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Studies on Bulblet Differentiation in Bulb-scale Segments of *Lilium longiflorum* IV. Involvement of calcium

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Summary

In lily bulb-scale segments cultured in vitro, adventitious bulblets were induced in the segments by treatment with cytokinin and auxin. Application of calcium ionophore A23187 promoted active meristematic divisions in the suprficial tissues of segments without phytohormones, resulting in the bulblet differentiation. The promotion was also induced by short-term treatment of A23187. The phytohormone-induced bulblet differentiation was suppressed by verapamil, a calcium channel inhibitor, or Quin II AM, a cytoplasmic calcium chelator. Intracellular Ca^{2+} contents increased in the local epidermal meristematic cells which divided to develop adventitious bulblets. These results support the idea that the phyrohormone-induced adventitious bulblets in lily bulb-scale segments may be mediated, at least partially, by an increase in the level of intracellular Ca^{2+}.

Key words: bulblet differentiation, calcium, *Lilium longiflorum*

Introduction

A number of physiological phenomena in higher plants are regulated through the action of phytohormones, such as auxin and cytokinin. We previously reported that treatment with cytokinin induced active merisematic divisions in the epidermis of *Torenia* stem segments, and that these meristematic zones developed into adventitious buds[21]. Simultaneous application of auxin and/or anti-cytokinin markedly inhibited this cytokinin -induced response, and application of anti-auxin showed an additive effect on cell division[22, 24].

In case of lily bulb-scale segments, adventitious bulblets differentiation was induced by application of auxin and cytokinin, and promoted by wounding, application of traumatic acid[5], some phospholipids and phorbol ester[7], and anaerobic treatment[6].

In *Torenia* stem segments, the adventitious bud induction was also attained by application of calcium ionophore A23187 and this differentiation process seemed to be mediated by intracellular Ca^{2+} level[26]. The effectiveness of A23187 may provide some clue to elucidate intriguing questions of plant organogenesis although its action mechanism is presently unknown.

The presence of calcium in the medium is known to enhance several cytokinin-regu-

lated responses, and treatment with certain calcium antagonists nullifies the action of cytokinin. For example, cytokinin-induced retardation of senescence in corn leaf discs[14], and cytokinin-stimulated ethylene production in mung bean hypocotyls[8] or in cucumber cotyledons[3], are significantly promoted by increasing the calcium concentration of the media.

In these calcium-stimulated physiological response, addition of ethyleneglycol-bis-(2-aminoethyl ether)-N, N, N', N', -tetraacetic acid (EGTA), a relatively specific chelator of extracellular Ca^{2+}, nullified the stimulation[19, 9, 10, 15]. A calcium channel inhibitor, verapamil, and a calcium antagonist, lanthanum, also suppressed several calcium-mediated phenomena[19, 9]. Saunders and Hepler[17, 18] postulated that the action of cytokinin as a mitotic regulator is mediated by an increase in intracellular free calcium. In fact, they demonstrated that the addition of calcium ionophore A23187 to cytokinin-free medium greatly stimulated budding in the filamentous protonema of moss *Funaria*[18].

The adventitious bulblet differentiation in lily bulb-scale segments was stimulated by application of phospholipids[7]. The Ca^{2+} accumulated in the organelle and vacuole was released by phospholipids in animal and microbial cells[4]. Intracellular concentration of free Ca^{2+} can be measured using Ca^{2+}-binding fluorescent indicators such as Quin II[27]. This chemical specifically bind with Ca^{2+} and the Ca^{2+} concentration can be detected by the intensity of fluorescence. According to the report of Tsien et al.[28], Quin II AM is acetoxymethyl ester of Quin II, readily permeates the membrane and is hydrolyzed in the cytoplasm. The hydrolyzed Quin II binds Ca^{2+} with 1: 1 stoichiometry.

