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# Evidence for a cellular gene with potential oncogenic activity in plants

(crown-gall transformation/cytokinins/habituated-leaf trait/tumor genes/tobacco)

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**ABSTRACT** **During tumor inception in crown gall disease, a portion of the tumor-inducing (Ti) plasmid, the transferred DNA (T-DNA), is integrated into the genome of the plant cell. Autonomous growth of the transformants requires expression of genes in the *tmr* and *tms* regions of the T-DNA, which code for enzymes concerned with biosynthesis of the plant growth hormones cytokinin and auxin, respectively. We show that a mutation of the Habituated leaf gene, *Hl*, of tobacco can compensate for a defective *tmr* locus in expression of the tumor phenotype. This provides evidence that a specific host–cell gene has an oncogenic function similar to *tmr* in the T-DNA.**

Neoplastic transformation in plants can be caused by plasmids of bacterial origin, double-stranded RNAs, spontaneous events in cell culture, and the combination of certain foreign genomes in interspecific sexual hybrids (1). Regardless of proximal cause, the autonomous growth of the tumors appears to result from the inappropriate production by the cells of growth-promoting substances. The most important of these are the plant growth hormones auxin and cytokinin (2).

In the case of crown gall disease, a portion of the tumor-inducing (Ti) plasmid, the transferred DNA (T-DNA), is transferred from the inciting bacterium, *Agrobacterium tumefaciens*, to the host cell, where it is integrated into the nuclear genome (3). Neoplastic growth of the transformed cells requires the expression of gene *4* in the *tmr* region and genes *1* and *2* of the *tms* region of the T-DNA (4). The *tmr* region codes for a key enzyme in cytokinin biosynthesis (5, 6); and at least one of the two genes in the *tms* region is involved in a biosynthetic pathway for auxin (7–9).

Tobacco cells transformed by the Ti plasmid lose their exogenous requirement for cytokinin and auxin for growth in culture and produce these hormones in amounts sufficient to support the proliferation of normal cells (10). Earlier, we identified a single, partially dominant Mendelian gene, habituated leaf (*Hl*), that regulates the cytokinin requirement of cultured tobacco cells. Whereas tissues from the pith parenchyma and leaf lamina of wild-type tobacco plants require cytokinin and auxin for growth in culture, comparable tissues from *Hl/Hl* and *Hl/hl* plants are cytokinin autotrophic (11). In this report, we show that the *Hl* allele can complement a defective *tmr* locus in expression of the tumor phenotype. This provides direct evidence that a specific cellular gene in tobacco has an oncogenic function similar to *tmr* in the T-DNA.

## MATERIALS AND METHODS

**Cell Lines.** Tumor-cell lines were established from 3-month-old wild-type *hl/hl Nicotiana tabacum* L. cv. Havana 425 plants and homozygous *Hl/Hl* habituated-leaf plants of the same variety (11) grown from seed in a greenhouse. Plants were inoculated (12) on the side of the stem with the strains A208 and A208 14a/a of *Agrobacterium tumefaciens* provided by A. N. Binns (University of Pennsylvania). The A208 strain carries the wild-type ($tmr^+$) pTiT37 plasmid, and the A208 14a/a strain carries a *tmr*-defective ($tmr^-$) derivative of this plasmid obtained by inserting the Tn*5* transposon into the *Bam*HI fragment 14a of the *tmr* region (13). Eight weeks after inoculation, tissues from the inoculation sites were surface-sterilized with 0.36% NaOCl and 0.1% of "7X" laboratory detergent (Tecnomara AG, Zürich), followed by 95% (wt/wt) ethanol. Axenic cultures were obtained by incubating tissue explants in the basal medium of Linsmaier and Skoog (14) as described by Hansen *et al.* (15) supplemented with 500 mg of the antibiotic Cefoxitin (Roussel, Paris) per liter.

