Various nopaline catabolism genes located outside the Ti-plasmids in *Agrobacterium tumefaciens* strains

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The structure and location of nopaline catabolism (*noc*) genes were examined in various *Agrobacterium tumefaciens* strains by plasmid transfer and by Southern hybridization analysis. In a pathogenic strain, PyTS3, the *noc* genes were identified both on the Ti-plasmid, pTiPyTS3, and on the cryptic plasmid, pCr140. Plasmid pCr140 was transmitted to another *A. tumefaciens* strain at a higher frequency than was pTiPyTS3. Southern hybridization analysis, using Ti-plasmid-derived *noc* genes as a probe, indicated that the *noc* genes of pTiPyTS3 differed structurally from those of pCr140. Three non-pathogenic strains possessed plasmids that hybridized to the probe, and all showed identical patterns of hybridization after restriction enzyme cleavage. However, this pattern was different from those of pTiPyTS3 and pCr140. Only pCr140 showed partial hybridization to the probe. A non-pathogenic strain, PyON8, that grew on both nopaline and octopine, showed no hybridization of its plasmid or chromosome to the probe. Since nopaline-utilizing transconjugants were not obtained from PyON8, the *noc* genes in PyON8 may be chromosomally located. These results indicate that *A. tumefaciens* contains diverse *noc* genes which differ in both organization and cellular localization.

Introduction

Crown gall tumours are induced on various plants upon infection by the soil bacterium *Agrobacterium tumefaciens*. Virulent *A. tumefaciens* strains contain a number of plasmids of various sizes, one of which is the tumour-inducing plasmid, or Ti-plasmid (Van Larebeke *et al.*, 1974). Part of this plasmid, known as T-DNA, is integrated into plant chromosomes (Chilton *et al.*, 1977), and expression of the genes on the T-DNA involved in phytohormone synthesis is required for initiation and maintenance of tumorous tissues (Akiyoshi *et al.*, 1984; Thomashow *et al.*, 1984). Except for a few instances, functions have not been ascribed to the other, as yet cryptic, plasmids in *A. tumefaciens*.

The tumorous tissues induced by *A. tumefaciens* produce and secrete unusual metabolites termed opines: nopaline, octopine, agropine and others (Bomhoff *et al.*, 1976; Nester *et al.*, 1984). The genes required for opine synthesis are found on the T-DNA, while the genes responsible for opine catabolism are located outside the T-DNA on the Ti-plasmid (Holsters *et al.*, 1980). In most cases, the type of opine catabolism encoded in a given Ti-plasmid is the same as that of opine synthesis. The importance of opine synthesis to plants has not yet been

established, but *A. tumefaciens* uses the opines as a source of carbon and nitrogen, and thus is able to survive in association with crown gall tumours. Another function of the opines is to promote conjugal transfer of Ti-plasmids between *A. tumefaciens* strains (Klapwijk *et al.*, 1978; Ellis *et al.*, 1982).

Some *A. tumefaciens* strains are non-pathogenic, and still capable of utilizing opines (Merlo & Nester, 1977). These strains also possess several plasmids, one of which carries opine catabolism genes (Hooykaas *et al.*, 1980). Some DNA sequence homology exists between Ti-plasmids and plasmids from non-pathogenic strains (Merlo & Nester, 1977), but the relationship between opine catabolism genes in pathogenic and non-pathogenic strains has not been well studied.

Here we report the distribution of the nopaline catabolism (*noc*) genes in *A. tumefaciens* and the structural relationship among these genes.

Methods

Bacterial strains, plasmids, media and chemicals. *Agrobacterium* strains used in this study are listed in Table 1. *A. tumefaciens* strains were isolated from crown gall tissues as reported previously (Wabiko *et al.*, 1989). The hybrid plasmid pGV0339, provided by Dr J. Schell (Max-Planck Institute, FRG), is composed of the vector pBR322 and *Hind*III

