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Timing of transposition of *Ac* mobile element in potato

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Abstract

The timing of excision of maize transposable element *Ac* was studied using visual histochemical assay based on *Ac* excision restoring activity of β-glucuronidase (GUS). The *Solanum tuberosum* L. cv. Bintje was used for *Agrobacterium*-mediated transformation with pTT230 plasmid harbouring *Ac*-interrupted *gus* A gene and *npt* II gene as a selectable marker gene. Twenty-eight out of 72 kanamycin resistant calli did not express any GUS activity, 31 calli showed partial GUS expression and 13 out of assayed calli revealed strong expression of *gus* A gene. Plants were regenerated from calli without and/or with partial expression of *gus* A gene. The regenerated transformants which did not express GUS during the callus phase often contained many small GUS expressing spots on leaves. A phenotypic selection assay for excision of *Ac* has been also used. This non-detectable excision of *Ac* in callus tissue could be followed by a "late" timing excision during leaf development. After transformation with pTT224 plasmid harbouring *Ac*-interrupted *hpt* II gene and *npt* II gene transgenic calli containing *Ac* within the hygromycin resistance gene were derived and hygromycin sensitive plants were regenerated from them. Protoplasts isolated from leaves of transgenic regenerated plants were selected on hygromycin. Hygromycin resistant minicalli showed to harbour multiple copies of *Ac* and mark out low uniqueness of integration sites.

Additional key words: Solanum tuberosum, β-glucuronidase, hygromycin resistance, transposon tagging.

Introduction

Since 1940s, when B. McClintock (1947, 1948) presented results of her studies concerning DNA mobile elements *Activator* (*Ac*) and *Dissociation* (*Ds*), it was shown that mobile elements are widespread in nature being found in all organisms examined (Kidwell and Lisch 1997). From 1960s, large effort has been devoted to examine structure and behaviour of the mobile elements both in host (endogenous) and heterologous species (for plant species see review Kunze *et al.* 1997). Transposable elements were discovered as powerful mutator mechanism which can be employed for gene isolation (transposon tagging) using the element as a probe. The method of isolation of gene by transposable elements was firstly described by Bingham *et al.* (1981) in *Drosophila* whereas Wienand *et al.* (1982) firstly reported method of transposon tagging in plant species. Maize gene *Bz1* was the first plant gene cloned by endogenous transposon tagging (Fedoroff *et al.* 1984), and *Ph6* gene of *Petunia* was the first plant gene cloned by transposon tagging in heterologous plants (Chuck *et al.* 1993).

The greatest advantage of utilisation of transposable elements for gene cloning, when compared with other methods of gene isolation, consists in that no information about gene of interest and its product are required. However, a prerequisite of multiple independent DNA mobile element reinsertions has to be fulfilled among others for successful transposon tagging. One out of possibilities how to reach such high uniqueness of mobile element reinsertions reported Rommens *et al.* (1991) in tobacco utilising of phenomenon of the "delayed" excision time.

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Abbreviations used: BAP - 6-benzylaminopurine; 2,4-D - 2,4-dichlorophenoxyacetic acid; GA_3 - gibberelic acid; Hg - hygromycin; IAA - indole-3-acetic acid; Kn - kanamycin; MES - 2-(N-morpholino)ethanesulphonic acid; NAA - α-naphthaleneacetic acid; X-gluc - 5-bromo-4-chloro-3-indolyl-β-D-glucuronide; Z - zeatin.

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Our study dealt with applicability of this phenomenon in potato, important crop plant. Visual assay with GUS activity as well as phenotype selection assay using hygromycin resistance were performed.

Materials and methods

Plant vectors: Plasmids pTT224 and pTT230 (Fig. 1) with *Ac* element inserted in the leader sequence of the hygromycin phosphotransferase gene (*hpt* II) and β-glucuronidase gene (*gus* A) (Haring *et al.* 1989) were obtained by courtesy of Dr. T. Kneppers from Free University Amsterdam.

