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Effect of IOA and Media on Production of Somatic Hybrid by the Asymmetric Protoplast Fusion Between Three *Solanum* Species

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Abstract - In this study, we surveyed the influence of IOA (iodoacetamide) and media upon protoplast fusion for the efficient production of the somatic hybrid among *S. sisymbriifolium* and other *Solanum* species (*S. intergrifolium* and *S. toxicarium*). Regardless of a breed, as the IOA concentration increases, the cell division tends to decrease. As the influence of the media on the colony formation, we could get 5 colonies from the fusion of *S. sisymbriifolium* and IOA-treated *S. integrifolium* protoplast, but none was observed in other fusions. As a result of analyzing their IDH isozyme, we found a somatic hybrid in 2 objects.

Key words - *Solanum* species, IOA concentration, Isozyme pattern

Introduction

The rearing of a new variety via inter-cell transplantation or absorption of gene information by cell fusion is the area which can possibly overcome the limitation of the existing breeding method, and active researches are being conducted currently in such area. In particular, because it is easy to insert an external gene material to the protoplast whose cell membrane is removed, the desirable genetic character is transplanted between varieties. Hess *et al.* (1973) opened a new way in the cytogenetics by reporting the absorption of exogenous DNA to the protoplast of petunia. Cooking (1976) reported the absorption of TMV (Tabacco mosaic virus) to the protoplast of a tomato thereby presenting the protoplast as a possible tool to describe the virus infection path. For the agricultural purpose, there are the attempts to fuse the root or protoplast of a legume containing Azotobacter with the protoplast of a non-legume (Holsten *et al.*, 1971; Davey and Cocking, 1972); and such transplantation attempts are being applied to the major economic plants efficiently to cultivate a new plant breed (Giles and Sarafis, 1971).

In this experiment, we isolated a protoplast from the mesophyll tissue of three *Solanum* species, and soaked it into IOA (iodoacetamaide) solution, and then surveyed their impact on the cell division, and the media impact on the colony formation upon protoplast fusion.

Materials and Methods

Plant material

Seeds of *Solanum sisymbriifolium*, *S. intergrifolium* and *S. toxicarium* were sterilized with sodium hypochlorite solution (2% active chlorine) for 20 min and washed three times with sterilized distilled water. They were aseptically sown in 100 mL conical flasks containing 30 mL of MS medium (Murashige and Skoog, 1962) supplemented with 1% sucrose and 0.25% gellan gum, and were incubated in a growth cabinet (light intensity was 30 $\mu E\ m^{-2}\ sec^{-1}$ for 16 hr day^{-1} and the temperature was kept at 25℃ both in the dark and the light). Proliferated leaves of 3-4 week-old plants were used for protoplast production.

Protoplast isolation

The sterile leaves were cut into stripes 1~2 mm in width and placed in 80×15 mm petri dishes containing 20 mL enzyme solution which contained 0.4 M mannitol, 0.03 M sucrose, 0.05-0.20% (w/v) Macerozyme and 0.25-1.0% (w/v) Meicerase and adjusted to pH 5.5. The dishes were sealed with parafilm and kept still at 25℃ in the dark. After 4-16 hr incubation, protoplasts were separated from undigested tissues and cells by successive passage through 50 μm nylon filter. They were then washed three times in culture medium by centrifugation (100 × g, 3min). Medium for the protoplast culture was Kao medium (Kao, 1997) modified by the addition of 0.4 M mannitol, 0.03 M sucrose, 5.0 mg/L 1-naphthaleneacetic acid (NAA), 1.0 mg/L 6-benzylaminopurine (BA).

The protoplasts were cultured as a thin layer (2 mL/dish) in 60 × 15 mm petri dishes at densities adjusting to 5.0×10^4 protoplasts per mL. The dishes were sealed with parafilm and kept at 30℃ for the first 7 days and thereafter at 25℃ in the dark. Two mL of fresh medium was added to each of culture dishes at 14 day intervals. Protoplast-derived colonies of 0.5-1.0 mm in diameter (5 weeks old) were counted.

