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Collado, Raúl; Bermudez-Caraballoso, Idalmis; Garcia, Lourdes; Veitía, Novisel; Torres, Damaris; Romero, Carlos; Angenon, Geert

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Epicotyl sections as targets for plant regeneration and transient transformation of common bean using *Agrobacterium tumefaciens*

R. Collado • I. Bermúdez-Caraballoso • L.R. García • N. Veitía • D. Torres • C. Romero • G. Angenon

R. Collado • I. Bermúdez-Caraballoso • L.R. García • N. Veitía • D. Torres • C. Romero
Instituto de Biotecnología de las Plantas, Universidad Central "Marta Abreu" de Las Villas. Carretera a Camajuaní km 5.5, Santa Clara, Villa Clara. Cuba. PC-54 830.

R. Collado • G. Angenon
Laboratory of Plant Genetics, Vrije Universiteit Brussel (VUB), Pleinlaan 2, B-1050 Brussels, Belgium.
Email: raulc@ibp.co.cu

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Abstract Common bean is recalcitrant to genetic transformation, due to limited regeneration capacity and low DNA transfer rates. The effect of different parameters on T-DNA transfer from *Agrobacterium tumefaciens*, was studied by measuring transient expression of the *β-glucuronidase* gene in *Phaseolus vulgaris* cv. CIAP7247F. Epicotyl containing seedling explants were inoculated with Agrobacterium EHA101 and C58C1Rif^R(pMP90) strains harboring the binary vector pTJK136 with *GusA* gene on the T-DNA. Parameters studied were temperature and light regime during co-cultivation, explant injury and the acetosyringone concentration for vir gene induction. The co-cultivation temperature and photoperiod had a significant effect on Agrobacterium DNA transfer. In addition, explant injury and supplementation of the co-cultivation medium with acetosyringone increased the GUS activity. Optimal T-DNA transfer was obtained under the following conditions: co-cultivation at 25°C in darkness, injuring the explants with carborundum, and supplementation of the co-cultivation medium with 200 µM acetosyringone. This T-DNA delivery system was combined with a direct organogenesis protocol using epicotyl explants and fertile regenerants were recovered from tissue transformed with Agrobacterium. However, no transmission of transgenes to progeny could be observed, suggesting that the obtained plants were chimeras.

Key words *Agrobacterium tumefaciens* • Genetic transformation • GUS activity • *Phaseolus vulgaris* • Epicotyl section

Introduction

The common bean, *Phaseolus vulgaris*, is an important dietary component especially in developing countries. This species is recalcitrant to genetic transformation. Somatic embryogenesis and shoot regeneration from callus is difficult to obtain in common bean and more genotype dependent than direct organogenesis. There are several reports describing regeneration from embryonic axes for common bean (Veltcheva et al., 2005; Arellano et al., 2009; Gatica Arias et al., 2010; Kwapata et al., 2012; Quintero-Jiménez et al., 2010). Despite the available information on *P. vulgaris in vitro* regeneration, none of the published protocols has been successfully used for common bean genetic transformation. The main disadvantage of these direct organogenesis systems is that the obtained shoots are of multicellular origin, which may prevent strict selection for transgenic shoots during the transformation procedure and may lead to high numbers of "escapes" (non-transgenic plants that survive selection) and chimeras (Angenon and Thu, 2011). Due to difficulties in identifying a

regeneration system compatible with transformation in common bean, an Agrobacterium-mediated transformation procedure has been developed that does not include *in vitro* culture (Liu et al., 2005). However, no further studies using this method have been reported.

Direct transformation of *P. vulgaris* has also been developed through particle bombardment to the apical meristem zone of seedlings using a gene gun (Aragão et al., 1996; Rech et al., 2008). Although transgenic plants have been obtained using above-mentioned protocols, these plants showed lower stable inheritance and the technique is expensive when compared to the Agrobacterium process. Agrobacterium remains the method of choice for insertion of transgenes into higher plants. It offers many advantages including integration of an accurate DNA sequence, usually low copy number, incorporation of the introduced genes into active sections of the genome and consistent gene expression over the generations (Opabode, 2006).

Optimisation of factors influencing Agrobacterium-mediated transformation using several genotypes of common bean was studied by Zhang et al. (1997). When explants derived from mature seeds of susceptible genotypes were injured, pre-cultured and then transformed with Agrobacterium strain A2760, a transformation efficiency of 4% was achieved as proven by GUS staining. However stable transformants were not obtained.

Mohamed et al. (2006) attempted to transfer a synthetic *Bacillus thuringiensis* (Bt) *crylC* gene (controlling Lepidopteran insect pests) to common bean 'Xan-159' via Agrobacterium-mediated transformation of cotyledonary explants. Hygromycin resistance was used as the selective marker. Although the shoots remained green for more than 2 months on medium with hygromycin, these shoots failed to elongate and establish plants. Amugune et al. (2011) also failed common bean transformation employing Agrobacterium, only shoots and roots with non-continuous histochemical GUS staining were observed.

Recently, factors determining Agrobacterium-mediated gene delivery, including strain of *Agrobacterium*, co-cultivation time, explant type, and bean genotype, were studied (Mukeshimana et al., 2013). Despite the high 'regeneration' frequency of the embryo axis explants obtained by Mukeshimana et al. (2013), stable transformation of common bean was inefficient. The authors concluded that the embryo axis-based regeneration system is not desirable for genetic transformation. Mukeshimana et al. (2013) attempted to recover transgenic plants from non-meristem containing tissues, but leaf explants and stem sections of common bean did not lead to any regeneration.

In this research, we report the regeneration of multiple buds from epicotyl sections and the application of epicotyl containing seedling explants for Agrobacterium-mediated transformation of common bean.

Materials and methods

Regeneration Seeds of P. vulgaris L. cv. CIAP7247F, a high yield cultivar widely used in Cuba, were disinfected and sowed *in vitro* (Fig. *1a*) on germination medium (*GM*, Table 1) as described in Collado et al. (2013).

Three-day-old seedlings without cotyledons, were placed in pre-culture medium (*PM*, Table 1) according to the methodology proposed by Varisai Mohamed et al. (2006). Ten seedlings were placed in Erlenmyer flasks containing 100 mL of this culture medium (Fig. *1b*) and placed in the dark for 5 d at a temperature of 26±2°C on an orbital shaker (INFORS HT) at 90 rpm.

Once the pre-culture period ended, a cut was made under the apex and the apex with the first pair of leaves was removed from the embryo axis (Fig. *1c)*. Another cut was made above the cotyledonary node to eliminate the cotyledonary node, the hypocotyl and roots (Fig. *1c*). The section of epicotyl (4–6 mm) located between the cotyledonary node and the apex (Fig. *1c*) was used as explant for multiple bud induction (Fig. *1d*). The epicotyl sections were placed on multiple bud induction medium (*MBIM*, Table 1) for 21 d.

To stimulate the differentiation of buds into shoots, multiple bud producing explants were placed on shoot induction medium (*SIM*, Table 1). Six explants were placed per culture vessel and the plant material was kept under conditions described above in the multiple bud induction. The subcultures were performed every 15 d for 60 d of cultivation. Differentiated shoots (Fig. *1e*) were separated and transferred to shoot elongation medium (*SEM*, Table 1). During elongation the shoots were subcultured every 15 d for 60 d. Elongated shoots (Fig. *1f*) were transferred to rooting medium (*RM*, Table 1) for 30 d. Rooted plantlets (Fig. *1g*) were placed in polystyrene foam containers with substrate composed of organic material mixture with zeolite in a ratio of 80:20% (*v/v*) respectively. The plant material was irrigated five times a day for two minutes each time. Acclimatized plants (Fig. *1*h) were used to produce fertile seeds (Fig. *1i*).

