Plant Cell Rep (2002) 21:333–340
DOI 10.1007/s00299-002-0518-0

J. De Clercq · M. Zambre · M. Van Montagu
W. Dillen · G. Angenon

An optimized *Agrobacterium*-mediated transformation procedure for *Phaseolus acutifolius* A. Gray

Received: 10 December 2001 / Revised: 1 August 2002 / Accepted: 7 August 2002 / Published online: 25 September 2002

Abstract To improve *Agrobacterium tumefaciens*-mediated transformation of *Phaseolus acutifolius*, we examined the effect of different factors on T-DNA transfer by measuring transient expression levels of an intron-containing β-glucuronidase gene. Improved transformation frequencies were obtained with an *A. tumefaciens* strain carrying nopaline-type virulence genes and when calli were infected with *Agrobacterium* cells in the early-log growth phase. Optimized co-cultivation was performed at 22°C under a 16/8-h (day/night) photoperiod in an acidic medium (pH 5.5) in the presence of 200 μ*M* acetosyringone. By combining the best treatments, an efficient and reproducible transformation procedure was established for the *P. acutifolius* genotype NI576. Southern and immunoblot analyses confirmed the stable integration and expression of the transgenes in the primary transgenic plants and their progeny.

Keywords *Agrobacterium* · Callus · *Phaseolus* · Tepary bean · Transformation

Communicated by K. Glimelius

J. De Clercq · M. Zambre · M. Van Montagu (✉) · W. Dillen
Department of Plant Systems Biology,
Flanders Interuniversity Institute for Biotechnology,
Ghent University, K.L. Ledeganckstraat 35, 9000 Gent, Belgium
e-mail: marc.vanmontagu@gengenp.rug.ac.be
Fax: +32-9-2645349

G. Angenon
Laboratory of Plant Genetics, Department
of Applied Biological Sciences,
Institute for Molecular Biology and Biotechnology,
Vrije Universiteit Brussel, Paardenstraat 65,
1640 Sint-Genesius-Rode, Belgium

Present address:
J. De Clercq, Vakgroep Moleculaire Biotechnologie,
Faculteit Landbouwkundige en Toegepaste
Biologische Wetenschappen, Universiteit Gent,
Coupure links 653, 9000 Gent, Belgium

Present address:
W. Dillen, CropDesign N.V., Technologiepark 3, 9052 Zwijnaarde, Belgium

Abbreviations *AS*: Acetosyringone · *CIM1*: Callus-inducing medium 1 · *GUS*: β-Glucuronidase · *MES*: 2-(*N*-Morpholino) ethanesulfonic acid · *nptII*: Neomycin phosphotransferase II gene · *uidA*: β-glucuronidase gene from *Escherichia coli*

Introduction

There are five cultivated species within the genus *Phaseolus* (*P. vulgaris*, *P. acutifolius*, *P. polyanthus*, *P. coccineus*, and *P. lunatus*), of which *P. vulgaris* is economically the most important. *Phaseolus* beans are an important food source, providing energy and protein for millions of people, mostly in Latin America and Africa. The world production of *Phaseolus* beans is approximately 20 million tons per year. To improve the crop, gene engineering is needed in addition to classical breeding techniques. Stable transformation of grain legumes, however, is difficult to achieve. With respect to *P. vulgaris* transformation, to date transgenic plants have been obtained only through particle bombardment of seedling apical meristems (Russell et al. 1993; Aragão et al. 1996), and the transformation frequencies obtained with this method were relatively low. More efficient production of transgenic plants can be expected from procedures based on rigorous selection for transformed tissues and subsequent regeneration from such tissues. Moreover, for the introduction of foreign genes into higher plants, *Agrobacterium tumefaciens*-mediated transformation is generally preferred to direct gene delivery methods, such as particle bombardment, because the former method offers several advantages – integration of a well-defined DNA sequence, potentially low copy number, high co-expression of the introduced genes and preferential integration into active regions of the chromosome (Birch 1997; Gheysen et al. 1998).

Within the genus *Phaseolus* Dillen et al. (1996) encountered regeneration capacity in the tepary bean, *P. acutifolius*, of which various genotypes could be re-

generated. This regeneration system was used to develop an *Agrobacterium*-based transformation procedure (Dillen et al. 1997a; Goossens et al. 1999).

The goal of the investigation reported here was to improve the transformation efficiency and to demonstrate the reproducibility of this transformation procedure for *P. acutifolius*. Therefore, the influence of different co-cultivation conditions on the efficiency of *Agrobacterium*-mediated gene transfer was assessed using the *uidA*-intron marker system. Secondly, the optimized transformation conditions were shown to facilitate the production of transgenic *P. acutifolius* plants. Transgene integration and inheritance in these transformants were examined.

