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ORIGINAL ARTICLE

Factors influencing regeneration and *Agrobacterium tumefaciens*-mediated transformation of common bean (*Phaseolus vulgaris* L.)

Gerardine Mukeshimana · Yumin Ma · Aaron E. Walworth · Guo-qing Song · James D. Kelly

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Abstract A systematic study was carried out to optimize regeneration and *Agrobacterium tumefaciens*-mediated transformation of four common bean (*Phaseolus vulgaris* L.) cultivars; Red Hawk, Matterhorn, Merlot, and Zorro, representing red kidney, great northern, small red, and black bean commercial classes, respectively. Regeneration capacity of leaf explants, stem sections, and embryo axes were evaluated on 30 media each containing Murashige and Skoog (MS) medium and different combinations of plant growth regulators. For stem sections and leaf explants, none of the media enabled plant regeneration from any of the four cultivars tested, indicating the recalcitrance of bean regeneration from these tissues. In contrast, several media enabled multiple shoot production from embryo axis explants, although optimal regeneration media was genotype-dependent. Under optimal regeneration conditions, multiple shoots, 2.3–10.8 on average for each embryogenic explant, were induced from embryo axis explants at frequencies of 93 % for 'Merlot', 80 % for 'Matterhorn', 73 % for 'Red Hawk', and 67 % for 'Zorro'. Transient expression studies monitored by an intron-interrupted *gus*A on explants transformed with *A. tumefaciens* strains GV3101, LBA4404, and EHA105 indicated that all three *A. tumefaciens* strains tested were efficient in gene delivery. Gene delivery depended on parameters including strain of *A. tumefaciens*, co-cultivation time, explant type, and bean genotype. Agroinfiltration also enhanced gene delivery. Kanamycin-resistant and GUS-positive calluses were induced from leaf, stem, and embryo axis explants. Chimeric transformants were obtained from embryo axis explants and showed partial GUS-staining. Lack of efficient regeneration from non-meristem containing tissues is the main limitation for stable transformation of common bean.

Keywords Common bean · Plant regeneration · Stable transformation · Transgenic common bean · Transient transformation

G. Mukeshimana · J. D. Kelly (✉)
Department of Crop and Soil Sciences, Michigan State University, 1066 Bogue St., East Lansing, MI 48824, USA
e-mail: kellyj@msu.edu

Y. Ma · A. E. Walworth · G. Song
Department of Horticulture, Plant Biotechnology Resource and Outreach Center, Michigan State University, 1066 Bogue St., East Lansing, MI 48824, USA

Introduction

Common bean (*Phaseolus vulgaris* L.) is the most important grain legume for direct human consumption (Broughton et al. 2003). As a desirable tool to complement conventional breeding techniques, genetic engineering provides the possibility to source genes from beyond the gene pool accessible only through sexual hybridization (Christou 1997; Somers et al. 2003; Dita et al. 2006). To date, creation of stable transgenic common bean at low frequencies has been achieved using particle bombardment-mediated transformation of meristematic tissues of cv. 'Seafarer' at 0.03 %, cv. 'Goldstar' at 0.05 %, and cv. 'Olathe' at 0.9 % (Aragão et al. 1992, 1998; Russel et al. 1993; Kim and Minamikawa 1996; Bonfim et al. 2007). The major challenge to the production of genetically engineered beans has been the lack of a stable genetic transformation system, due to their recalcitrance to in vitro regeneration and low rates of *Agrobacterium*-mediated transformation (Svetleva et al. 2003; Veltcheva et al. 2005).

Common bean regeneration has been extensively studied by many groups exclusively using MS (Murashige and Skoog 1962) medium. Some of these studies investigated the regeneration capacity of various bean explants such as cotyledonary nodes (Dang and Wei 2009), embryo axes (Zambre et al. 1998; Delgado-Sánchez et al. 2006; Quintero-Jimenez et al. 2010), immature embryos (Geerts et al. 2000), leaf sections and petioles (Crocomo et al. 1976; Malik and Saxena 1991), and thin-cell layers (Cruz de Carvalho et al. 2000). Others have investigated the influence of different growth regulators and/or their combinations on bean regeneration (Saunders et al. 1987; Malik and Saxena 1991; Dang and Wei 2009; Gatica Arias et al. 2010; Kwapata et al. 2010; Quintero-Jimenez et al. 2010). Despite these efforts, an efficient and repeatable system that can support the regeneration of transformed common bean cells does not exist.

Agrobacterium tumefaciens-mediated transformation is the gene delivery mode most preferred by plant breeders because of its easy accessibility and tendency to produce low- or single-copy insertion of the transgene (Somers et al. 2003). Historically, large-seeded legumes have been difficult to transform using *A. tumefaciens.* However, recent reports showing progress in this field suggest potential possibilities for common bean. For instance, pigeon pea (*Cajanus cajun* (L.) Millsp.) can now be easily regenerated through both organogenesis and somatic embryogenesis using various explants, and successful transformation has been attempted, although genotypic dependence still exists (Krishna et al. 2010). Chickpea (*Cicer arietinum* L.) regeneration is possible mainly through somatic embryogenesis and shoot organogenesis with varying degrees of success (Huda et al. 2003; Jayanand et al. 2003; Somers et al. 2003). Successful production of transgenic chickpea plants using *Agrobacterium*-mediated transformation has been reported (Polowick et al. 2004; Senthil et al. 2004; Indurker et al. 2010; Mehrotra et al. 2011). Regeneration and *Agrobacterium*-mediated transformation of peas (*Pisum sativum* L.) have been successful using immature cotyledons as explants (Grant and Cooper 2006). Cotyledonary nodes at various maturity stages are being routinely utilized for *Agrobacterium*-mediated transformation of soybean (*Glycine max* (L.) Merr.; Paz et al. 2006; Dang and Wei 2009). Peanut (*Arachis hypogaea* L.) has been easily transformable compared to other legume species (Sharma and Pooja 2006). *Agrobacterium*-mediated transformation of cotyledonary explants has led to the generation of stable transgenic plants in cowpea (*Vigna unguiculata* L.; Muthukumar et al. 1996; Popelka et al. 2006; Solleti et al. 2008; Bakshi et al. 2011).