**Tissue Culture and Grafting Methods.** Tumor cells were cloned and cultured as described (16). In brief, hormone-autotrophic tissue lines were subcultured at 21-day intervals on the basal medium of Linsmaier and Skoog (14) solidified with 1% agar ("hochrein" grade; Merck, Darmstadt, F.R.G.) and supplemented with 5 mg of the pH indicator chlorophenol red (Eastman) per liter. Hormone-requiring lines were maintained, as indicated, on the basal medium supplemented with the auxin α-naphthalene acetic acid at 2.0 mg/liter and the cytokinin kinetin at 0.3 mg/liter. Plants were regenerated from cultured tissues by incubating the tissues for 4–6 weeks on medium containing kinetin at 4.8 mg/liter as recommended by Binns (17). Growth is expressed on a fresh-weight basis as $(W - W_0)/W_0$, where $W$ is the tissue weight after 21 days and $W_0$ is the initial weight of the explant.

Tumorigenicity of cultured tissues was assayed in a grafting test. Cube-shaped explants, 2.3 mm on a side, from tissues 14–21 days after subculturing were grafted under the epidermis on the side of Havana 425 tobacco plants by the method of Meins (18).

**DNA Analysis.** DNA was isolated by the method of Murray and Thompson (19). In Southern blot analysis, 10 μg of plant DNA was digested completely with *Hin*dIII, fractionated by electrophoresis in a 0.8% agarose gel, and transferred to a nitrocellulose filter (20). Filters were hybridized with a nick-translated probe for the *tmr* region (21) under conditions that permit the detection of a single gene copy as determined by reconstruction experiments. The probe used for the *tmr* region of the T-DNA was a 1.98 kilobase (kb) *Hin*dIII–*Bam*HI subfragment from pTiT37, which spanned the *Hpa* I insertion site for the Tn*5* transposon. It was kindly provided by Michael Bevan (Plant Breeding Institute, Cambridge, U.K.).

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Abbreviations: Ti plasmid, tumor-inducing plasmid; T-DNA, transferred DNA; *Hl*, habituated leaf trait or gene; $tmr^+$, functional *tmr* region of the T-DNA; $tmr^-$, nonfunctional *tmr* region of the T-DNA containing a Tn*5* transposon insert; kb, kilobase(s).

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## RESULTS

**Infection of Plants.** To find out if the *Hl* gene had an effect on tumor inception, wild-type and *Hl/Hl* plants of the same age and comparable size were inoculated with *A. tumefaciens* strains carrying the *tmr*$^+$ and *tmr*$^-$ plasmids. Representative results are shown in Fig. 1. No difference in the response of the two plant genotypes was observed. In tests with 16 replicate inoculations, wild-type and *Hl/Hl* plants consistently formed large tumors with the *tmr*$^+$ strain and formed either very small tumors or no tumors with the *tmr*$^-$ strain. Therefore, the *Hl* gene did not replace *tmr* functions essential for tumor inception.

**Assays for the Neoplastic Phenotype.** Axenic cultures were established from tissue at the sites of bacterial inoculation on the plants. Cloned lines were isolated with the four possible combinations of plasmids and host cells: *hl/hl tmr*$^-$, *hl/hl tmr*$^+$, *Hl/Hl tmr*$^-$, and *Hl/Hl tmr*$^+$. We verified that the clones contained nopaline, the specific marker for cells transformed with pTiT37 plasmids (22).

Three commonly accepted criteria were used to judge the neoplastic phenotype of the cloned lines: capacity for hormone-autotrophic growth, inhibition of plant regeneration, and tumorigenicity in grafting tests (4). As expected the *hl/hl tmr*$^+$ and *Hl/Hl tmr*$^+$ lines with a functional *tmr*$^+$ were capable of growth on hormone-free medium; whereas the nine *hl/hl tmr*$^-$ lines required cytokinin for growth. The important point is that the 69 *Hl/Hl tmr*$^-$ lines were also hormone-autotrophic even though they did not contain the functional *tmr* locus.

