Transfer of resistance against *Phoma lingam* to *Brassica napus* by asymmetric somatic hybridization combined with toxin selection

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Summary. Irradiated mesophyll protoplasts from nine different accessions of *B. juncea*, *B. nigra* and *B. carinata*, all resistant to *Phoma lingam*, were used as gene donors in fusion experiments with hypocotyl protoplasts isolated from *B. napus* as the recipient. A toxin, sirodesmin PL, was used to select those fusion products in which the resistant gene(s) was present. In the fusion experiments different gene donors, various irradiation dosages and toxin treatments were combined. Symmetric and asymmetric hybrid plants were obtained from the cell cultures with and without toxin selection. Isozymes were used to verify hybrid characters in the symmetric hybrids, whereas two DNA probes were used to identify donor-DNA in the asymmetric hybrids. Resistance to *P. lingam* was expressed in all symmetric hybrids, and in 19 of 24 toxin-selected asymmetric hybrids, while all the unselected asymmetric hybrids were susceptible.

**Key words:** *Brassica* – Asymmetric hybrids – In vitro selection – Resistance – *Phoma lingam*

**Introduction**

Gene exchange between different species is normally restricted by incompatibility barriers. These problems can be overcome by using somatic cells for hybridization or DNA transformation. To transfer single, isolated and cloned genes into plants, methods such as the Agrobacterium system (Horsch et al. 1987), direct gene transfer (Potrykus et al. 1987), liposome-mediated transfer (Deshayes et al. 1985) and microinjection (Crossway et al. 1986; Neuhaus et al. 1987) could be used. However, when the goal is to transfer several genes or when the genes of interest are unidentified, asymmetric hybridization mediated by protoplast fusion may be a more efficient technique.

Asymmetric somatic hybrids, carrying the complete genome of the recipient partner plus a few chromosomes or chromosome fragments from a donor partner, can either arise spontaneously from fusions between phylogenetically remote species (Hoffmann and Adachi 1981; Schieder et al. 1985) or be induced. In the latter case, fragmentation of chromosomes in the donor plant material can be obtained after exposure to various physical or chemical mutagens (Blonstein and King 1985).

The most frequently used technique today, for induction of asymmetric somatic hybrids, is the donor-recipient method where the gene donor is irradiated. This method was originally developed for transfer of organelles (Zelcer et al. 1978), but is also used for transfer of nuclear DNA (Dudits et al. 1980). A number of different asymmetric hybrids have been produced by fragmentation of one fusion partner by irradiation (see Hinnidaels et al. 1988 for a review).

Since the elimination of DNA from the irradiated donor is variable and random, a selection pressure is required to ensure the maintenance of a defined trait in the recipient genome. Asymmetric hybridization in combination with in vitro selection has enabled a transfer of traits such as methotrexate and 5-methyltryptophan resistance from *Daucus carota* to *Nicotiana tabacum* (Dudits et al. 1987), as well as kanamycin resistance and the gene for nopaline synthesis from *N. plumbaginifolia* to wild-type protoplasts of *N. tabacum* (Bates et al. 1987).

The present study describes the transfer of resistance gene(s) against *Phoma lingam* from resistant accessions of *Brassica juncea*, *B. nigra* and *B. carinata* (Sjödin and Glimelius 1988) into a susceptible cultivar of *B. napus*. 
An in vitro selection was applied on the hybrid cell cultures with the toxin, sirodesmin PL, produced by *P. lingam* (Sjödin et al. 1988). This toxic compound has been shown to express selective properties (Sjödin and Glimelius 1989a) and was used in these experiments as a selective agent.

**Materials and methods**

**Plant material**

For the production of asymmetric hybrids, *B. napus* L. ssp. oleifera var. annua cv Hanna was used as recipient and five accessions of *B. juncea* (L.) Czern. & Coss., one of which was a rapid cycling line, two accessions of *B. nigra* (L.) Koch cv Junius and a rapid cycling line, and two *B. carinata* A. Braun accessions, all resistant to *Phoma lingam* (Sjödin and Glimelius 1988) were used as donor fusion partners. The origins of these have been described earlier by Sjödin and Glimelius (1988).

**Isolation and irradiation of protoplasts**

Mesophyll protoplasts were isolated from in vitro-grown plants according to Glimelius (1984). The X-rays were generated by a Siemens Stabilipan 200 apparatus. The tube (TR 200F) was operated at 180 kV, 10 mA and the radiation was filtered through 4 mm Al, with a dose rate of 5 Gy/min. Irradiation of the protoplasts was performed directly after enzyme treatment and enrichment of protoplasts, by floating on a CPW 16 solution (Banks and Evans 1976). After irradiation, the protoplasts were further washed with a salt solution W5 (Menczel et al. 1981).

**Protoplast fusion, selection and culture of heterokaryons**

Mesophyll protoplasts were isolated from in vitro-grown plants of the resistant accessions of *B. nigra*, *B. juncea* and *B. carinata*. The mesophyll protoplasts were treated with an X-ray dosage of 70, 100, 150, 180 and 200 Gy, as described above, before fusion with hypocotyl protoplasts isolated from *B. napus*. Growth of hypocotyls and isolation of hypocotyl protoplasts was performed according to Sjödin and Glimelius (1989b).

