Changes in Proteins during Bulblet Differentiation in *Lilium longiflorum*

Nahoko ISHIOKA and Shizufumi TANIMOTO
(Genetic Engineering Laboratory)
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Summary

Adventitious bulblets were successfully induced in lily bulb-scale segments, cultured cells and leaf segments cultured on the medium containing auxin and cytokinin. The differentiation was promoted by anaerobic treatment and by addition of some chemicals. In the bulb-scale segments, the differentiation induced by phytohormones or calcium ionophore was inhibited by such RNA and/or protein synthesis inhibitors as actinomycin-D and cycloheximide. During bulblet differentiation, protein contents progressively increased in the bulb-scale segments, cultured cells and leaf segments. The polyacrylamide gel electrophoresis profile of denatured proteins in the explants cultured on the differentiation and non-differentiation media differed to some extent. Three major bands of newly synthesized polypeptides having molecular weights of 17k, 32k and 65k daltons were detected in the explants with induced bulblets. We thought that these polypeptides are essential for adventitious bulblet differentiation in lily.

Key words : bulblet differentiation, calcium, cyclic AMP, *Lilium longiflorum,* protein synthesis.

Introduction

Several reports have demonstrated biochemical regulation of protein synthesis associated with organogenesis in several plant species[16]. Namely, Thorpe and Meier[15] pointed out higher rate of protein content in bud-forming tobacco callus than in non-bud-forming callus. The presence of specific proteins related to adventitious organ differentiation in carrot callus was suggested by Syono[9]. Similar observation was also made in the organogenesis from tobacco callus by Sekiya and Yamada[8]. In the bud-forming cultures of Douglas fir cotyledons, specific proteins of low molecular weight (MW) was synthesized[1, 17]. We have previously reported that 3 groups of polypeptides appearred during adventitious bud initiation in *Torenia* stem segments[10].

Adventitious bulblets were successfully induced in lily bulb-scale segments[2], cultured cells[14] and leaf segments[5] cultured on the medium containing auxin and cytokinin. The differentiation was promoted by anaerobic treatment[3, 5] and by addition of traumatic acid[2], phospholipid[4], calcium ionophore A23187[5, 11], dibutyryl cyclic AMP (Bt_2cAMP)[5, 13] or polyamine[5]. Therefore, important biochemical events could be responsible for organogenesis in the process of lily bulblet differentiation.

In this article, *de novo* protein synthesis in an early stage of bulblet differentiation is

presented in the bulb-scale segments, cultured cells and leaf segments of lily.

Materilas and Methods

Experimental materials

Plantlets of *Lilium longiflorum* Thunb. were grown *in vitro* as described previously[2] and the bulbs (about 15 mm in diameter) formed in the basal part of plantlets were harvested. The outer 2 scales of these bulbs were transversally cut into 6 segments and used as bulb-scale segments. The leaves formed in the apical part of outer scales were also harvested, cut into 5 mm width and used as leaf segments. For callus induction, bulb-scale segments were cultured on the medium consisting of Murashige and Skoog's mineral salts and vitamines[7], 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 as cultured cells.

Culture conditions

As bulblet-forming medium, MS medium was supplemented with 0.1 μM of NAA and 1 μM of BA for bulb-scale segments, done with 10 μM of NAA and 10 μM of BA for leaf segments, and done with 0.1 μM of NAA and 0.1 μM of BA for cultured cells. In some experiments, the medium was further supplemented with various concentrations of calcium ionophore A23187 (Calbiochem-Behring), Bt_2cAMP (Sigma), traumatic acid or putrescine (Sigma). For anaerobic treatment, the bulb-scale and leaf segments were arranged in open Petri dishes (9×6 cm), placed in a glass desiccator, and exposed for 1 hr to filter-sterilized N_2 gas at a flow rate of 300 ml/min. To examine the effects of RNA and protein synthesis inhibitors, actinomycin-D (Act-D) or cycloheximide (CHI) (both from Sigma) was added to the medium. The culture was maintained under 16 hr long day photoperiod (6,000 lux) at constant temperature of 25 ± 2°C.

