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Plant Regeneration from Mesophyll Protoplasts of *Dianthus superbus*

Eun Ae LEE, Joon Chul KIM*, and Won Bae KIM[1]
Department of Biology, Kangwon National University, Chuncheon, Kangwon-do, 200-701: and
[1]Alpine Experiment Station, RDA, Doam, Kangwon-do, 232-950. *Corresponding author.

술패랭이꽃(*Dianthus superbus*)의 엽육원형질체로부터 식물체 재분화

이은애 · 김준철* · 김원배[1]
강원대학교 자연과학대학 생물학과, [1]농촌진흥청 고령지시험장

Leaf mesophyll protoplasts of *Dianthus superbus* were cultured in MSP1 liquid medium supplemented with 0.5 mg/L BAP, 2.0 mg/L NAA and 9% mannitol. Protoplast-derived colonies were formed after 3 to 4 weeks of culture in the dark at 27° C. These colonies were kept under continuous illumination (21.5 μE · m^{-2} · sec^{-1}) for 2 weeks and finally most of the colonies became green microcalli, about 3 mm in diameter. When green microcalli were transferred to MS solidified medium with 2.0 mg/L 2,4-D, they formed embryogenic calli after 4 weeks of culture. These calli were then transferred onto N_6-2 medium containing 0.1 mg/L 2,4-D, 0.1 mg/L NAA, 2.0 mg/L kinetin and 2.0 g/L casein hydrolysate and cultured under illumination. After 5 weeks of culture the calli gave rise to multiple shoots of 10 to 15 per callus. Upon transfer onto MS medium containing 2.0 mg/L NAA, they were rooted. The regenerants were successfully transplanted into potting soil.

Key words: embryogenic callus, multiple shoots, N_6 medium, protoplast culture

To date, successful plant regeneration from protoplasts has been reported in several ornamental species (Griebach, 1988: Kunitake and Mii, 1990: Matthews et al., 1991: Nakano and Mii, 1992). With the development of plant molecular biology and genetic engineering, protoplast techniques have extensively offered many potential uses such as possible somaclonal variation, somatic hybridization and direct or vector mediated gene transfer (Lu et al., 1991: Nakano and Mii, 1993: Tsay, 1993). Especially protoplast culture and somatic hybridization may enable the genetic improvement of some ornamental species in relation to their floral and marketable qualities (Griesbach, 1988: Nakano and Mii, 1993).

The genus *Dianthus* contains a large number of commercially important ornamentals such as carnation, indian carpet, Chinese pink, *Dianthus superbus*, *Dianthus chinensis*. *Dianthus chinensis* and *Dianthus superbus* have been used widely for analgesic, sedative and urinary effects. But methods of cell, tissue and organ culture among them have been only developed to carnation. In case of carnation, adventitious shoots formation from petals (Kakehi, 1979: Gimelli et al., 1984: Frey and Janick, 1991: Lu et al., 1991), anthers (Villalobos, 1981), stem segments (Roest and Bokelmann, 1981: Radojevic et al., 1990: Frey and Janick, 1991) and axillary bud explants (Miller et al., 1991) have been reported. Also, carnation has been usually propagated vegetatively and meristem culture has been successfully incorporated for micropropagation and virus elimination (Mii et al., 1990). Such species can be realistically incorporated into somatic hybridization programmes aimed specifically to increase the diversity of flower colour and morphologies for some of the key members.

This report describes the condition for the effective isolation, culture and regeneration of *Dianthus superbus* protoplasts. In this respect, *Dianthus superbus* will be a potential sources for somatic hybrids as well as genetic transformation.

MATERIALS AND METHODS

Plant Materials

Seeds of *Dianthus superbus* were obtained from the Alpine Experiment Station, RDA, Korea. They were germinated in soil and grown in the greenhouse at 15-17°C under natural daylight (18 h day length). When the fourth-sixth leaves were unfolded, they were used for protoplast isolation.