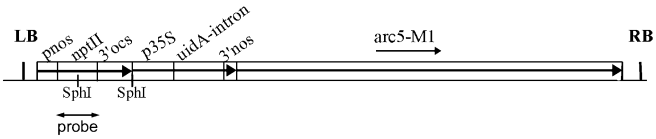

Fig. 1 The transferred region of plasmid pATARC3-M1. *RB* right border, *LB* left border, *pnos* promoter of the nopaline synthase gene, *nptII* neomycin phosphotransferase II gene, *3′ocs* 3′ signal of octopine synthase, *p35S* the cauliflower mosaic virus 35S promoter, *uidA-intron* GUS gene *Escherichia coli* with the potato *st-ls1* intron, *3′nos* 3′ signal of nopaline synthase, *arc5-M1* gene coding for the Arcelin 5a seed storage protein with four mutations in the coding sequence to increase the methionine content

Materials and methods

Bacterial strains and plasmids

In the initial experiments, we used *Agrobacterium tumefaciens* nopaline-type strain C58C1Rif^R that harbours the binary plasmid pTJK136 (Kapila et al. 1997). For the subsequent optimization experiments, we used binary plasmid pATARC3-B1b (Goossens et al. 1999) and for the stable transformation experiment, binary plasmid pATARC3-M1 (De Clercq et al., in preparation). All of these binary vectors contain the same P35S-*uidA*-intron-3′*nos* and P*nos*-*nptII*-3′*ocs* cassettes between the T-DNA borders. In addition, pATARC3-B1b contains a *Phaseolus vulgaris* genomic fragment that codes for the Arcelin 5a seed storage protein gene (*arc5-I*), whereas pATARC3-M1 contains an *arc5-I* gene with four mutations in the coding sequence to increase the methionine content (Fig. 1). Three types of non-oncogenic Ti plasmids were tested: the nopaline-type pMP90 (Koncz and Schell 1986), the octopine-type pGV2260 (Deblaere et al. 1985) and the agropine/succinamopine-type pEHA101 (Hood et al. 1986).

Agrobacterium transformation

All experiments were based on the transformation protocol previously described by Dillen et al. (1997a) and performed with regeneration-competent callus of the *P. acutifolius* A. Gray genotype NI576 (Dillen et al. 1996). Bud explants from greenhouse-grown plants were initiated on CIM1 medium [Murashige and Skoog salts (Murashige and Skoog 1962), 20 g/l sucrose, 8 g/l bacto agar (Difco, Detroit, Mich.), pH 5.7, 0.5 mg/l thidiazuron, and 0.25 mg/l indole-3-acetic acid] and subcultured on fresh CIM1 medium every 3 weeks. Five days after the third subculture, callus explants were cultured at a density of 15 pieces in 24 ml of co-cultivation medium. Agrobacteria were added to the calli to a final OD_{600} of 0.8, with 1 OD_{600} corresponding to $1.1\pm0.2\times10^9$ cells/ml for all *Agrobacterium* strains used. After 2 days of co-cultivation, the explants were washed twice and cultured on non-selective or selective media (20 mg/l geneticin) for optimization and stable transformation experiments, respectively.

GUS assay

Comparisons of the transient expression levels were made by assaying for expression of the *uidA*-intron gene in the explants 4 days after co-cultivation. GUS activity was localized histochemically as described previously (Dillen et al. 1997b).

Transformation frequency was evaluated as the total number of blue spots observed with the naked eye. A distinction was made between small spots (<0.5 mm in diameter), representing one or a few *uidA*-expressing cells, and large spots (>1 mm in diameter), representing a complete cell cluster expressing the *uidA* gene.

Factor evaluation

For each factor tested, at least two independent experiments were performed (with the exception of the pH parameter). In each experiment, a minimum of 30 callus pieces was used per treatment.

To test the effect of the growth phase of the *A. tumefaciens* inocula, we compared bacteria obtained from cultures grown for 36 h (late-log phase, $OD_{600}=2.5\pm0.3$) with those grown for 12 h (early-log phase, $OD_{600}=1.4\pm0.2$). A colony from a freshly streaked plate was used to inoculate 5 ml yeast extract broth medium (Grimsley et al. 1986) containing the appropriate antibiotics. The culture was incubated for exactly 12 h at 28°C under continuous shaking (150 rpm). This preculture ($OD_{600}=1.2\pm0.2$) was then diluted 20-fold in 100 ml yeast extract broth medium in a 500-ml Erlenmeyer and incubated for exactly 12 h or 36 h under identical conditions.

To examine the influence of light, we compared co-cultivation under total darkness and under a photoperiod of 16/8 h (light/dark) with light supplied at an intensity of 20 µmol/m^2 per second. To avoid temperature differences resulting from irradiation heating, the experiment was carried out in a water bath (Dillen et al. 1997b). Total darkness was achieved by wrapping the jars in aluminum foil.

Stable transformation and regeneration of transgenic plants

All selection and regeneration steps used to obtain transgenic *P. acutifolius* plants were performed as described by Dillen et al. (1997a). Regenerated shoots were grafted in vitro on *P. acutifolius* NI574 rootstocks as described by Zambre et al. (2001).

Analysis of transgenic plants

The presence of the *nptII* gene was analysed by Southern blot analysis. Genomic DNA was extracted from leaves of non-transformed and putative primary transformants by the procedure described by Dillen et al. (1997a). DNA (7 µg) was digested with *Sph*I, fractionated on a 1% agarose gel by electrophoresis, transferred to a Hybond-N$^+$ membrane (Amersham Pharmacia Biotech, Little Chalfont, UK) and hybridized at 65°C with a 0.8-kb *Bam*HI/*Bgl*II *nptII* fragment excised from pGemD (Ingelbrecht et al. 1989). The probe was radiolabelled with [^{32}P] using the T7 Quick Ready Prime II kit (Amersham Pharmacia Biotech).