This regeneration system was performed for four replicates comprising 100 seedlings as initial explants each. In this experiment the number of multiple buds, the number of shoots in shoot formation, shoot elongation or rooting phases, and the number of plants surviving in soil, as well as the number of plants that produced seeds were scored.

Genetic transformation, explant preparation. The seed coat was removed from 3-day-old seedlings using tweezers and cotyledons were gently pulled apart out from the embryonic axes. After removal of the root, the remaining part containing the cotyledonary node and the epicotyl (referred to as CNE) was used as explant for inoculation and co-cultivation steps in transformation experiments. All culture media were adjusted to the appropriate pH (Table 1) before autoclaving at 120 kg cm^{-2} for 20 min. Vitamins and antibiotics were filter sterilized and added to cooled (40-45ºC) media after autoclaving. The carborundum, needles, filter paper and flasks were also autoclaved at 120 kg cm^{-2}, but for 40 min.

Preparation of bacteria for co-cultivation. *Agrobacterium tumefaciens* strains EHA101 and C58C1Rif^R(pMP90), both containing the plasmid pTJK136 (Kapila et al. 1997) were used to inoculate the CNE in all experiments. The T-DNA of this vector contains the gene for neomycin phosphotransferase II (*nptII*) (EC 2.7.1.95) under control of the nopaline synthase promoter (*pnos*) and the terminator and polyadenylation signals of the octopine synthase gene (*3'ocs*). This gene confers resistance to the antibiotics kanamycin, neomycin, and geneticin. Furthermore, the construct contains the β-glucuronidase gene from *Escherichia coli* (*GusA*) (Jefferson et al., 1987) interrupted by the ST-LS1 intron of potato (*Solanum tuberosum*), ensuring that this gene is expressed only in plant cells. The *GusA* gene is under the control of the Cauliflower Mosaic Virus promoter (*p35S*) and terminator and polyadenylation signals from the *Nos* gene (Fig. 2). An Agrobacterium colony isolated from a fresh plate was suspended in 3 mL YEP culture medium (An et al., 1988), supplemented with antibiotics (300 mg L^{-1} streptomycin and 100 mg L^{-1} spectinomycin), and grown at 28ºC for 24 h in shaking conditions (120 rpm) and 100 µL of this culture was added in a 250 mL Erlenmeyer with 50 mL YEP medium and the above mentioned antibiotics. The culture was grown under previous mentioned conditions for 12-14 h to reach an optical density, A_{600} between 1.2 and 1.7. Aliquots of 45 mL Agrobacterium culture were centrifuged for 10 min at 5000 rpm at 21ºC to pellet the cells. The Agrobacterium pellet was washed twice and subsequently resuspended in 15 mL of liquid bacteria induction medium (BIM) containing 50% MS salts, 3.9 g L^1 MES, 1.98 g L^{-1} glucose, 2% (*w/v*) sucrose, pH 5.5. This concentrated bacterial suspension was diluted using antibiotic-free medium (BIM) to final A_{600} of 0.5 corresponding to $1.1 \pm 0.2 \times 10^9$ cells/mL. Acetosyringone (100 µM) was added to BIM and it was used to inoculate the explants.

Influence of co-cultivation temperature on DNA transfer. Explants were pricked gently 6-10 times on their epicotyl with a sterile fine needle without breaking the embryonic axis. After wounding, explants were inoculated in the Agrobacterium suspension by shaking them for 15 min at 28ºC in dark and then transferred to the liquid co-cultivation medium containing 100% MS salts, 100% H vitamins

(Heinz and Mee, 1969), 3% (*w/v*) sucrose, 0.1 g L^{-1} myo-inositol, 1.0 mg L^{-1} thidiazuron (TDZ), supplemented with acetosyringone (50 μM) and pH 5.5. Different temperatures (22±1ºC, 25±1ºC, 28±1ºC) were imposed during co-cultivation period. Each treatment had ten flasks containing 100 mL of co-cultivation medium with 10 explants each. The experiment was repeated four times. All treatments were kept shaking at 60 rpm in dark for five d, after which all explants were assayed for Gactivity (see below).

Influence of light conditions on DNA transfer. GUS activity in CNE explants co-cultivated either under a 16 h photoperiod or 24 h darkness were analysed. To evaluate the light effect, explants inoculated with Agrobacterium, were transferred to co-cultivation medium and shaken for five d at 60 rpm in flasks (10 explants/flask); 15 flasks were used per treatment. The experiment was repeated three times. The same temperature was used for all treatments (25±1ºC). GUS assays were done as described below.

Influence of explant injury on DNA transfer. Prior to co-cultivation, two different methods were used to injure the tissue: 1) The CNE explants were pricked gently in epicotyl region 6-10 times with a sterile fine needle without breaking the embryo; and 2) The areas described before were wounded with carborundum using a wet fine brush, running it three times on tissue. Explants without injuries were included as the control.

All explants, were inoculated with Agrobacterium suspension as above. These explants were transferred to liquid co-cultivation culture medium as previously described in a 250 mL Erlenmeyer for co-cultivation. These were kept in darkness at 25°C and shaking at 60 rpm for five d. Ten explants were placed per flask and 20 flasks were used for each treatment. The experiment was repeated three times.

Influence of acetosyringone concentration on DNA transfer. Different acetosyringone concentrations (0, 50, 100 and 200 μM) were incorporated in the co-cultivation medium. The explants used were wounded with carborundum as above. Inoculation and co-cultivation of explants were done in a similar way as in the previous experiment. Ten explants were placed per flask and 15 flasks per treatment were used. The experiment was repeated twice.

GUS assays and evaluations for parameter optimization. Expression of the *GusA-intron* gene was detected by means of histochemical assays (Mendel et al., 1989). After co-cultivation, tissues were washed three times with sterilized de-ionised water, blotted dry using sterile filter paper and then incubated in staining buffer (100 mM Na phosphate, pH 7.2, 2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid cyclohexylammonium salt, 0.1% (*w/v*) β-mercapto-ethanol, 0.1% (*w/v*) Triton X-100, 5mM K-ferricyanide, 5mM K-ferrocyanide) for 24 h at 37ºC. The reaction was stopped by adding 70% (*v/v*) ethanol. Stained green plant material was cleared with 90% (*v/v*) methanol.

In all experiments, the number of explants showing blue spots in the epicotyl region was scored. The percentage of explants with blue spots was calculated by the number of explants with blue spots divided by total number of explants multiplied by 100. The number of blue spots per explant was also examined.

Data collection and statistical analysis. The experiments to optimize all parameters studied were a completely randomized design. Statistical analyses were carried out by simple means comparison using Statistical Package for the Social Sciences (SPSS) version PASW Statistic 18 (SPSS Inc., Chicago, IL, USA). The significance of differences between mean values in the experiments (temperature influence, explants injury influence and light influence) were determined by non-parametric test Mann-Whitney and Kruskal-Wallis as data showed non-homogeneous variance. The significance of mean differences in the acetosyringone experiment were determined by Bonferroni test.

Transformation conditions, selection and analysis of putative transgenic plants. CNE, injured with carborundum in the epicotyl region, were inoculated with Agrobacterium strain EHA101(pTJK136) as above. Ten inoculated CNE explants were transferred to a flask (Erlenmeyer 250 mL capacity) containing 100 mL of CCM medium described in Table 1 for co-cultivation. The cultures were kept in dark at 25ºC and shaking at 60 rpm for five d. After co-cultivation, CCM medium was removed from the flasks using a transfer pipette and the explants were rinsed three times with 150 mL of washing medium (*WM*, Table 1), a cut was made under the apex, and the apex with the first pair of leaves was removed from the embryo axis. A second cut was made above the cotyledonary node to eliminate the cotyledonary node and the hypocotyl. The section of epicotyl (4–6 mm) between cotyledonary node and apex (termed *epicotyl section* in Fig. *1c*) was used as explant for multiple bud induction.

