J Appl Genet 46(2), 2005, pp. 139-147

Efficiency of transformation of Polish cultivars of pea (*Pisum sativum* L.) with various regeneration capacity by using hypervirulent *Agrobacterium tumefaciens* strains

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Abstract. An *Agrobacterium*-mediated transformation method of pea has been developed for several edible and fodder cultivars of pea (*Pisum sativum* L.), characterized previously in their potential for regeneration via organogenesis. The most appropriate explant, which was susceptible to *Agrobacterium* infection and capable of regenerating transgenic plants, turned out to be a slice of an immature embryo, including the embryo axis and the basal part of a cotyledon. Three hypervirulent strains of *A. tumefaciens* were tested: AgL0, AgL1 and EHA105. Each carried the binary vector pP35SGIB containing the *uid* gene, with an intron under control of the 35S promoter, and the *bar* gene conferring resistance to phosphinotricin. Strain AgL0 was found to be efficient for the majority of cultivars, followed by AgL1 and EHA105. Transformation efficiency varied from 0.7 to 4.1%, depending on cultivar and *Agrobacterium* strain. The transformation efficiency of particular pea cultivars did not clearly correspond to their regeneration capacity, which – although indispensable – was not a critical parameter of successful transformation. The presence of integrated genes in pea genomic DNA was detected by the PCR. T-DNA was stably transmitted to the progeny, as it was confirmed by Southern hybridization. The activity of introduced genes was analysed by the histochemical GUS assay and by painting leaves or by spraying transgenic plants with the herbicide Basta.

Key words: AgL0, AgL1, *Agrobacterium tumefaciens*, EHA105, pea, regeneration, transformation.

Introduction

Pea (*Pisum sativum* L.) is an important crop cultured worldwide for human consumption and for forage. The value of peas results from the high protein content and simultaneously the very low content or absence of anti-nutritive substances: alkaloids, protease inhibitors, haemagglutinins, etc. (Griga and Novák 1990; Casey and Davies 1993; Jasińska and Kotecki 1993). However, some traits of pea, e.g. the low content of sulphur amino acids or sensitivity to pests and diseases, require an improvement for cultivation needs. Contemporary breeding programs more and more often employ genetic transformation methods as an essential component. On the other hand, transgenic peas also can be successfully used for strictly biotechnological purposes, such as production of antibodies in seeds (Perrin et al. 2000).

The development of a transformation system is a prerequisite of biotechnological improvement or utilization of interesting pea cultivars. Although some sufficient protocols of pea transformation have been elaborated, the regeneration of transgenic peas still is not a routine. Two main factors are necessary to improve the pea transformation efficiency: (1) an efficient regeneration system and (2) an appropriate *Agrobacterium tumefaciens* strain. Transgenic peas have been regenerated mainly via organogenesis *de novo* (Puonti-Kaerlas et al.

Received: November 9, 2004. Accepted: December 28, 2004.
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1990; Schroeder et al. 1993; Grant et al. 1995) and also directly from meristems (Davies et al. 1993; Bean et al. 1997; Nadolska-Orczyk and Orczyk 2000). Transgenic pea plants were obtained after transformation conducted by hypervirulent *Agrobacterium* strains like EHA105 (Bean et al. 1997; Nadolska-Orczyk and Orczyk 2000) or AgL1 (Schroeder et al. 1993; Grant et al. 1995), but also by other strains. Unfortunately, the rate of pea transformation remains relatively low and reaches several per cent. For this reason, an efficient transformation protocol is still being searched for. The identification of suitable conditions of regeneration and transformation is especially necessary for cultivars of particular or practical importance, or for adaptation to specific environmental conditions (Polowick et al. 2000).

We present here a complete transformation protocol for several Polish pea cultivars of diverse applications: for either dry or fresh pulses or for forage. Three hypervirulent *Agrobacterium* strains, AgL1, EHA105 and the newly used AgL0, were used to transform pea cultivars differing considerably in regeneration capacity, and a previously developed method of regeneration via organogenesis was adapted to obtain transgenic peas (Pniewski et al. 2003). Transgenic plants stably transmitted integrated transgenes into the next generation and the introduced genes remained active.

