Cytokinin Antagonist Activity of Substituted Phenethylamines in Plant Cell Culture

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ABSTRACT

A series of structurally related substituted phenethylamines shows extreme toxicity toward wild-type callus tissue cultures of tobacco (Nicotiana tabacum), soybean (Glycine max), corn (Zea mays), and sunflower (Helianthus annuus L.), but tobacco crown gall cultures are resistant to the compounds. The essential components that result in toxicity of the phenethylamines include one aromatic hydroxyl and one primary aliphatic amino group. A series of attenuated crown gall cultures, generated by transformation of tobacco with various modified Agrobacterium strains, has been used to demonstrate that the resistance of crown galls to the phenethylamines is due to the expression in these tissues of isopentenyl transferase, a first step in cytokinin biosynthesis. The toxicity of the compounds to tissue cultures is very rapid, but can be overcome by prior exposure of the calli to exogenous cytokinin. Because of the relationships we have observed between cytokinins and these compounds, we propose that the substituted phenethylamines may represent a class of chemicals that can be used as specific probes to further an understanding of cytokinin metabolism in plant tissues.

A wide variety of substituted phenethylamines have been identified in plants, including norepinephrine (3, 12), tyramine (9, 24), dopamine (13, 19), adrenaline and dopa (11), N-methyl tyramine (22), and octopamine (16, 26). Although these compounds have been extensively studied in insects, mollusks, and crustaceans for their neurotransmitter properties (6, 18), their functions in plants remain relatively obscure. Several studies relate phenethylamine presence and activity to the actions of plant hormones, although the mechanisms have not been defined. Catecholamines enhance the induction of lettuce hypocotyl elongation by gibberellin, and such enhancement is subject to competitive inhibition by the known antiauxin, trans-cinnamic acid (20). However, because auxin does not by itself produce the gibberellin stimulation, the relationship between the catecholamines, gibberellin, and antiauxins is apparently quite complex. Work of Elstner et al. (10) has shown that endogenous dopamine in sugar beet leaves functions as a cofactor of monoxygen oxygen reduction, leading to the release of the plant hormone, ethylene. The pathway for ethylene biosynthesis in plants may, therefore, be influenced by phenethylamine levels and activities, as it is influenced by the activities of other plant hormones such as auxins (15). A further involvement of catecholamines in plant organs of movement such as tendrils, which are likely subject to rapid hormonal regulation, again relates these compounds to metabolic regulatory events in an as yet unrevealed way (3).

Unfortunately, the complex interactions of the various plant hormones with one another frequently complicate the development of a clear understanding of the underlying mechanisms of hormone activity. Because a number of seemingly unrelated studies have implicated the catecholamines in metabolic situations characterized by rapid hormone responses, we investigated a series of the compounds for growth altering characteristics on plant tissue cultures. In screening for cytokinin-active compounds, we initially observed that octopamine, a substituted phenethylamine, was exceedingly toxic to wild-type tobacco callus, but had no apparent effect on crown gall tissues. We explored this effect further by observing the effect of various related compounds on a variety of cell culture types, including a series of altered or attenuated crown galls. The results of these studies suggest that octopamine and the related compounds are antagonists of cytokinin metabolism in plant tissues, and may possibly be useful probes for further analysis of the details of cytokinin metabolism in plants.

MATERIALS AND METHODS

Plant Material

Tobacco (Nicotiana tabacum cv H425), cotton (Gossypium hirsutum cv DPL61), corn (Zea mays var A188), soybean (Glycine max cv Hardin, Williams, Peking, and Essex), and sunflower (Helianthus annuus L.) seeds were sterilized with commercial bleach (4–6% NaOCl) diluted 1:5 with water for 10 min, washed three times with sterile distilled water, and germinated on filter paper.

Callus Induction

Hypocotyl, stem and leaf derived callus was initiated and maintained on MS media (23) supplemented with kinetin at 0.2 mg/L and naphthalene acetic acid at 1 mg/L (23) in the case of tobacco, cotton, corn, and sunflower, and B5 media supplemented with 2,4-D at 1 mg/L (14) in the case of soybean.

