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Original Paper

Establishment of an *Agrobacterium*-mediated transformation system for *Periploca sepium* Bunge

Ren Chen[1], Mayumi Gyokusen[1], Yoshihisa Nakazawa[2], Yinquan Su[3], Koichiro Gyokusen[1,*]

[1] Department of Forest and Forest Products Sciences, Faculty of Agriculture, Kyushu University, Fukuoka 812-8581, Japan; [2] Department of Biotechnology, Graduate School of Engineering, Osaka University, Suita, Osaka 565-0871, Japan; [3] Faculty of Forestry, Northwest Sci-Tech University of Agriculture and Forestry, Yang Ling, Shanxi 712100, China
* E-mail: gyokusen@agr.kyushu-u.ac.jp Tel & Fax: +81-92-642-2873

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Abstract *Agrobacterium*-mediated transformation of *Periploca sepium* Bunge using proliferated clonal shoots was investigated to identify important factors affecting the transformation efficiency. *Agrobacterium tumefaciens* strains EHA105 and LBA4404 were used, both of which harbored a pKAFCR21 binary vector, which contained two reporter genes (*GUS* and *sGFP*, encoding β-glucuronidase and the synthetic green-fluorescent protein with S65T mutation) and two marker genes (encoding neomycin phosphotransferase II and hygromycin phosphotransferase). The factors evaluated were *Agrobacterium* strain, co-cultivation treatment, and antibiotic selection regime. The results revealed that the transformation efficiency could be synergistically increased to as high as 50–60% by infecting explants with *Agrobacterium* strain EHA105/pKAFCR21 and co-cultivating in the presence of 150 mg l^{-1} dithiothreitol, followed by selection at 100 mg l^{-1} kanamycin. Genomic DNA PCR, Southern hybridization, and quantitative real-time reverse transcription PCR analyses confirmed that the transgenes (*GUS* or *sGFP*) had presented, integrated, and expressed in all the tested transformant plants. The optimized protocol provides a basis for further genetic alteration of *P. sepium* for medicinal compounds and *cis*-polyisoprene production.

Key words: *Agrobacterium*-mediated transformation, *Agrobacterium* strain, antibiotic selection regime, co-cultivation treatment, *Periploca sepium* Bunge.

Periploca sepium Bunge is a perennial erect semi-woody liana belonging to the family *Asclepiadaceae*, which is widely distributed in temperate Asia, southern Europe, and tropical Africa (Yu et al. 2005). *P. sepium* is outstanding in its high resistance to cold, drought, saline-alkali (Han et al. 2003), and insects (Zhu et al. 2004). Moreover, phytochemical studies have reported that its bark, stem, leaf, and milky exudate contain various glycosides (periplogenins, strophanthidins, and steroidals), cardenoides, flavonoids, terpenes, and pregnanes, with various pharmacological functions such as cardiotonic, hypertensive, stomachic, anti-tumor, and rheumatoid arthritis (Li and Li 1992; Xu et al. 1990; Zhang and Wang 2003). In addition to producing bioactive substances, *P. sepium* is also known to produce a *cis*-polyisoprene, similar to that produced by *Hevea brasiliensis*, which is the only plant useful for commercial production of native rubber, mostly in the Southeast Asia area (Bamba et al. 2007).

P. sepium is interesting in view of its ecological and economic potential. To improve the plant species' genetic properties for medicinal compounds and *cis*-polyisoprene production, a genetic transformation system must be established. However, in our previous experiment, we found that *P. sepium* transformation frequency was low and transgenic plants were not readily reproducible (Miyabashira et al. 2003). In this paper, we focus on investigating the important factors (*Agrobacterium* strain, co-cultivation treatment, and antibiotic selection regime) that affected the transformation efficiency, and develop a rapid and efficient system for *P. sepium* transformation via an *Agrobacterium*-mediated method.

Abbreviations: BAP, 6-benzylaminopurine; DTT, dithiothreitol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GUS, β-glucuronidase; HPT, hygromycin phosphotransferase; intron, intron of the castor bean catalase gene *CAT-1*; MS medium, Murashige and Skoog medium; NAA, naphthaleneacetic acid; NOS-P, nopaline synthase promoter; NOS-T, nopaline synthase terminator; NPT II, neomycin phosphotransferase II; PPFD, photosynthetic photon flux density; real-time RT-PCR, real-time reverse transcription polymerase chain reaction; sGFP (S65T), synthetic green-fluorescent protein with S65T mutation; SEC, size exclusion chromatography; 35S-P, cauliflower mosaic virus (CaMV) 35S promoter; 35S-Ω-P, 35S promoter with an additional omega element translational enhancer; X-gluc, 5-bromo-4-chloro-3-indolyl-β-D-glucuronide cyclohexylammonium salt.
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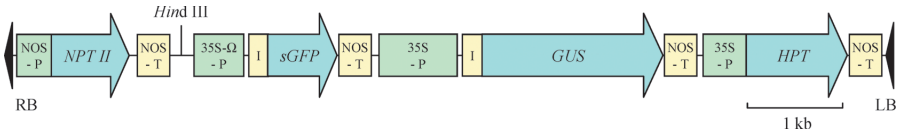

Figure 1. Schematic structure of the binary vector pKAFCR21 T-DNA region. RB, right border; LB, left border; NOS-P, nopaline synthase promoter; NOS-T, nopaline synthase terminator; 35S-P, cauliflower mosaic virus (CaMV) 35S promoter; 35S-Ω-P, 35S promoter with an additional omega element translational enhancer; *NPT II*, neomycin phosphotransferase II gene; *GUS*, β-glucuronidase gene; *sGFP*, synthetic green-fluorescent protein with S65T mutation gene; *HPT*, hygromycin phosphotransferase gene; I, intron of the castor bean catalase gene *CAT-1*.

Table 1. GUS transient expression after three days of co-cultivation.

