Gene transfer in plants of *Brassica juncea* using *Agrobacterium tumefaciens*-mediated transformation

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**Abstract.** An efficient system for gene transfer into plants of *Brassica juncea* var. India Mustard, mediated by *Agrobacterium tumefaciens*, was developed through the manipulation of the culture medium and the use of the appropriate *Agrobacterium* strain. High frequency shoot regeneration (90-100%) was obtained from hypocotyl explants grown on medium containing 0.9% agarose, 3.3 mg/L AgNO₃ and 0.5-2 mg/L BA in combination with 0.01-0.05 mg/L 2,4-D or 0.1-1 mg/L NAA. Of all the *Agrobacterium* strains tested, *A. tumefaciens* A208-SE, carrying the disarmed Ti plasmid and a binary vector pROA93, was the most effective for *B. juncea* transformation. pROA93 carries the coding sequences of the NPTII and the GUS genes, both driven by a common CaMV 35S promoter in two divergent directions. Inoculated explants grown on the selection medium in the presence of 0.5 mg/L BA and 0.1 mg/L NAA gave rise to transgenic shoots at the highest frequency (9%). All R₂ transgenic plants were phenotypically normal, but variation in expression patterns of the GUS gene occurred among the transgenic plants in an organ- and tissue-specific manner. Both the NPTII and the GUS genes were transmitted to the R₃ seed progeny and showed co-segregation.

**Abbreviations:** BA, benzyladenine; 2,4-D, 2,4-dichlorophenoxyacetic acid; NAA, naphthaleneacetic acid; NPTII, neomycin phosphotransferase type II; GUS, β-glucuronidase; CaMV, cauliflower mosaic virus; MS, Murashige and Skoog; X-Gluc, 5-bromo-4-chloro-3-indolyl-D-β-glucuronidase; IBA, indolebutyric acid; SDS, sodium dodecyl sulfate.

**Keywords:** *Agrobacterium tumefaciens; Brassica juncea*; genetic transformation; gene expression; transgenic plants.

**Introduction**

Recent advances in cell and molecular biology have facilitated the transfer of foreign genes into plants, which is the first step towards the genetic improvement of crops using the new biotechnological approach. To date plants from at least 26 genera belonging to 15 families have been successfully transformed and transgenic plants with agronomically important traits have been produced. These include plants resistant to viruses (Lawson et al., 1989; Hill et al., 1991), insects (Hilder et al., 1987; Fischhoff et al., 1989) or herbicides (Stalker et al., 1988; Lee et al., 1988). Of all the gene transfer methods available for plants (Potrykus 1989), *Agrobacterium tumefaciens*-mediated transformation has been most widely used.

The genus *Brassica* includes a diverse group of crop plants with great economic value worldwide. Various *Brassica* species have been shown to be highly susceptible to *A. tumefaciens* (Charest et al., 1989) and plants of *B. napus* (Pua et al., 1987; Fry et al., 1987; Moloney et al., 1989) *B. oleracea* (David and Tempe, 1988; Srivastava et al., 1988) and *B. juncea* (Mathews et al., 1990) have been transformed. *Brassica juncea* is an important oilseed and vegetable crop in Asia. Although Mathews et al. (1990) reported successful production of transgenic *B. juncea* plants, the frequency of plant transformation was low and the transgenic plants, carrying a chimeric NOS/NPTII gene, were not able to root on the selection medium. As part of a study to produce transgenic plants of *B. juncea* in this laboratory, we have developed methods for the regeneration of plants from cotyledonary explants (Chi et al., 1990) and hypocotyl protoplasts via somatic embryogenesis (Pua, 1990). In this study, we report an efficient gene transfer system for the production of normal, fertile transgenic plants of *B. juncea* using *Agrobacterium tumefaciens*-mediated transformation.

