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Agrobacterium-mediated transformation of *Phaseolus acutifolius* A. Gray

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Abstract *Agrobacterium*-mediated transformation of *Phaseolus acutifolius* A. Gray has been achieved. Regeneration-competent callus, obtained from bud explants of greenhouse-grown plants, was co-cultivated with *Agrobacterium tumefaciens* C58C1Rif^R(pMP90) harbouring a binary vector with the neomycin phosphotransferase II (*nptII*) and β-glucuronidase (*uidA*) marker genes. Transient expression of *uidA* was detected in five out of six genotypes tested. Transgenic callus lines of three genotypes were established on geneticin-containing medium. Plants were recovered from one line (genotype NI 576). This line had been transformed with a binary plasmid which, in addition to the marker genes, contained a genomic fragment encoding the *Phaseolus vulgaris* arcelin-5a protein. This seed storage protein presumably confers resistance to the insect *Zabrotes subfasciatus*, a major pest of *P. vulgaris*. Integration of foreign DNA was confirmed by molecular analysis. The introduced genes segregated as a single locus. Arcelin-5a was produced at high levels in seeds. The possibility of using *P. acutifolius* as a 'bridging' species to introduce transgenes into the economically more important species *P. vulgaris* is discussed.

Key words Arcelin · *Fabaceae* · Genetic transformation · Insect resistance · Tepary bean

Introduction

Transformation of leguminous species, and of large-seeded legumes (grain legumes) in particular, has so far been difficult to achieve. Proof of transformation requires that, at least for sexually propagated species, transmission of the introduced DNA be confirmed by molecular analysis of the offspring of primary transformants. In addition, to be of practical value, transformants should correctly express all the introduced genes. With these criteria in mind, transformation has now been convincingly demonstrated for *Pisum sativum* (Puonti-Kaerlas et al. 1990; Schroeder et al. 1993; Grant et al. 1995), *Vicia narbonensis* (Pickardt et al. 1991) and *Arachis hypogaea* (Cheng et al. 1996) with *Agrobacterium tumefaciens*, and for *Glycine max* (McCabe et al. 1988; Finer and McMullen 1991; Sato et al. 1993), *Arachis hypogaea* (Brar et al. 1994), and *Phaseolus vulgaris* (Russell et al. 1993) with particle bombardment. A drawback of biolistic gene delivery is the often complex and unpredictable pattern of DNA integration. As a consequence, large numbers of transformants have to be generated in order to obtain sufficient plants displaying a simple and correct integration pattern. There is a growing consensus among breeders that the precision of the *A. tumefaciens*-mediated integration mechanism and its tendency to produce low- or single-copy insertions constitute a considerable advantage over direct gene-transfer techniques. Therefore, it is important that efficient *Agrobacterium* protocols for the transformation of grain legumes be developed.

For *P. sativum*, a few approaches involving *Agrobacterium*-mediated transformation have so far been successful. Puonti-Kaerlas et al. (1992) obtained six transformants from shoot-derived callus but the regeneration procedure unfortunately caused ploidy alterations. Two studies reported the production of transgenic plants from the cotyledonary node region of seedlings. The transgenic nature of three shoots produced from callus was confirmed by progeny analysis (Davies et al. 1993). This was also the case for one shoot obtained from the cotyledonary node without callus formation, but this result could not be reproduced (Jordan and Hobbs 1993). The system developed by Schroeder et al. (1993) involves organogenesis from immature embryonic axis-derived callus and seems to be the most reproducible so far (Shade et al. 1994). More recently, transformants of four cultivars of *Pisum* were ob-

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tained by co-cultivation of immature cotyledon explants, callus induction, and shoot regeneration (Grant et al. 1995). In *V. narbonensis*, *Agrobacterium*-mediated transformation of shoot-tip and epicotyl explants and subsequent regeneration of plants from callus seems to be an efficient system (Pickardt et al. 1991, 1995; Saalbach et al. 1994, 1995). For *A. hypogaea*, the integration of foreign DNA was confirmed in the progeny of one plant, obtained after co-cultivation of wounded embryonic axes with *A. tumefaciens* (McKently et al. 1995). However, molecular analysis revealed that the introduced genes had undergone substantial rearrangement. More recently, Cheng et al. (1996) have reported the production of transgenic shoots from co-cultivated leaf explants. Analysis of the progeny confirmed both the integration and transmission of the transgenes. A 3:1 segregation ratio was often observed.

