Cytokinin production in relation to the development of pea root nodules[1]

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Quantitative changes in cytokinins were examined in developing root nodules on the lateral roots of seedlings of the garden pea *Pisum sativum* cv. Little Marvel infected with *Rhizobium leguminosarum* strain 128 C53.

Cytokinin activity was highest in 2- and 3-week-old nodules, when the growth rate was high, and decreased in older nodules. The cytokinin activities of 3-week-old nodules of various sizes were positively correlated with mitotic indices. In 3- and 4-week-old nodules most of the cytokinins were present in the white meristematic tip and not in the infected nitrogen-fixing or senescent cells. Since high cytokinin levels were associated with nodules having high mitotic rates or with the meristematic cells, it is proposed that cytokinins influence nodule morphogenesis by regulating the mitotic activity of the nodule meristem.

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Les auteurs ont examiné les changements quantitatifs des cytokinines des nodules racinaires se développant sur des racines latérales des plantules du pois de jardin, *Pisum sativum* cv. Little Marvel, inoculé par le *Rhizobium leguminosarum* lignée 128 C53.

L'activité des ines fut à son plus élève niveau dans les nodules âgés de 2 à 3 semaines, lorsque le taux de croissance fut élèvee, et l'activité décrut dans les nodules plus âgés. L'activité des cytokinines dans les nodules de diverses dimensions âgés de 3 semaines fut positivement associée avec leurs indices mitotiques. Dans les nodules ages de 3 à 4 semaines, la plupart des cytokinines furent présentes dans la partie blanche du sommet méristematique et non dans les cellules infectées fixatrices d'azote ou dans celles senscentes. Puisque de hauts niveaux des cytokinines sont associés avec des nodules ayant des taux élevés d'activité mitotique ou avec des cellules meristématiques, il est proposé que les cytokinines influencent la morphogénèse nodulaire en régularisant l'activité mitotique des méristemes nodulaires.

Introduction

The development of legume root nodules reflects the genetic, structural, and physiological complexities of an intimate symbiotic relationship between prokaryotic bacteria and the eukaryotic angiosperm partner. In the garden pea, *Pisum sativum* L., the bacteria invade a root hair by causing an invagination of the root hair cell wall and forming an infection thread which subsequently grows between and into cortical cells (Bond 1948; Libbenga and Harkes 1973). Nuclei of the cortical cells undergo endoreduplication and mitosis; eventually a polyploid nodule meristem is formed (Dart 1975). The meristem, which is itself not infected with rhizobia, gives rise by continued cell division to most of the cells of the nodule. Cells immediately proximal to and sometimes within the nodule meristem are invaded by infection threads and bacteria are released from the infection threads into the pea cell cytoplasm. Cells containing the free bacteria enlarge manyfold and differentiate into bacteroid-containing cells capable of fixing atmospheric nitrogen (Newcomb 1976).

Recently the occurrence of cytokinins, primarily zeatin and its riboside, was demonstrated within pea nodules (Syōno and Torrey 1976). Other cytokinins were present in minor amounts. The presence of isopentenyladenine (2iP) was reported in nodules of *Phaseolus vulgaris* L.

NOTE: The French translation of the abstract was provided by Jean Brisson.

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(Puppo *et al.* 1974) while high levels of zeatin and its riboside were found in nodules of *Vicia faba* L. (Henson and Wheeler 1976). In uninfected seedling pea roots, cytokinins occur both in the free form and in transfer ribonucleic acid (RNA) in the terminal meristematic millimetre (Short and Torrey 1972*b*). The experiments reported in this paper were designed to elucidate the role of cytokinins in nodule development. Structural aspects of pea nodule morphogenesis are described briefly in this paper; a detailed account of the morphology and ultrastructure of pea nodules harvested from the same plants as nodules studied in this paper may be found in a companion paper (Newcomb 1976).