Co-cultivation treatment		Co-1	Co-2	Co-3	Co-4
Acetosyringone ($mg\,l^{-1}$)		20	20	20	20
L-cysteine ($mg\,l^{-1}$)		400	400	400	
Sodium thiosulfate ($mg\,l^{-1}$)		150	150		150
DTT ($mg\,l^{-1}$)		150		150	150
A. tumefaciens EHA105/pKAFCR21	GUS+[a]/explants (%)[b]	0.0 ± 0.0*	2.5 ± 2.5	0.7 ± 0.7	12.9 ± 2.6
A. tumefaciens LBA4404/pKAFCR21	GUS+/explants (%)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	4.3 ± 0.2
Non-transformed control	GUS+/explants (%)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Co-cultivation treatment		Co-5	Co-6	Co-7	Co-8
Acetosyringone ($mg\,l^{-1}$)		20	20	20	20
L-cysteine ($mg\,l^{-1}$)		400			
Sodium thiosulfate ($mg\,l^{-1}$)			150		
DTT ($mg\,l^{-1}$)				150	
A. tumefaciens EHA105/pKAFCR21	GUS+[a]/explants (%)[b]	9.7 ± 2.7	10.4 ± 1.8	47.5 ± 6.6	25.9 ± 1.6
A. tumefaciens LBA4404/pKAFCR21	GUS+/explants (%)	0.0 ± 0.0	15.1 ± 2.9	14.8 ± 1.1	2.1 ± 1.8
Non-transformed control	GUS+/explants (%)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

[a] Scores of GUS+ that were assigned ranged from 0 to 100%; 0 for no GUS staining and 100% for complete GUS staining on the surface of explant
[b] GUS+/explants = total scores of GUS+/tested explant number × 100%
* Mean ± standard deviation, $n = 20 \times 3$

Materials and methods

Binary vector construction

A cassette comprising a 35S-Ω promoter (Benfey and Chua 1990)-driven *sGFP* (S65T) gene (Niwa et al. 1999) and a NOS terminator from pBIsGFP (S65T) (Kajiyama et al. 2007), was subcloned into the pIG121-Hm binary vector (Ohta et al. 1990). The resultant pKAFCR21 vector (Figure 1) has two reporter genes (*GUS* and *sGFP*) for monitoring, and two marker genes (*NPT II* and *HPT*) for selection. An intron from the castor bean catalase gene *CAT-1* was fused within the N-terminal part of the GUS or sGFP coding sequence for discrimination between *Agrobacterium* and plant expression (because the bacteria can not splice the intron). The resultant vector was introduced into *A. tumefaciens* strains, EHA105 (Hood et al. 1993) and LBA4404 (Bevan, 1984) using a freeze-thaw transformation (Chen et al. 1994).

Explant preparation and transformation

Seeds of *P. sepium* were surface sterilized in a laminar flow chamber by immersion in 70% ethanol (v/v) for one min followed by immersion in 3% (v/v) sodium hypocholorite solution for 5 min. The seeds were then rinsed five times with sterile distilled water, before being placed on a germination medium containing MS basal medium supplied with $20\,g\,l^{-1}$ sucrose for 4 weeks. The shoots of the seedlings were cut into segments about 10 mm in length, including the axillary buds, and were individually and perpendicularly planted on the same medium for shoot proliferation. The best-growing clone was selected and subcultured at an interval of every 6 weeks.

The proliferated clonal shoots were used for transformation. The two kinds of *A. tumefaciens* strains EHA105 or LBA4404 harboring pKAFCR21 vector were grown for overnight at 28°C in LB (Luria Bertani medium) liquid medium containing $50\,mg\,l^{-1}$ kanamycin (Wako) and $50\,mg\,l^{-1}$ hygromycin (Wako) shaken at $150\,rev\,min^{-1}$. Bacterial cells were collected by centrifugation and resuspended to a final $OD_{550} = 0.25$ in eight kinds of suspension solutions consisting MS basal medium, $20\,g\,l^{-1}$ sucrose, 3 μM BAP, 0.1 μM NAA and $20\,mg\,l^{-1}$ acetosyringone (Wako), in combination with 150 $mg\,l^{-1}$ DTT (Wako), $150\,mg\,l^{-1}$ sodium thiosulfate (Wako) and/or $400\,mg\,l^{-1}$ L-cysteine (Wako), respectively (Co-1–8, Table 1). The proliferated clonal shoots of *P. sepium* were cut into segments about 5–8 mm in length without axillary buds in the *Agrobacterium* suspensions and inoculated for 3 min. Then they were blotted dry with sterile filter papers to remove excess

bacteria, and were transferred to Petri dishes (90 mm×15 mm) with filter papers laid on the co-cultivation media consisting of the same compositions as the suspension solutions but solidified with 2.4 g l^{-1} Gelrite (Wako). After 3 days of co-cultivation at 22°C in the dark, some of the inoculated explants were used for transient GUS histochemical assay (Table 1).