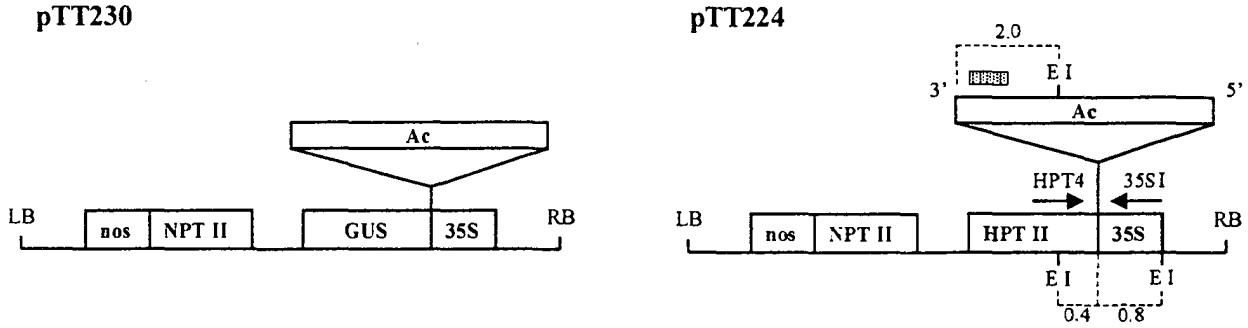

Fig. 1. Schematic presentation of the assay vectors containing *Ac* element. LB - left border; RB - right border; nos - nopaline synthase promoter; 35S - cauliflower mosaic virus promoter; NPT II - neomycin phosphotransferase gene; GUS - β-glucuronidase gene; HPT II - hygromycin phosphotransferase gene; E I - *Eco*R I restriction enzyme sites; HPT4 and 35S1 - primers for empty donor site PCR amplification; dotted box represents *Ac* 3' end probe; size of the *Ac*-border fragment (2.0) and *Eco*R I fragment in the T-DNA (0.4) is indicated in kb pairs.

Plant transformation: Overnight liquid cultures of *A. tumefaciens* strains were pelleted and resuspended in 10 mM $MgSO_4$. This bacterial suspension was used to infect internodes of potatoes (*Solanum tuberosum* L. cv. Bintje). After 30 min of cocultivation the internodes were placed for 24 h on agar MS medium (Murashige and Skoog 1962) free of growth regulators and antibiotics. Selection of transformed calli was performed on SH medium (Schenk and Hildebrandt 1972) supplemented with 2 mg dm^{-3} α-naphthaleneacetic acid (NAA), 0.5 mg dm^{-3} 6-benzylaminopurine (BAP) and 100 mg dm^{-3} kanamycin. To eliminate *Agrobacterium* 500 mg dm^{-3} ticarcilline and 200 mg dm^{-3} cefotaxime were added to the medium.

Culture of plants and calli: The potato plants were propagated on MS medium. Calli were derived from plant internodes on SH_2 medium (SH medium supplemented with 2 mg dm^{-3} NAA and 0.5 mg dm^{-3} BAP) and maintained on SH_3 medium (SH medium supplemented with 2 mg dm^{-3} NAA and 0.1 mg dm^{-3} BAP). To obtain regenerated transformed plants the transgenic calli were subcultivated three times on MS medium supplemented with 0.7 mg dm^{-3} zeatin (Z) and 0.5 mg dm^{-3} indole-3-acetic acid (IAA) and then on MS medium with addition of 1 mg dm^{-3} Z and 1 mg dm^{-3} gibberelic acid (GA_3).

Isolation and culture of protoplasts: Plant materials used for protoplast isolation were maintained on hormone free SH medium. Leaf mesophyll protoplasts were isolated and cultured as described by Bříza and Machová (1991). Treatment of donor plants for 2 - 3 weeks in a short daylength (6 h), a low irradiance (17 μmol m^{-2} s^{-1}) and a low temperature (10 °C) was applied. Isolation of protoplasts was carried out with enzyme mixture consisted of 1.4 % cellulase R-10, 0.2 % macerozyme R-10, 5 mM $CaCl_2$, 0.5 M sucrose, 10 mM MES and 2 mg dm^{-3} Z. Culture of protoplasts was performed in SW medium (Sidorov *et al.* 1987) containing 2 mg dm^{-3} NAA, 0.2 mg dm^{-3} dichlorophenoxyacetic acid (2,4-D) and 0.5 mg dm^{-3} Z. Hygromycin selection (20 mg dm^{-3}) was started at minicallus stage.

