Mol Gen Genet (1982) 186:16–22

MGG

DNA from *Agrobacterium rhizogenes* is Transferred to and Expressed in Axenic Hairy Root Plant Tissues

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Summary. Axenic root tissue cultures were established from primary hairy roots induced on carrot and potato by *Agrobacterium rhizogenes* strain 15834. cDNA made towards poly-A^+ RNA isolated from these tissues, hybridized with a limited number of well-defined fragments of the plasmid DNA present in the inciting *A. rhizogenes* strain. These data therefore demonstrate that at least part of the root-inducing (Ri) plasmid of *Agrobacterium rhizogenes* is transferred, stably maintained and expressed in hairy-root plant tissues and confirm that hairy roots are a special type of crown gall. The T-DNA in hairy-root cells appears to have several regions which are related in terms of sequence homology and probably also function to the T-DNA in octopine and nopaline crown gall tumours.

Introduction

The molecular mechanism responsible for tumorous growth of crown gall plant cells has recently been elucidated.

Ti-plasmids in *Agrobacterium tumefaciens* (Zaenen et al. 1974; Van Larebeke et al. 1974; Watson et al. 1975) are central in this process because they determine the transfer and integration of a specified DNA segment (the so-called T-region) of the Ti-plasmid into the nuclear DNA of the transformed plant cells (Chilton et al. 1977; Schell et al. 1979; Lemmers et al. 1980; Zambryski et al. 1980; Thomashow et al. 1980; Chilton et al. 1980; Willmitzer et al. 1980). The Ti-plasmid derived DNA sequence (the so-called T-DNA) is transcribed (Drummond et al. 1977; Gurley et al. 1979; Willmitzer et al. 1981a; Gelvin et al. 1981) by host RNA polymerase II (Willmitzer et al. 1981b) and at least partially translated (McPherson et al. 1980; Schröder et al. 1981; Schröder and Schröder 1982) in plant cells. Fine analysis of the different transcripts in octopine and nopaline tumours has shown the T-DNA to code for seven and nine well-defined polyadenylated transcripts respectively (Willmitzer et al. 1982; Willmitzer et al. in preparation). The functions of the different genes (transcripts) can be divided into two groups (Leemans et al. 1982):
– one group of transcripts directly and/or indirectly prevents differentiation of plant cells. Some of these transcripts prevent shoot formation while others prevent root formation
– another group of transcripts codes for enzymes involved in the synthesis of low-molecular-weight compounds called opines, which can be metabolized specifically by the tumour-inciting bacterium (for recent discussion see Guyon et al. 1980).

The hairy-root disease of a number of dicotyledonous plants is caused by *Agrobacterium rhizogenes*, a close relative of *Agrobacterium tumefaciens*.

The hairy-root disease is analogous to the crown gall disease in the following aspects:
– large plasmids (so-called root-inducing (Ri) plasmids) in *Agrobacterium rhizogenes* strains have been shown to be necessary for induction of hairy roots (Moore et al. 1979; White and Nester 1980a),
– the plant cells are forced into a defined developmental pathway, i.e. the formation of roots. It should be noted in addition that on several host plants *A. rhizogenes* produces crown gall like tumours rather than hairy roots (De Cleene and De Ley 1981),
– axenic hairy-root tissues produce agropine (Tepfer and Tempé 1981), an opine first discovered in octopine crown gall tumours (Firmin and Fenwick 1978).

These similarities have prompted us to test whether the formation of hairy roots is due to a similar molecular mechanism, i.e. the transfer to – and the expression of – a segment of the *A. rhizogenes* plasmid in the hairy-root cells. The results presented here based on the analysis of RNA present in axenic hairy-root tissues, support this hypothesis. After this work was completed independent evidence for a DNA transfer from *A. rhizogenes* 8196 to carrot hairy-root tissue was published (Chilton et al. 1982).

Materials and Methods

Isolation and Characterization of Plasmid DNA from Agrobacterium tumefaciens and Agrobacterium rhizogenes. Plasmid DNA was isolated using an alkaline denaturation-renaturation step essentially as described elsewhere (Casse et al. 1979).

Plasmid preparation were analyzed by electrophoresis in 0.7% agarose using 0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA pH 8.3 as buffer.