Materials and Methods

Estimation of Cytokinin Contents

Nodules which formed on the lateral roots of pea seedlings grown in aeroponic culture tanks (Zobel *et al.* 1976) and inoculated with a pure culture of *Rhizobium leguminosarum* strain 128 C53 were used as experimental material. The cytokinins of the nodules were extracted with 80% ethanol. After removal of the ethanol, the residual water layer was extracted sequentially with methylene chloride and 1-butanol. The concentrated butanol fraction was applied to silica gel GF_{254} plates for thin-layer chromatography and developed with chloroform–methanol (4:1, v/v). Zones were removed from the plates and cytokinin activity was detected by the soybean callus bioassay. Further details regarding the culture methods and conditions for the pea plants, methods of infection with *Rhizobium*, and the techniques of cytokinin extraction, fractionation, chromatography, and bioassay may be found in an earlier paper (Syōno and Torrey 1976).

Cell Number and Mitotic Activity

Cell number determinations were made by macerating the nodule tissue in 5% chromium trioxide in 5% hydrochloric acid (Fosket and Torrey 1969). Cells infected with large numbers of rhizobia turned brown during this treatment and were very distinct from the other cells of the nodule. Mitotic divisions were counted in tissues fixed in acetic alcohol (glacial acetic acid – absolute alcohol 1:3, v/v), squashed, and stained by the Feulgen technique (Fosket and Torrey 1969). For each developmental stage at least 2000 nuclei were counted in seven or more nodules; these data were expressed as mitotic indices, i.e., the percentage of nuclei undergoing mitosis.

Growth Measurements

The average fresh weight of shoots and roots was determined using 10 plants for each developmental stage. Similarly, the average fruit and flower length represents the average length of these organs from 10 plants. A minimum of 100 nodules for each stage was measured using a dissecting microscope to determine the average nodule diameter and height (distance from junction with lateral root or apical tip of nodule). At least 5000 nodules were used for the estimation of average fresh weight for each stage of the nodule.

Nitrogen Fixation Activity

Acetylene reduction to ethylene was used to measure nitrogen fixation activity. About 100 mg fresh weight of freshly harvested nodules was incubated in 1% acetylene and 10% air in argon in a 5-ml vial at room temperature. After an incubation period of about 1 h, a 0.5-ml sample was withdrawn for gas chromatography with a Varian aerograph, series 1400, with a stainless-steel column $\frac{1}{8}$ in. inside diameter by 6 ft long, packed with Poropak NC(800/100 mesh). Column temperatures were kept at 32 °C. Helium gas was used as the carrier at a flow rate of 30 ml/min at 40 lb/in.^{2} pressure. Under these conditions, the retention time of acetylene and ethylene were 7.6 and 3.8 min respectively. Ethylene generated from the reduction of acetylene by nodules was estimated by a comparison with known concentrations of ethylene.

Results

Morphogenesis

The first macroscopically visible nodules appeared on lateral roots on the 8th day after inoculation; at this stage the nodules were white and spherical in shape. An increase in nodule diameter coincided with the development of a pink-coloured zone proximal to the white tissue already present. The pink tissue, which consisted of cells infected with rhizobia and containing the red pigment leghemoglobin, was located in the central portion of the nodule. The pink tissue was enveloped by white tissue which consisted of the nodule meristem located at the apical tip of the nodule and the nodule cortex, the outer layer of tissues (Fig. 1). Just before full blossom, 17 days after inoculation with *Rhizobium*, the pink, infected tissues increased in volume. Three weeks after inoculation a green area consisting of senescent cells in which the leghemoglobin was degraded began to develop at the base of the nodule near the junction of the lateral root. The senescent tissue increased in volume until at 5 weeks it comprised much of the nodule, which at this stage lacked a nodule meristem (Fig. 2). The senescence of the nodule tissue coincided with a sharp increase in shoot fresh weight due largely to growth of the fruits, which accounted for about 35–50% of the total shoot fresh weight (Fig. 3, bottom).

