The EMBO Journal Vol. 1 No. 1, pp.139–146, 1982

The TL-DNA in octopine crown-gall tumours codes for seven well-defined polyadenylated transcripts

Lothar Willmitzer[1]*, Gisela Simons[1], and Jeff Schell[1,2]

[1]Max-Planck-Institut für Züchtungsforschung, D-5000 Köln 30, FRG, and [2]Laboratorium voor Genetika, Rijksuniversiteit Gent, B-9000 Gent, Belgium

Communicated by J.Schell
Received on 8 December 1981

Seven polyadenylated transcripts of significantly different relative abundance were detected in octopine crown-gall tissue after gel electrophoretic separation and subsequent transfer to diazobenzyloxymethyl paper. The transcripts range from 670 to 2700 bases long. The different transcripts were located using 19 different fragments of the TL-region as probes. By hybridizing labelled RNA to separated complementary strands of the T-DNA, and parallel determination of the chemical polarity of the strands, the 5′–3′ orientations of six of the seven transcripts was identified. Both strands of the T-DNA code RNA. Hybridization of octopine TL-DNA against poly A$^+$ RNA's present in two nopaline tumour-lines C58-S1 and BT37, and vice versa, reveals a minimum of two and possibly four transcripts common to both octopine and nopaline tumours. These transcripts originate from corresponding parts of the conserved region of the T-DNA and are of similar size.

Key words: crown-gall/transcripts/Northern analysis/common T-region

Introduction

Large plasmids (Ti-plasmids) present in all oncogenic *Agrobacterium tumefaciens* strains are responsible for crown-gall tumour induction by this soil bacterium on most dicotyledonous plants (Zaenen *et al.*, 1974; Van Larebeke *et al.*, 1974; Watson *et al.*, 1975). The plant tumour cells, unlike nontransformed plant tissues, can be cultured under axenic conditions on synthetic media in the absence of growth hormones, i.e., cytokinins and auxins (Braun, 1956); the tumour cells also produce low molecular weight compounds called opines not found in untransformed plant tissues. The opine produced defines crown-galls as octopine, nopaline, or agropine type tumours (Guyon *et al.*, 1980). Octopine and nopaline crown-gall tumours contain a DNA segment (called T-DNA) which is homologous and colinear with a defined fragment of the corresponding Ti-plasmid present in the tumour-inducing bacterium (Chilton *et al.*, 1977; Schell *et al.*, 1979; Lemmers *et al.*, 1980; Thomashow *et al.*, 1980a; De Beuckeleer *et al.*, 1981). The T-DNA is covalently linked to plant DNA (Zambryski *et al.*, 1980; Yadav *et al.*, 1980; Thomashow *et al.*, 1980b) in the nucleus of the plant cell (Chilton *et al.*, 1980; Willmitzer *et al.*, 1980).

The T-DNA is transcribed in the transformed plant cell (Drummond *et al.*, 1977; Gurley *et al.*, 1979; Willmitzer *et al.*, 1981a; Gelvin *et al.*, 1981), by the host RNA polymerase II (Willmitzer *et al.*, 1981b). T-DNA encoded proteins were found in several octopine crown-gall lines (McPherson *et al.*, 1980; Schröder *et al.*, 1981a), one of them being the octopine synthesizing enzyme lysopine dehydrogenase (LpDH) (Schröder *et al.*, 1981a).

To date, however, the number, sizes and locations of T-DNA transcripts were unknown, partly because the amount of T-DNA specific transcripts in the transformed plant cell is very low; the total population of T-DNA specific transcripts represents <0.001% of the poly A$^+$-RNA (Willmitzer *et al.*, 1981a). We report data on the number, size, and location of TL-DNA derived transcripts from Northern gel analysis of poly A$^+$ RNAs present in crown-gall cells. The direction of transcription was determined for six of the seven transcripts and the 5′ and 3′ ends of these transcripts were located. Hybridization of octopine TL-DNA fragments to poly A$^+$ RNA isolated from nopaline tumour tissues, and *vice versa,* revealed cross-hybridizing transcripts in both octopine and nopaline tissues.

We used the octopine tobacco crown-gall line A6-S1, which contains only the TL-DNA present in all octopine tumour lines analyzed, and thus represents the minimal T-DNA necessary for formation of a wild-type tumour (Thomashow *et al.*, 1980a; De Beuckeleer *et al.*, 1981). To compare transcripts in octopine and nopaline tissues, the nopaline tobacco tumours BT37 and C58-S1 were chosen because their T-DNA content is well characterized (Lemmers *et al.*, 1980). The function of the various transcripts is discussed in the accompanying paper by Leemans *et al.*

Results

TL-DNA of octopine tobacco crown-gall tumours codes for seven poly A-containing transcripts absent from habituated tobacco tissue cultures

Figure 1 shows the autoradiogram obtained after hybridizing nick-translated T-region clones pGV 0153 (containing BamHI fragment 8) and pGV 0201 (containing HindIII fragment 1 of pTi ACH5) to a diazobenzyloxymethyl (DBM) filter containing the separated poly A$^+$ fraction of the octopine line A6-S1 (lanes a–c) as well as the separated poly A$^+$ fraction of RNA isolated from untransformed habituated tobacco tissue (lane d). Six different RNA species can readily be detected, the seventh transcript, no. 5, is not visible due to its very low concentration (cf., however, Figure 2, lanes 17,19).

The sizes and relative abundance of the different transcripts vary considerably. Transcripts 3 and 7, 1400 and 670 nucleotides long, respectively, are present in the highest concentration, transcripts 4 and 6, of 1200 and 900 nucleotides, are less abundant, and transcripts 1 and 2, respectively, 2700 and 1600 nucleotides, have very low concentrations. The concentration of transcript 5 is even lower and its detection is possible only with small subfragments of the T-region probes. The terms high and low abundance are used only to compare the internal concentrations of the different TL-DNA transcripts; compared to other cellular transcripts, all TL-DNA transcripts belong to the very low abundance class. All seven transcripts contribute no more than 0.0005–0.001% of the total poly A$^+$ population (Willmitzer *et al.*, 1981a). The

*To whom reprint requests should be sent.