Material and methods

Bacterial strains and plasmid

The binary plasmid pP35SGIB was constructed for the experiments (Figure 1). The plasmid contained the marker gene *bar*, encoding phosphinotricin acetyltransferase (PAT), under the control of constitutive nopaline synthase promoter and the reporter gene *uid* encoding -glucuronidase (GUS) with an intron, under the control of constitutive 35S RNA CaMV promoter. The plasmid was mobilized into three hypervirulent strains of *Agrobacterium tumefaciens* AgL0, AgL1 and EHA105 by electroporation.

Plant material

The following Polish pea genotypes were used in the experiments: (1) edible cultivars for dry seeds: Agra, Kwestor; (2) edible cultivars for fresh (green) seeds: Pionier, Cud Kelwedonu, Delisa II, Konserwowy IHAR, Topaz; and (3) fodder cultivars: Grapis, Wiato.

Media and *in vitro* culture conditions

All media used in pea regeneration experiments consisted of MS salts (Murashige and Skoog 1962) and B5 vitamins (Gamborg et al. 1968). Most of the media were full-strength MS, except that the PKA medium contained half the amount of MS macroelements but a full dose of calcium. The media were supplemented with 3% (w/v) sucrose and plant growth regulators: P0 – 0.2 mgL^{-1} KIN, 1 mgL^{-1} 2,4-D; P1– 2.0 mgL^{-1} BAP, 2.0 mgL^{-1} NAA; P2 – 4.5 mgL^{-1} BAP, 0.02 mgL^{-1} NAA; P3 – 2.0 mgL^{-1} BAP; P4 – 0.02 mgL^{-1} BAP; PKA – 1.0 mgL^{-1} NAA. The pH was adjusted to 5.7 and agar (Serva) was added to 0.8% (w/v). Media were sterilized by autoclaving at 121°C for 20 minutes (106 kPa). Calli, regenerated plantlets and rooted shoots were cultured in approximately 4000 lx of light intensity at 16/8 h light/dark photoperiod and at 24/18°C day/night temperature regime.

Transformation procedure

Peas were grown in a growth chamber in 16/8 photoperiod (5000 lx of lights) and 18/12ºC day/night temperature regime. Immature pods containing seeds were harvested at the stage of maximum fresh weight when they started to dry. Pods were surface-sterilized by immersing for 30 s in 70% ethanol, followed by 20 min

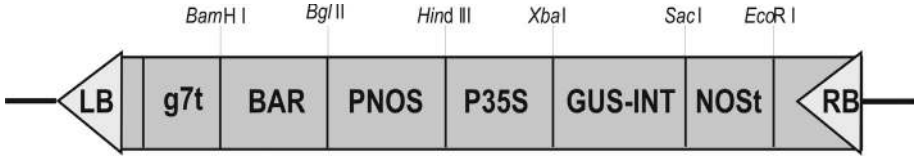

Figure 1. Organisation of T-DNA of the binary plasmid pP35SGIB

P35S = CaMV 35S promoter; PNOS = nopaline synthase promoter; GUS-INT = gene of β-glucuronidase with an intron; BAR = gene of phosphinotricin acetyltransferase; NOSt = nopaline synthase terminator; g7t = g7 terminator; LB and RB = left and right border sequences

in 25% commercial chlorine bleach (Clorox) with 0.01% Tween 20 and finally rinsed 4–5 times with sterilized distilled water. Pods were then cut longitudinally with a scalpel and seeds were released. The seed coat was removed and then the embryo root, apical bud and distal parts of cotyledons were excised. Finally, the remainder of the embryo was cut along the longer axis into two halves and each half was divided again, so four explants were derived from one seed. The explant consisted of a fragment of the embryo axis, cotyledonary node and the basal part of a cotyledon, adjacent to the meristematic region. Primary explants were directly immersed in *Agrobacterium* suspension for 40–45 min with light shaking. Inoculated explants were briefly dried on a sterile filter paper and then transferred onto the P0 medium and co-cultured for 3 days in darkness at 24/18°C day/night temperature regime. Explants were then washed 3–4 times in sterile water to remove bacteria, dried on a filter paper, and transferred onto the P1 medium.