Nitrogen Fixation in Relation to Nodulation Formation

Nitrogen fixation activity, determined by the acetylene reduction assay, was detectable in 2-week-old nodules, was highest in 3-week-old nodules, declined sharply in older nodules, and became negligible in 5-week-old nodules (Fig. 3). The rate of nitrogen fixation followed a pat-

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FIG. 1. Light micrograph montage of a 2-week-old pea nodule fixed in glutaraldehyde, postfixed in osmium tetroxide, embedded in Araldite, sectioned longitudinally at 1 micron (μ), and stained with toluidine blue O. Further details are provided on this method in the companion paper (Newcomb 1976). Shown are the nodule cortex, nodule meristem (NM), the region of thread invasion and subsequent release of bacteria (TI), infected cells in the early phase of symbiotic growth (ES), infected cells in the late phase of symbiotic growth (LS), uninfected cells (UI), vascular tissue (V), and the adjacent lateral tissue. The numbers with encircled areas refer to micrographs of higher magnification of the same areas in the companion paper. × 120.

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FIG. 2. Light micrograph montage of a 5-week-old pea nodule sectioned longitudinally showing the nodule cortex, living infected tissue in the late symbiotic phase of growth, and the dead or senescent tissue. Note the absence of a nodule meristem at this stage. × 70.

tern similar to the changes in volume of the pink, infected tissue. The average number of nodules per plant increased rapidly between 8 and 10 days and slowly thereafter. By 23 days no new nodules were formed. Because nodule initiation occurred over a period of several weeks, variation in nodule size was present at any stage with variability increasing with time (Figs. 4, 5). Heterogeneity in nodule diameter, length, and shape was regularly observed.

Nodules were subdivided into a series of morphological stages related to their height (Figs. 4,

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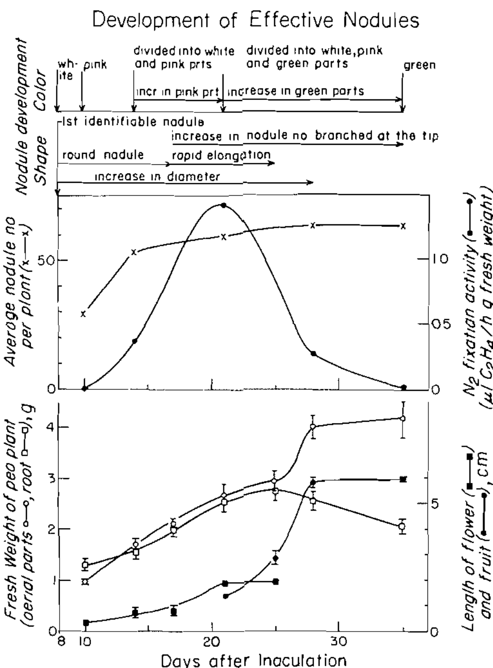

FIG. 3. The relationship between nodule and host plant development and nitrogen-fixing activity at various times after inoculation with *R. leguminosarum*. The diagram illustrates nodule color, size, and shape at various sampling times after inoculation. In the upper graph the relationship between the average number of nodules per plant (×) and nitrogen fixation activity (μl ethylene produced per gram fresh weight of nodules) (●) is presented. Shoot fresh weight (○), root fresh weight (□), flower length (■), and fruit length (●) are compared at various times in the lower graph. *incr.*, increase; *prt.*, part; *prts.*, parts.