 0261-4189/82/0101-0139$2.00/0

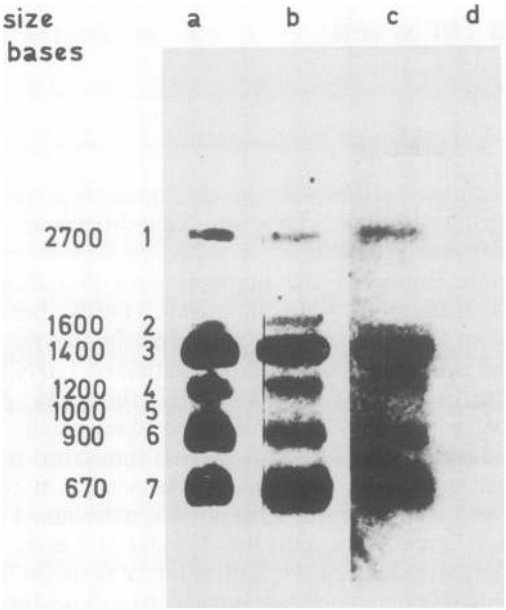

Fig. 1. Hybridization of octopine TL-region probes against poly A$^+$ RNA isolated from octopine crown gall tumour A6-S1 and from untransformed tissue NW-S1. Nick-translated plasmid clones pGV 0153 containing BamHI fragment 8 of pTi ACH5 cloned in pBR322 and pGV 0201 containing HindIII fragment 1 of pTi ACH5 cloned in pBR322 (De Vos *et al.*, 1981) (see Figure 2 for localization of these fragments) were hybridized against poly A$^+$ RNA isolated from three different batches of A6-S1 tissue (**lanes a – c**) as well as with poly A$^+$ RNA isolated from the untransformed habituated line NW-S1 (**lane d**). The amounts of RNA applied per lane are: **lane a**, 70 μg; **lane b**, 50 μg; **lane c**, 80 μg; **lane d**, 120 μg. The numbers 1 – 7 specify the transcripts according to diminishing size. The length of the transcripts was determined using denatured λ/PstI and λ/HindIII DNA fragments as well as the rRNAs as markers.

relative abundance of TL-DNA transcripts varies especially for transcript 6, which in some batches of poly A$^+$ RNA was found at the same concentration as transcripts 3 and 7 (Figure 1, lane a). This variation in transcription of TL-DNA may be related to the growth phase of the cultures (Willmitzer *et al.*, 1981a).

Arrangements of the different transcripts on the T-DNA

To determine from which regions of the TL-DNA the different transcripts arise, hybridization experiments were performed using 19 different subfragments of the TL-region as probes (see Figure 2.). The seven transcripts cover most of TL-DNA. The transcripts 3 and 7 with the highest relative concentration, are localized, respectively, near the right- and left-hand borders of TL-DNA. Transcript 7 hybridizes strongly with SmaI fragment 16a and HindIII fragment 18 of pTi ACH5 (cf., Figure 2, lanes 17,18) and must be derived from the overlapping part between HindIII fragment 18 and SmaI fragment 16a. This is consonant with the results of hybridizing labelled RNA to T-DNA fragments immobilized on nitrocellulose (Willmitzer *et al.*, 1981a). Transcript 3 hybridizes strongly with both the small EcoRI ω fragments and with EcoRI fragments 19a and 7 of pTi ACH5 but not with BamHI fragment 2 (Figure 2, lanes 1 – 3,5). The position of transcript 3 therefore confirms results of previous Southern-type hybridizations. Whereas transcript 3 is the main transcript hybridizing to the part of SmaI fragment 3b contained in HindIII fragment 1 (cf., Figure 2, lane 4) the neighbouring SmaI fragment, 10c, lights up transcripts 1, 4, and 6 (Figure 2, lane 8).

These transcripts were mapped more precisely with subfragments of EcoRI fragment 7 of pTi ACH5, obtained by digestion with KpnI (Figure 2, lanes 5 and 9), or BamHI (Figure 2, lane 7), or HpaI (Figure 2, lanes 6 and 10). The left border of transcript 1 hybridizes to the right border of BamHI fragment 8 (cf., Figure 2, lane 11). Transcripts 2 and 5, which are derived from the left-hand side of TL-DNA, were mapped using HindIII fragment 22 which lights up only transcript 2 (Figure 2, lane 16), and SmaI fragments 17, 16a and HindIII fragment 18 which light up transcript 5 (Figure 2, lanes 17 – 19). The extension of transcript 1 into BamHI fragment 8 was determined accurately using the overlapping parts of EcoRI fragment 7 and BamHI fragment 8, HindIII fragment 1 and BamHI fragment 8 as well as HindIII fragment 36b (Figure 2, lanes 13,11,12). Because EcoRI fragment 32 lights up only transcript 2 (Figure 2, lane 14) the left-hand border of EcoRI fragment 7 should determine the left-hand border of mature transcript 1. However, analysis of mutant crown gall lines suggests that the promoter region controlling the expression of transcript 1 is located on EcoRI fragment 32 g (see accompanying paper, Leemans *et al.*).

Determination of the direction of transcription

To determine the polarity of the different transcripts, restriction fragments of the T-region were denatured by alkali and the strands were separated by electrophoresis and blotted onto nitrocellulose. Subsequently poly A$^+$ RNA isolated from the A6-S1 line was labelled at its 5′-end with polynucleotide kinase and hybridized to the immobilized strands of the T-DNA fragments thus identifying the coding strand. In a second step the chemical polarity of the strands was determined by hybridizing T-region restriction fragments, specifically labelled at one 3′-OH end with terminal transferase, to the separated strands. The hybridization of RNA with the separated strands of BamHI fragment 8 of pTi ACH5, which codes for transcripts 5, 7, 2 and a part of transcript 1, is shown in Figure 3, group a, lane 2. The lower strand gives a very strong signal whereas only very weak hybridization was observed to the upper strand. The 3′-end of the lower strand is located at the right end because hybridization of the BamHI-KpnI fragment x (cf. Figure 3) which has been labelled at its left 3′-OH end with terminal transferase, specifically hybridizes only to the lower strand (cf. Figure 3, group a, lane 3). Therefore at least transcript 7, the most abundant transcript originating from BamHI fragment 8, must have its 5′ – 3′ direction from right to left. This was confirmed by hybridizing RNA to the separated strands of SmaI fragment 16a (Figure 3, group b) where only the lower strand hybridized. Hybridization of RNA to the separated strands of HindIII fragment 22, which is specific for transcript 2, again is with the lower strand only (cf. Figure 3, group c). Both these lower strands possess the same chemical polarity as the lower strand of BamHI fragment 8 (data not shown); thus transcripts 7 and 2 must both have a 5′ – 3′ orientation from right to left. Because we failed to separate the strands of SmaI fragment 17, the polarity of transcript 5 was not determined. Poly A$^+$ RNA from A6-S1 tissues hybridizes with both strands of EcoRI fragment 7 of pTi ACH5 (cf. Figure 3, group d, lane 2). EcoRI fragment 7 codes for the transcripts 1, 4, 6, and part of transcript 3. The chemical polarity of the strands was determined by hybridizing the KpnI-EcoRI fragment y (cf. Figure 3), labelled at its right 3′-OH end with terminal transferase, with the separated strands of EcoRI fragment 7; only the upper strand hybridized (cf. Figure 3, group d, lane 3). Thus, the upper strand must have its 5′ – 3′ direction from right to left. To determine the

