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Evidence for Cytokinin Involvement in *Rhizobium* (IC3342)-Induced Leaf Curl Syndrome of Pigeonpea (*Cajanus cajan* Millsp.)

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ABSTRACT

A uniquely abnormal shoot development (shoot tip-bending, leaf curling, release from apical dominance, and stunted growth) in pigeonpea (*Cajanus cajan* Millsp) induced by a nodulating *Rhizobium* strain, IC3342, is thought to be due to a hormonal imbalance. *Amaranthus* betacyanin bioassay indicated that xylem exudate and leaf extracts from pigeonpea plants with *Rhizobium*-induced leaf curl symptoms contained high concentrations of cytokinin relative to those in normal plants. Radioimmunoassay (RIA) of samples purified with high performance liquid chromatography revealed that zeatin riboside (ZR) and dihydrozeatin riboside (DZR) concentrations in xylem sap from plants with leaf curl symptoms were 7 to 9 times higher than those in the sap from symptomless, nodulated plants. The sap from symptomless plants nodulated by a Curl$^-$ mutant had ZR and DZR concentrations comparable to those in the normal plant sap. RIA indicated that the respective concentrations of zeatin and N^6-isopentenyladenine in culture filtrates of the curl-inducing strain IC3342 were 26 and 8 times higher than those in filtrates of a related normal nodulating strain (ANU240). Gas chromatographic-mass spectrometric analyses revealed similar differences. Gene-specific hybridization and sequence comparisons failed to detect any homology of IC3342 DNA to *Agrobacterium tumefaciens* or *Pseudomonas savastanoi* genetic loci encoding enzymes involved in cytokinin biosynthesis.

The interaction of *Rhizobium* with legumes has many features in common with plant parasitic infection and development (31). While the pathogenic *Agrobacterium*-plant interaction leads to production of nondifferentiated plant tissue, the *Rhizobium*-legume interaction usually leads to the development of highly organized root nodule tissue. A role for phytohormones in parasitic, bacterial-plant interactions is becoming more evident as a result of the discovery of genes involved in hormone biosynthesis or metabolism in those bacteria. Crown gall on most dicotyledonous plants, induced by infection of certain *Agrobacterium tumefaciens* strains, is due to the integration and subsequent expression of a portion of the Ti-plasmid, the transferred DNA (20). The transferred DNA contains genes encoding enzymes directly involved in IAA and cytokinin biosynthesis (8, 19). *Pseudomonas savastanoi,* which causes olive and oleander knot disease, was also shown to contain genes encoding enzymes involved in IAA and cytokinin biosynthesis (23, 34). In both cases, hormonal imbalance or overproduction was shown to be responsible for tumor induction and maintenance (19, 20).

Rhizobium species are known to produce IAA and a number of related compounds (2, 3). A gene encoding for one of the enzymes in the IAA biosynthetic pathway has been identified in *Bradyrhizobium japonicum* (26). Phytohormones are also implicated in the *Rhizobium* infection process and nodule morphogenesis, and nodules are known to contain much higher levels of cytokinins than root tissue (7), but to date no *Rhizobium* genes or genetic loci directly involved in cytokinin biosynthesis or in the maintenance of hormonal balance have been identified. Reports of cytokinins in *Rhizobium* culture media are contradictory. Some workers (22, 30) have detected low levels of zeatin-like compounds in culture media by bioassay; others (1, 32) have failed to detect cytokinins.

A recently discovered leaf curl syndrome of the legume pigeonpea (*Cajanus cajan*) is induced specifically by the *Rhizobium* strain IC3342 (17) and is thought to be due to *Rhizobium*-induced hormonal imbalance or overproduction of cytokinin. The leaf curl symptoms include tip bending followed by hyponasty, curling of the leaves (an upward inrolling of the margins of the blades), release from apical dominance, and proliferation of lateral buds (17). Grafting and sap-feeding studies indicated that a curl-inducing principle is produced in the root nodules and translocated through the xylem to the developing shoots to produce the symptoms (our unpublished data). Nodulation host range (effective and ineffective), the effect of nitrate supply, and studies with plasmid-cured, nonnodulating derivatives of the strain IC3342, showed that effective nodulation is a prerequisite for the development of leaf curl symptom (17; our unpublished data).

