Dual Genetic Pathways Controlling Nodule Number in *Medicago truncatula*[1]

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We report the isolation and characterization of a new *Medicago truncatula* hyper-nodulation mutant, designated *sunn* (super numeric nodules). Similar to the previously described ethylene-insensitive mutant *sickle*, *sunn* exhibits a 10-fold increase in the number of nodules within the primary nodulation zone. Despite this general similarity, these two mutants are readily distinguished based on anatomical, genetic, physiological, and molecular criteria. In contrast to *sickle*, where insensitivity to ethylene is thought to be causal to the hyper-nodulation phenotype (R.V. Penmetsa, D.R. Cook [1997] Science 275: 527–530), nodulation in *sunn* is normally sensitive to ethylene. Nevertheless, *sunn* exhibits seedling root growth that is insensitive to ethylene, although other aspects of the ethylene triple response are normal; these observations suggest that hormonal responses might condition the *sunn* phenotype in a manner distinct from *sickle*. The two mutants also differ in the anatomy of the nodulation zone: Successful infection and nodule development in *sunn* occur predominantly opposite xylem poles, similar to wild type. In *sickle*, however, both infection and nodulation occur randomly throughout the circumference of the developing root. Genetic analysis indicates that *sunn* and *sickle* correspond to separate and unlinked loci, whereas the *sunn/skl* double mutant exhibits a novel and additive super-nodulation phenotype. Taken together, these results suggest a working hypothesis wherein *sunn* and *sickle* define distinct genetic pathways, with *skl* regulating the number and distribution of successful infection events, and *sunn* regulating nodule organogenesis.

Legumes form a novel organ on their roots in response to lipooligosaccharide signals, the "Nod factors," delivered by specific soil bacteria called rhizobia. Nodules are colonized by the inciting rhizobia, and ultimately provide a physiological context necessary for symbiotic nitrogen fixation by the bacterium (Crawford et al., 2000). Key aspects of symbiotic metabolism include the supply of energy in the form of carbon, from the plant to the bacterium, and the return of reduced nitrogen in the form of ammonia, from the bacterium to the plant. The benefits of this cross-kingdom collaboration extend beyond the exchange of carbon and nitrogen between the symbiotic partners, to the ecosystem level where the increased abundance of biologically available nitrogen impacts coresident species across several trophic levels. Understanding the nodulation process represents an important objective for plant biologists, with significant implications for both agricultural and natural ecosystems.

Numerous genetic studies establish that the initiation of symbiotic development depends on the perception of Nod factor signals by the plant host. A description of the molecular mechanisms underlying this process is beginning to emerge primarily from studies involving two model legume species, *Medicago truncatula* and *Lotus japonicus*. Numerous nonnodulating mutants have been identified in these model legume systems, and in *M. truncatula*, several such mutants are implicated in transduction of the bacterial lipooligosaccharide signal (Catoira et al., 2000, 2001; Wais et al., 2000). Recently, Endre et al. (2002) demonstrated that the *dmi2* (doesn't make infections) locus of *M. truncatula* is a class I protein kinase. Orthologs of *dmi2* appear to regulate symbiotic development in alfalfa (*Medicago sativa*), pea (*Pisum sativum*; Endre et al., 2002), and *L. japonicus* (Stracke et al., 2002), providing the first functional proof of a common ancestry for nodulation in legumes. The likely complexity of Nod factor signaling is indicated by the ability of various ligand structures to incite common responses with varying efficiencies; a recent demonstration of such Nod factor structure-specific responses is the negative feedback of wild-type Nod factor perception involving the *dmi3* locus of *M. truncatula* (Oldroyd et al., 2002).

In addition to the perception of exogenous signals, it is now apparent that the perception of endogenous

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signals, particularly plant hormones, is important for proper symbiotic development. The best evidence to date involves the role of the plant hormone ethylene. In *M. truncatula*, genetic insensitivity to ethylene is correlated with an increase in the number of successful infections and differentiated nodules (Penmetsa and Cook, 1997). One model suggests that ethylene acts to negatively regulate the persistence of infection by *Sinorhizobium meliloti*. Thus, the numbers of successful bacterial infections are substantially increased in the *sickle* mutant, and chemicals that antagonize or promote ethylene synthesis (e.g. aminoethoxyvinylGly [AVG] and 1-aminocyclopropane carboxylic acid [ACC]) enhance or decrease infection by *S. meliloti*, respectively. Consistent with this model, Heidstra et al. (1997) determined that the distribution of transcripts for ACC oxidase, a committed step in ethylene synthesis, was negatively correlated with the spatial distribution of nodule development. Recently, Oldroyd et al. (2001) demonstrated that the sensitivity of root hair cells to Nod factor is significantly increased in the *sickle* mutant, and that modulation of ethylene synthesis in wild type had comparable effects on the sensitivity of Nod factor perception. It appears, therefore, that the symbiotic phenotypes of altered ethylene perception may arise from an interaction between pathways for perception of Nod factor and ethylene.

