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Regulation of Flowering in Stock [*Matthiola incana* (L.) R. Br.] by Manipulation of Gibberellin Biosynthesis

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Abstract

The role of gibberellins (GAs) in the control of growth and flowering in stock, *Matthiola incana* (L.) R. Br., and the possibility of their regulation by manipulation of GA biosynthesis were investigated. In *M. incana*, GAs were found to be necessary for stem elongation and flowering. GA_4 in the non-13-hydroxylation pathway of GA biosynthesis was the most active component for stem elongation and flowering. It was shown that GA biosynthesis inhibitors of acylcyclohexanedione type could promote stem elongation and flowering. These results indicate that the balance of activities among GA biosynthetic and catabolic enzymes is very important for stem elongation and flowering. Furthermore, development of responsiveness to active GAs for flowering may be one of the essential factors in the flower bud initiation process in *M. incana*. Based on the above results, we demonstrated that acceleration of the flowering time in summer-sown *M. incana* could be obtained by prohexadione-calcium (PCa) treatment under plastic-film greenhouse conditions.

Discipline: Horticulture
Additional key words: acylcyclohexanedione, cold requirement, inhibitor

Introduction

It has been generally recognized that endogenous gibberellins (GAs) play a regulatory role in stem elongation and flowering in some cold-requiring plants[12,16]. Although exogenous GAs induce flowering in several cold-requiring plants, the role of GAs in the flowering of cold-requiring plants is still controversial.

Stock, *Matthiola incana* (L.) R. Br., a member of Cruciferae, is an important ornamental plant in Japan. This species requires low temperature and long day for flowering. Genetic variations in flowering time can be attributed to differences in the sensitivity to low temperature and daylength, and to variations in the duration of the juvenile phase[1,4,14]. Low temperature requirement for flowering is more important for late-flowering cultivars than for early ones[1,7]. The observation that exogenous GAs accelerate flowering in *M. incana*[1,11,14] suggests the involvement of GAs in the flowering process, as had been noted for other photoperiodic or cold-requiring plants[12,16]. Therefore, we investigated the role of GAs in the control of the growth and flowering of *M. incana* and the possibility of their regulation by manipulation of GA biosynthesis.

Identification of endogenous GAs

We have identified several endogenous GAs including GA_1, GA_4, 3-*epi*-GA_4, GA_8, GA_{19}, GA_{20}, GA_{24}, GA_{34}, GA_{37}, GA_{53} and GA_{112} from *M. incana* by combined gas chromatography-mass spectrometry (GC-MS) and suggested that both the early C13-hydroxylation pathway and the C13-non-hydroxylation pathway of GA biosynthesis functioned in the plant (Fig. 1)[5]. The current knowledge of GA biosynthesis suggests that endogenously active GAs consist of GA_1 derived from the early C13-hydroxylation pathway and GA_4 from the C13-non-hydroxylation pathway[3].

Relationship between the structure of GAs and biological activity

GAs belong to a group of diterpenoid acids with an *ent*-gibberellane ring system. Although 126 GAs have been identified, not all the GAs display a high biological

