Asymmetric Somatic Cell Hybrids between Alfalfa and Birdfoot Trefoil

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Summary
Production of intergeneric somatic hybrids between two sexually incompatible leguminous species, alfalfa (*Medicago sativa* L.) and birdfoot trefoil (*Lotus corniculatus* L.) was carried out. Donor protoplasts of alfalfa were given lethal doses of X-irradiation, and recipient protoplasts of birdfoot trefoil were inactivated with iodoacetamide. Donor and recipient protoplasts were fused with PEG. Fusion products initiated cell division and colony formation in the culture medium and resulted in callus formation. By using isozyme analysis, it was confirmed that 17 out of 155 calli were somatic cell hybrids. Some of the hybrid calli regenerated shoots. By using isozyme analysis it was clarified that the chromosomes or chromosome segments were randomly eliminated from both parents at an early stage of hybrid callus culture. However, after a long period of subculture the isozyme pattern of the recipient type was seen in all of the regenerated shoots which were analyzed. This indicates that the genomes in the calli rapidly converged on the recipient type as subcultures progressed. Southern blot analyses of the chloroplast genomes indicated that either genome of both parents sorted out in all calli investigated.

Key Words: *Medicago sativa* L., *Lotus corniculatus* L., Somatic cell hybrids, Callus formation, Regenerated shoots, Chloroplast genome.

Introduction
Somatic cell hybrid production between distantly related species which are incapable of sexual hybridization has been widely carried out. However, agriculturally useful hybrid production has been very difficult, since there is an imbalance in the genomes of the parents in most cases and result in the rearrangement or partial elimination of the chromosomes of one parent and an incapability to achieve morphogenesis (Kao 1977, Chien *et al.* 1982, Sala *et al.* 1985).

Therefore, attempts have been made to fuse X-ray or γ-ray irradiated donor protoplasts with recipient protoplasts (Sidorov *et al.* 1981, Tanno–Suena *et al.* 1988).

In this case, the retention or integration of chromosome segments from the donor protoplasts can be expected in the recipient protoplasts and plant regeneration from the fused cells can also be expected.

Up to date, there have been few reports of successful hybridization between leguminous species (Sano *et al.* 1981, Niizeki and Saito 1989, Kihara *et al.* 1992). We report here, a successful production of calli and regenerated plants within the leguminous species through asymmetric protoplast fusion of X-irradiated alfalfa (*Medicago sativa* L.) with iodoacetamide (IOA)-treated birdfoot trefoil (*Lotus corniculatus* L.).

Materials and Methods

*Callus induction and culture*

The methods used to bring about the callus induction of alfalfa (cv. Rangelander) and birdfoot trefoil (cv. Viking) have been previously described by Niizeki and Saito in 1987 and 1986, respectively. The induced calli of alfalfa were subcultured on B5 basal medium (Gamborg and Ojima 1968) supplemented with 1.0 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.2 mg/l kinetin (B5h medium) at intervals of about 30 days. Birdfoot trefoil calli were subcultured on the MS basal medium (Murashige and Skoog 1962) supplemented with 4 mg/l 1-naphthaleneacetic acid (NAA) and 2.5 mg/l kinetin, also at intervals of about 30 days. This medium was described as M-1N (Niizeki and Saito 1989).

*Protoplast isolation and culture*

Alfalfa and birdfoot trefoil protoplasts were isolated from calli after 10–20 days of subculture. An enzyme solution containing 4% Cellulase Onozuka RS, 1% Macerozyme R-10, 0.2% Pectolyase Y-23 and 0.6–0.7 M mannitol (pH 5.3) was used for the protoplast isolation of both species. Alfalfa and birdfoot trefoil were incubated in this enzyme solution by shaking in the dark at 24 °C for 1–1.5 and 4 hr, respectively. After incubation, the isolated protoplasts were separated from undigested cell clumps by passage through eight layers of cotton gauze, followed by centrifugation at 80g for 5 min. The protoplasts were washed three times with washing solution containing 0.6–0.7 M mannitol and 50 mM CaCl₂ and cultured by a combination of the agarose-
bead method (Shillito et al. 1983) and the nurse culture method. The medium used was a modified KM8p (Kao and Michayluk 1975) containing 0.5 mg/l benzyladenine (BA) instead of zeatin, without adding coconut water. Isolated protoplasts were settled in this medium and solidified with 1.25% agarose (Sea Plaque agarose). The agarose was cut into blocks and transferred to a 60 mm plastic dish containing 5 ml of culture medium with the nurse cells of alfalfa or birdsfoot trefoil.