Agrobacterium-mediated transformation of *Phaseolus* species has been achieved with limited success. To date, only the tepary bean (*P. acutifolius* A. Gray) has a reproducible genetic transformation system (Dillen et al. 1997; Zambre et al. 2005). Liu et al. (2005) described the successful recovery of transgenic kidney bean (*P. vulgaris*) plants using sonification and vacuum infiltration techniques to transform bean seedlings using *A. tumefaciens*. However, the transformation rate was low and no subsequent studies using this protocol have been reported.

The possibility of transformation of *Phaseolus* species using *A. rhizogenes* has been demonstrated by Estrada-Navarrete et al. (2006). Although this *Agrobacterium* species may be useful for the production of hairy roots to enhance nitrogen fixation and functional genomics studies of root-expressed genes in common bean, the production of whole transgenic bean plants is not straightforward, since these composite plants do not transmit transgenic traits to their progenies. This reduces the utility *A. rhizogenes* for crop improvement purposes.

The present study was conducted to evaluate factors influencing transient and stable transformation of common bean using *A. tumefaciens.*

Materials and methods

Plant materials and culture media

Four common bean cultivars, Red Hawk, Matterhorn, Merlot, and Zorro, representing red kidney, great northern, small red, and black bean commercial common bean classes, respectively, were utilized in this study. These cultivars represent the racial and gene pool genetic diversity of common bean grown in North America (Broughton et al. 2003). 'Red Hawk' belongs to race Nueva Granada in the Andean gene pool, whereas 'Matterhorn' is race Durango, 'Merlot' is race Jalisco, and 'Zorro' is race Mesoamerica in the Middle American gene pool. Mature embryo axis, stem, and leaf explants were tested to determine their transformation and regeneration capacities.

Explant preparation

Mature, dry seeds were surface-sterilized with 3 % sodium hypochlorite with continuous shaking for 10 min in a 250-ml Erlenmeyer flask, followed by four rinses with sterile distilled water, and then soaked in sterile distilled water for approximately 16 h. The soaking water was then discarded; seeds were rinsed three times with sterile distilled water, and blotted dry on sterile filter paper. The seed coats were removed and the embryos were excised using a sterile scalpel. Embryo axes were obtained by cutting off radicles and leaflets.

To grow seedlings for stem and leaf explants, five sterile seeds were planted on half-strength MS medium in each Magenta® GA7 box (PhytoTechnology Laboratories, KS, USA). Seeds were germinated under a 16-h photoperiod of 30 μmol m^{-2} s^{-1} from cool white fluorescent tubes at 25 °C. Stem and leaf explants were prepared from 1-week-old seedlings. Stems were cut into 6- to 10-mm-length segments and were then cut in half longitudinally. Leaf explants of 5–7 × 5–7 mm were cut with a sterile scalpel after removing the outer leaf margins.

Regeneration experiments

All regeneration media contained Murashige and Skoog (1962) (MS) inorganic salts and B5 vitamins, 3 % sucrose, pH adjusted to 5.6, solidified with 0.8 % (w/v) Bacto agar unless otherwise mentioned, and autoclaved for 20 min.

Preliminary experiments were performed to evaluate regeneration capacity of three explant types (embryo axis, stem segments, and leaf explants) for each of the four cultivars (Red Hawk, Matterhorn, Merlot, and Zorro) on the selected media listed in Table 1. Fifteen explants were placed on each medium in Petri dishes (100 × 15 mm) with 3 replications. They were cultured at 25 °C under a 16-h photoperiod of 30 μmol m^{-2} s^{-1} for 4 weeks. Subcultures to fresh media were carried out at 4-week intervals. The explants with multiple shoot/bud formation were documented after 8 weeks. Regeneration capability of the calluses induced in some treatments were evaluated on both MS and elongation medium [herein EM: MS containing 1.45 μM gibberellic acid (GA_3)] after 4 more weeks of culture. For embryo axis explants, regeneration refers multiple shoot/bud formation from the areas adjacent to apical shoots and auxiliary buds.

Two more experiment replications were conducted on only four selected media, including DM4 (MS + 44.4 μM BAP + 2.27 μM TDZ), DM11 (MS + 4.94 μM 2ip +2.27 μM TDZ), DM42 (MS + 22 μM BAP), and DM43 (MS + 44.4 μM BAP) using embryo axis explants from 'Merlot'. The regeneration frequency was calculated as the number of regenerated explants/total number of explants × 100. The number of shoots/buds per explant was counted.