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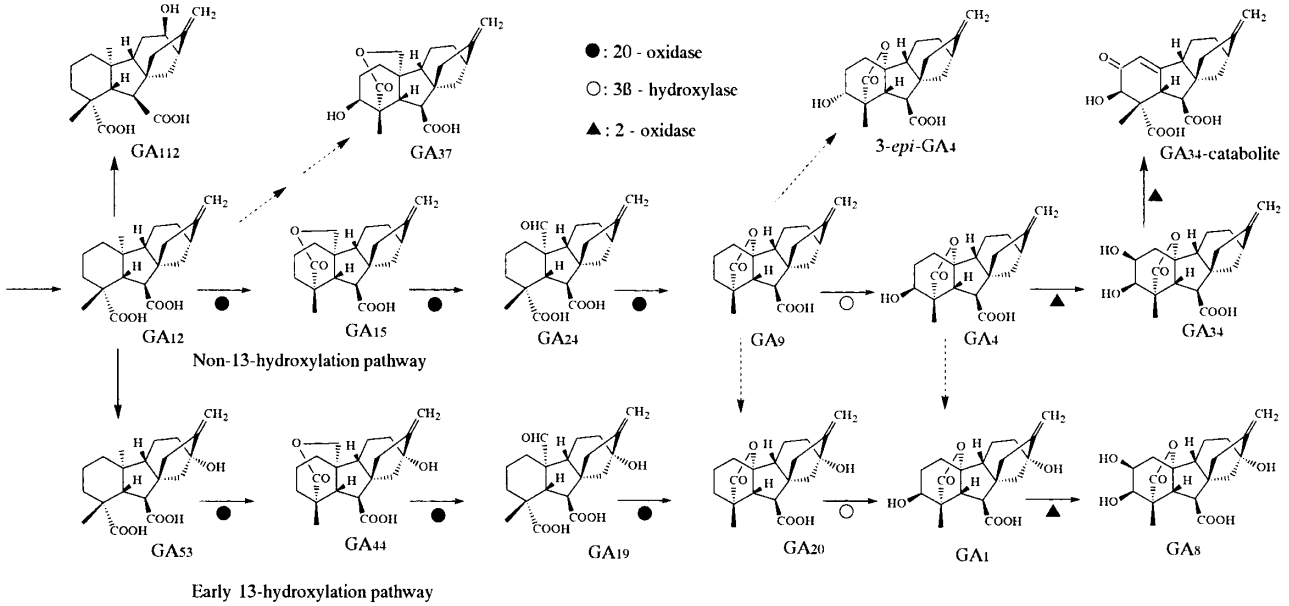

Fig. 1. Putative biosynthetic pathways of endogenous gibberellins in the shoots and flower buds of *M. incana*

activity. Many of the GAs in plants are precursors or deactivation products of the active GAs. We therefore undertook an analysis of the structural requirements for growth and flowering in *M. incana* (Fig. 2)[8].

Stem growth showed a sigmoidal pattern over time, reaching a plateau after 8 weeks in both control and GA-treated plants (Fig. 3-A, B). Plants treated with GA_1, GA_3, GA_9, GA_{13}, GA_{20} and 3-*epi*-2,2-diMe-GA_4 showed similar growth patterns to those of the control plants. GA_1 and GA_{20} did not promote significantly stem elongation, while GA_3, GA_9, GA_{13} and 3-*epi*-2,2-diMe-GA_4 slightly promoted stem elongation at the early stages of growth (Fig. 3-A, B). In contrast to these limited stem growth responses to GA_1, GA_3, GA_9, GA_{13}, GA_{20} and 3-*epi*-2,2-diMe-GA_4, stem elongation was substantially promoted by 2,2-diMe-GA_4, followed by GA_4. In the plants treated with 2,2-diMe-GA_4, flowering started 10 weeks after the onset of the treatment, so that stem elongation stopped at that time.

Flowering was not initiated by GA_1, GA_3, GA_9, GA_{13} and GA_{20} as in the case of the untreated control plants (Table 1). With the GA_4 and 3-*epi*-2,2-diMe-GA_4 treatments, 58 and 33% of the plants started to flower, respectively, whereas all the plants started to flower with the 2,2-diMe-GA_4 treatment (Table 1). Control and other non-flowering plants formed about 110 leaves at 80 or 90

