Strain-Dependent Temperature-Sensitive Phase in Crown Gall Tumorigenesis

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ABSTRACT

The effect of high temperature treatments on the early stages of crown gall tumorigenesis in sunflowers was investigated. Treatments of 32°C initiated at various times during the first ten days after infection had a similar effect on tumors induced by Agrobacterium tumefaciens strains B₅ and C58. Tumor growth was sensitive to 32°C until 60 hours after infection and was stimulated by 32°C at that time. Therefore, the "inception phase" for both C58 and B₅-induced tumors ends between 60 to 72 hours after infection. In contrast, B₅ and C58 tumors varied in their response to 37°C treatments during the first 168 hours after infection. Both C58 and B₅ tumors were sensitive to 37°C during the first 72 hours; however, B₅ tumors became resistant to 37°C after 96 hours, whereas C58-induced tumors remained sensitive until 144 to 168 hours after infection.

The growth rate of C58 and B₅ tumors in plants moved to 32°C at 90 hours after infection was the same. B₅ tumors in plants moved to 37°C at 90 hours after infection also continued to grow rapidly, whereas C58 tumors in plants moved to 37°C at 90 hours exhibited a complete cessation of tumor growth. The 37°C temperature-sensitive period specific to C58 tumors ceases by 168 hours after infection. This indicates that the temperature-sensitive functions are no longer required after 168 hours. The possible significance of this work with regard to the presence of a temperature-sensitive plasmid, pTIC58, in strain C58 and the mechanism of tumor-inducing plasmid DNA maintenance in incipient tumor cells is discussed.

The discovery of T° plasmids in all Agrobacterium tumefaciens strains and the proof of their involvement in crown gall disease (28, 29) has led to significant advances in our understanding of the mechanism of plant tumorigenesis. Most significantly, it has been shown that T-DNA is stably incorporated in plant tumor cells and is transcribed in tumor cells (9, 10, 12, 13, 19). At least 18 copies of a specific restriction endonuclease-generated fragment of Ti plasmid B₆-806 are present per tumor cell in the tumor line examined by Chilton et al. (9). Thomashow et al. (27) have shown that T-DNA segments appear to be integrated into plant DNA at multiple sites in the tumors they studied and the findings of Schell et al. (23) have also shown that T-DNA is integrated into plant DNA.

Once present in the plant cell, T-DNA induces the synthesis of various enzymes which catalyze the synthesis of "opine" derivatives of several amino acids and these derivatives are produced only in plant tumor cells (1, 20). Thus, the T-DNA is capable of bringing about the persistent alteration of biochemical pathways within the plant cell. The increased synthesis of other metabolic intermediates such as glutamine and plant hormones (auxin and cytokinins) are also characteristic of crown gall tumor cells (5, 7). These characteristics enable tumor cells to grow on tissue culture media in the absence of added hormones which are required for the growth of normal cells (7).

A new area of research has been made possible through the discovery of the Ti plasmids. It involves the use of temperature-sensitive plasmid mutations to study the role of specific plasmid genes during crown gall tumorigenesis. The first T° function of an Agrobacterium plasmid was identified by Hamilton (14) who showed that strain C58 loses its oncogenicity upon culturing at 37°C. Several reports (28, 29) have shown that this is due to loss of the Ti plasmid from this strain at 37°C. DNA-DNA hybridization studies have shown that the plasmid DNA is not integrated into the bacterial chromosome as a result of 37°C heat treatment but is completely lost from the cell (29). The replacement of the T° plasmid in strain C58 with a temperature-resistant plasmid (pTi B₅ 806) results in an exconjugant (strain A277) which does not lose its plasmid at 37°C. Therefore, the T° trait for plasmid maintenance is plasmid-coded in strain C58.