Transient transformation experiments

A. tumefaciens strains GV3101 (Koncz and Schell 1986), LBA4404 (Hoekema et al. 1983), and EHA105 (Hood et al. 1993), each harboring the pBISN1 plasmid, were tested for their capacity to infect common bean. The pBISN1 binary vector is a derivative of pBI101. It contains the neomycin phosphotransferase gene (*npt*II) driven by the *nos* promoter and an intron interrupted *β*-glucuronidase gene (*gus*A), which is controlled by the chimeric super promoter $(Aocs)_3$AmasPmas (Ni et al. 1995). Single colonies of each strain were cultured in 10 mL liquid yeast extraction broth (YEB) (Vervliet et al. 1975) containing 100 mg L^{-1} kanamycin monosulfate (Km) at 28 °C with constant shaking for 48 h. Then, 30 ml of the culture were inoculated into 15 mL of the same medium and grown to an OD_{600} of 0.8–1.0. Before transformation, the culture was centrifuged at 2,500*g* for 1 min. The bacterial pellets were resuspended to an OD_{600} of 0.5 in liquid callus-inducing medium (CIM) [MS + 3 % sucrose + 0.45 μM thidiazuron (TDZ) + 0.25 μM indole-3-acetic acid (IAA) + 100 μM acetosyringone (AS), pH 5.6]. Explants of four cultivars (Red Hawk, Matter Horn, Merlot, and Zorro) were incubated in the bacterial suspension for 30 min at room temperature, blotted dry on sterile filter paper, and placed on two layers of sterile filter paper saturated with liquid CIM + 100 μM AS in Petri dishes. Co-cultivation was carried out for 8 days at 25 °C in the dark. Explants were then washed in liquid CIM containing 500 mg L^{-1} timentin (Tn) for 10 min, rinsed three times in sterile water, and blotted dry on sterile filter paper.

Inoculated explants of four cultivars were either immediately assayed for the frequency of transient GUS expression or transferred to selection CIM containing 50 mg L^{-1} Km, 500 mg L^{-1} Tn, and solidified with 0.8 % (w/v) bacto agar for callus induction at 25 °C in the dark. After 2 weeks, a histochemical GUS assay was performed on the entire explants and the number of explants containing GUS-positive calluses was recorded. Stem, leaf, and embryo axis explants were tested in this manner with 10 explants per dish for callus induction.

To study the effect of co-cultivation period on bean transformation, leaf explants from 'Red Hawk' were used. After infection as described above, explants were co-cultivated on sterile filter paper saturated with liquid CIM + 100 μM AS in Petri dishes at 25 °C in the dark. Ten leaf explants were incubated in each dish and were assayed for GUS expression on days 2, 3, 4, 5, 6, 7, and 8 post-infection.

To determine the susceptibility of different genotypes and explants of common bean to *A. tumefaciens*, three different explants, including leaves and stems from 1-week-old seedlings and embryo axes, were co-cultivated with *Agrobacterium* strain GV3101 for 8 days.

In addition to the infection of explants by incubation in *Agrobacterium* solution, agro-infiltration was carried out. The *Agrobacterium* strain GV3101 harboring the pBISN1 plasmid was prepared as described above and used to infect embryo axes excised from 'Merlot' embryos grown on half-strength MS for 2 days. Explants were immersed in 15 mL of *Agrobacterium* solution in 50 mL Corning tubes (Denville Scientific, NJ, USA), which were placed in a

Table 1 Effect of plant growth regulators on shoot and bud production from embryo axes of four common bean cultivars

Code	Plant growth regulators and MES (μM)									Regeneration frequency (%) of different cultivars				Number of shoots per explant for each cultivar			
	BAP	2ip	Kinetin	TDZ	4-FA	NAA	2,4-D	IAA	MES	'Matter horn'	'Merlot'	'Red Hawk'	'Zorro'	'Matter horn'	'Merlot'	'Red Hawk'	'Zorro'
DM41	88.8									60.0ab	66.7bc	40.0bcd	33.3bcd	3.47ab	4.87bc	1.67cd	1.00bcde
DM4	44.4			2.27						73.3a	93.3a[a]	73.3a	53.3ab	5.93a	10.84a[a]	3.40a	1.80ab
DM43	44.4									80.0a	88.9a[a]	73.3a	66.7a	3.20bc	5.62b[a]	3.87a	2.33a
DM42	22.2									73.3a	86.7ab[a]	60.0ab	46.7abc	4.20b	4.51bc[a]	1.67cd	1.07bcde
DM18	8.88									46.7bc	66.7bc	40.0bcd	46.7abc	1.47def	2.53ef	1.27cdef	1.73abc
DM9	8.88							2.86		66.7ab	46.7cd	46.7bc	33.3bcd	2.53cd	1.80fgh	1.87bc	1.13bcd
DM6	4.44									26.7cde	26.7defg	20.0def	26.7bcde	0.93efgh	1.00ghi	0.60efgh	0.93cdef
DM1	4.44	4.92								46.7bc	40.0de	40.0bcd	26.7bcde	1.47def	1.33fghi	1.27cdef	0.80defg
DM5	4.44					2.69				13.3def	20.0efgh	6.7ef	20.0cde	0.33fgh	0.47i	0.20gh	0.53defg
DM83	4.44		4.65							33.3cd	40.0de	20.0def	13.3de	0.73efgh	0.93ghi	0.53fgh	0.33defg
DM21				9.08				0.57		33.3cd	46.7cd	20.0def	26.7bcde	1.47def	3.27de	0.67efgh	1.00bcde
DM20				9.08						26.7cde	33.3def	26.7cde	20.0cde	0.73efgh	1.20ghi	0.93defg	0.67defg
DM22				4.54				0.57		33.3cd	40.0de	13.3ef	33.3bcd	1.67ed	2.13efg	0.47gh	1.13bcd
DM2				4.54						20.0def	26.7defg	20.0def	6.7de	0.67efgh	0.73hi	0.53 fgh	0.13fg
DM11		4.92		2.27						60.0ab	84.4ab[a]	46.7bc	26.7bcde	1.73ed	3.75cd[a]	1.33cde	0.73defg
DM24				1.36				0.86		26.7cde	20.0efgh	13.3ef	6.7de	1.27efg	0.67hi	0.53fgh	0.27efg
DM23				1.36						6.7ef	13.3fgh	0.0f	0.0e	0.20gh	0.60 hi	0.00h	0.00g
DM10		9.84								33.3cd	46.7cd	53.3ab	33.3bcd	0.87efgh	1.87fgh	2.53b	1.13bcd
DM13		9.84						2.86		33.3cd	40.0de	20.0def	26.7bcde	0.87efgh	1.13ghi	0.47gh	0.73defg
DM85			4.65					2.86		20.0def	26.7defg	13.3ef	13.3de	0.67efgh	1.00ghi	0.53fgh	0.60defg
DM82			4.65			2.69				6.7ef	6.7gh	0.0f	13.3de	0.20gh	0.27i	0.00h	0.53defg
DM84		4.92	4.65							6.7ef	13.3fgh	6.7ef	13.3de	0.20gh	0.40i	0.20gh	0.33defg
DM12		4.92						2.86		0.0f	6.7gh	0.0f	0.0e	0.00h	0.20i	0.00h	0.00g
DM80			2.79			2.69				6.7ef	0.0h	0.0f	0.0e	0.13gh	0.00i	0.00h	0.00g
DM15					5.36				3.02	0.0f	0.0h	0.0f	0.0e	0.00h	0.00i	0.00h	0.00g
DM16					10.72				3.02	0.0f	0.0h	0.0f	0.0e	0.00h	0.00i	0.00h	0.00g
DM17							9.04			0.0f	0.0h	0.0	0.0e	0.00h	0.00i	0.00h	0.00g
DM19							4.52			0.0f	0.0h	0.0	0.0e	0.00h	0.00i	0.00h	0.00g
DM7							9.04		3.02	0.0f	0.0h	0.0	0.0e	0.00h	0.00i	0.00h	0.00g
DM81			4.65							0.0f	0.0h	0.0	0.0e	0.00h	0.00i	0.00h	0.00g

