control events activate developmental programs appropriate to neural tissue. The production of monoclonal antibodies that recognize other early-appearing markers of neural and epidermal development should facilitate the analysis of the events underlying ectodermal commitment.

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Molecular Basis for the Auxin-Independent Phenotype of Crown Gall Tumor Tissues

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The transfer of specific Ti (tumor-inducing) plasmid sequences, the T-DNA, from *Agrobacterium tumefaciens* to a wide range of plants results in the formation of crown gall tumors. These tissues differ from most plant cells in that they can be grown in vitro in the absence of added phytohormones. Here, data are presented that offer an explanation for the auxin-independent phenotype of crown gall tissues. It is shown that crude cell-free extracts prepared from three bacterial species harboring pTiA6 gene 1 could convert L-tryptophan to indole-3-acetamide; control extracts lacking gene 1 could not carry out the reaction. Other reports indicate that the pTiA6 gene 2 product can convert indole-3-acetamide to indole-3–acetic acid, a naturally occurring auxin of plants. It is concluded that the auxin-independent phenotype of crown gall tissue involves the introduction of Ti plasmid sequences encoding a two-step pathway for auxin synthesis.

CROWN GALLS ARE TUMORS THAT occur on a wide range of dicotyledonous plants as well as a few gymnosperms and monocots (*1*). They are caused by the soil bacterium *Agrobacterium tumefaciens*. The virulence mechanism responsible for this disease, the transfer of genetic information from bacterium to plant, is novel among described prokaryotic-eukaryotic host-pathogen interactions. All virulent strains of *A. tumefaciens* harbor a Ti (tumor-inducing) plasmid, a specific portion of which, the T-DNA, becomes integrated into the plant genome. Subsequent expression of the T-DNA "oncogenes" causes changes in the normal regulated metabolism of auxins and cytokinins, two classes of phytohormones that have key roles in controlling plant cell proliferation and development. Whereas normal plant cells generally require the addition of exogenous auxin and cytokinin for propagation in vitro, axenic cultures of crown gall tissues can be grown indefinitely without them (*2*). This discovery was made some 40 years ago, and since then a fundamental goal of crown gall research has been to understand the

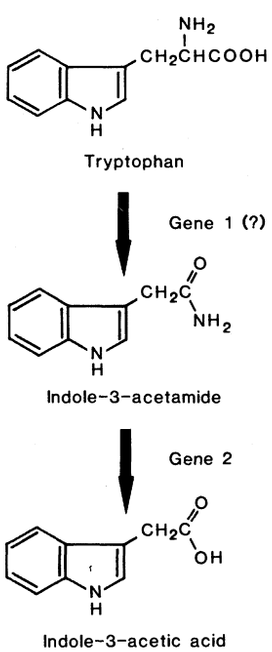

Fig. 1. Pathway for auxin synthesis in crown gall tumor tissues.

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molecular basis of the phytohormone autonomy. Significant progress has been made in understanding the cytokinin-independent phenotype. Three groups (*3*) have shown independently that T-DNA gene 4 encodes an isopentenyl transferase, an enzyme that catalyzes the first step in cytokinin biosynthesis. Here we describe experimental results that help explain the auxin-independent phenotype of crown gall tissues.

Genetic, transcription, and tissue culture studies (*4*) have shown that genes 1 and 2 encoded by the T-DNA regions of pTiA6 and related plasmids are responsible for the auxin-independent phenotype of crown gall tissues. We (*5*) and others (*6*) have demonstrated that gene 2 codes for an amidohydrolase that can convert indole-3-acetamide to indole-3–acetic acid, a natural auxin of plants (Fig. 1). Indole-3-acetamide, however, is not believed to be a common metabolite of plants. It was therefore suggested (*5, 6*) that the gene 1 product may catalyze its synthesis. Here we show that this hypothesis is correct.

We first constructed a plasmid that would express the pTiA6 gene 1 product in *Escherichia coli* (Fig. 2). The starting point for the construction was the plasmid pG1-Z, which contained almost all of gene 1 [approximately 1900 base pairs (bp) of coding sequence and an additional 264 bp of 5′ flanking sequence] fused in frame to the eighth codon of *lacZ* (*7*). When this plasmid was introduced into *E. coli* MC1061, a β-galactosidase–negative strain, only about 50 units of β-galactosidase activity (*8*) was detected. This suggested that the Ti plasmid sequences did not provide efficient transcription or translation signals (or both) for gene expression in *E. coli* (in wild-type *E. coli*, a fully induced *lac* operon produces about 1000 units of β-galactosidase activity). We therefore placed the *tac* promoter (*9*), which includes the *lacZ* ribosome binding site, at different positions in front of the gene 1 sequences and screened for plasmids producing high levels of β-galactosidase (*10*). One molecule chosen for further work, p23G1-Z, had the *tac* sequences placed approximately 100 bp in front of the gene 1 start codon. *Escherichia coli* MC1061 harboring this plasmid produced about 2000 units of β-galactosidase activity and synthesized a novel protein of about 178,000 daltons (178 kD), which is the mass expected for the gene 1-*lacZ* fusion protein. Finally, we reconstructed gene 1 by inserting the Eco RI–Sal I fragment of p23G1-Z, which contained the *tac* promoter and the 5′ portion of gene 1, into a derivative of pUC8, which contained the 3′ end of gene 1 (*11*). This molecule was designated p23G1.

