 26°C, whereas 34.2% of *mlf* plants produced malformed flowers at 14–16/12°C (day/night, natural light). Malformation frequency was slightly lower at a night temperature of 5°C compared with 14–16/12°C. When malformed *mlf* plants were transferred from 17/5°C to 23/18°C, flower malformation was alleviated. Conversely, when *mlf* plants grown under constant 26°C with a normal phenotype were transferred to 17/12°C, flower malformation was induced. Thus, flower malformation was reversible depending on the temperature regime. In Experiment 2, 92.2% of *mlf* plants produced malformed flowers under constant 15°C, whereas 3.1% and 1.3% showed flower malformation when grown under constant 20°C and 25/20°C, respectively. These findings suggested that the threshold for flower malformation is between 15°C and 20°C. Observation of shoot apices by optical microscopy and scanning electron microscopy revealed morphological differences between WT and *mlf* after sepal formation. Petal primordia were not visible in *mlf* plants at 15°C, although petal primordia were initiated in WT. After this stage, flower malformations observed in *mlf* included undeveloped petals, undeveloped or irregularly developed stamens, secondary flower primordia formation, and completely irregular arrangement of undeveloped flower organs. No phytoplasma was detected by PCR, indicating that it could not be the causal agent of the abnormal phenotypes. This is the first report of mutant flower phenotypes dependent on temperature and induced by only a 5°C difference within optimal growing-temperature regimes in carnations.

Key Words: flower primordia, malformed phenotypes, phytoplasma, secondary flower.

Introduction

Carnation (*Dianthus caryophyllus* L.) is an important ornamental plant used as a cut flower, potted plant, and bedding plant worldwide. As a potted plant, 5–6 million carnation plants are shipped before Mother’s Day in Japan. Malformed flowers on potted plants of the carnation ‘Cherry’ were found in Japanese production fields in 2011 and 2012. Several types of floral malformation, including flat-shaped buds, undeveloped petals, proliferation of petals, stamens, and pistils, and proliferate flower formation, were observed; these phenotypes appeared in spring and were alleviated in summer.

Blake (1962) reported diverse floral abnormalities in carnations, including development of adventitious buds within the flower, carpel-like structures in the stamen whorl, excessive development of ovules, and axillary secondary flower formation. Low temperature (5°C) promotes the formation of secondary growing centers...
within the carnation flower and thus the total petal number is markedly increased. The effects of low temperature on petal number are exerted in the early stages of flower development before the flower bud is visible at the shoot apex (Garrod and Harris, 1974).

In Antirrhinum, flowers of the temperature-sensitive DEFICIENS mutant display sepaloid petals and carpeloid stamens when grown at 26°C, whereas the floral morphology resembles that of the wild type at 15°C (Zachgo et al., 1995). The rose ‘Baccara’ forms many "bullhead" flowers, which contain a greater number of petals than normal flowers, at 12°C (Moe, 1971).

Phytoplasmas, formerly termed mycoplasmalike organisms (MLOs), induce a number of symptoms in infected plants, such as phyllody and proliferating growth (Namba et al., 1993; Schneider et al., 1997). In Japan, phytoplasmas are often detected in Hydrangea spp. (Sawayanagi et al., 1999). A type of jujube witches'-broom phytoplasma was identified from carnations with white leaves and stunt symptoms in China (Zhang et al., 2010).

In the present study, we hypothesized that carnation flower malformation is temperature-dependent. To verify this hypothesis, plants of normal and malformed lines were grown under several temperature regimes. Flower buds were observed to determine the stage of flower bud development at which abnormalities occurred. We investigated the effects of temperature on the occurrence of malformation, and the stage at which malformation phenotypes appeared during flower development. The possibility that malformation symptoms were caused by a phytoplasma was examined by PCR.

Materials and Methods

Plant materials

Potted plants of the carnation (Dianthus caryophyllus L.) ‘Cherry’ (hereafter referred to as the wild type; WT) and malformed-flowering lines (hereafter referred to as mlf) provided by the Japan Agribio Co., Ltd. (Shizuoka, Japan) were employed in all experiments. mlf plants which showed markedly malformed flowers were selected from farmers’ fields and propagated by cuttings.