Different letters in a column indicate a significant difference at $p \leq 0.05$ with Duncan's multiple range test

BAP 6-benzyl-aminopurine; *2ip* 6-(γ,γ-dimethylallylamino)purine, *TDZ* thidiazuron, *4-FA* 4-fluorophenoxyacetic acid, *NAA* α-naphthaleneacetic acid, *2,4-D*: 2,4-dichlorophenoxyacetic acid, *IAA* indole-3-acetic acid, *MES* 2-(*n*-morpholino)-ethanesulfonic acid

[a] The results of three experiments each with three replicates

vacuum chamber at 91 kPa for 3 min. Explants were then blotted dry on sterile filter paper for 5 min, and cultured at 25 °C in the dark on filter paper soaked with liquid medium (QL medium + 44.4 μM BAP + 100 μM AS). After 2 weeks, a histochemical GUS assay was performed on explants from each experiment.

Stable transformation experiments

'Merlot' was used for stable transformation. Whole embryos from mature seeds with seed coat and cotyledons removed after surface sterilization were used as initial explants. Five explants were transferred to each 60 × 15 mm petri dish containing about 10 ml DM4. They were cultured at 25 °C for 3 days under a 16-h photoperiod of 30 μmol m^{-2} s^{-1}. Sterile explants were used for transformation studies.

Preparation of GV3101:pBISN1 culture was performed as described above. The bacterial pellet was suspended to an OD_{600} of 0.5 in liquid CIM containing 100 μM AS. Sterile explants were immersed in *Agrobacterium* suspension and vacuumed at 91 kPa for 3 min. The explants were then blotted dry on sterile filter paper, transferred on filter paper overlaid on solidified CIM, and cultured at 25 °C in the dark for 8 days. After co-cultivation, the root parts and leaves of the co-cultivated explants were removed; the resulting axis parts were washed in liquid CIM three times (2 min/time) followed by one more wash in CIM supplemented with 500 mg L^{-1} Tn. The axis parts were dried on sterile filter paper and subsequently cultured on selection media containing 50 mg L^{-1} Km and 500 mg L^{-1} Tn. Subcultures of the explants to fresh selection media were performed at 3-week intervals. For the first two subcultures, all emerged shoots were removed and subjected to histochemical GUS assays. After three subcultures, regenerants were transferred to selection EM containing 50 mg L^{-1} Km, and 500 mg L^{-1} Tn. The entire selection and regeneration process was carried out at 25 °C under a 16 h photoperiod of 30 μmol m^{-2} s^{-1}. Two selection media, including DM4 and DM19 [MS + 4.52 μM dichlorophenoxyacetic acid (2,4-D)], were tested in six transformations. For each medium, 100–150 explants were used for each transformation and each experiment was repeated three times. Regeneration capability of the Km-resistant calluses induced on DM19 was evaluated on DM4 and EM, respectively. The number of explants producing either Km-resistant calluses or shoots was recorded after 16-week selection. Histochemical GUS assays were performed on randomly selected Km-resistant transformants.

Histochemical GUS assay

The histochemical assay of GUS activity was carried out following Jefferson et al. (1987). Explants were incubated overnight at 37 °C in 100 mM sodium phosphate buffer (pH 7.0) containing 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 10 mM Na_2EDTA, 0.5 % (v/v) Triton X-100, and 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc) at 0.5 mg L^{-1}. Following overnight incubation, chlorophyll was removed from the tissues using 70 % ethanol rinses. Transient *gus*A expression was measured by counting the number of explants and calluses with at least one blue focus. GUS assays were replicated three times with 10 or 12 explants per treatment. The frequency of transient GUS expression was the number of explants with at least one blue focus compared to the total number of explants, expressed as a percentage.

