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Studies on Bulblet Differentiation in Bulb-scale Segments of *Lilium longiflorum* III. Promotive effects of phospholipid

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Summary

In vitro cultured bulb-scale segments of *Lilium longiflorum* Thunb., adventitious bulblet differentiation can be induced when the segments were cultured on the medium containing some glycerophospholipid, such as phosphatidic acid, lysophosphatidic acid, phosphatidylinositol, phosphatidylserine, phosphatidylethanolamine, phosphatidylcholine, phosphatidylglycerol and dioleine. Intracellular concentration of free calcium ions in the segments was increased by the application of above phospholipid. Therefore, the promotive effects of phospholipid was thought to be due to the release of calcium ions from organelle or vacuole to cytosol, and the released calcium ions induced bulblet differentiation. The protein kinase C was thought to be stimulated by the released calcium ions, and the kinase was also stimulated by phorbol ester. The application of phorbol ester also promoted bulblet induction. These results support the idea that bulblet initiation induced by phospholipid or phorbol ester may be mediated, at least partially, by protein kinase C.

Key words: bulblet differentiation, intracellular calcium ion, *Lilium longiflorum,* phorbol ester, phospholipid

Introduction

Adventitious bulblet differentiation in the bulb-scale segments of *Lilium longiflorum* was induced by application of cytokinin and auxin, and stimulated by wounding treatment and addition of traumatic acid[2]. In the case of adventitious bud initiation in *Torenia* stem segments, additional wounding treatment[5] and application of traumatic acid[6] stimulated bud initiation, and increment in intracellular Ca^{2+} also promoted this phenomena[7]. Increment in Ca^{2+} was seemed to be essential for adventitious organ induction.

Intracellular concentration of free Ca^{2+} were maintained in relatively low levels by active transport systems in plasma membrane Ca^{2+}-ATPase and accumulation of Ca^{2+} in organelle and vacuoles[1]. The Ca^{2+} release from organelle and vacuoles to cytoplasm was attained by action of some phospholipid[1].

Intracellular concentration of free Ca^{2+} can be measured using Ca^{2+}-binding fluorescent indicators such as Quin II[8]. This chemical specificaly bind with Ca^{2+} and Ca^{2+} concentration can be detected by the intensity of fluorescence. According to the report of

Tsien *et al.*[9], 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[8].

Therefore, we tried to examine the effects of phospholipid on adventitious bulblet induction in lily bulb-scale segments, and measured intracellular concentration of Ca^{2+} in the segments.

The released Ca^{2+} by phospholipid was thought to stimulate protein kinase C (PK-C), and PK-C was also stimulated by phorbol ester[3]. Thus we tried to examine the effects of phorbol ester on bulblet initiation using 12-0-tetradecanoyl phorbol-13-acetate (TPA) and phorbol-12,13-dibutyrate (PBt_2).

Materials and Methods

Plantlets of *Lilium longiflorum* Thunb. were grown in vitro as reported previously[2], 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 culture medium containing Murashige and Skoog's mineral salts[4], 4 % sucrose and 0.25 % Gelrite with 0.1 μM naphthaleneacetic acid (NAA) and 1 μM benzyladenine (BA). Solutions of phosphatidic acid (PA), lysophosphatidic acid (LPA), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylglycerol (PG), diolein (DO), TPA and PBt_2 were sterilized through Millipore filter (0.22 μm) and added to the culture medium. The cultures were maintained under 16 hr long day photoperiod (6,000 lux) and constant temperature at 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.

To examine Ca^{2+} release to cytoplasm, $CaCl_2$ was eliminated from basal culture medium and ethyleneglycol-bis-(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) was added to medium at 0.8 mM.

Intracellular concentration of free Ca^{2+} were measured as follows. The explants cultured on the medium with phospholipids were sliced to sections (0.5 mm in deepth), and incubated in 50 μM Quin II AM (Dojin, Japan) 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 filter (Nikon, Japan) (broad band excitation peak at 340 nm) for Quin II AM excitation. Fluorescence of Quin II were monitored at the wavelength longer than 490 nm. Relative intensity for Quin II fluorescence was measured by a micro-photometer (Nikon P-1, Japan). For calculation of intracellular Ca^{2+} concentration, Ca^{2+} solutions at various concentrations were prepared, the relative intensity of Quin II fluorescence was measured (Fig. 1) and the Ca^{2+} concentrations were calculated by the intensity.