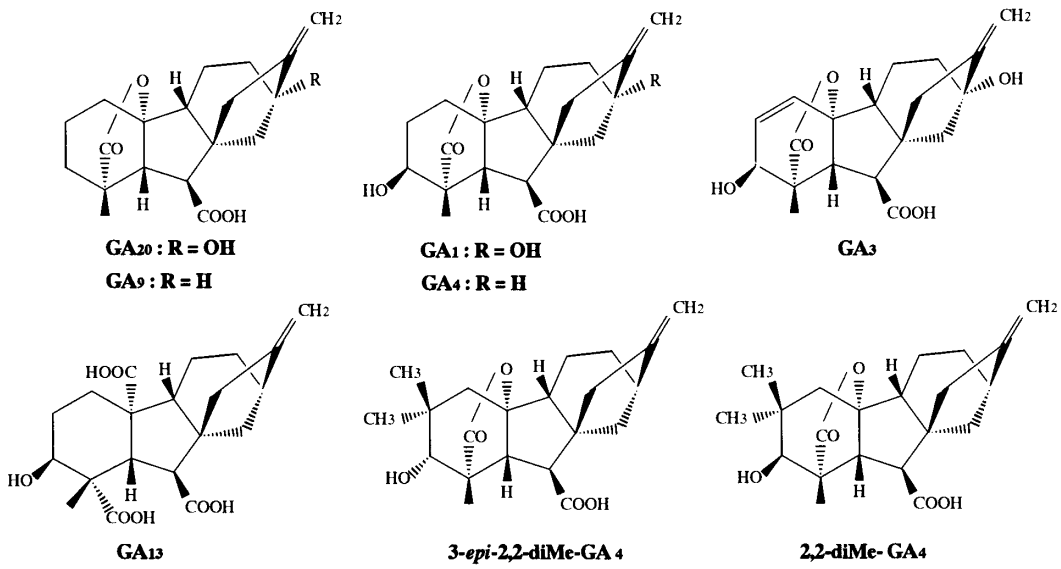

Fig. 2. Structure of gibberellins used in the experiments

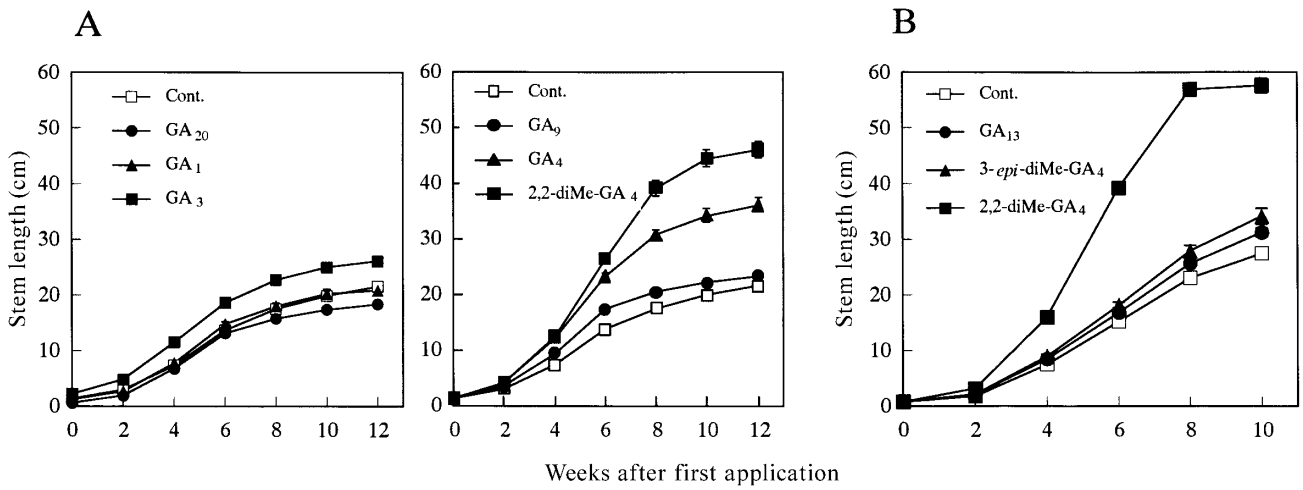

Fig. 3. Growth curves of stem of *M. incana* cv. Banrei plants as affected by treatment with several GAs
Plants were grown under the conditions described in Table 1. Data of A and B were obtained in different experiments.

days after the onset of the treatments (Table 1). In the flowering plants, the number of leaves formed in the 3-*epi*-2,2-diMe-GA_4 treatment was much higher than that in the GA_4 and 2,2-diMe-GA_4 treatments.

The structural characteristics of GAs for the promotion of stem elongation and flowering in *M. incana* can be described as follows:

(1) C-3ß hydroxylation is the key to GA activity for both stem elongation and flowering;

(2) C-3α hydroxylation is less active for both stem elongation and flowering than C-3ß hydroxylation;

(3) Inhibition of C-2ß hydroxylation (e.g. 2 methyl groups at C-2 or a double bond at C-1,2 of A ring) enhances stem elongation and flowering activity;

(4) C-13 hydroxyl group reduces stem elongation and flowering activity;

(5) C_{20}-GAs with a C-20 carboxylic acid group are biologically inactive.

