GENETIC TRANSFORMATION AND HYBRIDIZATION

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Agrobacterium-mediated genetic transformation of California poppy, Eschscholzia californica Cham., via somatic embryogenesis

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Abstract An efficient Agrobacterium-mediated protocol for the stable genetic transformation of Eschscholzia californica Cham. (California poppy) via somatic embryogenesis is reported. Excised cotyledons were co-cultivated with A. tumefaciens strain GV3101 carrying the pBI121 binary vector. Except for the co-cultivation medium, all formulations included 50 mg l⁻¹ paromomycin as the selective agent and 200 mg l⁻¹ timentin to eliminate the Agrobacterium. Four to five weeks after infection, paromomycin-resistant calli grew on 80% of explants in the presence of 2.0 mg l⁻¹ 1-naphthaleneacetic acid (NAA) and 0.1 mg l⁻¹ 6-benzylaminopurine (BAP). Calli were cultured on somatic embryogenesis induction medium containing 1.0 mg l⁻¹ NAA and 0.5 mg l⁻¹ BAP, and somatic embryos were visible on 30% of the paromomycin-resistant calli within 3–4 weeks. Three to four weeks after the somatic embryos were transferred to phytohormone-free plant regeneration medium, 32% converted to paromomycin-resistant plants. Detection of the neomycin phosphotransferase gene and high levels of β-glucuronidase (GUS) mRNA and enzyme activity, and the cytohistochemical localization of GUS activity in all plant tissues confirmed the integrative transformation of the regenerated plants. The normal alkaloid profile of California poppy was unaffected by the transformation process; thus, the reported protocol could serve as a valuable tool to investigate the molecular and metabolic regulation of the benzophenanthridine alkaloid pathway.

Key words Agrobacterium tumefaciens · Benzylisoquinoline alkaloids · California poppy · Eschscholzia californica Cham. · Genetic transformation · Somatic embryogenesis

Abbreviations BAP: 6-Benzylaminopurine · B5: Gamborg B5 basal medium · 2,4-D: 2,4-Dichlorophenoxyacetic acid · GUS: β-Glucuronidase · NAA: 1-Naphthaleneacetic acid · NPTII: Neomycin phosphotransferase · HPLC: High-performance liquid chromatography

Introduction

Eschscholzia californica Cham. (California poppy) is a traditional medicinal plant of many indigenous peoples in North America (Cheney 1964). The remedial properties of this common ornamental species are the result of its ability to synthesize a variety of benzophenanthridine alkaloids, which comprise a unique class of pharmacologically active compounds restricted in occurrence to higher plants. Sanguinarine, one of the principal benzophenanthridine alkaloids found in the roots of E. californica, is used commercially as an antiplaque agent in oral hygiene products due to its potent antimicrobial activity (Dzik and Socransky 1985). The biosynthesis of benzophenanthridine alkaloids in E. californica has been studied extensively (Kutchan 1998), especially since Penicillium-infected callus cultures of California poppy were first reported to accumulate copious amounts of these secondary metabolites (Schumacher et al. 1987). Recently, cDNAs encoding two alkaloid biosynthetic enzymes in E. californica have been cloned and shown to be transcriptionally induced in cultured cells in response to methyl jasmonate- or elicitor-treatment (Dittrich and Kutchan 1991; Pauli and Kutchan 1998).

Despite our appreciation for the chemistry, pharmacology, and commercial importance of benzophenanthridine alkaloids, the basic molecular and meta-
bolic mechanisms that regulate their biosynthesis remain mostly unknown. Attempts to understand the molecular regulation of genes encoding alkaloid biosynthetic enzymes in *E. californica* have relied on transient expression systems based on microprojectile bombardment of cell suspension cultures (Hauschild and Kutchan 1998). However, this approach precludes any investigation of developmental, and most inducible, aspects of regulation because many of the relevant genes are immediately activated by the wound signal that results from the entry of DNA-coated microcarriers into the cells. The establishment of an efficient genetic transformation protocol for *E. californica* will facilitate a more complete application of modern molecular and biochemical approaches to study the regulation of benzophenanthridine alkaloid metabolism and create opportunities for the development of metabolic engineering strategies designed to enhance the biotechnological potential of medicinal plants.

Recently, we reported the development and optimization of a rapid method for high-frequency somatic embryogenesis and plant regeneration from embryogenic callus cultures of *E. californica* (Park and Facchinini 2000). Here, we describe a simple and efficient *Agrobacterium*-mediated protocol for the stable genetic transformation of California poppy plants via somatic embryogenesis.

**Materials and methods**

Seed sterilization and germination

Seeds of *Eschscholzia californica* Cham. were purchased from Western Horticultural Limited (Calgary, Canada) and stored at 4°C. The seeds were surface-sterilized with 70% (w/v−1) ethanol for 30 s and 2% (w/v−1) sodium hypochlorite solution for 10 min, then rinsed three times in sterilized water. Approximately 30 seeds were placed on 25 ml of agar-solidified culture medium in petri dishes (100 x 15 mm). The basal medium consisted of B5 salts and vitamins (Gamborg et al. 1968) solidified with 0.8% (w/v−1) Phytagar (Gibco, Burlington, Canada). The medium was adjusted to pH 5.8 before adding the agar, and then sterilized by autoclaving at 1.1 kg cm−2 (121°C) for 20 min. The seeds were germinated in a growth chamber at 25°C under standard cool white fluorescent tubes (*Sylvania Gros-Lux Wide Spectrum, Mississauga, Canada*) with a flux rate of 35 μmol s−1 m−2 and a 16-h photoperiod.