Protoplast Isolation

The leaves were sterilized with 4% sodium hypochlorite for 10 min and then rinsed five times with sterile distilled water. After the lower epidermis was peeled off, the leaf fragments were transferred into petri dish containing CPW 13M solution. The CPW 13M solution was removed and replaced by 10 mL of filter sterilized enzyme solution of Cellulase R-10 in combination with Macerozyme R-10 and Pectolyase Y-23. The dishes were sealed with parafilm and they were incubated in static or shaking condition (60 rpm) at 27°C in the dark. After incubation, the entire subsequent mixture was passed through 45 μm nylon sieve to separate undigested debris and then the filtrate was centrifuged at 500 rpm for 3 min. Protoplast pellet was washed twice with CPW solution. The protoplast suspension was loaded gently on the top of 21% sucrose solution in a centrifuse tube and centrifuged at 800 rpm for 5 min. After centrifugation, the purified protoplasts floating on the sucrose solution were collected by using a pasteur pipette and they were washed by centrifusing twice with protoplast culture medium (MSP1 9M liquid medium containing 2.0 mg/L BAP, 0.5 mg/L NAA and 9% mannitol).

Protoplast Culture

Protoplast yield was calculated with a haemocytometer and the culture density of protoplasts was adjusted to 5×10^4/mL before plating. Protoplasts were cultured in 6×1.5 cm NUNCLON plastic petri dishes containing 5 mL MSP1 9M liquid medium with 2.0 mg/L NAA and 0.5 mg/L BAP at 27°C in the dark. In order to stimulate sustained division and colony formation, fresh liquid medium with a lower osmolarity was added at one-week intervals. At those processes, the influence of light conditions on protoplast division was investigated. The protoplast-derived colonies were transferred to continuous illumination (21.5 $\mu E \cdot m^{-2} \cdot sec^{-1}$) for 2 weeks.

Induction of Embryogenic Callus from Protoplast-Derived Colonies

When colonies became about 3 mm in diameter, in order to investigate induction of embryogenic calli, these green microcalli were placed on MS solid media (containing 0.8% agar) supplemented with 1 or 2 mg/L of 2,4-D or NAA, alone and in combination with 0.5 mg/L BAP or kinetin. Twelve microcallus pieces were used per treatment. Cultures were maintained at 27°C under continuous illumination for 4 weeks.

Primordial Shoot and Plant Regeneration

After 4 weeks of culture, the clones developing embryogenic calli were transferred onto regeneration media containing MS or N_6 basal salt and then kept at 27°C under continuous illumination. All the media were contained 0.1 mg/L NAA, 0.1 mg/L 2,4-D and 2 g/L casein hydrolysate. The effects of various cytokinin of 2 mg/L BAP, 2 mg/L kinetin and 1, 2 or 3 mg/L zeatin were investigated. Regeneration frequency was determined for each treatment after 2 months of culture. Regenerated shoots, 5 mm in height, were cut and cultured on MS medium with 0, 0.5, 1.0 or 2.0 mg/L NAA for further elongation of shoots and root formation. Regenerated plants were acclimatized to water and potted in sterile soil.

RESULTS AND DISCUSSION

Protoplast Isolation

For isolating the mesophyll protoplasts of *Dianthus superbus*, Cellulase R-10 in combination with Macerozyme R-10 and Pectolyase Y-23 was employed in a mixed enzyme technique. Enzyme solution 2 (ES 2) which contained 2% Cellulase R-10, 1% Macerozyme R-10, 0.1% Pectolyase Y-23, 13% mannitol and 5 mM MES buffer at pH 5.8 was more effective in degrading cell wall than enzyme solution 1 (ES 1) of 0.75% Cellulase R-10, 0.05% Macerozyme R-10. Optimum incubation condition for yielding intact protoplasts was 12 hours at 27°C in dark and stationary condition (Fig. 1). Tissue source for protoplast isolation is very important for both protoplast culture and subsequent recovery of plantlets (Xu, 1991: Xu and Wei, 1993). In *Dianthus superbus*, protoplast isolation from the full expanded fourth-sixth leaves showed higher yield (date not shown) than those of shoot

