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Stable transformation of lettuce cultivar South Bay from cotyledon explants[1]

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Abstract

Transgenic plants of lettuce cultivar (cv.) 'South Bay' were produced by using *Agrobacterium tumefaciens* vectors containing the β-glucuronidase (GUS) reporter gene and the NPT II gene for kanamycin resistance as a selectable marker. High frequency of transformation, based on kanamycin resistance and assays for GUS expression, was obtained with 24 to 72-h-old cotyledon explants cocultivated for 48 h with *Agrobacterium tumefaciens*. After the cocultivation period, the explants were placed in selection medium containing 50 or 100 mg l^{-1} of kanamycin, 100 mg l^{-1} cefotaxime and 500 mg l^{-1} carbenicillin for 10 days. Surviving explants were transferred every 14 days on shoot elongation medium. Progenies of R_0 plants demonstrated linked monogenic segregation for kanamycin resistance and GUS activity.

Introduction

The United States is the world's largest producer of lettuce with approximately 100,000 ha under cultivation (Anonymous 1992). Weeds remain the foremost problem for lettuce producers on organic soil (Dusky et al. 1988) and no safe and effective herbicides are available. The introduction of herbicide resistant lettuce would be a major benefit to growers. Developing fast, efficient and reproducible systems for transforming agronomically proven cultivars of lettuce is critical for rapid incorporation of engineered genes into field performance stocks. In this paper we describe a transformation and regeneration system for the lettuce cultivar South Bay, a head lettuce used in 80% of the production in Florida that completely failed to respond to previous transformation protocols developed for leafy lettuce types.

Materials and methods

Source of explants

Seeds of *Lactuca sativa* L. cv. South Bay were wrapped in cheesecloth squares, 10 × 10 cm, and disinfested in 1% sodium hypochlorite (5× diluted bleach) containing 2 drops of Tween 20 per 100 ml solution, under mild vacuum (−82 Kpa) for 30 min. After disinfestation, the solution was decanted and the seeds were rinsed 3 times with

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sterile distilled water. The seeds were placed on a filter paper platform in a sterile 250 ml Erlhenmeyer flask with the base immersed in 25 ml of a sterile nutrient solution containing MS salts (Murashige & Skoog 1962). The cultures were illuminated 16 h per day with 62 $\mu mol\ m^{-2}\ s^{-1}$ (cool white fluorescent light) and exposed to a constant temperature of 25°C. Cotyledons were excised about 2–3 mm above the cotyledonary node, from 24, 48, 72, 96 and 120-h old aseptically germinated seedlings, and used as explants for *Agrobacterium* infection.

Agrobacterium strain and growth conditions

Agrobacterium tumefaciens A208, carrying either an engineered Ti plasmid (pTiT37-SE) together with the plant transformation vector pMON 9749 or pMON 9793 conferring NPT II and β-Glucuronidase (GUS) activity (Hinchee et al. 1988), were used in experiments. The bacterial medium consisted of Luria broth (LB) (bacto-tryptone 10 $g\ l^{-1}$, bacto-yeast-extract 5 $g\ l^{-1}$ and sodium chloride 10 $g\ l^{-1}$ with Kan 50 $mg\ l^{-1}$ and spectinomycin (Spec) 100 $mg\ l^{-1}$. The medium pH was adjusted to 7.5 before autoclaving. Antibiotics were filter sterilized before adding to cooling medium. Cultures for transformation experiments were inoculated in 250 ml Erlhenmeyer flasks containing 50 ml of LB medium with Kan and Spec. Inoculated flasks were placed on an orbital shaker, 150 rpm, at 28°C and grown overnight to $A_{600} = 0.7$. Aliquots of 15 ml of these cultures were centrifuged at 5,000 rpm (Beckman J2-21, JA-20 rotor) for 10 min at 4°C. The supernatant was discarded and the pellets were resuspended in 15 ml of LB containing acetosyringone (AS) 0; 10 or 20 μM and syringaldehyde (SA) 0; 10 or 20 μM, pH 5.5, for 3–5 min prior to cocultivation.

