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Induction of early flowering in *Cymbidium niveo-marginatum* Mak in vitro

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Abstract Many orchids take several years to flower. We have been able to induce early flowering in the temperate orchid *Cymbidium niveo-marginatum* Mak in vitro. The combined treatment of cytokinin (6-benzylaminopurine), restricted nitrogen supply with phosphorus enrichment, and root excision (pruning) induced transition of the *Cymbidium* shoot from a vegetative to a reproductive stage. Nearly 100% of the plants flowered within 90 days only when the combined treatment was applied. When root excision and/or 6-benzylaminopurine were omitted from the combined treatments, flower induction was significantly reduced. The auxin transport inhibitor, 2,3,5-triiodobenzoic acid prevented flowering of *Cymbidium* in vitro, although auxin (α-naphthaleneacetic acid) itself did not induce flowering. Gibberellic acid markedly delayed flowering in C. *niveo-marginatum* even when the flower-promoting treatment was applied. Paclobutrazol, an anti-gibberellin agent, totally blocked the inductive effects of either cytokinin or pruning. These observations suggest that concerted actions of auxin, cytokinin, and gibberellin, as well as nutrient concentration and putative promoting/suppressing agents, determine the timing of *Cymbidium* orchid transition from the vegetative to reproductive stage.

Key words Orchid · In vitro flowering · Phytohormones · Nutrient application · Root excision

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Abbreviations *ABA* Abscisic acid · *BA* 6-Benzylaminopurine . GA_3 Gibberellic acid · *MS* Murashige and Skoog · *NAA* α-Naphthaleneacetic acid · *PBZ* Paclobutrazol · *TDZ* Thidiazuron · *TIBA* 2,3,5-Triiodobenzoic acid

Introduction

The transition from vegetative growth to flowering is a crucial point in higher plant development. It is known to be under the control of a so-called "switch-on" mechanism. Basically, this mechanism is governed by ensembles of flowering time and meristem identity genes with a complex hierarchy (Simon et al. 1996; Nilsson and Weigel 1997). This mechanism has not been fully elucidated. It may be influenced by various factors affecting plant development (Sachs and Hackett 1983; Chory et al. 1996), including hormones (Bonnet-Masimbert and Zaerr 1987; Dickens and Staden 1988, 1990) and nutrients (Dickens and Staden 1988; Bernier et al. 1993; Tretyn et al. 1994).

Many representatives of the large family, the Orchidaceae, usually have long juvenile periods - up to 13 years (Arditti 1992) - and delayed transition to reproductive development. Thus, the shortening of juvenility, especially in commercial varieties and hybrids, is of biotechnological interest.

The control of flowering in orchids is poorly understood, although several successful attempts to induce in vitro flowering of orchids have been described (Knudson 1930; Livingston 1962; Kerbauy 1984; Wang and Xiong 1988; Paek et al. 1989; Wang et al. 1993; Duan and Yazawa 1995). An in vitro system is considered a convenient tool to study the switch-on flowering mechanism. Establishing a reliable in vitro protocol to induce early flowering in orchids is important for the study of molecular and genetic mechanisms of flower induction and for assisting orchid breeding programs. This paper describes such a protocol for the commercially important orchid *Cymbidium niveo-marginatum*, which takes 4–7 years to flower in vivo.