Description of Crown Gall Tumour Lines. 1GV7: octopine type crown gall tumour induced on *Nicotiana tabacum* cv

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Wisconsin by *A. tumefaciens* carrying pTi2208 (Leemans et al. 1981)

1GV281: agropine type crown gall tumour induced on *N. tabacum* cv Wisconsin by *A. tumefaciens* strain A281

1GV23: octopine type crown gall tumour induced on *N. tabacum* cv Wisconsin by *A. tumefaciens* carrying pTi2206 (Leemans et al. 1981).

Description of Hairy-Root Plant Tissues. Primary hairy roots induced on potato tuber and carrot slices were cut off after they reached a length of 2–3 cm. They were cultured on solidified (1% agar) hormone-free medium (Linsmaier and Skoog 1965) in the dark at 25° C. Carbenicillin (1 mg/ml) was added to the medium for the first three to four passages. Bacteria-free tissue cultures were grown further in the absence of carbenicillin either in liquid or on solidified hormone-free medium and regularly transferred every four to six weeks.

The experiments were performed with the following two lines: P15834J: hairy-root tissue induced on potato tubers by *Agrobacterium rhizogenes* strain 15834 (january 1981). C15834J: hairy-root tissue induced on carrot slices by *A. rhizogenes* strain 15834 (april 1981).

Detection of Agropine. Plant tissue extracts were analyzed for the presence of agropine as described (Tepfer and Tempé 1981).

Analysis of Transcripts Present in Hairy-Root Tissues. Isolation of RNA, cDNA synthesis, digestion of plasmid DNA and blotting were performed as described previously (Willmitzer et al. 1981a). Hybridization was performed 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, 0.2% polyvinylpyrrolidon, 0.2% bovine serum albumin, 0.2% ficoll, 500 µg/ml denatured salmon sperm DNA. Filters were subsequently washed at 68° C for 1–2 h in 3 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M Na-citrate), 0.5% SDS with several changes. After drying, filters were exposed for autoradiography.

Hybridization of Ri-Plasmid DNA Against Octopine and Nopaline T-Region DNA. T-region fragments of the octopine plasmid pTiACH5 were obtained by digestion of plasmid DNA's with different restriction enzymes from pGV0201, pGV0153 and pGV120 [containing respectively *Hin*dIII fragment I, *Bam*H1 fragment 8 and *Bam*H1 fragment 2 of pTiACH5 (De Vos et al. 1981)]. T-region fragments of the nopaline plasmid pTiC58 were obtained by digestion of plasmid DNA's from pGV0369, pGV0351 and pGV0327 [containing respectively *Hin*dIII fragments 10, 15 and 14b of pTiC58 (Depicker et al. 1980)]. Restriction fragments were transferred to nitrocellulose filters and subsequently hybridized with nick-translated Ri-plasmid DNA.

Hybridizations were performed in the same buffer as described for cDNA-DNA hybridizations (cf above). Filters were subsequently washed for 2 h in 3 × SSC, 0.5% SDS at 70° C with 3–4 changes and for 1 h in 0.1 × SSC/0.1% SDS at 45° C (equivalent to $T_m - 30°$ C) with two changes. Filters were subsequently exposed to Kodak intensifying screens for 2–4 days.

Results

Description of the Hairy-Root Tissues

Carrot and potato discs were inoculated with a bacterial suspension of the inciting agent, i.e. *Agrobacterium rhizogenes* strain 15834. Our observation that this strain contains three plasmids with approximately sizes of respectively 90, 150 and $240 \cdot 10^6$ d confirms published data (White and Nester 1980a).

Two to four weeks after inoculation in the dark, massive amounts of roots developed on the carrot and potato slices. Bacteria-free, axenic cultures were initiated from 2–3 cm long roots as described in Materials and Methods. After three to four passages the roots were transferred to liquid or solidified media without carbenicillin and left in the dark. These root cultures gave vigorous growth (Fig. 1). No bacterial growth could be seen by visible inspection of the cultures, neither did plating of macerated cultures on rich medium produce any bacterial growth.

Although the vast majority of the plant tissues kept growing as roots, the formation of undifferentiated callus-like tissue was occasionally observed on carrots.

