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The conserved part of the T-region in Ti-plasmids expresses four proteins in bacteria

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The T-region of Ti-plasmids expresses four proteins (mol. wts. 74 000, 49 000, 28 000 and 27 000) in *Escherichia coli* minicells. Promoter activities are determined by sequences within the T-region, and the protein-coding regions map in that part of the T-region which is highly conserved in octopine and nopaline plasmids and which is responsible for shoot and root inhibition when expressed in plant cells. Three of the regions expressed in bacteria correlate with three regions which are transcribed in transformed plant cells; the fourth protein-coding region has no corresponding transcript in plants. At least three of the proteins synthesized in *E. coli* minicells are also expressed in cell-free systems prepared from *E. coli* and from *Agrobacterium tumefaciens*; the fourth protein (mol. wt. 49 000) is poorly expressed in both cell-free extracts. The possibility is discussed that the same genes are expressed in Agrobacteria and in transformed plant cells and that in both cases the gene products mediate growth regulatory effects to non-transformed plant cells.

Key words: Agrobacteria/crown gall/plant hormones/plant tumorigenesis/T-region

Introduction

The gram-negative soil bacterium *Agrobacterium tumefaciens* induces neoplastic growths called crown gall tumors on many dicotyledonous plants (Smith and Townsend, 1907; De Cleene and De Ley, 1976). Tumor formation is accompanied by transfer and integration of a specific part of the tumor-inducing (Ti) plasmid (Zaenen *et al.*, 1974) into the nuclei of the host cells (Chilton *et al.*, 1977; Schell *et al.*, 1979; Lemmers *et al.*, 1980; Thomashow *et al.*, 1980; Willmitzer *et al.*, 1980; Zambryski *et al.*, 1980). This part of the Ti-plasmid is termed the T-region in the bacteria and T-DNA in the plant cells. It controls, in plant cells, the formation of various compounds called opines which are metabolized by the bacteria. The opines produced define crown galls as octopine, nopaline or agropine type tumors (Guyon *et al.*, 1980). In octopine tumors it has been shown that the octopine-synthesizing enzyme is a gene product of the T-DNA (Schröder *et al.*, 1981a, 1981b; Murai and Kemp, 1982a; Schröder and Schröder, 1982).

The T-DNA is also responsible for undifferentiated, hormone-independent growth of the transformed plant cells. Experiments with Agrobacteria containing Ti-plasmids mutagenized in the T-region indicate that a set of genes rather than a single locus is responsible for the complex tumorous growth pattern, and these DNA regions are highly conserved in octopine and nopaline plasmids (Chilton *et al.*, 1978; Depicker *et al.*, 1978; Engler *et al.*, 1981). Two main actions in plant cells may be distinguished (Leemans *et al.*, 1982; Joos *et al.*, 1983): some of the genes suppress differentiation only in the cells in which they reside, while other genes suppress differentiation in both transformed and untransformed cells. The actions of those genes resemble the effects of supplying normal cells with suitable combinations of the growth hormones, auxin and cytokinins (Skoog and Miller, 1957; Ooms *et al.*, 1981), and thus they can be formally defined as genes exerting hormone-like effects on plant cells. The protein gene products and their roles in the control of hormone-independent growth and differentiation are not known. Therefore, there is no functional assay which could be conveniently used to isolate and purify these proteins, and attempts to identify them by translation *in vitro* of T-DNA derived mRNAs were of limited success (McPherson *et al.*, 1980; Murai and Kemp, 1982a; Schröder and Schröder, 1982).

To gain access to T-DNA encoded peptides we investigated whether genes from this part of the T-region could be expressed as protein in bacterial systems, either after fusion with bacterial genes or with promoter activities from the T-region. Here we report that the part of the T-region responsible for hormone-independent growth of plant cells contains a set of genes which can be expressed as protein in *Escherichia coli* and in a cell-free system from *A. tumefaciens*. Transcription appears to start within the T-region, and the expression is selective since other genes are not expressed at detectable levels. The possibility is discussed that these proteins are functional in the bacteria and that they participate in mediating hormone effects in plant cells in the early stages of tumor induction.

Results

Mol. wt. 49 000 protein

Plasmid pBR322 containing Ach5 *Hind*III fragments (14-18-22-38) (see Figure 1 and Table I for identification) expressed a protein of mol. wt. 49 000 in *E. coli* minicells (Figure 2, lane 2) which was also produced with Ach5 *Bam*HI

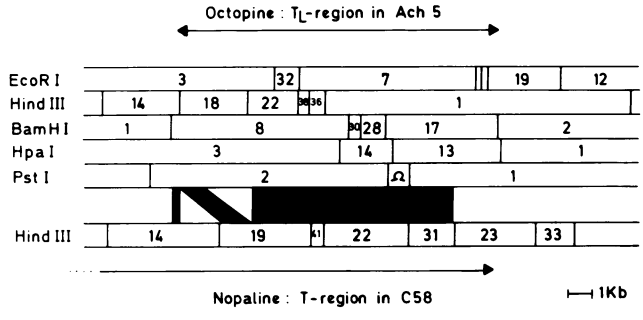

Fig. 1. Restriction map of the T_L-region in octopine plasmid pTi Ach5 **(top)** and *Hind*III map of part of the T-region in nopaline plasmid pTi C58 **(bottom)**. The black areas between the maps define the regions which are highly conserved in octopine and nopaline plasmids. The lines with an arrow indicate the extent of the T-DNA found most often in transformed plant cells.