For the detection of Arcelin 5, total protein extracts of T_1 seeds were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis according to Goossens et al. (1999). Accumulation levels were estimated by analysing a grey-scale image of the immunoblot using the IMAGEMASTER VDS software (Amersham Pharmacia Biotech). The concentration of the Arc5a-M1 proteins was determined relative to the concentration of the positive control B1b-8 (seed of a homozygous

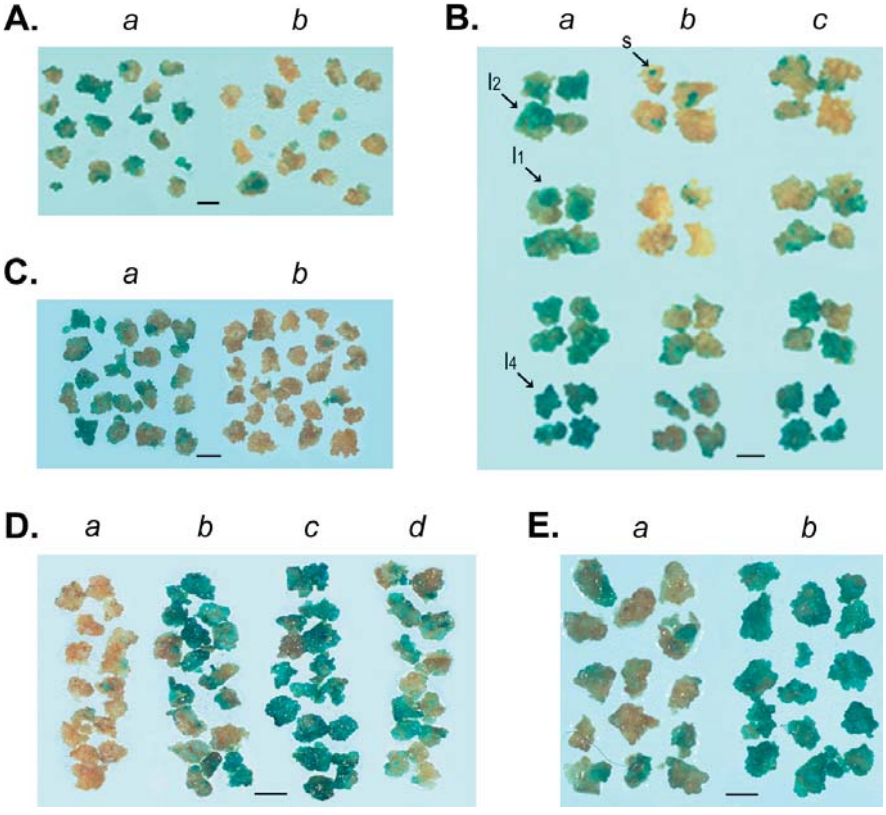

Fig. 2A–E Histological assay of *uidA* expression after co-cultivation with: **A** bacteria of strain C58C1Rif^R(pMP90) (pTJK136) harvested in the early-log phase (*a*) and in late-log phase (*b*), **B** bacteria of strain C58C1Rif^R (pMP90) (pTJK136) (*a*), EHA101 (pTJK136) (*b*) and C58C1Rif^R(pGV2260)(pTJK136) (*c*) at 25°C (*upper two rows*) and 22°C (*lower two rows*) (only the four highest expressing calli are shown), **C** bacteria of strain C58C1Rif^R(pMP90) (pATARC3-B1b) under a 16/8-h (light/dark) photoperiod (*a*) or complete darkness (*b*), **D** bacteria of strain C58C1Rif^R (pMP90)(pATARC3-B1b) in the presence of 0, 20, 200 and 2000 µ*M* AS (*a–d*), **E** bacteria of strain C58C1Rif^R(pMP90) (pATARC3-B1b) in co-cultivation medium without (*a*) and with (*b*) buffering with 20 m*M* MES. To evaluate the transformation frequency, we made a distinction between small (s) and large (*l*) spots. Each *uidA* expression zone covering approximately a quarter of the explant was counted as a large spot: l_1 one large spot, l_2 two large spots, l_4 four large spots. *Bar*: 3 mm

transformant accumulating 23.8% unmodified Arc5a; Goossens et al. 1999).

Results and discussion

The basic transformation procedure for *Phaseolus acutifolius* was as previously described (Dillen et al. 1997a). Briefly, regeneration-competent calli of the genotype NI576, established from bud explants (Dillen et al. 1996), were co-cultivated 5 days after the third subculture. The *Agrobacterium* inocula were pre-incubated with 200 µ*M* AS for 4 h and then added to the callus explants at an initial OD_{600} of 0.8. Co-cultivation was performed for 48 h in liquid co-cultivation medium containing 10 m*M* glucose, 20 µ*M* AS and 20 m*M* MES, pH 5.5. After co-cultivation, the calli were washed and cultured on non-selective medium. The influence of changing the transformation conditions was assessed by using *Agrobacterium* strains with a binary vector harbouring the *uidA*-coding sequence interrupted by an intron (pTJK136 or pATARC3-B1b). Transient expression of the *uidA*-intron gene was determined histochemically 4 days after co-cultivation of the calli.

In a first comparative experiment, calli were infected with *Agrobacterium* strain C58C1Rif^R(pMP90)(pTJK136) collected from either the early-log phase (12 h growth) or late-log phase (36 h growth) cultures. The bacteria were added at the same optical density (OD_{600}=0.8). Infection with bacteria from an early-log phase yielded many more *uidA* expression zones than infection with bacteria in a late-log phase (Fig. 2A, Table 1). This experiment was repeated three times and always gave the same result. In contrast to our results, tests performed by De Bondt et al. (1994) on the transformation of apple leaf explants with three different strains in either the lag phase, mid-log phase or stationary phase showed no significant effect of the bacterial growth phase. Because of the impact of the bacterial growth phase on the infection of *P. acutifolius* calli, all subsequent experiments were performed with early-log phase inocula.

