A DISSERTATION FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Flowering Inhibition by Intermittent High Temperature Treatment during Low Temperature Exposure in *Phalaenopsis*

호접란의 저온 기간 중 간헐적 고온처리에 따른 개화 억제

AUGUST, 2020

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UNDER THE DIRECTION OF DR. KI SUN KIM
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL OF SEOUL NATIONAL UNIVERSITY

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AUGUST, 2020

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ABSTRACTS

Phalaenopsis plants require low temperature exposure for their inflorescence initiation. However, since they are originated from tropical regions where a temperature is steadily warm without photoperiodic change throughout the year, this low temperature requirement is dissimilar to the low temperature requirements of vernalization-requiring plants which demand prolonged cold exposure as shown in a typical winter. In this study, we conducted a series of experiments to figure out the low temperature

requirement and to determine the inhibitory effects of discontinuous low temperature exposure by intermittent high temperature in *Phalaenopsis*. In Chapter I, the periods of low temperature exposure required for floral induction were observed at different cultivars and temperatures, and the effect of discontinuous low temperature exposure by intermittent high temperature treatment was also determined. In Chapter II, the correlation between inflorescence initiation and carbohydrate contents in the leaves was analyzed at different inducing temperatures. In Chapter III, metabolic changes under intermittent high temperature condition were identified by transcriptome analysis.

In this study, six to eight weeks of low temperature exposure were required to induce the competent inflorescence emergence regardless of cultivars. Also, there was no difference between the required periods at 20 and 23˚C. These results indicated that the low temperature accumulation for floral induction did not show a quantitative response to inducing temperatures although a certain period of low temperature is necessary for the inflorescence initiation of *Phalaenopsis*. Also, in Chapter II, a delay of inflorescence emergence was observed in the plants at 17˚C compared with at 20˚C and 23˚C. The inflorescence initiation induced the carbohydrate accumulation in the leaves, while the plants at vegetative temperature showed significantly

low carbohydrate contents. The inflorescence emergence was correlated with the photosynthetic ability and soluble sugar contents in the leaves at each temperature, not the amount of the cumulative low temperatures.

Low temperature disrupted by intermittent high temperature treatment significantly inhibited the inflorescence initiation despite sufficient low temperature period. Flowering-inhibited plants showed the increased number of new leaves, implying that vegetative growth was maintained by the intermittent treatment. In transcriptome analysis, down-regulation of carbohydrate metabolism was observed. Also, the expression levels of genes related to carbohydrate metabolism such as orthologs of fructose-1,6-bisphosphatase, glycoside hydrolase, G-3-P dehydronase, and sucrose transporter were decreased by intermittent high temperature treatment rather than continuous low temperature treatment. These levels were similar under continuous high temperature and intermittent high temperature conditions. Also, homologs of genes related to flowering, *PhalCOL* and *DhGI1*, showed decreased expression levels under continuous or intermittent high temperature conditions. Sugar contents in the leaves significantly increased under continuous low temperature condition rather than continuous high temperature condition. However, the contents were significantly decreased under intermittent high temperature condition rather than continuous low

temperature condition although the contents were higher than under continuous high temperature condition. These results indicated that discontinuous low temperature exposure by intermittent high temperature treatment might induce the down-regulation of sugar metabolism and inhibit the subsequent inflorescence initiation of *Phalaenopsis* plants.

In conclusion, *Phalaenopsis* plants showed carbohydrate accumulation in the leaves during inflorescence initiation. The inflorescence emergence timing was correlated with carbohydrate contents rather than the amount of low temperature exposure. Low temperature exposure disrupted by intermittent high temperature prevented the inflorescence initiation despite sufficient low temperature exposure and these inhibitory effects would be attributed to the down-regulation of carbohydrate metabolism by intermittent high temperature condition.

Keywords: carbohydrate, inflorescence initiation, intermittent high temperature, orchids, *Phalaenopsis*, RNA-seq

Student number: 2015-30374

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GENERAL INTRODUCTION

Flowering plants perceive environmental variations for their flowering. Low temperature is one of the most important environmental factors for floral transition of horticultural crops. Normally, vernalization-requiring plants respond to temperature ranging from 1 to 7°C (Chouard, 1960), and the effective temperatures can be expanded from -6 to 14°C among different species according to regions which species are native from (Chouard, 1960; Hackett and Hartmann, 1967; Malik and Perez, 2011; Michaels and Amasino, 2000).

Phalaenopsis requires temperature control for their inflorescence initiation (Sakanishi et al., 1980). *Phalaenopsis* shows a qualitative response in the low temperature requirement (Blanchard and Runkle, 2006). The inflorescence initiation could be induced at temperature below 25°C and the floral induction was prevented at temperature above 28°C (Sakanishi et al., 1980; Newton and Runkle, 2009). Although light conditions could influence the inflorescence development of *Phalaenopsis* (Kataoka et al., 2004; Lee et al., 2019; Wang, 1995b), temperature control is crucial to determine the inflorescence initiation being induced or not. Inflorescence meristem is considered to have eco-dormancy and it can be eliminated by low temperature exposure (Dueck et al., 2016; Runkle, 2010). The meristem pre-exists in the stem at the base of each leaf with juvenile or mature states (Rotor, 1952), and inflorescences at the third or fourth leaf from the apical leaf usually emerge

during flowering (Sakanishi et al., 1980).

However, the effective temperatures for *Phalaenopsis* inflorescence initiation are obviously higher than other vernalization-requiring species. *Phalaenopsis* originates from tropical or subtropical regions that are steadily warm all year round (Christenson, 2001), while many vernalization-requiring species have low temperature requirements of a typical winter (Sung and Amasino, 2004). No homologs of *FLOWERING LOCUS C* (*FLC*) or *VERNALIZATION1* (*VRN1*), which are known as genes related to vernalization, have been found in *Phalaenopsis* yet (Cai et al., 2015; Qin et al., 2012). Also, many vernalization-requiring species show long-day photoperiodic response in the floral transition (Kim et al., 2009), but it is unclear whether photoperiod has effects on the inflorescence initiation of *Phalaenopsis* (Lopez and Runkle, 2005). Instead, in recent studies, there was no significant difference in inflorescence emergence timing and expression pattern of *FLOWERING LOCUS T* (*FT*) homolog between short- and long-day conditions (Jang et al., 2015; Lee et al., 2019).

Phalaenopsis is one of the most economically important flowering crops and cultivated in many countries including Korea, The Netherlands, Taiwan, and the United States of America (De et al., 2014). These plants are propagated by tissue culture and grown in flasks for 10 to 12 months (Runkle et al.,2007), and go through long cultivation time for 50 to 70 weeks after de-flasking (Hückstädt and Torre, 2013). The cultivation period demands high production costs with heating throughout vegetative growth and cooling for flowering. Moreover, a long life cycle

also raises a problem to generate new varieties (Wang et al., 2017). Understanding the flowering mechanism can enable economically efficient flowering control and shortening the cultivation time for the desirable products.

In *Phalaenopsis*, previous studies reported that soluble sugars increased in the leaves during the inflorescence initiation (Chen et al., 1994) and the contents were related with inflorescence emergence timing (Kataoka et al., 2004). Several hormones such as gibberellin, cytokinin, auxin, and ethylene would be involved in the inflorescence initiation (Chou et al., 2000; Huang et al., 2016; Su et al., 2001; Qin et al., 2012). Nevertheless, the flowering mechanism of *Phalaenopsis* is not well understood due to little information to support the necessity of the substances. Based on functional studies of genes, several genes, e.g. *Phalaenopsis aphrodite FLOWERING LOCUS T1* (*PaFT1*) and *Phalaenopsis CONSTANS* (*CO*)-like (*PhalCOL*), have been suggested as genes controlling floral induction in *Phalaenopsis* (Jang et al., 2015; Zhang et al., 2011). In recent studies, genome-wide analyses were performed to identify the flowering mechanism in Orchidaceae plants (Huang et al., 2016; Qin et al., 2012; Wen et al., 2017). Further studies seem to be necessary to verify the putative roles of suggested genes or mechanisms.

Intermittent high temperature treatments during low temperature period could disrupt the floral transition (Malik and Perez, 2011; Purvis and Gregory, 1952). These treatments show whether the low temperature for floral induction is integrated regardless of the interrupted conditions or the floral induction can be prevented by the interrupted conditions. In a study by Angel et al. (2015), the vernalization being

registered at *FLC* was not different between interrupted and continuous cold conditions in *Arabidopsis*. Several weeks of low temperature period are normally required for inflorescence emergence of *Phalaenopsis* plants. The lack of the low temperature period completely inhibited achieving the competence for the inflorescence initiation in *Phalaenopsis* (Lee et al., 2015). We hypothesized that the intermittent treatment can induce metabolic changes which negatively affect the inflorescence initiation of *Phalaenopsis*.

This study focused on 1) the determination of flowering inhibition by intermittent high temperature treatment and 2) observation of metabolic changes by the intermittent treatment. The thesis consists of three chapters with following topics:

Chapter I: Low temperature requirement for inflorescence initiation and flowering inhibition by intermittent high temperature treatment in *Phalaenopsis*

Chapter II: Correlation between inflorescence initiation and carbohydrate contents in the leaves at different inducing temperatures in *Phalaenopsis*

Chapter III: Flowering inhibition by carbohydrate reduction in the leaves by intermittent high temperature treatment in *Phalaenopsis*

LITERATURE REVIEW

Horticultural Characteristics of *Phalaenopsis*

Phalaenopsis Blume, a genus of Orchidaceae, contains approximately 60-70 species and many generic hybrids such as *Doritaenopsis* (*Phalaenopsis* x *Doritis*), *Phalanetia* (*Phalaenopsis* x *Neofinetia*), *Rhyndoropsis* (*Phalaenopsis* x *Doritis* x *Rhyncostylis*), etc. (Chen and Chen, 2011; De et al., 2014). In some cases, *Doritis* are included in *Phalaenopsis* genus (Christenson, 2001; Tsai et al., 2010). These plants are included in Epidendroideae subfamily (Rundall and Bateman, 2002). Natural habitats of *Phalaenopsis* are usually found in tropical and subtropical regions including Asia, Australia, and South Pacific Islands, which are warm and humid throughout the year (Christenson, 2001; Pridgeon, 2000).

Most of *Phalaenopsis* species are monopodial and epiphytic orchids with thick and succulent leaves (Chen and Chen, 2011). The flower of these plants is zygomorphic with unique flower structure, just as other orchids (Su et al., 2013), and the expression patterns of *APETALA3* (*AP3*)/*AGAMOUS-LIKE6* (*AGL6*) homologs determine the perianth formation (Hsu et al., 2015). This flower has diverse colors and long longevity. *Phalaenopsis* appears to have eco-dormancy in flowering (Dueck et al., 2016). Temperature exposure below 25°C is a major factor for the inflorescence initiation (Lopez and Runkle, 2005). Inflorescence meristem for flower-stalk is differentiated in the stem at the base of each leaf (Rotor, 1952). Inflorescences usually emerge at the third or fourth leaf from the apical leaf

(Sakanishi et al., 1980).

Phalaenopsis shows typical crassulacean acid metabolism (CAM) photosynthesis (Endo and Ikusima, 1989). Daytime and nighttime photosynthetic characteristics are complementary and incomplete decarboxylation during daytime can limit the CO_2 uptake during nighttime (Guo et al., 2012; Lüttge, 2008). Although the photosynthetic capacity varies among cultivation conditions that plants have been acclimated, the daily CO_2 uptake or daytime electron transport become saturated at 200 to 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PPFD of light intensity (Guo et al., 2012; Lin and Hsu, 2004; Ota et al., 1991). Humidity and CO_2 enrichment also influence the photosynthesis (Lee et al., 2017; Lim et al., 2018; Lootens and Heursel, 1998; Song et al., 2019).

During the past several decades, *Phalaenopsis* becomes an economically important crop and has been commercially cultivated and traded in many countries (De et al., 2014). *Phalaenopsis* is normally micropropagated through flower-stalk culture. These plants require long cultivation time, approximately 50 to 70 weeks from *in vitro* plantlets to flowering, and the period is divided into three phases; vegetative, forcing, and finishing (Hückstädt and Torre, 2013). Temperature control enables the year-round production. During the vegetative period, temperature above 27 to 29°C for longer than 12 h during the day is necessary to prevent inflorescence initiation (Blanchard and Runkle, 2006; Newton and Runkle, 2009; Sakanishi et al., 1980). And, *Phalaenopsis* plants tolerate high temperature as high as 35°C for short time (Lopez and Runkle, 2005; Baker and Baker, 1991), but long-term exposure to

this temperature can induce high temperature stress (Jeong et al., 2020).

Environmental Requirements for Inflorescence Initiation of *Phalaenopsis*

Rotor (1952) reported that a drop in temperature induced the development of inflorescences in *Phalaenopsis*. These plants require relatively low temperature below 25°C although the effective temperature is higher than other veranlization-requiring plants and the temperature above 28°C inhibits the inflorescence emergence (Sakanishi et al., 1980). *Phalaenopsis* plants have a qualitative response to low temperature (Blanchard and Runkle, 2006), and approximately 3 to 10 weeks of low temperature exposure are necessary for the competence to flower (Lee et al., 2015). The inflorescence is primarily developed instead of vegetative organs, e.g. new leaves, during the reproductive period (Lee et al., 2020).

Within the effective range for the inflorescence initiation, relatively higher temperature accelerated the inflorescence emergence (Paradiso et al., 2012; Tsai et al., 2008). In several studies, daytime temperature above 28°C completely prevented inflorescence initiation although nighttime temperature was in the effective range (An et al., 2013; Blanchard and Runkle, 2006; Newton and Runkle, 2009), while cool-night condition could also induce flowering despite the high-daytime temperature in other studies (Chen et al., 2008; Pollet et al., 2011).

Little is known about the molecular mechanism regulating *Phalaenopsis* inflorescence initiation by low temperature exposure. *FLC* homologs have not been found in *Phalaenopsis* yet (Cai et al., 2015). Although not many functional studies

of *Phalaenopsis* flowering have been reported, several genes, e.g. *PaFT1,* an orthologue of *Arabidopsis FT*, *PhalCOL*, etc., have been identified (Jang et al., 2015; Zhang et al., 2011). *Doritaenopsis GIGANTEA* (*DhGI1*) and *Doritaenopsis EARLY FLOWERING 4* (*DhEFL*) were also characterized and the expression of these genes was regulated by low temperature exposure (Chen et al., 2015; Luo et al., 2011).

Phalaenopsis is regarded as day-neutral plant because this plant originated from regions close to the equator. Several studies referred that short-day conditions accelerated the spiking (Rotor, 1952; Yoneda et al., 1991). However, Jang et al. (2015) reported that there was no difference in *PaFT1* expression between short-day and long-day conditions. In a recent study, the increase of light amount by increasing photoperiod rather shortened the days to spiking and significant difference was not observed between short-day and long-day treatment with minimal daily light integral levels in flowering characteristics (Lee et al., 2019). Increasing light intensity could also accelerate inflorescence development (Kataoka et al., 2004; Wang, 1995b), but light levels over adequate ranges induced faded leaf color or leaf spots (Van der Knaap, 2005). Relative red:far-red ratio had positive effect on the inflorescence development (Dueck et al., 2016).

Metabolic Changes during Inflorescence Initiation of *Phalaenopsis*

The contents of soluble sugars such as sucrose, glucose, and fructose were accumulated in the leaves by low temperature exposure and sucrose synthase levels increased steadily during inflorescence development of *Phalaenopsis* (Chen et al.,

1994). Kataoka et al. (2004) reported that sucrose contents in the leaves were related to the inflorescence emergence timing. The sucrose contents were correlated with photosynthetic capacity (Lee et al., 2020). High temperature stress reduced CO_2 uptake rate and the sugar contents, and it could delay subsequent inflorescence initiation (Jeong et al., 2020). In the transcriptome analysis, the activation of starch degradation and sucrose biosynthesis pathways was observed during the inflorescence initiation (Qin et al., 2012). However, Ceusters et al. (2019) referred that there was no correlation between trehalose 6-phosphate (T6P) and sucrose, which is the role of T6P as a signal or regulator of sucrose levels, in *Phalaenopsis* unlike *Arabidopsis*.

