Plant Biotechnol Rep (2011) 5:147–156
DOI 10.1007/s11816-011-0166-3

ORIGINAL ARTICLE

Highly efficient production of transgenic *Scoparia dulcis* L. mediated by *Agrobacterium tumefaciens*: plant regeneration via shoot organogenesis

Mahender Aileni · Sadanandam Abbagani · Peng Zhang

Received: 8 May 2010 / Accepted: 26 December 2010 / Published online: 21 January 2011

Abstract Efficient *Agrobacterium*-mediated genetic transformation of *Scoparia dulcis* L. was developed using *Agrobacterium tumefaciens* strain LBA4404 harboring the binary vector pCAMBIA1301 with β-glucuronidase (GUS) (*uidA*) and hygromycin phosphotransferase (*hpt*) genes. Two-day precultured leaf segments of in vitro shoot culture were found to be suitable for cocultivation with the *Agrobacterium* strain, and acetosyringone was able to promote the transformation process. After selection on shoot organogenesis medium with appropriate concentrations of hygromycin and carbenicillin, adventitious shoots were developed on elongation medium by twice subculturing under the same selection scheme. The elongated hygromycin-resistant shoots were subsequently rooted on the MS medium supplemented with 1 mg l^{-1} indole-3-butyric acid and 15 mg l^{-1} hygromycin. Successful transformation was confirmed by PCR analysis using *uidA*- and *hpt*-specific primers and monitored by histochemical assay for β-GUS activity during shoot organogenesis. Integration of *hpt* gene into the genome of transgenic plants was also verified by Southern blot analysis. High transformation efficiency at a rate of 54.6% with an average of 3.9 ± 0.39 transgenic plantlets per explant was achieved in the present transformation system. It took only 2–3 months from seed germination to positive transformants transplanted to soil. Therefore, an efficient and fast genetic transformation system was developed for *S. dulcis* using an *Agrobacterium*-mediated approach and plant regeneration via shoot organogenesis, which provides a useful platform for future genetic engineering studies in this medicinally important plant.

Keywords *Scoparia dulcis* · *Agrobacterium*-mediated transformation · Shoot organogenesis · High efficiency · Molecular analysis

M. Aileni · P. Zhang (✉)
National Key Laboratory of Plant Molecular Genetics, Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 300 Fenglin Road, Shanghai 200032, China
e-mail: zhangpeng@sibs.ac.cn

M. Aileni · S. Abbagani
Plant Biotechnology Research Unit, Department of Biotechnology, Kakatiya University, Warangal 506009, India

P. Zhang
Key Laboratory of Synthetic Biology, Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200032, China

P. Zhang
Chenshan Botanical Garden, Shanghai Chenshan Plant Science Research Center, Chinese Academy of Sciences, 3888 Chenhua Road, Shanghai 201602, China

Introduction

Plant-based remedies have been highlighted due to their fewer side effects in comparison to synthetic drugs and antibiotics. It is estimated that 70–80% of people worldwide rely mainly on traditional, largely herbal, medicines to meet their primary health care needs (Farnsworth and Soejarto 1991). *Scoparia dulcis* L., commonly named sweet broomweed or vassourinha, is a perennial medicinal herb distributed in the tropical and subtropical regions of Asia and South America. This plant has been intensively studied as a traditional herbal medicine (Latha et al. 2004). Previous studies have revealed that the presence of numerous phytochemicals, such as coumarins, saponins,

tannins, flavonoids and various terpenoids like scoparic acid A, scoparic acid B, scopadulciol and scopadulin, in this plant species are responsible for its medicinal properties (Latha et al. 2006, 2009; Babincová et al. 2008; Riel et al. 2002; Porika et al. 2009; Ahsan et al. 2003). Some of these properties include antitumor-promoting activity, antihyperalgesic, antimalarial, antiulcer, antipyretic and aphrodisiac activities, as well as cytotoxicity activity against cancer cells (Taylor 2006). Because of these pharmaceutical functions, it is important to develop new lines with increased phytochemical content by either traditional breeding or biotechnological tools.

Genetic engineering is a powerful tool to study gene functions in plants and could help to understand the factors that control flux into specific routes of secondary metabolism in plants and microbes (Bohnert et al. 2008; Grotewold 2008; Kirby and Keasling 2009). Developing protocols for efficient genetic transformation of medicinal plants is an important tool to study their molecular basis and the regulation of metabolic pathways (Khan et al. 2009). It is also important for us to move from empirical to predictive metabolic engineering (Dixon 2005). Recently, various biotechnological approaches have been adopted for enhancing bioactive molecules in medicinal plants or microbes (Abdin 2007; Grotewold 2008; Schäfer and Wink 2009; Chemler and Koffas 2008). Through genetic manipulation of biosynthetic pathway via genetic transformation, success has been reported in enhancing the production of secondary metabolites in plants (Mahmoud and Croteau 2001; Franke et al. 2000; Rosati et al. 2000; Mann et al. 2000; Ye et al. 2000). Establishment of an efficient transgenic system is a prerequisite for genetic improvement of valuable medicinal plants rich in therapeutically active secondary metabolites.

In comparison with the abundant pharmacological data and clinical studies using *S. dulcis*, information concerning its in vitro plant regeneration and genetic engineering is rather sparse (Aileni et al. 2008). So far, there is only one report of production of transgenic herbicide-resistant *S. dulcis* plants using *Agrobacterium rhizogenes* (Yamazaki et al. 1996), and there are no reports available on *Agrobacterium tumefaciens*-mediated transformation for this valuable medicinal plant. Establishment of an efficient transformation method may facilitate its improvement in terms of the accumulation levels of secondary metabolites.

This study was carried out to obtain a fast, simple, reliable and high throughput method for genetic transformation of *S. dulcis*. We have shown that foreign genes were successfully integrated into the genome and expressed in the regenerated plants. This is the first report for developing transgenic *S. dulcis* plants via *A. tumefaciens*-mediated transformation.

