Occam's Razor Applied to Hormonology

Are Cytokinins Produced by Plants?

Mark A. Holland*

Department of Biological Sciences, Richard A. Henson School of Science and Technology, Salisbury State University, Salisbury, Maryland 21801

“One wonders if cytokinins may be important generally in associations between higher plants and microorganisms” (Miller, 1968).

Cytokinins are a class of compounds that are defined by their ability to stimulate cell division in plants. They are found throughout the plant, but are most abundant in actively growing tissues. Their isolation from corn (Letham, 1963; Miller, 1965), following the discovery of their effect on plant cells in culture (Miller et al., 1955), led to the assumption that cytokinins are endogenously produced plant-growth regulators. This assumption gained the status of fact, although it was never proven. Conversely, cytokinin production by plant-associated microorganisms is well documented. This Scientific Correspondence examines the assumption that plants produce cytokinins and presents an alternative hypothesis: cytokinins are produced exclusively by the microbial symbionts of plants.

CYTOKININS IN ROOTS

Root tissue is currently favored as the major site of cytokinin biosynthesis. Roots contain high concentrations of cytokinins, but it is not clear whether they are endogenous products. Many root-associated bacteria are demonstrated cytokinin producers, including Bradyrhizobium, Azotobacter, Arthrobacter, Streptomyces, and Frankia spp. (Greene, 1980). Mycorrhizal fungi also produce zeatin (Miller, 1967; Ng et al., 1982). The ubiquity of microbes associated with roots and their contribution to root function is better understood now than at any time since the discovery of cytokinins. It is an error to assume that microbes neither are present nor contribute to the cytokinin profile of root tissues, as some simple experiments illustrate.

By way of introduction, PPFMs of the genus Methylobacterium are found (10³–10⁷ colony-forming units/g fresh weight) on all plants (Corpe, 1985; Holland and Polacco, 1994). One study demonstrated that they make up more than 90% of the bacteria present on plant leaves (Hirano and Upper, 1992). Significantly, the PPFMs are not removed by washing or surface sterilization. Thus, they frequently inhabit tissues, including tissue cultures, presumed to be axenic (Basile et al., 1969; Holland and Polacco, 1992). We found as many as 10⁷ colony-forming units/g fresh weight in apparently uncontaminated callus, and we showed (Holland and Polacco, 1992) that PPFM enzymes are detectable in whole plants and in tissue culture.

When PPFM populations in seeds are reduced, seed germination declines. This effect can be reversed by inoculating seeds with PPFMs or by applying cytokinins (Holland and Polacco, 1994). Recently, Freyermuth et al. (1996) demonstrated that free-living PPFMs produce zeatin and zeatin riboside. Seedlings growing with reduced numbers of PPFMs have stunted roots. Inoculation of such plants with PPFMs or application of cytokinins to such plants restores normal root development (Holland, 1997). This experiment can be performed in soil or in autoclaved media with the same result. This is significant because the ability of whole plants in culture to produce cytokinins has been given as evidence of endogenous production.

CYTOKININS IN CULTURE

Tobacco (Nicotiana tabacum L.) pith callus is traditionally used in bioassays to measure cytokinin activity (for description, see Miller, 1965) because the cells require exogenously supplied cytokinins for growth. It is possible, however, to select cytokinin autotrophs from cytokinin-dependent cell lines. Such lines, “habitudated” to growth in culture, can be maintained for many generations and are relatively stable (for review, see Meins, 1989). Does cytokinin autotrophy in cultured plant cells prove endogenous production of cytokinins? It does not, because the presence of cytokinin-producing bacteria in cultures as covert contaminants is neither routinely checked for nor controlled. Covert contamination of cultures is
discussed by Leifert et al. (1991) and Holland and Polacco (1994). The probability that covert contaminants inhabit putatively axenic whole plants or tissues in culture represents an unrecognized cytokinin "wild card" that might resolve some cases of cell habituation and generally explain the uneven growth or performance of cultures (Holland and Polacco, 1994).

