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Changes of Growth and Inflorescence Initiation by Exogenous Gibberellic Acid₃ and 6-Benzylaminopurine Application in *Phalaenopsis* Orchids

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Abstract: This study was conducted to observe the effects of exogenous gibberellic acid₃ (GA₃) and 6-benzylaminopurine (BAP) treatments on the growth and inflorescence initiation of Phalaenopsis and to determine whether hormonal applications can substitute for low-temperature exposure for floral transition. Phalaenopsis Queen Beer 'Mantefon' clones were treated with no hormones (control), GA₃ 100 mg/L, GA₃ 200 mg/L, BAP 100 mg/L, and GA₃ 100 mg/L + BAP 100 mg/L by foliar spray. The treatments were carried at 28 °C for vegetative growth and 20 °C for forcing, respectively. At 28 °C of vegetative temperature, all exogenous hormonal treatments did not induce inflorescence initiation, but lateral shoots were observed in BAP-treated plants even though this plant is a monopodial orchid. GA₃ significantly increased leaf length and decreased leaf width, and consequently increased length:width (L:W) ratio compared with the control and BAP alone. The trend grew as GA₃ concentration increased. Also, the GA₃ increased stem length and decreased stem diameter. At 20 °C of forcing temperature, L:W ratio responded similarly to 28 °C in GA₃ treatments, but leaf size was smaller than for the control or BAP alone. BAP accelerated inflorescence emergence and significantly increased inflorescence numbers, whereas GA₃ and GA₃ + BAP slightly delayed inflorescence emergence. GA3 significantly promoted new leaf development at 20 °C of forcing condition. These results indicated that cytokinin was associated with the break of axillary vegetative and inflorescence meristems and exogenous GA₃ spray did not improve inflorescence initiation in Phalaenopsis. Although exogenous hormonal application did not substitute for low-temperature exposure, it showed a possibility in promoting the growth and inflorescence initiation.

Keywords: cytokinin; Doritaenopsis; floral transition; gibberellin; plant hormone

1. Introduction

Phalaenopsis is a monopodial orchid and includes approximately 60–70 species [1]. *Phalaenopsis* has unique flower shapes and colors with a long flowering time. There are many generic hybrids (e.g., *Doritaenopsis; Phalaenopsis x Doritis*) with diverse variations in flowers [2]. Because of these characteristics, *Phalaenopsis* is one of the most popular floricultural crops throughout the world and is cultivated commercially in many countries.

Plants perceive environmental changes, which in turn cause their floral transition. Temperature is an important environmental factor in many species and *Phalaenopsis* inflorescence initiation is also mainly controlled by exposure to temperature drop. Although *Phalaenopsis* originates from tropical and subtropical regions [3], common hybrids in this genus require several weeks of a prolonged period below 25 °C for inflorescence initiation, whereas temperatures above 28 °C prevent the floral transition [4]. Exposure to relatively low temperature from high temperature enables flowering time control and year-round cultivation. *Phalaenopsis* has meristems capable of developing into inflorescences in the stem



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Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). at the base of each leaf [5]. These meristems have eco-dormancy, which can be eliminated by low-temperature exposure [6,7].

Plant hormones play important roles in many developmental processes including morphogenesis, dormancy, flowering, etc. [8–10]. Among plant hormones, gibberellic acids (GAs) are involved in bolting of vernalization-requiring or long-day plants [11,12], and DELLA-mediated GA signaling is well understood [13]. In *Phalaenopsis*, GA contents in inflorescences increase simultaneously during inflorescence development and exogenous GA injection can induce flower primordia at vegetative high temperature, which normally inhibits flower induction [14–16]. The GA injection, however, does not induce inflorescence initiation [17]. Huang et al. [18] referred that the GA pathway may play a role in regulating inflorescence initiation in transcriptomic analysis. Another study, however, did not observe upregulations in transcription of genes related to GA biosynthesis at forcing temperature [19]. Thus, GA's role as a key regulator in *Phalaenopsis* floral transition is still unclear, including whether or not it can induce inflorescence initiation without low-temperature exposure.

