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Agrobacterium-mediated transformation of *Phaseolus vulgaris* L. using indirect organogenesis

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ABSTRACT

A protocol for genetic transformation of *Phaseolus vulgaris* L. cv. CIAP7247F *via Agrobacterium tumefaciens* was established. Primary green nodular calli and proliferative calli were used as target explants. Several factors such as *Agrobacterium* strain, plasmid, light conditions, bacterial concentration, co-cultivation period and type of callus were studied for optimization of the *Agrobacterium*-mediated transformation. The highest DNA transfer occurred when proliferative calli were inoculated with strain EHA105 harbouring pCAMBIA3301 plasmid at a density of OD_{600} = 0.5, and co-cultivated under 16 h light/8 h dark photoperiod for 6 days. The transformation system integrates *Agrobacterium*-mediated DNA transfer with efficient regeneration *via* indirect organogenesis, and using the *bar* gene as selectable marker and glufosinate ammonium (herbicide finale) for callus selection. The proposed system allowed obtaining transgenic lines with Mendelian inheritance of the transgenes, as demonstrated by PCR analyses. These results also validated the effectiveness of a regeneration protocol *via* indirect organogenesis for regeneration of transformed bean cells. This system constitutes a major initial step for common bean transformation using *A. tumefaciens*.

1. Introduction

Direct organogenesis has been the main route for plant regeneration in *Phaseolus vulgaris* L. (Gatica Arias et al., 2010; Kwapata et al., 2010; Quintero-Jiménez et al., 2010). Such a regeneration system coupled to genetic transformation by particle bombardment has been described by Aragão et al. (2002) and Rech et al. (2008) in *P. vulgaris*. However, this method often has the disadvantage of a complex pattern of transgene integration, thus increasing the probability of transgene silencing (Yang et al., 2005). Genetic transformation *via Agrobacterium tumefaciens*, on the other hand, is a biological method, which usually introduces few transgene copies in the genome of the plant, thus reducing problems such as gene silencing (Gelvin, 2000).

Regardless of available information on *P. vulgaris in vitro* regeneration, genetic transformation *via A. tumefaciens* still is a major challenge, mainly due to the lack of an adequate regeneration response. Two recent attempts to transform common bean using *Agrobacterium* (Amugune et al., 2011; Mukeshimana et al., 2013) failed, and transformed tissue could not be recovered mainly because of poor regeneration. Recently, a system combining direct organogenesis from hypocotyls, the *npt*II or *bar* gene as selectable marker and *Agrobacterium*-mediated transformation yielded transgenic plants (Espinosa-Huerta et al., 2013). Despite the ample use of embryo axes as explants for regeneration, these explants appear less suitable for genetic transformation in common bean (Mukeshimana et al., 2013). The main disadvantage of using such explants for genetic transformation is that the regeneration is usually from pre-existing meristematic tissue (Mukeshimana et al., 2013) and the shoots obtained are of multicellular origin which may inhibit strict selection for transgenic shoots leading to chimeric transformants (Angenon and Thu, 2011).

Production of chimeric transformants and escapes of non-transgenic materials that survive selection are problems that have often been described in legume transformation (reviewed by Angenon and Thu, 2011). These phenomena are linked to the mode of regeneration, but also the choice of the selective agent and its concentration can be important (Angenon and Thu, 2011).

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

Media	
GM	MS (Murashige and Skoog, 1962) basal salts, 1 mg l^{-1} thiamine, 1.13 mg l^{-1} 6-benzylaminopurine (BAP), 30 g l^{-1} sucrose and 3 g l^{-1} agar, pH 5.7
BIM	Half-strength MS salts, 3.9 g l^{-1} MES, 1.98 g l^{-1} glucose, 20 g l^{-1} sucrose, pH 5.5
CIM	MS basal salts, B5 vitamins (Gamborg et al., 1968), 0.2 mg l^{-1} thidiazuron (TDZ), 0.05 mg l^{-1} indole-3-acetic acid (IAA), 30 g l^{-1} sucrose and 6 g l^{-1} agar (Duchefa Biochemie B.V. Haarlem, The Netherlands), pH 5.7
CPM	MS salts, B5 vitamins, 0.05 mg l^{-1} of IAA, 20 g l^{-1} sucrose, 6.0 g l^{-1} agar, 0.04 mg l^{-1} of TDZ, pH 5.7
CPMAS	MS salts, B5 vitamins, 0.05 mg l^{-1} of IAA, 20 g l^{-1} sucrose, 6.0 g l^{-1} agar, 0.04 mg l^{-1} of TDZ, 200 μM acetosyringone, pH 5.5
CPMT	MS salts, B5 vitamins, 0.05 mg l^{-1} of IAA, 20 g l^{-1} sucrose, 6.0 g l^{-1} agar, 0.04 mg l^{-1} of TDZ, 200 mg l^{-1} timentin, pH 5.7
CPMTF	MS salts, B5 vitamins, 0.05 mg l^{-1} of IAA, 20 g l^{-1} sucrose, 6.0 g l^{-1} agar, 0.04 mg l^{-1} of TDZ, 200 mg l^{-1} timentin, 0.5 mg l^{-1} Finale pH 5.7
SRM	MS salts, B5 vitamins, 2.25 mg l^{-1} BAP, 30 g l^{-1} sucrose, 6 g l^{-1} agar and pH 5.7
SRMT	MS salts, B5 vitamins, 2.25 mg l^{-1} BAP, 30 g l^{-1} sucrose, 6 g l^{-1} agar, 100 mg l^{-1} timentin and pH 5.7
SERM	MS salts, B5 vitamins, 3% (w/v) sucrose, 0.4 mg l^{-1} indol butyric acid, 1.4 mg l^{-1} gibberellic acid, 3.18 mg l^{-1} silver nitrate and 6 g l^{-1} agar, pH 5.7