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Table I. List of fragments and recombinant plasmids

Ti-plasmid fragment	Name of recombinant plasmid	Vector plasmid	Reference
Ach5 *Hind*III (14-18-22-38)	pGV0219	pBR322	De Vos *et al.*, 1981
Ach5 *Bam*HI-8	pGV0153	pBR322	De Vos *et al.*, 1981
Ach5 *Hind*III-1	pGV0201	pBR322	De Vos *et al.*, 1981
Ach5 *Hind*III-22	pHD106.*1* pHD106.*2*	pUR51	This work
Ach5 *Eco*RI-7	pWK500.*1* pWK500.*2*	pACYC184	Schröder *et al.*, 1981b
Ach5 *Eco*RI-7 (Δ*Pst*IΩ)	pWK500.*2Pst*I	pACYC184	This work
Ach5 *Eco*RI-7 (Δ*Hpa*I-14)	pWK500.*2Hpa*I	pACYC184	This work
C58 *Hind*III (15-14-19-41-22)	pGV0354	pBR322	Depicker *et al.*, 1980
C58 *Hind*III-22 (*Eco*RI linkers)	pGC101.*1* pGC101.*2*	pACYC184	This work

Detailed maps of octopine plasmid pTi Ach5 (De Vos *et al.*, 1981) and nopaline plasmid pTi C58 (Depicker *et al.*, 1980) have been published; see Figure 1 for position of the fragments in the T-region. Affixes *.1* and *.2* to the plasmids indicate the orientation of the fragments in the vector plasmids (see Materials and methods).

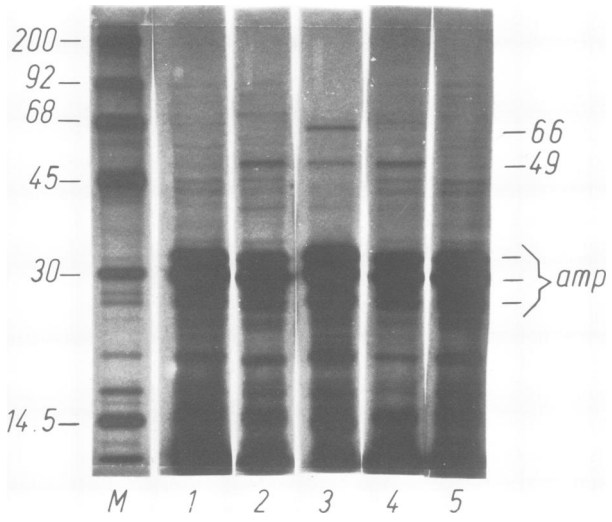

Fig. 2. Proteins synthesized in *E. coli* minicells with plasmids containing parts of the T_L-region of pTi Ach5. **Lane 1**: pBR322 (vector control); **2**: Ach5 *Hind*III (14-18-22-38) in pBR322; **3**: Ach5 *Bam*HI-8 in pBR322; **4**: Ach5 *Hind*III-22 in pUR51; **5**: pUR51 (vector control). M: Marker proteins, with their sizes in mol. wt. x 10^{-3} at the left side. The numbers at the right side indicate the size of proteins synthesized from the Ti-plasmid fragments. Amp: proteins produced from the ampicillin resistance genes on the vector plasmids.

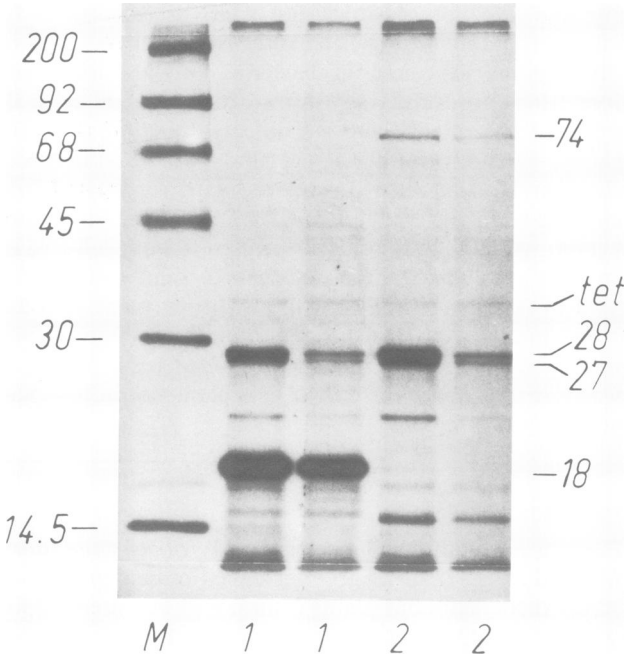

Fig. 3. Proteins synthesized in *E. coli* minicells with Ach5 *Eco*RI-7 inserted in both orientations in pACYC184. **Lane 1**: orientation *.1* (different experiments); **2**: orientation *.2* (different experiments). The numbers at the right side indicate the size (mol. wt. x 10^{-3}) of the proteins synthesized from the Ti-plasmid fragment. The 18 000 mol. wt. protein is a fusion of the octopine-synthesizing enzyme with the chloramphenicol acetyltransferase encoded on pACYC184 (Schröder *et al.*, 1981b). Tet: a 34 000 mol. wt. protein encoded by the tetracycline resistance on pACYC184.

fragment 8 (lane 3). The protein was not synthesized with plasmids containing *Hind*III-14 or 18 when cloned separately or together, suggesting that the gene was in *Hind*III-22 or close to it. Recloning of *Hind*III-22 followed by expression in minicells showed that the protein was synthesized in its entire length (Figure 2, lane 4), indicating that the complete coding region is in this fragment. Expression was independent of the orientation of the insert in the vector plasmid, suggesting transcription from a promoter within *Hind*III-22. This is supported by the finding that Ach5 *Bam*HI-8 and Ach5 *Hind*III (14-18-22-38) expressed the same protein, although they contain long stretches of DNA from the T-region either to the right or to the left of *Hind*III-22.

The gene coding for the 49 000 mol. wt. protein is in the highly conserved part of the T-region, and the corresponding DNA region from nopaline plasmid pTi C58 is in pGV0354 containing C58 *Hind*III fragments (15-14-19-41-22) (see Table I and Figure 1). This plasmid expressed a protein of very similar size in minicells (not shown). Although the coding region has not been mapped in more detail, this suggests that the highly conserved part of the nopaline plasmid codes for a similar protein.