The promotive effects of phospholipid were thought to be due to the release of Ca^{2+} from organelle to cytosol, and released Ca^{2+} induced bulblet differentiation. Therefore, we tried to examine the involvement of calcium in bulblet differentiation of lily bulb-scale segments, using calcium ionophore A23187, verapamil, Ca^{2+} channel inhibitor, Quin II AM, intracellular Ca^{2+} chelator.

Materials and Methods

Plantlets of *Lilium longiflorum* Thunb. were grown *in vitro* as reported previously[5], the bulbs (about 15 mm in diameter) formed in the basal part of plantlets were harvested and the outer 2 bulb-scales were used. The bulb-scales were transversally cut to 6 segments and the segments were used as explants. The explants were cultured on the basal medium containing Murashige and Skoog's mineral salts[11], 4 % sucrose and 0.25 % Gelrite (hereafter referred to as MS medium) with 0.1 μM naphthaleneacetic acid (NAA) and 1 μM benzyladenine (BA). The calcium ionophore A23187 (Calbiochem-Behring, USA) or 12-O-tetra-decanoyl phorbol-13-acetate (TPA; Funakoshi, Japan) was dissolved in dimethyl sulfoxide, then added to the culture medium at various concentrations. The final concentration of dimethylsulfoxide was adjusted to 0.3 % in all treatments. For pre-treatment with A23187, the explants were first suspended for 2 hr in the liquid MS medium containing A23187, then cultured further on the solid MS medium with 0.1 μM NAA and 1 μM BA.

To examine the effects of calcium concentration, explants were cultured on the modified MS medium containing 0 to 30 mM $CaCl_2$ (original MS medium contained 3 mM $CaCl_2$) in the presence of 0.1 μM NAA with 1 μM BA or 1 μM A23187. To examine the effects of EGTA, 1 mM EGTA was added to the MS medium or MS medium without $CaCl_2$. Verapamil (Sigma, USA) and Quin II AM (Dojin, Japan) were added individually at concentrations ranging from 0.1 μM to 1 mM to the MS medium with 0.1 μM NAA and 1 μM BA.

The cultures were maintained under 16 hr long-day photoperiod (6,000 lux) and constant temperature of 25 ± 2°C. After 3 weeks of culture, bulblet differentiation in the cultured explants and the number of bulblets formed in the explants were observed.

Intracellular accumulation of free Ca^{2+} was measured as reported previously[7]. The cultured explants were sliced to sections (0.5 mm in depth), and incubated in 50 μM Quin II AM for 1 hr, washed with water and then observed by a fluorescence microscope (Optiphot with EF, Nikon, Japan). For fluorescence excitation, an HBO 50-W mercury vapor lamp (Osram, FRG) was used with a U filer (Nikon, Japan) (broad band excitation peak at 340 nm) for Quin II AM excitation. Fluorescence of Quin II was monitored at the wavelength longer than 490 nm and photographed.

Results

Bulblet induction by calcium ionophore A23187

When lily bulb-scale segments were cultured on the MS medium with phytohormone, the average number of bulblets formed was always less than 2.5[5, 6, 7]. The number increased progressively as the concentration of A23187 added to the medium increased (Fig. 1); the largest number of bulblets, 6.4 per segment, was obtained with 1 μM A23187 (Fig. 2). In other experiments, pre-incubation with 10 μM A23187 for 2 hr caused bulblet induction (Fig. 3) comparable to that induced by application of 1 μM A23187 for 3 weeks.

Effects of $CaCl_2$ concentration

As shown in Fig. 4, the average number of bulblet formed on the MS medium containing pytohormone and no $CaCl_2$ was about 0.2 per segment. The number gradually increased as the $CaCl_2$ concentration increased, and the largest number of bulblet was observed when 3 mM of $CaCl_2$ was added to the medium. A similar response