**CYTOKININS IN SHOOTS**

Exogenously applied cytokinins are not mobile compounds. If sprayed on a leaf, for example, they produce only a local effect. This experiment can be done in a more sophisticated way by using tissue-specific promoters to drive cytokinin synthesis in transgenic plants. When such studies are performed (e.g. Gan and Amasino, 1995), the effect of cytokinin production is a tissue-specific (local) event. This is not surprising, since the introduction of a cytokinin synthesis gene from Agrobacterium tumefaciens to its host (Nester et al., 1984) results in tumor formation, not in a generalized effect. The immobility of cytokinins is at odds with the hypothesis that the plant synthesizes them in the roots and transports them throughout the plant. The hypothesis that shoot meristems must also produce cytokinins explains a paradoxical observation. However, shoot-associated bacteria also produce cytokinins. The activities of pathogens are obvious examples. In addition to A. tumefaciens, cytokinin production by Corynebacterium sp. results in fasciation of stems or in "witches broom" growth of shoots (Nester and Kosuge, 1981). However, not all cytokinin-producing, shoot-associated bacteria are pathogens.

A dramatic example is that of Ardisia crispa and its seed-transmitted leaf symbionts. At a low frequency, seeds of A. crispa produce seedlings with a "crippled" phenotype. Crippled seedlings stop growing shortly after germination. The apical meristems of the plants take on the appearance of callus tissue. In this state the plants can survive for 1 year or more. Eventually, they either recover and continue to grow normally or die. Rodrigues Pereira et al. (1972) demonstrated a clear link between the crippled phenotype and low numbers of seed-transmitted bacteria. They suggested that cripples are produced when bacteria are not packaged in the seed, and they were able to increase the frequency of cripples with treatments that reduced the numbers of bacteria. Significantly, Rodrigues Pereira and co-workers showed that the crippled phenotype could be corrected by applying cytokinins. They concluded that A. crispa is unable to synthesize cytokinins and that the symbionts are responsible for all cytokinin production in the plant. They further suggested that when cripples recover, it is because low-symbiont populations recover to functional levels. An interesting comparison can be made between crippled A. crispa plants and shoot-producing tissue cultures. The switch from callus or callus-like growth makes each of them cytokinin-autonomous. In A. crispa this switch is associated with a critical mass of bacteria. Normal numbers of PPFM bacteria were associated with regenerating shoots of tobacco in all such cultures we examined (M.A. Holland, unpublished data).

The distribution of PPFMs on shoots mirrors the distribution of cytokinins in plant tissue (Corpe and Rheem, 1989; Holland and Polacco, 1992). The same treatments used by Rodrigues Pereira et al. (1972) to lower symbiont populations on A. crispa also lower PPFM populations, resulting in lowered rates of seed germination and stunted growth. Either reintroduction of the bacteria or application of cytokinins reverses these effects.

**GENES AND ENZYMES FOR CYTOKININ METABOLISM**

The most satisfying evidence that plants produce cytokinins would be the isolation of a plant gene for cytokinin biosynthesis; however, the search for such a gene has been unsuccessful (Binns, 1994). Genes that have been isolated have all been cytokinin-response genes. If cytokinins are exogenously produced, the most effective regulation of the signal might be tissue-specific modification. This is, in fact, what has been found in examinations of the distribution of cytokinin-modifying enzymes (e.g. Brzobohaty et al., 1993). If plants make cytokinins, a puzzle results: The signal is inactivated in the same tissues that produce it and in which it acts.

Chen and Melitz (1979) reported cytokinin synthesis using enzymes derived from cytokinin-autotrophic tobacco cultures. Chen et al. (1983) reported cytokinin production in pea and carrot tissues. These results do not guarantee, however, that cytokinin-producing enzymes are of plant origin. Horsch and King (1983) demonstrated that leaky expression by an auxotrophic mutant in Datura innoxia (Mill.) cell cultures was produced by the bacterial species Hypromicrobium. Hill and Rogers (1972) found a bacterial l-Ser dehydratase in French beans. The PPFM urease is detectable in whole-plant and callus tissues of soybean (Glycine max L.) (Holland and Polacco, 1992). Tor et al. (1992) identified endophytic bacteria in Dioscorea sp. tissue as the origin of spurious GUS activity in transformation experiments.

