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Agrobacterium tumefaciens-mediated transformation of *Pisum sativum* L. using binary and cointegrate vectors

Andre De Kathen and Hans-Jörg Jacobsen

Institut für Genetik, Universität Bonn, Kirschallee 1, D-5300 Bonn 1, Federal Republic of Germany

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Abstract:
Epicotyl segments and nodus explants from etiolated seedlings of *Pisum sativum* were transformed using *Agrobacterium tumefaciens* strains GV 2260 (p35S GUS INT) and GV 3850 HPT carrying either a neomycin- or hygromycinphosphotransferase-gene as selectable markers. The transgenic character of hygromycin- or kananamycin-resistant tissue was confirmed by detection of nopaline or neomycinphosphotransferase-II- and ß-glucuronidase activity in crude extracts of resistant tissues. Up to 5 % of developing shoots from shoot proliferating nodi were regenerated via organogenesis to kanamycin-resistant plantlets. Transformation frequency *in vitro* was found to be influenced by explant source, *A.tumefaciens* strain, pea genotype and duration of cocultivation. Acetosyringone did not increase the transformation rate.

Abbrevations: GUS=ß-glucuronidase; NAA=1-naphthyl-acetic-acid; BA=6-benzylaminopurine; NPT-II=neomycinphosphotransferase-II; HPT=hygromycinphosphotransferase

Introduction:

With an annual production of around 15 million tons, *Pisum sativum* is one of the most important crop species and an eminent protein source for animal and human nutrition. The development of reliable transformation and regeneration systems would provide the opportunity to optimize protein content and amino acid composition as well as virus- and herbicide-resistance. Since 1907 it is known from the work of Smith and Townsend that "*Bacterium tumefaciens* " is capable of inducing tumors on higher plants. Lopatin (1936) demonstrated that pea belongs also to the host range of *Agrobacterium tumefaciens*. This was confirmed by a great number of authors (Kurkdijan et al. 1969, Broekart and van Parijs 1973, Kodama 1975, Hoekema et al. 1983, Bercetche et al. 1987, Hussey et al. 1989, Hobbs et al. 1989), but only Puonti-Kaerlas et al. (1989) reported on the production of kanamycin-resistant callus using disarmed, engineered *A.tumefaciens* strains. In an increasing number of so far recalcitrant grain legumes i.e. *Vigna aconitifolia* (Eapen et al. 1987), *Glycine max* (Hinchee et al. 1988), *Phaseolus vulgaris* (Mariotti et al. 1989) and *Vicia narbonensis* (Pickardt pers. comm.) at least transgenic plantlets were obtained. Up to now, attempts to regenerate plants from transgenic pea tissue have failed. Here we report on the susceptibility of pea nodi to disarmed, engineered *A.tumefaciens* vectors. Using an optimized regeneration protocol via organogenesis from axillary and adventitious buds derived from nodus explants we obtained kanamycin-resistant, GUS-positive plantlets of a proposed chimaeric character.

Materials and methods:

Bacterial strains: *Agrobacterium tumefaciens* binary vector p35S GUS INT carries a chimaeric NPT-II-gene and a GUS-gene construct with a ST-LS 1 gene derived intron in pBIN 19 (Vancanneyt et al. 1990). Vir-functions of the construct were provided by the plasmid pGV 2260 in a C58C1 chromosomal background (Deblaere et al. 1985). GV 3850 HPT is a GV 3850 (Zambryski et al. 1983) derived cointegrate vector. The HPT-gene was introduced by a double crossover event, resulting in pGV 3850 HPT (Altmann, pers.comm.). "Wildtype" strains C58C1, A281 and B6S3 were used for the determination of parameters affecting transformation efficiency.

Plant material: Certified seeds from the genotype Madria were kindly provided by Norddeutsche Pflanzenzucht Hans-Georg Lembke KG, Hohenlieth. GV 2260 p35S GUS transformed tobacco line J36, used as a control, was kindly provided by J. Stockhaus.

Culture conditions: *Agrobacterium* strains were grown on LB-medium (Maniatis et al. 1982) at 28°C with appropriate antibiotics. For explant transformation bacteria were grown overnight in liquid LB-medium, centrifuged and resuspended in a modified MS'-medium (Kysely et al. 1987) to a density of 1-5 * 10^8 living cells/ml and cultured for an additional 1-2 hours prior to cocultivation. Epicotyl and nodus explants were excised from 7 day old etiolated seedlings. Surface sterilisation and germination of seeds was carried out according to Kysely et al. (1987). Callus growth was induced on MS'-medium, supplemented with 0.1 mg/l BA and 0.1 mg/l picloram. For multiple shoot induction, nodi were placed on MS'-medium containing 1 mg/l NAA for one week prior to further culture on MS'-medium with 4.5 mg/l BA and 0.02 mg/l NAA. Transformed tissue was selected on 5-10 mg/l hygromycin or 50 mg/l kanamycin (G418: 30 mg/l). Shoot proliferating nodi were cultured under a 16/8 h light/dark regime at 26±2°C in 175 ml glass jars (Weck 202). Developing shoots with 2 visible nodi were excised, transferred on root induction

