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Enhancement of Colony Formation of Carnation (*Dianthus caryophyllus*) Mesophyll Protoplasts by Abscisic Acid Pretreatment

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Colony formation of mesophyll protoplasts isolated from micropropagated carnation plantlets was investigated. Abscisic acid (ABA) pretreatment of the leaf segments enhanced the colony formation of protoplasts while water pretreatment inhibited. When donor tissues were pretreated with 10 mg/*l* ABA for 12 hours, over 40% of the protoplasts successively divided and formed colonies.

There have been many reports on the isolation, colony formation and plant regeneration of protoplasts in various plant species. However, it is still difficult to obtain high plating efficiency in protoplast-culture in many species. Not only culture medium, but also the environmental and nutritive conditions for source plants affected the yield, viability and cell division of protoplasts.[1-3] Several other factors, such as sterilization procedures, growth stages (age) of the donor tissues, purification of the enzymes, and pretreatment of the donor tissue with mineral and hormonal solution also affect the yield, viability and cell division of protoplasts.[4] Cold conditioning of the donor tissue was also reported to enhance plant regeneration from protoplast cultures.[5]

The isolation and culture of carnation mesophyll protoplasts from preconditioned tissues were investigated.

Materials and Methods

Plant material. Rooted cuttings of *Dianthus caryophyllus* L. cv. Pallas (Miyoshi Nursery Co. Ltd., Tokyo, Japan) were grown in a greenhouse. The apical meristems (0.2-0.3 mm long) were aseptically excised and placed on half strength of Murashige and Skoog's[6] basal medium supplemented with 0.1 mg/*l* indole-3-acetic acid (IAA) and 2.5 mg/*l* gibberellic acid (GA_3) for 2-4 months at 25°C under continuous fluorescent light (about 3,000 lux). The developed plantlets were transplanted to Murashige and Skoog's medium containing 0.1 mg/*l* α-naphthaleneacetic acid (NAA) and subcultured every 2-3 month under the same conditions. Leaves of 2-3 months old plantlets *in vitro* were cut into small segments for the preparation of protoplasts.

Chemicals. Cellulase Onozuka R-10 and Macerozyme R-10 were purchased from Yakult Pharmaceutical Industry Co. Ltd., Nishinomiya, Japan. The pH of the enzyme solution was adjusted to 5.5 with 0.1 N NaOH or 0.1 N HCl and sterilized with 0.22 μm membrane filter (Millipore Corporation, Massachusetts, USA) prior to isolation of protoplasts. ABA, IAA, NAA, 2, 4-dichrolophenoxyacetic acid (2, 4-D), kinetin, GA_3 (Sigma Chemical Co. Ltd., St. Louis, USA) and agar (Shimizu Foods Co.

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Ltd., Shimizu, Japan) were used in this experiment. ABA solution adjusted to pH 5.75 with 0.1 N NaOH was filtered with the Millipore filter. The other media were autoclaved at 121°C for 15 min.

Pretreatment. Cold treatment; The leaf segments and the plantlets *in vitro* were incubated at 5°C in the dark or under continuous diffused light (about 50 lux).

ABA treatment; The leaf segments were incubated on a reciprocal shaker (60 strokes/min) at 25°C under fluorescent light (about 3,000 lux) in a medium with 0 mg/*l*, 1 mg/*l* or 10 mg/*l* ABA, respectively. After the pretreatment, the leaf segments were wiped to dry with sterilized filter papers.

Isolation of protoplasts. After the removal of upper epidermis with forceps, the leaf segments were incubated in an enzyme solution containing 2% (w/v) Cellulase Onozuka R-10, 0.5% (w/v) Macerozyme R-10 and 0.7 M mannitol at 30°C on a reciprocal shaker at 60 strokes/min for 4–5 hr. After filtration through a 40 μm nylon mesh, isolated protoplasts were washed twice with 0.5 M mannitol and once with culture medium by centrifugation at $60\times g$ for 5 min.