Genetic analysis of the leaf curl-inducing *Rhizobium* strain IC3342 revealed that at least five unlinked genetic loci are involved in the leaf curl induction (our unpublished data). Some of the mutations resulting in the Curl$^-$ phenotype had pleiotropic effects on nitrogen fixation. One of the genetic

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loci, designated *lcr1*, has the capacity to confer Curl$^+$ phenotype to a closely related noncurl-inducing strain ANU240. Sequence analysis of *lcr1* reveals five open reading frames (possible genes), of which two show structural homologies to *Escherichia coli* regulatory genes *omp*R and *fnr*. We postulate that these genes have regulatory significance in the expression of other genes involved in the leaf curl induction (our unpublished data).

In the present paper, we report experimental evidence for the involvement of cytokinin derivatives in the induction of leaf curl in pigeonpea. We report also the possible genetic control (mediated through the genes from the *lcr1* region) of *Rhizobium*-produced/induced cytokinin biosynthesis and the usefulness of this system in the study of the role of endogenous cytokinins in shoot development.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

Bacterial strains and plasmids used in the study are listed in Table I. *Rhizobium* cultures were maintained in tryptone yeast agar slopes and glycerol stocks (−70°C) as described elsewhere. For inoculation, cultures were grown in yeast extract mannitol medium at 30°C to late log phase (as shaken cultures, 250 rpm orbital). For cytokinin analysis of the culture filtrates, a starter culture (repeatedly subcultured) was inoculated (1:100) into eight 3-L conical flasks, each with 750 mL of Bergersen's defined medium (10), and grown at 30°C on an orbital shaking (200 rpm) incubator for 86 h. Appropriate antibiotics were included in the media to maintain the selection pressure if required.

Plant Growth Conditions

Plants were raised in a solar energy conserving greenhouse maintained at day temperatures between 25 and 33°C and a minimum night temperature of 15°C. Plastic pots (20 cm top diameter) were filled with a mixture of prewashed and steamed sand and vermiculite (60:40, v/v) as growth medium. One day prior to sowing, the growth medium was saturated with Fahraeus nutrient solution (12) containing 12.5 ppm N in the form of potassium nitrate. Seeds were surface-sterilized with 3% hydrogen peroxide and 4% sodium hypochlorite each followed by several washes with water. Pregerminated seeds were sown and immediately inoculated with 5 mL of *Rhizobium* cultures (about 10^9 cfu/mL), added to each pot by pipetting over the seeds. Nutrient solution was applied twice per week.

Table I. *Bacterial Strains and Plasmids Used in the Study*

Strains/Plasmids	Characteristics	Source/Reference
IC3342	Fast-growing, leaf curl-inducing, cowpea group wild-type *Rhizobium* strain	17
ANU240	Fast-growing, cowpea group wild-type *Rhizobium*	M. Trinick
IHP100	Fast-growing, cowpea group nodulates pigeonpea	17
ANU3003	A Tn5-induced mutant of the strain IC3342 (ANU1298)	N. M. Upadhyaya
pIPT/RR1	Cloned *ipt* gene from *A. tumefaciens* in pUC8	T. J. Higgins

Sap Collection and Preparation of Leaf, Nodule, and Cell-Free Culture Extracts

Pot-grown plants, 35 to 40 d old, were cut 3 cm above the ground level. A flexible silicone rubber tube was pushed over the stump of the stem to serve as a reservoir to collect the bleeding sap. Sap collection began at 11 AM on a bright day and was completed within 1 h of cutting the stem. To minimize enzyme activity during storage, the collected sap was stored at −20°C until used in a vial with twice its volume of methanol containing 5% (v/v) acetic acid.

Ten grams of fresh leaves or 5 g of fresh nodules were macerated in 100 mL of phosphate buffer (100 mM [pH 6.5]) and kept at 4°C overnight to allow debris to settle. The filtrate volume was reduced to 10 mL by vacuum evaporation (45°C). To prepare cell-free culture extracts, 100 mL of stationary phase culture in yeast extract mannitol broth was clarified by centrifugation, passed through a 0.22 μm membrane filter, and vacuum evaporated to 10 mL.

Amaranthus Betacyanin Bioassay for Cytokinin

Amaranthus seedling bioassays for cytokinins in xylem exudate (sap), leaf extract, nodule extract, and cell-free culture extracts were carried out essentially as described by Biddington and Thomas (11). BAPR[4] was used as a standard.