Materials and methods

Plant material, growth conditions and media composition

Mature seeds of *Scoparia dulcis* L. were collected from the medicinal arboretum maintained by the Forest Department, Warangal, India, in March 2009 and stored at −20°C until use. They were cleaned by tap water for 1 h, then surface sterilized by immersion in 70% ethanol for 30 s, followed by one rinse with sterile distilled water, additionally immersed in 0.1% mercuric chloride ($HgCl_2$) solution for 5 min followed by three rinses with sterile distilled water. After blotting on sterile filter paper, the surface-sterilized seeds were cultured aseptically in 9-cm Petri dishes containing 25 ml of seed germination medium composed of MS basal medium (Murashige and Skoog 1962) with 2% (w/v) sucrose and 0.3% (w/v) gelrite. After germination, the internodal segments were cut and cultured on MS medium containing 1 mg l^{-1} 6-benzylamino purine (BAP) and 1 mg l^{-1} kinetin for propagation. New expanding leaves excised from 1-month-old plants were cut into segments in the size of 1.0 cm^2 and used for transformation materials. The compositions of different media for preculture, cocultivation, shoot induction, shoot elongation as well as shoot rooting are listed in Table 1. Culture conditions were maintained at 25 ± 2°C under 16-h photoperiod provided by cool-white fluorescent lamps with an intensity of 30 μmol m^{-2} s^{-1}. All media were adjusted to pH 5.7

Table 1 Media used for transformation study of *Scoparia dulcis*

Stage	Medium composition	Medium abbreviation	Duration (days)
Preculture	MS + 5 mg l^{-1} BAP + 0.088 mg l^{-1} IAA	MSO	2
Cocultivation	MS + 5 mg l^{-1} BAP + 0.088 mg l^{-1} IAA + 200 μM acetosyringone	MSO + AS	3
Shoot induction	MS + 5 mg l^{-1} BAP + 0.088 mg l^{-1} IAA + 5 mg l^{-1} hygromycin + 400 mg l^{-1} carbenicillin	SSIM	30
Shoot elongation	MS + 3 mg l^{-1} BAP + 10 mg l^{-1} hygromycin + 200 mg l^{-1} carbenicillin	SSEM	20
Shoot rooting	MS + 1 mg l^{-1} IBA + 15 mg l^{-1} hygromycin	SSRM	15

before autoclaving at 121°C for 20 min. The antibiotics and acetosyringone (AS) were filter-sterilized after they were dissolved in appropriate solvents and added to the autoclaved medium when the temperature was about 40°C.

Numbers of responding explants and numbers of shoot buds per explant were recorded using a Nikon stereomicroscope (Olympus BX-50; Osaka, Japan) after 30, 40 and 60 days of culture following incubation. In addition, numbers of elongated shoots were also recorded.

Bacterial culture, cocultivation and hygromycin sensitivity

The *Agrobacterium tumefaciens* strain LBA4404 (Vander Fits et al. 2000) harboring a binary vector pCAMBIA1301 (CAMBIA, Australia) was used in this study. The T-DNA of pCAMBIA1301 contains an intron-interrupted β-glucuronidase (GUS) (*uidA*) and hygromycin phosphotransferase (*hpt*) genes under the control of cauliflower mosaic virus (CaMV) 35S promoter. Prior to transformation, a single colony of the LBA4404 from a fresh culture was picked and grown overnight in liquid YEB medium, pH 7.2, with rifampicin 25 mg l^{-1}, kanamycin 50 mg l^{-1} and chloramphenicol 75 mg l^{-1} at 28°C for 20–22 h at 180*g* in an incubator shaker.

The LBA4404 cells were harvested by centrifugation at 3,000*g* for 10 min and the pellet was resuspended and diluted to OD_{600} value of 0.6 with liquid MS medium containing 200 μM AS. Leaf explants of 1 cm^2 precultured on MSO medium composed of MS basic medium containing 5 mg l^{-1} BAP and 0.088 mg l^{-1} indole-3-acetic acid (IAA) for 0, 1, 2 or 3 days were inoculated in such bacterial suspension for 45 min with gentle shaking. After excess bacteria suspension had been blotted dry with sterile filter paper, leaf explants were transferred onto different media (Table 2) and co-cultivated in dark for 1, 2, 3, or 4 days at 25 ± 2°C. The beneficial effects of AS were also studied during cocultivation by using different media (Table 3) and MSO supplemented with AS at doses of 100, 200 or 300 μM (Table 4). Twenty-five explants were placed on each Petri dish in the transformation studies.

In order to use effective concentration of selection agents, the untransformed leaf segments were cultured on MS medium containing 5 mg l^{-1} BAP and 0.088 mg l^{-1} IAA with different concentrations of hygromycin (0, 2.5, 5, 7.5, 10, 12.5 or 15 mg l^{-1}) and carbenicillin (100, 200, 300, 400 or 500 mg l^{-1}), respectively.

Regeneration, subculturing and rooting of transgenic plants

Following cocultivation, the explants were washed twice with sterile distilled water. To further eliminate bacterial overgrowth, the infected leaf explants were washed once with MS basal medium containing 400 mg l^{-1} carbenicillin. The explants were then cultured on selective shoot induction medium (SSIM; Table 1) for the regeneration of

Table 2 Effect of preculture and cocultivation durations on transformation efficiency of *Scoparia dulcis*

Transformation parameters	No. of shooting explants	Transformation efficiency (%)[a]
Preculture duration (days)[b]		
0	6.60 ± 0.42	26.4 a
1	8.00 ± 0.48	32.0 b
2	14.20 ± 1.40	56.8 e
3	10.00 ± 1.15	40.0 d
Cocultivation duration (days)[c]		
0	8.00 ± 0.40	32.0 b
1	10.00 ± 1.15	40.0 d
2	12.40 ± 1.58	49.6 e
3	14.00 ± 1.46	56.0 e
4	9.00 ± 0.61	36.0 c

[a] Percent of explants producing hygromycin-resistant plants; means with different letters were significantly different ($p < 0.05$)

[b] Preculture was carried out using MSO (MS medium supplemented with 5 mg l^{-1} BAP + 0.088 mg l^{-1} IAA)

[c] Cocultivation was carried out using MSO without AS

Table 3 Comparison of effect of cocultivation media on transformation efficiency of *Scoparia dulcis*

Cocultivation medium	No. of shooting explants	Transformation efficiency (%)[a]
MS	6.20 ± 0.72	24.8 a
MSO	10.00 ± 1.15	40.0 b
MSO + acetosyringone[b]	14.20 ± 1.50	56.8 c