There is accumulating evidence that other plant hormones may also have important roles in the process of symbiotic development. In particular, the induction of nodule organogenesis in root cortical cells may be mediated by local changes in the auxin/cytokinin ratio (for review, see Hirsch and Fang, 1994). A role for auxin in nodule organogenesis was first suggested by Thimann (1936), and subsequently supported by physiological rather than genetic studies. For example, the localized application of auxin transport inhibitors to legume roots, thought to disrupt auxin transport and thereby modulate the endogenous auxin to cytokinin balance, induces nodule-like structures in several legumes (Allen et al., 1953; Hirsch et al., 1989; van de Weil et al., 1990). More recently Boot et al. (1999) obtained evidence for a reduction in endogenous auxin transport in response to bacterial Nod factors. Consistent with this finding, Mathesius et al. (1998) observed transient down regulation of the auxin-responsive *GH3:GUS* reporter gene fusion in white clover (*Trifolium repens*) roots acropetal to the site of rhizobial inoculation. Evidence suggestive of a role for cytokinin in nodule organogenesis includes the finding that cytokinin treatment induces *ENOD2* expression in *Sesbania rostrata* (Dehio and deBruijn, 1992) and cortical cell divisions and *ENOD12* induction in alfalfa (Bauer et al., 1996). Moreover, transformation of Nod factor-deficient *S. meliloti* strains with a gene for cytokinin synthesis (i.e. the trans-zeatin secretion gene) confers the ability to induce nodule-like structures on host alfalfa plants (Cooper and Long, 1994). Taken together, these studies suggest that a low ratio of auxin to cytokinin in legume roots may be sufficient to signal the induction of cortical cell divisions, with additional cues being required for the coordination of these cell division foci into organized nodule meristems.

In this study, we describe the analysis of a new hyper-nodulating mutant of *M. truncatula*, designated *sunn*. Genetic, physiological, anatomical, and molecular analyses distinguish *sunn* from the previously described hyper-nodulating mutant *sickle*. Although the nodulation phenotype of *sickle* is associated with altered ethylene perception, nodulation in *sunn* remains normally sensitive to ethylene. Instead, *sunn* exhibits root growth that is insensitive to ethylene. Similar to super-nodulation mutants described in soybean (*Glycine max*) and *L. japonicus* (Carroll et al., 1985; Wopereis et al., 2000), grafting experiments demonstrate that the *sunn* phenotype is determined by the genotype of the shoot, implicating a mobile signal in conditioning nodule number. We suggest a model for the genetic control of nodule number in *M. truncatula*, wherein rhizobial infection and nodule organogenesis are mediated by distinct genetic pathways involving *skl* and *sunn*, respectively.

RESULTS

Isolation of a Novel Super-Nodulation Mutant

Based on a visual screen for altered nodule development, we identified a mutant displaying roughly 70 nodules within the primary nodulation zone (Fig. 1, A and B). This compares with the parental genotype A17, which develops approximately eight nodules per root in response to inoculation with *S. meliloti* ABS7 (Penmetsa and Cook, 1997). We have designated this mutant "*sunn* " (super numeric nodules). Similar to wild-type plants, the nodulation response in *sunn* was transient, with an absence of bacterial infection and nodule development in the region of the root that develops subsequent to initial infection by *S. meliloti*. We used several criteria to assess the status of symbiotic development in *sunn*. In wild-type plants, the occurrence of leghemoglobin transcript serves as a molecular marker for the transition from nodule development to nodule function. As shown in Figure 1D, leghemoglobin transcript was readily detected in 23-d-old *sunn* nodules, although transcript levels were not increased in proportion to the increased mass of nodule tissue. Nevertheless, cytological analysis of *sunn* nodules indicated full differentiation of both symbionts. In particular, bacteriods, which represent the fully differentiated state of the bacterium, were abundant in the majority of cells within the nodule central tissue, surrounded by uninfected host cells as is typical of wild-type nodules (Fig. 1C). As an alternate measure of the differentiation of both partners, we compared

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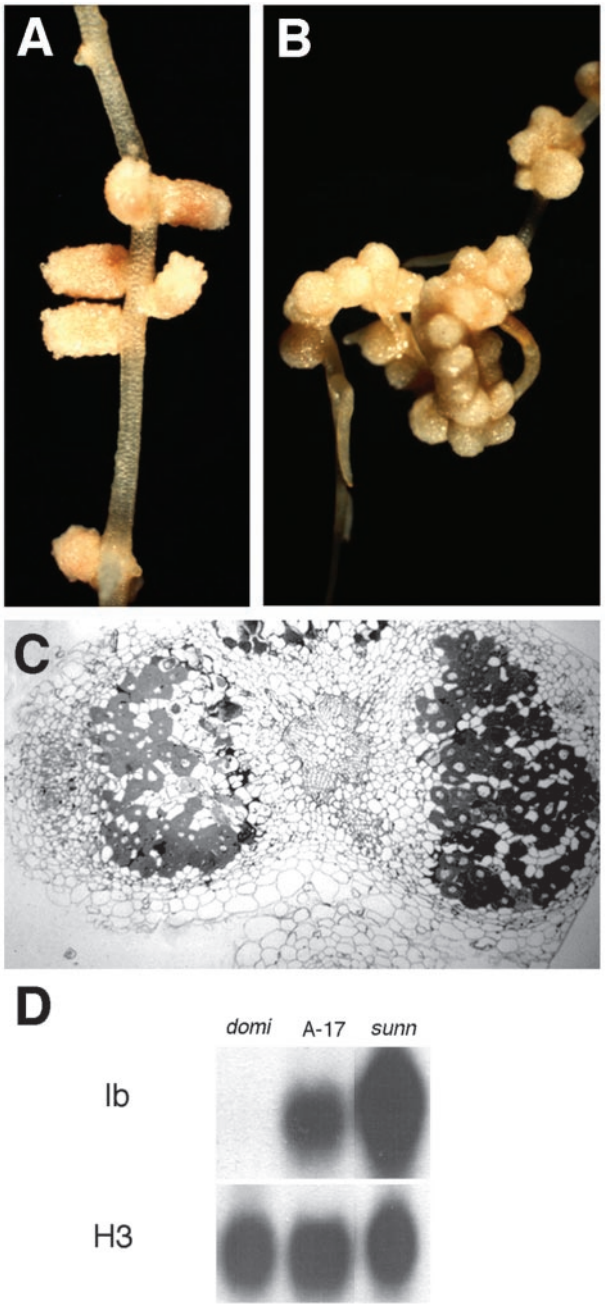