Materials and methods

Rhizomes (3–4 months old) of *C. niveo-marginatum* Mak and 3- to 5-month-old plants (3–5 months after the initiation of regeneration) regenerated from rhizomes of *C. niveo-marginatum* were used in the present experiments. The rhizomes had been cultured in vitro for about 22 years. Aseptic plant materials were purchased from Sunhwawon (Republic of Korea). The rhizomes were cultured on basal Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with 40 g l^{-1} of sucrose. α-Naphthaleneacetic acid (NAA) at 5 mg l^{-1} was added for rhizome propagation, and 6-benzylaminopurine (BA), triiodobenzoic acid (TIBA), gibberellic acid (GA_3), and paclobutrazol (PBZ) in various concentrations and combinations were added for plant regeneration and flowering induction. Gelrite (Duchefa, Netherlands) at 2.5 g l^{-1} was used as the solidifying agent. BA, NAA, and GA_3 were dissolved in 1 M NaOH and added to the media before autoclaving. TIBA and PBZ were dissolved in dimethyl sulfoxide. Water-soluble arginine, lysine, and L-pipecolic acid were filtered through a 0.22-μm Millipore filter and added after autoclaving. All explants (rhizomes, plants, and shoots) were cultured in 1-l autoclaved glass bottles filled with 250 ml of medium. Plants and rhizomes were maintained at 25–26 °C, 50 μmol m^{-2} s^{-1}, and a 16/8 h (day/night) photoperiod. The light was provided by cool-white fluorescent lamps. To simulate short-day condition, a 8/16 h (day/night) photoperiod was employed. The same light condition (8/16 h) was used for the low-temperature (4–6 °C) study.

In the experiments where pruning was applied, roots were cut off shoots immediately before transferring them to the experimental media. An explant was counted as a flowering plant when it produced a visible flower stalk. Twenty to 40 plants were examined in each experiment and for each treatment. All experiments were performed in duplicate. Percentage of plants committed to flowering represents the mean of two experimental replications. The Jandel Sigma Stat program was applied to calculate the standard deviation. To verify differences between the outcome of various treatments, a *t*-test at $P = 0.95$ was used (Williams 1993).

Results

To induce in vitro early flowering in temperate *Cymbidium* orchid, various conventional methods consisting of the following treatments were applied to rhizomes and to plants regenerated from rhizomes.

In the first experimental series: (1) in various combinations, low (4–6 °C) and high (30–32 °C) temperature, short-/long-day photoperiod, and sucrose enrichment (0.12, 0.18, 0.23 M); (2) phytohormones [BA 2, 5, 10, 20 mg l^{-1}; NAA 1, 3, 5, 10 mg l^{-1}; abscisic acid (ABA) 2, 5 mg l^{-1}, GA_3 5, 10, 15 mg l^{-1}]; (3) phytohormone biosynthesis and transport modulators (TIBA 1, 5, 10 mg l^{-1}; PBZ 25, 50 mg l^{-1}); (4) polyamine (spermidine 5, 10 mg l^{-1}); (5) amino acids (arginine, lysine, pipecolic acid, each at 5, 10 mg l^{-1}); (6) nitrate reductase suppresser (potassium ferricyanide 1, 5, 10 mM). No indication of flowering was observed within 4–5 months after the above treatments either in rhizomes or in plants regenerated from rhizomes.

In the second series of experiments, the following putative flower-inducing factors were applied to induce flowering in 3- to 5-month-old plants. The total nitrogen content in the inductive medium was reduced to 1/7–1/25 (8.6–2.4 mM) of nitrogen in basal MS medium. The total phosphorus content was increased to fivefold the level in MS medium. BA, thidiazuron (TDZ), TIBA and root excision were included in various combinations (Table 1).

We did not observe any flowering in the control plants cultured on MS medium during the 40-day period of observation (Table 1) or after up to 90 days (Table 2). The combined treatment, FI-12 (Table 1), was most effective for flower induction. However, in FI-12, TDZ caused poor plant growth, and floral buds withered soon after they appeared. Therefore, although the treatment with 2.5 mg l^{-1} TDZ was a strong flower inducer, TDZ was excluded from further experiments.