As opposed to *P. sativum*, *V. narbonensis* and *A. hypogaea*, efficient *Agrobacterium*-mediated transformation of other grain legumes has not yet been convincingly demonstrated. In an early report on *G. max* (Chee et al. 1989), seed inoculation yielded one putative transformant but segregation in the R_1 generation was aberrant and no data on the R_2 were provided. In another study on this species, two transformants were regenerated from cotyledon explants (Hinchee et al. 1988). It was not stated whether the regeneration involved a callus phase and so far the method has not been reproduced (Zhou and Atherly 1990). Recently, *G. max* transformants were produced at low frequency from cotyledonary node explants. Analysis of R_2 plants suggested that an introduced bean pod mottle virus (BPMV) coat protein precursor gene conferred resistance to BPMV (Di et al. 1996). For *Phaseolus*, *Lens*, *Cicer*, and *Vigna*, no *Agrobacterium*-based transformation protocols are available to-date.

The genus *Phaseolus* comprises five cultivated species, *P. vulgaris*, *P. acutifolius*, *P. coccineus*, *P. lunatus*, and *P. polyanthus*. *P. vulgaris* is the second most important grain legume after *G. max*. *P. acutifolius* (tepary bean) is nutritionally similar to *P. vulgaris* but, unlike it, is very well adapted to arid environments. In addition, it is a source of disease resistance traits that are not found in *P. vulgaris* (reviewed in Pratt and Nabhan 1988).

So far, genetic transformation of *P. vulgaris* has only been achieved through direct gene transfer into the apical meristem region of seedlings of one cultivar (Russell et al. 1993). The efficiency of recovery of transgenic progeny was low and the introduced DNA sequences were organised as arrays of, often incomplete, copies. Therefore, we concentrated our efforts on developing a way to introduce genes into the *Phaseolus* gene pool through *Agrobacterium*.

This study is the first report on *Agrobacterium*-mediated transformation of a member of the genus *Phaseolus* (*P. acutifolius* A. Gray), including evidence for transmission of the transgenes to the progeny. The transformation method is based on a previously described procedure that involves regeneration from callus (Dillen et al. 1996). We also demonstrate that the insertion of an arcelin-5-I genomic fragment (Goossens et al. 1994, 1995) results in high expression of this protein in seeds, which has potential agronomic value.

Materials and methods

Establishment of green nodular callus

Cultures of green nodular callus of *P. acutifolius* genotypes NI 574, NI 576, NI 589, NI 787, NI 843 and NI 845 (NI = collection number of the National Botanic Garden of Belgium, Meise, Belgium) were established as previously described (Dillen et al. 1996). Bud explants of greenhouse-grown plants were excised and cultured on CIM1 medium [Murashige and Skoog (1962) salts and organic addenda, 20 g/l sucrose, 8 g/l bacto-agar (Difco Labs, Detroit, Michigan, USA), 0.25 mg/l indole-3-acetic acid, and 0.5 mg/l thidiazuron, pH 5.7]. After three cultures lasting for 2, 1 and 1 months, approximately 20 (genotypes NI 574, NI 589, NI 787, NI 843, and NI 845) to 60 (NI 576) 5-mm callus pieces were used for co-cultivation. On average, five bud explants per genotype were used to obtain this amount of callus.

Co-cultivation of callus

Callus was pre-cultured for 5 days on CIM1, chopped into 2- to 4-mm pieces, and cultured in 10-cm Petri dishes sealed with gas-porous tape at a density of 20 pieces per 32 ml of co-cultivation medium in the presence of 20 μM of 3′,5′-dimethoxy-4′-hydroxy-acetophenone (acetosyringone). The co-cultivation medium was as CIM1 but without agar and including 10 mM of D(+)-glucose and 20 mM of 2-[N-morpholino]ethane sulphonic acid (MES), pH 5.5. *A. tumefaciens* inocula were obtained from cultures grown for 36-h on a gyratory shaker (150 rpm) at 28°C. Cells (100 ml) were collected by centrifugation (2000 *g*, 10 min), washed twice in 50 ml co-cultivation medium, and incubated for 4 h at room temperature in 5 ml of co-cultivation medium with 200 μM of acetosyringone. The concentrated *Agrobacterium* suspension was added to the explants to a final optical density (595 nm) of 0.8. The temperature was 24°C with a light period of 16 h (light intensity 20 μmol/m^2/s). After 2 days, explants were washed twice in co-cultivation medium containing 500 mg/l of cefotaxime, blotted dry, and cultured on selective medium.