After explant preparation, inoculated sections were briefly blotted dry on sterile filter paper. Six sections were placed in a glass culture vessel containing 30 mL of recovery medium (*ReM*, Table 1) for 28 d. The viable multiple buds were transferred to shoot induction medium plus selective agent (*SI+SA*, Table 1) for 60 d. The putative transgenic shoots selected further on shoot elongation medium plus selective agent (SE+SA) for 60 d. All the cultures were transferred to fresh medium every 15 d. The elongated shoots (>1 cm) were then rooted in rooting medium containing timentin (*RM+T*, Table 1) for 30 d. The rooted plantlets were transferred to 500 mL plastic pots containing substrate. This substrate was composed of organic material mixture with zeolite in a ratio of 80:20% (*v/v*) respectively. Putative transgenic plants (T_0) were grown in a phytotron under controlled conditions of 26±1°C, and a photoperiod of 13 h, with a light intensity 62–68 $\mu molm^{-2} s^{-1}$, and relative humidity 90 % the first wk, 80% second wk and 70–80% up to the harvest. This transformation experiment was done in four replicates with 110 initial explants each.

The number of viable multiple buds, the number of shoots surviving on geneticin-containing medium either in shoot formation or shoot elongation phases, and the number of plants surviving in soil were scored. The frequency of transformation was calculated as above (Table 6).

Observations during transformation. After co-cultivation, ten explants, randomly selected per replicate were assayed for GUS activity to confirm DNA-transfer efficiency. Histochemical analysis was also done to identify *GusA* expression in recovered multiple buds. In this case two multiple bud clusters were selected per replicate.

Analysis of putative transgenic plants. GUS activity in T_0 and T_1 plants was also assayed as described by Mendel et al. (1989).

Results

Regeneration. A procedure based on direct organogenesis described in Fig. 1 (see histological analysis in supplementary materials), was developed for regeneration of *P. vulgaris* cv. CIAP7247F. After 28 d on MBIM, 65.3% of the epicotyl sections formed multiple buds (Table 2). On SIM each explant with multiple buds produced 3–4 differentiated shoots, of which 84.9 % successfully elongated on SEM (Table 2). Of the elongated shoots 94.8% efficiently rooted on RM. After transfer to soil, 96.0 % of the plantlets survived in the greenhouse, where 99.9% of the plants produced seeds (Table 2).

Influence of co-cultivation temperature on T-DNA transfer. The difference in the percentage of explants with GUS activity after co-cultivation at 22, 25 and 28ºC was notable (Table 3). Irrespective of the Agrobacterium strain, the highest percentage of explants showing transient *GusA* expression was observed at 25ºC. Percentage of GUS expression at 28ºC was lower than at 25ºC. Low percentage of expression was also observed at 22ºC. The patches of tissue, showing GUS activity were also larger

and more intense in CNE cultivated at 25 and 28°C, whereas GUS activity in explants co-cultivated at 22°C was limited to small points (Fig. 3 *b, c, d*).

Influence of light conditions on T-DNA transfer. CNEs co-cultivated under light and dark conditions were evaluated with respect to GUS activity. No significant differences between the cultures in light and dark conditions were observed for the number of explants with blue spots. However, the average number of blue spots per explant in light and dark were statistically different (Table 4). GUS activity was three times higher in dark condition than in light photoperiod for CNEs inoculated with either Agrobacterium strains EHA101 or C58C1Rif^{R}pMP90 (Table 4).

Influence of explant injury on T-DNA transfer. In this experiment, the influence of wounding on transient GUS activity was assessed. The capability of carborundum as an alternative way to injure plant tissue for transformation, was compared to pricking with a needle. Explants from all tested treatments showed transient expression of the *GusA* transgene. Higher GUS activity was observed on explants wounded with carborundum followed, without statistical differences, by explants injured with a needle. The transient *GusA* expression was significantly less when explants were not injured (Table 5).

No significant difference was found in the percentage of GUS-positive CNE when comparing wounding with carborundum or needle. However, it was observed that the blue spots covered a larger area in CNEs wounded with carborundum than in those pricked with a needle (Fig. *3 f, g*). In explants without injuries, the GUS activity was confined to cut areas (Fig. *3e*); while at injured explants the penetration was focused mainly on injury sites (Fig. 3 *e, f, g*). Another important observation was that Agrobacterium penetrated deeply into explants treated with carborundum. Longitudinal and transversal cuts made to these explants revealed GUS activity inside the explants (Fig. 3 *h, i*).

Influence of acetosyringone concentrations on T-DNA transfer. GUS activity obtained from treatments with acetosyringone was significantly higher than treatment without it (Table 6, Fig. 3 *j-m*). The highest percentages of explants with blue spots were achieved after co-cultivation with Agrobacterium in presence of acetosyringone at 50–200 µM concentrations. Among these treatments, no significant differences appeared for CNEs inoculated with EHA101 (Table 6).

In the case of co-cultivation with C58C1Rif^R(pMP90), the percentage of explants showing GUS activity significantly differed depending on acetosyringone concentration added to the culture medium (Table 6). The increase of acetosyringone concentration in the co-cultivation culture medium provoked an increase in the number of blue spots per explant. Acetosyringone at a concentration of 200 µM gave a maximum response in the number of blue spots per explant (Table 5).

Selection and analysis of putative transgenic plants. Following co-cultivation, 80% (32/40) of the co-cultivated embryonic axes tested were GUS-positive. Blue staining covered almost the whole epicotyl region (Fig. 4 *a*), but was absent in non-transformed embryonic axes (Fig. 4 *b*). After 28 d on ReM, 32.8% of the epicotyl sections produced multiple buds (Table 7). Using GUS assays, strong blue staining was observed in some of these recovered multiple buds (Fig. 4 *c*). On the selective medium (*SI+SA,* Table 1), geneticin-resistant shoots were observed in 18.7% of the multiple buds, of which 78.3% successfully elongated. Some of these elongated shoots also formed roots on SE+SA media (Fig. 4 *d*). After transfer to soil, 77.8% of the putative transgenic plants survived under the conditions set in the phytotron, where they showed a healthy development (Fig. 4 *e*).

In three of the T_0 plants, *GusA* expression in various tissues was observed (Fig. 4 *f-n*). GUS assays revealed that just 21.4% of the plants surviving in soil were putative transgenic, the rest of the plants were escapes. Transformation frequency was low, since just three GUS-positive plants were obtained from a total 400 inoculated explants (Table 7). GUS staining in transformed plants varied among the tested tissue, expression was clearly seen in leaves; only few spots were localized in

flowers and small points in young tissues (Fig. 4 *f-n*). In tissues sampled from non-transformed plants, GUS blue spots were not observed (Fig. 4 *o-q*). Given that GUS-positive plants showed partial GUS-staining, the results suggested that these were chimeric transformants. The total progeny (41 plants) from the three T_0 GUS-positive plants were assayed. Unfortunately, these T_1 plants did not show GUS-positive tissue confirming that they came from chimeras.

Discussion

Regeneration. Currently, an efficient transformation protocol for common bean demands that a regeneration system be developed using non-meristem-containing tissues as explants (Mukeshimana et al., 2013). However, an efficient regeneration system for common bean using non-meristem-containing tissues as explants is a challenge (Mukeshimana et al., 2013). In the present study, an efficient regeneration procedure base on multiple bud formation from epicotyl sections was developed. This result, in agreement with Varisai Mohamed et al. (2006), clearly demonstrates the presence of totipotent cells capable of differentiating into adventitious buds in the section of epicotyl adjacent to the cotyledonary node or apex. Unlike the previously published regeneration protocols (Delgado-Sánchez et al., 2006; Gatica Arias et al., 2010; Kwapata et al., 2010; Quintero-Jiménez et al., 2010; Mukeshimana et al., 2013) where shoots were regenerated from embryonic axis and from calli from cotyledonary nodes and apical meristems (Arellano et al., 2009), this system is based on the production of multiple buds from non-meristematic tissue. The regeneration efficiency that we report is high compared with the results obtained previously by Delgado-Sánchez et al. (2006), Gatica Arias et al. (2010), and similar to those reported by Kwapata et al. (2012) and Quintero-Jiménez et al. (2010).