Mol. wt. 74 000 protein

Fragment Ach5 *Eco*RI-7 inserted into pACYC184 produced a 74 000 mol. wt. protein in minicells (Figure 3, lane 2), but only with orientation *.2* of the inserted DNA where transcription from the insertion-inactivated gene for chloramphenicol acetyltransferase reads into *Eco*RI-7 from left to right. It seemed possible, therefore, that the protein represented a fusion of the truncated vector plasmid encoded

protein with part of a protein encoded on *Eco*RI-7, like the fusion protein with the octopine-synthesizing enzyme described previously (Schröder *et al.*, 1981b). In that case it was shown that such fusion proteins react with antibodies against chloramphenicol acetyltransferase, and we therefore tested whether the 74 000 mol. wt. protein was precipitated with antiserum against this enzyme. The results were negative, suggesting that the protein was not a fusion product. The simplest explanation was therefore that transcription started on the vector plasmid, but that the ribosome-binding site and the complete protein-coding region are on the Ti-plasmid fragment. This was supported by more detailed mapping.

The coding region was mapped by Tn5 mutagenesis (Schröder *et al.*, 1981b) of *Eco*RI-7 cloned in orientation *.2* in pACYC184 (see Materials and methods for details). Insertion 1 (0.4 kb from left end of *Eco*RI-7) abolished formation of the protein (Figure 4, lane 3); insertion 2 (1.4 kb) produced a truncated 39 000 mol. wt. protein (lane 4); insertion 3 (1.8 kb) a truncated 52 000 mol. wt. protein (lane 5); and insertion 4 (2.4 kb) had no effect on formation of the complete protein (lane 6). The results confirm that transcription proceeded from left to right, and they localize the entire protein-coding region on *Eco*RI-7, as drawn in Figure 7. The location was confirmed by the following experiment: fragment *Hpa*I-14 was removed from *Eco*RI-7 (in pACYC 184) by excision and religation of the plasmid, and the effect on the formation of the protein was tested. Figure 4, lane 8, shows that the modified plasmid produced a 53 000 mol. wt. protein as would be expected from the position of the *Hpa*I fragment within *Eco*RI-7 (Figure 7).

The question of whether this gene could also be expressed from a promoter within the T-region was investigated with fragment Ach5 *Bam*HI-8. This fragment should contain ~85% of the protein-coding region of the 74 000 mol. wt. protein, and also a long stretch of DNA extending to the left of *Eco*RI-7 (Figure 1). When pBR322 containing this fragment was expressed in minicells, it synthesized the previously discussed 49 000 mol. wt. protein and a 66 000 mol. wt. protein (Figure 2, lane 3). The recombinant plasmid containing Ach5 *Hind*III fragments (14-18-22-38) which cover the left two thirds of *Bam*HI-8 did not synthesize a protein of 66 000 mol. wt. (Figure 2, lane 2), suggesting that this protein is encoded at the right end of *Bam*HI-8. The size of the protein and the position of the right *Bam*HI-8 site in the coding region indicate that the 66 000 mol. wt. protein represents a shortened form of the 74 000 mol. wt. protein, and therefore this result suggests that the gene can be expressed in *E. coli* with a promoter activity from the T-region located to the left of *Eco*RI-7.

Since the coding region for the 74 000 mol. wt. protein maps in the highly conserved 'common core' segment of the T-region, nopaline Ti-plasmid C58 should contain a large part of the corresponding gene in C58 *Hind*III-22 (Figure 1). This fragment was isolated, cloned in both orientations with *Eco*RI linkers into pACYC184, and the recombinant plasmids were tested in the minicell system. Figure 5, lane 2, shows that orientation *.2* (transcription from the vector reads from left to right into *Hind*III-22) produced a 60 000 mol. wt. protein which was not formed with orientation *.1* (lane 1). This protein reacted with antibodies against the chloramphenicol acetyltransferase encoded by pACYC184 (data not shown), and thus it represents a fusion protein. Since the *E. coli* gene and its protein product have been sequenced

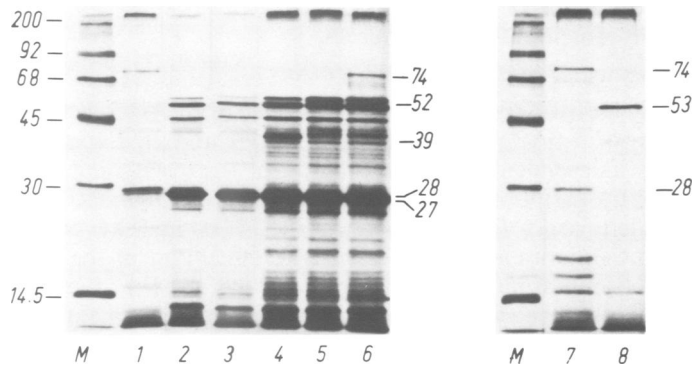

Fig. 4. Proteins synthesized in *E. coli* minicells with mutagenized fragment Ach5 *Eco*RI-7 in pACYC184. **Lane 1**: unmodified *Eco*RI-7; **2**: vector plasmid pACYC184 with Tn5 inserted in the chloramphenicol resistance (control for Tn5-encoded proteins). **Lanes 3–6**: Tn5 insertions in *Eco*RI-7; the numbers given here in the legend indicate the distance of the Tn5 insertion from the left *Eco*RI site. **Lane 3**: 0.4 kb (formation of the 74 000 mol. wt. protein abolished); **4**: 1.4 kb (truncated 39 000 mol. wt. protein); **5**: 1.8 kb (truncated 52 000 mol. wt. protein; difficult to visualize, since it is very close to a Tn5-encoded protein); **6**: 2.4 kb (formation of the 74 000 mol. wt. protein not affected). The apparent differences in the rate of expression of Tn5-encoded proteins in **lanes 2–6** are due to different exposure times which were necessary to visualize the comparably weak expression of the 74 000 mol. wt. protein or its truncated forms. **Lanes 7–8**: proteins synthesized with *Eco*RI-7 after mutagenization by excision of *Pst*I-Ω **(lane 7)** or *Hpa*I-14 **(lane 8)**.