Protein extraction and measurement of protein content

The explants were harvested and their fresh weights were determined. Extraction buffer containing 62.5 mM Tris (hydroxy-methyl) aminomethane-HCl buffer (pH 6.8) with 2% sodium dodecyl sulfate (SDS), 10% glycerol, 5% 2-mercaptoethanol, 1 μM (p-amidino-phenyl) methanesulfonyl fluoride (Wako) and 0.02% bromophenol blue was added to harvested explants. The material was homogenized and the filtrated through 4 layers of gauze and 2 layers of Miracloth (Calbiochem-Behring). The filtrate was boiled at 100°C for 10 min to denature proteins, followed by the centrifugation at 15,000 g for 15 min. The resulted supernatant was dialysed overnight against extraction buffer and used as crude soluble protein samples. The amount of soluble protein sample was estimated by DC Protein Assay kit from Bio-Rad, and the results were expressed in mg protein per g fresh weight tissues.

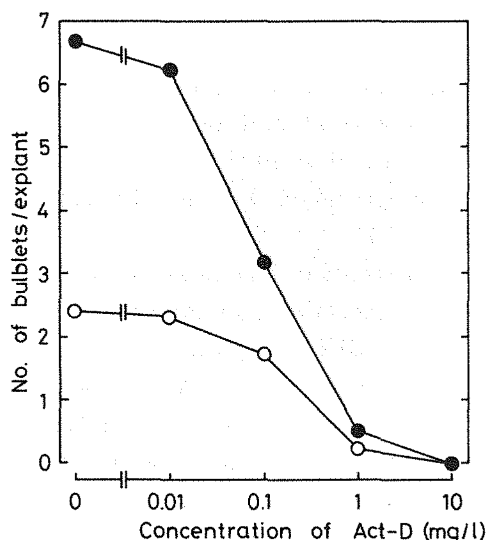

Fig. 1 Effects of actinomycin-D on bulblet differentiation in bulb-scale segments.
The bulb-scale segments were cultured for 3 weeks on the medium containing 0.1 μM of NAA and 1 μM of BA with (●) or without (○) 1 μM of A23187. Various concentrations of actinomycin-D was simultaneously added to the medium at the onset of segment culture.

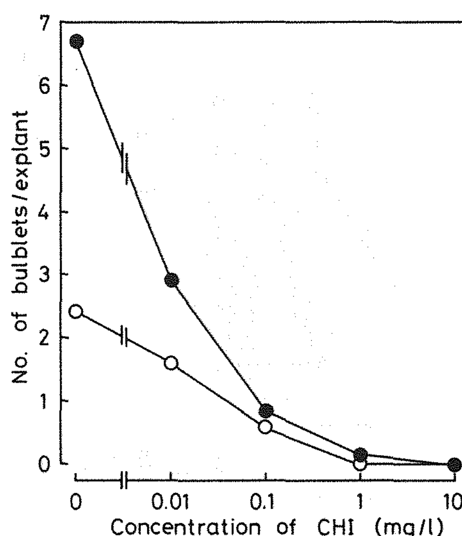

Fig. 2 Effects of cyclohexymide on bulblet differentiation in bulb-scale segments.
The bulb-scale segments were cultured for 3 weeks on the medium containing 0.1 μM of NAA and 1 μM of BA with (●) or without (○) 1 μM of A23187. Various concentrations of cyclohexymide was simultaneously added to the medium at the onset of segment culture.

SDS-polyacrylamide gel electrophoresis

One week cultures of bulb-scale and leaf segments and 3 weeks culture of callus of lily were harvested from respective media, and measured for their fresh weight. The soluble proteins were extracted by above procedure and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) following after Laemmli's method[6] with a slight modification; 0.1% tetramethylethylenediamine was used for polymerization of acrylamide. Separation gel (190×100×2 mm) was made with a gradient of acrylamide (12-20%). About 50 μg of a protein sample was applied on a gel. After SDS-PAGE at a constant current of 30 mA for 3 hr, the gel was fixed and stained for 1 hr with a solution containing 10% trichloroacetic acid, 10% acetic acid, 30% ethanol and 0.1% Coomassie Brilliant Blue R-250 (CBBR) and then washed overnight in the same solution without CBBR. Calibration proteins used for determination of MW were cytochrome C monomer (12,400 daltons), dimer (24,800 daltons), trimer (37,200 daltons), tetramer (49,600 daltons) and hexamer (73,800 daltons).