Agrobacterium strains were cultured on the solid YEB medium (Vervliet et al. 1975) with 50 mgL^{-1} of kanamycin and with 50 mgL^{-1} of rifampicin (only strains AgL1 and EHA105) at 28°C for two days. The liquid MG/L medium (Garfinkel and Nester 1980) was then inoculated with *Agrobacterium* and cultured overnight at 28°C with 280 rpm shaking, and bacteria were subsequently transferred onto the MG/L medium at a ratio of 1 : 100 and grown up to $OD_{600} \approx 0.6–0.8$. Then the culture was centrifuged at 4500 rpm and 4°C, and the bacterial pellet was resuspended in the MSGA solution consisting of MS salts, 10 mM glucose and 0.1 mM acetosyringone. The volume of MSGA solution was calculated to prepare the bacterial suspension of a density $\approx 10^8$ cells/mL. *Agrobacterium* suspension was then poured off onto Petri dishes and used for explant inoculation.

Plant regeneration

Transformed explants were cultured for 4–6 weeks on the P1 medium for callus growth. The shoots developed within the first 2–3 weeks of culture were cut out and discarded as they probably arose from pre-existing buds. Around 400 explants of each pea cultivar were used in two consecutive experiments. Callus pieces were moved onto the P2 medium to stimulate organogenesis. Calli were transferred onto a fresh medium every 4 weeks. Appearing plantlets were cut out from calli and moved for further development and multiplication into jars with the P3 medium. Developed plantlets, around 1.5–2 cm in length, were transferred onto the P4 medium to promote shoot elongation. Shoots of approximately 5–6 cm in length were then transferred onto the rooting medium PKA. Rooted plants were transferred into pots with a soil/perlite mixture (1 : 1), conditioned and grown in a greenhouse at 16/8 h light/dark photoperiod and at 22/16°C day/night temperature regime until the seed set. All media used for regeneration of transgenic plants were supplemented with 5 mgL^{-1} of phosphinotricin (PPT) and 300 mgL^{-1} (P1, P2) or 150 mgL^{-1} (P3) of timentin® (Beecham). Timentin was omitted in media P4 and PKA.

Transformation efficiency was calculated as percent representing the number of independent events, i.e. plants regenerated from separate adventitious buds per 100 primary explants.

Estimation of regeneration capacity of pea cultivars

The *in vitro* culture procedure omitting selection conditions was initially used for the regeneration of untransformed peas. The regeneration capacity was estimated on the basis of two values: percentage of regenerating explants and average number of adventitious buds formed by a single explant. Number of buds was calculated as an arithmetic mean together with its standard deviation for the population. The product of both parameters meant the total regeneration capacity, i.e. the number of individual regenerants likely to obtain from 100 primary explants.

PCR analysis of putative transformants

Genomic DNA from primary transgenic plants was isolated in a small-scale procedure according to the modified CTAB method (McGarvey and Kaper 1991). Polymerase chain reactions (PCR) were set up in a volume of 20 µL containing approximately 100 ng of plant genomic DNA, 0.2 mM of each dNTP, 10 pM of each primer and using 1 unit of *Taq* DNA polymerase (Biometra). The PCR temperature profile was set as follows: initial denaturation at 94ºC for 4 min, next 35 cycles of denaturation at 94ºC for 1 min, annealing for 45 sec, elongation at 72ºC for 1 min, and a final extension at 72ºC for 5 min. Primers were designed for the *uid* gene (primer F: 5'-GGA GTA TTG CCA ACG AAC C-3'; primer R: 5'-CGC CAG GAG AGT TGT TCA TTC-3'; primers annealed at 58ºC, amplified DNA frag-

ment of 606 bp in length) and the *bar* gene (primer F: 5'-TGA GCC CAG AAC GAC GCC-3'; primer R: 5'-GGA CTT CAG CCT GCC GGT AC-3'; primers annealed at 65ºC, amplified DNA fragment of 516 bp in length).

