Short root mutant of *Lotus japonicus* with a dramatically altered symbiotic phenotype

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**Summary**

Legume plants carefully control the extent of nodulation in response to rhizobial infection. To examine the mechanism underlying this process we conducted a detailed analysis of the *Lotus japonicus* hypernodulating mutants, *har*1-1, 2 and 3 that define a new locus, HYPERNODULATION ABERRANT ROOT FORMATION (*Har*), involved in root and symbiotic development. Mutations in the *Har*1 locus alter root architecture by inhibiting root elongation, diminishing root diameter and stimulating lateral root initiation. At the cellular level these developmental alterations are associated with changes in the position and duration of root cell growth and result in a premature differentiation of *har*1-1 mutant root.

No significant differences between *har*1-1 mutant and wild-type plants were detected with respect to root growth responses to 1-amino-cyclopropane-1-carboxylic acid, the immediate precursor of ethylene, and auxin; however, cytokinin in the presence of AVG (aminoetoxyvinylglycine) was found to stimulate root elongation of the *har*1-1 mutant but not the wild-type. After inoculation with *Mesorhizobium loti*, the *har*1 mutant lines display an unusual hypernodulation (HNR) response, characterized by unrestricted nodulation (hypernodulation), and a concomitant drastic inhibition of root and shoot growth. These observations implicate a role for the *Har*1 locus in both symbiotic and non-symbiotic development of *L. japonicus*, and suggest that regulatory processes controlling nodule organogenesis and nodule number are integrated in an overall mechanism governing root growth and development.

**Keywords:** root development, nodulation, *Rhizobium*, phytohormones.

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**Introduction**

Root development in plants is a complex process involving a high degree of morphological plasticity, which reflects inherent adaptive mechanisms to highly variable environmental conditions. Although the molecular determinants of root morphology and functioning are only starting to be understood, classical physiological experiments have clearly implicated both local and systemic regulatory circuits in the determination of root plasticity. In addition to its role in selective exploitation of specific soil domains for available nutrient and water sources, an important role of root developmental plasticity is to provide plants with the ability to recognize and respond to diverse biotic signals from soil microorganisms. Discriminative recognition of and appropriate responses to these biotic cues are essential for plant survival, and in the case of symbiotic plant-microbe interactions provides an additional means for selective exploitation of otherwise inaccessible nutrient...
sources. Nitrogen-fixing symbioses of legume plants provide an interesting example of the latter phenomenon (Vance, 1998). Upon infection with specific strains of rhizobia, root cortical cells of legume plants undergo deifferentiation, initiate cell divisions, and redirect their developmental fate towards the formation of nodule primordia. Thereafter, through a highly organized and controlled series of events, the nodule primordia develop into fully functional, nitrogen-fixing organs, root nodules (for recent reviews see Hadri et al., 1998; Hirsh, 1992). Nodule organogenesis is activated in response to specific lipo-chitooligosaccharide signal molecules (Nod factors) synthesized by compatible strains of rhizobia (Hadri and Bisseling, 1998; Spaink, 1996). Structural and functional adaptations of the root to Nod factors and rhizobial infection is controlled by the host plant and has been shown to be modulated by various environmental factors, including the availability of combined nitrogen, as well as developmental cues associated with plant growth (Caetano-Anolles and Gresshoff, 1991; Francisco and Harper, 1995; Nutman, 1952; Parsons et al., 1993; Streeter, 1988). One important aspect of this control process is the plant-mediated regulation of the extent of nodulation in response to rhizobial infection. The plant host actively controls the number of successful nodulation events on at least two different levels. One level involves a premature arrest of the majority of rhizobial infections, such that only a restricted number of nodules are formed within a highly specific susceptible zone, located just behind the growing root tip (Vasse et al., 1993). In Medicago truncatula, the sickle mutation has been shown to cause a dramatic increase in the number of persistent rhizobial infection, resulting in hypernodulation of the susceptible zone of the mutant root (Penmetsa and Cook, 1997). The latter phenotype was attributed to a second effect of the same mutation, namely overall insensitivity of the mutant plant to the hormone ethylene (Penmetsa and Cook, 1997). These results suggest that in addition to its well-characterized functions in plant development, ethylene is also involved in the signaling pathway controlling rhizobial nodulation of legumes. A role of the plant hormone ethylene in several other aspects of symbiotic development has been well documented for at least some legume plant species (Fernandez-Lopez et al., 1998; Grobbelaar et al., 1970; Heidstra et al., 1997; for a recent review see Hirsch and Fang, 1994). However, ethylene-insensitive mutants of soybean were shown to display a wild-type nodulation pattern (Schmidt et al., 1999). It is unclear at present whether the difference between the effects of ethylene and/or ethylene-sensitivity on nodulation in soybean and other legume plant species reflects a differential role of this hormone in regulating nodule development.

In addition to limiting the number of persistent rhizobial infections within the root susceptible zone, the plant also exerts a spatial and temporal control of root susceptibility to nodulation. This mechanism is referred to as auto-regulation or feedback regulation of nodulation, and involves inhibition of nodule formation on younger root tissues by prior nodulation events in older root regions (Kosslik and Bohlool, 1984; Nutman, 1952; Pierce and Bauer, 1983). Autoregulation renders the root cells only transiently susceptible to rhizobial infection, resulting in a narrow zone of infection and nodule differentiation (susceptibility zone; Bhuveswari et al., 1981). Plants defective in this mechanism continue to nodulate on newly developing roots and form a large number of nodules over the entire root system (hypernodulation or supernodulation phenotype). Based on experiments involving split root system and grafting between wild-type and supernodulation mutant plants, an interplay between local and systemic signaling events in establishing the autoregulatory control of nodulation has been postulated (Caetano-Anolles et al., 1991; Sheng and Harper, 1997). Similar experiments have identified leaf tissues as the major source of the systemic signal(s), implicating long distance communication between the root and shoot in autoregulation of nodule number. A local, non-systemic control exerted by fully mature nodules over the outgrowth of younger nodulation events has also been postulated (Caetano-Anolles et al., 1991; Nutman, 1952). The exact nature of the mechanisms involved in autoregulation is not understood and the identity of the postulated systemic and local signaling compounds remains unknown. However, it is tempting to speculate that the autoregulatory response relies, at least in part, on the mechanism of sensing and regulating cell divisions, and thus may constitute a part of a more general mechanism regulating plant growth. In this context it is interesting to note that plant developmental processes other than nodulation, such as those associated with the generation of apical root meristems, have been shown to influence nodule formation via a mechanism that resembles autoregulation (Gresshoff et al., 1989; Nutman, 1952). Conversely, mutations that impair the autoregulatory response have been found to exert various pleiotropic effects on plant growth and, almost invariably, lead to a high nitrate-tolerant symbiosis (efficient nodulation in the presence of high nitrate; nts phenotype). Nitrate (NO₃⁻) modulates plant growth and exerts complex effects on root development, symbiont recognition and nodulation (Dazzo and Brill, 1978; Gresshoff, 1993; Zhang et al., 1999). Common factors may be involved in the mechanisms regulating the extent of nodulation, nitrate inhibition and other related plant growth responses (e.g. lateral root formation). Alternatively, close interactions between specific regulatory pathways (e.g. autoregulatory and nitrate
inhibition pathways) may be sufficient to account for the pleiotropic effects of a single mutation in one of the controlling elements. Understanding the nature of the regulatory processes controlling nodule differentiation and nodule number, and integrating them in the overall mechanisms governing root growth and development constitute important elements of our quest to understand symbiotic nitrogen fixation in legumes.