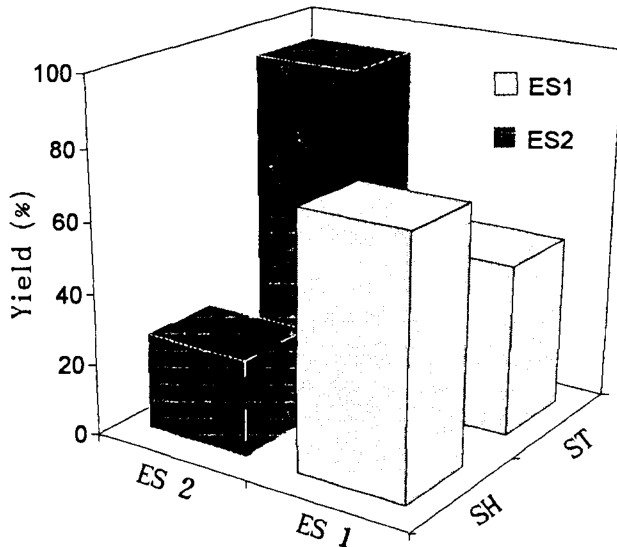

Figure 1. Yield of intact protoplasts. Protoplasts were isolated from the leaves of *D. superbus* in ES 1 (0.75% Cellulase R-10 and 0.05% Macerozyme R-10) and ES 2 (2% Cellulase R-10, 1% Macerozyme R-10 and 0.1% Pectolyase Y-23) with 5 h-shaking (SH) and 12 h-stationary (ST) conditions. ES 1 and ES 2 contained 13% mannitol, 5 mM MES buffer and 10 mL/L CPW solution.

Table 1. Influences of different culture methods on protoplast division.[a, b]

Culture Method	Time of Culture (day): 0 – 15	15 – 30	30 – 45
A	Division	Death	
B	Division	Colony	Callus
C	Division	Colony	

[a] ■ Dark, ▒ Light (21.5 μE · m^{-2} · sec^{-1}).
[b] A, 15 day-dark treated and then light treated: B, 30 day-dark treated and then light treated: C, Continuously dark treated.

tip-derived calli (Lee et al., 1994), and tissue maturity had a significant effect on the yield of isolated intact protoplasts (Fig. 2A).

Protoplast Culture

Protoplasts were initially cultured at a density of 5 × 10^4 protoplasts / mL (Fig. 2A). After 5 to 6 days of culture, the first mitotic divisions of cells (Fig. 2B) were observed on MSP1 9M liquid medium. As shown in Figure 2C, protoplasts were noticeably divided after 14 to 20 days of