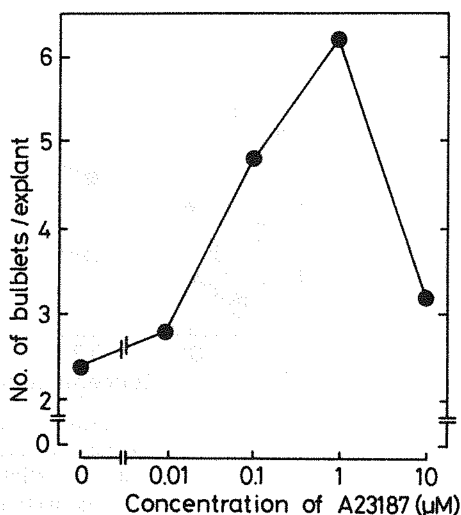

Fig. 1 Effects of A23187 on bulblet differentiation in lily bulb-scale segments.
The lily bulb-scale segments were cultured on the MS medium containing various concentrations of A23187.

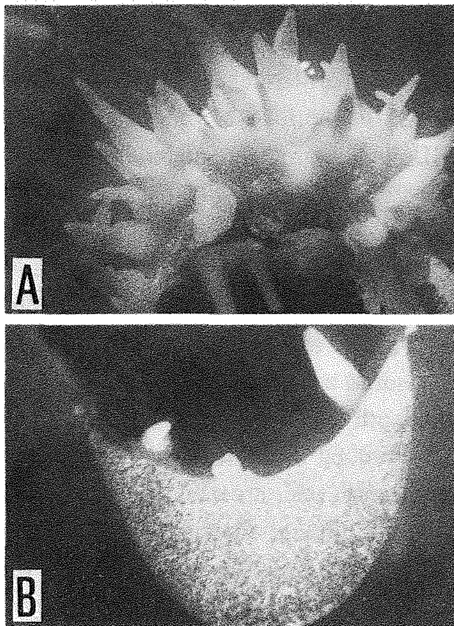

Fig. 2 Bulblet differentiation in lily bulb-scale segments.
The lily bulb-scale segments were cultured on the medium with (A) or without (B) A23187 (1μM).

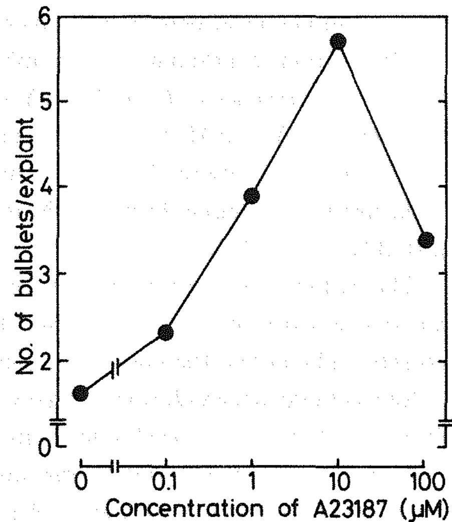

Fig. 3 Effects of pre-incubation with A23187 on bulblet differentiation in lily bulb-scale segments.
The lily bulb-scale segments were incubated for 2 hr with various concentrations of A23187, and then cultured on the basal MS medium.

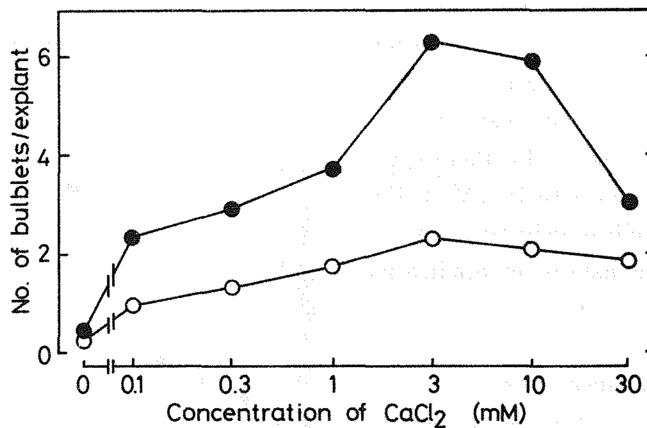

Fig. 4 Effects of $CaCl_2$ concentration in the medium on bulblet differentiation in lily bulb-scale segments.
The lily bulb-scale segments were cultured on the modified MS medium containing different concentrations of $CaCl_2$ with (●) or without (○) A23187 (1μM).