We have previously identified plant mutants from the diploid legume Lotus japonicus that define a locus controlling normal root development. The same mutations have been found to confer an aberrant response of the mutant plant to the challenge by symbiotic rhizobia, resulting in hypernodulation and abnormal plant growth phenotypes (de Bruijn et al., 1998; Schauer et al., 1998; Szczyglowski et al., 1998a; Szczyglowski et al., 1998b). Here we present a detailed characterization of these mutant lines and show that the underlying mutations affect plant development by changing the position and duration of root cell growth.

Results

Isolation and genetic analysis of hypernodulated aberrant root formation (Har) mutants of L. japonicus

We have previously described the isolation of two allelic EMS-induced mutant lines of L. japonicus ecotype Gifu (Ljsym34-1 and Ljsym34-2), which display both unusual symbiotic (hypernodulated) as well as drastically altered root developmental (aberrant root) phenotypes (de Bruijn et al., 1998; Szczyglowski et al., 1998a; see Figure 1). In addition, we identified an independent mutant line from a T-DNA mutagenesis experiment (sym16) with a highly similar phenotype, but the mutant phenotype was found to be genetically unlinked to the T-DNA insertion (Schauer et al., 1998). The allelic Ljsym34-1 and Ljsym34-2 mutations were found to be monogenic recessive with regard to the aberrant root phenotype and incomplete dominant in terms of the symbiotic hypernodulation phenotype (Szczyglowski et al., 1998a; Szczyglowski et al., 1998b, data not shown). The sym16 mutation was monogenic recessive for all phenotypes (Schauer et al., 1998). Reciprocal crosses revealed that the Ljsym34-1/2 and sym16 mutants belong to the same complementation group (data not shown). In accordance with the recently proposed guidelines for genetic nomenclature for L. japonicus (Stougaard et al., 1999), the corresponding alleles were re-named har1-1 (formerly Ljsym34-1), har1-2 (formerly Ljsym34-2), and har1-3 (formerly sym16), and the corresponding wild-type gene was named Har1. Based on its slightly stronger mutant phenotype, the har1-1 allele was chosen for further detailed analysis.

The symbiotic (hypernodulated) phenotype of the har1-1 mutant

Inoculation of L. japonicus har1-1 plants with Mesorhizobium loti strain NZP2235 resulted in an almost total inhibition of plant growth and the unusual hypernodulation phenotype previously described (Szczyglowski et al., 1998a; see Figure 1). Nodule-like structures covering nearly the entire short root system developed concomitantly with inhibition of plant growth and deterioration of overall plant vitality (hypernodulation response, HNR, phenotype; Szczyglowski et al., 1998a; Szczyglowski et al., 1998b). To further examine this novel hypernodulation phenotype, a derivative of M. loti strain NZP2235, carrying a constitutively expressed hemA::lacZ reporter gene fusion, was used to analyze early events during the infection of wild-type and har1-1 mutant plants. Microscopical analyses revealed that the mode of rhizobial primary entry into har1-1 mutant roots was through infection threads initiated within deformed root hairs, as in wild-type plants. Other early infection events, such as root hair deformation (Had), hair curling (Hac) and infection thread formation (Inf) were also similar in har1-1 mutant and wild-type plants (data not shown). However, subsequent stages of symbiotic development were found to differ significantly. In wild-type plants, the majority of primary infection events were found to be arrested early during symbiotic development, without advancing beyond the stage of a few cortical cell divisions, resulting in 9–15 nitrogen-fixing nodules on the upper portion of fully elongated 21-day-old L. japonicus wild-type roots (see Figure 1a,d). In contrast, roots of har1-1 mutant plants, at 11 days after inoculation with rhizobia, had much more abundant foci of cortical cell divisions than wild-type roots spanning almost the entire length of the root (Figure 2 and 3a,b). These initial cortical cell divisions gave rise to nodule primordia, and subsequently to a mass of nodules covering almost the entire root (Figures 1e,f and 3c,d). In addition, nodule morphogenesis on har1-1 mutant plants was found to be insensitive to normally inhibitory concentrations of combined nitrogen (5–15 mM NO3−). Six weeks after inoculation with rhizobia, har1-1 mutant plants developed approximately 40–60 nodules, in the presence of high concentrations of nitrate (5–15 mM), or ammonia (1–3 mM). In contrast, nodule development in control wild-type L. japonicus plants was found to be highly inhibited by combined nitrogen sources. For example, in the presence of 15 mM KNO3 only a few small nodule-like structures (bumps) were observed. When grown in the presence of a low concentration of KNO3 (0.5 mM), 21-day-old nodules on har1-1 mutant plants were significantly smaller than wild-type nodules of the same
age (data not shown). In spite of the size difference, light and transmission electron microscopic examination of the infected zone of nodules formed on har1-1 plants revealed a normal (wild-type like) cytology and histology (Figure 3e,f). Moreover, nodules formed on har1-1 mutant roots had the capacity to fix nitrogen (reduce acetylene) at levels comparable to wild-type nodules when calculated on a per plant basis (data not shown).

