Theor Appl Genet (2005) 110: 914–924
DOI 10.1007/s00122-004-1910-7

ORIGINAL PAPER

M. Zambre · A. Goossens · C. Cardona
M. Van Montagu · N. Terryn · G. Angenon

A reproducible genetic transformation system for cultivated *Phaseolus acutifolius* (tepary bean) and its use to assess the role of arcelins in resistance to the Mexican bean weevil

Received: 19 July 2004 / Accepted: 13 December 2004 / Published online: 9 February 2005

Abstract A reproducible *Agrobacterium tumefaciens*-mediated genetic transformation method that delivers fertile and morphologically normal transgenic plants was developed for cultivated tepary bean (*Phaseolus acutifolius* L. Gray). Factors contributing to higher transformation efficiencies include (1) a low initial concentration of bacteria coupled with a longer cocultivation period with callus, (2) an initial selection of callus on a medium containing low levels of the selectable agent, (3) omission of the selectable agent from the medium during callus differentiation to shoots and (4) the efficient conversion of transgenic shoots into fertile plants. All plants regenerated with this procedure (T_0) were stably transformed, and the introduced foreign genes were inherited in a Mendelian fashion in most of the 33 independent transformants. Integration, stable transmission and high expression levels of the transgenes were observed in the T_1 and/or T_3 progenies of the transgenic lines. The binary transformation vectors contained the β-glucuronidase reporter gene, the neomycin phosphotransferase II selectable marker gene and either an *arcelin 1* or an *arcelin 5* gene. Arcelins are seed proteins that are very abundant in some wild *P. vulgaris* L. genotypes showing resistance to the storage insect *Zabrotes subfasciatus* (Boheman) (Coleoptera, Bruchidae). Transgenic beans from two different cultivated *P. acutifolius* genotypes with high arcelin levels were infested with *Z. subfasciatus*, but they were only marginally less susceptible to infestation than the non-transgenic *P. acutifolius*. Hence, the arcelin genes tested here are not major determinants of resistance against *Z. subfasciatus*.

Electronic Supplementary Material Supplementary material is available for this article at http://dx.doi.org/10.1007/s00122-004-1910-7

Communicated by C. Möllers

M. Zambre · A. Goossens · M. Van Montagu · G. Angenon
Department of Plant Systems Biology,
Flanders Interuniversity Institute for Biotechnology (VIB),
Ghent University, Technologiepark 927, 9052 Gent, Belgium

M. Zambre · M. Van Montagu (✉) · N. Terryn
Institute for Plant Biotechnology for Developing Countries
(IPBO), Ghent University, K.L. Ledeganckstraat 35,
9000 Gent, Belgium
E-mail: marc.vanmontagu@ugent.be
Tel.: +32-9-2648727
Fax: +32-9-2648795

C. Cardona
Bean Entomology, Centro Internacional de Agricultura Tropical,
Apartado Aéreo, 6713 Cali, Columbia

G. Angenon
Laboratorium Plantengenetica, Vrije Universiteit Brussel,
Pleinlaan 2, 1050 Brussels, Belgium

Introduction

Grain legumes are a very valuable source of dietary proteins for both humans and animals and of these, *Phaseolus* beans represent a major legume with respect to nutrition (Singh 1999). Tepary bean (*Phaseolus acutifolius* L. Gray), one of the five cultivated species of the genus *Phaseolus*, is an annual legume adapted to the arid and semi-arid regions. Interest in *P. acutifolius* stems from its value as a source of several desirable genetic traits, such as its resistance to diseases, pests, high temperature and drought, that could be incorporated into its close relatives, such as *P. vulgaris* (Singh 1999).

To date, the *Phaseolus* species has been transformed genetically with limited success (Christou 1997), with most of the efforts being concentrated on the transformation of *P. vulgaris* (Nagl et al. 1997). Particle bombardment-mediated systems have been developed (Russell et al. 1993; Aragão et al. 1996) to obtain transgenic plants of *P. vulgaris*, but they are labor-intensive and the transformation frequencies obtained are rather low (Christou 1997). *Agrobacterium*-mediated transformation of *P. vulgaris* plants has been unsuccessful (Franklin et al. 1993; Zhang et al. 1997). At the present time, only one successful method has been reported for the *Agrobacterium*-mediated production of transgenic

plants in the large genus *Phaseolus*, namely in *P. acutifolius* (Dillen et al. 1997). Although this procedure has been improved (De Clercq et al. 2002), its use is restricted to a single, wild, small-seeded genotype and it did not allow us to obtain transgenic plants in elite genotypes of *P. acutifolius*. Consequently, no efficient genetic transformation method for any consumable large-seeded *Phaseolus* bean is available that can yield reasonably high numbers of transgenic plants to be used as a routine method. We describe here a reproducible and efficient procedure for obtaining morphologically normal, fertile and stable transgenic lines from cultivated *P. acutifolius*.

The transformation vectors used in this study contained marker genes as well as *arcelin 5* (*arc5*) or *arcelin 1* (*arc1*) genes. Arcelins are seed proteins found in some wild *P. vulgaris* genotypes that are resistant to the bruchid beetle *Zabrotes subfasciatus* (Mexican bean weevil). At least seven arcelin variants exist, each consisting of different isoforms. Resistance to *Z. subfasciatus* is genetically linked to the arcelin locus (Osborn et al. 1988; Cardona et al. 1990), and arcelin proteins have been regarded as its causal factors (Osborn et al. 1988; Sales et al. 2000), although direct evidence that the arcelin genes themselves are involved in resistance is lacking. To the contrary, we have shown that seeds from transgenic wild *P. acutifolius* (genotype NI576) expressing *arc5* genes at high levels are not significantly protected against *Z. subfasciatus* (Goossens et al. 2000). In the investigation reported here, we extended our previous analyses in two ways: (1) we tested a different arcelin variant (arcelin 1 versus arcelin 5 with 63% similarity at the amino acid level) and (2) we used a large-seeded, cultivated genotype instead of the wild genotype NI576. Because the wild genotypes have a hard seed coat that is difficult for the bruchid larvae to penetrate, the former may not be the ideal material for testing insect resistance. In addition, tests with large-seeded cultivated beans are more relevant with respect to applications in grain legumes.

An efficient transformation system for cultivated *P. acutifolius* will be useful in many respects. First, such a system is required to study gene functions in *Phaseolus*, which is especially important in view of the current genomics initiatives in *P. vulgaris* (http://www.phaseolus.net; Broughton et al. 2003). Second, an efficient system can be used for applied research—for example, for molecular farming (De Jaeger et al. 2002). Third, it can be helpful for tagging agronomically interesting genes that are present in *P. acutifolius* and, fourth, it will facilitate the development of *Agrobacterium*-mediated transformation systems for *P. vulgaris*, which is the second most important food legume of the world.