To determine the influence of temperature during co-cultivation, we performed experiments at temperatures ranging from 15°C to 29°C. In correspondence with our previous results (Dillen et al. 1997b), we found the optimum temperature for co-cultivation to be 22°C,

Table 1 Number of large and small *uidA* expression zones observed in two representative experiments with 30 explants each (*SD* standard deviation)

Treatment		*uidA* expression zones[a] (mean ± SD)	
		Large	Small
Growth phase[b]	Early-log (12 h)	12.5±2.1	30.5±3.5
	Late-log (36 h)	2.5±0.7	16.5±2.1
Plasmid (25°C)[c]	pMP90	12.5±2.1	30.5±3.5
	pEHA101	1.5±2.1	13.5±7.8
	pGV2260	3.5±2.1	24.0±0.0
Plasmid (22°C)[c]	pMP90	30.5±2.1	20.5±4.2
	pEHA101	2.5±0.7	16.5±2.1
	pGV2260	22.5±11.3	33.0±4.2
Light[d]	16/8 h (light/dark)	27.0±4.2	39.0±3.5
	24 h (dark)	0.5±0.7	4.5±3.5
AS concentration[e]	0 μ*M*	3.0±1.4	33.0±4.2
	20 μ*M*	32.0±2.8	46.0±16.9
	200 μ*M*	47.0±1.4	66.5±9.2
	2000 μ*M*	21.5±2.1	26.5±17.7

[a] See Materials and methods and Fig. 2 for definition
[b] Co-cultivation with C58C1Rif^R(pMP90)(pTJK136) harvested at different growth phases
[c] Co-cultivation with C58C1Rif^R(pTJK136) and three different non-oncogenic Ti plasmids
[d] Co-cultivation with C58C1Rif^R (pMP90)(pATARC3–B1b) under different photoperiods
[e] Co-cultivation with C58C1Rif^R(pMP90)(pATARC3–B1b) in the presence of different AS concentrations

irrespective of the type of previous non-oncogenic Ti plasmid. *UidA* expression markedly decreased when the temperature was increased to 25°C. Very low levels of *uidA* expression were observed at 27°C and 19°C, and no expression was observed at 29°C and 15°C. The difference in the number of *uidA* expression zones at 22°C and 25°C is notable (Fig. 2B; Table 1). Similar results were obtained when the effect of temperature was investigated in garlic transformation (Kondo et al. 2000). The highest transient *uidA* expression in garlic calli was observed at 22°C, whereas the ratio of GUS-stained calli to total calli decreased by 85% at 20°C and by 69% at 24°C. Results on the stable transformation of cotton were also in line with those of our experiments (Sunilkumar and Rathore 2001). Co-cultivation of cotyledon discs at 21°C, compared to 25°C, consistently resulted in higher transformation frequencies.

To examine the effect of the type of non-oncogenic Ti plasmid, we compared a nopaline-type plasmid (pMP90) with an octopine-type (pGV2260) and a succinamopine/agropine-type (pEHA101) together with the binary vector pTJK136. From experiments performed at 25°C, it was obvious that the efficiency of DNA transfer was highest with the nopaline helper plasmid. The number of *uidA*-expressing zones seen with pMP90 was much higher than that with pGV2260 and pEHA101 (Fig. 2B, upper rows; Table 1). Also at 22°C, the highest GUS activity was obtained with the strain carrying pMP90, although the difference with the strain carrying pGV2260 was not as marked as at 25°C (Fig. 2B, lower rows; Table 1). As a result, we decided to use only pMP90 as the helper plasmid in subsequent experiments. All of our experiments clearly showed that pEHA101 was the least efficient helper plasmid for *P. acutifolius* callus transformation. This observation is surprising because *Agrobacterium* strain EHA101 (or its derivative EHA105, or its oncogenic progenitor A281) is generally recognized as being superior in facilitating gene transfer to plant cells; for example, in leguminous species, such as soybean (Meurer et al. 1998; Donaldson and Simmonds 2000), pea (Nadolska-Orczyk and Orczyk 2000) and peanut (Egnin et al. 1998), but also in other crops, such as apple (De Bondt et al. 1994), cabbage (Takasaki et al. 1997), blueberry (Cao et al. 1998) and wheat and barley (Guo et al. 1998). However, the fact that this strain is not better than other strains, or even worse, has also been observed in *Phaseolus vulgaris* (Zhang et al. 1997) and in other plants, such as cotton (Sunilkumar and Rathore 2001), rice (Hiei et al. 1994, 1997), kalanchoe (Jia et al. 1989), black locust (Igasaki et al. 2000) and hibiscus (Srivatanakul et al. 2001). This underscores the importance of testing various *A. tumefaciens* strains for every species and genotype under study.

To test the effect of light conditions during co-cultivation on the gene transfer, we co-cultivated calli in complete darkness or under a 16/8-h (light/dark) photoperiod. In darkness, almost no GUS activity could be detected (Fig. 2C, Table 1). Moreover, the explant survival rate was drastically reduced. We concluded that co-cultivation in darkness was deleterious for the *P. acutifolius* calli. Many other *A. tumefaciens*-mediated transformation protocols specifically use dark co-cultivation conditions and, on the basis of the present results, it may be useful to reconsider the light parameter in such procedures. Because the influence of different light conditions during co-cultivation has not received much attention until now, subsequent light experiments were conducted and will be published elsewhere (Zambre et al. 2002).