[a] Percent of producing hygromycin-resistant and GUS-positive plants; means with different letters were significantly different ($p < 0.05$)

[b] MS medium supplemented with 200 μM acetosyringone

Table 4 Effect of acetosyringone (AS) concentration in cocultivation on transformation efficiency of *Scoparia dulcis*

AS concentration (μM)[a]	No. of shooting explants	Transformation efficiency (%)[b]
100	9.0 ± 1.2	33.3 a
200	14.0 ± 2.5	56.0 b
300	10.0 ± 1.2	40.0 a

[a] Acetosyringone was added to MSO medium

[b] Percent of producing hygromycin-resistant and GUS-positive plants; means with different letters were significantly different ($p < 0.05$)

shoot buds and retained for 30 days, which includes two more subcultures onto the same medium at 10-day intervals. This medium was supplemented with 0.5 mg l^{-1} BAP, 0.088 mg l^{-1} IAA, 5 mg l^{-1} hygromycin as selective agent, and 400 mg l^{-1} carbenicillin as bactericide. Adventitious shoot buds developed from the leaf segment in due course.

For shoot elongation, the regenerated cultures were transferred to the selective shoot elongation medium (SSEM; Table 1) and subcultured twice at 10-day intervals. In this medium, the concentrations of the growth regulator (3 mg l^{-1} BAP) and carbenicillin (200 mg l^{-1}) were decreased while hygromycin concentration was increased to 10 mg l^{-1}. Hygromycin-resistant, healthy and elongated shoots of 2.0–2.5 cm with 2–3 leaves were excised and transferred to selective shoot rooting medium (SSRM; Table 1). The medium was supplemented with 1 mg l^{-1} indole-3-butyric acid (IBA) and 15 mg l^{-1} hygromycin. Plantlets were gently rinsed under running tap water to remove residual medium, and transferred to plastic pots containing a mixture of vermiculite and perlite (1:1). During the process of acclimatization, the plantlets were covered with polythene bags to maintain humidity for 1 week and irrigated with tap water.

Each of these experiments was repeated thrice and two replicates kept per treatment. The significance of the results obtained for transformation efficiency was verified by Duncan's test ($p < 0.05$).

β-Glucuronidase histochemical assay

Transient GUS expression was screened in the leaf explants just after cocultivation, while stable GUS expression was observed in the hygromycin-resistant transgenic plants. The assay for the GUS activity was carried out as previously described (Jefferson et al. 1987). This assay gives a blue coloration in the presence of the β-GUS enzyme. After incubating overnight in the substrate solution at 37°C, the stained tissues were rinsed several times with 75% ethanol to bleach chlorophyll, and observed under a light microscope. The transformation efficiency was calculated by percent of explants showing GUS positive shoots.

Molecular analyses of transformants

Total genomic DNA from randomly selected hygromycin-resistant plants and control plants were isolated according to the method described by Kim and Hamada (2005). The presence of transgenes (*uidA* and *hpt*) in the transformants was confirmed by PCR amplification using gene-specific forward primer 5′-CGACGGCCTGTGGGCATTTCA-3′, and reverse primer 5′-TGGTCGTGCACCATCAGCAC-3′ designated to amplify 900 bp of *uidA* gene and forward primer 5′-TAGAAAAAGCCTGAACTCACCG-3′ and reverse primer 5′-TATTTCTTTGCCCTCGGACG-3′ amplifying 1 kb of *hpt* gene. PCR reactions were carried out in 20 µl reaction mixture containing 0.5 units of Takara Ex Taq polymerase and 1× *Taq* buffer (Takara, Dalian, China), 0.2 mM of each dNTP, 0.5 µM of each primer, and 50 ng of template DNA. The PCR conditions for *uidA* gene detection was pre-denaturation at 95°C for 5 min, then followed by 30 cycles of strand separation of 94°C for 40 s, annealing at 58°C for 40 s and extension at 72°C for 1 min, and 10 min at 72°C as final extension. The PCR conditions for *hpt* gene detection was pre-denaturation at 94°C for 5 min, then followed by 30 cycles of strand separation at 95°C for 40 s, annealing at 56°C for 1 min and extension at 72°C for 40 s, and 10 min at 72°C final extension. Amplified DNA fragments were electrophoresed in 1% agarose gel, detected by ethidium bromide staining.

To detect the expression of transgenes by RT-PCR, total RNA was isolated from young shoots of transgenic lines using TRNzol reagent (Tiangen Biotech, Beijing, China) according to the manufacturer's protocol and treated with DNase I (Takara) to remove contaminant DNA traces. Total RNA (2 µg) was used as template to synthesis first-strand cDNA with oligo $(dT)_{18}$ (First strand cDNA synthesis kit; Takara). PCR of the *uidA* gene was carried out according to the conditions described above. The housekeeping gene *actin* was used as an internal control to check the expression levels of transgenes. The primer sequences of *actin* gene were forward: 5′-CACCACCACAGCCG AACGGG-3′, and reverse: 5′-ACCCGGGAACATGGTG GAACC-3′, which give a 325-bp product with cDNA. The PCR products were subjected to electrophoresis in 1.0% agarose gel.

For Southern blot analysis, 20 µg of DNA was digested with *Eco*RI, which cuts once within the T-DNA region, in 400 µl of the manufacture's buffer (Takara, Japan). After digestion, the DNA fragments were size-fractioned by electrophoresis on 0.8% (w/v) agarose gel and subsequently transferred to a Hybond^{+} nylon membrane (Amersham Pharmacia Biotech). A 500 bp DIG- labeled DNA fragment of *hpt* gene amplified by PCR was used as probe. Labeling, hybridization and detection were performed according to the manufacturer's instructions (Roche Applied Science).