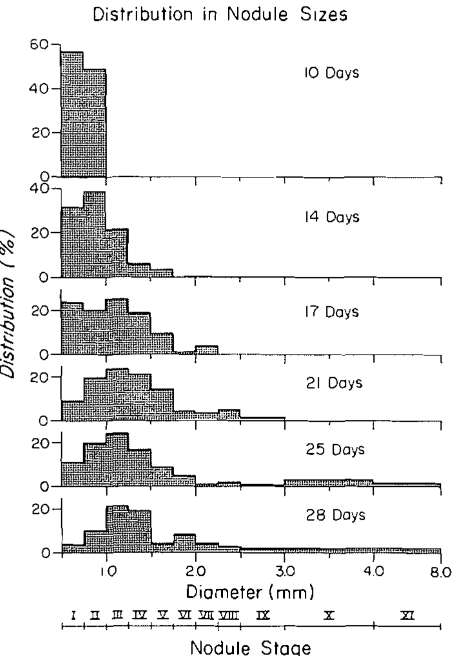

FIG. 4. Graph of the distribution or percentage of nodules according to nodule diameter (stage) between 10 and 28 days after inoculation.

5) rather than age. Many nodules formed two or more meristematic areas in their later development (Fig. 5) but were scored simply by height. Some nodules in stages I and II developed and then were arrested in their development, persisting at that size for most of the life of the plant. Most passed through a regular developmental pattern (Fig. 3).

Nodule Development

Nodule growth involves a complex sequence of events including cell division of the meristematic cells, infection of many of the new derivatives of the meristem, and the subsequent enlargement and differentiation of the bacteria-containing cells which become capable of fixing molecular nitrogen. Average nodule volume (Fig. 6) increased at a fairly constant rate from the 10th day until the 30th day and then leveled off. By the 5th week nodule enlargement had almost ceased. Nodule fresh weight increased rapidly between 10 and 21 days after inoculation but increased only slightly in older nodules (Fig. 6). This growth in fresh weight occurred during the most active period of increase of the pink, infected tissues while smaller increases occurred after senescence had begun.

The mitotic indices of 3-week-old nodules of various sizes were determined and compared with total nodule cell number of infected cells (Fig. 7). The highest mitotic indices were observed in small (or young, possibly initiated later than the larger ones) stage I and stage II nodules, which coincided with the period of rapid increase in the total number of nodule cells. The number of infected (bacteroidal) cells increased at a lower rate than total cell number and only very slowly after stage V. Meristematic cells differentiate into nodule cortex, uninfected parenchymatous cells, and vascular tissues in addition to infected cells. Infected cells arise only from the continuous invasion by bacterial infection threads at the base of the nodule meristem. The infected cells once invaded do not undergo mitosis, which occurs only within the nodule meristem.

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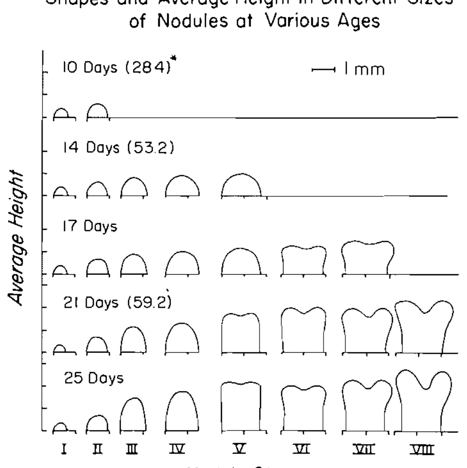

FIG. 5. Diagram of the relationships between nodule height, diameter (stage), and shape between 10 and 25 days after inoculation. Numbers in parentheses indicate the average number of nodules per plant.

Cytokinin Formation in Relation to Nodule Development

Data for concentrations of total extractable cytokinins at different stages of nodule development are given in Fig. 6 and Fig. 7. In Fig. 6, cytokinin content shows an inverse relationship to changing nodule size measured either by volume or by fresh weight. Fourteen-day-old nodules showed the highest cytokinin content; nodules sampled at 35 days showed almost no extractable cytokinin.

