Analysis of the T-DNA structure in a large number of transgenic petunias generated by *Agrobacterium*-mediated transformation

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**Abstract**

Southern hybridisation was performed on ninety-six transgenic petunias that had been selected for resistance to kanamycin. Just over half of the plants contained intact copies of the T-DNA. The most common rearrangements (at least 24 plants out of 96) were simple deleted derivatives that had lost one or both ends of the T-DNA. T-DNAs lacking the left border occurred at a frequency of 20%, and estimates of the frequency of T-DNAs lacking the right border were at least this high. Three plants contained grossly rearranged T-DNAs, of which all expressed the kanamycin resistance gene but only one transmitted the gene to progeny. Two plants lacked T-DNA homology altogether and did not express kanamycin resistance in their leaves or their progeny. Circumstantial evidence suggests that plants containing a chimaeric kanamycin resistance gene driven by the ocs promoter do not root efficiently in the presence of kanamycin. There was no correlation between intactness of the T-DNA and Mendelian inheritance of the kanamycin-resistance phenotype. However, a disproportionate number of plants showing non-Mendelian inheritance had a high copy number of their T-DNA.

**Introduction**

*Agrobacterium tumefaciens* has evolved a natural gene transfer system to transfer a region of its DNA (the T-DNA) into plant cells (for reviews see [12, 14, 36]). The T-DNA region is bounded by 23 base pair (bp) direct repeats [1, 16, 46]. These ‘border’ sequences have been implicated in excision of a single-stranded DNA intermediate that is subsequently transferred into the plant cell [38, 39]. The right border may act as the initiation point for transfer since its orientation determines the direction of transfer [4, 43]. A short sequence located adjacent to the right border increases the efficiency of transfer [22, 34]. Integration of the T-DNA appears to be more precise at the right border than the left border [19, 46].

Early analysis of T-DNA structures in plant tumour tissue showed that the T-DNA region was intact and was present in several copies per plant genome [27]. In several tumour lines the multiple T-DNA copies were present in a tandem configuration [27, 45]. From these observations it was concluded that the T-DNA usually undergoes no major rearrangements during transfer and integration into the plant genome. Later results revealed the presence of truncated T-DNA insertions missing the right border region and some rearranged plant T-DNA inserts [6, 17, 30]. Van Lijsebettens *et al.* [41] and Peerbolte *et al.* [32, 33] have studied in detail the T-DNA struc-
tures in *Agrobacterium*-induced tumour lines. They found a high frequency of deleted and rearranged T-DNA structures in their cell lines, and suggested that the changes occurred at the time of transfer, rather than subsequent to integration.

At the time this work was initiated little information was available on the structure of plant T-DNA inserts in transgenic plants generated using disarmed *Agrobacterium* T-DNA vectors carrying foreign chimaeric genes. In most cases Southern analysis of the plant T-DNA had shown that the gene of interest was intact in a few individuals [2, 11, 20, 47].

To better understand the process of *Agrobacterium*-mediated gene transfer and to apply it to plant breeding, it is important to determine the frequency with which single copies of unrearranged versions of the gene are introduced and the frequency with which transgenic plants transmit their genes in a stable manner to their progeny. Analysis of the frequency of T-DNA rearrangements may also shed more light on the mechanism of integration. In this paper, we describe the results of an analysis of a large number of transgenic petunia plants generated by *Agrobacterium* cocultivation of leaf discs using a binary vector system carrying a disarmed T-DNA.

**Materials and methods**

*pCGN200 binary vector*

The binary vector used, pCGN200, was constructed by forced recombination between two precursor plasmids, pCGN587 and pCGN167 (Knauf and Gardner, unpublished). Plasmid pCGN587 is a pRK290-based wide host range vector that contains an ocs-kan gene between T-DNA borders (described in [9]). pCGN167 is a pACYC184-based vector containing a 35S-kan gene (which differs from pCGN149a [13] by deletion of the ATG upstream of the NPTII coding region). Recombination between the two plasmids occurred in the chloramphenicol genes (refer to Fig. 1).

**Southern hybridisation of plant genomic DNA**

Plant DNA was isolated using the method of Laporta *et al.* [7], digested with EcoRI and HindIII (Promega Biotec), and electrophoresed on horizontal 0.7% agarose gels. After EtBr staining and photography, the gel was pretreated with TS solution (0.4 M NaOH, 0.6 M NaCl) for 30 minutes. DNA was transferred overnight onto “Genescreen Plus” nylon membranes using TS solution, and then filter-neutralised in NS solution (0.5 M Tris-HCl pH 7, 1 M NaCl) for 15 minutes. Plasmid DNA for use as a probe was isolated from *E. coli* using the method of Ish-Horowitz *et al.* [21] with solution III taken from Birnboim [3], followed by CsCl density gradient centrifugation [28]. Radiolabelling of the probe DNA (a supercoiled preparation of pCGN167) was carried out using a “BRL” nick translation kit. Prehybridisation and hybridisation solutions were as described in Maniatis *et al.* [28] with 10% dextran sulphate added to the hybridisation solution. Two washes were performed: 1 × SSC, 0.5% (w/v) SDS at room temperature and 0.2 × SSC, 0.5% SDS at 60 °C (both for 30 minutes). Filters were exposed to X-ray film with DuPont Cronex Lightning Plus screens for 12-36 hours at -70 °C.

**Results**

One hundred and four transgenic petunias were obtained by leaf disc cocultivation with *Agrobacterium tumefaciens* strain LBA4404 [18] containing a binary vector pCGN200. The plasmid pCGN200 contains two chimaeric kanamycin resistance genes located between the left and right borders of the T-DNA. The plants were characterised with respect to their transmission of kanamycin resistance phenotype, and placed into seven inheritance categories [8]. DNA was isolated from 96 of the plants and subjected to Southern hybridisation. The results are presented below. A summary of the data for individual plants can be found in Table 1 of Deroles and Gardner [8].

**Southern hybridisation of transgenic plants**

Figure 1 shows the EcoRI and HindIII restriction