Materials and methods

Plant material and media used

Two cultivated genotypes of *Phaseolus acutifolius*, TB1 (Kumar et al. 1988) and PI440795 (Scott and Michaels 1992), were obtained from the Centro Internacional de Agricultura Tropical (CIAT; Cali, Colombia) and the United States Department of Agriculture (Agricultural Research Station, Pullman, Wash.), respectively. Breeding lines of *P. vulgaris* containing either the Arcelin 1 protein (RAZ2) or no arcelin (Emp175) and wild genotype G02771 containing Arcelin 5 protein have been described previously (Cardona et al. 1990).

The composition of the different culture media used is summarized in Table 1. All media were adjusted to the appropriate pH (see Table 1) before autoclaving at 120 kg/cm^2 for 20 min. The hormones [with the exception of thidiazuron (TDZ)], acetosyringone (AS), vitamins and antibiotics were added following autoclaving to cooled (35–40°C) media. All chemicals were from Sigma-Aldrich (St. Louis, Mo.), and the agar was from Difco (Detroit, Mich.), except for that used in the germination media (GM) (Invitrogen, Carlsbad, Calif.). All callus induction media (CIM) were poured into petri dishes (9 cm or 14.5 cm in diameter; 40 ml or 75 ml per plate, respectively); GM, shoot induction (SIM) and shoot development media (SDM) into glass jars [8 cm (diameter) × 8.5 cm (height); 50–60 ml per jar] with white transparent screw caps; and all callus cocultivation media (CCM) were dispensed in similar jars [8 cm (diameter) × 14.5 cm (height); 200 ml per jar].

Bacteria and plasmids

Agrobacterium tumefaciens strain C58C1Rif^R (pMP90) harboring the binary plasmids pATARC3-B1b or pATARC3-B52b (Goossens et al. 1999) or pATARC3-B515b was used in all experiments. These binary vectors contained the neomycin phosphotransferase II (*nptII*) gene under control of the nopaline synthase promoter and the *β*-glucuronidase (GUS)-encoding gene (*uidA*) with an intron under control of the cauliflower mosaic virus (CaMV) 35S promoter. In addition, the vectors carried three different *arcelin* gene sequences: *arc5-I* encoding Arcelin 5a in pATARC3-B1b, *arc5-II* encoding Arcelin 5b and Arcelin 5c in pATARC3-B52b and *arc1* encoding a dimeric Arcelin 1 protein in pATARC3-B515b (Fig. S1, Electronic Supplementary Material). The *arc1* coding region was obtained through PCR using genomic DNA from the *P. vulgaris* genotype RAZ2 as a template and specific primers based on the sequence reported by Anthony et al. (1991). All *arcelin* coding sequences were under the control of the seed-specific promoter region and the 3′ flanking sequence from *arc5-I* (Goossens et al. 1999).

Explant and bacterial preparation for coculture

Green nodular callus from seeds germinated in vitro from a cultivated genotype (TB1) was induced (Fig. 1a,b) and maintained for four passages as

Table 1 Composition of media used for transformation of *Phaseolus acutifolius*

Constituents[a]	Media										
	Germination media (GM)		Callus induction and maintenance media (CIM)			Callus cocultivation media (CCM)		Callus wash media (CWM)		Shoot induction and development media (SIDM)	
	GM1	GM2	CIM 1	CIM1AS	CIM1/5	CCM1	CCM1/5	CWM1	CWM1/5	SIM	SDM
MS salts and vitamins	+	+	+	+	+	+	+	+	+	+	+
Sucrose (g/l)	30	30	20	20	20	20	20	20	20	20	20
Glucose (g/l)	–	–	–	–	1.98	1.98	1.98	1.98	–	–	–
Agar (g/l)	6	6	8	8	8	–	–	–	–	8	8
Phytagel (g/l)	–	–	3[b]	–	3[b]	–	–	–	–	–	–
MES (g/l)	–	–	–	–	–	3.9	3.9	3.9	3.9	–	–
Acetosyringone (μM)	100	–	–	100	–	200	200	–	–	–	–
IAA (mg/l)	–	–	0.25	0.25	0.05	0.25	0.05	0.25	0.05	–	–
TDZ (mg/l)	–	–	0.50	0.50	0.10	0.50	0.10	0.50	0.10	–	–
BAP (mg/l)	1.13	–	–	–	–	–	–	–	–	1	0.10
Geneticin (mg/l)[c]	–	–	–	–	–	–	–	–	–	–	–
Carbenicillin (g/l)	–	–	–	–	–	–	–	–	–	0.05	0.05
Coconut water (%)	–	–	–	–	–	–	–	–	–	10	10
pH	5.7	5.7	5.7	5.7	5.7	5.5	5.5	5.5	5.5	5.7	5.7

[a] MS, Murashige and Skoog 1962; MES, 2-(*N*-morpholino)ethanesulfonic acid; IAA, indole-3-acetic acid; TDZ, thidiazuron; BAP, 6-benzylaminopurine; [b]phytagel, for the first passage following cocultivation, the media used contained phytagel as a solidifying agents instead of agar; [c]geneticin, for details on geneticin addition, see text

described by Zambre et al. (1998) on callus-inducing media (CIM1 and CIM1/5). Callus for *Agrobacterium* infection was used 5–6 days after the fourth subculture. The calli were chopped into small (2–5 mm) pieces, mixed together and distributed into jars for cocultivation. For genotype PI440795, sterilized seeds (Zambre et al. 1998) were first sown in germination medium (GM1) for 3 days, following which the cotyledons were pulled apart. Embryo axes were cultured on CIM1AS medium, taking care that the nodal area and root were in good contact with the medium, and incubated for 10 days in the dark. Prior to cocultivation the nodal areas were pricked gently with a sterile fine-gauge sewing needle without breaking the embryo and cocultivated as described below.

Agrobacterial colonies from fresh plates were suspended in 50 ml yeast extract liquid broth (YEB) to a concentration of $OD_{600} = 0.1–0.2$ (an OD_{600} of 0.8 corresponds to 10^9 cfu/ml) and grown at 28°C for 12 h while shaking (150 rpm). This culture was inoculated in 1 l YEB supplemented with antibiotics and grown for an additional 12 h under the same conditions. Cultures were washed twice by centrifugation and then resuspended in 50 ml of liquid CCM in which the phytohormone levels were the same as in the CIM media. This concentrated bacterial suspension was diluted to either an OD of 0.8 (for embryo axes) or an OD of 0.05 (for calli) in the corresponding CCM media and used for cocultivation (200 ml/jar for 3 g of callus or 25 embryos). Embryo and callus were cocultivated for 2 days and 7 days, respectively. Cocultivated cultures were shaken by hand once a day for a few seconds to avoid any formation of bacterial films on the liquid medium. Following cocultivation, the explants were washed three times with the corresponding callus wash media (CWM) (Table 1), with the help of a sterilized metal sieve, blotted briefly on sterile paper and cultured. For embryos, CIM1/5 was supplemented with 500 mg/l carbenicillin and 5 mg/l geneticin; for callus, CIM1/5 or CIM1 were supplemented with 500 mg/l carbenicillin and either 5 mg/l or 20 mg/l geneticin depending on the treatment (Table 1; Table S3 of Electronic Supplementary Material) for 3 weeks (first passage). For transient *uidA* expression, callus was cultured on corresponding CIM media without geneticin but with 500 mg/l carbenicillin for 4 days and used for GUS staining (see below).