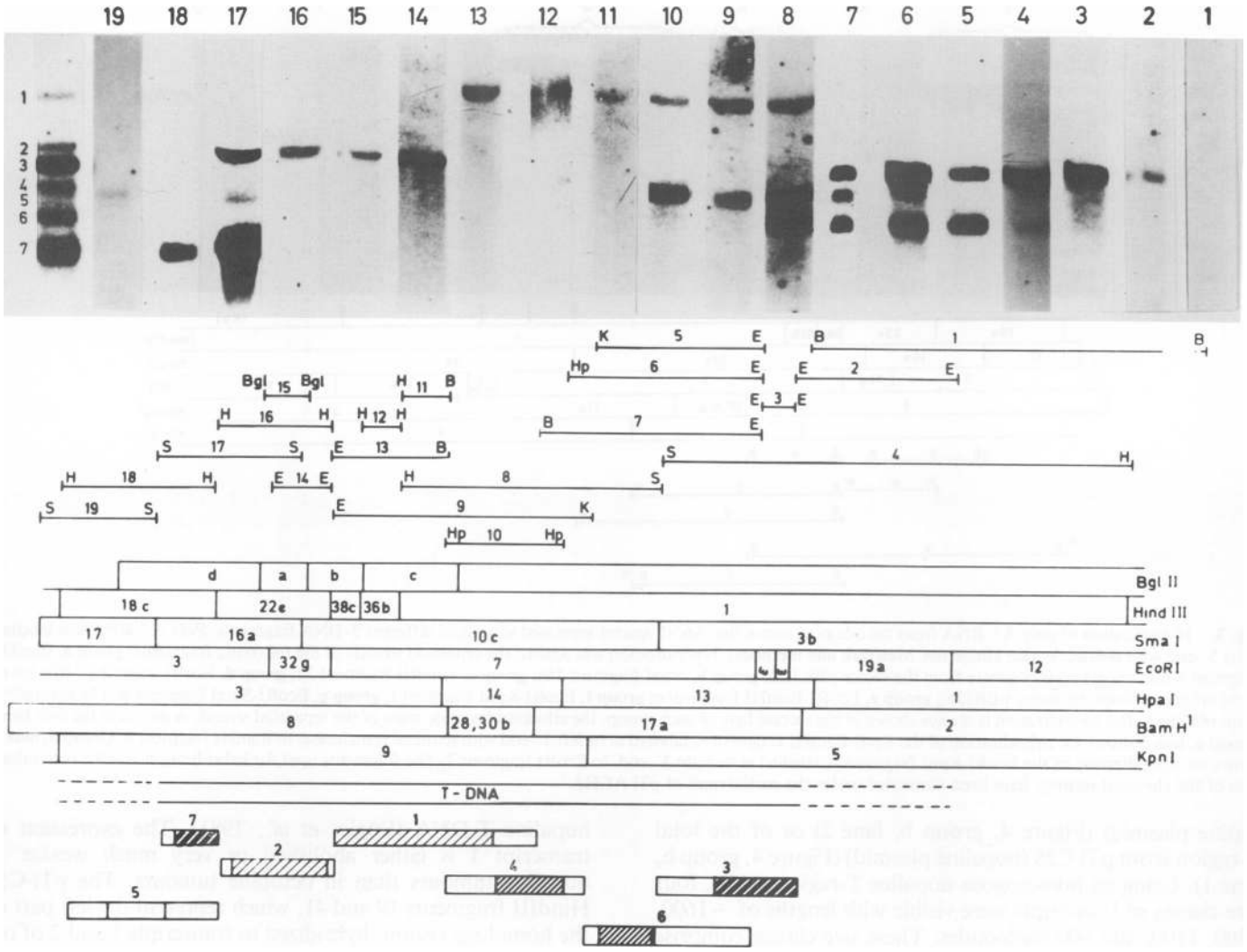

Fig. 2. Hybridization of probes of subfragments of the octopine TL-region against poly A$^+$ RNA isolated from the octopine crown gall tumour A6-S1. Subfragments obtained by digestion of the plasmids pGV 0153, pGV 0201, and pGV 0507 (containing the fragments BamHI, 8; HindIII, 1 and EcoRI, 7 of pTi ACH5 (De Vos *et al.*, 1981)) with different restriction enzymes and isolation after gel electrophoretic separation. **Above,** hybridizations, first lane to the left, probes overlapping the total TL-region. Numbered lanes correspond to different fragments used as probes as indicated above the partial map of pTi ACH5. Restriction sites that determine the end points of the fragments used as probes: Bg = BglII, B = BamHI, E = EcoRI, H = HindIII, Hp = HpaI, K = KpnI, S = SmaI. **Below:** the partial map of pTi ACH5 with approximate location of the different transcripts. The shaded area of each box indicating the size and the most probable map position of each transcript under the untested assumption that no splicing occurs. Intensity of shading is proportional to the relative concentration of the different transcripts. The extent of the T-DNA in the line A6-S1 is indicated below the partial map of pTi ACH5 (De Beuckeleer *et al.*, 1981).

polarity for each of the four transcripts encoded by EcoRI fragment 7, RNA was hybridized to separated strands of T-region fragments specific for either one or a group of transcripts. Thus, the EcoRI-BamHI fragment e is specific for transcript 1, the EcoRI-KpnI fragment f is specific for transcripts 1 and 4, and the EcoRI-SmaI fragment g encodes transcripts 1, 4, and 6. Separation of the complementary strands was achieved for these fragments: Figure 3, group e, f, and g. In all cases hybridization was observed to the upper strand only, indicating that the transcripts 1, 4, and 6 are of the same polarity. As the polarity of the upper strands in group e, f, and g is the same as the polarity of the upper strand of EcoRI fragment 7, the 5′−3′ orientation of transcript 1, 4, and 6 must be from left to right. The hybridization to the lower strand of EcoRI fragment 7 (cf. Figure 3, lane d) must, therefore be due to the other transcript, 3, encoded by EcoRI fragment 7. The 5′−3′ polarity of transcript 3 must, therefore, be the opposite of transcripts 1, 4, and 6, i.e., from right to left.