In more detailed experiments, the kinetin dose–response relationship of representative *hl/hl tmr*$^-$, *hl/hl tmr*$^+$, and *Hl/Hl tmr*$^-$ lines was measured in the absence of added auxin (Fig. 2). The *hl/hl tmr*$^-$ line required the same optimum kinetin concentration as did the nontransformed *hl/hl* lines cultured in the presence of auxin (23). The *Hl/Hl tmr*$^-$ transformants, like wild-type cells transformed with *tmr*$^+$ plasmids, were inhibited in their growth by concentrations of kinetin optimal for the *hl/hl tmr*$^-$ line. These results show that with regard to cytokinin requirement, the presence of the *Hl* gene can complement the T-DNA and restore the hormone-autotrophic phenotype typical of crown gall cells.

Normal, cultured tobacco cells can give rise to complete plants when incubated on medium containing a high cytokinin-to-auxin ratio (24). As a result of transformation by the Ti plasmid, tobacco cells lose this regenerative capacity. Cloned lines of pTiT37 transformed cells can form shoots when grafted onto the cut surface of decapitated tobacco plants, but they do not form roots (25). The presence of a functional *tmr* locus is required to block regeneration of plants from tobacco cells (26). We confirmed that *hl/hl tmr*$^-$ clones are capable of regeneration. Plantlets were obtained from 15 clones incubated on an inductive medium containing high concentrations of kinetin (17). Under the same conditions, 35 *Hl/Hl tmr*$^-$ clones failed to form plantlets, showing that the *Hl* gene can compensate for a defective *tmr* in suppressing regeneration.

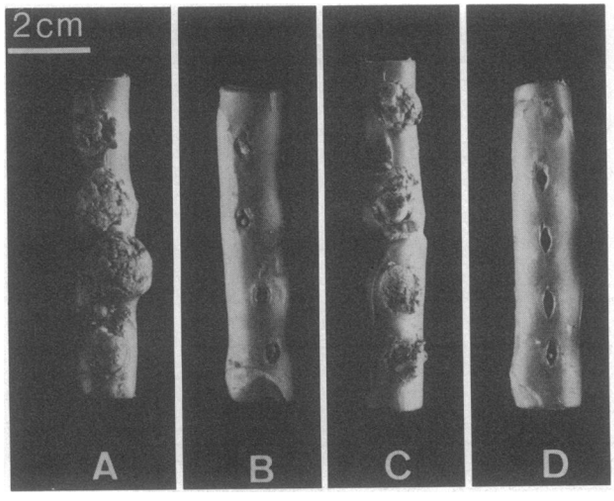


FIG. 1. Inoculation of Havana 425 tobacco plants with *A. tumefaciens* strains carrying *tmr*$^-$ and *tmr*$^+$ plasmids. *Hl/Hl* host with *tmr*$^+$ (*A*) and *tmr*$^-$ (*B*) strains; wild-type *hl/hl* host with *tmr*$^+$ (*C*) and *tmr*$^-$ (*D*) strains. Suspensions of bacteria were introduced into punctures made through the stems of 3-month-old plants. The stems were photographed 8 weeks after inoculation.

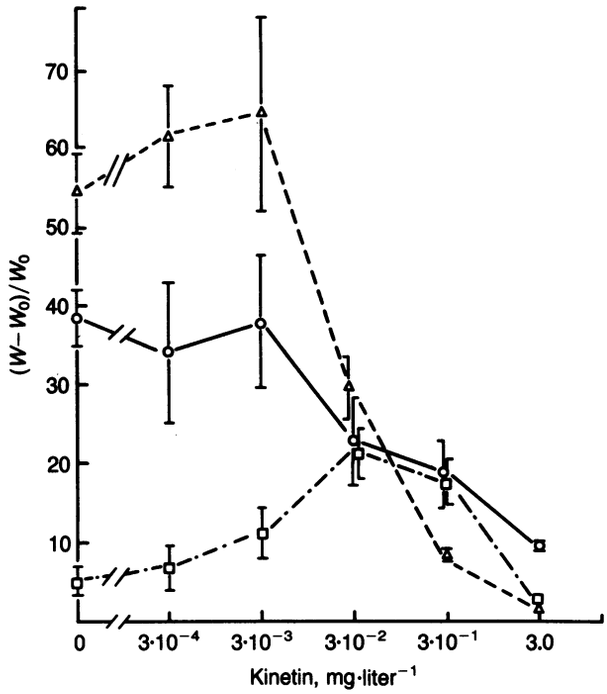


FIG. 2. The growth response of cloned lines of *hl/hl tmr*$^-$ (□), *hl/hl tmr*$^+$ (○), and *Hl/Hl tmr*$^-$ (△) tissues to different concentrations of the cytokinin kinetin. Growth is expressed as the relative increase in fresh weight after 21 days of incubation. Error bars show SEM for eight to nine replicate explants.