Quantification of Cytokinins in Xylem Exudates (Sap)

Preserved sap samples from several plants were pooled into three replicates for each treatment, and to known quantities of the pooled sap (about 2 mL each), [^{3}H]DZ and [^{3}H]DZR were added to serve as indicators of recovery. The sap was adjusted to pH 2.8 to 3.0 with 1.5 N formic acid and centrifuged for 5 min at 13,500 rpm in an Eppendorf centrifuge. The supernatant was fractionated on a cellulose phosphate column essentially as described by Badenoch-Jones *et al.* (4). The ammonia eluate, which contained cytokinin bases and ribosides, was collected in fractions, and the radioactive fractions were combined and evaporated. The residue was subjected to HPLC (column: Waters μBondapak C18 semipreparative column, 7.8 × 300 mm; solvent: methanol/water/

[4] Abbreviations: BAPR, 6-benzylaminopurine 9-riboside; RIA, radioimmunoassay; ZR, zeatin 9-riboside; DZR, dihydrozeatin 9-riboside; Z, zeatin; iP, N^6-(2-isopentenyl)adenine; DZ, dihydrozeatin; iPA, N^6-(2-isopentenyl)adenosine; *t*BuDMS, *t*-butyldimethylsilyl; ZNT, zeatin nucleotide, *i.e.* ZR-5′-phosphate; DZNT, dihydrozeatin nucleotide, *i.e.* DZR-5′-phosphate; iPNT, iP nucleotide, *i.e.* iPA-5′-phosphate; ORF, open reading frame; ESE, effective standard error; [^{3}H]DZ, [2′,3′-^{3}H]dihydrozeatin; [^{3}H]DZR, [8-^{3}H]dihydrozeatin riboside.

acetic acid, 25:74:1, v/v; flow rate: 3.5 mL per min) to give two cytokinin fractions: Z plus DZ in one, and ZR plus DZR in the other (retention times 6.6 and 10.0 min, respectively) for RIA. Cytokinin nucleotides were not held on the cellulose phosphate column. To determine these cytokinins, the column effluent was evaporated, and the residue was dissolved in water (pH 7) and extracted with water-saturated *n*-butanol (three times with an equal volume). The extracts were discarded and the aqueous layer was evaporated for RIA of the nucleotides. RIA was performed with antibodies and methods as described previously (4, 6).

Quantification of Cytokinins in the Culture Supernatants by RIA

The cells were pelleted from 6-L shake cultures of IC3342 and ANU240 (stationary phase) by centrifugation. The supernatant was stored at 4°C for 2 d after addition of *n*-butanol (10% v/v). Recovery markers, [^{3}H]DZ and [^{3}H]DZR, and methanol (6 L) were added to each of the culture supernatants, which were then left to stand for 48 h at 4°C with occasional stirring. The supernatants were clarified by centrifugation at 8000*g* for 20 min, and their volume was reduced to 400 mL of aqueous solution by rotary evaporation under vacuum. The cytokinins were salted out of the aqueous phase into ethyl acetate with KH_2PO_4 essentially as described by Miller (18). Because of solubility problems, the salt was added to the aqueous solution at a concentration of 0.25 g/mL. Ethyl acetate extraction was repeated six times, and the pooled extracts were evaporated to dryness. The back extraction was achieved by redissolving this extract in 100 mL of ethyl acetate and extracting six times with an equal volume of water. The extracted fraction was then subjected to cellulose phosphate ion exchange column chromatography as described earlier. The NH_4OH eluate containing the radioactive cytokinin markers was fractionated on a Whatman Partisil CCS C-8 semipreparative column (9.2 mm × 50 cm) that was eluted with 50% methanol (v/v) containing 0.2 M acetic acid. An aliquot was taken from each fraction for liquid scintillation counting to determine the location of the cytokinin markers. This chromatographic system resolved the cytokinins into three groups, which were eluted in the following order: Z/DZ/*cis*-Z, ZR/DZR/*cis*-ZR, and iP/iPA. The last group, although not containing a radioactive marker, could be located reliably by reference to the location of the radioactive DZR in the previous group. These three groups of cytokinins were then further resolved by previously published methods: *cis*-ZR was resolved from ZR plus DZR, and *cis*-Z was resolved from Z plus DZ according to scheme B described by Parker *et al.* (21), whereas iP was resolved from iPA according to the scheme in Badenoch-Jones *et al.* (6). RIA was carried out with antibodies and procedures detailed previously (4, 6, 21).