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Iodoacetamide treatment

Protoplasts were treated with four levels of concentrations (5.0, 7.5, 10.0 and 12.5 mM) of iodoacetamide (IOA) solution containing 0.4 M mannitol and 0.03 M sucrose (pH 5.5) at 5℃ for 10 min. Then they were washed three times by centrifugation (100 × g, 3 min.) with culture medium. Protoplasts of *S. sisymbriifolium*, *D. integrifolium* and *S. toxicarium*, which were all treated with IOA solution, were cultured in media F (Kao medium containing 5.0 mg·L^{-1} NAA, 1.0 mg·L^{-1} 2,4-D and 1.0 mg·L^{-1} BA), G (a half strength MS medium containing 0.03 M sucrose, 0.4 M mannitol, 1.0 mg·L^{-1} NAA and 1.0 mg·L^{-1} kinetin) (Sadohara, 1993) and H (a half strength MS medium containing 0.03 M sucrose, 0.4 M mannitol, 0.1 mg·L^{-1} NAA and 0.1 mg·L^{-1} kinetin) (Sadohara, 1993; Sadohara *et al.*, 1993), respectively. Frequency of cell division was examined after 10 days of protoplast culture.

Protoplast fusion

The isolated protoplasts were suspended in fusion solution containing 0.225 M glucose, 0.225 M mannitol and 2.5 mM $CaCl_2$. The protoplast density was adjusted to 2.0 × 10^5 protoplasts per mL. The protoplast suspensions of *S. sisymbriifolium* and *S. integrifolium*, or those of *S. sisymbriifolium* and *S. toxicarium* were mixed at a ratio of 1 : 1 (v/v). The protoplasts were fused by means of electro fusion using a Somatic Hybridizer SSH-1 (Shimadzu Corp., Japan). A 0.8 mL aliquot of mixed protoplast suspension was introduced into the fusion chamber FTC-03 (Shimadzu Corp., Japan), aligned with a voltage alternating current of 40 v, frequency of 1.0 MHz, voltage direct current of 150 v (750 V cm^{-1} field strength), pulse width of 400 μs and pulse of 1 ~ 2 time. Following the fusion treatment, the protoplast suspension was transferred to 60 × 15 mm petri dish and an equal volume of two-fold strength culture medium was added. Selection of the fused heterokaryon protoplast from homokaryon or nonfused protoplast was performed by means of the combination of IOA inactivation and the difference in cell division ability in the selective media (see Fig. 1 A-C, Table 1).

Plant regeneration

Protoplast-derived calli of 1-3 mm in diameter (8 weeks after culture) were transferred to 100 mL conical flasks containing 30 mL MS medium supplemented with 30 g·L^{-1} sucrose, 7.0 g·L^{-1} agar, 0.1 mg·L^{-1} IAA and 1.0 mg·L^{-1} zeatin for shoot regeneration. After 4 weeks of culture, regenerated plants were transferred for rooting on a half strength MS medium with 5.0 g·L^{-1} sucrose and 2.5 g·L^{-1} gellan gum, which is good for rooting from the regenerated plants as described by Sadohara (1993).

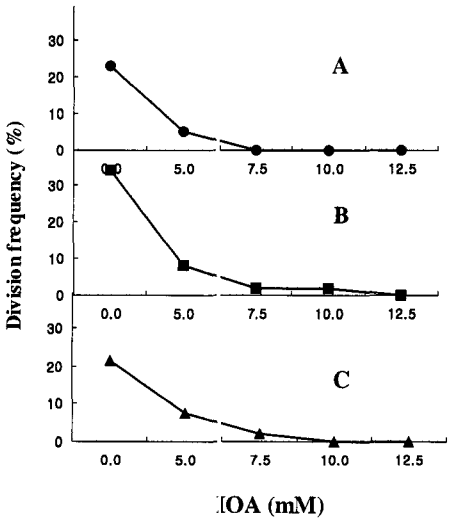

Fig. 1. Effect of IOA on the cell division of *S. sisymbriifolium* (A), *S. integrifolium* (B) and *S. toxicarium* (C).

Table 1. Effect of media on colony formation from protoplast mixture fused between *Solanum sisymbriifolium*, *S. integrifolium* and *S. toxicarium*

	Medium	Cell division[z]	No. of colonies
*S. sisymbriifolium** + *S. integrifolium*	F[x]	-	-
*S. sisymbriifolium** + *S. integrifolium*	G[w]	+ (4)[y]	5
*S. sisymbriifolium** + *S. toxicarium*	F	-	-
*S. sisymbriifolium** + *S. toxicarium*	H[v]	-	-

[z] Volum of protoplast suspension = 6.0, protoplast density = 5.0 × 10^4·mL^{-1}. +: cell division, -: no cell division.
[y] Days to first division.
[x] Kao medium containing 5.0 mg·L^{-1} NAA, 1.0 mg·L^{-1} 2,4-D and 1.0 mg·L^{-1} BA.
[w] A half strength MS medium containing 0.03 M sucrose, 0.4 M mannitol, 1.0 mg·L^{-1} NAA, 1.0 mg·L^{-1} kinetin.
[v] A half strength MS medium containing 0.03 M sucrose, 0.4 M mannitol, 0.1 mg·L^{-1} NAA, 0.1 mg·L^{-1} kinetin.
* IOA treatment.