Nowadays, the procedure for regenerating transgenic plants in *Phaseolus acutifolius* (Dillen et al., 1997; De Clercq et al., 2002; Zambre et al., 2005) is still the only successful protocol to reproducibly recover genetically modified plants for a *Phaseolus* species, using *Agrobacterium*-transformation. The success of this system was mainly based on the use of an indirect regeneration method coupled to the *A. tumefaciens* genetic transformation and effective selection of transformed tissues. An efficient protocol for *in vitro* regeneration of *P. vulgaris* via indirect organogenesis was described by Collado et al. (2013). This callus-phase regeneration procedure was succesfully applied in five commercial *P. vulgaris* varieties. To date, a transformation system using organogenic callus as the target explant for *P. vulgaris* transformation using any DNA delivery method has not been reported.

The objective of our study was to develop a protocol for *Agrobacterium*-mediated transformation of *P. vulgaris* var. CIAP7247F. Therefore, several factors to enhance T-DNA transfer were assessed. Secondly, by combining *Agrobacterium*-mediated transformation techniques, the *bar* gene as selectable marker system and regeneration through indirect organogenesis, a work scheme for *P. vulgaris* transformation was established; and the use of this scheme allowed to obtain transgenic common bean plants.

2. Material and methods

2.1. General procedures

The instruments used for the aseptic handling of plant material were sterilized in a stove at a temperature of 180 °C for two hours before each work session. All dissection and transfer operations of the explants were performed in horizontal laminar flow cabinets. The pH of all media was adjusted with NaOH or HCl prior to autoclaving and the optimum pH for each medium is detailed in Table 1. The culture media were autoclaved at 121 °C and 1.2 kg/cm^2 pressure for 20 min.

2.2. Plant material

Mature seeds of *P. vulgaris* L. var. CIAP7247F, obtained by selection from the 7247 line in the bean genebank from Centro de Investigaciones Agropecuarias (CIAP), Universidad Central "Marta Abreu" de Las Villas were used as initial plant material. Seeds were harvested in the greenhouse and disinfected as described below.

2.3. In vitro *culture*

2.3.1. Seed disinfection and germination

Seeds were washed in running tap water and disinfected by a quick rinsing (10 s) in 70% (v/v) ethanol and 10 min in a solution containing 3% (v/v) NaOCl. Seeds were rinsed three times with sterile deionized water to remove any residue of disinfectant. They were placed on germination medium (GM, Table 1). Ten seeds were cultured in 250 ml glass culture vessels containing 30 ml of GM at 25 ± 2 °C in darkness for three days.

2.3.2. Callus induction and proliferation

After germination, explants were dissected from 3-day-old germinated seeds; the cotyledonary node with one cotyledon (CN-1, Collado et al., 2013) was used for callus induction (Fig. 1). Explants were grown on callus induction medium (CIM, Table 1). Plant material was maintained in a growth chamber at 25 ± 2 °C; during the first 7 days the culture vessels were covered with black bags to keep the crop in darkness, after this period the bags were removed and the plant material was under 16 h light/8 h dark photoperiod of 45 μmol m^{-2} s^{-1} for 14 days. Twenty one-day-old primary calli induced on CN-1 explants were chopped into small (0.2–0.3 g) pieces, mixed together and transferred to callus proliferation medium (CPM, Table 1) for 30 days. Proliferative calli 5–7 days after the second subculture on CPM were chopped into small (3–5 mm) fragments and used as target explant for *Agrobacterium* inoculation.

2.4. Genetic transformation

2.4.1. T-DNA description

Two plasmids, pTJK136 (Kapila et al., 1997) and pCAMBIA3301 (CAMBIA, 1997), were used for genetic transformation. The pTJK136 T-DNA contains the gene for neomycin phosphotransferase II (*npt*II) (EC 2.7.1.95) under control of the promoter of the nopaline synthase (*nos*) and the terminator and polyadenylation signals of the octopine synthase. Furthermore, the construct contains the β-glucuronidase gene from *Escherichia coli* (*gus*A) (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 *gus*A gene is under the control of the Cauliflower Mosaic Virus promoter (CaMV-35S) and terminator signals from the *nos* gene (Fig. 2a).

The pCAMBIA3301 T-DNA contains the *bar* gene (Thompson et al., 1987) that encodes the enzyme phosphinothricin *N*-acetyl transferase which confers resistance to the herbicides containing glufosinate ammonium as active ingredient. The *bar* gene is regulated by the 35S promoter and 35S terminator from CaMV (t-35S). This construct also contains the *gus*A reporter gene (Jefferson et al., 1987) encoding for the β-glucuronidase enzyme which is regulated by CaMV-35S promoter and the terminator sequence of the nopaline synthase gene of *Agrobacterium* (Fig. 2b).

2.4.2. Preparation of bacterial suspension

A. tumefaciens strains EHA105 (Hood et al., 1993) and C58C1Rif^R(pMP90) (Koncz and Schell, 1986), both containing the plasmid pTJK136 or pCAMBIA3301 were used to inoculate the pieces of callus. An *Agrobacterium* colony isolated from a fresh plate was suspended in 3 ml YEP culture medium (An