Results

Optimization of hygromycin selection, preculture duration and cocultivation conditions

Explants sensitivity to hygromycin was studied by culturing the uninfected leaf segments on MSO medium

supplemented with 0, 2.5, 5, 7.5, 10, 12.5 and 15 mg l^{-1} hygromycin. Without hygromycin selection, a maximum of 95% shoot organogenesis was observed on MSO medium after 4 weeks of culture (Fig. 1a). Shoot regeneration was inhibited from all explants when increasing selection pressure to 10 mg l^{-1} hygromycin. A few explants having

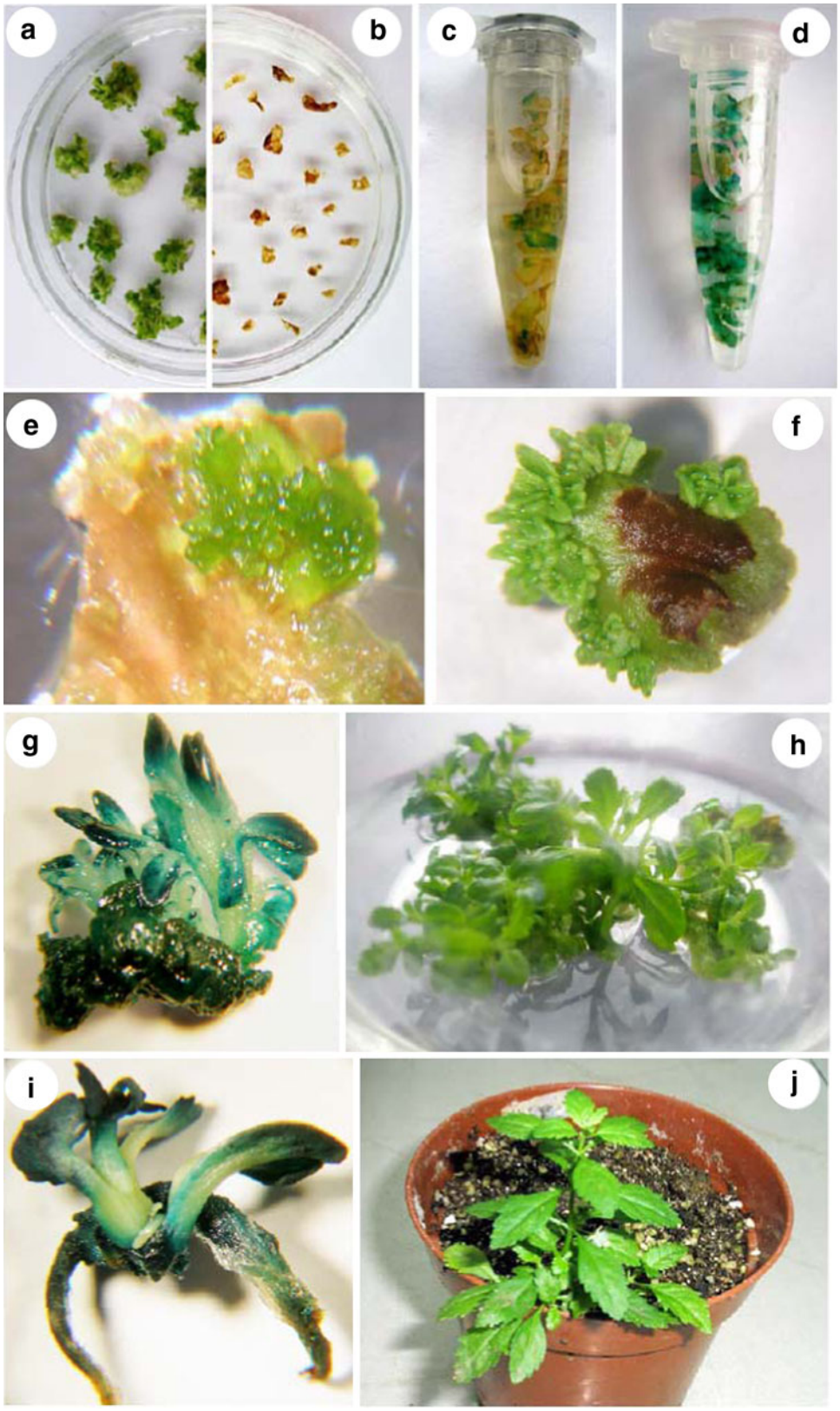

Fig. 1 Production of transgenic *Scoparia dulcis* using axenic leaf explants via *Agrobacterium tumefaciens*-mediated transformation. **a** Profuse shoot organogenesis from 95% of leaf explants after 4 weeks of incubation on MSO medium; **b** complete inhibition of shoot organogenesis in all leaf explants after 4 weeks of selection on the MSO medium supplemented with 12.5 mg l^{-1} hygromycin; **c** transient GUS expression shown by infected leaf explants without any preculture after 3 days of cocultivation; **d** transient GUS expression shown by infected leaf explants with 2 days preculture after 3 days of cocultivation; shoot primordia developed from mid-portion (**e**) and cut-end (**f**) of leaf explants after 20–30 days of culture on SSIM; **g** shoot cluster showing GUS expression after first 10 days of subculture on SSEM for elongation; **h** elongation of putative transgenic shoots after second 10 days of subculture on SSEM; **i** GUS-positive transgenic plantlet after 10 days of rooting on SSRM; **j** phenotypically normal transgenic plant growing on soil in the greenhouse

shoot bud initiation showed necrosis by the end of the third week. Hygromycin concentrations at/above 12.5 mg l^{-1} completely inhibited shoot organogenesis (Fig. 1b). In the present study, we used 15 mg l^{-1} of hygromycin for the final selection of transformants in order to eliminate escapes. With the fresh leaf explants without preculture, transient GUS expression assay showed 26.4% transient transformation efficiency (Fig. 1c). Our results revealed that a preculture period of 2 days was effective for the improvement of *S. dulcis* transformation (Table 2), producing 56.8% of transient GUS assay (Fig. 1d). Preculture for shorter or longer than 2 days reduced the transformation efficiency (Table 2).

Various concentrations of carbenicillin were also tested to find a proper dose, which could give rise to maximum rate of shoot survival during the whole selection procedure. It was found that selection medium should contain at least 400 mg l^{-1} carbenicillin to suppress the overgrowth of *A. tumefaciens* during the selection procedure. Carbenicillin concentration of more than 400 mg l^{-1} has resulted in tissue necrosis in *S. dulcis*. During the selective shoot regeneration, elongation and rooting of transformants on different media (Table 1), the concentration of hygromycin was gradually increased from 5 to 15 mg l^{-1} with diminishing usage of carbenicillin from 400 to 0 mg l^{-1}.