Table 1. *Agrobacterium* strains

Strain	Relevant phenotype*	Opine type†	No. of plasmids	Origin	Reference
<i>A. tumefaciens</i>					
PyTS1	Non-pathogenic	N	3 (pPyTS1‡, cryptic plasmids)	Pear	Wabiko <i>et al.</i> (1989)
PyTS2	Non-pathogenic	N	3 (pPyTS2‡, cryptic plasmids)	Pear	Wabiko <i>et al.</i> (1989)
PyTS3	Pathogenic	N	4 (pTiPyTS3‡, pCr140‡, other cryptic plasmids)	Pear	Wabiko <i>et al.</i> (1989)
PyTS4	Non-pathogenic	N	2 (pPyTS4‡, cryptic plasmid)	Pear	Wabiko <i>et al.</i> (1989)
PyON8	Non-pathogenic	N, O	1 (cryptic plasmid)	Pear	This study
PO31	Pathogenic	N	4 (pTi PO31‡, cryptic plasmids)	Poplar	Wabiko <i>et al.</i> (1989)
PO201	Pathogenic	N	3 (pTi PO201‡, cryptic plasmids)	Poplar	Wabiko <i>et al.</i> (1989)
RE1	Non-pathogenic, Rif ^R Ery ^R	—	1 (pAtC58)		Wabiko <i>et al.</i> (1989)
RE1TS3	Pathogenic	N	2 (pAtC58, pTiPyTS3‡)		This study
RE1Cr140	Non-pathogenic	N	2 (pAtC58, pCr140‡)		This study
PO31Ag ^R	Non-pathogenic, resistant to Agr84	—	3 (cryptic plasmids)		This study
PO201Ag ^R	Non-pathogenic, resistant to Agr84	—	2 (cryptic plasmids)		This study
A208	Pathogenic	N	2 (pAtC58, pTiT37‡)		Sciaky <i>et al.</i> (1978)
A277	Pathogenic	O	2 (pAtC58, pTiB ₆ 806§)		Sciaky <i>et al.</i> (1978)
<i>A. radiobacter</i>					
K84	Non-pathogenic	—	2 (pAgK84 , cryptic plasmid)	Soil	Slota & Farrand (1982)

* Rif^R, rifampicin resistance; Ery^R, erythromycin resistance.

† N, nopaline type; O, octopine type.

‡ Nopaline utilization.

§ Octopine utilization.

|| Agrocin 84 producer.

fragment 2 (11 kb) of the nopaline-type Ti-plasmid pTiC58 (Depicker *et al.*, 1980). This 11 kb fragment contains *nocB* and *nocC* genes required for synthesis of the protein involved in nopaline uptake (termed n1 protein) and nopaline oxidase respectively (see Fig. 1 of Schardl & Kado, 1983). The T-DNA clone pT37P1 (provided by Dr Y. Machida, Nagoya University, Japan) was derived from pTiT37 (Wabiko *et al.*, 1989). Minimal media were AB (Chilton *et al.*, 1974), SMNO (Lichtenstein & Draper, 1985) and the mineral salt mixture of Petit & Tempe (1978). Nopaline and octopine were purchased from Sigma.

Bacterial conjugation and transformation. Cells of donor strains were mixed with cells of the recipient RE1 (Table 1) as described by Lichtenstein & Draper (1985), and transconjugants capable of utilizing nopaline were selected on SMNO plates containing 100 µg rifampicin ml⁻¹, 150 µg erythromycin ml⁻¹ and 100 µg nopaline ml⁻¹ (as a nitrogen source). Transformation of RE1 by plasmid DNA was done by the freeze-thaw method (Holsters *et al.*, 1978), and nopaline-utilizing transformants were selected on SMNO plates containing 100 µg nopaline ml⁻¹ as a nitrogen source.

Selection of derivatives resistant to agrocin 84 (Agr84). Bacterial cells were streaked close to a lawn of the Agr84-producing strain *A. radiobacter* K84 that had been grown at 29 °C for 2 d on AB medium (Unger *et al.*, 1985). Colonies surviving in the area where normal growth was inhibited were isolated and tested for Agr84 resistance in a similar manner. The selected colonies, regarded as resistant clones, were used for further analysis.

Test of opine and arginine utilization. Bacterial cells were streaked onto the mineral salt mixture supplemented with nopaline, octopine or arginine (each 10 mM) as a carbon source, and 1.8% (w/v) Noble agar (Difco) which had been washed with deionized water prior to use.