A series of experiments were aimed at a better induction of the *vir* genes. The *vir* genes of the Ti plasmid mediate the transfer of T-DNA from *A. tumefaciens* to plant cells and the steps preceding integration into the plant genome (for review, see Gelvin 2000). Transcription of these *vir* genes can be induced by various related phenolic compounds, such as AS (Stachel et al. 1985), and certain sugars act synergistically with these phenolic inducers (Cangelosi et al. 1990). In addition, an acidic pH is needed for optimal expression of the *vir* genes (Stachel et al. 1986). Turk et al. (1991) found a maximum *vir* induction response at pH 5.3 for an octopine and leucinopine strain and at pH 5.8 for a nopaline and agropine strain. In many systems, the addition of AS to the co-cultivation medium and/or *Agrobacterium* pre-culture have proven to be beneficial. Pretreating the explants with AS can also enhance the transformation (Guivarc'h et al. 1993; Boase et al. 1998). In our standard transformation protocol, the agrobacteria are pre-cultured for 4 h in the presence of 200 μ*M* AS and co-cultivated in medium supplemented with 20 μ*M* AS. To investigate whether AS is needed in the co-cultivation medium to obtain *Agrobacterium*-mediated gene transfer to *P. acutifolius* calli and whether the efficiency can be improved, AS was added at different concentrations (0, 20, 200 and 2,000 μ*M*) to the co-cultivation medium. This experiment was repeated more than five times, and always gave consistent results: the number of *uidA*-expressing zones increased with increasing AS concentration and reached a maximum at 200 μ*M* AS (Fig. 2D, Table 1). When 2,000 μ*M* AS was used, less blue staining was observed and the calli had necrotic zones; more than 10% of the explants did not survive this co-cultivation. A positive effect of AS on *Agrobacterium*-mediated gene transfer to *Phaseolus* cells has been suggested in previous experiments (Becker et al. 1994), where increased tumour proliferation on cotyledonary node explants of *P. vulgaris* was observed after co-cultivation with *A. tumefaciens* in the presence of 100 μ*M* AS.

The necessity of an acidic pH during co-cultivation was also tested. In the experiments of Turk et al. (1991), the nopaline strain showed a maximum response at pH 5.8 and still displayed significant *vir* induction at a higher pH. Our co-cultivation medium was prepared with an initial pH of 5.5, which, however, may change during the co-cultivation period. Consequently, we determined whether it was necessary to stabilize the pH of the co-cultivation medium during the whole co-cultivation period, which was achieved by adding 20 m*M* MES. When 20 m*M* MES was supplemented to the co-cultivation medium, the pH of the medium after the co-cultivation period was still 5.5–5.6; without buffering, the pH rose to 7.2 during co-cultivation. Figure 2E shows that this high pH seemed to prevent for the most part gene transfer. Many more *uidA*-expressing zones (46 large and 73 small zones) were observed on 30 explants co-cultivated with bacteria of strain C58C1Rif^{R}(pMP90)(pATARC3-B1b), in medium with than in medium without buffering (four large and 16 small zones). Our results are in contrast to those obtained by Becker et al. (1994) in *P. vulgaris* with an octopine-type bacterial strain; here, buffering with MES had an inhibitory effect, whereas the pH was not a critical factor for *Agrobacterium* infection.

Other factors tested were age and size of the calli, density of the calli in co-cultivation medium and time between subculturing and co-cultivation, but none of these parameters seemed to substantially affect the efficiency of transformation. We also carried out experiments in which a higher density of bacterial inoculum (1.6–2.4 OD_{600}) was used and a longer co-cultivation period (4 days) applied. These circumstances prevented proper killing of the bacteria after the co-cultivation and were also detrimental for the calli, resulting in a drastically decreased explant survival rate already 4 days after co-culivation.

Systematic evaluation of the various parameters enabled us to improve the transformation procedure and to obtain a very high level of transient *uidA* expression in the calli. This co-cultivation protocol includes the following conditions: (1) an *Agrobacterium* strain with pMP90 as helper plasmid; (2) harvesting *Agrobacterium* cells in the early-log phase; (3) co-cultivation under a 16/8-h (light/dark) photoperiod at 22°C; (4) co-cultivation medium buffered at pH 5.5 with 20 m*M* MES; (5) co-cultivation in the presence of 200 μ*M* AS.

Transient expression studies may not always be relevant to stable transformation. The results from some investigations have made it clear that while T-DNA transfer from *A. tumefaciens* to the plant cell can occur in an appropriate manner, as demonstrated by efficient transient transformation, T-DNA integration may be limiting, resulting in poor stable transformation. In these cases, an improvement in the transient expression levels will not necessarily result in better stable transformation (Shen et al. 1993; Ishida et al. 1996; Maximova et al. 1998). However, most studies have shown that conditions leading to enhanced transient expression do result in a higher number of transformed plants (Cao et al. 1998; Kondo et al. 2000; Niu et al. 2000; Zhang et al. 2000; Choi et al. 2001; Trifonova et al. 2001; Suzuki and Nakano 2002).

Our results also show improved rates of stable transformation using the procedure that gives optimum transient expression. Before optimization, one to two independent transformants could be produced in one transformation experiment with 200 explants. When the improved procedure was used, ten independent transgenic lines of the *P. acutifolius* wild genotype NI576 were obtained from a single experiment with 150 explants. Following co-cultivation, the callus explants were transferred to selective medium supplemented with 20 mg/l geneticin. During four subcultures, each lasting 3 weeks, selection took place. Transgenic shoots developed on these selected callus lines. The number of phenotypically normal shoots produced varied among the different transgenic lines from five to more than 50 shoots. Because survival of the rooted shoots was poor, the shoots were in vitro-grafted when three trifoliate leaves

had been produced. From one independent callus line, different clonal transformants could be established. All of the transgenic plants were fully fertile and produced seeds in quantities comparable with those of non-transformed plants. The time from co-cultivation to obtaining transgenic seeds varied from 9 months to 12 months.

