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Protoplast Culture by Electrofusion of Protoplasts from *Solanum sisymbriifolium* and Other *Solanum* Species

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Abstract - This research was conducted to get the basic materials necessary to obtain the somatic hybrid plant between *Solanum sisymbriifolium* and other *Solanum* species (*S. integrifolium* and *S. toxicarium*). Regarding the formation of colony from the protoplast in *S. sisymbriifolium*, *S. integrifolium* and the fused protoplast mixture; for the *S. sisymbriifolium*, a colony was observed in F medium(Kao medium containing 5.0 mg·L^{-1} NAA, 1.0 mg·L^{-1} 2,4-D and 1.0 mg·L^{-1} BA); and for the *S. integrifolium*, in G medium (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) respectively. In mixed cultured protoplast after electriofusion treatment, the cell division and colony formation were observed in both media F and G. For the shoot and root formation rate, there was no difference between the parent of each breed and mixed protoplast regardless of the medium. In the fused protoplast mixture of *S. sisymbriifolium* and *S. toxicarium*, a colony formation was also observed in both media F and H(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); and there was no difference in the shoot and root formation rate between the parent and the mixed protoplast.

Key words - *Solanum* species, Culture media, Isozyme pattern

Introduction

The genus *Solanum*, predominantly distributed in Central and South America (Khan, 1979), is a very large genus containing many species. They include a lot of commercially important vegetables for their useful leaves, fruits and tubers.

Generally speaking, the wild *Solanum* species had been an important source for quality traits, and thus a number of cultivated varieties have been improved. Backcross is the most appropriate method to combine resistant gene (s) of wild species into the commercial susceptible varieties. Ordinarily, materials have been advanced up to BC_5 to BC_6 generations and therefore homozygous resistant plants were selected in the case of resistance controlled by single dominant gene (Kalloo, 1992). Three wild *Solanum* species, *S. sisymbriifolium*, *S. integrefolium* and *S. toxicarium*, however, are perfectly cross incompatible with each other, so that the optimal rootstock has not been produced until now (Nishio *et al.*, 1984; Ali and Fujieda, 1990; Ali, 1991).

Somatic hybridization is a technique which allows the manipulation of cellular genomes by protoplast fusion. Its major contribution to plant breeding is in overcoming common crossing barriers among plant species and in organelle genetics and breeding. In this study, We experimented with electrofusion for the production of somatic hybrid plants of *S. sisymbriifolium* and other *Solanum* species (*S. integrifolium* and *S. toxicarium*).

Materials and Methods

Plant material

Seeds of *S. sisymbriifolium*, *S. integrifolium* and *S. toxicarium* were surface sterilized with sodium hypochlorite solution (2% active chlorine) for 20 min and washed three times with sterilized water. They were aseptically sown in 200 mL conical flasks containing 40 mL of MS medium (Murashige and Skoog, 1962) supplemented with 1% sucrose and 0.25% gellan gum, and incubated in the growth cabinet. Plants of *S. toxicarium* were subcultured by leafy node cuttings on the fresh medium of the same composition at 3-weeks intervals. Leaves of *S. sisymbriifolium*, *S. integrifolium* and *S. toxicarium* were taken from 3-4 week-old, 2 week-old and 6 week-old plants, respectively.

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

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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, 3 min). 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} 1-naphthaleneacetic acid (NAA), 1.0 mg·L^{-1} 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.

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 electrofusion 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.

Protoplast culture

Fused protoplasts between *S. sisymbriifolium* and *S. integrifolium* were cultured in medium 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) or G (1/2 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 those between *S. sisymbriifolium* and *S. toxicarium* were cultured in medium F or 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). Protoplasts of both the fusion pairs were cultured in the dark at 30℃ for the first 7 days and thereafter placed at 25℃ in the dark. Two mL of fresh media (same as each initial medium but modified with 0.2 M mannitol) were added at an interval of 14 days.

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).