Offprint requests to: A. De Kathen

medium for 6 weeks and rooted shoots were planted in a sterile 1:1 soil-vermiculite mixture in a cotton capped jar, 25 cm in height. The cap was gradually removed to harden the plantlets against low moisture conditions. Bacterial growth was controlled by addition of 300 mg/l carbenicilin (Pfizer) and 200 mg/l claforan (Hoechst) to the medium.

Transformation: Explants from etiolated seedlings were soaked for one hour in the bacterial suspension described above, blotted dry on sterile filter paper and were incubated on solid MS'-medium with or without acetosyringone (50 µM) for 24 to 120 h in the dark. After cocultivation explants were washed three times in sterile water and placed on selective media.

Enzyme assays: For enzyme assays tissue was homogenized according to Bertoni and Mills (1987). Hygromycin-resistant plantlets and callus were tested for nopaline production according to Otten and Schilperoort (1978). In kanamycin-resistant tissues NPT-II activity was monitored after native polyacrylamid-gel-electrophoresis of crude extracts as described by Reiss et al. (1984). GUS-activity was detected according to Jefferson (1987). GUS-kinetics were measured by a Perkin-Elmer LS-5B fluorimeter. Protein concentration was estimated according to Bradford (1976).

Results and Discussion:

Strain and cultivar specificity: Our susceptibility tests on epicotyl segments from etiolated seedlings *in vitro* showed the transformation rate and the vir-region of the *Agrobacterium tumefaciens* strain used for inoculation to be correlates. The chromosomal background as well as the vector construction (cointegrate vs. binary) did not influence the transformation frequency significantly (table 1). Similar findings were made by Hood et al.(1986) and Otten et al.(1985). Both groups were able to increase the virulence of a given strain by transformation of *Agrobacterium* with plasmids containing vir-region fragments of more virulent strains.

Table 1: Percentage of tumor formation on medium without hormones, or callus growth on medium with selective antibiotics after 48 h cocultivation of Madria epicotyl-explants with different *A.tumefaciens* strains.

strain	chr.bg.	vir Ti	tf %
C58C1	C58C1	C58	36
GV 3851	C58C1	C58	26
GV 3850 HPT	C58C1	C58	35
B6S3	B6S3	B6	8
35S GUS INT	C58C1	B6	5

chr.bg.=chromosomal background; vir Ti=Ti-plasmid from which vir-region is derived; tf=transformation rate; - Results from at least 2 independent experiments with more than 50 explants, monitored 8 weeks after cocultivation.

Explant source and duration of cocultivation: We were not able to induce tumors either with C58C1, or B6S3 respectivily, on wounded immature zygotic embryos or the apical dome of etiolated seedlings which were the main source for the production of somatic embryos according to Kysely et al.(1987). Leaf discs and immature leaflets showed a low tumor formation frequency. Nodi, epicotyl segments and excised shoot tips exhibit the highest sensitivity for transformation (table 2).

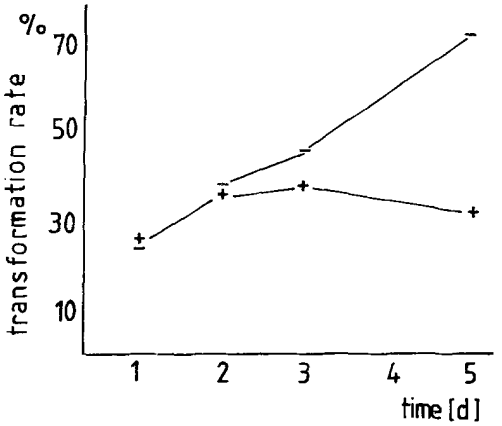
Table 2: Tumor induction frequency on different explants of Madria after 4d of cocultivation with C58C1.

explant	percentage of tumors
immature embryo	0 %
apical dome	0 %
immature leaflet	5 %*
leaf disc	11 %*
epicotyl segment	68 %
nodus explant	47 %
whole apex	63 %

Results from at least 2 independent experiments with more than 20 explants, monitored after 6 weeks in culture; (*= no opine-assay done).