The stable integration of the introduced transgenes was confirmed by Southern analysis. DNA isolated from leaves of all ten independent primary transformants was digested with *Sph*I and probed with an *nptII* fragment (Fig. 1). This probe can hybridize to an internal 1.4-kb T-DNA fragment and to a junction of plant DNA and a left-border fragment of the integrated T-DNA. As shown in Fig. 3, these fragments were found for nine of the ten transformants. Transformants 2, 3, 4 and 6 probably harbour one T-DNA insertion, and the other transformants harbour two or more. In plant no. 10, the *nptII* gene was not present, and *uidA* expression could not be detected in a histochemical assay (data not shown), suggesting that this plant was not stably transformed. To further confirm transformation, T_1 seeds were harvested and seed proteins extracted and analysed by SDS-PAGE and immunoblotting. The binary vector used in this stable transformation experiment, pATARC3-M1, contains between the T-DNA borders a modified *arc5-I* gene coding for the *P. vulgaris* seed storage protein Arcelin 5a enhanced with four methionine codons (De Clercq et al. in preparation). This protein was found in seeds of eight out of the ten transgenic plants (Fig. 4). As expected, the modified *arc5-I* gene was absent in transformant no. 10, but because the T_1 seeds were not tested for the presence of the *nptII* or *uidA* marker genes before extraction, a negative result could also be related to the segregation of the transgenes, which was probably the case in transformant no. 2. The relative concentration of Arcelin 5-M1 in the T_1 seeds varied from 11% to 29% of the total extracted seed protein. These results confirmed the transmission of the transgenes to the offspring.

In the investigation reported here we were able to establish an improved *Agrobacterium*-mediated transformation system for *P. acutifolius* that allows the efficient production of transgenic plants. The optimized conditions were also used to produce several series of transformants of the *P. acutifolius* cv. TB1 (Zambre et al. in preparation). Within the grain legumes, *P. acutifolius* is now one of the few species for which the number of transformed plants that can be generated is large enough to enable the application of transgenic approaches for applied and fundamental research. *P. acutifolius* can be used to test gene engineering strategies, such as enhancing resistance against pests and diseases, enhancing tolerance against abiotic stresses and improving seed nutritional quality (for example, increasing the methionine content and reducing antinutritional factors).

The parameters that we examined only concern the 48 h of the co-cultivation period and should thus have a minimal impact on tissue culture and regeneration response. Similar optimizations may therefore be useful for improving other transformation protocols.

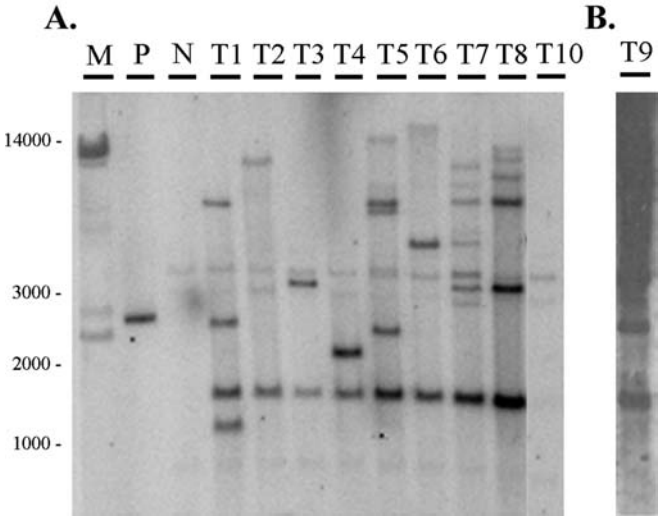

Fig. 3A, B Southern analysis of a non-transformed NI576 plant (*lane N*) and primary transformants (*lanes T1–T10*). *Sph*I-digested DNA was probed with an *nptII* fragment hybridizing to an internal 1.4-kb fragment and LB fragments in transgenic plants (see Fig. 1). T2, T3, T4, and T6 probably harbour one T-DNA insertion and T1, T5, T7, T8 and T9 two or more. T10 did not react with the probe. The digest of T9 was not successful in Southern blot **A**, so part of another Southern blot (**B**) is attached. *Lane P* contains an *nptII* plasmid fragment of 2.5 kb. *Lane M* contains marker DNA of which the size is indicated on the *left* (in basepairs)

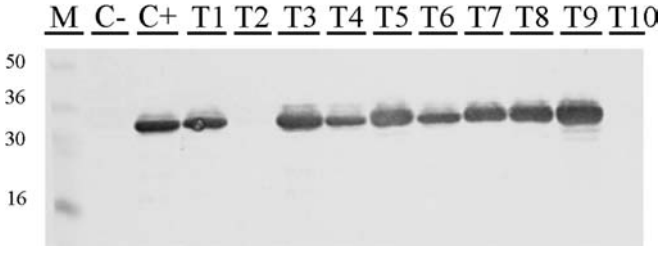

Fig. 4 Immunoblot analysis of crude protein extracts of progeny (T_1) seeds of non-transformed NI576 (*C–*) and primary transformants transformed with the unmodified *arc5–I* gene (*C+*) and the modified *arc5I-M1* gene (*T1–T10*). *Lane M* contains the marker proteins of which the molecular mass is indicated on the *left* (in kiloDaltons)

Acknowledgements The authors thank Christa Verplancke for technical assistance with Southern analysis, Alain Goossens for assistance with the protein work, Karel Spruyt for help with the preparation of the figures and Martine De Cock for help in preparing the manuscript. J.D.C. is indebted to the Vlaams Instituut voor de Bevordering van het Wetenschappelijk-Technologisch Onderzoek in de Industrie for a pre-doctoral fellowship.