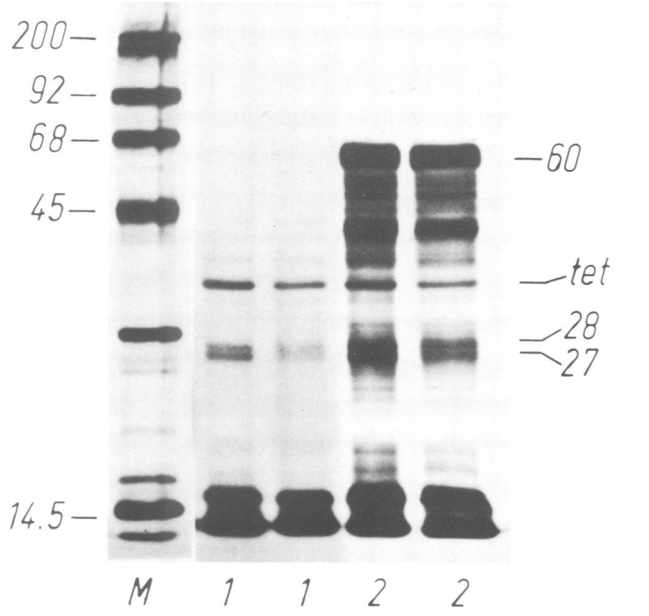

Fig. 5. Proteins synthesized in *E. coli* minicells with C58 *Hind*III-22 cloned in both orientations with *Eco*RI linkers into pACYC184. **Lane 1**: orientation *.1* (different experiments); **2**: orientation *.2* (different experiments). M: marker proteins (left side).

(Alton and Vapnek, 1979; Shaw *et al.*, 1979), the contribution from the gene in the T-region can be calculated. The results (Figure 7) show that the position of the coding region expressed as fusion protein corresponds to the region expressed as complete protein with Ach5 *Eco*RI-7. Plasmid pGV0354 which contains, in addition to C58 *Hind*III-22, several *Hind*III fragments to the left also synthesized a protein of very similar size (data not shown), suggesting that the

T-region of the nopaline Ti-plasmid can also express the complete protein in *E. coli* minicells.

Mol. wt. 27 000 and 28 000 proteins

These two proteins were synthesized with Ach5 *Eco*RI-7 cloned in pACYC184 (Figure 3, lanes 1 and 2). Expression was independent of the orientation of the insert, indicating that transcription started within the T-region fragment. Since the left part of *Eco*RI-7 codes for the 74 000 mol. wt. protein and the right end for the carboxy-terminal part of the octopine-synthesizing enzyme (Schröder *et al.*, 1981b), it seemed likely that the two proteins would be encoded by the middle of the fragment. This was confirmed by the following experiments: the small *Pst*I fragment in *Eco*RI-7 cloned in pACYC184 was excised by restriction and religation, and the resultant plasmid was tested in the minicell system. Figure 4, lane 7, shows that the 28 000 mol. wt. protein was still synthesized but not the complete 27 000 mol. wt. protein. Although this was not investigated further, the smaller proteins found instead may represent truncated forms of this protein, suggesting that only part of the gene is on the excised *Pst*I fragment. The position was confirmed by the following experiment: fragment *Hpa*I-14 was removed from *Eco*RI-7 cloned in pACYC184 by excision and religation, and the new plasmid was tested. Figure 4, lane 8, shows that this led to a truncated 74 000 mol. wt. protein (as discussed before) and to disappearance of both the 27 000 and 28 000 mol. wt. proteins. This indicates that part of the gene for the smaller protein is localized in the *Hpa*I/*Pst*I overlap. Since formation of the 28 000 mol. wt. protein was eliminated after the removal of *Hpa*I-14 but not of the small *Pst*I fragment, its gene must be localized to the left of the *Pst*I site, but within *Hpa*I-14, as drawn in Figure 7. These results support the interpretation that the two proteins are encoded by different genes and that the 28 000 mol. wt. protein is not a slightly larger form of the 27 000 mol. wt. protein encoded by the same gene. However, it cannot be excluded that the two genes overlap to some degree, and other experiments are necessary to map more precisely the 28 000 mol. wt. protein.

The regions corresponding to these locations in nopaline Ti-plasmid C58 are in fragment C58 *Hind*III-22. Figure 5 shows that this fragment expressed two proteins of very similar size independent of the orientation of the fragment in the vector plasmid pACYC184. Since the position of the *Pst*I and *Hpa*I sites in octopine plasmid Ach5 and nopaline plasmid C58 correspond precisely in this part of the T-region, the coding regions were also localized by deletion mapping in the same manner as performed with Ach5 *Eco*RI-7. The results (data not shown) obtained with the nopaline Ti-plasmid fragment corresponded to those obtained with the octopine plasmid fragment, and since this region belongs to the highly conserved part of the T-region, we assume that the locations of the two genes correspond in Ach5 and C58, as drawn in Figure 7.

Expression of plasmids and fragments in cell-free systems from E. coli and A. tumefaciens

We also investigated whether the four proteins produced in minicells were synthesized in a cell-free system prepared from *E. coli*. The results (data not shown) indicate that the 27 000, 28 000, 74 000 and 66 000 (= truncated 74 000) mol. wt. proteins were expressed in the extracts, but synthesis of the 49 000 mol. wt. protein was poor when compared with the minicells, and it was clearly visible only after long exposure

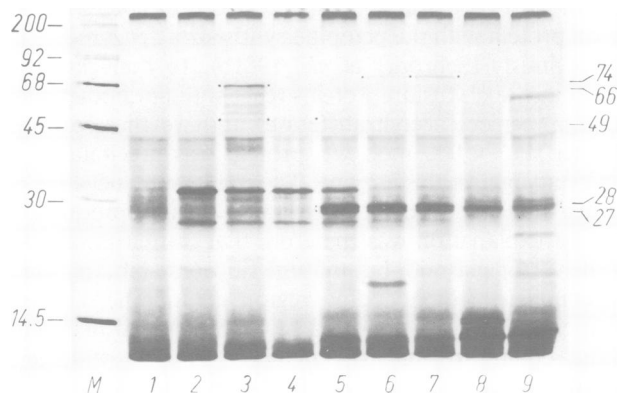

Fig. 6. Expression of T-region-encoded proteins in a cell-free system for coupled transcription/translation prepared from *A. tumefaciens*. **Lane 1**: no DNA added (control); **2**: pBR322 (vector control); **3**: Ach5 *Bam*HI-8 in pBR322; **4**: Ach5 *Hind*III-22 in pUR51; **5**: Ach5 *Hind*III-1 in pBR322; **6**: Ach5 *Eco*RI-7 in orientation *.1* in pACYC 184; **7**: Ach5 *Eco*RI-7 in orientation *.2* in pACYC184; **8**: C58 *Hind*III-22 in orientation *.1* in pACYC184; **9**: C58 *Hind*III-22 in orientation *.2* in pACYC184. The numbers at the right side indicate the size of the proteins (mol. wt. x 10^{-3}) synthesized with promoter activities from within the T-region.