Culture of protoplasts. The protoplasts were suspended and diluted to 5×10^4/ml with Murashige and Skoog's medium containing 0.25 M mannitol, 1 mg/*l* 2, 4-D and 0.1 mg/*l* kinetin. Five ml of the protoplast suspension was cultured in glass petri dishes (6 cmϕ) at 25°C under diffused continuous light (about 500 lux).

Determination of plating efficiency. After 2 weeks of culture, the numbers of colonies, protoplasts and dead cells were counted under an inverted microscope. Plating efficiency was calculated as follows; plating efficiency = number of colonies $\times$ 100 (%)/total numbers of colonies, protoplasts and dead cells.

Results and Discussion

In the first step of the study, protoplasts were isolated from leaves of plants grown in the greenhouse. In this case, however, yields of the protoplasts were not stable. Therefore, the method of *in vitro* cultivation of plantlets were established to grow under regulated conditions. The plantlets from apical meristems were micropropagated at regular intervals. Although total yield of protoplasts is low due to their small leaves, it was shown that micropropagated plantlets are a better source for the isolation of protoplasts than lea es of house-grown plants for the constant yields of protoplasts.

Cold treatment (3–7 days) of the leaf segments prior to isolation of protoplasts enhanced the yields of protoplasts up to 4.5 times, although cell division was not increased by this treatment (**Table 1**). The same treatment on the plantlets affected both the yield and plating efficiency of the protoplasts. In the plantlets, however, the efficiency was not stable from experiment to experiment, suggesting the implication of some other factor(s) in the cold treatment of plantlets (**Table 2**). These facts suggest that the cold treatment changes the composition of the cell walls and/or enhances the stability of the protoplasts. But the effect was lowered by the treatment longer than 7 days. Other factors, such as synthesis of ethylene under the stress, may lower the cell viability.[7]

ABA treatment enhances the yield of the protoplasts (**Table 3**). Water treatment was reported to

Table 1. Effect of cold treatment of carnation leaf segments on the yield and plating efficency of protoplasts.

Treatment (days)	Yield ($\times10^5$ protoplasts/g FW leaves)	Plating efficiency (%)	
		Light	Dark
0	22.6± 6.6	1.6±0.8	1.5±0.5
1	37.6± 6.1	2.3±0.8	2.1±0.7
3	98.5±14.3	2.8±0.6	4.6±1.4
7	91.8±15.4	1.4±0.8	2.7±0.8
14	72.8±11.8	1.2±0.4	1.6±1.3
28	15.2± 3.1	1.1±0.2	not tested

Values are means±standard deviation(SD), $n=5$. At least 2,000 protoplasts were counted in each determination.

Table 2. Effect of cold treatment of carnation plantlets *in vitro* on the yield and plating efficiency of protoplasts.

Treatment (Days)	Yield ($\times 10^5$ protoplasts/g FW leaves)	Plating efficiency (%)	
		Experiment I	Experiment II
0	22.6± 6.6	7.4±1.4	4.4±0.6
3	78.8±11.4	18.1±3.3	3.9±1.0
7	77.7± 7.7	5.7±1.2	28.2±5.5
14	75.5±10.2	7.7±1.4	8.0±3.0
28	57.2± 9.4	22.8±6.2	5.0±1.4

Values are means±SD, $n=5$. At least 2,000 protoplasts were counted in each determination.

Table 3. Effect of abscisic acid pretreatment* on the yield and plating efficiency of carnation mesophyll protoplasts.

Concentration of ABA (ppm)	Yield ($\times 10^5$ protoplasts/g FW leaves)	Plating efficiency (%)	
		Light	Dark
0	96.3±16.1	0.7±0.2	5.7±2.2
1	125.0±13.9	3.3±0.9	6.2±4.1
10	118.5±12.5	44.7±5.6	35.3±3.5

* Source leaves were treated with ABA before isolation of protoplasts for 12 hr.

