An efficient Agrobacterium tumefaciens-mediated transformation and regeneration system for cotyledons of spinach (Spinacia oleracea L.)

Abstract An efficient transformation and regeneration system was established for the production of transgenic spinach (Spinacia oleracea L.) plants. Cotyledon explants were infected with Agrobacterium tumefaciens strain LBA4404 carrying the selectable marker gene, neomycin phosphotransferase II (nptII), and the reporter gene smgfp, encoding soluble-modified green-fluorescent protein, driven by the cauliflower mosaic virus 35S promoter. The infected explants were cultured on Murashige and Skoog medium, containing 1 mg/l benzyladenine and 0.4 mg/l naphthaleneacetic acid. Shoots were regenerated on selection medium containing 50 mg/l kanamycin. Regenerated kanamycin-resistant shoots were rooted on medium containing 1 mg/l indolebutyric acid and subsequently grown in soil in the greenhouse. Southern blot analysis indicated that the smgfp gene had been integrated into the spinach genome. Northern and Western blots showed that the smgfp gene was expressed in progeny plants.

Key words Spinach (Spinacia oleracea L.) · Regeneration · Green-fluorescent protein · Transformation · Cotyledon

Abbreviations BA 6-Benzyladenine · CaMV Cauliflower mosaic virus · GA 3 Gibberellic acid · GUS β-Glucuronidase · IBA Indolebutyric acid · MS medium Murashige and Skoog medium · NAA Naphthaleneacetic acid · smGFP Soluble-modified green-fluorescent protein

Introduction

Recent advances in molecular genetics and biotechnology have made plant transformation and regeneration a powerful tool for crop improvement. In many species, transgenic plants have been obtained by Agrobacterium tumefaciens-mediated transformation. In spinach, a vegetable crop which is rich in vitamins and other essential nutrients, plant regeneration was reported only recently by organogenesis from leaf discs, hypocotyl segments (Al-Khayri et al. 1991a, b, 1992a, b; Xiao and Branchard 1993; Molvig and Rose 1994; Sakaki et al. 1994), and root sections (Knoll et al. 1997). Al-Khayri (1995) transformed spinach with the GUS reporter gene. However, the stable incorporation of the GUS gene into the spinach genome was not verified. More recently, two cucumber mosaic virus coat protein genes were expressed in spinach (Yang et al. 1997). In all these reports, gibberellic acid (GA 3 ) was found to be necessary for shoot regeneration from leaf discs, hypocotyl, and root sections. We report here a rapid and efficient method for producing transgenic plants from spinach cotyledons which does not require addition of GA 3 for shoot regeneration.

Materials and methods

Plant material

Spinach (Spinacia oleracea L.) plants of the cultivars Longstanding Bloomsdale Dark Green (Stokes Seeds, Buffalo, N. Y.) and Melody (Harris Seeds, Rochester, N. Y.) were used. Seeds were surface-sterilized in germicidal bleach (Clorox, Oakland, Calif.) for 25 min, followed by immersion in 70% (vol/vol) ethanol for 20 min, and rinsed three times in sterile water. Seeds were germinated on Murashige and Skoog (1962) (MS)-based medium with 3% (wt/vol) sucrose and 0.8% (wt/vol) Difco agar in petri dishes in short-day conditions consisting of 8 h light from 40-W cool white Duke fluorescent tubes (80–90 μmol m −2 s −1 ) and 16 h of darkness at 23°C. Cotyledons were aseptically excised from 5-day-old seedlings for infection with A. tumefaciens.
Transformation of LBA4404 with p35S/smGfp

*Agrobacterium tumefaciens* LBA4404 was cultured in YEP medium (Sambrook et al. 1989) containing 100 mg/l rifampicin (Sigma, St. Louis, Mo.). An overnight culture of 2 ml was added to 50 ml fresh YEP medium and incubated at 28°C with vigorous shaking. When the optical density at 600 nm reached 0.5, the culture was kept on ice for 30 min, and the bacteria were pelleted by centrifugation and washed once with 10 ml 0.15 M NaCl. Finally, the collected bacteria were suspended in 1 ml 600 nm reached 0.5, the culture was kept on ice for 30 min, and the bacteria were pelleted by centrifugation and washed once with 10 ml 0.15 M NaCl. Finally, the collected bacteria were suspended in 1 ml 600 nm reached 0.5, the culture was kept on ice for 30 min, and the bacteria were pelleted by centrifugation and washed once with 10 ml 0.15 M NaCl. Finally, the collected bacteria were suspended in 1 ml 0.1 ml of solution I (4 mg/ml lysozyme, 50 mM sucrose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0) at 37°C for 5 min. This was mixed with 0.2 ml of solution II (1% SDS, 0.2 M NaOH) and 0.15 ml of 3 M KAc (pH 4.8). The mixture was kept at 20°C for 25 min and then centrifuged in a microcentrifuge at 7,850 g for 3 min. The supernatant was mixed with two volumes of 100% ethanol and precipitated at –20°C for 24 h. Spinach cotyledons were ground in liquid nitrogen using a motor driven homogenizer (Waring). The homogenate was filtered through 1% agarose gels in the presence of formaldehyde (Strommer et al. 1995). The blots were then exposed to phosphor screens, and checked for amplification of the 35S promoter and the nos terminator signal were inserted.