After 3 days of cocultivation on MS medium, the infected explants showed 24.8% of transformation efficiency. MS medium with inclusion of plant growth regulators (5 mg l^{-1} BAP + 0.088 mg l^{-1} IAA) gave a transformation efficiency of 40%. Moreover, the promoting effect of AS in the medium (MSO + AS) was also noticeable with 16.8% increase of transformation efficiency (Table 3). Among the different concentrations of AS tested, 200 μM was found to be the optimal concentration for maximum transformation efficiency (Table 4). Optimal transformation efficiency could be achieved by using leaf explants after 2 days of preculture followed by 3 days of cocultivation on MSO supplemented with 200 μM AS.

Regeneration of transgenic plants

On average, 70% of leaf explants (data not shown) started to initiate the shoot organogenesis from the mid-portions and cut ends of leaf explants (Fig. 1e, f) after 20–30 days of incubation on SSIM. Every 10 days, it was necessary to subculture thrice onto the same medium for healthy and maximum numbers showing shoot formation. During shoot organogenesis, shoots were formed directly from infected leaf segments without an intervening callus phase. Because these cultures with shoot primordia did not elongate further if transferred onto the same SSIM medium, the cultures were subcultured onto elongated medium (SSEM), which was supplemented with a reduced concentration of BAP (Table 1). The elongated shoots displayed dark-blue after GUS assay, showing their transgenic nature (Fig. 1g, h). On SSEM medium during 20 days of culture (including another subculture after 10 days), we observed that the percent of elongating shoots in clumps started to decline from 70 to 60%, possibly due to the elimination of untransformed shoots. The elongated hygromycin-resistant shoots were finally transferred onto the rooting medium (SSRM) containing 15 mg l^{-1} of hygromycin and proved to be transgenic by GUS assay (Fig. 1i). During this culture period, only putatively transformed shoots survived to rooting, the rest were finally eliminated. Similar results of rooting capability and reduction of non-transgenic escapes were observed with the use of 20 mg l^{-1} hygromycin in the rooting medium (SSRM). Based on the percent of co-cultivated leaf explants producing shoots on hygromycin-containing medium, the average transformation efficiency of 54.6% was achieved (Table 5). A mean of 3.9 ± 0.39 transgenic plantlets per explant were recorded. After 15 days on SSRM medium, complete transgenic plantlets ready for transfer to the greenhouse were produced. The plantlets survived with 80% transplant success in the greenhouse and flowered in due course (Fig. 1j). The morphology of the transformed plants resembled those of the parent.

Table 5 Transformation efficiency mediated by *Agrobacterium tumefaciens* strain LBA4404 (pCAMBIA1301) in *S. dulcis*

Experiment[a]	No. of cocultivated explants	No. of shooting explants under hygromycin selection	No. of transgenic plants		No. of transgenic plants/explant	Transformation efficiency (%)[c]
			Hygromycin resistant	GUS positive[b]		
1	50	27	108	96	4.0 ± 0.51	54.0 a
2	50	30	105	98	3.5 ± 0.43	60.0 b
3	50	25	105	94	4.2 ± 0.25	50.0 a
Mean	50	27.3 ± 2.51	106 ± 1.73	96 ± 2.00	3.9 ± 0.39	54.6

[a] Plants grown on regeneration medium containing 15 mg l^{-1} hygromycin

[b] Plants showing GUS gene expression at the fully mature stage

[c] Means with different letters were significantly different ($p < 0.05$)

Confirmation of transgenic plants

In comparison with untransformed wild-type plants which did not contain any expression of GUS (Fig. 2a, b), the transgenic plant lines showed the typical dark-blue (Fig. 2c, d) after the GUS assay. Upon histochemical staining of leaves and/or roots from 68 hygromycin-resistant plant lines randomly selected from the pool of rooted plants, except for 5 lines all the others showed expression of *uidA* gene (Fig. 2e). These 5 lines were even found to be positive for *hpt* PCR analysis, but did not show any histochemical staining similar to untranformed control plantlets (Fig. 2e, upper left corner). The presence of the transgenes was further confirmed by PCR analysis using total genomic DNA isolated from leaves of transformed and untransformed (control) plants using specific primers designed to amplify *uidA* and *hpt* genes. Five GUS-positive lines were selected randomly and used for PCR analysis with one non-transgenic line as negative control. Fragments of 900-bp *uidA* (Fig. 3a) and 1-kb *hpt* (Fig. 3b) were amplified from genomic DNA of all the transgenic plants, whereas corresponding bands were not detected in the untransformed control plant (Fig. 3a, b).

