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Studies on the structure of cointegrates between octopine and nopaline Ti-plasmids and their tumour-inducing properties

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Abstract. Stable cointegrates between *inc*Rh-1 octopine (Ach5) and nopaline (C58) Ti-plasmids, present in ten independently isolated *Agrobacterium tumefaciens* strains, showed identical restriction endonuclease patterns. Each cointegration event had taken place in the common sequence between the T-regions of both Ti-plasmids. This illustrates a high preference for this region when used in the formation of cointegrates. Four crown gall tissues, obtained after transformation of *Nicotiana tabacum* cells by one of the mutants, were analysed by using Southern blot analysis for their T-DNA structure. The borders of T-DNA frequently appeared to differ from T-DNA borders previously detected in tumour tissues that had been induced by *Agrobacterium* strain C58 or Ach5. Therefore, it was concluded that possibly a less stringent mechanism exists for the integration into plant DNA of T-DNA, derived from a composite (octopine/nopaline) T-region than for integration of T-DNA from a normal (octopine or nopaline) T-region.

Introduction

Plasmid incompatibility has been defined as the inability of two plasmids to coexist in the same host bacterium in the absence of selection pressure [18, 19]. Generally, incompatible plasmids are functionally similar and have large regions of considerable DNA homology [9]. Octopine and nopaline tumor-inducing (Ti) plasmids in *Agrobacterium tumefaciens* have extensive regions of DNA homology [2, 7] and are functionally similar in that they both confer the ability to induce crown galls on dicotyledonous plants to their host strain [27, 28]. Indeed, these Ti-plasmids are incompatible with each other and have been classified as *inc*Rh-1 type plasmids [12]. It was shown, however, that *Agrobacterium* strains, that carried Ti-plasmid functions coded for by both Ti-plasmids could be isolated with a low frequency. In such

Abbreviations

Agr, sensitivity to agrocin 84,
Ape, phage Apl exclusion,
Cb, resistance to carbenicillin,
Occ, octopine catabolism,
Ocs, octopine synthesis,
Noc, nopaline catabolism,
Nos, nopaline synthesis,
Rec, recombination,
Tra, transfer,
Vir, virulence.

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bacteria the Ti-plasmids had apparently formed stable cointegrates [12]. Investigations of the structure of one of these cointegrates showed that the cointegration event had taken place in one of the regions of high DNA homology. This specific region of homology is essential for tumour development and is transferred into plant cells upon tumour induction [3, 15, 26]. The transferred plasmid fragment has been called the T-region, if part of a Ti-plasmid, and T-DNA, when integrated in the nuclear genome of crown gall cells [4, 29]. The sequence homology between the T-regions in octopine and nopaline Ti-plasmids has been called 'the common sequence'. Normally, both in octopine and nopaline Ti-plasmids the confines of the T-regions are located within well defined Ti-plasmid restriction endonuclease fragments [5, 15, 22, 26]. This suggests that specific nucleotide sequences are preferred for the integration of T-DNA in the plant genome. DNA sequence analysis of T-DNA borders in an octopine and a nopaline tumour tissue showed that inverted and direct repeats are present at the confines of the T-DNA, indicating that indeed specific primary and/or secondary structures might be involved in the integration event [24, 32]. In the present study we show that the cointegration event for independently isolated cointegrates had taken place repeatedly in the 'common sequence'. We localised the position of the integration event more precisely within this area, using a cointegrate Ti-plasmid deleted for one of its composite T-regions that was analysed with various restriction endonucleases. Furthermore, it was shown by Southern hybridisation of DNA restriction patterns from four tobacco crown gall tissues that segments of the cointegrate Ti-plasmids were present in the plant genome, but that the borders of the transferred composite (octopine/nopaline) segments frequently differed from the borders of normal (octopine or nopaline) T-DNA.

Materials and Methods

Isolation of agrobacteria with cointegrated Ti-plasmids

Following procedures summarised and described in a previous study [12], cointegrates were obtained that originated from octopine Ti-plasmid pAL657 (Cb^R, Ape^+, Occ^+, Ocs^+, Vir^+) and nopaline Ti-plasmid pTiC58 (Ape^+, Noc^+, Nos^+, Vir^+, Agr^+). In short, the octopine Ti-plasmid pAL657 was introduced via bacterial conjugation into a pTiC58 carrying *Agrobacterium* strain LBA298 and transconjugants were selected on medium containing carbenicillin (75 mg/liter). Transconjugants were screened for their ability to degrade nopaline and octopine. Presumptive cointegrate plasmids were checked for co-transfer of octopine- and nopaline. Ti-plasmid coded markers in further crosses. Strains that showed co-transfer of all markers were used in subsequent DNA restriction enzyme analyses.