Regeneration of transgenic shoots

After the first passage, the callus from genotype TB1 was sequentially transferred four or five times to CIM1/5 or CIM1 supplemented with geneticin and carbenicillin. A constant geneticin level (20 mg/l) was maintained in these media, while the carbenicillin level was reduced progressively by 100 mg/l at each passage (Table 1). In PI440795, green meristematic tissues that originated at nodal areas of the embryos following the first passage were cut out together with some of the original nodal tissue. These green tissues were transferred to CIM1/5 supplemented with geneticin and carbenicillin for five passages. At each passage, the geneticin level was increased by 5 mg/l to a maximum of 15 mg/l, while

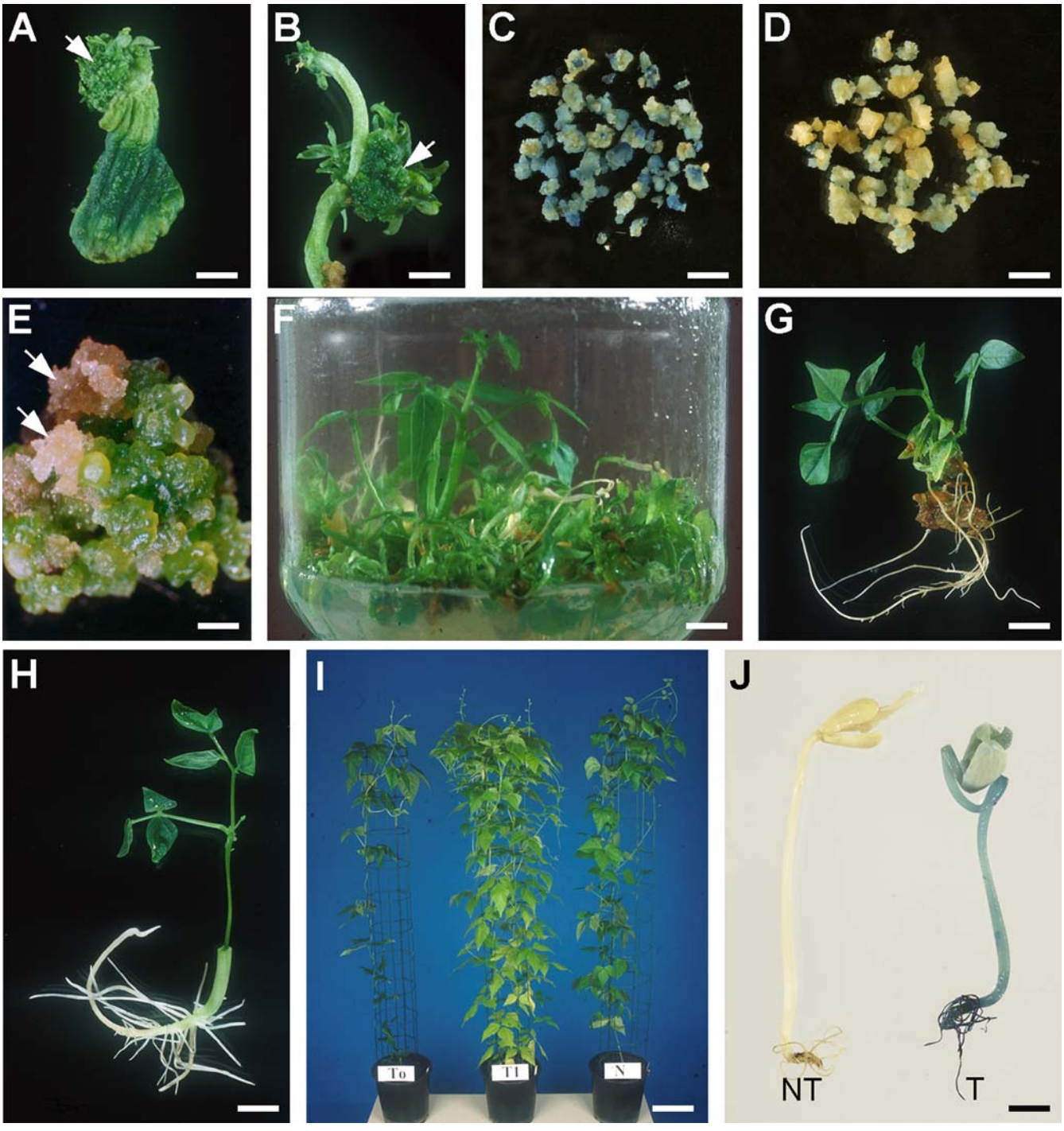

Fig. 1 In vitro regeneration and *Agrobacterium*-mediated transformation procedure for *Phaseolus acutifolius*. **a**,**b** Induction of green tissues from cotyledons (**a**) and embryos (**b**) at the embryo attachment sites (*arrows*) at the end of the first culture passage that evolved as transformation- and regeneration-competent green nodular callus in subsequent passages (not shown), **c**,**d** transient *uidA* expression in calli cocultivated with *Agrobacterium* at OD = 0.05 for 1 week (**c**) and OD = 0.8 for 48 h (**d**), **e** formation of yellow and brown, loose callus (*arrows*) on the green-nodular callus following cocultivation, **f** prolific in vitro transgenic shoot regeneration response on shoot development medium (SDM), **g** spontaneously rooted transgenic shoots on SDM, **h** established graft with transgenic shoot just before being transplanted into the soil, **i** morphology of primary transformant (T_0) and progeny of T_0 (T_1) and non-transgenic plant (*N*), **j** GUS staining of GUS-positive T_1 seedlings (*T*) and non-transgenic control (*NT*). All panels show genotype TB1, except for **g** (genotype PI440795). *Bars*: 3.2 mm (**a**,**b**), 1 mm (**c**,**d**), 1.6 mm (**e**), 8.0 mm (**f**), 3.7 mm (**g**), 3.5 mm (**h**), 70 mm (**i**), 5 mm (**j**)

carbenicillin was reduced at the same rate as for TB1. At each transfer, green nodular callus was selected for further culture. All callus cultures that survived selection were transferred to SIM twice for 2 weeks for shoot initiation and then successively for shoot development onto SDM for two or more passages. Geneticin was omitted from SIM and SDM media, while carbenicillin was added at a concentration of 50 mg/l. Each cultural passage lasted 2–3 weeks unless otherwise indicated.

Grafting and establishment of shoots in the greenhouse

Transgenic shoots regenerated in vitro were grafted as described by Zambre et al. (2001). In vitro-established grafted shoots and rooted shoots were transplanted into 4.5-l pots containing soil with basal nutrients (1.25 kg/m^3 N-P-K 14:16:18%). The plants were initially covered with transparent plastic to maintain humidity, but over a period of time the humidity was lowered slowly by cutting holes in the plastic for acclimatization of the plants. All plants were fertilized with 2 g urea 1 month after transplantation and again with 2 g urea after the induction of flowering. The greenhouse was maintained at 25 ± 2°C, 60–70% relative humidity and a photoperiod of 16/8 h (light/dark). Non-transgenic control plants were grown concurrently under the same growth conditions.