Bacterial strains and plasmids

Co-cultivation was carried out with the *A. tumefaciens* strain C58C1Rif^R containing the helper plasmid pMP90 (Koncz and Schell 1986). Two-thirds of the material was infected with this strain harbouring the binary plasmid pTJK136 (Kapila et al., in press) which between the T-DNA borders contains the neomycin phosphotransferase II (*nptII*) gene under control of the nopaline synthase (*nos*) promoter and the octopine synthase 3′ termination and polyadenylation signals, as well as the *Escherichia coli* β-glucuronidase (*uidA*) gene (Jefferson 1987) with the potato *st-ls1* intron (Vancanneyt et al. 1990) under the control of the CaMV 35S promoter and the *nos* 3′ processing and polyadenylation signals. One third of the material was treated with the same strain harbouring the binary plasmid patarc3-B1b. This plasmid contains the same marker genes as pTJK136 and in addition a genomic fragment presumably coding for the *P. vulgaris* arcelin-5a seed storage protein (Goossens et al. 1995) (Fig. 1).

Selection and regeneration

All media used for selection and regeneration of shoots contained either 300 mg/l of kanamycin or 20 mg/l of geneticin (antibiotic G418). After washing, the callus was transferred to CIM1 supplemented with 500 mg/l of cefotaxime. After 14 days, surviving tissue was subcultured at 3-week intervals on the same medium. The cefotaxime con-

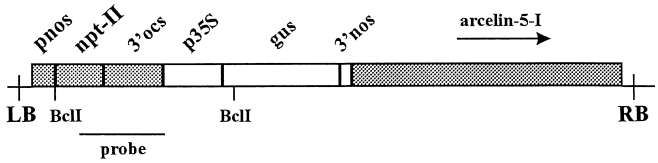


Fig. 1 The transferred region of plasmid patarc3-B1b

centration was reduced to 400 mg/l in the first subculture, to 300 mg/l in the second subculture, to 200 mg/l in the third subculture, and cefotaxime was omitted from the fourth and subsequent subcultures. In the fifth subculture the callus was transferred to shoot induction medium [SIM; Murashige and Skoog (1962) salts and organic addenda, 20 g/l sucrose, 8 g/l bacto-agar, 10% coconut water (Sigma, St. Louis, Missouri, USA), and 1.0 mg/l benzyl amino purine, pH 5.7] for 1 month followed by two 2-week passages to shoot-development medium (SDM; as SIM but with benzyl amino purine reduced to 0.1 mg/l). Shoots were excised from the last culture on SDM and grafted.

Grafting of regenerated shoots

The base of apical or nodal segments (approximately 5 mm) of 2- to 4-cm shoots was trimmed to obtain a wedge shape. NI 574 seeds were germinated on MS medium [Murashige and Skoog (1962) salts and organic addenda, 30 g/l sucrose, and 8 g/l bacto-agar, pH 5.8] until the root emerged from the seed coat. The latter was removed and a transverse cut was made in the epicotyl region to remove the plumule. The scion base was inserted into a vertical incision in the epicotyl region of the root stock. The resulting grafts were cultured on MS medium and buds emerging from the cotyledonary node axils were carefully removed at regular intervals.

Flowering and seed set

Established grafts were transferred to potting soil [conductivity 500 microsiemens, pH (H_2O) 5.5–6.5, fertilised with 83 g NPK 14-46-18 per kg] in a greenhouse and covered with plastic foil. The relative humidity was reduced by gradually removing the foil. Hardened plants were grown in the greenhouse for 1 month and then transferred to a growth chamber and provided with short-day conditions (8 h light, 50 μmol/m^2/s, 24°C) to induce flowering. Seeds were harvested between 2 and 3 months after transfer to the greenhouse.