Overall, these results indicate that GAs with 3ß-OH and without 13-OH groups (e.g. GA_4) are the most important compounds for stem elongation and flowering in *M. incana*. Thus, the structural requirements for flowering are the same as those for stem elongation in *M. incana*.

Table 1. Effect of several GAs on flowering in *M. incana*

Treatment	Total number of leaves		Number of	Days to visible
	Flowering	Non-flowering	flowering plants (%)	flower buds
A				
Control	–	109.2 ± 1.8	0	–
GA_{20}	–	112.4 ± 1.3	0	–
GA_1	–	109.1 ± 2.0	0	–
GA_3	–	108.2 ± 2.3	0	–
GA_9	–	113.3 ± 2.0	0	–
GA_4	64.1 ± 1.4	112.9 ± 3.5	58.3 (54.2)	60.5 ± 1.9
2,2-diMe-GA_4	61.5 ± 1.7	–	100 (95.8)	51.6 ± 2.0
B				
Control	–	107.1 ± 2.0	0	–
GA_{13}	–	111.3 ± 1.4	0	–
2,2-diMe-GA_4	54.3 ± 1.4	–	100 (100)	48.2 ± 2.6
3-*epi*-2,2-diMe-GA_4	84.5 ± 4.7	107.0 ± 3.0	33.3 (25.0)	77.3 ± 5.4

Plants were grown in a phytotron maintained at 20 / 15ºC (day / night) under a 12 h photoperiod (PPFD 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Data were collected 90 days (A) or 80 days (B) after the first application. GAs were applied 10 times (2 times a week) for a total dose of 10 µg per plant, respectively. Values are means ± SE. Percentage of plants with flower buds is shown in parentheses.

Effect of acylcyclohexanedione compounds

As mentioned above, the active GAs play an important role in the growth and flowering of *M. incana*. Acylcyclohexanediones such as prohexadione-calcium (PCa) and trinexapac-ethyl (TNE) inhibit late stages of GA biosynthesis. They act as competitors of 2-oxoglutarate which is an essential co-factor for 20-oxidase, 2ß- and 3ß-hydroxylase enzymes of GAs[13], because of the similarity in their chemical structure to 2-oxoglutarate. Hydroxylation, particularly at C-2ß and C-3ß of the gibberellane skeleton, appears to be the primary target of these acylcyclohexanedione compounds. Hydroxylation at C-3ß leads to the formation of biologically active GAs such as GA_1 and/or GA_4 from their precursors, GA_{20} and/or GA_9, respectively. On the other hand, hydroxylation at C-2ß leads to the formation of inactive GAs such as GA_8 and/or GA_{34} from their corresponding biologically active GAs, GA_1 and/or GA_4, respectively[3]. Recently, we have observed that flowering was promoted by PCa and TNE as well as GA_4[6,9].

Stem growth assumed a sigmoidal pattern over time, reaching a plateau after 8 weeks in the control plants (Fig. 4). PCa promoted stem elongation at all doses, and the PCa-treated plants continued to grow even 12 weeks after the first application. Treatments with 20 µg and 200 µg of PCa showed the same promotive effect, while the promotive effect of 2 µg of PCa decreased at 8 weeks after the first application. Treatment with 1 µg and 10 µg of TNE promoted stem elongation. Plants treated with 1 µg of TNE showed a continuous stem elongation for 12 weeks after the first application. Plants treated with 10 µg of TNE reached a plateau at 10 weeks after the first application, which can be related to blooming. Application of 100 µg of TNE inhibited stem elongation at the early stage (Fig. 4), while promoted it at the late stage, and the plants showed a continuous stem elongation for 12 weeks after the first application.