Carrot roots transformed by *Agrobacterium rhizogenes* line A4 contain agropine (Tepfer and Tempé 1981). We therefore used paper electrophoresis to test for agropine in our root tissue. The electropherogram (Fig. 2) shows the presence of agropine as well as the presence of a second compound with a lower mobility which was also detected in two octopine tumour line 1GV7 and 1GV23 and in an agropine tumour line 1GV281. This compound, which was not detected in non-transformed tissues, was first observed by J. Tempé and is thought to represent the open chain precursor of agropine called mannopine (Tate et al. 1982).

DNA Homologous to the Plasmids of Agrobacterium rhizogenes Strain 15834, is Expressed in Axenic Root Tissue Cultures

If hairy-root tissues are produced by a mechanism similar to the formation of crown galls by *Agrobacterium tumefaciens*, RNA of transformed roots should contain Ri-plasmid derived transcripts which would be expected to be partially poly-adenylated (Willmitzer et al. 1981a). To assess this question, total RNA was isolated from axenic hairy-root tissues of potato and carrot as well as from untransformed potato roots. By reverse transcription, using oligo-dT as primer, radioactive cDNA probes were obtained for the poly-A^+ population of the RNA's. These were subsequently used for hybridization against total plasmid DNA isolated from strain 15834 digested by *Sma*I.

The result is shown in Fig. 3 lanes a–d. Strong hybridization was observed to a well-defined and limited number of plasmid bands with RNA from both transformed potato (lane b) and transformed carrot (lane c) roots. No hybridization was visible using RNA from untransformed potato roots (lane d). The same amount of radioactivity was offered in both potato experiments. In addition the same pattern of hybridization was observed irrespective of whether the RNA was isolated from potato or carrot hairy-root tissue.

These data therefore demonstrate that the hairy roots formed upon inoculation of certain plants with *Agrobacterium rhizogenes* do contain and transcribe Ri-plasmid derived sequences into poly-adenylated RNA.

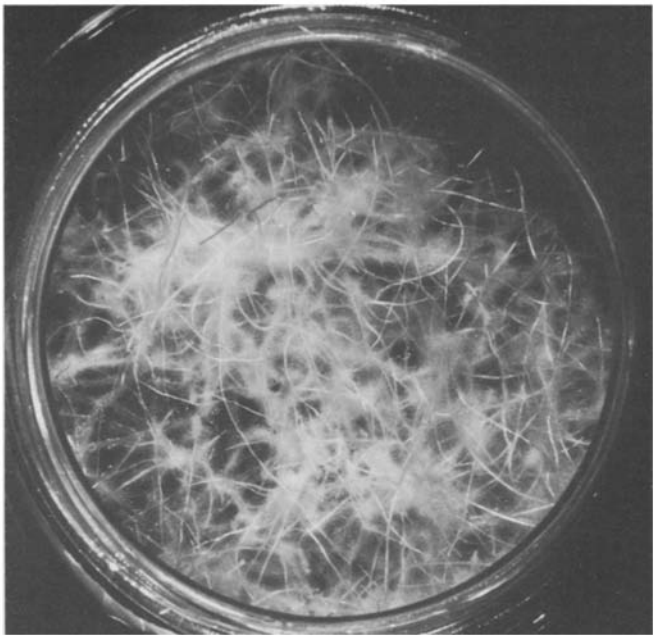

Fig. 1. Axenic hairy-root tissues obtained from primary hairy roots incited on potato by *Agrobacterium rhizogenes* strain 15834

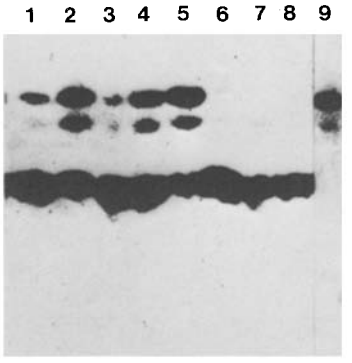

Fig. 2. Presence of agropine in hairy-root tissues. Plant cell extracts were analyzed by paper electrophoresis for the presence of agropine as described in Materials and Methods. 5 µl of extract were applied per lane. Extracts from the following tissues culture lines were applied: lane 1: 1GV23 (agropine producing octopine tumour); lane 2: 1GV7 (agropine producing octopine tumour); lane 3: carrot hairy-root tissue C15834J; lane 4: potato hairy-root tissue P15834J; lane 5: 1GV281 (agropine tumour); lane 6: untransformed, habituated tobacco tissue; lane 7: untransformed carrot; lane 8: untransformed potato roots; lane 9: authentic agropine

