Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 1660–1664, March 1983
Genetics

Multiple mutations in the T region of the *Agrobacterium tumefaciens* tumor-inducing plasmid

(crown gall tumor/transposon/phytohormone)

LLOYD W. REAM*, MILTON P. GORDON†, AND EUGENE W. NESTER*

Departments of *Microbiology and Immunology and of †Biochemistry, University of Washington, Seattle, Washington 98195

Communicated by Herschel L. Roman, November 9, 1982

ABSTRACT **Three genetic loci affecting tumor morphology lie within pTiA6NC T-DNA: *tms*, *tmr*, and *tml*. Using deletions and multiple transposon insertions, we constructed tumor-inducing (Ti) plasmids representing every possible double and triple mutant combination. *tms tmr* and *tms tmr tml* mutants did not incite tumors on most plants and produced a very weak response on a few other hosts but *tms tml* and *tmr tml* mutants were virulent. Thus, either tms^+ or tmr^+ alone can promote significant tumor growth but tml^+ by itself is not sufficient. On hosts where *tms* mutants induce tumors accompanied by shoot proliferation, addition of a *tml* mutation reduces or eliminates shoot proliferation, suggesting that tml^+ promotes shoot development. The small calli incited by *tms tmr* and *tms tmr tml* mutants contain agropine, an indication that these plant cells incorporate T-DNA in the absence of substantial tumor growth.**

During crown gall tumor induction by *Agrobacterium tumefaciens*, a specific segment of the tumor-inducing (Ti) plasmid called the T-DNA integrates into plant nuclear DNA (1–3). Three genetic loci affecting tumor morphology lie within pTiA6NC T-DNA: *tms*, *tmr*, and *tml* (4–8). Crown gall tumors incited on tobacco by strains harboring pTiA6NC appear unorganized, but *tms* mutations incite tumors that have shoots proliferating from the callus and *tmr* mutations incite tumors showing root proliferation (4–8). *tml* mutations result in abnormally large tumors (5).

Tumor morphologies resulting from *tms* and *tmr* mutations resemble morphologies of nontransformed tobacco tissue grown *in vitro* on media containing different amounts of auxins and cytokinins (9). Unlike normal plant tissue, crown gall tumor tissue contains sufficient amounts of auxins and cytokinins to grow *in vitro* on media lacking added phytohormones (10). When host plants with tumors induced by *tms* or *tmr* mutants are treated with auxin or cytokinin, respectively, tumor growth is stimulated (7). These data suggest that tms^+ and tmr^+ increase levels of auxins and cytokinins, respectively, in crown gall tumors. Based on this hypothesis, mutations in both *tms* and *tmr* should block the increase in both auxin and cytokinin, thereby preventing tumor growth. In this paper, we report the analysis of the tumor-inducing ability of strains with multiple mutations in the T region.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. *A. tumefaciens* strains used are described in Table 1. Plasmids used are described in Table 2.

Media. AB minimal agar (13) and peptone broth and nutrient agar (Difco) were used. For selection of drug-resistant *Escherichia coli*, antibiotics were used at the following concentrations: tetracycline, 10 μg/ml; kanamycin, 25 μg/ml; ampicillin, 50 μg/ml; gentamicin, 50 μg/ml. For selection of drug-resistant *A. tumefaciens*, gentamicin was used at 50 μg/ml, and carbenicillin and kanamycin were used at 100 μg/ml.

Transformation. Transformation of *A. tumefaciens* (14) and *E. coli* (15) with plasmid DNA was carried out essentially as described.

Bacterial Conjugation. Bacterial matings using *E. coli* strain 2174 (pPH1JI) as donor and *A. tumefaciens* strains as recipients were carried out as described (5).

A Deletion Affecting *tms* and *tmr*. Portions of *tms* and *tmr* lie in *Hpa* I fragment 14 of pTiA6NC (5). The recombinant plasmid pNW34D7, containing pTiA6NC *Eco*RI fragment 7, was digested with *Hpa* I to produce two restriction fragments: *Hpa* I fragment 14 and the remainder of the original plasmid, which codes for ampicillin and tetracycline resistance. The *Hpa* I digestion products were incubated with T4 DNA ligase and then transformed into MM294 (F^- *pro hsdR endA*; ref. 16). One ampicillin/tetracycline-resistant transformant harbored a derivative of pNW34D7 (pWR1) having a deletion, del(*tms-tmr*)2, removing *Hpa* I fragment 14.