DNA hybridisations

Total Agrobacterium DNA was isolated from 5 ml cultures of bacteria grown overnight at 29 °C (250 rpm) in TY medium (5 gl^{-1} tryptone and 3 gl^{-1} yeast extract). The DNA was subjected to Southern blot analysis following techniques described previously [20] and autoradiography was performed at −70 °C, −20 °C or room temperature using Ilford intensifying screens and Kodak XRI films. Plant DNA was screened for the presence of T-DNA under identical experimental conditions except that 14 μg of plant DNA was used per gel lane and this DNA was isolated from 3–5 g lyophilised tobacco crown gall tissue [3].

Bacterial strains and tumor tissues

From strain LBA4057, carrying the cointegrate plasmid pAL672, a derivative had been isolated, LBA4080, which carried cointegrate plasmid pAL4080, that is missing genes for octopine synthesis (Ocs) and sensitivity to agrocine 84 (Agr) as a result of a large deletion [12]. LBA4057 has been used to transform tobacco protoplasts (*Nicotiana tabacum* var. Petit Havana) by means of co-cultivation [30]. From this study and from a later performed identical co-cultivation experiment a number of tissues were recovered that grew in the absence of phytohormones under axenic conditions. A limited number of the tissues produced both octopine and nopaline implying that the T-DNA-coded enzymes lysopine dehydrogenase and nopaline dehydrogenase were present. Three of the octopine positive/nopaline positive tissues were selected for further analysis in the present study: SR_1-4057-11, SR_1-4057-7 and SR_1-4057-9. A fourth crown gall tissue, $SR_1$4057, was induced by infecting stems of *N. tabacum* var. Petit Havana plants with LBA4057. Axenic tissue from this tumour was obtained after excision of the tumour tissue from the plant two months after induction. Subsequent growth in vitro was on L + S medium [16] without hormones, but initially supplemented with 250 μg/ml carbenicillin.

Results

Agrobacteria carrying cointegrated Ti-plasmids

From ten independent conjugation experiments ten mutant strains of *Agrobacterium* were isolated that carried, on the basis of genetic evidence, cointegrates between octopine and nopaline Ti-plasmids. When these strains were used as donors in further crosses they showed 100% cotransfer of octopine and nopaline Ti-plasmid coded markers. That the plasmids were cointegrated was further substantiated when total lysates from both parental strains and strains carrying presumptive cointegrates were analysed by gel electrophoresis according to Casse et al. [1] Lysates of strains carrying presumed cointegrates

lacked bands of DNA which comigrated with pAL657 or pTiC58, although such bands were present in lysates of the corresponding parental strains (results not shown). Total DNA was isolated from the ten mutants and samples of the DNA preparations were treated with each of the restriction endonucleases *Bam*HI, *Sma*I, *Hpa*I, *Kpn*I, *Sal*I, *Pst*I, and *Hind*III. Southern blots of the digests were hybridised with ^{32}P-labelled plasmid pAL3252 DNA (which contains *Bam*HI fragment 8 of pTiAch5 and which shares DNA homology with the T-region of the nopaline Ti-plasmid pTiC58; see also Figure 1). Some of the results obtained after autoradiography of the Southern blots are illustrated in Figure 2. It appeared that for all but *Kpn*I digests, the hybridisation patterns are a summation of patterns obtained for an octopine- and a nopaline Ti-plasmid plus an additional band (see e.g. Figure 2B, lanes, 1, 2 and 3 representing *Bam*HI digests and Figure 2C, lanes 1, 2 and 3 representing *Sma*I digests; digests for other restriction endonucleases used are not shown). These additional bands resulted from hybridisations between pBR322 and Tn*1* inserted in the octopine Ti-plasmid (pAL657) used in this study (results not shown). Presumably the observed additional hybridisation is a result of homology between the β-lactamase genes coded for by both Tn*1* and pBR322. It is noted that the Tn*1* insertion was not in or close to the T-region of the octopine Ti plasmid and therefore did not affect the migration properties of any of the T-region restriction endonuclease fragments. All ten cointegrate carrying strains gave rise to exactly the same hybridisation patterns as is illustrated for LBA 4057 in Figure 2A, 2B, and 2C, lanes 3 (results for other DNA preparations are not shown). As *Kpn*I digests also gave identical patterns, which were not a summation of patterns for octopine and nopaline T-regions, the cointegrate plasmids in the newly isolated strains most likely evolved via the same mechanism resulting in identical structures.