Optimisation of Agrobacterium transformation. Influence of temperature on Agrobacterium-mediated gene transfer was previously observed in *P. acutifolius* and *Nicotiana tabacum* (Dillen et al., 1997). Authors described that the optimum temperature for DNA transfer from Agrobacterium to *P. acutifolius* calli was 22ºC, based on transient expression of the *GusA* transgene. However, this does not seem to be the case for P. vulgaris CNE explants, or at least cv. CIAP7247F, as low transient expression of the *GusA* transgene was observed during co-cultivation at 22ºC.

Temperature influence on plant cell development was noticed in this study. GUS staining in CNE co-cultivated tissues at 25 and 28ºC were almost three times larger than CNE co-cultivated at 22ºC, suggesting much more active cell division in tissue co-cultivated at 25 and 28ºC than at 22ºC. The possible reason for the observed differential response of Agrobacterium at temperatures of 22 and 25ºC could be given by changes in the composition of cells wall that occur at different physiological and developmental stages. As the plant cell dedifferentiates and starts dividing, its cell wall composition changes and this may affect Agrobacterium binding. Furthermore, Villemont et al. (1997) have indicated the T-DNA only is integrated into DNA-duplicating cells, a phenomenon that depend on the phase of host cell cycle.

For *P. vulgaris* CNE explants co-cultivated with Agrobacterium EHA101 and C58C1Rif^R(pMP90) strains, transient expression also decreased when the temperature was increased from 25 to 28ºC. Expression was also markedly lower when temperature decreased from 25ºC to 22ºC. Similar effects of temperature on both transient expression, and stable transformation were observed in other laboratories using cotyledonary node and nodular calli of soybean as model system. The optimal temperature for both T-DNA delivery and stable transformation was 23-25ºC for soybean (Hoa et al., 2008).

Thermo-sensitivity of crown gall development and of Agrobacterium-mediated transient gene expression has been regarded as a consequence of the dependence of T-DNA transfer on temperature, and thus essentially a characteristic of the Agrobacteria (Riker, 1926; Fullner and Nester, 1996; Dillen et al., 1997). However, co-cultivation temperature can also affect plant development and in an indirect

manner could influence Agrobacterium attachment to host plant cells and T-DNA transfer. Furthermore, another important aspect that requires optimization for successful Agrobacterium-mediated genetic transformation is the influence of co-culture conditions on interaction of the bacterial cells with plant cells that are in a competent physiological state. Finally, in particular for *P. vulgaris*, for which Agrobacterium-mediated genetic transformation remains challenging and co-cultivation temperature is still not defined, 25ºC during Agrobacterium co-cultivation was beneficial.

The fact that GUS activity was three times higher in explants co-cultivated in the dark compared to those explants grown under light, might be connected to the induction of flagella in Agrobacterium. It has been demonstrated that light limits the expression of flagella genes in Agrobacterium and dark culture conditions increase bacteria motility, virulence and adherance to tissue (Oberpichler et al., 2008). On the other hand, Agrobacterium mediated transformation of *P. acutifolius* calli benefited from a 16 h light photoperiod, and co-cultivation in dark was deleterious (De Clercq et al., 2002; Zambre et al., 2003). Light conditions may indeed affect both bacterial virulence and plant cell competence for transformation and the net outcome may not be the same in all transformation systems. The influence of different photoperiod conditions during co-cultivation has received attention in some plant species, but for *P. vulgaris* it has not been published until now. Therefore the light experiment was conducted and it demonstrated that co-cultivation in darkness increases GUS activity for CNE explants of the cv. CIAP7247F.

Many mechanical treatments may injure plant tissue to create attachment sites for Agrobacterium infection. It has been reported to increase transformation frequency in different legume species (Thu et al., 2003; Zambre et al., 2005). However, it has not been established whether wounding the tissue is necessary for transformation in common bean or not. In this study, non-injured explants inoculated with two Agrobacterium strains EHA101 and C58C1Rif^R(pMP90) showed GUS activity. Therefore, wounding is not an absolute prerequisite for T-DNA transfer to common bean cells; however, it appears to promote preferential attachment of the bacterial cells at the wounded site, because GUS activity increased significantly in common bean wounded explants (Table 5). Injury of explants in dicots incites wound responses in the form of secretion of *vir* gene inducing phenolics and multiple cell divisions at the site of injury. It also may increase the Agrobacterium attachment to plant cells (Gupta et al., 2006). On the basis of results from bean transformation experiments (Lippincott and Lippincott, 1969) it was proposed that wounding is an essential step in the infection process. This proposal was backed up by reports that plant cells with incomplete cell walls are more suitable for bacterial adherence than intact cells (Sen et al., 1986). Results of this study indicate that wounding is not a prerequisite for bacterial attachment. However, wounding increased *GusA* expression. Therefore, it is concluded for common bean that wounding improves DNA transfer at regeneration zones.

Transient *GusA* expression was studied to evaluate the optimal concentration of acetosyringone for T-DNA delivery into common bean cells during Agrobacterium culture. The GUS assay of Agrobacterium infected CNE explants showed that adding acetosyringone in co-cultivation medium, increased the size and intensity of patches having GUS activity (Fig. 3 *j, k, l, m*). The addition of acetosyringone in Agrobacterium culture medium should enhance gene transfer efficiency (Stachel et al., 1985; Riva et al., 1998; Zupan et al., 2000). Our results showed that acetosyringone 50-200 µM, in all cases, enhanced the transient *GusA* expression. Similar results have been reported for callus of *P. acutifolius* (De Clercq et al., 2002). Acetosyringone levels up to 200 µM were considered not significantly toxic to Agrobacterium cells (Stachel et al., 1985), whereas a concentration of 2000 µM was found to be deleterious for *P. acutifolius* callus. It remains to be determined whether acetosyringone concentrations higher than 200 µM would be beneficial in our transformation system.

Selection and analysis of putative transgenic plants. The optimization of several parameters involved with DNA-transfer combined with an efficient multiple bud regeneration from epicotyl sections

allowed generation of chimeric plants in common bean. However, Agrobacterium inoculation contributed to detrimental effects on common bean regeneration. The recent attempts to transform common bean using Agrobacterium failed (Amugune et al., 2011; Mukeshimana et al., 2013), and transformed tissue could not be recovered mainly due to the poor regeneration. In this study, after Agrobacterium inoculation the percentage of multiple bud formation diminished from 65.5 to 32.8%. This reduction in the regeneration efficiency may be related to Agrobacterium overgrowth or a hypersensitive response of common bean cells to repel the bacterial attack. The use of less aggressive Agrobacterium strains than EHA101, or lowered concentration of bacterial suspension may help to improve the regeneration efficiency of transformed tissue. On the other hand, the morphogenetic response of shoots was drastically affected by the selective agent, the percentages of shoot formation and shoot elongation for putative transgenic shoots were lower compared with those obtained from non-transformed shoots.