GUS assay

GUS activity was histochemically localised according to Jefferson (1987). Tissue was incubated in staining buffer (100 mM $NaPO_4$, 50 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid cyclohexylammonium salt, 0.1% β-mercaptoethanol, 0.1% Triton X-100, pH 7.2) for 16 h. Stained plant material was cleared with 70% ethanol.

Callus induction assay

Leaves of greenhouse-grown plants were immersed in 70% ethanol followed by a 10-min sterilisation in 0.75% sodium hypochlorite. After removal of the mid vein, leaf discs of approximately 1 cm^2 were excised and cultured, abaxial side down, on callus culture (CC) medium (Franklin et al. 1993).

Southern analysis

DNA was prepared from leaves with a modification of the RNA preparation procedure described by Jones et al. (1985). After precipitation of RNA with lithium acetate, DNA was recovered from the supernatant by the addition of an equal volume of isopropanol. The DNA pellet was washed with 70% ethanol, re-suspended in water, precipitated by adding 0.1 vol of 2 M sodium acetate pH 4.8 and 2 vol of ethanol, washed with 70% ethanol, and re-suspended in water. Approximately 10-μg samples of plant DNA were digested with the appropriate restriction enzymes and analyzed by Southern-blot hybridisation (Sambrook et al. 1989).

Detection and quantification of the arcelin-5a protein

Crude protein samples were prepared by briefly vortexing 1- to 2-mg pieces of dry seed cotyledon tissue in 20 μl of extraction buffer (10 mM sodium chloride, 50 mM glycine, pH 2.4). After centrifugation for 10 min at 20000 *g*, proteins in the supernatant were prepared and separated by SDS/PAGE (Hames and Rickwood 1990). The sampling did not affect the germination capacity of the seeds. The arcelin content of the seeds was estimated on Coomassie blue-stained SDS/PAGE using BioImage software.