Statistical analyses

All experiments were arranged in completely randomized designs. Data were analyzed using PROC GLM or ANOVA of SAS 9.2 (SAS institute, Cary, NC, USA). Means were separated by the Duncan's multiple range test at $p \leq 0.05$.

Results

Optimization of shoot regeneration systems

Our regeneration results provide one more piece of evidence that common bean cultivars are recalcitrant for regeneration from meristem-free tissues. For stem sections and leaf explants, none of the 30 media tested enabled plant regeneration from any of the four cultivars used. The DM19 induced friable calluses from stem and embryo axis explants, but the calluses could not further develop into somatic embryos or plants after they were transferred on either PGR-free MS or DM43 for shoot induction from embryo axis explants.

For embryo axis explants, 20 out of 30 media enabled multiple shoot/bud production for each cultivar (Table 1). Both genotype and culture medium had a significant impact on regeneration frequency as well as the mean number of shoots/buds per explant. 'Merlot' and 'Matterhorn' were more amenable to shoot production than 'Red Hawk' and 'Zorro' (Fig. 1a–d). A high level of BAP (44.4 μM), either alone (DM43) or combined with 2.27 μM TDZ (DM4), resulted in the best shoot and bud production for each cultivar. When TDZ was included, it inhibited shoot elongation but promoted more bud production (Fig. 1e, f). Under the optimal conditions, multiple shoots and buds, an average of 2.3–10.8 for each embryogenic explant, were induced from embryo axis explants at frequencies of

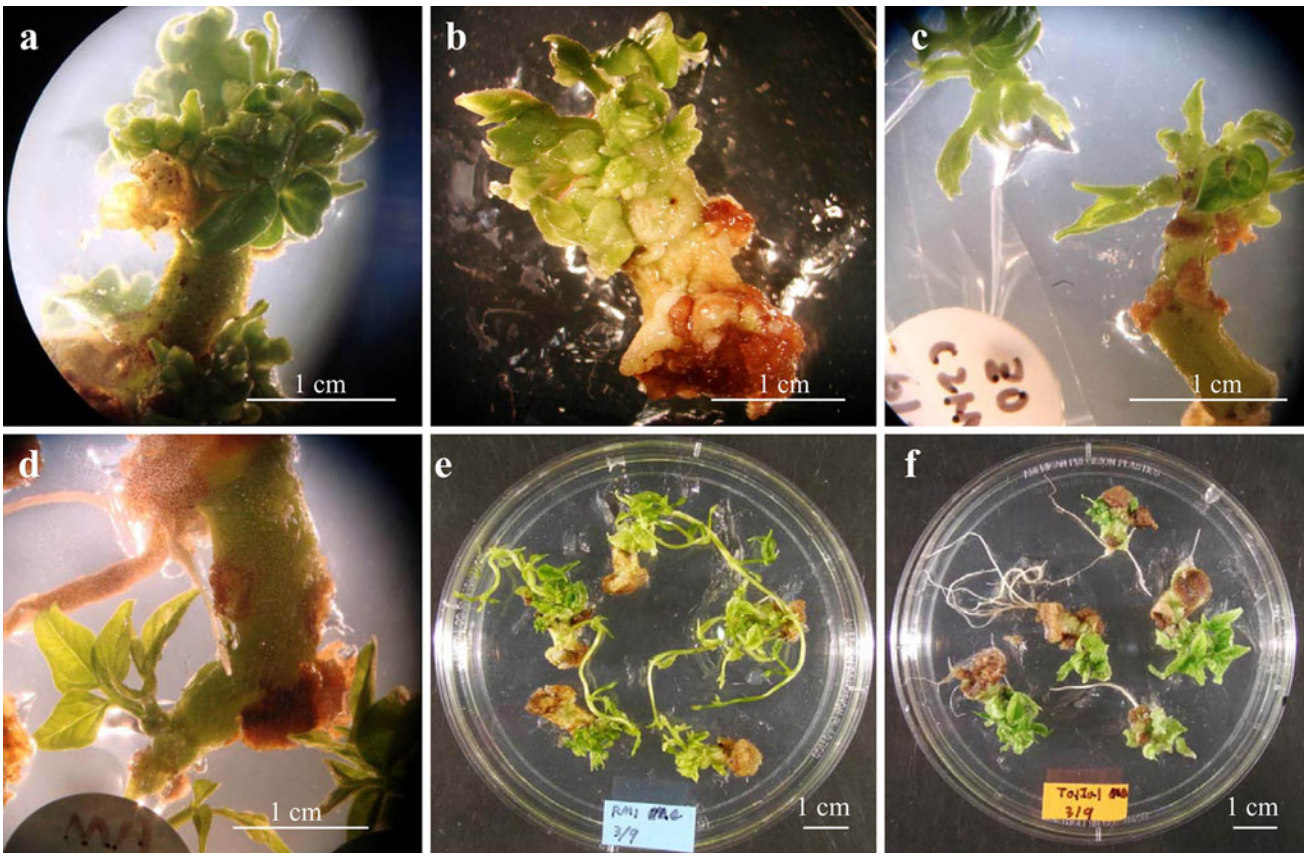

Fig. 1 Shoot/bud production patterns of embryo axes of four common bean cultivars after 8 weeks of culture. **a** Merlot on DM4; **b** Red Hawk on DM43; **c** Zorro on DM 43; **d** Matterhorn on DM43; **e** Merlot on DM43; **f** Merlot on DM4

Table 2 Effect of co-cultivation period on GUS expression in leaf explants of 'Red Hawk'