During the inflorescence initiation, gibberellic acid (GA) contents in the leaves increased simultaneously (Chen et al., 1994). GA pathway may play a role in regulating the inflorescence initiation in *Phalaenopsis* (Huang et al., 2016). According to Su et al. (2001), flowering inhibition under high temperature condition might be attributed to lowered levels of endogenous GA contents. However, Wang (1995a) reported that the injection of GA did not induce the inflorescence initiation. The up-regulation of genes related to GA biosynthesis was not observed under reproductive condition in transcriptome analysis (Qin et al., 2012). Thus, it is controversial whether GA is a key regulator although the GA contents increase during inflorescence initiation.

Other hormones also have been considered to play potential roles in inflorescence initiation of *Phalaenopsis*. In a study by Chou et al. (2000), cytokinins

in the leaves were converted into inactive glucoside forms under vegetative conditions, while remaining active at inducing temperature. In *Dendrobium nobile*, an orchid, exogenous cytokinin application could induce inflorescence initiation (Wen et al., 2017). In *Phalaenopsis*, benzyladenine treatment by a foliar spray accelerated inflorescence emergence timing and promoted breaking dormancy of inflorescence meristems during low temperature exposure (Blanchard and Runkle, 2008). However, it is unknown whether the exogenous cytokinin application can substitute for low temperature exposure in the floral induction of *Phalaenopsis*. Qin et al. (2012) suggested that auxin and ethylene also could be involved in the inflorescence initiation.

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CHAPTER I

Low Temperature Requirement for Inflorescence Initiation and Flowering Inhibition by Intermittent High Temperature Treatment in *Phalaenopsis*

ABSTRACT

This study was conducted to verify whether *Phalaenopsis* plants show quantitative response to the low temperature accumulation and to determine the inhibitory effects by discontinuous low temperature exposure by intermittent high temperature treatments on the floral induction. In the first experiment, 'Mantefon', 'Hong Seol', and 'Cutie' plants were exposed to 0, 2, 4, 6, 8, and 10 weeks of low temperature at 20°C and, after each treatment, the plants were moved to 28°C of high temperature condition to prevent further low temperature accumulation. Also, 'Mantefon' plants were treated with the same methods at 20 and 23°C. In the second experiment, four different temperature regimes [continuous low temperature at 25/20°C, continuous high temperature at 28/28°C, one week of high temperature every one (1L+1H) and two (2L+1H) weeks] were treated to four cultivars ('Mantefon', 'Hong Seol', 'Bravo Star', and 'Cutie'). Zero or two weeks of low temperature period did not induce the inflorescence initiation, but the percent flowering increased with increasing the low temperature period over four weeks. In plants exposed to four or six weeks of low temperature period, the percent flowering was over 50%, but further inflorescence elongation was prevented by the following high temperature conditions. When different low

temperatures, 20 and 23°C, were treated in the same cultivar, there was no significant difference between two temperature groups. Thus, *Phalaenopsis* plants required six to eight weeks of low temperature exposure for their complete inflorescence initiation regardless of two inducing temperatures. It indicated that the low temperature accumulation does not show a quantitative response to inducing temperatures although sufficient low temperature periods were necessary for the competent floral induction. In the second experiment, inflorescence initiation was inhibited by intermittent high temperature treatment in all cultivars. The more frequent treatments, the more inhibitory effects. For example, in 'Mantefon', the percent inflorescence emergence was 100, 0, 80, and 55% at continuous low and high temperatures, 2L+1H, and 1L+1H, respectively. Under the intermittent conditions, the plants in each treatment group received six weeks of low temperature period after eight and eleven weeks of experiment period in '1L+1H' and '2L+1H', respectively. Flowering-induced plants showed reduced the number of new leaves compared with non-induced plants regardless of treatments, implying that vegetative growth can be maintained by the intermittent treatments. These results demonstrate the inhibitory effects of intermittent high temperatures on the floral induction of *Phalaenopsis* plants despite sufficient low temperature exposure.

INTRODUCTION

Many flowering plants sense environmental variations, especially temperature or photoperiodic changes, for their floral transition to reproductive development. Low temperature is the most important environmental factor for the transition in many horticultural crops. The floral induction by the exposure to the prolonged low temperature is normally called as 'vernalization' (Chouard, 1960). *Phalaenopsis,* a genus of Orchidaceae, is one of the most popular floricultural crops and also requires exposure to an ambient low temperature for the inflorescence initiation (Sakanishi et al., 1980). Several weeks of an ambient temperature below 25°C is necessary for inflorescence initiation, while temperatures above 28°C prevent the floral induction (Blanchard and Runkle, 2006; Lee et al., 2015; Sakanishi et al., 1980). Temperature is considered as the primary factor in *Phalaenopsis* flowering, and light conditions and CO_2 concentration also have an influence (Kataoka et al., 2004; Lopez and Runkle, 2005).

In vernalization-responsiveness, the epigenetic repression of *FLOWERING LOCUS C* (*FLC*), which acts as a repressor of genes required for the switch to floral induction (Michaels and Amasino, 1999; Song et al., 2012). The *FLC* silencing is quantitative to the length of low temperature exposure (Sheldon et al., 2000). The temperature for vernalization ranges from 1 to 7°C (Chouard, 1960). The effective temperatures vary among different species and the limits can be expanded from -6

to 14°C according to the origin of the species (Chouard, 1960; Hackett and Hartmann, 1967; Malik and Perez, 2011; Michaels and Amasino, 2000). In *Arabidopsis*, lower temperatures were more effective for vernalization (Wollenberge and Amasino, 2012), while the rate of vernalization increased with increasing temperatures in wheat (Brooking, 1996).

Nevertheless, effective temperature ranges for *Phalaenopsis* species are too high compared to the temperatures of other vernalization-requiring plants although these plants demand low temperatures for their inflorescence initiation. In molecular and genetic studies, genes related to vernalization such as *FLC* or *VERNALIZATION1* (*VRN1*) were not identified in *Phalaenopsis* (Cai et al., 2015; Qin et al., 2012). Most of the vernalization-requiring species show low temperature requirements of a typical winter (Sung and Amasino, 2004), but *Phalaenopsis* originated from tropical and subtropical regions where temperatures are steadily warm throughout the year (Christenson, 2001). Thus, more detailed studies are needed to understand the floral induction by low temperature exposure in *Phalaenopsis*.

Previous studies reported that high temperature treatment during low temperature exposure could interrupt the floral transition (Malik and Perez, 2011; Purvis and Gregory, 1952). However, according to Angel et al. (2015), in *Arabidopsis*, vernalizing low temperature is registered at *FLC* with a digital manner (all-or-nothing) and similar responses were observed between the interrupted and continuous cold conditions. Although *FLC* homolog was not found in *Phalaenopsis*

yet, an epigenetic modification during the floral induction was also observed in *Doritaenopsis* which is an intergeneric hybrid between *Doritis* and *Phalaenopsis* (Qin et al., 2012).

Phalaenopsis has dormant-state inflorescence meristems (Rotor, 1952), and the meristems are regarded as having eco-dormancy (Dueck et al., 2016). For breaking this dormancy, several weeks of low temperature exposure is required. In our previous study, insufficient length of low temperature exposure can prevent the inflorescence initiation of *Phalaenppsis* (Lee et al., 2015). To our knowledge, no data have been reported on whether the low temperature accumulation is a quantitative response to treatment temperatures or duration. In this chapter, a series of experiments were conducted 1) to verify whether *Phalaenopsis* plants show a quantitative response to the low temperature accumulation and 2) to determine the inhibitory effects by discontinuous low temperature exposure by intermittent high temperature treatments on the floral induction.

MATERIALS AND METHODS

Plant materials and growth conditions

Clones of twelve-month-old *Phalaenopsis* Queen Beer 'Mantefon', *P*. hybrid 'Hong Seol', 'Bravo Star', and 'Cutie', were purchased from a commercial grower (Sang Mi Orchids, Taean, Korea), and plants were transplanted into 10-cm (approximately 500 mL volume) plastic pots filled with 100% sphagnum moss. Before the start of treatments, these plants were cultivated in a walk-in chamber and were acclimatized at 28°C, 60% relative humidity, and 12 h of photoperiod with 130 $\mu mol \cdot m^{-2} \cdot s^{-1}$ by fluorescent lamps (TL-D 32W RS865, Philips Lighting Co., Ltd., Eindhoven, Netherlands) for six weeks. The plants were fertigated once a week with water soluble fertilizer (EC 0.8-1.0 $mS \cdot cm^{-1}$; Hyponex professional 20N-20P-20K, Hyponex Japan, Osaka, Japan) using hand-drip irrigation.

Low temperature treatments with different durations (Expt. 1)

After the acclimatization period, the plants were treated with low temperature at 20°C for 0, 2, 4, 6, 8, and 10 weeks in 'Mantefon', 'Hong Seol', and 'Cutie'. After each treatment period, the plants were moved to 28°C of high temperature condition in a separate chamber to prevent further low temperature accumulation. The average temperatures in chambers were 19.4 and 28.3°C, respectively. At the beginning of treatments, the mean plant span, which is the distance between the tips of the longest

leaves, was 20.2, 21.6, and 21.8 cm in ‘Mantefon’, ‘Hong Seol’, and ‘Cutie’, respectively. Also, ‘Mantefon’ plants were treated with 20 and 23°C of low temperatures for 0, 2, 4, 6, 8, and 10 weeks. Likewise, after each treatment, the plants were moved to 28°C high temperature condition. Cultivation conditions were the same as the above experiment. The mean plant span was 20.3 cm. The average temperatures in chambers were 20.2, 22.4, and 28.3°C in each temperature group, respectively. Plants with visible inflorescence (VI, > 0.5 cm in length) was counted as flowering.

Intermittent high temperature treatment (Expt. 2)

Twelve-month-old ‘Mantefon’, ‘Hong Seol’, and ‘Bravo Star’ in replication 1 and ‘Mantefon’, ‘Hong Seol’, and ‘Cutie’ in replication 2, i.e. four different cultivars were used in this experiment. The mean plant span was 19.7, 22.7, and 24.5 cm in replication 1 and 20.2, 21.6, and 21.8 cm in replication 2, respectively. The plants were cultivated in environment-controlled growth chambers with 250-W metal halide lamps (Han Young Electrics Co., Gwangju, Korea) and other growing conditions were the same as above. The plants were treated with four different temperature regimes: continuous low temperature at 25/20°C (12 h day/12 h night), continuous high temperature at 28/28°C, one week of high temperature every one (1L+1H) or two weeks (2L+1H) of low temperature. Each treatment was maintained for 15 weeks and. After that, it was considered that the flowering initiation was no longer induced. Ten plants were placed in each treatment. The actual average

temperatures in each treatment group were described in Table I-1. Plants with VI and the days to VI were counted in all cultivars. Total days to flowering, days from VI to flowering, and the number of flowers were counted in 'Hong Seol' because the plants flowered during the experiment period. The number of total leaves and new leaves, plant span, and length and width of the uppermost mature leaf were measured at the end of experiment.

Experimental design and statistical analysis

Completely randomized design was used in all experiments. Statistical analysis was performed using ANOVA with SAS (Windows version 9.3; SAS Institute Inc., Cary, NC, USA). In Expt. 1, homogeneity of variance in percent plants with VI was analyzed by GLM with Levene's test in cultivar and temperature variables. In Expt. 2, comparisons of parameters among temperature regimes were performed by Duncan's multiple range test with $p < 0.05$ as the threshold for statistical significance. SigmaPlot (version 10.0; Systat Software, Chicago, IL, USA) was used for graph module analyses.

Table I-1. Actual average temperatures in each treatment group in the second experiment.

Treatment	Replication 1		Replication 2	
	25/20˚C	28/28˚C	25/20˚C	28/28˚C
Low (L)	24.8/19.4˚C	-	25.4/20.3˚C	-
High (H)	-	28.6/28.0˚C	-	28.8/28.4˚C
2L+1H	24.8/20.3˚C	28.6/28.3˚C	25.3/20.2˚C	28.8/28.1˚C
1L+1H	25.1/19.9˚C	28.6/28.4˚C	25.7/20.2˚C	29.1/28.8˚C

Low (L), 25/20°C; High (H), 28/28°C

Low, continuous low temperature; High, continuous high temperature; 2L+1H, one week of high temperature every two weeks of low temperature;1L+1H, one week of high temperature every one week of low temperature

RESULTS

Inflorescence initiation by different duration of low temperature exposure

'Mantefon', 'Hong Seol', and 'Cutie' plants at least required eight, six, and six weeks of low temperature exposure at 20°C, respectively, for inflorescence emergence (Fig. I-1A). Zero and two weeks of low temperature did not induce inflorescence initiation in all cultivars. More than 50% of plants showed the inflorescence emergence in treatment with four weeks of low temperature, but further inflorescence development was prevented by following high temperature period at 28°C. Therefore, six to eight weeks of low temperature exposure at 20°C were necessary for the competence to induce inflorescence initiation and development. When two low temperatures (20 and 23°C) were compared in 'Mantefon' plants, the required period was six and eight weeks, respectively (Fig. I-1B). In statistical analysis, homogeneity of variance for the percent flowering-induced plants to different cultivars and inducing temperatures showed no significance, and *p*-values were 0.9104 (F=0.09) and 0.6041 (F=0.29), respectively (data not shown).

Flowering inhibition under discontinuous low temperature condition

Intermittent high temperature decreased the percent plants with VI and delayed

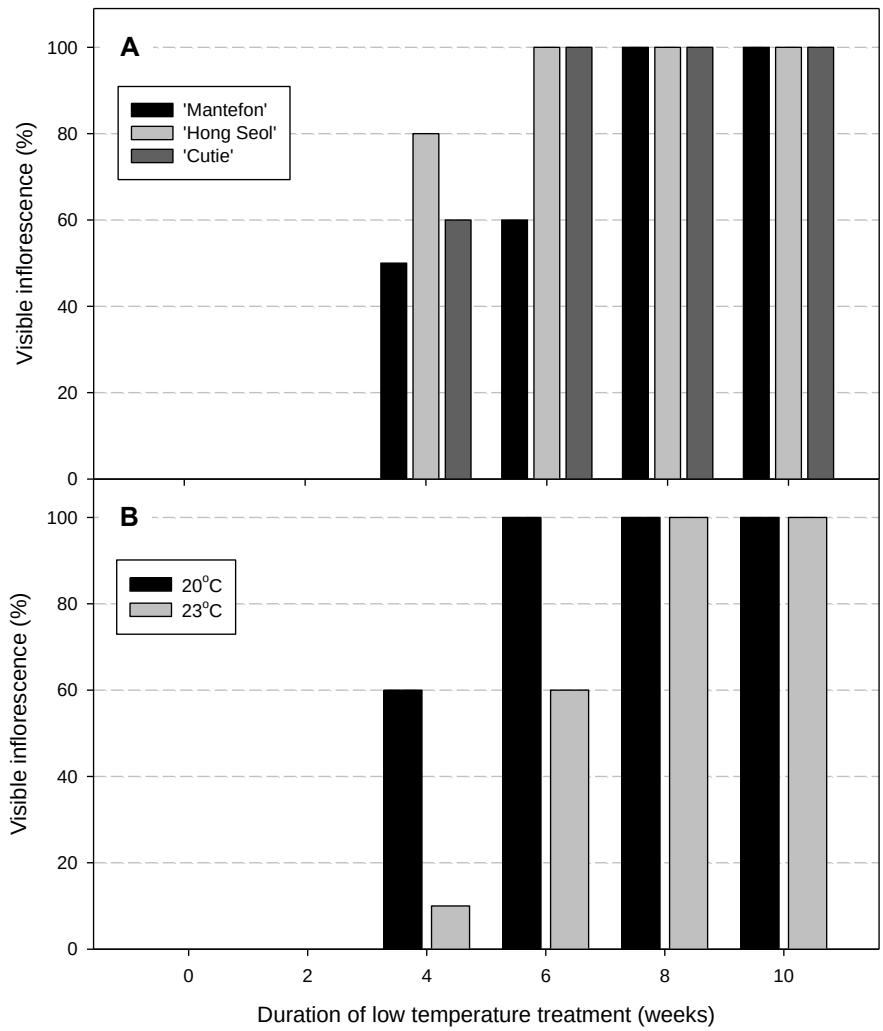

Figure I-1. Percent plants with visible inflorescence (VI) of plants treated with different durations of low temperature exposure. 'Mantefon', 'Hong Seol', and 'Cutie' at 20˚C (A) and of 'Mantefon' at 20 and 23˚C (B).

inflorescence initiation in all cultivars although the inhibitory effects varied among cultivars (Fig. I-2A-D). More frequent high temperature conditions, i.e. '1L+1H' treatment, had more effects compared to '2L+1H' treatment. On the contrary, the intermittent treatment decreased total days to flowering and days from VI to flowering compared with continuous low temperature condition in 'Hong Seol' (Table I-2). The days from VI to flowering in '1L+1H' treatment was significantly lower than the days in '2L+1H' treatment. There was no difference in the number of flowers among continuous low temperature and intermittent treatments.