Explant transformation

Cotyledons were dipped into the bacterial suspension for 10 min, blotted with sterile filter paper and cocultivated for either 24, 48, 72, 96 or 120 h on a shoot initiation medium containing MS salts and (in $mg\ l^{-1}$): sucrose 30,000; i-inositol, 100; thiamine HCl, 1.0; pyridoxine HCl, 0.05; nicotinic acid, 0.05; glycine, 2.0; naphthaleneacetic acid (NAA), 0.05; benzyladenine (BA), 0.2 and phytagar, 7,000. Medium pH was adjusted to 5.7. After cocultivation, explants were immersed for 30 min in 200 ml of basal medium supplemented with 100 $mg\ l^{-1}$ of cefotaxime (Cef) and 500 $mg\ l^{-1}$ of carbenicillin (Carb). Then explants were placed in selection/shoot initiation medium containing Kan at 0, 25, 50, 100 or 200 $mg\ l^{-1}$, Cef 100 $mg\ l^{-1}$ and Carb 500 $mg\ l^{-1}$ for 14 days. Following shoot initiation, surviving explants were transferred every 14 days to shoot elongation medium that had the same composition as selection/shoot initiation medium, except that BA was decreased to 0.1 $mg\ l^{-1}$, NAA was replaced by 0.1 $mg\ l^{-1}$ isopentenyladenine (2ip) and 0.1 $mg\ l^{-1}$ zeatin (ZEA).

Histochemical staining for GUS activity

The GUS staining procedure was adapted from Jefferson (1987) (Jefferson et al. 1987). The substrate was 5-bromo-4-chloro-3-indolyl-glucuronide (X-Glu; Jersey Lab and Glove Supply, Livingston, NJ). The tissue was placed in enough staining solution to cover the entire section or organ and incubated at 37°C for 2 to 4 h.

Southern hybridization analysis of transformed plants

Genomic DNA was extracted according to the protocol described by Paul & Ferl (1991). Approximately 10 μg of total genomic DNA was digested with Hind III, then separated by electrophoresis through 0.7% agarose. The DNA was capillary blotted to GeneScreen (NEN-DuPont) and attached by UV crosslinking. The blot was then probed with random primed-labelled plasmid pBI 221 (Clontech, which contains the GUS gene coding sequence) according to the protocol of Church & Gilbert (1984). Plants transformed with pMON 9749 should all release a common, internal 2.1 Kb fragment that hybridizes with the GUS gene probe.

Statistical analysis of data

In all experiments, 30 explants were used per treatment and each treatment was replicated 4 times. The percent transformation was recorded and 95% confidence limits were calculated.

Results and discussion

Adventive shoot formation on cotyledon explants

The procedure described by Enomoto et al. (1990) for transformation of lettuce was unsatisfactory for 'South Bay' lettuce, as it induced only extensive callusing and very poor shoot proliferation. A series of experiments were conducted to establish culture conditions that would minimize callus production and enhance shoot regeneration in 'South Bay'. These experiments revealed that cotyledons excised from 24 to 72-h-old aseptically germinated lettuce seedlings were the most efficient explant to obtain shoot regeneration. Shoots (3–4 per explant) were obtained after 14 days in a medium supplemented with 0.05 mg l^{-1} NAA and 0.2 mg l^{-1} BA (shoot initiation medium). After shoot induction, the explants were transferred every two weeks on shoot elongation medium.

Development and elongation of shoots occurred in the next 14-28 days. Individualized shoots between 5–10 mm in length were transferred to a medium containing 0.5 mg l^{-1} indolylbutyric acid (IBA). Root formation was observed in 70% of the shoots and normal development of shoots and roots followed. Shoots rarely arose from callus tissue. Shoots did, however, originate directly at the cut surface on the basal end of cotyledons within 10–14 days of culture. Some callus was formed but the amount was greatly reduced relative to explants grown on Enomoto et al. (1990) medium.