Experiment 1: Effect of temperature regime on flower malformation

WT and mlf plants were transplanted into 12 cm pots on 19 December 2012 and grown under six temperature regimes (T1 to T6) in glasshouses under natural light (NL) at the Center for Bioscience Research and Education, Utsunomiya University (36°32′55″ N, 139°54′42″ E) or in growth chambers under artificial light (AL) (LH-350S; Nippon Medical & Chemical Instruments Co., Ltd., Osaka, Japan). The temperature and light conditions in each treatment were as follows: T1: 26°C constant (NL); T2: 14–16/12°C (day/night; NL); T3: 14–16/12°C (NL) (17/5°C [AL] from 1 January to 10 February 2013); T4: 14–16/12°C (NL) (17/5°C [AL] from 10 February to 10 March 2013); T5: 17/12°C (AL); T6: 17/12°C (AL). Normal and malformed flowers were counted on 25 May 2013, except T1 for which counts were undertaken on 5 May because anthesis was earlier than in the other temperature regimes.

On 31 May 2013, mlf plants grown under the T1 regime were pinched and transferred to the T6 regime, and thereafter malformed flowers were counted on 5 November 2013. On 19 July, mlf plants in the T6 regime were pinched and transferred to a growth chamber maintained at 23/18°C (AL) and thereafter malformed flowers were counted on 12 October 2013.

Artificial light was supplied by cool white fluorescent light and day length with AL was set at 12 h/12 h (day/night). Fifteen plants were used for each line and treatment. The growth medium was standard peat moss (pH 5.0–6.0, N 180 mg·L⁻¹, P 120 mg·L⁻¹, K 220 mg·L⁻¹; Sakata Seed Corp. Ltd., Yokohama, Japan). The plants were fed with a commercial tablet fertilizer (Promic, N:P:K 8:12:10; Hyponex Japan Corp. Ltd, Osaka, Japan) and nutrient solution (Hyponex, N:P:K 6:10:5; Hyponex Japan Corp. Ltd).

Experiment 2: Effect of temperature regime on development of flower buds and flower malformation

Cuttings of WT and mlf were planted in mid-February 2015, transplanted into 12 cm pots, and transferred to three temperature regimes in June 2015: T7: 15°C constant (AL); T8: 20°C constant (AL); T9: 25/20°C (NL: 2015 only). Twelve plants were used per treatment. Occurrence of malformed flowers was observed in WT and mlf plants from August to October 2015. In 2016, ten plants of WT and mlf lines were grown under T7 (15°C constant; AL) and T8 (20°C constant; AL).

To follow flower development, shoot apices and young flower buds were sampled from WT and mlf plants lines at various developmental stages and observed with an optical microscope in 2015 and a scanning electron microscope (SEM) in 2016.

Observation of flower bud morphology

1. Optical microscope

In 2015, thin sections of flower buds of WT plants at different developmental stages grown under T7 and T8, and mlf plants grown under T7 were observed with an optical microscope. Sections were prepared in accordance with Shabman (1943) as follows. Flower buds (2–10 mm diameter) were fixed in FAA (5% formaldehyde, 5% acetic acid, 45% ethanol, and 45% deionized water) under a vacuum. The samples were dehydrated in an ethanol series (50%, 70%, 90%, and 100%) and then substituted with lemosol (Wako Pure Chemical Industries, Ltd., Osaka, Japan)/paraffin (1:1) solution...
and finally embedded in paraffin. Sections (10 μm thickness) were cut with a microtome (PM2255; Leica Microsystems, Wetzlar, Germany) and fixed on glass slides. The sections were stained with iron alum, safranin, and orange G. Longitudinal sections were observed under an optical microscope (BX60; Olympus Corp. Ltd., Tokyo, Japan).