Strains	Percentage of GUS-positive explants after different co-cultivation time						
	2 days	3 days	4 days	5 days	6 days	7 days	8 days
GV3101	3.8	18.9	25.3	56.4	83.3	88.8	90.2
LBA4404	1.0	7.9	11.8	37.9	62.5	57.1	60.0
EHA105	3.3	12.8	19.9	49.5	70.9	75.3	79.4
Mean ± SD	2.7 ± 0.9d	13.2 ± 3.2c	19.0 ± 3.9c	47.9 ± 5.4b	72.3 ± 6.1a	73.7 ± 9.2a	76.5 ± 8.8a

Means followed by the same letter are not significantly different ($\alpha = 0.05$)

93.3 % for 'Merlot', 80.0 % for 'Matterhorn', 73.3 % for 'Red Hawk', and 66.7 % for 'Zorro' (Table 1).

Influence of co-cultivation period on transient GUS expression

The influence of co-cultivation period on transient GUS expression was determined on 'Red Hawk' leaf explants (Table 2). Almost no visible GUS expression was observed following 2 days of co-cultivation with all three strains. The frequency of transient GUS expression increased with increasing co-cultivation time to a maximum mean of 76.5 % for all *Agrobacterium* strains after 8 days.

Influence of *A. tumefaciens* strains, explant type, and genotype on GUS expression

Among the strains tested, GV3101 induced the highest level of GUS expression in all cultivars, followed by EAH105 and LBA4404 after a co-cultivation period of 8 days (data not shown)

Of the three explant types tested, the frequency of transient GUS expression was highest in leaf and embryo axis explants. Stem explants exhibited a significantly lower level of GUS expression. The average transient GUS expression frequencies for leaf, stem, and embryo axis explants were 92.5, 21.0, and 89.1 %, respectively

Table 3 Effect of explant type on GUS expression assayed after 8 days of co-cultivation with GV3101 and again on calluses formed after co-cultivation plus 2 weeks on callus inducing medium

Cultivars	Percent of GUS expression					
	Explants (8 days of co-cultivation)			Calluses (2 weeks post co-cultivation)		
	Leaf	Stem	Embryo axis	Leaf	Stem	Embryo axis
Red Hawk	93.8	19.8	91.4	4.7	1.3	4.5
Zorro	92.9	22.1	90.6	4.4	1.5	4.2
Matterhorn	91.6	24.2	82.1	3.6	1.2	3.7
Merlot	91.9	17.9	92.4	4.5	1.4	4.4
Mean ± SD	92.5 ± 0.5a	21.0 ± 1.4b	89.1 ± 2.4a	4.3 ± 0.5a	1.4 ± 0.1b	4.2 ± 0.2a

Means followed by the same letter (within explant type and within calluses) are not significantly different ($\alpha = 0.05$)

Fig. 2 Effect of agroinfiltration on bean transformation after 2 weeks of co-cultivation. **a** Embryo axes transformed by incubation in *Agrobacterium* solution for 30 min; **b** embryo axes transformed by infiltration

(Table 3). When callus was induced in each explant type under selection conditions of 50 mg L^{-1} of Km, the frequency of calluses expressing GUS decreased considerably for all explants (Table 3).

Effect of agroinfiltration on transient GUS expression

Infiltration increased transgene delivery and resulted in 100 % of embryo axes expressing GUS compared to 55 % obtained by regular *Agrobacterium* incubation. In addition, more blue spots with intense blue color were observed in the infiltrated explants (Fig. 2).

Stable transformation

Agroinfiltration followed by 8 days co-cultivation did not lead to necrosis of the explants (Fig. 3a). After 16 weeks of selection on DM19, 23.6 % (150/635) of embryogenic axes produced Km-resistant calluses, of which 65 % of callus clusters tested were GUS-positive (Table 4). The calluses showed some embryogenic characteristics, but none of the calluses further developed into plants when they were transferred onto either DM4 or EM. On the selection DM4, Km-resistant shoots or buds were observed in 33 % (289/876) of explants, of which 22 % of explants tested had GUS positive shoots or buds (Fig. 3b; Table 4). After transfer to selection EM, 2.8 % (5/174) of the explants, which had Km-resistant shoots or buds, developed into plantlets after 6 weeks (Fig. 3c). Unfortunately, these plantlets stopped growing and subsequently did not develop into normal plants after they were transplanted into soil.

Using histochemical GUS assays, blue staining was observed in some Km-resistant calluses, shoots, or buds, but was absent in nontransformed tissues (Fig. 3d–f). For some Km-resistant callus clusters, co-existence of blue and white cells was observed (Fig. 3d). Similarly, unevenly distributed blue staining was observed in leaf and root tissues from Km-resistant transformants obtained after 16 weeks selection (Fig. 3f). These results indicate the expression of the *gus*A reporter in transgenic tissues. The variations in blue staining might be due to the uneven penetration of X-gluc or chimeric tissues. In addition, all the early induced shoots/buds obtained within 6 weeks of selection were not transgenic based on GUS staining, because they either were GUS-negative or had only a few

