Development of Ectopic Roots from Abortive Nodule Primordia

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The symbiotic phenotype of five Tn5-induced mutants of *Rhizobium etli* affected in different anabolic pathways (namely, gluconeogenesis and biosynthesis of lysine, purine, or pyrimidine) was analyzed. These mutants induced, on the root of *Phaseolus vulgaris*, a normal early sequence of morphogenetic events, including root hair deformation and development of nodule primordia. Later on, however, from the resulting root outgrowths, instead of nodules, one or more ectopic roots (spaced closely related and agravitropic) emerged. Therefore, this group of mutant was collectively called “root inducer” (RIND). It was observed that the RIND-induced infection threads aborted early inside the invaded root hair, and that the resulting abortive nodules lack induction of late nodulin genes. Moreover, experiments performed using a conditional mutant (a methionine-requiring invader) revealed that bacterial invasion plays a key role in the maintenance of the program of nodule development and, in particular, in the differentiation of the most specific symbiotic tissue of globose nodules, the central tissue. These data indicate that, in *P. vulgaris*, the nodule primordium is a root-specified pro-meristematic tissue.

Additional keywords: root development.

N₂-fixing nodules histologically are well organized organs generated on the roots of leguminous plant through a complex process of organogenesis (Brewin 1991; Franssen et al. 1992; Patriarca et al. in press). Nodule development begins with the deformation of root hair and follows with the mitotic reactivation (dedifferentiation) of different types of cortical and vascular cells leading to the formation of a nodule primordium, from which a nodular meristem or foci of meristematic cells arise (Newcomb et al. 1979). In developing nodules, the cells derived from the mitotic activity of meristematic cells undergo differentiation, thus forming the nodule tissues; namely, the central tissue, including the invaded cells (i.e., harboring the symbiotic form of rhizobia), and the peripheral tissues, including the endodermis, parenchyma, and vascular bundles.

The root cells (cortical and vascular) reactivated to form a nodule primordium as well as the final shape of a developed nodule are different in different rhizobia–legume pairs. In particular, the nodule shape depends on the form, localization, and persistence of the nodular meristem. For instance, the nod-

ules induced by *Rhizobium etli* on *Phaseolus vulgaris* are globose (also called the determinate type of nodules) because they are derived from the mitotic activity of a globularly organized meristematic zone formed by the reactivation of outer cortical cells and active for only few days (transient) (Tatè et al. 1994). Other nodules, such as those induced by *Sinorhizobium meliloti* on *Medicago sativa*, are cylindrical or elongated (also called the indeterminate type of nodules) because they are derived from an apically located meristematic zone arising from reactivated cells of the middle cortex and active for several weeks (persistent) (Timmers et al. 1999).

Nodule and lateral root development have many common features, which are obvious when elongated nodules and lateral roots are compared; namely, the presence of a persistent apical meristem (Verma 1992). However, it was established that these two root organs have distinct ontogeny; in particular, the differences are most remarkable when the early sequence of morphogenetic events leading to the generation of their meristem are considered. In fact, the root meristem arises from a primordium originated from the vascular pericycle, a single-cell layer located at the periphery of the stele, whereas the meristem of elongated nodules arises from a primordium formed by the reactivation of inner and middle cortical cells (Laskowski et al. 1995; Timmers et al. 1999).

The analysis of an interesting phenotype elicited on the roots of *P. vulgaris* by a group of genetically uncorrelated mutant strains of *R. etli* is reported here. In fact, instead of globose nodules, all these mutants induced the formation of root outgrowths from which usually ectopic roots emerged. A set of histological and molecular analyses was performed, allowing us to conclude that (i) the mutants induced the formation of nodule primordia following the same program of cortical cells reactivation induced by the wild-type strain; (ii) developing nodule primordia are not invaded as usual due to the early abortion of the infection threads; (iii) specific markers of the central tissue (invaded and uninvaded cells) are not induced in abortive nodules; and (iv) from the undeveloped apical zone of abortive nodules, one or several root meristem arise, thus leading to the formation of aberrant roots showing the anastomosis of their basal region. Finally, we have shown that bacterial invasion plays a key role in maintaining the developmental fate of a nodule primordium.

RESULTS

Mutant-induced ectopic roots.