Comparison of transcripts in octopine and nopaline tumours originating from T-DNA segments that are homologous in both tumours

Octopine and nopaline Ti-plasmids share several regions of homology, including a segment within the T-region, which has been accurately mapped (Depicker *et al.*, 1978; Chilton *et al.*, 1978; Engler *et al.*, 1981). It was reasonable to assume that this conserved region codes for oncogenic functions expressed in both octopine and nopaline tumours. To verify this we made a series of heterologous hybridizations. First the seven separated poly A$^+$ transcripts derived from the TL-DNA of line A6-S1 (Figure 4, group a) were hybridized to the total nopaline T-region. Transcripts 1, 2, 4 and 6 hybridized clearly, whereas transcripts 3 and 7, the most prominent ones in the octopine tissue, did not. No hybridization to transcript 5 was observed, either because of lack of homology and/or its low concentration. Then, poly A$^+$ RNA from the nopaline tumour tissue BT37 was isolated, separated on gels, and hybridized to either the total TL-region from pTi ACH5 (oc-

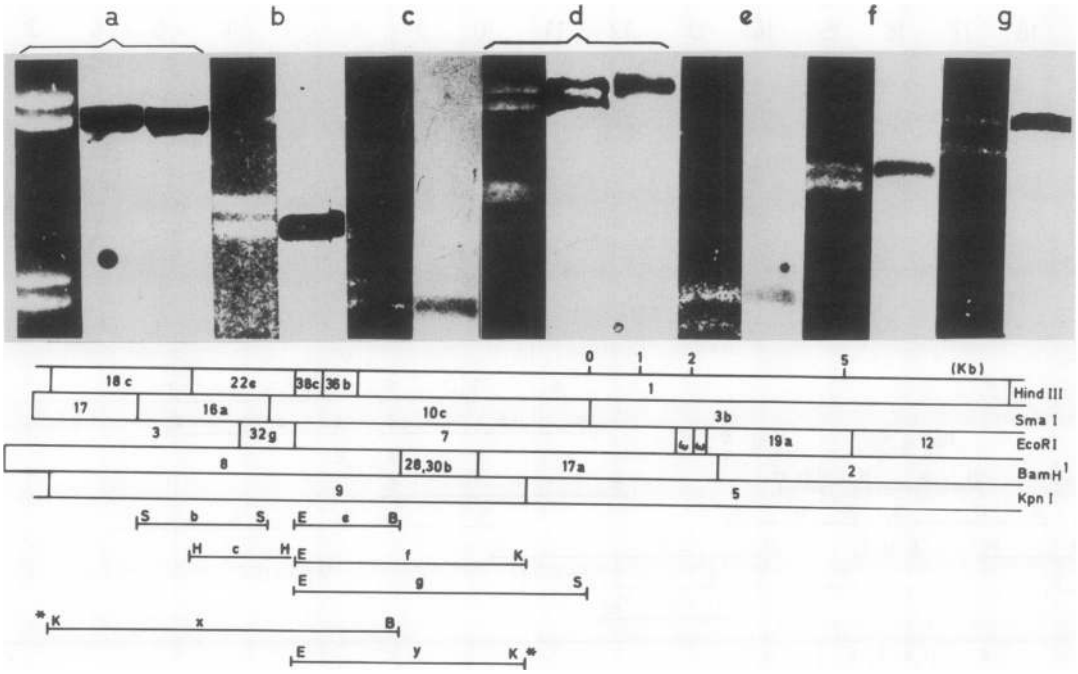

Fig. 3. Hybridization of poly A$^+$ RNA from the octopine tumor line A6-S1 against separated strands of different T-DNA fragments. Poly A$^+$ RNA was labelled at its 5'-end with polynucleotide kinase (see **Materials and methods**). Hybridization was against the separated strands of the following fragments: **group a**, BamHI fragment 8 (the lower bands originate from the vector pBR322); **group b**, SmaI fragment 16a; **group c**, HindIII fragment 22; **group d**, EcoRI fragment 7 (the lower band originates from the vector pBR325); **group e**, EcoRI-BamHI fragment e; **group f**, EcoRI-KpnI fragment f; **group g**, EcoRI-SmaI fragment g. The autoradiogram obtained after hybridization is always shown in the second lane of each group, the ethidium bromide stain of the separated strands is shown in the first lane. **Group a, lane 3** shows the hybridization of the KpnI-BamHI fragment x, labelled at its left 3'-end with terminal transferase, to BamHI fragment 8. **Group d, lane 3** shows the hybridization of the EcoRI-KpnI fragment y, labelled at its right 3'-end, to EcoRI fragment 7. The fragments used for hybridization and for determination of the chemical polarity have been illustrated under the partial map of pTi ACH5.

topine plasmid) (Figure 4, group b, lane 2) or of the total T-region from pTi C58 (nopaline plasmid) (Figure 4, group b, lane 1). Using an homologous nopaline T-region probe, four size classes of transcripts were visible with lengths of ~1600, 1200, 1100, and 900 nucleotides. These size classes comprise at least nine different transcripts (L. Willmitzer *et al.*, in preparation). The octopine TL-DNA probes specifically hybridized to three transcripts of 1200, 1100, and 900 nucleotides. These nopaline tumour transcripts therefore correspond in size to transcripts 4, 5, and 6 of octopine tumours.