These media contained (NH₄)₂SO₄ (2 g l⁻¹) as a nitrogen source. The bacteria were incubated at 29 °C for 4–7 d.

Plasmid preparation and hybridization. To identify the number of plasmids, the rapid alkali/SDS lysis method (Kado & Liu, 1981) was employed. To isolate plasmid DNA, total DNA was briefly sheared, denatured with alkali, and the plasmid DNA was purified by CsCl/ethidium bromide density-gradient centrifugation as described by Currier & Nester (1976). Upon shearing, intact molecules of the large cryptic plasmid pAtC58 represented less than 1% of the plasmid preparation (determined by visual inspection of ethidium-bromide-stained purified DNA on agarose gel); this did not interfere with subsequent restriction enzyme digestion analysis or hybridization analysis under our experimental conditions.

To purify chromosomal DNA, the same procedure was followed except that the shearing and alkali denaturation steps were omitted. The purified DNA was digested with appropriate restriction enzymes and subjected to Southern hybridization analysis (Maniatis *et al.*, 1982). Hybridization was performed at 42 °C for 15 h in a solution consisting of 50 mM-Tris/HCl pH 7.5, 1 mM-EDTA, 0.5% SDS, 100 µg sonicated calf thymus DNA ml⁻¹, 20 µg *E. coli* tRNA ml⁻¹, 1 × Denhardt's solution (0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrrolidone), 3 × SSC (1 × SSC is 0.15 M-NaCl, 0.015 M-sodium citrate), 50% (v/v) formamide, 10% (w/v) dextran sulphate, and radioactive probe labelled by nick-translation (1–5 × 10⁵ c.p.m. ml⁻¹; 1–4 × 10⁷ c.p.m. µg⁻¹). Washing was carried out at high stringency in 0.1 × SSC, 0.1% SDS at 65 °C, or at low stringency in 2 × SSC, 0.1% SDS at 65 °C. The low-stringency washing conditions allowed about 24% more base-pair mismatch than the high-stringency conditions. Colony hybridization was performed with Gene Screen Plus filters (New England Nuclear) according to the manufacturer's instructions.

Tumorigenicity assay. Intact tomato stems were inoculated with 10^4 – 10^5 cells of *A. tumefaciens* using needles as described previously (Wabiko *et al.*, 1989).

Results

noc genes on a cryptic plasmid

A nopaline-type pathogenic strain, PyTS3, possessed four plasmids ranging in size from 140 to 350 kb. Of these, a 260 kb plasmid was established as the Ti-plasmid (pTiPyTS3) since it hybridized to a known T-DNA clone (Wabiko *et al.*, 1989). In order to obtain a strain carrying only pTiPyTS3, a mating experiment was performed using PyTS3 as donor and RE1 (Rif^R Ery^R) as recipient, and RE1 transconjugants capable of growing on nopaline (*noc*⁺) were selected. The frequency of transfer was 1 – 10×10^{-6} per recipient RE1; similar frequencies were observed when the donor was substituted by other pathogenic or non-pathogenic strains (data not shown). Preliminary screening showed that several *noc*⁺ transconjugants unexpectedly contained the 140 kb cryptic plasmid (pCr140) but did not contain pTiPyTS3 and did not induce tumours on tomato. We examined 459 *noc*⁺ transconjugants by colony hybridization using two different probes: a part of T-DNA (T37P1), which hybridizes only to pTiPyTS3, and pCr140, which hybridizes only to pCr140. Of the 459 *noc*⁺ transconjugants, 452 hybridized to pCr140, while only seven hybridized to T-DNA; the latter class possessed only the 260 kb pTiPyTS3. Thus pCr140 was transferred from PyTS3 to RE1 about 65 times (452/7) more efficiently than pTiPyTS3 in the presence of nopaline. These results indicated that in strain PyTS3, the *noc* genes resided both on the non-pathogenic cryptic plasmid, pCr140, and on the Ti-plasmid, pTiPyTS3.