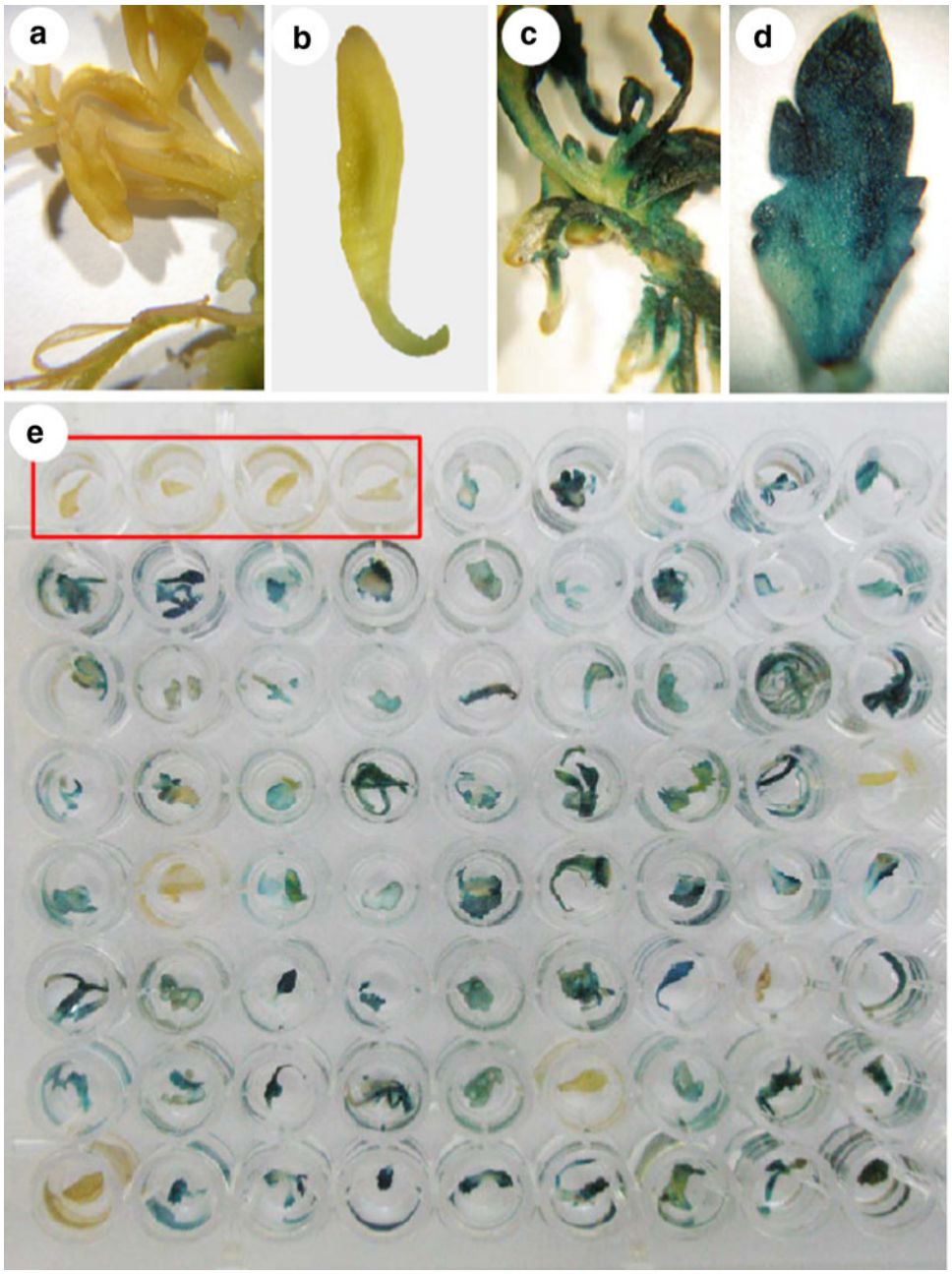

Fig. 2 Histochemical analysis of GUS expression in *Scoparia dulcis*. Non-transformed control plantlet (**a**) and young leaf (**b**); GUS-positive transgenic plantlet (**c**) and leaf (**d**); **e** evaluation of GUS expression in leaves/roots from 68 hygromycin-resistant plant lines and 4 wild-type control (*red square boxed*) (color figure online)

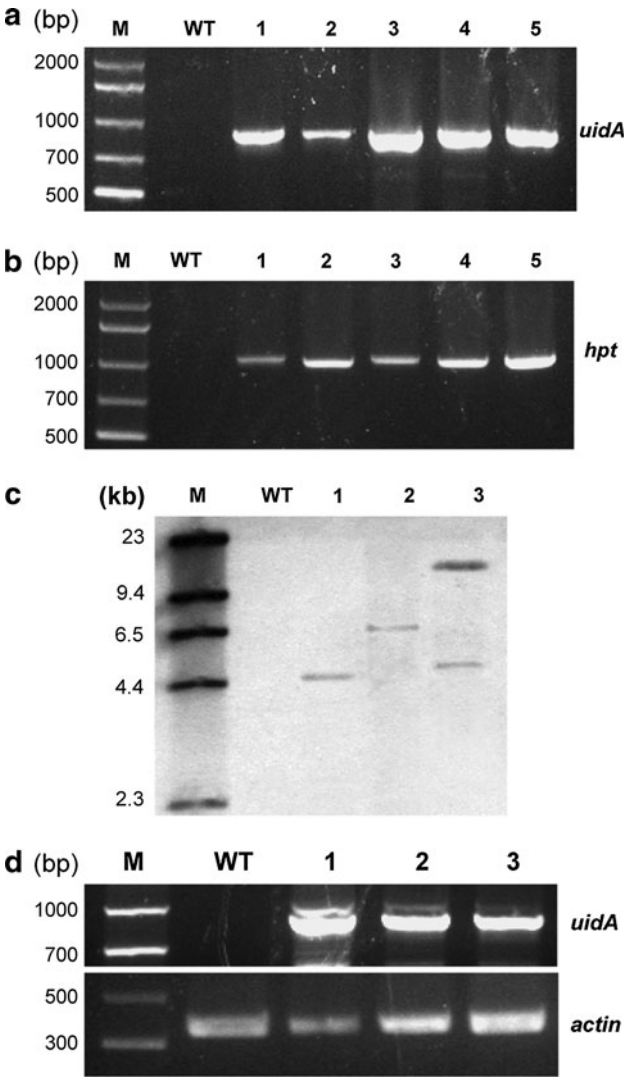

Fig. 3 Molecular analyses of transgenic plant lines of *Scoparia dulcis*. **a** PCR detection of *uidA* gene; **b** PCR detection of *hpt*; **c** Southern blot detection of transgene integration in transgenic lines; **d** RT-PCR detection of *uidA* and native *actin* expression. *M* molecular marker, *WT* wild-type control, *1–5* different transgenic plant lines

The integration of the *hpt* gene into the genomic DNA was further confirmed by Southern blot analysis of transformed plants. The genomic DNA was digested with *Eco*RI, which cuts only once in the T-DNA region of pCAMBIA1300 plasmid. The DIG-labeled *hpt* gene was used as a probe. Among the tested three lines, two lines (Fig. 3c, lanes 1 and 2) showed single integration and the other one (lane 3) showed two insertional integration. As expected, no integration was observed in the control plants (WT). Besides the GUS assay, the expression of *uidA* gene was also carried out by RT-PCR analysis. The presence of a band of 900 bp was amplified from the cDNA products of three transgenic plants, while the control DNA showed no amplification (Fig. 3d).

Discussion

The objective of the present study is to develop an efficient *A. tumefaciens*-mediated transformation using axenic leaf segments of *S. dulcis*. In order to achieve the maximum transformation efficiency in *S. dulcis*, a number of transformation parameters were studied and optimized, including different media used for preculture, cocultivation and shoot primordia induction and elongation, culture duration for each phase, and selection strategy to eliminate non-transgenic escapes.