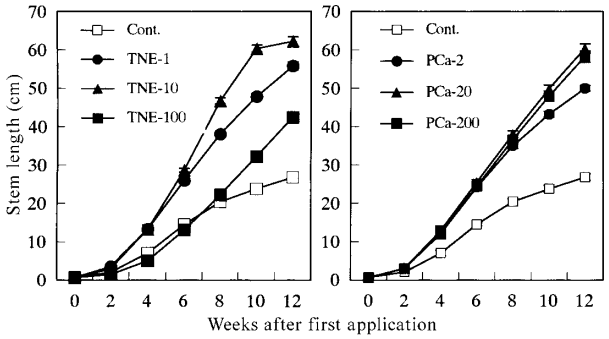

Fig. 4. Growth curves of stem of *M. incana* cv. Banrei plants as affected by the dose of prohexadione-calcium (PCa) and trinexapac-ethyl (TNE) administered

Plants were grown under the conditions described in Table 3.

Our data revealed that these inhibitors of GA biosynthesis and catabolism either inhibited or promoted stem elongation depending on the dose applied. A high dose of TNE inhibited stem elongation. TNE blocked 3ß-hydroxylation of GA_9 to its active product GA_4 and resulted in the accumulation of GA_9 and its precursor, GA_{24} (Table 2). In the plants treated with a high dose of TNE, the formation of GA_{34} by the reduction of precursor and/or blocking of 2ß-hydroxylation of GA_4 to GA_{34} by TNE decreased (Table 2). Such a 2ß-hydroxylation occurs in a number of plant species[6] and is known to be

Table 2. Effect of different doses of TNE on GA levels (ng g^{-1} fresh weight) in *M. incana*

Treatment	GA levels (ng g^{-1} fresh weight)					
	GA_{24}	GA_9	GA_4	GA_{34}	GA_{20}	GA_1
First analysis						
Control	–	1.18	0.60	2.68	0.08	0.06
TNE-low	–	0.82	2.05	2.56	0.06	0.05
TNE-high	–	6.45	0.21	0.80	0.25	0.06
Second analysis						
Control	1.15	1.48	0.89	3.04	0.08	0.05
TNE-low	0.53	0.59	1.82	2.43	0.04	0.03
TNE-high	5.33	6.84	0.38	0.60	0.20	N.D.

Plants were grown in a phytotron maintained at 20 / 15ºC (day / night) under a 12 h photoperiod (PPFD 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$). TNE was applied 6 times (2 times a week) for a total dose of 0.6 (low dose) or 60 (high dose) µg per plant. Stems including folded leaves (less than 5 mm) were collected at the beginning of the light period 3 weeks after the first treatment. The levels of GAs were determined by GC-MS-selected ion monitoring using deuterated internal standards. The analysis was performed twice.

Table 3. Effect of different doses of PCa and TNE on flowering in *M. incana*

Treatment	Total number of leaves		Number of	Days to visible
	Flowering	Non-flowering	flowering plants (%)	flower buds
Control	–	113.3 ± 2.28	0	–
PCa-2	–	105.5 ± 1.15	0	–
PCa-20	81.3 ± 2.22	–	100 (91.7)	75.8 ± 2.20
PCa-200	84.8 ± 1.21	–	100 (66.7)	77.3 ± 2.13
TNE-1	86.0	108.8 ± 1.70	8.3 (0)	–
TNE-10	62.4 ± 1.04	–	100 (100)	54.6 ± 1.19
TNE-100	78.2 ± 1.59	–	100 (100)	77.1 ± 1.89

Plants were grown in a phytotron maintained at 20 / 15ºC (day / night) under a 12 h photoperiod (PPFD 200 μmol·m^{-2}·s^{-1}). Data were collected 90 days after the first application. PCa and TNE were applied 10 times (2 times a week) for a total dose of 2, 20, or 200 μg per plant and 1, 10, or 100 μg per plant, respectively. Values are means ± SE (n=12). Percentage of plants with flower buds is shown in parentheses.

regulated by multifunctional 2-oxidases catalyzing successive oxidations at C-2, leading to the formation of 2ß-hydroxy GAs and GA catabolites[15]. The enzymes are inhibited by PCa and TNE[13]. In contrast to this high dose, the increase in the formation of GA_4 apparently due to enhanced conversion from its precursors GA_{24} and GA_9 was observed when low doses of TNE were administered (Table 2). Catabolism of GA_4 to GA_{34} was not significantly affected by the low doses of TNE as the GA_{34} levels in the treated plants were close to those of the control plants (Table 2). It could be argued that the low doses of TNE were partially blocking GA_{34} accumulation but this effect was counterbalanced by a 3-fold increase of the GA_4 level which could lead to an increase of GA_4 catabolism to GA_{34}. Our best evidence for the role of TNE and PCa in the blocking of the 2ß-hydroxylation step involving GA_4 catabolism to GA_{34}, is the finding that simultaneous application of PCa and GA_4 enhanced stem elongation compared to GA_4 or PCa alone[8].