The crucial test for neoplastic growth is the ability of tissues to form tumors when grafted onto the host plant (10). No tumors were formed in control grafts with nontransformed *Hl/Hl* and *hl/hl* tissues. The *hl/hl tmr*$^-$ clones were weakly tumorigenic (Fig. 3). Most grafts (29 of 38) did not form tumors, several (5 of 38) formed very small tumors, and 2 grafts formed tumors comparable in size to those obtained with *hl/hl tmr*$^+$ clones. In contrast, 39 of 41 grafts of *Hl/Hl tmr*$^-$ clones formed large tumors. Therefore, the *Hl* gene can restore the tumorigenicity of cells transformed with the *tmr*$^-$ plasmid.

**DNA Hybridization.** It could be argued that the autonomous growth of *Hl/Hl* cells transformed with the *tmr*$^-$ plasmid results from elimination of the Tn*5* insert to give a functional *tmr*. To test for this, DNA from nontransformed, *tmr*$^-$-transformed and *tmr*$^+$-transformed cells of both plant genotypes was analyzed by Southern blot hybridization. The 1.98-kb *Hin*dIII–*Bam*HI subfragment from the intact *tmr* used as a probe spanned the *Hpa* I cloning site where Tn*5* was inserted (13) (Fig. 4). No hybridization was detected with DNA from nontransformed *hl/hl* or *Hl/Hl* cells of leaf origin, confirming that tobacco cells do not contain DNA sequences homologous to *tmr* (30). The expected 3.8-kb *Hin*dIII fragment of the intact *tmr* locus (27) was found in DNA from lines transformed with the *tmr*$^+$ plasmid (Fig. 5). If cells contain

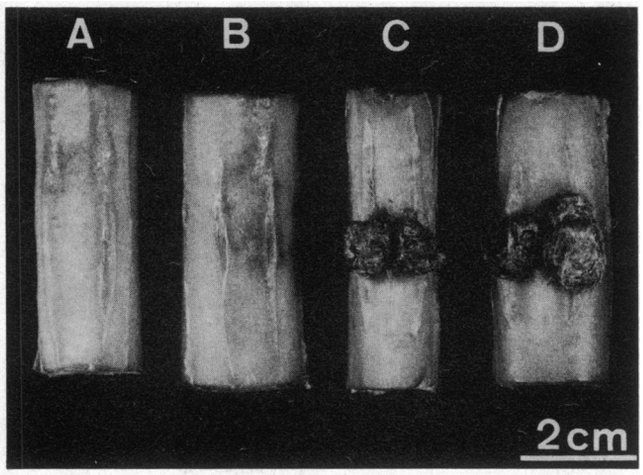


FIG. 3. Tumorigenicity of representative *hl/hl tmr*$^-$ (*A* and *B*) and *Hl/Hl tmr*$^-$ (*C* and *D*) clones. Small explants of the cloned tissues were grafted under the epidermis of the stem of Havana 425 tobacco plants. The stems were photographed 8 weeks after grafting.