2. Scanning electron microscopy

In 2016, flower buds of WT plants at different stages grown under T7 and T8, and mlf plants grown under T7 were dissected under a stereomicroscope (M205 FA; Leica Microsystems) and observed with a SEM (Miniscope TM3030; Hitachi High-Technologies Corp. Ltd., Tokyo, Japan).

Definition of flower bud developmental stages

Stages of flower bud development were defined in accordance with the methods of Blake (1962) and Furusato (1967) with modifications as follows. Stage 1 (S1): vegetative apex before initiation of sepal primordia; S2: formation of sepal primordia; S3: initiation of petal primordia; S4: formation of petals and initiation of stamens and pistil; S5: formation of stamens and pistil; and S6: formation of ovules in pistil and pollen grains in anthers.

Phytoplasma detection by PCR

Total DNA from 100 mg of WT fresh young buds and three mlf lines was extracted using the cetyltrimethylammonium bromide method (Doyle and Doyle, 1987). The phytoplasma-specific forward primer (SN910601: 5'-GTTTGATCCTGGCTCAGGATT-3') and reverse primer (SN920204: 5'-CCTCAGCGTCAGTAA-3') were used for PCR (Namba et al., 1993). The PCR assay was performed with a thermal cycler (DNA Engine PTC-200; BioRad Laboratories Corp. Ltd, Tokyo, Japan) and KAPA Taq EXtra HotStart ReadyMix (NIPPON Genetics Corp. Ltd, Tokyo, Japan). The PCR was carried out for 50 cycles under the following conditions: the first cycle, denaturation 90 s at 94°C; ramping over 40 s to 60°C, annealing 2 min at 60°C, ramping 30 s to 72°C, extension for 3 min at 72°C; the continuing cycles, denaturation 2 min at 94°C; the final cycle, extension for 7 min at 72°C (Namba et al., 1993). DNA extracted from leaves of Hydrangea macrophylla, which exhibits phyllody, was used as a positive control. The PCR products were visualized by agarose gel electrophoresis in 1.0% agarose gel and stained with ethidium bromide.

Statistical analysis

The percentage of malformed flowers per plant was arcsine transformed and analyzed by one-way analysis of variance (ANOVA) and Tukey’s honestly significant difference (HSD) test using the JMP Ver. 6.0 software package (SAS Institute Inc., Cary, NC, USA).

Results and Discussion

Phenotypes of flowers

Phenotypes of florets of normal (WT) and malformed flower (mlf) lines are shown in Figure 1. WT showed only normal phenotypes (Fig. 1A) and the flower contained five sepals, 30–40 petals, 20–30 stamens, and one pistil. By contrast, mlf lines showed a variety of malformed floral structures, including proliferated sepaloids (sepal-like structures) and phyllody-like secondary flower formation in the flower center (Fig. 1B), proliferated petals and petaloids (petal-like structures) with several pistillodes (carpel-like structures) (Fig. 1C), proliferated pistillodes without petals (Fig. 1D), proliferated sepaloids and pistillodes (Fig. 1E), and proliferated pistillodes and petals (Fig. 1F). Florets in mlf lines tended to be flatter because the proliferated central secondary flowers enlarged the receptacle. Phenotypes of malformed florets did not change back to normal after formation and vice versa. Similar malformed floral phenotypes in carnations were reported by Blake (1962), who noted a variety of abnormalities, including adventitious buds within the flower, carpel-like structures in the stamen whorl, excessive development of ovules, and axillary secondary flower formation in ‘Maytime’, ‘Puritan’, and ‘Miller’s Yellow’.