In comparison with growth characteristics, flowering-induced plants showed relatively reduced growth, e.g. plant span, leaf length and width, compared with non-induced plants in all cultivars (Table I-3). However, the leaf thickness increased in flowering-induced plants. These parameters showed cultivar-dependent responses. Interestingly, significant differences in the number of total and new leaves were observed between flowering-induced and non-induced plants. Although the difference was relatively small in 'Cutie' compared with other cultivars, the leaf numbers significantly decreased in flowering-induced plants regardless of treatments. When the floral induction was inhibited by intermittent high temperatures, new leaf development of non-induced plants was maintained in common with plants with continuous high temperature condition. In two-way ANOVA, the interaction between treatment and floral induction was not observed in most of the parameters in all cultivars except for leaf thickness of 'Hong Seol' ($p < 0.001$) and plant span of 'Cutie' ($p < 0.05$).

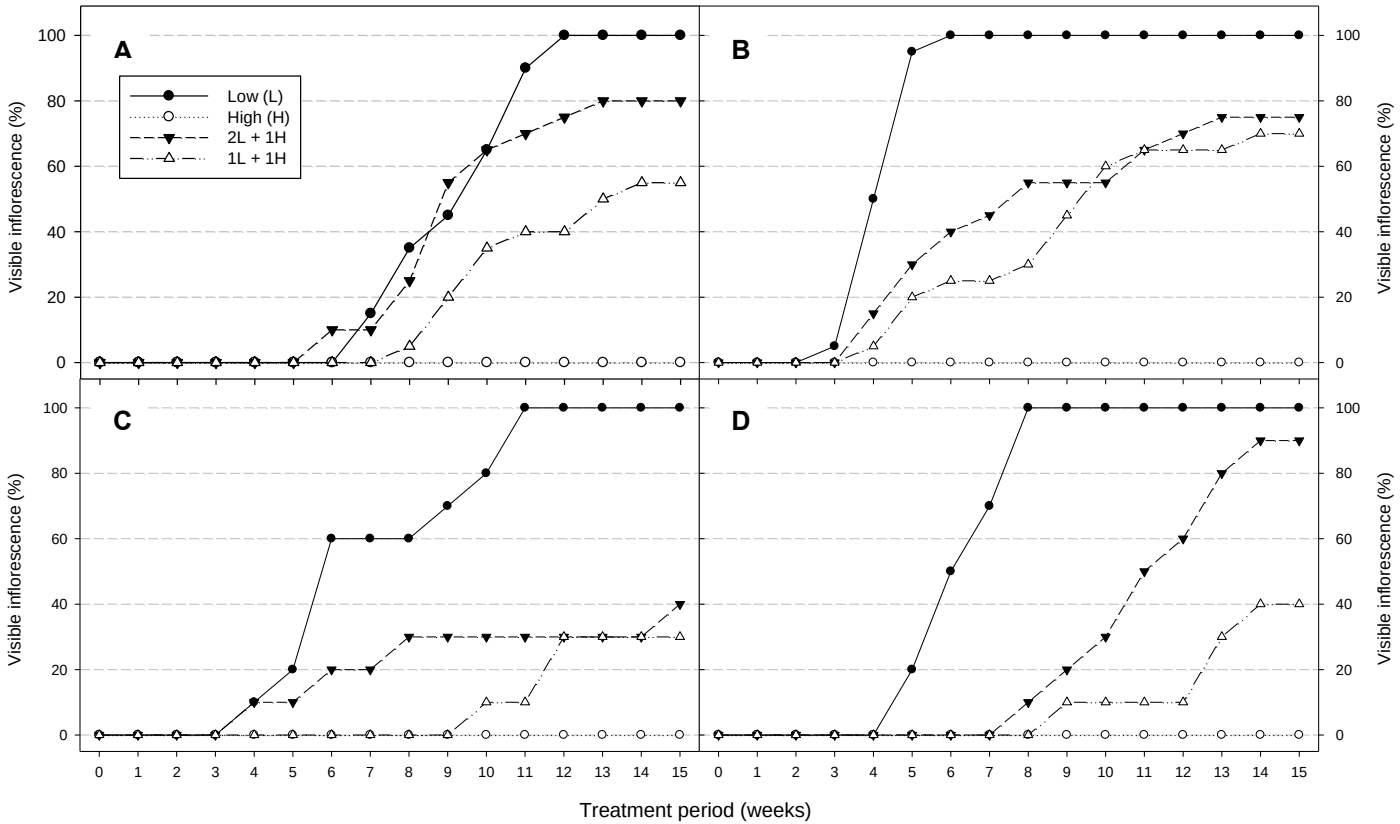

Figure I-2. Percent plants with visible inflorescence of 'Mantefon' (A), 'Hong Seol' (B), 'Bravo Star' (C), and 'Cutie' (D)

plants treated with different temperature regimes. Low (L), 25/20°C; High (H), 28/28°C. Low, continuous low temperature; High, continuous high temperature; 2L+1H, one week of high temperature every two weeks of low temperature;1L+1H, one week of high temperature every one week of low temperature. Data of 'Mantefon' and 'Hong Seol' in replication 1 and 2 were pooled because there was no significant difference between replications.

Table I-2. Flowering characteristics of 'Hong Seol' plants treated with different temperature regimes after 15 weeks of treatment.

Treatment	Total days to flowering	Days from VI to flowering	No. of florets
Low (L)	106.1a[z]	73.7a	7.8
Hight (H)	-	-	-
2L + 1H	97.3b	64.0b	6.7
1L + 1H	91.6b	55.2c	6.4
Significance	***	***	NS

Low (L), 25/20°C; High (H), 28/28°C. Low, continuous low temperature; High, continuous high temperature; 2L+1H, one week of high temperature every two weeks of low temperature;1L+1H, one week of high temperature every one week of low temperature.

[z]Means within columns followed by different letters are significantly different by Duncan's multiple range test at $p < 0.05$.

[NS], [***] non-significant or significant at $p < 0.001$, respectively.

Table I-3. Vegetative growth characteristics of 'Mantefon', 'Hong Seol', 'Bravo Star', and 'Cutie' plants treated with different temperature regimes after 15 weeks of treatment.

Treatment (T)	Floral induction (FI)[z]	No. of total leaves	No. of new leaves	Plant span (cm)	Uppermost mature leaf		
					Length (cm)	Width (cm)	Thickness (mm)
				'Mantefon'			
Low (L)	FI	5.60bc[y]	1.00d	21.07b	13.05b	6.56ab	2.05a
	Non-FI	-	-	-	-	-	-
High (H)	FI	-	-	-	-	-	-
	Non-FI	6.20ab	1.90ab	24.76a	16.90a	6.57ab	1.90b
2L+1H	FI	5.50c	1.25cd	23.73ab	14.30b	6.49b	1.94ab
	Non-FI	-	-	-	-	-	-
1L+1H	FI	6.25ab	1.50bc	24.85a	14.15b	7.20a	2.01ab
	Non-FI	6.60a	2.20a	25.22a	15.02ab	6.82ab	2.00ab
Significance	T	**	***	*	**	NS	*
	FI	**	***	*	**	NS	NS
	T*FI	NS	NS	NS	NS	NS	NS
				'Hong Seol'			
Low (L)	FI	5.80c	0.80b	25.50	14.69b	6.34ab	2.16ab
	Non-FI	-	-	-	-	-	-
High (H)	FI	-	-	-	-	-	-
	Non-FI	7.00a	2.10a	26.18	16.34a	6.55a	2.11b
2L+1H	FI	6.29abc	1.00b	25.40	14.71b	5.93b	1.97c
	Non-FI	6.67ab	2.00a	26.33	17.23a	6.47a	2.26 a
1L+1H	FI	6.17bc	1.00b	25.12	13.80b	6.27ab	1.98c
	Non-FI	7.00a	2.25a	25.10	14.95b	6.43ab	2.11b
Significance	T	***	***	NS	***	NS	*
	FI	***	***	NS	***	*	*
	T*FI	NS	NS	NS	NS	NS	***

				‘Bravo Star’			
Low (L)	FI	5.50a	1.40b	32.51	21.45ab	7.51	2.12a
	Non-FI	-	-	-	-	-	-
High (H)	FI	-	-	-	-	-	-
	Non-FI	6.10a	2.40a	34.83	22.36a	7.04	1.92b
2L+1H	FI	5.50a	1.75ab	32.88	19.58b	7.50	2.07a
	Non-FI	5.83a	1.83ab	31.13	19.75b	7.13	2.08a
1L+1H	FI	5.67a	2.00ab	31.50	20.20ab	7.47	2.01ab
	Non-FI	6.00a	2.29a	32.77	21.43ab	7.46	2.04ab
Significance	T	NS	***	NS	*	NS	**
	FI	*	***	NS	NS	*	*
	T*FI	NS	NS	NS	NS	NS	NS
				‘Cutie’			
Low (L)	FI	7.00	1.40d	24.05	13.12c	5.24bc	2.14a
	Non-FI	-	-	-	-	-	-
High (H)	FI	-	-	-	-	-	-
	Non-FI	7.70	2.90a	23.16	15.78a	5.67a	1.88c
2L+1H	FI	7.33	1.78cd	23.64	13.88bc	5.20c	2.00b
	Non-FI	-	-	-	-	-	-
1L+1H	FI	7.50	2.00bc	24.85	14.53ab	5.58ab	2.00b
	Non-FI	7.67	2.33b	23.27	15.07ab	5.57ab	1.93bc
Significance	T	NS	**	NS	**	NS	****
	FI	NS	NS	NS	NS	NS	NS
	T*FI	NS	NS	*	NS	NS	NS

Low (L), 25/20°C; High (H), 28/28°C. Low, continuous low temperature; High, continuous high temperature; 2L+1H, one week of high temperature every two weeks of low temperature;1L+1H, one week of high temperature every one week of low temperature.

[z]FI, data from treatments with ≥ 30% flowering induced; non-FI, data from treatments with ≥ 30% flowering inhibited.

[y]Means within columns followed by different letters are significantly different by Duncan’s multiple range test at $p < 0.05$.

[NS], [*], [**], [***] non-significant or significant at $p < 0.05$, 0.01 or 0.001, respectively.

DISCUSSION

Phalaenopsis shows an obligate response in inflorescence initiation to a temperature below 25°C (Blanchard and Runkle, 2006; Sakanishi et al., 1980). Six to eight weeks of low temperature exposure were needed for the competent floral induction irrespective of cultivars (Fig. I-1A). However, these low temperature requirements may be different from the general vernalization response. Natural habitats of *Phalaenopsis* plants are usually found in tropical or subtropical regions (Christenson, 2001).

The effective temperature ranges for inducing the inflorescence initiation are higher than those of other species requiring vernalization, which is a cumulative process (Chouard, 1960; Hackett and Hartmann, 1967; Malik and Perez, 2011; Michaels and Amasino, 2000). In *Arabidopsis*, the effectiveness of vernalization increased with decreasing temperature (Wollenberge and Amasino, 2012). Lower inducing temperatures might accelerate inflorescence emergence if *Phalaenopsis* showed a quantitative response to low-temperature exposure like other vernalization-requiring plants. However, in this study, there was no significant difference in the duration for the floral induction between 20 and 23°C (Fig I-1B). It indicated that the low temperature accumulation for the floral induction did not show a cumulative response to inducing temperatures.

Paradiso et al. (2012) reported that temperature variations during inflorescence

initiation influenced inflorescence emergence timing, and lower temperatures led to longer emergence time in *Phalaenopsis*. Kataoka et al. (2004) suggested that the inflorescence emergence timing would be related to carbohydrate contents under different environmental conditions. Therefore, the relatively higher inducing temperature seems to rather accelerate the inflorescence initiation by the support of higher carbohydrate contents than less effective in the low temperature requirements. To verify this phenomenon, further experiments were designed in the next chapter.

Intermittent high temperature interrupts to achieve the low temperature requirement for the inflorescence initiation. In our previous study, the intermittent high temperature treatment could inhibit the floral induction of *Phalaenopsis*, but further study was required since the effects showed a cultivar-dependent response (Lee et al., 2015). In this study, four different cultivars with two replications were used to verify the reproducibility of the inhibitory effects of the intermittent treatment in *Phalaenopsis*. A decrease in the percent plants with visible inflorescence (VI) observed in all cultivars (Fig I-2A-D).

The inflorescence development might be delayed, not inhibited, by the reduced low temperature period. However, in case of 'Hong Seol', six weeks of continuous low temperature could induce the inflorescence initiation in Expt. 1 (Fig. I-1A and 2B). Under the intermittent conditions, after eight and 11 weeks of experiment period in '1L+1H' and '2L+1H', respectively, plants in each treatment group received the same period of low temperatures in plants with continuous low temperature. Nonetheless, the percent plants with VI under the intermittent high temperature

conditions did not reach 100% inflorescence emergence at the end of the experiment. The cumulative low temperature period could not account for the low temperature requirements in *Phalaenopsis*. Thus, the intermittent high temperature treatment induces not only a delay but also the inhibition of the inflorescence initiation. These inhibitory effects also have been reported in olive (Malik and Perez, 2011).

Also, flowering-induced plants showed reduced new leaf development regardless of cultivars, while the number of new leaves significantly increased in non-induced plants under both continuous high and intermittent high temperature conditions (Table I-3). The number of new leaves can be an indicator of vegetative growth of *Phalaenopsis* plants. During the reproductive period, inflorescence development primarily occurs rather than new leaf development (Lee et al., 2015; Newton and Runkle, 2009). In this study, the increase of the newly-developed leaves in plants under the intermittent high temperature conditions demonstrated that vegetative growth was maintained and the floral induction was prevented by the intermittent treatment.

Nevertheless, some plants treated with the intermittent high temperatures showed the inflorescence emergence (Fig. 2A-D). In a study by Angel et al. (2015) in *Arabidopsis*, there was no difference in the spliced *FLC* mRNA levels between interrupted (similar to the intermittent treatment in this study) and continuous cold conditions. Although an epigenetic modification was reported in *Doritaenopsis* inflorescence initiation (Qin et al., 2012), *FLC* homolog was not found in *Phalaenopsis* in the genome-sequencing analysis (Cai et al., 2015). Instead,

supplemental studies will be required to verify the regulation of flowering related genes that switch the floral induction under the interrupted conditions in *Phalaenopsis*.

Once inflorescence emerged, plants treated with intermittent high temperatures showed a shortened time to flowering (Table I-2). Also, more frequent high temperature condition (1L+1H) significantly accelerated the inflorescence emergence compared with 2L+1H treatment. Normally, higher temperatures during inflorescence development reduce the time to flowering in *Phalaenopsis* (Blanchard and Runkle, 2006; Paradiso et al., 2012). The intermittent high temperature treatment increased the average temperature during the inflorescence development.

Phalaenopsis plants required sufficient low temperature periods, six to eight weeks in this study, for their competent inflorescence initiation. However, this low temperature requirement did not show a cumulative response to inducing temperatures. Discontinuous low temperature exposure by intermittent high temperature treatment partially inhibited the floral induction in spite of the sufficient low temperature periods. Molecular genetic or metabolic approaches seem to be necessary to determine the causes of the inhibitory effects of intermittent high temperature treatments.

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CHAPTER II

Correlation between Inflorescence Initiation and Carbohydrate Contents in the Leaves at Different Inducing Temperatures in *Phalaenopsis*

The research described in this chapter has been published in

Scientia Horticulturae (2020, volume 265 109270).

