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Agrobacterium-mediated transformation of *Pisum sativum* *in vitro* and *in vivo*

L. ŠVÁBOVÁ, P. SMÝKAL, M. GRIGA* and V. ONDŘEJ[1]

AGRITEC Ltd., Plant Biotechnology Department, Zemědělská 16, CZ-78701 Šumperk, Czech Republic

Abstract

Six pea (*Pisum sativum* L.) cultivars (Adept, Komet, Lantra, Olivín, Oskar, Tyrkys) were transformed via *Agrobacterium tumefaciens* strain EHA105 with pBIN19 plasmid carrying reporter *uidA* (β-glucuronidase, GUS, containing potato ST-LS1 intron) gene under the CaMV 35S promoter, and selectable marker gene *nptII* (neomycin phosphotransferase II) under the *nos* promoter. Two regeneration systems were used: continual shoot proliferation from axillary buds of cotyledonary node *in vitro*, and *in vivo* plant regeneration from imbibed germinating seed with removed testa and one cotyledon. The penetration of *Agrobacterium* into explants during co-cultivation was supported by sonication or vacuum infiltration treatment. The selection of putative transformants in both regeneration systems carried out on media with 100 mg dm^{-3} kanamycin. The presence of introduced genes was verified histochemically (GUS assay) and by means of PCR and Southern blot analysis in T_0 putative transformants and their seed progenies (T_1 to T_3 generations). Both methods, but largely *in vivo* approach showed to be genotype independent, resulting in efficient and reliable transformation system for pea. The *in vivo* approach has in addition also benefit of time and money saving, since transgenic plants are obtained in much shorter time. All tested T_0 - T_3 plants were morphologically normal and fertile.

Additional key words: grain legumes, pea, transgene integration, transgene stability.

Introduction

First studies on experimental induction of crown gall tumors in pea seedlings *in vitro* were reported by Manigault and Kurkdjian (1967) and Kurkdjian *et al.* (1968), systematic research of pea and *Agrobacterium tumefaciens* and *A. rhizogenes* wild strains interactions *in vitro* started Hobbs *et al.* (1989), Hussey *et al.* (1989), Schaerer and Pilet (1991) and Lutova and Sharova (1993), followed by pea modification by *Agrobacterium* bearing artificially adjusted plasmids (Filippone and Lurquin 1989, Puonti-Kaerlas *et al.* 1989, Schaerer and Pilet 1991). This effort culminated by obtaining first complete transgenic pea plants (Puonti-Kaerlas *et al.* 1990, De Kathen and Jacobsen 1990). Last decade of the 20th century was devoted to development and optimization of pea transformation protocols, but also to the pea modification by constructs with "useful" genes, namely conferring for herbicide tolerance, insect and virus resistance (Shade *et al.* 1994, Grant *et al.* 1995, Jones *et al.* 1998, Chowrira *et al.* 1998, Charity *et al.* 1999). Recently, this research resulted in successful proof of pea insect and virus resistance in the field conditions (Morton *et al.* 2000, Timmerman-Vaughan *et al.* 2001). During last decade, the problem of pea transformation was discussed in several review papers (De Kathen and Jacobsen 1993, Davies and Mullineaux 1993, Malysheva *et al.* 2001, Morton *et al.* 2002, Grant and Cooper 2003).

Up to date, only *Agrobacterium*-based protocols led to successful pea transformation, the attempts to use

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Abbreviations: BAP - 6-benzylaminopurine; CN - cotyledonary node; GUS - β-glucuronidase; LK medium - medium according to Langley and Kado (1972); MSB medium - medium with mineral salts according to Murashige and Skoog (1962), vitamins according to Gamborg *et al.* (1968); MS-salts - mineral salts according to Murashige and Skoog (1962); NAA - α-naphthaleneacetic acid; PCR - polymerase chain reaction; S - seed.

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[1] Present address: Institute of Biophysics, Czech Academy of Sciences, Královopolská 135, CZ-612 65 Brno, Czech Republic

* To whom correspondence should be addressed; fax: (+420) 583 382 998, e-mail: griga@agritec.cz

biolistics were unsuccessful, yet (Warkentin *et al*. 1992). A specific approach is *in vivo* transformation (DNA microinjection into intact nodal meristems followed by electroporation) – a technique which does not need an *in vitro* culture (Chowrira *et al.* 1995). In *in vitro* systems, the protocols are mostly based on organogenesis in callus or on stimulation of proliferation of organized meristematic tissue contained in transformed explants (stem nodal explants, cotyledonary nodes, immature embryo segments/slices, immature cotyledons – see review of Malysheva *et al*. 2001), only exceptionally on regeneration via somatic embryogenesis (Nicolas *et al*. 1995).

Despite mentioned progress in pea transformation, the available protocols still do not represent a quite routine technology. There is a number of factors which may affect the success and efficiency of transformation procedure as well as the subsequent behaviour of transgenes (stability/instability) in a sexual progeny. The co-cultivation procedure and its modifications, the regeneration system used connected with genotype-dependent regeneration potential, as well as efficiency of putative transformants selection may play an important role.

In this paper we describe a genotype-independent protocol of *Agrobacterium*-mediated transformation of pea *in vitro* and also so far unpublished protocol of pea transformation *in vivo*.