References

Aragão FJL, Barros LMG, Brasileiro ACM, Ribeiro SG, Smith FD, Sanford JC, Faria JC, Rech EL (1996) Inheritance of foreign genes in transgenic bean (*Phaseolus vulgaris* L.) co-transformed via particle bombardment. Theor Appl Genet 93:142–150

Becker J, Vogel T, Iqbal J, Nagl W (1994) *Agrobacterium*-mediated transformation of *Phaseolus vulgaris*. Adaptation of some conditions. Ann Rep Bean Improv Coop 37:127–128

Birch RG (1997) Plant transformation: problems and strategies for practical application. Annu Rev Plant Physiol Plant Mol Biol 48:297–326

Boase MR, Bradley JM, Borst NK (1998) An improved method for transformation of regal pelargonium (*Pelargonium* × *domesticum* Dubonnet) by *Agrobacterium tumefaciens*. Plant Sci 139:59–69

Cangelosi GA, Ankenbauer RG, Nester EW (1990) Sugars induce the *Agrobacterium* virulence genes through a periplasmic binding protein and a transmembrane signal protein. Proc Natl Acad Sci USA 87:6708–6712

Cao X, Liu Q, Rowland LJ, Hammerschlag FA (1998) GUS expression in blueberry (*Vaccinium* spp.): factors influencing *Agrobacterium*-mediated gene transfer efficiency. Plant Cell Rep 18:266–270

Choi YE, Yang DC, Kusano T, Sano H (2001) Rapid and efficient *Agrobacterium*-mediated transformation of *Panax ginseng* by plasmolyzing pre-treatment of cotyledons. Plant Cell Rep 20:616–621

De Bondt A, Eggermont K, Druart P, De Vil M, Goderis I, Vanderleyden J, Broekaert WF (1994) *Agrobacterium*-mediated transformation of apple (*Malus* × *domestica* Borkh.): an assessment of factors affecting gene transfer efficiency during early transformation steps. Plant Cell Rep 13:587–593

Deblaere R, Bytebier B, De Greve H, Deboeck F, Schell J, Van Montagu M, Leemans J (1985) Efficient octopine Ti plasmid-derived vectors for *Agrobacterium*-mediated gene transfer to plants. Nucleic Acids Res 13:4777–4788

Dillen W, De Clercq J, Van Montagu M, Angenon G (1996) Plant regeneration from callus in a range of *Phaseolus acutifolius* A. Gray genotypes. Plant Sci 118:81–88

Dillen W, De Clercq J, Goossens A, Van Montagu M, Angenon G (1997a) *Agrobacterium*-mediated transformation of *Phaseolus acutifolius* A. Gray. Theor Appl Genet 94:151–158

Dillen W, De Clercq J, Kapila J, Zambre M, Van Montagu M, Angenon G (1997b) The effect of temperature on *Agrobacterium tumefaciens*-mediated gene transfer to plants. Plant J 12: 1459–1463

Donaldson PA, Simmonds DH (2000) Susceptibility to *Agrobacterium tumefaciens* and cotyledonary node transformation in short-season soybean. Plant Cell Rep 19:478–484

Egnin M, Mora A, Prakash CS (1998) Factors enhancing *Agrobacterium tumefaciens*-mediated gene transfer in peanut (*Arachis hypogaea* L.). In Vitro Cell Dev Biol Plant 34:310–318

Gelvin SB (2000) *Agrobacterium* and plant genes involved in T-DNA transfer and integration. Annu Rev Plant Physiol Plant Mol Biol 51:223–256

Gheysen G, Angenon G, Van Montagu M (1998) *Agrobacterium*-mediated plant transformation: a scientifically intriguing story with significant applications. In: Lindsey K (ed) Transgenic plant research. Harwood Academic, Amsterdam, pp 1–33

Goossens A, Dillen W, De Clercq J, Van Montagu M, Angenon G (1999) The *arcelin-5* gene of *Phaseolus vulgaris* directs high seed-specific expression in transgenic *Phaseolus acutifolius* and Arabidopsis plants. Plant Physiol 120:1095–1104

Grimsley N, Hohn B, Hohn T, Walden R (1986) "Agroinfection", an alternative route for viral infection of plants by using the Ti plasmid. Proc Natl Acad Sci USA 83:3282–3286

Guivarc'h A, Caissard J-C, Brown S, Marie D, Dewitte W, Van Onckelen H, Chriqui D (1993) Localization of target cells and improvement of *Agrobacterium*-mediated transformation efficiency by direct acetosyringone pretreatment of carrot root discs. Protoplasma 174:10–18

Guo G, Maiwald F, Lorenzen P, Steinbiss H-H (1998) Factors influencing T-DNA transfer into wheat and barley cells by *Agrobacterium tumefaciens*. Cereal Res Commun 26:15–22

Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J 6:271–282

Hiei Y, Komari T, Kubo T (1997) Transformation of rice mediated by *Agrobacterium tumefaciens*. Plant Mol Biol 35:205–218