**Materials and methods**

**Plant materials.** Seeds of *Brassica juncea* var. India Mustard (from Sunrise Enterprises, Elmwood, CT) were surface-sterilized as previously described (Pua 1989) and then germinated on half-strength hormone-free Murashige and Skoog's (MS) medium (1962). In all the experiments reported here, all the constituents were added to the medium and the pH adjusted to 5.8 before autoclaving at 1 kg/cm² and 121°C for 15 min, except AgNO₃, kanamycin, carbenicillin, spectinomycin and chloramphenicol, which were filter-sterilized. Cultures were maintained in a tissue culture room under a 16 h light and 8 h dark cycle with a photon fluence rate of 55-65 μE/m²/s from Sylvania cool white F37T8/CW fluorescent lamps. The day and night temperatures were 27±1°C and 23±1°C, respectively.

**Shoot regeneration.** To optimise the culture medium for high frequency shoot regeneration, hypocotyls excised from 3-4 d-old aseptically germinated seedlings were used. These explants were cultured in 100 x 25 mm Petri dishes each containing MS medium supplemented with different combinations of BA, i.e. 0.5, 1 and 2 mg/L, and 2,4-D, i.e. 0.01, 0.05 and 0.1 mg/L, or NAA, i.e. 0.01, 0.1, 0.5 and 1 mg/L, and 0.9% agarose (SeaPlaque® FMC Bioproducts, Rockland, ME) in the presence or absence of 3.3 mg/L AgNO₃ and 500 mg/L carbenicillin. There were two replicates each with 10-12 explants. Explants were evaluated for percent shoot
regeneration and number of shoots per explant after 3-5 weeks in culture.

**Agrobacterium strains.** Four *A. tumefaciens* strains, namely, A208-SE (Sciaky et al., 1977), GV3111-SE (DeGreve et al., 1981), EHA101 (Hoed et al., 1986) and A348 (Sciaky et al., 1977), and *A. rhizogenes* R1000 (White et al., 1985) were used in this study. EHA101, A348 and R1000 are the generous gift from N. H. Chua of the Rockefeller University, New York. A binary vector pROA93 (Jia et al., 1989) in E. coli LE392 was mobilized into various *Agrobacterium* strains using triparental mating (Rogere et al., 1986). pROA93 carries the coding sequences of the NPTII and the GUS genes both driven by a common CaMV 35S promoter in two divergent directions (Figure 1). Transformed cells expressing both chimeric genes are resistant to kanamycin and stain blue in the presence of X-Gluc.

**Co-cultivation of explants with agrobacteria.** The overnight grown bacteria were suspended in liquid shoot induction medium consisting of MS salts and vitamins, 30 g/L sucrose, 3.3 mg/L AgNO₃, supplemented with 2 mg/L BA and 1 mg/L NAA (medium 1), 0.5 mg/L BA and 0.1 mg/L NAA (medium2) or 1 mg/L BA and 0.01 mg/L 2,4-D (medium 3). The density of the bacteria was adjusted to A₆₀₀ = 0.1. Cotyledons and hypocotyls (the two 5 mm long segments closest to the shoot apex), excised from 3-4 d-old aseptically germinated seedlings were pre-cultured for one day in the appropriate liquid medium at 28°C in the dark. Explants were then inoculated with agrobacteria for 3-5 min, blotted dry and co-cultured at 25°C in the dark on a layer of sterile filter paper placed on top of the medium solidified with agarose. After three days of co-culture, explants were washed overnight in liquid medium containing 500 mg/L carbenicillin. For early assessment of the transformation efficiency, we employed a modified version of the method described by Janssen and Gardner (1989), by hand sectioning the cut ends of 10 inoculated explants grown on medium 1 (2-3 sections per end), and assaying histochemically for GUS activity as described below.

**Histochemical assay for β-glucuronidase (GUS).** Histochemical determination of GUS activity in the plant tissues was generally conducted according to Jefferson et al., (1987). Tissue sections or organs were incubated overnight at 37°C in X-Gluc solution consisting of 10 mM ethylenediaminetetraacetic acid, 100 mM sodium phosphate (pH 7.0), 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide and 0.1% (w/v) X-Gluc (Research Organics, Inc., Ohio). Chlorophyll was removed by treating the tissue for 15 min with FAA (10 mL formaldehyde : 5 mL glacial acetic acid : 50 mL ethyl alcohol : 35 mL water), followed by 2-10 min each with 70%, 90% and 100% ethanol and water before examination microscopically.