times. The reason for this difference is not known, but a similar situation has been described recently for another gene (Trinks *et al.*, 1981). The cell-free system was also used to test whether expression of the T-region genes was independent of the vectors used for cloning. In these experiments, a mixture of fragments obtained by complete digestion of the plasmid with the appropriate restriction enzymes still synthesized the proteins, confirming the previous conclusion that the genes were expressed from promoters within the T-region. Again, the 49 000 mol. wt. protein was synthesized very poorly.

The natural host of these genes is *A. tumefaciens*, and a discussion of functions for the genes is only possible if it can be demonstrated that Agrobacteria also express these proteins. This was investigated with a cell-free system for coupled transcription/translation prepared from Agrobacteria. Figure 6 shows the results obtained with a variety of plasmids containing fragments which produced the proteins in *E. coli*. The 27 000, 28 000, 74 000 and 66 000 (= truncated 74 000) mol. wt. proteins were expressed in this cell-free system, but again formation of the 49 000 mol. wt. protein was poor. Interestingly, the results also show that Ach5 *Eco*RI-7 cloned in pACYC184 expressed the 74 000 mol. wt. protein with both orientations of the fragment (Figure 6, lanes 6,7). This is in contrast to the *E. coli* system, where it was produced only with orientation *.2* and expression was strictly dependent on the promoter of the vector plasmid. This indicates that *Eco*RI-7 contains a promoter activity for expression of the 74 000 mol. wt. protein which is recognized in Agrobacteria, but not in *E. coli*. Taken together, the results suggest that the proteins expressed in *E. coli* can also be expressed in *A. tumefaciens*. The only exception may be the 49 000 mol. wt. protein. However, this seems to be a property of the cell-free system rather than the gene, since the *E. coli* extract showed similarly poor expression of this gene when compared with the minicells.

Discussion

Our results show that the center part of the T-region can express a set of proteins in bacteria. The same part is

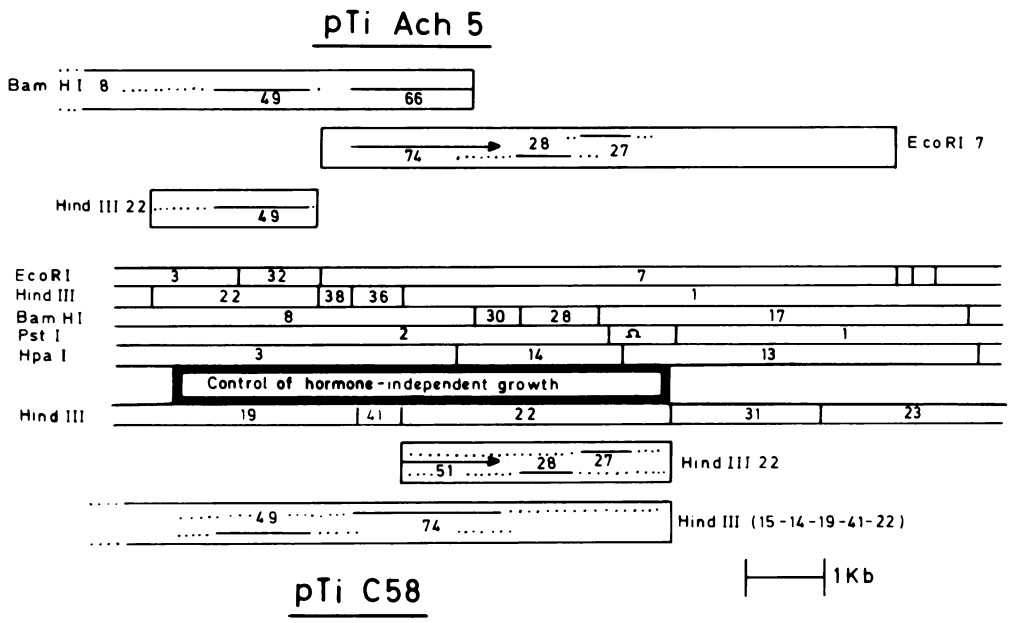

Fig. 7. Proteins expressed in bacteria from the highly conserved part of the T-region. The boxes show the restriction fragments most often used in the experiments. The solid lines within indicate the most likely location of the coding region, and the dotted lines the limits of mapping accuracy. Arrows define the direction of transcription where known, and the numbers in the boxes indicate the sizes of the proteins (mol. wt. x 10^{-3}) synthesized with the fragments, either as complete (74,49,28,27) or as truncated (66) or fusion (51) proteins. The black box in the middle of the figure shows the highly conserved part of the T-region which is responsible for the control of hormone-independent growth of plant cells.