The combination of plant growth regulators and appropriate transformation strategy is critical for a successful production of transgenic plants. As in many transformation studies such as in *Lycium babarum* (Hu et al. 2006), preculturing of *S. dulcis* leaf explants has also shown a promoting effect on transformation efficiency in our study (Table 2). The beneficial effect of preculture on transformation frequency in the presence of plant growth regulators might be attributed to promoting effects of plant growth regulators on cell division, as actively dividing cells are more vulnerable to delivery and integration of T-DNA (An 1985). Cocultivation is one of the most important steps for *Agrobacterium*-mediated transformation of plants (James et al. 1993). The presence of plant growth regulators in the cocultivation medium also stimulated the dividing cells, resulting in a high regeneration frequency of transgenic shoots as compared with the usage of cocultivation medium without plant growth regulators (Table 3; Wang and Fang 1998). Cocultivation medium supplemented with optimum AS and phytohormone concentrations has remarkably increased the efficiency of transformation in *S. dulcis* (Tables 3 and 4). This is in good agreement with the previous studies such as in *Malus domestica* (James et al. 1993) and Indica rice (Rashid et al. 1996). The phenolic compound AS has been known to induce the expression of virulence genes of *A. tumefaciens* and increase transformation frequency in many plant transformations (Shimoda et al. 1990).

Based on our prior report in regenerating 14 ± 1.14 plantlets per mature leaf explant (Aileni et al. 2008), in the present investigation we have also used the optimal combination and concentrations of plant growth regulators for regeneration of shoot primordia after cocultivation. Different from our previous regeneration studies of *S. dulcis* in which we had used basal medium for elongation of shoots after shoot induction (Aileni et al. 2008), in the present report the transformed shoot primordia were transferred onto the medium with lower concentration of BAP for promoting shoot elongation (Fig. 1g). This is in accordance with previous reports on medicinal plants, in which multiple transgenic shoots with normal phenotype regenerated

very well in the lower dose of plant growth regulators (Bhattacharya and Bhattacharya 2001; Ghanti et al. 2004).

It is important to eliminate non-transgenic escapes efficiently during shoot organogenesis and select true transgenic plants for further molecular analysis. Because in our genetic transformation system lots of shoots were regenerated from each leaf segment on the regenerative selection media (Fig. 1e–g), these putative transgenic plants were further screened in rooting medium containing higher concentrations of hygromycin. Such rooted plant lines were proved to be transgenic in nature after PCR and GUS analyses for the transgenes (Figs. 1i and 3a, b). Some hygromycin-resistant transformants confirmed by PCR did not express GUS (Fig. 2e), probably due to the genomic position effects, deletion of promoter or part of *uidA* coding region, or gene silencing due to DNA methylation (Baulcombe 2004).

Because of the diverse medicinal values of *S. dulcis*, whose functions have been well evaluated and documented in the literature (Latha et al. 2004), more attention has to be paid towards engineering its metabolic pathways for value-addition via biotechnology approaches. Recently, large-scale production of terpenoid scopadulcic acid B from *Scoparia dulcis* was reported using luffa sponge bioreactor (Mathew and Jayachandran 2009). Production of transgenic *S. dulcis* conferring herbicide resistance using Ri binary vector was the only documented transformation study (Yamazaki et al. 1996). No reports are available on genetic transformation using *A. tumefaciens* in *S. dulcis*. The *Agrobacterium*-mediated transformation system remains the favorite for many researchers, as it does not involve sophisticated equipment and more frequently produces clean transgenic integration events (intact integration and single copy; Fig. 3c). The leaf-based genetic transformation via shoot organogenesis also proved to be more efficient and practical, as leaf is the most favorable source of explant for plant genetic transformation studies. In our system, transformation efficiency of *S. dulcis* was 54.6% with mean number of 3.9 transgenic plantlets per explants (Table 5). The high transformation efficiency observed in *S. dulcis* may be contributed by the efficient regeneration response, similar to the cases of model systems like tobacco and *Arabidopsis*. This is the first report on successful transformation of *S. dulcis* using leaf-based transformation. The platform of *A. tumefaciens*-mediated transformation in *S. dulcis* has laid the foundation towards metabolic engineering by expression of metabolic pathway genes.

In conclusion, a simple, efficient and reproducible transformation protocol for *S. dulcis* has been developed using *A. tumefaciens*-mediated gene delivery. It only took 2–3 months from seed germination to transgenic plants transplanted to soil. The optimized transformation protocol will also allow us to produce transgenic *S. dulcis* with higher contents of pharmaceutical compounds or modified secondary metabolic profiles in future using approaches of specific metabolic engineering.

Acknowledgments This work was supported by grants from the National Basic Research Program (2007CB108805), the Shanghai Pujiang Talent Program (08PJ14109) and the CAS-TWAS Post-graduate Fellowship.

References

Abdin MZ (2007) Enhancing bioactive molecules in medicinal plants. In: Zhu Y, Tan B, Bay B, Liu C (eds) Natural products—essential resources for human survival. World Scientific, Singapore

Ahsan M, Islam SK, Gray AI, Stimson WH (2003) Cytotoxic diterpenes from *Scoparia dulcis*. J Nat Prod 66:958–961

Aileni M, Kokkirala VR, Kota SR, Umate P, Abbagani S (2008) Efficient in vitro regeneration from mature leaf explants of *Scoparia dulcis* L., an ethnomedicinal plant. J Herbs Spices Med Plants 14:200–207

An G (1985) High-efficiency transformation of cultured tobacco cells. Plant Physiol 79:568–570

Babincová M, Schronerová K, Sourivong P (2008) Antiulcer activity of water extract of *Scoparia dulcis*. Fitoterapia 79:587–588

Baulcombe D (2004) RNA silencing in plants. Nature 431:356–363

Bhattacharya R, Bhattacharya S (2001) High frequency in vitro propagation of *Phyllanthus amarus* Schum. & Thonn. by shoot tip culture. Indian J Exp Biol 39:1184–1187

Bohnert H, Nguyen H, Lewis NG (2008) Bioengineering and molecular biology of plant pathways. Advances in plant biochemistry and molecular biology, vol 1. Elsevier, Amsterdam