ABSTRACT

This study was conducted to determine the effects of temperature variations on inflorescence initiation and to verify the correlation of carbohydrate contents and floral induction in *Phalaenopsis*. Twelve-month-old *Phalaenopsis* Queen Beer 'Mantefon' clones were grown at 17, 20, 23, or 28°C (Expt. 1). Photosystem II (PSII) operating efficiency and CO_2 uptake rate were measured at 3, 7, 14, and 28 days after treatment (DAT), and carbohydrate content in the stem and leaf was analyzed at 14 and 28 DAT. In a supplemental experiment (Expt. 2), 0, 10, 20, and 40 $g \cdot L^{-1}$ of sucrose solutions were treated by foliar spray under vegetative and inducing conditions at 28 and 20°C, respectively. Plants at 17, 20, and 23°C of inducing temperatures showed inflorescence emergence, while vegetative temperature at 28°C completely inhibited inflorescence initiation. The flowering-inhibited plants developed significantly increased the number of new leaves compared with plants at inducing temperatures. Days to visible inflorescence (VI) were 85.7, 44.2, and 45.8 at 17, 20, and 23°C, respectively. PSII operating efficiency and CO_2 uptake rate significantly decreased at 17°C irrespective of measurement timings, and these values were similar between plants at 20 and 23°C. Starch and soluble sugars content in the stem showed no difference

among temperature or duration variable, while the content of soluble sugars in the leaves increased in flowering-induced plants at inducing temperatures. Sucrose content in the leaf at 14 DAT significantly was higher at 20 and 23°C than at 28°C, and a similar trend was observed in glucose, fructose, and sucrose at 28 DAT. Soluble sugar content at 17°C also slightly increased compared with that at 28°C. Although plants at 28°C showed higher PSII operating efficiency and CO_2 uptake rate, lower soluble sugar contents were observed than those in flowering-induced plants. There was a negative correlation in the days to VI with photosynthetic parameters and sucrose content in the leaves. Although exogenous sucrose treatment at 28°C did not induce the inflorescence initiation, the treatment at 20°C accelerated inflorescence emergence. These results indicate that inflorescence initiation induces carbohydrate accumulation in the leaves and is correlated with the contents of soluble sugars.

INTRODUCTION

In the preceding study, a lower inducing temperature could not promote the inflorescence initiation. In *Phalaenopsis*, inducing temperatures can influence inflorescence emergence timing and relatively higher inducing temperature seems to accelerate the inflorescence initiation (Blanchard and Runkle, 2006; Paradiso et al., 2012). It has been reported that the inflorescence emergence is related with soluble sugar contents during the floral induction (Katakoka et al., 2004). Thus, it can be considered that the inflorescence initiation is determined by the carbohydrate contents at the inducing temperatures, not the cumulated low temperature amount.

Normally, diurnal temperature fluctuation is recommended to promote inflorescence development (Lee and Lin, 1984). However, in a study by Blanchard and Runkle (2006), diurnal fluctuation was not required for the inflorescence initiation, and daytime temperatures above 26°C inhibited the inflorescence emergence despite a cool night temperature. Nevertheless, because *Phalaenopsis* is a crassulacean acid metabolism (CAM) plant and absorbs CO_2 during the night (Endo and Ikusima, 1989; Guo and Lee, 2006), it requires a cool nighttime temperature (Chen et al., 2008). For CAM plants, a cool nighttime temperature or diurnal fluctuation are required for enhancing CO_2 uptake or nocturnal accumulation of malic acid (Lüttge, 1988; 2008; Osmond, 1978).

Sucrose is the main photosynthetic product, and its metabolism is essential for

plants based on allocation of carbon sources and sugar signals (Koch, 2004; Ruan, 2014). Sugar content is also correlated with flowering in *Phalaenopsis* (Kataoka et al., 2004). During inflorescence development at low ambient temperatures, sucrose synthase level increases steadily (Chen et al., 1994), and sugars, especially sucrose, accumulate in the leaves (Konow and Wang, 2001). This accumulation of sugars shows a negative correlation with inflorescence emergence timing (Kataoka et al., 2004).

Many orchids like *Cymbidium* and *Oncidium* have pseudo-bulbs that store water, minerals, and carbohydrates to support inflorescence development (Kim et al., 2013; Ng and Hew, 2000; Wang et al., 2008). *Phalaenopsis* plants do not have pseudo-bulbs but reserve carbohydrates in their stems (Liu et al., 2013) by developing undifferentiated and dormant buds in the stem at the base of each leaf (Rotor, 1952). Based on this information, carbohydrate accumulation in the leaves under reproductive conditions may be attributed to use of energy sources for inflorescence development.

The impacts of exogenous sucrose treatments have been reported in many plants including *Brassica campestris* and chrysanthemum (Friend et al., 1984; Roldán et al 1999; Sun et al., 2017). For example, the spraying of 50 mM sucrose accelerated flowering of chrysanthemum ‘Floral Yuuka’ under night interruption condition and increased *CmFTL2*, *FT-like* gene, expression levels (Sun et al., 2017).

We hypothesized that photosynthetic ability, carbohydrate accumulation, and inflorescence initiation are closely correlated, and the inflorescence initiation is

affected by carbohydrate status, not simply the amount of low temperatures. This study was performed to determine the effects of temperature variations on photosynthetic ability, carbohydrate contents in the leaf and stem, and inflorescence initiation (Expt. 1), and the effects of exogenous sucrose treatment on the inflorescence initiation in *Phalaenopsis* (Expt. 2).

MATERIALS AND METHODS

Plant materials and growth conditions

Twelve-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 (Sang Mi Orchids, Taean, Korea). Plants were acclimatized in growth chambers (HB-301MP; Hanbaek Scientific Co., Bucheon, Korea) with 250-W metal halide lamps (Han Young Electrics Co., Gwangju, Korea) for four weeks. Other acclimatization conditions were the same as those in Chapter I.

Temperature treatments at different inducing temperatures

After the acclimatization period, plants were treated with four different temperatures (17, 20, 23, or 28 ± 1°C) for 15 weeks. Each temperature was set in one of four growth chambers. The actual average temperatures were 17.0, 20.2, 23.6, and 28.8°C, respectively, during the treatment period. Except for temperature condition, other growth conditions were maintained at the same values as during the acclimatization period. There were three replications per each temperature group with at least five plants. At the beginning of treatments, the mean plant span was 20.5 cm. The number of new leaves, plant span, and length, width, and thickness of the uppermost mature leaf were measured at the end of treatments. The percent plants

with VI, days to VI, and number of VIs were also counted.

Measurement of photosynthetic ability during the day- and nighttime

Chlorophyll fluorescence and CO_2 uptake rate of the uppermost mature leaves were measured using a PAM chlorophyll fluorometer (PAM 2000; Heinz Walz, Effeltrich, Germany) and a portable photosynthesis system (LI-6400XT; Li-Cor Co., Inc., Lincoln, NE, USA) at 3, 7, 14, and 28 days after treatment (DAT). The measurements were conducted during the middle of the light and dark period, respectively. Three plants from each treatment group were randomly chosen, and light-adapted leaves were induced with a saturating light pulse for 0.8 s to obtain maximum fluorescence (F_m'). In addition, F_q,' the difference between F_m' and F' (steady-state level of fluorescence), was calculated to determine photosystem II (PSII) operating efficiency (F_q'/F_m,' Yield). In the measurement of CO_2 uptake rate, temperatures inside the leaf chamber were maintained at that used for treatment. Relative humidity and CO_2 concentration were set at approximately 60% and 400 $\mu mol \cdot mol^{-1}$, respectively. The gas exchange of each leaf was measured every minute for 10 minutes, and the average value of the 10 data points was used for further analysis.

Analysis of carbohydrate contents in the stem and leaf

The plant stem and the middle part of the uppermost mature leaf were sampled from three replications at 14 and 28 DAT at different inducing temperatures. Fresh

biomass of each sample was weighed and immersed in liquid N_2 and homogenized samples were stored at -80˚C. Contents of soluble sugars of glucose, fructose, and sucrose were analyzed by the method of González-Rossia et al. (2008) with slight modifications. Briefly, 200 mg of stored sample was incubated in 80% ethanol at 85˚C for 15 minutes. The supernatant was collected after centrifuging at 3,600 g for 30 minutes. The sugar extracts were evaporated by a N_2 evaporator (N-EVAPTM; Organomation Associates, Inc., Berlin, MA, USA) at 60˚C. Ethanol-insoluble material was dried and stored for starch analysis. The evaporated supernatant was dissolved in 3 mL of distilled water and filtered through a 0.45-μm nylon filter (Acrodisc® 13-mm syringe filter; Pall Co., Port Washington, NY, USA) with a C18 Sep-Pak cartridge (Waters Corp., Milford, MA, USA). The contents were analyzed using ion chromatography with a pulsed amperometric detector (ICS-5000; Thermo Dionex, Sunnyvale, CA, USA) on a Dionex CarboPac PA1 column using 50 mM NaOH (1 $mL \cdot min^{-1}$).

The method of Smith and Zeeman (2006) was used for analysis of starch content. The remaining sediment for analysis of soluble sugar content was dissolved in 1 mL of distilled water. The pellets were boiled to gelatinize starch granules, and 0.5 mL of 0.2 M Na-acetate (pH 5.5) buffer, 15 units of amyloglucosidase (A7095; Sigma-Aldrich Korea Ltd., Yongin, Korea), and 5 units of α-amylase (A4862; Sigma-Aldrich Korea Ltd.) were added to each sample. Samples were incubated at 55˚C for two hours and centrifuged at 3,600 g for 30 minutes. The supernatant was collected two times and dissolved in 3 mL of distilled water. The extract was filtered through

a 0.45-μm nylon filter with a C18 Sep-Pak cartridge. The released glucose was analyzed using an UltiMate 3000 HPLC instrument (Dionex) equipped with a Shodex RI-101 detector (Showa Denko K.K., Kawasaki, Japan) on a Waters Sugar-Pak column using distilled water (0.5 $mL \cdot min^{-1}$).

Exogenous sucrose treatment under vegetative and reproductive conditions

Clones of 15-month-old *Phalaenopsis* Queen Beer 'Mantefon' were purchased from a commercial grower (Sam Hyeon Orchids Farm, Goyang, Korea) in 10-cm (approximately 500 mL volume) plastic pots filled with 100% sphagnum moss. Before the start of treatments, these plants were cultivated in a walk-in chamber with 130 $\mu mol \cdot m^{-2} \cdot s^{-1}$ by fluorescent lamps (TL-D 32W RS865, Philips Lighting Co., Ltd., Eindhoven, Netherlands) and were acclimatized for four weeks. The acclimatization conditions were the same as the preceding experiments. After the acclimatization period, plants were moved to 28 and 20°C of vegetative and inducing temperatures, respectively. The actual average temperatures were 28.2 and 20.9°C, respectively, at vegetative and inducing temperatures. Other growth conditions were maintained at the same values as during the acclimatization period. Five plants with three replications were treated with four different concentrations (0, 10, 20, and 40 $g \cdot L^{-1}$, w/v) of sucrose (S7903, Sigma-Aldrich Korea Ltd., Yongin, Korea) solution with 0.1% Tween-20 (P1379, Sigma-Aldrich Korea Ltd., Yongin, Korea) by foliar spray. Each plant received 20-25 mL of solutions. The solutions

were treated two times at one-week interval. At the beginning of treatments, the mean plant span was 27.1 cm. At vegetative temperature, the percent plants with visible inflorescence (VI), number of new leaves, plant span, and length and width of the uppermost mature leaf were measured after 20 weeks of temperature treatment. At inducing temperature, the number of new leaves, percent plants with VI, days to VI, and number of VIs were observed for 15 weeks.

Experimental design and statistical analysis

Completely randomized design was used in all experiments. Statistical analysis was performed using ANOVA in SAS (Windows version 9.3; SAS Institute Inc., Cary, NC, USA). Comparisons among treatment groups were performed by Tukey's HSD test for evaluating the growth and flowering characteristics, PSII operating efficiency, CO_2 uptake rate, and carbohydrate contents with $p < 0.05$ as the threshold for statistical significance. Also, Pearson's correlation coefficients (r) of photosynthetic ability and soluble sugar content in the leaf with days to were calculated to determine the relationships between inflorescence emergence timing and parameters. Graph module analyses were performed using SigmaPlot software version 10.0 (Systat Software, Inc., Chicago, IL, USA).

RESULTS

Growth characteristics at different inducing temperatures

There were no differences among temperature treatments in growth characteristics except for number of new leaves (Table II-1). New leaves significantly increased at 28°C compared with other temperatures, and plants at 17, 20, and 23°C showed similar values to one another.

Inflorescence initiation at different inducing temperatures

Inflorescence initiation was prevented at 28°C. Although a slightly smaller percentage of plants with visible inflorescence (VI) (87%) was observed at 17°C, this was not statistically different from plants at 20 and 23°C (100%) (Table II-2). Number of VIs was also similar irrespective of inducing temperature. However, days to first VI was significantly delayed at 17°C compared with 20 and 23°C (85.7, 44.2, and 45.8 at 17, 20, and 23°C, respectively). Inducing temperature had a negative correlation with days to first VI (r = -0.84) (Table II-4).

Photosynthetic ability at different inducing temperatures

Photosystem II (PSII) operating efficiency (Yield) during the day and CO_2 uptake rate during the night at 17°C were significantly lower than at other temperatures throughout the experiment. Among 20, 23, and 28°C conditions, there

Table II-1. The number of new leaves, plant span, and length, width, and thickness of the uppermost mature leaf of 'Mantefon' plants after 15 weeks of 17, 20, 23, and 28°C treatments.

Temperature (°C)	No. of new leaves	Plant span (cm)	Uppermost mature leaf		
			Length (cm)	Width (cm)	Thickness (mm)
17	1.13b[z]	23.57	14.98	6.78	2.00
20	1.20b	22.83	13.67	6.79	2.01
23	1.53b	23.86	14.65	6.99	2.04
28	2.83a	22.03	13.32	7.12	1.98
Significance	***	NS	NS	NS	NS

[z]Means within columns followed by different letters are significantly different by Tukey's HSD test at $p < 0.05$.

NS, *** non-significant or significant at $p < 0.001$, respectively.

Table II-2. Percent visible inflorescence (VI) of 'Mantefon' plants, days to VI, and number of VIs after 15 weeks of 17, 20, 23, and 28°C treatments.

Temperature (°C)	Visible inflorescence (%)	Days to VI	No. of VIs
17	87a[z]	85.7a	1.67
20	100a	44.2b	1.80
23	100a	45.8b	1.73
28	0b	-	-
Significance	***	***	NS

[z]Means within columns followed by different letters are significantly different by Tukey's HSD test at $p < 0.05$.

NS, *** non-significant or significant at $p < 0.001$, respectively.

was no significant difference in Yield value except at 14 DAT (Fig. II-1A). Under the 17°C condition, the value slightly increased at 28 DAT compared with 14 DAT. The CO_2 uptake rates showed similar trends (Fig. II-1B). These photosynthetic parameters at 14 and 28 DAT showed $|r| \geq 0.8$ with days to first VI (Table II-4).

Carbohydrate contents in the stem and leaf

Soluble sugars and starch contents in the stem showed no differences among temperature and duration variables (Table II-3). However, in leaves, the content of soluble sugars increased at low ambient temperatures. Significant differences by temperature were observed in glucose ($p < 0.05$), fructose ($p < 0.05$), and sucrose ($p < 0.001$), while there was a difference by duration in sucrose ($p < 0.01$) and in starch ($p < 0.05$). After two weeks of treatment, sucrose significantly increased at 20 and 23°C compared with 28°C. This response was similar in glucose, fructose, and sucrose after four weeks of treatment. Although slightly higher contents of soluble sugars were observed at 17°C than at 28°C, there was no significant difference. The total content of soluble sugars was highest at 20°C, followed by 23, 17, and 28°C. Glucose and fructose contents in the leaves only showed significant correlation with days to first VI at 28 DAT, while sucrose content had significance at both 14 and 28 DAT (Table II-4). Days to first VI decreased with increasing soluble sugar content in the leaves at all inducing temperatures.