GUS assay: GUS activity was determined using a histochemical assay (Jefferson 1987). Transformed calli or leaves of transgenic plants were vacuum infiltrated with 50 mM $NaPO_4$ containing 250 μg cm^{-3} X-gluc, pH 7, for 20 - 30 min and then overnight incubated at 37 °C in the same solution. Green tissues were clearing in 96 % ethanol.

PCR analysis: The samples for polymerase chain reaction (PCR) were prepared essentially as described by Klimyuk *et al.* (1993). Briefly, a small parts of tissue were incubated in 0.04 cm^3 0.25 M NaOH for 30 s in boiling water and immediately transferred on ice. Afterwards, 0.04 cm^3 0.25 M HCl and 0.02 cm^3 0.5 M TrisHCl (pH 8) with 0.25 % Nonidet P-40 were added, DNA was denatured by boiling for 2 min and quickly cooled on ice.

Ac excisions from construct pTT224 were detected using primers 35S1 (5'-AAG CTT ACA GTC TCA GAA GAC CAA AG-3') and HPT4 (5'-CCC AAA GCA TCA GCT CAT CGA GAG CC-3') which amplified 1050-bp fragment between CaMV 35S promoter and *hpt* II gene. Perkin-Elmer thermal cycler was used to perform 35 cycles using the following program: 45 s at 94 °C, 30 s at 55 °C and 2 min at 72 °C. The products were separated on 1.5 % agarose/ethidium bromide gels.

Southern blot analysis: Genomic DNA was extracted from calli as described Tai and Tanksley (1991). About 15 μg of DNA were digested with *Eco*R I restriction enzyme, resolved overnight in 1.0 % agarose gel with TBE buffer (Sambrook *et al.* 1989) and transferred to nylon *Hybond-N* (*Qiagen*, Hilden, Germany) membranes. Southern hybridisations were performed according to Church and Gilbert (1984). The membranes were probed with the 334-bp 3' end of *Ac* element which contained no *Eco*R I restriction site. The probe was labeled with REDIVUE™ [α-^{32}P]dCTP (110 Bq mmol^{-1}) using random prime labelling kit REDIPRIME™ II (*Amersham Pharmacia Biotech*, Freiburg, Germany). The blots were autoradiographed for 5 h using phosphoimager system STORM (*Molecular Dynamics*, Sunnyvale, USA).

Results and discussion

Transformation using plasmid pTT230: *Agrobacterium* strain harbouring pTT230 was used for visual assay based on *Ac* excision restoring GUS activity. Successive steps of experiments are demonstrated on Fig. 2*A*. Totally, we obtained 99 kanamycin resistant (*i.e.* transgenic) calli and 72 out of them were GUS assayed. Three different groups of calli were revealed: *1*) 28 "completely blue" calli with strong expression of GUS. In this case *Ac* element excised in very early period of callus development; *2*) 31 "partially blue" calli when the expression of GUS was observed only in some parts of the calli; *3*) 13 "white" calli in which no GUS expression was detected. In these calli *Ac* element remained in T-DNA.

The plants were regenerated from 9 "white", 13 "partially blue" and 7 "completely blue" calli and leaves of 730 regenerated transformants were histochemically assayed (Fig. 3). Whole blue leaves or large blue sectors were visualised on those regenerants which were derived from completely GUS$^+$ calli. This is in contrary to results of Rommens *et al.* (1991), who found no transgenic tobacco leaves with staining of all cells of specific tissue, but in agreement with results of Finnegan *et al.* (1989), who revealed complete staining in leaves of 2 out of 3 assayed plants when *Ac* excision from *gus A* gene in tobacco was studied. It is clear that origin of regenerated plants displaying whole blue leaves lies in cells where *Ac* excision occurred during callus growth stage.