**X-irradiation to alfalfa protoplasts**
Isolated alfalfa protoplasts were X-ray irradiated at four levels of 100, 200, 300 and 400 Gy and then washed once before giving protoplast fusion treatment.

**IOA-treatment of birdsfoot trefoil protoplasts**
Birdsfoot trefoil protoplasts were treated with 4 concentrations (2.5, 5, 7.5 and 10 mM) of IOA solution containing 0.6 M mannitol and 50 mM CaCl₂ (pH 5.3) for 15 min at 4 °C. They were then washed 3 times by centrifugation (80g) with a washing solution.

**Protoplast fusion and culture of fused protoplasts**
Protoplast fusion by polyethylene glycol (PEG) was carried out using the method of Niizeki et al. (1985).

The fused protoplasts were cultured using the same method as was used for alfalfa and birdsfoot trefoil protoplast when cultured separately. After two weeks the agarose blocks were then washed with 0.5 M sucrose solution in order to remove the nurse cells, and were again transferred to 5 ml of modified KM8p medium. When the colonies became visible, they were transferred to the M-1N medium containing 3.5 g/l agar. The calli obtained were subcultured at intervals of about one month.

**Plant regeneration from the calli**
When vigorous shoot formation occurred, excised shoots were transferred to the basal medium of Nitsch and Nitsch (1969) without growth regulators solidified with 0.2% Gelrite (Scott Laboratory, Inc., California, USA). After root proliferation, the plantlets were transferred to the sterilized soil in pots and 1/2 MS basal medium was added to the soil.

**Isozyme analysis**
Four kinds of isozymes in the calli and regenerated plants were analyzed using the method described by Ishikawa (1994). They were namely; aminopeptidase (AMP), esterase (EST), glutamate dehydrogenase (GDH) and catalase (CAT).

**Southern blot analysis**
The preparation of total DNA from calli was performed according to the method of Varadarajan and Prakash (1991). The 5 µg of total DNA were digested with 20 units of Hind III. The electrophoresis was carried out on 0.8% agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). The Southern blots were prepared by transferring the DNA to a nylon membrane (Duralon-UV membrane; Stratagene) and covalently binding the DNA to the membrane by ultraviolet irradiation. The P2 fragment, which is one of the fragment derived from the rice chloroplast DNA, was used as a probe. The probe was radioactively labeled using Prime-It II random primer labeling kit (Stratagene). The blots were hybridized with the labeled probe in 20 ml of hybridization buffer (6 x SSC, 2 mM EDTA pH 8.0, 10 mM Tris-HCl pH 7.5, 5 x Denhardts, 0.2 mg/ml salmon spermary DNA, 10 mM Na₃PO₄, 1% Na N-Lauroyl Salcosinate) and autoradiographed using Kodak XJB-1 X-ray film.

**Results**

**Effect of X-irradiation on the division of alfalfa protoplasts**
X-ray irradiated protoplasts were cultured using the agarose-bead method with alfalfa nurse cells. The results are indicated in Fig. 1. Protoplast division ceased at a X-ray irradiation level of 300 Gy. In this study protoplasts X-ray irradiated at a level of 400 Gy were predominantly used for protoplast fusion.

**Effect of IOA treatment on the division of birdsfoot trefoil protoplasts**
Using the agarose-bead method, protoplasts of birdsfoot trefoil treated with IOA were cultured with the nurse cells of birdsfoot trefoil. The results are shown in Fig. 2. Protoplast division was completely inhibited after the treatment with 7.5 mM IOA, but protoplasts treated with 10 mM IOA were in the protoplast fusion.

