

Full Length Research Paper

Composite *Phaseolus vulgaris* plants with transgenic roots as research tool

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Large seeded grain legumes such as the common bean (*Phaseolus vulgaris*) and cowpea (*Vigna unguiculata*) are very important crops with seeds that are major protein source for people in developing countries, but their yields and improvement lag behind the economically more important cereals. For research purposes, genetic transformation is a powerful tool to obtain valuable information on gene expression and putative gene functions. In addition, through genetic transformation, candidate genes can be evaluated for their potential in agricultural biotechnology applications, such as resistance against biotic stresses. However, it remains difficult to stable genetically transform large seeded grain legumes such as *Phaseolus* and cowpea using *Agrobacterium tumefaciens*. In this paper a system is described to obtain so-called transgenic composite plants from *P. vulgaris*. These have a transgenic root system, obtained through *Agrobacterium rhizogenes* transformation of de-rooted seedlings. Their potentials for studies on important processes in the root system will be discussed.

Key words: Genetic transformation, *Phaseolus vulgaris*, *Agrobacterium rhizogenes*.

INTRODUCTION

Grain legumes are important agricultural crops, especially for developing countries, where they provide proteins in vegetarian or meat-poor diets. In addition, they are easily stored in a dry form and their interactions with symbiotic nitrogen-fixing bacteria make them valuable crops for

sustainable agriculture. Among the grain legumes, *Phaseolus vulgaris*, the common bean, is the most important as a food crop. Common bean was introduced to Africa in the 16th century, and today it is the second most important source of human dietary protein. Per capita bean consumption is highest in Africa, reaching 66 kg/yr in Western Kenya. Over 4 million hectares of beans are sown each year in Africa (Buruchara, on <http://www.africancrops.net>).

Despite their major role as food crop, research in grain legumes has always been somewhat behind the economically more important cereals, but has recently started to make a big leap forward. Several international initiatives

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Abbreviations: WT, Wild type; GFP, Green fluorescent protein; GUS: β -glucuronidase; ER, endoplasmic reticulum; OD, optical density.

Table 1. Composition of the media used for seed germination and transformation (per liter).

Component	GM	CM	WM	SM
MS basal (g)	2.15	4.3	4.3	4.3
MES (g)	-	0.5	0.5	0.5
Sucrose (g)	20	-	-	-
Glucose (g)	-	20	20	20
pH	¹ 5.8	² 5.8	² 5.8	² 5.8
Bacto Agar (g)	6	-	-	-
Phytigel (g)	-	3	-	3
AS (1M) (μl)	-	200	-	-
Cb (200 mg/ml) (ml)	-	-	2.0	1.5
G418 (20 mg/ml)(ml)	-	-	-	1.5
Recipient	³ 16 pots	⁴ 16 plates	-	³ 16 pots

¹pH was set with 1 M NaOH before adding agar.

²pH was set with 10 M NaOH, before adding Phytigel if required.

³Sterile plastic pots with filter system in cover (height 13.5 cm, diameter 9 cm (Duchefa®)).

⁴Petridisches, diameter 14.5 cm and height 2.0 cm.

have been started, aiming at more concerted actions in the field of legume genomics and genetics. Important to mention in this context are the *Medicago* genome consortium (<http://www.medicago.org/genome>), the International Conferences on Legume Genomics and Genetics (ICLGG), a large EU funded Integrated Project focusing on legumes (www.eugrainlegumes.org) and consortia focusing on *Phaseolus* (www.phaseolus.net) and cowpea (<http://www.entm.purdue.edu/ngica/>).

In spite of these efforts, two of the more important food legumes, common bean (*P. vulgaris*) and cowpea (*Vigna unguiculata*), still miss some practical tools for genomic research, such as genetic transformation. Although progress has been achieved in *Phaseolus acutifolius* (Zambre et al., 2005) and cowpea (Popelka et al., 2006), it may still take some time for these protocols to be used routinely in several labs. Furthermore the protocols are laborious and have a rather long time frame.

For these reasons we have investigated the use of composite plants as described for *Medicago* (Boisson-Dernier et al., 2001) and soybean (Collier et al., 2005) for their potential to be used in *P. vulgaris*. These composite plants can be seen as semi-transgenic plants, obtained by using *Agrobacterium rhizogenes* to form transgenic roots on a wild type de-rooted seedling. An optimized protocol for *P. vulgaris* is described and the possibilities on the use of this system are discussed.

MATERIAL AND METHODS

Growth of *P. vulgaris* seedlings

P. vulgaris seeds of the cultivars Carioca and Xan (obtained from Prof. Zuilly, Univ. Paris 12, France and CIAT, Columbia, respectively) have been used. Thirty seeds were rinsed in 70% EtOH (100ml) for 20 s and vigorously shaken, after which they were immersed in 3% NaOCl (300 ml) with 6 drops Tween 20 for 10 m with gentle shaking. Seeds were washed 4 times with sterile water (about 500 ml in total), and distributed in pots with GM medium (Table 1). Seeds were pushed in the medium about 3/4 of the seed size deep, with the hilum side up and incubated at 25°C for 7 days. The first 3 days the pots were covered with aluminium foil to keep the seeds in the dark, the next four days a 16 h light/8 h dark cycle was used.