The observation of GUS-staining during the transformation procedure in CNE explants or in multiple buds (Fig. 4 *c*) clearly demonstrated DNA transfer by the Agrobacterium strain (EHA101) used in this work. However, different blue staining patterns observed amongst the tested tissue strongly suggested that these plants were chimeric, later confirmed since none of the progenies were GUS-positive. Although high *GusA* expression was found in several steps of the procedure, the genetic transformation protocol did not yield permanent transgenic events. Several factors can affect the efficiency of a transformation system, including regeneration and selection procedure (Angenon and Thu, 2011). Despite the formation of multiple buds from non-meristematic tissue, stable transformation of common bean was inefficient. The main reason could be that multiple buds were derived from a group of predetermined cells (see Supplementary Material). This fact could promote chimera formation in this study. Moreover *nptII* as selectable marker is possibly not appropriate to select transgenic cells in the transformation system employed in this study. Selection systems based on antibiotic resistance are most effective in those tissue parts nearer to the selective medium (Faize et al., 2010). In our experiment, the selection was carried out on shoots regenerated from multiple buds. Transformed cells in the basal area of the multiple buds may have protected the growth of non-transformed distal tissue of those shoots. Another aspect that could increase the development of chimeric plants in the present transformation system was the use of a recovery phase on ReM without selective agent. Since the primary multiple buds could have a multicellular origin, omission of a selective agent could promote the escape of chimeric shoots capable of elongation and rooting in presence of the selective agent. Absence of a selective agent into RM could have limited the strict selection.

Shoots which were regenerated from meristematic-free tissue, do not originate from single cells – this reduces the efficiency of transformation. Alternatively, a selectable marker system based on a herbicide, such as bialaphos-resistance (*bar*) gene and/ or imidazolinone-resistance (*ahas*) gene could be more effective. Repetitive multiplication of the primary multiple buds on selective medium could also reduce the regeneration of escapes and chimeric plants. Rooting shoots on media containing selective agent may contribute to further screening of transformants. On the other hand, another solution comprises using a regeneration system based on indirect organogenesis with a callus phase, which shoot may arise from a single cells as was described before for *P. acutifolious* (Zambre et al., 2005) and *Glycine max* (Hong et al., 2007). This is further supported by recent results in our lab that green nodular calli showed high regeneration capability (Collado et al., 2013) and they were also susceptible to Agrobacterium inoculation (data not shown).

Conclusions

The *in vitro* regeneration via direct organogenesis reported here, is efficient and reproducible for *P. vulgaris* cv. CIAP7247F. This protocol shows the advantage of using non-meristimetic tissue as initial explant, being useful and applicable for genetic transformation. Explant inoculation with

Agrobacterium drastically reduced multiple bud regeneration from epicotyl sections. All evaluated parameters had significant effect on DNA transfer from Agrobacterium to common bean epicotyl sections. The combination of the best treatment from each parameter studied (temperature of 25°C, darkness, injuring explants with carborundum and supplementation of co-cultivation medium with 200 µM acetosyringone) established a reproducible and efficient Agrobacterium DNA transfer procedure. Although GUS assays only demonstrated T-DNA delivery into common bean cells, the transformation method described in this study has the potential to enhance Agrobacterium techniques. Even though the recovered putative transgenic plants were chimeras, this established protocol shows the advantage of being efficient for the regeneration of transformed common bean tissue. Since none of the published common bean transformation procedures using Agrobacterium have recovered transformed plants–either chimeric or completely transgenic – due to the inefficient regeneration (Amugune et al., 2011; Mukeshimana et al., 2013), the protocol described here is an advance compared with the previous attempts above. This is an important step concerning Agrobacterium-mediated transformation in *P. vulgaris* and it can also be used to assist follow-up research regarding genetic transformation and breeding of this important legume species.

Acknowledgements
This research was financially supported by the Institutional University Collaboration programme with Universidad Central "Marta Abreu" de Las Villas from the Flemish Interuniversity Council (VLIR IUC UCLV). Thanks also go to Edilio Quintero from CIAP for kindly supplying seeds of common bean cv. CIAP7247F.

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Figures

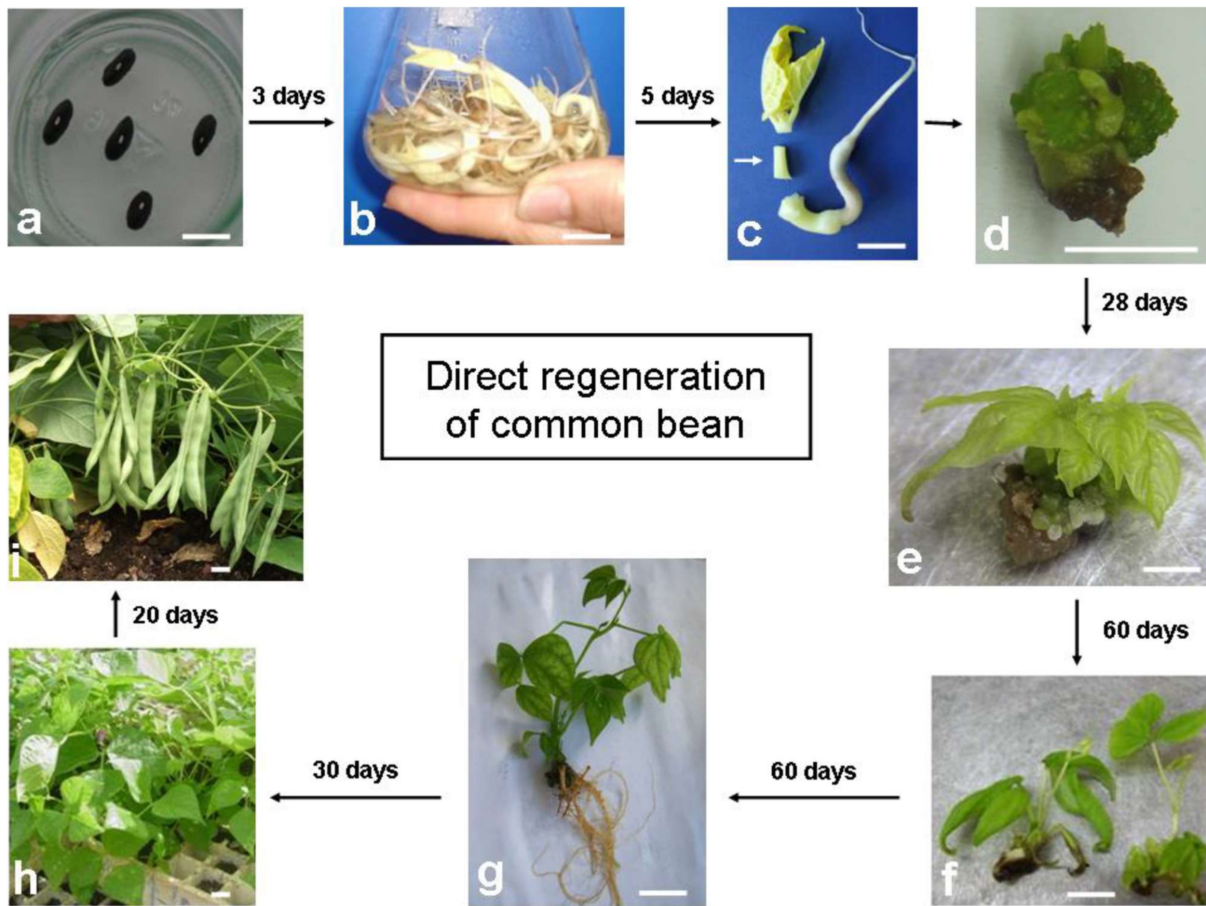

Figure 1. Regeneration protocol of common bean cv. CIAP7247F (*Bars*, 1 cm). (**a**) Seeds sowed *in vitro*. (**b**) Seedlings pre-cultured in liquid culture medium. (**c**) Explant preparation for multiple bud induction, epicotyl section (*arrowed*). (**d**) Multiple buds formed on epicotyl section. (**e**) Shoots formed from multiple buds. (**f**) Elongated shoots. (**g**) Rooted plantlet. (**h**) Plants acclimatized in boxes of polystyrene foam. (**i**) Plants acclimatized in greenhouse with healthy pods.