**Culture of fused protoplasts**
Selection of the fused hybrid protoplasts was performed by means of metabolic and physical complementation of IOA-treated and X-ray irradiated protoplasts. Nurse

![Fig. 1. Division rate of the cells derived from alfalfa protoplasts after irradiation of X-rays. This investigation was carried out after two weeks of culture.](image-url)
cells were very effective for cell division in both the parent protoplasts themselves and the fused protoplasts. The fused protoplasts cultured in the M-1N medium formed 2-3 mm colonies after about one month. These colonies were transferred to the solid M-1N medium. 155 calli were obtained from these colonies which were subcultured at intervals of one month.

Regenerated plants
Shoots regenerated from the calli resembled the deep green leaf of birdsfoot trefoil. The shoots regenerated at an early stage of callus culture were frequently teratogenic (Fig. 3). However, the number of teratogenic
Table 1. Isozyme analysis of asymmetric somatic cell hybrids of alfalfa and birdsfoot trefoil

<table>
<thead>
<tr>
<th>Hybrid calli</th>
<th>Plant regeneration</th>
<th>Isozyme pattern I</th>
<th>Isozyme pattern II</th>
<th>Isozyme pattern III</th>
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<tr>
<td></td>
<td></td>
<td>AMP</td>
<td>EST</td>
<td>GDH</td>
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<tr>
<td>I - 1</td>
<td></td>
<td>A+B</td>
<td>-</td>
<td>A</td>
</tr>
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<td>I - 3</td>
<td></td>
<td>A+B</td>
<td>-</td>
<td>A</td>
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<td>I - 7</td>
<td>+</td>
<td>A</td>
<td>-</td>
<td>B</td>
</tr>
<tr>
<td>I - 16</td>
<td>-</td>
<td>A+B</td>
<td>-</td>
<td>A</td>
</tr>
<tr>
<td>I - 20</td>
<td>-</td>
<td>B</td>
<td>B</td>
<td>A</td>
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<tr>
<td>I - 27</td>
<td>+</td>
<td>A+B</td>
<td>B</td>
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<td>I - 29</td>
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<tr>
<td>I - 31</td>
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<td>II - 14</td>
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<td>A+B</td>
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<tr>
<td>II - 63</td>
<td>+</td>
<td>A+B</td>
<td>A+B</td>
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1) +, plant regeneration; -, no plant regeneration. 2) Isozyme pattern I, investigation of the calli cultured for one month, Isozyme pattern II, investigation of the calli cultured for two months, Isozyme pattern III, investigation of the regenerated plants. A: Isozyme band pattern of alfalfa, B: Isozyme band pattern of birdsfoot trefoil, A+B: Isozyme band pattern of both parents. *: Indistinct band pattern or no investigation, AMP: Aminopeptidase, EST: Esterase, GDH: Glutamate dehydrogenase, CAT: Catalase.

Fig. 6. Southern blot analyses of chloroplast genomes in the 14 hybrid calli and their parents. Total DNA was digested with Hind III and P2 fragment of rice chloroplast DNA was used as a probe. A1: Alfalfa, Bt: birdsfoot trefoil, I - 1~ - II - 63: Hybrid calli. The II - 44 and II - 46 hybrid calli show identical banding pattern to alfalfa and the other to birdsfoot trefoil.

regenerated shoots decreased as time proceeded and the morphology of regenerated plants growing in soil resembled to those of birdsfoot trefoil.

Isosyme analysis

Out of 155 calli, 17 calli were identified as being somatic cell hybrids through the use of isosyme analysis (Table 1, Fig. 4). After the culture of hybrid calli for a month, the banding pattern of both parents were seen for four isozymes which were analyzed. In addition, an isosyme banding pattern of one parent was seen in a certain callus, while in the other isosyme, banding pattern of the other parent was also found in the same callus. After another month of subculture on the medium M - 1N, most of the banding patterns had altered to those of birdsfoot trefoil in most of the calli, although only one callus indicated the banding pattern of both parents. In another experiment at early stage of cultured hybrid calli, seven hybrid calli had a catalase with the banding pattern of alfalfa and five calli were found to have the banding pattern of the other parent of birdsfoot trefoil (Fig. 5 (1)). Only one callus exhibited the banding pattern of both parents. However, all of the isosyme banding patterns found in shoots regenerated were the same as those of birdsfoot trefoil (Fig. 5 (2)).