T° mutants have been used successfully to elucidate the function of SV40 genes during the early and late stages of viral infection of permissive monkey AGMK cells (16, 24, 25). Early studies with SV40 showed that T° mutants could be divided into three groups based on their functional defects (24). Two groups involved T° defects in structural components of the virus and one group involved defective synthesis of viral DNA. Experiments with the mutant NTG-7, defective in viral DNA synthesis, provided direct evidence that a viral function is required for the replication of SV40 DNA in monkey cells. This was done with temperature shift experiments which showed an inhibition of viral DNA synthesis at the restrictive temperature. These experiments defined the period during which the expression of the viral replication functions began and ended, thus revealing the time course of expression of T° genes during SV40 transformation of monkey cells.

This paper reports the results of temperature shift experiments aimed at studying the expression of pTIC58 T° functions during crown gall tumorigenesis. A correlation has been drawn between the presence of T° genes for plasmid maintenance, located on pTIC58, and the inhibition of tumorigenesis during a specific period after infection by high temperatures. This period immediately follows the completion of the inception phase as described by Braun (3, 4, 6, 8) and, therefore, is believed to be due to the T° function of plasmid genes within incipient plant tumor cells. Furthermore, the T° period ends 7 to 8 days after infection illustrating a transient requirement for the T° function during tumorigenesis. The rationale and implications of this work are discussed.

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2 Abbreviations: Ti, tumor-inducing; T-DNA, tumor-inducing plasmid DNA; T°, temperature-sensitive.
MATERIALS AND METHODS

Bacterial Strains. *A. tumefaciens* strain C58 was obtained from R. H. Hamilton, Penn State University. It, as well as strain B6, was maintained on Y medium (1% yeast extract, 2% glucose, 2% calcium carbonate, 1.5% agar) and transferred to nutrient agar plates for single colony isolation before use in experiments. *A. tumefaciens* strain B6 was obtained from A. Braun, Rockefeller University, NY. In some experiments, the initial culture of strain B6 was grown in octopine medium (15) at 37°C before plating on nutrient agar at 28°C for use as inoculum.

Sunflower Crown Gall Tumor Assay.

Plant Material. Seeds of *Helianthus annuus* var. Mammoth Russian, were planted 1.27 cm deep in 10.2 cm square plastic pots in coarse, packed vermiculite and germinated in a lighted growth chamber at 26°C with watering and fertilizing (Peters soln, 10-10-10) from the bottom. Seedlings were grown under a mixture of fluorescent and incandescent light at 2500 lux, in controlled environment chambers with a 17-h daylength. The internal chamber temperature at plant height was measured with mercury thermometers placed directly below the position of stem wounds. Five days after planting seedlings were thinned to 5 to 7 uniform plants.

Inoculation. Stationary L Broth- or octopine-grown cultures were plated on nutrient agar (0.1 ml/plate) and grown overnight at 28°C. Bacterial inoculum was prepared by harvesting cells from 1-day-old lawns grown on nutrient agar and suspending them in water to a density of approximately $1 \times 10^{11}$ cells/ml. Five μl of inoculum was placed in fresh wounds which were made in the hypocotyl of sunflower seedlings, 3 mm below the cotyledonal node. Wounding was accomplished by making one longitudinal slice in the center and completely through the hypocotyl with a flame-sterilized No. 11 scalpel blade. The size of the wound was determined by the dimensions of the blade and was uniform from seedling to seedling. Inoculated plants were watered only from the bottom. Three to four days after inoculation the entire stem above the cotyledons, including epicotyl, primary leaves, and apical meristem was carefully pinched off. This allowed tumor growth to occur without competition from shoot growth. Some early experiments were conducted without pinching the tops or fertilizing the seedlings. The results of those experiments (Figs. 1, 6, and 8) were qualitatively the same as with pinched and fertilized seedlings, except that the tumor size was smaller.

**RESULTS**

Sunflower Tumor Assay. Six-day-old sunflower seedlings were inoculated with increasing amounts of *A. tumefaciens* strain C58 to determine the minimum number of bacteria needed to saturate the wound sites. The dose response curve shown in Figure 1 illustrates that maximum tumor size was obtained with $10^6$ or more viable bacteria inoculated per wound. The dose response curves for other bacteria including strains 209 and B6 were similar to that of strain C58 (data not shown).