Additional screening of plant genomic DNA samples by the PCR analysis was done to exclude *Agrobacterium* DNA residual contamination. Internal primers (primer F: 5'-TTG ACC TTG TTT CAG GTT TAC ACA-3'; primer R: 5'-GAC GAG GAT AAT CAT CAT CGA AAC-3'; primers annealed at 62ºC) for the *Agrobacterium* chromosomal *chvA* gene were designed to amplify the 777 bp DNA fragment. The PCR reactions were set up as previously. All the PCR reactions were performed in the PTC-200 MJ Research thermal cycler. Amplification products were analysed by agarose gel electrophoresis. Molecular weight of products was estimated with the use of a 1 kb ladder (Sigma) as a standard.

Southern hybridization

Genomic DNA from T1 plants was isolated according to the modified CTAB method (Chee and Slightom 1994). 20–25 µg of DNA was digested with *Eco*R I and after electrophoresis blotted onto a positively charged nylon membrane (Boehringer Mannheim). The probe was prepared by the PCR amplification of the *bar* gene with the use of a DIG labelling kit (Boehringer Mannheim) and hybridized according to the manufacturer's protocol. CSPD was used as a chemiluminescent substrate and the signals were detected on an X-ray film.

GUS assay and Basta tests

The histochemical GUS assay was carried out on leaf fragments according to Jefferson (1987). Four-week-old T1 plants were sprayed with the solution of herbicide Basta containing 200 mgL^{-1} of phosphinotricin. Alternatively, leaflets were painted with the solution on the upper side.

Results and discussion

Regeneration capacity of pea cultivars

The studied cultivars differed distinctly in regeneration capacity (Figure 2). The highest percentage (81–97%) of regenerating explants was noted for cultivars Wiato, Agra and Kwestor, and medium values (62–87%) were recorded for Konserwowy IHAR, Topaz and Delisa II. However, the highest average number of adventitious buds formed by a single explant (5.1–5.8) was characteristic for Wiato, Grapis, Pionier, Konserwowy IHAR. The product of both parameters, namely the number of individual regenerants per 100 primary explants, reflected the total regeneration capacity. Therefore, the highest regeneration capacity appeared in the cultivars Wiato and Konserwowy IHAR. A reasonable regeneration capacity was also noted for Agra and Kwestor.

Effect of explant type on transformation efficiency

Mainly immature embryos were earlier reported as an appropriate source of explants. However, different types of embryo-derived explants were also used: slices of embryo axis (Schroeder et al. 1993; Polowick et al. 2000), cotyledons (Nadolska-Orczyk and Orczyk 2000), basal parts of the embryo (Grant et al. 1995), or cotyledonary nodes (De Kathen and Jacobsen 1990; Davies

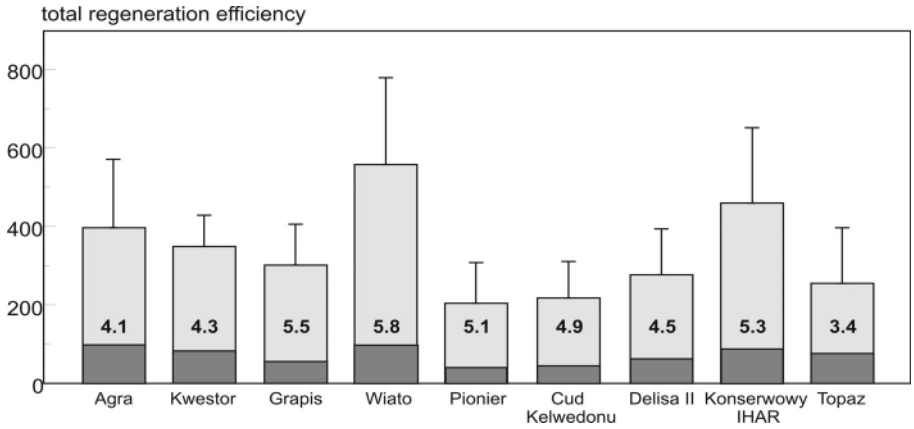

Figure 2. Regeneration capacity of pea cultivars, i.e. number of regenerated shoots per 100 primary explants (light bars), being a product of two factors: percentage of regenerating explants (dark bars) and the average number of buds formed by a single explant (numbers in the bars)

et al. 1993). We tested both axis and cotyledon slices as well as explants consisting of both the embryo axis and the basal part of a cotyledon (Figure 3). In our experiments the embryo axis was found to be unsuitable for transformation, as it died soon, especially on the selection medium, whereas cotyledon slices formed callus but had a very limited regeneration capacity. Conceivably, intact cotyledons could regenerate more efficiently than thin slices, as previously reported (Grant et al. 1995; Nadolska-Orczyk and Orczyk 2000). However, we obtained satisfactory results