As previously described (Tatè et al. 1994), after inoculation of *P. vulgaris* roots with strain CE3 (wild type) of *R. etli*, the development of globose nodules was observed. In contrast, after inoculation with strains CTNEX1(*lysA*), CTGUT5(*fbaB*), CTGUT26(*purF*), CTGUT28(*pyrB*), or CTGUT42(*pckA*), the

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formation of morphologically not well-defined root outgrowths from which variable numbers of roots emerge was observed. In uninoculated plants, these aberrant structures were not observed. Thus, the group of mutants was called “root inducer” (RIND). For instance, the structures developed after inoculation with strain CTNUX1 are shown in Figure 1. The number of roots extruding from a single root outgrowth was variable and related to the dimension reached before root induction. In fact, several roots were observed emerging from the larger ones (Fig. 1D), whereas a single root often emerged from the smaller ones (Fig. 1B, C, and E). Sometimes, the most proximal region of ectopic roots was fused (anastomosis), whereas the most distal regions (meristematic regions) were unconnected and strongly stained by acetocarmine (Fig. 1D) (Wopereis et al. 2000). Moreover, in most cases, the most proximal region of RIND-induced root outgrowths remained reactive to acetocarmine even after the extrusion of the roots. Finally, ectopic roots were characterized by the lack of gravitropism, elongating in all directions and sometimes parallel to the main root (Fig. 1E).

Lateral roots, nodules, and ectopic roots.

To compare the ontogeny of lateral root, nodule, and ectopic root in *P. vulgaris*, a histological analysis was performed. Lateral roots at different stages of development are shown in Figure 2. Lateral roots were initiated from a primordium that was first evident closely related to the vascular system of the parent root (Fig. 2A). Usually, the root primordium arises from cells located adjacent to a xylem pole (data not shown). Later, a meristematic region located in the most distal part emerged and, as a consequence of its activity, developing roots (usually elongating perpendicularly to the parent root) extruded from the root cortex (Fig. 2B). Finally, the apical meristematic region of emerging and developed roots was strongly stained by acetocarmine (Fig. 2C and D), whereas; unlike ectopic roots, the most proximal or basal region (i.e., the region of connection with the parent root) was regular in size and weakly reactive or not reactive to acetocarmine (Fig. 1C–E).

Nodule formation is a multistep process that begins with the formation of a nodule primordium through the reactivation of

different cell types. In roots of *P. vulgaris* inoculated with strain CE3 (wild type) of *R. etli*, the first reactivated cells were observed in the root outer cortex (Fig. 3A). Every single reactivated cell was subjected to successive rounds of divisions, generating a mitotic island, and a group of mitotic islands created an apical zone of mitotic activity (Fig. 3B). Later, a second zone of mitotic reactivation involving inner cortical cells as well as vascular cells was visible (Fig. 3B). Finally, the mitotic reactivation involved a progressive high number of middle cortical cells (Fig. 3C). Significantly, the pattern of cell divisions was different in the different cell types involved. Outer cortical cells (apical zone) divided in all directions, thus generating a globular meristematic zone. Conversely, inner cortical cells (proximal region) divided in a regular fashion (i.e., anticlinal or periclinal), thus generating the vascular tissue. Consequently, developing primordia began to adopt a light bulb shape (i.e., a globular structure connected to the central stele of the parent root by a vascular system) (Fig. 3D).

The early sequence of histologically relevant events induced by the RIND mutants was comparable with that induced by strain CE3 and, in particular, the timing of root cortical cells reactivation (outer, inner, and middle cortex) was maintained. For instance, the early developmental stages of nodule primordia induced by strain CTNUX1 are shown in Figure 4. Remarkably, in the CTNUX1-induced primordia, the most apical meristematic activity was arrested (or proceeded at very low rates), whereas the mitotic activity of inner and middle cortical cells continued (Fig. 4A and B). Thus, the primordia adopted an elongated shape with prominent vascular bundles (Fig. 4C). Later, in the distal region of these aberrant nodule primordia, multicellular structures, organized as conical-shaped root meristems and strongly stained by acetocarmine, emerged (Fig. 4D). Finally, as a consequence of the meristematic activity, developing roots extruded from the nodule primordia, remaining connected with the root stele by irregularly curved vascular bundles (Fig. 4E and F). Frequently, more than one interconnected apical meristematic region deriving from a single tissue (strongly stained by acetocarmine) could be ob-

