Bulblet Differentiation in Cultured Cells of *Lilium longiflorum*

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Summary

Adventitious bulblets could be induced in lily cells cultured on medium without phytohormone. The bulblet differentiation was not stimulated by anaerobic treatment and application of traumatic acid. The differentiation was promoted by addition of phytohormones (auxin and cytokinin) or calcium ionophore A23187. The simultaneous application of calcium channel inhibitor or intracellular Ca^{2+} chelator caused inhibiting effects on bulblet initiation induced by phytohormones and A23187. Intracellular Ca^{2+} contents increased in the cells treated by above chemicals. The Ca^{2+}-binding mediater protein calmodulin was present in cells, and the calmodulin inhibitor strongly inhibited bulblet differentiation. These results support the idea that adventitious bulblet differentiation in lily cultured cells may be mediated, at least partially, by calmodulin through an increase in the level of intracellular Ca^{2+}.

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

Introduction

Adventitious bulblet differentiation in lily bulb-scale segments could be induced by phytohormones, and promoted by application of calcium ionophore A23187[10]. The bulblet differentiation induced by phytohormones or A23187 in bulb-scale segments was inhibited by simultaneous application of calcium channel inhibitor, verapamil, and intracellular Ca^{2+} chelator, Quin II AM[11]. Many physiological phenomena induced by intracellular Ca^{2+} were controlled by Ca^{2+}-binding protein, calmodulin (CAM)[1]. Adventitious bud initiation in *Torenia* stem segments[10] and bulblet formation in lily bulb-scale segments[22] were also promoted by A23187 and application of CaM inhibitor, N-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide hydrochloride (W-7), suppressed the initiation. Therefore, we tried to examine the effects of A23187, verapamil, Quin II AM or W-7 on bulblet differentiation in cultured cells of *Lilium longiflorum*.

In *Torenia* stem segments[12] and lily bulb-scale segments[3], application of traumatic acid stimulated bud and bulblet differentiation, respectively. This chemical was thought to be one of the plant wound hormones[2]. Furthermore, wounding treatment given to the explants stimulated bud formation in *Torenia*[7] and bulblet initiation in lily[3]. Although the anaerobic treatment was also promoted bud[8] and bulblet differentiation[4], the treatment was effective only when it was given to the explants just after the excision from mother

plants. Thus, anaerobic treatment seemed to be closely related with wounding. We also investigated the effects of traumatic acid and anaerobic treatment on bulblet differentiation in the cultured cells of *L. longiflorum*.

Materials and Methods

Plantlets of *Lilium longiflorum* Thunb. were grown *in vitro* as reported previously[3], the bulbs formed in the basal part of plantlet were harvested and were cut to 6 segments. For callus induction, the segments were cultured on the basal medium containing Murashige and Skoog's mineral salts[6], 4% sucrose and 0.25% Gelrite (Merck) (hereafter referred to as MS medium) with 1 μM of naphthaleneacetic acid (NAA) and 1 μM of benzyladenine (BA). The callus tissues were transferred to fresh medium with same composition at every 2 months and used experimental materials.

The 32 callus pieces (about 20 mg fresh weight) were cultured in a Petri dish (9×6 cm). To examine the effects on bulblet differentiation, various concentrations and combination of NAA and BA, A23187, traumatic acid, verapamil or Quin II AM were added to the MS medium. For anaerobic treatment, the callus pieces were arranged in open Petri dishes, the Petri dishes were places in a glass desicator and given N_2 treatment for various periods. Filter-sterilized N_2 at 1 bar was flowed (300 ml/min) through the desicator.

The cultures were maintained under 16 hr long-day photoperiod (6,000 lux) at constant temperature of 25±2°C. After 6 weeks of culture, number of bulblet formed in a piece of callus were observed.

Intracellular accumulation of free Ca^{2+} was measured as reported previously[5]. The cells cultured for 3 weeks were incubated in 50 μM Quin II AM for 1 hr, washed with water and then observed by a fluorescence microscope (Optiphot with EF, Nikon). For fluorescence excitation, an HBO 50-W mercury vapor lamp (Osram, FRG) was used with a U filter (Nikon) (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. Relative intensity for Quin II fluorescence was measured by a micro-photometer (P-1, Nikon). For calculation of intracellular Ca^{2+} concentration, Ca^{2+} solutions at various concentrations were prepared, the relative intensity of Quin II fluorescence was measured and the Ca^{2+} concentrations were calculated by the intensity[5].

Results and Discussion

Effects of phytohormones on bulblet differentiation

As shown in Table 1, application of NAA and BA to MS medium stimulated the adventitious bulblet induction. The best result was obtained when 0.1 μM of NAA and 0.1 μM of BA were added to the medium, and about 1.6 bulblets were formed in a callus piece. Some bulblets could be induced even when lily cells were cultured on medium without any phytohormones. The results suggested that endogenous contents of phytohormones in cultured cells seemed to be sufficient to initiate bulblets.