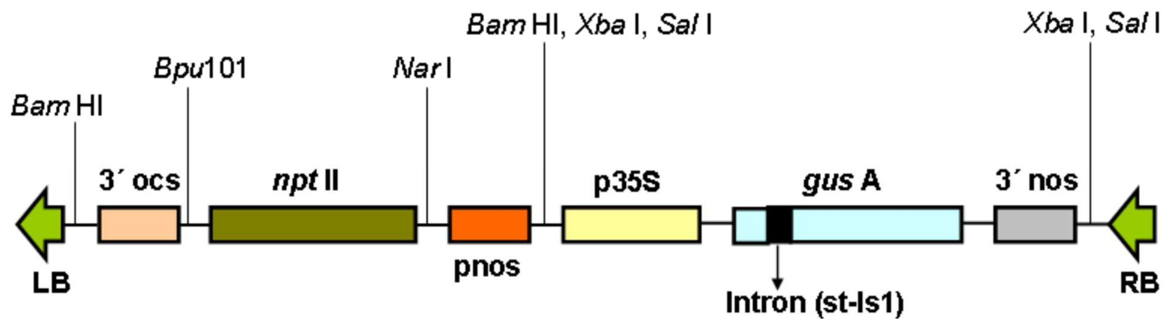

Figure 2. Schematic representation of T-DNA from pTJK136 vector.

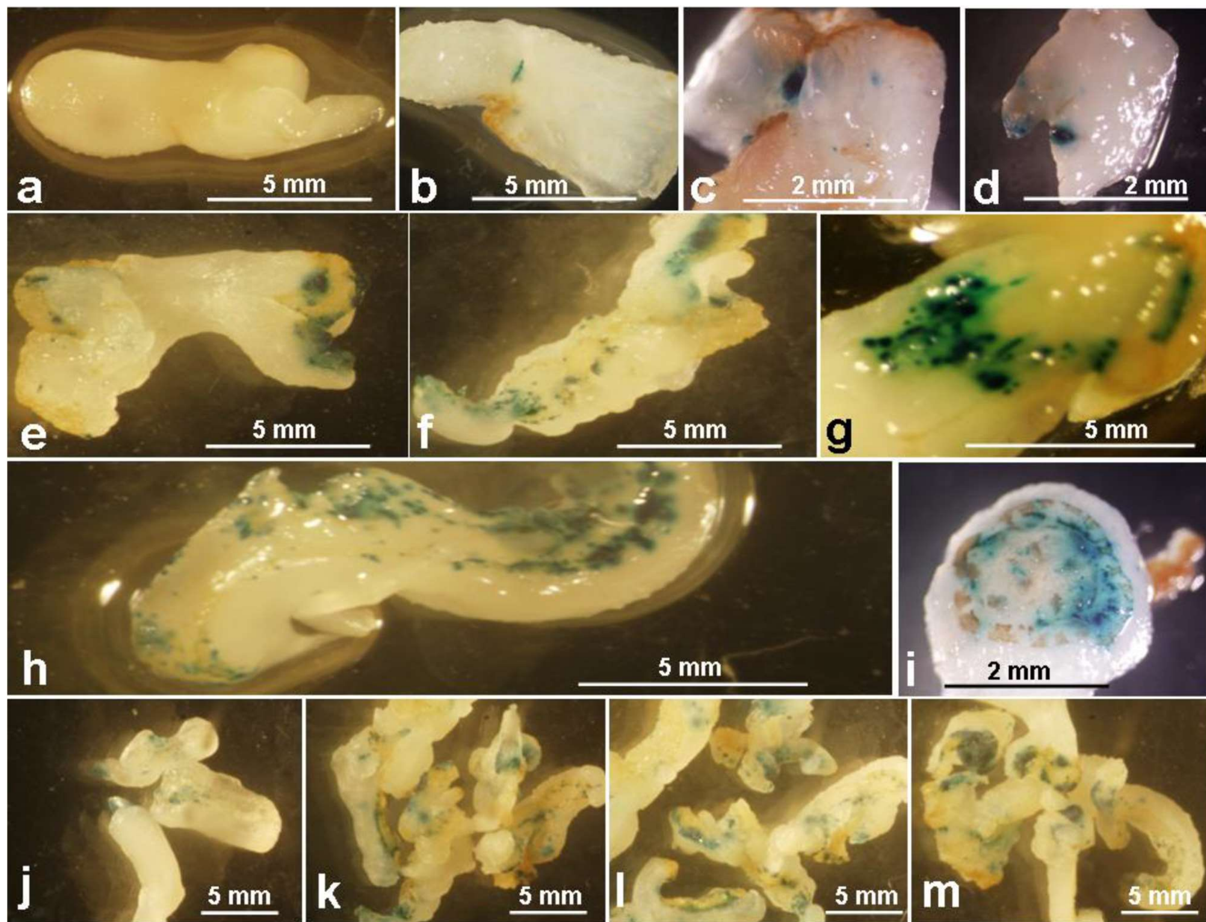

Figure 3. Transient GUS assay of common bean CNE explants co-cultivated with Agrobacterium EHA101(pTJK136). (**a**) Negative control; **b-d** explants co-cultivated at different temperatures, (**b**) 22ºC, (**c**) 25ºC, (**d**) 28ºC. **e-g** Explants injured in different manners; (**e**) Explant without injuries, (**f**) Explant pricked with a needle, (**g**) Explant wounded by brushing with carborundum, (**h**) Explant wounded by brushing with carborundum with a longitudinal cut, (**i**) Explants wounded by brushing with carborundum with a transversal cut. **j-m** explants co-cultivated with increasing concentrations of acetosyringone in co-cultivation medium, (**j**) Without acetosyringone, (**k**) 50 μM, (**l**) 100 μM, (**m**) 200 μM.