Isozyme analysis

At the leaf regeneration stage, one leaf of each plant was examinated for isozymes by starch gel electrophoresis. The gels were stained for isocitrate dehydrogenase (IDH) or phosphogluconate dehydrogenase (PGD) by means of the methods of Wendel and Parks (1982) and Wendel (1983).

Results

The process of electro fusion took less than 2 min per dish. Pearl chains of 6 ~ 7 protoplasts were formed within 50 sec. of an A.C. field application at 1.0 MHz and 750 v·cm^{-1}. Fusion between protoplasts occurred when D.C. square pulses were applied. The exposure of samples of protoplast mixture to a train of 2 ~ 3 D.C. pulses of 400 μs, each 750 v·cm^{-1}, resulted in a fusion rate of 40-50%. At least 20% of the fusion products were in the 1 : 1 (binary) category. The frequency of heterokaryons was not determined, since it was unable to distinguish one parent protoplasts from the other parent.

Solanum sisymbriifolium (+) *S. integrifolium*

Effect of media on colony formation from protoplasts in *S. sisymbriifolium*, *S. integrifolium* and fused protoplast mixture is shown in Table 1. In medium G, protoplast division was not found in *S. sisymbriifolium* but found in *S. integrifolium*, whereas, in medium F, protoplasts division was not observed in *S. integrifolium* but observed in *S. sisymbriifolium*. In mixed cultured protoplasts after electro fusion treatment, cell division and colony formation was observed in both media F and G.

The rates of shoot and root formation from the protoplast-derived calli in the fusion plates were not much different in comparison with those of the control plates of *S. sisymbriifolium* or *S. integrifolium* parental protoplasts (Table 2).

Two hundred thirty eight shoots transferred to rooting medium were used for analyzing isozyme patterns of IDH. The isozyme pat-

Table 1. Effect of media on colony formation from protoplasts of *S. sisymbriifolium* and *S. integrifolium* and from fused protoplast mixture[z]

	Medium	Volume of protoplast suspension[y](ml)	No. of colonies
S. sisymbriifolium	F[x]	2.0	7
	G[w]	2.0	0
S. integrifolium	F	2.0	0
	G	2.0	211
Fused protoplast mixture	F	6.0	13
	G	6.0	280

[z] Protoplasts obtained after electro protoplast fusion between *S. sisymbriifolium* and *S. integrifolium.*
[y] Protoplast density = 5.0 × 10^4·mL^{-1}.
[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.

Table 2. Comparison of shoot formation from protoplast-derived calli and rooting of the shoots between *S. sisymbriifolium, S. integrifolium* and fused protoplast mxture[z]

	Colony induction medium	No. of calli		No. of shoots	
		Transferred	Forming shoots	Transferred	Forming roots
S. sisymbriifolium	F[w]	52	27 (51.9)[y]	50	43 (86.0)[x]
S. integrifolium	G[v]	52	23 (44.2)	48	39 (81.3)
Fused protoplast mixture	F	13	6 (46.2)	28	20 (71.4)
	G	280	98 (35.0)	210	173 (82.4)

[z] Protoplasts obtained after electro protoplast fusion between *S. sisymbriifolium* and *S. integrifolium.*
[y] Percent calli forming shoots.
[x] Percent shoots forming roots.
[w] Kao medium containing 5.0 mg·L^{-1} NAA, 1.0 mg·L^{-1} 2,4-D and 1.0 mg·L^{-1} BA.
[v] 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.