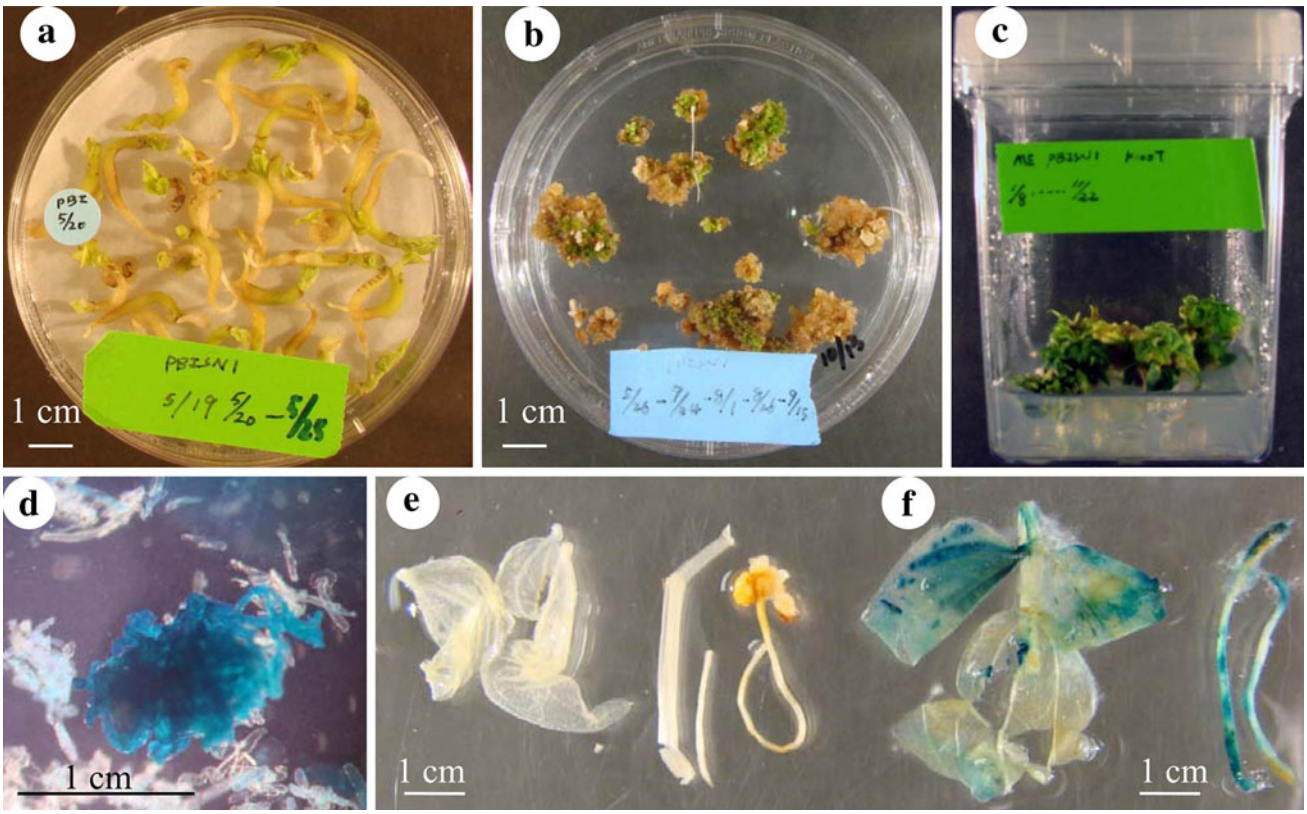

Fig. 3 Transformation of common bean cultivar Merlot using embryo axes as explants. **a** Explants after 8 days of co-cultivation; **b** Selection of Km-resistant shoots on selection DM4; **c** Growth of Km-resistant shoots and buds on selection EM; **d** GUS staining in the calluses induced on selection DM19; **e** GUS staining in non-transformed tissues; **f** GUS staining in tissues of Km-resistant transformants

Table 4 Transformation of embryo axes of common bean cultivar Merlot

Selection medium	Experiment	Total number of explants	Number of explants producing Km-resistant shoots	Number of explants producing Km-resistant calluses	Number of explants producing GUS-positive calluses (number of Km-resistant calluses assayed)	Number of explants producing GUS-positive shoots (number of Km-resistant shoots assayed)	Transformation frequency (%)[a]
DM19	Exp 1	110	0	25	8 (10)	NA	18.2
	Exp 2	100	0	22	7 (10)	NA	15.4
	Exp 3	105	0	32	5 (10)	NA	15.2
	Exp 4	100	0	29	6 (10)	NA	17.4
	Exp 5	100	0	21	7 (10)	NA	14.7
	Exp 6	120	0	21	6 (10)	NA	10.5
	Total	635	0	150			15.2 (average)
DM4	Exp 1	150	43	NA	NA	3 (21)	4.1
	Exp 2	145	55	NA	NA	4 (26)	5.8
	Exp 3	133	38	NA	NA	5 (20)	7.1
	Exp 4	150	48	NA	NA	8 (30)	8.5
	Exp 5	150	69	NA	NA	NA	NA
	Exp 6	148	36	NA	NA	5 (18)	6.8
	Total	876	289				6.5 (average)

NA not analyzed

[a] Transformation frequency (%) $= \left[\frac{\text{Number of explants producing GUS positive tissues}}{\text{Number of Km resistant tissues assayed}}\right] \times \left[\frac{\text{Total number of explants producing Km resistant tissues}}{\text{Total number of explants}}\right] \times 100$

blue spots, which were similar to the pattern of transient GUS expression.

Discussion

Our attempt to induce plant regeneration from non-meristem containing tissues, such as leaf explants and stem sections of common bean, did not lead to any regeneration. The results are consistent with most of the previous reports, in which embryo-axes of common bean were amenable for multiple shoot and bud production (Zambre et al. 1998; Delgado-Sánchez et al. 2006; Arellano et al. 2009; Kwapata et al. 2010; Quintero-Jimenez et al. 2010). However, 'shoot proliferation' instead of 'regeneration' is a more accurate term for this type of shoot production, since the shoots or buds appeared only in the adjacent areas of apical meristems or auxiliary buds (Fig. 1a–d). It is not very clear whether the newly formed shoots are derived from a group of predetermined regenerable cells or from single cells. A single cell-derived plant regeneration system is desirable for genetic transformation, since it can minimize the production of chimeric transformants. However, such a single cell-derived regeneration system from mersitem-free tissues is still lacking for common bean cultivars.