To analyze whether these transcripts, which correspond in size, also originate from corresponding locations on the T-DNA, different subfragments of the nopaline Ti-plasmid C58, which are homologous with the TL-region of octopine Ti-plasmids, were hybridized to poly A$^+$ RNA isolated from the octopine tumour A6-S1 and the nopaline tumours C58-S1 and BT37. Transcript 6 of octopine tumours gave a strong hybridization signal with HindIII fragment 31. In both nopaline tumour lines (C58-S1 and BT37) a transcript of 900 nucleotides hybridized to this probe (Figure 4, group c). HindIII fragment 22 of pTi C58 hybridized strongly with transcripts 4 and 1 of octopine tumours and with the 1200 nucleotide transcript of nopaline tumours. In addition, transcript 6 of octopine tumours as well as the 900-base transcript of nopaline tumours is weakly visible (Figure 4, group d). Thus, the 900 and 1200 nucleotide transcripts of nopaline tissues which hybridize with octopine T-DNA probes have the same size as octopine transcripts 4 and 6 and originate from homologous sequences in the respective T-DNAs. We were unable to detect a counterpart of octopine transcript 1 in nopaline tumours which is unexpected because the TL-DNA segment coding for transcript 1 in octopine tumours is homologous to the corresponding region in nopaline T-DNA (Engler *et al.*, 1981). The expression of transcript 1 is either abolished or very much weaker in nopaline tumours than in octopine tumours. The pTi-C58 HindIII fragments 19 and 41, which represent the left part of the homology region, hybridized to transcripts 1 and 2 of octopine tumours. A very weak hybridization was also observed in the region of transcript 5 which indicates that transcript 5 might be only partially homologous to the HindIII fragments 19 and 41 of pTi C58. A 1100 nucleotide transcript in the poly A$^+$ RNA isolated from the nopaline tumours strongly hybridized with these probes. This transcript corresponds in size to transcript 5 of octopine tumours. In addition a 1600 nucleotide transcript of lower abundance was detected (Figure 4, group e), which had escaped detection with a total octopine T-region probe. This finding might be explained by the much lower concentration of this transcript compared with the 1200, 1100, and 900 nucleotide transcripts, but our data do not unambiguously establish whether the 1600 nucleotide transcript of nopaline tumours corresponds to transcript 2 of octopine tumours. The same holds true for the 1100 nucleotide transcript of nopaline tumours and transcript 5 of octopine tumours. However, two independent results support the idea that transcripts 2 and 5 of octopine tumours have their counterparts in the nopaline tumour transcripts of 1600 and 1100 nucleotides respectively. Firstly, a more precise mapping of both nopaline transcripts has shown that the 1100 nucleotide transcript is located to the left of the 1600 nucleotide transcript, i.e., they possess the same relative order as transcripts 5 and 2 of octopine tumour. Secondly, inactivation of the region coding for the 1600 nucleotide transcript in nopaline tumours leads to the development of shoots from the corresponding tumours (unpublished data) and this same phenotype arises in octopine tumours on inactivation of

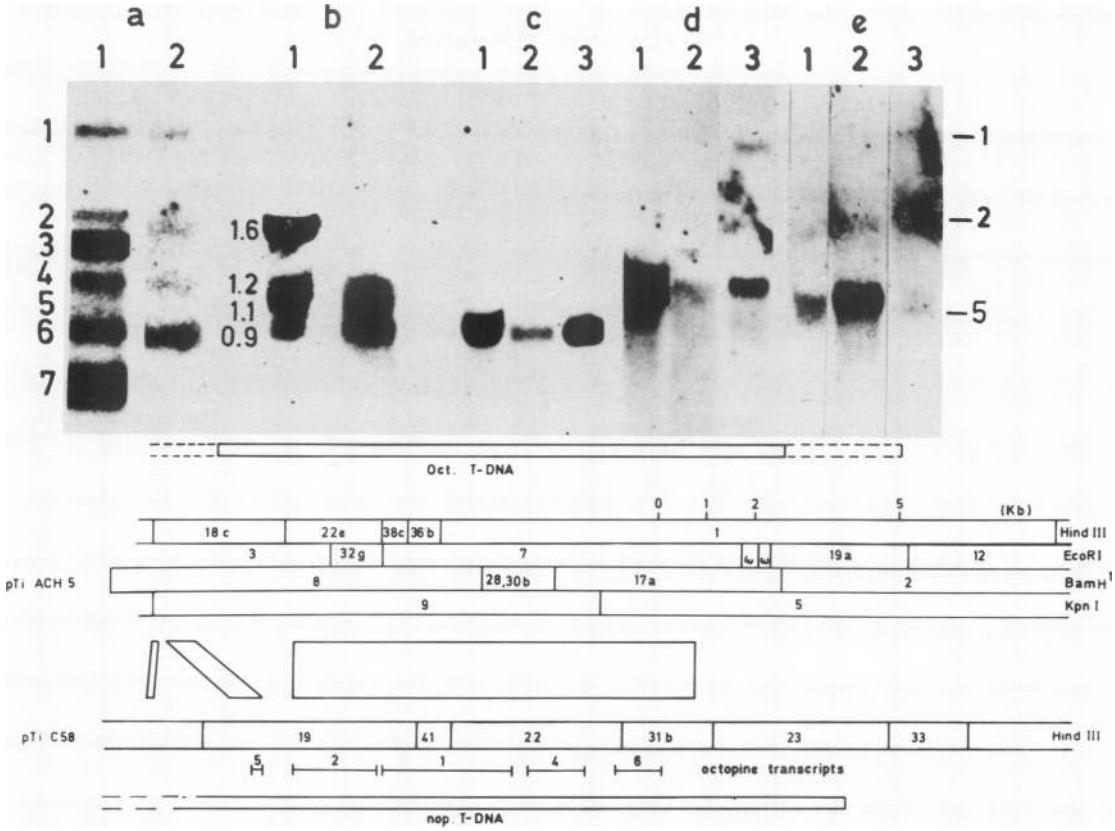

Fig. 4. Homologous and heterologous hybridization of octopine and nopaline T-region DNA fragments against poly A$^+$ RNAs isolated from the octopine crown gall tumour A6-S1 and the nopaline crown gall lines C58-S1 and BT37. **Group a**: driver, poly A$^+$ RNA (A6-S1); *probe,* lane 1: total octopine TL-region; lane 2: total nopaline T-region. **Group b**: driver, poly A$^+$ RNA (BT37) *probe,* lane 1: total nopaline T-region; lane 2: total octopine TL-region. **Group c, d,** and **e**: driver, poly A$^+$ RNA from BT37 (lane 1), C58-S1 (lane 2), A6-S1 (lane 3); *probe,* **group c**: HindIII fragment 31 of pTiC58, **group d**: HindIII fragment 22 of pTiC58, **group e**: HindIII fragments 19 + 41 of pTiC58. **Below**: partial map of the octopine pTi ACH5 and the nopaline pTiC58 indicating the extent of the respective T-regions. The homologous part of both T-regions is indicated by the boxes drawn between both maps (Engler *et al.*, 1981). The position of the octopine transcripts on the nopaline T-region obtained by superimposing the conserved regions of the octopine and nopaline T-regions.