Bacteria

Agrobacterium tumefaciens strain LBA4404 was provided by A. Hoekema (17). Agrobacterium strains A208 and A208 (pTIT3ADH) have been previously described (5). The binary vector pMC92/14a was constructed by ligating the BamHI
fragment 14a of pTiT37, which encodes IPTase1 (5), into the vector pCMC92 (27). Agrobacterium strain LBA4404 (92/14a) was constructed by conjugation of the plasmid pCMC92/14a into LBA4404 from Escherichia coli as described elsewhere (5).

Inoculations and Recovery of Transformants

All inoculations were performed in vitro as described earlier (5). Crown gall cultures were maintained on phytohormone-free media, whereas wild-type callus tissue as well as tissue derived from transformations using disarmed vectors were grown in the presence of auxin and cytokinin, as described in the callus induction section.

Octopamine Selections

The effects of octopamine on callus tissue were investigated by subculturing tissue on octopamine enriched media (0.01 mM), in the presence or absence of phytohormones; similar results were obtained in the presence or absence of auxin. Wild-type callus tissue (approximately 13 mg) when plated on hormone-free media in the presence of octopamine, turned purple in color in about 30 min. In 1 to 2 d the tissue was totally black and no further growth was observed.

Extraction of Octopamine and Its Metabolites—Analytical Procedures

Fresh tissue (50–100 mg) grown on octopamine-enriched media, was homogenized in 1 mL of 2 N NaOH. Following clarification by centrifugation, 50 L of the supernatant were spotted on Whatman 3MM paper and subjected to electrophoresis (analytical reagent grade formic acid/glacial acetic acid:water, 1:3:16 v/v [pH 1.8], 400 V for 1.5 h). Octopamine and at least one of its metabolites were isolated by column chromatography (silica, MeOH/0.1 N NaOH, 95:5 v/v, following exhaustive Soxhlet extraction (24 h) with methanol (100–300 mg of tissue), concentration on a rotary evaporator and activated charcoal decolorization (Aldrich Chemical Co.). Similar fractions were pooled, evaporated on a rotary evaporator, and lyophilized. The two compounds were subjected to NMR spectroscopic analysis using a Varian EM360L spectrometer. The identity of octopamine was established by comigration with authentic material (Aldrich Chemical Co.) using paper electrophoresis, and it was also confirmed by NMR spectroscopy. The second compound had an octopamine substructure, but its full structure was not pursued further in this investigation.

RESULTS AND DISCUSSION

Inoculation of tobacco stem segments with virulent Agrobacterium initiates the formation of a crown gall, capable of sustained growth in axenic culture without exogenous auxins or cytokinins. The transfer of T-DNA from the inciting bacteria into the plant nucleus provides for hormone biosynthesis, and genes in T-DNA have been identified which encode enzymes of both auxin and cytokinin biosynthesis (2, 7, 25). Unless measures are taken to clone single cells, primary crown galls are generally composed of sectors of cells resulting from various transformation events, interspersed with wild-type sectors that cross-feed for the hormones of crown gall cells. After a number of subcultures, such a culture represents an equilibrium that can be altered in growth rate by the addition of exogenous chemicals affecting any of a wide variety of cellular growth activities. In our initial testing of octopamine for effects on such a system, we observed that octopamine rapidly killed isolated sectors of all uncloned crown galls (Fig. 1). Within one day after plating the galls on 10 mM octopamine, distinct black necrotic lesions appeared. If the necrotic sectors were quickly excised and replated on fresh media without octopamine, they would generally recover. However, the recovering calli required both auxin and cytokinin for sustained growth. The sectors of a primary gall that did not become necrotic when octopamine was added remained hormone autonomous, and could be cultured for 12 months or longer in the presence of octopamine. The hormonal requirements of the two tissue types suggested that crown gall cells, but not the wild-type sectors, were resistant to the octopamine.