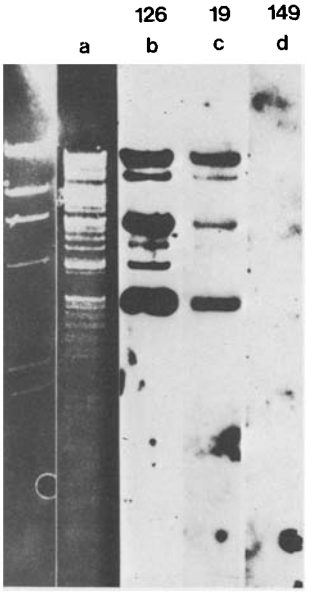

Fig. 3. Hybridization of cDNA made towards total RNA isolated from hairy-root tissues (lane b, c) and untransformed tissue (lane d) against total plasmid DNA from *Agrobacterium rhizogenes* strain 15834. cDNA obtained by oligo dT primed reverse transcription of total RNA preparations from untransformed potato roots (lane d), and the axenic hairy-root tissue lines C15834J (carrot, lane c) and P15834J (potato line, lane b) was hybridized against a nitrocellulose filter containing a *Sma*I digest of total plasmid isolated from *Agrobacterium rhizogenes* strain 15834, the pattern of which is shown in lane a. The size markers to the left indicate the position of a λ/*Hin*dIII digest. The amount of radioactivity offered in each experiment is indicated above lane b–d ($\times 10^6$ cpm)

The size of the transferred DNA can be estimated roughly by adding up the molecular weights of all the DNA-fragments which hybridize with the RNA from transformed tissues. This leads to a molecular weight of about 40–50 kb which is double the molecular weight of the T-region of nopaline plasmids (Lemmers et al. 1980). However, assuming that the transferred DNA is integrated into the plant host genome, at least two of the DNA fragments should represent plant DNA-T-DNA junctions. This could reduce the estimated size by as much as 20 kb thus reaching the size of T-DNA's of octopine and nopaline tumours. The amounts of T-DNA derived transcripts in transformed root tissue were estimated by determining the ratio of specifically hybridized transcripts (radioactivity) to the amount of total radioactivity offered for hybridization. Our data indicate that 0.0006–0.002% of total poly-A^+ transcripts are derived from the transferred part of the Ri-plasmid. This is roughly the same amount as found in octopine crown gall tumours for T-DNA derived transcripts (0.0006–0.001% of the total poly-A^+ RNA population) (Willmitzer et al. 1981 a).

Some of the T-DNA Transcripts in Hairy-Root Tissues are Related by Sequence Homology to T-DNA Present in Octopine and/or Nopaline Crown Gall Tumours

In crown gall tumours T-DNA codes for two categories of functions (cf Introduction), opine synthesis and suppression of normal plant development. In view of the similarities between crown galls and hairy root equivalent functions are expected to be determined by Ri-derived T-DNA sequences. The question therefore arises whether these functions are coded for by homologous DNA sequences in both crown galls and hairy roots. Concerning opine synthesis the gene(s) involved in agropine synthesis represents an obvious candidate to look for homologous DNA sequences. As for functions acting to suppress plant development the following reasoning was applied: Tissues induced by *A. rhizogenes* on carrot, potato or tobacco grow as roots. This might be due either to an active principle inducing root development or to a specific suppression of shoot formation or to a combination of both mechanisms.