Localisation of the cointegration event

To define more precisely the location of the position of the cointegration, restriction endonuclease digests of total DNA from strain LBA 4080

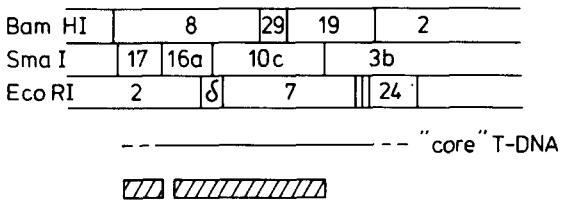

Figure 1. Restriction endonuclease map of the octopine Ti-plasmid T-region. The recognition sites for restriction endonucleases *Hpa*I, *Kpn*I, *Bam*HI, *Sma*I and *Eco*RI are shown. Below the map, the extent of 'core' T-DNA usually detected in octopine crown gall tissues, is indicated by a solid line, whereas the hatched areas refer to regions with homology with the nopaline Ti-plasmid pTiC58.

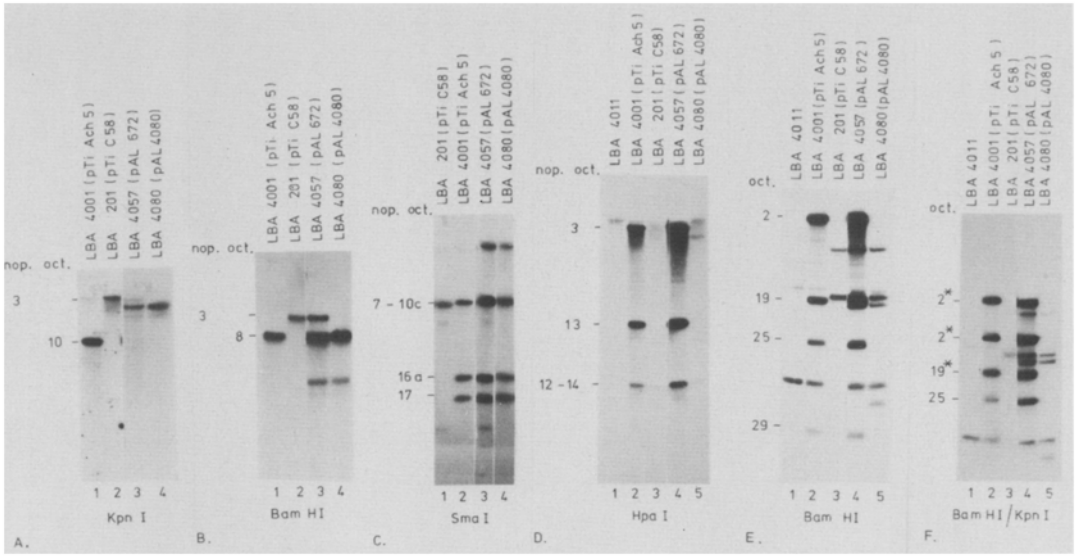

Figure 2. Autoradiograms of Southern-type blots carrying total *A. tumefaciens* DNA, that were hybridised with ^{32}P-labelled octopine Ti-plasmid T-region DNA. Total *A. tumefaciens* DNA was digested with *Kpn*I (A), *Bam*HI (B), *Sma*I (C), *Hpa*I (D), *Bam*HI (E) or with both *Bam*HI and *Kpn*I (F). Only bands corresponding with the most relevant Ti-plasmid fragments are numbered. The indications 2* and 19* in Fig. F refer to *Bam*HI fragments that have been cleaved by *Kpn*I as well. As a ^{32}P-labelled probe, either octopine Ti-plasmid fragment *Bam*HI 8, cloned in pBR322, has been used (A, B and C) or octopine Ti-plasmid *Bam*HI fragment 29, 19, 2 and 25 cloned in pBR322 (D, E and F).