Figure 1. Infection and nodulation phenotypes of wild-type *M. truncatula* and mutant *sunn*. Light micrographs of primary nodulation zones from wild-type (A) and *sunn* (B) roots. C, Bright-field micrograph of a transverse section of 21-d-old *sunn* nodules from the primary nodulation zone. X, Root xylem tissue; C, nodule central tissue; E, nodule endodermis; M, nodule meristem. D, Leghemoglobin expression in wild type and mutant *sunn*. Lanes contain total RNA from roots 23 d after inoculation with *S. meliloti*. Comparable root tissues were assayed for nitrogenase activity (Table I) and tissue differentiation (C). lb, Leghemoglobin; H3, histone expression.

nitrogenase activity in *sunn* and wild type based on the ability of excised nodulation zones to reduce acetylene to ethylene. On a per root basis, *sunn* possessed similar nitrogenase activity as observed in wild type (Table I). The absence of a correspondence between nitrogenase activity and nodule number in *sunn* is consistent with previously described hypernodulating mutants in both *M. truncatula* (Penmetsa and Cook, 1997) and in other legumes (Carroll et al., 1985; Wopereis et al., 2000).

In both the soybean *Nts* mutant (Mathews et al., 1990) and the *L. japonicus har1* mutant (*Wopereis* et al., 2000), the super-nodulation phenotype is controlled by the genotype of the shoot. To determine if the *sunn* hyper-nodulation phenotype might also be shoot genotype controlled, we used a simple grafting protocol for *M. truncatula* to construct chimeric plants with opposing root and shoot genotypes. As shown in Table II, the nodulation phenotype of the shoot was dominant, with *sunn* shoots conferring excessive nodulation on wild-type root systems, and wild-type shoots conferring reduced nodule numbers on *sunn* root systems. Despite the similarity of increased nodule number and shoot control over nodulation that are common to *sunn* and the *Har1* mutant of *L. japonicus*, the phenotype of *sunn* is distinct from that of *Har1*. Although both mutants exhibit retarded root growth in the absence of rhizobia, the short root of the *sunn* mutant is not accompanied by increased lateral root number that has been observed in *Har1*. Moreover, whereas the *Har1* mutant exhibits a pronounced retardation of root growth after rhizobial inoculation, root growth in *sunn* continues largely unabated after inoculation (J.A. Frugoli, unpublished data).

Genetic Analysis of *sunn* and Comparison with the Previously Described Nodulation Mutant Sickle

To determine the genetic nature of the *sunn* mutation, pollen from wild-type Jemalong (A17) was used in crosses with homozygous *sunn* individuals. F_2 seedlings (288) were scored for nodule number at 7 to 10 d subsequent to inoculation with *S. meliloti*, revealing 77 plants that contained an estimated >70 nodules/plant. The remaining 211 individuals were either wild type or had intermediate numbers of nodules; thus, whereas wild-type plants consistently produce a range of three to 12 nodules per plant, with an average of 7.6 ± 1.5, the F_2 population displayed an average of 11.4 ± 5.9 nodules (excluding the strong *sunn* phenotype of >50 nodules per plant) with a range of three to 28 nodules. The phenotypic

Table I. *Nitrogenase activity in select M. truncatula genotypes determined by the acetylene reduction assay*

Genotype	Acetylene Reduction, Mean + SE
	pmol ethylene min^{-1} root^{-1}
A17 (wild type)	233 ± 54
sunn	374 ± 145
skl	200 ± 87
Double mutant	265 ± 32

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Table II. *Nodulation in grafted plants of sunn and wild type, 12 to 17 d postinoculation*

Shoot Genotype	Root Genotype	No. of Plants	No. of Nodules
A17 (wt)	A17	6	18 ± 5
A17	*sunn*	11	18 ± 7
sunn	A17	5	45 ± 11
sunn	*sunn*	7	50 ± 12

ratio of 77:211 is consistent with a single gene ($\chi^2 = 0.463$, $P > 0.475$), whereas the observations that nodule number is significantly increased in a portion of F_2 individuals and in F_1 heterozygotes derived from the *sunn* mutant (data not shown), are suggestive of a semidominant mutation at the *sunn* locus.

To test if the *sunn* mutation was allelic to the previously described hyper-nodulation mutant *sickle*, we crossed the two mutants and examined phenotypes in F_1 and F_2 populations. F_1 individuals possessed an intermediate number of nodules similar to the inferred *sunn* F_2 heterozygotes described above, suggesting that these mutants were not allelic. This conclusion was supported by the occurrence of wild-type and both hyper-nodulation phenotypes in F_2 populations, by extensive test cross analysis (see below), and by genetic mapping of the two mutants to different linkage groups (H.K. Choi and D.R. Cook, unpublished data).