Callus selection and plant regeneration

After co-cultivation, to continuously observe the GUS expression frequency, some of the explants inoculated with the two kinds of *A. tumefaciens* strains (using Co-7, 8 suspension solutions and co-cultivation media) were transferred to a callus/adventitious shoot induction and selection medium. This comprised MS basal medium supplied with 20 g l^{-1} sucrose, 3 μM BAP, 0.1 μM NAA, 250 mg l^{-1} carbenicillin (Wako) combined with 100 mg l^{-1} kanamycin and solidified with 2.4 g l^{-1} Gelrite (Table 2). To determinate the effective concentration of selection agents, the explants inoculated with *A. tumefaciens* strain EHA105 (using Co-7 suspension solution and co-cultivation medium) were transferred to various callus/adventitious shoot induction and selection media containing the same composition as mentioned above but combined with 50–200 mg l^{-1} kanamycin, or 6.25–50 mg l^{-1} hygromycin (Table 3). They were subcultured at an interval of every 3 weeks. At the end of each culture stage (3, 6 and 9 weeks), the induced callus weight, regenerated adventitious shoot number, GUS histochemical assay and sGFP fluorescence observation were recorded (Table 3). After the calli regenerated adventitious shoots, only one well grown shoot from each induced callus was harvested and transferred to a shoot elongation medium containing the same composition and the same concentration of kanamycin and/or hygromycin as the callus/adventitious shoot induction and selection media but without any plant growth regulator for 3 weeks. The shoots stained to GUS blue (GUS+) and showed sGFP fluorescence (sGFP+) longer than 4 cm were transplanted to pots (φ90 mm×90 mm) containing mixed soil (red gravel : pearlite : peat moss:barnyard manure=2 : 1 : 1 : 1) for producing roots and acclimatization in a culture-room. Initially, the pot was covered with a transparent plastic cup to prevent desiccation and was watered with tap water every day. The cup was then gradually opened week by week. After 4 weeks the cup was completely removed, and the plantlets were moved to the greenhouse.

All of the media mentioned above were adjusted to pH 5.8 prior to the addition of Gelrite. The co-cultivation media, the callus/adventitious shoot induction and selection media were dispensed into 25 ml per Petri dish. All other media were dispensed into 30 ml per 100 ml Erlenmeyer flasks and capped with aluminum foil before being autoclaved at 121°C for 15 min. Antibiotics (kanamycin, hygromycin, and carbenicillin) and certain supplementary substances (DTT, sodium thiosulfate, L-cysteine, and acetosyringone) were added after the media were cooled to 60°C. The cultures were incubated at 25°C under a 16-hour photoperiod using cool, white fluorescent light irradiance of 50 μmol $m^{-2}s^{-1}$ PPFD. For each experiment, a least 20 explants were taken, and each experiment was repeated in triplicate. Some of the shoot segments (explants) that had not been inoculated with *Agrobacterium*, were also cultured on the co-cultivation or selection media and analyzed at each experiment stage as non-transformed negative controls (Table 1–3).

GUS assay

The inoculated explants after 3 days of co-cultivation, the calli selected on the callus/adventitious shoot induction and selection media for 3, 6 and 9 weeks, and the leaves cut from regenerated shoots were assessed for GUS activity using a histochemical staining (Jefferson, 1987) overnight at 37°C. The GUS histochemical staining solution contained 100 mM Na-phosphate buffer (pH 7.0), 1 mM X-gluc, 0.2 mM $K_3Fe(CN)_6$, 0.2 mM $K_4Fe(CN)_6$, and 0.1% (v/v) Triton X-100.

sGFP fluorescence observation

The calli selected on the callus/adventitious shoot induction and selection media for 3, 6 and 9 weeks, and the leaves cut from regenerated shoots were observed for sGFP expression with a fluorescence microscope (SMZ800, Nikon with a U-GFP-A mirror set). Micrographs were obtained using a digital camera system (CoolPix8400, Nikon).

DNA analysis of the transgenic plantlets

PCR analysis was conducted to screen transgenic plants and

Table 2. GUS and sGFP expression after six weeks of selection[a].

Co-cultivation treatment		Co-7	Co-8
Acetosyringone (mg l^{-1})		20	20
L-cysteine (mg l^{-1})			
Sodium thiosulfate (mg l^{-1})			
DTT (mg l^{-1})		150	
A. tumefaciens EHA105/pKAFCR21	GUS+[b]/explants (%)[c]	49.0 ± 18.8*	22.9 ± 9.5
	sGFP+/explants (%)	44.8 ± 4.8	19.8 ± 3.6
A. tumefaciens LBA4404/pKAFCR21	GUS+/explants (%)	16.8 ± 4.7	6.8 ± 0.5
	sGFP+/explants (%)	15.1 ± 6.6	8.0 ± 2.4
Non-transformed control	GUS+/explants (%)	0.0 ± 0.0	0.0 ± 0.0
	sGFP+/explants (%)	0.0 ± 0.0	0.0 ± 0.0

[a] Explants co-cultured on Co-7 and Co-8 were selected on the callus/adventitious shoot induction and selection medium CS-CK100 (see Table 3; supplemented with 100 mg l^{-1} kanamycin) for six weeks

[b] Scores of GUS+ (or sGFP+) that were assigned ranged from 0 to 100%; 0 for no GUS staining (or sGFP fluorescence) and 100% for complete GUS staining (or sGFP fluorescence) on the surface of callus induced from explant

[c] GUS+ (or sGFP+)/explants=total scores of GUS+ (or sGFP+)/tested explant number×100%

* Mean±standard deviation, n=20×3

Table 3. Effects of selection agents on callus induction, shoot regeneration, GUS and sGFP expression after three, six and nine weeks of selection[a].

Callus/adventitious shoot induction and selection medium			CS-CK200	CS-CK150	CS-CK100
Carbelicillin (mg l^{-1})			250	250	250
Kanamycin (mg l^{-1})			200	150	100
Hygromycin (mg l^{-1})					
3 weeks	*A. tumefaciens* EHA105/pKAFCR21	callus weight/explants (mg)[b]	41.0 ± 1.9*	79.3 ± 18.9	89.0 ± 10.3
		GUS+[c]/explants (%)[d]	61.5 ± 3.3	38.8 ± 4.5	41.5 ± 2.0
		sGFP+[c]/explants (%)[d]	55.2 ± 5.0	40.0 ± 8.1	38.3 ± 6.4
	Non-transformed control	callus weight/explants (mg)	28.5 ± 2.7	28.7 ± 0.8	35.2 ± 1.4
		GUS+/explants (%)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		sGFP+/explants (%)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
6 weeks	*A. tumefaciens* EHA105/pKAFCR21	callus weight/explants (mg)	322.7± 19.5	410.7 ± 26.8	433.7 ± 14.4
		GUS+/explants (%)	47.8 ± 5.6	44.0 ± 11.3	54.2 ± 3.1
		sGFP+/explants (%)	49.2 ± 4.4	44.8 ± 6.4	58.9 ± 7.6
	Non-transformed control	callus weight/explants (mg)	– –[g]	– –	25.5 ± 6.7
		GUS+/explants (%)	– –	– –	0.0 ± 0.0
		sGFP+/explants (%)	– –	– –	0.0 ± 0.0
9 weeks	*A. tumefaciens* EHA105/pKAFCR21	shoots/explants[e]	1.1 ± 0.5	1.5 ± 0.3	3.4 ± 0.3
		GUS+/shoots (%)[f]	44.4 ± 21.0	47.9 ± 24.7	61.7 ± 12.6
		sGFP+/shoots (%)[f]	50.0 ± 22.0	50.2 ± 14.6	60.0 ± 17.3

