55. Secondary Plant Products from Cultured Hybrid Cells

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Great interest in the production of such useful plant products as alkaloids, steroids, vitamins, and pigments has arisen with the development of plant cell technology. Recently, several examples have been reported of the establishment of stable cell lines that produce large amounts of secondary metabolites.

Yamada and Sato^{1),2)} have reported the establishment of *Coptis japonica* (Polycaypiidae) cell lines that produce high amounts of berberine in the dark. Using the small cell aggregate selection method, they obtained one cell line whose average productivity over two years is 8.2% (D.W.) and 0.90 g/liter of culture medium, the highest production being 13.2% (D.W.) and 1.39 g/liter of culture medium. This berberine content is several times higher than that in the parent plant. Berberine is a useful antibacterial and antityphoid agent and its anti-inflammatory potential has recently been reported.

Yamamoto *et al.*³⁾ have also established cell lines of *Euphorbia millii* (Sapindaifloriidae) that produce seven times as much anthocyanin as the original non-selected calluses. The production of this red pigment in the cells was observed only with illumination.

Here we report on the somatic hybridization of protoplasts isolated from these two high-metabolite producing cells.

We fused the protoplasts isolated from *Coptis* and *Euphorbia* cells by using polyethylene glycol (PEG) and by electrofusion with previously reported methods.^{4),5)} Briefly, the protoplast mixture was placed between parallel platinum electrodes in a fusion chamber (2×2×10 mm) and a 1 to 50 protoplast layer formed giving rise to membrane-membrane contact in the absence of an applied AC field (multi-protoplast layer method). A single pulse (1.5 to 3.5 kV/cm, $\tau=CR=100$ to 200 us) was applied at room temperature by the capacitor-discharge method.

We succeeded in forming callus colonies from a mixture of protoplasts of *Coptis* and *Euphorbia* that were electrically fused. The colonies cultured in the light were composed both of red (anthocyanin-producing) and white (no anthocyanin-producing) cells, while those cultured in the dark were composed both of yellow (berberine-producing) and white (no berberine-producing) cells. Fig. 1 shows typical cell colonies that formed from culture of the electrically-fused protoplast mixture in the light.

Interestingly, we found that dark-red vesicles formed in the heteroplasmically fused protoplast induced by PEG and electrofusion between *Coptis* and *Euphorbia*. Based on the detailed analyses of the fusion process, this vesicle formation was considered to occur at the stage of vacuolar fusion following the initial cytoplasmic fusion. Fig. 3 shows typical heterokaryons with such vesicles. Clearly,

the heterokaryons can easily be distinguished from non-fused protoplasts or homokaryons.

Fig. 2 shows viable heterokaryons 18 hr after electrical treatment plotted against the initial intensity of the capacitor-discharge pulse. The protoplast mixture containing an equal number of protoplasts of both species was added to the chamber so that the number of total protoplasts in the chamber was 8×10^3 , 4×10^4 and 4×10^5 . The number of protoplast layers in the chamber was

Fig. 1. Cell colonies formed from mixture of *Coptis japonica* and *Euphorbia millii* protoplasts that had been electrically fused. The protoplasts were cultured in the light condition.

Fig. 2. Per cent viable heterokaryons between *Coptis japonica* and *Euphorbia millii* (18 hr after electrical treatment) plotted against the initial intensity of the capacitor-discharge pulse. The number of protoplast layer was ca. 1 ($\circ-\circ$), 5 ($\triangle-\triangle$) and 50 ($\square-\square$). See text for detail.

Fig. 3. Heterokaryons between *Coptis japonica* and *Euphorbia millii*. Note that heterokaryons have dark-red vesicles as indicated by arrows.

estimated to be 1, 5 and 50 respectively, since the average diameter of the protoplasts was ca. 40 μm and the volume of the chamber was 40 μl . We placed sedimented pellet of the protoplast mixture to the chamber to make 50 protoplast layers. Clearly, a maximum percentage of the viable heterokaryons was obtained at the initial intensity of 2 kV/cm and this value did not change appreciably with the number of protoplast layers in the chamber (see Fig. 2). The present result indicates that the highest number of heterokaryons can be obtained when the electric pulse is applied to a sedimented pellet of protoplast mixture using the multi-protoplast layer method.

Employing the vesicles as a marker, we picked up the inter-infraclass heterokaryons with a micropipette and transferred them to a nurse culture.⁽⁶⁾ Altogether 2830 electrically-induced heterokaryons were recovered. The heterokaryons fluoresced yellow (berberine-producing) when cultured in the dark and became red (anthocyanin-producing) when cultured in the light. Three of the heterokaryons showed cell division and one developed into a small cell colony of 10 to 20 cells. This is the first report of the successful cell division and colony formation of inter-infraclass hybrids between high-metabolite-producing cells.

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