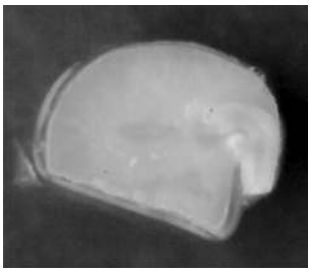

Figure 3. Primary pea explant – immature embryo slice containing the embryo axis and the basal part of

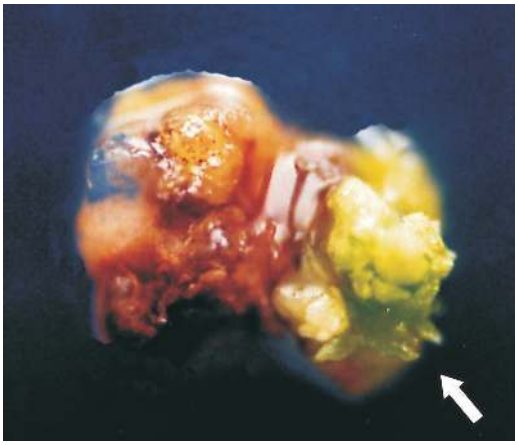

Figure 4. Adventitious bud formation in transgenic callus via organogenesis (the arrow indicates a bud)

(see below) using "mixed" explants, containing both the embryo axis and the basal part of a cotyledon. A problem of using such explants is the possible development of non-transgenic or chimeric shoots arising from the remaining cotyledonary nodes. To reduce this obstacle, we removed any shoots developing during the first 2–3 weeks of culture, i.e. of callus formation.

Transgenic plants were regenerated via organogenesis in callus (Figures 4, 5). Although such a system of regeneration extended plant recovery to several months, as in other reports (Puonti-Kaerlas et al. 1992; Polowick et al. 2000),

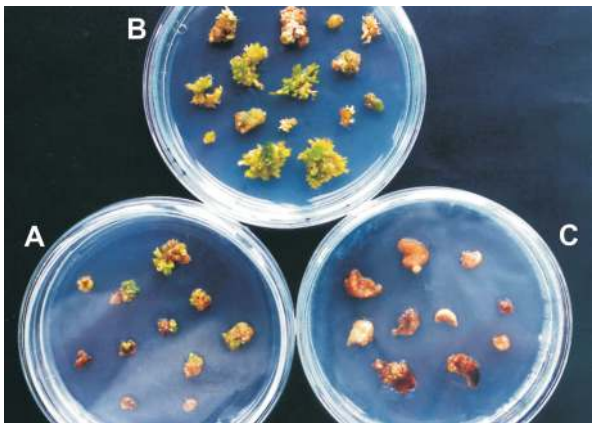

Figure 5. Regeneration of transgenic pea cv. Wiato
A = shoot/bud initiation on a selection medium; B =control explants on a non-selection medium; C = control explants on a selection medium

the plants obtained were most likely not chimeric but developed *de novo* from one initial cell.