**A HYPOTHESIS**

Cytokinins are produced by the microbial symbionts of plants, not by plants themselves. Plants have long-standing, symbiotic relationships with specific
cytokinin-producing microbes; the PPFMs are an example. We know this by their distribution on all plants, and because development of reliable mechanisms for their transmittance in seeds are neither accidental nor trivial. Populations of these organisms are greatest at the growing points of the plant, their distribution mimicking the distribution of cytokinins in plant tissue.

The relationship between plants and their microbial symbionts is incompletely understood, but it must be mutually beneficial. Symbionts depend on the plant for their nutritional needs and as a physical habitat. I speculate that the plant depends on the bacteria for the removal of metabolic waste products that are generated during plant growth. Methanol is an example of such a waste product, which is produced by the plant and consumed by the PPFMs. It is reasonable that metabolically active cells generate substantial waste. I suggest that bacteria remove these materials from the apoplast and, using them as a nutritional resource, degrade them into ever simpler compounds, such as ammonium, which eventually are returned to the plant. This was demonstrated with the PPFMs in soybean (Holland et al., 1992; Stebbins et al., 1992).

Because they are immobile, the ability of plants to grow depends on their ability to deal with the waste products generated during the growth process. Cytokinins are a signal from microbes to the plant telling it that its waste managers are present and active, in essence that growth can take place. Plants respond to this signal by initiating growth and by deactivating the signal molecule as it is received.

This hypothesis explains why different tissues are implicated as sites for cytokinin production and why absolute levels of cytokinin seem less important than the balance among growth regulators. When cytokinins are viewed as a molecular signal from microbe to plant, the idea of balance between auxins and cytokinins makes a lot of sense. It also explains why modification of the cytokinin signal occurs in the same tissues in which the signal is apparently produced. The distribution of bacteria on the plant obviates the requirement for an integrative mechanism of cytokinin production by the plant. It also explains what appear to be low levels of circulating cytokinins, compounds that, when applied exogenously, appear immobile.

**IMPLICATIONS AND OBSERVATIONS**

Impediments to acceptance of the hypothesis that cytokinins are of microbial origin are accepting that cytokinin-producing microbes are regularly and reliably associated with all plants, and demonstrating that these microbes produce the cytokinin normally found in plant tissue. PPFM bacteria address both of these problems; not only are they abundant and ubiquitous, but they produce zeatin. If the cytokinin signals produced by them are not significant to the plant, it is justifiable to ask how the plant ignores them.

The microbial symbionts of plants are not accidental visitors. They are coevolved participants in plant physiology. Further study of microbes such as the PPFMs should yield insights into plant metabolism. Do they produce other “plant products”? PPFM strains are known to produce triterpenoids (Zundel and Rohmer, 1985) and vitamins (Basile et al., 1985). *A. tumefaciens* transforms plant tissues with genes for opine biosynthesis. Are bacteria responsible for the production of some of the nonprotein amino acids contained in plant tissues?

Whether microbes are solely responsible for cytokinin production, manipulating them through engineering or selection may be a legitimate strategy for “plant” improvement. Stimulating bacterial growth is probably an inadvertent goal of foliar feeding. Perhaps fertilizer regimes will be improved by recognizing that they are feeding microbes. Is it possible that the nonpurine cytokinins such as dihydroxyurea actually work by stimulating bacteria to make the real thing? The success of cell culture might be improved by manipulation of microbial populations. Bacteria may also solve problems of poor storability and low germinability of some seeds.

The maxim known as Occam’s Razor, that unnecessary assumptions should be abandoned, argues against cytokinin production by plants. The assumption that cytokinins are plant products is unnecessary. Moreover, it is counterproductive to downplay the importance of microbial symbionts to plants. Whereas it is not possible to prove by experiment that plants do not produce cytokinins, it is easy to demonstrate that bacteria do produce them. Microbes have the means, the motive, and the opportunity to generate the cytokinin signal. The same has not been demonstrated of plants.

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**LITERATURE CITED**

Hill HM, Rogers LJ (1972) Phytochemistry 11: 9–18
Miller CO (1965) Proc Natl Acad Sci USA 54: 1052–1058
Miller CO (1968) Biochemistry and Physiology of Plant Growth Substances. The Runge Press Ltd., Ottawa, Canada, pp 33–45
Rodrigues Pereira AS, Houwen PJW, Deurenberg-Vos HWJ, Pey EBF (1972) Z Pflanzenphysiol 68: 170–177