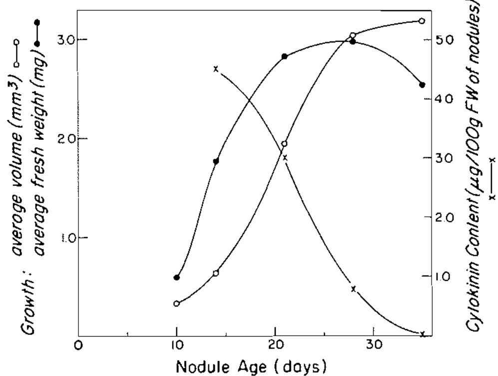

FIG. 6. Graph illustrating the relationships between nodule growth (volume (○) and fresh weight (●)) and cytokinin contents (µg per 100 g fresh weight of nodules) (×) between 10 and 35 days after inoculation.

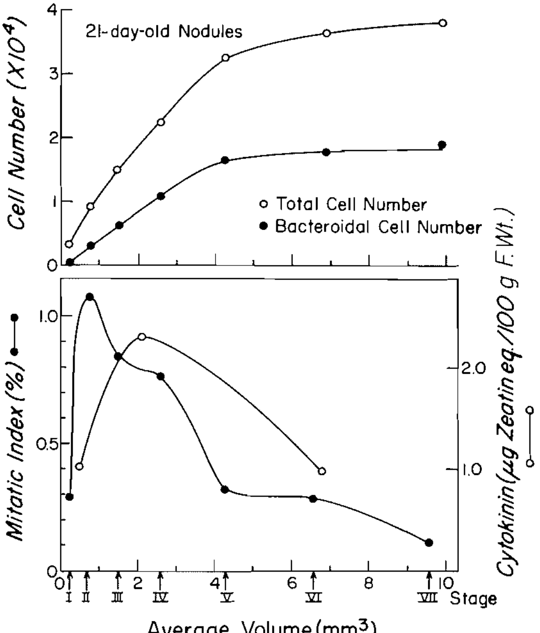

FIG. 7. Upper graph shows the relationship between total cell number (○) and infected or bacteroidal cell number (●) at various times after inoculation. The lower graph compares the mitotic index (percentage of cells in mitosis) (●) with cytokinin contents (µg zeatin per 100 g fresh weight of nodules) (○) at various times after inoculation. Because of the necessity to extract sizeable amounts of nodule tissue, nodules of different ages were pooled. See text for further details. *eq.*, equivalent, F.Wt., fresh weight.

Cytokinin levels were measured in 3-week-old nodules that were separated on the basis of their diameters into three groups; stages I and II, stages III and IV, and stages V to VII (Fig. 7). The cytokinins of each group were extracted and estimated by means of the soybean callus bioassay. Highest cytokinin content was present in nodules of stages III and IV which exhibited a high mitotic index. A lower level of cytokinins was extracted from nodules of stages III to VII. The cytokinin content of nodules in stages I and II was low despite the high mitotic index of stage II nodules. This result may be due to dilution by the less mitotically active nodules of stage I and the presence of cortical tissues of the lateral root; it is technically difficult to separate the very small young nodules completely from the lateral root tissues.

Morphological Distribution of Cytokinins

Three-week-old nodules 1.5–2.0 mm in dia-

Distribution of Mitotic Cells and Cytokinins in the Pea Nodule

Nodule age (weeks)	Size of nodules used for experiment (mm)	Color of nodule parts	Mitotic Index (%)	Cytokinin content (μg/100 g F.Wt.)
3	20~30 × 1.5~2.0	white + pink	0.43	4.18
		pink	0.00	1.78
		pink or pale green	0.00	1.29
4	2.5~3.5 × 1.5~2.5	white	0.58	2.36 (—)*
		pink	0.00	0.26 (0.33)*
		green	0.00	— (0.23)*

*Calculated from crude n-BuOH extract

FIG. 8. Table showing distribution of cytokinins and mitotic cells in white, pink, and green tissues of 3- and 4-week-old nodules.