Materials and methods

Plants and preparation of explants for transformation: *Pisum sativum* L. cultivars from Agritec Pea Collection were used in the experiments: five dry seed peas cv. Adept (yellow seed, leaf type, bred in Czech Republic), cv. Komet (yellow seed, leaf type; CR), cv. Lantra (green seed, semi-leafless type; the Netherlands), cv. Olivín (green seed, leaf type; Slovakia), cv. Tyrkys (green seed, leaf type; CR) and canning pea cv. Oskar (green seed, leaf type; CR). Mature seeds were surface sterilised with 96 % ethanol for 30 s and 10 % chloramine for 20 min. Then the seeds were rinsed 3 times in sterile deionized water and germinated aseptically for 4 - 5 d in the dark at room temperature in 250 cm^3 flasks on a layer of cellulose wadding covered with filter paper and soaked with water. Cotyledonary nodes containing axillary meristems (Fig. 1*A*,*B*) were isolated for transformation procedure and subsequent multiple shoot regeneration *in vitro via* induction of proliferation of axillary meristems and *de novo* adventive shoot organogenesis (Griga *et al*. 1986, Jackson and Hobbs 1990). For *in vivo* transformation, surface sterilized mature seeds (S) were imbibed for 24 h in the dark at room temperature. Seed coat and nearly whole one cotyledon was removed and the embryo axis with remaining cotyledon was used for co-cultivation with *Agrobacterium* (Fig. 1*C*,*D*).

***Agrobacterium* and plasmid characterization, maintenance and multiplication:** A hypervirulent strain of *Agrobacterium tumefaciens* EHA 105 (Hood *et al.* 1993) with plasmid pBIN19 (Bevan 1984) derivative carrying reporter *uidA / gus-int* (β-glucuronidase - GUS) gene containing potato ST-LS1 intron under the control of CaMV 35S promoter and *nptII* (neomycin phosphotransferase II) selectable marker gene conferring resistance to kanamycin under the control of *nos* promoter, both genes with polyadenylation signal from nopaline synthase gene (Vancanneyt *et al.* 1990) was used. *Agrobacterium* was cultured in liquid LK medium (Langley and Kado 1972) supplemented with 100 mg dm^{-3} rifampicin and 50 mg dm^{-3} kanamycin. Before long-term preservation, the concentration of *Agrobacterium* suspension was adjusted to 10^9 cells cm^{-3}, split into 1 cm^3 epruvete tubes and stored at -80 °C. Immediately before use the content of one epruvete tube was resuspended in 100 cm^3 of LK medium during 24 - 48 h on a rotary shaker (120 rpm) at 28 °C. Revitalised faint milky suspension was centrifuged 15 min in 3 000 *g* and resuspended in fresh LK medium one hour before the co-cultivation with explants.

Transformation methods: Cotyledonary nodes (CN) and trimmed seeds (S) were co-cultivated in suspension of 100 cm^3 liquid co-cultivation media (MS-salts, supplemented with 100 μM acetosyringone) and 10 cm^3 of freshly revitalised *Agrobacterium* suspension. The penetration of *Agrobacterium* into plant cells was supported with vacuum infiltration (15 min) or sonication (30 s). Co-cultivation proceeded 1 h on a rotary shaker (120 rpm), afterwards on solid co-cultivation media 48 h at room temperature (CN) or at 4 °C (S) in a refrigerator. *Agrobacterium* was then eliminated from the cultures by washing in MS-liquid medium with antibiotics (augmentin 500 mg dm^{-3}, timentin 500 mg dm^{-3}). The solution was changed three times after 20, 40 and 60 min, meanwhile the flasks containing explants were placed on rotary shaker (120 rpm). Then both types of explants were dried on filter paper and transferred onto regeneration/selection media.

Selection and regeneration: For determination of selective kanamycin concentrations in both trans-formation systems, the preliminary screening of natural tolerance of pea to kanamycin has been done (concentration range 50 to 250 mg dm^{-3}). Serial proliferation of shoot buds and *de novo* adventive organogenesis *via* multiple-shoot culture was induced

from axillary meristems of CNs on MSB medium with 20 μM BAP, 0.01 μM NAA, 3 % sucrose, 0.7 % agar, pH 5.8 (Griga *et al*. 1986). Developing shoots 20 - 25 mm long were cut off and transferred on rooting medium (half-strength MS-salts, B5 vitamins, 5 μM FeEDTA, 10 μM inositol, 1 μM NAA, 4 % sucrose, 0.7 % agar, pH 5.8). Both media for shoot regeneration and rooting were supplemented with antibiotics (400 mg dm^{-3} augmentin, 400 mg dm^{-3} timentin) for bacteria elimination and 100 mg dm^{-3} kanamycin for transformant selection. Shoots with developed roots were transferred into 250 cm^3 Erlenmayer flasks filled with perlite and soaked with half strength MS-salts for one month. In these conditions, the T_0 plants reached 10 to 15 cm in height (4 - 5 true leaves) and fully developed root system. Thereafter, the plants surviving selection pressure were grown in the pots with pre-sterilised sandy-loam substrate in culture room, temperatures 20 to 22 °C and 16-h photoperiod (irradiance 20.4 μmol m^{-2} s^{-1}). The plants were regularly watered, fertilised and treated with pesticides up to flowering and seed setting. Mature seeds (T_1 generation) were then sown in a glasshouse to produce T_1 plants bearing T_2 seeds and subsequently further progenies (T_2, T_3).