The non-symbiotic (aberrant root formation) phenotype of the har1-1 mutant

Uninoculated L. japonicus har1-1 mutant plants develop a significantly shortened root system and an enhanced number of lateral roots as compared to wild-type plants (Szczyglowski et al., 1998a). To further analyze this phenotype, we examined lateral root formation in uninoculated har1-1 and wild-type primary roots and found no significant differences in their position relative to the root tip, as almost all LRP s were found in a region located 0.75–4.5 cm from the root apex. However, the density of lateral root primordia and emerged lateral roots (number per unit length of root) were at least three times higher in the har1-1 mutant than in wild-type plants (data not shown). Detailed examination of median longitudinal sections of uninoculated har1-1 root samples approximately 2 cm above the root tip revealed a significantly higher level of mitotic activity in the root pericycle layer of har1-1 versus wild-type roots (Figure 4a,b). In 9-day-old roots of the har1-1 mutant, periclinal cell divisions in the pericycle, as well as the development of two or three new cell layers, were

![Figure 1. Root and nodulation phenotypes of wild-type and har1 mutant L. japonicus plants.](image)

Plants were grown for 21 days in the presence (a; wild-type and b; har1-1) or absence (c; har1-1) of Mesorhizobium loti NZP2235, with 0.5 mM KNO₃ in the watering solution. Panels (d) and (e) show a close-up of the nodulated roots shown in (a) and (b), respectively. Panel (f) shows har1-1 grown for 3 weeks in the presence of rhizobia and for an 8 additional weeks in nitrogen-rich Hormum growth medium containing 8 mM KNO₃ and 5 mM NH₄⁺ (Thykjaer et al., 1998).

![Figure 2. Infection and nodulation events in wild-type and har1-1 plants upon inoculation with M. loti strain NZP2235 carrying a hemA:lacZ reporter gene fusion.](image)

Roots were stained for β-galactosidase activity and examined using brightfield microscopy. Open bars denote the number of root hairs with visible infection threads; solid bars indicate the number of nodules and nodule primordia. Each value represents the mean of measurements from 7 to 15 plants. Error bars represent 95% confidence intervals.

Figure 3. Microscopic analysis of symbiotic development in wild-type and har1-1 plants. (a,b) Brightfield micrographs of cleared wild-type and har1-1 mutant roots 11 days after infection with M. loti NZP2235. Nodule primordia are indicated by arrows. (c,d) Montages of the nodule phenotypes of wild-type and har1-1 mutant plants 14 days after inoculation with M. loti NZP2235 carrying a hemA: lacZ reporter gene fusion. Roots were stained for β-galactosidase activity and examined using brightfield microscopy. (e,f) Transmission electron micrographs of the central zone of wild-type and har1-1 mutant nodules showing infected host cells filled with bacterial endosymbionts, and a portion of adjacent highly vacuolated uninfected cells.

detected in all sections examined. Abundant anticlinal cell divisions in the pericycle, as well as in the neighboring cortical cell layers, could also be detected in sections of har1-1 mutant roots, and in several cases well-developed lateral root primordia were observed. In contrast, wild-type roots of a similar age exhibited only limited mitotic activity in the pericycle, which was mostly composed of a single cell layer (Figure 4a,b). Lateral root primordia were only rarely observed in sections of wild-type roots.

**Root and shoot phenotype of the har1-1 mutant**

The short root phenotype of *L. japonicus* har1-1 plants has been documented previously (de Bruijn et al., 1998; Szczyglowski et al., 1998a; Szczyglowski et al., 1998b). To further assess this phenotype quantitatively, the longitudinal growth of uninoculated and inoculated har1-1 and wild-type roots was measured. An average root length of 61.3 ± 0.2 mm was observed for uninoculated har1-1 plants 21 days after sowing versus 131.9 ± 1.0 mm for wild-type uninoculated control plants (Figure 5a). In addition, the root mass of uninoculated har1-1 plants at 21 days after sowing (26 ± 2 mg fresh weight) was significantly smaller than that of the wild-type plants (45 ± 6 mg). The aberrant har1-1 root growth phenotype was even more extreme when plants were inoculated with *M. loti* strain NZP2235. Root growth of har1-1 plants ceased entirely within the first days after inoculation, and the roots did not exceed an average length of 20 ± 1.5 mm (Figure 5b). The shoot mass of inoculated har1-1 plants was also significantly reduced in infected plants, while it was comparable in uninoculated har1-1 versus wild-type shoots (Figure 5c,d).

**Cytology of har1-1 mutant roots**

Having established that several root developmental parameters (root length/elongation, lateral root formation and overall root mass accumulation) were significantly altered in the har1-1 mutant line, the phenotype of the har1-1 mutant roots was further analyzed. First, the cellular organization of wild-type *L. japonicus* roots was examined. Primary *L. japonicus* roots were found to contain an outer single layer of epidermal cells, 3–5 irregularly shaped cortical cell layers surrounding a single layer of endodermal cells, an innermost region consisting of a single layer of pericycle cells enclosing the vascular cylinder, and a...
Figure 6. Histology of non-symbiotic root development.
(a,b) Median longitudinal sections of the root tip regions of 9-day-old wild-type and har1-1 mutant plants showing the overall anatomy of the meristematic region and the position of the cell elongation/vacuolation zone. (c,d) Cross-sections of roots of 22-day-old plants at approximately 800 μm above the root tip, showing differences in the extent of cell vacuolation and root diameter. (e,f) Cross-sections of mature root regions of 6-day-old plants approximately 1 cm above the root tip.

Table 1. Morphometric measurements of root cross-sections [area ± SD (μm²)] at a distance of 1 cm from the root tip

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Wild type (a)</th>
<th>har1-1 (b)</th>
<th>b/a ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total section</td>
<td>62357 ± 14772</td>
<td>33786 ± 4845</td>
<td>0.54</td>
</tr>
<tr>
<td>Epidermis</td>
<td>10073 ± 2250</td>
<td>5626 ± 760</td>
<td>0.56</td>
</tr>
<tr>
<td>Cortex</td>
<td>45228 ± 11283</td>
<td>24433 ± 3820</td>
<td>0.54</td>
</tr>
<tr>
<td>Endoderm</td>
<td>2097 ± 373</td>
<td>1387 ± 175</td>
<td>0.66</td>
</tr>
<tr>
<td>Stele</td>
<td>9070 ± 1033</td>
<td>2655 ± 513</td>
<td>0.52</td>
</tr>
</tbody>
</table>

Table 1. Morphometric measurements of root cross-sections [area ± SD (μm²)] at a distance of 1 cm from the root tip

Distally located area of root cap cells. The same general organization of root cell layers was found in har1-1 mutant roots (Figure 6). However, several significant differences were observed. Vacuolation, which typically accompanies cell expansion, occurred closer to the root tip in har1-1 versus wild-type roots (Figure 6a,b). In addition, the diameter of mutant har1-1 roots (0.23 ± 0.03 mm), measured using digitized micrographs of living roots at 3–6 mm from the root tip, was significantly smaller than the average diameter of the corresponding region of wild-type L. japonicus roots (0.31 ± 0.02 mm; see also Figure 6c,d). Based on these results, the following two hypotheses were formulated and tested: (1) the har1-1 mutation affects the radial organization of the L. japonicus root, and/or (2) the decreased root length and diameter is a result of abnormal (reduced) cell expansion.