Effect of *Agrobacterium tumefaciens* strain and plant genotype

It is commonly assumed that virulence of the strain of *Agrobacterium* sp. against a particular plant genotype is crucial for successful transformation. Initial studies on pea transformation were conducted to compare the ability of *Agrobacterium tumefaciens* strains to induce tumour or callus growth. Results showed that strains virulent for other legumes, as C58C1 (De Kathen and Jacobsen 1990), GV3101 (Puonti-Kaerlas et al. 1992), or EHA 101 (Lulsdorf et al. 1991), were also effective for pea. Transgenic peas were regenerated when hypervirulent *Agrobacterium* strains, like EHA105 (Bean et al. 1997; Nadolska-Orczyk and Orczyk 2000; Polowick et al. 2000) or AgL1 (Schroeder et al. 1993; Grant et al. 1995), were used for transformation. In our study, these strains were also efficient, but we obtained some improved results using the hypervirulent strain AgL0. Strain AgL0 was earlier successfully used for the transformation of other recalcitrant grain legumes: narrow-leaved lupin and yellow lupin (Pigeaire et al. 1997; Li et al. 2000). Strain AgL0 was able to transform six out of the nine tested pea cultivars and it was the only effective strain for three of them (Figure 6). The transformation rates were also relatively high, from 0.7 to 3.3%. Conceivably, the strain could be even more efficient, but it appeared to be especially difficult to eliminate by using the antibiotic timentin®. Secondary bacterial contaminations occurred relatively often and caused a rejection of a part of regenerating

explants. Among other strains used, AgL1 was more efficient than EHA 105. AgL1 was virulent for four cultivars and it was the only strain able to transform cv. Kwestor. Transformation efficiency using strain AgL1 (1.4–4.1%) was comparable to AgL0; for cv. Wiato it was even higher and for cv. Kwestor the transformation rate was the highest observed. Strain EHA 105, previously reported as virulent for pea (Nadolska-Orczyk and Orczyk 2000), was not so efficient in our experiments. It transformed only three cultivars, with the efficiency range of 0.6–0.9%, but it was the only one capable to transform cv. Cud Kelwedonu.

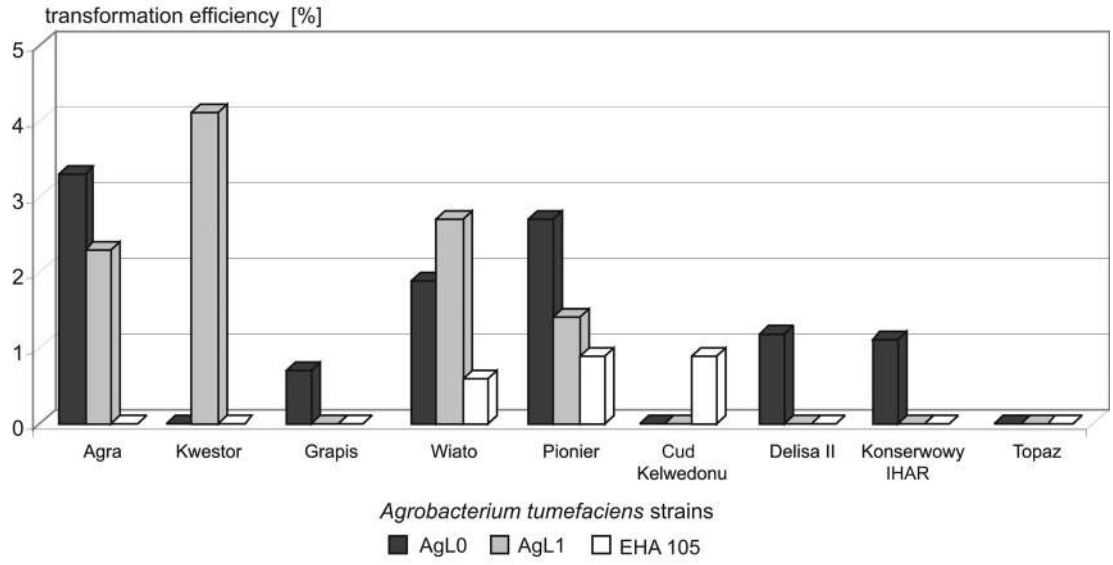

Figure 6. Efficiency of transformation of pea cultivars by using *Agrobacterium tumefaciens* strains, i.e. number of independent transformation events per 100 explants

Plant genotype often affects transformation efficiency like the virulence of *Agrobacterium* strains. In our experiments the pea cultivar clearly affected the transformation rate. Regeneration capacity varied visibly among the cultivars (Figure 2) and it appeared to be not clearly corresponding to *Agrobacterium*-mediated transformation efficiency (Figure 6). Three of the cultivars Agra, Kwestor and Wiato were characterized by both a relatively high regeneration capacity and a high transformation rate. Cultivar Delisa II, with moderate regeneration capacity, was also moderately susceptible to *Agrobacterium* strains. The low regeneration capacity of another cultivar, Cud Kelwedonu, was correlated with the low transformation efficiency. However, the transformation rates for four cultivars were opposite to their regeneration capacity. Cultivars Konserwowy IHAR, Grapis and Topaz, which regenerated high or average numbers of buds, were weakly sensitive to *Agrobacterium* strains or completely insusceptible, e.g. Topaz. On the other hand, cv. Pionier, initially characterized by the lowest regeneration capacity, was one of the most suitable targets for *Agrobacterium*- mediated transformation.