Double Insertion Mutants. We constructed *tms tmr*, *tms tml*, and *tmr tml* mutants by using the strains and plasmids listed in Table 1.

Translocation of Mutant Alleles from Cloned T-DNA to Ti Plasmids. Mutations in cloned T-DNA restriction fragments were translocated to Ti plasmids by homologous recombination essentially as described (5, 16). For example, pRK290 derivatives carrying Tn5 insertions (kanamycin resistant) in cloned T-DNA fragments were transformed into an *A. tumefaciens* strain harboring a Ti plasmid with a Tn3 insertion (carbenicillin resistant) and pPH1JI (gentamicin resistant), a plasmid incompatible with pRK290 (5, 16). We identified gentamicin-sensitive transformants and reintroduced pPH1JI into these strains by using 2174(pPH1JI) (met^- pro^-) as conjugal donor. We selected Met^+ Pro^+ transconjugants that retained Tn5 and Tn3 on AB minimal agar containing gentamicin, kanamycin, and carbenicillin. The incoming pPH1JI excluded the Tn5-containing pRK290 derivative and, usually, the Tn5 had translocated to the Ti plasmid by homologous recombination. We introduced a deletion removing *Hpa* I fragment 14 into a Ti plasmid that had originally carried a Tn5 insertion in this fragment; in this case, we screened gentamicin-resistant transconjugants for loss of Tn5. Strain construction details are in Table 1.

DNA Isolation. Plasmid DNA was isolated from *E. coli* as described (17) and centrifuged in cesium chloride/ethidium bromide density gradients. Total *A. tumefaciens* DNA was isolated as described (18).

Restriction Endonuclease Digestion and Ligation. Restriction endonucleases were supplied by Bethesda Research Lab-

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Abbreviation: Ti plasmid, tumor-inducing plasmid.

Table 1. *A. tumefaciens* strains used

Strain	*tms*	*tmr*	*tml*	Derivation
A136*	–	–	–	Ref. 11
328†	*328*::Tn*5*	+	+	Ref. 5
334†	+	+	+	Ref. 5; *Hpa* I fragment 14::Tn*5*
338†	+	*338*::Tn*5*	+	Ref. 5
A348‡	+	+	+	Ref. 5
358†	+	+	*358*::Tn*5*	Ref. 5
393†	*393*::Tn*3*	+	+	Ref. 5
A1079§	+	*25*::IS*66*¶	+	Ref. 4
A3000†§	*328*::Tn*5*	*25*::IS*66*¶	+	Km^R Gm^R trans of 2174 (pPH1JI) × A3003
A3004†	del2	del2	+	Gm^R Km^S Cb^S trans of 2174 (pPH1JI) × A3002
A3017†	*393*::Tn*3*	+	*358*::Tn*5*	Km^R Cb^R Gm^R trans of 2174 (pPH1JI) × A3008
A3019†	*393*::Tn*3*	*338*::Tn*5*	+	Km^R Cb^R Gm^R trans of 2174 (pPH1JI) × A3012
A3025†	del2	del2	*358*::Tn*5*	Km^R Gm^R trans of 2174 (pPH1JI) × A3016
A3044†§	+	*25*::IS*66*¶	358::Tn*5*	Km^R Gm^R trans of 2174 (pPH1JI) × A3035
A3088†	*393*::Tn*3* *337*::Tn*5*	+	+	Km^R Gm^R recombinant‖

R indicates resistance and S indicates sensitivity to carbenicillin (Cb), gentamicin (Gm), or kanamycin (Km). Trans indicates Met^+Pro^+ transconjugant produced by using *E. coli* strain 2174 (pPH1JI) (*met*$^-$*pro*$^-$$Gm^R$; ref. 12) as donor and one of the following *A. tumefaciens* strains as recipient: A3002, a Cb^R Gm^S pWR1 transformant of 334; A3003, a Km^R pDG289 transformant of A1079; A3008, a Km^R Cb^R Gm^S pDG83 transformant of 393; A3012, a Km^R Cb^R Gm^S pDG85 transformant of 393; A3016, a Km^R Cb^R Gm^S pDG83 transformant of A3004; A3035, a Km^R Cb^R pDG83 transformant of A1079. Plasmids pDG83(Km^R Cb^R), pDG84(Km^R Cb^R), pDG85(Km^R Cb^R), pDG289 (Km^R), and pWR1 (Cb^R) are described in Table 2. Strains harbor derivatives of pTiA6NC unless otherwise indicated.