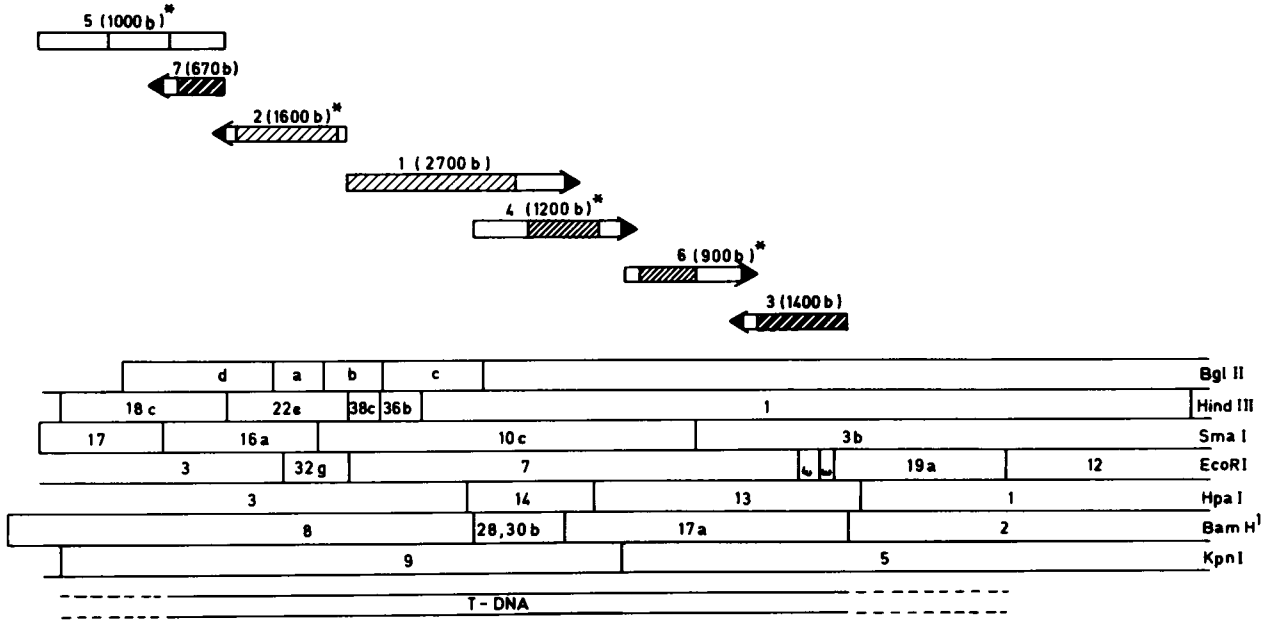

Fig. 5. Size, location, and direction of transcription of octopine TL-DNA derived polyadenylated transcripts. The location of each transcript is indicated by a separate box, the **shaded area** indicates size and the most probable map position of transcripts under the untested assumption that no splicing occurs; the intensity of shading is proportional to the relative concentration of each transcript. The size in bases is given for each transcript, these numbers are not corrected for the length of the poly A tail. The 5′ −3′ polarity is indicated by an arrow. Transcripts which probably have functional counterparts in nopaline crown galls are marked by an asterisk. Transcript 1, although hybridizing strongly to nopaline T-DNA probes, did not appear to have an homologous counterpart in nopaline crown galls. For the final location of transcript 6 and 3 the fact was taken into account that transcript 3 originates from the non-homologous region whereas transcript 6 arises from the homologous region of octopine and nopaline T-DNA's. The lower part shows a partial map of pTi ACH5 as well as the extent of the TL-DNA in the octopine line A6-S1 (De Beuckeleer *et al.*, 1981).

transcript 2 (see accompanying paper, Leemans *et al.*).

Discussion

Crown gall tumours are caused by the transfer and stable maintenance in the nucleus of transformed plant cells, of a defined segment (T-DNA) of the Ti-plasmid. Since T-DNA sequences are transcribed, it was important to determine whether or not these transcripts are mRNAs. Different approaches have been used to answer this question. We have made a detailed analysis of the number, size, and location of T-DNA transcripts (Figure 5). Seven distinct transcripts were detected in the poly A^+ fraction of RNA from the octopine producing crown gall line A6-S1 which contains only the TL fragment of the octopine T-region (Thomashow *et al.*, 1980a; De Beuckeleer *et al.*, 1981). These transcripts differ markedly in their relative abundance and vary in size from 2700 to 670 nucleotides. They all bind to an oligo-dT cellulose column, indicating that they are at least partially polyadenylated and hence, that the T-DNA, although of a prokaryotic origin, must provide specific poly A addition sites. We have determined the polarity of six of the seven transcripts and have approximately located their respective 5′ and 3′ ends on the physical map of the TL-DNA.

All seven transcripts mapped within the TL-DNA sequence. This observation and the inhibition of T-DNA transcription by low concentrations of α-amanitin (Willmitzer *et al.*, 1981b), may mean that each transcript is determined by a specific promotor site recognized by host RNA polymerase II. Alternatively, a group of transcripts may use a common promotor site on the T-DNA, and then be processed to provide independent 5′ and 3′ ends. Transcription studies with isolated nuclei (Willmitzer *et al.*, 1981a) make the theoretical possibility that transcription of T-DNA sequences proceeds by readthrough from host DNA promotor sequences, unlikely. Willmitzer *et al.*, found that different parts of the T-DNA are reproducibly transcribed to different degrees, whereas a readthrough mechanism should have given at most two different levels of transcripts in isolated nuclei. The data presented here do not rule out that some T-DNA promotors serve for the transcription of more than one RNA. Considering the groupwise orientation of transcripts 7 and 2 as well as 1, 4, and 6, the simplest model would assume one promotor per group of transcripts. If so, one would expect that inactivation of a 5′-proximal transcript of one group would lead to the disappearance of the 5′-distal transcripts. Analysis of mutant lines, however, indicates that the expression of 5′-distal transcripts is independent of the expression of 5′-proximal transcripts (see accompanying paper, Leemans *et al.*). Thus, most of the transcripts seem to have an independent promotor sequence as well as independent 3′ poly A addition sites.