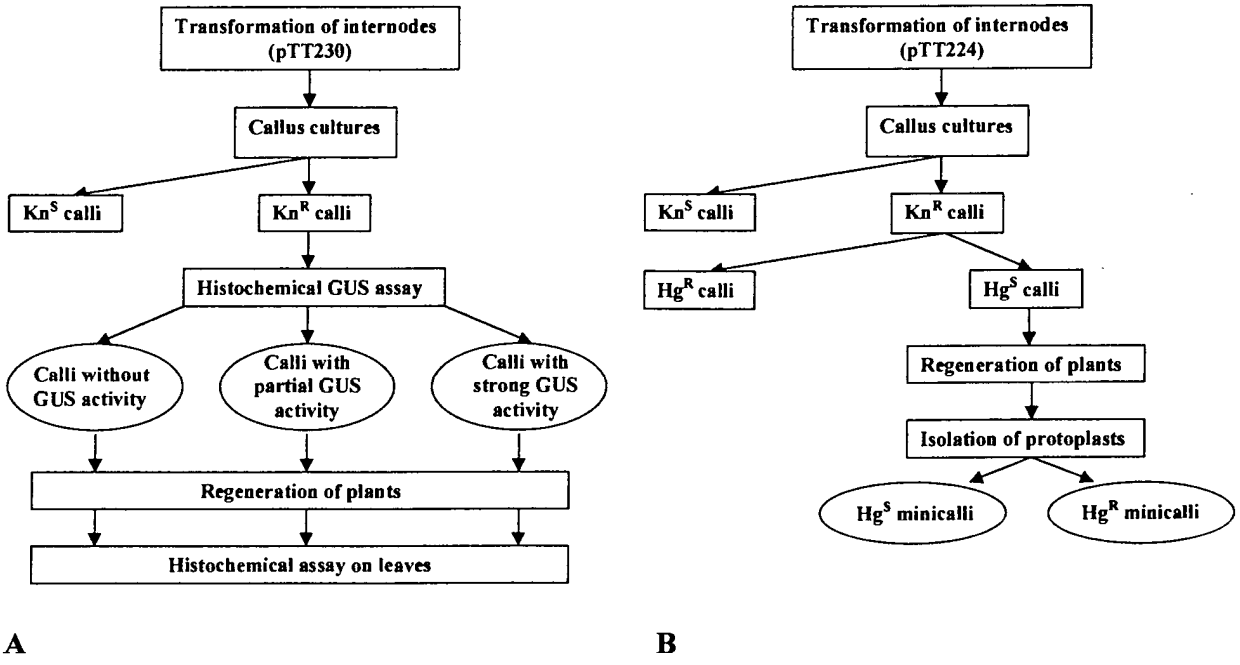

Fig. 2. Experimental strategies for visual assay (*A*) and phenotypic selection assay (*B*).

Similar pattern was found on the leaves of plants regenerated from calli showing partial GUS expression but plants with pattern of small blue spots were also found among these regenerants. Any plants derived from both "partially blue" and "completely blue" calli were not found that completely lacked GUS activity.

Leaves of 191 plants regenerated from calli which did not express GUS ("white" calli) largely exhibited small blue spots with high variation of number of spots among different regenerants. Mostly (51 %) high or very high number of spots was observed whilst 25 % of regenerants showed a few spots only. The blue spots were observed in such plants after two or three vegetative propagations within a few months. On the contrary, 24 % of the plants regenerated from "white" calli never showed any blue sectors, *i.e.* no *Ac* transposition occurred. Presence of small blue spots on leaves of the assayed regenerants implies *Ac*-activation occurring not during regeneration by organogenesis but in later stages of shoot development.