* This strain does not harbor a Ti plasmid.

† Strain harboring pPH1JI.

‡ Strain harboring wild-type pTiA6NC.

§ Strain harboring a derivative of pTiB6806.

¶ Formerly called *onc-25* (4).

‖ Formed after transformation of 393 with pDG84.

oratories and T4 DNA ligase was supplied by New England BioLabs. They were used under the reaction conditions specified in the New England BioLabs catalog (1979). The restriction endonucleases were inactivated by incubation at 65°C for 10 min prior to ligation.

Agarose Gel Electrophoresis. Horizontal agarose slab gels (1.0%) were formed using Tris/EDTA/borate buffer (4).

DNA Blotting and Filter Hybridizations. After agarose gel electrophoresis, DNA restriction fragments were eluted onto nitrocellulose filters essentially as described (19). Radioactive DNA fragments were hybridized to the nitrocellulose-bound DNA under conditions based on the procedure of Wahl *et al.* (20) as modified by Gillen *et al.* (21). DNA was labeled with [α-^{32}P]dATP, [α-^{32}P]dCTP, and [α-^{32}P]dTTP (each, 300 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear) by nick-translation (22).

Tumor Morphology Determination. We tested our single and multiple tumor morphology mutants on stems of *Kalanchoë daigremontiana*, *Kalanchoë tubiflorae*, *Nicotiana tabacum* var. xanthi n.c. (tobacco), *Nicotiana glauca*, *Nicotiana langsdorfii*, *Datura stramonium*, *Lycopersicum esculentum* var. early girl (red cherry tomato), and *Helianthus annus* var. mammoth Russian (sunflower) and on root slices of *Daucus carota* (carrot) and tuber slices of *Helianthus tuberosis* (Jerusalem artichoke). We found that *K. tubiflorae* stems gave tumors with the most obvious morphological differences for each type of inciting strain.

Table 2. Plasmids used

Plasmid	Vector	T-DNA insert	Drug resistance	Ref.
pDG83	pRK290–pBR325 hybrid	*Eco*RI 7-*tml-358*::Tn*5*	Ap, Km, Tc	5
pDG84	pRK290–pBR325 hybrid	*Eco*RI 7-*tms-337*::Tn*5*	Ap, Km, Tc	5
pDG85	pRK290–pBR325 hybrid	*Eco*RI 7-*tmr-338*::Tn*5*	Ap, Km, Tc	5
pDG289	pRK290	*Bam*HI 8-*tms-328*::Tn*5*	Km, Tc	5
pNW34D7	pRK290–pBR325 hybrid	*Eco*RI 7	Ap, Tc	5
pNW31C8	pBR322	*Bam*HI 8	Ap	5
pNW33D7	pBR325	*Eco*RI 7	Ap, Tc	5
pPH1JI		—	Gm, Sm, Sp	12
pWR1	pRK290–pBR325 hybrid	*Eco*RI 7-del(*tms-tmr*)2	Ap, Tc	This work

Drug resistance: Ap, ampicillin; Gm, gentamicin; Km, kanamycin; Sm, streptomycin; Sp, spectinomycin; Tc, tetracycline.