Quantification of Cytokinins in Culture Supernatants by GC-MS

*t*BuDMS derivatives of cytokinins for GC-MS were prepared according to Hocart *et al.* (16). GC-MS was performed with a Finnigan 4530 mass spectrometer fitted with a BP1 capillary column (15 m × 0.32 mm i.d.; carrier gas helium, 60 kPa; 220°C for 0.5 min, 220–268°C at 8°C/min) obtained from SGE (Ringwood, Victoria, Australia). An electron energy of 70 eV was used for chemical ionization (reagent gas NH_3, 0.25 torr) and electron impact spectra when the source temperatures were 100 and 150°C, respectively. For quantification with deuterium-labeled internal standards, the following ions produced by electron impact were assessed by selected ion current monitoring: [$^2H_6$]iP *m/z* 323 (M^+), 305; [$^2H_0$]iP (endogenous) *m/z* 317, 302; [$^2H_5$]Z *m/z* 395, 320; [$^2H_0$]Z (endogenous) *m/z* 390, 315. The structures of the fragment ions monitored are given by Hocart *et al.* (16).

Hybridization Analysis and Sequence Comparison

Total genomic DNA from *Rhizobium* strain IC3342 and *Agrobacterium tumefaciens* C58 was isolated and used for Southern blotting onto Nylon membrane filters (Hybond-N, Amersham, International Ltd, England) as described previously (25). Probes were prepared from plasmid pIPTi (kindly provided by T. J. Higgins, CSIRO, ACT, Australia) by the random priming method and hybridizations were carried out according to Schofield *et al.* (25).

DNA sequence data, either from the databases (GENBANK or EMBL) or from respective publications, were analyzed with the iterative tree multiway alignment program (28) to derive a hypothetical phyletic tree of the genes concerned.

RESULTS

Cytokinin Activity in Extracts

The *Amaranthus* seedling bioassay was used to measure the cytokinin activity in the xylem exudates, nodule extracts, and leaf extracts of pigeonpea plants. Samples were collected from 60-d-old pigeonpea plants nodulated by leaf curl-inducing strain IC3342 and from normal plants nodulated by the strain IHP100. Detectable levels of betacyanin production were observed at a BAPR concentration of 10 nM and increased as the concentration of BAPR was increased to 10 μM. Sap and leaf extracts from leaf curl plants showed a higher cytokinin activity than those collected from normal plants (Fig. 1). Xylem exudate contained the highest concentration of cytokinin activity among the samples tested. However, undiluted nodule extracts and leaf extracts induced less pigment production than the 10-fold diluted samples, suggesting the presence of some inhibitory substance(s) in those extracts.

Quantification of Cytokinins in Xylem Exudates by RIA

To determine the type of cytokinins overproduced, sap samples, collected by stem bleeding from 35-d-old pigeonpea plants, were purified and fractionated by column chromatography on cellulose phosphate and by HPLC. Appropriate fractions, located by the presence of ^{3}H-labeled cytokinins added to the original samples as markers, were assayed for Z, DZ, ZR, and DZR by RIA. Cytokinins in sap obtained from the following plants were compared: plants inoculated with (a) the leaf curl-inducing strain IC3342; (b) a Tn5-induced, Curl$^-$ mutant (*lcr1* locus) of the strain IC3342 (ANU3003); (c) the nonleaf curl strain IHP100; and (d) uninoculated