Results

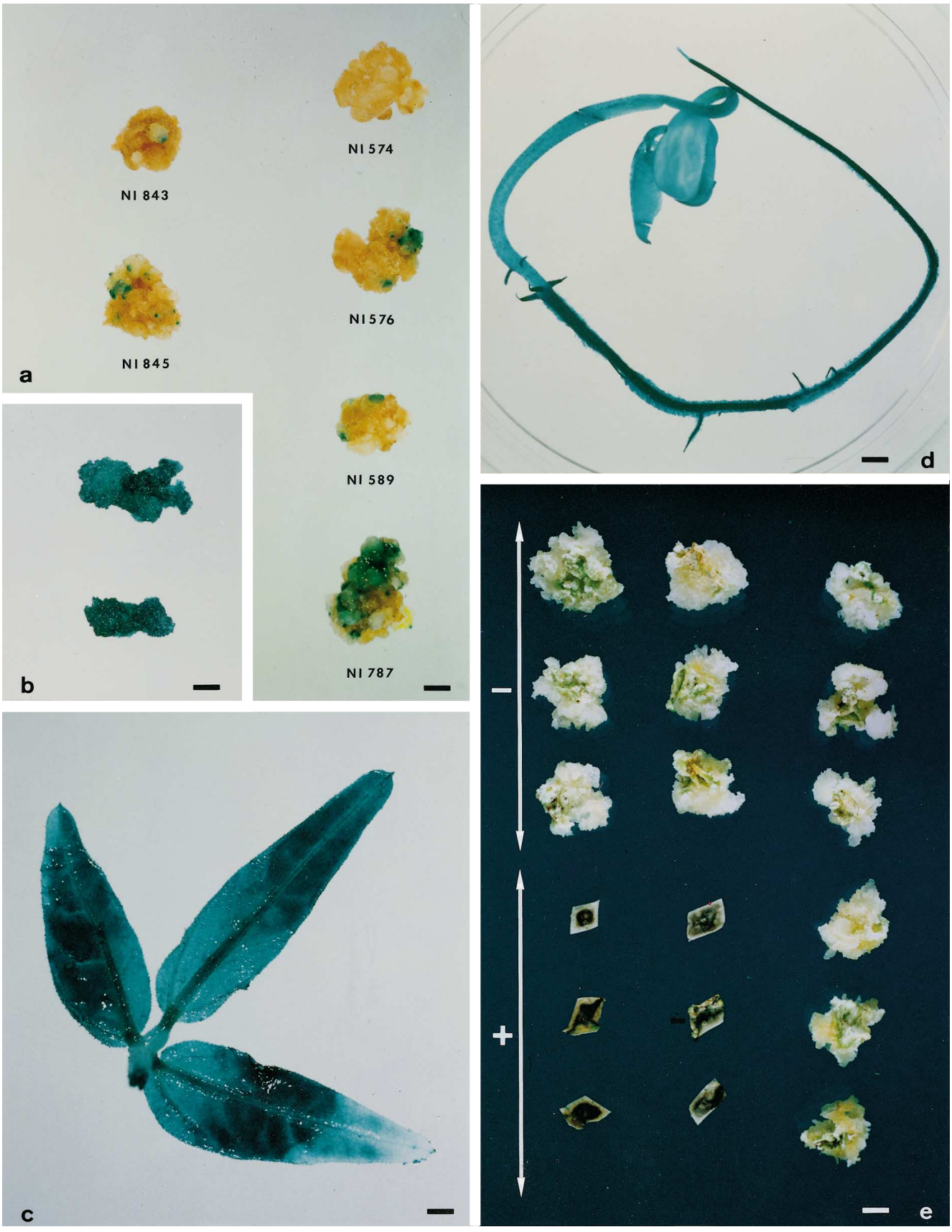
Transformation of organogenic callus

Green nodular callus with a capacity to regenerate shoots was obtained from bud explants of several *P. acutifolius* genotypes as previously described (Dillen et al. 1996). Five days after the third subculture on CIM1, the callus was co-cultivated with *A. tumefaciens* strain C58C1Rif^R(pMP90) (pTJK136) or C58C1Rif^R(pMP90)(patarc3-B1b). After co-cultivation, explants were put on CIM1 with 500 mg/l of cefotaxime containing either 300 mg/l of kanamycin or 20 mg/l of geneticin as a selective agent. The toxic levels of these antibiotics to non-transformed callus tissue had been determined in preliminary experiments (data not shown). In five out of six tested genotypes, transient *uidA* expression was detected 4 days after co-cultivation (Fig. 2 a). The recalcitrance of the callus of genotype NI 574 for transformation with *A. tumefaciens* C58C1Rif^R(pMP90) was confirmed in later experiments involving more plant material.

At the end of the third subculture of co-cultivated material on CIM1, 13 callus lines of genotype NI 576, two of NI 787, and seven of NI 843 had survived selection on geneticin whereas all material on kanamycin had died. Eighteen of these lines (ten of NI 576, one of NI 787, and seven of NI 843) proved positive in a GUS histochemical assay (Fig. 2 b). In the following two subcultures on CIM1, material of genotype NI 843 deteriorated (although GUS assays remained positive). Lines of genotypes NI 787 and NI 576, however, continued to proliferate.

Regeneration of transgenic shoots

When transferred to SIM, callus became organised and meristems and leaf-like structures developed. After three transfers on SDM, one line of NI 576 produced viable GUS-positive shoots (Fig. 2 c) that could be grafted and hardened. This line was derived from callus that had been


NI 843
NI 574
NI 845
NI 576
a
NI 589
NI 787
b
c
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–
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e