As in alfalfa (Chabaud et al. 1988), genotype (data not shown) but also explant source, type of vector and the duration of cocultivation influenced the transformation efficiency. In contrast to the observation of Chabaud et al. (1988) we found a nearly linear relation between transformation frequency and duration of cocultivation (figure 1). An extension of cocultivation for more than 5 d led to an explant-abortion caused by bacterial contamination. Acetosyringone was used to induce the vir-genes (Stachel et al. 1985) but was found to decrease the transformation rate. This finding was also observed by Puonti-Kaerlas (pers. comm.).

Figure 1: Transformation frequency after inoculation of epicotyl-segments of Madria with C58C1 as a function of duration of cocultivation in the presence and absence of acetosyringone.



"+"= with, "-" = without acetosyringone (50 µM)

Plant regeneration via organogenesis was improved by modifying the protocol of Griga et al. (1986). Preculture on 1 mg/l NAA for one week led to a nearly 100 % increase in shoot formation. Usually two shoots per explant and month in culture were excised over a period of at least one year whereby 85-90% could be rooted. Resistant plantlets were obtained following two different selection procedures. The first approach allowed shoots to develop under non-selective conditions. Selection pressure was applied when excised shoots were rooted on MS'-medium supplemented with 1 mg/l NAA, containing 5 mg/l hygromycin or 50 mg/l kanamycin. Only 1 % of shoots obtained after transformation of nodus-explants gave rise to resistant plantlets under these conditions. Following the second procedure, nodi were placed on selective media immediately after cocultivation

to inhibit development of non-transformed shoots. Up to 5 % of excised shoots were regenerated under selective conditions (table 3).
One should be aware, however that it is not possible to culture nodus-explants for more than a few weeks under these conditions without death of the whole explant.

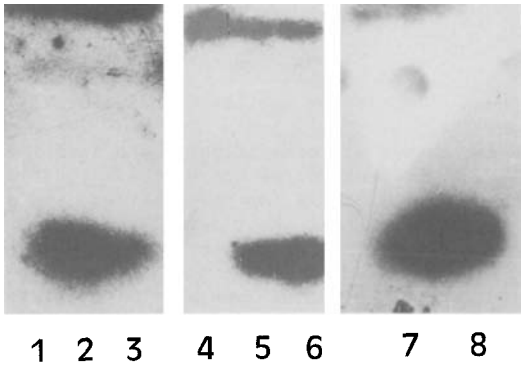
Table 3: Summary of transformation efficiencies, following two different selection procedures.

strain	GV 3850 HPT		p35S GUS INT	
selec. lev.	I	II	I	II
cocultivated nodi	223	373	245	234
excised shoots	122	1869	391	1182
resistant shoots	6	16	8	13
% resistant	4.9	0.9	2.5	1.1
enzyme NOS	n.d.	n.d.	-	-
positive GUS	-	-	4/5	2/2
NPT	-	-	5/8	-

I= selection at the level of shoot induction
II= selection at the level of root induction
n.d.= no data, see text

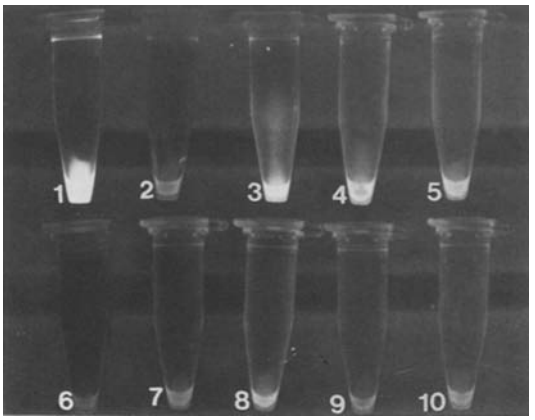
The transgenic character of the plantlets obtained following the described selection procedures was confirmed by measuring the activity of the enzymes coded by the introduced genes (figure 3, 4). Crude extracts of hygromycin-resistant plantlets often showed no clear nopaline signal mainly because of the insensitivity of the assay. Therefore it was not possible to determine the percentage of transformed plantlets by nos-activity

Figure 3: Autoradiograph showing NPT-II activity after native disc-polyacrylamid-gel-electrophoresis of crude extracts of callus, leafs and/or tendrils using a minigel-system (CTI).



