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A transposon tagging strategy with Ac on plant cell level in heterologous plant species

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The maize transposable element Ac can have a 'late' excision time during leaf development in certain transgenic tobacco plants. This was visualized with an assay based on Ac-excision restoring GUS-expression. Leaves of the described plants contain over 10^3 small blue spots, each of these spots representing an independent excision event. Leaves showing this 'late' excision phenomenon may be used for transposon tagging experiments at plant cell level. Plants which display 'late' Ac-excision do not detectably express GUS during the preceding callus phase, thus allowing transformants to be preselected for a 'late' timing of excision. To examine the applicability of this phenomenon a phenotypic selection assay for excision of Ac was used. Transformed calli containing Ac within the hygromycin resistance gene were regenerated and protoplasts isolated from leaves of regenerated plants were selected on hygromycin. Up to 0.8% of these protoplasts displayed hygromycin resistance. The hygromycin resistant derivatives analyzed were shown to represent independent transposition events. Ac-insertions which can be generated in this way may be used for transposon tagging experiments at cell level.

Key words: Ac transposable element; transgenic tobacco; transposition assay; transposon tagging strategies

Introduction

Transposition of Ac in various heterologous plant species [1—5] suggests that gene tagging with Ac, as has succesfully been carried out in maize [6—10], may be extended to these plant species. For this reason, a number of transposon tagging strategies are at the moment being developed.

In maize, frequencies with which insertions into a gene of interest can be obtained usually vary from 10^{-3} to 10^{-6} [11]. Probably, these frequencies are similar in other plant species, indicating that the ability to generate over 10^3 independent insertions is an important step towards transposon tagging. Screening procedures for visible mutations can be carried out at the plant level in resemblance to experiments in maize (for detailed description see Ref. 12).

Alternatively, it might be envisaged that transposon insertion mutants can be selected for at the plant cell level. For such a procedure leaf mesophyll protoplasts isolated from plants harbouring an Ac-containing T-DNA can be used. However, an efficient procedure also requires the timing of Ac-excision to be considered. As has been reported previously, Ac excises with high frequency relatively soon after its introduction into heterologous plant species [13]. 'Early' transposition of Ac may result in entire leaves or a large leaf sector representing a single transposition event. Protoplasts obtained from leaves of such plants may contain Ac at predominantly one specific position. Therefore, plants should be used in which Ac transposes 'late' during leaf development. The timing of Ac-excision can be studied with visual assays. These assays are based on Ac-excision restoring activity of β-glucuronidase (GUS) [14], *rolC* [15] or streptomycin resistance [16].

In this study, a transposon tagging strategy at

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plant cell level will be described. This strategy is based upon the use of transgenic tobacco plants in which Ac excises 'late' during leaf development. We show that transformants which did not express GUS during the callus phase often contained many small GUS expressing spots on leaves. Thus, nondetectable excision of Ac in callus tissue can be followed by a 'late' timing of excision during leaf development.

Protoplasts isolated from leaves of the described plants may be useful to screen for mutants at the somatic cell level. In a similar way, a phenotypic selection assay for excision of Ac has been used.

Methods

Construction of plant vectors

To visualize Ac excision from GUS, pBI 121 [17] was used. pBI 121 contains the GUS gene fused to the 35S promoter from Cauliflower Mosaic Virus (CaMV 35S promoter) in the polylinker of pBIN19 [18]. The 4.6 kb Ac-element containing BglII linkers at the ends [5] was inserted into the BamHI site between the 35S promoter and GUS. The resulting plasmid was called pTT230.

To be able to select for excision of Ac, pTT224 was used. This plasmid contains, apart from an intact neomycin phosphotransferase gene (NPT II), Ac inserted in the leader sequence of the hygromycin phosphotransferase gene (HPT II) [5].