The har1-1 mutation results in a diminished radial expansion of root cells

To test the first hypothesis (a defect in the radial organization of roots), a large number of root sections were examined microscopically. No clear evidence for one or more missing cell layers or a diminished number of root cells in har1-1 mutant roots was found (Figure 6). Therefore, we tested the second hypothesis (changes in root cell expansion), by analyzing sections of the fully differentiated region of primary roots (Figure 6e,f). The average total cross-sectional area of har1-1 mutant roots was almost two times smaller than that of the wild-type.
root (Table 1). Individual cell layers (epidermis, cortex, endodermis and root stele) of har1-1 mutant roots showed a similar reduction in size, contributing nearly equally to the overall decrease in root diameter (Table 1). Subsequently, the projected cross-sectional area of individual root cells was analyzed to determine whether cell radial expansion was altered in har1-1 mutant roots. The radial surface areas of individual cells of the epidermis, cortex and endodermis of roots had a frequency distribution that was confined to a much smaller size range for the har1-1 mutant (Figure 7), suggesting that the har1-1 mutation limits the ability of root cells to expand.

The length of the meristematic region is shortened in har1-1 mutant roots

To examine whether the short root phenotype of har1-1 mutant plants was caused by alteration in the primary direction of cell expansion along the apical–basal axis, the length of har1-1 root epidermal cells was measured. The average length of fully expanded epidermal cells of har1-1 mutant roots (138 ± 30 µm) was nearly equal to that of wild-type roots (132 ± 30 µm), indicating that the har1-1 mutation did not affect longitudinal cell expansion of the epidermis (Figure 8a). These results suggest that the short root phenotype of the mutant plant is unlikely to be due to an abnormal longitudinal expansion of root cells.

![Figure 7](image_url) **Figure 7.** Root cell radial expansion in wild-type and har1-1 mutant plants. The frequency distribution of the cross-sectional area of root epidermal (a), cortical (b) and endodermal (c) cells of 6-day-old plants measured at the location of approximately 1 cm above the root tip is shown. Ten wild-type and mutant plants were analyzed and each point represents a frequency value for cell size range increments of 35 µm (epidermis), 20 µm (cortex) and 20 µm (endodermis); n represents the number of cells measured.

![Figure 8](image_url) **Figure 8.** Root cell elongation and size of meristematic region in wild-type and har1-1 mutant plants. (a) Epidermal cell length along the root axis. Each point represents the mean cell length value for range increments of 250 µm (for the first 4 mm from the root tip), and 500 µm (between 4 and 11 mm from the root tip), along the root axis. Single and double arrows indicate significant differences between the mean epidermal cell length at two consecutive and equivalent positions in wild-type and har1-1 mutant roots. (b) Intact roots of 14-day-old plants stained with acetocarmine. The red-stained meristematic regions of the wild-type and har1-1 mutant roots appear as dark areas, and their extent is indicated by the brackets.
However, consistent with our earlier observations (see above), epidermal cells of the har1-1 mutant roots showed evidence of elongation along the longitudinal axis significantly closer to the root tip (hence earlier in development) than wild-type roots (Figure 8a). Transmitted brightfield microscopy and laser scanning microscopy of whole cleared roots stained with acetocarmine revealed an area of densely cytoplasmic cells constituting the root meristematic region whose borders could be defined by interactive thresholding techniques using digital image processing. In independent experiments using both types of microscopy, an approximately 2.6-fold reduction in the projected area of the har1-1 mutant versus wild-type root meristematic regions was detected (45082 ± 4373 μm²; n = 11 versus 121635 ± 12545 μm², n = 19; see also Figure 8b). This reduction in the size of the root meristematic region was invariably associated with an acropetal displacement of the root cell elongation/vacuolation and vascularization zones (Figure 8b). har1-1 mutant roots also showed an inferior root cap structure in comparison with the wild type, but remained gravitropic (data not shown). In addition, the mitotic index was measured in order to estimate the proportion of mitotic cells in the root meristematic regions of har1-1 versus wild-type plants of an equal age, but no significant differences were found (har1-1 mutant MI = 3.8 ± 0.8 versus wild-type MI = 3.96 ± 0.7).

**Effect of hormones on har1-1 mutant root elongation**

The gross changes in har1-1 root morphology suggested that the hormonal regulation of the mutant root development could be disturbed. In order to test this hypothesis the effects of exogenous hormone applications on root elongation of wild-type and har1-1 mutant roots were investigated using a plate bioassay specifically developed for this purpose. Sucrose was found to be required at a relatively high concentration (4.5%) to support the maximal and uniform root elongation of both wild-type and har1-1 mutant roots. The roots of wild-type plants elongated more rapidly in the dark than in the light especially during the first 2–3 days of growth, after which time the difference in growth rate disappeared (Figure 9a). har1-1 mutant plants formed short roots when grown in the dark under conditions that provided maximal wild-type root growth. A dramatic inhibition or retardation of root elongation was observed after 2 days of incubation in the dark. Interestingly, the short root phenotype was partially suppressed when har1-1 mutant plants were grown in the light (Figure 9b).

Since har1-1 roots exhibited an abnormal pattern of radial expansion of root cells, as well as a hypernodulation response (see Figures 1, 3d and 6), and since the hypernodulation phenotype of a *Medicago truncatula* mutant (*sickle*) had been correlated with a change in ethylene sensitivity (Pennetsa and Cook, 1997), the sensitivity of wild-type and har1-1 seedlings to the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) was examined. When grown vertically in the dark on agar plates containing increasing concentrations of ACC, both wild-type and har1-1 mutant seedlings showed the same overall sensitivity pattern to ethylene inhibition of root growth (Figure 10a). However, in two independent experiments, har1-1 mutant roots displayed a slightly increased resistance to certain concentrations of ACC as compared with wild-type roots (e.g. 1 × 10⁻⁷ to 1 × 10⁻⁶ M ACC in Figure 10a). To further evaluate the observed decrease in

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**Figure 9.** Effect of light on the elongation rates of wild-type and har1-1 mutant roots.

(a) The rates of wild-type root elongation. (b) The rates of har1-1 mutant root elongation. Each value is the mean of measurements on 20 plants. Error bars represent 95% confidence intervals.
sensitivity of the har1-1 mutant line to ACC, we examined the responses of entire seedlings to exogenously applied ACC. When grown in the dark in the presence of ACC, both wild-type and har1-1 seedlings showed a typical triple response (Guzman and Ecker, 1990), consisting of a shortening of the hypocotyl, inhibition of root elongation, and exaggeration of apical hook curvature (Figure 11). Both genotypes exhibited similar levels of sensitivity to ACC in terms of hypocotyl length (data not shown).