![Fig. 1. Flower phenotypes of normal (WT) and malformed flower (mlf) lines of the carnation ‘Cherry’. A: Normal flower in WT under T8. B: Proliferated sepaloids with other undevolved organs (phyllody-like) in mlf under T6. C: Proliferated petals and petaloids with pistils in mlf under T7. D: Proliferated pistillodes with no petals in mlf under T7. E: Proliferated sepaloids and pistillodes in mlf under T7. F: Proliferated pistillodes with petals in mlf under T6. G: Plants of mlf grown under 23/18°C (day/night) transferred from T5. H: Malformed flowers differentiated continuously for more than 1 year in mlf under T7. Bars are 500 μm.](image-url)
Effect of temperature regime on occurrence of malformed flowers

In Experiment 1, all WT plants produced normal flowers under the temperature regimes (data not shown). In *mlf* plants under T1, the regime with the highest temperature, no malformed flowers were observed, whereas malformed flowers developed in the other treatments (Fig. 2). *mlf* plants under T2 (14–16/12°C) and T6 (17/12°C) produced 34.2% and 24.4% malformed flowers, respectively. We hypothesized that different low night temperature regimes during flower bud formation may have influenced malformation and therefore set the night temperature of the regimes T3, T4, and T5 with different periods of chilling at 5°C. However, occurrence rates of malformed phenotypes were 27.0% and 19.4% under T3 and T4 which incorporated a night temperature of 5°C for one month (data not shown), and were significantly lower under T5 (17/5°C), i.e. 7.6%, than under T2 and T6 (Fig. 2). These results indicated that the malformed flowers in *mlf* developed at 5–17°C and most frequently at 14–17°C, but rarely at temperatures higher than 20°C. These results supported the hypothesis that the malformed floral phenotypes of *mlf* lines are temperature-dependent.

Five months after transfer from T1 into T6, *mlf* plants had 6.6% malformed flowers, that is, 53.3% plants produced more than one malformed flower (data not shown), which indicated that the change from high temperature to a low-temperature regime may induce malformed floral phenotypes. In contrast, *mlf* plants grown under 23/18°C transferred from T5 (17/5°C) produced 2.4% malformed flowers, which was lower than the 7.2% produced under T5 (data not shown). The percentage of plants that produced malformed flowers decreased from 80% to 13.3% after transfer from T5 (17/5°C) to 23/18°C (data not shown). An *mlf* plant transferred from T5 to 23/18°C had old dwarf shoots and malformed flowers formed under T5, and new healthy shoots and normal flowers formed under 23/18°C (Fig. 1G). These results suggested that flower malformation is reversible depending on the temperature conditions.

In Experiment 2, we assumed the threshold for flower malformation was between 15 and 20°C, and therefore applied the regimes T7 (15°C constant) and T8 (20°C constant) as well as T9 (25/20°C). Under the three temperature regimes, all WT plants produced normal flowers (data not shown), whereas more than 90% of *mlf* plants under T7 produced malformed flowers. However, significantly fewer malformed flowers on *mlf* plants were observed under T8 and T9, i.e. 3.1% and 1.3%, respectively (Fig. 3), which supported the hypothesized threshold for flower malformation between 15 and 20°C.

Garrod and Harris (1974) reported that low temperature (5°C) promoted the formation of secondary flowers in the flower center and thus the total petal number was markedly increased in carnations. On the other hand, flower malformation in *mlf* increased at about 15°C, which is within the temperature range suitable for carnation growth. It is thus difficult to suppress the occurrence of flower malformation at production sites. These results indicate that flower malformation in *mlf* is caused by a unique mutation in carnations and is fundamentally different from those reported previously (Blake, 1962; Garrod and Harris, 1974).
Development of floral buds in WT

Longitudinal sections and SEM micrographs of flower buds are shown in Figures 4 and 5, respectively. The vegetative shoot apical meristem (SAM) of carnation is a morphologically simple structure with leaf primordia initiated in opposite and alternate pairs, and two pairs of bracts are initiated before sepal primordia (Blake, 1962). At stage 1 (S1) the vegetative SAM appeared to elongate prior to formation of sepal primordia (Fig. 4A). Flower bud initiation started with initiation of sepal primordia near the tip of the broader and flatter stamen and carpel primordia proceeded simultaneously at S4 (Figs. 4G and 5D). Anthers and the ovary cavity were initiated in the inner whorl of sepals at S3 (Figs. 4C and 5B). Formation of petals and initiation of stamen and carpel primordia proceeded simultaneously at S4 (Figs. 4G and 5D). Anthers and the ovary cavity were formed at S5 (Figs. 4H and 5G). Finally, ovules, stigmas, and pollen grains in the anthers were formed at S6 (Fig. 4I).