Chemler JA, Koffas MAG (2008) Metabolic engineering for plant natural product biosynthesis in microbes. Curr Opin Biotechnol 19:597–605

Dixon RA (2005) Engineering of plant natural product pathways. Curr Opin Plant Biol 8:329–336

Farnsworth NR, Soejarto DD (1991) Global importance of medicinal plants. In: Akerele O, Heywood V, Synge H (eds) Conservation and medicinal plants. Cambridge University Press, Cambridge, pp 25–51

Franke R, Michael CM, Meyer K, Shirley AM, Cusumano JC, Chapple C (2000) Modified lignin in tobacco and poplar plants over-expressing the *Arabidopsis* gene encoding ferulate 5-hydroxylase. Plant J 22:223–234

Ghanti KS, Govindaraju B, Venugopal RB, Ramgopal RS, Kaviraj SP, Jabeen FTZ, Barad A, Rao S (2004) High frequency shoot regeneration form *Phyllanthus amarus* Schum. & Thonn. Indian J Biotechnol 3:103–107

Grotewold E (2008) Transcription factors for predictive plant metabolic engineering: are we there yet? Curr Opin Biotechnol 19:138–144

Hu Z, Wu YR, Li W, Gao H (2006) Factors effecting *Agrobacterium tumefaciens*-mediated gentic transformation of *Lycium babarum* L. In Vitro Cell Dev Biol Plant 42:461–466

James DJ, Uratsu S, Cheng J, Negri P, Viss P, Dandekar AM (1993) Acetosyringone and osmprotectants like betaine or proline synergistically enhance *Agrobacterium*-mediated transformation of apple. Plant Cell Rep 12:559–563

Jefferson RA, Kavanagh TA, Bevan MW (1987) Gus fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901–3907

Khan MY, Aliabbas S, Kumar V, Rajkumar S (2009) Recent advances in medicinal plant biotechnology. Indian J Biotechnol 8:9–22

Kim SH, Hamada T (2005) Rapid and reliable method of extracting DNA form RNA form sweet potato [*Ipomea batatas* (L.) Lam.]. Biotechnol Lett 27:1841–1845

Kirby J, Keasling JD (2009) Biosynthesis of plant isoprenoids: perspectives for microbial engineering. Annu Rev Plant Biol 60:35–355

Latha M, Pari L, Sitasawad S, Bhonde R (2004) *Scoparia dulcis*, a traditional antidiabetic plant, protects against streptozotocin induced oxidative stress and apoptosis in vitro and in vivo. J Biochem Mol Toxicol 18:261–272

Latha M, Ramkumar KM, Pari L, Damodaran PN, Rajeshkannan V, Suresh T (2006) Phytochemical and antimicrobial study of an antidiabetic plant: *Scoparia dulcis* L. J Med Food 9:391–394

Latha M, Pari L, Ramkumar KM, Rajaguru P, Suresh T, Dhanabal T, Sitasawad S, Bhonde R (2009) Antidiabetic effects of scoparic acid D isolated from *Scoparia dulcis* in rats with streptozotocin-induced diabetes. Nat Prod Res 14:1–13

Mahmoud SS, Croteau RB (2001) Metabolic engineering of essential oil yield and composition in mint by altering expression of deoxyxylulose phosphate reductoisomerase and menthofuran synthase. Proc Natl Acad Sci USA 98:8915–8920

Mann V, Harker M, Pecker I, Hirschberg J (2000) Metabolic engineering of astaxanthin production in tobacco flowers. Nat Biotechnol 18:888–892

Mathew AJ, Jayachandran K (2009) Production of scopadulcic acid B from *Scoparia dulcis* Linn. using a *luffa* sponge bioreactor. Plant Cell Tissue Organ Cult 98:197–203

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Porika M, Aileni M, Gadidasu K, Kokkiral VR, Umate P, Rao AV, Devarakonda RK, Abbagani S (2009) In vitro HIV type 1 reverse transcriptase inhibitory acitivity of leaf extract of *Scoparia dulcis* L. J Herbs Spices Med Plants 15:241–247

Rashid H, Yokoi S, Toriyama K, Hinata K (1996) Transgenic plant production mediated by *Agrobacterium* in indica rice. Plant Cell Rep 20:701–705

Riel MA, Kyle DE, Milhous WK (2002) Efficacy of scopadulcic acid A against *Palsmodium falciparum* in vitro. J Nat Prod 65:614–615

Rosati C, Aquilani R, Dharmapuri S, Pallara P, Marusic C, Tavazza R, Bouvier F, Camara B, Giuliano G (2000) Metabolic engineering of beta-carotene and lycopene content in tomato fruit. Plant J 24:413–419

Schäfer H, Wink M (2009) Medicinally important secondary metabolites in recombinant microorganisms or plants: progress in alkaloid biosynthesis. Biotechnol J 4:1684–1703

Shimoda N, Toyoda-Yamamoto A, Nagamine J, Usami S, Katayama M, Sakagami Y, Michida Y (1990) Control of expression of *Agrobacterium* vir genes by synergistic action of phenolic signal molecules and monosaccharides. Proc Natl Acad Sci USA 87:6684–6688

Taylor L (2006) Vassourinha monograph 4/*Scoparia dulcis*. In: The rainforest, pharmacy to the world. Raintree Nutrition, Inc., Carson City. http://www.rain-tree.net/Vassourinha-Monograph.pdf

Vander Fits L, Deakin EA, Hoge JH, Memelink J (2000) The ternary transformation system : constitive *virG* on a compatible plasmid dramatically increases *Agrobacterium*-mediated plant transformation. Plant Mol Biol 43:495–502

Wang GL, Fang HL (1998) Mechanism and technology of plant genetic engineering. Science, Beijing

Yamazaki M, Lin S, Hayashi T, Morita N, Asamizu T, Mourakoshi I, Saito K (1996) Transgenic fertile *Scoparia dulcis* L., a folk medicinal plant, conferred with a herbicide-restistant trait using an Ri binary vector. Plant Cell Rep 15:317–321

Ye X, Al-Babili S, Klöti A, Zhang J, Lucca P, Beyer P, Potrykus I (2000) Engineering of povitamin A (beta-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. Science 287:303–305