**Construction of the soluble-modified green-fluorescent protein (smGfp) gene expression vector p35S/smGfp**

Plasmid CD3–326 (Arabidopsis Biological Resource Center, The Ohio State University, Columbus, Ohio) which contains a more soluble version of the codon-modified green-fluorescent protein, called smGfp (Davis and Vierstra 1998), was digested with HindIII and EcoRI. To produce the expression vector p35S/smGfp (Fig. 1), the cauliflower mosaic virus (CaMV) 35S promoter and the GUS gene (Jefferson et al. 1987) in the A. *tumefaciens*-based binary vector pBI121 were replaced with the 35S/smGfp-nosT region as a HindIII-EcoRI fragment from CD3-326.

**Spinach transformation and regeneration**

*A. tumefaciens* LBA4404 containing p35S/smGfp was cultured in YEP medium (pH 7.0) and 1 mM ethanol, kept in –80°C for 8 min, then precipitated. Finally, the pellets were dissolved in 0.5 ml of 0.3 M NaAc (pH 7.0) and 1 mM EDTA. Filters were washed twice for 25 min in 0.4 M Na2HPO4 (pH 7.2), 10 mM EDTA, and 5% SDS at 68°C (Xu et al. 1995). The blots were then exposed to phosphor screens, and checked with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.).

**Southern blot analysis of transgenic plants**

For Southern blot experiments, total genomic DNA was isolated from wild-type spinach and kanamycin-resistant *T*1 progeny (different regenerated lines). About 2.0 g of leaves was ground in liquid nitrogen, suspended in 2 ml DNA isolation buffer (1% sarkosyl, 0.25 M sucrose, 50 mM NaCl, 20 mM EDTA, 50 mM Tris-HCL, pH 8.0) and 2 ml phenol (saturated with Tris-HCl, pH 8.0). The mixture was kept in ice water for 10 min, and centrifuged for 5 min at 13,200 g. The aqueous phase was transferred to a new tube which contained an equal volume of 2-propanol, and mixed gently by inverting the tube. Genomic DNA was removed with a toothpick, rinsed in 5 ml 70% (vol/vol) ethanol, and dissolved in 0.5 ml distilled water. About 20 mg DNA was digested with *ClaI*. The digest was electrophoresed through a 0.8% agarose gel and blotted onto nylon membranes (Sambrook et al. 1989).

**Western blot verification of the smGFP protein in transgenic plants**

Spinach leaves (1.0–1.5 g) from Southern and Northern positive *T*1 plants, and from wild type spinach plants were ground in liquid ammonia (Stasinopoulos and Hangarter 1990; Hangarter and Stasinopoulos 1991). The extracts were transferred to fresh selection medium biweekly. Regenerated shoots were separated from the callus and left on root-inducing medium [MS+20 mg/l indolebutyric acid (IBA)] for 2 h, then planted on rooting medium (Table 1). When roots had formed, the plants were further grown in soil in the greenhouse.

**Segregation of kanamycin resistance in progeny spinach plants**

Because spinach is dioecious, regenerated kanamycin-resistant plants, *T*0, were crossed with wild-type spinach to obtain *T*1 seeds. *T*1 kanamycin-resistant seedlings were screened on MS medium containing 50 mg/l kanamycin, transferred into soil, and the population was self-pollinated to obtain *T*2 seeds. For blot analysis, *T*1 kanamycin-resistant seedlings were also screened on MS medium containing 50 mg/l kanamycin, transferred to soil, and grown in the greenhouse.

**Table 1 Media used for the transformation and regeneration of spinach**

<table>
<thead>
<tr>
<th>Medium</th>
<th>IBA</th>
<th>Kanamycin</th>
<th>Cefotaxime</th>
<th>Carbenicillin</th>
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<tr>
<td>MS +</td>
<td>–</td>
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<td>CM +</td>
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<td>RM +</td>
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Medium IBA Kanamycin Cefotaxime Carbenicillin

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<tr>
<th>MS salts</th>
<th>1 mg/l</th>
<th>50 mg/l</th>
<th>100 mg/l</th>
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<tr>
<td>RM +</td>
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**Fig. 1** Schematic presentation of the construct p35S/smGfp used for expression of soluble-modified green-fluorescent protein in spinach. The GUS gene was removed from pBI121 by digestion with HindIII and EcoRI, and the smGfp gene with the CaMV 35S promoter and the nopaline synthase (nos) terminator signal were inserted.