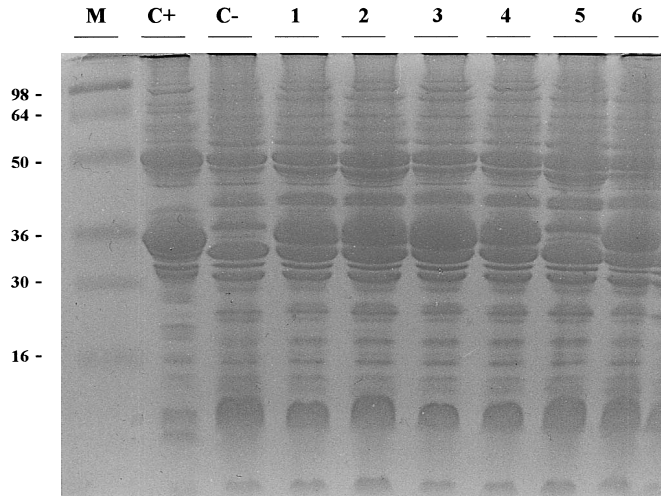

Fig. 3 SDS/PAGE (13.5%, Coomassie blue-stained) of crude protein extracts of progeny (T_1) of transformed *P. acutifolius* NI 576. *Lane M*, marker proteins (molecular mass indicated on the left in kDa). Extracts from seeds that produced GUS-positive plants (*lanes 1, 2, 3, 4, and 6*) display a band (32.2 kDa) identical to that of the arcelin-5 protein in an arcelin-containing wild *P. vulgaris* accession, G02771 (*C+*). The band is absent in non-transformed NI 576 (*C–*) and in GUS-negative progeny (*lane 5*)

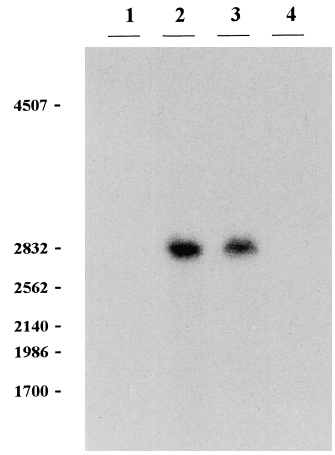

Fig. 4 Southern analysis of non-transformed NI 576 (*lane 1*), primary transformant (*lane 2*), GUS-positive progeny (T_1) of primary transformant (*lane 3*), GUS-negative progeny (T_1) of primary transformant (*lane 4*). *Bcl*I-digested DNA was probed with the fragment indicated in Fig. 1. The size of the marker DNA is indicated on the left in bp

transformed with *A. tumefaciens* C58C1Rif^R(pMP90) (patarc3-B1b). PCR analysis of in vitro-formed stem and leaf structures was indicative of the presence of the transgenes (data not shown). Different parts of the primary transformants (leaves, petals, pods) were tested for GUS activity and all proved positive. To further confirm transformation, primary transformants were subjected to Southern analysis. A probe covering the 3′ octopine synthase region and part of the *nptII*-coding region of the T-DNA hybridised to two plasmid-plant DNA junction fragments (data not shown). This indicates that two copies of the T-DNA had integrated. The different transformants showed identical integration patterns confirming that they were derived from the same integration event. This was expected since they arose from the same callus line.

Analysis of progeny

Five grafts (T_0) were established in the greenhouse and, when provided with short-day conditions, flowered, and set seed. Seeds (T_1) were harvested and tested for the presence of arcelin. Of 18 seeds tested, 12 apparently expressed the protein (Fig. 3). The relative concentration of arcelin-5a was in the order of 20% of total seed protein. Seeds were subsequently germinated and assayed for *uidA* expression (Fig. 2 d). There was an absolute correlation between GUS expression and the arcelin phenotype.

Of a total of 42 seedlings (T_1) tested for *uidA* expression, 30 were positive. This corresponds to a 3:1 segregation ratio (χ^2 test at the 5% significance level), indicating that the transgenes were inherited as a single locus. The T_2 generation segregated accordingly (data not shown).

Leaf discs of 20 T_1 plants were cultured in vitro on CC medium containing 20 mg/l of geneticin. This antibiotic concentration completely inhibited callus formation on non-transformed leaf discs. Callus production from transformed GUS-positive progeny in the presence or absence of the antibiotic was similar (Fig. 2 e). For all plants tested, the resistance phenotype correlated with the GUS phenotype.

The presence or absence of hybridising fragments in a Southern analysis of progeny plants correlated with the presence or absence of transgene expression (Fig. 4).