Results and Discussion

Effects of Act-D and CHI

Effects of Act-D and CHI on bulblet differentiation were examined using bulb-scale segments as experimental materials. As shown in Figs. 1 and 2, application of Act-D and/

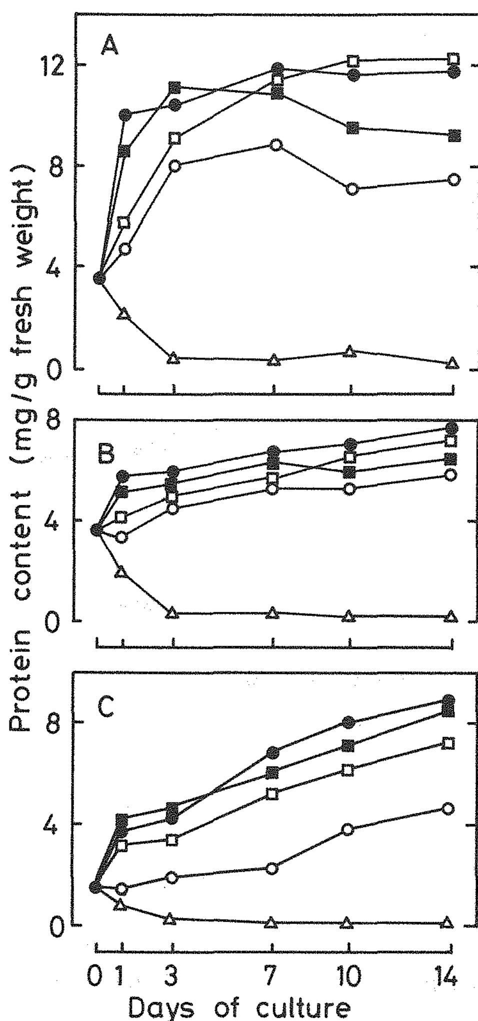

Fig. 3 Changes in protein contents during bulblet initiation.
A; The bulb-scale segments were cultured on the medium containing 0.1 μM of NAA and 1 μM of BA without (○) or with 1 μM of A23187 (●), 0.1 μM of Bt_2cAMP (□) or 1 mg/l of CHI (△), or the segments were treated with N_2 stream (■) for 1 hr. The protein contents in the segments were measured.
B; The leaf segments were cultured on the medium containing 10 μM of NAA and 10 μM of BA without (○) or with 10 μM of A23187 (●), 1 μM of Bt_2cAMP (□) or 1 mg/l of CHI (△), or the segments were treated with N_2 stream (■) for 1 hr. The protein contents in the segments were measured.
C; The callus cells were cultured on the medium containing 0.1 μM of NAA and 0.1 μM of BA without (○) or with 1 μM of A23187 (●), 0.1 μM of Bt_2cAMP (□) or 1 mg/l of CHI (△), or the cells were treated with N_2 stream (■) for 1 hr. The protein contents in the cells were measured.

or CHI strongly inhibited bulblet differentiation. The concentrations of inhibitors which substantially suppressed bulbet formation were 10 mg/l for Act-D and 1 mg/l for CHI. Both Act-D and CHI inhibited A23187-promoted bulblet differentiation (Figs. 1, 2).

Adventitious bud initiation in *Torenia* stem segments were also suppressed by Act-D and CHI; complete inhibition was recorded at 3 mg/l for Act-D and at 0.3 mg/l for CHI[10]. The RNA synthesis inhibitor, Act-D, inhibited bud and bulblet formation. These facts indicated that transcription of new mRNAs and ensuing protein *de novo* synthesis may be essential for the differentiation.