Southern blot analysis of the chloroplast genome

Southern blot analyses of 14 hybrid calli indicated that two hybrid calli, II - 44 and II - 46, have the chloroplast genomes of alfalfa and the other 12 hybrid calli have those of birdsfoot trefoil (Fig. 6). According to the isosyme analysis, it was confirmed that the callus of II - 44 has nuclei of the birdsfoot trefoil (Table 1). Therefore, the II - 44 callus seems to have the nuclei of birdsfoot trefoil and the genomes of alfalfa chloroplast. The color of two hybrid calli with the alfalfa chloroplast genomes was green which was intermediate between the dark green calli of birdsfoot trefoil and yellow one of alfalfa. No shoot regenerated from these hybrid calli.

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Discussion

Some successes in asymmetric protoplast fusion based on the complementation of X-ray or γ-ray irradiated and IOA-treated protoplasts have been reported (Sidorenkov et al. 1981, Tanno–Suenaga et al. 1988). Our results also support these results.

Many hybrid calli cultured for one month were found to have isozymes indicating the banding patterns of both parents. Certain calli were found to have the banding pattern of one parent in some isozymes and the banding pattern of another parent in other isozymes. These facts may suggest that chromosomes or chromosome segments of both parents may be eliminated randomly at the early stage of callus culture. On the other hand, most of the banding patterns had altered to those of the birdsfoot trefoil in two months of culture. Therefore, the number of calli having the chromosomes of both parents would seem to decrease and most of the calli had only birdsfoot trefoil chromosomes. This may indicate that callus cells with birdsfoot trefoil genomes rapidly came to be selected dominantly.

Niizeki et al. (1989) reported that the shoot regeneration did not occur from the symmetric somatic hybrid calli of birdsfoot trefoil and alfalfa. This fact might be attributed to the imbalance of birdsfoot trefoil and alfalfa genomes or incomplete genome of alfalfa, some of which chromosomes were eliminated after protoplast fusion. On the other hand, in this study normal shoot regeneration occurred from asymmetric somatic cell hybrid calli. All of the isozyme banding patterns in the shoots investigated so far were the same as those of birdsfoot trefoil. This result indicates that most of the alfalfa chromosomes irradiated by X-rays degenerated during the subcultures. Accordingly, it may be possible to regenerate the shoots from the calli derived from asymmetric hybrids with X-ray irradiated donor protoplasts, while it is unlikely impossible to regenerate the novel shoots from symmetrical somatic hybrids carrying complete chromosomes of both parents or some chromosomes of one parent.

Niizeki et al. (1990) and Kihara et al. (1992) reported that X-ray irradiated soybean protoplasts and IOA-treated birdsfoot trefoil protoplasts were fused in order to transfer the desirable germplasm from soybean into birdsfoot trefoil. The morphology of the regenerated plants resembled to that of birdsfoot trefoil. However, five regenerated plants were teratological with an erect and short plant height even though all plants had no soybean chromosomes. In this study, the morphology of all plant regenerated at the late stage of hybrid callus culture resembled to that of normal birdsfoot trefoil. This discrepancy may be caused by the difference of phylogenetic distances for the genomes of organelle. The distance of soybean and birdsfoot trefoil may be more remote than that of alfalfa and birdsfoot trefoil.

The isozyme and Southern blot analyses indicated that some hybrid calli had the nuclei of birdsfoot trefoil and chloroplast genomes of alfalfa. This fact proved that the calli obtained were the real asymmetric hybrids. The recalcitrance in shoot regeneration from the calli may be caused by the imbalance of the nucleus and chloroplast genome of concerned two species for the morphogenetic potential. However, improvement or modification of culture media may provide a break through for the regeneration of novel plants.

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Literature Cited