Large callus-like sunflower tumors were formed in response to both *A. tumefaciens* strains C58 and B6. In no instance were teratomas observed on sunflower. The growth rate of tumors induced by strains C58 and B6 was the same during the first 13 days after inoculation (Fig. 2), and all experiments were conducted within this period. A delay of only 2 h between wounding and inoculation causes a 40% reduction in the final tumor size as shown in Figure 3. If bacteria are withheld from the wound about 80 h, no tumor formation is observed in the wound area after 12

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*Fig. 1.* Dose-response curve for crown gall tumor formation. Sunflower wounds were inoculated with the designated amounts of *A. tumefaciens* strain C58, and the resultant tumors were harvested and weighed 10 days later.

*Fig. 2.* Growth rate of crown gall tumors on sunflower seedlings. Tumors induced by *A. tumefaciens* strain B6 (O—O) and C58 (---).

*Fig. 3.* Final tumor weight as a function of delay in inoculation time after wounding. *A. tumefaciens* strain C58 was applied to wounds at the specified intervals after wounding without disturbing the tissue. Tumors were harvested 10 days after inoculation.

Data. Tumors were harvested by excising the tumor directly above and below the primary wound site. Stem tissue below the wound which had swelled was not included as tumor tissue. Net tumor weight was determined by subtracting the fresh weight of stem sections of wounded, uninoculated controls kept at 26°C from the fresh weight of the experimental tumors. The net tumor weight of experimental tumors in temperature shift experiments is expressed as percentage of the net tumor weight of 26°C controls (kept at 26°C for the entire test period).
days. Extended growth of these plants results in the production of small slow-growing tumors. Presumably the lack of tumor formation when inoculations are delayed longer than 80 h is due to the masking of binding sites for *A. tumefaciens* as the wound response progresses.

**Time Course of 32 C Sensitivity.** Braun and others (4, 6, 18) have reported that temperature treatments ≥ 30°C prevent crown gall tumor formation when they are applied immediately after wounding, and this period in which treatments of 30°C or above inhibit crown gall tumor formation has been operationally defined as the “inception” phase. In *Vinca rosea*, this period occurs between 2 to 5 days after infection (2, 3).

The point at which tumor formation becomes resistant to 30°C treatments has been designated the beginning of the “developmental” phase (8). Living bacteria are not required in the wound during the developmental phase for tumor growth to occur (3, 4). Therefore, it is believed that transfer of T-DNA to plant cells must occur during the inception phase.

To define the end of the inception phase in sunflower, according to the method of Braun, temperature shift experiments were conducted. The point at which tumor formation was no longer inhibited by 32°C treatments was taken as the end of the inception phase and beginning of the developmental phase. The final size of tumors on sunflower seedlings which were kept at 26°C for various intervals before being shifted to 32°C are shown in Figure 4. The average fresh weight per tumor (10–15 tumors/group) at the end of the 10-day period was used as an assay of the effectiveness of a particular temperature regime either to inhibit or stimulate tumor formation. The results of this experiment show that tumor formation was inhibited by 32°C treatments initiated up to 60 to 72 h after infection.

The period of 32°C sensitivity was similar for tumors induced by both strains C58 and B6. Shifts to 32°C after 72 h stimulated the growth of B6 tumors to a greater extent than C58 tumors. The switch from temperature sensitivity to stimulation is consistent with Braun’s findings that after the inception phase is complete, the developmental phase is stimulated by temperatures of 32°C (6).