Plant transformation

The constructed binary vectors were transferred by conjugation to Agrobacterium tumefaciens LBA 4404 [19] using the helper plasmid pRK2013 [20]. Overnight cultures of the Agrobacterium strains were used to infect explants of Nicotiana tabacum cv. Petit Havanna SR1 [21]. Infected explants were dried briefly on filter paper and incubated on feeder layers of Petunia Albino Comanche suspension cells for 3 days. Selection for transformed cells was performed by placing infected explants on R3B medium containing kanamycin (100 mg/l). Selection for excision of Ac or Ds from HPT II was performed by subjecting the infected explants to hygromycin B (20 mg/l). To kill off Agrobacterium, carbenicillin (200 mg/l) and vancomycin (200 mg/l) were added to the medium. To induce shoot formation, zeatin (1 mg/l)

was added to the medium. All chemicals were supplied by Sigma.

PCR analysis of Ac-excision

The polymerase chain reaction (PCR) technique has been applied to detect Ac-excision events in transgenic tobacco plants. Primers were made for the (-)strand of the CaMV35S promoter: AAG CTT ACA GTC TCA GAA GAC CAA AG and the (+)strand of HPT II: GAA TTC CCC AAT GTC AAG CAC TTC CG. Plant DNA (2 μg) was mixed with 100 pmol of each primer in a reaction buffer (10 mM Tris—HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.05% Tween 20) containing 2.5 units Cetus Taq polymerase. The final reaction volume was 100 μl. A 'Perkin Elmer Cetus' thermoblock was used to perform 30 cycles using the following regime: (a) 1 min at 94°C, (b) 2 min at 55°C and (c) 3 min at 72°C. Precipitated products were separated on a 3% agarose gel.

Southern blot analysis of total plant DNA

Tobacco DNA (10 μg) was isolated as described by Dellaporta et al. [22] and digested with 50 units of restriction enzyme as described by the manufacturer (Biolabs Research Laboratories). Following electrophoresis on a 1% agarose gel, DNA was transferred to Hybond-N membrane (Amersham) using 20 × SSC as a transfer buffer. Hybridization was at 60°C for 24 h in 10% Dextran sulphate, 1 M NaCl, 1% SDS and 200 μg Salmon sperm DNA. The filters were washed twice in 2 × SSC, once in 1 × SSC/0.1% SDS and once in 0.1 × SSC/0.1% SDS at 60°C. The washed filters were exposed to Kodak X-Omat AR films for 1—5 days at -70°C using intensifying screens. DNA for the 2.0 kb EcoRI probe containing the 3′ sequence of Ac was isolated from pJAC [7] and labeled with [32P]dATP (spec. act., >10^8 cpm/μg) using random primers.

Fluorometric GUS-assay

GUS-assays were performed on 100 mg callus tissue, as described by Jefferson et al. [17], using the substrate methyl umbelliferone glucuronide (MUG, Sigma). Enzyme activity was visualized by fluorescence of the product, MU, on a long-wavelength transilluminator.

In situ GUS assay

Leaves or seedlings were incubated in GUS assay buffer (0.1 M NaH_2PO_4 (pH 7.0), 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid) for 16 h at 37°C in the dark. Plant pigments were partially removed in 70% ethanol. Tissues were photographed with Kodak EPY-50 using an 'Olympus B071' microscope.

Results and Discussion

A visual assay based on Ac-excision restoring GUS activity was used to examine whether transgenic plants could be identified in which Ac excises 'late' in leaf development. For this purpose *Nicotiana tabacum* SR-1 was transformed with an Agrobacterium strain harbouring pTT230. The T-DNA of this plasmid contains NPT II and an Ac-interrupted GUS gene. A fluorometric GUS assay was performed on kanamycin resistant calli 6 weeks after transformation. Four calli which strongly expressed GUS and seven calli in which no GUS expression could be detected were regenerated. Leaves of regenerated transformants were histochemically assayed. This lead to visualization of clearly distinguishable sectors and/or spots (Fig. 1).