Cytokinin (CK) is also considered to act as a stimulus for floral transition [20]. In rapeseed (*Brassica napus*), an increase of CK levels was observed in reproductive plants when compared with vegetative plants [21]. CK also promoted flowering via transcriptional activation of a paralogue of *FLOWERING LOCUS T* (*FT*), *TWIN SISTER OF FT* (*TSF*), in *Arabidopsis* [22]. In *Dendrobium nobile* orchid, exogenous CK treatment could induce inflorescence initiation without vernalization [23]. CKs remained in active forms under forcing condition, while the CKs in leaves were converted from active to inactive glucoside forms at vegetative high temperature in *Phalaenopsis* [24]. Exogenous 6-benzylaminopurine (BAP) spray during a low-temperature exposure accelerated inflorescence emergence and increased inflorescence numbers [25,26], and inflorescence length decreased with increasing BAP concentrations [27]. Also, CKs can be used in callus formation and shoot regeneration for vegetative propagation from inflorescences in *Phalaenopsis* [28–30]. To our knowledge, however, there is no data verifying whether exogenous CK treatment can substitute for low-temperature requirement in *Phalaenopsis* floral transition.

Long cultivation time and a cooling requirement for inflorescence initiation demand high production costs in *Phalaenopsis* production. Alternative techniques that replace temperature-induced floral transition can save energy costs and improve production rate in *Phalaenopsis* [6,31]. Plant hormones and its synthetic compounds have been widely used in the horticultural industry for a long time. The use of the compounds can provide insights into further development in *Phalaenopsis* cultivation. However, as mentioned above, there is a lack of information about the roles of GAs and CKs in *Phalaenopsis*. Also, the effects of these hormones on growth characteristics during a vegetative period have not been discussed yet. In this study, GA₃ and BAP were sprayed under vegetative and forcing conditions to observe their effects on the growth and inflorescence initiation of *Phalaenopsis* and to determine whether the exogenous hormonal application can substitute for low-temperature requirements in floral transition.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

Fifteen-month-old *Phalaenopsis* Queen Beer 'Mantefon' clones transplanted into 10 cm (approximately 500 mL volume) plastic pots filled with 100% sphagnum moss were purchased from a commercial grower (Samhyeon Orchid Farm, Goyang, Korea). To prevent premature flowering, these plants were acclimatized for four weeks in environment-controlled growth chambers (HB-301MP; Hanbaek Scientific Co., Bucheon, Korea). Temperature and relative humidity were maintained at 28 ± 1 °C and $60\% \pm 10\%$, respectively. The photoperiod was 12 h with $130 \pm 10 \ \mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetic photon flux density (PPFD) provided by 250 W metal halide lamps (Han Young Electrics Co., Gwangju, Korea). Plants were fertigated once a week with a water-soluble fertilizer (electrical conductivity $0.8 \ mS \cdot cm^{-1}$, Hyponex professional 20N-20P-20K; Hyponex Japan, Osaka, Japan) by hand-

drip irrigation. At the end of the acclimatization period, the mean plant span was 27.1 cm with 5–6 fully developed leaves.

2.2. Exogenous GA₃ and BAP Treatment under Vegetative Condition

After acclimatizing, plants were treated with five different experimental combinations: no hormones (distilled water; control), GA₃ (Duchefa Biochemie, Haarlem, The Netherlands) 100 mg/L, GA₃ 200 mg/L, BAP (6-Benzylaminopurine, Duchefa Biochemie, Haarlem, The Netherlands) 100 mg/L, and GA₃ 100 mg/L + BAP 100 mg/L. Just prior to foliar spray, 5 mL of GA₃ and BAP solutions dissolved in 100% ethanol and 1 M NaOH respectively, were blended with distilled water and 0.1% Tween-20 (P1379, Sigma-Aldrich Korea Ltd., Yongin, Korea). Each plant received 20–25 mL of each solution and the solutions were treated two times with a one-week interval. There were three replications per each treatment and each replication contained five plants. After treatment, plants were placed in a 28 °C growth chamber (average temperature 28.4 °C) for 15 weeks. All other cultivation conditions were maintained at the same levels as during the acclimatization period. Completely randomized design was used in this experiment.