Using probes derived from the T-region of nopaline Ti-plasmids, four of the seven octopine TL-DNA transcripts (1, 2,4, and 6) were found to hybridize. These transcripts are coded by the T-region sequences which are homologous in octopine and nopaline Ti-plasmids. This conservation of genes argues that they have important functions. The very low level of cross-hybridization with transcript 5 could be due to its very low abundance and/or only partial homology (Depicker *et al.*, 1978; Chilton *et al.*, 1978; Engler *et al.*, 1981). Transcripts 3 and 7, the most abundant TL-DNA transcripts, do not hybridize with nopaline T-region probes and must therefore code for functions specific for octopine tumours. For transcript 3 this was proved directly by another approach. Figure 5, shows that transcript 3 completely covers two small EcoRI fragments (called ω). These fragments, which specifically hybridize to transcript 3, were therefore used (Schröder and Schröder, submitted) to isolate transcript 3 from mRNA preparations from octopine tumours A6-S1 and B6S3-S1. Transcripts gave rise, in an *in vitro* wheat germ translation system, to a 39 kd protein which was immunoprecipitated by antibody against LpDH, the octopine synthesizing enzyme. Transcript 3 is, therefore, the mRNA coding for LpDH. As expected, crown galls, induced by Ti-plasmids from which the small EcoRI ω fragments together with EcoRI fragment 19 were deleted, do not synthesise octopine (Leemans *et al.*, 1981). The function of transcript 7, the second "octopine specific" transcript remains unknown. *In vitro* translation of RNAs, selected by hybridization to BamHI fragment 8, yields a protein of mol. wt. 14 500 (McPherson *et al.*, 1980). As transcript 7 is the most prominent transcript coded for by BamHI fragment 8 we may assume that transcript 7 codes for this 14.5 kd protein. More direct evidence for this has recently been obtained by *in vitro* translation of hybrid-selected RNAs using HindIII fragment 18c as a probe (Schröder and Schröder, submitted). Tumours induced by Ti-plasmid mutants with Tn5 inserts in the TL-DNA segment from which transcript 7 is transcribed were normal with respect to morphology and opine synthesis (Garfinkel *et al.*, 1981) and gave no clue as to the function of the 14.5 kd protein.

The function of the octopine TL-DNA transcripts coded by the DNA sequences that are totally or largely conserved in both octopine and nopaline crown galls, i.e., trancripts 1, 2, 4, 5, and 6, has been studied by constructing T-DNA mutations resulting in the specific elimination of one or of a combination of these transcripts from octopine crown gall tumours. These studies (see accompanying paper, Leemans *et al.*) indicate that transcripts 1 and 2 and possibly 5 are involved in the negative control of shoot formation, whereas transcript 4 negatively controls root formation. Teratoma-like shoots appear to be prevented from developing further to normal shoot and root-forming plants by transcript 6. A direct analysis of the functions is complicated by the very low concentrations of these transcripts and of some of their resulting proteins in crown gall tissues (Willmitzer *et al.*, 1981a; Schröder and Schröder, submitted). Expression of these functions in *Escherichia coli* minicells is, therefore, being attempted (Schröder *et al.*, 1981b). The Ti-plasmid-*A. tumefaciens* system provides a natural host-vector system for introducing foreign DNA into plants. DNA, inserted into the T-region is co-transferred into the nucleus of the plant cells (Hernalsteens *et al.*, 1980; Leemans *et al.*, 1981) where it is stably maintained. Using mutated Ti-plasmids the regeneration of normal fertile plants from tumours and the Mendelian transmission of T-DNA genes through meiosis is possible (Otten *et al.*, 1981; De Greve *et al.*, submitted). The data reported here support the idea that the T-DNA contains several promotor sites active in the plant cell; it may be possible to use these to express foreign DNAs in plant cells.

Materials and methods

Description of the plant tissue cultures

A6-S1: octopine tumour line induced on *Nicotiana tabacum* var. White Burley by *A. tumefaciens* strain A6. NW-S1: untransformed tobacco line (*N. tabacum* var. White Burley) selected for phytohormone autotrophy. C58-S1: nopaline crown gall tumour incited on *N. tabacum* var. White Burley by *A.*

tumefaciens strain C58. BT37: nopaline line incited on *N. tabacum* var. Havanna by *A. tumefaciens* strain T37.

BT37, A6-S1, and NW-S1 cells were cultured in liquid, C58-S1 cells on solidified (1.0% agar) hormone-free medium (Linsmaier and Skoog, 1965). The cells were cultured at 27°C, with a day-night rhythm of 16 h day (3000 lux)/8 h night.

Electrophoresis of RNA and transfer to DBM paper

Isolation of total RNA and selection of the poly A^+ fraction was as described previously (Willmitzer *et al.*, 1981a). After denaturation with glyoxal/dimethylsulfoxide poly A^+ RNA was separated by electrophoresis in 1.2% agarose gels (McMaster and Carmichael, 1977). Subsequent transfer to DBM paper and pretreatment prior to hybridization was performed according to published procedures (Alwine *et al.*, 1979). Usually 50 – 150 μg poly A^+ RNA were applied per lane.

Hybridization of DNA to RNA coupled to DBM filters

DNA fragments used were either available as fragments cloned in pBR322 (de Vos *et al.*, 1981; Depicker *et al.*, 1980) or were isolated from agarose gels after restriction endonuclease digestion of cloned T-region segments and separation of the fragments by electrophoresis (Tabak and Flavell, 1978). DNA fragments were labelled by nick translation (Rigby *et al.*, 1977) to specific activities of $1-2 \times 10^8$ c.p.m./μg. Hybridization was performed in sealed plastic bags for 3 days at 42°C in a buffer containing 50% formamide, 0.9 M NaCl, 50 mM NaH_2PO_4 pH 7.0, 5 mM EDTA, 10 x Denhardt (0.2% polyvinylpyrrolidon, 0.2% bovine serum albumin, 0.2% ficoll), 500 μg/ml denatured sonicated salmon sperm DNA, 30 μg/ml *E. coli* chromosomal DNA. Filters were subsequently washed at 70°C in 3 x SSC (1 x SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.5% SDS for 1 – 2 h with several changes and subsequently at 60°C in 0.1 x SSC/0.1% SDS (corresponding to $T_m - 15$°C) for 1 h with one change. After drying filters were exposed to Kodak X-omat XR-5 films using Kodak intensifying screens for 2 – 10 days.

Separation of complementary DNA strands and hybridization with labelled RNA

The isolated DNA fragments were denatured in 0.2 N NaOH and directly applied to a second, strand-separating electrophoresis in 0.9% agarose gels in a low-salt buffer (6 mM Tris-HCl pH 7.7, 7 mM NaH_2PO_4, 0.2 mM EDTA) at 4°C at a voltage of 3V/cm (Perlman and Huberman, 1977). After staining with ethidium bromide, DNA was blotted onto nitrocellulose filters (Southern, 1975). Labelling of poly A^+ RNA by polynucleotide kinase and hybridization to DNA cellulose filters were as described previously (Willmitzer *et al.*, 1981).