Acylcyclohexanediones could also promote flowering in *M. incana*. Based on the morphological characteristics in the sequence of flower formation, floral stages were determined as follows[8]: 0, vegetative stage; 1, dome-forming stage; 2, floret primordia initiation stage; 3, sepal-forming stage; 4, stamen and pistil-forming stage (single-flowered plants, SFP) or early petal-forming stage (double-flowered plants, DFP); 5, stamen and pistil development stage (SFP) or late petal-forming stage (DFP); 6, early petal elongation stage; 7, late petal elongation stage; and 8, blooming. We considered that plants shifted to reproductive development when they reached stage 2 or beyond. Control plants and plants treated with 2 μg of PCa and with 1 μg of TNE did not flower (except for one plant treated with 1 μg of TNE), whereas all the other plants treated with PCa and TNE flowered. Control and other non-flowering plants formed about 110 leaves 90 days after the first application (Table 3). In the flowering plants, the number of leaves formed was the smallest in the 10 μg of TNE treatment. Other treatments led to the formation of almost the same number of leaves. Days to visible flower buds were also similar in the flowering plants (Table 3). Promotion of flowering by acylcyclohexadiones seemed to be caused by an inhibition of 2ß-hydroxylation of endogenous GA_4 and resulting in the accumulation of endogenous GA_4 as well as promotion of stem elongation. Treatments with 2 μg of PCa and 1 μg of TNE promoted stem elongation but they did not induce flowering, although treatments with higher doses of these compounds induced flowering (Table 3). These phenomena were ascribed to the fact that the inhibitory effect of 2ß-hydroxylation was not pronounced at low doses, and consequently, a sufficient level of biologically active GAs which is required for the initiation of flowering could not be reached at the late stages of growth. The above results may also suggest that the accumulation of biologically active GAs is required for flowering at some developmental stages which may be related to the development of the responsiveness to biologically active GAs for flowering.

Involvement of GAs in flowering

A triazol type inhibitor, uniconazole (UCZ), and an acylcyclohexanedione type inhibitor, TNE, can be used to block early steps and late steps of GA biosynthesis, respectively[13]. Thus, UCZ induces a reduction of endogenous GA levels, and TNE inhibits both GA_4 formation from GA_9 and GA_4 catabolism to GA_{34}[2,8]. These applications may reflect the growth response depending on the amounts of applied GA_4. Application of UCZ completely

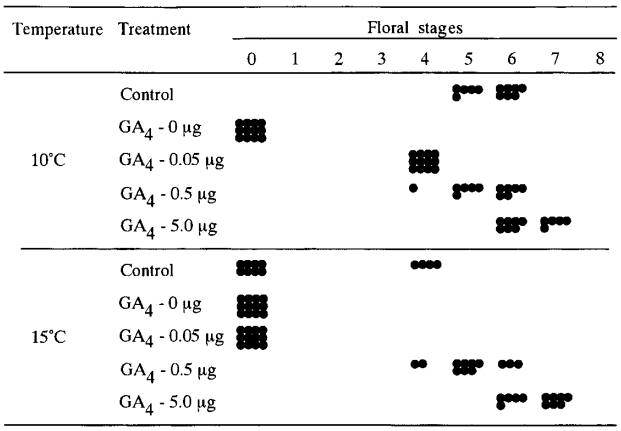

Fig. 5. Effect of temperature and GA_4 levels on flower differentiation in *M. incana* cv. Banrei

● : One plant. Ten mL of UCZ (100 $mg \cdot L^{-1}$) solution were applied twice to the medium 1 and 2 weeks before the onset of the cold treatment. Ten μL of TNE (0.1 $\mu g \cdot \mu L^{-1}$) and GA_4 (0, 0.0025, 0.025 or 0.25 $\mu g \cdot \mu L^{-1}$) were applied to shoot tips of the plants. TNE was applied 3 days before the onset of the cold treatment and again 2 days after the first treatment. GA_4 was applied 1 and 3 days after the first TNE treatment. No chemicals were applied to the control plants. Cold treatments were conducted in growth chambers maintained at 10ºC and 15ºC under a 12 h photoperiod for 20 days.