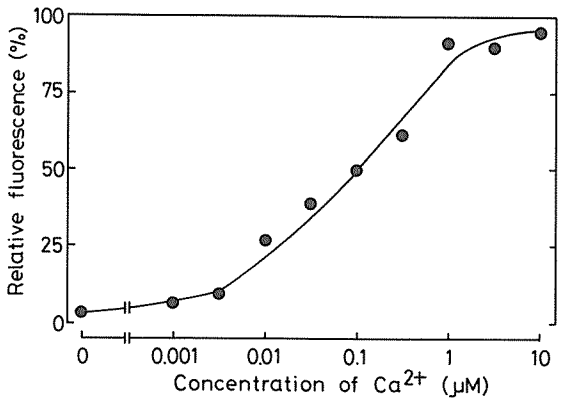

Fig. 1. Correlation between Ca^{2+} concentrations of solutions and relative flourescence. The Ca^{2+}-binding fluorescent indicator, Quin II was added to the various concentrations (0.001 to 10 μM) of Ca^{2+} solution, and the relative fluorescence intensity was measured using fluorescence microscope and micro-photometer.

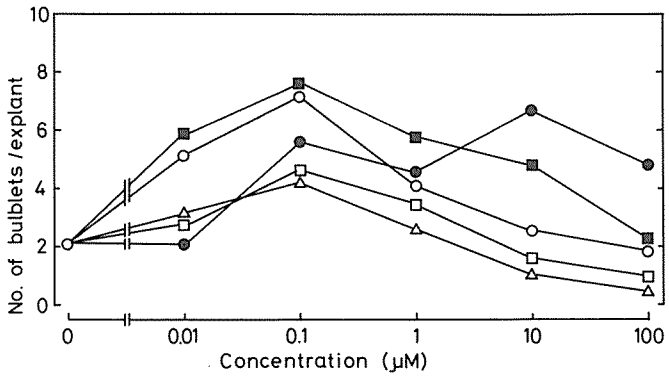

Fig. 2. Effects of phospholipid on bulblet initiation in lily bulb-scale segments (1). The bulb-scale segments were cultured for 3 weeks on the medium containing phosphatidylinositol (○), phosphatidylethanolamine (□), phospatidylcholine (△), phosphatidylserine (●) or phosphatidylglycerol (■).

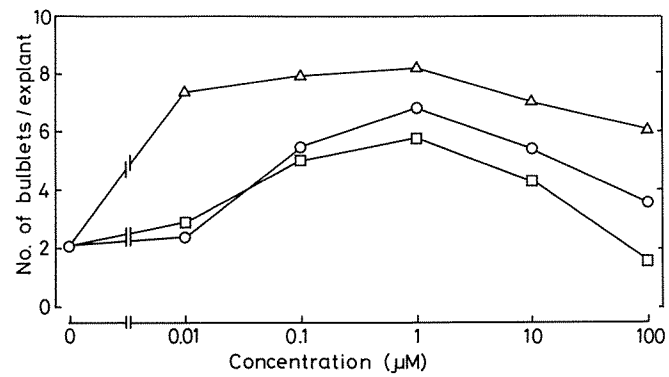

Fig. 3. Effects of phospholipid on bulblet initiation in lily bulb-scale segments (2). The bulb-scale segments were cultured for 3 weeks on the medium containing phosphatidic acid (○), lysophosphatidic acid (□) or diolein (△).

Table 1. Intracellular concentrations of Ca^{2+} in bulb-scale segments cultured on medium containing phospholipid.

Phospholipid	Concentration (μM)	Intracellular concentration of Ca^{2+} (μM)	No. of bulblets per explant
No addition	0	0.02	1.8
PA	1	0.9	8.2
LPA	1	0.8	7.2
PI	0.1	1.2	8.6
PE	0.1	0.8	5.8
PC	0.1	0.7	5.2
PS	10	0.9	8.0
PG	0.1	1.0	8.4
DO	1	1.4	8.8

The bulb-scale segments were cultured on the medium without Ca^{2+} and added 0.8 mM EGTA with some phospholipids such as phosphatidic acid (PA), lysophosphatidic acid (LPA), phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylglycerol (PG) or diolein (DO). The cultured segments were sliced 0.5 mm in deepth and incubated with 50 μM Quin II AM for 1 hr, and observed fluorescent microscope. The relative intensity of fluorescence was measured by micro photometer and then Ca^{2+} concentration was calibrated by Fig. 1.

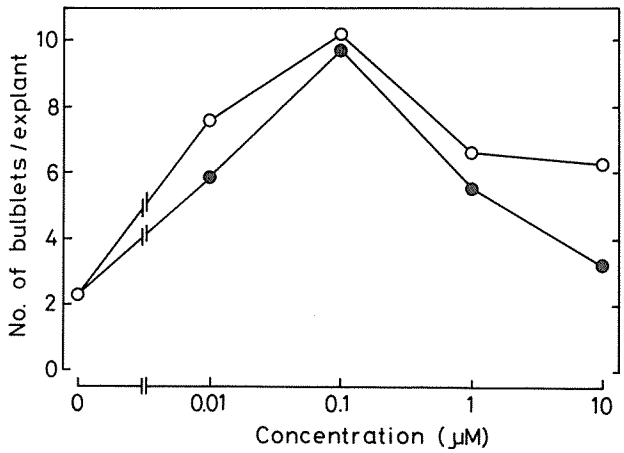

Fig. 4. Effects of phorbol ester on bulblet initiation in lily bulb-scale segments. The bulb-scale segments were cultured on the medium containing 12-0-tetradecanoyl phorbol-13-acetate (○) or phorbol-12,13 -dibutyrate (●).

