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Studies on Bulblet Differentiation in Bulb-scale Segments of *Lilium longiflorum*

I. Effects of wounding and traumatic acid

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Summary

In 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 cytokinin and auxin. If the bulb-scale was transversally cut off to several fragments. the number of bulblets per bulb-scale progressively increased in proportion to wounding area of bulb-scale. When one of the presumed wounding hormones, traumatic acid, were added to the culture medium, the bulblet differentiation was significantly promoted. The short term treatment with solution of traumatic acid was also effective only when it was given to the explants just after their excision from bulb-scales. Simultaneous application of anti-auxin or anti-cytokinin did not affect the promotive action of wounding and traumatic acid in bulblet differentiation.

Key words: bulblet differentiation, bulb-scale culture, *Lilium longiflorum*, traumatic acid, wounding.

Introduction

Since Skoog and Miller[6] reported that adventitious organ differentiation in callus culture was controlled by a balance between auxin and cytokinin applied to the culture medium, many researchers reported that same phenomena were observed in a large number of plant species (review by Thorpe[18]). When organ fragments were used as explants, however, adventitious buds could be formed by the addition of cytokinin alone to the medium as reported in *Torenia*[10], *Perilla*[11] and *Rudbeckia*[12]. Promotive effects of cytokinin on bud initiation in stem segments of *Torenia* was counteracted by auxin which was either applied or already present in the explant[13, 14, 16]. The differences between a piece of callus and an organ explant with regard to their response to phytohormones may arise from the fact that the former is not wounded while the latter is damaged during excision.

The adventitious bulblet was usually formed by addition of phytohormones such as auxin and cytokinin[19]. About 4 bulblets were formed per bulb-scale explant in *L. longiflorum*[7, 8] and about 5 in *L. speciosum*[20]. The number increased significantly when wounding was given to the explants, about 13 bulblets were formed in the explants cultured with naphthaleneacetic acid (NAA), and application of triiodobenzoic acid (TIBA) also stimulated the bulblet differentiation in non-wounded explants[21]. Van Aartrijk and Blom

-Barnhoorn[22] demonstrated that wounding and NAA interacted in influencing the regeneration process, and NAA could be substituted for wounding.

Wounding treatment applied to plant tissues is known to cause several changes at different levels such as protein synthesis[2], DNA synthesis[23] and cell division[1]. These results suggest that excising organ explants from mother plants may provoke a series of biochemical events which are closely related to adventitious organ differentiation. In fact, adventitious bud initiation in *Torenia* stem segments was induced when an anaerobic treatment was given just after the excision of explants[15]. The treatment may favourably modify some physiological or biochemical processes which are triggered by wounding, leading to the formation of adventitious buds. Furthermore, additional wounding treatment promoted the bud initiation and the wounding treatment did not affect the uptake into explants and the distribution pattern of cytokinin applied to the culture medium[9]. Therefore, we tried to examine the exact roles of wounding on bulblet differentiation in bulb-scale explants of *L. longiflorum*.

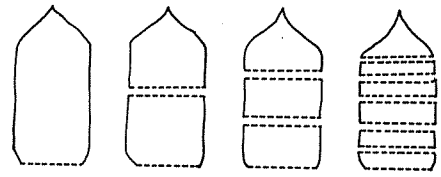
In *Torenia* stem segments, application of traumatic acid (TA) stimulated bud initiation[17]. This chemical was thought to be one of the plant wound hormones[3], and Margara[4] found that TA promoted bud formation in explants of cauliflower heads. We also investigated the effects of TA on bulblet differentiation in the bulb-scale cultures.

In case of that wounding and TA stimulated bulblet differentiation, the effects seem to be interacted in phytohormones such as auxin or cytokinin. Thus, correlative effects of anti-cytokinin, 4-cyclobutylamino-2-methylpyrrolo [2, 3-d] pyrimidine (CB-P) or anti -auxin, TIBA, and wounding or traumatic acid on bulblet differentiation was also examined.

Materials and Methods

Apical parts of *Lilium longiflorum* Thunb. plants were sterilized with 15% NaOCl solution for 30 min and rinsed with sterilized water. These apices were excised from the apical parts and cultured on medium containing Murashige and Skoog's mineral salts[5], 4% sucrose and 0.25% Gelrite (hereafter referred to as MS medium) with 0.1 μM NAA. After 1 months of culture, bulbs formed in the basal part of regenerated plantlet. The bulbs were developed to 15 mm in diameter after 3 months, the outer 2 bulb-scales were used as materials. The size of material bulb-scale was about 12 mm in length, 8 mm in width and 6 mm in depth.