In order to determine whether the occurrence of *noc* genes on cryptic plasmids is a general phenomenon, we examined two other pathogenic strains, PO31 and PO201, from poplar galls, which contained four and three plasmids, respectively. The *noc*⁺ transconjugants obtained from crosses between either of these strains and RE1 possessed only 200 kb Ti-plasmids. Furthermore, as most nopaline-type Ti-plasmids confer sensitivity to the antibiotic Agr84, two Agr84-resistant derivatives, PO31Ag^R and PO201Ag^R (Table 1), were isolated as a result of spontaneous curing of the Ti-plasmids from the sensitive parents PO31 and PO201 respectively. These isolates had lost only the Ti-plasmids and had also lost the capacity to grow on nopaline, indicating that in the case of PO31 and PO201, the *noc* genes resided exclusively on the Ti-plasmids. These results suggested that the cryptic plasmid encoding nopaline utilization existed specifically in PyTS3.

In strains containing nopaline-type Ti-plasmids, nopaline is cleaved to give arginine, and subsequent arginine catabolism is induced by nopaline (Ellis & Kerr, 1979). To see whether similar mechanisms exist in strains containing pCr140, the transconjugants RE1Cr140 (pCr140) and RE1TS3(pTiPyTS3), and one typical nopaline-type strain, A208(pTiT37), were examined for arginine utilization. As shown in Fig. 1(a–c), none of these strains grew on the medium containing only 10 mM-arginine, but all grew when the medium was also supplemented with 0.1 mM-nopaline. This result indicated that arginine utilization was also induced by nopaline in the cryptic plasmid, pCr140, and suggested that the regulation of *noc* gene expression in pCr140 is similar to that in Ti-plasmids.

Hybridization analysis of *noc* genes on pCr140

The structure and location of the *noc* genes of pCr140 and pTiPyTS3 were examined by Southern hybridization analysis. Plasmid DNA was digested with *Bam*HI and *Hind*III, and the restriction patterns were analysed by hybridization to a probe consisting of a part of the *noc* operon (containing the *nocB* and *nocC* genes) from the well-known Ti-plasmid pTiC58. High- and low-stringency washing conditions were employed so as to determine the extent of DNA sequence homology between pCr140 and pTiPyTS3.

Under high-stringency conditions, 1.7 kb *Bam*HI and 11 kb *Hind*III fragments of pCr140 hybridized with the probe (Fig. 2b). Under low-stringency conditions, however, additional *Bam*HI fragments of 3.8 and 1.3 kb became visible (Fig. 2c), indicating that these fragments are also part of the *noc* genes, but with low homology to the probe. In addition, the 1.7 kb *Bam*HI and the 11 kb *Hind*III fragments showed stronger hybridization intensities under low-stringency conditions (Fig. 2c), suggesting that these fragments are also only partially homologous to the probe. The total sum, 6.8 kb (3.8 + 1.7 + 1.3 kb), of the hybridized *Bam*HI fragments was less than that of the probe (11 kb), suggesting that some regions of the pCr140 *noc* genes contained unique DNA base sequences that were not homologous to the probe. In contrast to the results with pCr140, 11 kb and 10 kb *Bam*HI and 11 kb *Hind*III fragments of pTiPyTS3 showed strong hybridization to the probe under both high- and low-stringency conditions (Fig. 2b, c). Similarly, another well-studied Ti-plasmid, pTiT37, also showed strong hybridization signals to the probe under both washing conditions (Fig. 3). These results indicate that the *noc* genes of the cryptic plasmid pCr140 differ at least partly from those of Ti-plasmids, while the *noc* genes of the Ti-plasmids from different origins closely resemble each other.

Fig. 1. Growth of *A. tumefaciens* strains with arginine, nopaline or octopine as sole carbon source. The culture medium contained the mineral salt mixture (see Methods) supplemented with the compounds indicated in the figure. Bacterial strains are: PyTS3 (1), RE1Cr140 (2), RE1TS3 (3), RE1 (4, 7), A208 (5, 9), PyON8 (6) and A277 (8). The bacteria were incubated for 5 d at 29 °C.

