The transformation of Zea mays seedlings with *Agrobacterium tumefaciens*

Detection of T-DNA specific enzyme activities

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Summary

Virulent strains of the soil bacterium *Agrobacterium tumefaciens* infect dicotyledonous plants and elicit a profound neoplastic response which results in crown gall formation (18). The inciting agent has been shown to be a high molecular weight plasmid (Ti) a section of which, the T-DNA, integrates into the host plant's genome (4, 28, 30). Although transformation of this kind was presumed to be limited to dicots, the detection of enzyme activities linked to the expression of T-DNA has been demonstrated in monocots from the families Liliaceae and Amaryllidaceae (10, 11).

In this communication, we present evidence that a member of the commercially important Gramineae also is subject to *A. tumefaciens* directed transformation. This conclusion is based on two observations. First, seedlings of *Zea mays* that have had the bacteria introduced into wound sites defined by a region which includes the scutellar node and mesocotyl express the activity of enzymes whose synthesis is associated with the translation of T-DNA transcripts. Specifically, strain specific lysopine dehydrogenase activity has been detected in B6 infected material, whereas nopaline dehydrogenase activity is reported only in those plants inoculated with C58N. Second, the detection of either of these activities in extracts made from infected maize plants requires that the assaulting bacterial strain be competent with respect to the transfer of T-DNA. The vir strains, JK195 and 238MX, are not, and transformation does not seem to occur. In this connection, the corresponding opine synthase activities are not observed.

Introduction

When plant wound sites are infected with *Agrobacterium tumefaciens* carrying a Ti plasmid, crown gall tumor results (18). This response is coincident with the specific integration of the plasmid's T-region into the chromosomes of recipient plants and constitutes a naturally evolved transformation system (4, 28, 30). Linked to this DNA segment are genes that affect tumor morphology and the synthesis of a specialized class of amino acids called opines (19, 20). Opine synthase genes are expressed only in infected plant tissue and not in the host bacterium. This is consistent with the observation that both 'TATA' and polyadenylation-like termination sequences have been identified with these genes. Such characteristics are known to be associated with the regulation of eukaryotic rather than prokaryotic genes (2, 6, 7). The identification of a particular opine synthase activity in extracts from infected plants may be taken as evidence that T-DNA has entered a cell and is expressed.

Since monocotyledonous plants do not form crown galls, it has been assumed that the host range of *A. tumefaciens* is restricted and that transformation could not be effected with this bacterium. Such a conclusion has been recently proven false. For example, strain specific opine synthesis has been demonstrated in extracts of successfully transformed members of both the Liliaceae and Amaryllidaceae, indicating the production of T-linked gene product (11). Furthermore, these
changes are not transient but involve actual additions to the plant's genetic complement. Tissue cultured from tumorous proliferations in *Asparagus*, for example, continues to express functions encoded by genes in the T-region for several cell generations (10). This expression includes not only opine synthesis but phytohormone independent growth as well. In principle then, no impediment to the introduction of T-DNA into representatives of the commercially important Gramineae should exist.

**Materials and methods**

Single colonies of the *Agrobacterium tumefaciens* strains B6, C58N, A348, 238MX, and JK195 were inoculated into a yeast extract broth containing 0.1% yeast extract, 0.8% nutrient broth, and 0.5% sucrose. The cells were incubated at 27°C for 48 hours, reaching a final titre of $3.8 \times 10^9$ cells/ml (14). The standard strains, B6 and C58N, that produce lysopine and nopaline dehydrogenase activities, respectively, in suitable plant hosts, were provided by Lippincott and Kado. A348 carries the broad host range plasmid pTiA6NC marked by $Vir^+$, $Oec^+$, and $Tra^+$ as described by Sciaky (27) and was donated by Nester. 238MX is similar in background and source but carries a Tn3 insertion in the $virB$ gene thus rendering it avirulent (Nester, per. comm.). JK195 is a C58N derived $vir^-$ mutant which carries a Tn5 insertion in complementation group VI. It was provided by Kado and a detailed description may be found in Kao (12) and Lindquist (16). Each strain was selected on media containing the appropriate antibiotics and supplements.

Seeds of the hybrid yellow Iochief were sterilized using the procedure outlined in Hernalsteens (10). The protocol includes an initial 2 minute treatment in 70% v/v ethanol, followed by a 5 minute in cubation in 0.5% w/v HgCl$_2$, and a final 30 minute wash in 15% v/v of commercial bleach containing 0.1% liquid dishwashing detergent. Following the sterilization, the seeds were washed in sterile double distilled water.

The treated seeds were placed on sterile moistened Whatman 3 filter paper and incubated in constant darkness at 25°C for four days. A total of $10^8$ cells were dripped into four wound sites on the surface of the germinating seedling, involving an area which extends from the base of the scutellar node through the mesocotyl. The location of each incision is shown in Fig. 1. The precise number of bacteria that remain in each wound site after infection is unknown. After receiving an inoculum, these seedlings, five per plate, were incubated on agar at 27°C under conditions of controlled humidity for an additional 7–14 days.

The seedlings were then ground in a Tris · HCl-Sucrose extraction buffer (pH 8.0) and a portion of the homogenate added to an equal volume of an appropriate reaction medium for the detection of lysopine or nopaline dehydrogenase activity. The products of the enzyme activity were separated electrophoretically on Whatman 3MM paper in formic acid/acetic acid/water (5:15:80) buffer pH 1.8 for 2.5 hr. at 450 volts. The presence of the opines was demonstrated following staining with phenanthrenequinone (20).

**Results**

By assaying for the expression of T-DNA linked markers, the transformation of *Zea mays* seedlings was confirmed. Octopine production can be demonstrated in cell-free extracts of B6 transformed material but not its buffered control (Fig. 2a). Furthermore, the amount of opine produced, as measured by an increase in phenanthrenequinone fluorescence, increases in proportion to time of incubation. While no nopaline can be detected in a reaction mixture that includes arginine and $\alpha$-keto glutarate at time zero, it is clearly present after 15 hours of incubation at room temperature (Fig. 2b). Such results are congruent with those expected if the reaction is enzyme catalyzed. In addition, the opine dehydrogenases of strain B6 or C58N are substrate specific. Thus extracts from transformed B6 material can not use $\alpha$-keto glutarate in the condensation reaction with arginine to produce an opine (Fig. 2b).

The synthesis of nopaline by extracts transformed with C58N has been confirmed by criteria other than electrophoretic mobility. When this compound is eluted from a paper chromatogram and treated with hot 2M acetic acid, pyronopaline is formed (Fig. 2c). The conversion reaction is diagnostic for nopaline and no other opine. Finally, *in vitro* directed opine synthesis results in the produc-