If pTiA6 genes 1 and 2 provide a pathway for indole-3–acetic acid synthesis with indole-3-acetamide as an intermediate, a likely beginning substrate is L-tryptophan (Fig. 1). Such a pathway exists in *Pseudomonas savastanoi*, a plant pathogen that causes hyperplasias on olive and oleander (*12*). In this case, it has been shown that the first step is catalyzed by a monooxygenase that converts L-tryptophan to indole-3-acetamide. We therefore transformed p23G1 into *E. coli*, prepared crude cell-free extracts, and incubated them with L-[^{14}C]tryptophan (*13*). Extracts of cells containing p23G1 produced material that comigrated with authentic indole-3-acetamide, whereas control extracts did not (Fig. 3A, panel 1). Synthesis of this material was not peculiar to *E. coli* because it was also catalyzed by extracts prepared from *P. putida* and *A. tumefaciens* A136 (a strain lacking the Ti plasmid) when the strains contained gene 1 constructions (Fig. 3A, panels 2 and 3, respectively). That the material synthesized was indeed indole-3-acetamide was confirmed by fractionating the samples by means of a second thin-layer chromatography (TLC) developing system and by high-performance liquid chromatography (HPLC) (*14*). In both cases, the material comigrated with authentic indole-3-acetamide. In addition, when cell-free extracts prepared from *E. coli* producing the pTiA6 gene 2 product were added to the ether extracts containing the putative indole-3-acetamide (Fig. 3B) or to the material after purification by HPLC, the material

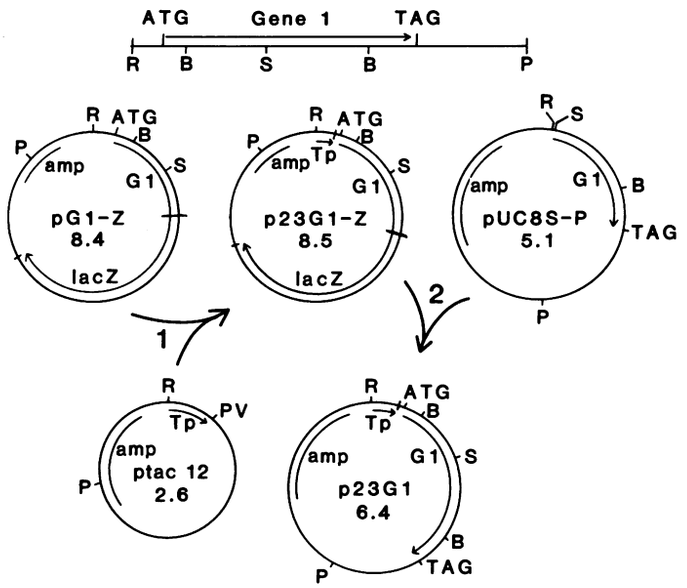

Fig. 2. Construction of a plasmid to produce the pTiA6 gene 1 product in *E. coli*. (Top) A restriction map for the region of pTiA6 encoding gene 1. The gene 1 open reading frame is 2268 bp long (*19*). (Step 1) Placement of the *tac* promoter 5′ to the coding sequence of gene 1. (Step 2) Reconstruction of intact gene 1 (see text for details). Abbreviations: amp, ampicillin resistance; B, Bgl II; G1, pTiA6 gene 1; P, Pst I; PV, Pvu II; R, Eco RI; S, Sal I; Tp, the *tac* promoter. Numbers below the names of the plasmids denote their size (in kilobase pairs).

