Selection of a universal hybridizer in *Sinapis turgida* Del. and regeneration of plantlets from somatic hybrids with *Brassica* species

Kinya Toriyama¹, Toshiaki Kameya² and Kokichi Hinata¹

¹ Faculty of Agriculture and ² Institute for Agricultural Research, Tohoku University, Sendai 980, Japan

**Abstract.** A double-mutant cell line, which was unable to grow in a medium with NO₃ as the sole nitrogen source and was resistant to 5-methyltryptophan (5MT), was selected from cell suspensions of *Sinapis turgida* Del. (Brassicaceae) by culturing the cells in AA medium (Toriyama and Hinata, 1985, Plant Sci. 41, 179–183) supplemented with 50 mM chlorate and 229 μM 5MT. Protoplasts of this cell line were fused with mesophyll protoplasts of *Brassica oleracea* L. with dextran, and six somatic hybrids were selected initially by culture in the NO₃ medium and then by transfer to the NO₃ medium supplemented with 229 μM 5MT. The somatic hybrids produced embryoids, leaves and plantlets on a regeneration medium. The hybrid characters were confirmed by investigations of acid phosphatase (EC 3.1.3.2) and peroxidase (EC 1.11.1.7) isoenzymes, chromosome number, growth on NO₃ medium, 5MT resistance, and capacity to regenerate plants. Somatic hybrids between *S. turgida* Del. and *B. nigra* (L.) Koch were also obtained using this method. These results indicate that the double-mutant cell line established here will be able to serve as a universal hybridizer to select somatic hybrids after protoplast fusion with any other wild-type partner.

**Key words:** *Brassica* (somatic hybridization) - Hybridizer, universal - Protoplast fusion - *Sinapis* - Somatic hybrid.

**Introduction**

Somatic hybridization by protoplast fusion is a new method for increasing genetic variability in higher plants. Several intra- and interspecific somatic hybrids have been recovered by this method, and some examples of morphogenesis in intergeneric somatic hybrids have also been reported (Melchers et al. 1978; Gleba and Hoffmann 1978, 1980; Hoffmann and Adachi 1981; Krumbiegel and Schieder 1979, 1981; Dudits et al. 1979, 1980; Nagao 1982; Gleba et al. 1982). Most of the somatic hybrids were produced using selection systems such as biochemical mutants, inactivation of protoplasts, shoot morphology, and mechanical isolation. Among the biochemical mutants, antimetabolite-resistant strains, like strains resistant to amino-acid analogs, were often used effectively as dominant selection markers to select somatic hybrids (White and Vasil 1979; Kameya et al. 1981; Harms et al. 1981; Horn et al. 1983). Counterselectable markers, such as inability to utilize nitrate (Glimelius et al. 1978; Gupta et al. 1982; Pental et al. 1984) and inability to grow in HAT medium (LoSchiavo et al. 1983) are also considered to be very useful. If a double-mutant having both dominant and counter-selectable markers can be established, it should be able to serve as a universal hybridizer to select somatic hybrids exclusively after protoplast fusion with any other wild-type partner. Such double mutants have been reported in *Daucus* (LoSchiavo et al. 1983) and in *Nicotiana* (Pental et al. 1984).

In this paper we report the isolation and characterization of a double mutant of *Sinapis turgida* Del. which was unable to grow on medium containing nitrate as the sole nitrogen source and was resistant to 5-methyltryptophan (5MT). This double mutant was used to produce intergeneric somatic hybrids with *Brassica oleracea* L. and *B. nigra* (L.) Koch, to determine if it could be used as a universal hybridizer.

*Sinapis turgida* Del. is a members of the Brassicaceae and has been classified in the cytode of...
The selected double mutant, which was resistant to 50 mM chlorate and 229 μM 5MT, was designated as "35-i" and used for protoplast preparation. The cell line "35-k", which was isolated from 35-i as a single colony on AA agar medium containing both 50 mM chlorate and 229 μM 5MT, was also used.

Suspension cultures of the mutants were maintained with 40 ml of 5MT medium (Table 1) in 100-ml flasks on a rotary shaker at 120 rpm and subcultured every 14 d at a 1:4 dilution (suspension inoculum/fresh medium), as reported in Toriyama and Hinata (1985).

Protoplast isolation, fusion, selection and culture. Protoplasts of S. turgida were isolated by incubating cells of 35-i or 35-k in an enzyme solution consisting of 4% Cellulase Onozuka R10 (Yakult Biochemicals, Nishinomiya, Japan), 0.2% Macerozyme R10 (Yakult Biochemicals), 10% mannitol and 0.1% CaCl₂·2H₂O (pH 5.5) for 3 h as described in Toriyama and Hinata (1985). The protoplasts were passed through a 30-μm-mesh nylon net and washed three times with a washing solution consisting of 10% mannitol and 0.1% CaCl₂·2H₂O by centrifugation (100 g, 3 min). The floating protoplasts were collected and washed again with the washing solution by sedimentation.