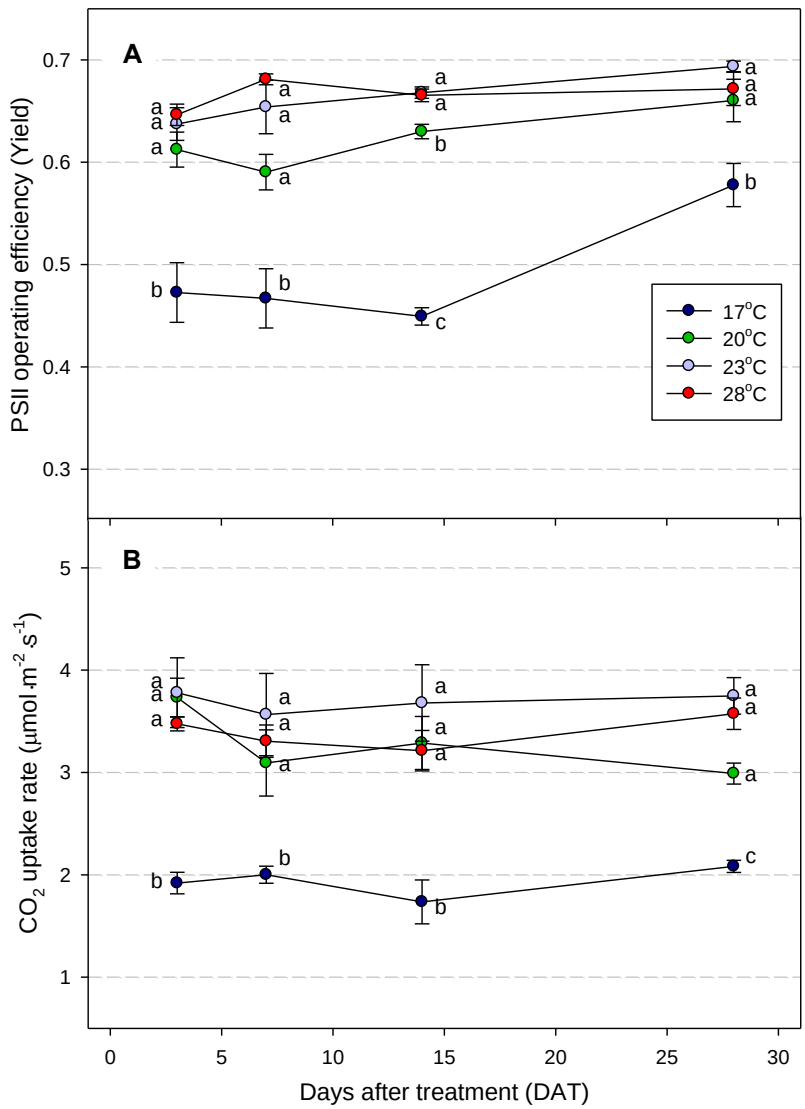

Figure II-1. Photosystem II operating efficiency (Yield) (A) and CO_2 uptake rate (B) of the uppermost mature leaves of 'Mantefon' plants after 3, 7, 14, and 28 days at 17, 20, 23, and 28°C. The data shown are the mean (n=3) ± standard error. Different letters are considered significantly different at $p < 0.05$ by Tukey's HSD test.

Table II-3. Contents of soluble sugars and starch in the stem and leaves of 'Mantefon' plants after 2 or 4 weeks of 17, 20, 23, and 28°C treatments.

Temperature (°C)	Stem (mg·g^{-1} FW)								Leaf (mg·g^{-1} FW)							
	Glucose		Fructose		Sucrose		Starch		Glucose		Fructose		Sucrose		Starch	
	2 w	4 w	2 w	4 w	2 w	4 w	2 w	4 w	2 w	4 w	2 w	4 w	2 w	4 w	2 w	4 w
17	0.47	0.31	0.22	0.12	3.45	3.95	18.34	19.64	1.55	1.55c[z]	1.20	1.15ab	2.25ab	1.51c	19.48	23.93
20	0.47	0.50	0.22	0.27	4.37	3.59	17.99	20.32	2.85	3.79a	1.64	1.80a	3.08a	2.80a	19.50	21.68
23	0.28	0.29	0.12	0.26	3.73	2.99	18.87	18.90	2.22	2.98ab	1.27	1.40ab	2.27a	2.24ab	21.80	19.88
28	0.37	0.33	0.17	0.16	3.60	3.60	20.65	21.06	2.47	1.79bc	1.13	0.56b	1.61b	1.58bc	20.36	24.09
Significance																
Temperature (T)	NS		NS		NS		NS		*		*		***		NS	
Duration (D)	NS		NS		NS		NS		NS		NS		**		*	
T * D	NS		NS		NS		NS		NS		NS		NS		NS	

[z]Means within columns followed by different letters are significantly different by Tukey's HSD test at $p < 0.05$.

NS, *, **, *** non-significant or significant at $p < 0.05$, 0.01, or 0.001, respectively.

Table II-4. Pearson's correlation coefficients (*r*) of photosynthetic ability and soluble sugar content in a leaf with days to visible inflorescence (VI) at inducing temperatures.

Parameters	Days to VI	
	r	*p*-value
Inducing temperature	-0.84	**
Yield at 14 DAT	-0.95	***
Yield at 28 DAT	-0.81	**
CO_2 uptake rate at 14 DAT	-0.85	**
CO_2 uptake rate at 28 DAT	-0.86	**
Glucose at 14 DAT	-0.52	NS
Glucose at 28 DAT	-0.83	**
Fructose at 14 DAT	-0.37	NS
Fructose at 28 DAT	-0.68	*
Sucrose at 14 DAT	-0.77	*
Sucrose at 28 DAT	-0.87	**

Data at 28°C were not included in the analysis because inflorescence initiation at 28°C was completely prevented.

NS, *, **, *** non-significant or significant at $p < 0.05$, 0.01, or 0.001, respectively.

Growth and inflorescence initiation by exogenous sucrose treatment

Exogenous sucrose treatment during vegetative period at 28˚C could not induce inflorescence initiation (Table II-5). The treatment increased the leaf width, but there was no significant difference in other growth parameters. The number of new leaves slightly increased by the exogenous sucrose treatment. However, at 20˚C, all plants showed inflorescence emergence regardless of sucrose concentrations and treatment timing (Figure II-2). The sucrose treatment accelerated the inflorescence emergence regardless of sucrose concentrations compared with 0 g/L of solution (Fig. II-1). The days to visible inflorescence (VI) were 29.3, 27.4, 26.2, and 26.7 in plants treated with 0, 10, 20, and 40 g/L of solutions, respectively.

Table II-5. Growth and inflorescence initiation of 'Mantefon' plants treated with exogenous sucrose application after 15 weeks of treatments.

Sucrose concentration (g/L)	Percent flowering (%)	No. of new leaves	Plant span (cm)	Leaf length (cm)	Leaf width (cm)
0	0	3.5	32.9	17.1	6.9b[z]
10	0	3.9	32.2	17.0	7.4a
20	0	3.8	33.3	17.9	7.3a
40	0	3.9	32.6	17.3	7.2ab
Significance	NS	NS	NS	NS	*

[z]Means within columns followed by different letters are significantly different by Tukey's HSD test at $p < 0.05$.

[NS], [*] non-significant or significant at $p < 0.05$, respectively.

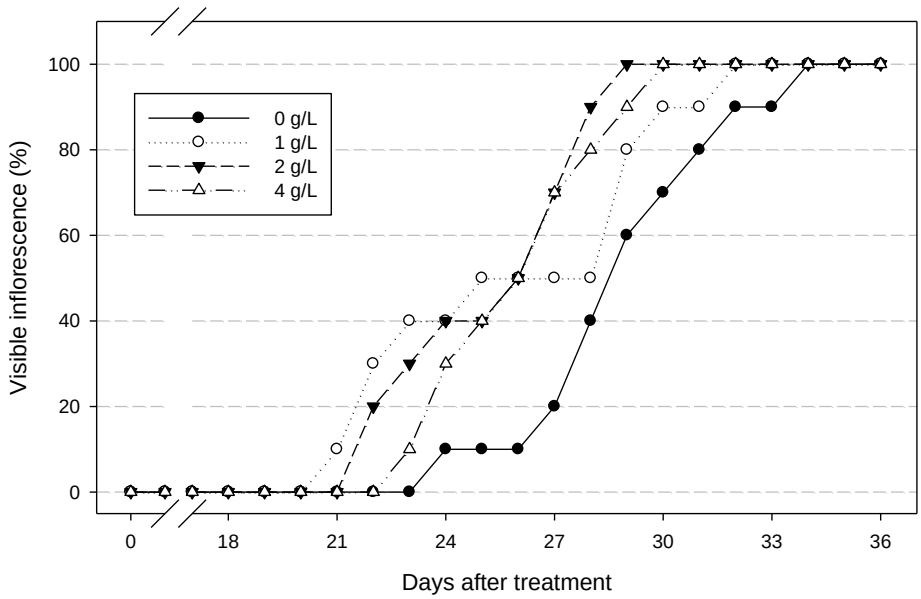

Figure II-2. Percent plants with visible inflorescence of 'Mantefon' plants treated with 0, 10, 20, and 40 g/L of sucrose solutions.

DISCUSSION

The number of new leaves significantly increased at 28°C compared with other inducing temperatures (Table II-1). Monopodial plants like *Phalaenopsis* have one growing stem, and bud primordia for inflorescences are differentiated in the stem at the base of each leaf (Rotor, 1952). During the reproductive period, inflorescence development primarily occurs rather than new leaf development. This phenomenon has been observed in several studies on *Phalaenopsis* (Lee et al., 2015; Newton and Runkle, 2009). After the reproductive period, new leaf development resumes to differentiate further inflorescences. Thus, the number of new leaves can be an indicator of vegetative growth of *Phalaenopsis* plants, and the increase in new leaf development in this study indicated that vegetative growth is maintained by flowering inhibition at 28°C.

Phalaenopsis shows a qualitative response in floral transition to ambient temperatures below 25°C (Blanchard and Runkle, 2006; Sakanishi et al., 1980). However, this low-temperature requirement may be different from the general vernalization response. Natural habitats of *Phalaenopsis* plants are usually tropical or subtropical (Christenson, 2001). The effective temperature ranges for inducing floral transition of *Phalaenopsis* plants are higher than that of other species requiring vernalization, which is a cumulative process (Chouard, 1960; Hackett and Hartmann, 1967; Malik and Perez, 2011; Michaels and Amasino, 2000). Therefore, lower

inducing temperatures might accelerate inflorescence emergence if *Phalaenopsis* showed a quantitative response to low-temperature exposure like other vernalization-requiring plants. But, in this study, a significant delay in inflorescence emergence was observed at 17°C compared with 20 and 23°C (Table II-2). Paradiso et al. (2012) reported that temperature variations during inflorescence initiation influenced inflorescence emergence timing, and lower temperatures led to longer emergence time.

Because the bud primordia are connected to the stem, the carbohydrate status of the stem may contribute to inflorescence development (Liu et al., 2013). However, there was no difference in stem carbohydrate content among treatments in this study (Table II-3). Carbohydrates, especially sucrose, are used as an energy source for floral organ development during reproductive growth. Generally, plant growth depends on the translocation of carbohydrates from 'source' to 'sink' (Bernier et al., 1993; Ruan, 2014). In *Phalaenopsis*, the leaf is the 'source' organ supporting inflorescence development during the flowering process. Inflorescence originates in the stem, where energy is stored, similar to the pseudo-bulbs of other orchids. Nevertheless, carbohydrate status of the leaf also seems to play a major role in *Phalaenopsis* flowering. In a study by Liu et al. (2016), shade treatment decreased the sucrose content in the leaf but not in the stem, resulting in a low inflorescence emergence ratio. On the other hand, sucrose accumulation in the leaf during floral transition has been reported by other authors (Kataoka et al., 2004; Konow and Wang, 2001).

PSII operating efficiency and CO_2 uptake rate represent photosynthetic ability during day and night, respectively, and the two parameters showed similar trends (Fig. 1A and B). *Phalaenopsis* has a typical CAM photosynthetic pathway (Endo and Ikusima, 1989). These CAM plants fix CO_2 absorbed during the night into malic acid, which is stored in the vacuole (Osmond, 1978), and released CO_2 is re-fixed via the Calvin cycle during the day (Dodd et al., 2002). During the diel CAM cycle, the metabolic flux between day and night involves strict interactions (Cheung et al., 2014; Maxwell et al., 1999). Decarboxylation during the day is a light-dependent reaction (Dodd et al., 2002). Incomplete decarboxylation can inhibit stomatal opening during the night (Lüttge, 2008), while sufficient light for decarboxylation does not affect CO_2 absorption in *Phalaenopsis* (Guo et al., 2012).

Temperatures at 17, 20, and 23°C induced sugar accumulation in the leaf (Table II-3), while sugar contents were lower at 28°C even though relatively higher PSII operating efficiency and CO_2 uptake rate were observed throughout the treatment period (Fig. II-1A and B). These results can be attributed to development of the main organ during the vegetative or reproductive period. At 28°C, plants made more leaves, while inflorescence mainly occurred at the inducing temperatures. This indicates that newly developing leaves are the 'sink' organs at 28°C, whereas inflorescences are the 'sink' organs at inducing temperatures. Also, accumulation of sucrose or starch in the leaves has been associated with decreased activity of 'sinks' (Herold, 1980). During the reproductive period, growth of vegetative organs – newly emerging leaves in the case of *Phalaenopsis* – stops. Moreover, it takes

approximately 3 to 10 weeks of low temperature period to achieve inflorescence emergence (Blanchard and Runkle 2006; Lee et al., 2015).

Similar to the delay in inflorescence emergence, sucrose content in the leaves was significantly decreased at 17°C compared with the other inducing temperatures (Table II-3). Also, sucrose content in the leaves showed a positive correlation with inflorescence emergence timing regardless of treatment duration (Table II-4). The correlation between sucrose content and inflorescence emergence timing in *Phalaenopsis* has been reported by other authors (Kataoka et al., 2004; Konow and Wang, 2001; Qin et al., 2012). However, Liu et al. (2013) suggested that the relationship between sucrose content and inflorescence emergence may be more complex than believed. Considering the importance of sucrose as a photosynthetic product, the significant decrease in sucrose content at 17°C could be explained by lower photosynthetic ability. Significantly lower PSII operating efficiency and CO_2 uptake rate were observed at 17°C compared to 20 and 23°C (Fig. 1A and B). In a study by Konow and Wang (2001), low irradiance levels decreased malic acid and sucrose contents in the leaves. In other previous studies, inflorescence emergence was accelerated and sucrose contents in the leaves increased with increased light, temperature, and atmospheric CO_2 concentration (Kataoka et al., 2004), and relatively higher CO_2 uptake rates were also observed under those conditions (Guo and Lee, 2006; Lootens and Heursel, 1998; Yun et al., 2018). Therefore, although further studies may be required to understand how sucrose acts in floral transition and inflorescence development, the delay of inflorescence emergence at 17°C could

be attributed to low sucrose content in the leaves due to low photosynthetic ability. Similar to our findings, low irradiance levels negatively affected photosynthetic performance and decreased carbohydrate content in the leaves, delaying subsequent inflorescence development (Liu et al., 2016).

In this study, exogenous sucrose treatment did not substitute for an inducing low temperature (Table II-5), indicating that the carbohydrate accumulation in the leaf is a subsequent phenomenon of inflorescence initiation by low temperatures. Several studies have reported that exogenous sucrose treatment influenced flowering process (Friend et al., 1984; Roldán et al 1999; Sun et al., 2017). In *Brassica campestris*, the sucrose application promoted flowering initiation and it was not due to osmotic stress (Friend et al., 1984). The spraying of 50 mM sucrose accelerated flowering of chrysanthemum 'Floral Yuuka' under night interruption condition and increased *CmFTL2*, *FT-like* gene, expression levels (Sun et al., 2017). However, the promotive effects decreased in plants treated with 40 $g \cdot L^{-1}$ of sucrose solution compared with plants treated with 20 $g \cdot L^{-1}$ of sucrose solution. It would be attributed to too high concentration of solutions over the optimum concentration for the effects. For example, foliar ion uptake rate such as potassium and chelated iron decreased as concentrations increased (Chamel, 1988; Schlegel et al., 2006), and high viscosity decreased the absorption of zinc (Rathore et al., 1970).

During floral transition, sugars are needed as energy sources to support reproductive development. Sugar metabolism also plays a role as a signal in flowering timing (Bolouri Moghaddam and Van den Ende, 2013). This response is

complicated, interacting with expression of genes, transcription factors, microRNAs, and hormones (Ruan, 2014; Yu et al., 2015). In recent studies, trehalose 6-phosphate rather than sucrose was shown to affect flowering (Ponnu et al., 2011; Wahl et al., 2013). Further studies are necessary to determine how sucrose accumulation in the leaves acts as a signal in inflorescence emergence timing.