In vivo system was based on the direct development of plants from treated seeds. Trimmed seeds after co-cultivation were grown in sterile flasks with artificial substrate (perlite) soaked with half strength MS-salts, antibiotics and kanamycin (the same concentrations as in CN system; in later experiments kanamycin concentration was elevated to 220 mg dm^{-3}) for 3 - 4 weeks since the plants reached the top of the flasks. Then the T_0 plants were transferred to sterilised sandy-loam substrate where they flowered and set T_1 seeds and – similarly as mentioned above – T_1, T_2 and T_3 plants.

GUS histochemical assay of putative transformants: For GUS assay, the explants after co-cultivation (3 - 4 weeks) and leaves from plants in seed green maturity stage were taken. Histochemical staining solution was prepared by *Springer* laboratory manual (Fütterer *et al*. 1995), samples soaked in staining solution were put on vacuum for 1 h and incubated at 37 °C overnight. To reduce natural endogenous GUS activity, pH was increased to 7.0 - 7.2 and 20 % methanol was added into extraction buffer. Fixation solution was applied for 10 min, at the end solution of concentrated acetic acid and 96 % ethanol in ratio 1:3 was used for better chlorophyll removing. GUS positive plants (blue colour of cells/tissues) were then tested with PCR.

PCR analyses of transformed plants: Genomic DNA was isolated from pea leaves by modified CTAB procedure (Doyle and Doyle 1987). In short: 50 to 100 mg (f.m.) of leaf tissue was homogenized by mortar and pistil in 0.5 cm^3 of extraction buffer containing 100 mM Tris-HCl pH 7.5, 1.4 M NaCl, 20 mM EDTA, 2 % CTAB, 1 % PVP Mr. 360 000, 0.4 % mercapto-ethanol, incubated at 65 °C for minimum 60 min with occasional vortexing. Subsequently 0.5 cm^3 of chloro-form was added, vortexed and centrifuged. Obtained aqueous phase was precipitated with 0.5 × volume 5 M NaCl and 1 × volume iso-propanol and finally upon centrifugation dissolved in TE buffer. Two transgenes were detected by PCR analyses: *uidA* and *nptII*. The sequences of particular primers are shown in Table 1. Aliquots for PCR reaction (0.025 cm^3) contained the 1× PCR buffer (TAKARA), 1.5 mM $MgCl_2$, 100 mM each of deoxynucleoside triphosphates, 7.5 nmol of each primer (*Generi-Biotech*, Hradec Králové, Czech Republic), 20 ng of genomic DNA and 0.5 units of *Tag* DNA polymerase (*TAKARA*). DNA was amplified for 40 cycles of 30 s at 96 °C, 40 s at 55 °C for *nptII* detection, 60 °C for *gus-int* detection, 1 min at 72 °C with *Master* thermal cycler (*Eppendorf AG*, Hamburg, Germany). An initial denaturation for 5 min at 96 °C and final extension step of 10 min at 72 °C were included. Amplified fragments were subjected to electrophoresis on 1 % agarose (*Serva*, Heidelberg, Germany) gels and visualised by ethidium bromide.

Table 1. Primer sequences used for PCR detection of transgenes and lectin A gene and length of PCR amplified fragment.

Gene	DNA sequence	[bp]
uidA	5´-TAATCAGGAACTGTTGGCCC-3´	460
	5´-CAACGAACTGAACTGGCAGA-3´	
nptII	5´– ACAAGATGGATTGCACGCAGG –3´	780
	5´–AACTCGTCAAGAAGGCGATAG –3´	
lecA	5´ - GATCTAAACCGAACAACCTCG - 3´	350
	5´ - CAAGAAGGAAGTGGTTTCAGTTG - 3´	

Southern hybridization: The 30 μg of genomic DNA isolated according to *CTAB* protocol was subjected to restriction with *EcoRI* enzyme (*Fermentas*, Praha, Czech Republic), subjected to electrophoresis and capillary blotted onto nylon hybridization membrane (*Roche*, Manheim, Germany) according to standard Southern blotting by Sambrook *et al.* (1989). As molecular mass standard, DIG labelled marker II was used (*Roche*). After fixing of transferred DNA by baking at 120 °C for 30 min, the blots were overnight hybridized with corresponding PCR digoxigenin labelled (DIG) gene probes according to manufacturer protocol (*Roche*) in DIG EasyHybridization buffer at 42 °C in hybridization oven (*Amersham Pharmacia Biotech*, Uppsala, Sweden). After washing, the chemiluminiscent signal generated by CSPD substrate (*Roche*) was detected on X-ray film (*Medix B*, *Foma*, Hradec Králové, Czech Republic).