noc genes in non-pathogenic strains

Previously, we isolated three non-pathogenic (to tomato, petunia, poplar, kalanchöe, apple and grape), nopaline-type *A. tumefaciens* strains from crown gall tissues of pear that occurred in Ten-no, Japan (Wabiko *et al.*, 1989). These strains contained either two (in PyTS4) or three (in PyTS1 and PyTS2) plasmids ranging from 140 to 350 kb in size, and the distribution of the plasmids was strain-specific. The *noc* genes were located on a 200 kb plasmid in all three strains, as recognized by hybridization to the *noc* probe, and by isolation of *noc*⁺ transconjugants which had acquired only the 200 kb plasmids, pPyTS1, pPyTS2 and pPyTS4 (Table 1). The *noc* gene probe hybridized to 7.4, 4.2 and 0.64 kb *Hind*III fragments of plasmid DNA from these strains, under both high- and low-stringency conditions (Fig. 3, lanes 6–8, and data not shown). The hybridization pattern was identical among these three strains, but was different from those of pCr140 and Ti-plasmids (Fig. 2, Fig. 3).

Possibly chromosomally located *noc* genes

We have isolated an anomalous strain, PyON8, from crown gall tissue of pear in Ohgata, Japan. This strain, containing only one 50 kb plasmid, did not induce tumours on tomato, petunia, poplar, kalanchöe, apple or grape, and utilized not only nopaline but also octopine and arginine as carbon sources (Fig. 1*d–g*). Growth on nopaline and arginine was more pronounced than that on octopine. In order to determine whether the *noc* genes were located on the 50 kb plasmid, we attempted to transfer this plasmid into RE1 and then to screen for the acquisition of the *Noc*⁺ phenotype. However, neither mating between PyON8 and RE1, nor direct transformation of RE1 with the 50 kb plasmid, produced any *Noc*⁺ strains. No hybridization of the *noc* probe either to the 50 kb plasmid or to chromosomal DNA was detected, even under low-stringency conditions and with prolonged exposure of X-ray film to the nitrocellulose filter (Fig. 3, lanes 5 and 9). These results indicate that PyON8

Fig. 2. Southern hybridization analysis of plasmids pTiPyTS3 and pCr140. Plasmids pCr140 and pTiPyTS3 were purified from RE1Cr140 and RE1TS3 respectively, and then 1 μ g DNA of pCr140 or 1.6 μ g DNA of pTiPyTS3 was digested with *Bam*HI or *Hind*III. The DNA fragments were separated by 0.8% agarose gel electrophoresis, and stained with ethidium bromide (1 μ g ml⁻¹) (a). The fragments were then transferred to nitrocellulose filters and subjected to hybridization with a radioactively labelled 11 kb *Hind*III fragment of pGV0339 (1.5 $\times 10^6$ c.p.m. per filter; specific activity 1.3 $\times 10^7$ c.p.m. μ g⁻¹). The filters were washed either under high-stringency (b) or low-stringency (c) conditions, and were then exposed to X-ray film for 24 h at -80°C . Ti and Cr refer to plasmids pTiPyTS3 and pCr140 respectively. The molecular sizes indicated on the right were determined from *Hind*III digests of phage λ DNA. Part of the restriction map of pTiC58, the fragment numbers (Depicker *et al.*, 1980; Holsters *et al.*, 1980), and the locations of T-DNA, *noc* genes and the probe used in this study, are also shown.

possesses structurally unique *noc* genes, and that these genes reside either on a non-conjugative plasmid, or possibly on the chromosome.

Discussion

This paper describes three different types of *noc*⁺ *A. tumefaciens* strains: (1) one strain (PyTS3) contained *noc* genes at two loci, one on the Ti-plasmid and the other on the non-Ti cryptic plasmid; (2) three strains (PyTS1, PyTS2, PyTS4) contained *noc* genes only on non-Ti plasmids; (3) one strain (PyON8) possibly contained *noc* genes either on a non-conjugative plasmid or on the chromosome. Structural similarities and differences of these genes were demonstrated. The nopaline-type Ti-plasmid, pTiPyTS3, and the non-Ti cryptic plasmid, pCr140, existed as separate replicons in strain PyTS3.

This is consistent with a previous report that some non-pathogenic *A. tumefaciens* strains possess plasmids that carry genes for nopaline or octopine utilization, and that these plasmids are compatible with Ti-plasmids coding for nopaline or octopine utilization (Hooykaas *et al.*, 1980).