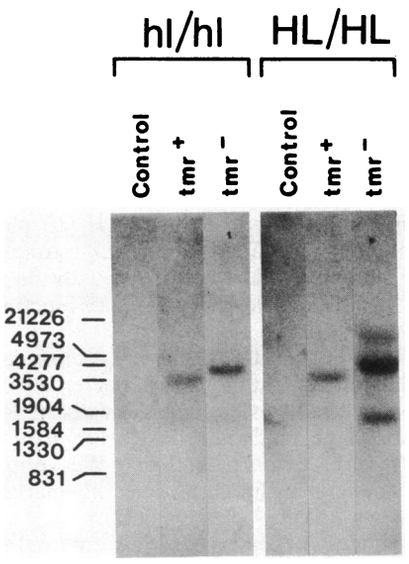


FIG. 5. Southern hybridization of DNA from transformed and nontransformed *hl/hl* and *Hl/Hl* tobacco cells using a probe for the *tmr* locus. The plant DNA was digested completely with *Hin*dIII and was hybridized with a *Hin*dIII–*Bam*HI subfragment spanning the *tmr* region. Control DNA was obtained from leaf tissues cultured, as indicated, from *hl/hl* and *Hl/Hl* plants. The DNA from transformed cells was obtained from cloned lines that were used in grafting tests. Scale at the left shows the size in bp of a DNA standard.

*tmr* with the Tn*5* insertion, then two restriction fragments, 1.7 and 4.2 kb, should hybridize with the probe. The expected fragments were detected in DNA from both *Hl/Hl* and *hl/hl* cells transformed with the *tmr*$^-$ plasmid. Although the 1.7-kb band obtained with DNA from *hl/hl tmr*$^-$ cells is faint, it is clearly visible on the original autoradiograms. The weak hybridization signal obtained with the 1.7-kb fragment probably reflects the short region of homology (0.65 kb) of the fragment with the probe used. An additional >4.9-kb fragment was detected in DNA from *Hl/Hl tmr*$^-$ cells. Similar large bands have been reported by others (26, 31) and have been attributed to incomplete nuclease digestion or aberrant integration events (32). The important point is that our results show that restoration of tumor autonomy in *Hl/Hl* cells transformed with the *tmr*$^-$ plasmids is not accompanied by loss of the Tn*5* insert in the *tmr* region.

## DISCUSSION

There is considerable indirect evidence for cellular genes in plants with an oncogenic function. In crown gall transformation, expression of the tumor phenotype depends on the epigenetic state of the host cell and the species of the host plant as well as the strain of the Ti plasmid used to incite the tumor (33). It appears that both the *tmr* region and a gene concerned with cytokinin synthesis in the *vir* region of the Ti plasmid, which is not integrated into the host cell, affect the host range of *A. tumefaciens* strains (34, 35). Finally, neoplastic transformation of plant cells can occur in the absence of plasmids or other readily identified infectious agents. Interspecific sexual hybrids obtained with certain *Nicotiana* species form spontaneous tumors (36). On prolonged culture, plant cells sometimes become neoplastic; they lose their requirement for both auxin and cytokinin and are tumorigenic in grafting tests (37). Thus, the presence of T-DNA in the host cell is neither necessary nor sufficient to account for neoplastic growth.