(pAL4080) were analysed. Plasmid pAL4080 derives directly from pAL672 and differs from pAL672 in that one of the two composite T-regions present in pAL672 is deleted in pAL4080 [12]. Following the same procedure as described above, autoradiograms were obtained representing the T-region patterns for pAL4080 as they became evident upon hybridisation with pAL3252 (Figure 2A, 2B, and 2C lane 4). From these, it can be concluded that octopine Ti-plasmid fragments *Bam*HI 8 (Figure 2B) and *Sma*I 16a and 17 (Figure 2C) are still present, whereas nopaline Ti-plasmid fragment *Bam*HI 3 was missing (Figure 2B). From the three bands detected in a *Kpn*I digest, the upper (faint) band was absent on the autoradiogram obtained for LBA4080 DNA. As it was known that the lower (faint) band was due to the above mentioned additional hybridisation between Tn*1* and pBR322 (not shown), the most predominant band most likely represented a fusion fragment between the octopine and nopaline Ti-plasmid with a large extent of homology with octopine Ti-plasmid fragment *Bam*HI 8. Therefore this fusion fragment presumably contains a significant part of the overlap between octopine Ti-plasmid fragment *Kpn*I 10 and octopine Ti-plasmid fragment *Bam*HI 8 (see Figure 1). The location of the region involved in cointegration was determined more accurately when plasmid pAL3076, carrying *Bam*HI fragments 29, 19, 2 and 25 of the octopine Ti-plasmid [20], was used as a ^{32}P-labelled probe in the hybridisation experiments. As can be seen from Figure 2D, 2E and 2F, which shows autoradiograms of hybridisations between pAL3076 DNA and Southern blots of *Hpa*I, *Bam*HI and *Bam*HI/*Kpn*I double digested total *A. tumefaciens* DNA preparations, it is clear that most octopine Ti-plasmid restriction endonuclease fragments, that show homology with pAL3076, are absent in plasmid pAL4080. Among these missing fragments are octopine Ti-plasmid fragments *Hpa*I 13 and 14 (Figure 2D) and *Bam*HI fragments 25, 2, 19 and 29 (Figure 2E and 2F). *Bam*HI/*Kpn*I double digests were carried out to obtain a more unambiguous view of octopine Ti-plasmid fragment *Bam*HI 19 in pAL672 and pAL4080, since the presence or absence of this fragment was obscured by other fragments with similar mobility that hybridised with pAL3076 as well. It was noted that pAL3076 showed homology with total *Agrobacterium* DNA of a cured derivative of wild type strain Ach5 (i.e. LBA4011), which is in agreement with previous observations [21]. From the data described above and published restriction endonuclease maps of the octopine and nopaline Ti-plasmid T-regions [6, 21], a restriction endonuclease map was constructed for the cointegrate plasmid pAL672 (Figure 3). The region involved in the cointegration event is indicated on the map. In plasmid pAL4080 the entire lower composite T-region was absent.

T-DNA analysis

As the integration of T-DNA in the plant genome, in both octopine and nopaline crown gall tissues, usually takes place within specific Ti-plasmid restriction endonuclease fragments [5, 15, 22, 26], the mechanisms of

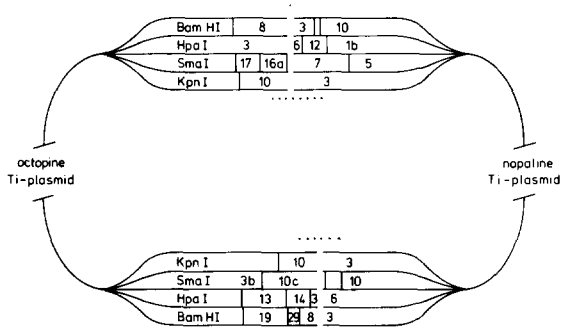

Figure 3. Restriction endonuclease map of the composite T-regions in a Ti-plasmid in which an octopine- and nopaline Ti-plasmid are cointegrated. The integration positions are indicated by interruptions on the map: the exact position of the integration event lies within regions of high DNA homology between octopine and nopaline Ti-plasmids as are indicated by dotted lines. The numbering is taken from previously published maps for the individual Ti-plasmids (see text).

