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Nonspecific transmission of the NPTII gene from *E. coli* helper plasmid to a plasmid of *Agrobacterium tumefaciens*

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The transmission of ColE1/pMB1-derived plasmids, such as pBR322, from *E. coli* donor strains into *Agrobacterium tumefaciens* is accomplished by using *E. coli* carrying the helper plasmids pGJ28 and R64*drd*11 which provide the Col EI *mob* functions and *tra* functions, respectively [1]. The helper plasmids themselves are not stable when they are transferred into *A. tumefaciens*. However, van Haute *et al.* [1] have observed that the cross of *E. coli* strain HB101 having the plasmids pGV1150, pGJ28 and R64*drd*11, with *A. tumefaciens* strain GV3101 cured for its Ti plasmid gives nonspecific exconjugants with a frequency of 1.6×10^{-5}. These particular exconjugants contain a new cointegrate plasmid pGV1150::pGJ28.

The aim of our studies was to investigate whether these phenomena also appeared in the case when the acceptor Ti plasmid pGV3850 HPT [2] (a kind gift from Prof. H. Saedler, Max-Planck Institut für Zuchtungsforschung, Kćln) and the pBR322 derivative plasmid containing the sequence of lupin plasmid-like mitochondrial DNA-K1 [3] were used.

Plasmid transfers were performed by conjugation. The first step of mating included conjugation between *E. coli* strains as follows:

GJ23(pGJ28, R64*drd*11) X DH5α(pBR322)

and GJ23(pGJ28, R64*drd*11) X DH5α (pBR322/K1)

The exconjugants were selected on kanamycin (25 μg/ml), tetracyklin (10 μg/ml), ampicillin (70 μg/ml) and streptomycin (100 μg/ml). The second step of mating was performed between *E. coli* strain and *A. tumefaciens* strain:

DH5α(pGJ28, R64*drd*11, pBR322) X C58C1(pGV3850 HPT)

and DH5α(pGJ28, R64*drd*11, pBR322/K1) X C58C1(pGV3850 HPT)

The exconjugants were selected on rifampicin (100 μg/ml), spectinomycin (100 μg/ml), hygromycin (10 μg/ml) and kanamycin (20 μg/ml). As an alternative way of introduction of the mentioned above plasmids into *A. tumefaciens*, triparental matings were performed. It included the following strains:

DH5α(pBR322) X GJ23 (pGJ28, R64*drd*11) X C58C1(pGV3850 HPT)

and DH5α(pBR322/K1) X GJ23 (pGJ28, R64*drd*11) X C58C1(pGV3850 HPT)

The exconjugants were selected of the same type and on the same amount of the antibiotics as in the case of the second step of matings.

All exconjugants obtained from diparental and triparental matings were probed in colony hybridization experiments [4] with ^{32}P-labelled [5] fragment of NPTII gene derived from pGJ28 plasmid (other parts of this plasmid are not useful as hybridization probe [1]). Some exconjugants which showed the positive hy-

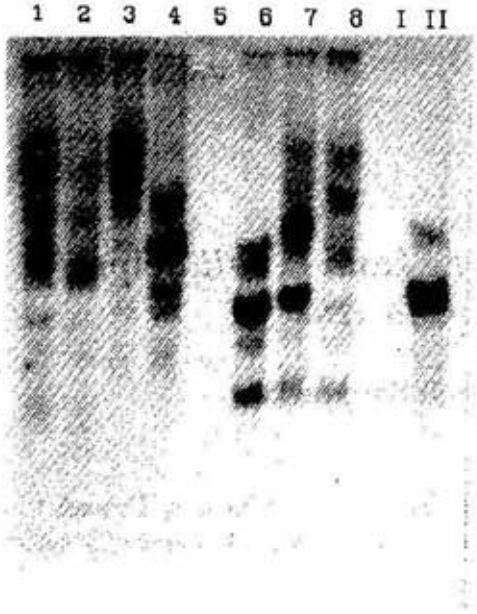

Fig. 1. Southern blot hybridization of eight selected exconjugants (1–8).
DNAs were digested with *Hin*dIII and hybridized with NPTII probe for 16 h at 66°C in 3 × SSC, 10 × Denhardt solution, 0.1% SDS and 100 μg/ml salmon sperm DNA. The filter was washed twice in 3 × SSC, 0.1% SDS and in 0.3 × SSC for 15 min at 65°C. I, DNA from *A. tumefaciens* C58C1(pGV3850HPT). II, DNA from *A. tumefaciens* C58C1(pGV3850HPT/NPTII) which confirmed the presence of NPTII gene in the earlier obtained exconjugant.

bridization signals were checked in ketolactose test (not shown) and then taken for DNA isolation and Southern blot hybridizations (Figs. 1, 2A, B) as well as determination of the neomycin phosphotransferase activity [6] (Table 1). To determine the place of integration of pGJ28 plasmid to Ti plasmid, total DNA from one of the obtained exconjugants (No. 4, see Fig. 1) was digested by a few restriction endonucleases and analysed in Southern blot hybridization. Hybridization probes were: 1, NPTII gene fragment alone (the same as in the colony hybridization experiment); 2, pBR322 and pGJ28 plasmids labelled separately and mixed in proportion 1:1. The latter probe provides information about the sequence of the whole region of T-DNA (except the fragments No. 10 and 23). The results presented in Fig. 2A, B and Fig. 3 agreed well with the data published by Van Haute *et al.* [1], Depicker *et al.* [7] and Zambryski *et al.* [8] as far as type, approximate place and size of restriction fragments were concerned. Thus they confirmed that the integration place of pGJ28 plasmid lay within the T-DNA fragment of the pGV3850HPT vector.