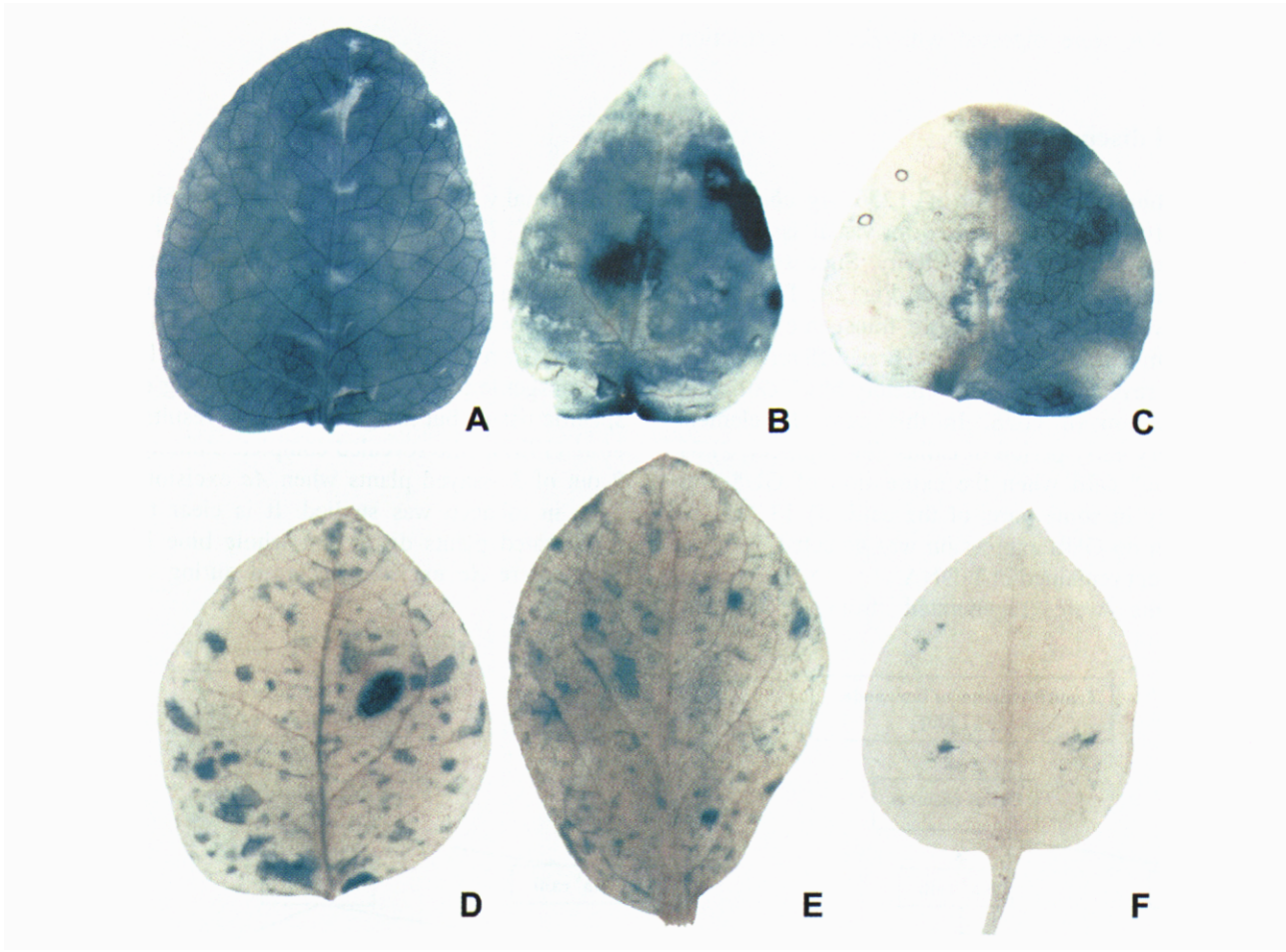

Fig. 3. Histochemical assay for GUS activity in leaves of potato plants regenerated from Kn^R calli exhibiting different activity of *gus* A gene. *A* and *B* represent leaves of regenerants derived from completely GUS^+ calli. *C* and *D* represent regenerants derived from "partially blue" calli while panels *E* and *F* show examples of regenerants derived from "white" calli.

The visual assay used demonstrated clearly differences in timing of *Ac* transposition. The question is what is cause such behaviour. First, we have to think about position effect when structure of the nearly chromatin is different in individual transformants and can affect *Ac*-excision timing and frequency (Schwartz 1986, Heinlein 1995). Second, Peterson and Yoder (1995) which observed high variability of germinal excision frequency in tomato reported high or low activity state of the *Ac* element and connected it with amplification of the element. The same high variability of germinal excision frequency between individual tomato plants was observed by Bříza *et al.* (2000). Third, methylation of the *Ac* element seemed to be cause for different transposition activity of the element in maize (Schwartz and Dennis 1986). Finally, we have to take into account possibility of different number of *Ac* element in individual transformants owing to multiple T-DNA transfer and integration. As Jones *et al.* (1989) reported copy number of *Ac* affected its transposition activity.