Table 1 Effects of NAA, BA, verapamil and Quin II AM on bulblet differentiation in lily cultured cells.

Concentration (μM)				No. of bulblet
NAA	BA	Verapamil	Quin II AM	per callus piece
0	0	0	0	1.2
0.1	0	0	0	1.2
0.1	0.1	0	0	1.6
0.1	0.1	100	0	0.2
0.1	0.1	0	10	0
0.1	1	0	0	0.9
1	0	0	0	1.4
1	0.1	0	0	1.0
1	0.1	100	0	0
1	0.1	0	10	0
1	1	0	0	0.6
0	0.1	0	0	1.3
0	0.1	100	0	0
0	0.1	0	10	0
0	1	0	0	1.1
0	1	100	0	0
0	1	0	10	0

The 32 callus pieces (about 20 mg fresh weight) were cultured on the medium contained NAA, BA, verapamil and Quin II AM. After 6 weeks of culture, number of bulblets formed in a callus piece was observed.

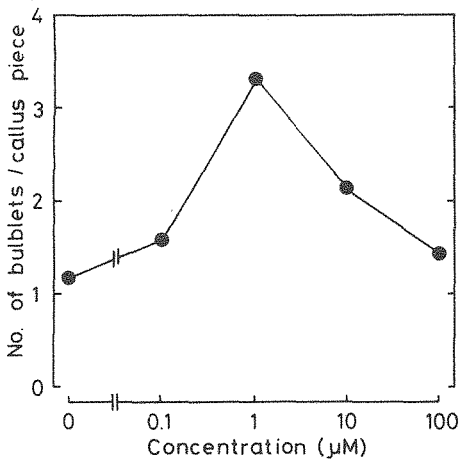

Fig. 1 Effects of calcium ionophore A23187 on bulblet differentiation in lily cultured cells.

The 32 callus pieces (about 20 mg fresh weight) were cultured on the medium contained various concentrations of A23187. After 6 weeks of culture, number of bulblets formed in a callus piece was observed.

The bulblet formation was suppressed by simultaneous addition of verapamil or Quin II AM (Table 1). Therefore, the roles of phytohormones for bulblet initiation seemed to be increase in endogenous Ca^{2+} concentration.

Involvement of Ca^{2+} in bulblet differentiation

Bulblet differentiation in cultured lily cells was strongly promoted by application of A23187 to the medium without phytohormone (Fig. 1), the largest number of bulblets, 3.6 per callus piece, was obtained with 1 μM A23187.

Another series of experiments was conducted using a Ca^{2+} channel inhibitor, verapamil, and a intracellular Ca^{2+} chelator, Quin II AM. As shown in Fig. 2, both chemicals strongly suppressed phytohormone-and A23187-induced bulblet for-

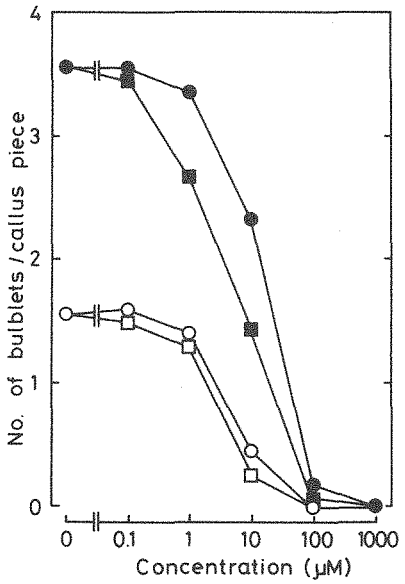

Fig. 2 Effects of verapamil and Quin II AM on bulblet differentiation in lily cultured cells.

The 32 callus pieces (about 20 mg fresh weight) were cultured on the medium contained various concentrations of verapamil (○, ●) or Quin II AM (□, ■) with 1 μM of A23187 (●, ■) or 0.1 μM of NAA and 0.1 μM of BA (○, □). After 6 weeks of culture, number of bulblets formed in a callus piece was observed.

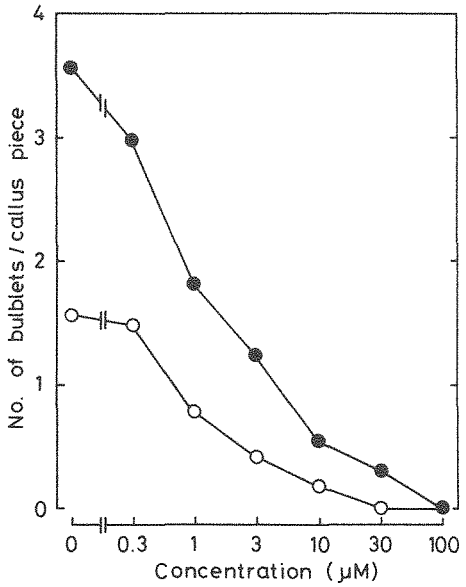

Fig. 3 Effects of W-7 on bulblet differentiation in lily cultured cells.