Fig. 1. Nodule-like structures (pseudonodules) induced by strain CTNUX1 of *Rhizobium etli* on the roots of *Phaseolus vulgaris* A, B, and C, 2 or D and E, 3 weeks post inoculation. The root system was harvested fixed and stained with acetocarmine. RO = root outgrowth, ER = ectopic root, MZ = meristematic zone of developing ectopic roots, and LR = lateral root.

served in sections of nodule primordia (Fig. 4F). This analysis clearly shows that ectopic roots emerge from rhizobia-induced nodule primordia. Moreover, because the central tissue of nodules (formed by invaded and uninvaded host cells) arises from the apical zone of nodule primordia (Cermola et al. 2000; Tatè et al. 1994), these data support the idea that RIND-induced nodule primordia were partially invaded or not invaded (empty).

Root-hair deformation and infection thread (IT) formation.

Root-hair deformation is the first morphological event induced by rhizobia on their specific legume host. As previously observed in other rhizobia–legume combinations, different types of hair deformation are induced by *R. etli* on different zones of *P. vulgaris* roots. In fact, 2 to 3 days after inoculation with strain CE3, fully developed root hairs (the longer ones) become twisted, developing hairs are ballooned or branched,

Fig. 2. Lateral root development in *Phaseolus vulgaris*. The complete root system of uninoculated plants, grown as in a symbiotic test, was harvested, fixed, and stained with acetocarmine. **A, B, C, and D,** Photographs show lateral roots at successive stages of development. **E,** The most proximal zone (connecting tissue) of two developed lateral roots is shown. CS = central stele, DR = developing lateral roots, VB = vascular bundles, MZ = meristematic zone, and BZ = basal zone of developed lateral roots.

Fig. 3. Development of nodule primordium elicited by strain CE3 (wild type) of *Rhizobium etli* on the roots of *Phaseolus vulgaris*. **A, B, C, and D,** Photographs depict nodule primordia at successive stages of development. Transversal sections of inoculated roots were fixed and stained with methylene blue. **E,** Longitudinal section of a developed nodule stained with acetocarmine. CS = central stele, IC = inner cortex, MC = middle cortex, OC = outer cortex, VB = vascular bundles, AM = apical meristematic zone, CT = central tissue, and PT = peripheral tissue.

while emerging root hairs are curled (Fig. 5A; data not shown). Comparable phenotypes were observed after inoculation with the RIND mutants (data not shown), even though aberrant root-hair deformations appearing to be a consequence of a sequence of abortive deformation events (enlargement, branching, and so on) separated by events of hair growth re-initiation also frequently were induced (Fig. 5A).

To test the capacity of the RIND mutants to induce ITs, bacteria were highlighted by assaying in situ the β -galactosidase activity expressed therein. *R. etli* strains were transformed with plasmid pAR66 carrying the *ntrBC* promoter fused to the *lacZ* gene (Patriarca et al. 1996) and used to inoculate roots of *P. vulgaris* seedlings. Then, 3 to 4 days later, the complete root system was harvested and the *lacZ* expression was analyzed.

Strain CE3 induced the development of highly ramified ITs elongating toward the reactivated outer cortical cells (Fig. 5B). In contrast, the RIND mutants elicited the formation of a few undeveloped ITs showing a low number of short ramifications. For instance, the ITs induced by strain CTNUX1 are shown in Figure 5B. These data indicate that the ITs elicited by the mutants grow at slower rates compared with normal ITs, or abort early after formation. Frequently, the RIND-induced ITs elongate in a direction opposite to normal (Fig. 5B). Aberrant ITs with similar features and named “reversed” threads were observed previously, analyzing the invasion capacity of *nodE-nodO* double mutants of *R. leguminosarum* (Walker and Downie 2000). The arrest of IT elongation was confirmed by analyzing the degree of invasion of the nodule primordia. Due

Fig. 4. Development of nodule primordium induced by strain CTNUX1 of *Rhizobium etli* on the roots of *Phaseolus vulgaris*. **A, B,** and **C,** Photographs depict nodule primordia at successive stages of development. Transversal sections of inoculated roots were fixed and stained with methylene blue. **D, E,** and **F,** Longitudinal sections of ectopic roots emerging from pseudonodules stained with acetocarmine. CS = central stele, VB = vascular bundles, and MZ = meristematic zone of developing ectopic roots.