The physical and genetic analysis of octopine (Willmitzer et al. 1982; Leemans et al. 1982) and nopaline tu-

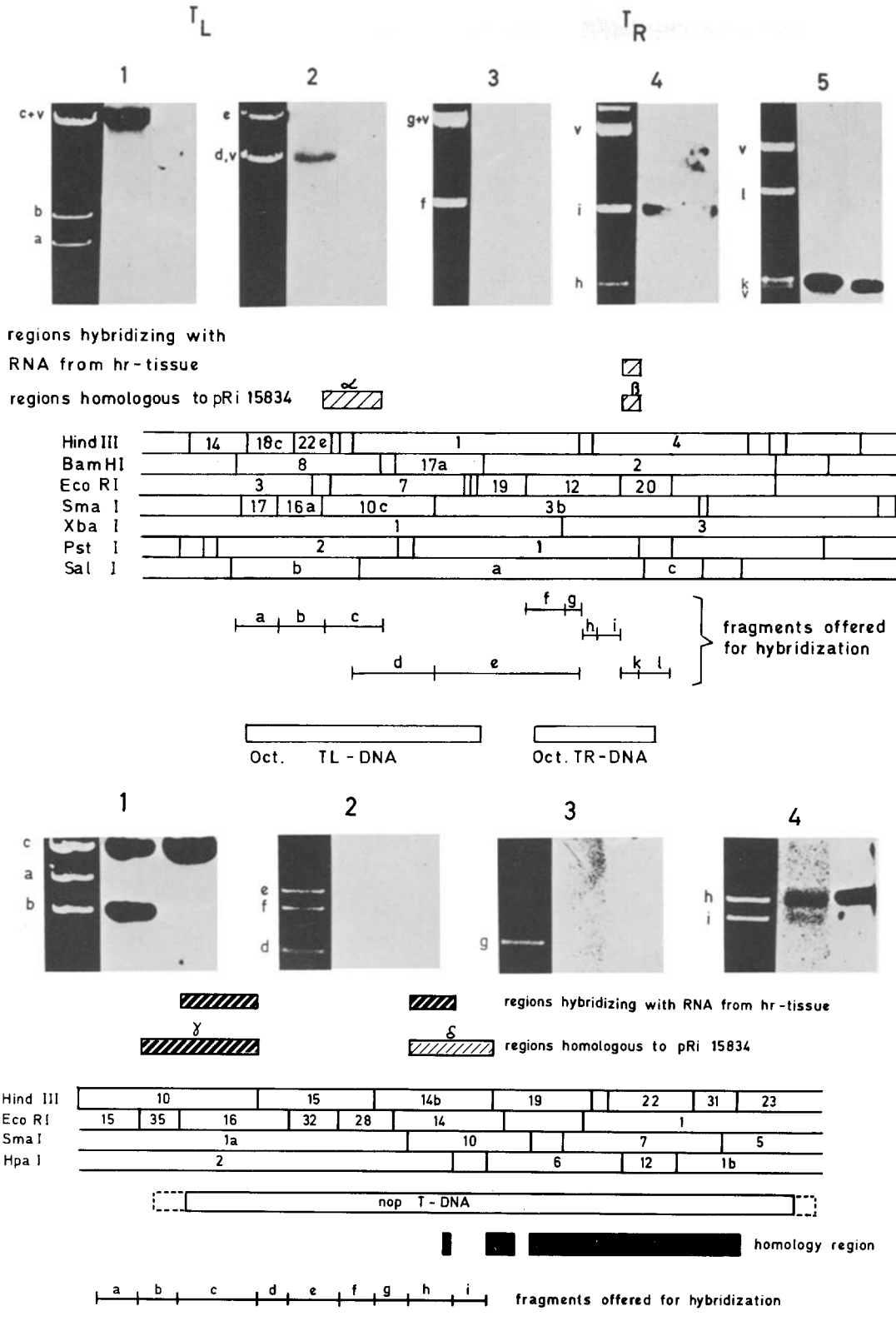

Fig. 4. Hybridization of pRi15834 (second lane of each group) and cDNA made towards poly-A$^+$ RNA present in hairy-root tissue line P15834J (third lane of each group) towards fragments covering the total TL- and TR-region of the octopine plasmid pTiACH5. Lane 1 shows the fragments of pTiACH5 offered for hybridization; the lettering refers to the letters in the lower part of the figure showing the location of the different fragments; v=pBR322. The lower part of the figure shows a partial map of pTiACH5 (De Vos et al. 1981) together with the extension of the TL- and TR-region (Thomashow et al. 1980a; De Beuckeleer et al. 1981) in octopine tumours. The regions of homology between pRi15834 and octopine T-region as well as the regions giving rise to hybridization with RNA from P15834J tissue are indicated by shaded areas above the partial pTiACH5 map. ▨= strong homology; ▧= partial homology. **b** Hybridization of pRi15834 and cDNA made towards poly-A$^+$ RNA present in hairy-root tissue line P15834J towards fragments covering the nonconserved part of the T-region of pTi C58 (Lemmers et al. 1981; Engler et al. 1981). Further details are as for Fig. 4a