Figure 11. Triple response to ACC of wild-type and har1-1 mutant plants. Wild-type and har1-1 mutant seeds were germinated on MS medium in the dark at 28°C in the absence (0) or presence of increasing concentrations (1–100 mM) of ACC. The photograph was taken 6 days after incubation in the dark. The triple response is characterized by shortened hypocotyls, roots and exaggerated apical hook formation.

Figure 10. Wild-type and har1-1 root growth in the presence of increasing concentrations of exogenously applied plant hormones. The relative elongation of wild-type and har1-1 mutant roots in the presence of (a) 1-amino-cyclopropane1-carboxilic acid (ACC); (b) α-naphthalene-acetic acid (NAA); or 6-benzylaminopurine (BA) is shown. Each value is the mean of measurements on 20 plants. Error bars represent 95% confidence intervals. The mean value of 100% root growth in (a) wild-type, 44.5 ± 3.4 mm; har1-1 21.8 ± 1.2 mm; in (b) wild-type, 56.9 ± 2.4 mm; har1-1, 25.8 ± 1.6 mm; in (c) wild-type 37.1 ± 1.9; har1-1, 21.6 ± 1.1 mm.
Subsequently, the effect of the auxin α-naphthalene-acetic acid (NAA) on root growth was examined. Wild-type and har-1-1 mutant roots were found to display a similar overall NAA sensitivity pattern but, as had been observed with ACC, roots of har-1-1 mutant plants showed a slight NAA-insensitive phenotype at higher concentrations of NAA (Figure 10b).

In Arabidopsis, cytokinin inhibits root elongation in light- and dark-grown seedlings due to the stimulation of endogenous ethylene production (Cary et al., 1995). Therefore, the sensitivity of wild-type and har-1-1 mutant roots to exogenously applied cytokinin (thus endogenously produced ethylene) was also examined. The presence of even very low concentrations (10–50 nM) of the synthetic cytokinin, 6-benzylaminopurine (BA), significantly reduced root growth in both genotypes. However, again the har-1-1 mutant roots exhibited a moderately higher resistance to a wide range of BAP concentrations than wild-type roots (Figure 10c). This slightly elevated resistance of har-1-1 mutant root could be due to either an altered ethylene-independent response to cytokinin, diminished cytokinin-stimulated ethylene production, and/or an attenuated response to endogenously produced ethylene. To distinguish between these possibilities, the influence of exogenously added cytokinin on inhibition of root growth was examined in the presence silver ions, applied as silver thiosulfate, to inhibit ethylene binding (Beyer, 1979), or aminoethoxyvinylglycine (AVG; Yang and Hoffman, 1984) to inhibit ethylene biosynthesis. Since 50 nanomolar BA almost maximally inhibited root elongation in both genotypes, we used this concentration of cytokinin in combination with variable concentrations of inhibitors.

In the absence of BA, Ag⁺ only had a limited stimulatory effect on the growth/elongation of wild-type and har-1-1 mutant roots (Figure 12a). Five μM Ag⁺ was sufficient to overcome all the inhibitory effects of cytokinin on har-1-1 mutant roots, whereas 5 μM Ag⁺ restored wild-type root growth to a level of about 60% of that of untreated control roots (Figure 12b).

A similar set of experiments was conducted with an inhibitor of ethylene synthesis, AVG. In control experiments, an AVG concentration equal to or lower than 0.1 μM had no measurable effect on root growth in both genotypes (Figure 12c), whereas higher concentrations were strongly inhibitory (data not shown). In contrast to the silver-mediated phenotypic suppression, a low concentration of AVG (0.1 μM) was found to relieve all of the inhibition caused by cytokinin, and to restore a normal long-root phenotype to wild-type plants (Figure 12d). However, the har-1-1 mutant plants responded differently to the same treatment, not only by recovering a short root phenotype, but also by an additional stimulation of root growth. The latter effect resulted in the mutant plants developing long roots that elongated at the same rate as wild-type roots. However, a prolonged incubation (more than 10 days) of both wild-type and mutant har-1-1 plants in the presence of cytokinin and AVG resulted in the total arrest of their root growth. This effect was found to be associated with a terminal differentiation of the root meristem, thus

Figure 12. Effect of BA on elongation of the wild-type and har-1-1 mutant roots in the presence of ethylene perception/synthesis inhibitors.

The relative elongation of wild-type and har-1-1 mutant roots in the presence of (a) Ag⁺; (b) BA plus Ag⁺; (c) AVG; and (d) BA plus AVG is shown. Mean value of 100% root growth in (a) and (b) wild-type, 41.6 ± 3.0 mm; har-1-1, 23.3 ± 1.2 mm; in (c) and (d) wild-type, 47.5 ± 4.0 mm; har-1-1, 23.2 ± 1.6 mm. For further details see legend to Figure 10.
precluding experiments with longer treatment times (data not shown).

Discussion

We have shown here that the Har1 locus of L. japonicus is critical for both root and symbiotic development by analyzing three mutant alleles at this locus, designed har1-1, har1-2 and har1-3. The har1-1 allele has the most pronounced phenotype and therefore was examined in most detail. However, a similar range of defined mutant phenotypes was found during a parallel analysis of har1-3, and also a partial analysis of har1-2 mutant lines (data not shown). Under axenic growth conditions, the most extreme phenotypic effects of har1 mutations are the restriction of root elongation and the promotion of lateral root growth. In the presence of rhizobia, har1 mutants display an unusual hypernodulation (HNR) response, characterized by unrestricted nodule formation (hypernodulation), and a concomitant drastic inhibition of root and shoot growth (Figure 1). Other legume symbiotic mutants with a pleiotropic phenotype have been described suggesting that at least in some cases the corresponding genes may function in both symbiotic and non-symbiotic processes (Delves et al., 1986; Duc and Messager, 1989; Kneen et al., 1994; Lee and Larue, 1992; Penmetsa and Cook, 1997).

Since the har1 mutant root phenotype is observed in the absence of rhizobia, we postulate that a primary role of the Har1 locus is to control root development. A specific defect in the root developmental program, such as the one caused by the har1-1 mutation, may indirectly affect the ability of the mutant plant to control nodule organogenesis by, for example, antagonizing one or more functions by which the host plant autoregulates nodulation. Alternatively, the L. japonicus Har1 gene product may play a direct role in mechanism(s) controlling nodulation, and thus could represent a common regulatory element for both root and symbiotic development.