In vitro culture growth conditions

All of the cultures were grown under a photoperiod of 16/8 h (light/dark) in two growth chambers equipped with cool-white lighting (35–40 μmoles/m^2 per second). Growth chambers 1 and 2 were kept at 22 ± 0.4°C and 25 ± 1°C during the day, respectively, and 23 ± 1°C during the night. All cultures were sealed with gas-porous tape (Urgopore, Chenoves, France). The seed and cocultivated cultures were incubated in growth chamber 1; all other cultures were incubated in growth chamber 2.

Southern analysis

Genomic DNA (7 μg) from leaves of 2- to 3-week-old progeny plants (T_1 and T_3) was digested with *Sph*I and fractionated by electrophoresis on a 0.9% agarose gel. DNA gel blot analysis was performed according to standard procedures using a [^{32} P]-labeled *nptII* fragment as a probe.

Detection of arcelin proteins in T_1 or T_3 progeny seed

Individual seeds were soaked overnight in distilled water, and small pieces of seed-coat-free cotyledons (30–50 mg) were macerated in a microfuge tube with protein extraction buffer (10 m *M* NaCl and 50 m *M* glycine, pH 2.4). Proteins were extracted in two successive rounds (Goossens et al. 1999). Total proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized either by Coomassie blue staining or by protein gel blot staining with a rabbit polyclonal anti-Arcelin 5 antiserum (for details, see Goossens et al. 1999).

uidA expression in transgenic tissues

Expression of the *uidA* gene was detected by histological assays of GUS activity (Jefferson et al. 1987). Callus and plant tissues were incubated in sodium phosphate buffer (0.1 *M*) with substrate for 6–12 h (6 h for transient expression studies) at 37°C, and the reaction was stopped by the addition of 70% ethanol. Chlorophyll from the tissues was removed by the 70% ethanol. *uidA* expression in seed-coat-free cotyledon pieces was scored following a 2-h incubation. The data generated were used for the χ^2 test.

Resistance of the transgenic lines to *Zabrotes subfasciatus*

The techniques we used to maintain insect cultures and to test the selected lines were identical to those described previously (Schoonhoven and Cardona 1982; Cardona et al. 1989). All experiments were done at 27°C and 70% relative humidity in a controlled environment chamber. Wild-type and transgenic seeds were tested in five replicates, with each replicate consisting of 50 seeds infested with seven pairs of newly emerged Mexican bean weevil adults. Insect development was compared based on days to adult emergence and percentage emergence. Percentage adult emergence (transformed to arcsine square root of proportion) and days to adult emergence were submitted to an analysis of variance, and treatment means were separated with the Least Significant Difference computation (SAS Institute 1985). Resistant *P. vulgaris* lines were used as controls (Cardona et al. 1990; Goossens et al. 2000).

Results

Influence of bacterial concentration and duration of cocultivation on transformation

Transient *uidA* gene expression was visualized through histochemical staining to monitor early transformation events in callus of the cultivated *P. acutifolius* genotype TB1 and to assess the effects of bacterial concentration and cocultivation duration. Higher transformation rates (Fig. 1c) were obtained following a 1-week cocultivation at a low initial concentration of bacteria ($OD_{600} = 0.05$). Therefore, this combination was used for all subsequent experiments.

Selection of callus and effect of two selection regimes on the production of transgenic calli

The effective concentration of the antibiotic geneticin for selecting transgenic callus was determined by assessing the sensitivity of non-transgenic callus. Geneticin almost totally inhibited the proliferation of green nodular callus of genotypes TB1 and PI440795 at 20 mg/l and 15 mg/l, respectively (Table S1, Electronic Supplementary Material).

Following cocultivation, callus from genotype TB1 was cultured (first passage) on media containing either a low (5 mg/l) or a lethal (20 mg/l) level of geneticin (Table S2, Electronic Supplementary Material). During the first passage, calli became yellowish with green nodular sectors (Fig. 1e). At the end of the first passage on 5 mg/l geneticin, most calli had green, and occasionally dead, sectors, whereas following the first passage on 20 mg/l geneticin, many calli underwent rapid necrosis and gradually died. After the first passage, calli were transferred sequentially to the corresponding CIM media which contained a constant level of geneticin (20 mg/l) and a progressively lower level of carbenicillin. At each passage, surviving green nodular calli were separated from other callus types and chopped into small pieces, when necessary, to facilitate selection. Subculturing was continued for four to five passages, depending on the proliferation rate of each callus line. Uniformly intense GUS staining was observed in all callus lines at this stage, indicating effective selection and stable co-expression of both the *uidA* and *nptII* genes.

As mentioned above, for transformation of genotype TB1, two selection schemes were compared. In scheme 1 (direct selection), callus was exposed to a lethal dose of geneticin (20 mg/l) from the first passage onwards. In scheme 2 (delayed selection), callus was initially exposed to a low level of selection (5 mg/l) that was increased to 20 mg/l in the second passage and kept constant in subsequent callus maintenance passages. Both selection schemes produced stable transgenic callus lines. However, we obtained a higher number of callus lines and more independent transgenic shoots using the second scheme (Table S2, Electronic Supplementary Material).

For genotype PI440795, a similar procedure did not allow recovery of any transgenic callus despite transient *uidA* expression in calli comparable to that of genotype TB1. Therefore, embryo explants were used instead of callus. During the first passage following cocultivation, explants formed variable amounts of green tissues on the nodal areas on CIM1/5, which contained 500 mg/l carbenicillin and 5 mg/l geneticin (Table 1). These tissues were not visible on some of the explants before the end of the first passage. From all explants, regardless of the presence or absence of green tissues, nodal areas were carefully separated along with a small portion of the original explant tissues. These explants were cultured further (second passage) so that the cut portion was in contact with the medium. After the second passage, a selection procedure was followed comparable to that described for genotype TB1 to obtain stably transformed callus, except that the maximum level of geneticin was maintained at 15 mg/l instead of 20 mg/l due to the higher sensitivity of genotype PI440795 to geneticin (Table S1, Electronic Supplementary Material). The procedure for genotype PI440795 has not been optimized yet and, hence, only a few callus lines were obtained.