The hypocotyl protoplasts were stained for 20 min by 5(6)-carboxylfluorescein diacetate (Molecular Probes, Inc.) in a concentration of 0.05 mM per ml of hypocotyl protoplast suspensions. The stain was dissolved in W5 (Menczel et al. 1981) as a stock solution of 0.22 mM.

The protoplast fusions were performed with polyethylene glycol (PEG), both as described by Sundberg and Glimelius (1986), and with the following modification. The protoplasts were mixed to a final concentration of 4.8 x 10^5/ml of hypocotyl protoplasts and 2.5 x 10^7/ml of mesophyll protoplasts. The protoplast mixture was heated in a 45°C water-bath for 5 min before being spaced as droplets in 10-cm petri dishes. The PEG solution and the CaCl_2 washing solutions were also heated to 45°C before addition to the protoplast droplets. A solution with the modified 8 pm culture medium with 0.4 M mannitol and W5 (1:1 v/v), containing 4.5 µM 2,4-D, 2.2 µM BAP and 0.5 µM NAA, was finally added to the PEG-treated protoplasts.

**Enrichment, selection and culture of heterokaryons**

After fusion treatment the cultures were stored in darkness, for at least 4 h, in a refrigerator. Before selection of the heterokaryons with flow sorting (Glimelius et al. 1986), the protoplasts were washed off the petri dishes and centrifuged at 75 g for 5 min. The protoplast pellet was suspended in the 8 pm culture medium to a density of about 75 x 10^9 protoplasts per ml.

The enriched heterokaryons were cultured after flow sorting in the 8 pm medium. When the cell divisions had started, fresh culture medium without hormones was added. The volumes and the hormone concentrations used were as described above.

When small cell aggregates were obtained (15–20 cells), the cell cultures were embedded according to Sjödin and Glimelius (1989a). After gelling, the beads were placed in 2.0-cm petri dishes with liquid 8 pm medium, containing the same hormones as the beads. Sirodesmin PL was added to the bead cultures to give a final concentration of 3.0 µM.

Differentiation of the small calli was performed according to the methods described by Sjödin and Glimelius (1989b). The hormone concentrations used during these steps were 0.6 µM IAA, 2.2 µM BAP and 2.3 µM zeatin, or 0.6 µM IAA, 4.4 µM BAP and 4.6 µM zeatin. During the whole culture period, 3.0 µM sirodesmin PL was present in the medium. Light and temperature conditions for the cell cultures were as described by Sjödin and Glimelius (1989b). Shoots were transferred to hormone-free medium with salts from the root-inducing medium M4 (Installé et al. 1985), supplemented with vitamins from the K_5 medium, 0.03 M sucrose and 0.3% Gellan Gum (Kelco). The shoots were cultured under sterile conditions (Sjödin and Glimelius 1989b) in a temperature of 15°C. When roots had developed, the established plants were planted in the greenhouse.

**Confirmation of hybrid character**

**Isozyme analysis**

Isozyme analyses were performed according to Sundberg and Glimelius (1986), using the buffer G and N according to Shields et al. (1983). The enzymes examined were phosphoglucomutase (PGM), leucine aminopeptidase (LAP), phosphoglucomerase (PGI), aspartate aminotransferase (AAT), 6-phosphogluconate dehydrogenase (6-PGDH), glucose-6-phosphate dehydrogenase (G-6-PDH), shikimate dehydrogenase (SHDH), malate dehydrogenase (MDH), and triosephosphate isomerase (TPI), which gave distinct isozyme patterns between *B. napus* and the other fusion parents used.

**RFLP analysis**

**DNA isolation from plants.** Total DNA was isolated according to a modified method of DellaPorta et al. (1983). One gram of tissue from each of the young greenhouse-grown plants of *B. napus* cv Hanna, the 9 donor accessions and 23 putative hybrid plants was homogenized in liquid nitrogen. The homogenate was suspended in a buffer (15% sucrose, 50 mM TRIS-HCl pH 8, 50 mM NaEDTA), and 200 µl of a 10% SDS solution was added to the suspension. The sample was incubated at +70°C for 20 min before addition of 1 ml 5 M KAc. After incubation on ice for 60 min, the sample was centrifuged at 1,000 x g for 10 min, and the supernatant was collected and filtered through one layer of Miracloth. DNA was ethanol-precipitated, dried and dissolved in 900 µl TE-buffer (1 mM NaEDTA, 10 mM TRIS-HCl, pH 7.5) and 100 µl 3 M NaCl. The sample was then treated with RNase, precipitated again and the final pellet was dissolved in 100-200 µl TE-buffer.

**Southern blot and hybridization.** About 10 µg DNA of each sample was digested with BamHI, EcoRV and HaeIII (Pharmacia), and fragments were separated by electrophoresis in 0.5% agarose gel (40 V, 40 mA, 20 h).

The Southern blot analysis was a modified method of Maniatis et al. (1982). The DNA fragments were transferred