The bulb-scale was transversally cut to several fragments (Fig. 1), and cultured on the basal culture medium containing MS medium with 0.1 μM of NAA and 1 μM of benzyladenine (BA). Solutions of TA, TIBA and CB-P (kindly supplied from Dr. H. Iwamura, Kyoto University) were sterilized through a Millipore filter (0.22 μm) and added to the culture medium. The short term treatment with TA was performed as follows. The explants were taken from bulb-scales, cultured on the basal medium for various durations (0 to 24 hr), then incubated in 1 μM of TA solution for 1 hr and then cultured on the basal medium. The cultures were maitained under 16 hr long day photoperiod (6,000 lux) and



Explant	A	B	C	D
Wounding area (cm^2)	0.38	1.14	1.90	4.18
No. of bulblets / explant	1.4±0.1	2.1±0.2	2.3±0.2	2.5±0.3
No. of bulblets / bulb-scale	1.4±0.1	4.2±0.4	6.9±0.5	15.0±0.9

Fig. 1 Bulblet differentiation in various kinds of bulb-scale explants. The bulb-scales were transversally cut to several fragments (explants), and the explants were cultured on the medium containing 0.1 μM of NAA and 1 μM of BA. For each treatment, at least 200 explants were used, the experiments were repeated at least 3 times and the standard errors were then calculated.

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.

Results and Discussion

Effects of wounding

The material bulb-scales were transversally cut to several fragments (shown in Fig. 1 as explants A, B, C and D), the wounding area per bulb-scale was measured, and cultured on the MS medium with 0.1 μM NAA and 1 μM BA. After 3 weeks, bulblet number initiated in each explant was observed. As shown in Fig. 1, the numbers of bulblet formed were 1.4 per explant in the explant A to 2.5 in the explant D, and 1.4 per bulb-scale in the explant A to 15.0 in the explant D (Fig. 1). Correlation between wounding area of bulb-scale and the number of bulblet was shown in Fig. 2, and the bulblet number increased in proportion to increment in wounding area.

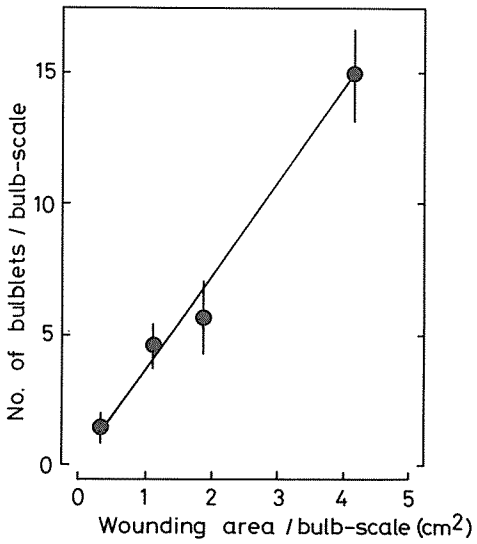


Fig. 2 Relations between wounding area applied to the bulb-scale and number of bulblets formed in the bulb-scale explants. The bulb-scales were transversally cut to several fragments, and the fragments were cultured on the medium with 0.1 μM of NAA and 1 μM of BA. After 3 weeks of culture, the number of bulblets formed in the fragment was counted. Relationships between wounding area applied to the bulb-scale and number of bulblets formed was expressed. For each treatment, at least 200 fragments were used, the experiments were repeated at least 3 times and the standard errors were then calculated.

From above experimental results, dependency of bulblet formation in wounding area was clear. In *Torenia* stem segments, additional wounding given to the middle part of explant caused a significant increase in the number of buds and this treatment did not affect the uptake of cytokinin into explants[9]. These results suggested that the wounding treatment induced some chemicals favorable for adventitious organ differentiation.

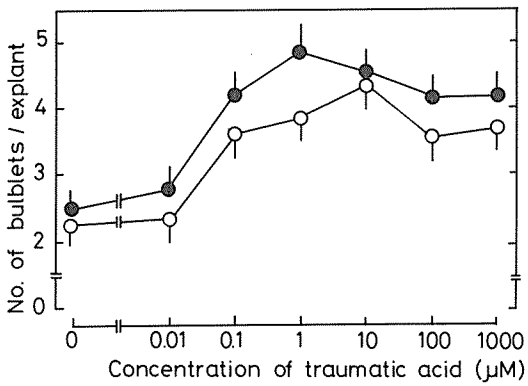


Fig. 3 Effects of traumatic acid (TA) on bulblet differentiation in bulb-scale explants. The explants C (○) and D (●) (shown in Fig. 1) were cultured on the medium contained 0.1 μM of NAA 1 μM of BA and various concentrations of TA. For each treatment, at least 200 explants were used, the experiments were repeated at least 3 times and the standard errors were then calculated.

Effects of traumatic acid

As the wounding treatment stimulated bulblet differentiation in the above experiments, effects of TA, one of the wound hormones, on bulblet formation were investigated using the explant C and D. The number of bulblet formed increased in the explant cultured on the medium containing more than 0.1 μM of TA (Fig. 3).