The 'C/N ratio,' the ratio of carbohydrates and nitrogen, is another factor that regulates flowering, and a high ratio promotes flowering (Corbesier et al., 2002; Upreti et al., 2013). In *Phalaenopsis*, the inflorescence emergence was hastened by reduced nitrogen fertilization level, implying increased C/N ratio (Lin et al., 2019). Sugar accumulation, especially sucrose in this study, was correlated with transition from the vegetative to the reproductive period and inflorescence emergence timing in *Phalaenopsis*. Variation in temperatures for the inflorescence initiation induced changes in photosynthetic ability and subsequent changes in carbohydrate content in the leaves. Although further investigation is needed to understand the function of sugar metabolism in inflorescence initiation, sugar content in the leaf during low temperature exposure seems to have an important role in the flowering process of *Phalaenopsis* plants. In conclusion, *Phalaenopsis* inflorescence initiation induces sugar accumulation in the leaves, and inflorescence emergence timing is correlated with sugar content from photosynthetic performance.

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CHAPTER III

Flowering Inhibition by Carbohydrate Reduction in the Leaves by Intermittent High Temperature Treatment in *Phalaenopsis*

ABSTRACT

To observe the metabolic changes by intermittent high temperature treatment, transcriptomes from plants under vegetative, inflorescence initiation, and intermittent high temperature conditions were analyzed in this study. Continuous high temperature at 28°C (HT+HT), continuous low temperature at 20°C (LT+LT), and two weeks of intermittent high temperature every two weeks of low temperature (LT+HT) were treated to *Phalaenopsis* Queen Beer 'Mantefon' plants. A delay of inflorescence emergence and decreased percent plants with visible inflorescence were observed in plants treated with intermittent high temperatures. Also, these plants showed relatively higher number of new leaves compared with flowering-induced plants at continuous low temperature. There were 154, 156, and 1,308 differentially expressed genes (DEGs) identified in RNA-seq analysis. 28 gene ontology (GO) terms were annotated in GO enrichment between LT+LT and LT+HT treatments and carbohydrate phosphatase activity and fructose 1,6-bisphosphate 1-phosphatase activity showed down-regulation by intermittent high temperature treatment. Especially, the expression of DEGs for enzymes related to carbohydrate hydrolysis and gluconeogenesis was relatively lower in HT+HT or LT+HT treatments compared with LT+LT

treatment. In the qRT-PCR analysis, orthologs of fructose-bisphosphatase, glycoside hydrolase, G-3-P dehydronase, and sucrose transporter showed low expression levels in HT+HT and LT+HT treatments. The expression of homologs of *PhalCOL* and *DhGI1* was also down-regulated by HT+HT or LT+HT treatments. However, *DhEFL4* homolog showed increased expression levels in these treatments. The expression levels analyzed in this experiment were similar between HT+HT and LT+HT treatments. In an analysis of carbohydrate contents in the leaves, the contents of soluble sugars significantly decreased in plants with intermittent high temperature compared with plants at continuous low temperature. These results indicated that the carbohydrate metabolism is involved in the inflorescence initiation process of *Phalaenopsis*, and the flowering inhibition by intermittent high temperature treatment would be attributed to the reduction of carbohydrate contents in the leaves by the down-regulation of carbohydrate metabolism.

INTRODUCTION

Temperature control is generally used to induce flowering of many horticultural crops. Especially, chilling treatment has a forcing capacity on physiological development like flowering and breaking dormancy, and this effect is called as 'vernalization' (Chouard, 1960). Vernalization-requiring plants normally originate from temperate regions where a typical winter exists and these plants show long-day requirements for their floral transition (Kim et al., 2009; Sung and Amasino, 2004). *Phalaenopsis*, a monopodial epiphytic orchid, also require low temperature for inflorescence initiation (Chen and Chen, 2011). This plant has dormant-state meristems in the stem at the base of leaves (Rotor, 1952), and a temperature drop from over 28°C to below 25°C induces the inflorescence emergence (Sakanishi et al., 1980). However, in spite of low temperature requirements for the inflorescence initiation, *Phalaenopsis* species are distributed throughout tropical and subtropical regions where the temperature is steadily warm and there is little photoperiodic change throughout the year (Christenson, 2001). These habitat characteristics are dissimilar to other vernalization-requiring plants.

No homologs of *FLOWERING LOCUS C* (*FLC*) have been reported in orchids including *Phalaenopsis*. Recently, functional studies suggested key genes regulating floral induction of *Phalaenopsis* like *Phalaenopsis aphrodite FLOWERING LOCUS T1* (*PaFT1*) and *Phalaenopsis CONSTANS* (*CO*)-like (*PhalCOL*) (Jang et al., 2015;

Zhang et al., 2011). Also, *GIGANTEA* (*DhGI1*) and *Doritaenopsis EARLY FLOWERING 4* (*DhEFL*) were regulated by low temperature exposure (Chen et al., 2015; Luo et al., 2011). Homologs of cereal *VERNALIZATION1* (*DnVRN1*) and *Arabidopsis AGMOUS-like19* (*DnAGL19*) were recognized in *Dendrobium nobile* (Liang et al., 2012). However, genetic interactions among flowering-regulating genes or comparative responses are not well understood Therefore, the flowering mechanism of *Phalaenopsis* still remains elusive.

Genetic studies reported that hormones might play roles in the regulation of the inflorescence initiation or development (Huang et al., 2016; Qin et al., 2012). Especially, gibberellic acid (GA) contents in the leaves increased simultaneously during low temperature exposure and GA injection could induce the inflorescence initiation (Chen et al., 1994; Su et al., 2001). However, in a study by Wang (1995), the GA application could not induce the inflorescence initiation. Also, up-regulations in the transcription of genes related to GA biosynthesis were not observed under inducing conditions (Qin et al., 2012). Although it was also suggested that cytokinin or other hormones were involved in the floral induction (Blanchard and Runkle, 2008; Chou et al., 2000; Qin et al., 2012), further studies seem to need to support the correlation between the hormones and inflorescence initiation. Thus, it is still controversial whether hormones are key regulators for the inflorescence initiation of *Phalaenopsis*.

On the other hand, carbohydrate contents are related to inflorescence initiation of *Phalaenopsis* by low temperature exposure. Soluble sugars were accumulated and

sucrose synthase levels also increased during the inflorescence initiation (Chen et al., 1994). The time to inflorescence emergence was shortened as sucrose contents in the leaves increased (Kataoka et al., 2004). Although exogenous sucrose application could not induce flowering under vegetative conditions, the treatment at inducing temperature increased the sucrose content in the leaves and accelerated the inflorescence emergence (Lee et al., 2020). Starch degradation and sucrose biosynthesis pathways were activated during the inflorescence initiation (Qin et al., 2012). Also, in our previous experiment, the inflorescence initiation was highly correlated with soluble sugar contents in the leaves (Chapter II).

Preceding experiments showed that *Phalaenopsis* plants required a sufficient period of low temperature exposure for their competent inflorescence initiation, but this low temperature accumulation was not quantitative with inducing temperatures. Discontinuous low temperature exposure by intermittent high temperature treatment had an inhibitory effect on the inflorescence initiation although a sufficient low temperature period was satisfied. In the case of *Arabidopsis*, the spliced *FLC* mRNA levels showed similar responses between under continuous and discontinuous cold conditions (Angel et al., 2015). Nevertheless, the interrupted low temperature accumulation would prevent floral transition (Malik and Perez, 2011; Purvis and Gregory, 1952). We assumed that these inhibitory effects by discontinuous low temperature exposure interrupted by intermittent high temperature treatment would be attributed to the reduction of carbohydrate metabolism by the intermittent high temperature condition. This study was conducted to determine the relationship

between flowering inhibition by intermittent high temperature treatment and carbohydrate metabolism in *Phalaenopsis*.

MATERIALS AND METHODS

Plant materials and growth conditions

Twelve-month-old *Phalaenopsis* Queen Beer 'Mantefon' clones in 10-cm transparent plastic pots filled with 100% sphagnum moss were purchased from a commercial grower (Sang Mi Orchids, Taean, Korea). Plants were acclimatized in growth chambers (HB-301MP; Hanbaek Scientific Co., Bucheon, Korea) with 250-W metal halide lamps (Han Young Electrics Co., Gwangju, Korea) for four weeks. Other acclimatization conditions were the same as those in Chapter I and II.

Intermittent high temperature treatment

After acclimatization period, plants were grown under three different temperature conditions; continuous low and high temperature at 20 (LT+LT) and 28°C (HT+HT), respectively, and two weeks of intermittent high temperature every two weeks of low temperature (LT+HT). Other growth conditions were the same as acclimatization period. At the start of temperature treatment, the mean plant span was 26.5 cm. Each treatment was maintained for 15 weeks. The actual average temperatures in each treatment group were 20.1°C of continuous low temperature, 27.7°C of continuous high temperature, and 20.4 and 27.7°C of low and high temperatures during intermittent high temperature treatment, respectively. There were three replications per treatment and each replication contained five plants. The

percent plants with visible inflorescence and the number of new leaves were counted during experiment period.

RNA-seq analysis

The middle part of the uppermost mature leaves was sampled from randomly-chosen three plants in every replication after four weeks of each treatment. Then, the samples were pooled in one replication and three replications were used in the analysis. RNA extraction, library construction, and sequencing using NEXTflex™ rapid directional mRNA-seq (Bioo) protocol were performed at NICEM (Seoul, Korea) by HiSeq2500 (Illumina, San Diego, CA, USA). *Phalaenopsis equestris* genome was used as reference genome (Cai et al., 2015). Quality scores of the sequencing were described in Table III-1.

Expression values in reads per kilobase transcript per million (RPKM) were calculated. Orthologs of differentially expressed genes (DEGs) were identified from homology database OrthoDB v10 (https://www.orthodb.org; Kriventseva et al., 2019). DEGs with $\log_2$ fold change > |1| and the false discovery rate (FDR) *p*-value < 0.05 were used in gene ontology (GO) enrichment analysis. GO terms were obtained by using InterProScan version 5.34-73.0 program (Finn et al., 2017), and DEGs between LT+LT and LT+HT treatments were analyzed by using the Singular Enrichment Analysis (SEA) Tool in agriGO v2.0 (http://bioinfo.cau.edu.cn/agriGO/) with the *Arabidopsis* gene model (TAIR9) as a reference (Tian et al., 2017). Also, the list of annotated GO terms was summarized by REVIGO (https://revigo.irb.hr/)

Table III-1. Summary of RNA-seq analysis data of the 'Mantefon' leaves treated with 4 weeks of continuous low, continuous high, and intermittent high temperatures.

Sample	Total reads	Total bases (Gb)	Q20 (%)	Q30 (%)	GC content (%)
HT+HT-1	37,392,414	5.61	96.90	91,78	49.60
HT+HT-2	36,317,708	5.45	96.30	90.50	49.83
HT+HT-3	40,949,508	6.14	96.79	91.64	49.11
LT+LT-1	48,618,684	7.29	97.44	92.92	50.21
LT+LT-2	39,160,852	5.87	96.99	92.08	51.00
LT+LT-3	36,076,026	5.41	96.64	91.28	48.87
LT+HT-1	35,132,830	5.27	96.78	91.63	49.21
LT+HT-2	56,391,076	8.46	96.49	91.00	48.30
LT+HT-3	34,399,556	5.16	96.90	91.87	48.56

Q20 and Q30 are the percentages of bases with Phred quality scores >20 and 30, respectively.

GC content is the percentage of G + C bases.

LT+LT, continuous low temperature at 20°C; HT+HT, continuous high temperature at 28°C; LT+HT, two weeks of high temperature after two weeks of low temperature.

to remove redundant GO terms (Supek et al., 2011).

qRT-PCR array

To validate the transcriptome data, qRT-PCR array was conducted with the same samples used in RNA-seq analysis. Three genes from DEGs for Fructose-bisphosphatase (PE00374, Bioneer), Glycoside hydrolase (PE00375, Bioneer), and G-3-P dehydronase (PE00376, Bioneer) were analyzed, and homologs of *PhalCOL* (PE00369, Bioneer) (Zhang et al., 2011), *DhGI1* (PE00370, Bioneer) (Luo et al., 2011), and *DhEFL4* (PE371, Bioneer) (Chen et al., 2016) also were analyzed to determine the expression of genes related with regulating the floral induction. The information of genes used in this study were described in Table III-2. 5 ng RNA samples were amplified in a final volumn of 50 μL by employing *AccuPower*® GreenStar™ RT-qPCR MasterMix (Bioneer, Daejeon, Korea) with *Exicycler*™ 96 Real-Time Quantitative Thermal Block (Bioneer, Daejeon, Korea). PCR condition was 15 min at 55°C, 5 min at 95°C of pre-denaturation, and 40 cycles of 5 s at 95°C and 5 s at 60°C, and then a final step was performed at 60~94°C with a thermal transition of 1°C/s for melting curve analysis. 2^(-ΔΔCT) values were used to determine the relative fold change data. All data were normalized to the housekeeping *Actin* expression level (Luo et al., 2011). All reactions were conducted in three replications.

Table III-2. Information of selected genes used in qRT-PCR array.

Gene symbol	Putative function	Accession No.	Reference
LOC110025886	Fructose-bisphosphatase	XM_020726571.1, XM_020726569.1, XM_020726570.1, XM_020726568.1	GO enrichment
LOC110030889	Glycoside hydrolase	XM_020733861.1, XM_020733860.1	GO enrichment
LOC110027345	G-3-P dehydrogenase	XM_020728727.1, XM_020728726.1, XM_020728724.1, XM_020728723.1	GO enrichment
LOC110037752	*CONSTANT-like*	XM_020742459.1	Zhang et al., 2011
LOC110019219	*GIGANTEA*	XM_020716802.1, XM_020716800.1	Luo et al., 2011
LOC110021895	*EARLY FLOWERING 4-like4*	XM_020720602.1, XM_020720601.1	Chen et al., 2016
LOC110026462	*ACTIN*	XM_020727406.1	Luo et al., 2011

Analysis of carbohydrate contents in the leaves

Same samples used in RNA-seq analysis and qPCR array were used in this analysis. Fresh biomass of each sample was weighed and immersed in liquid N_2 and homogenized samples were stored at -80˚C. Contents of soluble sugars of glucose, fructose, and sucrose were analyzed by the method of González-Rossia et al. (2008) with slight modifications. Briefly, 200 mg of stored sample was incubated in 80% ethanol at 85˚C for 15 minutes. The supernatant was collected after centrifuging at 3,600 g for 30 minutes. The sugar extracts were evaporated by a N_2 evaporator (N-EVAPTM; Organomation Associates, Inc., Berlin, MA, USA) at 60˚C. Ethanol-insoluble material was dried and stored for starch analysis. The evaporated supernatant was dissolved in 3 mL of distilled water and filtered through a 0.45-μm nylon filter (Acrodisc® 13-mm syringe filter; Pall Co., Port Washington, NY, USA) with a C18 Sep-Pak cartridge (Waters Corp., Milford, MA, USA). The contents were analyzed using ion chromatography with a pulsed amperometric detector (ICS-5000; Thermo Dionex, Sunnyvale, CA, USA) on a Dionex CarboPac PA1 column using 50 mM NaOH (1 $mL \cdot min^{-1}$).

The method of Smith and Zeeman (2006) was used for the analysis of starch content. The remaining sediment for analysis of soluble sugar content was dissolved in 1 mL of distilled water. The pellets were boiled to gelatinize starch granules, and 0.5 mL of 0.2 M Na-acetate (pH 5.5) buffer, 15 units of amyloglucosidase (A7095; Sigma-Aldrich Korea Ltd., Yongin, Korea), and 5 units of α-amylase (A4862; Sigma-Aldrich Korea Ltd.) were added to each sample. Samples were incubated at

55˚C for two hours and centrifuged at 3,600 g for 30 minutes. The supernatant was collected two times and dissolved in 3 mL of distilled water. The extract was filtered through a 0.45-μm nylon filter with a C18 Sep-Pak cartridge. The released glucose was analyzed using an UltiMate 3000 HPLC instrument (Dionex) equipped with a Shodex RI-101 detector (Showa Denko K.K., Kawasaki, Japan) on a Waters Sugar-Pak column using distilled water (0.5 $mL \cdot min^{-1}$).

Experimental design and statistical analysis

Completely randomized design was used in all experiments. Statistical analysis was performed using ANOVA in SAS (Windows version 9.3; SAS Institute Inc., Cary, NC, USA). Comparisons among treatment groups were performed by Tukey's HSD test for evaluating the growth and flowering characteristics and carbohydrate contents in the leaves with $p < 0.05$ as the threshold for statistical significance. Graph module analyses were performed using SigmaPlot software version 10.0 (Systat Software, Inc., Chicago, IL, USA).