Values are means±SD, $n=5$. At least 2,000 protoplasts were counted in each determination.

enhance the yield of protoplasts from leaves of deciduous trees, and it was suggested that the treatment changes the composition of the cell walls.[8] In the present study, a similar effect was also shown by the water treatment, but ABA treatment resulted in the higher yield. This fact suggests that ABA changes the cell wall composition or the stability of the cell, too, but the effect was a little.

Additionally, the ABA treatment remarkably promoted a colony formation of the mesophyll protoplasts (**Table 3**). When the donor tissues were preincubated in 10 mg/*l* ABA for 12 h, 44.7% of the protoplasts divided and most of them formed colonies (**Fig. 1**). Preincubation of the leaves in water as a control experiment allowed only 1.2% of the protoplasts to divide. The pretreatment with 1 mg/*l* ABA enhanced cell division slightly. When the donor tissues were pretreated with water or 1 mg/*l* ABA, the protoplasts well divided in the dark rather than in the continuous light. The effect of the pretreatment with 1 mg/*l* ABA was as the same as that of the pretreatment with water when the protoplasts were cultured in the dark. Pretreatment with 10 mg/*l* ABA promoted the plating efficiency as far as 35.3% in the dark, and higher plating efficiency (44.7%) was obtained in the continuous light. Moreover, the ABA pretreatment did not affect further growth of the colonies. These results offer several assumptions. The first one is that the ABA pretreatment enhances mitotic activity of the cells and the effect is not counteracted by the preparation of protoplasts. The second is that the stress by the pretreatment lowers mitotic activity of the cells in continuous light but did not in the dark. The third is that ABA reduces the damages of the cells by the stress. The fourth is that the effect of ABA is higher in the continuous light than in the dark.

Using cotyledonary protoplasts of *Pinus pinaster*, David and David suggested that the pretreatment with the mineral salts and hormonal solution stimulated cell division of the protoplasts.[9] Since the solution used by them contained many mineral salts and hormones, it is not clear which factor was the most effective. In the present study, pretreatment with ABA solution is needed for isolation and culture of the protoplasts reproducibly. The protoplasts from the plantlets grown under the regulated conditions

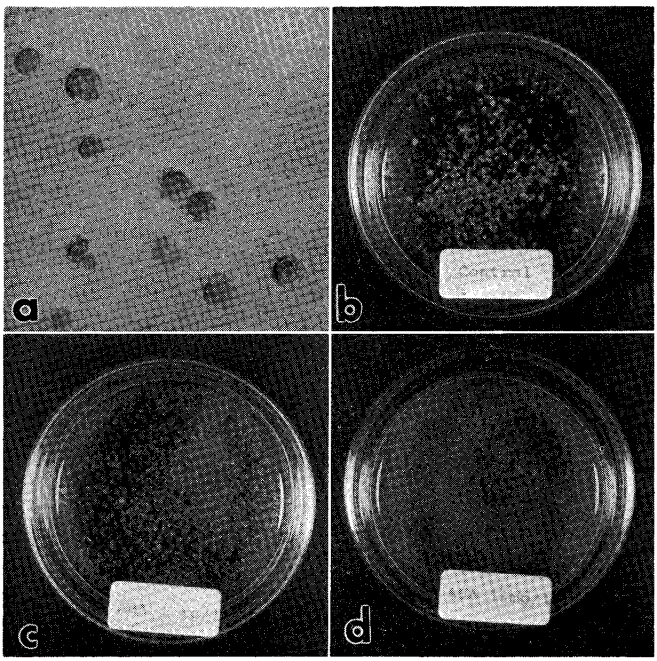

Fig. 1. Carnation mesophyll protoplasts and the colonies from the protoplasts. a) Freshly isolated mesophyll protoplasts, b) colonies derived from the protoplasts isolated from leaves which had been pretreated with water for 12 hr, c) colonies derived from the protoplasts isolated from leaves which had been pretreated with 1 mg/*l* ABA for 12 hr, d) colonies derived from the protoplasts isolated from leaves which had been pretreated with 10 mg/*l* ABA for 12 hr.