Results and discussion

Effect of transformation procedure on plant regeneration and selection: Both culture and wild peas express a natural tolerance to kanamycin (Davies *et al.* 1993, De Kathen and Jacobsen 1993, Schroeder *et al.*

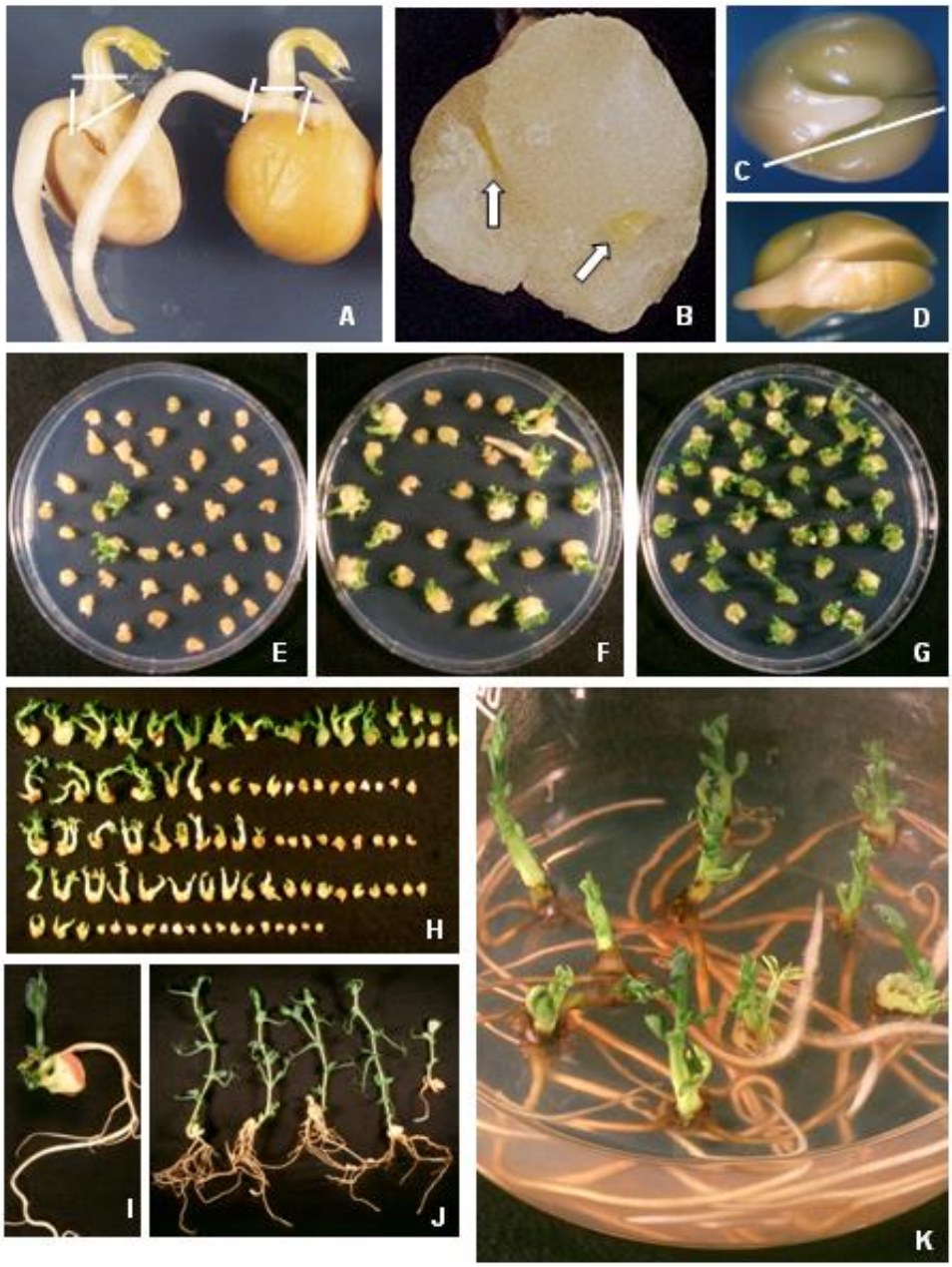

Fig. 1. *In vitro* and *in vivo Agrobacterium*-mediated transformation of pea. *A* - germinating etiolated pea seedlings at the stage (4-d-old) proper for isolation of cotyledonary node segments – lines mark cut areas. *B* - isolated cotyledonary node with axillary buds (arrows) used for cocultivation. *C* - imbibed pea seed with partially removed one cotyledon *(D)* ready for cocultivation. *E, F, G* - effect of vacuum infiltration *(E)* and sonication *(F)* during cocultivation on regeneration of transformed cotyledonary nodes on selection Km^+ medium; control culture *(G)* - sonication, Km^- medium. *H* - test of intrinsic kanamycin tolerance in pea cotyledonary nodes *in vitro*; from the top to bottom: 0 (control), 50, 100, 150 and 200 mg(Km) dm^{-3}(medium). *I, J* - development of *in vivo* infected trimmed seeds in perlite saturated with selection Km^+medium. *K* - root induction on shoots isolated from cotyledonary nodes on selection Km^+ medium; second selection step.

1993, Griga and Švábová, unpublished data with *P. jomardi* and *P. elatius*) and this may result in a number of regenerated non-transformed shoots which escape kanamycin selection. The concentrations 40, 50, 60, 75 or 100 mg dm^{-3} kanamycin were routinely used for pea transformant selection by several laboratories (De Kathen and Jacobsen 1990, Puonti-Kaerlas *et al*. 1990, Davies *et al*. 1993, Schroeder *et al*. 1993), which, however, were not able to guarantee 100 % selection efficiency. Nevertheless, the dramatic increase of kanamycin concentration led to severe decrease of regeneration potential and even to production of phenotypically abnormal plants (Bean *et al*. 1997, Nadolska-Orczyk and Orczyk 2000). Our tests of intrinsic kanamycin resistance confirmed data mentioned above (Fig. 1*F*). *In vitro* explants responded more sensitively than complete plants from trimmed seeds. Selective kanamycin concentration for CNs was determined as 100 mg dm^{-3}, for shoot apical meristems (not presented in this study) as 25 mg dm^{-3}, but 220 mg dm^{-3} for trimmed S. Roots of complete plantlets were more sensitive to kanamycin treatment as compared to shoots. Thus, two-step selection procedure (during shoot formation followed by root formation) should minimise the number of "escape" individuals in our experiments.