Fig. 2 a–e GUS activity in callus of different *P. acutifolius* genotypes 4 days after co-cultivation with *Agrobacterium* **(a)**, in geneticin-resistant callus of genotype NI 843 **(b)**, in a leaf of an in vitro-formed shoot of NI 576 **(c)**, and in a progeny (T_1) seedling of NI 576 **(d)**. Callus development on leaf discs of NI 576 in the presence (+) or absence (–) of 20 mg/l of geneticin **(e)**. Each column contains discs of one leaf. From left to right: non-transformed, GUS-negative progeny (T_1), and GUS-positive progeny (T_1). Bars = 1.2 mm **(a, b)**, 1.3 mm **(c)**, 2.1 mm **(d)**, and 4.0 mm **(e)**

Discussion

Regeneration of grain legumes, and of *Phaseolus* in particular, has met with considerable difficulties. With respect to transformation, it is useful to discriminate between two types of regeneration. One type is characterised by the absence of unorganised growth (a callus phase). Shoots proliferate in a region adjacent to meristems, or pre-existing meristems, that are contained in the explants. This type of regeneration has been repeatedly described for most grain legumes. For *Phaseolus*, explants have been used that contained seedling apical meristems (Kartha et al. 1981; Allavena and Rossetti 1986; Malik and Saxena 1992) or meristems located in the cotyledon axils (McClean and Grafton 1989; Franklin et al. 1991; Malik and Saxena 1992; Mohamed et al. 1992 a, b; Vaquero et al. 1993). Explant integrity is a prerequisite for this type of regeneration to occur (Malik and Saxena 1992) and, therefore, the selec-

tion of transformed sectors in explants is perilous (McClean and Grafton 1989). As a consequence, screening rather than selection for transgenic shoots is inevitable and transformation efficiencies are accordingly low. Moreover, the recovery of transgenic offspring through this type of regeneration necessitates L_2-layer transformation in order to achieve germ line transformation (Satina et al. 1940). Obviously, cells in the deeper layers of organised tissues may not be physically accessible to *Agrobacterium*. Consequently, success in combining this type of regeneration with *Agrobacterium*-mediated gene transfer for the genetic transformation of grain legumes has been very limited (McClean et al. 1991; Jordan and Hobbs 1993; McKently et al. 1995; Di et al. 1996) and the use of particle bombardment may be more appropriate (McCabe et al. 1988; Russell et al. 1993; Brar et al. 1994).

A second type of regeneration system is characterised by the occurrence of a phase of unorganised callus from which shoots or embryos develop. Although presumably more genotype-dependent and implying a higher risk for somaclonal variation, this type of regeneration seems much more suited for generating transgenic plants. Selection of transgenic callus is usually straightforward and should allow for efficient enrichment of transformed tissue, irrespective of the gene delivery method employed. In addition, rapidly dividing cells in the initial explant, or in the callus itself, are likely targets for *Agrobacterium*. Indeed, where such regeneration systems have been used, the efficiency with which transgenic progeny was obtained was often relatively high and *Agrobacterium* could be used (Pickardt et al. 1991; Puonti-Kaerlas et al. 1992; Schroeder et al. 1993; Cheng et al. 1996). Gene transfer through the latter strategy to 'tissue culture-competent' genotypes with *Agrobacterium*, and subsequent breeding to introgress the transgenes into agronomically important lines, may after all prove more efficient than particle bombardment of organised meristematic tissues and screening for germ line transformation events.

P. vulgaris is susceptible to *A. tumefaciens* infection (McClean et al. 1991; Franklin et al. 1993; Lewis and Bliss 1994). Although callus-derived shoot regeneration had been demonstrated for two *P. vulgaris* lines (Mohamed et al. 1993), the efficiency of the reported procedure was, in our hands, too low to attempt *Agrobacterium* transformation. The two regenerable *P. vulgaris* lines were constructed from a *P. vulgaris* × *P. acutifolius* interspecific cross (Mohamed et al. 1993). We demonstrated that the regeneration capacity most probably derived from *P. acutifolius* and we identified several *P. acutifolius* genotypes that exhibited a superior regeneration capacity (Dillen et al. 1996). Based on these findings, we attempted to genetically transform *P. acutifolius*. In the present report, we demonstrate that callus cultures of five out of six tested *P. acutifolius* genotypes are susceptible to *Agrobacterium* infection. Of several genotypes examined, resistance to the antibiotic geneticin and positive histological GUS assays indicated that stably transformed callus lines had been established. Callus from NI 576, a wild genotype identified as possessing superior regeneration capacity (Dillen et al. 1996), continued to proliferate under selective conditions and finally produced five primary transformants. Southern analysis of these plants revealed that they were most probably of clonal origin. Mendelian segregation in the offspring for GUS activity, geneticin resistance, and the occurrence of an introduced foreign seed storage protein, the *P. vulgaris* arcelin-5a protein (Goossens et al. 1995), constitute proof for stable integration and transmission of the transgenes.