Nodule Position Is Different between *sunn* and *sickle*

In pea, nodule organogenesis typically initiates in the root cortex across the protoxylem poles (Libbenga et al., 1973; Heidstra et al., 1997). Based on visual inspection, nodules in *sunn* appear to be arranged in parallel files along the length of the root (Fig. 1B). This phenotype contrasts with the *sickle* mutant, where the nodulated root zone expands radially and discrete nodule foci are not evident until several weeks after infection. To further characterize the spatial pattern of nodulation in these two mutants, we prepared transverse sections of entire nodulation zones from seedlings 4 to 6 d after rhizobial inoculation and examined the position of nodule development relative to subtending protoxylem poles and phloem tissue. Scoring of early nodule development was aided by using an *S. meliloti* ABS7 strain that expresses *lacZ* and, therefore, stains blue upon treatment with the X-Gal substrate. In both the wild-type and *sunn* genotypes, the majority of nodules (approximately 80%) developed within a 30° arc centered on the protoxylem poles (Table III). In contrast, this spatial restriction was absent in *sickle*, where nodules were developed with approximately equal frequencies throughout all portions of the root (Table III). In fact, in many cases, scoring nodule position in *sickle* was not possible because single infection events often ramified throughout the entire circumference of the root cortex. Thus, the two super-nodulating mutants exhibit distinct nodule zone anatomies, with nodule position in *sunn*, but not *sickle*, spatially restricted to sites across protoxylem poles.

Marker Gene Expression Distinguishes Super-Nodulators from Wild Type, and *sunn* from *sickle*

In an effort to determine whether the hyper-nodulating mutants (i.e. *sunn* and *sickle*) could be distinguished from one another and either mutant from wild type on the basis of their molecular phenotypes, we examined the expression of four genes in *S. meliloti*-inoculated roots of *sunn*, *sickle*, and wild type. Of the two nodulin genes we examined, expression of the *S. meliloti*-induced peroxidase homolog *RIP1* is correlated with early responses in the root epidermis and at sites of infection (Cook et al., 1995; Peng et al., 1996), whereas the expression of *ENOD40* is correlated with nodule organogenesis (Yang et al., 1993; Charon et al., 1997). As shown in Figure 2, there was close correspondence in the onset of *RIP1* induction between wild type and both hyper-nodulation mutants, with increases in transcript first evident at 12 h. However, the transience of *RIP1* expression, which is characteristic of wild-type plants (see Fig. 2; Cook et al., 1995), was absent from either mutant. In the case of *ENOD40*, increased transcript levels were first evident between 12 and 24 h for each of the genotypes, although in contrast to wild type, the *ENOD40* transcript continued to increase in both mutants. The expression of these early nodulin genes was most enhanced in the *sickle* background, with transcript levels increasing earlier and generally to a higher level. The observation that *sickle* plants display a significant increase in the extent of the root inner cortex involved in symbiotic development (see above) compared with both wild type and *sunn* may

Table III. *Frequency distribution of nodule development in relation to the root vascular tissues in wild type, sunn, sickle, and the sunn/skl double mutant*

Also see Figure 5.

Genotype	No. of Nodules Analyzed	Nodules across Pole of		% of Nodules across Xylem Pole
		Xylem	Phloem	
A17 (wild type)	32	26	6	81.25
sunn	*56*	43	13	76.79
sickle	*58*	31	27	56.34
Double mutant	80	45	35	56.25

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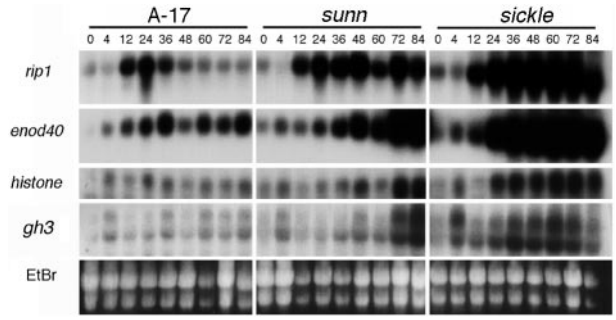

Figure 2. Symbiotic gene expression in wild-type, *sunn*, and *sickle* genotypes. Gel blots were prepared from total RNA isolated from *S. meliloti*-inoculated roots at the indicated times (hours postinoculation). Blots were hybridized sequentially with radiolabeled *rip1*, *enod40*, *histone H3*, and *gh3* probes. EtBr, Image of ethidium bromide-stained gel of the RNA samples before transfer to membrane and hybridization.

explain the increased abundance of nodulation transcripts.

Nodule organogenesis involves the reactivation of the cell cycle in the root cortex, including both active cell division in inner cortical cells and S-phase activation without cell division in outer cortical cells. As a measure of the timing and extent of cell cycle activation, we analyzed the abundance of histone H3 transcript. In wild-type roots, the level of histone *H3* transcript appeared to be slightly elevated after inoculation with *S. meliloti*, but in general, expression was low and varied little over the time course of the experiment (Fig. 2). We assume that the inability to detect significant increases in histone H3 expression in wild-type inoculated roots is a consequence of dilution by the much larger portion of the root not involved in symbiotic development. In contrast, both *sunn* and *sickle* exhibited increased levels of H3 transcript between 24 and 72 h after inoculation with *S. meliloti*, with significantly earlier induction evident in *sickle* compared with *sunn*.

Mathesius et al. (1998, 2000b) used the auxin-responsive *GH3:GUS* fusion from soybean (Gee et al., 1991; Hagen et al., 1991) to infer changes in auxin activity during nodulation of white clover by *Rhizobium leguminosarum*. In particular, their results suggest that *GH3* expression is a useful marker for cortical cell activation during the formation of nodule primordia. To investigate whether the expression *GH3* was also correlated with nodule development in *M. truncatula*, we examined the expression of a *GH3* sequence homolog in *M. truncatula* in inoculated roots of wild-type, *sunn*, and *sickle* genotypes. The Institute for Genomic Research gene index for *M. truncatula* (http://www.tigr.org/tdb/mtgi) suggests that *GH3* genes comprise a small gene family in *M. truncatula*; thus, RNA blots were hybridized and washed at moderate stringency to allow detection of multiple transcripts with similarity to Gm*GH3*. As shown in Figure 2, this strategy revealed two distinct messages with differing electrophoretic mobilities. In wild-type roots, transcript levels were low and only slightly increased after inoculation with *S. meliloti*. In contrast, significant increases in *GH3* transcripts were observed in both *sunn* and *sickle*, with a time course essentially identical to that observed with histone H3. These results are consistent with the results of Mathesius et al. (1998, 2000b), indicating a temporal relationship between induction of *GH3* and cell cycle activation.