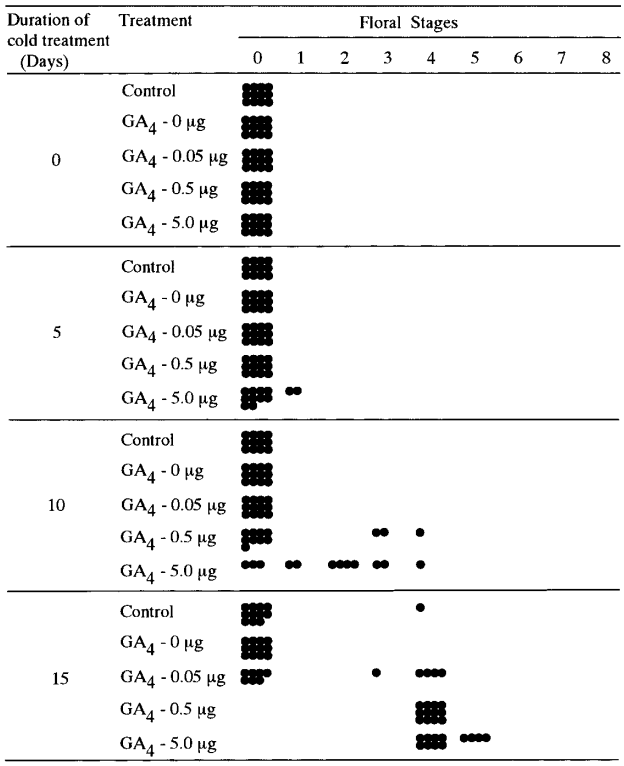

Fig. 6. Effect of duration of cold treatments and GA_4 levels on flower differentiation in *M. incana* cv. Banrei

● : One plant. The solutions of UCZ, TNE and GA_4 were applied to plants as described in Fig.5. No chemicals were applied to the control plants. Cold treatments were conducted in a growth chamber maintained at 10ºC under a 12 h photoperiod for 0, 5, 10 and 15 days.

inhibited flower bud initiation under inductive conditions (Fig. 5). The inhibition of flower bud initiation by UCZ was alleviated by GA_4 treatments, indicating that endogenous GAs must be involved in the regulation of flowering in *M. incana*, as had been reported for other long-day and cold-requiring plants[12,16]. The GA_4 level necessary for flower bud initiation was lower in the 10ºC cold-treated plants compared to that in the 15ºC cold-treated plants (Fig. 5) and it decreased with the increase of the duration of the cold treatment (Fig. 6). These results indicate that cold treatments enhance the responsiveness to GA_4 in the flower bud initiation process, and the development of responsiveness to GA_4 may be correlated with the degree and duration of cold temperature exposure. At a high temperature, GA_4 promoted stem elongation but not flower bud initiation in *M. incana*[6]. Therefore, the development of the responsiveness to GA_4 for flowering may be one of the essential factors in the flower bud initiation process in *M. incana*[10].

Conclusion

In summary, our findings highlight 3 important features of GA response in *M. incana*. Firstly, the presence or absence of 3-OH and 13-OH groups in the GA structure influences its activity in *M. incana*. C_{19}-GA with a 3ß-OH group and without a 13-OH group (e.g. GA_4) is most active in the promotion of stem elongation and flowering in *M. incana*. Secondly, the activity of 2ß-hydroxylases controlling GA catabolism exerts significant effects on the level of active GA, GA_4. Thus, it appears that the balance of activities between GA biosynthetic and catabolic enzymes is very important for stem elongation and flowering. Thirdly, the development of responsiveness to active GAs for flowering may be one of the essential factors in the flower bud initiation process in *M. incana*. Based on the above results, we demonstrated that acceleration of the flowering time in summer-sown *M. incana* could be obtained by PCa treatment under plastic-film greenhouse conditions[7].

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