Results of the visual assay also showed high transposition activity of *Ac* element in potato. This high activity is obvious from high number of blue spots on leaves of transgenic plants as well as from low proportion (18 %) of transgenic calli without any GUS activity. Similarly, high transposition activity of *Ac* in potato was reported by Knapp *et al.* (1988).

Transformation using plasmid pTT224: Timing of *Ac* element transposition was further studied using a phenotypic selection assay established on *Ac* excision reinstalling resistance to hygromycin (Fig. 2*B*). After transformation with pTT224 kanamycin resistant calli were obtained. Half of each such callus was subjected to hygromycin selection while other half of the callus grew on medium without hygromycin. Thirty four calli with a hygromycin sensitive phenotype indicating that *Ac* element had not excised yet were selected among 225 kanamycin resistant calli. Out of them 16 calli were used for regeneration of 410 plants and mesophyll protoplasts were successfully isolated from 35 randomly selected regenerants. When hygromycin selection has been performed at the minicalli derived from mesophyll protoplasts we did not find any hygromycin resistant calli among calli derived from 18 plants (like as in control) but we found from 2 to 217, *i.e.* up 0.075 %, hygromycin resistant calli derived from the other 17 hygromycin sensitive plants. Total of 1157 hygromycin resistant calli were isolated and PCR assayed to confirm resistance to hygromycin as a consequence of *Ac* excision (Fig. 4). All calli contained 1050 bp empty donor site affirming *Ac* excision.

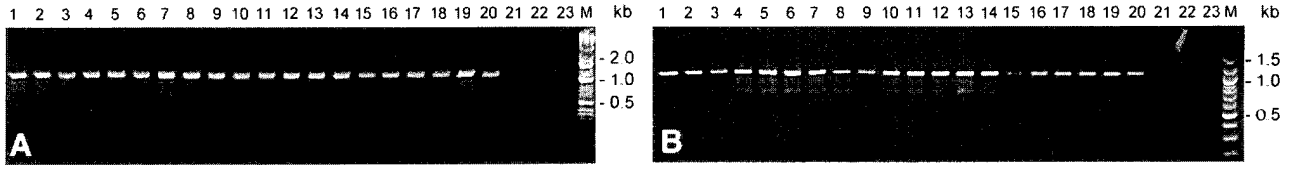

Fig. 4. Example of ethidium bromide stained products of PCR analysis for detection of empty donor site in Hg^R minicalli derived from mesophyll protoplasts. Fragments of the expected size (1050 bp) were amplified in all samples containing transgenic minicalli. *A) Lanes 1 - 20* - minicalli derived from piant 87/16; *lane 21* - nontransformed potato; *lane 22 - A. tumefaciens* harbouring pTT224; *lane 23* - negative control; *lane M* - marker DNA (1 kb ladder, BRL). *B) Lanes 1 - 3* - minicalli derived from plant 45/21; *lanes 4 - 14* - minicalli derived from plant 45/30; *lanes 15 - 20* - minicalli derived from plant 100/18; *lanes 21, 22* and *23* are identical with that on panel *A*; *lane M* - marker DNA (100 bp ladder, NEB).