**Time Course of 37°C Temperature Sensitivity.** After having established that the inception phase was the same duration for both C58- and B6-induced tumors, additional work was carried out to determine the time course of sensitivity to 37°C treatments. This was of interest because pTiC58 carries a gene which confers temperature sensitivity for plasmid maintenance on the plasmid at 37°C. In contrast, strain B6 does not contain a T′ plasmid. If the T′ gene for plasmid maintenance, located on pTiC58, functions during tumorigenesis, then one would expect 37°C treatments to inhibit tumor formation during the period in which that gene is being expressed. In contrast, B6 tumors would be expected to be temperature-resistant during the same period because the plasmid maintenance functions of B6 are temperature-resistant.
The 37°C temperature shift experiments were conducted in the same manner as the 32°C experiments, and the combined results from three separate experiments are shown in Figure 5. Slight variations between the experiments cause the data to appear more variable than they were in any individual experiment. In contrast to the 32°C sensitivity periods, which were similar for C58 and B6, C58-induced tumor formation was inhibited by 37°C treatments up to 168 h after infection. B6 tumors exhibit a rapid transformation to temperature resistance between 60 and 100 h after infection which corresponds closely to the 32°C sensitivity period for this bacterium. C58-induced tumors become temperature-resistant gradually between 72 and 168 h after infection. This represents a lag of approximately 3 days before C58-induced tumors exhibit the same degree of resistance to 37°C as do B6 tumors.

Experiments were conducted to determine the effect of 32 or 37°C treatments on the growth rate of C58 and B6 tumors. Treatments of 32 or 37°C were initiated at 90 h after infection with the goal of determining if the difference in tumor size observed in earlier experiments (Figs. 4 and 5) was due to differences in the growth rate of the tumors or a qualitative prevention of tumor formation. The results of Figure 6 show that at 32°C, C58 tumors exhibit growth kinetics similar to those of B6 tumors. In contrast, plants infected with C58 and moved to 37°C after 90 h exhibited a complete inhibition of tumor growth whereas B6 tumors at 37°C grew the same amount as tumors kept at 32°C.

The 37°C temperature shift experiment indicated that C58 tumors become resistant to 37°C after 168 h. To quantitate this result, C58 tumors were moved to 37°C at 168 h, and tumor growth kinetics were determined (Fig. 7). As expected, the C58 tumors moved to 37°C at 168 h have a growth rate identical to that of controls kept at 26°C. The conclusion is that the 37°C sensitivity period is a transient effect which is expressed before 168 h after infection. After 168 h, C58 tumors behave as do temperature-resistant B6 tumors with regard to growth rate.

A reverse temperature shift experiment was conducted for B6 and C58 induced tumors in which infected seedlings were kept at 37°C for varying periods immediately following infection before transfer to 26°C (Fig. 8). A gradual reduction in final tumor size was observed according to the length of time the plants were kept at 37°C before transfer to 26°C. No tumors were formed on plants kept at 37°C for 88 or more hours after infection and then moved to 26°C. Saturating levels of virulent bacteria (10⁶ or greater, data not shown) were reisolated from the wounds of treated plants kept at 37°C for 100 h. Similar results were obtained for a 32°C reverse temperature shift experiment conducted in a similar manner (data not shown). These data further support the conclusion that the inceptive phase of crown gall disease in sunflowers is complete by approximately 80 h after wounding because virulent bacteria present within the tissue after that time cannot induce tumor formation at permissive temperatures.

**DISCUSSION**

To detect the expression of a temperature-sensitive gene during crown gall tumor formation, a rapid, high efficiency assay for tumor formation was necessary. Since a suitable in vitro assay is not available, an assay using intact sunflower plants was developed in which large tumors form in a 10-day period. The effect of a particular temperature treatment on tumor formation is determined by measuring the fresh weights of tumors produced under various temperature regimes and comparing them to those of control tumors kept at 26°C. Although direct measurements of the numbers of cells transformed in a particular wound have not been made, the tumor growth data of Figure 2 show that tumor growth rate is nearly linear during the experimental period. The tumor size in the assay is probably directly related to the number of tumor cells initially formed.

