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INDOLEACETIC ACID PRODUCTION: A PLASMID FUNCTION OF AGROBACTERIUM TUMEFACIENS C58

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Summary: Indoleacetic acid (IAA) production is regulated by the 117 Mdalton pTi plasmid that is harbored by the crown gall bacterium Agrobacterium tumefaciens strain C-58. In the presence of tyrosine, about 5- to 10-fold higher IAA production (0.8-0.9 μg/ml) occurs in strain C-58 than in the plasmidless avirulent derivative strain NT1. The high level of IAA production is restored when the plasmid is reintroduced into strain NT1.

INTRODUCTION

The transformation of higher plant cells into crown gall tumor cells results from infection by Agrobacterium tumefaciens. Oncogenicity is conferred by large pTi plasmids harbored in strains of this organism (12, 30, 32). Portions of this plasmid are apparently stably maintained in tumor cells based on the detection of plasmid DNA sequences in periwinkle and tobacco crown gall cells (5, 6, 26, 27) and on tumor RNA hybridizing to specific plasmid segments (9, 18). These findings suggest that A. tumefaciens actively transfers its plasmid DNA into host plant cells. It is still unclear whether the stably maintained plasmid segments themselves directly play a regulatory role in initiating neoplastic growth or whether such segments code for products that perturb the normal cell machinery. We have therefore surveyed the possibilities of plant growth regulating substances as potential candidates. Evidence is presented in this paper showing that indoleacetic acid (IAA) production (a phytohormone) is regulated by the same plasmid that confers oncogenicity.

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MATERIALS AND METHODS

Bacterial strains. The oncogenic A. tumefaciens strain C-58 was obtained from R. S. Dickey, Cornell University, Ithaca, New York. Strain NT1, a plasmidless derivative of C-58 was obtained from M.-D. Chilton, University of Washington, Seattle, Washington. Other derivatives of strain C-58 were constructed in our laboratory at Davis. Strain 1D1274 is a transconjugant obtained by mating strains C-58 and NT1 that carried rifampicin and neomycin resistance markers for counterselection. In planta mating procedures were employed as described elsewhere (14).

Media and Growth Conditions. The strains were grown in basal medium 925 which contains per liter: 10 g sucrose, 3 g K_2HPO_4, 1 g NaH_2PO_4, 1 g NH_4Cl and 0.3 g $MgSO_4 \cdot 7H_2O$. When required, this medium was supplemented with either L-tryptophan or L-tyrosine at a concentration of 0.2 g/liter. The cells were grown in 250 ml of medium 925 for 48 hours at 25°C on a rotary shaker. They were harvested by centrifugation (10,000 g, 10 min, 4°C) and stored in the frozen state. The supernatant was collected and used immediately for analysis of IAA.

Extraction of Indoleacetic Acid. The culture supernatants were individually processed as follows: they were acidified to pH 2.9 with 1 N HCl at 4°. Immediately thereafter, they were extracted four times with 5 volumes of peroxide-free redistilled diethylether that was pretreated with $FeSO_4$ after distillation to minimize IAA degradation. The ether extracts and the ether was removed by flash evaporation as described by Liu et al. (21, 22). The residue was dissolved in 20 ml of 2% Na_2CO_3 and then adjusted to pH 2.9 with 1 N HCl. This solution was extracted with diethylether as described above and the ether was removed by flash evaporation. The residue was dissolved in 2 ml dry methanol. The recovery of IAA by this procedure was 96% based on β-[2-^{14}C]-IAA (New England Nuclear, sp. act. 50 mCi/mmol) used as an internal standard. In several experiments the methanol and any residual H_2O was removed by lyophilization.

In some experiments, washed cells were sonicated in fresh medium at 0° for 5 min at 1 min intervals. Cellular debris were precipitated with acetone and removed by centrifugation (10,000 g, 10 min, 4°C). The supernatant was processed as described above.