The symbiotic phenotype of the har1-1 mutant indicates a defect in the autoregulatory mechanism controlling nodulation

M. loti induces the HNR response on L. japonicus har1-1 mutant plants. The most striking phenotypic effect associated with this response is an almost total inhibition of shoot and root growth that results in an overall small stature of inoculated har1-1 mutant plants. On a cytological level, this rapid growth arrest appears to coincide with the initiation of abundant cortical cell divisions and the formation of numerous nodule primordia (Figure 3). The hypothesis that the HNR is directly linked to nodulation is further strengthened by the identification of a partially epistatic mutation in the har1-2 line (LjEMS40; Szczygielowski et al., 1998a; Szczygielowski et al., 1998b). In this double mutant line (LjEMS40), the second unlinked allele (Ljsym22-2) confers a non-nodulating (Nod-) phenotype and suppresses the HNR, without affecting root morphology. Introduction of the Ljsym22-2 allele into a har1-1 genetic background suppresses both nodulation and the HNR responses, but does not alter the root morphology phenotype of the har1-1 mutant (Szczygielowski et al., 1998a; K. Szczygielowski et al., unpublished data). Although the exact mechanism responsible for HNR remains unknown, it appears likely that the abundant early nodulation events in har1-1 mutant roots are directly responsible for triggering the HNR.

Reduced plant growth has been previously observed in supernodulating/hypernodulating mutants of soybean and other legume plant species, although the overall growth retardation was never as extreme as that observed in L. japonicus har1-1 plants (Carroll et al., 1985; Day et al., 1986; Gremaud and Harper, 1988; Novak et al., 1997; Sheng and Harper, 1997). Unlike the HNR response of L. japonicus har1-1 mutant plants, and in spite of the abundant level of nodulation, the initial growth rate of the soybean supernodulating nts384 mutant is greater than that of the parental soybean line cv. Bragg. Only at a later stage, when nts384 roots become even more extensively nodulated, does a significant reduction in plant growth rate and size occur.

In Vicia sativa subsp., nigra infection with Rhizobium leguminosarum induces the formation of thick and short roots (Tsr phenotype; Van Brussel et al., 1982; Van Brussel et al., 1986), somewhat resembling the short root phenotype of inoculated har1-1 plants. However, the Tsr response has been shown to be associated with an ethylene-related increase in root cell radial expansion and to be accompanied by the inhibition of nodulation on primary roots and restricted nodule formation on lateral roots (Van Brussel et al., 1982; Van Brussel et al., 1986). In contrast, the primary roots of har1-1 mutant plants, as well as infrequently formed lateral roots, remain fully susceptible to rhizobial infection. The latter characteristic strongly suggests that the har1-1 mutants may be impaired in the mechanism(s) that autoregulate nodulation. This is further supported by the observation that har1-1 mutants, similar to other previously described mutants defective in autoregulation of nodulation, is able to be nodulated in the presence of high nitrate concentrations. The latter conditions support better root elongation and more frequent lateral root formation in har1-1 mutant plants in spite of the presence of rhizobia. These new root tissues remain susceptible to rhizobial infection and nodulation, regardless of the presence of mature nodules on older parts of har1-1 mutant roots (Figure 1f). Nitrate represents a major environmental factor that negatively regulates nodulation.
in legumes, including *L. japonicus* (Carroll and Mathews, 1990; Streeter, 1988). A connection between the mechanisms underlying nitrate inhibition and autoregulation of nodule development has been hypothesized previously (Delves et al., 1986; Gresshoff, 1993). Thus, the observed coexistence of nitrate-tolerance and hypernodulation (lack of autoregulation) phenotypes in the har1-1 mutant line lends additional support to this hypothesis. Results of preliminary grafting experiments have revealed that it is shoots and not roots that determine the altered symbiotic phenotype of the har1-1 mutant plants (data not shown). Altogether, these observations strongly suggest that the har1-1 mutation results in an impairment of the mechanism(s) responsible for systemic regulation of nodule number in *L. japonicus*.

The har1-1 mutation affects root growth by changing the position and duration of root cell growth

Changes in root morphology have been associated previously with hypernodulating (supernodulating) mutations in legumes, but have not been analyzed in detail. In the case of the soybean supernodulating mutant nts382, early (prior to nodule emergence) lateral root formation is increased, independent of the application of rhizobia and/or nitrate (Day et al., 1986). The pea hypernodulating mutant nod5 also displays an altered root morphology phenotype. This mutant line shows a reduced tap root growth pattern, forms more lateral roots, and during growth on nitrate produces more tertiary roots than the wild-type plant (Jacobsen and Feenstra, 1984).

A primary function of the *L. japonicus* Har1 locus in the mechanism(s) regulating root development is suggested by two independent observations. First, the mutations in this locus significantly alter root development in the absence of rhizobia. Under the same growth conditions, in the absence of rhizobia, the aerial portions of har1-1 plants continue to have a wild-type appearance. Second, nodulation of har1-1 mutant plants can be specifically suppressed by the presence of additional unlinked mutations (e.g. the *LjSYM22-2* allele) without a noticeable effect on the altered root morphology phenotype (see above). These results clearly show that the *Har1* gene product is involved in the non-symbiotic development of *L. japonicus* roots. All three har1 mutations described above drastically diminish elongation of both main and lateral roots, decrease root diameter, and promote lateral root differentiation. The latter phenomenon is particularly intriguing since development of lateral roots also involves the initiation of new meristems (Steeves and Sussex, 1989).

It has been suggested that lateral root formation in *Arabidopsis* involves at least two distinct stages. During the first stage, the lateral root primordium (LRP) is formed by stimulation of dedifferentiation and proliferation of cells in the root pericycle layer. This is followed by the second phase, which involves the organization of an autonomous lateral root meristem that is capable of perpetuating LRP structures and producing lateral roots (Celenza Jr et al., 1995; Cheng et al., 1995; Laskowski et al., 1995; Malamy and Benfey, 1997). The development of LRP appears to be significantly stimulated in har1-1 mutant versus wild-type roots. The activated pericycle in har1-1 mutant roots forms an uninterrupted file of two to three cell layers suggestive of abundant lateral root formation events (Figure 4). Assuming that all these pericycle cell divisions indeed reflect initiation of LRP in har1-1 mutant root, the approximately threefold increase in the number of emerged lateral roots suggests that in the har1-1 mutant line a certain amount of control of lateral root differentiation and growth still exists. har1 mutants do not show enhanced proliferation of adventitious roots (roots derived from hypocotyl) or distortion of root and/or hypocotyl structures, phenotypes associated with increased lateral root development in the *Arabidopsis* rooty mutant (Boerjan et al., 1995; Celenza Jr et al., 1995; King et al., 1995; Lehman et al., 1996). har1 mutants are also phenotypically different from other previously reported root mutants of *Arabidopsis* that display alterations either in the radial organization of the root (e.g. shortroot mutant; Scheres et al., 1995) or root cell expansion (e.g. CORE mutants; Hauser et al., 1995; see also Nicol and Hofte, 1998). Unlike shortroot, which lacks an endodermis, har1 mutants have the same root cell layers as wild-type plants (Figure 6). In contrast to the CORE mutants, har1 mutations result in a reduction, rather then increase, in root cell radial expansion, and are not associated with a defect in the orientation of root cell growth (Figures 7 and 8). The primary direction of growth of har1-1 mutant root cells remains longitudinal, along the apical–basal axis, and the extent of cell elongation does not appear to be affected by the har1-1 mutation. However, the position at which root cells start to elongate and become vacuolated is significantly altered, being closer to the root tip in the har1-1 mutant than in wild-type roots. This is accompanied by a significant reduction in the size of the area of the cytoplasmically dense cells in har1-1 mutant roots (Figure 8).