Hood EE, Helmer GL, Fraley RT, Chilton M-D (1986) The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded in a region of pTiBo542 outside of T-DNA. J Bacteriol 168: 1291–1301

Igasaki T, Mohri T, Ichikawa H, Shinohara K (2000) *Agrobacterium tumefaciens*-mediated transformation of *Robinia pseudoacacia*. Plant Cell Rep 19:448–453

Ingelbrecht ILW, Herman LMF, Dekeyser RA, Van Montagu MC, Depicker AG (1989) Different 3′ end regions strongly influence the level of gene expression in plant cells. Plant Cell 1:671–680

Ishida Y, Saito H, Ohta S, Hiei Y, Komari T, Kumashiro T (1996) High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*. Nat Biotechnol 14:745–750

Jia S-R, Yang M-Z, Ott R, Chua N-H (1989) High frequency transformation of *Kalanchoe laciniata*. Plant Cell Rep 8:336–340

Kapila J, De Rycke R, Van Montagu M, Angenon G (1997) An *Agrobacterium*-mediated transient gene expression system for intact leaves. Plant Sci 122:101–108

Koncz C, Schell J (1986) The promoter of T_L-DNA gene *5* controls the tissue-specific expression of chimaeric genes carried by a novel type of *Agrobacterium* binary vector. Mol Gen Genet 204:383–396

Kondo T, Hasegawa H, Suzuki M (2000) Transformation and regeneration of garlic (*Allium sativum* L.) by *Agrobacterium*-mediated gene transfer. Plant Cell Rep 19:989–993

Maximova SN, Dandekar AM, Guiltinan MJ (1998) Investigation of *Agrobacterium*-mediated transformation of apple using green fluorescent protein: high transient expression and low stable transformation suggest that factors other than T-DNA transfer are rate-limiting. Plant Mol Biol 37:549–559

Meurer CA, Dinkins RD, Collins GB (1998) Factors affecting soybean cotyledonary node transformation. Plant Cell Rep 18:180–186

Murashige T, Skoog F (l962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–493

Nadolska-Orczyk A, Orczyk W (2000) Study of the factors influencing *Agrobacterium*-mediated transformation of pea (*Pisum sativum* L.). Mol Breed 6:185–194

Niu X, Li X, Veronese P, Bressan RA, Weller SC, Hasegawa PM (2000) Factors affecting *Agrobacterium tumefaciens*-mediated transformation of peppermint. Plant Cell Rep 19:304–310

Russell DR, Wallace KM, Bathe JH, Martinell BJ, McCabe DE (1993) Stable transformation of *Phaseolus vulgaris* via electric-discharge mediated particle acceleration. Plant Cell Rep 12:165–169

Shen W-H, Escudero J, Schläppi M, Ramos C, Hohn B, Koukolíková-Nicola Z (1993) T-DNA transfer to maize cells: histochemical investigation of β-glucuronidase activity in maize tissues. Proc Natl Acad Sci USA 90:1488–1492

Srivatanakul M, Park SH, Salas MG, Smith RH (2001) Transformation of parameters enhancing T-DNA expression in kenaf (*Hibiscus cannabinus*). J Plant Physiol 158:255–260

Stachel SE, Messens E, Van Montagu M, Zambryski P (1985) Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens*. Nature 318: 624–629

Stachel SE, Nester EW, Zambryski PC (1986) A plant cell factor induces *Agrobacterium tumefaciens vir* gene expression. Proc Natl Acad Sci USA 83:379–383

Sunilkumar G, Rathore KS (2001) Transgenic cotton: factors influencing *Agrobacterium*-mediated transformation and regeneration. Mol Breed 8:37–52

Suzuki S, Nakano M (2002) *Agrobacterium*-mediated production of transgenic plants of *Muscari armeniacum* Leichtl. ex Bak. Plant Cell Rep 20:835–841

Takasaki T, Hatakeyama K, Ojima K, Watanabe M, Toriyama K, Hinata K (1997) Factors influencing *Agrobacterium*-mediated transformation of *Brassica rapa* L. Breed Sci 47:127–134

Trifona A, Madsen S, Olesen A (2001) *Agrobacterium*-mediated transgene delivery and integration into barley under a range of in vitro culture conditions. Plant Sci 161:871–880

Turk SCHJ, Melchers LS, den Dulk-Ras H, Regensburg-Tuïnk AJG, Hooykaas PJJ (1991) Environmental conditions differentially affect *vir* gene induction in different *Agrobacterium* strains. Role of the VirA sensor protein. Plant Mol Biol 16:1051–1059

Zambre M, Geerts P, Maquet A, Van Montagu M, Dillen W, Angenon G (2001) Regeneration of fertile plants from callus in *Phaseolus polyanthus* Greenman (Year Bean). Ann Bot 88:371–377

Zambre M, Terryn N, De Clercq J, De Buck S, Dillen W, Van Montagu M, Van Der Straeten D, Angenon G (2002) Light strongly promotes gene transfer from *Agrobacterium tumefaciens* to plant cells. Planta (in press)

Zhang F-L, Yakahata Y, Watanabe M, Xu J-B (2000) *Agrobacterium*-mediated transformation of cotyledonary explants of Chinese cabbage (*Brassica campestris* L. ssp. *pekinensis*). Plant Cell Rep 19:569–575

Zhang Z, Coyne DP, Mitra A (1997) Factors affecting *Agrobacterium*-mediated transformation of common bean. J Am Soc Hortic Sci 122:300–305