2.3. Exogenous GA₃ and BAP Treatment under Forcing Condition

Treatment solutions and methods were the same as described in Section 2.2. After treatment, plants were placed in a 20 °C growth chamber (average temperature 19.5 °C) for 15 weeks. There were three replications per each treatment and each replication contained five plants. All other cultivation conditions were maintained at the same levels as during the acclimatization period. Completely randomized design was used in this experiment.

2.4. Data Collection and Statistical Analysis

After 15 weeks at 28 °C (vegetative condition), the number of new leaves (leaves longer than 0.5 cm that developed after treatments), length and width of the uppermost mature leaf, stem length and diameter, and percent of plants with visible inflorescence (VI, flower-stalk longer than 0.5 cm) were measured and the ratio between leaf length (L) and width (W) (L:W ratio) was calculated. Under 20 °C forcing condition, the number of new leaves, length and width of the uppermost mature leaf, a ratio between leaf length and width, and percent of plants with VI were measured after 15 weeks of temperature treatment, and the days to VI and number of VIs were also counted every day during the experiment period. Statistical analysis was performed using analysis of variance (ANOVA) in SAS (Windows version 9.3; SAS Institute Inc., Cary, NC, USA). A comparison of parameters among treatment groups was performed by Tukey's honestly significant difference (HSD) test for evaluating the growth and flowering characteristics with $\alpha = 0.05$ as the threshold for statistical significance. Graph module analyses were performed using SigmaPlot software version 10.0 (Systat Software, Inc., Chicago, IL, USA).

3. Results

3.1. Growth Responses by Exogenous GA₃ and BAP Treatment

Under the 28 °C vegetative condition, GA₃ significantly increased leaf length (p < 0.001) and decreased leaf width (p < 0.001) (Table 1). Consequently, a L:W ratio (p < 0.001) increased compared with the no hormone and BAP alone. Similarly, stem length (p < 0.001) and diameter (p < 0.001) increased and decreased respectively, by the exogenous GA₃ treatments. As the GA₃ concentration increased, the L:W ratio increased significantly. When the parameters were compared between plants with no hormone and BAP alone, there was no significant difference, although the leaf length was slightly shorter in BAP-treated plants. The mixed treatment of GA₃ and BAP yielded a reduced L:W ratio and stem length but also increased leaf width and stem diameter compared with the GA₃ treatments. The BAP treatment also induced lateral shoots emerged from axillary meristems (Figure 1). Of the plants, 68.3% had one or two lateral shoots. No other treatments resulted in lateral shoots. Under the 20 °C forcing condition, although the leaf length (p < 0.05) and width (p < 0.001)

were shorter in GA₃ treatments than the no hormone and BAP alone, the L:W ratio (p < 0.01) still slightly increased, similar to the results under the 28 °C vegetative condition.

Table 1. Growth characteristics of *Phalaenopsis* Queen Beer 'Mantefon' treated with exogenous gibberellic acid₃ (GA₃) and 6-benzylaminopurine (BAP) at either 28 °C (vegetative condition) or 20 °C (forcing condition) for 15 weeks.

Treatment	Leaf Length (cm)	Leaf Width (cm)	L:W ratio	Lateral Shoot (% of Plants)	Stem Length (cm)	Stem Diameter (mm)
Vegetative condition at 28 °C						
Control	23.1bc ^z	8.2a	2.8d	0b	6.8c	17.1a
GA100	27.0a	5.7b	4.8b	0b	9.1a	13.7c
GA200	28.0a	5.1b	8.5a	0b	9.6a	12.6d
GA100 + BAP100	25.3ab	7.2a	3.5c	0b	7.9b	15.2b
BAP100	20.4c	8.3a	2.4d	68.3a	6.6c	17.1a
Significance	***	***	***	***	***	***
			Forcing condi	tion at 20 °C		
Control	22.1a	7.9ab	2.8abc	-	-	-
GA100	18.7ab	6.3c	2.9ab	-	-	-
GA200	19.3ab	6.3c	3.0a	-	-	-
GA100 + BAP100	18.2b	7.0bc	2.6bc	-	-	-
BAP100	21.3ab	8.6a	2.5c	-	-	-
Significance	*	***	**	-	-	-

Control: no hormones; GA100: GA₃ 100 mg/L; GA200: GA₃ 200 mg/L; GA100 + BAP100: GA₃ 100 mg/L + BAP 100 mg/L; BAP100: BAP 100 mg/L. ^z Means within columns followed by different letters are significantly different by Tukey's range test at p < 0.05. *, **, *** significant at p < 0.05, 0.01, or 0.001, respectively. Length:Width (L:W) ratio.