Shoot regeneration from infected CNs on medium with high cytokinin content, antibiotics and kanamycin carried out *via* proliferation of axillary buds and *de novo* formation of adventive shoot buds (Griga *et al*. 1986). Vacuum infiltration decreased significantly regeneration potential on selection medium (12 % of untreated control) as compared to sonication treatment (36 %) in CNs (Fig. 1*E*,*F*,*G*); this reduction of explant survival was not so dramatic in trimmed seeds (67 % in vacuum infiltration; 77 % in sonication). The regeneration from CNs was accompanied by explant swelling and negligible callogenesis. Regenerated shoots which survived kanamycin selection were rooted on selection rooting medium (Fig. 1*K*). *In vivo* infected trimmed S developed more or less similarly as complete (undamaged) S (Fig. 1*I*,*J*). Putative (GUS and PCR positive) *in vitro* and *in vivo* transformants (T_0) were grown and multiplied in the greenhouse (T_1, T_2, T_3 - seed generation). The time needed from co-cultivation to mature seeds was 5 to 6 months for CNs and 3 - 4 months for trimmed S. As compared to time needed usually from co-cultivation to *ex vitro* transfer in other reports dealing with pea transformation (more than 9 months – Puonti-Kaerlas *et al*. 1990, 1992; 9 months – Schroeder *et al*. 1993; 7 months – Grant *et al*. 1995; 11 months – Bean *et al*. 1997; more than 6 months – Polowick *et al*. 2000; 5.5 to 10 months – Nadolska-Orzczyk and Orczyk 2000), these intervals, particularly in case of trimmed S, seem to be very efficient. We did not observe any morphological and cytological abnormalities or fertility disorders in regenerants selected on kanamycin media as reported for pea elsewhere (De Kathen and Jacobsen 1990, 1993, Bean *et al*. 1997, Nadolska-Orzczyk and Orczyk 2000).

GUS assay of putative transformants: Despite of the use of a standard protocol for qualitative GUS assay, there was a tendency for acidification of X-Gluc staining solution with pea tissue samples (below pH 7.0), which resulted in endogenous GUS expression. This was more pronounced in the organs of plants in green seed maturity stage (light blue coloration of veins of leaflets, flower petals and pod walls; dark blue coloration of seed coats of immature seeds – Fig. 2*D*) as compared to 4-week-old CNs after co-cultivation. The endogenous enzyme is active at pH 4 - 5, but some plant species or specific cells/tissues (trichomes, epidermis, cells of vascular system) with acidic cell content may decrease initial pH 7 of X-Gluc assay and thus trigger endogenous GUS expression (Hodal *et al*. 1992, Wozniak and Owens 1994). The necessity for exact control of X-Gluc assay pH 7 or even its increase in order to avoid endogenous GUS activity was reported in several plant species (Van Wordragen *et al*. 1992, Boase *et al*. 1998, Krasnyanski *et al*. 2001). Thus, the endogenous GUS activity generated some proportion of false GUS positives in our initial experiments, when adult plants were assayed. Both better pH control (7.0 to 7.5) and the use of 20 % methanol led to the reduction of endogenous GUS activity in our later experiments and only typical strong dark blue GUS signal was taken into account.

Histochemical detection of intron-containing *uidA* gene expression in CNs early after cocultivation (3 - 4 weeks) visualized GUS mainly in developing axillary buds (Fig. 2*A*,*B*) and in the vascular tissue area (Fig. 2*C*). The observation that CaMV 35S promoter-driven *uidA* gene exhibits the strongest expression at the vascular bundle cells (and tissues active in cell division) was reported in some leguminous plants (Bhagarva and Smigocki 1994, Shao *et al*. 2000, Jaiwal *et al*. 2001) including pea (De Kathen and Jacobsen 1993). Fig. 2 *A*,*B*,*C* shows ways of *Agrobacterium* spreading in CN tissues after co-cultivation. Suitability of axillary meristems/buds for successful transformation and production of non-chimaeric shoots may be based on the fact that axillary meristems are relatively undifferentiated and contain fewer cells than meristem of the primary shoot (Davies *et al*. 1993). Fig. 2*E*,*F*,*G* illustrates GUS expression in plant parts (shoot apex, mature leaf, pod wall) of putatively transformed T_0 regenerants of both origin (CN, S). The *in vivo* treatment (31.6 %) yielded 1.7 fold higher frequency of GUS positive plants in T_0 generation as compared to *in vitro* treatment (18.1 %). Cv. Komet yielded the highest and cv. Tyrkys the lowest frequency of GUS positive plants in both transformation procedures (Table 2).