A. rhizogenes

A. rhizogenes WT strain 15834 (Lippincott and Lippincott, 1969) was grown overnight at 28°C in 100 ml liquid YEB medium. For the transformation, an *A. rhizogenes* strain obtained through tri-parental mating the Gateway vector pK7WG2D (Karimi et al., 2002), containing an *NPTII* antibiotic resistance marker with a cloned *uidA* (GUS marker gene with an intron) under the control of the 35S promoter was used. The pK7WG2D vector has the *EGFP-ER* gene, containing a signal to direct GFP to the ER under the control of the *RoiD* promoter.



Figure 1. **A:** *P. vulgaris* (genotype Xan) one week after germination; **B:** derooted *P. vulgaris* (genotype Carioca left and Xan right) before *Agrobacterium* infection; **C:** hairy roots of *P. vulgaris* (genotype Xan) forming on the inoculated derooted seedlings (view from the top in large pot).

Co-transformation

7-day old seedlings were de-rooted about 4 cm under the cotyledonary nodes. The cotyledons were also removed. The de-rooted seedlings were put vertically in an *A. rhizogenes* suspension of 1 OD, so that only the lower 2 cm of the de-rooted seedlings were in touch with the solution during 5 m. The de-rooted seedlings were dipped on sterile absorbing paper, to remove excess bacteria. They were then incubated on plates (2 seedlings per plate) with co-cultivation medium (CM, Table 1). Seedlings were put slightly inclined, so that the leaves did not touch the medium but the region of the removed cotyledons and the cut root did. The infected seedlings were incubated for 72 h at 22°C in a 16 h light/8 h dark cycle.

Selection

After the 72 h incubation, the de-rooted seedlings were washed in 100 ml wash medium (WM, Table 1) with careful shaking. This step was repeated twice, after which the de-rooted seedlings were dipped on sterile absorbing paper to remove excess liquid. Then they were transferred to pots with selection medium (SM, Table 1), taking care that the stem was not fully immersed in the medium, to allow the generation of hairy roots. Carbenicillin was added to the medium as an antibiotic to stop growth of *A. rhizogenes*. As selective antibiotic for the presence of the T-DNA, geneticin G418 was used at a concentration of 30 mg/l. Controls were included to check for the potential effect of geneticin on hairy root formation. Pots were incubated at 22°C in a 16 h light/8 h dark cycle. At the cut region, usually first yellow-green callus-like tissue appeared, from which roots then emerged.

GFP and GUS expression

GFP fluorescence was visualised with a fluorescence microscope

Leica, with excitation at 480/40 nm and emission at 510 nm and photographed with a digital camera. *GUS* expression was analysed as described in Zambre et al. (2005). Incubation was done for 1 h. These assays were done 3 weeks after transfer to selective medium at which time hairy roots were about 4 cm long. Agar was removed and GFP visualised followed by *GUS* staining.

RESULTS AND DISCUSSION

Since stable transformation of large grain legumes is still quite troublesome, we have optimized a protocol to obtain so-called transgenic composite plants from *P. vulgaris*. It had been demonstrated before that *P. vulgaris* was sensitive to *A. rhizogenes* infection (Barros et al., 1997), but there are no reports that it has been possible to regenerate transgenic plants from these roots. In this work we have used de-rooted seedlings that were inoculated with *A. rhizogenes*, which resulted in a rapid formation of hairy roots, and the establishment of so-called composite plants (Figure 1).

Hairy roots were obtained in 50% of the plants for genotype Xan and to 75% for genotype Carioca. The used *A. rhizogenes* strain in this work carried, besides its Ri plasmid, a T-DNA vector with an antibiotic resistance gene (*NPTII*) and a *GFP* and/or *GUS* gene. Geneticin was added to the medium to select for roots co-transformed with this T-DNA, as it had been observed in previous work from the group that geneticin generally gives better results than the traditionally used kanamycin (Dillen et al., 1997). Controls were included to check for the potential effect of geneticin on hairy root formation. No negative effect on hairy root formation was observed.