Treatments FI-2, -3, and -4 (Table 1) induced over 50% flowering within 40 days after the plants were transferred to inductive media. There were no significant differences ($P = 0.95$) among results from FI-2, -3,

Table 1 Experimental series 2: the influence of macronutrients and growth regulators on the induction of the reproductive stage in *Cymbidium niveo-marginatum* (– no treatment, + treatment). Data for induced shoots (mean ± SD) were collected 40 days after plants had been transferred to the inductive medium

Treatment	N[a]	P[b]	Root excision	BA (mg/l)	TDZ (mg/l)	TIBA (mg/l)	Number of explants	Induced shoots (%)
MS-control	×1	×1	–	–	–	–	60	0
FI-1	×1/7	×5	+	5	–	–	40	10.0 ± 1.6
FI-2	×1/7	×5	+	10	–	–	50	54.5 ± 1.4
FI-3	×1/20	×5	+	5	–	–	45	55.6 ± 1.4
FI-4	×1/20	×5	+	10	–	–	52	53.8 ± 3.1
FI-5	×1/25	×5	+	5	–	–	56	12.5 ± 1.8
FI-6	×1/25	×5	+	10	–	–	51	29.4 ± 2.0
FI-7	×1/25	×5	+	15	–	–	53	32.0 ± 5.5
FI-8	×1/7	×5	+	10	–	1	54	0
FI-9	×1/20	×5	+	10	–	1	60	25.0 ± 4.4
FI-10	×1/25	×5	+	–	0.1	–	61	6.6 ± 0.14
FI-11	×1/25	×5	+	–	0.5	–	60	13.3 ± 3.1
FI-12	×1/25	×5	+	–	2.5	–	64	75.0 ± 7.4

[a] Final nitrogen concentration in the inductive medium compared to that of basal MS medium

[b] Final phosphorus concentration in the inductive medium compared to that of basal MS medium

and -4. The different concentrations of nitrogen in FI-2 and FI-4 combined with 10 ml l^{-1} BA did not affect flower induction. However, when the nitrogen level was lowered in FI-5, FI-6, and FI-7, the promotive action of cytokinin and other inductive factors for flowering dramatically decreased. Interestingly, in FI-1, FI-2, FI-5, FI-6, and FI-7, the *Cymbidium* plants showed cytokinin-dose-dependent transition to reproductive development. In contrast, treatments FI-3 and FI-4 resulted in approximately the same level of floral induction despite different concentrations of cytokinin.

When 1 mg l^{-1} TIBA was applied to the inductive media, it either totally (FI-8) or partially (FI-9) blocked *Cymbidium* transition to flowering (Table 1). In contrast auxin (NAA) did not induce the transition to reproductive development when applied alone either to rhizomes or shoots (data not shown).

In the third series of experiments, we studied the importance of various factors including gibberellin and anti-gibberellin for flower induction in *Cymbidium* (Table 2). The combined treatment of nutrient (nitrogen and phosphorus) application (NP), root excision (RE) and cytokinin (BA) resulted in almost 90% of plants flowering at 60 days after treatment in FI-4 (Fig. 1). No sign of flowering was observed in the controls during the whole experimental period (90 days). A few plants in FI-4 set fruits when self-pollinated (Fig. 2).

Deletion of the NP treatment factors (FI-13) from the inductive medium slightly reduced flowering compared to that in FI-4 (Table 2). The exclusion of RE significantly ($P=0.95$) reduced the transition to flowering, especially during the early days of the FI-14 treatment. In FI-15 with NP and RE, and without BA, only a few shoots displayed floral bud development. However, BA itself in FI-20 promoted flowering even though the induction was low and delayed compared to FI-4 (NP, RE, and BA), FI-13 (BA and RE) and FI-14 (BA and NP). MS medium modified with an NP supplement was unable to induce in vitro flowering of *C. niveo-marginatum* in FI-16.

Fig. 1 In vitro flowering of *Cymbidium niveo-marginatum*

PBZ completely abolished flowering in FI-17 and did not induce flowering when it was applied along with RE in FI-18. When the inductive medium was supplemented with GA_3 in FI-19, the transition to flowering was delayed and the percentage of shoots induced to flower decreased.