Molecular detection and characterization of transformants (PCR analysis, Southern blot): Isolated genomic DNA of 89 T_0 putative transformants, 99 T_1, 327 T_2 and 59 T_3 plants of cultivars Adept, Komet, Lantra, Olivín, Oskar, Tyrkys, was analyzed by PCR for the presence of *uidA*, *npt II* transgenes which are in T-DNA of pBIN19 binary vector (Bevan 1984, Frisch *et al.* 1995, Vancanneyt *et al.* 1990). The *uidA* gene is under the control of CaMV 35S promoter and *npt II* gene is under *nos* promoter located towards the right border of T-DNA sequence, respectively. All tested plants were previously histochemically assayed for GUS protein activity in mature leaves. PCR analysis showed differences in transgene T-DNA integration mainly in T_0, but also in the following seed generations. The 50 % of analyzed T_0 plants contained both tested transgenes, presence of only *uidA* gene was detected in 22 %, *nptII* in 17 %, respectively (Fig. 3), remaining 11 % represents plants upon *in vitro* kanamycin selection and exhibiting histochemical GUS positive reaction but being PCR negative for both transgenes. The T-DNA cassette fragmentation results could be attributed to incomplete T-DNA integration into genome, as reported for soybean and *nptII* gene located also near to the right T-DNA border by Olhoft *et al.* (2003) and other species (Kumar and Fladung 2002, Kim *et al.* 2003, Forsbach *et al.* 2003).

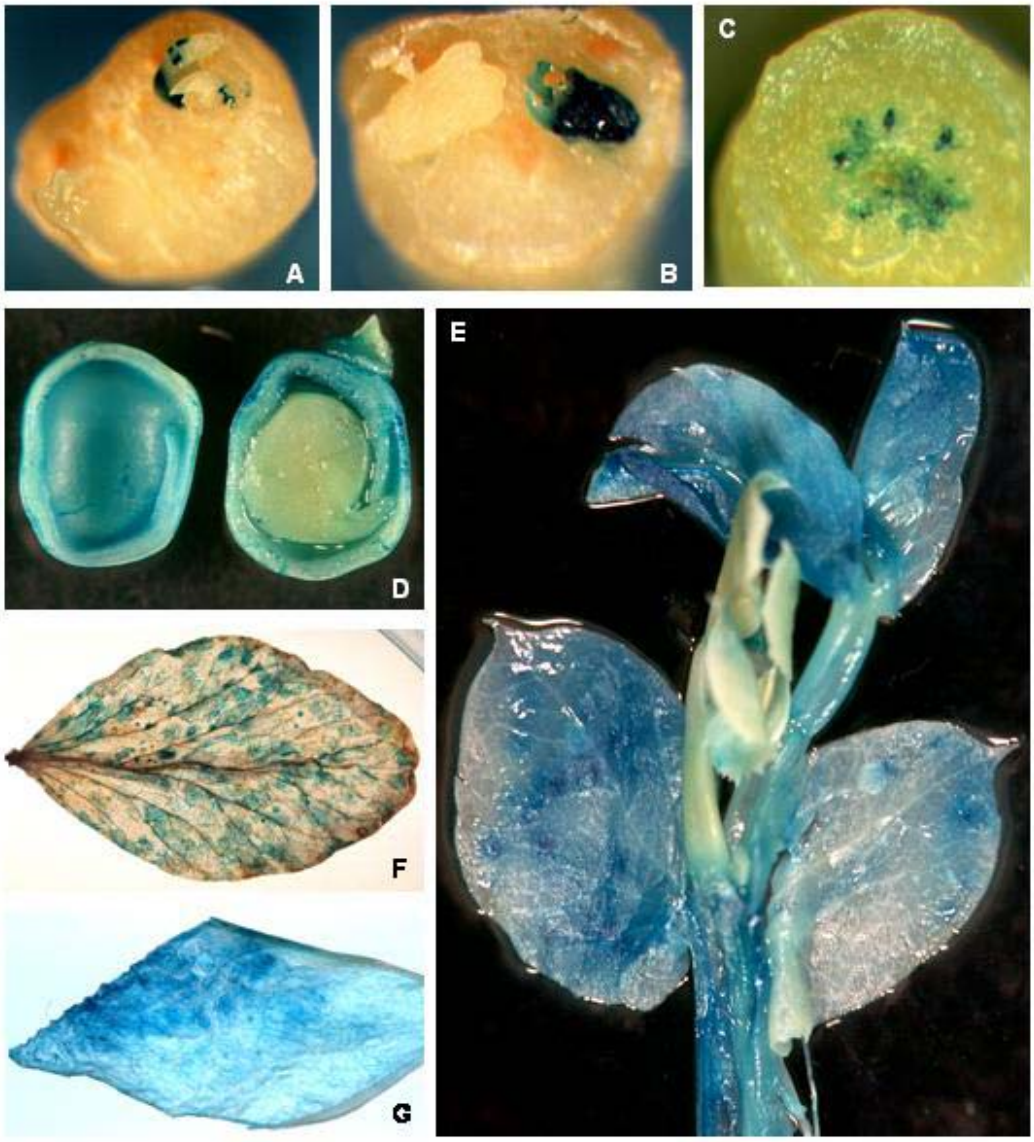

Fig. 2. Histochemical detection of intron-containing *uidA* gene expression. *A, B* - transient GUS expression in developing axillary buds of cotyledonary nodes, 3 to 4 weeks after co-cultivation. *C* - transient GUS expression in vascular tissue area of cotyledonary nodes, 3 to 4 weeks after cocultivation. *D* - endogenous GUS expression in control, non-transformed plant, dark blue coloration of seed coats of immature seeds. *E, F, G* - GUS expression in plant parts (shoot apex, mature leaflet, pod wall) of putatively transformed T_0 regenerants of both origins (*in vitro*, *in vivo*); the presence of *uidA* gene was later confirmed by molecular analysis.