Table. 1 Effects of $CaCl_2$ and EGTA on bulblet differentiation in lily bulb-scale segments.

Chemicals or Treatment	No. of bulblets/explant		
	$+CaCl_2$ (3mM) $-$EGTA	$-CaCl_2$ $-$EGTA	$-CaCl_2$ $+$EGTA (1 mM)
NAA (0.1 μM) + BA (1 μM)	2.3	0.2	0
A23187 (1 μM)	6.2	0.4	0
Traumatic acid (1 μM)	4.4	0.3	0
TPA (0.1 μM)	9.6	0.6	0
Anaerobic treatment (1 hr)	5.2	0.4	0

The lily bulb-scale segments were cultured on $CaCl_2$-free MS medium containing NAA and BA, A23187, traumatic acid or TPA with or without adding $CaCl_2$ and EGTA to the medium. In another series of experiments, the segments were exposed to N_2 stream for 1 hr and then cultured on $CaCl_2$-free MS medium with or without adding $CaCl_2$ and EGTA.

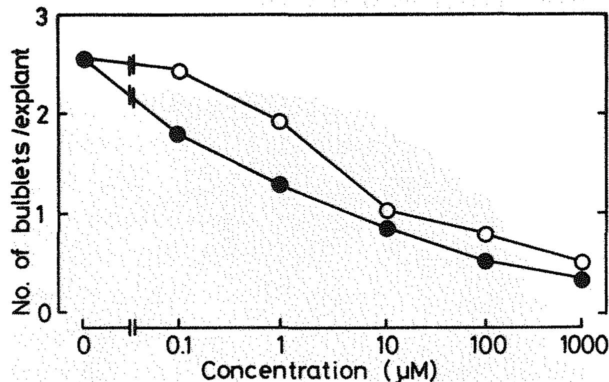

Fig. 5 Effects of verapamil and Quin II AM on bulblet differentiation in lily bulb-scale segments.
The lily bulb-scale segments were cultured on the MS medium with 0.1μM NAA and 1μM BA containing various concentrations of verapamil (○) or Quin II AM (●).

to $CaCl_2$ was seen on the media containing 1 μM A23187 instead of phytohormone, but the bulblet number formed was about 6. Although $CaCl_2$ was completely omitted from the medium, 0.2 bulblets were formed per segment. In order to remove extracellular Ca^{2+}, 1 mM EGTA was added to the calcium-free medium. Application of EGTA completely inhibited bulblet induction by phytohormone, A23187, TPA, traumatic acid or anaerobic treatment (Table 1).

Effects of Ca^{2+} uptake inhibitor and intracellular Ca^{2+} chelator

Another series of experiments was conducted using a Ca^{2+} channel inhibitor, verapamil, and a intracellular Ca^{2+} chelator, Quin II AM. Fig. 5 shows that treatment with 1 mM verapamil strongly inhibited bulblet differentiation in the explants cultured on the MS medium with phytohormone. Application of Quin II AM strongly suppressed phytohormone -induced bulblet induction; 1 μM Quin II AM suppressed bulblet initiation by 50 %, and 1

Table. 2 Effects of verapamil and Quin II AM on bulblet differentiation induced by some chemicals and anaerobic treatment.

Chemicals or Treatment	No. of bulblets/explant		
	−Verapamil −Quin II AM	+Verapamil (10 μM) −Quin II AM	−Verapamil +Quin II AM (10 μM)
NAA (0.1 μM)+BA (1 μM)	2.6	1.2	0.6
A23187 (1 μM)	6.2	1.9	1.4
Traumatic acid (1 μM)	4.6	2.5	1.3
TPA (0.1 μM)	9.8	6.4	0.8
Anaerobic treatment (1 hr)	5.4	2.3	1.3

The lily bulb-scale segments were cultured on the MS medium containing NAA, BA, A23187, traumatic acid or TPA with or without verapamil and Quin II AM. In another series of experiments, the segments were exposed to N_2 stream for 1 hr and then cultured on the MS medium with or without verapamil and Quin II AM.