Generally, the growth rate of a plant organ is regulated by the combined activity of two linked processes, cell production and cell expansion (Beemster and Baskin, 1998). Kinematic methods have been used recently to show that the root meristem of *Arabidopsis*, defined on the basis of distribution of mitotic events in the root apical region, extends well into the region of rapid cell elongation (Beemster and Baskin, 1998). Assuming a similar situation in *L. japonicus*, the size of such meristematic regions appears to be reduced in har1-1 mutant roots. Therefore, we postulate that the short root phenotype of har1-1 plants...
is likely to be due to a decrease or modification of root meristem activity. One attractive possibility is that the Har1 gene product is a component of the mechanism(s) that control(s) meristematic activity and/or orchestrates(s) cell division and cell differentiation in the root meristem. The reduction of the har1-1 mutant root meristem area, as well as an acropetal displacement of the cell elongation zone, indeed suggest an impairment in these processes, resulting in an inhibition of har1-1 mutant root elongation. The latter may result in the generation of a physiological signal to stimulate lateral development, in a manner similar to the promotion of lateral root formation upon physical removal of the root tip (Steeves and Sussex, 1989), or inhibition of root meristematic activity by a genetic ablation of root cap cells (Tsugeki and Fedoroff, 1999). Increased lateral root development, in turn, may exert an additional negative effect on the elongation of har1-1 mutant roots.

The decreased radial expansion of har1-1 mutant roots may also be a direct consequence of the reduction of har1-1 mutant root elongation. It is generally assumed that once root cells are present in the zone of rapid elongation, they cease radial expansion (Pysh and Benfey, 1998). Thus, the acropetal displacement of the rapid cell elongation zone in har1-1 mutant roots may limit the duration of the period of cell expansion, resulting in an overall smaller diameter of har1-1 mutant roots.

Of course, alternative scenarios can also be envisaged. For example, the observed deregulation of lateral growth may be a primary, rather then secondary effect of the har1-1 mutation. Some of the developmental aberrations observed in har1-1 mutant roots may also be due to independent effects of the har1-1 mutation. In any case, the har1-1 mutation clearly affects root elongation and promotes lateral root initiation. Therefore, it is tempting to speculate that nodule formation represents an alternative type of lateral root growth, and therefore the physiological conditions that promote lateral root formation during nonsymbiotic development in har1-1 mutant roots may be the same as those stimulating nodule formation, while antagonizing autoregulation of nodulation. Thus, the Har1 gene product appears to be an essential component of the regulatory mechanism responsible for maintaining root growth rates and suppressing lateral root and nodule formation.

### Physiological aspects of the har1-1 mutant phenotype

It has been established previously that plant growth regulators affect both root development and nodulation. For example, ethylene is a potent inhibitor of cortical cell division in roots and nodule formation (Hirsch and Fang, 1994; Lee and LaRue, 1992). Ethylene also exerts a strong inhibitory effect on elongation of primary roots, which is usually accompanied by an increase in radial root cell expansion (Jackson et al., 1992). As mentioned above, a recessive mutation in M. truncatula (sickle) results in overall plant ethylene insensitivity, as well as hyperinfection by rhizobia, resulting in a significant increase in the number of root nodules formed (Penmetsa and Cook, 1997). Moreover, Caba et al. (1999) have suggested recently that the sensitivity of nodulation to ethylene might be specifically decreased in soybean supernodulation mutants, although a significant difference in ethylene perception between wild-type and supernodulating mutants on a whole-plant basis was not found. No significant differences in sensitivity to exogenously applied ACC were observed in har1-1 mutant versus wild-type L. japonicus plants (Figure 10). Although a slightly elevated resistance of har1-1 roots to particular concentrations of ACC was observed, this effect was not specific for ACC since similar differences were observed with auxin. Inhibitors of ethylene perception (Ag+) or ethylene synthesis (AVG) were found to have a limited stimulatory effect on elongation of both har1-1 and wild-type roots. In addition, 0.1 μM AVG did not significantly affect root growth, nodulation or the development of the HNR response in inoculated har1-1 mutant plants (data not shown). The latter result suggests that, unlike the tsr phenotype (Zaat et al., 1989; see also above) and the recently described inhibitor growth responses of white clover (Trifolium repens L) roots to the external application of diglycosyl diacylglycerol membrane glycolipids derived from Rhizobium leguminosarum bv. trifolii (Orgambide et al., 1994), the HNR response is ethylene-independent.

Cytokininin appears to stimulate root growth of the dark-grown uninoculated har1-1 mutant plants in an ethylene-independent manner, such that the length of the main har1-1 roots is almost identical to that of the wild-type roots after 10 days of incubation in the presence of 0.05 μM cytokinin and 0.1 μM AVG (Figure 12). Interestingly, light also partially restores the elongation of the har1-1 mutant root (Figure 9). Cytokininin, together with auxin, regulate cell division and differentiation processes (D’Agostino and Kieber, 1999). In addition, cytokinin promotes cell expansion and exerts an inhibitory effect on pericycle cell division (Cary et al., 1995; Coenen and Lomax, 1997). All these functions can be pertinent to the observed stimulatory effect of cytokinin on har1-1 mutant root elongation. In fact, we observed a strong inhibition of pericycle cell divisions in the har1-1 and wild-type roots when incubated in the presence of 0.05 μM cytokinin and 0.1 μM AVG (data not shown).

The Har1 locus has been mapped to the linkage group LG2 of an L. japonicus F2 genetic map derived from a cross between homozygous har1-1 (Gifu) and the polymorphic L. japonicus ecotype B-581 Funakura (data not shown). Molecular cloning and studies on the expression of the Har1 locus in response to applied cytokinin are in progress and will help to uncover its function in the cytokinin-
mediated control of root growth and nodulation in legumes.