Large blue sectors could be visualized in leaves of those transformants which already expressed GUS in callus tissue (AAN 4008, AAN 4201, AAN 4210 and AAN 4221). However, we did not find staining of all cells of specific tissues. This last observation is in agreement with the results of earlier studies on both Ac-excision from the *rolC* gene [15] and Spm-excision from the GUS gene [23] in tobacco. Our results diverge from those obtained by Finnegan et al. [14] who studied excision of Ac from the GUS gene in tobacco. They found extensive or complete staining of certain tissues in leaves of 2 out of 3 analyzed plants. Their results indicate excision of Ac to have occurred in progenitor cells of shoots which were used to obtain the described plants.

Leaves of seven out of eight plants which did not express GUS relatively soon after transformation predominantly showed small blue spots. The number of spots varied among different transformants. In four transformants (AAN4203, AAN4204, AAN4212 and AAN4222) at least 10^3 clones of blue cells could be identified in each leaf. Leaves of two other plants (AAN4227 and AAN4230) contain less blue spots while from six leaves of AAN4202 only once a small blue spot could be detected. Leaves of one plant (AAN4220) like leaves of non transformed plants never could be stained at all, while leaves of plants transformed with pBI121, harbouring an intact GUS gene, showed a uniform distribution of stain. The presence of only small spots in leaves of the described transformants indicates that activation of Ac was not due to general processes underlying regeneration. If such an activation would occur, also large sectors should be expected.

There are various explanations possible for the observed differences in timing of transposition. One of the possibilities is that the timing of transposition is affected by the copynumber of Ac in the individual plants. An effect of the number of Ac elements on the frequency of transposition has already been indicated by Jones et al. [16]. They showed that progeny plants homozygous for Ac are more variegated than heterozygous progeny plants derived from the same primary transformed tobacco plant. Late excision could therefore be due to low Ac copy numbers. Alternatively, as suggested by Taylor et al. [24], the timing of transposition may be dependent on the structure of the surrounding chromatin.

Protoplasts isolated from leaves of plants like AAN4203, AAN4204, AAN4212 and AAN4222 in which Ac excises with high frequency after a period of Ac-inactivity will contain Ac at many different positions. Thus, the use of protoplasts from such plants may allow efficient screening procedures at the plant cell level.

The applicability of 'delayed' excision was further examined with a phenotypic selection assay based on Ac excision restoring hygromycin resistance. For this purpose, tobacco plants were transformed with an Agrobacterium strain containing pTT224. This plasmid contains NPT II and an Ac-interrupted HPT II. Kanamycin-resistant calli were cut in halves and subjected to either kanamycin or hygromycin selection. Calli with a hygromycin sensitive phenotype, indicating that Ac had not excised yet, were regenerated. Six independently