Responsiveness to Ethylene Differs between *sunn* and Wild Type

Previous analyses suggest that the plant hormone ethylene plays a central role in the regulation of the number of successful infections in *M. truncatula* (Penmetsa and Cook, 1997; Oldroyd et al., 2001). To determine whether the *sunn* mutant might be perturbed in ethylene responses, hypocotyl and root length of wild-type and *sunn* seedlings were measured in the presence of various concentrations of ACC or pure ethylene. ACC is a biosynthetic precursor that is readily converted to ethylene by endogenous ACC oxidase activity. In the presence of ACC or ethylene, *sunn* plants displayed most aspects of the "triple response" in a dose-dependent manner. For example, a reduction of hypocotyl length in both wild type and *sunn* was correlated with increasing doses of ACC and ethylene (Fig. 3, A and B, respectively). In contrast, *sunn* root growth was significantly less sensitive than wild-type roots to ACC or ethylene (Fig. 3,

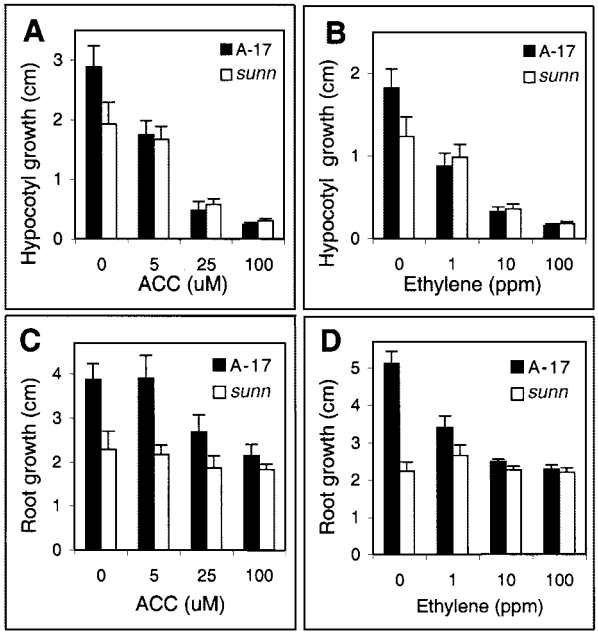

Figure 3. Sensitivity of wild-type and *sunn* seedlings to exogenous ACC and ethylene gas. Hypocotyl growth response of wild-type (solid bars) and *sunn* (white bars) seedlings to exogenous ACC (A) and to ethylene gas (B). Root growth response of wild-type (solid bars) and *sunn* (white bars) seedlings to exogenous ACC (C) and to ethylene gas (D).

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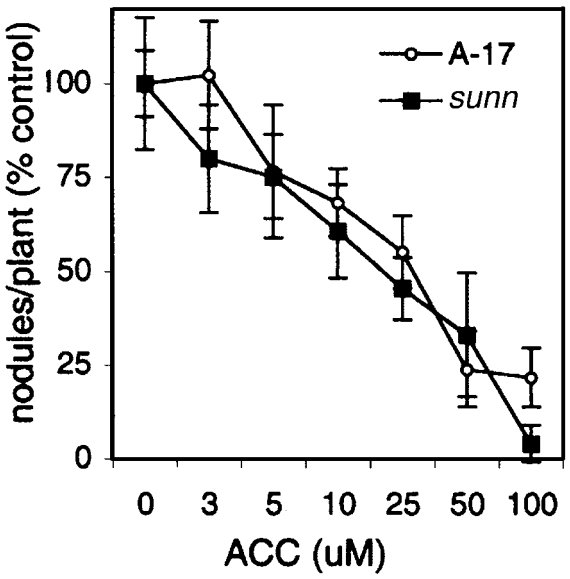

Figure 4. Effect of ACC on nodulation in wild-type and *sunn* genotypes of *M. truncatula*. Seedlings were grown in growth pouches and ACC applied at 48 h after inoculation with *S. meliloti*, a time previously determined to provide maximal inhibition. Nodule numbers at different concentrations of ACC are presented as percentage of response relative to untreated plants. Nodule numbers of pouch grown seedlings in the absence of ACC were 7.6 ± 0.7 in wild type and 23.6 ± 4.2 in *sunn*.

C and D). Other components of the ethylene response syndrome in *sunn* were similar to wild type, including increased root diameter and increased root hair density (data not shown), suggesting that with the exception of elongation, roots of *sunn* are not generally insensitive to ethylene.

To determine whether the root growth insensitivity to the ethylene phenotype of *sunn* might have a direct role in the increased nodule number phenotype, we analyzed the nodulation response of *sunn* in the presence of increasing concentrations of ACC. As shown in Figure 4, ACC treatment reduced nodulation in a dose-dependent manner, with similar sensitivities in both *sunn* and wild type (Fig. 4). Thus, the nodulation response of *sunn* to exogenous ACC is indistinguishable from that in wild type. Taken together, these results suggest that the altered ethylene response of *sunn* roots is not causal to the hypernodulation phenotype, and serve to further distinguish *sunn* from the *sickle* mutant, where ethylene insensitivity is pleiotropic for many aspects of plant development, including the nodulation response (Penmetsa and Cook, 1997).