Selection of the fused heterokaryon protoplast from homokaryon or nonfused protoplast was performed by means of the combination of IOA inactivation and the difference in cell division ability in the selective media (see Fig. 1 A-C, Table 1).

Isozyme analysis

Samples were extracted from calli and leaves of regenerated plants, which were examinated for isozymes by starch gel electrophoresis. The gels were stained for Isocitrate dehydrogenase

(IDH), Phosphoglucomutase (PGM) and Shikimate dehydrogenase (SKDH) by means of the methods of Wendel and Parks (1982) and Wendel (1983).

Results

Protoplasts of *S. sisymbriifolium*, *S. integrifolium* and *S. toxcarium* treated with IOA were cultured in the optimal culture conditions, respectively. The results are shown in Fig. 1. Cell division of *S. sisymbriifolium*, *S. integrifolium* and *S. toxicarium* protoplasts were completely inhibited at the IOA concentration of 7.5, 12.5 and 10.0 mM respectively.

The results of fused protoplast selection are indicated in Table 1. The only fusion pair of *S. sisymbriifolium* and IOA treated *S. integrifolium* protoplasts formed five colonies, whereas the other pairs did not. Five calli (Table 1) were transferred to the regeneration medium, among which these calli regenerated shoots.

Before the shoot regeneration, their parentage of these three calli were investigated by isozyme analysis for IDH, PGM and SKDH. Two calli were somatic cell hybrids (Fig. 2 ; data of PGM and SKDH not shown) while the others were the same as *S. integrifolium*. The shoots regenerated from these two somatic hybrid calli, however, showed the only one isozyme pattern, that the same as the pattern of *S. sisymbriifolium* (data not shown). These two plants also had the similar morphology as *S. sisymbriifolium*.

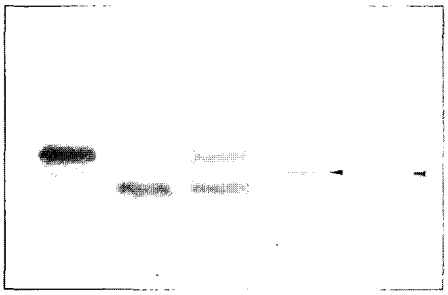

Fig. 2. Electrophoretic banding patterns of IDH isozymes, in leaf tissue of *S. sisymbriifolium* (SS), *S. integrifolium* (SI) and their mixture (Mix), and in calli of somatic hybrids (1 and 2). Arrows indicate heterodimer bands.Discussion

When the parental species have no selective markers at the cellular level, efficient procedures for the hybrid cell selection must be established. In this study, IOA treatment (Nehls, 1978) of the protoplasts of one parental species (*Solanum integrifolium*) was successfully combined with another untreated parent (*S. sisymbriifolium*) in the medium G. The IOA treated protoplast of *S. integrifolium* occasionally divided when they were cocultured with untreated protoplast of *S. sisymbriifolium*, even at the high IOA concentration enough to kill all the *S. integrifolium* protoplasts when cultured alone. This 'nurse effect' of untreated protoplasts explains the appearance of a escaped callus of S. *integrifolium* even at the 12.5 mM concentration of IOA (Fig. 1).

Inability of cell division of one parental species has been utilized for selection of somatic hybrids (Kameya *et al.*, 1990). In this study, two calli which formed shoots were somatic hybrid. But the shoots regenerated from them showed the same IDH, PGM and SKDH isozyme patterns and morphology as *S. sisymbriifolium* did. This result may be caused by further elimination of *S. integrifolium* chromosomes in the plants regenerated from the hybrid calli, i.e., *S. integrifolium* chromosomes in hybrid calli disappeared during mitotic division at regenerating stage because the parents' chromosomes were unbalanced. There are numerous reports that interfamilial or intergeneric somatic hybrids were genetically unstable and morphologically abnormal and showed the elimination of specific chromosome (s) and the disappearance of specific isozyme bands of a parent (Krumbiegel and Schiedr, 1979; Gleba and Hoffmann, 1979; 1980; Chien *et al.*, 1982; Niizeki *et al.*, 1985; Gleba *et al.*, 1988). I considered that the similar phenomenon occurred in this study.

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