Results and Discussion

The number of bulblets formed in the explants cultured on basal culture medium without phospholipid was about 2.1. The application of all of phospholipids examined significantly increased the number of bulblets (Fig. 2, 3). The most effective concentration was different in each chemical, 0.1 μM in PI, PE, PC and PG, 1 μM in PA, LPA and DO, and 10 μM in PS. The largest number of bulblets, 8.2 per explant, was obtained at 1 μM of DO. (Fig. 3). The Ca^{2+} accumulated in the organelle and vacuoles was released by phospholipid in animal and microbial cells[1]. To examine the roles of phospholipid, the intracellular concentrations of Ca^{2+} was measured. The explants were cultured on the

medium without Ca^{2+}, and containing 0.8 mM of EGTA and various phospholipids. As shown in Table 1, the endogenous concentrations of Ca^{2+} were remarkably increased by the application of phospholipids.

The released Ca^{2+} by phospholipid was thought to stimulate PK-C activity, and PK-C acted phosphorylation of some proteins. Stimulation of PK-C activity was also attained by phorbol ester[3]. Therefore, we examined the effects of phorbol ester such as TPA and PBt_2. The results was shown in Fig. 4, and both TPA and PBt_2 strongly promoted bulblet differentiation at very low concentration (0.1 μM). These results support the idea that bulblet initiation induced by phospholipid or phorbol ester may be mediated, at least partially, by protein kinase C.

References

1. Hirata, M., T. Ishimatsu, T. Sasaguri and H. Kuriyama (1986). Ca^{2+} release by inositol 1,4,5 -triphosphate. *Protein Nucleic acid and Enzyme* **31**, 1761-1770.
2. Ishioka, N. and S. Tanimoto (1990). Studies on bulblet differentiation in bulb-scale segments of *Lilium longiflorum* I. Effects of wounding and traumatic acid. *Bull. Fac. Agr., Saga Univ.* **69**, 27-33.
3. Kajikawa, N. and K. Ase (1986). The role of phospholipid dependent protein kinase in stimulus -response coupling. *Protein Nucleic acid and Enzyme* **31**, 1871-1878.
4. Murashige, T. and F. Skoog (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15**, 473-497.
5. Takeuchi, N., S. Tanimoto and H. Harada (1985). Effects of wounding on adventitious bud formation in *Torenia* stem segments cultured in vitro. *J. Exp. Bot.* **36**, 841-847.
6. Tanimoto, S. and H. Harada (1984). Stimulation of adventitious bud initiation by cyclic AMP and traumatic acid in *Torenia* stem segments. *Biol. Plant.* **26**, 337-341.
7. Tanimoto, S. and H. Harada (1986). Involvement of calcium in adventitious bud initiation in *Torenia* stem segments. *Plant Cell Physiol.* **27**, 1-10.
8. Tsien, R. Y., T. Pozzan and T. J. Rink (1982). T-cell mitogens cause early changes in cytoplasmic free Ca^{2+} and membrane potential in lymphocytes. *Nature* **295**, 68-71.
9. Tsien, R. Y., T. Pozzan and T. J. Rink (1982). Calcium homeostasis in intact lymphocytes: Cytoplasmic free calcium monitored with a new, intracellularly trapped fluorescent indicator. *J. Cell Biol.* **94**, 325-334.

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

III. リン脂質の分化促進効果

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摘　　要

鉄砲ユリの鱗片切片培養において，切片をリン脂質を含む培地で培養することにより，球根分化が促進される．効果のあるリン脂質としては，フォスファチジン酸，リソフォスファチジン酸，フォスファチジルイノシトール，フォスファチジルセリン，フォスファチジルエタノールアミン，フォスファチジルコリン，フォスファチジルグリセロール，ディオレインなどがある．これらのリン脂質の添加によって細胞内のカルシウムイオン濃度が上昇するので，リン脂質の効果は細胞内小器官や液胞に蓄積されているカルシウムイオンを細胞質へ動員することによるものと思われる．動員されたカルシウムイオンは蛋白質燐酸化酵素C（PK—C）を活性化し，またPK—Cはホルボールエステルによっても活性化されることが知られている．そこで，ホルボールエステルの球根分化に対する影響を調べたところ，球根分化は著しく促進された．従って，鉄砲ユリの球根分化はPK—Cにより制御されているのではないかと考えられた．