Initiation and elongation of transgenic shoots

Transgenic calli from both genotypes were transferred to SIM medium containing 1 mg/l 6-benzylaminopurine (BAP) for two passages and successively for one to several passages to SDM medium containing only 0.1 mg/l BAP. Both media contained 50 mg/l carbenicillin but no geneticin (Table 1). Callus cultured on SIM proliferated vigorously and profusely produced leafy clusters. Vigorous proliferation and differentiation of organized clusters continued during all successive passages on SDM (Fig. 1f). Most of the lines from both genotypes produced 10–150 shoots that were more than 2 cm long. This range depended on the initial amount of callus that was transferred onto SIM. Some lines did not produce any shoots, although the clusters remained organized (Table S2, Electronic Supplementary Material). The shoots (Fig. 1g) in clusters rooted spontaneously and readily on SDM medium. Individually separated shoots obtained by cutting at the most basal portion did not root on SDM, indicating that structural integrity of shoot clusters is required to form roots. In general, younger shoots in earlier passages had no roots, while older shoots had roots in the late passages. The shoots did not root in approximately one-third of the lines; in the remaining lines rooting occurred at a frequency of 25–40%. Non-rooted shoots were used for grafting.

Grafting and establishment of the transgenic plants

The grafting of non-rooted transgenic shoots (Fig. 1h) was performed essentially as reported by Zambre et al. (2001). The grafts showed in vitro juvenile growth within 3–6 weeks following planting on GM2 medium, and the in vitro establishment rate for the grafts was 80–100% regardless of genotype. This procedure enabled the recovery of plants from all of the callus lines that produced shoots without roots or even from those that produced shoots unable to differentiate fully or with a stunted (1–2 mm) morphology. All of the assayed plant parts tested GUS-positive in GUS histochemical assays. All lines produced seeds, usually 100–400 per plant. Seeds from

all of the transgenic plants had germination rates of more than 80% and segregated for *uidA* expression (Fig. 1j; Table S3, Electronic Supplementary Material). The plants developing from these seeds showed no apparent morphological variations (Fig. 1i), and their seed yield was comparable to that of non-transformed control plants. The procedure took approximately 8–11 months from callus initiation to progeny of primary transgenic plants.

Inheritance of the transgenes in the progeny

The progeny seeds (T_1) of 33 self-fertilized transgenic plants were subjected to GUS assays and the segregation data analyzed by the χ^2 test (Table S3, Electronic Supplementary Material). These data indicated that 88% of the transgenic lines contain one transgene locus (3:1 segregation), and that three lines (1-2, 1-12 and 2-6) may have two transgene loci (15:1 segregation). In line 1-6, all of the 230 seeds tested were found to be positive for *uidA* expression in the cotyledons, which may be due to the presence of three or more transgene loci or to T-DNA integration in two homologous chromosomes at linked positions.

Expression of the *arcelin* genes in progeny seeds of transgenic plants

Three binary vectors containing either the *arc5-I*, *arc5-II* or *arc1* gene in the T-DNA were used for transformation. *Arc5-I* encodes the protein Arcelin 5a (Arc5a; 32.2 kDa), whereas *arc5-II* encodes the glycosylation variants Arcelin 5b (Arc5b) and Arcelin 5c (Arc5c) (31.5 kDa and 30.8 kDa, respectively) (Goossens et al. 1999). The *arc1* coding region used is expected to encode a 36.1-kDa protein (Osborn et al. 1988; Anthony et al. 1991; Hartweck et al. 1991). The expression of these genes was assessed in progeny seeds (T_1 and/or T_3 generation) from all 33 transgenic *P. acutifolius* lines by means of immunoblots (for the lines expressing *arc5-I* and *arc5-II*; Fig. 2) or Coomassie blue-stained gels (for the lines expressing *arc1*; Fig. 3). The expression levels in the transgenic lines were compared with those in a wild non-transgenic *P. vulgaris* genotype (G02771) containing Arc5 polypeptides (Goossens et al. 1999) and a breeding line (RAZ2) containing Arc1 polypeptides (Cardona et al. 1990). All of the lines expressed the introduced *arcelin* genes, except for line 3-8 (Fig. 3). Many lines had *arcelin* expression levels comparable to those of the standard *P. vulgaris* genotypes G02771 and RAZ2. For several lines, we found similar or increased levels of Arc5 protein accumulation relative to those found in a previously quantified transgenic line (8.1.22) of the wild *P. acutifolius* genotype (NI576) in which Arc5a protein accounted for 24% of the total protein (Goossens et al. 1999, 2000).

Southern analysis

To confirm the transgenic nature of progeny (T_1) plants, Southern blot analysis was performed on 11 transgenic lines representing all experiments and constructs. For each line, *Sph*I-digested genomic DNA (Fig. S1, Electronic Supplementary Material) from a GUS-positive and a GUS-negative T_1 segregant was used. Hybridization was done with a radioactively labeled *nptII* probe (Fig. S1, Electronic Supplementary Material) that is expected to hybridize to an internal T-DNA fragment of 1.4-kb as well as to junctions of plant DNA with the left border fragment of the integrated T-DNA. The Southern blot indeed revealed the internal T-DNA fragment of 1.4 kb (Fig. S2, Electronic Supplementary Material) in all *uidA*-expressing T_1 plants. This fragment was not detectable in lanes corresponding to GUS-negative T_1 plants nor in non-transgenic TB1 or PI440795 plants (Fig. S2, Electronic Supplementary Material). Four T_1 plants had two or three border fragments and thus probably more than one T-DNA copy, whereas a single border fragment was detected in the other T_1 plants. The results of the Southern blots confirm the segregation of the transgenes in the progeny of the transgenic *P. acutifolius* plants.

Resistance of *arcelin*-expressing beans to *Z. subfasciatus* (Mexican bean weevil)

To analyze the involvement of Arcelin 5 and Arcelin 1 in bruchid resistance, we infested seeds from several transgenic lines with the Mexican bean weevil. For each transgenic line, seeds from homozygous progeny were tested concurrently with seeds from azygous progeny (i.e. a segregant that does not contain the transgene) that derived from the same primary transformant as a control. Both susceptible and resistant *P. vulgaris* varieties were included as well as non-transformed *P. acutifolius* TB1 and PI440795.

In most of the lines the emergence of adult insects was delayed and/or the percentage emergence was lower in seeds homozygous for the arcelin transgene than in the corresponding azygous control seeds (Table 2). However, the differences were small, especially when compared to the resistant *P. vulgaris* lines included as a control. Therefore, although the presence of the *arc1* or *arc5* genes did influence Mexican bean weevil biology, it cannot explain the high levels of resistance seen in wild *arcelin*-containing bean genotypes nor is it sufficient to achieve adequate control of this insect.