integration most likely involves specific Ti-plasmid nucleotide sequences. However, transformation of protoplasts by co-cultivation with a strain carrying a cointegrate plasmid often results in tissues that do not show both octopine and nopaline synthesis and therefore, most likely, lack the structural genes for one or both of the corresponding enzymes [30]. This might be the result of integrations in which the normally preferred Ti-plasmid sequences have not been used. On the other hand, in tissues that show both octopine and nopaline synthesis it is possible that the borders of one of the T-DNA's are normally present (e.g. for the nopaline T-DNA) but that in a (nopaline) T-DNA an entire (octopine) Ti-plasmid (or vice versa) is integrated. This would be similar to what has been observed for transposons Tn*7* [9], Th*5* [8] and Tn*904* [22], insertion sequence IS*60* [20] or R702 fragments [14], all of which were shown or inferred to be co-transferred into the plant genome as part of T-DNA. All four tumour tissues selected for analysis in the present study, showed both octopine and nopaline synthesis, and consequently it was assumed that the structural genes for the corresponding enzymes were present as well. Therefore, at least these parts of the two T-regions shown in Figure 3 were expected to have been transferred into the plant cell. The locus for the enzyme lysopine dehydrogenase (LpDH) catalysing the synthesis of octopine synthesis, is known to be situated at the extreme left end of *Bam*HI fragment 19 of the octopine Ti-plasmid present in the lower T-region [8, 13, 21, 22], whereas the locus for nopaline dehydrogenase (NpDH) is situated at the extreme left of *Sma*I fragment 5 of the nopaline Ti-plasmid in the upper T-region [11].

Using a mixture of ^{32}P-labelled plasmids pAL3252 and pAL3076, Southern blots of *Eco*RI and *Sma*I digests of crown gall DNA isolated from SR_1 4057-4, SR_1-4057-9, SR_1-4057-11 and SR_1-4057, were screened (Figure 4A, lanes 5, 6,7 and 8; Figure 4B, lanes 2, 3, 4 and 5). From these hybridisations it can

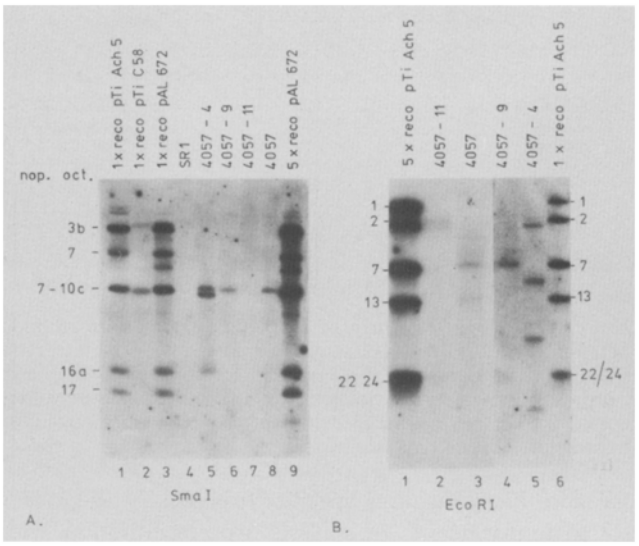

Figure 4. T-DNA banding patterns of tobacco crown gall tissues transformed with *A. tumefaciens* strain LBA4057. The transformed tissues are indicated by 4057, 4057-4, 4057-9 and 4057-11. As control experiments hybridisations with untransformed tobacco tissue (SR1; Fig. A, lane 4) and reconstruction experiments with purified Ti-plasmid DNA (Fig. A. lane 1, 2, 3, 9; Fig. B, land 1 and 6) were used. As a ^{32}P-labelled probe a 1:1 mixture was used of plasmids pAL3252 and pAL3076, both of which carry T-region derived restriction endonuclease fragments from octopine Ti-plasmid pTiAch5.

be concluded that *Sma*I fragment 16a was present only in SR_1-4057-7 whereas *Sma*I fragment 17 was absent in this tissue. This suggests that one of the T-DNA borders in tissue 4057-4 only, is located within *Sma*I fragment 17. In tissues SR_1-4057-9 and SR_1-4057, fragments co-migrating with octopine Ti-plasmid fragment *Sma*I 1OC and nopaline Ti-plasmid fragment *Sma*I 7, were present. These fragments were regarded as both or only one of the two composite fragments in which *Sma*I fragments 1OC (octopine) and 7 (nopaline) are joined together (see above). None of these fragments were detected in SR_1-4057-11 DNA although in this tissue some T-DNA could be detected, with the probes used, in an *Eco*RI digest (Figure 4B lane 2). *Eco*RI digests for the three other crown gall tissues showed the presence of a fragment comigrating with *Eco*RI fragment 7 of the octopine Ti-plasmid in tissues SR_1-4057 and SR_1 4057-9. In tissue SR_1-4057-4, T-DNA was clearly present, but none of the bands appeared at the position of octopine Ti-plasmid *Eco*RI fragment 7. Although these results do not allow the construction of a detailed physical map of the T-DNA organization in the various crown gall tissues, they do give answers to the questions we posed. The presence of octopine Ti-plasmid *Sma*I fragment 16a in combination with the absence of *Sma*I fragment 17 in only one out of four tissues, shows that octopine