Our results suggest that the regeneration capacity of a particular cultivar is not sufficient to predict transformation efficiency, although sometimes the two values may be correlated. Similar research on regeneration capacity of pea genotypes affecting transformation efficiency has been rarely reported. Polowick et al. (2002) examined the transformation susceptibility of 8 Canadian pea genotypes. However, in their experiments only *Agrobacterium* strain EHA105 was tested. Those authors observed that plant genotype affected the transformation rate, which depended also on the vector used.

Transformation efficiency of pea cultivars, observed in our experiments, corresponded to results of other authors for strain AgL1: 1.4–4.1% versus 0.7–2.5% (Schroeder et al. 1993; Grant et al. 1995); and for strain EHA105: 0.6–0.9% versus 0.1–2.4% (Polowick et al. 2000). However, some publications showed a higher efficiency of strain EHA105: up to 3.6% (Nadolska-Orczyk and Orczyk 2000). The newly used strain AgL0, characterized by the virulence of 0.7–3.3% and comparable to AgL1, seems to be worthy

of wider utilization because of its broader range of host genotype suitability.

Some publications showed a more satisfactory transformation efficiency of peas. The higher transformation rate resulted probably from the selection system utilizing antibiotics like kanamycin: from 3.4% (Grant et al. 1998) to 5% (De Kathen and Jacobsen 1990) or 8.2% (Nadolska-Orczyk and Orczyk 2000); or hygromycin: from 4.9% (De Kathen and Jacobsen 1990) to 15% (Puonti-Kaerlas et al. 1992). However, plants regenerated on kanamycin sometimes were not transgenic or chimeric, as proved in PCR tests (Nadolska-Orczyk and Orczyk 2000). We decided to use phosphinotricin for selection as a more strict agent, to decrease the risk of regeneration of chimeric plants. The herbicide-resistant transgenic plants are also more suitable for commercial applications.

T0 plants were grown in a greenhouse until seed set. Usually the number of seeds set by a single transgenic plant was low, from 1 to 8. To increase the number of seeds, we micropropagated the T0 transformants during *in vitro* culture on the P3 medium (see Materials and methods). Finally we obtained 20–30 seeds per individual transgenic line. The seeds germinated and T1 plants were analysed similarly to T0 parental plants.

Approximately 75% of progeny plants were PCR-positive for *uid* and *bar* genes, proving Mendelian inheritance of transgenes. However, in some lines, T-DNAs were transmitted inconsistently to the 3:1 pattern, indicating that primary transformants in those events were presumably chimeric. Southern hybridization showed 1–5 copies of the gene *bar* in plant genomic DNA (Figure 9). As expected, the number of gene copies

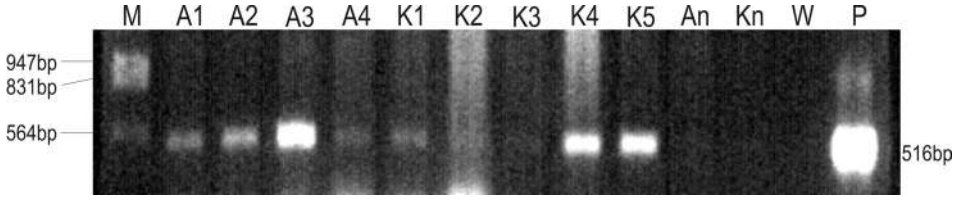

Figure 7. PCR analysis of the *bar* gene in putative T0 transformants
Lanes: M = DNA size marker; A1 to K5 = putative transformants of cv. Agra and Kwestor; An and Kn = non-transformed Agra and Kwestor (negative control); W = water (negative control); P = plasmid P35SGIB (positive control)