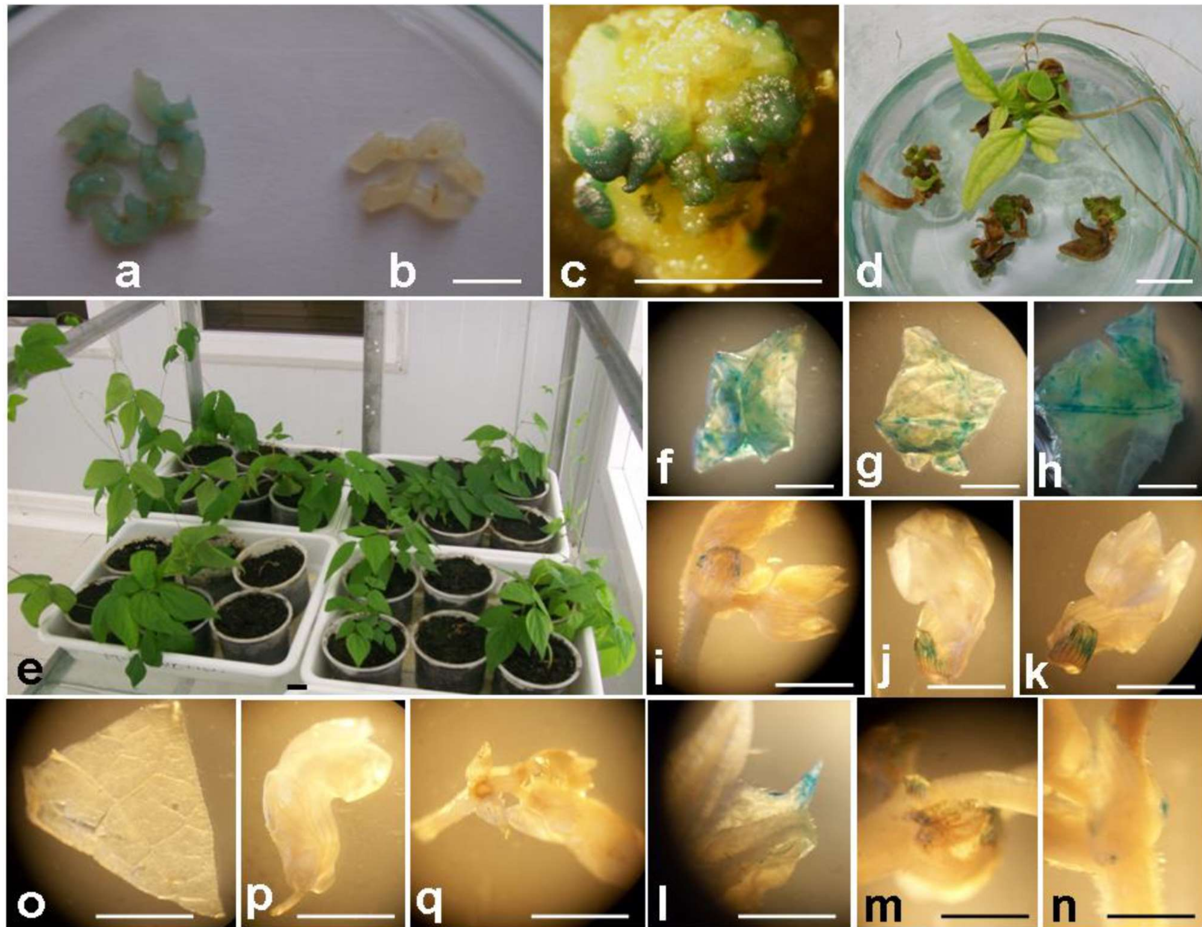

Figure 4. Transformation of common bean cv. CIAP7247F using CNE as target explants, and multiple bud regeneration from epicotyl sections (*Bars*, 1 cm). (**a**) GUS staining in the CNE after 5 d of co-cultivation. (**b**) GUS staining in non-transformed CNE. (**c**) GUS staining in recovered multiple buds. (**d**) Geneticin-resistant shoots on SE+SA medium. (**e**) Putative transgenic plants growing in soil in the phytotron. (**f-n**) GUS staining in collected tissue from transformed plants. (**o-q**) GUS staining in collected tissue from non-transformed plants.

Tables

Table 1. Media for culture, selection, and regeneration of common bean plants

Designation	Media	Composition
GM	Germination medium	50 % (Murashige and Skoog, 1962) salts (MS), 2 % (*w/v*) sucrose and 1.13 mg L^{-1} 6-benzylaminopurine (BAP), 0.25 % (*w/v*) Gel-rite, pH 5.7
PM	Pre-culture medium	100 % MS salts supplemented with H vitamins (Heinz and Mee, 1969), myo-inositol (100 mg L^{-1}), sucrose 3% (*w/v*) and TDZ (1.0 mg L^{1}), pH 5.7
MBIM	Multiple bud induction medium	100 % MS salts, H vitamins, 3 % (*w/v*) sucrose, 100 mg L^{-1} myoinositol, 0.5 mg L^{-1} TDZ, 0.25 % (*w/v*) Gel-rite, pH 5.7
SIM	Shoot induction medium	100 % MS salts, H vitamins, 3 % (*w/v*) sucrose, 100 mg L^{-1} myoinositol, 20 mg L^{-1} adenine sulphate, 0.25 % (*w/v*) Gel-rite, pH 5.7
SEM	Shoot elongation medium	100 % MS salts, H vitamins, 3 % (w/v) sucrose, 100 mg L^{-1} myoinositol, 20 mg L^{-1} adenine sulphate, 1.4 mg L^{-1} GA_3, 0.4 mg L^{-1} IBA, 2.12 mg L^{-1} $AgNO_3$, 0.25 % (w/v) Gel-rite, pH 5.7
RM	Rooting medium	100 % MS salts, H vitamins, 3 % (*w/v*) sucrose, 1.0 mg L^{-1} IBA, 2.12 mg L^{-1} $AgNO_3$, 0.25 % (*w/v*) Gel-rite, pH 5.7
CCM	Co-cultivation medium	PM, supplemented with acetosyringone (200 μM) and pH 5.5
WM	Washing medium	50 % MS salts, 100 % H vitamins, 3 % (*w/v*) sucrose, 0.1 g L^{-1} myo-inositol, 1.0 mg L^{-1} TDZ, 200 mg L^{-1} timentin[1], pH 5.7 (for washing the explants after co-culture)
ReM	Recovery medium	MBIM + 200 mg L^{-1} timentin
SI+SA	Shoot induction medium plus selective agent	SIM + 150 mg L^{-1} timentin, 50 mg L^{-1} geneticin[2]
SE+SA	Shoot elongation medium plus selective agent	SEM + 100 mg L^{-1} timentin, 50 mg L^{-1} geneticin
RM+T	Rooting medium plus timentin	RM + 50 mg L^{-1} timentin

[1] Timentin (antibiotic) is a mixture of ticarcillin and clavulanic acid and is commonly used at a ratio of 50 (ticarcillin):1 (clavulanic acid) (*w/w*) effectively used to suppress Agrobacterium growth (Cheng et al., 1998)

[2] Geneticin (antibiotic) commonly know as G418 or G-418, Geneticin® (INVITROGEN) reagent is an aminoglycoside related to Gentamicin and is commonly used as a selective agent for eukaryotic cells. The minimal inhibitory concentration used for common bean in this work was determined in previous study (Bermúdez-Caraballoso et al., 2007)

Table 2. Summary of direct organogenesis procedure using multiple buds regeneration from epicotyl section in common bean cv. CIAP7247F

Replica	Number of epicotyl sections	Number of multiple buds	Number of shoots	Number of elongated shoots [1]	Number of rooted plantlets	Number of surviving plants in soil	Number of plants that produced seeds
1	100	61	224	192	183	172	172
2	100	66	246	218	209	202	202
3	100	63	231	187	171	167	167
4	100	71	254	214	206	197	196
Total	400	261	955	811	769	738	737

[1] Number of elongated shoot refers to shoots ≥20 mm

Table 3. Influence of co-cultivation temperature on transformation of *P. vulgaris* cv. CIAP7247F CNE explants co-cultivated with two Agrobacterium strains

Temperature (°C)	Percentage of explants showing transient GUS activity (%)[1]	
	EHA101(pTJK136)	C58C1Rif^R(pMP90) (pTJK136)
22	31.0±2.3c	6.0±1.0c
25	80.0±2.2a	46.0±1.6a
28	62.0±2.5b	17.0±1.0b

Different letters in the same column indicate significant differences (*P< 0.05*) by non-parametric Kruskal-Wallis test; Data are means ± Standard Error

[1] Percentage of GUS-positive explants in 363-400 Agrobacterium-inoculated CNE per treatment.