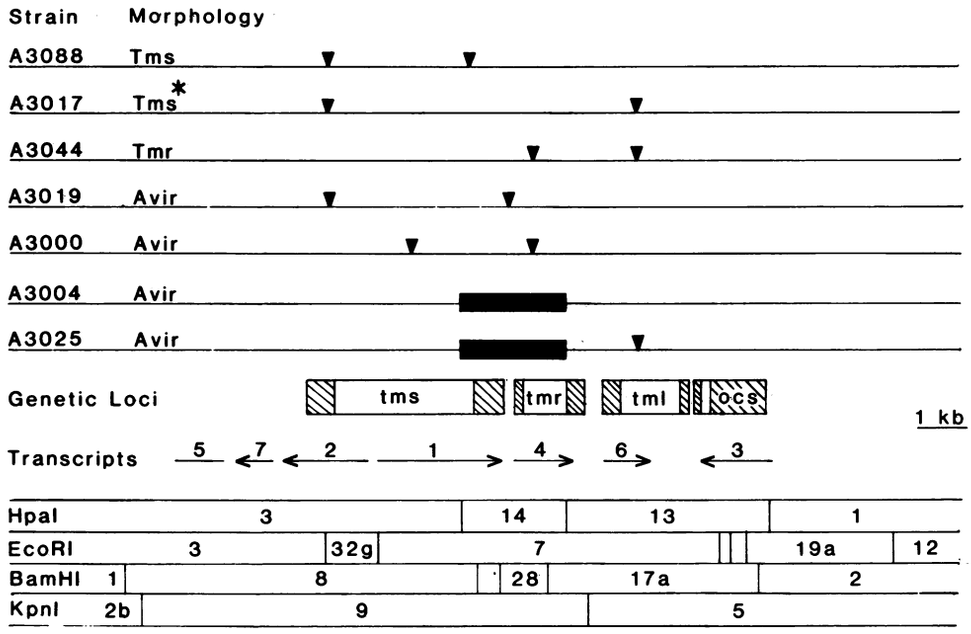

FIG. 1. Locations of the multiple mutations. The genetic (5), physical (24–26), and transcriptional (27, 28) maps have been adapted from previous publications. ▼ indicates a transposon insertion, bold lines indicate a deletion, boxes indicate a genetic locus, hatched boxes designate the region between the outermost mutation that defines a locus and the nearest phenotypically silent mutation, and arrows indicate the locations and directions of transcription.
* This strain is avirulent on *N. tabacum* and incites tumors with reduced shoot formation on *K. tubiflorae*.

AB minimal agar plates (without antibiotics) were inoculated with *A. tumefaciens* strains and incubated at 28°C for 3 days. A mucoid mass containing approximately 5×10^8 bacteria was scraped from each AB agar plate with a sterile toothpick and smeared into a fresh wound on a *K. tubiflorae* stem. Stems were wounded in the middle of an internode region by puncturing