Development of malformed flower buds in mlf

No notable differences in flower bud morphology were observed between WT and mlf at S1 (Fig. 4A, D) and S2 (Fig. 4B, E). Subsequently, petal primordia were initiated at S3 in WT (Figs. 4C and 5B); however, the primordia were not obvious as a whorl on the SAM in mlf plants (Figs. 4F and 5C). There are several malformed floral phenotypes in mlf and all contained more than five sepals (data not shown). Blake (1962) mentioned that the calyx was the most developmentally stable of the floral whorls, although occasionally four or six sepals were recorded. These results suggest that the malformed flower phenotypes arose after sepal formation.

After S2, various malformed flower structures, including undeveloped petals (Figs. 4F, 5C, E, and F), undeveloped or irregularly developed stamens (Figs. 4K, L, 5E, F, and H), secondary flower primordia formation (Figs. 4I, L, and 5H), and completely irregular arrangement of undeveloped flower organs (Figs. 4L and 5I), were observed in mlf plants grown under T7. Blake (1962) described that a petaloid organ, transitional between the true petal and true stamen, often appeared, and in some cases adventitious buds developed and initiated petals continuously in abnormal carnation flowers. Although it is very difficult to predict final forms from flower primordia in mlf, it is expected that a phenotype of proliferated pistillodes and petaloids on the flatter receptacle (Fig. 1F) probably reflects repeated differentiation of secondary flower primordia (Figs. 4I, L, and 5H). Several malformed flowers differentiated continuously for more than 1 year and resembled indeterminate inflorescences in mlf under T6 (Fig. 1H),

Fig. 4. Longitudinal sections of flower buds at six developmental stages in wild type (WT) and mlf lines of the carnation ‘Cherry’. A: WT—Stage (S) 1: Elongated stem apical meristem (SAM) with leaf primordium (LP) in vegetative state before initiation of sepal primordia. B: WT—S2: Apical meristem with sepal primordia (Se) protected by bracts (Br). C: WT—S3: Initiation of petal primordia (Pe). D: mlf—S1. E: mlf—S2. F: mlf—S3: Petal initiation is not clear. G: WT—S4: Formation of petals and initiation of stamens and carpels (Ca). H: WT—S5: Formation of stamens (St) and pistil (Pi). I: WT—S6: Formation of ovules (Ov) in the pistil and pollen grains in anthers (An). J: mlf—S4: Axillary central flowers apparent. K: mlf—S5: Pistil with stunted petals and stamens. L: mlf—S6: Multiple pistils. Bars are 100 μm in A–F, 200 μm in G, H, J, and K, and 500 μm in I and L.

Fig. 5. Scanning electron micrographs of flower buds at different developmental stages in WT and mlf lines of the carnation ‘Cherry’. A: WT—S2. Sepal primordia (Se). B: WT—S3. Initiation of petal primordia (Pe). C: mlf—S3. Petal initiation is not clear. D: WT—S4. Formation of petals and initiation of stamens (St) and carpels (Ca). E: mlf—S4. Petals and stamens are not clear. F: mlf—S4. Petals and stamens are not clear. G: WT—S5. Formation of stamens, anthers (An), and pistil. H: mlf—S4–5. Petals and stamens are irregular and a secondary flower primordium (SF) is apparent. I: mlf—S4–5. Irregular and non-distinguishable organs. Bars are 200 μm in A–E, 500 μm in F–H, and 400 μm in I.
which suggested that control of differentiation in apical tissues was lost in this line. On the other hand, WT plants under T6 had continuously normal flowers for more than 1 year, except that a few florets had a small secondary floral organ.