meter and 2.0–3.0 mm in height and 4-week-old nodules 1.5–2.5 mm in diameter and 2.5–3.5 mm in height were separated into white, pink, and green tissues. Mitotic figures were observed in the tip regions in nodules of both ages but not in the proximal tissues. In 3-week-old nodules the highest levels of cytokinins were found in the white meristematic tissues (Fig. 8); moderately high cytokinin activities were also present in the pink and green tissues. There was 42 and 31% of the activity present in the tip portion present in the pink and green tissues respectively in 3-week-old nodules. In 4-week-old nodules the highest cytokinin activity was found in the white tip tissues while only $\frac{1}{10}$ of the activity was present in the pink and green tissues.

Inhibition Studies

The observed decrease in extractable cytokinin activity in older nodules as detected by the soybean callus bioassay (Fig. 6) could be due either to a real decrease in cytokinin levels or to the presence of an inhibitor or conceivably a combination of both. To test for the presence of inhibitors, 4- and 5-week-old nodules and 1-cm segments of lateral roots adjacent to the nodules were extracted with 1-butanol. All three butanol fractions were tested without further purification in the soybean callus bioassay in the presence or in the absence of 0.2 μg/litre zeatin. Extracts from 4-week-old nodules showed cytokinin activity, but none was detected in extracts from 5-week-old nodules or from lateral roots. No inhibitory activity was detectable in any of the extracts in the presence of added zeatin (Fig. 9).

FIG. 9. Graph of the growth responses of soybean callus tissue to extracts of 4- and 5-week-old nodules and adjacent lateral roots in the presence and absence of exogenous zeatin in a test for the presence of inhibitors in these pea tissues.

These results demonstrate that the decrease in the cytokinin activity of extracts from 4- and 5-week-old nodules is due to reduced cytokinin concentrations and not to the presence of inhibitory substances. The lack of cytokinin activity in the tissues of the lateral root segments points out the contrast between young developing nodules (4 weeks old) as sites of cytokinins and mature root tissues.

Discussion

Numerous studies have demonstrated the requirement of exogenous cytokinins for cell proliferation in tissues cultured in vitro (Miller 1961). The dependence of tobacco pith parenchyma cells upon a proper balance and concentration of auxin and cytokinin is well known (Das *et al.* 1956). Exogenous cytokinins promote the formation of polyploid cells (Torrey 1961) and in fact are required for the mitotic activity of

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these cells in cultured pea root segments (Matthysse and Torrey 1967). This fact is especially relevant to the morphogenesis of pea root nodules because the infected cells are polyploid (Mitchell 1965) and diplochromosomes are frequently observed in division figures in the cells of nodule primordia (Torrey and Barrios 1969). The hormonal stimulation of cortical cells leading to nodule formation was discussed in detail recently by Libbenga and Harkes (1973).

Several studies have shown a correlation between growth and cytokinin content in various tissues of *Pisum sativum* and *P. arvense*. In growing seeds of *P. arvense* the three peaks of cytokinin activity are reported to coincide with the maximum volume of the endosperm and with two maxima in growth rates of the embryo and whole seed (Burrows and Carr 1970). In cultured root callus tissue of *P. sativum* cytokinin production was highest when the frequency of mitosis was high and dropped when fewer cells were undergoing mitosis (Short and Torrey 1972*a*). In root tips of *P. sativum* the greater amount of cytokinin was found in the terminal millimetre, which also contained most of the dividing meristematic cells (Short and Torrey 1972*b*). The cytokinins of pea root tips were identified as zeatin and its riboside and ribotide. Kinetin, the synthetic analog of zeatin, was effective in producing polyploid cell divisions and tracheid differentiation in cultured pea root segments (Torrey and Foskat 1970). The finding of the present study that the highest concentrations of cytokinins in the nodule occur when and where the mitotic index is high agrees with earlier results with other tissues of pea. The correlation of cytokinin level with the mitotic rate in pea root nodules may be due in part to the vigorous infection thread growth and subsequent infection of cells being derived from the nodule meristem during this period, as well as the mitotic activity of the nodule meristem itself. The site of mitotic activity in the nodule meristem is immediately contiguous with the site of progressive bacterial thread invasion into cells formed by the meristem (Newcomb 1976). Cytokinin formation by meristematic cells could be supplemented by cytokinin release from invasive bacterial threads as argued by Phillips and Torrey (1970, 1972) for nodule initiation.