Genetic Interaction between *sunn* and *sickle*

To test if the *sunn* and *sickle* genes function as part of a single genetic pathway or act in independent genetic pathways, the genetic interaction between these loci was examined by crossing *sickle* into *sunn*. The nodulation phenotypes of F_1 individuals were similar to previously characterized *sunn* heterozygotes, suggesting that these mutants were not allelic. This inference was further verified by the segregation of wild-type nodulation in the F_2 generation and the corresponding F_3 progeny. Four phenotypic classes were scored in F_2 populations: wild type (including putative *sunn* heterozygotes), *sunn*, *sickle*, and a novel super-nodulation phenotype corresponding to the presumptive double mutant (see below), in a ratio of 186:53:47:12. Putative *sunn* heterozygotes were pooled with the "wild-type" class, because the range of the number of nodules on presumptive heterozygotes significantly overlaps with bona fide wild-type individuals (see above), complicating precise phenotypic assignment. The observed ratio of F_2 pheno-

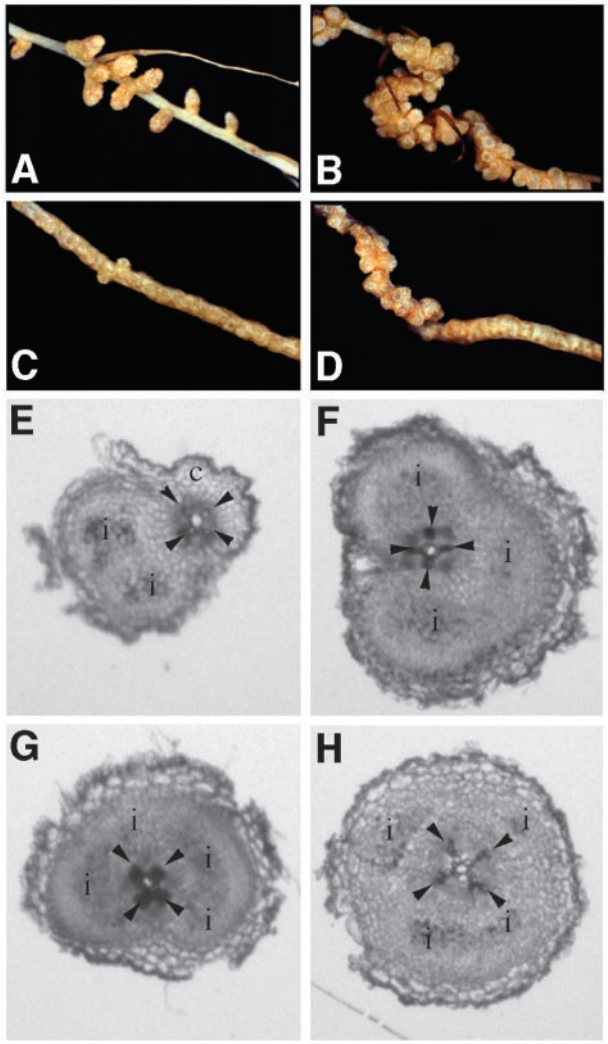

Figure 5. Distribution of rhizobial infections and nodules in wild type, *sunn*, *sickle*, and the *sunn/skl* double mutant. Typical macroscopic nodulation zones of wild type (A), *sunn* (B), *sickle* (C), and the *sunn/skl* double mutant (D), and transverse sections through the nodulation zone of wild type (E), *sunn* (F), *sickle* (G), and the *sunn/skl* double mutant (H) are shown. c, Root cortex; i, rhizobial infection. Arrowheads mark position of xylem poles.

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types most closely approximates 9:3:3:1 ($\chi^2 = 5.9$, $P > 0.1$), suggesting interaction between the two homozygous loci. Testing F_2 phenotypic ratios for gene action models of either *sunn* epistatic to *sickle* or *sickle* epistatic to *sunn* (i.e. 9:3:4) led to a rejection of both hypotheses ($P \ll 0.01$; data not shown).

Progeny testing and test cross analysis of selected individuals representing F_2 phenotypes allowed us to subsequently confirm the inferred F_2 genotypes. For example, of 23 independent F_3 populations derived from F_2s with the *sunn* nodulation phenotype, approximately two-thirds (16 of 23) of the F_3 populations segregated for the *sunn* and the double-mutant phenotype. The remaining one-third (seven of 23) contained only *sunn* homozygous phenotypes. Chi-square analysis of the proportions of lines segregating for *sunn* and double mutant phenotypes (16 of 23 lines) or containing only *sunn* phenotypes (seven of 23 lines) was consistent with the expected 2:1 ratio ($\chi^2 = 0.087$, $P > 0.750$). Similarly, F_3 populations derived from multiple F_2 individuals with the novel super-nodulation phenotype bred true, yielding exclusively individuals with the double-mutant phenotype in the F_3. To directly verify the genotype of the inferred double mutant, we performed test crosses to homozygotes of the parental *sunn* and *sickle* super-nodulation loci. To eliminate confounding effects produced by seed set from self-pollination in these crosses, we took advantage of a *M. truncatula* male-sterile, female-fertile floral homeotic mutant *tap*, which in itself does not affect nodulation (Penmetsa and Cook, 2000). The *tap* mutation was moved into both *sunn* and *sickle* backgrounds to obtain male-sterile, female-fertile individuals to serve as pollen recipients. All F_1 progeny obtained using pollen from the double mutant in test crosses to either *tap/sunn* or *tap/skl* pollen recipients exhibited phenotypes of the corresponding single super-nodulation locus, thereby confirming the genotype of the double mutant. Taken together, these genetic data unambiguously establish that the novel super-nodulation phenotype corresponds to that of the *sunn/skl* double mutant.