Effect of kanamycin

We examined the effects of Kan on South Bay lettuce cotyledons in a concentration range of 0, 25, 50, 100 and 200 mg l^{-1}. At 25 mg l^{-1} Kan, 'South Bay' cotyledons lost green pigmentation and died within 30 to 40 days in culture. Michelmore et al. (1987) also reported that Kan concentrations above 25 mg l^{-1} inhibited callus formation. Therefore, Kan at 50 and 100 mg l^{-1} was chosen for selection of transformants (Table 1).

In the presence of Kan, small compact callus staining positive for GUS developed around the proximal edge of cotyledons in a large number of explants. Over 80% of the cotyledons produced GUS positive callus at Kan concentrations of 50 and 100 mg l^{-1}. However, only 6% of the cotyledons gave rise to shoots (Table 1). Using our protocol a higher population of cells were apparently competent for transformation and callus formation than were competent for transformation and shoot regeneration.

Effect of period of cocultivation

The period of cocultivation has been reported to affect the process of transformation in lettuce (Michelmore et al. 1987; Enomoto et al. 1990) and other plant species (Fillati et al. 1987; Chabaud et al. 1988). Although the percent of explants producing transformed callus was similar at 48, 72, 96 and 120 h of cocultivation (Table

Table 1. Effect of kanamycin concentration on the percent of GUS positive callus and GUS positive shoots in 'South Bay' lettuce.

Kanamycin (mg l^{-1})	% Explants with GUS$^+$ callus		% Explants with GUS$^+$ shoots	
	pMON 9749	pMON 9793	pMON 9749	pMON 9793
25	66(47–82)	46(28–65)	3(0.08–17)	0(0–11)*
50	90(73–97)	80(61–92)	6(8–22)	3(0.08–17)
100	86(69–96)	86(69–96)	6(0.8–22)	6(0.8–22)
200	66(47–82)	50(31–68)	0(0–11)	0(0–11)

*Numbers in parentheses denote 95% confidence limits.
Cotyledons were excised from 72-h-old aseptically germinating seedlings. *Agrobacterium* culture was harvested at $A_{600} = 0.7$. Cocultivation period was 48 h.

Table 2. Effect of cocultivation period on the percent of GUS positive shoots and GUS positive callus in 'South Bay' lettuce.

Cocultivation period (h)	% Explants with GUS$^+$ callus		% Explants with GUS$^+$ callus	
	pMON 9749	pMON 9793	pMON 9749	pMON 9793
24	26(12–45)	43(25–62)	3(0.08–17)	3(0.08–17)*
48	93(77–99)	80(61–92)	6(0.8–22)	6(0.8–22)
72	93(77–99)	80(61–92)	3(0.08–17)	3(0.08–17)
96	90(73–97)	83(65–94)	0(0–11)	0(0–11)
120	90(93–77)	90(73–97)	0(0–11)	0(0–11)

*Numbers in parentheses denote 95% confidence limits.
Cotyledons were excised from 72-h-old aseptically germinated seedlings. Kanamycin concentration was 50 mg l^{-1}.

2), GUS positive shoots were produced only after cocultivation of 24, 48 and 72 h. Prolonged cocultivation of 96 or 120 h had an inhibitory effect on shoot formation in the selection medium.

Effect of explant age

GUS positive callus and shoots using both vectors pMON 9749 and pMON 9793 were produced with cotyledons excised from 24 to 72-h-old seedlings (Table 3). While there was no statistical difference among treatments of 24–72 h, a trend toward decrease in transformation was observed with older explants and no transformed shoots were recovered from the cotyledons of 96 and 120 h seedlings. This corresponds with the best time period determined previously for optimal shoot production in control explants on non selective media. These data demonstrate a sharp, variety-dependent difference with the Enomoto (1990) transformation protocol that utilized 5-day explants.