In order to further investigate the cause for the differential effect on crown gall versus hormonally dependent cells, we generated tissue cultures containing various Agrobacterium T-DNA complements. The tissue types (Table 1) varied specifically with respect to the input dosage of the gene encoding IPTase (7). This gene in T-DNA is responsible for production of isopentenyl adenosine, a first step in cytokinin biosynthesis (4). The distinctive features of the tissues were: WT, wild-type callus of tobacco; T37, crown gall tissue generated by inoculation of tobacco with A. tumefaciens strain A208 harboring the wild-type pTiT37 (5); T37-IPTase, a modified crown gall tissue lacking the IPTase gene, generated by transforming tobacco with attenuated Agrobacterium strain A208(HADH) (5), a strain with an insertional mutation that inactivates only

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1 Abbreviations: IPTase, isopentenyl transferase; 16-Ade, N6(2-isopentenyl)-adenosine; T-DNA, tumor-inducing DNA.
the IPTase gene; IPTase, a callus generated by transformation with a binary vector (27) containing the IPTase gene of pTiT37, but not the two auxin loci (2, 25); and T37+IPTase, a crown gall tissue generated by inoculation of tobacco with Agrobacterium A208 (pCMC92:14a). This strain contains the wild-type Ti plasmid pTiT37, but in addition has a binary T-DNA vector (27) containing the isopentenyl transferase gene of pTiT37 (19). The IPTase present on the binary vector is transferred into plant cells at high frequency relative to the wild-type T-DNA (1).

Phenotypes and growth requirements of each of the above cultures varied depending upon the input complement of Agrobacterium hormone genes: the wild-type calli require both exogenous auxin and cytokinin for sustained growth; T37 calli are hormonally autonomous and undifferentiated; T37-IPTase calli require exogenous kinetin, but not auxin; IPTase calli require auxin but have a “shooty” phenotype characteristic of cytokinin overproduction; while T37+IPTase calli are hormonally autonomous and also have a distinctly “shooty” phenotype. The five callus types were plated on various media, and growth was monitored. When plated on MS medium supplemented with 1 mM kinetin, all tissue types survived and continued growth even in the presence of 10 mM octopamine. However, when cytokinin was omitted from the culture media only the T37, T37+IPTase, and IPTase calli survived exposure to octopamine. The other two types of calli, WT and T37-IPTase, turned red or purple in color within 30 min (Fig. 2). The color deepened to black within 24 h, and by 48 h no live cells could be recovered. We concluded that the basis for the necrotic sectoring we first observed in primary galls is closely related to the cytokinin activity of the affected cells. The toxicity can be overcome by either endogenous generation of cytokinins due to the T-DNA encoded IPTase, or alternatively by exogenous kinetin.

In order to determine whether the response is specific to tobacco, or is more general to plant metabolism, we tested several alternative dicots and monocots. Wild-type calli of cotton, sunflower, soybean, and corn were all rapidly killed following exposure to 10 mM octopamine. Once again, addition of exogenous kinetin overcame the toxicity (data not shown).

To better define the structural features of octopamine that contribute to the cytokinin-antagonistic toxicity, compounds that are structurally or metabolically related to octopamine were tested (at the same concentration) for differential effects on wild-type and crown gall cultures (Fig. 3). Although the pathways in plants are not documented, catecholamine biosynthesis in mammals proceeds primarily from tyrosine through dopa, dopamine, norepinephrine, and then to epinephrine. An apparent lack of enzyme specificity in mammals permits some alternative paths as well, including a pathway from tyrosine to tyramine, octopamine, synephrine, and then to epinephrine (21). We, therefore, tested a variety of compounds that are structurally related to various of the potential intermediates in these pathways, in order to reveal the essential components resulting in the toxic activity. Ethanolamine and 2-amino-1-propanol did not show any toxicity toward wild-type tobacco calli, suggesting that an aromatic ring is essential. Phenethyl alcohol and sec-phenethyl alcohol were also nontoxic, although some growth inhibition was noted in the wild-type calli. This implied that the position and nature of substitution pattern on the aromatic ring alone did not account for the toxicity of the compound. Phenethyl ethanolamine and phenethyl amine showed a stronger growth inhibition than the previous two compounds, but still no severe toxicity, suggesting that the aliphatic amine moiety alone was not responsible for the octopamine activity. Combining both functional groups in one structure (aromatic hydroxyl and aliphatic amino groups), as in tyramine and octopamine, resulted in severe toxicity to the wild-type tobacco cultures. The two compounds had comparable effects on the tissue, although octopamine was more rapid in its action. The addition of a second hydroxyl group on the aromatic ring, as is found in dopamine, enhanced toxicity to levels higher than those exhibited by octopamine, and some growth inhibition in cultures containing the T-DNA IPTase gene was also noted with this compound. Epinephrine, with two hydroxyl groups on the benzene ring and a methyl group on the amine, functionality proved to be lethal to all types of tissue tested, including crown gall. From this study, we concluded that