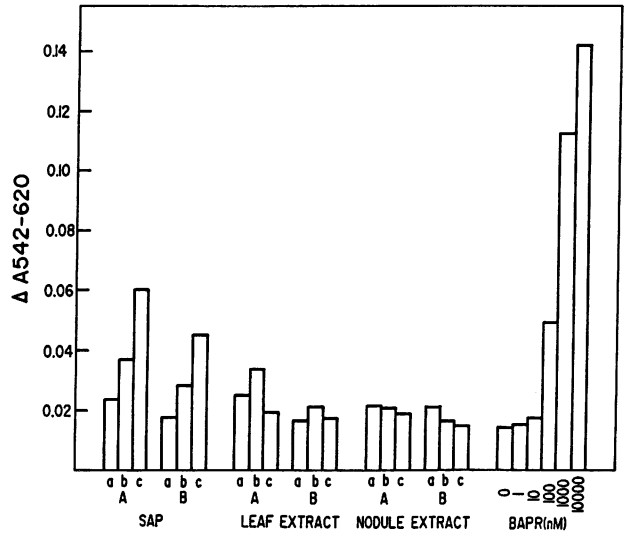

Figure 1. *Amaranthus* seedling betacyanin bioassay for cytokinins. Betacyanin pigment production (as measured by $OD_{542-620}$ nm) with sap, leaf, and nodule extracts from (A) the leaf curl plant and (B) the normal plant at dilutions of (a) 10^{-2}, (b) 10^{-1}, and (c) 10^{0} are represented by histograms, along with those of standard BAPR (0–10000 nM).

nitrate control plants. The results presented in Table II show that the concentrations of ZR, DZ, and DZR in leaf-curl plant sap were markedly greater than those in the sap collected from normal, nodulated plants, or nitrate control plants, which did not differ in their cytokinin levels. Relative to sap from the leaf-curl plants, the sap from plants nodulated by the Curl$^-$ Tn*5* mutant strain, ANU3003, had significantly reduced concentrations of DZ, ZR, and DZR. However, the Z concentrations in the sap from inoculated plants (treatments a, b, and c) did not vary significantly at $P \leq 0.01$.

The trends observed in the concentrations of Z and DZ nucleotides (ZNT and DZNT) among the above four sap samples (Table III) followed those found for ZR and DZR.

Table III. *Cytokinin Nucleotide Concentrations in Xylem Exudates of Plants Nodulated by the Leaf Curl-Inducing Strain (IC3342), a Tn5-Induced Curl$^-$ Mutant (ANU3003), and a Normal Wild-Type Curl$^-$ Strain (IHP100), and a Nonnodulated Nitrate Control Plant*

Each value is an average of three replicates.

Nodulating Strain/ Nitrate Treatment	Cytokinin Nucleotide Levels in Xylem Exudate		
	iPNT	ZNT	DZNT
		ng/mL	
IC3342	0.34	3.48	2.50
ANU3003	0.26	1.30	0.93
IHP100	0.28	0.63	0.56
Nitrate (100 μg/mL)	0.18	0.86	0.58
Effective standard error (ESE)	0.058	0.284	0.179
LSD ($P \leq 0.01$)	NS	1.489	0.938

The concentrations of iP nucleotides did not vary significantly among the different sap samples tested.

Cytokinin Overproduction by the Strain IC3342

The identities of cytokinins in the culture media of the curl-inducing strain IC3342 were compared with those in a closely related, noncurl-inducing strain ANU240 by RIA. The putative cytokinins were resolved by HPLC into groups suitable for analysis by RIA, and the results are presented in Table IV. In the media of both strains, iP was the predominant cytokinin. The culture medium of strain IC3342 contained iP and Z at concentrations which were, respectively, 8- and 26-fold higher than those in strain ANU240. The concentrations of DZ and iPA in IC3342 supernatant were 2 to 3 times those in ANU240, but the concentrations of ZR, DZR, and *cis*-Z did not vary appreciably between strains.

Z and iP in the culture media were also identified and quantified by GC-MS. After RIA, [$^2H_5$]Z and [$^2H_6$]iP were added as internal standards to the HPLC fractions in which these cytokinins had been detected. The cytokinins were then converted to *t*BuDMS derivatives, and the fractions were

Table II. *Cytokinin Concentrations in Xylem Exudates of Plants Nodulated by the Leaf Curl-Inducing Strain (IC3342), a Tn5-Induced Curl$^-$ Mutant (ANU3003), and a Normal Wild-Type Curl$^-$ Strain (IHP100), and a Nonnodulated Nitrate Control Plant*

Each value is an average of three replicates.