Table 2 Experimental series 3: the influence of macronutrients, pruning and growth regulators on the transition to flowering of *C. niveo-marginatum* (− no treatment, + treatment)

Treatment	N[a]	P[b]	Root excision	BA (mg/L)	PBZ (mg/L)	GA_3 (mg/L)	Percentage induced shoots (mean ± SD)							Number of explants
							30 days	40 days	50 days	60 days	70 days	80 days	90 days	
MS-control	×1	×1	−	−	−	−	0	0	0	0	0	0	0	79
FI-4	×1/20	×5	+	10	−	−	47.5 ± 3.5	62.5 ± 7.1	77.5 ± 3.5	87.5 ± 3.5	92.5 ± 7.1	97.5 ± 3.5	97.5 ± 3.5	80
FI-13	×1	×1	+	10	−	−	45.0 ± 3.6	57.5 ± 3.6	67.5 ± 7.0	75.0 ± 3.5	80.0 ± 3.5	90.0 ± 3.6	94.7 ± 3.6	80
FI-14	×1/20	×5	−	10	−	−	22.5 ± 4.0	27.5 ± 3.5	40.0 ± 0.7	52.5 ± 3.5	70.0 ± 4.1	85.0 ± 6.7	92.6 ± 0.6	76
FI-15	×1/20	×5	+	−	−	−	2.6 ± 0.0	2.6 ± 0.0	2.6 ± 0.0	5.2 ± 3.7	5.2 ± 3.7	5.2 ± 3.7	5.2 ± 3.5	76
FI-16	×1/20	×5	−	−	−	−	0	0	0	0	0	0	0	78
FI-17	×1	×1	−	10	50	−	0	0	0	0	0	0	0	77
FI-18	×1	×1	+	−	50	−	0	0	0	0	0	0	0	80
FI-19	×1/20	×5	+	10	−	15	0	3.3 ± 0.1	10.0 ± 4.7	16.6 ± 4.7	23.3 ± 4.8	33.3 ± 9.4	36.7 ± 4.7	60
FI-20	×1	×1	−	10	−	−	0	7.6 ± 0.8	19.2 ± 3.4	26.9 ± 2.9	30.8 ± 3.4	34.6 ± 1.7	38.5 ± 6.8	52
FI-21	×1	×1	+	−	−	−	0	0	0	0	0	0	0	64

[a] Final nitrogen concentration in the inductive medium compared to that of basal MS medium

[b] Final phosphorus concentration in the inductive medium compared to that of basal MS medium

Fig. 2 In vitro fruit onset of *C. niveo-marginatum*

Discussion

Various conventional approaches have been reported to induce in vitro and ex vitro flowering in different species (Scorza 1982; Bonnet-Masimbert and Zaerr 1987; Dickens and Staden 1988, 1990; Fujioka and Sakurai 1992; Bernier et al. 1993). However, in our experiments, they were ineffective in inducing flowering on rhizomes, shoots, and plants regenerated from *C. niveo-marginatum* rhizomes. Although spermidine in *Dendrobium candidum* (Wang et al. 1993), and lysine (Tanaka et al. 1997), pipecolic acid (Fujioka and Sakurai 1992), and ferricyanide (Tanaka and Asagami 1986) in *Lemna* species (family Lemnaceae) were reported to induce flowering, these agents did not induce flowering in *Cymbidium* orchids.

There are several reports on in vitro induction of early flowering in orchids (Kerbauy 1984; Paek et al. 1989; Arditti and Ernst 1992; Wang et al. 1993; Duan and Yazawa 1994, 1995). Kerbauy (1984) reported that 8- to 9-month-old *Oncidium varicosum* seedlings produced flower stalks on Knudson (1946) hormone-free medium. Flower buds were evoked directly from rhizomes of *C. niveo-marginatum* and hybrid Dogui (*C. ensifolium* × *C. kanran*) after treatment with a high cytokinin/auxin ratio (Paek et al. 1989). However, only two of the above studies used vegetative orchid plant material which was not preconditioned for flowering (Paek et al. 1989; Wang et al. 1993). The remainder used reproductive-related explants, for example flower stalks, as an initial source. In our experiments, we used rhizomes as well as 3- to 5-month-old plants regenerated from them. Both had been previously cultured in vitro for several years without flowering and flower bud formation. Therefore, we suggest that our plant material was not preconditioned for flowering and thus responded sensitively to the flower-inducting stimuli.