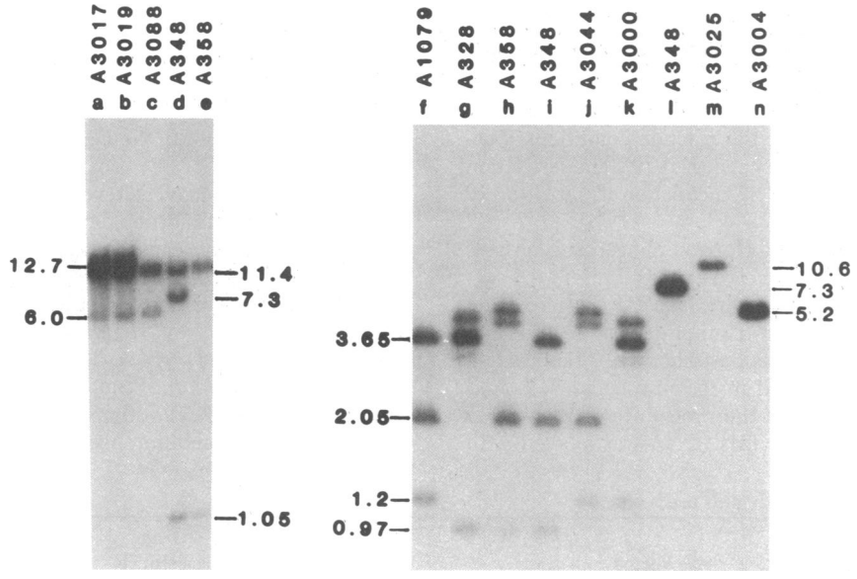

FIG. 2. Verification of multiple Ti plasmid mutations. The figure is an autoradiogram of a Southern blot of restriction digests of total *A. tumefaciens* DNA hybridized with ^{32}P-labeled plasmids containing T-DNA. Lanes: a–e, probed with labeled pNW31C8 (pTiA6NC *Bam*HI fragment 8), contain *Eco*RI digests of DNAs from strains A3017 (*tms-393*::Tn*3* *tml-358*::Tn*5*), A3019 (*tms-393*::Tn*3* *tmr-338*::Tn*5*), A3088 (*tms-393*::Tn*3* *tms-337*::Tn*5*), A348 (wild-type pTiA6NC), and 358 (*tml-358*::Tn*5*); f–k, probed with pTiA6NC *Eco*RI fragment 7, contain *Bam*HI/*Eco*RI double digests of DNAs from strains A1079 (*tmr*-25::IS*66*), 328 (*tms-328*::Tn*5*), 358 (*tml-358*::Tn*5*), A348 (wild-type pTiA6NC), A3044 (*tmr-25*::IS*66* *tml-358*::Tn*5*), and A3000 (*tms-328*::Tn*5* *tmr-25*::IS*66*); l–n, probed with labeled pNW33D7 (pTiA6NC *Eco*RI fragment 7), contain *Eco*RI digests of DNAs from strains A348 (wild-type pTiA6NC), A3025 [del(*tms-tmr*)2 *tml-358*::Tn*5*], and A3004 [del(*tms-tmr*)2]. In A3017, A3019, and A3088, a Tn*3* [4.957-kilobase (kb)] insertion increased the size of *Eco*RI fragment 32 from 1.05 to 6.0 kb. Tn*5* (5.4-kb) insertions increased the size of *Eco*RI fragment 7 from 7.3 to 12.7 kb in A3017, A3019, and A3088. In A3000 and A3044, an IS*66* (1.5-kb) insertion altered the size of *Bam*HI fragment 30 (0.97 kb). Tn*5* insertions in the *Eco*RI fragment 7 altered the sizes of the 3.65-kb (in A3044) and 2.05-kb (in A3000) *Bam*HI/*Eco*RI fragments. The 2.1-kb deletion in A3004 decreased the *Eco*RI fragment 7 from 7.3 to 5.2 kb. When *tml-358*::Tn*5* was introduced into this deletion mutant, the size of the *Eco*RI fragment 7 del(*tms-tmr*)2 increased from 5.2 to 10.6 kb in A3025.

them with sterile toothpicks to a depth of 3–5 mm. Each stem received only one wound, and each mutant was tested on at least four plants.

Agropine Production. Tissue (0.1 g) excised from *K. tubiflorae* stems was examined for agropine essentially as described (23).

Physical and Biological Containment. Containment levels used during experiments involving recombinant DNA conformed to the guidelines of the National Institutes of Health.

RESULTS

Locations of Mutations. The positions of the multiple mutations are shown in Fig. 1. Verification of the presence of the mutations was obtained from Southern filter hybridization (Fig. 2).

Tumor Morphology of Single Mutants. Tumors incited by a wild-type strain, A348, on *K. tubiflorae* stems produced a large gall with one or two shoots developing from lateral buds located near the top and roots proliferating from the bottom (Fig. 3*a*). Inoculation of a wound with a Ti-plasmidless strain, A136, produced a very weak response, and an uninoculated wound gave no response.

Tumors incited by *tmr* mutants produced a small gall with roots proliferating from the bottom but no shoots (Fig. 3*c*), whereas a *tms* mutant produced a large gall with one or two shoots arising from lateral buds located near the top but no roots (Fig. 3*d*). Tumors incited by *tml* mutants produced a very large gall with roots proliferating from the bottom but no shoots (Fig. 3*e*) or one or two small shoots developing from nearby lateral buds (data not shown).

Tumor Morphology of Virulent Double Mutants. Tumors incited by *tmr tml* mutants resembled those incited by a *tmr* mutant except that more roots proliferated from the galls (Fig. 3*f*). Tumors incited by *tms tml* mutants produced a large gall lacking roots and shoots (Fig. 3*g*) or having one very small shoot (data not shown). Thus, a *tml* mutation reduces shoot production from lateral buds.