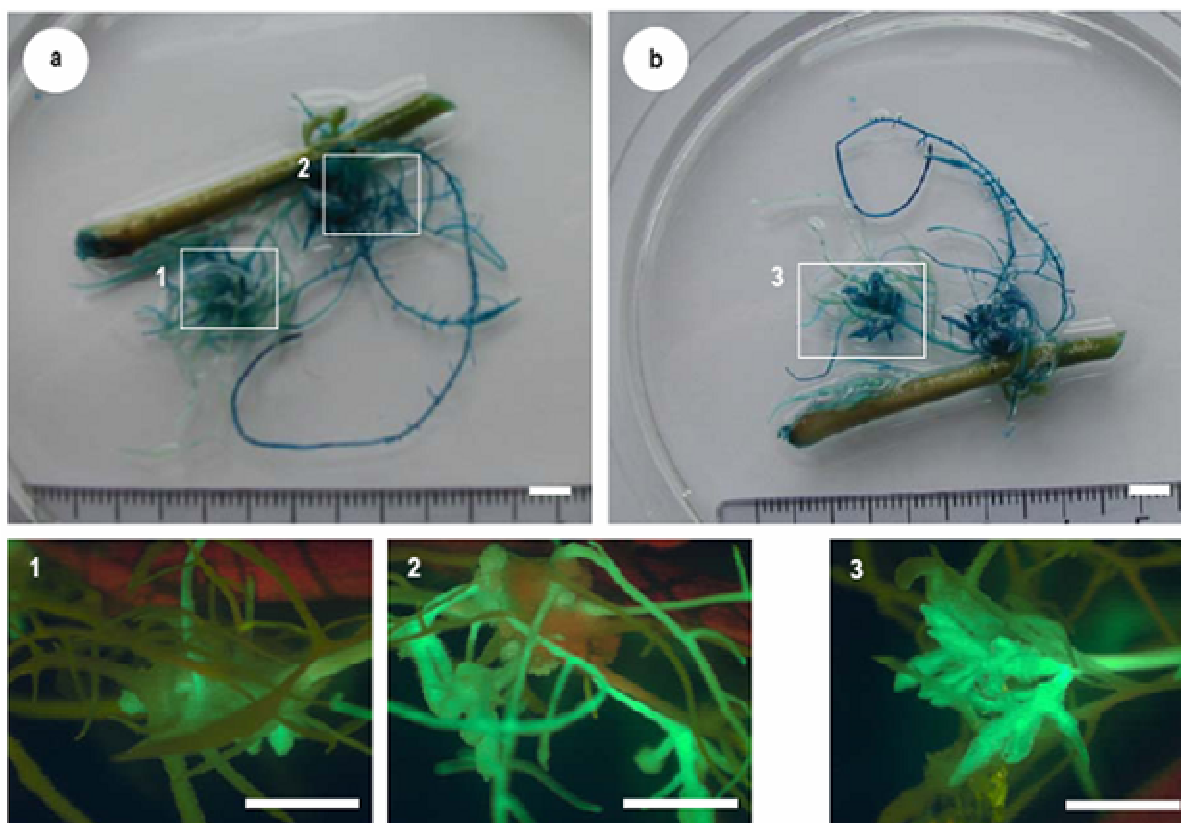


Figure 2.a, b: *GUS* expression of hairy roots of *P. vulgaris* genotype Xan (photo taken from 2 different angles). **1, 2, 3:** *GFP* -expression of the indicated regions of the hairy roots from *P. vulgaris* genotype Xan (bar corresponds to 5 mm).

Transformation of the *P. vulgaris* hairy roots was detected using two visible markers, GFP and GUS. It has been described that co-transformation of both the Ri and a binary T-DNA is possible due to the high homology of these plasmids (Simpson et al., 1986). Three weeks after transformation, hairy roots were tested for *GFP* and *GUS* expression. The hairy roots at that point reached about 4 cm. Staining with GUS and visualization of GFP gave in principle identical results, although sometimes it was observed that roots that were negative for GFP expression, were stained light blue in the GUS assay. This could be due to a higher sensitivity of the GUS assay or a specific staining, resulting from diffusion of the X-Gluc monomer after cleavage of the β -glucuronide group, followed by dimerisation at that site. As GFP is an *in vivo* marker, it can be used to screen for the co-transformed roots, and discard the non-transformed ones before biological assays are done. Based on *GFP* and *GUS* expression analysis (Figure 2) it was estimated that about

75% of the roots were co-transformed.

It has been shown that hairy roots either as such or as part of a composite plant, can behave similarly to wild type roots in many root relevant processes such as nodulation or colonization by endomycorrhizal fungi (Boisson-Dernier et al., 2001; Estrada-Navarette et al., 2006), pathogen infection (Fang et al., 2006) and nematode infection (Narayanan et al., 1999; Williamson and Kumar, 2006). Thus we believe that this protocol can have wide applications in *Phaseolus* research. Many problems in bean cultivation arise from biological processes at the root level. Research in this area could already benefit by this fast transgene approach as a research tool as long as a rapid and reproducible stable transformation system is not at hand for *P. vulgaris*. Recent techniques such as gene silencing also allows for this system to provide a rapid tool for analysis of the role of specific genes in certain processes.

The described protocol is a full *in vitro* one, which also

makes it possible to select and work with single transformed hairy roots and assay them *in vitro* for their properties. *In vitro* tissue culture is still preferred in studies related to plant-pathogen interaction or nodulation, as well as in studies related to environmental, hormonal and nutritional conditions.

As the recalcitrant nature of *P. vulgaris* has been a rate limiting step in *Phaseolus* research, this fast and easy approach, complementary to the protocol by Estrada-Navarette et al. (2006) to obtain *in vivo* composite plants, offers a new avenue for the genetic analysis of this most important food legume.

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