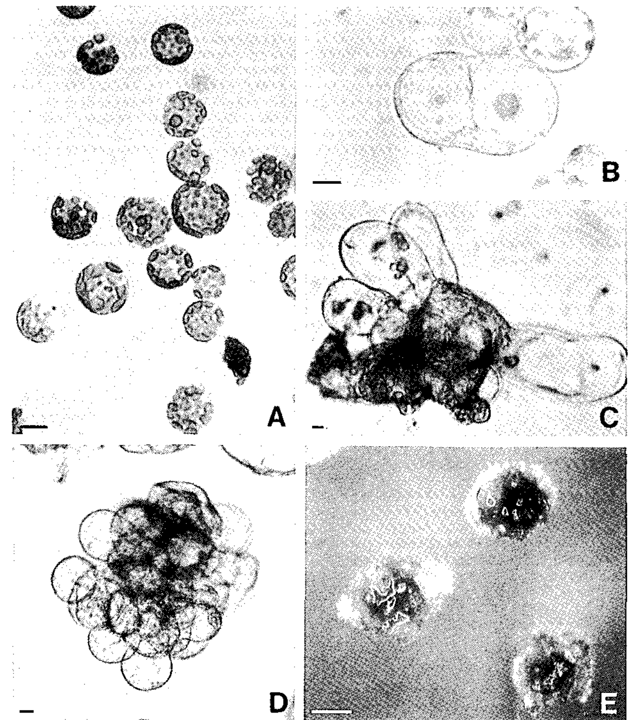

Figure 2. Mesophyll protoplasts and protoplast-derived microcalli in *D. superbus*. A, Intact purified protoplasts by the gradient of 21% sucrose in CPW solution: B, Protoplast division after 5 to 6 days of culture: C, Noticeable division of protoplast after 14 to 20 days: D, Colony formed after 30 days: E, Protoplast-derived green microcalli from cultured cell colony. Bars in A, B, C and D indicate 30 μm, and Bar in E indicates 1 mm.

culture. Some of the protoplasts in the culture medium settled and later adhered to the bottom of petri dish.

The protoplast-derived colonies became visible with naked eye within 30 days of culture (Fig. 2D) in the dark at 27°C, but they did not grow further in this condition. For further proliferation, the 30 day-dark treated colonies should be transferred to continuous illumination (21.5 μE · m^{-2} · sec^{-1}) for 2 weeks. The protoplast viability and division frequency could be dramatically increased in the illuminated condition and protoplast-derived green microcalli reached about 3 mm in diameter (Fig. 2E). However, as in Table 1, 15 day-dark treated colonies were cultured continuously in the light, most of the colonies turned dark-brown and eventually degenerated to death. When microcolonies continuously cultured in the dark, they formed calli, but degenerated to necrosis suggesting illumination appear necessary to sustainable cell division in the colony stage of protoplast culture in this species.

Induction of Embryogenic Callus from Protoplast-Derived Colonies

Table 2. Effect of plant growth regulators on embryogenic callus induction in *D. superbus.*

Plant growth regulators[a] (mg/L)				*Dianthus superbus*
2,4-D	NAA	BAP	kinetin	Embryogenic callus induction[b]
1.0	0	0	0	++
2.0	0	0	0	+++
1.0	0	0.5	0	+
2.0	0	0.5	0	+
1.0	0	0	0.5	+
2.0	0	0	0.5	+
0	1.0	0	0	-
0	2.0	0	0	-
0	1.0	0.5	0	-
0	2.0	0.5	0	-
0	1.0	0	0.5	-
0	2.0	0	0.5	-

[a] Plant growth regulators were added in MS basal medium.
[b] -, none: +, less than 30%: ++, 30-70%: +++, more than 70%.

Table 3. Effect of plant growth regulators on shoot formation from protoplast-derived callus in *D. superbus.*

culture media[a]	Regulators (mg/L)	Frequency of shoot regeneration(%)[d]
MSB 2[b]	2.0 BAP	0
MSK 2[b]	2.0 kinetin	18.0
MSZ 1[b]	1.0 zeatin	0
MSZ 2[b]	2.0 zeatin	8.3
MSZ 3[b]	3.0 zeatin	16.7
N6 - 1[c]	2.0 kinetin + 1.0 ABA	41.7
N6 - 2[c]	2.0 kinetin	75.0

[a] All the media contained 0.1 mg/L NAA, 0.1 mg/L 2,4-D and 2 g/L casein hydrolysate.
[b] Based on MS salt and vitamin mixture.
[c] Based on N6 salt and modified vitamin mixture.
[d] For each experiment 12 protoplast-derived calli were tested with 10 replications.

Auxin is the most important growth regulator for regulation of induction and development of embryogenesis (Fujimura and Komamine, 1979). In all the successful cases of somatic embryogenesis, a high auxin concentration is required for the formation of embryogenic callus, whereas a lower concentration of auxin favours the development of somatic embryo from embryogenic callus (Fujimura and Komamine, 1979: Chen et al., 1985: Kawahara and Komamine, 1991).