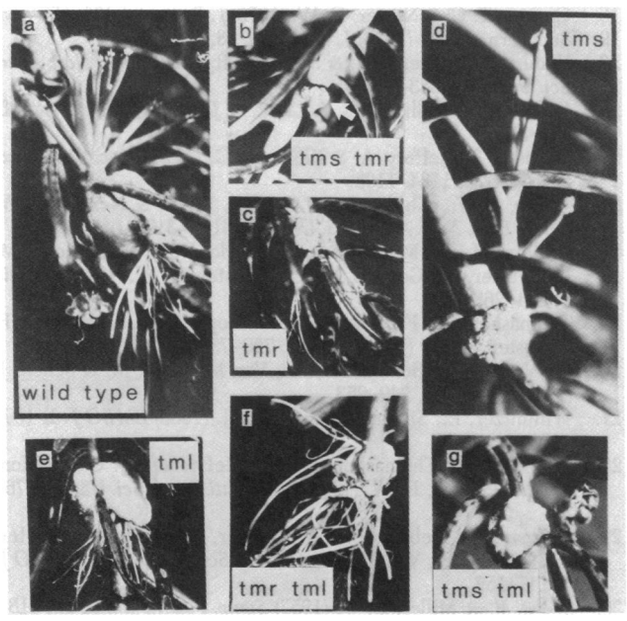

FIG. 3. Tumor morphology on *K. tubiflorae*. Tumors incited by various strains were photographed 48 days after inoculation. (*a*) A348 (wild-type pTiA6NC). (*b*) A3004 [del(*tms-tmr*)2]. (*c*) 338 (*tmr-338*::Tn*5*). (*d*) 328 (*tms-328*::Tn*5*). (*e*) 358 (*tml-358*::Tn*5*). (*f*) A3081, a *tmr-25*::IS*66* *tml-358*::Tn*5* strain indistinguishable from A3044. (*g*) A3017 (*tms-393*::Tn*3* *tml-358*::Tn*5*).

Avirulent Multiple Mutants. Wounds on *K. tubiflorae* inoculated with *tms tmr* or *tms tmr tml* mutants gave a weak response without root or shoot production (Fig. 3*b*). These strains induced a somewhat greater response than the Ti-plasmidless control strain. The *tms tmr* and *tms tmr tml* mutants produced similar responses when inoculated onto stems of *K. daigremontiana* and *H. annus*, but these strains produced no apparent response on the other hosts tested.

The small callus appearing on *K. daigremontiana* stems inoculated with a *tms tmr* or a *tms tmr tml* strain reached its maximum size within approximately 1 month, and no significant growth occurred during an additional 8 months of cultivation. Thus, mutations in both *tms* and *tmr* block or strongly suppress tumorous growth. The weak responses induced by *tms tmr* and *tms tmr tml* strains required the Ti plasmid and may result from expression of T-DNA genes (other than *tms*, *tmr*, and *tml*) integrated into plant DNA or from expression of Ti-plasmid genes outside the T region (for example, *vir* genes).

Agropine Production. We examined a small callus incited by the *tms tmr tml* triple mutant on a *K. tubiflorae* stem for accumulation of agropine. This tissue contained agropine, as did tissue from a tumor incited by a *tms* mutant, but no agropine was detected in normal stem tissue (Fig. 4). Production of agro-

FIG. 4. Paper electrophoresis of agropine. Extracts of *K. tubiflorae* tissue infected with 393 (*tms-393*::Tn*3*; lane 1) and A3025 [del(*tms-tmr*)2 *tml-358*::Tn*5*; lane 3] contain material comigrating with purified agropine (100 ng) (lane 4). Normal tissue extract (lane 2) lacks agropine. The spot farthest from the origin (top) is agropine, a cyclization product of mannopine (the other spot in lane 4). The large uppermost spots in lanes 1–3 presumably represent glucose or glucose-containing compounds. Plant tissue (0.1 g) was ground in 0.2 ml of 95% ethanol and centrifuged for 2 min in an Eppendorf microcentrifuge. The supernatant was concentrated to 20 μl and applied to a 56 × 15 cm sheet of Whatman 3mm paper. The paper was moistened with electrophoresis buffer [formic acid/acetic acid/water, 1:3:16 (pH 1.8)] and subjected to electrophoresis (with the anode at the origin) at 1,000 V for 2 hr. After electrophoresis, the paper was air dried, immersed in $AgNO_3$ solution (12.5 g of $AgNO_3$ in 1 liter of acetone and 50 ml of water), air dried, immersed in NaOH reagent (10 ml of 20% NaOH and 90 ml of methanol), air dried, submerged in 5% aqueous sodium thiosulfate for 2 min, and rinsed briefly with water.

pine by this small callus suggests that this plant tissue contains T-DNA.