Ti-plasmid sequences present in SmaI fragment 17, that are presumably preferred for integration, have not been used frequently. Furthermore, the absence of Ti-plasmid fragments normally not detected in crown gall tissue, but visible in reconstruction experiments in which a digest of the entire Ti-plasmid was used (Figure 4A, lanes 1, 2, 3 and 9; Figure 4B, lanes 1 and 6), shows that an entire Ti-plasmid had not been co-transferred as part of the T-region of another Ti-plasmid.

Discussion

Octopine and nopaline Ti-plasmids share various regions of weak or strong homology. However, it appears that a cointegration between both types of Ti-plasmids normally takes place exclusively within only one of these regions, the mutual T-regions. This event seems very specific, since all ten, independent cointegrates isolated under identical conditions showed identical restriction endonuclease patterns. Therefore, it seems likely that the mechanism by which these cointegrates arise is highly specific. It is noted that if cointegrates with a different structure would have been formed that were Tra^-, our isolation procedure would have selected against bacteria with such plasmids and they would have escaped analysis. Furthermore, it remains to be investigated whether in a Rec^- *Agrobacterium* strain identical cointegrate structures are formed as those described in this study.

The integration of Ti-plasmid DNA from cointegrated Ti-plasmids into plant DNA does not appear to be very precise. In three out of four tissues analysed, at least one of the T-DNA borders normally present in octopine crown gall tissue, was absent. This indicates that specific Ti-plasmid sequences, presumably frequently involved in integration of octopine T-DNA in the plant genome, are not involved as frequently in the integration of composite octopine/nopaline T-DNA. The results show that in tissues with LpDH and NpDH activities, at least two separate Ti-plasmid derived fragments are present in the plant genome and apparently not an entire (octopine) Ti-plasmid is transferred as part of a (nopaline) T-region (or vice versa). Among the possible explanations of the results observed is that a left-hand octopine T-region in combination with a right-hand nopaline T-region is not as efficient in promoting integration of T-DNA into plant DNA than a normal octopine or nopaline T-region itself. Such an explanation would agree with previous results suggesting that direct and/or indirect repeats, with unique sequences are involved in the integration mechanism of T-DNA in plant DNA [24, 32]. It can be postulated that end sequences between the T-region in octopine and nopaline Ti-plasmids are not interchangeable without a reduction in, or loss of, specificity by which defined Ti-plasmid sequences are involved in the integration of T-DNA in the plant genome. This apparent lowered specificity for integration is not associated with a reduction in virulence because infection of wounded plants with the cointegrate carrying *Agrobacterium*

strain LBA 4057 causes morphologically normal tumour development. The present results do not contradict the conclusion, reached in a previous study [22], that transformation after co-cultivation, or after plant infection, results in transformed cells with similar T-DNA organisations: in the present study the one crown gall tissue obtained after plant infection has an abnormal left hand T-DNA border that was derived from an octopine Ti-plasmid and so do two out of the three tissues obtained after co-cultivation experiments.

The demonstration that two Ti-plasmid derived T-DNA's of different origin exist in a single plant cell, has implications for introducing specified genetic information into plants. As it appears possible to construct Ti-plasmids with two functionally different T-regions then one could expect that in a number of transformed cells both corresponding T-DNA's may have become integrated in the plant genome. Independent of this observation it has been demonstrated that T-DNA may be lost upon meiosis, presumably due to deletion, from transformed plants that are recovered from crown galls induced by *Agrobacterium* strain T37 [15, 31]. The parental plants lack a root system, whereas the offspring produces a normal root system.

Therefore, if plants are recovered with two T-DNA's, one of which has had the region responsible for hormonal disturbances inactivated or deleted but containing desired additional genetic information, whilst the second T-DNA was similar to that found in tumourous plants without roots, then it may be possible via this route to isolate normal plants that carry the first T-DNA fragment only, therefore without hormonal disturbances, but with introduced foreign DNA.

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