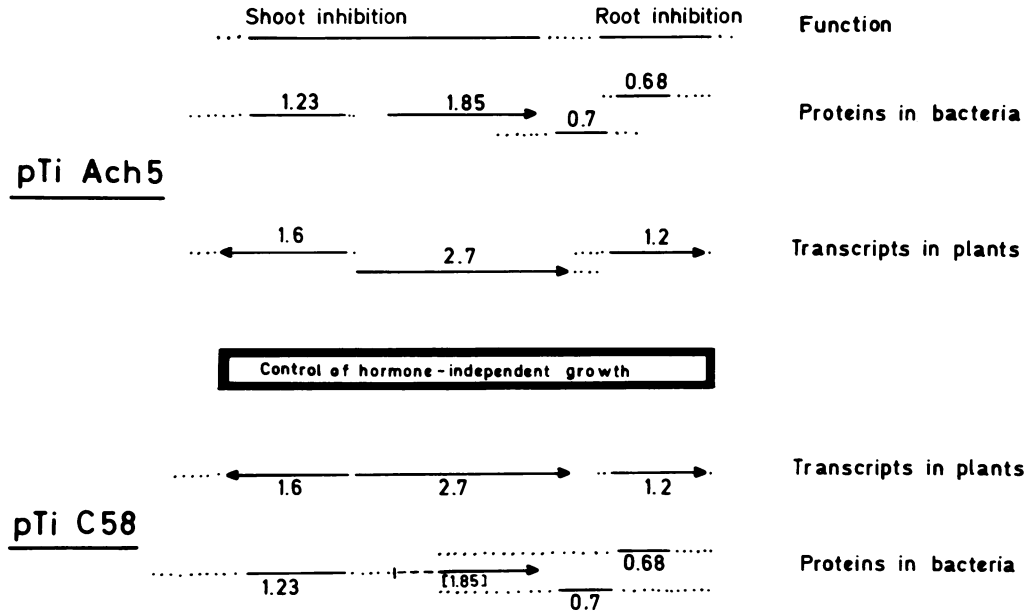

Fig. 8. Comparison of regions expressed in bacteria and in plants. Proteins: the lines represent the coding regions expressed in bacteria with promoters from the T-region. The numbers indicate the minimum length of the coding region, as calculated from the size of the proteins, and assuming that the average mass of amino acids is 120. In pTi C58, 1.85 is given in brackets since the mapping is based on expression of the gene as fusion protein (see Results section). Transcripts: the data were taken from the work of Willmitzer *et al.* (1982, and in preparation) and redrawn under the assumption that the genes are not spliced. Function: the parts of the T-region responsible for suppression of root or shoot formation in plant cells, as defined by genetical analysis (Leemans *et al.*, 1982; Joos *et al.*, 1983).

transcribed in plant cells (Gelvin *et al.*, 1982; Willmitzer *et al.*, 1982, and in preparation), but the protein products have not yet been identified. Thus, the T-region contains DNA sequences expressed in procaryotic and also DNA sequences expressed in eucaryotic cells, and it is an important question whether they are the same or different genes. Figure 8, a comparison of the regions expressed in bacteria and in plants, indicates that three of the protein-coding regions in bacteria correlate with three transcript-coding regions in plants in several points: the locations correspond within the limits of mapping accuracy, the sizes correspond, and in each case the transcript is larger than required for the protein. The comparison is particularly striking with the 74 000 mol. wt. protein (calculated minimum size of coding region 1.85 kb): the protein-coding region is within the transcript-coding region, and the coding strand is the same in bacteria and in plants.

This suggests, as a working hypothesis, that the same genes are expressed in procaryotic and eucaryotic cells, but further evidence is necessary to support this hypothesis. It will be of special interest to discover whether the two systems use the same or different signals for transcription, since our data indicate that even *E. coli* and *A. tumefaciens* can utilize different promoter sequences to express the 74 000 mol. wt. protein.

Figure 8 also shows that the fourth protein expressed in bacteria (mol. wt. 28 000; calculated minimum size of coding region 0.7 kb) appears to have no corresponding transcript in plant cells. Since some genetic evidence is consistent with a function (see later), this could mean that expression is either not necessary or occurs only under special conditions. A similar situation has been described for the gene expressing the 2.7-kb transcript: it was detected by some authors in all octopine tumors but rarely in nopaline tumors (Willmitzer *et al.*, 1982, and in preparation), while others failed to find it in octopine tumors (Gelvin *et al.*, 1982; Murai and Kemp, 1982b). Also, recent results indicate that another transcript (1.6 kb, see Figure 8) was often not detectable in nopaline tumors (Willmitzer *et al.*, in preparation). This suggests that some genes of the T-DNA need not be expressed continuously to maintain hormone-independent growth of plant cells.

The four proteins expressed in the bacteria are encoded in the part of the T-region which is responsible for hormone-independent growth of plant cells. According to the genetic identification of functions, the 74 000 and 49 000 mol. wt. proteins are encoded in the part responsible for suppression of shoot formation and the 27 000 mol. wt. protein in the part responsible for suppression of root formation in plant morphogenesis (Leemans *et al.*, 1982; Joos *et al.*, 1983). The assignment of the 28 000 mol. wt. protein is difficult since the coding region may belong to either one of the functions (see Figure 8). However, the genetic data of Garfinkel *et al.* (1981) suggest that there may be a second locus for root suppression to the left of the region coding for the 27 000 mol. wt. protein, and the 28 000 mol. wt. protein possibly represents expression from this second locus. It should be stressed, however, that all of these functions were attributed to these parts of the T-region because of their effects in plant cells, not in Agrobacteria. Our result indicating that the same genes can possibly also be expressed in bacteria, adds a new aspect. Agrobacteria do produce plant hormones like cytokinins and auxin, and Ti-plasmids influence their metabolism (Claeys *et al.*, 1978; Liu and Kado, 1979; McCloskey *et al.*, 1980; Weiler and Spanier, 1981; Liu *et al.*, 1982; Regier and Morris, 1982). Moreover, recent experiments indicate that at least one locus in the T-region is involved in *trans*-zeatin production in Agrobacteria (Regier *et al.*, in preparation). Since the gene products from this part of the T-region, when expressed in plant cells, mediate hormone-like effects to untransformed cells (Leemans *et al.*, 1982; Joos *et al.*, 1983), it is tempting to speculate that the gene products in the bacteria may fulfill a similar function. Such a role could be envisioned in the following model: efficient tumor induction requires a wound in plant tissue which induces cell divisions, and transformation is believed to occur in this period (see review by Braun, 1978). In non-infected tissue, cell divisions cease after a few rounds while they continue in infected tissue. It seems possible that the bacterial gene products participate in mediating hormone-like effects from the bacteria to plant cells such that wound-induced cell division is maintained until the T-region is transferred and becomes operative in the plant cells. Certain plant pathogenic bacteria do exert hormone effects on plants; and in at least one case it has been shown that plasmid-coded genes are involved (Comai and Kosuge, 1980, 1982; Comai *et al.*, 1982), but the genes are not known to be transferred to plants. It seems possible that Agrobacteria use similar mechanisms in the early stages of infection, and in addition succeed in transferring the genes into the plant cells.