Determination of the chemical polarity of separated DNA strands

Probes for determining the chemical polarity of DNA strands were obtained as follows: BamHI fragment 8 as well as EcoRI fragment 7 of pTi ACH5 contain only one assymetric KpnI site (cf. Figure 5). Both fragments are available cloned in pBR322 respectively pBR325. As both vectors do not contain any additional KpnI site, cleavage of the respective DNA clones with KpnI will result in linearization of both plasmids. This linear DNA contains a KpnI site with 3'-overlapping ends on each side, which are easily labelled by terminal transferase. After labelling, and ethanol precipitation DNA is cleaved a second time using BamHI and EcoRI, respectively. Thus, the vector will be excised from both linearized DNAs leaving two T-DNA fragments which are labelled at only one 3'-OH end. Due to the assymetric location of the KpnI site the labelled fragments can easily be separated by gel electrophoresis and used as probes for hybridization against the separated DNA strands after isolation from the gel.

Labelling of the 3'-OH ends of DNA fragments with terminal transferase

Labelling of the 3'-OH ends of DNA fragments with terminal transferase was performed in 100 mM Na cacodylate pH 7.0, 0.2 mM dithiothreitol, 1 mM $CoCl_2$ containing 20 μCi α-[^{32}P]dCTP (specific activity: 2 – 3000 Ci/mMol) (Deng and Wu, 1981).

Acknowledgements

We thank W. Schmalenbach for excellent technical assistance, Ms. M.Kalda for photographic work and Ms. E.Schölzel and Mrs. E.Kath for preparing the manuscript. The authors express their sincere gratitude to their collaborators at the Department of Genetics, Rijksuniversiteit Gent and at the unit for Genetische Virologie VV Brussel, who provided cloned Ti-plasmid fragments, good advice and stimulating discussions.

References

Alwine,J.C., Demp,D.J., Parker,B.A., Reiser,J., Renart,J., Stark,G.R., and Wahl,G.M. (1979) in Wu,R. (ed.), *Recombinant DNA, Methods in Enzymology*, **68**, Academic Press, NY, pp. 220-242.

Braun,A.C. (1956) *Cancer Res.*, **16**, 53-56.

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.

Chilton,M.-D., Saiki,R.K., Yadav,N., Gordon,M.P., and Quetier,F. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 4060-4064.

De Beuckeleer,M., Lemmers,M., De Vos,G., Willmitzer,L., Van Montagu, M., and Schell,J. (1981) *Mol. Gen. Genet.*, **183**, 283-288.

Deng,G., and Wu,R. (1981) *Nucleic Acids Res.*, **9**, 4173-4188.

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.

Drummond,M.H., Gordon,M.P., Nester,E.W., and Chilton,M.-D. (1977) *Nature*, **269**, 535-536.

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., Gordon,M.P., Nester,E.W., and Aronson,A.I. (1981) *Plasmid*, **6**, 17-29.

Gurley,W.B., Kemp,J.D., Albert,M.J., Sutton,D.W., and Callis,J. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 2828-2832.

Guyon,P., Chilton,M.-D., Petit,A., and Tempé,J. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 2693-2697.

Hernalsteens,J.P., Van Vliet,F., De Beuckeleer,M., Depicker,A., Engler,G., Lemmers,M., Holsters,M., Van Montagu,M., and Schell,J. (1980) *Nature*, **287**, 654-656.

Leemans,J., Shaw,C., Deblaere,R., De Greve,H., Hernalsteens,J.P., Maes, M., Van Montagu,M., and Schell,J. (1981) *J. Mol. Appl. Genet.*, **1**, 149-164.

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.

Linsmaier,E.M., and Skoog,F., (1965) *Physiol. Plant.*, **18**, 100-127.

McMaster,G., and Carmichael,G. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 4835-4838.

McPherson,J.C., Nester,E.W., and Gordon,M.P. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 2666-2670.

Otten,L., De Greve,H., Hernalsteens,J.P., Van Montagu,M., Schieder,O., Straub,J., and Schell,J. (1981) *Mol. Gen. Genet.*, **183**, 209-213.

Perlman,D., and Huberman,J.A., (1977) *Anal. Biochem.*, **83**, 666-677.

Rigby,P.W.W., Dieckmann,M., Rhodes,C., and Berg,P. (1977) *J. Mol. Biol.*, **113**, 237-251.

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. Lond., B*, **204**, 251-266.

Schröder,J., Schröder,G., Huisman,H., Schilperoort,R.A., and Schell,J. (1981a) *FEBS Lett.*, **129**, 166-168.

Schröder,J., Hillebrandt,A., Klipp,W., and Pühler,A. (1981b) *Nucleic Acids Res.*, **9**, 5187-5202.

Southern,E.M. (1975) *J. Mol. Biol.*, **98**, 503-518.

Tabak,H.F., and Flavell,R.A. (1978) *Nucleic Acids Res.*, **5**, 2321-2332.

Thomashow,M.F., Nutter,R., Montoya,A.L., Gordon,M.P., and Nester, E.W. (1980a) *Cell*, **19**, 729-739.

Thomashow,M.F., Nutter,R., Postle,K., Chilton,M.-D., Blattner,F.R., Powell,A., Gordon,M.P., and Nester,E.W. (1980b) *Proc. Natl. Acad. Sci. USA*, **77**, 6448-6452.

Van Larebeke,N., Engler,G., Holsters,M., Van den Elsacker,S., Zaenen,I., Schilperoort,R.A., and Schell,J. (1974) *Nature*, **252**, 169-170.

Watson,B., Currier,T.C., Gordon,M.P., Chilton,M.-D., and Nester,E.W. (1975) *J. Bacteriol.*, **123**, 255-264.

Willmitzer,L., De Beuckeleer,M., Lemmers,M., Van Montagu,M., and Schell,J. (1980) *Nature*, **287**, 359-361.

Willmitzer,L., Otten,L., Simons,G., Schmalenbach,W., Schröder,J., Schröder,G., Van Montagu,M., De Vos,G., and Schell,J. (1981a) *Mol. Gen. Genet.*, **182**, 255-262.

Willmitzer,L., Schmalenbach,W., and Schell,J. (1981b) *Nucleic Acids Res.*, **9**, 4801-4812.

Yadav,N.S., Postle,K., Saiki,R.K., Thomashow,M.F., and Chilton,M.-D. (1980) *Nature*, **287**, 458-461.

Zaenen,I., Van Larebeke,N., Teuchy,H., Van Montagu,M., and Schell,J.

(1974) *J. Mol. Biol.*, **86**, 109-127.
Zambryski,P., Holsters,M., Kruger,K., Depicker,A., Schell,J., Van Montagu,M., and Goodman,H.M. (1980) *Science (Wash.)*, **209**, 1385-1391.