Discussion

Agrobacterium-mediated transformation, regeneration and expression of transgenes in large-seeded legumes have proven difficult, although considerable progress has been obtained for some species (for example, pea,

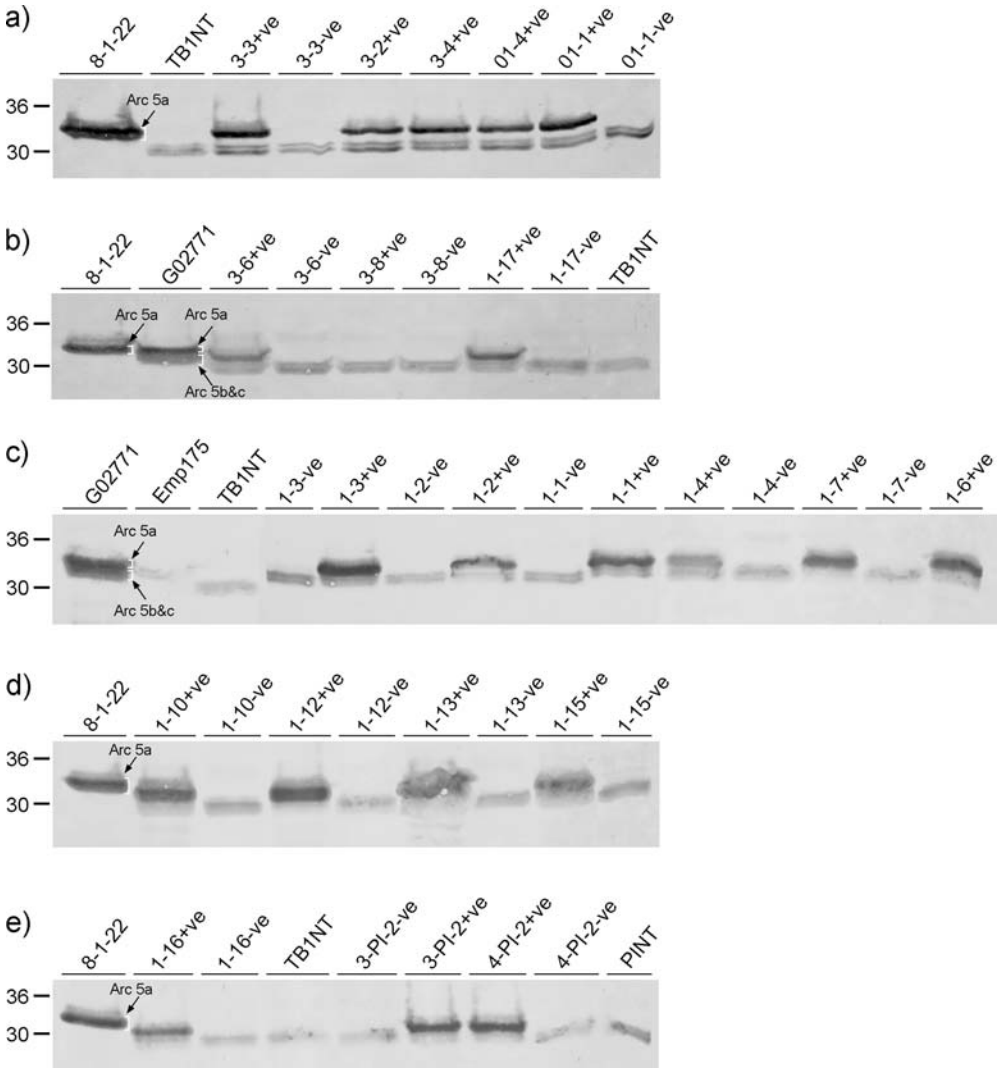

Fig. 2 Protein gel blot analysis of seed proteins from transgenic lines of *P. acutifolius*. Proteins were separated by SDS-PAGE (13%) and probed with antiserum raised against the protein Arcelin 5 (Arc5). **a** Transgenic lines for *arc5-I* (encoding Arc5a), **b–e** transgenic lines for *arc5-II* (encoding Arc5b and Arc5c). From each transgenic line, GUS-positive (*+ve*) and GUS-negative (*−ve*) seeds were analyzed. Total seed protein (2 µg) was loaded on each lane. Seeds from the T_3 generation were used for lines 01-1 and 01-4, and seeds from the T_1 generation were used for the other lines. Molecular mass is indicated on the *left*. *TB1NT*, *PINT* Non-transgenic controls of genotypes TB1 and PI440795, respectively, *G02771*, *RAZ2*, *Emp175* wild genotype of *P. vulgaris* or breeding lines containing Arc5, Arc1 and no Arc, respectively, *8.1.22* homozygous transgenic wild *P. acutifolius* genotype (NI576) in which Arc5a makes up 24% of the total protein (Goossens et al. 2000). In all of the immunoblots, other proteins than arcelins cross-reacted with the Arc5 antiserum; these may be lectin or lectin-like proteins and appear to be specifically present in genotypes TB1 and PI440795 as these bands are absent in all other genotypes

peanut and soybean). We report here an *Agrobacterium*-mediated method for the transformation of two cultivated genotypes (TB1 and PI440795) of *P. acutifolius*, which constitutes the most efficient *Agrobacterium*-mediated transformation system to achieve transformation in agronomically important and edible beans in the large *Phaseolus* genus. The system is useful as a biotechnological and breeding tool because of the prolific production of transgenic shoots without "escapes", the efficient conversion of shoots regenerated in vitro, the yield of plenty viable progeny seeds, the lack of morphological variation among the progenies and, most importantly, the stable and high levels of co-expression of the transgenes introduced into most lines.

In our system, a 1-week cocultivation period with a low initial concentration of *Agrobacterium* was found to be important for obtaining higher callus transformation. Another important factor we use to improve the transformation procedure is to reduce the selection pressure in the first passage after cocultivation. The rationale for delayed selection is to allow the transformed cells to proliferate and form cell clusters that are able to survive within a surrounding mass of dying or dead cells before subculture onto a lethal antibiotic concentration. The low and delayed selection probably prevents excessive death of non-transformed cells that leads to high toxicity levels for the transformed cells developing within the callus. Indeed, when gradual instead of direct selection is applied (Table S2, Electronic Supplementary Material), the number of geneticin-resistant transformed calli and transgenic plants is consistently higher.

Although both cultivated bean genotypes TB1 and PI440795 proved to be amenable to transformation, the sensitivity of the callus to the *Agrobacterium* procedure differed. At the end of the cocultivation, callus from PI440795 was softer than that of TB1. This difference may result in more dead tissue or in more yellowish callus in PI440795 that can prevent further recovery of regenerable callus, although we did obtain comparable transient *gus* expression in callus from both genotypes. Therefore, we select intact embryo axes of genotype PI440795 as explants in our system as these are supposed

to better withstand the negative effects of *Agrobacterium* infection. This choice allowed recovery of transgenic plants of genotype PI440795, although the procedure for this genotype should be optimized further to increase transformation efficiency. Nevertheless, it is important to note that our regeneration procedure—variations of which are applicable for a range of grain legumes genotypes (Zambre et al. 1998, 2001, 2002)—can be combined with *Agrobacterium* infection in at least two different stages (original embryo explant and propagated callus) to produce transgenic plants. This increases the versatility of the system and might be relevant for developing efficient transformation of other *Phaseolus* genotypes and other grain legumes.

Although this protocol uses callus for regeneration, somaclonal variation does not seem to pose a problem given that all transgenic plants were fertile and appeared to be morphologically normal. On the contrary, the rather long callus phase on selective medium (four to five passages) is an apparently highly effective means to completely remove non-transgenic callus tissues. Progeny analysis proved that all of the shoots analyzed were transgenic, and no indication of chimerism was encountered. In addition, the strict selection protocol during the callus phase enables geneticin to be omitted from all subsequent culture passages on SIM and SDM, which in turn may be beneficial to the production of prolific and vigorously growing shoots.