The *Agrobacterium* strain carrying cointegrative plasmid pGV3850 HPT:NPTII was used for leaf disc infection of diploid potato H-120. The infection and regeneration of transformed plants were carried out according to the established methods. Transformants were maintained on MS media containing an appropriate amount of antibiotics: kanamycin sulfate (200 μg/ml), hygromycin (20 μg/ml), and cefotaxime (500 μg/ml), (amounts at the starting time). Three-month-old transformants were taken for DNA isolation [9] and Southern blot hybridization (Fig. 4).

The results of all the above mentioned experiments revealed that almost all exconjugants which appeared in diparental and triparental matings showed positive hybridization signals which suggested the presence of the NPTII gene in them (not shown). The latter suggests also that the method of matings had no in-

Table 1
Activity of the neomycin phosphotransferase in some exconjugants of A. tumefaciens

No. of *A. tumefaciens* clone	Protein concentration in extracts (μg/ml)	NPTII activity 10^{-3} c.p.m./mg protein
1	355	70
2	290	310
3	405	250
4	340	290
5	455	0
6	385	2500
7	385	520
8	385	120

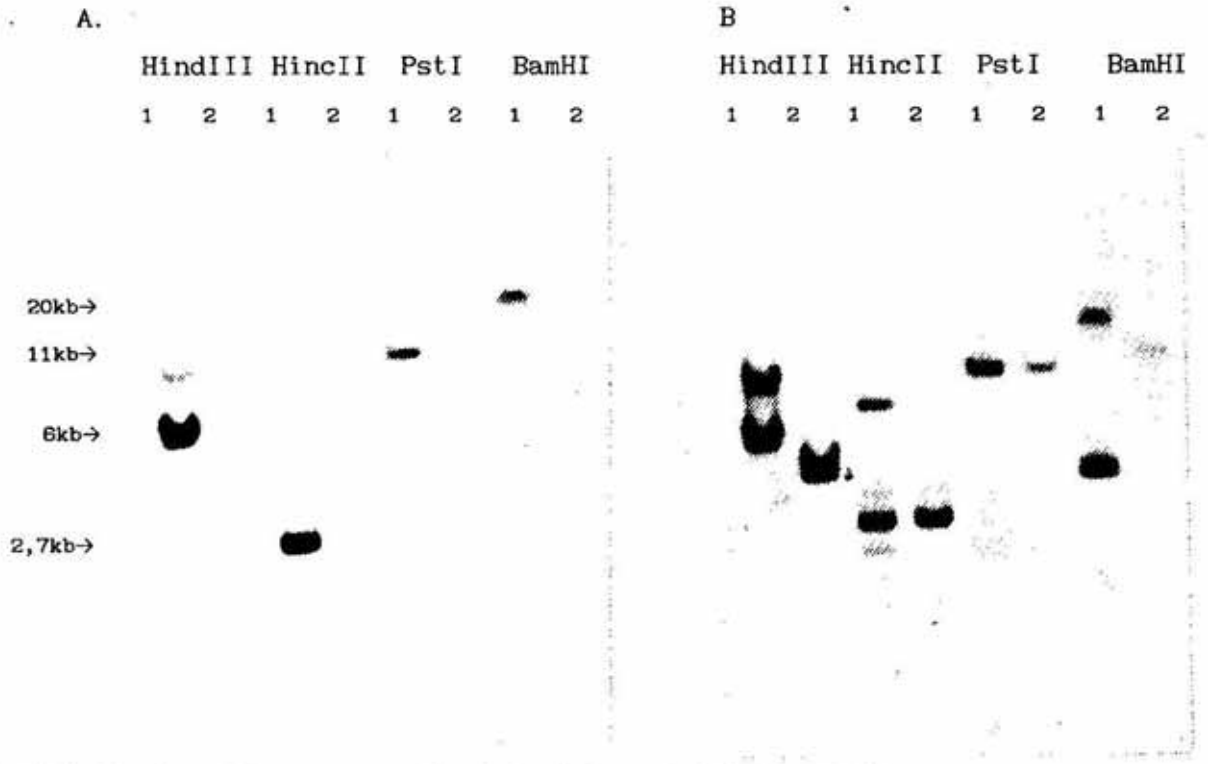

Fig. 2. Verification of the structure of the obtained plasmid pGV3850 HPT/NPTII.
Hybridization: A, with NPTII gene fragment as a probe; B, with both pGJ28 and pBR322 DNAs as a probe. Lanes 1, total DNA of *A. tumefaciens* C58C1(pGV3850 HPT/NPTII); lanes 2, total DNA of *A. tumefaciens* C58C1(pGV3850 HPT). The same blot was used in hybridization A and B. The obtained fragments are depicted in Fig. 3B. DNAs were digested by the indicated restrictases.