Callus/adventitious shoot induction and selection medium			CS-CK50	CS-CH50	CS-CH25
Carbelicillin (mg l^{-1})			250	250	250
Kanamycin (mg l^{-1})			50		
Hygromycin (mg l^{-1})				50	25
3 weeks	*A. tumefaciens* EHA105/pKAFCR21	callus weight/explants (mg)[b]	143.0 ± 39.5	32.0 ± 2.3	32.8 ± 9.1
		GUS+[c]/explants (%)[d]	32.2 ± 4.2	32.0 ± 5.2	72.6 ± 16.8
		sGFP+[c]/explants (%)[d]	36.1 ± 5.1	32.0 ± 8.4	43.3 ± 4.4
	Non-transformed control	callus weight/explants (mg)	46.7 ± 5.3	32.0 ± 2.1	28.3 ± 4.2
		GUS+/explants (%)	0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		sGFP+/explants (%)	0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
6 weeks	*A. tumefaciens* EHA105/pKAFCR21	callus weight/explants (mg)	522.8 ± 64.3	48.3 ± 13.8	52.1 ± 7.8
		GUS+/explants (%)	37.4 ± 3.9	43.3 ± 7.6	55.0 ± 7.1
		sGFP+/explants (%)	38.9 ± 4.2	37.6 ± 4.5	45.0 ± 7.1
	Non-transformed control	callus weight/explants (mg)	49.2 ± 9.3	– –	– –
		GUS+/explants (%)	0.0 ± 0.0	– –	– –
		sGFP+/explants (%)	0.0 ± 0.0	– –	– –
9 weeks	*A. tumefaciens* EHA105/pKAFCR21	shoots/explants[e]	4.2 ± 0.6	0.0 ± 0.0	0.0 ± 0.0
		GUS+/shoots (%)[f]	47.1 ± 15.3	– –	– –
		sGFP+/shoots (%)[f]	41.1 ± 6.7	– –	– –

Callus/adventitious shoot induction and selection medium			CS-CH12.5	CS-CH6.25	CS-CK50H25
Carbelicillin (mg l^{-1})			250	250	250
Kanamycin (mg l^{-1})					50
Hygromycin (mg l^{-1})			12.5	6.25	25
3 weeks	*A. tumefaciens* EHA105/pKAFCR21	callus weight/explants (mg)[b]	39.2 ± 5.9	47.5 ± 0.0	33.3 ± 5.5
		GUS+[c]/explants (%)[d]	55.6 ± 7.9	43.3 ± 1.7	52.3 ± 2.7
		sGFP+[c]/explants (%)[d]	41.7 ± 9.3	41.7 ± 2.9	50.1 ± 3.0
	Non-transformed control	callus weight/explants (mg)	36.8 ± 5.8	36.2 ± 10.3	23.2 ± 1.8
		GUS+/explants (%)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		sGFP+/explants (%)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
6 weeks	*A. tumefaciens* EHA105/pKAFCR21	callus weight/explants (mg)	43.6 ± 27.8	71.0 ± 8.1	56.7 ± 17.4
		GUS+/explants (%)	38.1 ± 2.7	27.4 ± 14.4	52.1 ± 5.1
		sGFP+/explants (%)	36.9 ± 7.6	34.5 ± 14.9	47.3 ± 10.5
	Non-transformed control	callus weight/explants (mg)	– –	– –	– –
		GUS+/explants (%)	– –	– –	– –
		sGFP+/explants (%)	– –	– –	– –

Table 3. Continued

Callus/adventitious shoot induction and selection medium			CS-CH12.5	CS-CH6.25	CS-CK50H25
9 weeks	*A. tumefaciens* EHA105/pKAFCR21	shoots/explants[e]	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		GUS+/shoots (%)[f]	− −	− −	− −
		sGFP+/shoots (%)[f]	− −	− −	− −

Callus/adventitious shoot induction and selection medium			CS-CK50H12.5	CS-CK50H6.25	CS-C
Carbelicillin (mg l^{-1})			250	250	250
Kanamycin (mg l^{-1})			50	50	
Hygromycin (mg l^{-1})			12.5	6.25	
3 weeks	*A. tumefaciens* EHA105/pKAFCR21	callus weight/explants (mg)[b]	37.3 ± 3.7	44.7 ± 1.8	217.3 ± 47.5
		GUS+[c]/explants (%)[d]	60.6 ± 3.5	49.4 ± 3.8	26.7 ± 2.9
		sGFP+[c]/explants (%)[d]	48.9 ± 3.5	47.8 ± 3.5	18.3 ± 1.7
	Non-transformed control	callus weight/explants (mg)	26.8 ± 2.5	44.5 ± 3.9	269.7 ± 33.5
		GUS+/explants (%)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		sGFP+/explants (%)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
6 weeks	*A. tumefaciens* EHA105/pKAFCR21	callus weight/explants (mg)	57.3 ± 9.5	84.9 ± 15.9	758.8 ± 74.2
		GUS+/explants (%)	46.7 ± 5.8	46.1 ± 14.6	23.6 ± 10.3
		sGFP+/explants (%)	36.7 ± 5.8	34.2 ± 3.8	12.8 ± 9.7
	Non-transformed control	callus weight/explants (mg)	− −	− −	766.7 ± 65.5
		GUS+/explants (%)	− −	− −	0.0 ± 0.0
		sGFP+/explants (%)	− −	− −	0.0 ± 0.0
9 weeks	*A. tumefaciens* EHA105/pKAFCR21	shoots/explants[e]	0.0 ± 0.0	0.0 ± 0.0	3.9 ± 0.7
		GUS+/shoots (%)[f]	− −	− −	13.3 ± 11.5
		sGFP+/shoots (%)[f]	− −	− −	12.2 ± 10.7