Preparation of *Agrobacterium tumefaciens*

The binary vector pBI121 (Jefferson et al. 1987) was mobilized by electroporation in *Agrobacterium tumefaciens* strain GV3101 carrying the helper plasmid pMP90 (Kounei and Schell 1986). *A. tumefaciens* cultures were grown at 28°C on a gyromatic shaker at 180 rpm in liquid Luria-Bertani medium [1% (w/v−1) tryptone, 0.5% (w/v−1) yeast extract, and 1% (w/v−1) NaCl, pH 7.0] containing 50 mg l−1 kanamycin, to mid-log phase (A590=0.5). The bacterial cells were collected by centrifugation for 10 min at 1500 rpm and resuspended at a cell density of A600=1.0 in liquid inoculation medium (B5 salts and vitamins containing 20 g l−1 sucrose).

Production of transgenic plants

Excised cotyledons from 5-day-old seedlings were isolated by longitudinal bisection of the hypocotyl. The cotyledons were dipped into the *A. tumefaciens* culture in liquid inoculation medium for 15 min, blotted dry on sterile filter paper, and incubated in the dark at 25°C on primary callus induction medium (B5 medium containing 30 g l−1 sucrose, 2.0 mg l−1 NAA, and 0.1 mg l−1 BAP, 8 g l−1 Phytagar). After 2 days of co-cultivation with *A. tumefaciens*, the cotyledons were transferred to fresh primary callus induction medium containing 50 mg l−1 paromomycin and 200 mg l−1 timentin. After 4–5 weeks of incubation, primary calli were subcultured on somatic embryo induction medium (B5 medium containing 1.0 mg l−1 NAA, 0.5 mg l−1 BAP, 50 mg l−1 paromomycin, 200 mg l−1 timentin, and 8 g l−1 Phytagar). After 3–4 weeks of cultivation on induction medium, somatic embryos were transferred to phytohormone-free plant regeneration medium (B5 medium containing 50 mg l−1 paromomycin, 200 mg l−1 timentin, and 8 g l−1 Phytagar). Regenerated putative transgenic plantlets were grown in a growth chamber for 3–4 weeks at 25°C under standard cool-white fluorescent tubes (*Sylvania Gros-Lux Wide Spectrum*) with a flux rate of 35 μmol s−1 m−2 and a 16-h photoperiod. Plantlets were then transferred to pots containing autoclaved vermiculite, covered with polyethylene bags for 1 week to sustain high humidity, and maintained in the growth chamber at 25°C for 4 weeks before the plants were transferred to the greenhouse (Park and Facchinini 2000).

Polymerase chain reaction (PCR) analysis of transformation

Plant genomic DNA for PCR analysis was extracted as described by Edwards et al. (1991). Tissues (50 mg fresh weight) were homogenized in 200 μl of extraction buffer [0.5% (w/v−1) SDS, 250 mM NaCl, 100 mM Tris-HCl, pH 8.0, and 25 mM EDTA] and centrifuged at 13 000 rpm for 5 min. The supernatant was transferred to a new tube, and an equal volume of isopropanol was added. The sample was incubated on ice for 5 min and then centrifuged for 10 min at 13 000 rpm. The pellet was dried at 60°C for 10 min and then resuspended in 100 μl of TE buffer (10 mM Tris-HCl, pH 7.4 and 1.0 mM EDTA). PCR was performed for 30 thermal cycles (denaturation at 95°C for 1 min, primer annealing at 55°C for 1 min, and primer extension at 72°C for 1 min) using primers specific to sequences found in the *NPTII* selectable marker gene (*5'-TATGTTATGTATGGCAGATGATT-3', and 5'-GTCGACTCTACCCGAAAGAACTGTC-3'). Amplification products were analyzed on 1% (w/v−1) agarose gels.

Assay of GUS activity

*E. californica* tissues were ground with extraction buffer (50 mM KPO4 buffer, pH 7.0, 1 mM EDTA, and 10 mM β-mercaptoethanol) in an Eppendorf tube. 4-Methylumbelliferyl-β-D-glucuronide was added at a final concentration of 0.44 mg ml−1 to the GUS fluorometric assay buffer (50 mM NaPO4 buffer, pH 7.0, 10 mM β-mercaptoethanol, 10 mM EDTA, 0.1% (w/v−1) sodium lauryl sarcosine, and 0.1% (w/v−1) Triton X-100). Assays were performed on 50 μl of tissue extract for 3 h at 37°C and stopped with a 10× volume of 0.2 M Na2CO3. A fluorescence spectrophotometer (Hitachi F-2000, Tokyo, Japan) was used to quantify the amount of 4-methylumbelliferone (MU) cleaved from MUG. The protein concentration was determined by the method of Bradford (1976) using BSA as the standard.

RNA gel blot hybridization

Total RNA for gel blot hybridization analysis was isolated using the method of Loganmann et al. (1987), and 15 μg was fraction-