mours (unpublished data) have produced no evidence for an activator function inducing root formation on potato or tobacco. On the contrary these data indicated that both shoot and root formation were independently suppressed by T-DNA functions. Three genes involved in the suppression of shoot formation have been mapped by transcript analysis and were located on the TL-DNA segment of octopine Ti-plasmids (Willmitzer et al. 1982; Leemans et al. 1982). If the model discussed above is correct these genes represent possible candidates as homologous functions expressed in hairy-root tissue.

In a first step we looked for homologies between plasmid DNA of *A. rhizogenes* strain 15834 and the T-regions of both octopine and nopaline Ti-plasmids. Total Ri-plasmid DNA was labelled by nick translation and hybridized to a blot containing DNA fragments representing both the TL and TR region of octopine Ti-plasmids (Thomashow et al. 1980; De Beuckeleer et al. 1981) as well as the non-conserved part of the T-region of nopaline Ti-plasmids (Lemmers et al. 1980; Engler et al. 1981). A specific hybridization to two well-defined parts of the octopine T-region was observed: one region (α) was located in the right part of *Bam*HI fragment 8 and the other (β) mainly in the left part of *Eco*RI fragment 20 of pTiACH5 (cf Fig. 4). It should be stressed that the observed hybridizations represent only partial homology since the intensity in this heterologous hybridization was significantly weaker than a homologous hybridization (data not shown). These observations are therefore not necessarily in contradiction with those published previously (White and Nester 1980b). Under the fairly stringent conditions used for hybridization ($T_m - 30°$ C) no other segments of the octopine T-region were found hybridize to Ri-plasmid DNA.

Synthesis of agropine in octopine tumours was shown to be encoded by T_R-DNA (Leemans et al. 1982). In addition analysis of T_R-derived transcripts present in different agropine producing octopine tumour lines showed that a 1.6 kb transcript mainly derived from the 1.7 kb *Eco*RI-*Sal*I subfragment of *Eco*RI fragment 20 of pTiACH5 (cf Fig. 4) is involved in agropine synthesis (unpublished data). The same DNA subfragment was found to be involved in the observed hybridizations of Ri-plasmid 15834 DNA, towards region β.

The other region of homology (α) involved the part of *Sma*I fragment 10c which is contained in *Bam*HI fragment 8. This TL-DNA segment codes for transcript 1 of octopine tumours and is involved in the suppression of shoot development (Leemans et al. 1982; Willmitzer et al. 1982).

In addition it was observed that pRi15834 DNA contains sequences homologous to two well-defined fragments of the non-conserved part of the T-region of nopaline Ti-plasmids.

Indeed pRi15834 was found to hybridize to *Eco*RI fragment 35 and to part of the neighbouring fragment *Eco*RI 16 (region γ) of pTiC58 and also to the overlapping part of *Sma*I fragment 10 and *Hpa*I fragment 2 (region δ). Hybridizations performed under different stringencies (data not shown) indicated that region δ of pTiC58 is only partially homologous with pRi15834 sequences, whereas region γ is highly conserved.

Both the γ and δ regions are actively transcribed in the nopaline tumour line BT37 (unpublished data) and give rise to at least three defined poly-adenylated transcripts.

Mutations in the right-hand part of region δ were found to inactivate agrocinopine synthesis (H. Joos et al. in preparation), no function can presently be ascribed to region γ.

If this partial physical homology between the T-regions of octopine and nopaline Ti-plasmids on the one hand and the *A. rhizogenes* plasmid pRi 15834 on the other hand is of functional importance, one would expect these sequences to be part of the T-region of Ri-plasmids and thus to be transferred and possibly expressed in hairy-root tissues.