[a] Explants inoculated with *Agrobacterium* strain EHA105 and co-cultured on Co-7 were used.
[b] Callus weight/explants=total weight of induced calli (with explant)/tested explant number.
[c] Scores of GUS+ (or sGFP+) that were assigned ranged from 0 to 100%; 0 for no GUS staining (or sGFP fluorescence) and 100% for complete GUS staining (or sGFP fluorescence) on the surface of callus induced from explant.
[d] GUS+ (or sGFP+)/explants=total scores of GUS+ (or sGFP+)/tested explant number×100%.
[e] Shoots/explants=total number of regenerated adventitious shoots/tested calli number.
[f] GUS+/shoots (or sGFP+/shoots)=total scores of GUS+ (or sGFP+)/tested shoot number×100%.
[g] Almost no explants or calli survived on the selection media and were excluded from further experiment.
* Mean ± standard deviation, n=20×3.

investigate the presence of the transgenes. Total genomic DNA were isolated from the leaves of regenerated plantlets (GUS+ and sGFP+, as only one well grown shoot derived from each induced callus was regenerated to a plantlet, the plantlets were independent clones) using the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instruction. Based on the *GUS* and *sGFP* gene sequences, two pairs of primers (GUS forward primer 5′-TTT AAC TAT GCC GGG ATC CAT CGC-3′ and GUS reverse primer 5′-CCA GTC GAG CAT CTC TTC AGC GTA-3′; sGFP forward primer 5′-AGC TGG ACG GCG ACG TAA A-3′ and sGFP reverse primer 5′-CAG GAC CAT GTG ATC GCG CTT-3′) were synthesized for amplification of the 529 bp *GUS* fragment and the 611 bp *sGFP* fragment, respectively. The PCR reaction comprised 20 ng of total isolated DNA as a template and amplification was performed by 5 min at 95°C for preheating, 30 cycles of 1 min at 95°C for denaturation, 1 min at 60°C for annealing, 2 min at 74°C for synthesis, and 7 min at 74°C for final extension.

Southern hybridization analysis was conducted to confirm the stable integration of the transgenes. Total genomic DNA (10 μg) from the leaves of 10 PCR-positive (PCR+) plantlets (clones) were completely digested with *Hin*d III and *Spe* I (Toyobo), separated by electrophoresis on 0.6% agarose gel, and then transferred onto a Hybond-N$^+$ membrane (GE Healthcare). The membrane was hybridized with a *GUS* or *sGFP* labeling probe amplified by a PCR DIG Probe Synthesis Kit (Roche) using the primer pairs above and pKAFCR21 DNA as a template. Signal detection was performed with anti-digoxigenin-AP Fab fragments (Roche) and its substrate, CDP-*Star* (Roche) under the conditions specified by the manufacturer.

RNA analysis of the transgenic plantlets

Total RNA were isolated from the leaves of 10 PCR+ plantlets (clones) using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instruction. To eliminate the residual genomic DNA, the RNA samples were treated with the RNase-Free DNase I (Qiagen) and were tested by real-time RT-PCR using 50 ng RNA as template under the same conditions as described below. The RNA was then quantified using an ND-1000 Spectrophotometer (Nanodrop) and six diluting concentrations of RNA (400, 100, 25, 6.25, 1.56, 0.39 ng μl^{-1}) were prepared to generate a standard curve. All the RNA samples (including the samples for standard curve) were reverse transcribed into first strand cDNA using the High Capacity Reverse Transcription Kit (Applied Biosystems).

The cDNA samples were used as templates to quantify target gene (*GUS* or *sGFP*) expression levels. An endogenous gene,

GAPDH, was used as the housekeeping gene to calibrate for experimental variability. The primer pairs for real-time RT-PCR of the three genes were designed using Primer Express (Applied Biosystems; GUS-RT forward primer 5′-CAA AGC GGC GAT TTG GAA-3′, GUS-RT reverse primer 5′-GCC AGG CCA GAA GTT CTT TTT-3′; sGFP-RT forward primer 5′-GTC CGC CCT GAG CAA AGA-3′, sGFP-RT reverse primer 5′-TCC AGC AGG ACC ATG TGA TC-3′; GAPDH-RT forward primer 5′-GGC ATT GTT GAG GGT CTT ATG AC-3′, GAPDH-RT reverse primer 5′-TGG TCC ATC AAC AGT CTT CTG AGT-3′). The resulting PCR products had approximate sizes of 54–69 bp with melting temperatures of 58–60°C.

Real-time PCR was performed in a 25 μl volume containing 150 nM of each primer, 50 ng of cDNA sample and 1×SYBR Green PCR Master Mix (Applied Biosystems) on the ABI Prism 7300 Sequence Detection System (Applied Biosystems) in a 96-well reaction plate. The RT-PCR parameters were those recommended by the manufacturer (50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min, and a dissociation stage of 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s, 60°C for 15 s). Each PCR reaction was performed in triplicate and a no-template control was included.