DISCUSSION

Transposon insertion mutations define three T-DNA loci (*tms*, *tmr*, and *tml*) that affect tumor morphology, but a mutation in any of these loci does not abolish virulence (5). *tms tml* and *tmr tml* mutants also remained virulent, indicating that either tms^+ or tmr^+ alone promoted significant tumor growth. This result suggests that two separate pathways of tumorigenesis exist: a *tms* pathway and a *tmr* pathway. Mutations in both *tmr* and *tms* prevented significant tumor growth for 9 months on *K. daigremontiana* stems and abolished virulence completely on six other hosts, indicating that tml^+ alone did not promote tumor growth. Thus, blocking both tumorigenesis pathways prevented tumor growth. A small unorganized callus induced on *K. tubiflorae* by the *tms tmr tml* mutant contained agropine, suggesting that these plant cells incorporated T-DNA in the absence of substantial tumor growth. This observation, also reported by Leemans *et al.* (29), might facilitate genetic engineering of plants. Foreign genes inserted into the T region of a *tms tmr* mutant Ti plasmid might be incorporated into the plant genome. Presumably, these nontransformed T-DNA-containing cells would regenerate easily into morphologically normal plants that retain the entire T-DNA because the deleterious T-DNA genes have already been removed.

Crown gall tumor tissue contains sufficient amounts of auxins and cytokinins to grow *in vitro* on media lacking added phytohormones (10). Normal tobacco tissue forms unorganized callus when grown *in vitro* on media containing specific amounts of auxins and cytokinins (30). Increasing the auxin/cytokinin ratio in the medium promotes root proliferation, and decreasing this ratio promotes shoot proliferation (30). Thus, a *tmr* mutation in the T-DNA of a tumor cell may increase the auxin/cytokinin ratio and a *tms* mutation may decrease this ratio. In support of this hypothesis, when tomato stems inoculated with *tms* or *tmr* mutants are treated with auxin or cytokinin, respectively, normal tumor growth resumes (7). Also, *tms* and *tmr* mutations alter the levels of auxins and cytokinins detected in tumor galls (31, 32). We presume that mutations in both *tms* and *tmr* prevent tumor growth by keeping the phytohormone levels in the infected plant cells at the levels found in nontransformed tissue.

Our results allow us to speculate on the nature of the *tml* gene product. The tml^+ allele failed to promote tumor growth by itself and affected tumor morphology only in strains also having tms^+, tmr^+, or tms^+ tmr^+ alleles. Thus, the tml^+ gene product apparently requires phytohormones for its activity and probably does not produce increased levels of auxin or cytokinin in plant cells. In tumors induced by *tml* or *tml tms* mutants, shoot production from lateral buds (on *K. tubiflorae*) and from tumor galls (on *N. tabacum*) was reduced, suggesting that tml^+ stimulates shoot development. Addition of a *tml* mutation to a *tmr* mutant stimulated root proliferation from tumors on *K. tubiflorae* stems and *H. tuberosis* slices. Thus, tml^+ could conceivably promote shoot production and reduce root proliferation by enhancing cytokinin activity, inhibiting auxin activity, or both.

Crown gall tumors produce at least seven polyadenylylated RNA transcripts that hybridize to the T region of the Ti plasmid (ref. 27; Fig. 1). Transcripts 1 and 2 are both associated with *tms* (29). We constructed a *tms*-393::Tn*3* *tms*-328::Tn*5* mutant strain (A3088) that should interrupt both *tms* transcripts. This strain induced tumors morphologically indistinguishable from tumors induced by either single mutant parent on *K. daigremontiana*, *K. tubiflorae*, and *N. langsdorfii*. A deletion mutation known to eliminate both transcripts 1 and 2 gave similar results (29). In addition, *tms tmr* mutants resulted in the same tumor phenotype regardless of which transcript the *tms* mutation presumably affects. Thus, the proteins specified by these transcripts probably affect the same biochemical pathway.

We thank D. Garfinkel and R. Amasino for critically reading and R. Finical for typing this manuscript, B. Watson and G. Ghidossi for maintaining the plants, and M. Tate for agropine. This work was supported by National Cancer Institute Grant CA 13015 and American Cancer Society Grants IN-26V and PF-1913 (to L.W.R.).