It was reported by Petit *et al.* (1983) that a few pathogenic *A. rhizogenes* strains (closely related to *A. tumefaciens*) contained not only an agropine-utilizing Ri (root-inducing) plasmid but also another plasmid required for catabolism of other opines – mannopine, mannopinic acid and agropinic acid – in a single cell. Together with these results, our findings establish that the opine catabolism can exist independently of pathogenicity in *Agrobacterium* strains.

Conjugation experiments showed that pCr140 was preferentially transferred to another strain over the Ti-plasmid pTiPyTS3. This result implies that, in nature,

Fig. 3. Hybridization analysis of plasmids and chromosomal DNA. Plasmids and chromosomal DNA were prepared from the strains indicated, and were digested with *Hind*III. Lane 1, a 0.05 µg sample of the 11 kb *Hind*III fragment of pGV0339; lane 2, 0.5 µg plasmid DNA from A208; lane 3, 0.8 µg plasmid DNA from RE1TS3; lane 4, 0.5 µg plasmid DNA from RE1Cr140; lane 5, 0.5 µg plasmid DNA from PyON8; lane 6, 0.5 µg plasmid DNA from PyTS1; lane 7, 0.5 µg plasmid DNA from PyTS2; lane 8, 0.5 µg plasmid DNA from PyTS4; lane 9, 3 µg chromosomal DNA from PyON8. The probe was the same as described in the legend to Fig. 2. Radioactivities of the probe were 1.5×10^6 c.p.m. per filter for lanes 1–8, and 2×10^7 c.p.m. for lane 9. After hybridization, the sheets were washed under low-stringency conditions. Films were exposed at -80°C for 24 h (lanes 1–4, 6–8) or for 7 d (lanes 5 and 9).

pCr140 would spread to other strains more efficiently than pTiPyTS3, particularly in tumorous plant tissues, where nopaline promotes conjugal transfer. This efficient transfer system would also help to generate non-pathogenic *A. tumefaciens* strains which would be able to survive in crown galls.

The *noc* genes on the plasmids, pPyTS1, pPyTS2, and pPyTS4, from three avirulent strains hybridized strongly to the *noc* probe. Their hybridization patterns after restriction enzyme digestion were indistinguishable, suggesting that the *noc* genes of these three plasmids had the same origin. However, the patterns were quite distinct from those of the *noc* genes borne by the Ti-plasmid and cryptic plasmid pCr140. Thus pCr140 was apparently not the result of a transfer of one of the three

avirulent, nopaline-type plasmids to the virulent strain PyTS3, even though these strains originated from the same crown gall tumour. The restriction patterns of the *noc* genes from pCr140, pTiPyTS3 and pPyTS1 were all different, indicating that the structure of these genes was highly diverse. However, the hybridization analyses under low- and high-stringency washing conditions revealed that the *noc* genes of pTiPyTS3, pPyTS1, pPyTS2 and pPyTS4 were all closely related to the well-known probe of pTiC58 and that the *noc* genes of pCr140 were distantly related to the same probe.

The third type of strain, PyON8, was proposed to have *noc* genes in the bacterial chromosome, since *noc* genes were not identified on the 50 kb plasmid in this strain. Although the possibility remains that the 50 kb plasmid does possess these genes but that it could not be stably maintained in the RE1 recipient, or that the *noc* genes are located on a large plasmid which could not be isolated intact, our data are consistent with the observation that, in octopine-type strains, the genes for octopine degradation reside in the chromosome in addition to the plasmids (Montoya *et al.*, 1978). To our knowledge, there is no previous report suggesting that the *noc* genes are chromosomally located.