Green microcalli derived from the 30 day-dark treated colonies under illumination were transferred onto the solidified medium with 2,4-D (Table 2) for stimulating embryogenic callus induction (Lee et al., 1994). They became nodular, mucilaginous, pale-yellow and knobby embryogenic calli (Fig. 3A). However, in case of using the medium of 1 or 2 mg/L NAA alone and combination with 0.5 mg/L BAP or kinetin, they formed non-embryogenic calluses as dark-green and compact. That is, morphogenic capacity was observed only in subsequent culture on MS medium with 2,4-D (Table 2). This morphological appearance presenting organogenesis seems to be the key factor for the success of obtaining plantlets from intact protoplasts of *D. superbus.*

Primordial Shoot and Plant Regeneration

After 4 weeks of culture on MS growth medium with 2 mg/L 2,4-D, embryogenic calli were transferred to regeneration medium (Table 3) supplemented with different combinations and concentrations of cytokinins and cultured under illumination (21.5 $\mu E \cdot m^{-2} \cdot sec^{-1}$). After 7 to 8 weeks of subculture, green spots appeared on embryogenic calli and regenerated primordial shoots (Fig. 3B and 3C). They grew eventually forming 10 to 15 shoots per callus (Fig. 3D). N6 medium with 2.0 mg/L kinetin was more suitable than MS medium for achieving rapid primordial shoots from such protoplast-derived embryogenic calli. The difference in the total nitrogen ratio of these two media suggests that the nitrogen source plays an important role in obtaining plant regeneration from *Dianthus superbus* protoplasts. The type and concentration of cytokinins have a critical effect on plant regeneration from embryogenic callus of soybean (Wei and Xu, 1988). As shown in Table 2, kinetin was more effective than zeatin and BAP for shoot formation from the protoplast-derived calli in *D. superbus.*

All the shoots of about 5 mm in height were cut and rooted on MS medium with 2.0 mg/L NAA, resulting in the regeneration of whole plants (Fig. 3E). They were acclimatized to water and then transferred to potting soil (Fig. 3F). The reproducible plant regeneration from the cultured protoplasts of *D. superbus* is a prerequisite for utilization of genetic manipulation techniques such as somatic hybridization and gene transfer. Protoplast culture system described above is the first report in *D. superbus* and the ability to regenerate *D. superbus* protoplasts into plants has been employed to protoplast culture of *D. caryophyllus* and somatic hybridization between *D. superbus* and *D. caryophyllus.*