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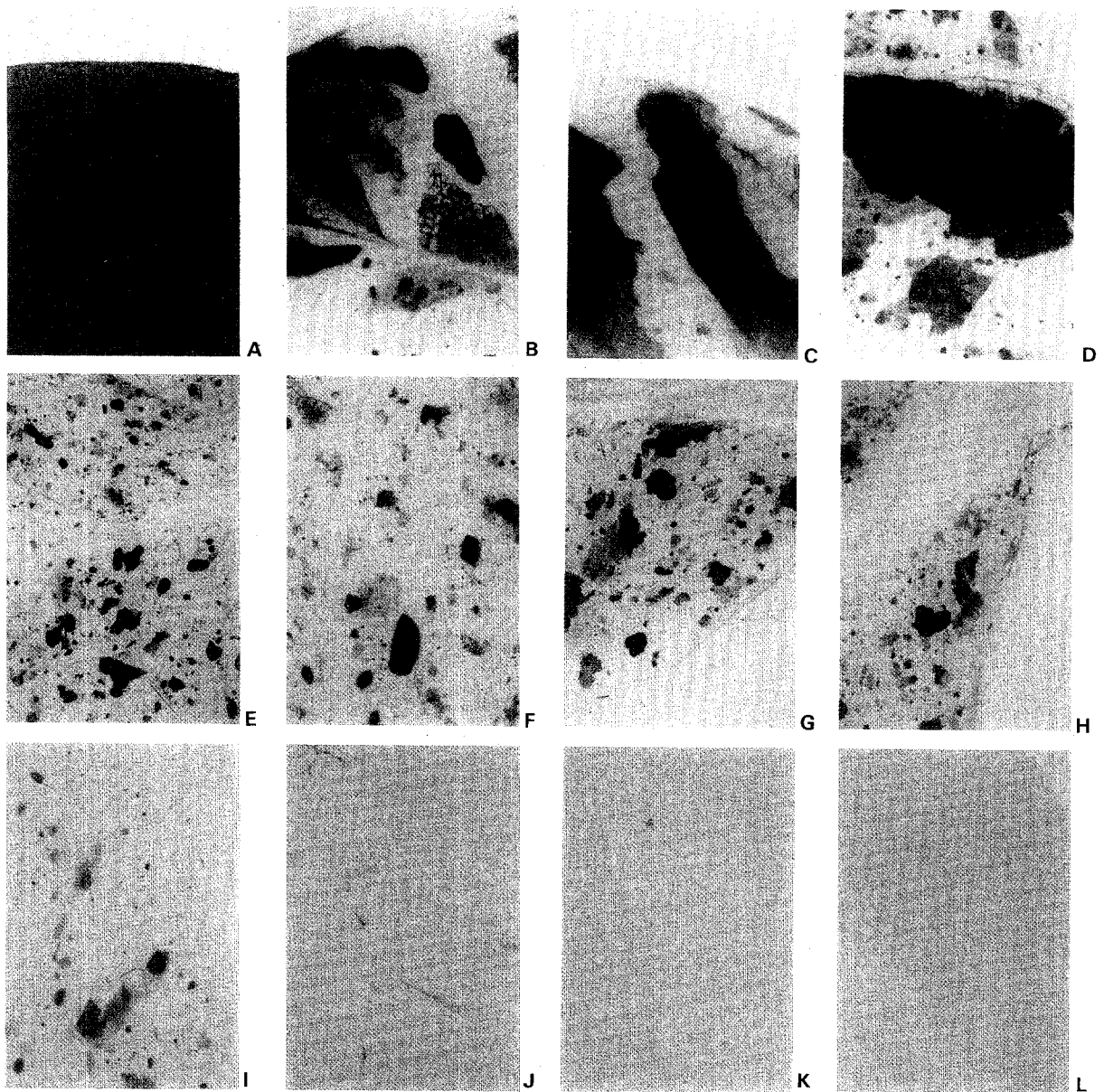
Fig. 1. Histochemical localisation of GUS activity in segments of leaves of transgenic tobacco plants. Plants contained the T-DNA of: pBI121 (intact GUS; A) or pTT230 (Ac inserted within GUS; B—K). A leaf segment of a non-transformed plant is presented in (L). Plants were regenerated from calli which did (A—D) or did not (E—K) express GUS. Leaf segments were magnified 7.5×, except for F, I and K (10×) and J (15×). (B) AAN4008, (C) AAN4210, (D) AAN4221, (E) AAN4203, (F) AAN4212, (G) AAN4222, (H) AAN4204, (I) AAN4227, (J) AAN4230 and (K) AAN4202.

transformed and regenerated plants were used to select for excision events occurring after many cell divisions. Protoplasts were isolated from leaves and cultured to minicalli stage. Minicalli were subjected to 20 mg/l hygromycin. Up to 0.8% of the minicalli derived from five out of six plants displayed hygromycin resistance (Table I). This implies that the late excision events which were visualized with

Table I. Frequency of calli displaying hygromycin resistance as a consequence of Ac excision. Hygromycin resistant (Hyg^r) calli were derived from protoplasts isolated from leaves of plants transformed with pTT224. These plants did not display hygromycin resistance (Hyg^s plants), indicating that Ac had not excised yet from HPT II.