Analysis of T0 and T1 plants

The regenerated T0 plants growing in a greenhouse were analysed by the PCR, the histochemical GUS assay, and Basta painting. Most of the regenerated plants were PCR-positive for both genes: *bar* and *uid* (Figure 7). Additional screening of *Agrobacterium* residual contaminations (see Materials and methods) proved that PCR products were derived only from the transgenes integrated into the plant genome. PCR-verified plants were tested in respect of GUS expression and resistance to Basta. GUS expression was unexpectedly low (data not presented), but the expression of PAT was sufficient. Leaflets painted with Basta solution containing 200 mgL^{-1} of PPT, survived the herbicide treatment, but some injuries were observed (Figure 8). Individual transgenic plants varied in susceptibility to the herbicide, from partial sensitivity to complete resistance to the applied dose.

was the same in progenies coming from a single T0 plant cloned, proving that multiplication had no effect on genetic stability of primary transgenic plants.

Results of Basta spraying tests were not completely correlated with results of the PCR analysis. Some T1 plants were not fully resistant to the herbicide, although they were PCR-positive. How-

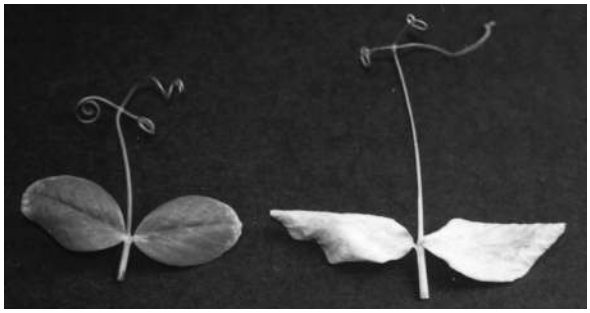

Figure 8. Herbicide test: painted leaflets 5 days after herbicide application: transgenic pea cv. Kwestor line K1a (on the left) and control plant (on the right)

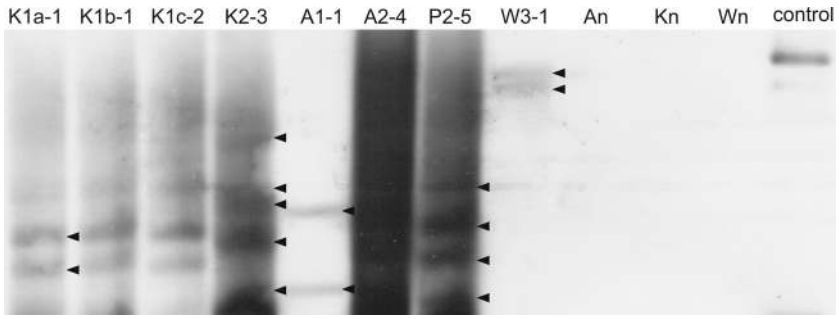

Figure 9. Southern analysis of transgenic T1 plants
Lanes: K1a-1 to W3-1 = transgenic plants; An to Wn = non-transformed plants of cvs. Agra, Kwestor and Wiato; control = plasmid P35SGIB. Note: K1a to K1c = micropropagated clones of K1 line

ever, these plants were the progeny of T0 plants that were also sensitive to Basta. The fact of differentiated PAT expression could be caused by the position effect of T-DNA.

Conclusions

We developed a transformation protocol for several pea cultivars adapted to Polish growing conditions. The presented method introduced two methodological modifications, increasing the extent of plant recovery: type of explant and multiplication of primary transformants. Our results confirmed the high virulence of *Agrobacterium tumefaciens* strain AgL1 and the newly used strain AgL0 for peas, allowing to achieve a transformation efficiency of 3–4%. We noticed that the regeneration capacity of pea genotype/cultivar, although indispensable, was not a critical parameter for efficient transformation.

Acknowledgments. This work was supported by funds of the Polish State Committee for Scientific Research granted to J. K. (project No. 6 P04B 012 016), and partially was executed in the Institute of Bioorganic Chemistry, Polish Academy of Sciences.

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