Table 2. The effect of transformation procedure (cotyledonary nodes *in vitro* versus trimmed seeds *in vivo*) on explants/plants survival and the results of histochemical GUS assay in T_0 generation of five pea cultivars.

Cultivar	Cotyledonary nodes							Trimmed seeds						
	cocultivated explants	explants after selection		regenerated plants		GUS positive		cocultivated seeds	seeds after selection		regenerated plants		GUS positive	
	No.	No.	[%]	No.	[%]	No.	[%]	No.	No.	[%]	No.	[%]	No.	[%]
Adept	79	65	82.3	28	35.4	18	22.8	46	43	93.5	37	80.4	16	37.2
Komet	44	35	79.5	19	43.2	15	34.1	30	30	100.0	29	96.7	13	43.3
Lantra	114	51	44.7	45	39.5	25	21.9	30	30	100.0	25	83.3	12	40.0
Olivín	20	9	45.0	8	40.0	6	30.0	52	51	98.1	40	76.9	12	23.1
Oskar	68	64	94.1	19	27.9	9	13.2	46	41	89.1	36	78.3	15	32.6
Tyrkys	100	32	32.0	4	4.0	4	4.0	30	25	71.4	10	33.3	6	20.0
Total	425	256	60.2	123	28.9	77	18.1	234	220	94.0	177	75.6	74	31.6

In contrast to PCR test negatives but histochemically GUS positive, there were detected PCR *uidA* gene positive plants from randomly selected T_0 samples which previously did not show positive GUS reaction. In the first case, data could be explained as escapes from *in vitro* kanamycin selection and false GUS assay positives due to endogenous plant GUS genes activity. The second case apparently represents transformants whose integrated T-DNA is not expressed due to gene transcriptional silencing or post-transcriptionally by RNA interference (co-supression) mechanism. Frequency of both events is reported to be promoted by higher transgene copy number and by presence of viral 35S CaMV promoter (Olhoft *et al*. 2003, Qin *et al*. 2003).

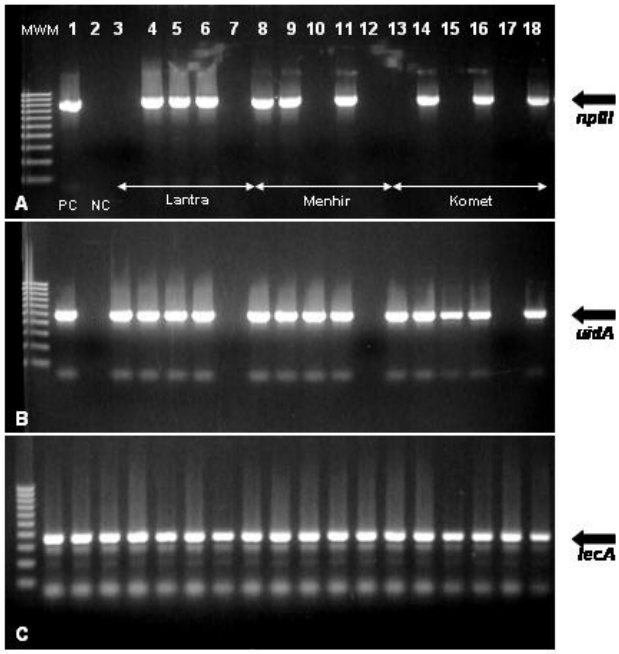

Fig. 3. Results of PCR testing for *nptII* (*A*), *uidA* (*B*) and *lecA* internal gene control (*C*) in 16 T_2 plants originated from independent T_0 lines of cv. Lantra (3 - 7), Menhir (8 - 12) and Komet (13 -18). Line No. 1 is positive and No. 2 negative pea control, respectively. Lines 7, 12, 17 are segregating non-transgenic plants, lines 3, 10, 13, 15 display only *uidA* and not *nptII* gene, the rest have both transgenes. MWM - markers.

The aberrant transgene arrangement was much rarely detected in subsequent generations (8 out of 64 PCR positive from 327 tested T_2 plants, which represents 12 % displayed just *uidA* gene and no *nptII* transgene). As previous corresponding T_0 - T_1 generations plants were not completely molecularly tested, and the selection criteria was based on GUS histochemical assay, consequently we can not rule out already aberrant integration in primary T_0 transformants. This would be more plausible explanation, on the other hand, illegitimate recombination reported especially in case of several transgene copies could be a reason. In addition, frequently used CaMV 35S promoter which belongs to pararetroviral promoters, is considered as fragmentation hotspot together with T-DNA borders (Kohli *et al*. 1999, Ho *et al*. 2000, Matzke *et al*. 2000, Vain *et al*. 2002). These hypotheses and PCR derived results could not be definitely proven by Southern hybridization and are subject of our further investigation.