Fig. 5. **A,** Root hair deformation, **B,** infection threads development, **C,** and primordia invasion during *Rhizobium etli*–*Phaseolus vulgaris* symbiosis. Strains CE3 (wild type) and CTNUX1 were transformed with plasmid pAR66 (*ntrBCp-lacZ*) and used to inoculate roots of *P. vulgaris* seedlings. At **A**, 2 or **B**, 4 days post inoculation, the complete root system was harvested and fixed and β -galactosidase activity was assayed. **C,** Developing nodules (1 week old) were sectioned before analyzing the *lacZ* expression. Arrows indicate the infection threads, VB = vascular bundles, and CT = central tissue.

to the presence of invaded cells, the central tissue of normal (CE3-induced) nodule primordia was stained (Fig. 5C). In contrast, the RIND-induced nodule primordia remained unstained (Fig. 5C). At a higher magnification, a few blue spots located in the apical periphery of these empty primordia and corresponding to abortive ITs could be observed (data not shown). These data confirm the early arrest of bacterial invasion occurring inside developing ITs.

Expression of nodulins in abortive nodules.

In order to confirm the absence of a differentiated central tissue in the abortive nodules induced by the RIND mutants, we analyzed the expression of two genes, leghemoglobin and uricase II, specifically induced in the invaded and uninvaded cells, respectively, of *P. vulgaris* nodules (Tatè et al. 1994). RNA was extracted from (i) secondary root (meristematic region), ii) normal developing nodules induced by strain CE3 (wild type), and (iii) abortive nodules induced by strain CTNUX1, and the level of leghemoglobin and uricase II transcripts in these tissues was compared by a real-time reverse-transcription polymerase chain reaction (RT-PCR) analysis. In

all the reported experiments, we used as internal standard the 18S ribosomal RNA primers or competitors in a ratio of 4:6. The designed primers bracket intronic sequences, and genomic-amplified fragments never were observed with the cDNA samples. The two molecular markers clearly were induced in normal nodules when compared with both CTNUX1-induced abortive nodules and secondary roots (Table 1). As expected, the uricase II gene showed a basal level of expression in the *P. vulgaris* root tissue (Capote-Mañez and Sánchez 1997), whereas leghemoglobin transcript was merely detectable (Table 1). These data confirm the lack of a well-differentiated central tissue in the RIND-induced structures.

Bacterial invasion and developmental fate of nodule primordium.

To restore the invasion capacity of RIND mutants, nodulation experiments in the presence of the corresponding nutritional requirement (lysine, glucose, or nucleotides) were performed. However, in all cases, the rescue was only partial or not evident. Thus, to test a putative link between the absence of bacterial invasion and the induction of lateral roots from nodule primordia,

Table 1. Real-time reverse-transcription polymerase chain reaction (RT-PCR) analysis of late nodulin genes^a

Gene	CE3-induced developing nodules	Secondary root (meristematic region)	CTNUX1-induced abortive nodules
Uricase II	1	0.18 ± 0.04	0.07 ± 0.005
Leghemoglobin	1	<0.05	<0.05

^a Leghemoglobin and uricase II gene expression levels were normalized with respect to the internal control 18S and are indicated relative to their expression in nodules. Numbers represent the mean and standard deviations of data obtained from three real-time PCR experiments.

Fig. 6. Bacterial invasion and nodule development. Strain CTNUX23-pAR66 (*ntrBCp-lacZ*) of *Rhizobium etli* was used to inoculate *Phaseolus vulgaris* seedlings. A solution of L-methionine was added for the first 3 days after inoculation; later, the plants were maintained on Jensen medium. Two-week-old nodules or nodule-like structures were harvested, assayed for β -galactosidase activity, and photographed. The concentration of L-methionine was **A** and **A'**, 5 $\mu\text{g ml}^{-1}$, **B** and **B'**, 25 $\mu\text{g ml}^{-1}$, and **C** and **C'**, 125 $\mu\text{g ml}^{-1}$, as indicated on the left. CS = central stele, CT = central tissue, and ER = ectopic root.