Results

Effect of Agrobacterium strains and co-cultivation treatments

The GUS transient expression after 3 days of co-cultivation (Figure 2D) varied with the *Agrobacterium* strains and co-cultivation treatments (the supplements of the suspension solutions and the co-cultivation media). Between the two kinds of *A. tumefaciens* strains, the strain EHA105 consistently showed higher level of GUS transient expression in almost all the co-cultivation treatments compared to LBA4404 (Table 1). The addition of DTT alone in the co-cultivation treatment (Co-7) significantly increased GUS transient expression, especially in the explants inoculated with strain EHA105 (which showed the highest GUS transient expression, GUS+/explants=47.5%, Table 1). Moreover, there was less browning on the two incision sites of the explants after co-cultivation. The addition of sodium thiosulfate alone (Co-6) could also improve GUS transient expression inoculated with LBA4404, whereas it had no effect on that of EHA105. However, the combination of DTT and sodium thiosulfate (Co-4), or the addition of L-cysteine (Co-1, 2, 3, 5) reduced GUS transient expression compared to the non-thiol treatment (Co-8).

As it could not excluded the possibility that the increase in GUS histochemical staining detected after 3 days of co-cultivation was caused by the externally added DTT in the co-cultivation treatment (exposing explants to DTT may enhance GUS activity and intensify the blue staining), the explants co-cultured on Co-7 and Co-8 were GUS stained again (Figure 2E, F) after 6 weeks of selection on the callus/adventitious shoot induction and selection medium CS-CK100 (Table 2, 3; supplemented with 100 mg l^{-1} kanamycin) to assay for stable transformation. The GUS expression of induced calli was more than 2-fold higher when the explants were treated

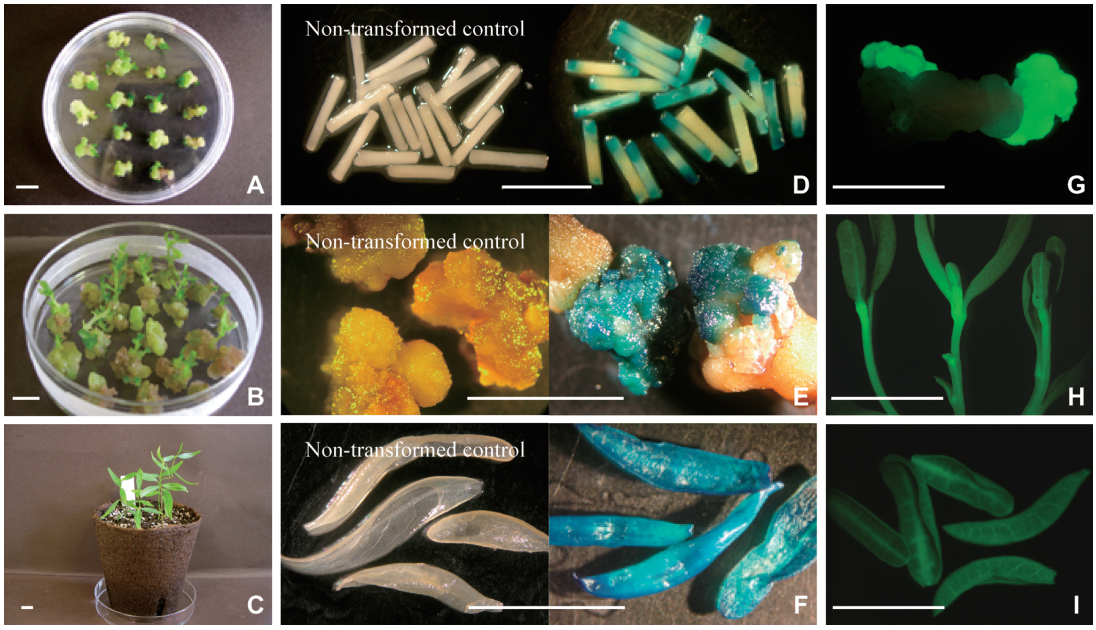

Figure 2. Regeneration of transgenic *P. sepium* plants and monitoring of GUS and sGFP expressions at different developmental stages. (A), (B) The inoculated explants were selected on the callus/adventitious shoot induction and selection medium supplemented with 100 mg l^{-1} kanamycin for 6 and 9 weeks; (C) The GUS+ and sGFP+ shoots were transplanted to pots to produce roots and for acclimatization; (D), (E) GUS histochemical assays after 3 days of co-cultivation and 6 weeks of selection; (F) GUS histochemical assays of the leaves from the regenerated shoots; (G), (H) sGFP fluorescence observation after 6 and 9 weeks of selection; (I) sGFP fluorescence observation of the leaves from the regenerated shoots. Non-transformed control, Non-transformed explants cultured at the same stages. Scale bars, 1 cm.

with DTT (Co-7) compared to those treated without DTT (Co-8) in co-cultivation (Table 2). The results were also proved by sGFP fluorescence observation at the same stage (Table 2; Figure 2G, H, I). This indicated that addition of DTT in co-cultivation treatment not only increased the amount of GUS transient expression but also resulted in an obvious increase in the number of transformed callus after long-term selection. The enhancement was not due to a direct effect of DTT on GUS histochemical staining. Therefore, *Agrobacterium* strain EHA105 and co-cultivation treatment Co-7 were used for optimization of selection agents in the next stage.

Neither GUS nor sGFP expression could be detected in non-transformed control explants nor in their induced calli and shoots (Table 1–3; Figure 2). As the non-transformed control explants barely survived on the selection media containing kanamycin or hygromycin, they were excluded from further experiment after 3–6 weeks of selection.