Doriella Tiny (*Doritis pulcherrima* × *Kingiella philippinensis*) and *Phalaenopsis* Pink Leopard ("Petra") plants were induced to flower by cytokinin and low nitrogen supply (Duan and Yazawa 1994, 1995). In our experiments, very low nitrogen supply alone did not induce reproductive development in *C. niveo-marginatum*. Nevertheless, the low nitrogen supply clearly provided favorable conditions for the inductive action of other promotive agents, such as BA and RE (Table 2). In general, the nutritional status of plants greatly affects the sensitivity of the major flowering mechanism to both suppressive and inductive stimuli (Sachs and Hackett 1983; Kerbauy 1984; Yoneda 1989; Hirst and Ferree 1995), although the interactions between nutrition and flowering mechanism are still unclear.

Cytokinin promotes the transition in some higher plants to their reproductive stage in vitro (Dickens and Staden 1988; Paek et al. 1989; Wang et al. 1993). In our experiments, BA alone induced around 40% of shoots to flower within 90 days of treatment. When BA combined with NP and RE was used, the flower induction was enhanced to 97.5% (Table 2). However, there was no indication of flowering when either RE or NP were used alone. These data suggest the important role of cytokinin in activating the switch-on mechanism for flower induction under the present experimental conditions.

The flower-promoting effect of root pruning is well-known in some woody plants (Fossard 1972; Meilan 1997). Although cytokinin synthesis occurs in the root of higher plants (Davies 1995), a specific signal in tobacco roots was also reported to prevent flowering (McDaniel 1996). Thus, the abolishment of anti-floral signals by root excision may be the reason we observed slight induction of orchid flowering in vitro, even when BA was not employed (Table 2, FI-15).

Gibberellins are thought to play an essential role in vegetative and reproductive development of plants (El-Antably 1976; Tymoszuk et al. 1979; Cecich 1985; Arditti 1992; Eysteinsson and Greenwood 1995; Meilan 1997). In our experiments, a high concentration of GA_3 delayed flowering and decreased the percentage of plants exhibiting flower induction. However, it did not totally block flower induction. When PBZ was applied with BA, it totally abolished the promotive effect of

cytokinin. These observations suggest that gibberellin interacts with other plant hormones in flower induction of *Cymbidium* in vitro.

TIBA reduced flower induction of *C. niveo-marginatum* orchid, significantly ($P=0.95$) in FI-9 and completely in FI-8 (Table 1). In contrast auxin itself did not induce even flower bud formation. Auxins play some inductive (Metzger 1995) or inhibitory (Applewhite et al. 1994) roles to initiate reproductive development in higher plants. In our experiments, *C. niveo-marginatum* flowering was not induced by either TIBA or a high level of NAA in the medium.

In addition, we have applied combined FI-4 treatment to some other orchid species (*C. goeringii*, *C. kanran*, and *Dendrobium phalaenopsis* hybrid). The induction of flowering was achieved in all these species although at a lower level than with *C. niveo-marginatum* (data not shown). This implies possible similarity in the mechanism controlling transition to flowering in the orchid species tested.

In conclusion, we observed that concerted actions of cytokinin, root excision and restricted nitrogen supply resulted in early flowering of *C. niveo-marginatum* in vitro. When root excision and/or BA were omitted from the combined treatments, the percentage of plants exhibiting floral induction was significantly lower ($P=0.95$). Gibberellin and auxin, as well as their inhibitors also affected flower induction in *Cymbidium* orchid.

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