Materials and methods

Isolation and analysis of plasmids

Small amounts of plasmids were routinely isolated from purified minicells according to Birnboim and Doly (1979). For large scale preparations, plasmids were prepared with the cleared lysate technique and purified with two sequential centrifugations to equilibrium in CsCl (Bolivar, 1978). Plasmids were analyzed by digestion with the appropriate restriction enzymes, followed by electrophoresis in agarose gels and staining with ethidium bromide as recommended by the enzyme manufacturer (Boehringer, Mannheim, FRG).

Recombinant plasmids

Several plasmids containing fragments from the T-regions (pGV0219, pGV0153, pGV0201, pGV0354; compare Table I and Figure 1) have been described (Depicker *et al.*, 1980; DeVos *et al.*, 1981) and were provided by the crown gall research group in Gent (Belgium). Plasmids pWK500.*1* and pWK500.*2*, which contain Ach5 *Eco*RI-7 cloned in pACYC184 in opposite orientations, have been described (Schröder *et al.*, 1981b).

Construction of new plasmids

pHD106: plasmid pGV0219 (20 μg) was digested to completion with *Hind*III, and the fragments were separated on agarose gels. Fragment *Hind*III-22 was isolated by electroelution and ligated with *Hind*III-digested, phosphatased vector pUR51 (Rüther, 1980). After plating, colonies resistant to ampicillin (100 μg/ml) were screened for plasmids containing *Hind*III-22 inserts in pUR51. The orientation of the insert was determined by restriction analysis with *Sma*I and *Pst*I which cut asymmetrically in insert and vector, respectively.

pWK500.*2Pst*I and pWK500.*2Hpa*I: plasmid pWK500.*2* contains no restriction sites for *Pst*I or *Hpa*I in vector plasmid pACYC184, but two for both enzymes in the inserted Ach5 *Eco*RI-7. Thus, it was possible to mutagenize *Eco*RI-7 with the following steps: plasmid pWK500.*2* was digested to completion with either *Pst*I or *Hpa*I, extracted with phenol and religated. After plating, colonies resistant to tetracycline (20 μg/ml) were screened for plasmids containing only one *Pst*I or *Hpa*I site and the *Eco*RI-7 fragment shortened by the excised *Pst*I or *Hpa*I fragment.

pGC101: plasmid pBR322 containing C58 *Hind*III-22 (= pGV0423, Depicker *et al.*, 1980) was digested with *Hind*III, and fragment *Hind*III-22 was isolated from an agarose gel by electroelution. It was then ligated with *Eco*RI linkers (destroying the *Hind*III sites and creating new *Eco*RI sites at the end of the fragment) and cloned into the *Eco*RI site of pACYC184. Tetracycline-resistant colonies (20 μg/ml) were screened for plasmids containing the correct insert, and the orientation of the insert was determined by restriction analysis with *Pst*I and *Hind*III which cut asymmetrically in insert and vector, respectively.

Ligation mixes were routinely used to directly transform the minicell-producing strain *E. coli* DS410 (Dougan and Sherratt, 1977) with the calcium technique (Cohen *et al.*, 1972).

Mutagenesis of multicopy plasmids by transposon Tn5 was carried out with a chromosomally located Tn5 transposon (*E. coli* C600, met::Tn5). Spontaneously occurring transpositions were selected directly by using gene dosage effects from the multicopy plasmids. Cells containing multicopy plasmids with Tn5 inserts form large colonies on agar plates with enhanced neomycin concentrations (150 – 500 μg/ml). Plasmids with Tn5 inserts band – as a result of their increase in size of ~6 kd – at a position different from that of the original plasmid. The location of Tn5 inserts in Ti-plasmid fragments was determined as described previously (Schröder *et al.*, 1981b).

All cloning experiments were carried out under L1/B1 conditions as specified by the German Central Commission for Biological Safety (Berlin).

Definition of insert orientation

Vector plasmids pUR51 (in pHD106) and pACYC184 (in pWK500, pGC101) possess strong promoter activities reading into the cloned Ti-plasmid fragments. Since this may affect gene expression from the cloned fragments, it is necessary to define the orientation of the insert in the vector. For the purpose of this work, the orientations are defined by the presence of a promoter

in the vector plasmid which reads from right to left (orientation *.1*) or from left to right (orientation *.2*) into the Ti-plasmid fragment as drawn in Figure 1.

Synthesis of radioactive proteins

Isolation and labeling of *E. coli* minicells was carried out as described (Schröder *et al.*, 1981b). Cell-free extracts for coupled transcription/translation were prepared from *E. coli* according to Wetekam *et al.* (1971, 1972), and *in vitro* protein synthesis was performed essentially as described by these authors, but all assays contained, in addition, polyethyleneglycol at a final concentration of 2.5%. The same protocol was followed in the preparation and use of the cell-free system from *A. tumefaciens* (strain B6S3), but the amount of methionine was reduced to 2 μM final concentration. Proteins were analyzed in slab gels containing 12% polyacrylamide and 0.1% dodecylsulfate (Schröder *et al.*, 1981b).