To find out the most effective period of TA treatment, the explants were initially cultured on the basal medium for various durations (0 to 24 hr), then incubated with solution of 1 μM TA for 1 hr, and then cultured on the basal medium. The promotive effects of TA were obtained only when the incubation with TA was given just after the excision of the explants (Table 1).

Table 1 Effects of incubation with traumatic acid (TA) during various periods of cultures on bulblet differentiation in bulb-scale explants.

Period of TA incubation	No. of bulblets per explant	Cultures with bulblets (%)
No incubation	2.8±0.4	90.4±2.6
0-1 hr	5.2±0.6	100
1-2 hr	3.6±0.4	98.8±1.0
6-7 hr	2.7±0.2	96.2±1.6
12-13 hr	2.8±0.4	100
24-25 hr	2.6±0.2	94.8±2.0

The explants used were D as Fig. 1. Firstly, explants were cultured on the basal medium for 0, 1, 6, 12 or 24 hr, then incubated with 1 μM TA solution for 1 hr. For each treatment, at least 200 explants were observed, the experiment were repeated at least 3 times and the standard errors were then calculated.

English et al.[3] reported that TA was one of the plant wound hormones, and Margara[4] demonstrated that TA stimulated bud formation in cauliflower head explants. We also examined the effects of TA in *Torenia* stem segments and found that TA enhanced adventitious bud initiation[17]. This promotive effects of TA seemed to be essential for adventitious organ initiation from organ fragments.

The most effective period

Table 2 Correlative effects of anti-auxin (TIBA) or anti-cytokinin (CB-P) and wounding on bulblet differentiation in bulb-scale explants.

Kinds of explant	No. of bulblets per explant		
	−CB−P, −TIBA	+CB−P (10 μM)	+TIBA (10 μM)
A	1.2±0.2	1.0±0.2	1.4±0.4
B	2.0±0.2	1.9±0.2	2.4±0.2
C	2.2±0.3	2.4±0.2	2.6±0.2
D	2.6±0.3	2.5±0.2	3.2±0.4

The explants were cultured on the medium containing 0.1 μM of NAA and 1 μM of BA with or without CB-P (10 μM) or TIBA (10 μM). For each treatment, at least 200 explants were used, the experiments were repeated at least 3 times and the standard errors were then calculated.

Table 3 Correlative effects of anti-auxin (TIBA) or anti-cytokinin (CB-P) and traumatic acid (TA) on bulblet differentiation in bulb-scale explants.

Concentration of TA (μM)	No. of bulblets per explant		
	−CB−P, −TIBA	+CB−P (10 μM)	+TIBA (10 μM)
0	2.4±0.2	2.2±0.2	3.2±0.4
0.1	4.2±0.4	4.3±0.4	4.6±0.4
1	4.4±0.4	4.6±0.4	5.2±0.6

The explants used were explant D as Fig. 1. The explants were cultured on the medium containing 0.1 μM of NAA, 1 μM of BA and various concentrations of TA with or without CB-P (10 μM) or TIBA (10 μM). For each treatment, at least 200 explants were used, the experiments were repeated at least 3 times and the standard errors were then calculated.

of TA treatment was observed only when TA incubation was given just after the excision of the explants (Table 1). The action of excision (wounding) itself may affect the initial process of bulblet differentiation, and this effect can be amplified by the treatment with TA.

Correlative effects of wounding, traumatic acid and anti-auxin, anti-cytokinin

Using several kinds of explants (explants A, B, C and D), effects of anti-auxin (TIBA) and anti-cytokinin (CB-P) on wounding-promoted bulblet initiation were examined. Furthermore, correlative effects of TIBA or CB-P and TA were also investigated. The results (Table 2 and 3) showed that CB-P did not affect wounding- and TA -promoted bulblet initiation, and TIBA was slightly stimulated the bullet differentiation.

Van Aartrijk and Blom-Barnhoorn[22] demonstrated that the effects of wounding and TIBA were similar and additive, and depended on the presence of NAA in *L. speciosum*. In our results, however, application of TIBA slightly promoted bulblet initiation, and did not show the additive promotion (Table 2 and 3).

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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
I．傷害及びトラウマチン酸の影響

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摘　　要

鉄砲ユリの鱗片切片培養において，切片をサイトカイニンとオーキシンを含む培地で培養することにより球根が分化する．鱗片を横方向に切断して切片を調製すると，鱗片当りの球根分化数は傷害面積に比例して増加した．傷害ホルモンの一つと考えられているトラウマチン酸を添加した場合にも球根分化は著しく促進された．短時間トラウマチン酸溶液で切片を処理した後に培養した場合には，切片を鱗片から切り出した直後に処理した場合のみ効果があった．同時に添加した抗サイトカイニンや抗オーキシンは傷害及びトラウマチン酸の分化促進効果に影響しなかった．