The source of the cytokinins in pea root nodules is particularly interesting because *R. leguminosarum* in culture produces different cytokinins from those found in pea root nodules and uninfected pea root tips. Uninfected pea root meristems contain zeatin and its riboside and ribotide (Short and Torrey 1972*b*) while pea root nodules contain small amounts of isopentenyladenine (2iP) and its derivatives in addition to the zeatin compounds (Syōno and Torrey 1976). Because of the close relationship of zeatin and 2iP it has been suggested that 2iP may be a precursor of zeatin and its derivatives (Miura and Miller 1969). Cultured *R. leguminosarum* produce 2iP, isopentenyladenosine (IPA), as well as two unknown cytokinins (Giannattasis and Coppola 1969; Phillips and Torrey 1970, 1972).

The high concentration of cytokinins in the meristematic regions of pea root nodules, their lower concentrations in other nodule tissues, and their apparent absence in 5-week-old pea root nodules that lack a nodule meristem suggest that the nodule meristem is the source of zeatin and its derivatives while the bacteria produce 2iP and its derivatives. Presumably both cytokinins could function in the stimulation of deoxyribonucleic acid (DNA) synthesis and mitosis in the host pea cells. However, two other possibilities as suggested earlier (Syōno and Torrey 1976) must also be considered. The pea nodule cells may convert the bacterial-produced 2iP to zeatin and its derivatives, which then function in nodule development. Alternatively, the bacterial cytokinin 2iP may initiate nodule development, which is subsequently controlled by zeatin produced by the host cells. Ineffective pea nodules produced by another strain of *R. leguminosarum* contain another unidentified cytokinin (unpublished observations). This is added support that the bacterial cytokinin differs from that produced by the host. Clearly more work remains to be done on the origin of the cytokinins in the nodule. The intimate symbiotic interrelations of nodule development may also involve the synthesis of different hormones from the endophyte and angiosperm host; this would represent another example of the complex control by two genomes of nodule morphogenesis.

The decline in levels of cytokinin in pea nodules is interesting and of some significance to the development of the nodule. It is well known that cytokinins are produced in roots and transported to the shoot (Kende and Sitton 1967). However, in the case of pea it is not known if cytokinins are exported out of the nodule or degraded within them. If the former

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alternative were the case, it could provide an explanation for the presence of cytokinins in the pink and green tissues of 3-week-old nodules and the lack of cytokinins in the same tissues of 4-week-old nodules. While it is conceivable that cytokinin export could occur faster than cytokinin synthesis, it seems an unlikely explanation for the decreased concentrations in older nodules. Breakdown of cytokinins and the export of the breakdown products seems more reasonable. It is suggested that the decreased cytokinin levels are responsible for the lower mitotic indices. The decreased rate of mitotic activity in the nodule meristem enables the infection process to proceed faster than the formation of derivatives from the meristem. As a result the meristematic cells become invaded by infection threads and differentiate into infected cells capable of fixing molecular nitrogen. Since these cells have a short life span, the whole nodule becomes senescent and nitrogen fixation soon ceases. Thus, it would seem advantageous to prolong the period of nitrogen fixation. One way might be by prolonging the meristematic activity of the nodule meristem. Since there is a correlation between cytokinin levels and meristematic activity, chemical modification of the plant or genetic selection might produce plants having a longer period of meristematic activity and hence a longer time of molecular nitrogen fixation.

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