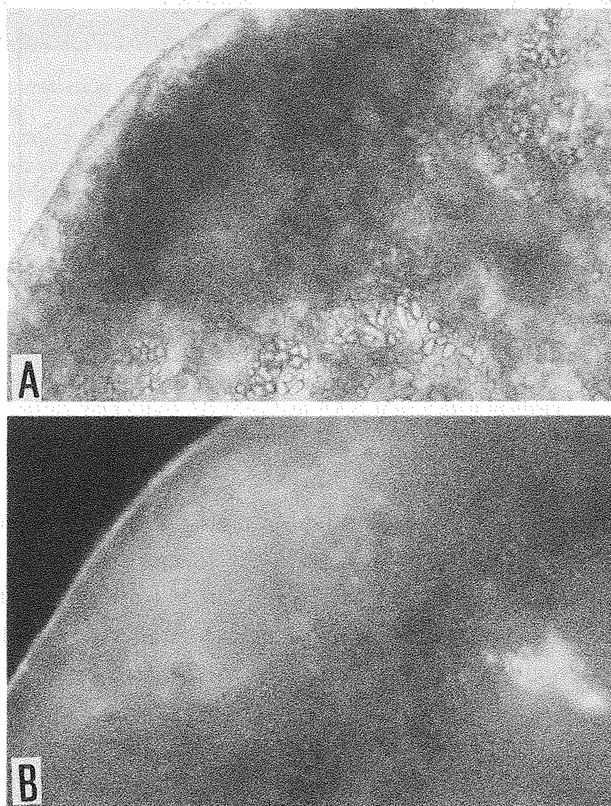

Fig. 6 Intracellular localization of Ca^{2+} in lily bulb-scale segments. The lily bulb-scale segments were cultured on the MS medium with 1μM A23187 for 1 week. The segments were sliced to sections, incubated with 50μM Quin II AM for 1 hr, and then observed with fluorescence microscope. Light (A) and fluorescence (B) microphotographs.

mM Quin II AM inhibited it completely, despite the presence of NAA, BA and 3 mM $CaCl_2$ in the medium.

Bulblet differentiation induced by traumatic acid, A23187 or TPA application or by anaerobic treatment was also suppressed by simultaneous addition of 10 μM verapamil or 10 μM Quin II AM (Table 2).

Accumulation of Ca^{2+} in meristematic divided zone

Intracellular Ca^{2+} was accumulated in the meristematicallly dividing zone of the explants cultured on the MS medium with phytohormones or A23187 (Fig. 6).

Discussion

In our experimental system, application of 1 μM A23187 to the culture medium induced 6.4 bulblets per explant (Fig. 1). A23187 has been reported to be most effective at concentrations of 1 to 10 μM in peroxidase secretion[1, 13], at 1 μM in the circadian rhythm of conidiation[12], at 10 μM in morphogenesis of *Microsterias*[9], at 10 μM in leaflet movement of *Cassia*[15], and at 10 μM in adventitious bud initiation in *Torenia*[26]. The concentration of A23187 effective for promoting adventitious bulblet differentiation in lily bulb-scale segments was at a similar level.

Recently, we reported that 2 hr pre-treatment with traumatic acid[5] and 1 hr pre-treatment with N_2[6] stimulated bulblet differentiation. A 2 hr incubation with A23187 also stimulated bulblet differentiation (Fig. 3). These treatments, however, were only stimulative when they were applied to the explants immediately after their excision from mother bulbs. Bulblet differentiation was also stimulated by wounding[5]. In case of *Torenia* stem segments, pre-treatment with N_2[23] and traumatic acid[25], and additional wounding[20] also promoted adventitious bud differentiation.

Based on the results mentioned above, it is proposed that the initial process of adventitious organ differentiation is an induction of meristematic division which can be elicited by excising explants from mother bulbs or stems. As well as the treatments with N_2, traumatic acid, or phytohormone, A23187 treatment can amplify the wounding effect.