Figure 1. Growth and inflorescence emergence of *Phalaenopsis* Queen Beer 'Mantefon' plants treated with exogenous gibberellic acid₃ (GA₃) and 6-benzylaminopurine (BAP) at either 28 °C (vegetative condition) or 20 °C (forcing condition) for 15 weeks. Control: no hormones; GA100: GA₃ 100 mg/L; GA200: GA₃ 200 mg/L; GA100 + BAP100: GA₃ 100 mg/L + BAP 100 mg/L; BAP100: BAP 100 mg/L. Circle and arrows indicate lateral shoot and inflorescences, respectively.

All exogenous hormonal treatments slightly increased the number of new leaves at 28 °C vegetative temperature, although there was no significant difference among treatments (Figure 2). The average new leaf numbers were largest in plants with GA₃ + BAP. On the other hand, at 20 °C forcing temperature, the average new leaf numbers significantly increased in GA₃ 100 mg/L and GA₃ + BAP treatments compared with the no hormone and BAP alone ones.

3.2. Inflorescence Initiation by Exogenous GA₃ and BAP Treatment

All exogenous hormonal treatments did not induce inflorescence initiation under the 28 °C vegetative condition (Figure 1). Only BAP alone accelerated inflorescence emergence compared with the no hormone condition, while GA_3 and GA_3 + BAP delayed the emergence and reduced the percentage of plants with inflorescences (Figure 3). The

4.0

3.5

3.0

2.5

2.0

1.5

1.0

0.5

0.0

b

Control

GA100

Number of new leaves



average days to visible inflorescence were 47.4, 59.5, 63.7, 58.3, and 42.9 in no hormone, GA₃ 100 mg/L, GA₃ 200 mg/L, GA₃ + BAP, and BAP treatments, respectively.

Figure 2. Number of new leaves of *Phalaenopsis* Queen Beer 'Mantefon' treated with exogenous gibberellic acid₃ (GA₃) and 6-benzylaminopurine (BAP) at either 28 °C (vegetative condition) or 20 °C (forcing condition) for 15 weeks. Control: no hormones; GA100: GA₃ 100 mg/L; GA200: GA₃ 200 mg/L; GA100 + BAP100: GA₃ 100 mg/L + BAP 100 mg/L; BAP100: BAP 100 mg/L. Different letters above bars are considered significant at *p* < 0.05 by Tukey's honestly significant difference (HSD) test.

GA100 + BAP100 BAP100

GA200



Figure 3. Percent of *Phalaenopsis* Queen Beer 'Mantefon' plants with visible inflorescence treated with exogenous gibberellic acid₃ (GA₃) and 6-benzylaminopurine (BAP) at 20 °C forcing temperature. Control: no hormones; GA100: GA₃ 100 mg/L; GA200: GA₃ 200 mg/L; GA100 + BAP100: GA₃ 100 mg/L + BAP 100 mg/L; BAP100: BAP 100 mg/L.

Inflorescence numbers demonstrated opposite trends to the number of new leaves (Figures 2 and 4). BAP-treated showed significantly more inflorescence numbers than



plants treated with GA₃ and GA₃ + BAP. Although there was no significant difference, a 50% increase was observed in BAP treatment compared with no hormone.

Figure 4. Number of inflorescences of *Phalaenopsis* Queen Beer 'Mantefon' plants treated with exogenous gibberellic acid₃ (GA₃) and 6-benzylaminopurine (BAP) at 20 °C forcing temperature for 15 weeks. Control: no hormones; GA100: GA₃ 100 mg/L; GA200: GA₃ 200 mg/L; GA100 + BAP100: GA₃ 100 mg/L + BAP 100 mg/L; BAP100: BAP 100 mg/L. Different letters above bars are considered significant at p < 0.05 by Tukey's HSD test.