Effective concentration of selection agents

Different types and concentrations of antibiotics (kanamycin and hygromycin) were applied to select transformed calli and regenerate shoots (Table 3). Although selection at high kanamycin concentrations (150–200 mg l^{-1}) exhibited higher GUS or sGFP expression in the early selection stage (3 weeks), the trend reversed after continuous selection (6 weeks). Furthermore, selection at high kanamycin concentrations obviously inhibited callus growth and shoot regeneration resulting in lower callus weights and shoot regeneration rates (adventitious shoot number/tested calli) than those selected at lower kanamycin concentrations (50–100 mg l^{-1}). Selection at 100 mg l^{-1} kanamycin was determined to be the most suitable because it exhibited the highest GUS or sGFP expression and the highest shoot regeneration rates after 6 weeks of selection. At 50 mg l^{-1} kanamycin, both the GUS+ or sGFP+ score was reduced and more non-transformed calli or shoots (escape) regenerated. This 'escape' increased visibly when selected on the CS-C medium, which was only supplemented with carbenicillin and no other antibiotic (Table 3). Few explants selected with hygromycin alone or in combination with kanamycin developed healthy calli. The growth of induced calli was slow and they nearly stopped growing after 6 weeks of selection. Shoots regeneration was reduced to almost nil, even though at a low concentration of 6.25 mg l^{-1} hygromycin. This result indicated that *P. sepium* was very sensitive to hygromycin. Thus, 100 mg l^{-1} kanamycin was used throughout the selection. Under the selection pressure, part of the inoculated explants on callus/adventitious shoot induction and selection medium remained green and developed small nodular protuberances in the two incision sites in the second-third week. These protuberances then developed into calli and adventitious shoots in the fourth-sixth week (Figure 2A). Adventitious shoots elongated during the subculture stage after the calli were excised from the explants (Figure 2B). Over 60% of shoots were stained to GUS+ blue and showed sGFP+ fluorescence (Table 3), and 33 independent GUS+ and sGFP+ shoots (clones) were successfully regenerated into plantlets (Figure 2C) from 60 tested explants (55%).

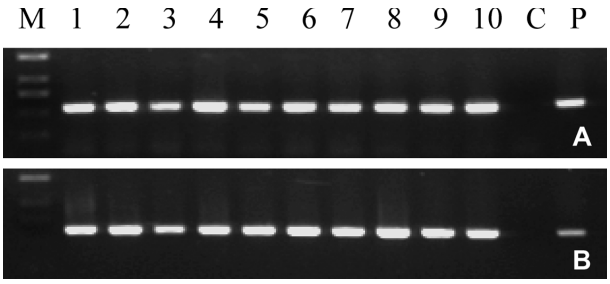

Figure 3. PCR analysis of *GUS* and *sGFP* genes in regenerated *P. sepium* plantlets. (A) *GUS* PCR (529 bp); (B) *sGFP* PCR (611 bp). Lane M, DNA marker; Lane 1-10, GUS+ and sGFP+ plantlets 1–10; Lane C, Non-transformed control; Lane P, pKAFCR21 (positive control).

Confirmation of transformation events in regenerated plantlets

Genomic DNA from the leaves of GUS+ and sGFP+ plantlets (clones) were analyzed by PCR for the presence of the transgenes. PCR amplification exhibited that all the tested GUS+ and sGFP+ plantlets produced the predicted *GUS* fragment (529 bp) and *sGFP* fragment (611 bp), respectively (Figure 3A, B). Neither *GUS* nor *sGFP* fragments could be detected in non-transformed controls plantlets.

The results of the genomic Southern hybridization analysis (Figure 4A, B) of all the tested PCR+ plantlets (clones) with the DIG labeled *GUS* or *sGFP* probes confirmed that the transgenes were stably integrated. The hybridization patterns ranged from single to three bands, with predicted sizes of more than 6.7 kb, and there were no significantly different bands between the *GUS* and *sGFP*. As there was only one *Hin*d III site in the T-DNA region of pKAFCR21 (Figure 1) and the total DNA was completely digested with *Hin*d III, the hybridizing bands indicated that at least 1–3 copies of *GUS* and *sGFP* transgenes were inserted into the transgenic plantlet genomes. No signal was detected in non-transformed control plantlets.

Expression analysis of transgenes in transgenic plantlets

The result of quantitative real-time RT-PCR detection (Figure 5) showed that the transgenes, *GUS* and *sGFP*, were transcribed and expressed in all the tested PCR+ plantlets (clones), but the transcript levels (calibrated to

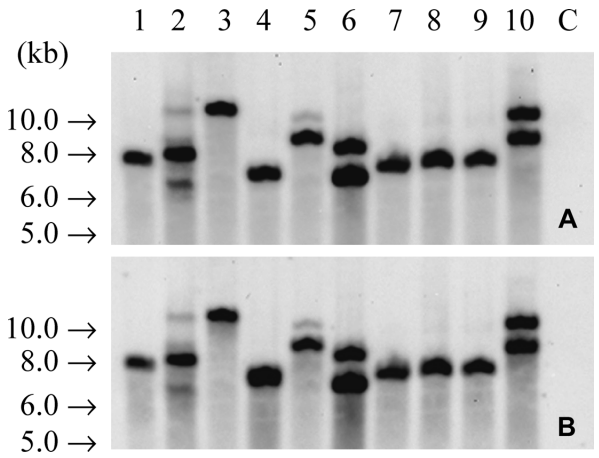

Figure 4. Southern hybridization analysis of *GUS* and *sGFP* genes in PCR+ *P. sepium* plantlets. (A) Probing with a *GUS* probe (529 bp); (B) Re-probing with an *sGFP* probe (611 bp). Lane 1–10, PCR+ plantlets 1–10; Lane C, Non-transformed control.