In the early stage of bulblet differentiation in lily bulb-scale segments, meristematic divisions could be induced by calcium ionophore A23187 (Figs 1, 2, 3), as was the case of moss protonema[18] and *Torenia* stem segments[26]. These observations suggest that the level of intracellular free calcium is closely related to meristematic divisions in lower and higher plants, and that the initial action of phytohormone on adventitious organ induction may also be mediated through an increase in intracellular Ca^{2+}. In fact, the accumulation of intracellular Ca^{2+} in meristematically dividing zones of superficial tissues was observed (Fig. 6).

The number of bulblets induced by A23187 was not increased by increasing $CaCl_2$ concentrations to 10 and 30 mM (Fig. 4). There is accumulating evidence that the intracellular concentration of Ca^{2+} is kept at a relatively low level by a balance between passive influx via calcium channel and active efflux via plasma membrane Ca^{2+}-ATPase.

Therefore, it is possible that the amount of Ca^{2+} imported via calcium channel from the culture medium is sufficient to enhance bulblet initiation. It has been reported that $CaCl_2$ stimulates α-amylase synthesis, although this effect levels off at about 0.5 mM $CaCl_2$[10].

When A23187 was added to the $CaCl_2$-free medium 0.4 bulblets differentiated per explant (Fig. 4, Table 1). It is known that the bulk of calcium in plant tissues is accumulated in the intercellular space, cell wall and/or plasma membrane[16], from where it may be incorporated into cytoplasm. In fact, addition of EGTA to remove the extracellular Ca^{2+} caused complete inhibition of bulblet differentiation induced by A23187, traumatic acid, TPA or anaerobic treatment (Table 1). In moss protonema, completely eliminating calcium from the medium did not affect BA-induced budding; the budding was inhibited only when the protonema was pre-washed with EGTA and then cultured on a calcium-free medium with EGTA[19].

Similar inhibitory effects were also obtained by application of verapamil or Quin II AM (Fig. 5). Verapamil is thought to be an inhibitor of calcium influx through the calcium channels located in the plasma membrane[2]. the concentration of verapamil effective in our system is similar to those in other experimental systems[9, 19]. Quin II AM is acetoxymethyl ester of Quin II, readily permeates the membrane and is hydrolyzed in the cytoplasm[28]. The hydrolyzed Quin II binds Ca^{2+} with 1: 1 stoichiometry. Therefore, Quin II AM can be used as intracellular Ca^{2+} chelator. Inhibitory effects of Quin II AM on bulblet differentiation was stronger than verapamil (Fig. 5). Verapamil and Quin II AM also inhibited bulblet differentiation by traumatic acid, TPA, or anaerobic treatment (Table 2). Therefore, these treatments were effective through increasing concentration of intracellur Ca^{2+}.

We conclude that adventitious bulblet initiation in lily bulb-scale segments is mediated through an increase in intracellular free calcium levels, which can also be induced by the applications of traumatic acid, TPA, calcium ionophore A23187, wounding, and anaerobic treatment.

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鉄砲ユリの鱗片切片培養における球根分化に関する研究
IV. カルシウムの関与

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摘　　要

鉄砲ユリの鱗片切片培養において，球根分化は通常サイトカイニンとオーキシンによって誘導される。培地中にカルシウムイオノフォアA23187を添加すると植物ホルモンなしでも分裂が促進され，球根分化が起こる。このA23187の効果は，切片を短時間A23187溶液に浸漬した後に培養することによっても達成される。植物ホルモンによる球根分化はカルシウムチャンネルの阻害剤であるverapamilや細胞内カルシウムのキレート剤であるQuin II AMによって抑制される。細胞内のCa^{2+}濃度は球根分化が起こる表層細胞において増加する。これらの結果から鉄砲ユリの球根分化は細胞内Ca^{2+}濃度の上昇によって引き起こされるものと考えられる。