Arcelins are lectin-like proteins that presumably confer resistance to the Mexican bean weevil, *Zabrotes subfasciatus* (Osborn et al. 1988), a major cause of post-harvest losses in cultivated common beans in tropical regions. Of the six known allelic variants (Osborn et al. 1986; Lioi and Bollini 1989; Santino et al. 1991), arcelin-5 is very promising with respect to conferring insect resistance (Kornegay et al. 1993). The SDS/PAGE data obtained with transformed seeds confirm that the previously isolated fragment (Goossens et al. 1995) contains an actively expressed arcelin-5 gene. Breeding experiments and tests with artificial seeds strongly suggest, but do not prove, that arcelins are involved in insect resistance (Osborn et al. 1988). The introduction of the presumed resistance gene through genetic transformation will allow an unambiguous assessment of the role of arcelin-5a. *P. acutifolius* NI 576 is a host for *Z. subfasciatus* and insect tests on transgenic seeds are underway.

This work started before the differences in the regeneration capacity of the different genotypes had been fully established. Therefore, we used only a limited amount of callus of each genotype for co-cultivation. For NI 576, the callus was derived from at most five initial bud explants. We have repeated the initiation of green nodular callus from this genotype several times and find it very reliable. Transient *uidA* expression levels similar to those described here are routinely achieved. We are currently optimising the procedure with respect to helper plasmid type, co-cultivation conditions, choice of marker genes, and selection strategy. It is expected that further improvements in the regeneration procedure for NI 576 will enhance the efficiency of recovery of transgenic plants.

The introduction of transgenes from *P. acutifolius* into the economically more important species *P. vulgaris* would be a way to genetically engineer the latter. Hybridisation between *P. vulgaris* and *P. acutifolius* has been attempted over a long period with the aim of transferring desirable characters from *P. acutifolius* to *P. vulgaris*. Such traits include drought and heat resistance and several disease resistances (reviewed by Pratt and Nabhan 1988). Artificial cross-pollination and fertilisation do not pose problems. However, post-zygotic barriers usually prevent normal development of the embryo so that, as a rule, no viable hybrids are obtained (Pratt 1983). In vitro embryo culture has proven a useful tool for rescuing both hybrid embryos from initial crosses as well as the progeny of more advanced backcross generations. Recently reported improvements in embryo-culture procedures (Mejía-Jiménez et al. 1994), the use of congruity backcross programs (Haghighi and Ascher 1988; Mejía-Jiménez et al. 1994), and the identifi-

cation of *P. vulgaris* (Parker and Michaels 1986) and *P. acutifolius* (Mejía-Jiménez et al. 1994) facilitator genotypes, should further improve the efficiency of hybridisation. The production of large numbers of F_1 seeds *in planta* from a cross involving *P. acutifolius* NI 576 (Haghighi et al. 1984) and the observation that in vitro culture was not required to rescue backcross embryos (Haghighi and Ascher 1988), are indicative of a high cross-hybridising capacity for this genotype. In view of its amenability for *Agrobacterium*-mediated transformation, NI 576 could serve as a 'bridging genotype' for gene transfer to *P. vulgaris*. Introgression of a simply inherited trait from *P. acutifolius* to *P. vulgaris* is feasible as has been demonstrated for *Xanthomonas campestris* resistance (Scott and Michaels 1992).

P. acutifolius is an important source of resistance traits that awaits further exploitation for the improvement of *P. vulgaris* and possibly other crops. The availability of a transformation system for *P. acutifolius* opens up the possibility of introducing transposon systems for the tagging and cloning of resistance genes.

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