Protoplast fusion was carried out by dextran treatment (Kameya et al. 1981) after mixing equal parts of protoplasts of B. oleracea and 35-i, or B. nigra and 35-k. These protoplasts (2·10⁵ ml⁻¹) were cultured in 2 ml of B5+AA medium (Table 1) supplemented with 8% mannitol. After two weeks, fresh B5+AA medium (1 ml) containing 5% mannitol was added and after another one to two weeks, fresh B5 medium (1 ml) containing neither the amino acids nor mannitol was again supplemented with 229 μM 5MT. One week later, the remaining colonies were transplanted to NB medium (Table 1) for plant regeneration. Growing calli were transplanted every three weeks to fresh NB medium.

For the fusion experiment between B. nigra and 35-k, the selection by 5MT was omitted, because the fusion treatment resulted in destruction of all nonfused mesophyll protoplasts of B. nigra.

The suspension and protoplast cultures were grown under diffuse light (10 μmol m⁻² s⁻¹) and plant regeneration was performed under light (60 μmol m⁻² s⁻¹) from fluorescent lamps (Sunline, Hitachi, and Plantlux, Toshiba, Japan) with a light period of 14 h daily, at 25°C.

Isoenzyme analysis. Isoenzymes were studied in calli of the parents and the hybrids, in embryos that were regenerated by the hybrids, and in the leaves of parents. The tissues (callus, 20 mg; embryos, 20 mg; leaf, 10 mg) were homogenized separately, using mortar and pestle, with 500 μl of 50 mM 2-amino-2-hydroxymethyl-1,3-propanediol (Tria)-HCl buffer (pH 7.5). After centrifugation at 10000·g for 15 min, 20 μl of the super-

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**Table 1. Culture media and their use**

<table>
<thead>
<tr>
<th>Medium</th>
<th>Composition</th>
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<tbody>
<tr>
<td><strong>Suspension culture of Sinapis turgida</strong></td>
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<tr>
<td>AA</td>
<td>Modified B5 medium*, containing an amino-acid mixture* as the sole nitrogen source (Toriyama and Hinata 1985)</td>
</tr>
<tr>
<td>SMT</td>
<td>Modified AA medium, supplemented with 229 μM 5MT</td>
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<tr>
<td><strong>Protoplast culture and selection of hybrids</strong></td>
<td></td>
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<tr>
<td>B5 + AA</td>
<td>Modified B5 medium, supplemented with the amino-acid mixture</td>
</tr>
<tr>
<td>NO₃</td>
<td>Modified B5 medium, lacking (NH₄)₂ SO₄ (contains KNO₃ as sole nitrogen source)</td>
</tr>
<tr>
<td><strong>Plant regeneration</strong></td>
<td></td>
</tr>
<tr>
<td>NB</td>
<td>Modified MS medium*, supplemented with 1 mg·l⁻¹ 3-naphthaleneacetic acid, 1 mg·l⁻¹ N₂-benzylaminopurine and 100 mg·l⁻¹ casamino acids</td>
</tr>
</tbody>
</table>

* Medium of Gamborg et al. (1968), supplemented with 1 mg·l⁻¹ 2,4-dichlorophenoxyacetic acid, 0.2 mg·l⁻¹ kinetin (N⁶-furfuryliminopurine) and 0.1 mg·l⁻¹ gibberellic acid

b The amino-acid mixture of Müller and Grafé (1978): 876 mg·l⁻¹ glutamine, 266 mg·l⁻¹ aspartic acid, 174 mg·l⁻¹ arginine, 7.5 mg·l⁻¹ glycine

c Medium of Murashige and Skoog (1962)

S. arvensis L. (Harberd 1976). There have been no reports of sexual hybrids between S. turgida Del. and Brassica species although hybrids between S. arvensis L. and B. oleracea L., and between S. arvensis L. and B. nigra (L.) Koch have been obtained (Tsunoda et al. 1980).

Materials and methods

Selection of a double mutant. A strain of Sinapis turgida that was preserved in our laboratory (accession No. 1) was used as material. Callus of S. turgida (2n = 18) was initiated by inoculating anthers on MS medium (Murashige and Skoog 1962) supplemented with 1 mg·l⁻¹ 2,4-dichlorophenoxyacetic acid (2,4-D). This callus was maintained as a cell suspension culture (200 mg FW) in 40 ml of 5MT medium, in a Petri dish (90 mm diameter, 15 mm high), according to Toriyama and Hinata (1985). Cells growing in 5MT medium were again plated on 5MT (229 μM) agar (0.8%) medium.