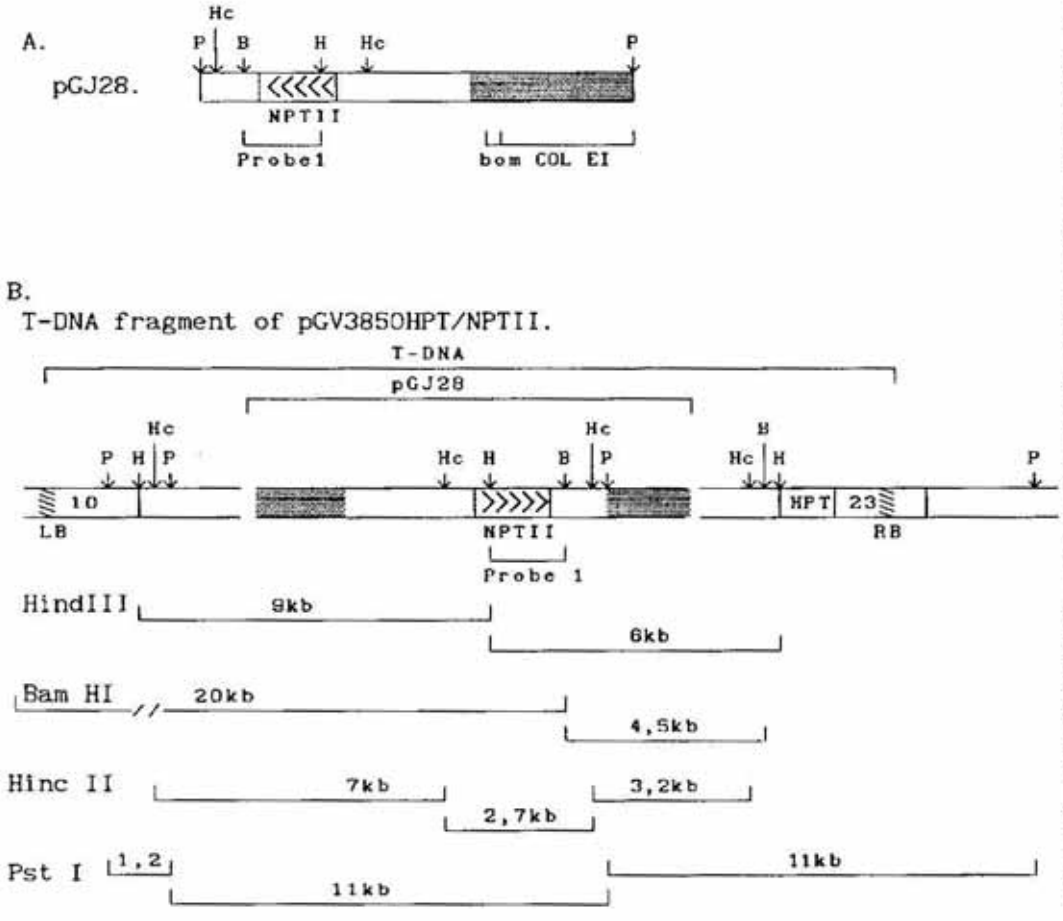

Fig. 3. Comparison the structures of the pGJ28 plasmid and T-DNA fragment of pGV3850 HPT/NPTII.
A, pGJ28 plasmid according to [1]. B, T-DNA fragment of pGV3850 HPT/NPTII. Restriction fragments according to [1, 8, 9] and Fig. 2A, B. Probe 1, fragment used as a probe of NPTII in hybridization experiments (Fig. 2a). The restriction sites are indicated as arrows: H, *Hin*dIII; B, *Bam*HI; Hc, *Hin*cII; P, *Pst*I.

Fig. 4. Southern blot of DNAs from transgenic potatoes transformed by A. tumefaciens C58C1 (pGV3850 HPT/NPTII).
Lane 1, DNA fragment containing NPTII gene; lane 2, DNA from nontransformed plant; lane 3, DNA from transformed plant.

fluence on the transmission of the NPTII gene. There was no distortion in the transmission of this gene, either when lupin mtDNA was present in a donor pBR322 plasmid. The neomycin phosphotransferase activity determined in protein extracts from some exconjugants pointed to the positive expression of NPTII gene. In one of the transformed diploid potatoes H-120 the positive hybridization signal suggested the presence of this gene.

Further work has to be carried out to elucidate the mechanism of the NPTII gene insertion.

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