1. Chilton, M.-D., Drummond, M. H., Merlo, D. J., Sciaky, D., Montoya, A. L., Gordon, M. P. & Nester, E. W. (1977) *Cell* **11**, 263–271.
2. Chilton, M.-D., Saiki, R. K., Yadav, N., Gordon, M. P. & Quetier, F. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 4060–4064.
3. Willmitzer, L., DeBeuckeleer, M., Lemmers, M., Van Montagu, M. & Schell, J. (1980) *Nature (London)* **287**, 359–361.
4. Garfinkel, D. J. & Nester, E. W. (1980) *J. Bacteriol.* **144**, 732–743.
5. Garfinkel, D. J., Simpson, R. B., Ream, L. W., White, F. F., Gordon, M. P. & Nester, E. W. (1981) *Cell* **27**, 143–153.
6. Ooms, G., Klapwijk, P., Poulis, J. A. & Schilperoort, R. A. (1980) *J. Bacteriol.* **14**, 82–91.
7. Ooms, G., Hooykaas, P. J. J., Moolenaar, G. & Schilperoort, R. (1981) *Gene* **14**, 33–50.
8. De Greve, H., Decraemer, H., Seurinck, J., Van Montagu, M. & Schell, J. (1981) *Plasmid* **6**, 235–248.
9. Amasino, R. M. & Miller, C. O. (1982) *Plant Physiol.* **69**, 389–392.
10. Braun, A. C. (1958) *Proc. Natl. Acad. Sci. USA* **44**, 344–349.
11. Watson, B., Currier, T. C., Gordon, M. P., Chilton, M.-D. & Nester, E. W. (1975) *J. Bacteriol.* **123**, 255–264.
12. Beringer, J. E., Beynon, J. L., Buchanan-Wollaston, A. V. & Johnston, A. W. B. (1978) *Nature (London)* **276**, 633–634.
13. Chilton, M.-D., Currier, T. C., Farrand, S. K., Bendich, A. J., Gordon, M. P. & Nester, E. W. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 3672–3676.
14. Holsters, M., de Waele, D., Depicker, A., Messens, E., Van Montagu, M. & Schell, J. (1978) *Mol. Gen. Genet.* **163**, 181–187.
15. Brown, M., Weston, A., Saunders, J. & Humphreys, G. (1979) *FEMS Lett.* **5**, 219–222.
16. Ruvkun, G. B. & Ausubel, F. M. (1981) *Nature (London)* **289**, 85–88.
17. Birnboim, H. C. & Doly, J. (1979) *Nucleic Acids Res.* **7**, 1513–1525.
18. Currier, T. C. & Nester, E. W. (1976) *J. Bacteriol.* **126**, 157–165.
19. Southern, E. M. (1975) *J. Mol. Biol.* **98**, 503–517.
20. Wahl, G. M., Stern, M. & Stark, G. R. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 3683–3687.
21. Gillen, J. R., Willis, D. K. & Clark, A. J. (1981) *J. Bacteriol.* **145**, 521–532.
22. Maniatis, T., Jeffrey, A. & Kleid, D. G. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 1184–1188.
23. White, F. F., Ghidossi, G., Gordon, M. P. & Nester, E. W. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 3193–3197.
24. Chilton, M.-D., Montoya, A. L., Merlo, D. J., Drummond, M. H., Nutter, R., Gordon, M. P. & Nester, E. W. (1978) *Plasmid* **1**, 254–269.
25. Thomashow, M. F., Nutter, R., Montoya, A. L., Gordon, M. P. & Nester, E. W. (1980) *Cell* **19**, 729–739.
26. De Vos, G., DeBeuckeleer, M., Van Montagu, M. & Schell, J. (1981) *Plasmid* **6**, 249–253.
27. Willmitzer, L., Simons, G. & Schell, J. (1982) *EMBO J.* **1**, 139–146.
28. Gelvin, S. B., Thomashow, M. F., McPherson, J. C., Gordon, M. P. & Nester, E. W. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 76–80.
29. Leemans, J., Deblaere, R., Willmitzer, L., DeGreve, H., Hernalsteens, J. P., Van Montagu, M. & Schell, J. (1982) *EMBO J.* **1**, 147–152.
30. Skoog, F. & Miller, C. O. (1957) *Symp. Soc. Exp. Biol* **11**, 118–131.
31. Morris, R. O., Akiyoshi, D. E., MacDonald, E. M. S., Morris, J. W., Regier, D. A. & Zaerr, J. B. (1982) in *Plant Growth Substances*, ed. Wareing, P. F. (Academic, New York), pp. 175–183.
32. Akiyoshi, D. E., Morris, R. O., Hinz, R., Mischke, B. S., Kosuge, T., Garfinkel, D. J., Gordon, M. P. & Nester, E. W. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 407–411.