Effect of phenolic compounds

Improvement in the frequency of transformation has been obtained by adding AS (Sheikholeslam & Weeks 1987; Bevan & Goldsbrough 1988; Owens & Smigocki 1988; Zambryski 1988; Godwin et al. 1991) or SA (Bevan & Goldsbrough 1988; Owens & Smigocki 1988; Zambryski 1988) in the *Agrobacterium* culture prior to incubation

Table 4. Segregation of GUS staining and Kan resistance in R_1 seedlings.

R_0 Plant #	GUS$^+$ Kan Resistant	GUS$^-$ Kan Sensitive	X^2	p
1	54	15	0.3914	0.5–0.7
2	49	18	0.1243	0.7–0.8
3	44	12	0.3809	0.5–0.7
4	15	9	2.0	0.1–0.2
5	29	7	0.5924	0.3–0.5
6	22	8	0.04	0.8–0.9

Table 3. Effect of explant age on the percent of GUS positive callus and GUS positive shoots in 'South Bay' lettuce.

Explant age (h)	% Explants with GUS$^+$ callus		% Explants with GUS$^+$ callus	
	pMON 9749	pMON 9793	pMON 9749	pMON 9793
24	80(61–92)	83(65–94)	6(0.8–22)	3(0.08–17)*
48	76(57–90)	83(65–94)	6(0.8–22)	3(0.08–17)
72	73(54–87)	79(60–92)	3(0.08–17)	3(0.08–17)
96	66(47–82)	60(40–77)	3(0.08–17)	0(0–11)
120	66(47–82)	53(34–71)	0(0.0–11)	0(0–11)

*Numbers in parentheses denote 95% confidence limits.
Kanamycin concentration was 50 mg l^{-1}. Cocultivation period was 48 h.