The segregation data suggest that the transgenes in most of the lines were integrated as a single locus (Table S3, Electronic Supplementary Material). All transformants expressed *arcelin* in the cotyledons of T_1 and/or T_3 seeds, except for one line (3-8). However, this line was resistant to geneticin and showed the active expression of *uidA* in all of the plant tissues as well as in the progeny seeds. Consequently, we conclude that the three introduced genes were co-expressed in all but one of the transgenic lines. Many of the lines consistently showed Arcelin 5 accumulation comparable to that of the native line (G02771) and the transgenic line (8.1.22); the latter two express *arcelin* at very high levels (35–40% and 24% of the total soluble protein, respectively; Goossens et al. 1999, 2000). In addition, four of the 12 *arc1*-containing lines accumulated exceptionally high levels of Arcelin 1 that were comparable to or higher than those in the control line RAZ2 (Fig. 3). Expression of *arc5-I* was also obtained in the two lines that were advanced to the T_3 generation (Fig. 2a; Table S3, Electronic Supplementary Material), indicating the stability of transgene expression over generations.

Our transformation procedure was used to introduce *arcelin* genes into *P. acutifolius*. We concentrated on the *arc5* and *arc1* genes because these *arcelin* variants are considered to be the most interesting with respect to bruchid resistance. Backcrossing of *arcelin*-containing wild *P. vulgaris* accessions with bean cultivars has shown that only backcross lines with *arc5* and *arc1* maintain high levels of resistance against the Mexican bean weevil (Kornegay et al. 1993). The results of our experiments with large-seeded *P. acutifolius* expressing the *arc1* and *arc5* genes at high levels, together with results from previous experiments involving wild *P. acutifolius* transformed with *arc5* genes (Goossens et al. 2000), clearly show that single *arc5* or *arc1* genes do not provide high levels of resistance to the Mexican bean weevil. Although insect development was delayed and/or emergence of adults was reduced significantly in most of the transgenic lines when compared with the azygous siblings, this effect can be considered to be very limited when compared to highly resistant lines (Table 2). Thus, arcelins may play an accessory role in resistance against bruchids, but the generally held view

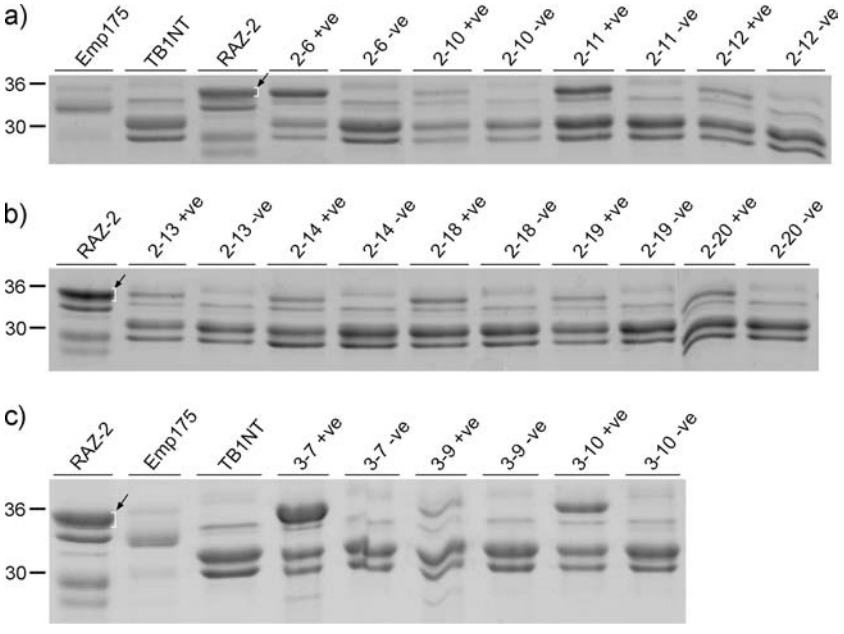

Fig. 3 SDS-PAGE analysis of seed protein extracts of transgenic *P. acutifolius* lines for *arc1*. GUS-positive (*+ve*) and GUS-negative (*−ve*) seeds were analyzed from each line. On each lane, 5 μg (**a**,**b**) and 4 μg (**c**) of protein was loaded. Seeds from the T_1 generation were used for all lines. Molecular mass is indicated on the *left*. *G02771*, *RAZ2*, *Emp175*, *TB1NT* (see Fig. 2 for details) were used as controls. The *arrow* indicates the Arc1 protein band

Table 2 Larval development of *Zabrotes subfasciatus* on transgenic *P. acutifolius* plants (T_3 generation)

Genotype[a]	Line[b]	Days to adult emergence[c]	Emergence (%) [c]
TB1	01-1 (Arc5a)	33.7 ± 0.11h,i	92.9 ± 1.4a
TB1	01-4 (Arc5a)	34.3 ± 0.51h,i	72.9 ± 3.9c
TB1	01-1 (azygous)	32.4 ± 0.18i	97.9 ± 0.7a
TB1	1-12 (Arc5bc)	37.3 ± 0.19e	96.4 ± 0.5a
TB1	1-12 (azygous)	33.7 ± 0.41h,i	95.9 ± 2.1a
TB1	1-6 (Arc5bc)	35.1 ± 0.19f,g	96.6 ± 1.4a
TB1	1-6 (azygous)	34.4 ± 0.37h,i	97.3 ± 1.5a
PI440795	3-PI-2 (Arc5bc)	39.7 ± 0.23d	95.9 ± 1.4a
PI440795	3-PI-2 (azygous)	34.0 ± 0.17h,i	97.9 ± 1.1a
PI440795	4-PI-2 (Arc5bc)	38.8 ± 0.23d,e	95.8 ± 1.6a
PI440795	4-PI-2 (azygous)	34.7 ± 0.19h	95.2 ± 1.3a
TB1	2-11 (Arc1)	34.1 ± 0.19h,i	95.1 ± 1.5a
TB1	2-11 (azygous)	32.4 ± 0.09i	94.8 ± 0.8a
TB1	2-6 (Arc1)	37.1 ± 0.28e,f	82.5 ± 1.6b
TB1	2-6 (azygous)	33.8 ± 0.21h,i	95.4 ± 1.6a
TB1	3-7R (Arc1)	40.4 ± 0.26d	66.0 ± 4.5d
TB1	3-7R (azygous)	38.4 ± 0.44d,e	82.5 ± 6.8b
TB1	Non-transgenic (no arcelin)	33.2 ± 0.19h,i	95.4 ± 1.6a
PI440795	Non-transgenic (no arcelin)	34.2 ± 0.18hi	96.9 ± 0.4a
Emp175	Non-transgenic (no arcelin)	33.0 ± 0.22hi	97.0 ± 1.2a
RAZ2	Non-transgenic (Arc1)	51.7 ± 1.35c	9.8 ± 3.2e
Ica Pijao	Non-transgenic (no arcelin)	33.5 ± 0.15hi	97.1 ± 0.7a
RAZ44	Non-transgenic (Arc1)	50.8 ± 1.48c	6.4 ± 0.8ef
G12882	Wild genotype (Arc1)	51.1 ± 0.94c	7.5 ± 1.4e
G02771	Wild genotype (Arc5)	56.4 ± 3.79b	1.1 ± 0.3f
G12952	Wild genotype (Arc4)	59.5 ± 0.55a	10.8 ± 1.5e
RAZ136	Non-transgenic (Arc1)	50.3 ± 0.66c	5.4 ± 0.7ef