Experimental procedures

Plant material and growth conditions

The har-1-1 (Lisym34-1) and har-1-2 (Lisym34-2) mutant alleles were generated by ethylmethanesulfonate (EMS) mutagenesis of L. japonicus seeds (Gifu ecotype), as previously described (Szczyglowski et al., 1998a). The har-1-3 (sym10) mutant allele was isolated from a population of L. japonicus (ecotype Gifu) containing T-DNA insertions, but the T-DNA insertion was found to be unlinked to the har-1-3 mutation (Schauser et al., 1998). L. japonicus seeds were surface sterilized and germinated as described previously (Szczyglowski et al., 1998a). For root and stem growth measurements, 7-day-old axenic seedlings were transferred to pots containing a 6:1 mixture of vermiculite and sand, and grown as described by Krapanj et al. (1997). At given time-points, plants were carefully uprooted, washed, blotted dry, and the masses of the whole root and shoot were determined.

The length of primary roots was determined by direct measurements. The Mesorhizobium loti wild-type strain NZP2235 was used in nodulation experiments, as described previously (Szczyglowski et al., 1997). For microscopical studies, L. japonicus seedlings were grown in nylon ‘pillow systems’, as described previously by Szczyglowski et al. (1998a). For hormone treatments, surface sterilized wild-type and har-1-1 mutant seeds were germinated on filter papers soaked with sterile H2O for 2 days under light, and sown onto 100 cm² agar plates (five seedlings per plate) containing 1/2 strength Gamborg’s B5 basal medium with minimal organics (Sigma), 0.8% phytagel (Sigma), 2.5 mM 2-N-morpholinoethanesulfonic acid (MES) and 4.5% sucrose. The pH of the medium was adjusted to 6.0 with NaOH. The plates were incubated in the dark at 22°C for 10 days, after which time the change in main root length was measured. Root growth was expressed as relative growth (i.e. the ratio of root length in the presence and absence of hormones × 100).

Complementation and genetic segregation analyses

Complementation analyses and dominance tests were conducted by cross-pollinating homozygous plants and analyzing both root and symbiotic phenotypes of the F1 and F2 seedlings. In addition, homozygous har-1-1 (Gifu) plants were crossed with polymorphic L. japonicus B-581 Funakura plants to produce F1 seeds. A mapping population of 242 F2 plants was established. Preliminary linkage analysis was performed using randomly chosen, representative markers from the skeletal F2 map derived from a cross between L. japonicus ecotype B129-S9 Gifu and B-581 Funakura (Jiang and Gresshoff, 1997), utilizing the MAPMAKEr version 1.0 Program.

Microscopic examination of symbiotic development

Primary root infections were examined by brightfield microscopy, using M. loti strain NZP2235 carrying a hemA:JacZ reporter gene fusion. Seven-day-old wild-type and mutant L. japonicus seedlings were inoculated and transferred to the nylon pillow systems. Seven to 11 days after inoculation wild-type and har-1-1 mutant plants were vacuum infiltrated for 15 min with fixative solution (1.25% v/v glutaraldehyde solution buffered with 0.2 M sodium cacodylate pH 7.2), and incubated in the same buffer for an additional 1 h. Fixed specimens were rinsed twice with 0.2 M sodium cacodylate pH 7.2 for 15 min, and stained for β-galactosidase activity using a staining solution that contained 800 μM sodium cacodylate (0.2 M, pH 7.2), 50 μl of 100 mM K2Fe(CN)6, 50 μl of 100 mM K4Fe(CN)6, and 40 μl of 5-bromo-4-chloro-3-indolyl-β-d-galactoside (2%) substrate in N,N-dimethylformamide. After overnight incubation at room temperature, the roots were rinsed successively with 0.2 M sodium cacodylate buffer (pH 7.2; 3 × 10 min), distilled water (2 × 5 min), and cleared following the protocol described by Malamy and Benfey (1997). Specimens were examined by brightfield light microscopy. Color micrographs were digitized and images processed into montages using Adobe Photoshop 5.0 software.

Foci of cortical cell divisions and nodule primordia were examined by brightfield microscopy of whole roots 7–11 days after inoculation with wild-type M. loti, as described previously (Szczyglowski et al., 1998a). Histology and cytology of nodules harvested 21 days after inoculation with M. loti strain NZP2235 were examined by combined light and transmission electron microscopy, as described previously (Szczyglowski et al., 1998a).

Microscopic analysis of non-symbiotic plant development

In order to evaluate their non-symbiotic root phenotype, wild-type and har-1-1 mutant L. japonicus plants were grown in the absence of symbiotic bacteria and in the presence of a low concentration of combined nitrogen (0.5–1.0 mM KN03). Root specimens were examined by brightfield light microscopy to document their morphological and developmental characteristics.

The distribution of events of lateral root development, including pericycle cell divisions, formation of lateral root primordia, and emerged lateral roots, were evaluated using Chlorox-cleared L. japonicus roots as previously described (Szczyglowski et al., 1998a).

For cytological and histological examination of L. japonicus wild-type and har-1-1 mutant roots, 4 mm long root segments were vacuum infiltrated for 2 h in 2.5% glutaraldehyde, 50 mM sodium cacodylate buffer (pH 6.8), rinsed for 30 min in 50 mM sodium cacodylate buffer (pH 6.8; 3 × 15 min) and postfixed for 2 h in 1% osmium tetroxide in the same buffer. After washing in 50 mM sodium cacodylate (pH 6.8; 3 × 15 min) and dH2O (3 × 5 min), the root segments were dehydrated in an acetone series and embedded in Spurr’s epoxy resin. Two μm semi-thin sections were stained with 0.02% toluidine blue and examined by light microscopy. A series of micrographs from longitudinal sections derived from either the meristematic region of the root or a mature portion located 2 cm above the root tip were reconstructed into montages using Adobe Photoshop software. Root cell layers, cell numbers and projected cell areas were measured using micrographs of semi-thin cross-sections taken from the differentiated zone of the root (1 cm above the root tip) by image analysis by Bioquant System IV program (Dazzo and Petersen, 1989). The diameter of the living root was measured by image analysis of a series of micrographs covering a distance up to approximately 1 cm from the root tip. The length of root epidermal cells was measured from digitized videotape micrographs of unstained wet mounts of 5–10 whole roots using CMEIAS ImageTool software (Liu et al. 2000).

The size of the root meristematic region was estimated using intact 10-day-old roots stained with acetocarmine (1% carmine in 45% acetic acid). The projected areas of the red-stained meristematic regions of wild-type and har-1-1 mutant roots were measured from digitized videotape micrographs using CMEIAS/


References


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