The 32 callus pieces (about 20 mg fresh weight) were cultured on the medium contained various concentrations of W-7 with 1 μM of A23187 (●) or 0.1 μM of NAA and 0.1 μM of BA (○). After 6 weeks of culture, number of bulblets formed in a callus piece was observed.

mation, and application of 0.1 mM verapamil or Quin II AM inhibited it completely, despite the presence of phytohormone and A23187 in the medium. Intracellular Ca^{2+} concentrations were increased in the cells cultured on the medium contained A23187 or phytohormones (Table 2), 1.0 μM and 0.6 μM Ca^{2+}were accumulated in cells cultured with A23187 and phypohormones (NAA and BA), respectively. In the case of lily bulb-scale segments, endogenous levels of Ca^{2+} were increased to 0.7 to 1.4 μM in segments cultured on the medium with phospholipids[5].

Table 2 Intracellular Ca^{2+} concetrations in lily cells.

Chemicals (Concentration)	Ca^{2+} concentration (μM)
None	0.3
NAA (0.1 μM) +BA (0.1 μM)	0.6
A23187 (1 μM)	1.0
A23187 (1 μM) +verapamil (0.1 mM)	0.01

The lily cells were cultured on the medium contained NAA, BA, A23187 and verapamil. After 3 weeks of culture, intracellular Ca^{2+} concentrations in cells were measured.

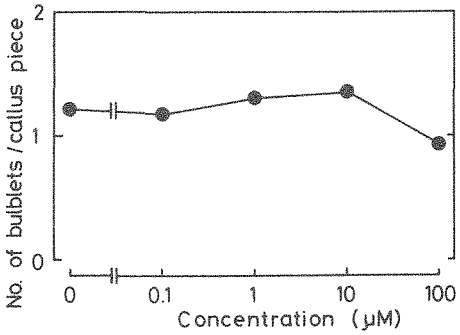

Fig. 4 Effects of traumatic acid on bulblet differentiation in lily cultured cells.
The 32 callus pieces (about 20 mg fresh weight) were cultured on the medium contained various concentrations of traumatic acid. After 6 weeks of culture, number of bulblets formed in a callus piece was observed.

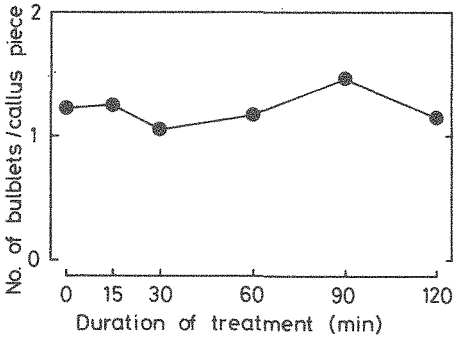

Fig. 5 Effects of anaerobic treatment on bulblet differentiation in lily cultured cells.
The lily callus pieces were arranged in Petri dishes with basal medium, treated with N_2 stream for various period, and then cultured on the basal medium. After 6 weeks of culture, number of bulblets formed in a callus piece was observed.

Adventitious bulblet differentiation in the cells cultured on the medium with phytohormone or A23187 was strongly suppressed by simultaneous application of W-7 (Fig. 3). The 50% inhibition was apparent in 1 μM W-7, and addition of 100 μM W-7 completely inhibited bulblet induction (Fig. 3). We tried to isolate CaM in lily cells and purified it. The content of lily CaM was about 3 μg per g fresh weight and the molecular weight was 17,000. The CaM was seemed to be same that purified from lily bulbs[12]. These results suggested that CaM is a main regulator for bulblet induction in lily bulb-scale segments and cultured cells.

Effects of traumatic acid and anaerobic treatment on bulblet differentiation

When lily cells were cultured on the basal MS medium contained various concentrations of traumatic acid, the number of bulblets formed in a callus piece was less than 2, and promotive effects can not be observed (Fig. 4). The anaerobic treatment was also ineffective (Fig. 5). Traumatic acid was thought to be one of the plant wound hormones[2]. Although the anaerobic treatment was promoted bud[8] and bulblet differentiation[4], the treatment was effective only when it was given to the explants just after the excision from mother plants. Therefore, application of traumatic acid and anaerobic treatment seemed to be closely related with wounding. In cultured cells, wounding was not related to bulblet differentiation.

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鉄砲ユリ培養細胞からの球根分化

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摘　　要

鉄砲ユリ培養細胞からの球根分化は植物ホルモン無添加の場合にも誘導されるが，植物ホルモン（オーキシンとサイトカイニン），Ca^{2+}イオノフォアなどの添加によって促進される．Ca^{2+}チャンネルの阻害剤や細胞内Ca^{2+}のキレート剤は分化を阻害する．細胞内のCa^{2+}濃度は分化促進処理によって著しく上昇する．Ca^{2+}-結合蛋白質であるカルモデュリンが培養細胞に存在すること，カルモデュリン阻害剤が分化を阻害することなどから，Ca^{2+}濃度の上昇がカルモデュリンを活性化させ，そのカルモデュリンが球根分化を制御しているという機構が考えられる．