RESULTS

Growth characteristics and inflorescence initiation

28°C of continuous high temperature completely prevented inflorescence initiation while 20°C of continuous low temperature induced 100% inflorescence emergence (Fig. III-1). Intermittent high temperature treatment delayed inflorescence emergence. Under the vegetative conditions at 28°C, plants showed significantly increased new leaf emergence (Fig. III-2). Although there was no significant difference between continuous low temperature and intermittent high temperature groups, the increased number of new leaves was observed in plants treated with intermittent high temperatures.

Transcriptome analysis

There were 154, 156, and 1,308 DEGs with $\log_2$ fold change > |1| and FDR *p*-value < 0.05 between HT+HT vs. LT+LT, HT+HT vs. LT+HT, and LT+LT vs. LT+HT were obtained (data not shown). The DEGs between continuous low temperature and intermittent high temperature were used in GO enrichment and 28 GO terms were annotated with FDR $p < 0.05$ (Table III-3). Especially, carbohydrate metabolism like carbohydrate phosphatase activity and fructose 1,6-bisphosphate 1-phosphatase activity showed down-regulation under intermittent high temperature condition. Relatively low expression of DEGs of enzymes related to hydrolysis

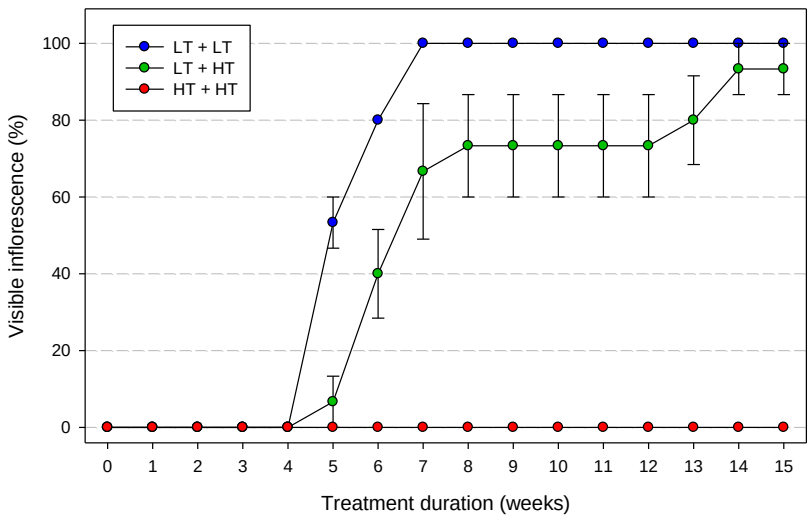

Figure III-1. Percent plants with visible inflorescence of 'Mantefon' plants treated with continuous low (LT+LT), continuous high (HT+HT), and intermittent high (LT+HT) temperatures. LT+LT, continuous low temperature at 20°C; HT+HT, continuous high temperature at 28°C; LT+HT, two weeks of intermittent high temperature every two weeks of low temperature.

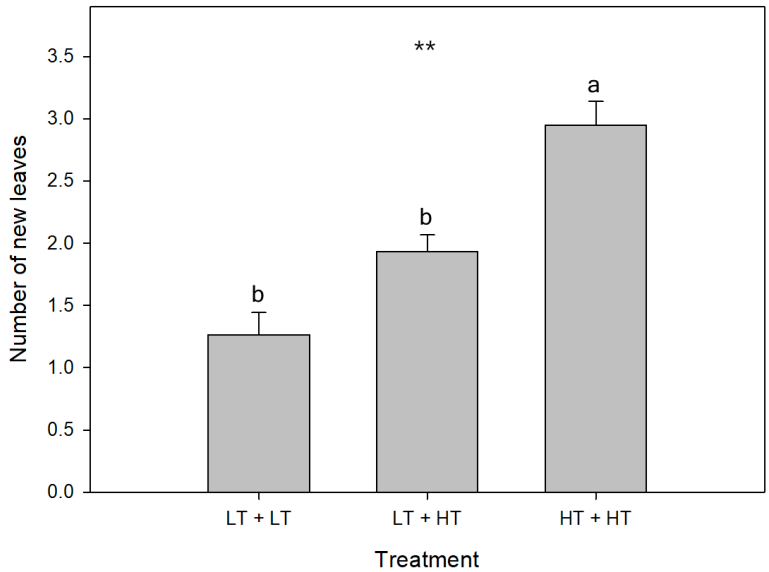

Figure III-2. The number of new leaves of 'Mantefon' plants treated with continuous low (LT+LT), continuous high (HT+HT), and intermittent high (LT+HT) temperatures. LT+LT, continuous low temperature at 20°C; HT+HT, continuous high temperature at 28°C; LT+HT, two weeks of intermittent high temperature every two weeks of low temperature. The data shown are the mean (n=3) ± standard error. Different letters are considered significantly different at $p < 0.05$ by Tukey's HSD test. ** significant at $p < 0.01$ in ANOVA.

Table III-3. GO terms annotated in GO enrichment between continuous low and intermittent high temperatures.

GO terms	Ontology	Description	Frequency	Log_{10}(*p*-value)
GO:0000184	P	nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	0.03%	-2.6021
GO:0006310	P	DNA recombination	1.64%	-2.1308
GO:0006259	P	DNA metabolic process	5.61%	-1.6198
GO:0006270	P	DNA replication initiation	0.14%	-1.6198
GO:0006260	P	DNA replication	1.58%	-2.1308
GO:0019203	F	carbohydrate phosphatase activity	0.07%	-3.7959
GO:0008168	F	methyltransferase activity	2.78%	-1.4318
GO:0042623	F	ATPase activity, coupled	2.50%	-1.9586
GO:0016741	F	transferase activity, transferring one-carbon groups	3.04%	-1.4318
GO:0042132	F	fructose 1,6-bisphosphate 1-phosphatase activity	0.04%	-3.7959
GO:0004386	F	helicase activity	1.22%	-1.4318
GO:0008026	F	ATP-dependent helicase activity	0.49%	-1.6198
GO:0005623	C	cell	53.55%	-1.4949
GO:0016021	C	integral component of membrane	55.87%	-1.3872
GO:0031974	C	membrane-enclosed lumen	2.74%	-1.4949
GO:0032991	C	macromolecular complex	14.01%	-1.4949
GO:0042555	C	MCM complex	0.06%	-3.1805
GO:0044427	C	chromosomal part	1.12%	-2.1612
GO:0048046	C	apoplast	0.07%	-1.4949
GO:0030312	C	external encapsulating structure	0.92%	-1.4949
GO:0044464	C	cell part	52.39%	-1.4949
GO:0032993	C	protein-DNA complex	0.42%	-1.4949
GO:0044425	C	membrane part	57.39%	-1.4949
GO:0005618	C	cell wall	0.29%	-1.4949
GO:0031224	C	intrinsic component of membrane	55.98%	-1.3468
GO:0043234	C	protein complex	6.42%	-1.4949
GO:0031981	C	nuclear lumen	2.29%	-1.4949
GO:0005694	C	chromosome	1.51%	-1.5376

P, Biological process; F, Molecular function; C, Cellular component.

of carbohydrates, e.g. glycoside hydrolase, and gluconeogenesis, e.g. fructose 1,6-bisphosphatase, was observed in continuous high and intermittent high temperature treatments (Fig. III-3).

qRT-PCR array

To validate the results of RNA-seq analysis, four transcripts, orthologs of fructose-bisphosphatase, glycoside hydrolase, and G-3-P dehydronase were selected based on GO enrichment (Fig. III-4). These genes were down-regulated by intermittent high temperature treatment (LT+HT) compared with continuous low temperature treatment (LT+LT). Also, the expression levels of these genes were similar with levels in continuous high temperature treatment (HT+HT).

The expression of homologs of *PhalCOL*, *DhGI1*, and *DhEFL4* were also analyzed (Fig. III-5). The expression levels of these genes were similar between HT+HT and LT+HT. The levels of *PhalCOL* and *DhGI1* homologs increased under inducing condition (LT+LT) compared with vegetative (HT+HT) or intermittent condition (LT+HT), while the level of *DhEFL4* homolog decreased by inducing treatment.

Carbohydrate contents in the leaves

Soluble sugar accumulation was observed in flowering-induced plants under continuous low temperature or intermittent high temperature conditions compared with plants under continuous temperature conditions (Table III-4). However,

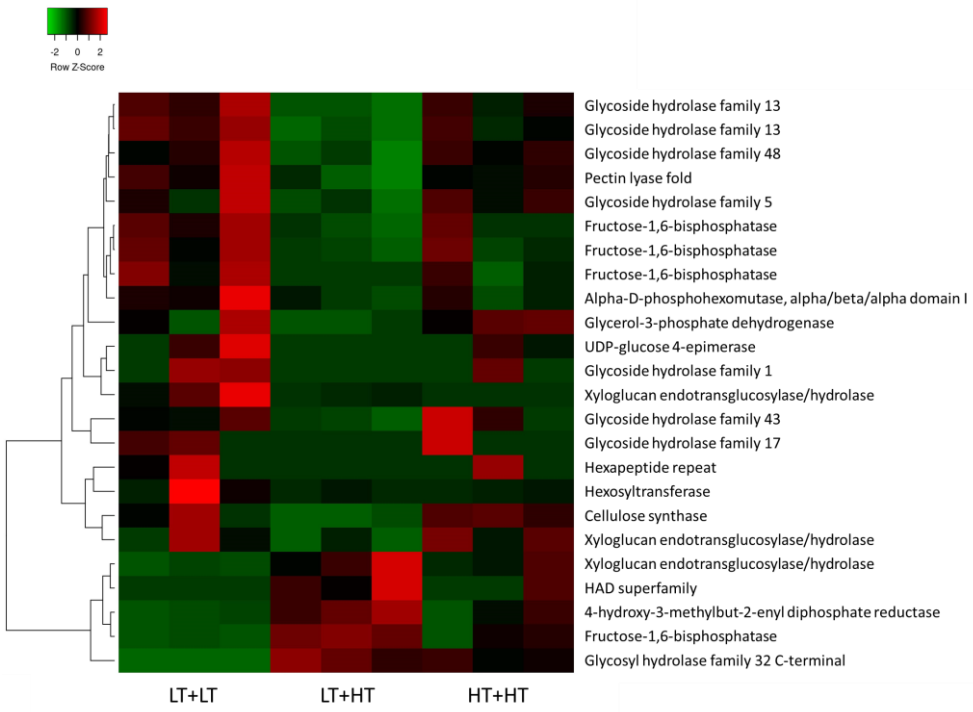

Figure III-3. Differentially expressed genes of 'Mantefon' plants related with carbohydrate metabolism under continuous low (LT+LT), continuous high (HT+HT), and intermittent high (LT+HT) conditions. LT+LT, continuous low temperature at 20°C; HT+HT, continuous high temperature at 28°C; LT+HT, two weeks of high temperature after two weeks of low temperature.

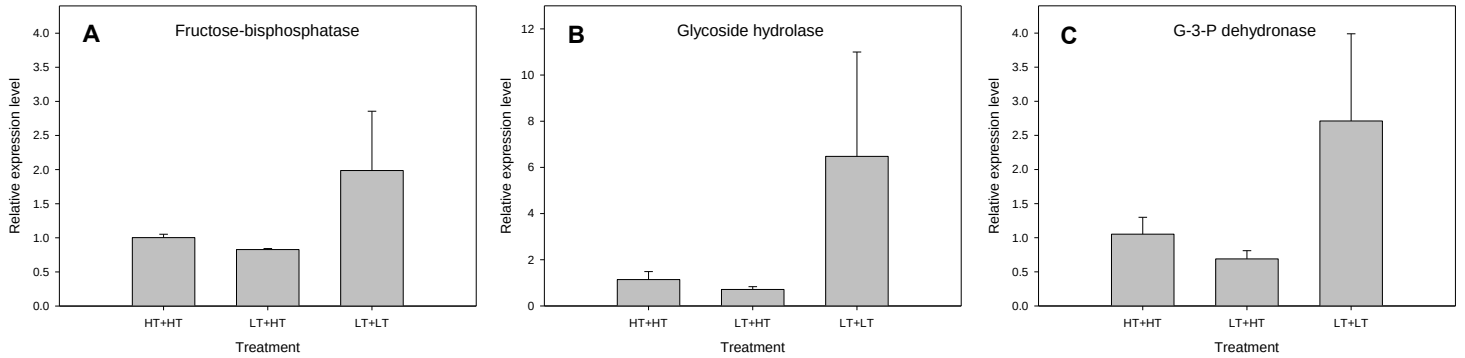

Figure III-4. Gene expression for fructose-bisphosphatase (A), glycoside hydrolase (B), and G-3-P dehydrogenase (C) in the leaves of 'Mantefon' treated with continuous low (LT+LT), continuous high (HT+HT), and intermittent high (LT+HT) temperatures. LT+LT, continuous low temperature at 20°C; HT+HT, continuous high temperature at 28°C; LT+HT, two weeks of high temperature after two weeks of low temperature.

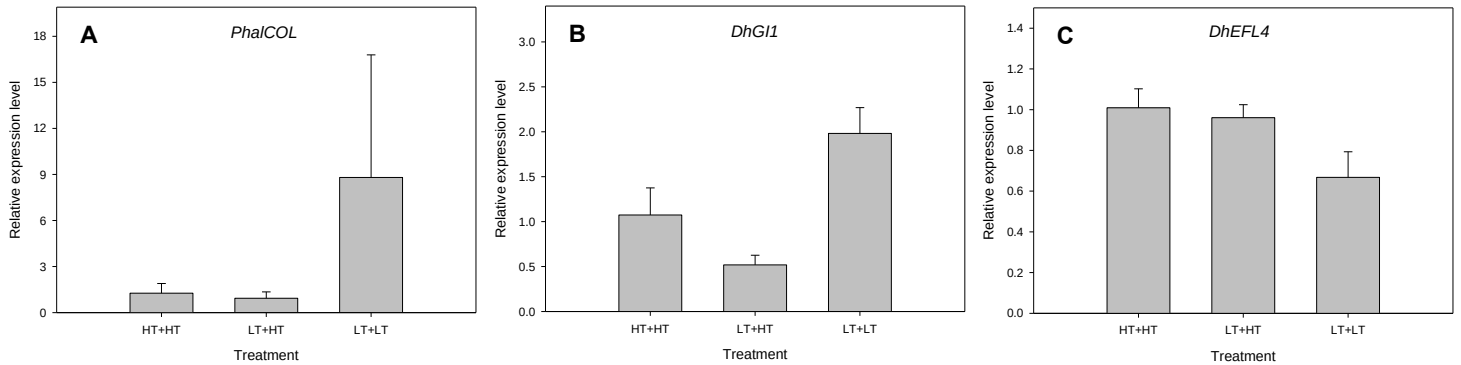

Figure III-5. Gene expression of *PhalCOL* (A), *DhGI1* (B), and *DhEFL4* (C) homologs in the leaves of 'Mantefon' treated with continuous low (LT+LT), continuous high (HT+HT), and intermittent high (LT+HT) temperatures. LT+LT, continuous low temperature at 20°C; HT+HT, continuous high temperature at 28°C; LT+HT, two weeks of high temperature after two weeks of low temperature.

Table III-4. Carbohydrate contents of the 'Mantefon' leaves treated with four weeks of continuous low and high temperature and intermittent high temperature.

Treatment	Glucose	Fructose	Sucrose	Total soluble sugars	Starch
HT+HT	1.78	0.87b[z]	1.50c	4.15c	60.27
LT+HT	2.49	1.53a	2.18b	6.20b	59.65
LT+LT	2.85	1.62a	3.27a	7.74a	56.50
Significance	NS	**	***	**	NS

[z]Means within columns followed by different letters are significantly different by Tukey's HSD test at $p < 0.05$.

[NS], [**], [***] non-significant or significant at $p < 0.01$ or 0.001, respectively.

Unit: mg/g FW

LT+LT, continuous low temperature at 20°C; HT+HT, continuous high temperature at 28°C; LT+HT, two weeks of high temperature after two weeks of low temperature.

although plants treated with intermittent high temperature showed inflorescence emergence, sucrose or total soluble sugar contents significantly decreased compared with plants treated with continuous low temperature. Glucose and fructose contents showed similar responses, but there was no difference between plants under continuous low and intermittent high temperature conditions. The fructose contents under continuous high temperature condition were significantly lower than flowering-induced plants under other conditions. The opposite trend compared with sugar content was observed in starch contents although there was no significant difference.