[a] EMP175 and Ica Pijao are *P. vulgaris* genotypes containing no arcelin and susceptible to *Z. subfasciatus*; RAZ2, RAZ44 and RAZ136 are *P. vulgaris* genotypes with similar genetic background as EMP175, but containing Arc1 and resistant to *Z. subfasciatus*; G12882, G02771, and G12952 are wild *P. vulgaris* accessions that contain arcelin and are resistant to *Z. subfasciatus*

[b] For each transgenic *P. acutifolius* line, an azygous segregant derived from the same primary transformant was used as negative control

[c] Means within a column followed by the same letter are not significantly different by the Least Significant Difference computation at the 5% level

that arcelin proteins themselves provide bruchid resistance has to be revised. It now appears that a gene closely linked to the *arcelin* gene is the most likely source of bruchid resistance. *Arcelin* genes are part of complex loci that contain genes encoding lectins, α-amylase inhibitors, arcelins and related proteins. Sequence analysis of these complex loci may reveal candidate bruchid resistance genes, which could be transformed in *P. acutifolius* to determine which gene or gene combination provides resistance towards the Mexican bean weevil.

Because of the large seed size (10–20 g/100 TB1 seeds) and high transgene expression level conferred by the *arcelin* regulatory sequences, this system has already been tested in view of large-scale production of high-value heterologous proteins (De Jaeger et al. 2002). Studies related to improvement of the nutritional quality, such as increasing methionine levels in seeds (De Clercq et al., manuscript in preparation) and reducing anti-nutritional factors, are also feasible because the seeds from the transformed genotypes are edible.

Acknowledgements The authors thank Martine De Cock for help in preparing the manuscript and Rebecca Verbanck and Karel Spruyt for the artwork. This work was supported by a grant from the Belgian Directorate General for Development and Co-operation.

References

Anthony JL, Vonder Haar RA, Hall TC (1991) Nucleotide sequence of a genomic clone encoding arcelin, a lectin-like seed protein from *Phaseolus vulgaris*. Plant Physiol 97:839–840

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

Broughton WJ, Hernandez G, Blair M, Beebe S, Gepts P, Vanderleyden J (2003) Beans (*Phaseolus* spp.)—model food legumes. Plant Soil 252:55–128

Cardona C, Posso CE, Kornegay J, Valor J, Serrano M (1989) Antibiosis effects of wild dry bean accessions on the Mexican bean weevil and the bean weevil (Coleoptera: Bruchidae). J Econ Entomol 82:310–315

Cardona C, Kornegay J, Posso CE, Morales F, Ramirez H (1990) Comparative value of four arcelin variants in the development of dry bean lines resistant to the Mexican bean weevil. Entomol Exp Appl 56:197–206

Christou P (1997) Biotechnology applied to grain legumes. Field Crops Res 53:83–97

De Clercq J, Zambre M, Van Montagu M, Dillen W, Angenon G (2002) An optimized *Agrobacterium*-mediated transformation procedure for *Phaseolus acutifolius* A. Gray. Plant Cell Rep 21:333–340

De Jaeger G, Scheffer S, Jacobs A, Zambre M, Zobell O, Goossens A, Depicker A, Angenon G (2002) Boosting heterologous protein production in transgenic dicotyledonous seeds using

Phaseolus vulgaris regulatory sequences. Nat Biotechnol 20:1265–1268

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

Franklin CI, Trieu TN, Cassidy BG, Dixon RA, Nelson RS (1993) Genetic transformation of green bean callus via *Agrobacterium* mediated DNA transfer. Plant Cell Rep 12:74–79

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

Goossens A, Quintero C, Dillen W, De Rycke R, Valor JF, De Clercq J, Van Montagu M, Cardona C, Angenon G (2000) Analysis of bruchid resistance in the wild common bean accession G02771: no evidence for insecticidal activity of arcelin 5. J Exp Bot 51:1229–1236

Hartweck LM, Vogelzang RD, Osborn TC (1991) Characterization and comparison of arcelin seed protein variants from common bean. Plant Physiol 97:204–211

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901–3907

Kornegay J, Cardona C, Posso CE (1993) Inheritance of resistance to Mexican bean weevil in common bean, determined by bioassay and biochemical tests. Crop Sci 33:589–594

Kumar AS, Gamborg OL, Nabors MW (1988) Regeneration from long-term cell suspension cultures of tepary bean (*Phaseolus acutifolius*). Plant Cell Rep 7:322–325

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473–497

Nagl W, Ignacimuthu S, Becker J (1997) Genetic engineering and regeneration of *Phaseolus* and *Vigna*. State of the art and new attempts. J Plant Physiol 150:625–644

Osborn TC, Alexander DC, Sun SSM, Cardona C, Bliss FA (1988) Insecticidal activity and lectin homology of arcelin seed protein. Science 240:207–210

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

Sales MP, Gerhardt IR, Grossi-de-Sá MF, Xavier-Filho J (2000) Do legume storage proteins play a role in defending seeds against bruchids? Plant Physiol 124:512–522

SAS Institute (1985) SAS user's guide: statistics version 5. SAS Institute, Cary, N.C.

Schoonhoven AV, Cardona C (1982) Low levels of resistance to the Mexican bean weevil in dry beans. J Econ Entomol 75:567–569

Scott ME, Michaels TE (1992) *Xanthomonas* resistance of *Phaseolus* interspecific cross selections confirmed by field performance. Hortic Sci 27:348–350

Singh SP (1999) Integrated genetic improvement. In: Singh SP (ed) Common bean improvement in the twenty-first century. Developments in plant breeding, vol 7. Kluwer, Dordrecht, pp 133–165

Somers DA, Samac DA, Olhoft PM (2003) Recent advances in legume transformation. Plant Physiol 131:892–899

Zambre MA, De Clercq J, Vranová E, Van Montagu M, Angenon G, Dillen W (1998) Plant regeneration from embryo-derived callus in *Phaseolus vulgaris* L (common bean) and *P. acutifolius* A. Gray (tepary bean). Plant Cell Rep 17:626–630

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, Chowdhury B, Kuo Y-H, Van Montagu M, Angenon G, Lambein F (2002) Prolific regeneration of fertile plants from green nodular callus induced from meristematic tissues in *Lathyrus sativus* L. (grass pea). Plant Sci 163:1107–1112

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