While friable calluses showing some embryogenic characteristics were induced by using 2,4-D (Fig. 3d), we have not found a method that enables the conversion of these calluses to somatic embryos. However, this may indicate that it could be possible to attain common bean regeneration through somatic embryogenesis.

There is no question that common bean regeneration depends on many factors, such as genotype, explant type, and medium formula (Malik and Saxena 1991; Delgado-Sánchez et al. 2006; Dang and Wei 2009; Gatica Arias et al. 2010; Kwapata et al. 2010; Quintero-Jimenez et al. 2010). Most of the previous studies focused on investigating regeneration capacity of different genotypes and explants as well as different plant growth regulators (PGRs). Few studies have been undertaken to evaluate the impact of other factors, such as basal salts, vitamins, and carbon sources, on common bean regeneration. Quintero-Jimenez et al. (2010) reported that Gamborg's (1968) B5 medium resulted in a higher regeneration frequency than MS medium. More recently, we evaluated six basal media on regeneration of 'Merlot'. Our preliminary data showed that two basal media, Lloyd and McCown's (1980) woody plant medium (WPM) and Quorin and Lepoivre medium (QL) (Quoirin and Lepoivre (1977), showed potential for further improvement of shoot production from embryo axes (data not shown).

Common bean is susceptible to *Agrobacterium* spp. (Mariotti et al. 1989; McClean et al. 1991; Lewis and Bliss 1994; Brasiliero et al. 1996). Various factors influencing transient and stable transformation of common bean have been investigated in this study. A co-cultivation period of 2–3 days is generally considered to be suitable for *Agrobacterium*-mediated transformation in many other plant species (Hiei et al. 1994; Li et al. 1996; Cheng et al. 1997; Uranbey et al. 2005). In this study, we found that 8-day co-cultivation yielded the best transient GUS expression and did not cause necrosis of the embryo axes in the bean cultivars tested. This result is similar to some previous reports (Zhang et al. 1997; Zambre et al. 2005). Our data indicate that it is possible to improve *Agrobacterium*-mediated gene delivery by extending the co-cultivation time, especially when embryo axes are used as explants.

The virulence of *Agrobacterium* strains varies widely among host plant species depending on the interaction between the *Agrobacterium* strain and host plant (Davis et al. 1991; Zhang et al. 1997). In this study, the *Agrobacterium* strain GV3101 yielded stronger intensity of GUS staining and more GUS foci per explant than other strains under the same co-cultivation conditions for the four cultivars. This is comparable to the results obtained in other legume crops such as soybean (Paz et al. 2006). These results indicate that common beans are probably more susceptible to this nopaline type of *Agrobacterium* strain and highlight the importance of using suitable virulent strains in bean transformation.

Explant tissues are important for both regeneration and *Agrobacterium* infection. Although leaf explants of common bean are not yet regenerable, they showed the highest susceptibility to *A. tumafaciens* in all genotypes tested (Table 3). Prior studies in other legume crops, such as lentil (*Lens culinaris* M.; Mahmoudian et al. 2002), showed that agroinfiltration resulted in higher transient GUS expression than regular inoculation. More importantly, agroinfiltration did not cause overgrowth of *Agrobacterium* cells during co-cultivation (Fig. 3a). Washing of the agroinfiltrated explants following co-cultivation is necessary in order to keep *Agrobacterium* growth well controlled during the selection stage.

To date, embryo axes of common bean are still the optimal explants that enable *Agrobacterium*-mediated gene transformation and subsequent shoot production. Despite the high 'regeneration' frequency of the embryo axis explants (Table 1), stable transformation of common bean is still inefficient. The main reason is that the embryo axis-based regeneration system is not desirable for genetic transformation. In this study, although 6.5 % of explants had GUS-positive shoots or buds after 16 weeks selection, we could not exclude the possibility that some of these were chimeric transformants. Since early formed shoots during the first 6 weeks of selection were putative non-transgenics, removal of these shoots could promote the

development of transformed cells and increase the chance of obtaining common bean transformants. Alternatively, since effective selection is critically important for stable transformation when meristem-containing tissues are the only regenerable explants available, the bialaphos resistance (*bar*) gene and hygromycin phosphotransferase (*hpt*) gene could be more effective selectable markers. This is further supported by recent results in our lab that non-transformed embryo axes of common bean were extremely sensitive to glufosinate ammonium and hygromycin B (data not shown).

Conclusions

To optimize the regeneration system for common bean cultivars, regeneration capacities of leaf explants, stem sections, and embryo axes were evaluated on 30 media containing different PGRs. Although none of the media enabled plant regeneration from leaf explants or stem sections, several media enabled multiple shoot production from embryo axes for each genotype. Under optimal regeneration conditions, *A. tumefaciens*-mediated gene delivery parameters, including strain of *A. tumefaciens*, co-cultivation time, explant type, and bean genotype, were optimized. Both agroinfiltration and an 8-day co-cultivation period enhanced gene delivery. For stable transformation, GUS-positive transformants were obtained after 16 weeks selection. Removal of early formed shoots during the first 6 weeks of selection could increase the chance of obtaining transformants. In order to develop an efficient transformation protocol for common bean, more efforts are still needed to develop an efficient regeneration system using nonmeristem-containing tissues as explants.

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