Nodulating Strain/ Nitrate Treatment	Cytokinin Concentrations in Xylem Exudate			
	Z	ZR	DZ	DZR
		ng/mL		
IC3342	1.81	10.50	2.44	8.92
ANU3003	1.95	1.68	0.96	1.89
IHP100	0.13	1.23	0.82	1.34
Nitrate (100 μg/mL)	0.06	0.58	0.23	0.66
Effective standard error (ESE)	0.764	0.829	0.280	0.830
LSD ($P \leq 0.01$)	NS	4.346	1.468	4.351

Table IV. *Concentration of Cytokinins in the Culture Supernatants of the Strains IC3342 and ANU240*

The values in parentheses were determined by GC-MS; the other values were obtained by RIA.

Cytokinins	Concentration in the Culture Supernatants of	
	ANU240	IC3342
	ng/L	
Z	83 (105)	2,188 (2,410)
DZ	54	133
ZR	9	7
DZR	8	9
cis-Z	166	176
cis-ZR	37	2
iP	5,191 (7,128)	41,420 (44,356)
iPA	123	329

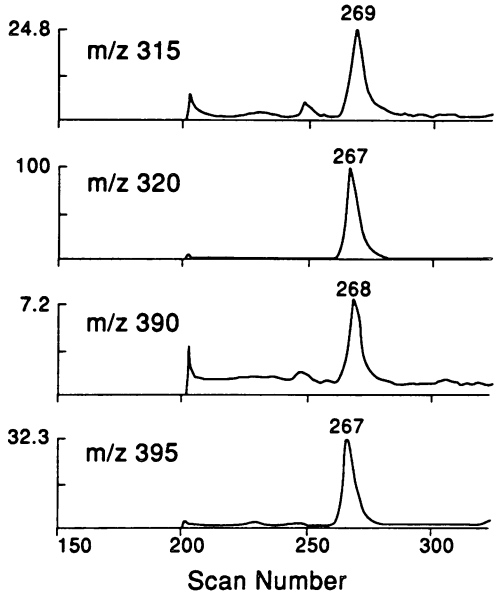

Figure 2. Positive ion currents induced by electron-impact ionization during GC-MS and used for quantification of Z in the culture supernatant of IC3342. Ions of *m/z* 320 and 395 are derived from $[^2H_5]Z$; those of *m/z* 315 and 390 arise from endogenous Z, $[^2H_0]Z$. Scan number 269 corresponds to a retention time of 3.83 min, the predicted time for $[^2H_0]Z$, which was 0.03 min longer than that of $[^2H_5]Z$ (maximum at scan 267).

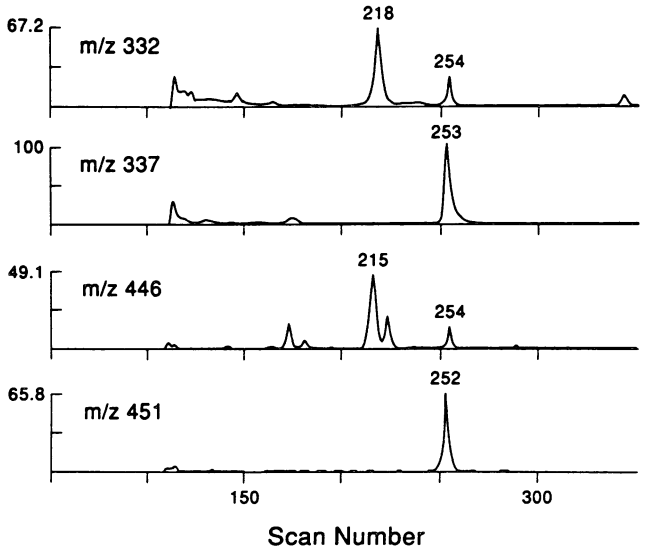

Figure 3. Negative ion currents produced by chemical ionization and used for confirmation of the presence of Z in culture supernatants of IC3342. Ions of *m/z* 337 and 451 are derived from $[^2H_5]Z$ and are $[M\text{-}t\text{BuDMS}]^-$ and $[M\text{-}H]^-$ ions, respectively. The ions of *m/z* 332 and 446 are the analogous ions derived from endogenous Z. Scan numbers 252 and 254 correspond to retention times of 3.78 and 3.82 min, the retention times for $[^2H_5]Z$ and $[^2H_0]Z$, respectively. The ions which peak at scans 215 and 218 are due to unknown compounds.

subjected to GC-MS with selected ion current monitoring. Ion currents obtained by electron impact and chemical ionization which were used for identification and quantification of Z are presented in Figures 2 and 3. From the amounts of iP and Z determined by GC-MS in the HPLC fractions, the concentrations in the original culture media were calculated (Table IV, values in parentheses) using the recovery correction (based on $[^3H]DZ$ recovered) applied to the RIA data.