As shown in Figure 5, the phenotype of the double mutant (Fig. 5D) was a novel super-nodulation phenotype with aspects of phenotypes observed in the single super-nodulation mutants (Fig. 5, B and C). In particular, nodules at the proximal end (toward the hypocotyl) of the nodulation zone were well developed and discrete, similar to nodules of *sunn* homozygotes at a comparable age, whereas nodule development at the distal end (toward the root tip) was characterized by radial expansion of the root without discrete nodule foci, reminiscent of nodule development observed in similarly aged *sickle* plants. In contrast to either of the single mutants or wild type, root growth was arrested subsequent to nodulation in the double mutant. We scored the position of rhizobial infection and inner cortical cell division in the double mutant and determined that, like *sickle*, nodule foci occurred throughout the circumference of the root (Fig. 5, G and H; Table II). Moreover, nitrogenase activity in nodules of the double mutants, as measured by the acetylene reduction assay, was similar to that observed in wild type and the single parental mutants (Table I).

DISCUSSION

Based on a visual screen for nodulation mutants in *M. truncatula*, we identified a novel hyper-nodulation mutant, designated *sunn*. Similar to the previously reported hyper-nodulation mutant *sickle*, *sunn* is characterized by an approximately 10-fold increase in nodule number. Despite this similarity, *sunn* and *sickle* exhibit distinct symbiotic, genetic, anatomical, molecular, and physiological properties that readily distinguish them from one another. In addition to the genetic separation of the mutant alleles based on complementation analysis and genetic mapping (data not shown), the *sunn/skl* double mutant exhibits a novel hyper-nodulation phenotype. The additive nature of the double mutant phenotype suggests that the *sickle* and *sunn* genes may act in separate genetic pathways, underscoring the likely complexity of host control of symbiotic establishment.

Using a set of marker genes as probes in northern-blot analysis, we profiled gene expression in *S. meliloti*-inoculated roots of *sunn*, *sickle*, and wild type. In the case of the early nodulin genes, *RIP1* and *ENOD40*, transcript levels were significantly elevated in both mutants, with the highest transcript levels observed in *sickle*, although the timing of the onset of gene expression was not changed. Histone transcripts increase specifically during S phase of the eukaryotic cell cycle (e.g. Meskiene et al., 1995) and have been used previously as a specific marker of S phase during early nodulation (Yang et al., 1994). In the current study, we observed that histone H3 transcript was induced to high levels earlier in *sickle* than in *sunn* (see Fig. 2). This result suggests that S phase is activated precociously in *sickle*. S-phase markers have been correlated with infection thread formation during nodulation, where cells enter the cell cycle but arrest in G2 (Yang et al., 1994), and should also be expressed in association with actively dividing cells in the nodule primordium and nodule meristem. Both bacterial infection and inner cortical cell division occur in near coincidence throughout the circumference of the root in *sickle*; thus, it is likely that both contribute to the observed increase in histone H3 transcript. Increased infection and inner cortical cell division in *sickle* compared with *sunn* may also explain the generally higher levels of *RIP1* and *ENOD40* transcription in *sickle*. In contrast to *sickle*, histone H3 transcription was not substantially elevated in *sunn* until 48 to 72 h postinoculation. This timing is consistent with the appearance of abundant

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infection and the onset of inner cortical divisions in wild-type plants, suggesting that the timing of symbiotic development in *sunn* is largely similar to wild type. In the case of wild-type plants, the relatively small amount of root involved in symbiotic development (compared with the hyper-nodulating mutants) is likely to obscure our ability to observe increased histone H3 transcript, especially in RNA prepared from whole roots as was the case here.

Altered levels of *ENOD40* have been correlated positively with changes in nodule number in *M. truncatula*; in particular, accelerated nodulation was observed in lines with elevated levels of *ENOD40* and decreased nodulation was associated with lowered *ENOD40* transcript abundance (Charon et al., 1997, 1999). Genetic mapping of *sunn* and *sickle* relative to *ENOD40* demonstrates that *ENOD40* is genetically distinct from either mutant locus (H.K. Choi and D.R. Cook, unpublished data). Moreover, although we cannot formally rule out the possibility that increased expression of *ENOD40* is causal to the hyper-nodulation phenotypes of either mutant, the similar increases in *ENOD40* and *RIP1* expression suggests that both genes are downstream of the causal events.

In pea, nodule organogenesis typically initiates in the root cortex across the protoxylem poles (Libbenga et al., 1973). Two lines of evidence suggest that ethylene might provide a spatial cue to limit the occurrence of infection across phloem poles in the pea system (Heidstra et al., 1997). First, transcripts for ACC oxidase are highly localized to cells opposite phloem poles in pea roots, with the resulting production of the poorly diffusible ethylene gas presumed to establish a gradient of ethylene opposite phloem poles. Second, treatment of pea roots with either Ag^{+} or AVG, inhibitors of ethylene perception and synthesis, respectively, was shown to substantially increase the occurrence of nodulation across phloem poles. Based on these results in pea, we analyzed the position of nodule development in wild-type *M. truncatula*, and in the *sickle* and *sunn* mutants. We determined that although nodulation occurred preferentially opposite xylem poles in wild type, there was a complete loss of spatial control in the *sickle* mutant. The fact that *sickle* is a strong ethylene-insensitive mutant suggests that pea and *M. truncatula* possess similar mechanisms to regulate nodule position, and that both require ethylene perception. In contrast to *sickle*, we observed that nodulation in *sunn* was restricted primarily to locations opposite xylem poles. In fact, nodules in *sunn* were often evident as parallel files along the long axis of the root, and spatial constraints due to nodule expansion are presumed to cause the characteristic distortion-induced twist in the nodulation zone of *sunn* (see Fig. 1).