The multiple locations and structural diversity of the *noc* genes in the various *A. tumefaciens* isolates raise several questions. First, were *noc* genes from both pTiPyTS3 and pCr140 expressed in strain PyTS3, and if so, is there a dosage effect upon nopaline catabolism? The similar growth rate of strains PyTS3, RE1Cr140 and RE1TS3 on nopaline may be explained if we assume that the rate of nopaline degradation was also similar in these strains. Second, is there any similarity among the metabolic pathways encoded by these *noc* genes? In the case of pCr140 and pTiPyTS3, induction of catabolism of nopaline and arginine (a cleavage product of nopaline) appears to be under identical regulation. In the case of PyON8, however, a unique catabolic pathway may exist since its *noc* genes did not show any homology with the known *noc* genes. Third, how did diverse *noc* genes originate? Cointegration of nopaline- and octopine-type Ti-plasmids does occur in *A. tumefaciens* (Hooykaas *et al.*, 1980), and an agropine-type Ri-plasmid and a non-pathogenic mannopine type-plasmid form a cointegrate in *A. rhizogenes* (Petit *et al.*, 1983). An insertion sequence (IS66) has been discovered in several locations both on plasmids and on the chromosome of *A. tumefaciens* (Machida *et al.*, 1984). Such genetic rearrangements might account for different locations of the *noc* genes. Further, different structures of the *noc* genes may reflect the divergence of these genes during the evolution of *Agrobacterium*. The isolation and characterization of the *noc* operons from the strains described in this paper may well answer these questions.