Hyg^s plants	No. of calli tested	No of Hyg^r calli	% Hyg^r calli
AAN 1548	71 000	501	0.7
AAN 1566	50 000	12	0.02
AAN 1574	25 000	1	0.004
AAN 1585	7000	20	0.2
AAN 1615	72 000	557	0.8
AAN 1626	80 000	0	0

GUS (blue sectors) could be selected for with HPT II (hygromycin resistance).

In order to confirm that hygromycin resistance is a consequence of Ac-excision, a PCR reaction was performed with DNA from five independent hygromycin resistant calli derived from one hygromycin sensitive plant (AAN 1566). Using primers flanking the original integration site of Ac between the CaMV 35S promoter and the HPT II coding region, an empty donor site fragment of 0.7 kb could be visualized in the DNA of all five calli. This implies that hygromycin resistance is a consequence of Ac-excision. DNA of the same calli was also used for a Southern blot analysis to examine reintegration of Ac into the genome. Along this line it can be investigated whether the hygromycin resistant calli originated from one sector of cells in which Ac had transposed or whether they represent independent Ac transposition events. Hybridization with a 3′ sequence of Ac enabled detection of the 1.6 kb internal HindIII fragment of Ac in 4 out of 5 calli (see Fig 2). The absence of any Ac-hybridizing sequences in one callus indicates that in this case Ac did not reintegrate into the genome after excision.

From four of the calli which still contained Ac, two contained one new and specific Ac-homologous fragment, indicating integration of Ac at predominantly one new position. The ability to generate such non-chimaeric derivatives is impor-

tant for experiments set up to tag genes which can only be assayed biochemically, because inactivation of such genes in only a fraction of the cells may be difficult to distinguish from normal expression levels. This is unlike genes involved in plant morphology. Such genes can also be scored for inactivation in chimaeric plants (variegated phenotype). The insertion of Ac at predominantly one new position was also demonstrated in related progenies of a transgenic tomato plant [25].

The other two calli which have been analyzed at the molecular level and which do contain an internal Ac-fragment probably contain Ac at many different positions as no new location was frequent enough to allow detection of new bands. Comparable results have been described by Lassner et al. [25]. Our results demonstrate that Ac-excision has occurred independently in all analyzed cases.

It can be concluded that plants in which Ac transposes 'late' in development can be used to generate independent insertions. As millions of protoplasts can be screened relatively easy, it will be possible to generate in the order of thousands of calli containing transposed Ac elements. Therefore, the described procedure may enable transposon tagging of genes at the plant cell level. Transposon tagging strategies at protoplast level in heterologous plant species have not been reported previously.

Many functions which are, in principle, accessible for transposon tagging at plant cell level can be envisaged referring to in vitro selection experiments (see e.g., Refs. 26 and 27). Usually resistance to toxic concentrations of amino acids or amino acid analogs or other compounds was employed. The molecular basis of most of the obtained variants is unknown. However, the availability of a cDNA probe for the structural nitrate reductase (NR) gene allowed NR-deficient tobacco mutants to be molecularly analyzed. These mutants were obtained by subjecting mesophyll cells of a somatically instable tobacco line to chlorate. It was shown that one-third of the mutants contained a previously uncharacterized retrotransposon inserted into the NR gene [28]. This experiment thus showed the applicability of insertion mutagenesis at the plant cell level.