The usefulness of the sunflower system in elucidating the expression of plasmid T' genes during tumor formation depends on two primary criteria. First, the cells in the wound vicinity must become "conditioned" or "competent" to be transformed in a synchronous manner. Second, the transfer of the Ti principle (T-DNA) must
occur in a synchronous manner, so that one can identify the point at which the inception phase is complete. This was done according to the criteria of Braun which define the end of the “inception” phase as the point at which tumor formation becomes resistant to temperatures above 30°C (7, 8). T* functions observed after 30°C resistance is established may be due to T-DNA directed events occurring in the incipient plant tumor cell rather than the bacterium.

The tumor formation response described in this paper is compatible with the criteria stated above. A rapid change from temperature sensitivity to temperature resistance occurs in the 32°C temperature shift experiments indicative of a synchronous population of responding cells. The shift occurs at different times after infection according to which strain of A. tumefaciens is used and which temperature regime is selected. A summary of the temperature-sensitive periods affecting tumor formation by strains C58 and Bk are shown in Figure 9. The 32°C T* period is similar for both strains C58 and Bk and is believed to represent the period up to the completion of T-DNA transfer. The 37°C T* period for strain Bk coincides with that of the 32°C period. This is expected because pTiBk does not have any known T* genes which might function during tumor induction. On the other hand, the 37°C T* period for C58-induced tumors extends 80 to 96 h past that of the 32°C sensitivity period. This lag in the acquisition of temperature-resistance is specific for strain C58. A possible explanation for this lag is that a plasmid-coded T* gene product is required during this stage of tumorigenesis in plant cells. It is possible that high temperature (37°C) causes its inactivation thus preventing further continuation of cell transformation and tumor growth.

Braun has shown that when tumor formation is interrupted at different times during infection by high temperature treatment, the final growth rate and phenotype of the tumors produced are affected (7). For example, tumors formed after high temperature treatment early during infection are slow-growing and require hormone supplements in the medium to reach the growth rate of fully transformed tumor cells. Furthermore, high temperature treatments initiated later in tumor formation result in fast-growing tumors which are independent of added hormones for maximum growth in tissue culture. It is possible that part of the difference in final tumor size in the temperature shift experiments reported here is due to a difference in the growth rate of tumor cells interrupted at different stages of tumor formation. Differential growth rate of tumors is not sufficient to explain the differential response to C58 tumors to 32 or 37°C treatments between 60 and 144 h after inoculation when Bk tumors behave identically to 32 or 37°C treatments during that same period.

The fact that C58-induced tumors become resistant to 37°C between 144 and 168 h after infection indicates that the T* gene products are no longer required after 168 h. The transient expression of T* functions similar to those observed in the sunflower system have also been reported for T* mutations of SV40 and avian sarcoma virus during cell transformation (17, 24, 25). One model to explain the results presented is that there are two temperature-sensitive processes occurring during C58-induced cellular transformation. The first one is sensitive to 32°C and is common to all tumors induced by A. tumefaciens (8). This could possibly be the T-DNA transfer step as has been suggested by Tempe et al. (26). The second process is sensitive to 37°C but not 32°C and is specific to strain C58. This step requires the function of the T* plasmid-coded gene products within the incipient plant tumor cell in order for cell transformation to reach completion. Genetic evidence directly showing that the 37°C T* period is coded by the pTiC58 plasmid has been recently presented and supports this model (22).

The exact function of the T* genes in strain C58 is not known at this time. Data on the incorporation of 3H thymidine into plasmid DNA of strain C58 show that incorporation into plasmid DNA is inhibited at 37°C but not at 32°C (22). These findings along with the known curing of pTiC58 from strain C58 at 37°C make it attractive to speculate that the T* genes are involved in the replication of pTiC58 and that they are responsible for the extension of the 37°C T* phase in C58-induced tumorigenesis. Molecular approaches to this problem should now be used to investigate the mechanism by which temperature sensitivity occurs.

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