To answer this question oligo-dT primed cDNA made towards total RNA isolated from hairy-root tissue was hybridized to nitrocellulose filters containing the restriction fragment of octopine and nopaline T-region as described above. The result is shown in Fig. 4 (always third lane). All fragments of nopaline T-region found to be homologous to pRi15834 also hybridized to RNA isolated from hairy-root tissues with the exception *Eco*RI fragment 35. These fragments must therefore be part of the T-region of Ri-plasmids since they are transferred and expressed in hairy-root tissues.

RNA isolated from hairy-root tissues hybridized also to fragment β of TR-DNA of octopine Ti-plasmid. Thus, these sequences must also be part of the T-region of Ri-plasmids since they are transferrred and expressed in hairy-root tissues. In octopine tumours this fragment of TR-DNA has been shown to be involved in the synthesis of agropine (cf above). As hairy-root tissues do contain agropine (cf Fig. 2; Tepfer and Tempé 1981) it is reasonable to postulate that the homologous region of pRi15834 codes for this function. Thus, this segment of the T-region of pRi15834 seems to be related both in terms of sequence homology and function to the T-DNA present in octopine tumours.

We were unable to detect any RNA hybridizing to the partially homologous sequence contained in the TL-region of octopine Ti-plasmids (region α). This finding is somewhat unexpected as the model outlined above predicts that the T-region of *A. rhizogenes* would contain genes active in shoot suppression, a function coded for by this region in octopine tumours (Leemans et al. 1982; Garfinkel et al. 1981). The absence of hybridization between RNA from hairy-root tissues and this segment of octopine TL-region may be due either to the fact that this part is not contained in the T-region of Ri-plasmids or that it is transferred to hairy-root tissues but its expression is too low to be detected by the methods used. In this respect it is interesting to note that also in nopaline tumour lines no RNA could be detected derived from this segment of the T-region (Willmitzer et al. 1982) despite the fact that genetic data (H. Joos, in preparation) indicate that this T-DNA segment must be functional in nopaline tumours.

Discussion

The data presented in this paper confirm the hypothesis that the hairy-root disease of plants induced by *Agrobacterium rhizogenes* is a special type of crown gall and that the so-called Ri-plasmids of *Agrobacterium rhizogenes* (Tepfer and Tempé 1981) are a special class of Ti-plasmids. Total RNA isolated from axenic hairy-root tissues of potato and carrot contains sequences that hybridize with a well-defined and limited number of DNA fragments derived from *Agrobacterium rhizogenes* plasmid. These data indicate therefore that hairy roots cultured in vitro contain and actively tran-

scribe DNA sequences that are homologous with and therefore presumably derived from sequences carried by large Ti-like plasmids present in *A. rhizogenes* strains.

Several lines of evidence indicate that these transcripts are equivalent to the T-DNA transcripts that have been previously shown to be responsible for tumorous transformation and for opine synthesis in crown gall tumours.

First of all the same Ri-DNA derived transcripts were found in both carrot and potato hairy-root tissues induced by the same *Agrobacterium rhizogenes* strain (15834), thus indicating that the same region of the Ri-plasmid is involved in hairy-root formation in different plants cells. In other words both the Ti-plasmids of *A. tumefaciens* and the Ri-plasmids of *A. rhizogenes* contain specific DNA segments (T-region) that are somehow transferred to – and maintained and expressed in – transformed plant cells. It is therefore an important question to ask whether Ri-plasmids represent a totally independent class of bacterial plasmids capable of transferring genes to plants or whether they fall within the general class of Ti-plasmids.