4. Discussion

Compared to the no hormone or BAP alone treatments under the 28 °C vegetative condition, GA₃ increased a L:W ratio by increasing leaf length and decreasing leaf width. Given GA's role in vegetative elongation [8], these results are easily understood. In our previous study, an exogenous paclobutrazol, an anti-GA, reduced leaf length in *Phalaenopsis* [32]. Stem growth also showed similar responses. GA₃ increased stem length but decreased stem diameter. However, when using GA₃ and BAP together, the GA₃ effects significantly weakened, although the L:W ratio of plants with the GA₃ + BAP was still higher than for plants treated with the no hormone or BAP alone. GA and cytokinin (CK) are normally considered hormones that promote plant growth, but these hormones sometimes showed antagonistic effects on various developmental processes [33]. In tomato, for example, there was a negative interaction in hypocotyl elongation between plants treated with GA and BAP [34].

All exogenous hormonal treatments increased the number of new leaves. However, an abnormal increase in leaf and stem length by GA₃ is not desirable in *Phalaenopsis*. In previous studies, mature *Phalaenopsis* plants generally showed a L:W ratio below 3.0 [35,36]. On the other hand, BAP increased new leave numbers with no significant difference in leaf shape (Table 1). The increase of leaf numbers by exogenous BAP treatment has been reported in many plants, including *Dendrobium* and *Epipremnum* [37,38]. CK-deficient transgenic tobacco plants showed decreasing leaf numbers [39]. Interestingly, a synergistic effect was observed in GA₃ + BAP treatment compared with GA₃ or BAP alone. Although there was no significance, the increase of approximately 0.9 in the number of new leaves was a remarkable difference, considering the slow growth of *Phalaenopsis* plants. Because *Phalaenopsis* develops inflorescence meristem at the base of each leaf in stem [5], the number of new leaves is an important indicator for vegetative growth. Exogenous hormonal application has not been used for commercial *Phalaenopsis* production yet, but the growth-promoting effects by a balanced use of GA and CK can give new insights into improvement of *Phalaenopsis* cultivation.

Contrary to results at 28 °C, relatively little differences in leaf shape among treatment groups were observed at the 20 °C forcing temperature. It could be attributed to the growth characteristics during reproductive development. In *Phalaenopsis*, inflorescence is a developing organ during the reproductive period at forcing temperature, whereas vegetative development mainly occurs at high temperatures [40].

 GA_3 and GA_3 + BAP slightly increased a L:W ratio at the forcing temperature, but the leaves were shorter than for the no hormone or BAP alone, unlike the results at 28 °C, indicating that plants treated with GA_3 under forcing condition developed small leaves. The GA_3 increased the number of new leaves. Assimilates are partitioned by the 'source–sink' relationship [41]. Since a newly developing leaf is the main 'sink' organ in *Phalaenopsis* [42], increasing new leaf numbers could limit leaf growth. In previous studies, GAs promoted sucrose translocation from 'source' leaves to 'sink' organs [43,44]. Also, in perennials, exogenous GA treatment can prevent transition from the vegetative to reproductive stage by promoting vegetative organs like leaves and roots [11]. In this study, the inflorescence emergence was delayed by GA_3 and GA_3 + BAP treatments (Figure 3).

Previous studies reported that exogenous BAP treatment enhanced inflorescence emergence and this promotive effect increased with increasing BAP concentrations in *Phalaenopsis* [25,27]. In this study, BAP also increased inflorescence numbers (Figure 4). CK can play a role as a stimulus for floral transition [20]. In *Arabidopsis*, CK induced transcriptional activation of a paralogue of *FLOWERING LOCUS T* (*FT*), *TWIN SISTER OF FT* (*TSF*) [22]. Also, exogenous CK treatment could induce the biosynthesis of endogenous CKs [45,46]. In general, *Phalaenopsis* inflorescences at the leaf axil of the third or fourth nodes from apical leaf are differentiated during inflorescence initiation [4]. BAP spray could induce more inflorescences below the fourth nodes [25,47]. However, GA₃ + BAP did not increase inflorescence number (Figure 4). This result is consistent with a study by Blanchard and Runkle [25]. It indicated that the addition of GA attenuated the promotive effect of BAP in inflorescence emergence. Also, in this study, GA₃ alone elicited no promotion in inflorescence initiation, irrespective of solution concentrations.