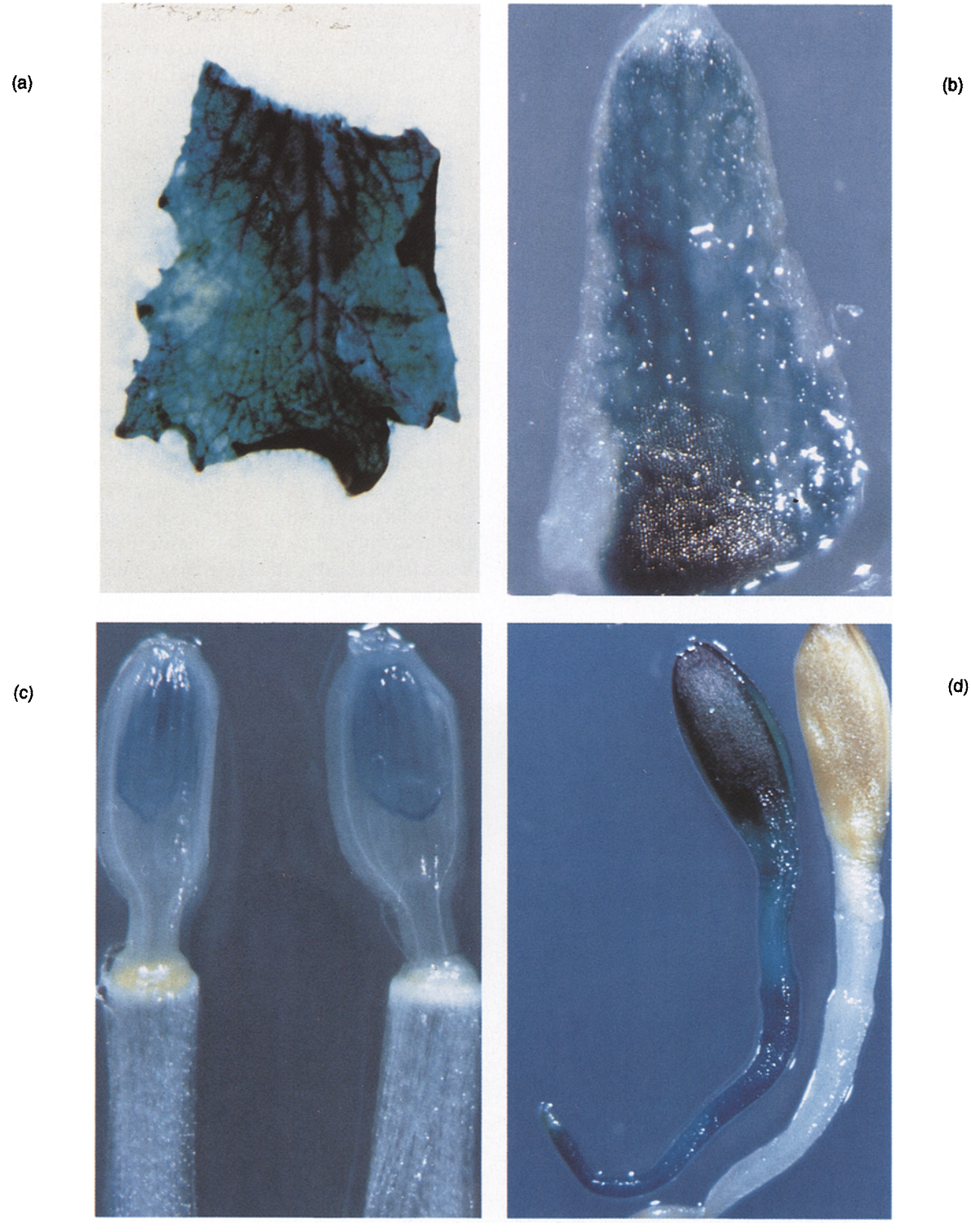

Fig. 1. Staining of GUS positive plants in different stage of development after transformation with pMON 9749. (*a*) Leaf from *in vitro* growing R_0 plant; (*b*) bractea from greenhouse R_0 plant; (*c*) ovaries from R_0 flowering plant; (*d*) germinating R_1 seedling from selfed R_0 plant (left) and control seedling (right).

with the explant. Various combinations of AS and SA resulted in no stimulatory benefit for increasing the percent of transformation.

Analysis of transgenic plants

Leaves, bracts and ovaries (Fig. 1a, b, c) of transgenic lettuce South Bay R_0 plants stained intensely blue when incubated with the X-GLU substrate, while the control, non transformed tissue did not stain. Seeds of 12 self-fertilized R_0 plants were aseptically germinated and 48-h-old seedlings were incubated with X-GLU. R_1 seedlings stained prominently blue (Fig. 1d) and the segregation patterns in these progenies suggested inheritance of GUS activity was by a single dominant gene. Examples of segregation data from the progenies of six R_0 plants are presented in Table 4 and demonstrated in Fig. 2. In all cases, kanamycin resistance in a recallusing assay was linked to GUS staining. Clear monogenic segregation is statistically supported in most cases. Analysis of R_2 seedlings indicated that the genes continued to be seed transmissible.

Southern hybridization of total genomic DNA isolated from 3 of the GUS positive R_0 plants revealed a release of a common 2.1 Kb Hind III fragment that hybridized with the GUS gene probe (Fig. 3). These data indicate the presence of GUS gene sequences within the kan resistant R_0 plants, and the absence of those sequences from a control plant. The presence of NPT II sequences was confirmed by PCR analysis (data not shown). The segregation analysis presented above clearly indicates the linked, chromosomal integration of the GUS and NPT II sequences.