1-3: p35S GUS transformed tobacco line J36, protein amount: 1) 45 µg 2) 30 µg 3) 15 µg
4: untransformed callus of the genotype Madria
5,6: p35S GUS INT transformed callus of Madria
7,8: p35S GUS INT transformed, kanamycin-resistent plantlets of the genotype Madria

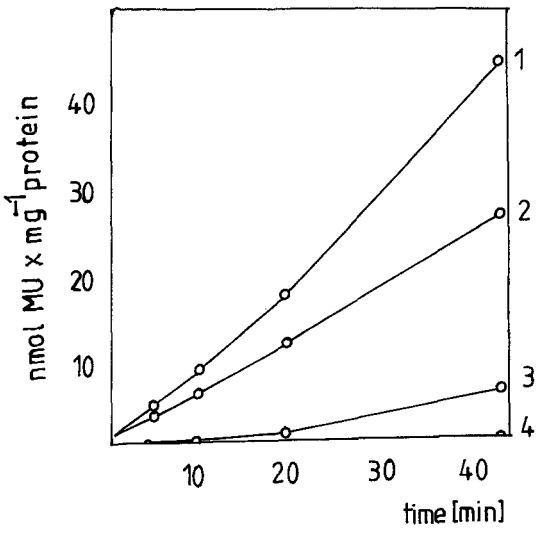
Figure 4: Qualitative GUS-assay showing ß-Glucuronidase activity in crude extracts after 40 min incubation with 2 mM substrate.



1: p35S GUS transformed tobacco line J36
2-5: p35S GUS INT transformed callus of Madria
6: untransformed callus of Madria
7-10: leaf/tendril extracts of regenerated, kanamycin-resistant plantlets after transformation of nodus-explants with p35S GUS INT

12 out of 43 resistant plantlets obtained so far flowered but none of the plants set seed, so the R1 could not be analysed. Because of the weak signals of ß-glucuronidase activity in the qualitative test, activity was quantified and found to be more than 12 fold higher than background activity (figure 5). NPT-II activity was detected in 5 out of 8 resistant plants tested.

Figure 5: ß-glucuronidase kinetics in crude extracts of leaf/tendril or callus, (MU= 4-methyl -umbelliferon).



1: p35S GUS transformed tobacco line J36
2: p35S GUS INT transformed, epicotyl-derived callus of the genotype Madria
3: p35S GUS INT transformed plantlet of the genotype Madria
4: nodus-explants, callus, immature embryos and leafs of genotypes Birte, Belman and Madria

Kanamycin- and hygromycin-resistant plantlets from *Pisum sativum* were obtained at low frequencies using binary and cointegrate vectors.

We have presented evidence that the introduced chimaeric genes were correctly processed and translated into functional proteins. The intron-containing GUS-gene is also functional in pea and so we could exclude false positive GUS-signals due to bacterial contamination. The low percentage of recovered GUS- and NPT-II gene expressing plantlets is due to the low virulence of the vectors and the proposed lower susceptibility of highly meristematic tissue in the explants used for transformation. It turns out, that the up to 7 fold higher virulence of GV 3850 HPT on epicotyl segments in comparison to GV 2260 p35S GUS INT does not result in a comparable increase in recovered hygromycin-resistant plantlets. Since hygromycin B showed a 5 fold higher toxicity than kanamycin, regeneration of chimaric, hygromycin-resistant plants obviously needs a more sophisticated selection procedure. Therefore, the observed discrepancy is not a result of a GV 3850 HPT-specific lower virulence on regenerative tissue. The low GUS-activity in crude extracts of these plants is likely a result of the chimaeric character of the tissues, since regeneration of plants via organogenesis from chimaeric tissue leads to chimaeric plants. This, however, will be insignificant if either transgenic plants can be regenerated from chimaeric tissues via an additional tissue culture cycle, as it has been shown by Mc Hughen and Jordan (1989) for flax, or if germ-line cells were transformed. Regeneration via somatic embryogenesis seems to be advantageous, because somatic embryos develop from a few or even only one cell as was shown for walnut by Polito et al. (1989). But until now, there is no evidence for a single cell origin of somatic embryos in pea. Attempts to regenerate soybean plants after transformation via somatic embryogenesis led to chimaeras (Parrot et al. 1989), whereas Hinchee et al. (1988) were successful in producing transgenic plants by regeneration via organogenesis. We found that cells with a highly meristematic character (i.e. cells of immature zygotic embryos or the apical dome of etiolated seedlings) were not susceptible to *Agrobacterium* transformation. This is in contrast to Hussey et al.(1989), but they did not confirm the transformation either at the DNA or at the protein level. Nevertheless, in the work presented here it is shown that pea tissue with some regenerative capacity can be used successfully for *Agrobacterium* -mediated transformation.

It is now possible to regenerate pea plants from protoplasts (Lehminger-Mertens and Jacobsen 1989) which is a prerequisite to use free DNA delivery methods. Nevertheless in the present study we choose the *Agrobacterium* -system for transformation because the regeneration protocol presented here is efficient and less laborious than somatic embryogenesis from complex explants and protoplasts, and applicable to more genotypes. In addition the integration of foreign DNA into the plant genome is relativly precise and of a defined nature (Gasser and Fraley 1989). Therefore it is furthermore important to optimize this method by increasing the virulence of vectors and the susceptibility of regenerative tissue. Experiments to test these opportunities are in progress.

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