Changes in protein contents

In the case of bulb-scale segments, they initially contained about 3.5 mg of

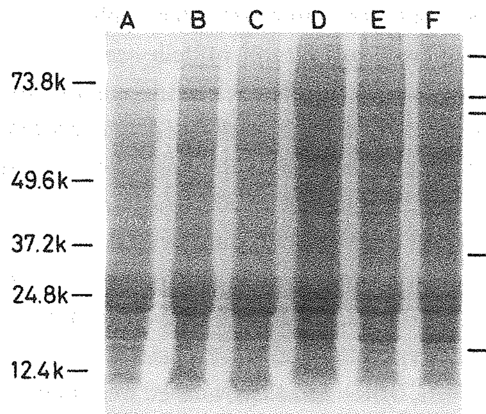

Fig. 4 The SDS-PAGE profile of polypeptides in the bulblet-initiating bulb-scale segments.
The proteins were extracted from initial bulb (A) and the bulb-scale segments cultured on the medium containing 0.1 μM of NAA and 1 μM of BA with or without (B) 1 μM of A23187 (C), 0.1 μM of Bt_2cAMP (D) or 1 μM of traumatic acid (E), or the segments were treated with N_2 stream (F) for 1 hr. The proteins were denatured and applied to SDS-PAGE. Bars indicate newly synthesized polypeptides.

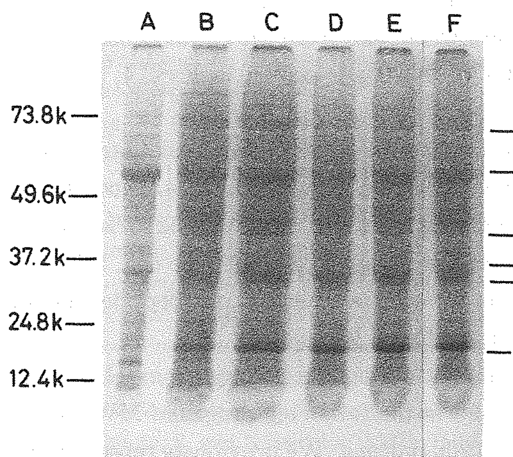

Fig. 5 The SDS-PAGE profile of polypeptides in the bulblet-initiating leaf segments.
The proteins were extracted from initial leaf (A) and the leaf segments cultured on the medium containing 10 μM of NAA and 10 μM of BA with or without (B) 10 μM of A23187 (C), 1 μM of Bt_2 cAMP (D) or 0.1 mM of putrescine (E), or the segments were treated with N_2 stream (F) for 1 hr. The proteins were denatured and applied to SDS -PAGE. Bars indicate newly synthesized polypeptides.

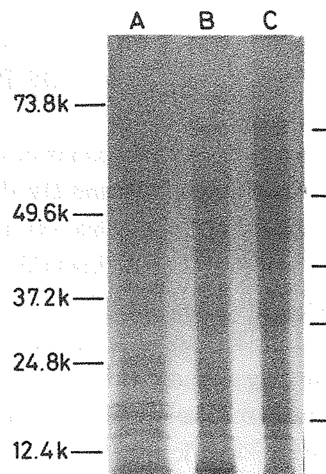

Fig. 6 The SDS-PAGE profile of polypeptides in the bulblet-initiating callus cells.
The proteins were extracted from initial callus (A) and tthe callus cells cultured on the medium containing 0.1 μM of NAA and 0.1 μM of BA with or without (B) 1 μM of A23187 (C). The proteins were denatured and applied to SDS-PAGE. Bars indicate newly synthesized polypeptides.

proteins per g fresh weight. On the medium with NAA and BA, the protein content progressively increased in an early period of culture and gradually decreased toward the 10th day of culture (Fig. 3A). Although the increasing rate of protein content in the segments cultured on the medium containing 0.1 μM of NAA, 1 μM of BA and 0.1 μM of Bt_2cAMP was almost same to that recorded in the explants cultured on the medium without Bt_2cAMP. When the segments were cultured on the medium containing 1 mg/l of CHI, the content was substantially decreased. Further addition of A23187 to the medium containing NAA and BA or the anaerobic treatment of the segments increased remarkably protein content at the 1st day of culture, about 12 mg proteins per g fresh weight were accumulated (Fig. 3A).