![Image](https://academic.oup.com/plphys/article-lookup/doi/10.1104/pp.89.2.564)

**Table 1.** Tissue Types Varying Specifically with Respect to the Input Dosage of the Gene Encoding IPTase

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Description</th>
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<tbody>
<tr>
<td>WT</td>
<td>Wild-type callus of tobacco H425</td>
</tr>
<tr>
<td>T37</td>
<td>Crown gall tissue (A208)</td>
</tr>
<tr>
<td>T37-IPTase</td>
<td>Modified crown gall tissue lacking the isopentenyl transferase gene</td>
</tr>
<tr>
<td>IPTase</td>
<td>Callus containing the isopentenyl transferase gene but not the auxin genes</td>
</tr>
<tr>
<td>T37+IPTase</td>
<td>Crown gall tissue containing pTiT37 and isopentenyl transferase gene</td>
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**Figure 2.** Various tissue types on octopamine-enriched media (10 mM) in the absence of exogenously supplied cytokinin. A, wild type; B, T37; C, T37 + IPTase; D, IPTase.
octopamine may be the most useful compound for further studies into the cytokinin antagonistic effect.

The biosynthesis and metabolic activities of cytokinins in plants are only superficially understood at this time. Several primary metabolic pathways apparently exist for the cytokinin I6-Ade and its derivatives, with hormonal activity levels regulated by the synthesis, degradation, and interconversion of the compounds (8). Intact plants have no requirement for an exogenous supply of cytokinins, due to sufficient levels maintained by the flux of endogenous pathways. However, plant cells in tissue culture generally require exogenous hormones, for proper cell growth and differentiation. Endogenous levels of I6-Ade in tissue culture cells are generally quite low, although whether this is due to inefficient synthesis, or to degradation or conversion to alternative compounds is not known (8). The isopentenyltransferase gene of A. tumefaciens T-DNA, present in crown gall cells and in the various tissues described in this study, substitutes for the cytokinin requirement of these cultures by the production of greatly elevated levels of cytokinin (2). We have shown that pretreatment of the tissues in this study with kinetin (6-furfuryl-aminopurine, a synthetic cytokinin), or the presence in the tissues of the T-DNA IPTase, both protect against the rapid discoloration and toxicity caused by exposure to octopamine. While the association of catecholamine metabolism with cytokinin activities is therefore apparent, the mechanism remains to be resolved.

The rapid activity of octopamine (several minutes) makes it unlikely that the synthesis of new enzymes is induced in resistant cells, but instead suggests that alternative enzymes are operational in the resistant versus the sensitive tissues at the time of octopamine exposure. To test whether transport and uptake differences exist for the catecholamines, we used chromatographic, electrophoretic, and spectroscopic techniques as described in "Materials and Methods" to determine whether octopamine was accumulated within resistant calli. These studies showed that even the resistant tissues could readily accumulate octopamine despite the lack of toxicity of the compound to those cells. Tissues grown on media containing octopamine were found to contain significant amounts of octopamine, in addition to a second compound with an octopamine substructure, as determined by NMR spectroscopy. It is, therefore, apparent that prior exposure of cells to cytokinins, whether as exogenously supplied kinetin or the T-DNA encoded I6-Ade, activates a resistance mechanism to the catecholamines, and the resistance does not appear to involve inhibition of uptake of the compound. Whether the octopamine becomes compartmentalized or undergoes modifications in either resistant or sensitive cells remains to be elucidated. In either case, an investigation of the underlying mechanisms of the relationship between cytokinin and octopamine activities may well provide insight into the broader mechanisms of cytokinin actions on plant development.

ACKNOWLEDGMENT

We would like to thank Barbara Keller for excellent technical assistance.

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