Taken together 45 out of 89 T_0 transformants (50 %), 50 from 99 T_1 (50 %), 64 from 327 T_2 (20 %) and 10 from 59 T_3 plants (17 %) were proven to be PCR positive for both transgenes. From 327 T_2 plants only 77 (23.5 %) or 64 (19.5 %) were PCR positive for *uidA* or *nptII* genes, respectively. Remaining 15 plants (5 %) of T_2 generation showed difference between *uidA* and *nptII* PCR results. They contained only *uidA* and not *nptII* gene after repeated testing. These results suggesting partial integration of the T-DNA especially next to the RB sequence, are comparable to soybean transformation results of Olhoft *et al*. (2003). As previous T_0 - T_1 selection was based on GUS assay, this would explain discarding of just *nptII* gene containing plants. Negative PCR results were not due to problems with PCR amplification or DNA quality as demonstrated by internal gene (lectinA) (Kaminski *et al*. 1987) (Fig. 3) and

transgenic positive-negative controls. In agreement with Nadolska-Orczyk and Orczyk (2000), PCR-based screening proved to be reliable and time-saving technique of transformants identification, since escapes from *in vitro* selection can not be reduced further by increasing the concentration of selecting agents, which is true especially in the case of antibiotic selection. Proportion of positive T_0 plants is in a good agreement with published data (Nadolska-Orczyk and Orczyk 2000, Grant *et al*. 1995, Puonti-Kaerlas *et al*. 1990).

Stable integration of transgenes in selected 30 T_1 and 30 T_2 plants was confirmed by genomic DNA *EcoRI* digest and Southern detection of both transgenes. Since *EcoRI* enzyme cuts only once in T-DNA near to left border of T-DNA sequence and then in adjacent plant DNA, the corresponding hybridizing bands are of minimal size 4.5 kb. Obtained data demonstrate true genome integration, the intactness of T-DNA cassette as well as sexual transmission to next generations. All selected PCR positive plants hybridized with fragments of *uidA* (Fig. 4) and *nptII* genes, supporting the reliability of fast and less expensive PCR screening method. Based on the pattern of Southern detection, copy numbers varied from 1 to 4 T-DNA cassettes per genome, with one copy (Fig. 4) being the most frequent number (Nadolska-Orczyk and Orczyk 2000, Grant *et al*. 1995, Puonti-Kaerlas *et al*. 1990, Schroeder *et al*. 1993). Importantly, all tested T_0 - T_3 plants produced morphologically normal, fertile plants.

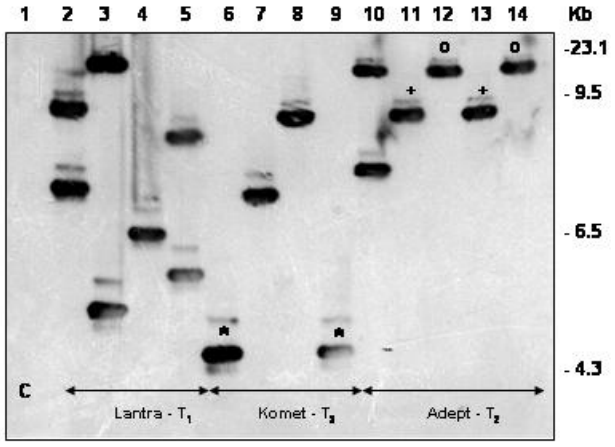

Fig. 4. Isolated genomic DNA from selected PCR positive T_1, T_2 and T_3 plants of cv. Lantra (2 - 5; T_1, *in vivo*, sonication), Komet (6 - 9; T_3, *in vivo*, sonication) and Adept (10 - 14; T_2, *in vitro*, sonication) was digested with *EcoRI*, electrophoresed on 0.8 % agarose gel and upon transfer onto membrane hybridized with DIG-labelled *uidA* probe. Line No. 1 is non-transgenic pea control. Plants No. 4, 6, 7, 8, 9, 11, 12, 13, 14 have likely single copy T-DNA insertion, the others at least 2 copies. T_3 plants of cv. Komet No. 6 and 9 (asterisk), T_2 plants of cv. Adept No. 11 and 13 (cross) and No. 12 and 14 (circle) originated from the same T_0 transformants, respectively; all other plants represent progenies of independent T_0 transformants.

Absence of complete T_0 - T_1 generations molecular testing could lead to incorrect estimations of transformation efficiency, therefore no precise percentage for each genotype and transformation protocol are given. Resulting presence of false positives (escapes from *in vitro* kanamycin selection and propagation of T_0 plants or from histochemical GUS T_0 and T_1 testing), indicates T_1 - T_2 plants results, where not all histochemically strong GUS positives were truly PCR transgenics. In spite of this, histochemical GUS assay estimation showed to be reliable criteria in about 80 % cases, supporting the usefulness of this fast first screening method. Remaining 20 % of false GUS positives could be explained by strong endogenous GUS genes activity in pea tissue as reported previously (Hodal *et al*. 1992).

Conclusions: We have developed a reliable transformation system based on kanamycin selection for pea using either *in vitro* axillary buds transformation and proliferation or simpler *in vivo* plant regeneration protocol. Both methods but largely *in vivo* approach showed to be genotype-independent, resulting in efficient and reliable transformation system for pea. The latter one may belong to „non-tissue culture" methods which would be a great boost in progress toward development of high-throughput transformation systems. Stable transgene integration and transmission to subsequent generations (in several lines to T_3) was demonstrated in 50/99 (number of transgene positive from total) plants of T_1 generation representing 13 original independent T_0 lines, 64/327 T_2 plants of 18 original T_0 lines, and 10/59 T_3 plants from original 2 T_0 lines of six commercially used pea cultivars. All tested T_0-T_3 plants produced were morphologically normal and fertile.

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