the *metZ* mutant strain CTNUX23 (methionine auxotroph) of *R. etli* was used. It previously has been shown that this mutant is unable to grow to produce Nod factors and, thus, to induce nodulation (Nod⁻) unless the plant growth medium is supplemented with methionine (Tatè et al. 1999). Strain CTNUX23 was transformed with plasmid pAR66 (*ntrBCp-lacZ* transcriptional fusion) and used to inoculate roots of *P. vulgaris* seedlings. Nodulation assays were performed in the presence of increasing concentrations of methionine (up to 125 $\mu\text{g ml}^{-1}$). After inoculation, the plants were watered for 3 days with Jensen medium supplemented with methionine and, after that, with Jensen medium without methionine. After 11 days, the resulting nodules or nodule-like structures were harvested, sectioned (if required), and their invasion (in situ β -galactosidase assay) was analyzed (Fig. 6). At a low methionine concentration (5 $\mu\text{g ml}^{-1}$), strain CTNUX23 induced uninvaded pseudonodules from which developing lateral root emerged (Fig. 6A and A'). At a higher methionine concentration (25 $\mu\text{g ml}^{-1}$), the mutant induced genuine nodules, characterized by the presence of invading bacteria in their apical or distal zone (Fig. 6B and B'). Moreover, the number of nodules induced, as well as the degree of invasion reached (revealed by the staining intensity), increased with the methionine concentration used (Fig. 6C and C'). These data indicate that, when the ITs and, thus, the invading bacteria reach the apical meristematic region of a developing nodule primordium, the nodular tissues are formed while the induction of ectopic roots is inhibited. The degree of bacterial invasion (namely, the number of ITs or bacteria and their localization) required to sustain the process of nodule organogenesis was not established, but the experiment performed allowed us to conclude that a partial invasion is sufficient.

DISCUSSION

On the roots of *P. vulgaris*, the RIND mutants of *R. etli* analyzed in this study elicited the same early sequence of morphogenetic events induced by the wild-type strain until nodule primordia formation, thus indicating that they are able to produce biologically active Nod factors (lipochitooligosaccharides

derivatives of N-acetyl glucosamine) (Spaink 2000; Spaink and Lugtenberg 1994). In correlation, it was observed that, when growing in the presence of the corresponding nutritional requirement, the RIND mutants released Nod factors with chromatographic features similar to those produced by strain CE3 (data not shown).

The mutants elicited root-hair deformation as well as the parent strain CE3, even though the deformations induced sometimes were abnormal (Fig. 5A). Why aberrant or exaggerated root hair deformations are induced presently is unknown, but a correlation with the incapacity of RIND mutants to grow efficiently in the rhizosphere is not excluded. In fact, entrapped bacteria growing associated or attached to the cell wall may induce the completion of the first deformation event, leading to extreme hair curling. In correlation, root-hair deformation but not curling is induced by purified Nod factors (Miller et al. 2000; Niwa et al. 2001) and, moreover, most auxotrophs of *R. etli* elicited the formation of a low number of nodules or showed a lower competitiveness (Ferraioli et al. 2002; Tatè et al. 1997).

The RIND mutants were able to initiate, although rarely, the infection process by inducing the formation of ITs, but their elongation was not sustained. The early abortion of the ITs may be due to the lack of production of a signal molecule, such as particular decorated Nod factors (Walker and Downie 2000) or 4-aminoimidazole-5-carboxamide (AICAR) (Newman et al. 1995). However, in the RIND mutants, the Tn5 insertions disrupt different (and almost unrelated) metabolic genes. Thus, their inability to produce a common signal molecule is unpredictable. On the other hand, the early abortion of the ITs may be related to nutritional requirements. In fact, it was proposed that the bacterial division is the driving force for IT elongation (Bladergroen and Spaink 1998). Nevertheless, a suggestive hypothesis is a strict correlation between bacterial division and production of a signal molecule that stimulates the plant to maintain the process of IT elongation.