Table 4. Yield and plating efficiency of protoplasts from various developmental stages of carnation leaves.

Developmental stage	Yield ($\times 10^5$ protoplasts/g FW leaves)	Plating efficiency (%)	
		Light	Dark
Not fully expanded young leaves[a)]	41.9±9.1	4.4±1.5	2.6±1.7
Fully expanded young leaves[b)]	26.8±5.2	3.8±1.0	5.3±1.1
Mature leaves[c)]	16.7±6.0	14.4±3.5	10.9±2.2

Values are means±SD, $n=5$. At least 2,000 protoplasts were counted in each determination.

[a)] The youngest (uppermost) three pairs of the leaves in each plantlet *in vitro* which is 8–12 cm tall and has 8–12 pairs of leaves.

[b)] Intermediate 2–6 pairs of the leaves in each plantlet *in vitro* which is 8–12 cm tall and has 8–12 pairs of leaves.

[c)] The oldest (lowest) three pairs of the leaves in each plantlet *in vitro* which is 8–12 cm tall and has 8–12 pairs of leaves.

may not need so many factors as their experiments for preconditioning.

Stimulation of senescence by ABA have been reported in excised leaf segments.[10] Our result that the plating efficiency of mesophyll protoplasts of old leaves was higher than that of young leaves (**Table 4**) suggests some changes induced in the cells or tissues by ABA may promote cell division of the protoplasts. Several workers suggested that cold treatment affected the component of plasma mem-

branes in which ABA binding sites were located.[11-14] In addition, a similar effect of ABA on plasma membranes was reported.[13,14] The effects of cold conditioning and ABA pretreatment on colony formation from the mesophyll protoplasts suggest that the mitotic activity of protoplasts closely relates to the statement of the plasma membranes. In order to obtain high plating efficiency of protoplasts, ABA pretreatment may be an advantageous method. Further investigation is required to discuss the mechanism of the effect of ABA on cell regeneration from mesophyll protoplasts.

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References

1) Shepard, J. F., R. E. Totten, 1975. Plant Physiol., **55**: 689-694.
2) Shepard, J. F., R. E. Totten, 1977. Plant Physiol., **60**: 313-316.
3) Tal, M., J. W. Watts, 1979. Z. Pflanzenphysiol., **92**: 207-214.
4) David, H., A. David, T. Mateille, 1982. Physiol. Plant., **56**: 108-113.
5) Saxena, P. K., 1985. J. Plant Physiol., **119**: 385-388.
6) Murashige, T., F. Skoog, 1962. Physiol. Plant., **15**: 473-497.
7) Cassels, A. C., F. M. Cooker, S. Austin, 1980. Plant Sci. Lett., **19**: 169-173.
8) Butt, A. D., 1985. Plant Sci., **42**: 55-59.
9) David, A., H. David, 1979. Z. Pflanzenphysiol, **94**: 173-177.
10) Milborrow, B. V., 1974. Ann. Rev. Plant Physiol., **25**: 259-307.
11) Hornberg, C., E. W. Weiler, 1984. Nature, **310**: 321-324.
12) Lynch, D. V., P. L. Steponkus, 1987. Plant Physiol., **83**: 761-767.
13) Lalk, I., K. Dörffling, 1985. Physiol. Plant., **63**: 287-292.
14) Mohapatra, S. S., R. J. Poole, R. S. Dhindsa, 1988. Plant Cell Physiol., **29**: 727-730.

≪和文要約≫

カーネーション葉肉プロトプラストのコロニー形成に及ぼすアブシジン酸前処理の効果

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無菌的に増殖したカーネーションの葉肉プロトプラストのコロニー形成を調べた．葉切片をアブシジン酸で前処理するとコロニー形成率は高まったが，水での前処理では阻害された．葉切片を 10 mg/*l* のアブシジン酸で 12 時間前処理した場合，40% 以上のプロトプラストが分裂し，コロニーを形成した．