Huang et al. [18] reported that DELLA genes were suppressed, and floral pathway integrator genes were upregulated by cool temperature exposure in *Phalaenopsis*, indicating that the GA pathway may be involved in floral transition. However, in a previous study, upregulations of genes related to GA biosynthesis were not identified under forcing condition [19]. In addition, inflorescence initiation by GA_{4+7} injection was not observed at vegetative high temperature [17]. In this study, exogenous GA_3 treatments also had no effect on the substitution of a low-temperature exposure (Figure 1). However, in our previous study, anti-GA (paclobutrazol) drenching inhibited the inflorescence initiation under forcing condition [32]. Low-temperature exposure is necessary for GA to be active in floral transition even though it is involved in *Phalaenopsis* inflorescence initiation. Exogenous GA treatment could induce flowering in vernalization-requiring or long-day plants [11,48,49]. Although *Phalaenopsis* also requires a temperature drop for inflorescence initiation, its effective temperature ranges from 15 to 25 °C and is higher than for other vernalization-requiring plants [4,50]. Furthermore, this plant is regarded as a short-day or day-neutral plant [51–53]. Considering these flowering responses to temperature and photoperiod, it would be expected that the GA pathway is not the main factor in controlling floral transition of *Phalaenopsis*.

Exogenous BAP treatment at 28 °C vegetative temperature also did not induce inflorescence initiation (Figure 1). Under vegetative high-temperature condition, CKs were converted from active to inactive forms in *Phalaenopsis* [24]. Because several weeks of a prolonged forcing period are required for inflorescence initiation, the action of exogenous BAP would not last long enough. On the other hand, in *Dendrobium*, which similarly requires low-temperature exposure for floral transition, exogenous CK could induce inflorescence initiation [23]. Therefore, it is necessary to determine whether the substitution of low-temperature exposure is possible through repeated BAP treatments. On the other hand, lateral shoots were observed in BAP-treated plants under vegetative condition (Figure 1). Maintenance of shoot apical meristem is normally regulated by a crosstalk between auxin and CK [54]. CK is directly involved in the outgrowth of axillary buds [55], and auxin inhibits the development of axillary buds by repressing CK biosynthesis [56]. *Phalaenopsis* develops two bud primordia at each node during vegetative growth period: one can be differentiated to inflorescence and the other to vegetative bud [5]. The newly developed shoots might be differentiated from the vegetative bud by the BAP treatment.

Unlike other sympodial orchids such as *Cymbidium*, *Oncidium*, etc., *Phalaenopsis* is a monopodial orchid. Single stem has a limitation in producing a lot of inflorescences. Because the number of inflorescences is an important indicator of commercial quality in *Phalaenopsis* production, multiple shoots can increase its horticultural value by the potential to make many inflorescences. To our knowledge, no data has been published about induction of multiple shoots by exogenous CK treatment during a vegetative cultivation period. Therefore, from the results of this study, further research is required to identify the detailed method for multi-shoot *Phalaenopsis* production.

5. Conclusions

In this study, we observed the effects of exogenous application of GA₃ and BAP on leaf development and on changes of leaf and stem shapes in *Phalaenopsis*. Interestingly, BAP induced the development of lateral shoots even though this plant is a monopodial orchid. Although the exogenous hormonal application at vegetative high temperature could not substitute for a low-temperature exposure in floral transition, BAP treatment under forcing condition accelerated inflorescence emergence and increased inflorescence numbers. These findings can be of help to use the growth-promoting hormones on quality *Phalaenopsis* production.

Author Contributions: K.S.K. and H.B.L. conceived and designed the study. H.B.L. and N.H.I. carried out the experiments. H.B.L. and S.K.A. analyzed the data. All authors contributed to data interpretation. H.B.L. wrote the manuscript. K.S.K. provided guidance on the whole study and improved the manuscript. All authors have read and agreed to the published version of the manuscript.

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