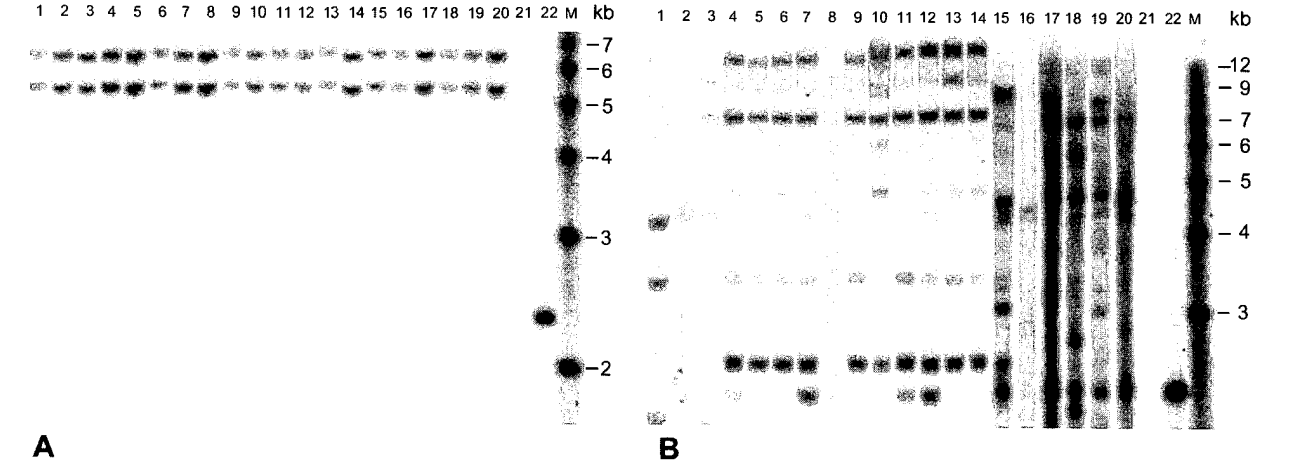

Fig. 5. Southern hybridisation analysis of DNA isolated from in Hg^R minicalli derived from mesophyll protoplasts. DNA was digested with *Eco*R I, electrophoresed, blotted and probed with 334-bp 3' end of *Ac* which contained no *Eco*R I site. Band of 2.4 kb representing original position of *Ac* in T-DNA is evident in *lane 22* (DNA of *A. tumefaciens* harbouring pTT224) on both panels. DNA samples in *lanes 1 - 21* of both parts of the figure are the same as on Fig. 4. *Lane M* - marker DNA (1 kb ladder, BRL).

Part of hygromycin resistant minicalli derived from mesophyll protoplasts of 4 different plants were subjected to Southern hybridisation with 334-bp long probe of 3' end of *Ac* element to detect uniqueness of transposition events and number of *Ac* elements. All 20 minicalli derived from protoplasts isolated from plant 87/16 harboured 2 identical *Ac* containing fragments at new integration sites and no fragment indicating original position of *Ac* in the T-DNA (Fig. 5*A*). This means that all these minicalli carried the same transposition events.

Hygromycin resistant minicalli coming from protoplasts isolated from plants 45/21, 45/30 and 100/18 revealed both new *Ac* integration sites and original T-DNA position (Fig. 5*B*). Number of *Ac* elements was

high, mostly 4 and more, but uniqueness of transposition sites among minicalli derived from the same plant was small. If we could conclude, when using visual GUS assay, that non-detectable excision rate in calli was proceeded frequent excision events at level of the regenerated plants arising high level of transposition uniqueness, then using of phenotypic selection assay showed low uniqueness of new *Ac* positions. Main difference between both assays lay in calli used for plant regeneration. Calli without any GUS activity was used for regeneration of plants displaying many small independent spots directly while in other assay selection on hygromycin was performed on one half of transgenic callus and other half was used for plant regeneration when the half on hygromycin medium was shown as hygromycin sensitive. Therefore, both halves of the callus could not be identical, *Ac* transposition could occur in part used for plant regeneration without recognising. When plant was regenerated from such part of calli all plant cells as well as all protoplasts isolated from it harboured identical transposition event.

The use of phenotypic selection assay showed high transposition activity of *Ac* in potato alike to Knapp *et al.* (1988). This high activity is evident from presence of multiple copies of the *Ac* element in individual hygromycin resistant calli. This increasing of copy number of DNA mobile elements is caused by transposition of the elements from replicated to nonreplicated DNA (Greenblatt 1984, Chen *et al.* 1992). The high *Ac* transposition activity led to early *Ac* element excision in the callus stage followed by low uniqueness of transposition events harboured by hygromycin resistant minicalli. On contrary, Rommens *et al.* (1991) reported, when used phenotypic selection assay in tobacco, that independent transposition events were found. Unfortunately, they analysed only 5 hygromycin resistant calli and out of them only 2 contained one new *Ac* insertion position. Our results show that strategies relying on „delayed" excision of DNA element during leaf development of transgenic plants should not be applicable in all plant species of interest.

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