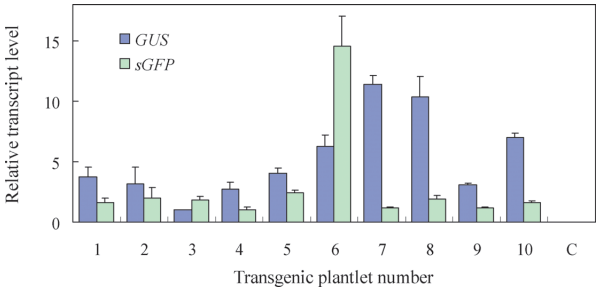

Figure 5. Quantitative real-time RT-PCR analysis of *GUS* and *sGFP* genes in PCR+ *P. sepium* plantlets. 1–10, PCR+ plantlets 1–10; C, Non-transformed control; Bars, mean ± standard deviation.

the housekeeping *GAPDH* gene expression level) were different. The highest transcript level for *GUS* was measured in transgenic plantlet 7, while the highest transcript level for *sGFP* was observed in transgenic plantlet 6. No expression of *GUS* or *sGFP* was detected in non-transformed control plantlets.

Discussion

Plant genetic transformation using *Agrobacterium* is an integrated process that involves a judicious choice of many variables, including the bacterial strain, co-cultivation conditions, selective agents, and selective pressure. The data presented above clearly demonstrated that the *P. sepium* transformation efficiency varied with *Agrobacterium* strain. The use of strain EHA105 resulted in consistently higher transformation efficiency than LBA4404. This might be due to the different virulence (*vir*) regions and different chromosomal backgrounds of the two strains (Gelvin 1990; Hood et al. 1993; Hellens et al. 2000). The EHA105 succinamopine strain contains a disarmed pEHA105 plasmid present in the C58 chromosomal background. The LBA4404 octopine strain contains a disarmed pAL4404 plasmid in the TiAch5 chromosomal background. Thus, the differences in *vir* region and chromosomal background between *A. tumefaciens* EHA105 and LBA4404 may affect the range of plants susceptible to T-DNA transfer via their *vir* genes (Hood et al. 1993).

Besides the bacterial strain, the *P. sepium* transformation efficiency also varied with co-cultivation treatments. The addition of DTT to the suspension solution and the co-cultivation medium significantly increased the frequency of stable transformation. As the explants have to be cut into segments before *Agrobacterium* infection, the wounding typically causes an extensive enzymatic browning and cell death at the two incisions of the explant. Thiol compounds, such as DTT, L-cysteine, and sodium thiosulfate, appear to improve T-DNA delivery by inhibiting the activity of explant wound- and pathogen-response enzymes, such as peroxidases (PODs) and polyphenol oxidases (PPOs) (Olhoft et al. 2001, 2003). This resulted in greater transformation efficiencies for soybean (Olhoft et al. 2001, 2003), grape (Perl et al. 1996), and rice (Enríquez-Obregón et al. 1999).

The effectiveness of tissue-culture selection regimes is dependent on many factors, including type of explant, the chemical properties of the selective agents, and time of application (Bowen 1993). Usually, higher selection pressure enhances the selection efficiency, but the regeneration ability will also be reduced, making it difficult to regenerate plantlets after selection. Proper balance between selection and regeneration has to be considered. In the present research, the addition of 100 mg l^{-1} kanamycin to the *P. sepium* callus/adventitious shoot induction and selection medium or the shoot elongation medium was adequate for selection without jeopardizing the survival of the transformed tissues. Kanamycin is an aminoglycoside antibiotic inhibiting protein synthesis and generating errors in the transcription of the genetic code. In contrast, hygromycin inhibits polypeptide chain elongation in protein synthesis (Gonzalez et al. 1978). Selection with hygromycin evidently stunted *P. sepium* callus induction, development, and differentiation. In previous transformation experiments in our laboratory, hygromycin (25 mg l^{-1}) had been used for selection throughout the callus/adventitious shoot induction and shoot elongation stages (Miyabashira et al. 2003). As *P. sepium* was very sensitive to hygromycin, few explants developed healthy calli and only seven plantlets were regenerated from 100 inoculated explants.

In the present research, we developed several efficient methods that resulted in a synergistic increase in the production of transgenic *P. sepium* plants to efficiencies exceeding 55% of explants. By treatment with DTT during the co-cultivation stage, the transformation efficiency was significantly increased. Based on the kanamycin (100 mg l^{-1}) selection system, we rapidly and

efficiently selected transgenic calli and shoots with a very low selection escape frequency. Moreover, using a binary vector, pKAFCR21 (Figure 1), which contains two reporter genes (*GUS* and *sGFP*), and two marker genes (*NPT II* and *HPT*), we optimized the conditions for *Agrobacterium* infection and selection by monitoring GUS and sGFP expression at different developmental stages. The GUS staining assay can be performed as early as the infection stage, while the sGFP fluorescence observation avoids destroying the live tissue at the selection and regeneration stage. Furthermore, because an intron of the castor bean catalase gene *CAT-1* was fused within the N-terminal part of the GUS or sGFP coding sequences and *Agrobacterium* cannot splice the intron, we could discriminate between *Agrobacterium* and plant expression. As expected, the GUS+ and sGFP+ plants were positive, while the non-transformed control plants were negative for genomic DNA PCR, Southern hybridization, and quantitative real-time RT-PCR analyses. The results revealed that using the two reporter genes for preliminary appraisal of the transgene expression at early transformation stages was effective.

The protocol described above provides a basis for the further genetic alteration of *P. sepium* for medicinal compounds and *cis*-polyisoprene production. Bamba et al. (2007) reported that the polyisoprene extracted from the milky exudate or the seedlings of *P. sepium* was confirmed to be *cis*-polyisoprene by ^{1}H-NMR analysis, and the content was estimated to be about 0.2% (based on calculation of the peak area in the SEC chromatogram). *H. brasiliensis* is not a good experimental plant because it only grows in the tropics (mostly in the Southeast Asia area) and is difficult to transform (Bamba et al. 2007). *P. sepium*, on the other hand, outstanding in its wide acclimatization, fast growth, and easy receptibility to genetic transformation, may be an excellent experimental model plant for rubber biosynthesis research and related gene characterization. By overexpression of the genes relating to rubber content and rubber quality, transgenic *P. sepium* may become a commercial rubber producing plant that can be planted from tropical to temperate zones.

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