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Indole-3-Acetic Acid and Indole-3-Butyric Acid in Tissues of Carrot Inoculated with *Agrobacterium rhizogenes*

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Abstract. The role of auxins in induction of roots by *Agrobacterium rhizogenes* was studied in carrot root disks. Transformed roots were produced on root disks by inoculation with *A. rhizogenes*, A4. Measurement of indole-3-acetic acid (IAA) by gas chromatography–mass spectrometry (GC-MS) indicated that there was a significant increase in the concentration of IAA in transformed callus and induced roots compared with initial IAA concentrations in carrot disks. Indole-3-butyric acid (IBA) was found to occur naturally in carrot roots. The presence of IBA, a potent root inducer, must be taken into account when assessing the role of auxin during transformation and induction of roots by *A. rhizogenes*.

Agrobacterium rhizogenes is a naturally occurring bacterium which produces "hairy root disease" in dicotyledonous plants. It has been shown to infect intact plants, excised organs, and cultured cell lines of a wide range of plant species (DeCleene and DeLey 1981). *A. rhizogenes* induces root formation in host plants by inserting a section of its Ri plasmid, the T-DNA, into the plant cell. The T-DNA is stably integrated into the genome of the plant cell (White et al. 1985) in a process known as transformation. The T-DNA contains a left (TL) and right (TR) portion. The TR-DNA contains sequences homologous to the *tms*, auxin-producing, genes of *A. tumefaciens* (Huffman et al. 1984; Jouanin 1984). The TL-DNA contains the *rol* genes, four loci known to be involved in root induction (Boulanger and Berkaloff 1986, White et al. 1985).

The mechanism by which *A. rhizogenes* transforms cells is not known. It has been postulated that the transformation event is independent of root formation and that the primary effect of transformation is to increase the sensitivity of the infected cells to auxin (Shen et al. 1988). The role of indole-3-acetic acid (IAA), an auxin known to be intimately involved with adventitious root formation, also has not been defined in this process since roots can be induced without the presumptive IAA synthesis genes (Ryder et al. 1985, Vilaine and Casse-Delbart 1987). Research on root induction by *A. rhizogenes* has focused on IAA as the sole auxin involved in hairy root disease and only one paper has reported measuring IAA in tissues transformed by *A. rhizogenes* (Hwang et al. 1986). Indole-3-butyric acid (IBA), an auxin known to be much more effective than IAA in adventitious root formation, has been identified recently as an endogenous auxin in pea (Bandurski and Schulze 1977, Schneider et al. 1985), tobacco (Bayer 1969), cypress (Epstein et al. 1988), and maize (Epstein et al. 1989). Studies in which auxins are identified and quantitated using gas-chromatography coupled with mass spectrometry (GC-MS) are lacking, but are necessary to determine the presence and amounts of auxins in root induction by *A. rhizogenes*. The objectives of this study were to determine whether IBA was present in carrot disks naturally and to measure the concentrations of IAA in carrot root disk tissues prior to and after transformation with *A. rhizogenes*.

Materials and Methods

Plant Material

Carrots (*Daucus carota* L.) were purchased from a local market and stored in plastic bags at 4°C for the duration of the experiment. Most carrots were sampled from a single purchased lot. The carrot roots were washed with soapy water prepared with a mild detergent (0.1% Lux) and were then rinsed with tap water. They were dipped in 70% ethanol for 10 s, peeled, and then soaked in 20% laundry bleach (1.05% sodium hypochlorite) for

20 min with intermittent shaking. The roots were rinsed four times with sterile, distilled water, ends were removed and the carrot roots were then cut transversely into 0.5-cm sections and were placed, distal side down, on sterile filter paper moistened with sterile water in petri dishes.

Agrobacterium rhizogenes Cultures and Inoculation

Agrobacterium rhizogenes strain A4, obtained from A. Dandekar, Department of Pomology, University of California, Davis, was stored frozen in sterile 60% glycerol. Cultures were plated out 4 days prior to use on Kado 323 medium (Kado et al. 1972). Carrot disks were inoculated by spreading approximately 20 μl of bacteria colonies on the exposed surface of the disk. The inoculated carrot disks were incubated in a dark incubator maintained at 25°C.