With regard to sensitivity to temperature at different stages of flower development, the effects of low temperature on petal number were marked in the early stages of flower development before the flower bud was visible at the shoot apex (Garrod and Harris, 1974). In the present study, the petal initiation stage (S3) seemed to be sensitive to the temperature regime, whereas secondary flower primordia often arose at later stages. These findings suggest that the SAM of mlf plants may maintain continuously high sensitivity to temperature.

Causes of flower malformation in mlf

A phytoplasma was detected from leaves of Hydrangea macrophylla that exhibited phyllody, but not from the WT and mlf carnation lines (Appendix 1), which suggested that phytoplasma does not cause flower malformation in mlf.

In carnations, formation of secondary growth centers and additional petals in flowers may be promoted by application of gibberellin A₃ or indoleacetic acid, and the number of petals was increased by application of kinetin (Garrod and Harris, 1974). In the rose ‘Motrea’, the cytokinin content in leaf-like style-tubes in malformed flowers, as well as in partially malformed ovaries at the base of phyllodes, was significantly lower (Chmelinský et al., 2001). These reports suggest that phytohormones regulate flower malformation.

In Antirrhinum, flowers of the temperature-sensitive DEFICIENS mutant display sepaloid petals and carpeloid stamens when grown at 26°C, whereas the flower morphology resembles that of the wild type at 15°C (Zachgo et al., 1995). These responses to temperature regimes are opposite to those observed in the present study. Zachgo et al. (1995) described that a combination of regulatory interactions between the MADS-box proteins DEFICIENS and GLOBOSA are involved in the DEFICIENS mutant. The carnation floral-specific MADS box gene CMB2, which is homologous to DEFICIENS, has been isolated (Baudinette et al., 2000).

A similar temperature-dependent phenomenon in cyclamen has been reported (Mizuno and Ozaki, 2015); the frequency of complete petals (petaloid-stamens) was greatest at 15°C, followed by 25°C and 20°C. Mizuno and Ozaki (2015) noted the possibility that low temperature induces (or high temperature suppresses) petaloidy and additional organ formation in double-flowered cyclamen with petaloid-stamens by reduction (or expansion) of floral homeotic gene (C-class gene) expression. It was similarly reported that low and high temperatures affect the expression of C-class genes in cucumbers and tomatoes respectively (Kater et al., 2001; Lozano et al., 1998). In roses, suppression of C-class genes leads to altered expression of APETALA1 in whorls 3 and 4, which leads to a mutant flower phenotype (Mibus et al., 2011). In Arabidopsis, Wollmann et al. (2010) showed that APETALA2 can cause striking organ proliferation defects that are not limited to the center of the floral meristem, where its antagonist AGAMOUS is required for termination of stem cell proliferation. For mlf, a substantial mutation including MADS-box genes may be involved in flower malformation. Additional studies of molecular aspects of flower malformation are required.

Living organisms perceive temperature using thermo-sensory molecules, of which various types have been identified, including nucleic acid thermometers, thermo-sensing via protein conformational change, or via changes in membrane properties (Sengupta and Garrity, 2013). Recently, phytochrome B was shown to directly associate with the promoters of key target genes in a temperature-dependent manner and it functions as a temperature sensor in plants (Jung et al., 2016; Legris et al., 2016). Fujii et al. (2017) reported that phototropin perceives temperature based on the lifetime of its photoactivated state and acts as a sensor of temperature in Marchantia polymorpha. Mutation in genes associated with these thermo-sensory mechanisms may be involved in flower malformation in mlf.

In conclusion, the present results suggest that malformation of carnation flowers is temperature-dependent. A temperature of about 15°C causes malformed flower phenotypes, but such phenotypes are rarely exhibited at 20°C. The malformed flower phenotypes were apparent after the stage of sepal formation. This is the first report on flower malformation dependent on temperature and induced by optimal temperature regimes in carnations. A phytoplasma is not indicated to be the causal agent of this syndrome. Additional studies are needed to elucidate the underlying regulatory mechanism of flower malformation.

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