The idea that hairy-root disease induced by Ri-plasmids represents an independent type of plant cell transformation or an independent class of transforming plasmids is based mainly on two observations:

i: the phenotype of the hairy-root disease i.e. the massive formation of roots. – The assumption, that tumorigenic activity is restricted to *A. tumefaciens* strains and rhizogenic activity to *A. rhizogenes* strains (Moore et al. 1979) is mainly due to the fact that experimental work has been carried out on a small selection of host plants with a small selection of *Agrobacterium* strains. Actually the phenotypes induced by one Ti-plasmid on different plants and by different Ti-plasmid on the same host vary considerably. Thus wild-type nopaline Ti-plasmids such as pTiT37 produce transformed cells that grow into teratoma-like shoots (Braun and Wood 1976) on tobacco; the octopine wild-type strain B6 induces root-teratomata on *Kalanchoe daigremontiana*. In addition *A. rhizogenes* is known to induce typical neoplasms without external roots and shoots on several hosts, e.g. *Vicia faba* or *Phaseolus vulgaris* (De Cleene and De Ley 1981).

ii: the low degree of homology observed between *A. rhizogenes* plasmid 15834 and the octopine type plasmid pTiB6-806 (White and Nester 1980b). – It should be stressed that this observation is based on hybridization experiments performed under highly stringent conditions (White and Nester 1980b). The authors state that as much as 65% of the rhizogenic plasmid is partially homologous to the octopine type plasmid based on hybridization experiments performed under lower stringency.

Using total plasmid DNA from *A. rhizogenes* strain 15834 as probe four well-defined regions of homology have been detected with the T-region of octopine and nopaline Ti-plasmid (cf Fig. 4). Three regions can only be detected using hybridization conditions which allow a base-pair mismatch of up to 25%. These regions therefore are only partially homologous. The fourth region (γ) located at the very left of the nopaline T-region however was found to be highly conserved between nopaline and rhizogenic plasmids.

For the parts of pRi15834 homologous to *Eco*RI fragment 35 of pTiC58 and region α of pTiACH5 (cf Fig. 4) it remains unknown whether they constitute part of the T-region of the Ri-plasmid DNA.

*Eco*RI fragment 35 of pTiC58 forms part of the left-hand border of the T-DNA in nopaline tumours (Lemmers et al. 1980). It contains a 22 base-pair repeat as well as the chi-sequence which are both thought to be important for the integration of the T-DNA into the plant chromosome (Zambryski et al. in prep.). It remains to be determined whether the homologous region in pRi15834 is also important for integration of the T-region of the Ri-plasmid into the plant DNA.

As outlined above region β of the pTiACH5 is involved in the synthesis of agropine. In view of the ability of hairy-root tissues to produce agropine it is reasonable to assume that the RNA in hairy-roots found to be partially homologous with region β also codes for agropine synthesis.

One transcript corresponding to fragments i and h of region δ of pTiC58 in nopaline tumours is involved in agrocinopine synthesis (H. Joos et al. in prep.) RNA from hairy-root tissues hybridizes to the left-hand part h of region δ. No hybridization was observed to the neighbouring fragment i (cf Fig. 4) although a partial homology between pRi15834 and region i was found. Thus although the RNA from hairy-root tissues hybridizes to only part of the region coding for agrocinopine synthesis in nopaline tissues, these data suggested that agrocinopine might be a second opine produced in hairy-root tissues. This possibility was further supported by the fact that *A. rhizogenes* strain 15834 is sensitive to agrocin 84 (White and Nester 1980a), a trait associated with the synthesis of agrocinopine (Ellis and Murphy 1981). Recently J. Tempé (personal communication) reported the finding of agrocinopine in several hairy-root tissues.

In conclusion our data indicate that the T-region in Ri-plasmids is not only functionally but also phylogenetically related to the T-region of octopine and nopaline Ti-plasmids and hence that the so-called Ri-plasmids should be regarded as a special group of Ti-plasmids, possibly derived by recombination from the other Ti-plasmids.

Acknowledgement. We would like to thank G. Simons and C. Skroblin for expert technical assistance. The help of Mrs. E. Kath and Mrs. E. Schölzel in typing the manuscript and Mrs M. Kalda in preparing the figures is gratefully acknowledged. We thank J. Tempé for valuable discussions as well as for communication of results prior to publication. The continued help and advise from our colleagues in Gent and Brussels was essential for this work. In particular we thank Dr. J.-H. Hernalsteens (Brussels) who first introduced J. Sanchez-Serrano to the *A. rhizogenes* system. J. Sanchez-Serrano was the recipient of a fellowship of the Max-Planck-Gesellschaft, München.

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Communicated by G. Melchers

Received March 15 / 26, 1982