**Selection of stable transformants.** Hypocotyls co-cultivated with A208-SE as previously described were transferred to the appropriate selection medium 1, 2 or 3, containing 500 mg/L carbenicillin and 20 mg/L kanamycin to optimise for transgenic shoot production. Explants were subcultured at 2-3 week intervals. As green shoots emerged from the explants over a period of 1-3 months, they were tested for GUS activity. The transformation frequencies were calculated according to the number of explants forming GUS-positive shoots. These GUS positive shoots were transferred to selection rooting medium containing 0.1 mg/L IBA plus carbenicillin and kanamycin at the same concentrations as used for the selection medium. For plant acclimatization, the rooted shoots were transferred to plastic pots (6 in diameter) each containing Levingtons® potting compost (Fisons plc, Ipswich, U.K.). After 2-3 d in dim light, plants were transferred to an environmentally controlled growth chamber with 75-80% relative humidity, 12 h photoperiod and a light intensity of 170-280 µE/m²/s from the combination of fluorescent and incandescent lamps. The temperatures were 20°C in the light and 15°C in the dark. Plants were grown to maturity, with a weekly application of nutrient 20N:20P:20K. The inflorescence of each flowering plant was bagged to ensure self pollination. Seeds were collected and stored at room temperature.

Various organs, i.e. leaf, root, sepal, petal, anther, pistil, mature pollen and seed pod, of 18 transgenic plants were tested for the histochemical assay of GUS activity to provide an indication of the uniformity of GUS gene expression in different organs among individual transgenic plants.

**Evaluation of seed progeny from transgenic plants.** Seed progeny of 19 transgenic plants of *B. juncea* were evaluated for kanamycin sensitivity by aseptically germinating the seeds on hormone-free MS medium supplemented with 200 mg/L kanamycin. The sensitivity of seedlings was evaluated after one week. The GUS activity in cotyledons, hypocotyls, shoot apices and roots of transgenic seedlings was also determined as previously described.

**DNA isolation and Southern blot analysis.** DNA was isolated from frozen leaf tissues originating from 6-8 week-old flowering plants according to the method of Eichholtz et al. (1987). Southern hybridization was conducted according to Sambrook et al. (1989) with modification. For each plant, 5 µg genomic DNA was digested with restriction endonuclease EcoRI and separated by electrophoresis in a 0.8% agarose gel. Prehybridization was performed by incubating the membrane in 50% formamide - 1% SDS, 1 M NaCl and 10% dextran sulfate at 42°C for 45 min, followed by hybridization at 42°C overnight with labelled probe in prehybridization solution containing 100 µg/ml denatured salmon sperm DNA. The probe was prepared from the HindIII/BamHI fragment of pROA93 containing approximately 1 kb of the NPTII coding sequence, then labelled with ³²P(dCTP) using a random primed DNA labelling system (Boehringer Mannheim) according to the manufacturer's instructions. After hybridization, the membrane was washed twice at room temperature for 5 min in a solution containing 2xSSC, then twice at 65°C for 30 min in 2xSSC and 1% SDS, followed by two 30 min washes in 0.1xSSC at room temperature and autoradiography with an intensifying screen at -80°C.

**Results**

Preliminary studies in the optimization of the culture medium indicated that the use of agarose as a gelling agent rather than Difco bacto-agar was beneficial for shoot regeneration from seedling explants of *B. juncea*, since agarose resulted in a higher regeneration frequency and a greater number of shoots per explant (results not shown). Bacto-agar is therefore replaced by agarose in all subsequent studies. Although SeaPlaque agarose was used throughout this study, we later found that a more economic form of gelling agent, 0.4% ultra-pure agarose (electrophoresis grade, from BRL Life Technologies Inc., MD 20877), could be used as a substitute for SeaPlaque agarose. With regard to growth regulators, high frequency shoot regeneration (90-100%) from hypocotyl explants occurred on media containing 0.5-2 mg/L BA in combination with 0.01-0.05 mg/L 2,4-D or 0.1-1 mg/L NAA in the presence of 3.3 mg/L AgNO₃, with each explant producing 5-13 shoots (Table 1). Explants were poorly regenerative in