Analysis of Indoleacetic Acid. Each sample was prepared for gas liquid chromatography for analysis of IAA as described elsewhere (21, 22). Known quantities of indolepropionic acid were used as internal standards. The samples with added indolepropronic acid were derivatized with N,O-bis (trimethylsilyl)-trifluoroacetamide or N-methyl-N-trimethylsilyl-trifluoroacetamide (Pierce) in acetonitrile at 100°C for 30 min. About 0.4 µl of sample was analyzed under the conditions described previously (22). IAA was also analyzed by high performance thin layer chromatography as described by Liu et al. (22).

Plasmid Characterization. The pTi plasmid was isolated by lysing lysozyme digested cells with Sarkosyl NL-97 (Geigy) followed by alkali and sodium perchlorate treatment and extraction of the lysate with redistilled phenol as described previously (17). The final purification was performed by standard isopycnic centrifugation of the plasmid in CsCl containing ethidium bromide (23). Molecular weights were determined by measuring the contour lengths of fully relaxed circles of each plasmid preparation. Plasmid Col E1 was used as an internal standard of measurement (mol. wt. = 4.2 mdal).

The oncogenic phenotype was assayed on tobacco, tomato, sunflower, cowpea, and pea plants as described previously (23). The appearance of well defined tumors after 3-4 weeks were scored as positive for oncogenicity. Phage AP1, obtained from J. Schell and M. Van Montagu, State University Gent, Belgium, was used to assay phage exclusion (10). The presence of nopaline was determined by extracting tumor tissue that was homogenized in 60% ethanol. The ethanol extract was concentrated by flash evaporation at 50° and the basic amino acids including nopaline were resolved by paper electrophoresis using 50 mM ammonium formate buffer, pH 3.5, at 300 volts, 15 hr. Nopaline migrates toward the cathode under these conditions whereas most other amino acids and unknown compounds migrate toward the anode. Known nopaline standards were used each time. Nopaline was detected as a yellow green fluorescent band under long wave ultraviolet after spraying the electrophoretogram with a solution containing 1 part 0.02% alcoholic phenanthrenequinone and 1 part 10% NaOH in 60% ethanol. Nopaline was eluted from the paper electrophoretogram and again subjected to electrophoresis on 46 X 57 cm Whatman 3 MM paper.

The utilization of nopaline was determined by observance of growth on basal mineral medium containing 50 µg/ml nopaline as the sole carbon and nitrogen source.

RESULTS AND DISCUSSION

Strain C-58 was compared in its ability to produce IAA relative to its derivative strains. Data presented in Table 1 show that all strains release no detectable IAA in the medium in the absence of tryptophan or tyrosine. Cell extracts of these strains had very little IAA. On the other hand, very high amounts of IAA were present in the culture medium when cells of strain C-58, NT1 or 1D1214 were grown in the presence of tryptophan. This appears to result from the conversion of tryptophan to indolepyruvic acid by means of a transaminating enzyme present in a number of bacteria such as *Agrobacterium* and the subsequent enzymatic or nonenzymatic oxidative decarboxylation of indolepyruvic acid to IAA (33; T. Kosuge, personal communication). Thus, it was expected that all strains tested here would show IAA production in the presence of an exogenous supply of tryptophan. The production of IAA by oncogenic and non-oncogenic *Agrobacterium* has been observed previously (1, 4, 13, 28) but because tryptophan was used in the earlier work, no consistent differences were observed at that time. It was

Table 1. Indoleacetic acid production by Agrobacterium tumefaciens in relation to plasmid content and its phenotype.