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References

- AKIYOSHI, D. E., KLEE, H., AMASINO, R. M., NESTER, E. W. & GORDON, M. P. (1984). T-DNA of *Agrobacterium tumefaciens* encodes an enzyme of cytokinin biosynthesis. *Proceedings of the National Academy of Sciences of the United States of America* **81**, 5994–5998.
- BOMHOFF, G., KLAUWIJK, P. M., KESTER, H. C. M. & SCHILPEROORT, R. A. (1976). Octopine and nopaline synthesis and breakdown genetically controlled by a plasmid of *Agrobacterium tumefaciens*. *Molecular and General Genetics* **145**, 177–181.
- CHILTON, M.-D., CURRIER, T. C., FARRAND, S. L., BENDICH, A. J., GORDON, M. P. & NESTER, E. W. (1974). *Agrobacterium tumefaciens* DNA and PS8 bacteriophage DNA not detected in crown gall tumors. *Proceedings of the National Academy of Sciences of the United States of America* **71**, 3672–3676.
- CHILTON, M.-D., DRUMMOND, M. H., SCIACKY, D., MONTOYA, A. L., GORDON, M. P. & NESTER, E. W. (1977). Stable incorporation of plasmid DNA into higher plant cells. *Cell* **11**, 263–271.
- CURRIER, T. C. & NESTER, E. W. (1976). Isolation of covalently closed circular DNA of high molecular weight from bacteria. *Analytical Biochemistry* **76**, 431–441.
- DEPICKER, A., DE WILDE, M., DE VOS, G., DE VOS, R., VAN MONTAGU, M. & SCHELL, J. (1980). Molecular cloning of overlapping segments of the nopaline Ti-plasmid pTiC58 as a means to restriction endonuclease mapping. *Plasmid* **3**, 193–211.
- ELLIS, J. G. & KERR, A. (1979). Arginine catabolism: a new function of both octopine and nopaline Ti-plasmids of *Agrobacterium*. *Molecular and General Genetics* **173**, 263–269.
- ELLIS, J. G., KERR, A., PETIT, A. & TEMPE, J. (1982). Conjugal transfer of nopaline and agropine Ti plasmids – the role of agrocinopines. *Molecular and General Genetics* **186**, 269–274.
- HOLSTERS, M., DE WAELE, D., DEPICKER, A., MESSENS, E., VAN MONTAGU, M. & SCHELL, J. (1978). Transfection and transformation of *Agrobacterium tumefaciens*. *Molecular and General Genetics* **163**, 181–187.
- HOLSTERS, M., SILVA, B., VAN VLIET, F., GENETELLO, C., DE BLOCK, M., DHAESE, P., DEPICKER, A., INZE, D., ENGLER, G., VILLAROEL, R., VAN MONTAGU, M. & SCHELL, J. (1980). The functional organization of the nopaline *A. tumefaciens* plasmid pTiC58. *Plasmid* **3**, 212–230.
- HOYKAAS, P. J. J., DEN DULK-RAS, H., OOMS, G. & SCHILPEROORT, R. A. (1980). Interactions between octopine and nopaline plasmids in *Agrobacterium tumefaciens*. *Journal of Bacteriology* **143**, 1295–1306.
- KADO, C. I. & LIU, S.-T. (1981). Rapid procedure for detection and isolation of large and small plasmids. *Journal of Bacteriology* **145**, 1365–1373.
- KLAUWIJK, P. M., SCHEULDERMAN, T. & SCHILPEROORT, R. A. (1978). Coordinated regulation of octopine degradation and conjugative transfer of Ti plasmids in *Agrobacterium tumefaciens*: evidence for a common regulatory gene and separate operons. *Journal of Bacteriology* **136**, 775–785.
- LICHTENSTEIN, C. & DRAPER, J. (1985). Genetic engineering of plants. In *DNA Cloning*, vol. 2, pp. 67–119. Edited by D. M. Glover. Washington, DC: IRL Press.
- MACHIDA, Y., SAKURAI, M., KIYOKAWA, S., UBASAWA, A., SUZUKI, Y. & IKEDA, J. (1984). Nucleotide sequence of the insertion sequence found in the T-DNA region of mutant Ti plasmid pTiA66 and distribution of its homologues in octopine Ti plasmid. *Proceedings of the National Academy of Sciences of the United States of America* **81**, 7495–7499.
- MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- MERLO, D. J. & NESTER, E. W. (1977). Plasmids in avirulent strains of *Agrobacterium*. *Journal of Bacteriology* **129**, 76–80.
- MONTOYA, A. L., MOORE, L. W., GORDON, M. P. & NESTER, E. W. (1978). Multiple genes coding for octopine-degrading enzymes in *Agrobacterium*. *Journal of Bacteriology* **136**, 909–915.
- NESTER, E. W., GORDON, M. P., AMASINO, R. M. & YANOFSKY, M. F. (1983). Crown gall: a molecular and physiological analysis. *Annual Review of Plant Physiology* **35**, 387–413.
- PETIT, A. & TEMPE, J. (1978). Isolation of *Agrobacterium* Ti-plasmid regulatory mutants. *Molecular and General Genetics* **167**, 147–155.
- PETIT, A., DAVID, C., DAHL, G. A., ELLIS, J. G. & GUYON, P. (1983). Further extension of the opine concept: plasmids in *Agrobacterium rhizogenes* cooperate for opine degradation. *Molecular and General Genetics* **190**, 204–214.
- SCHARDL, C. L. & KADO, C. I. (1983). A functional map of the nopaline catabolism genes on the Ti-plasmid of *Agrobacterium tumefaciens* C58. *Molecular and General Genetics* **191**, 10–16.
- SCIACKY, D., MONTOYA, A. L. & CHILTON, M.-D. (1978). Fingerprints of *Agrobacterium* Ti plasmids. *Plasmid* **1**, 238–253.
- SLOTA, J. E. & FARRAND, S. K. (1982). Genetic isolation and physical characterization of pAgK84, the plasmid responsible for agrocin 84 production. *Plasmid* **8**, 175–186.
- THOMASHOW, L. S., REEVES, S. & THOMASHOW, M. F. (1984). Crown gall oncogenesis: evidence that a T-DNA gene from the *Agrobacterium* Ti plasmid pTiA6 encodes an enzyme that catalyses synthesis of indoleacetic acid. *Proceedings of the National Academy of Sciences of the United States of America* **81**, 5071–5075.
- UNGER, L., ZIEGLER, S. F., HUFFMAN, G. A., KNAUF, V. C., PEET, R., MOORE, L. W., GORDON, M. P. & NESTER, E. W. (1985). New class of limited-host-range *Agrobacterium* mega-tumor-inducing plasmids lacking homology to the transferred DNA of a wide-host-range, tumor-inducing plasmid. *Journal of Bacteriology* **164**, 723–730.
- VAN LAREBEKE, N., ENGLER, G., HOLSTERS, M., VAN DEN ELSACKER, S., ZAENEN, I., SHILPERPOORT, R. A. & SCHELL, J. (1974). Large plasmid in *Agrobacterium tumefaciens* is essential for crown gall-inducing ability. *Nature, London* **252**, 169–170.
- WABIKO, H., KAGAYA, M., KODAMA, I., MASUDA, K., KODAMA, Y., YAMAMOTO, H., SHIBANO, Y. & SANO, H. (1989). Isolation and characterization of diverse nopaline type Ti plasmids of *Agrobacterium tumefaciens* from Japan. *Archives of Microbiology* **152**, 119–124.