The root outgrowths induced by the RIND mutants are characterized by an underdeveloped apical region reached by a well-differentiated vascular system (Fig. 4). Similar structures

Fig. 7. Lateral root, nodule, and ectopic root development, illustrated schematically.

were induced either by noninfective strains (including purine auxotrophs and lipopolysaccharides mutants) or by purified Nod factors of *R. etli* (Cardenas et al. 1995; Noel et al. 1988; VandenBosch et al. 1985). Moreover, by comparing the structure of nodules induced by a purine auxotroph of *R. etli* in the presence or absence of AICAR, a correlation between IT elongation and nodule development was suggested (Newman et al. 1995). Finally, the analysis of specific molecular markers in the RIND-induced abortive nodules (Table 1) and the experiments performed using a methionine-requiring mutant (Fig. 6) showed that bacterial invasion is essential to specify (or to maintain) the commitment of the apical meristematic cells of a nodule primordium toward the development of the most specific nodular tissue; namely, the central tissue (formed by invaded and uninvaded cells). Taken together, these data support the notion that, in the *R. etli*-*P. vulgaris* symbiosis, i) the program of nodule development requires additional bacterial factors, even though the local production of Nod factors (i.e., by bacteria growing inside the elongating ITs) is not excluded; and ii) globose nodules are derived from two distinct meristematic centers (Tat et al. 1994). From the most proximal or basal one (arising from vascular and inner cortical cells), the connecting nodule vasculature differentiates; whereas, from the most distal or apical one (derived from the reactivated outer cortical cells), the invaded central tissue is formed. In contrast, in elongated nodules, all the tissues are derived from a single apical meristem formed by a group of reactivated cells of the middle cortex (Timmers et al. 1999), although bacterial invasion is required for the differentiation of the invaded tissue (Mitra and Long 2004).

Frequently, from the empty nodule primordia induced by the RIND mutants, the formation of ectopic roots, aberrantly localized and agravitropic, was observed. This abnormal developmental process is illustrated schematically in Figure 7. To our knowledge, the formation of nodule-derived ectopic roots was not observed in other rhizobia-legume pairs. Conversely, the induction of nodules at sites of lateral root emergence was described previously (Mathesius et al. 2000). Usually, the meristem of a lateral root arises from a root primordium formed by rapidly dividing cells originating from the reactivation of pericycle cells (Laskowski et al. 1995). Thus, in abortive nodule primordia, the meristem of ectopic roots may arise from a developing vascular bundle, as previously suggested (VandenBosch et al. 1985). Auxins are inducers of lateral root formation (Rashotte et al. 2001), and the level of auxins decrease during nodule development (Mathesius et al. 1998). Thus, it is reasonable to speculate that bacterial invasion directly or indirectly alters the hormone balance of the most apical region of nodule primordia, inhibiting the formation of ectopic roots.

MATERIALS AND METHODS

Growth conditions and bacterial strains.

R. etli strains were grown at 30C in TYR medium containing tryptone at 5 g liter⁻¹, yeast extract at 3 g liter⁻¹, and CaCl₂ · 2H₂O at 0.88 g liter⁻¹. Antibiotics were added to the medium as needed at the following concentrations: tetracycline at 5 g ml⁻¹, nalidixic acid at 20 g ml⁻¹, and kanamycin at 50 g ml⁻¹. The isolation and molecular characterization of strain CTNUX1(*lysA*), CTGUT5(*fbaB*), CTGUT26(*purF*), CTGUT28(*pyrB*), and CTGUT42(*pckA*) was described elsewhere (Ferraioli et al. 2002; Tat et al. 2004).

Transformation of *R. etli*.

Strains were grown at 30C with shaking (250 rpm; rotary shaker, New Brunswick, NY) in TYR medium, and competent

cells for electroporation were prepared as described previously (Tat et al. 1997). DNA of plasmid pAR66 (Patriarca et al. 1996) was purified with a Qiagen column (Promega Corp., Madison, WI, U.S.A.). Electroporation was performed with a Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, CA, U.S.A.). Electroporated cells were resuspended in 1 ml of a rich medium containing tryptone at 20 g liter⁻¹, yeast extract at 3 g liter⁻¹, KCl at 0.19 g liter⁻¹, CaCl₂ at 2.1 g liter⁻¹, MgSO₄ at 2.5 g liter⁻¹, and mannitol at 10 g liter⁻¹. The cells were incubated for 4 h at 30C and then plated on TYR agar containing tetracycline at 5 g ml⁻¹ plus kanamycin at 50 g ml⁻¹. Colonies appearing after incubation for 3 days at 30C were streaked on plates of rich (TYR) and minimal media.

Nodulation test and preparation of fixed, sectioned material.