In leaf segments, protein content was not increased remarkably during bulblet formation, and not significantly promoted by A23187, Bt_2cAMP and anaerobic treatment (Fig. 3B).

As shown in Fig. 3C, the protein content in the initial callus cells was 1.5 mg per g fresh weight, and the content was almost same as in the bulb-scale segments. The cells cultured on the medium with A23187 or Bt_2cAMP, or with anaerobic treatment, 8 mg of proteins were accumulated after 2 weeks of culture.

Qualitative changes in newly synthesized proteins

Qualitative changes in *de novo* synthesized proteins during an initial stage of bulblet

differentiation were examined by SDS-PAGE. The protein samples were prepared from the segments of bulb-scale and leaf cultured for 1 week, and from the callus cells cultured for 3 weeks. The typical SDS-PAGE profiles of them are shown in Figs. 4, 5 and 6, respectively.

There were 5 major bands of newly synthesized polypeptides corresponding to MW of about 80k, 65k, 60k, 32k and 17k daltons for the bulb-scale segments (Fig. 4). In the case of leaf segments, the *de novo* synthesized polypeptides were 65k, 56k, 40k, 36k, 32k and 17k daltons (Fig. 5). The polypeptides newly synthesized in cultured cells were 65k, 56k, 40k, 32k and 17k daltons (Fig. 6). Three major bands of newly synthesized polypeptides having MW of 17k, 32k and 65k daltons were common to the all samples.

Working with an early stage of organogenesis in the Douglas fir cotyledons, Hasegawa et al.[1] reported the presence of major polypeptides in the following ranges of MW; 76k to 84k, 52k to 58k, 37k to 42k, 24k to 27k, and 16k to 20k daltons. We previously reported that the 3 peaks of polypeptides ranging from 50k to 60k, 35k to 40k and 24k to 30k were found in *Torenia* stem segments during bud initiation[10] hae similar MW as those reported in Douglas fir cotyledons. However, the low MW polypeptides (16k to 20k daltons) which seemd to be related to bud formation in the Douglas fir explants[1, 17] were not detected in *Torenia* stem segments[10]. In lily explants, specific low MW polypeptide (17k daltons) was appeared in bulb-scale, leaf and callus explants. Previously we elucidated that the possible involvement of calmodulin (CaM) in bulblet differentiation and extracted this mediator protein from lily bulb[12], leaf[5] or callus cells[14]. It is interesting fact that the MW of CaM in lily is 17k[5, 12, 14] and the same MW to the newly synthesized polypeptide noted above. Although CaM was thought to be house-keeping protein and could not be synthesized by any stimulus, it may present certain relations between CaM synthesis and organogenesis.

We conclude that this low MW polypeptide may be essential for adventitious organ differentiation in higher plants. Efforts are directed to obtain more information regarding the newly synthesized proteins during bulblet differentiation, and try to isolate the genes encoding these proteins.

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鉄砲ユリの球根分化時における蛋白質の変動

石岡奈穂子・谷本　靜史
(生物工学講座・遺伝子工学研究室)
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摘　　要

鉄砲ユリの鱗片切片，培養細胞，葉切片からの球根分化はオーキシンとサイトカイニンを添加した培地で培養することにより達成された．その分化は，嫌気処理やいくつかの化学物質によって促進された．鱗片切片では，植物ホルモンやカルシウムイオノフォアによる分化誘導は(RNAあるいは蛋白質合成の阻害剤である) actinomycin-D あるいは cycloheximide の添加によって阻害された．鱗片切片，培養細胞及び葉切片からの球根分化時には蛋白質含量は急速に増加した．分化処理を行った外植体からのポリペプチドの電気泳動パターンは分化処理を行わなかったものと比べて有意に異なっていた．球根が分化する外植体において分子量17k，32k，65kのポリペプチドが特異的に出現し，これらのポリペプチドが球根分化に対してなんらかの役割を担っているものと思われた．