DISCUSSION

Discontinuous low temperature by intermittent high temperature treatment delayed inflorescence emergence compared with continuous low temperature exposure (Fig. III-1). However, the inhibitory effects were relatively lower than the results of Chapter I (Fig. I-2). The low impacts would be attributed to the length of intervals of low temperature period. In Chapter I, one week of intermittent high temperature every one week of low temperature, i.e. the same period of low and high temperature, reduced the percent plants with visible inflorescence (VI) from 100% to 55% after 15 weeks of treatment. In this chapter, plants treated with the intermittent high temperatures showed approximately 90% of plants with VI. Although the intermittent treatment had the same periods of low and high temperature, the length of low and high temperature periods was two weeks in this experiment. These results indicated that the inhibitory effects would be bigger by shorter low temperature period with more frequent intermittent high temperature treatment. Nevertheless, the intermittent high temperature treatment in this experiment delayed inflorescence emergence and increased the number of new leaves (Fig. III-2), implying that the inhibitory effects by discontinuous low temperature exposure still existed.

GO enrichment between continuous low temperature and intermittent high temperature showed changes in carbohydrate metabolism like carbohydrate

phosphatase activity and fructose 1,6-bisphosphate 1-phosphatase activity (Table III-3). DEGs for enzymes related to the hydrolysis of carbohydrates (glycoside hydrolases) and gluconeogenesis (fructose-1,6-bisphosphatase) had low RPKM values in the intermittent treatment compared with continuous low temperature treatment (Fig. III-3). The expression showed a similar trend in RT-PCR analysis (Fig. III-4). Glycoside hydrolase families are involved in the enzymatic systems for the breakdown of di- or polysaccharides, and these enzymes have various functions like cell wall metabolism, defense, signaling (Henrissat et al., 2001; Minic, 2008). In this study, the contents of monosaccharides like glucose and fructose were similar between plants treated with LT+LT and LT+HT (Table III-4). But, the sucrose contents were significantly low in plants treated with LT+HT. Therefore, the low expression of carbohydrate hydrolysis would act in low sucrose accumulation, not monosaccharides. Also, fructose-1,6-bisphosphatase is an enzyme to form fructose 6-phosphate and play a role in cytosolic gluconeogenesis for synthesizing sucrose or starch (Anderson et al., 2004; Cha-um et al., 2009).

In a transcriptomic study in *Phalaenopsis* (*Doritaenopsis*, an intergeneric hybrid between *Doritis* and *Phalaenopsis*) by Qin et al. (2012), carbohydrate metabolisms like starch degradation and sucrose metabolism pathways were up-regulated in cold-treated leaves. Sucrose accumulation in the *Phalaenopsis* leaves was induced during inflorescence initiation by low temperature exposure (Kataoka et al., 2004; Konow and Wang, 2001). Similarly, the contents of sugars, especially sucrose, were highly correlated with inflorescence emergence in Chapter II (Table

II-4). Also, the exogenous sucrose treatment could increase the sucrose contents in the leaves and accelerate the inflorescence emergence (Fig. II-2; Lee et al., 2020).

Intermittent high temperature treatment significantly reduced the sucrose contents (Table III-4). High temperature stress could decrease photosynthetic ability and the sugar contents and delay the subsequent inflorescence initiation (Jeong et al., 2020). However, 28°C of high temperature used in this study did not induce a decrease of photosynthetic ability in Chapter II (Fig. II-1). Therefore, the reduction of sucrose in the leaves seems to be due to the flowering inhibition by the intermittent high temperature condition rather than the stress phenomenon.

In orchids, pseudo-bulb is the main organ for storing water, minerals, or carbohydrates to support inflorescence development during the long flowering phase (Kim et al., 2013; Ng and Hew, 2000). In a study by Wang et al. (2008), polysaccharide mobilization and enzymatic changes were observed in pseudo-bulb during the inflorescence development. However, our preceding results showed no changes in carbohydrate contents between vegetative and reproductive phases (Table II-3). On the other hand, carbohydrate status in the leaves was correlated with floral induction or inflorescence emergence of *Phalaenopsis* in previous studies (Jeong et al., 2020; Kataoka et al., 2004; Konow and Wang, 2001; Liu et al., 2016). Starch, a common storage polysaccharide is synthesized and degraded to sucrose during the day and nighttime, respectively (Ceusters et al., 2019; Okita, 1992), and the sucrose is transported from the leaves to other organs (Bernier et al., 1993; Mérida et al., 1999; Ruan, 2014). Although a significant decrease was not observed in starch

contents in the leaves, the starch contents showed an opposite trend with sucrose contents in this study (Table III-4). Thus, in *Phalaenopsis*, the carbohydrates in the leaves, not the stem, are the main energy sources for the inflorescence initiation and development.

The reduction of sugars in the leaves by intermittent high temperature treatment could be attributed to the sugar translocation. Under vegetative condition at 28°C, *Phalaenopsis* plants developed significantly more leaves in previous studies (Table I-3 and Table II-1). During vegetative growth, newly-developing leaves are 'sink' organs (Susilo et al., 2013). On the other hand, nutrients are transported to inflorescences during reproductive growth (Susilo et al., 2014). However, because it takes several weeks for emerging inflorescences in *Phalaenopsis* (Blanchard and Runkle, 2006; Lee et al., 2015), there is a time interval for the transition of 'sink' organs. Also, during the reproductive period, the new leaf development generally stops. The decreased activity of 'sink' organs has been related to the accumulation of carbohydrates in the leaves (Herold, 1980).

Sugars not only are essential components for growth and development as providers of energy sources but also act as signal molecules in various developmental processes such as floral transition, seed development, senescence, and stress response (Bolouri Moghaddam and van den Ende, 2013; Rolland et al., 2006; Smeekens and Hellmann, 2014). Sugar mobilization from 'source' to 'sink' organs through carbohydrate partitioning is important to sugar signaling (Bihmidine et al., 2013; Corbesier et al., 1998; Gibson, 2005). Especially, the carbon allocation and

sugar signals are involved in the transition from vegetative to reproductive phase. The 'C/N ratio' of the phloem sap increased during the floral induction (Corbesier et al., 2002). However, the signaling of sugars in the flowering process is intricate by interacting with gene expression, microRNAs, and hormones (Ruan, 2014; Yu et al., 2015). Recent studies proposed that the floral transition by sugar metabolism is associated with trehalose 6-phosphate rather than sucrose in signaling (Bolouri Moghaddam and van den Ende, 2013; Ponnu et al., 2011).

The relation between sugars and photoperiod pathway has been discussed (Bolouri Moghaddam and van den Ende, 2013; Wahl et al., 2013). In *Phalaenopsis*, early studies reported that inflorescence development was enhanced by short-day conditions (Rotor, 1952; Yoneda et al., 1991). However, recent studies referred that there was no photoperiodic response in inflorescence initiation (Jang et al., 2015; Lee et al., 2019). Gibberellins (GA) also has been known to affect carbohydrate status during flowering (Iqbal et al., 2011; Ranwala and Miller, 2008; Yim et al., 1997). GA levels increased simultaneously during the inflorescence initiation of *Phalaenopsis* (Chen et al., 1994; Su et al., 2001), and the role of the GA pathway in floral induction has been suggested (Huang et al., 2016). But, exogenous GA injection could not substitute for low temperature exposure (Wang, 1995). Also, the genes related to GA biosynthesis were not activated during floral induction (Qin et al., 2012). In our preliminary study, exogenous GA application delayed inflorescence initiation and decreased the percent flowering-induced plants (unpublished).

Homologs of *PhalCOL* and *DhGI* were relatively repressed by intermittent high temperature treatment (Fig. III-5A and B). These genes were activated by floral induction by low temperature exposure in *Phalaenopsis* (Luo et al., 2011; Zhang et al., 2011). In *Arabidopsis*, *FT* is activated by *CO*, which is affected by *GI*, and GI-CO-FT module is the major pathway in photoperiodic response (Putterill et al., 1995; Cho et al., 2017). On the other hand, the expression of a *DhEFL4* homolog increased by the intermittent treatment (Fig. III-5C). The over-expression of *DhEFL4* delayed the flowering of transgenic *Arabidopsis* (Chen et al., 2016). These genes were regulated during floral induction by low temperature exposure (Chen et al., 2015; Luo et al., 2011; Zhang et al., 2011). Although our preceding results in Chapter II showed the correlation between inflorescence emergence timing and sugar contents in the leaves, it is still uncertain whether the sugar content is a key factor for controlling the inflorescence initiation of *Phalaenopsis* or not.

In this study, the decrease of sugar contents by intermittent high temperature treatment might only delay the inflorescence emergence rather than work together in switching off the floral induction. However, changes in the expression of flowering-related genes proved the flowering inhibition in the molecular level by the intermittent high temperature treatment. Several review studies suggested hypothetical models of the flowering mechanism in *Phalaenopsis* (Wang et al., 2017; Wang et al., 2019), however, to our knowledge, the regulatory network between sugar signaling and flowering-related genes had not been discussed in *Phalaenopsis*.

Further studies are required to determine whether the sugars in the leaves interact with flowering-related genes in the floral induction of *Phalaenopsis*.

In this study, intermittent high temperature treatment induced the down-regulation of carbohydrate metabolism. In transcriptome analysis, the expression of genes for enzymes like glycoside hydrolases and fructose-1,6-biphosphatase was reduced by discontinuous low temperature exposure. Continuous low temperature increased the sugar contents in the leaves, while significantly low contents were observed under continuous high temperature condition. Under the intermittent high temperature condition, the sucrose contents significantly decreased compared with under continuous low temperature condition. Subsequently, the intermittent treatment delayed inflorescence emergence and maintained vegetative growth. These results indicated that the flowering inhibition by intermittent high temperature treatment would be attributed to the down-regulation of sugar metabolism. Also, changes in the molecular mechanism for floral induction were observed and further studies are expected to give a better understanding of the regulatory networks in *Phalaenopsis* inflorescence initiation.

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CONCLUSIONS

Low temperature accumulation response was determined to understand the low temperature requirements of *Phalaenopsis* plants for inflorescence initiation. The orchids required a sufficient period of low temperature exposure for their competent inflorescence initiation, but the floral induction did not show a cumulative response to inducing temperatures which were the amount of low temperature. On the other hand, the inflorescence emergence was determined by photosynthetic ability and subsequent sugar contents in the leaves. Although inflorescence meristems are differentiated at the stem, the inflorescence initiation was correlated with the carbohydrate status in the leaves.

Discontinuous low temperature exposure could partially inhibit the inflorescence initiation and maintained vegetative growth, e.g. new leaf development, regardless of cultivars. These inhibitory effects were induced by intermittent high temperature treatment in spite of sufficient periods of low temperature exposure. Transcriptome analysis implied the down-regulation of expression in enzymes for sugar accumulation under intermittent high temperature condition. Carbohydrate metabolisms like polysaccharide hydrolysis and gluconeogenesis were involved in the flowering inhibition by intermittent high temperature treatment. As a result, the decrease of sucrose contents in the leaves was observed in plants treated with the intermittent treatment compared with non-treated plants under continuous low

temperature condition. Interestingly, the expression of genes related to floral induction, e.g. homologs of *CONSTANT-like* and *GIGANTEA*, was repressed by the intermittent high temperature treatment, while the expression of *EARLY FLOWERING4-like*, which may repress the *COSTANT* activity, was relatively induced. These results indicated that discontinuous low temperature exposure by intermittent high temperature treatment could inhibit flowering and these inhibitory effects would be attributed to the down-regulation of the molecular mechanism for floral induction as well as sugar accumulation.

It is difficult to understand the regulatory networks of orchid flowering because of the long life cycle and diversity of orchid. *Phalaenopsis* is regarded as a suitable material for studying the flowering mechanism since this orchid shows unique environment requirements for floral induction. Although *Phalaenopsis* plants are distributed throughout the tropical regions, low temperature exposure is a primary factor for inflorescence initiation. In this study, the correlation between carbohydrate metabolism and the inflorescence initiation was discussed by physiological and transcriptomic approaches. Also, an interesting treatment, intermittent high temperature, was used to understand the low temperature requirements. These data can be useful to reveal the flowering mechanisms of orchids and also to develop new cultivation techniques, e.g. exogenous sucrose treatment or energy-saving by intermittent heating for preventing premature flowering during the winter season.

ABSTRACT IN KOREAN

호접란의 화경 유도를 위해서는 저온 노출이 필요하다. 하지만, 호접란은 일장의 변화가 없고 일년 내내 따뜻한 열대 지역에 자생하는 식물이라는 점이 특징이다. 이러한 특징은 겨울의 연속적인 저온 노출을 필요로 하는 일반적인 춘화 식물들의 특징과는 다르다. 따라서, 본 연구에서는 호접란의 저온요구도를 이해하고 불연속적인 저온 노출 환경에서의 개화 억제 효과를 알아보고자 하였다. 제 1 장에서는 호접란의 화경유도를 위해 필요한 저온 노출 기간을 여러 품종 및 저온 처리 온도에서 확인하였으며, 또한 간헐적 고온 처리를 통한 불연속적인 저온 노출 환경에서의 화경유도 반응을 관찰하였다. 제 2 장에서는 화경유도와 잎에서의 탄수화물 함량 간의 상관관계에 대하여 분석하였으며, 제 3 장에서는 전사체 분석을 통한 간헐적 고온 처리 환경에서의 대사 변화를 확인하였다.

본 연구에서는 호접란의 화경유도를 위해서는 품종에 상관없이 6~8 주 동안의 저온 노출 기간이 필요하였으며, 저온 기간이 부족할 경우 화경이 출현하지 않거나 화경 발달이 멈추는

현상이 관찰되었다. 20℃와 23℃의 저온 처리에서는 화경유도 반응에 차이가 없었으며, 이를 통해 화경유도를 위한 저온 충족 반응이 처리 온도에 양적인 반응을 나타내지 않는다고 판단되었다. 두번째 연구에서 17℃의 저온 처리를 하였을 때 20℃나 23℃에 비해 화경 출현이 유의성 있게 늦게 나타났다. 저온에 의해서 화경이 유도될 때 영양생장 환경과 비교하여 잎에서의 탄수화물 축적이 일어나는 것을 관찰할 수 있었는데, 이는 각 온도에서의 화경 출현 일수는 그 온도에서의 광합성 능력과 잎에서의 당 함량과 상관관계가 있었다는 것을 나타낸다. 따라서 호접란의 화경유도는 저온의 양 보다는 유도 환경에서의 탄수화물 함량이 중요한 것으로 나타났다.

간헐적 고온 처리로 인한 불연속적인 저온 노출 환경에서 저온 노출 기간이 충분했음에도 불구하고 화경유도가 억제되었고 이때 신엽의 수가 유의성 있게 증가하였는데, 이를 통해 영양생장이 유지되었음을 알 수 있었다. 전사체 분석을 통해 간헐적 고온 처리 환경에서의 탄수화물 대사의 저하를 확인하였고, fructose-bisphosphatase, glycoside hydrolase, G-3-P dehydrogenase orthologs 의 발현량이 감소하였다. 또한 호접란의

개화 관련 유전자들의 발현을 비교하였을 때 연속적 고온 또는 간헐적 고온 처리 환경에서 연속적 저온 환경에 비해 *PhalCOL* 과 *DhGI1* homolog 의 발현이 감소하였고, *DhEFL4* homolog 의 상대적인 발현이 증가하였다. 이러한 유전자들의 발현량은 연속적 고온과 간헐적 고온 환경에서 유사한 것으로 나타났다. 당의 함량을 확인하였을 때 연속적 저온 환경에서 앞선 실험과 마찬가지로 높게 유지되었다. 간헐적 고온 환경에서는 연속적 고온 환경에 비해 당 함량이 높았고, 연속적 저온 환경에 비해서는 유의성 있게 낮았다. 이러한 결과는 간헐적 고온 처리로 인한 호접란의 화경유도 억제가 탄수화물 대사의 저하로 인한 잎에서의 당 함량 감소 때문일 수 있음을 의미한다.

따라서, 호접란의 화경유도 과정에서 잎에서의 탄수화물 축적이 일어나며, 화경유도는 저온의 양 보다는 각 온도에서의 당 함량과 상관관계가 있음을 알 수 있었다. 불연속적인 저온 노출은 호접란의 화경유도를 억제하며, 이러한 개화억제 효과는 탄수화물 대사의 저하로 인한 것으로 판단된다.