Although transposon insertions predominantly lead to recessive mutations in maize, insertions can
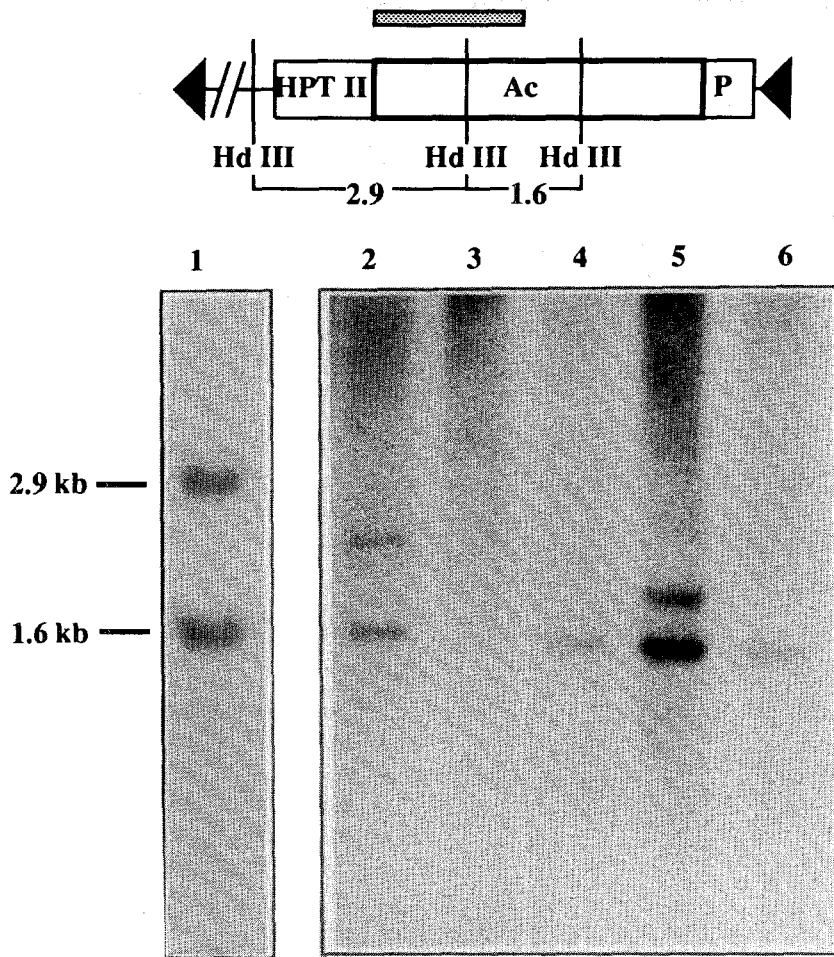
Fig. 2. Blot hybridization analysis of DNA isolated from AAN1566 and five derivative plants. AAN1566 displays hygromycin sensitivity while derivative plants display hygromycin resistance. The top figure shows a schematic representation of part of the T-DNA of plasmid pTT224, containing Ac between the CaMV35S promoter and HPT II. The sizes of the internal Ac-fragment and the Ac-border fragment with HindIII in the T-DNA of pTT224 are indicated below the diagram. The Southern blot contains HindIII-digested DNA isolated from AAN1566 (lane 1) and 5 derivatives (lane 2—6). The probe which has been used is a 2.3-kb EcoRI-fragment of pJAc [7] containing a 2.0-kb 3′ sequence of Ac (represented by a dotted box above the diagram). AAN1566 contains 2 fragments with a size expected for the presence of Ac at the original position in the T-DNA. Four out of 5 variants (lanes 2, 4, 5 and 6) contain the internal Ac-fragment. New integration sites can be visualized in 2 out of 5 variants (lanes 2 and 5).

also cause (semi) dominant mutations [29]. This further indicates that both haploid and diploid cells might be used for in vitro transposon mutagenesis experiments.

Before carrying out transposon tagging experiments, the potential of the presented procedure will be further examined. For this purpose, T-DNA gene 2 will be used. This gene is located 1.8 kb from the original integration site of Ac between the borders of the T-DNA of pTT224 [5]. It encodes an enzyme converting naphtalene acetamid (NAM) into naphtalene acetic acid (NAA) [31]. High levels of NAA are toxic to plant cells. Thus, selection of protoplasts on media with NAM enables recovery of T-DNA gene 2 mutants [30]. Selection of protoplasts obtained from transgenic plants contain-

ing the T-DNA of pTT224 on NAM may allow the extent in which somaclonal variation contributes to the mutation frequency to be determined. Furthermore, selection of T-DNA gene 2 mutants on hygromycin may enable the frequency of Ac-insertion into a closely linked gene to be determined.

Acknowledgements

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