Our results provide direct evidence for a cellular gene in

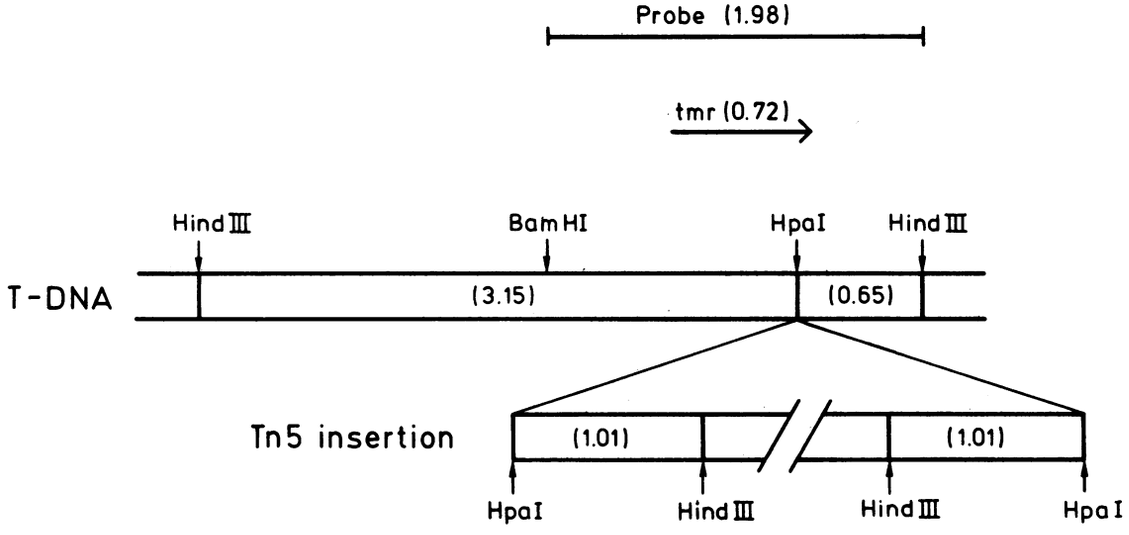


FIG. 4. Partial restriction maps showing the 1.98-kb *Hin*dIII–*Bam*HI subfragment used as a probe in DNA hybridization experiments and the position of the Tn*5* insert in the *tmr* region. The maps were constructed with data from refs. 27, 28, and 29. The numbers in parentheses are distances in kb.

tobacco that can be activated by mutation to have an oncogenic function similar to the *tmr* locus borne on the Ti plasmid. Three characteristics of autonomous crown gall cells—hormone autotrophy, inability to regenerate plants, and tumorigenicity in grafting tests—are restored in combinations of *Hl/Hl* cells with T-DNA containing a defective *tmr* locus.

Although there were clear cut differences of *Hl/Hl tmr*$^-$ and *hl/hl tmr*$^-$ clones in the hormone autotrophy and regeneration tests, we found that some *hl/hl tmr*$^-$ lines were tumorigenic in grafting tests. This suggests that the *tmr* locus may not be absolutely essential for tumorigenicity. Alternatively, on prolonged culture, the host cells may have undergone mutation at the *Hl* locus to give heterozygous *Hl/hl* cells. It has been shown that this mutation can occur at very high rates—*ca.* $5 \times 10^{-3}$ mutations per cell generation in culture (38).

In animals, specific cellular genes with an oncogenic function, the oncogenes, have been identified that exhibit sequence homology with oncogenes borne by oncogenic viruses (39, 40). It is unlikely that a similar relationship exists between *Hl* and the *tmr* locus. There is no evidence from our studies or from the literature (30) for *tmr* sequences in the DNA of normal tobacco cells as judged by hybridization. Moreover, *Hl* and *tmr* have different functions in tumor development. *Hl* can replace the requirement for *tmr* in expression by cells of the tumor phenotype; but it cannot replace the requirement of *tmr* for tumor inception. The *A. tumefaciens* strain carrying *tmr*$^-$ plasmids does not induce tumors on either *hl/hl* plants or *Hl/Hl* plants.

This observation emphasizes the importance of developmental regulation in the formation of crown gall tumors. Even though tumors do not form when *Hl/Hl* plants are inoculated with the *tmr*$^-$ strain of bacteria, tissues cultured from the inoculation site are autonomous and tumorigenic when grafted on the host plant. Evidently, culturing the tissues triggers the persistent expression of the tumor phenotype. Similar conclusions may be drawn from studies of shoots regenerated from cloned lines of tobacco cells transformed with wild-type pTiT37 plasmids (41, 42). Although these shoots appear to be composed of tumor cells (43), they often exhibit normal morphology and do not form tumors either spontaneously or in response to wounding. Nevertheless, when normal-appearing tissues are cultured, they exhibit the tumor phenotype and form tumors when grafted on the host plant. There is good evidence that this culture-induced change depends on both the epigenetic state of the cell and the hormonal constitution of the culture medium (17, 41, 42).

The physiological basis for the oncogenic function of the *Hl* gene is not known. Cultured crown gall cells produce several metabolically related cytokinins (44). Nontransformed tobacco cells have the genetic potential to produce these cytokinins but do not normally do so in culture (15, 45). The *tmr* locus codes for just one enzyme in the biosynthetic pathway, isopentenyl transferase. Therefore, additional T-DNA and/or host genes must be involved.

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