Cytokinin Gene Sequence Homology Search in Strain IC3342

To determine whether any of the identified genetic loci of the strain IC3342, and in particular the *lcr1* locus, have similarity with genes involved in cytokinin biosynthesis, genomic DNA and cosmids containing DNA from strain IC3342 were probed with a sequence of the cloned cytokinin biosynthetic gene (*ipt*) from *A. tumefaciens*. Hybridization, even under conditions of low stringency, revealed no homology with the *ipt* gene probe (data not shown). Computer assisted sequence comparison, both at the nucleotide and the amino acid levels, using the Wilber-Lipman program (33) failed to detect homology of any of the identified ORFs of the *lcr1* region to the known genes involved in the cytokinin biosynthesis, *viz.* dimethylallyl transferase genes *ipt* and *tzs* from *A. tumefaciens* (8, 9) and *ptz* from *Pseudomonas savastanoi* (23). Furthermore, homology was not detected with genes involved in IAA synthesis, *viz.* tryptophan 2-monooxygenase (*iaaM*) and indoleacetamide hydrolase (*iaaH*) genes from *P. savastanoi* (34). The unrelatedness of putative *lcr1* genes (ORF1, ORF3, and OFR5) to those genes is depicted in a hypothetical phyletic tree derived from an iterative tree

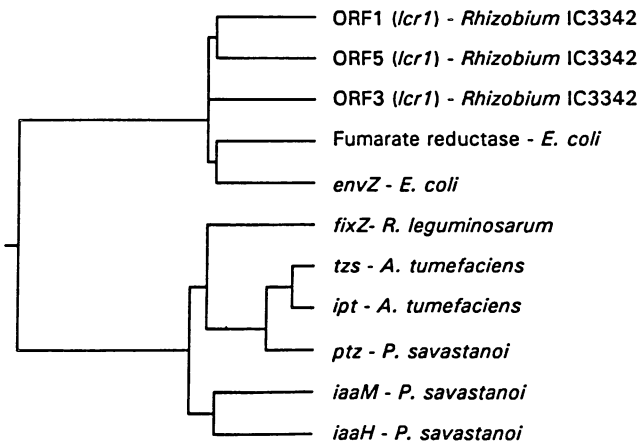

Figure 4. Hypothetical phyletic tree of regulatory genes and cytokinin biosynthesis genes from different systems. Iterative tree multiway alignments were performed with the program developed by Smith (28) by the K-tuple method. McLachlan's conservative changes were used as weight matrix to provide amino acid similarity scores. Alignments were performed with the following settings: -K-tuple size 2, window size 20, penalty for the existence of gap 10.0, gap penalty of 11 per unit length. No weightage was given to the leading or trailing gaps. Maximum iterations allowed were three. Based on the sequence similarities and the extent of conservative amino acid changes, a hypothetical phyletic tree was generated.

multiway sequence alignment, and these data are presented in Figure 4. These data suggest that, if the *Rhizobium* strain IC3342 has a cytokinin biosynthetic gene(s), the extent of its sequence homology with that of *ipt* gene is not sufficient to be detected by DNA hybridization.

DISCUSSION

The *Amaranthus* betacyanin bioassay detected high cytokinin activity in sap and leaf extracts derived from leaf-curl plants when compared to those obtained from normal plants. This suggests a possible role for cytokinins in leaf curl induction. Some, but not all, of the symptoms produced in a plant nodulated by strain IC3342 could be mimicked by feeding the cytokinin BAPR (our unpublished data). Observed differences in the symptoms induced by IC3342 nodulation and those induced by BAPR might be due to the differences in the effects of exogenously supplied cytokinins and endogenous cytokinins on plant development.