Table 4. Transient *gusA* expression in *P. vulgaris* cv. CIAP7247F CNE co-cultivated with two Agrobacterium strains under light and dark conditions

Photoperiod condition	Average blue spots per explant	
	EHA101(pTJK136)	C58C1Rif^R(pMP90)(pTJK136)
16h light /8h dark	1.00±0.27b	1.20±0.39b
Dark	3.25±0.80a	4.52±0.46a

Different letter in the same column indicate significant difference (*P< 0.05*) based on non-parametric Mann-Whitney test; Data are means ± Standard Error

Table 5. Influence of two kinds of mechanical damage on transformation of *P. vulgaris* cv. CIAP7247F CNE explants co-cultivated with two Agrobacterium strains

Treatments	Percentage of explants showing transient GUS activity (%)[1]	
	EHA101(pTJK136)	C58C1Rif^R(pMP90)(pTJK136)
Explants pricked with a needle	67.0±4.2a	41.2±2.3a
Explants wounded with carborundum by brushing	86.0±2.6a	56.0±4.0a
Explants without injuries	34.8±1.9b	17.3±1.4 b

Different letters in the same column indicate significant difference (*P< 0*.05) by non-parametric Kruskal-Wallis test; Data are means ± Standard Error

[1] Percentage of explants showing GUS-activity in 581-600 Agrobacterium-inoculated CNE per treatment

Table 6. Influence of three acetosyringone concentrations on GUS activity in *P. vulgaris* cv. CIAP7247F CNE explants co-cultivated with two Agrobacterium strains

Acetosyringone concentration (μM)	Percentage of explants showing GUS activity (%)[1]		Number of blue spots per explant[2]	
	EHA101 (pTJK136)	C58C1Rif^{R}(pMP90) (pTJK136)	EHA101 (pTJK136)	C58C1Rif^{R}(pMP90) (pTJK136)
0.0	54.0±3.2b	32.0±1.7c	1.86±0.21c	1.04±0.19c
50	83.0±2.2a	58.0±2.1b	4.11±0.74b	4.37±0.28b
100	88.0±2.9a	61.0±1.3b	3.70±0.39b	4.63±0.26b
200	90.0±3.3a	67.0±1.3a	6.30±0.76a	5.72±0.18a

Different letters in the same column indicate significant difference by parametric Bonferroni test (*0.95*); Data are means ± Standard Error

[1] Percentage of explants showing GUS-activity in 300 Agrobacterium-inoculated CNE per treatment

[2] Transient *gusA* expression is represented as number of blue spots per explant; only explants with at least one blue spot were taken into account.

Table 7. Summary of transformation procedure using CNE as target explants, and the multiple buds regeneration from epicotyl section in common bean cv. CIAP7247F

Replica	Total number of explants	Number of multiple buds recovered	Number of geneticin resistant shoots	Number of geneticin resistant elongated shoots	Number of surviving plants in soil	Number of GUS^{+} plants	Transformation frequency (%)[1]
1	110	25 (100)	6 (23)	5	3	1	1
2	110	31 (100)	4 (29)	4	3	0	0
3	110	46 (100)	8 (44)	6	5	1	1
4	110	29 (100)	5 (27)	3	3	1	1
Total	440	131 (400)	23 (123)	18	14	3	0.75

[1.] Transformation frequency (%) is (number of plants producing GUS positive tissue/ total number of plants) x 100.

Epicotyl sections as target for plant regeneration and transient transformation of common bean using *Agrobacterium tumefaciens*

R. Collado[1, 2], I. Bermúdez-Caraballoso[1], L.R. García[1], N. Veitía[1], D. Torres[1], C. Romero[1], G. Angenon[2]

[1]*Instituto de Biotecnología de las Plantas, Universidad Central "Marta Abreu" de Las Villas. Carretera a Camajuaní km 5.5, Santa Clara, Villa Clara. Cuba. PC-54 830.*

[2]*Laboratory of Plant Genetics, Vrije Universiteit Brussel (VUB), Pleinlaan 2, B-1050 Brussels, Belgium.*

Supplementary material

Histological analysis

Methodology

Tissues from cultures in various stages ranging from multiple bud formation to the shoot regeneration stage were sampled. Longitudinal sections of stem with small multiple buds were cut into approximately 4 mm cubes and were fixed in 2.5% glutaraldehyde (in phosphate buffer, pH 7) overnight. Tissue sections were stained with safranin and then multiple bud formation on the tissue was observed under a stereoscopic microscope at 10x plus the objective 40x.

To observe the organization of shoot regeneration, longitudinal sections of multiple buds were fixed as described for the stem sections. Then, they were infiltrated and embedded in hydroxyethylmethacrylate (Technovit 7100, Kulzer, Wehrheim, Germany) and kept at 4ºC. Samples were embedded in a sucrose gradient prior to tissue sectioning: (30% (w/v) for six hours, 60% for six hours and finally 100% overnight). Samples were included in Tissue-Tek O.C. T. compound (Sakura Finetek Europe B.V.) and frozen at –20ºC. Tissue was sectioned at 100 µm thickness using a cryomicrotome CH1510-1 (Leica Microsystems GmbH Wetzlar, Germany). Sections were stained with lactophenol cotton blue or safranin (in the case of well differentiated shoots arising from a multiple bud cluster).

Results

Regenerable multiple buds were estimated to be arising from stem sections at 15 days of culture. After this period multiple buds were observed on the epidermis of the stem sections. However none of these structures grew from the cut ends of the explants. Histological analysis shows that such multiple buds were formed directly from the stems without callus phase (Fig. 1 a-b). Small buds were formed separately from each other and they were not associated to pre-existing meristems (Fig. 1 b).

The multiple buds showed clear organization from early developmental stage on (Fig. 1 c-d). Moreover, well differentiated unipolar adventitious shoots that emerged from the parental tissue (bud) were also observed (Fig. 1 e).

The multiple bud formation directly from stem tissue followed by early organization resulting in well differentiated shoots clearly demonstrated a morphogenesis *via* direct organogenesis.

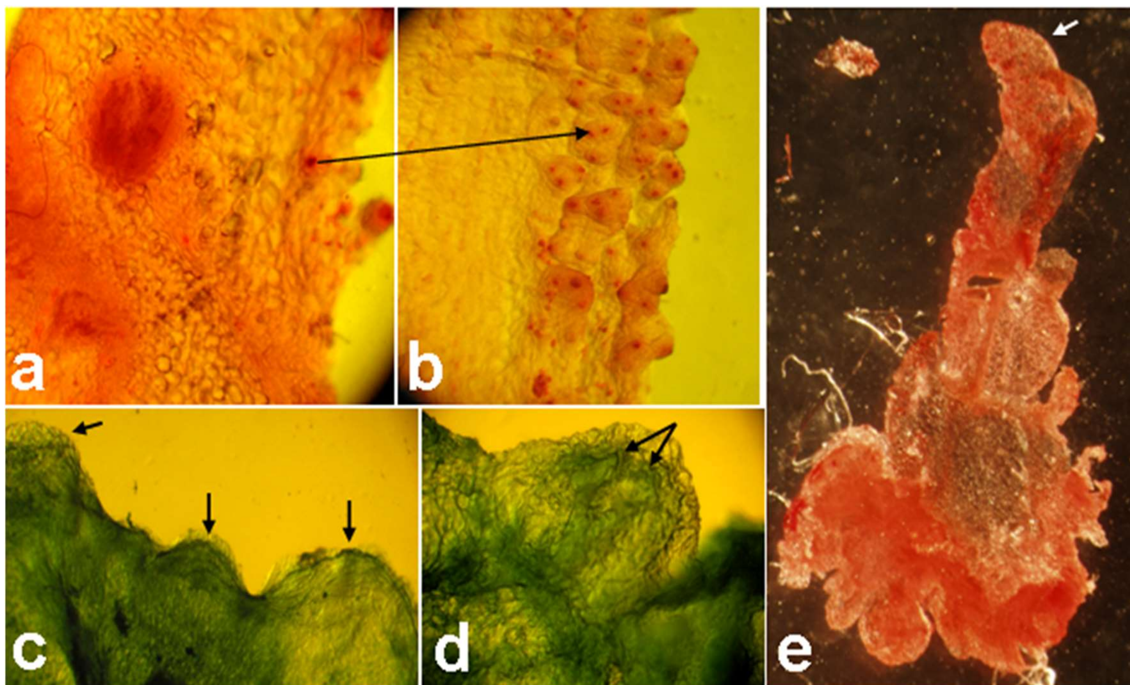

Fig. 1 Histological characterization of multiple bud formation and differentiation of buds to shoots; (a, b) longitudinal section of stem with multiple buds, (c) longitudinal section from a bud initiating shooting (arrow points to the dome of the shoot), (d) longitudinal section of the bud-shoot with leaflet protuberances (arrows point the leaflet), (e) longitudinal section of a well-developed shoot (arrow points to the shoot apex)