Strain	pTi plasmid present	Mol wt of pTi plasmid (X 10^6d)	Onco-genic	Phage API exclu-sion	Nopaline		Indoleacetic acid in culture medium and cell extract (total µg[a]) with cells grown in specified aromatic amino acids					
							Culture medium			Cell extract		
					Syn-thesis	Utili-zation	Trypto-phan	Tyro-sine	None	Trypto-phan	Tyro-sine	None
C-58	yes	117	yes	yes	yes	yes	352.4	21.0	0.0	1494.0	108.4	1.0
NT1	no	0	no	no	no	no	167.6	3.0	0.0	680.0	9.6	3.0
1D1274	yes	117	yes	yes	yes	yes	343.5	29.0	0.0	1490.0	109.0	1.0

[a]Total average amount extractable from 250 ml of culture medium freed of cells that had grown for 48 hr at 25 C with shaking. Averages based on three experiments.

therefore important to use an aromatic amino acid other than tryptophan. Tyrosine was chosen because it presumably inhibits tyrosine synthesis and therefore diverts chorismic acid for the endogenous synthesis of tryptophan. Indeed a dramatic difference in IAA production between strain C-58 and its plasmidless derivative NT1 was observed when these bacteria were grown in the presence of tyrosine (Table 1). The oncogenic strain C-58 showed high level (7 to 10-fold) of IAA production relative to the non-oncogenic strain NT1. This difference is heavily masked when they were grown in medium containing tryptophan [about twofold difference and no difference in other experiments (data not shown)]. The marked difference in IAA production is striking when tyrosine was used. These data indicate that oncogenic strain C-58 produce more IAA than the non-oncogenic strain NT1. These results strongly suggest that IAA production is regulated by the pTi plasmid that is harbored in strain C-58. To test this hypothesis further, the pTi plasmid was reinserted into plasmidless strain NT1 by mating NT1 with strain C58. The resulting transconjugant, strain 1D1274 [which showed restored oncogenicity and had reacquired the pTi plasmid (Table 1)] produced IAA at levels comparable to the wild-type oncogenic strain C-58 (Table 1). When this transconjugant was cured of its plasmid, IAA levels were again equivalent to that in NT1. Furthermore various phenotypes and the presence of the pTi plasmid confirmed that transconjugant 1D1274 had indeed reacquired the pTi plasmid (Table 1). Thus, it seems that IAA production is regulated by the pTi plasmid of A. tumefaciens. The lowered levels of IAA produced in the plasmidless strain suggests that there may be two operons for IAA synthesis: one of chromosomal origin and the other of the pTi plasmid. The

plasmid regulation of IAA appears to be dominant over that of the chromosomal IAA regulation. It also could mean that IAA regulation is reflected by the inability of strain NT1 to take up IAA precursors and that the pTi simply confers a permease for the precursor. This latter hypothesis is less attractive because two different aromatic amino acids were employed and both showed differences in IAA production between strains.

The implications of our findings may be far reaching, in that elevated IAA production has been noted in crown gall tumor cells and that bacteria-free crown gall tissues are IAA autotrophic (3, 8, 11, 16, 20, 29). Thus, plasmid genes for IAA production (and perhaps its regulation) may be inserted along with genes necessary for maintaining the oncogenic state in plant cells and thereby function in crown gall tumor cells apart from the eukaryotic regulatory restraints. The reported presence of plasmid sequences in crown gall cells (5, 6, 26, 27), lends support to our hypothesis that IAA genes on the plasmid may be inserted during infection by A. tumefaciens. Furthermore, it has been demonstrated that avirulent strains of A. tumefaciens, such as AA6 and IIBNV6, are capable of forming large tumors on host plants when supplemented with IAA (2, 7, 15, 24, 25). Strain IIBNV6 carries a plasmid about 40% the size of the pTi plasmid and is able to enhance tumor initiation on coinoculation with various tumorigenic strains (19). Although the complementation factor is unknown, the possibility exists that IIBNV6 plasmid is a vestigial pTi plasmid lacking the genes for IAA regulating function normally carried on wild-type plasmids in oncogenic strains. We caution here that IAA production is probably not the sole basis for crown gall oncogenesis and the possibility of other components needed for

oncogenesis that is conferred by the pTi plasmid is not ruled out.

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