Extraction of IAA

Roots and callus under the roots were removed from carrot disks with a razor blade, weighed, and dropped immediately into liquid N_2. Tissue samples (1 g) were ground to a fine powder and extracted over night at 0°C in 5 ml of 80% methanol/water (vol/vol) to which 290 ng of [$^{13}C_6$]IAA was added as internal standard (Cohen et al. 1986). Samples were then centrifuged at 2000 *g*, supernatants removed and evaporated to the aqueous phase in a Speed Vac Concentrator (Savant Instruments, Farmingdale, NY, USA). The aqueous phase was then diluted with 2 ml of 0.1 M imidazole buffer, pH 7.0. IAA was purified from the extracts using SPE amino columns (J.T. Baker, Phillipsburg, NJ, USA) prewashed with hexane, acetonitrile (ACN), water, and imidazole buffer according to the method of Chen et al. (1988). The extracts were passed through amino columns and the columns were washed with 2 ml each of hexane, ACN, ethyl acetate, and methanol. IAA was eluted from the columns with 2 ml of methanol containing 2% acetic acid (vol/vol). The acidic methanol solutions were evaporated to approximately 200 μl and then diluted with 2 ml of high-performance liquid chromatography (HPLC) grade water (EM Science, Gibbstown, NJ, USA). IAA was extracted using 1 ml C_{18} SPE columns (J.T. Baker) according to the procedure of Nissen and Foley (1987). IAA was eluted from the C_{18} columns with 200 μl HLPC grade methanol. Sample volumes were reduced to 50 μl under a stream of N_2 so that the entire sample could be purified further by HPLC.

HPLC Purification

Reverse-phase HPLC was used to purify samples for GC-MS analysis. The HPLC was an LDC Gradient Master with two Constametric II pumps coupled to an Applied Biosystems Spectroflow 980 fluorescence detector (Applied Biosystems, Foster City, CA, USA). The column was a 5 μm 4.6 × 250 mm Baker-Bond C18 (J.T. Baker). Solvents were HPLC grade water (EM Science) containing 0.1% HPLC grade acetic acid (J.T. Baker) and HPLC grade ACN (American Burdick & Jackson, Muskegon, MI, USA). The HPLC was programmed for a 15-min linear gradient from 10/90 ACN/water to 50/50 ACN/water (vol/vol) at a flow rate at 1.5 ml/min. The fluorescence detector excitation wavelength was at 220 nm and emission was monitored with a 350-nm band pass filter. IAA had a retention volume of 15.75 ml under the conditions described. Peaks eluting at the retention volume of authentic IAA were collected and diluted with HPLC grade water to reduce the ACN concentration below 10%. IAA was then extracted using 1 ml C_{18} SPE columns as previously described.

Peaks eluting with the same retention volume as authenic IBA were also collected and extracted from the HPLC solvent by the method previously described for IAA.

GC-MS Analysis

IAA and IBA samples were methylated by the procedure of Cohen (1984), evaporated to dryness under N_2, and resuspended in 10 μl of ethyl acetate for GC-MS analysis. The mass spectrometer was a VG Trio-2 quadrupole coupled to a Hewlett Packard 5890 gas chromatograph (GC). The GC was equipped with a DB-5 (J & W Scientific, Folsom, CA, USA) 30-m column (0.25 mm ID, film thickness 0.25 μm) using He_2 as the carrier gas at a linear velocity of 32 cm/s. The injector, GC transfer line, and source were set at 200, 250, and 200°C, respectively. The emission current was 800 μA with an electron energy of 70 eV. The trap current was regulated at 100 μA, while the photomultiplier was maintained at 500 V. Parameters used for tuning the mass spectrometer, such as ion focus, ion extraction, ion repeller, and ion energy voltages were adjusted by maximizing the signal for several ions of perfluorotributylamine.

IAA quantitation was by selected ion monitoring (SIM) of m/z 130, 189 for authentic IAA-ME and m/z 136, 195 for [$^{13}C_6$]IAA-ME. The dwell time for each ion was 80 ms with 20 ms to switch between ions. Splitless 1-μl injections were made. The ratio of ion current for each ion pair was used to determine the amount of IAA according to Cohen et al. (1986). IBA-ME from the carrot tissue was identified by retention time with authentic IBA-ME and comparison of mass spectra. The mass spectrum of IBA-ME was determined from m/z 100–300 using the same GC-MS conditions previously described.