Table 3. Effect of media on colony formation from protoplasts of *S. sisymbriifolium* and *S. toxicarium* and from fused protoplast mixture[z]

	Medium	Volume of protoplast suspension[y](ml)	No. of colonies
S. sisymbriifolium	F[x]	2.0	6
	H[w]	2.0	0
S. toxicarium	F	2.0	0
	H	2.0	11
Fused protoplast mixture	F	6.0	18
	H	6.0	13

[z] Protoplasts obtained after electro protoplast fusion between *S. sisymbriifolium* and *S. toxicarium.*
[y] Protoplast density = 5.0 × 10^4·mL^{-1}.
[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, 0.1 mg·L^{-1} NAA, 0.1 mg·L^{-1} kinetin.

tern of 28 shoots regenerated in medium F was all identical and it was the same as that of *S. sisymbriifolium.* The only one same isozyme pattern was obtained in 210 shoots regenerated in medium G and in the shoots of *S. integrifolium.*

Solanum sisymbriifolium (+) *S. toxicarium*

Results of colony formation from protoplasts in *S. sisymbriifolium, S. toxicarium* and fused protoplast mixture on various media were indicated in Table 3. Cell division and colony formation from the protoplasts of *S. sisymbriifolium* of *S. toxicarium* were not observed in either medium F or H, whereas those in mixed cultures of both protoplasts after electro fusion treatment, were observed in both media F and H.

Compared the control plates of *S. sisymbriifolium* or *S. toxicarium*

Table 4. Comparison of shoot formation from protoplast-derived calli and rooting of the shoots between *S. sisymbriifolium*, *S. toxicarium* and fused protoplast mixture[z]

	Colony induction medium	No. of calli		No. of shoots	
		Transferred	Forming shoots	Transferred	Forming roots
S. sisymbriifolium	F[w]	52	25 (48.1)[y]	46	41 (89.1)[x]
S. toxicarium	H[v]	52	16 (30.8)	30	30 (100)
Fused protoplast mixture	F	18	11 (61.1)	29	20 (82.8)
	H	13	5 (38.5)	14	173 (92.9)

[z] Protoplasts obtained after electro protoplast fusion between *S. sisymbriifolium* and *S. toxicarium*.
[y] Percent calli forming shoots.
[x] Percent shoots forming roots.
[w] Kao medium containing 5.0 $mg \cdot L^{-1}$ NAA, 1.0 $mg \cdot L^{-1}$ 2,4-D and 1.0 $mg \cdot L^{-1}$ BA.
[v] A half strength MS medium containing 0.03 M sucrose, 0.4 M mannitol, 0.1 $mg \cdot L^{-1}$ NAA, 0.1 $mg \cdot L^{-1}$ kinetin.

to the fusion plates of *S. sisymbriifolium* or *S. toxicarium* to the fusion plates, the rates of shoot and root formation from protoplast-derived calli was so much different (Table 4).

Forty three shoots transferred to rooting medium were used for analyzing isozyme pattern of PGD. Twenty four shoots regenerated from the calli cultured in medium F and 13 shoots in medium H showed the same isozyme patterns of *S. sisymbriifolium* and *S. toxicarium*, respectively.

Discussion

In this study, somatic hybrid production by symmetric fusion was attempted using the protoplast culture systems of *Solanum sisymbriifolium*, *S. integrifolium* and *S. toxicarium* (Sadohara, 1993). There are reports concerning the selection of somatic hybrids by combination of media (Carlson *et al.*, 1972), of irradiation and chemical treatment (Sidorov *et al.*, 1981; Kihara *et al.*, 1992), or of chemical treatment and selective media (Terada *et al.*, 1987).

The present results indicate that fusion rates of 40~50% were obtained, which were similar to those in the fusion between *S. integrifolium* and *S. toxicarium* (Sadohara, 1993). Heterokaryon frequency was not determined since the protoplasts of the fusion partners were of the same type. However, at least 20% of the fusion products were estimated to be binary fusions. In a previous fusion experiment using the same electric apparatus (unpublished results) with green mesophyll protoplasts of *S. integrifolium* or *S. toxicarium* and colorless cell suspension protoplasts of *S. sisymbriifolium*, heterokaryons formed one quarter of the fusion products (50% fusion rate overall). Therefore, at least 10% of the fusion products were estimated to be heterokaryon. This frequency was 5~6% higher than that reported by Sadohara (1993) for the electro fusion between *S. integrifolium* and *S. toxicarium* protoplasts.