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References

Alton,N.K. and Vapnek,D. (1979) *Nature,* **282**, 864-869.
Birnboim,H.C. and Doly,J. (1979) *Nucleic Acids Res.*, **1**, 1513-1523.
Bolivar,F. (1978) *Gene,* **4**, 121-136.
Braun,A. (1978) *Biochim. Biophys. Acta,* **516**, 167-191.
Chilton,M.-D., Drummond,H.J., Merlo,D.J., Sciaky,D., Montoya,A.L., Gordon,M.P. and Nester,E.W. (1977) *Cell,* **11**, 263-271.
Chilton,M.-D., Drummond,M.H., Merlo,D.J. and Sciaky,D. (1978) *Nature,* **275**, 147-149.
Claeys,M., Messens,E., Van Montagu,M. and Schell,J. (1978) *Quant. Mass Spectr. Life Sci.,* **2**, 409-418.
Cohen,S.N., Chang,A.C.Y. and Hsu,L. (1972) *Proc. Natl. Acad. Sci. USA,* **69**, 2110-2114.
Comai,L. and Kosuge,T. (1980) *J. Bacteriol.,* **143**, 950-957.
Comai,L. and Kosuge,T. (1982) *J. Bacteriol.,* **149**, 40-46.
Comai,L., Surico,G. and Kosuge,T. (1982) *J. Gen. Microbiol.,* **128**, 2157-2163.
De Cleene,M. and De Ley,J. (1976) *Bot. Rev.,* **42**, 389-466.
Depicker,A., Van Montagu,M. and Schell,J. (1978) *Nature,* **275**, 150-153.
Depicker,A., De Wilde,M., De Vos,G., De Vos,R., Van Montagu,M. and Schell,J. (1980) *Plasmid,* **3**, 193-211.
De Vos,G., De Beuckeleer,M., Van Montagu,M. and Schell,J. (1981) *Plasmid,* **6**, 249-253.
Dougan,G. and Sherratt,D. (1977) *Mol. Gen. Genet.,* **151**, 151-160.
Engler,G., Depicker,A., Maenhaut,R., Villarroel-Mandiola,R., Van Montagu,M. and Schell,J. (1981) *J. Mol. Biol.,* **152**, 183-208.
Garfinkel,D.J., Simpson,R.B., Ream,L.W., White,F.F., Gordon,M.P. and Nester,E.W. (1981) *Cell,* **27**, 143-153.
Gelvin,S.B., Thomashow,M.F., McPherson,J.C., Gordon,M.P. and Nester, E.W. (1982) *Proc. Natl. Acad. Sci. USA,* **79**, 76-80.
Guyon,P., Chilton,M.-D., Petit,A. and Tempé,J. (1980) *Proc. Natl. Acad. Sci. USA,* **77**, 2693-2697.
Joos,H., Inze,D., Caplan,A., Sormann,M., Van Montagu,M. and Schell,J. (1983) *Cell,* in press.
Leemans,J., Deblaere,R., Willmitzer,L., De Greve,H., Hernalsteens,J.P., Van Montagu,M. and Schell,J. (1982) *EMBO J.,* **1**, 147-152.
Lemmers,M., De Beuckeleer,M., Holsters,M., Zambryski,P., Depicker,A., Hernalsteens,J.P., Van Montagu,M. and Schell,J. (1980) *J. Mol. Biol.,* **144**, 353-376.
Liu,S.-T. and Kado,C.I. (1979) *Biochem. Biophys. Res. Commun.,* **90**, 171-178.
Liu,S.-T., Perry,K.L., Schardl,C.L. and Kado,C.I. (1982) *Proc. Natl. Acad. Sci. USA,* **79**, 2812-2816.
McCloskey,J.A., Hashizume,T., Basile,B., Ohno,Y. and Sonoki,S. (1980) *FEBS Lett.,* **111**, 181-183.
McPherson,J.C., Nester,E.W. and Gordon,M.P. (1980) *Proc. Natl. Acad. Sci. USA,* **77**, 2666-2670.
Murai,N. and Kemp,J.D. (1982a) *Proc. Natl. Acad. Sci. USA,* **79**, 86-90.
Murai,N. and Kemp,J.D. (1982b) *Nucleic Acids Res.,* **10**, 1679-1689.
Ooms,G., Hooykaas,P.J., Moleman,G. and Schilperoort,R.A. (1981) *Gene,* **14**, 33-50.
Regier,D.A. and Morris,R.O. (1982) *Biochem. Biophys. Res. Commun.,* **104**, 1560-1566.
Rüther,U. (1980) *Mol. Gen. Genet.,* **178**, 475-477.
Schell,J., Van Montagu,M., De Beuckeleer,M., De Block,M., Depicker,A., De Wilde,M., Engler,G., Genetello,C., Hernalsteens,J.P., Holsters,M., Seurinck,J., Silva,B., Van Vliet,F. and Villarroel,R. (1979) *Proc. R. Soc. London, B,* **204**, 251-266.
Schröder,J., Schröder,G., Huisman,H., Schilperoort,R.A. and Schell,J. (1981a) *FEBS Lett.,* **129**, 166-168.
Schröder,J., Hillebrand,A., Klipp,W. and Pühler,A. (1981b) *Nucleic Acids Res.,* **9**, 5187-5202.
Schröder,G. and Schröder,J. (1982) *Mol. Gen. Genet.,* **185**, 51-55.
Shaw,W.V., Packman,L.C., Burleigh,B.D., Dell,A., Morris,H.R. and Hartley,B.S. (1979) *Nature,* **282**, 870-872.
Skoog,F. and Miller,C.O. (1957) *Symp. Soc. Exp. Biol.,* **11**, 118-131.
Smith,E.F. and Townsend,C.O. (1907) *Science (Wash.),* **25**, 671-673.
Thomashow,M.F., Nutter,R., Montoya,A.L., Gordon,M.P. and Nester, E.W. (1980) *Cell,* **19**, 729-739.
Trinks,K., Habermann,P., Beyreuther,K., Starlinger,P. and Ehring,R. (1981) *Mol. Gen. Genet.,* **182**, 183-188.
Weiler,E.W. and Spanier,K. (1981) *Planta,* **153**, 326-337.
Wetekam,W., Staack,K. and Ehring,R. (1971) *Mol. Gen. Genet.,* **112**, 14-27.
Wetekam,W., Staack,K. and Ehring,R. (1972) *Mol. Gen. Genet.,* **116**, 258-276.
Willmitzer,L., De Beuckeleer,M., Lemmers,M., Van Montagu,M. and Schell,J. (1980) *Nature,* **287**, 359-361.
Willmitzer,L., Simons,G. and Schell,J. (1982) *EMBO J.,* **1**, 139-146.
Zaenen,I., Van Larebeke,N., Teuchy,H., Van Montagu,M. and Schell,J. (1974) *J. Mol. Biol.,* **86**, 109-127.
Zambryski,P., Holster,M., Kruger,K., Depicker,A., Schell,J., Van Montagu, M. and Goodman,H. (1980) *Science (Wash.),* **209**, 1385-1391.