Opine Analysis

Callus and root tissue of carrot disks were assayed for the presence of the agropine family of opines according to the method of Petit et al. (1983) with slight modifications. A 500 keV power source was used rather than a 5000 keV source and the time of electrophoresis was increased to 45 min.

Results and Discussion

Callus formed first over the vascular ring in both control and treated carrot disks. The callus continued to proliferate slowly in controls and eventually covered the stele of the root. No roots formed on the surface of control disks. In treated disks, callus formed initially over the vascular ring and then formed over the entire surface. Roots arose first from the callus over the vasculature and eventually covered the entire surface of the disk. A sample of induced root tissue was tested for opines at each harvest. Evidence for transformation was indicated

by positive tests for agropine and mannopine/mannopinic acid in roots and callus of treated disks.

In this study, IAA was identified by GC-MS and quantitated by GC-SIM-MS using [$^{13}C_6$]. In controls the concentration of IAA was 12.7 ± 2.2 ng/g fresh weight (FW) (±SE). The amount of IAA in callus of treated disks was significantly greater than in controls, averaging 31.5 ± 1.9 ng/g FW. Induced roots contained the greatest amount of IAA, 81.1 ± 8.9 ng/g FW. These results agree with those of Hwang et al. (1986) who, using HPLC for quantitation, reported an increase from approximately 12 ± 5 ng/g FW IAA to a maximum of 55 ng/g FW IAA in carrot disks 13 days after treatment with *A. rhizogenes*.

Our results fall within the range of concentrations of IAA obtained by others. Ishikawa et al. (1988) reported 2.2 ng/g FW in control carrot root disks and 6.8–12.8 ng/g FW IAA in carrot callus transformed by *A. tumefaciens*. Pence and Caruso (1986) obtained a range of 37–176 ng/g FW IAA in tumors incited by *A. tumefaciens*. Other researchers measuring IAA in roots of nontransformed plants obtained values of 50 ng/g FW IAA in barley roots (Tagliani et al. 1986) and 30–60 ng/g FW IAA in pea roots (Pengelly and Torrey 1982).

The presence of significantly higher amounts of endogenous free IAA in transformed carrot root tissue compared to controls indicates that hairy root formation is accompanied by increased levels of IAA. Quattrocchio et al. (1986) showed that an increase in IAA synthesis played a key role in the formation of hairy roots in potato. Cardarelli et al. (1987) concluded that a threshold level of auxin must be present in carrot root disks in order for root induction to occur after inoculation with *A. rhizogenes*. They postulated that auxin accumulated on the distal surface of carrot disks due to polar transport of IAA towards the root apex. Cardarelli et al. (1987) suggested that auxin synthesis might also be stimulated in cell transformed with the TR-DNA, which contains the putative auxin genes.

In these studies IBA was identified by GC-MS (Fig. 1) in both normal and transformed tissues of carrots. We could not measure changes in the concentration of IBA due to lack of an appropriate internal standard for quantitation by GC-SIM-MS. To our knowledge this is the first report that IBA is a naturally occurring, endogenous auxin in carrot. IBA, a potent root inducer, may be important in hairy root formation. It is known, for example, that exogenous IBA is converted to IAA in tissues undergoing adventitious root formation (Alvarez et al. 1989, Epstein and Lavee 1984). Additional research focusing on IBA was well as on cytokinins is required to elucidate the role of plant growth regulators during transformation and adventitious root formation by *A. rhizogenes*.

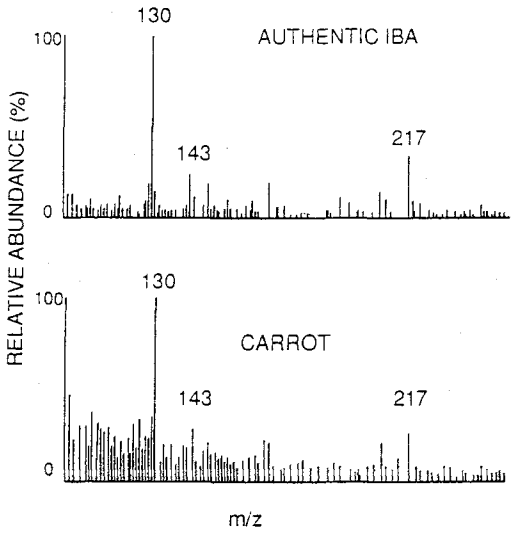

Fig. 1. Mass spectra of authentic IBA and putative IBA from carrot root extract.

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