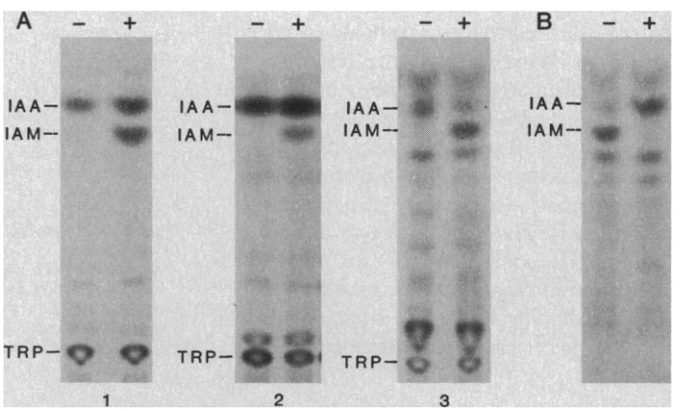

Fig. 3. (A) Conversion of tryptophan to indole-3-acetamide by bacterial extracts containing pTiA6 gene 1. Crude bacterial extracts were prepared and incubated with L-[^{14}C]tryptophan, and the indole compounds were extracted and separated by TLC (*13*). The autoradiograms of the fractionated samples are shown, and the migration positions of authentic indole-3–acetic acid (IAA), indole-3-acetamide (IAM), and tryptophan (TRP) are indicated. (Panel 1) *Escherichia coli* MV17 (W3110 Δ*trpE5 tna2*) (*20*) containing placI^{q} (*21*) plus pUC8 (−) or p23G1 (+). (Panel 2) *Pseudomonas putida* A100 containing either p23G1-Z (−) or p23G1 (+) inserted into the Eco RI site of the wide–host range cloning vector pJBK68 (*22*). (Panel 3) *Agrobacterium tumefaciens* A136 containing pJBK68 (−) or p23G1 inserted into the Eco RI site of pJBK68 (+). (B) Conversion of the putative indole-3-acetamide to indole-3–acetic acid. Ether extracts containing the putative indole-3-acetamide were treated with crude extracts of *E. coli* harboring either pBR322 (−) or pMTlacT2 (+), a plasmid that codes for the synthesis of the pTiA6 gene 2 product (*15*). The indole compounds were extracted and separated by TLC, and the plates were developed by autoradiography.

was converted to a compound that comigrated with authentic indole-3–acetic acid (*15*).

These data indicate that the gene 1 product can catalyze the synthesis of indole-3-acetamide. This result is consistent with two recent observations. First, Onckelen and colleagues (*16*) have shown that *Nicotiana tabacum* tissues transformed with gene 1 have elevated levels of indole-3-acetamide compared to control cells. Second, Yamada and co-workers (*17*) have found that the pTiA6 gene 1 product shares considerable amino acid sequence homology with the tryptophan monooxygenase of *P. savastanoi*. Thus, the gene 1 product is probably a tryptophan monooxygenase that, in combination with the gene 2 product, catalyzes the synthesis of indole-3–acetic acid. It should be pointed out, however, that the gene 2 product has been shown to have a relatively broad substrate specificity (*18*). It can, for example, convert phenylacetamide to phenylacetic acid, another compound with auxin activity. The gene 1 product may also have a broad substrate specificity. One interesting possibility is that it may convert phenylalanine to phenylacetamide. If so, this would mean that the T-DNA could direct the synthesis of at least two potent auxins.

The basic mechanism by which *A. tumefaciens* transforms plant cells to auxin independence is now established. It involves the introduction of a two-step pathway for auxin biosynthesis. Whether additional T-DNA gene products can alter the auxin metabolism of plant cells remains to be determined. It is clear, however, that the response of different plant species to genes 1 and 2 can vary. For example, Binns and colleagues (*4*) have shown that a Ti plasmid with a nonfunctional gene 2 cannot transform *N. tabacum* tissues to auxin independence but can transform *N. glutinosa* tissues to auxin autonomy. The molecular basis for this complementation is not known. Its elucidation should not only lead to a more complete knowledge of the interaction between *A. tumefaciens* and plants but should also shed light on the mechanisms involved in regulating auxin metabolism in plants.

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11. Plasmid pUC8S-P consisted of the Sal I–Pst I fragment of pTiA6 containing the 3′ end of gene 1 inserted into pUC8. The plasmid was digested with Eco RI and Sal I, and the large fragments were gel-purified. These fragments were mixed with p23G1-Z that had been digested with Eco RI and Sal I, and the mixture was ligated and transformed into *E. coli* MC1061. Clones that produced white colonies (β-galactosidase–negative) on LB plates containing ampicillin and X-gal were selected, and the plasmid DNA was subjected to restriction analysis.
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15. L-[^{14}C]tryptophan was incubated with crude extracts of *A. tumefaciens* A136 containing pTiA6 gene 1 as described above (*13*). The indole products were extracted with ether, dried, rehydrated in 50 m*M* tris-HCl (*p*H 8.0), and treated with crude extracts of *E. coli* harboring pBR322 or pMTlacT2 prepared as described (*5*). The *p*H of the reactions was lowered to approximately 3.0 by the addition of hydrochloric acid, and the samples were extracted with ether. The indole compounds were then fractionated on TLC plates (*13*).
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22. pJBK68 is a derivative of pRK290, which has the kanamycin resistance gene from transposon *Tn5* inserted into the Bgl II site (J. Kaper, unpublished results).

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