적 요

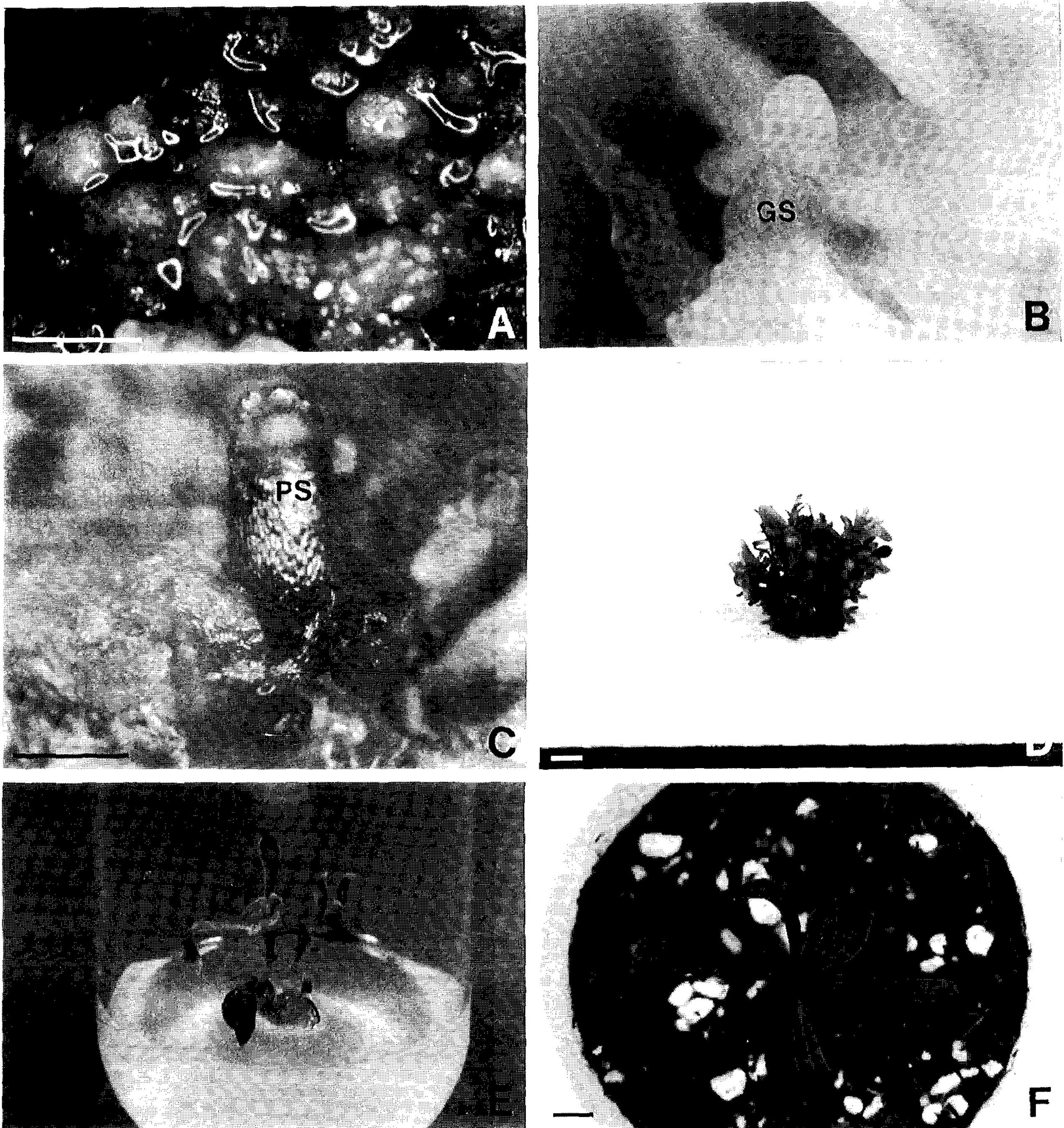

Figure 3. Shoot and plant regeneration from protoplast-derived embryogenic callus in *D. superbus*. A, Pale-yellowed embryogenic callus was formed on MS medium containing 2 mg/L 2,4-D under continuous light condition at 27°C; B and C, Green spot (GS) was formed on embryogenic callus and grew eventually forming primordial shoots (PS); D, Regenerated 10 to 15 shoots per callus; E, Regenerated plantlet derived from protoplast growing in MS medium supplemented with 2.0 mg/L NAA; F, A regenerant potted in soil. Bars indicate 1 mm in A, B and C, and 5 mm in D, E and F.

술패랭이꽃의 엽육원형질체를 2.0 mg/L NAA, 0.5 mg/L BAP 및 9%의 mannitol이 포함된 MSP1 9M 액체배지에서 배양하였다. 27°C, 암조건에서 배양 3-4주일 후 활발한 분열과정을 거쳐 원형질체로부터 colony가 형성되었으며 이들 colony는 연속광 (21.5 $\mu E \cdot m^{-2} \cdot sec^{-1}$)하에서 배양 2주일 후 직경 약 3 mm의 녹색 microcallus로 생장하였다. 녹색 microcallus는 2.0 mg/L 2,4-D가 첨가된 고체배지에서 배양 30일 후 배발생 캘러스를 형성하였으며 이들 배발생 캘러스는 0.1 mg/L 2,4-D, 2.0 mg/L kinetin 및 2.0 g/L casein hydrolysate가 포함된 N_6-2 배지에서 캘러스당 10-15개의

multiple shoot의 분화가 이루어졌다. 재분화된 shoot는 2.0 mg/L NAA가 포함된 MS 배지에서 뿌리가 유도되었으며 이들을 pot로 이식하여 재분화 개체를 획득할 수 있었다.

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