The objective of this study was to develop a rapid, efficient and effective system to transform an important commercial cultivar of lettuce and to obtain active expression of the newly introduced genes. This work was necessary in that previous protocols proved totally ineffective in the transformation of the major Florida crisphead lettuce cultivar, South Bay. This technology can now be used to introduce agronomically

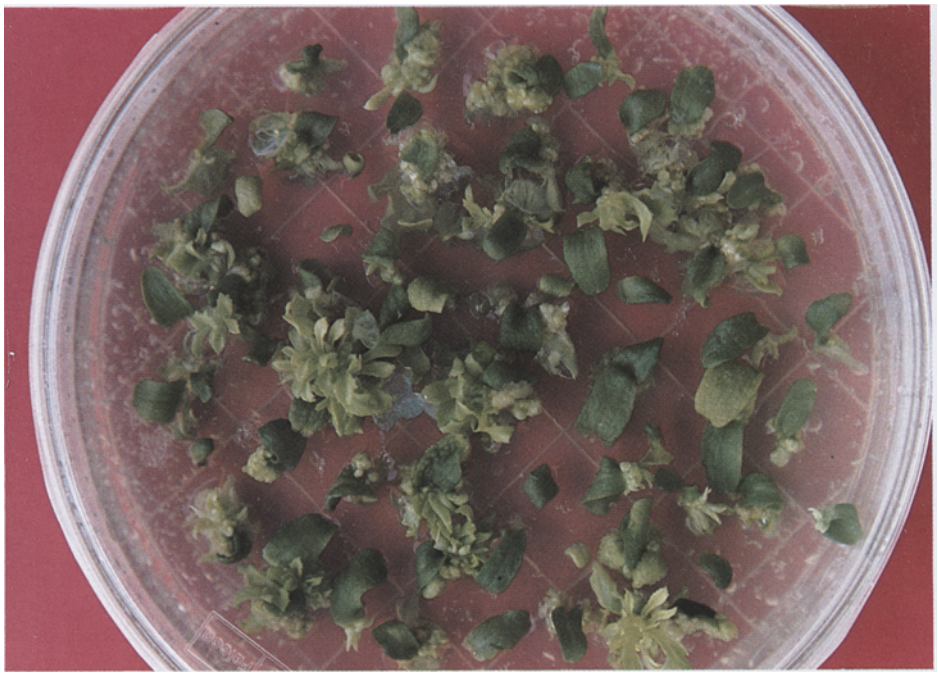

Fig. 2. Segregation of Kan^R in seeds derived from an R_0 plant. Cotyledons were excised from 48-h-old aseptically germinating R_1 seedlings and placed on medium with 50 mg l^{-1} of Kan. Kan Res. = 49; Kan Sens. = 18; $X^2 = 0.1248$; p (0.7–0.8).

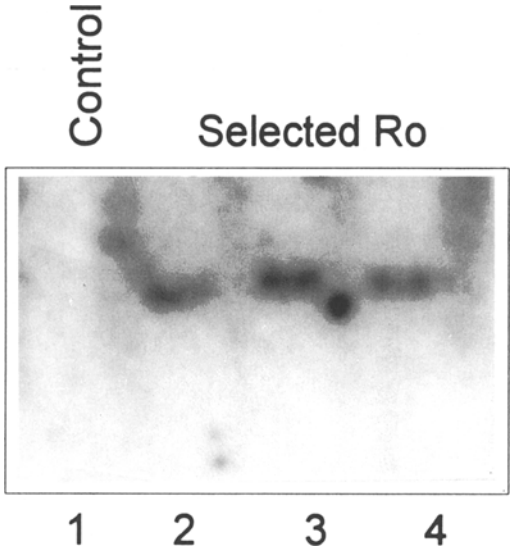

Fig. 3. Southern hybridization of genomic DNA from lettuce plants transformed with pMON 9749 (lanes 2–4) and control plant (lane 1). The 2.1 Kb fragment released from DNA from blue staining transformed plants marks hybridization with the GUS gene probe.

important traits into a species with a limited genetic base. By using a commercially important cultivar we hope to overcome major field production problems within a fairly short time period and to utilize such materials in the development of new germplasm.

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