The selection of somatic hybrids by combination of media has been reported (Carlson et al., 1972). In the present experiments, however, the medium for selection of only hybrid could not be found. But I found the media such as F, G and H that one parent protoplasts could not form colonies and the other parents could do. It is unable to determine in these media whether the calli were from one parent or somatic hybrid but is possible to do that they were not from th other parent.

Shoots regenerated from the calli cultured in these media were all identified to be one parent by isozyme analysis. There are two possible reasons that the somatic hybrids could not be obtained. One is that the only one parent protoplasts divided but heterokaryons did not divide or not form colonies after division. The other is that the one parent's chromosome (s) in hybrid cell disappeared during mitotic division at division or regenerating stage.

In this study, I considered that it was necessary for production of somatic hybrids to improve culture conditions, or to use other methods as asymmetric electro fusion.

Literature Cited

Ali, M. 1991. Breeding eggplant rootstock for multiple disease resistance. Dr. Thesis, Kyushu Univ.

Ali, M. and K. Fujieda. 1990. Cross compatibility between eggplant (*Solanum melongena* L.) and wild relatives. J. Japan. Soc. Hort. Sci. 58: 977-984.

Carlson, P.S., H.H. Smith and R.D. Dearing. 1972. Parasexual interspecific plant hybridization. Proc. Nat. Acad. Sci. U.S.A. 69: 2292-2294.

Kalloo. G. 1992. Utilization of wild species. p.149-167. In: G. Kalloo

and J. B. Chowdhury (eds.). Distant hybridization of crop plants. Springer-Verlag, Berlin Heidelberg.

Kao, K.N. 1977. Chromosomal behaviour in somatic hybrids of *Soybean-Nicotiana glauca*. Molec. Gen. Genet. 150: 225-230.

Khan, R 1979. *Solanum melongena* and its wild ancestral forms. p.629-636. In: J. Hawkes, R. Lester and A. Skelding (eds.). The biology and taxonomy of the Solanaceae. Academic Press, London.

Kihara, M., K. Cai. R. Ishikawa. T. Harada, M. Niizeki and K. Saito. 1992. Asymmetric somatic hybrid calli between *Leguminous* species of *Lotus corniculatus* and *Glycine max* and regenerated plants from the calli. Japan. J. Breed. 42: 55-64.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth bioassays with tabacco tissue cultures. Physiol. Plant. 15: 473-479.

Nishio, T., H. Mochizuki and K. Yamakawa. 1984. Inter specific cross of eggplants and related species. Bull. Veg. & Ornam. Crops Res. Stn. Japan. Ser. A. 12: 57-64.

Sadohara, T. 1993. Studies on the breeding of *Solanum* species by protoplast fusion. Thesis of the master degree, Kyushu Univ. (In Japanese).

Sadohara. T., H. Okubo and K. Fujieda. 1993. Plant regeneration from mesophyll protoplasts of *Solanum toxicarium*. Sci. Bull. Fac. Agr., Kyushu Univ. 48: 13-19. (In Japanese).

Sidorov. V.A., L. Menczel, F. Nagy and P. Maliga. 1981. Chloroplast transfer in *Nicotiana* based on metabolic complementation between irradiated and iodoacetate treated protoplasts. Plants 152: 341-345.

Terada, R., Y. Yamashita, S. Nishibayashi and K. Shimamoto. 1987. Somatic hybrids between *Brassica Oleracea* and *B. Campestris*: selection by the use of iodoacetamide inactivation and regeneration ability. Theor. Appl. Genet. 73: 379-384.

Wendel, J.F. 1983. Electrophoretic analysis of genetic variation in wild and cultivated *Camellia japonica* L. Ph. D. Thesis, University of North Carolina, Chapel Hill, North Carolina.

Wendel, J.F. and K. Parks. 1982. Genetic control of isozyme variation in *Camellia japonica* L. J. Hered. 73: 197-204.

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