Further evidence for the involvement of cytokinins in leaf curl induction was obtained from determination of cytokinin concentrations in xylem sap samples of plants nodulated with wild-type and mutant strains. The results suggested that DZ, ZR, DZR, ZNT, and DZNT were overproduced in plants nodulated by the curl-inducing strain IC3342. A particular mutation in the *lcr1* locus (our unpublished data) affecting the Curl phenotype also affected ZR, DZR, and DZ production, but not Z production. No data have been collected on the other four Tn5-induced mutants. It is not known whether the levels of ZR and DZR in leaf-curl plants are sufficient to cause the abnormal development of the plant. However, in soybeans, a 10-fold increase in xylem cytokinin level markedly decreased leaf senescence (13). A 7- to 9-fold increase in ZR and DZR concentrations was observed in leaf-curl sap over the normal sap. Since 10-fold increases in BAPR supplied to the xylem of pigeonpea induced hyponasty and lateral bud development (our unpublished data), it is likely that such a level of ZR and DZR overproduction would be sufficient to cause abnormal effects on shoot development.

Interestingly, the culture medium of strain IC3342 showed elevated levels of iPA and DZ and especially of iP and Z. The low levels of ZR and DZR in the medium of IC3342 were similar to those in the strain ANU240. Thus, while the principal cytokinins in sap of IC3342-inoculated plants were ribosides (ZR, DZR), the main cytokinins in IC3342 culture medium were the bases iP and Z. Modification (reduction and especially ribosylation) of *Rhizobium*-produced cytokinin bases in pigeonpea nodules or root tissue would explain this difference in cytokinin type. Relevant to this suggestion are the observations in other plant species that radioactive cytokinin bases supplied exogenously to the roots appear in the xylem sap as ribosides (15, 24), and that reduction of ZR to DZR occurs actively in pea roots and nodules (5). In the above explanation, it is assumed that the pathway of cytokinin biosynthesis in cultured *Rhizobium* cells is the same as in the symbiotic association. However, it is also possible that *Rhizobium*-controlled, symbiotically induced synthesis occurs by a pathway different from that in free-living cells.

In the present studies, cytokinins were quantified by RIA in *Rhizobium* culture media, and the results obtained for iP and Z (the principal cytokinins in IC3342 medium) were verified by GC-MS analysis. Additional mass spectrometric studies concerning cytokinins in the culture media of ANU240 and IC3342, which further substantiate the present results, will be presented elsewhere. Wang *et al.* (32) identified iP in culture media of a Ti-plasmid-containing *Rhizobium leguminosarum* and very low levels in wild-type *R. leguminosarum*. However, they could not confirm the results from the wild-type strain by the chemical ionization spectrum due to limited quantities of the sample. The studies described in the present paper provide the first identification of cytokinins in culture media of naturally occurring *Rhizobium* strains.

A detailed genetic analysis (our unpublished data) revealed that several genetic loci of the *Rhizobium* strain IC3342 are involved in leaf-curl induction, and some of them have pleiotropic effects on nitrogen fixation. The genetic region (comprising *lcr1* locus) which conferred leaf curling ability on a nonleaf-curl strain upon transfer has been found to contain five putative genes. Two of them showed significant structural homology with regulatory genes *ompR* and *fnr* (*nirR*) of *Escherichia coli*. These regulatory genes are required for the activation of several other genes (14, 27, 29). As discussed earlier, the *lcr1* mutation results in the loss of leaf curl-inducing ability and a reduction in the endogenous cytokinin level in sap. Furthermore, leaf-curl induction occurs only when there is effective nodulation. Thus, it is possible that products of these genes regulate the expression of genes involved in the biosynthesis or metabolism of phytohormones in the symbiotic association. It is tempting to suggest that a gene(s) involved in cytokinin biosynthesis is (are) also located in the *lcr1* region. However, failure to detect DNA homology between IC3342 and the *Agrobacterium* cytokinin biosynthetic gene by specific probing suggests that the extent of sequence homology is not sufficient to be detected by hybridization. DNA sequences of the *lcr1* region showed no homology with any of the available sequences of cytokinin biosynthetic genes from other microorganisms. Thus, it is also possible that the *lcr1* genes, the expression of which is symbiotically induced, either induce plant genes to overproduce cytokinins or themselves produce cytokinins by pathways quite different from those of *Agrobacterium* or *Pseudomonas*.

These molecular aspects require further investigation, but the IC3342 nodulated pigeonpea plant offers a novel system to study the role of endogenous xylem cytokinins in aspects of shoot development, especially apical dominance, lateral shoot proliferation, and hyponasty.

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