Seed of *P. vulgaris* cv. Negro Jamapa were sterilized and germinated as described previously (Tat et al. 1997). Following germination, seed were transferred to sterile plant growth pouches (Mega International, Minneapolis, MN, U.S.A.) and root tips were inoculated with *R. etli* cells resuspended in inoculation buffer (50 mM phosphate buffer, pH 7.0) at the required density (10⁴ to 10⁶ ml⁻¹). Plants were grown in a growth chamber maintained at 65 to 75% relative humidity, 23C in light and 19C in dark with a 16-h photoperiod, and with the N-free Jensen medium (Jensen 1942).

For histological analysis, the roots were collected at different times after inoculation and fixed as previously described (Tat et al. 1994). Portions of roots (0.8 to 1 cm long) were excised with a razor blade and embedded in 4% agarose. Sections (50 to 80 m) were obtained using a Leica VT1000 S (Nussloch, Germany) vibrating-blade microtome. Specimens (whole roots or sections) were stained with methylene blue (0.01% in distilled water) or acetocarmine (0.5% carmine red, 45% acetic acid in distilled water) and photographed with a Zeiss (Jena, Germany) Axiophot microscope equipped with a digital camera (Coolpix995; Nikon Corporation, Tokyo).

To test the nodulation ability of strain CTNUX23 of *R. etli* in the presence of methionine, bacterial cells were applied (spotted) to the root of 5-day-old *P. vulgaris* seedlings. First, inoculation bacteria were grown in TYR medium, centrifuged at low speed, and resuspended in inoculation buffer (50 mM phosphate buffer, pH 7.0) with or without supplemental L-methionine. L-methionine also was added to the sterile basal medium (Jensen medium).

Histochemical localization of -galactosidase activity.

Whole roots were collected and fixed with a solution containing 1% paraformaldehyde, 0.3 M mannitol, and 10 mM 2-N-morpholino ethanesulfonic acid (pH 5.6) for 1 h at room temperature under a brief and gentle vacuum. Whole root fragments or nodule sections were washed three times with 50 mM phosphate buffer, pH 7.2, and immersed in a staining solution containing 10 mM phosphate buffer (pH 7.2), 150 mM NaCl, 1 mM MgCl₂, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.03% Triton-X100, and 1 mM 5-bromo-4-chloro-3-indolyl -galactopyranoside (X-Gal). The samples were incubated at 37C overnight in the dark, then briefly cleared with sodium hypochlorite, washed in phosphate buffer, and photographed with a Zeiss Axiophot microscope equipped with a Coolpix995 digital camera.

Real-time RT-PCR.

Total RNA was prepared from secondary root (meristematic region), CE3-induced developing nodules and CTNUX1-induced abortive nodules by using the RNeasy Plant minikit (Clontech Laboratories, Palo Alto, CA, U.S.A.). To remove

contaminating DNA, the samples were treated with DNase I. Total RNA (1.5 µg) was annealed to random decamer and reverse-transcribed by using RT (Ambion, Austin, TX, U.S.A.) to obtain cDNA. Real-time PCR assays were performed with a DNA Engine Opticon 2 System (MJ Research, Waltham, MA, U.S.A.) using SYBR to monitor double-stranded DNA synthesis. As internal standard, the 18S ribosomal RNA primers or competitors (Ambion) in a ratio of 4:6 were used. Every reaction was set up in three replicates. The program used was as follows: 95°C for 2 min and 35 cycles of 95°C for 20 s, 60°C for 15 s, and 72°C for 20 s. Data were analyzed using Opticon Monitor Analysis Software (version 2.01; MJ Research). The relative level of expression was calculated with the following formula: relative expression ratio of the gene of interest was $2^{-\text{DCT}}$, where $\text{DCT} = \text{Ct}_{\text{gene}} - \text{Ct}_{18\text{S}}$. The PCR-amplified fragments from total cDNA were gel purified and sequenced to assure accuracy and specificity. The sequences of the gene-specific oligonucleotides used for real-time RT-PCR were as follows: LbcPvforw, 5'-TCTGGTGAACAGCTCATGGG-3'; LbcPvrev, 5'-AACCAAGTGCAGCATCAGCG-3'; UriPvforw, 5'-ACAGGGGAGTCTATAGCCCAT-3'; and UriPvrev, 5'-CATGAGGCTCGTCCGTTGGTA-3'.

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