The conclusion that ethylene insensitivity is causal to the hyper-nodulation phenotype of *sickle* (Penmetsa and Cook, 1997) provided the rationale to examine ethylene responses in *sunn*. We determined that, unlike *sickle*, nodulation in *sunn* was efficiently suppressed by ACC, with a dose sensitivity indistinguishable from wild-type plants (Fig. 4). Most aspects of seedling responses to ethylene were normal in *sunn*, including stem responses such as exaggeration of the apical hook and reduced hypocotyl growth, and root responses such as radial enlargement and ectopic root hair production. These data suggest that hyper-nodulation in *sunn* is unlikely to be mediated by a defect in ethylene perception, which is consistent with the observation that spatial control of nodulation is not affected in *sunn* (as described above). However, *sunn* root growth was insensitive to concentrations of ACC or ethylene that were inhibitory to root growth in wild-type plants.

The Arabidopsis *eir1/agr1* mutants, which affect the putative auxin efflux carrier *PIN2*, also display an ethylene-insensitive root phenotype (Luschnig et al., 1998; see also Chen et al., 1998). Several physiological studies have suggested that lowering the ratio of auxin to cytokinin in the root serves as a cue for the activation of nodule organogenesis (Hirsch and Fang, 1994). Experimental manipulations that lower this ratio either by increasing the level of cytokinin (Cooper and Long, 1994) or decreasing auxin concentrations from the application of auxin transport inhibitors (Hirsch et al., 1989) result in the development of nodule-like structures on legume roots. Whether *sunn* might exhibit defects in sensitivity to effectors of auxin action and transport, or be altered in auxin transport directly, is a topic under investigation. However, our determination that the *sunn* super-nodulation phenotype is influenced by the genotype of the shoot suggests that a mobile shoot-derived signal (potentially auxin?) is altered in the *sunn* genotype.

Indirect evidence that a reduction in root auxin transport precedes nodulation in white clover was provided by studies of an auxin-responsive *GH3:GUS* gene fusion (Hagen et al., 1984; Mathesius et al., 1998; Boot et al., 1999; Li et al., 1999). Inoculation with *R. leguminosarum* was correlated with an initial down-regulation of *GH3:GUS* expression in the root zone acropetal to the point of inoculation, with a subsequent up-regulation at the point of inoculation during formation of nodule primordia (Mathesius et al., 1998). Application of auxin transport inhibitors resulted in a similar down-regulation of the *GH3:GUS* reporter gene as obtained with *R. leguminosarum*, suggesting that the changes in gene expression might be mediated by *R. leguminosarum*-induced alteration of auxin transport. Flavonoids have been suggested to act as negative regulators of auxin transport in planta (Jacobs and Rubery, 1988; Brown et al., 2001; Peer et al., 2001), and rhizobial inoculation is associated with increased gene expression and flux through the phenyl-propanoid biosynthetic pathway (Recourt et al., 1992; Lawson et al., 1994,

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1996), providing a potential mechanism to integrate perception of the rhizobial partner with nodule organogenesis. In the current study, we observed increased expression, in both *sickle* and *sunn*, of transcripts with homology to the soybean *GH3* gene. Altered expression was correlated with changes in histone H3, with earlier induction in *sickle* than *sunn*, presumably reflecting cell cycle activation during the nodulation response. Mathesius et al. (1998) observed increased *GH3:GUS* expression in nascent nodule primordia in white clover, establishing a spatial and temporal correlation between *GH3* expression and the initial activation of the cell cycle. Moreover, auxin and cytokinin are implicated (probably in concert) in regulating the G1 to S transition (den Boer and Murray, 2000). Thus, the finding that increases in GH3 transcripts are tightly correlated with increases in histone H3 transcripts is not surprising, although our current results are inadequate to distinguish whether GH3 induction preceded histone H3 accumulation. One might speculate that a rapid auxin response in *sickle* is causal to the apparently precocious entry into S phase (indicated by histone H3 expression).

The recent positional cloning of genes underlying the super-nodulating *Nts* mutant of soybean (Searle et al., 2002), and its candidate orthologous mutants *Har1* of *L. japonicus* and *Sym29* of pea (Krusell et al., 2002; Nishimura et al., 2002), suggest commonalities between the regulation of apical meristem proliferation and the regulation of nodule number in legumes. In particular, the *Nts*, *Har1*, and *Sym29* genes encode receptor kinases most similar to the *Clavata1* receptor kinase of Arabidopsis, providing additional evidence of an overlap between symbiotic functions and the control of plant growth and development (Szczyglowski and Amyot, 2003). If *sunn* is orthologous to the *Nts* family, then the phenotypic differences between *sunn* and *Har1* may reflect differences in the species context or correspond to allele-specific effects; alternatively, *sunn* could encode an unrelated protein.

In this study, we describe a new *M. truncatula* super-nodulation mutant, designated *sunn*. Although root growth in *sunn* is insensitive to exogenous ethylene or ACC, nodulation in *sunn* remains sensitive to these treatments, suggesting that altered ethylene perception is not causal to the *sunn* phenotype. Together with the ethylene-insensitive mutant *sickle*, this study suggests that the control of nodule number in *M. truncatula* occurs by two genetic pathways, with the *sickle* pathway mediated by perception of the pant hormone ethylene (Penmetsa and Cook, 1997). Whether *sunn* might have altered auxin phenotypes similar to certain ethylene-insensitive root mutants in Arabidopsis (Luschnig et al., 1998) is a topic under active investigation in our laboratories. The discovery that these mutants display both symbiotic and nonsymbiotic phenotypes provides a valuable opportunity to dissect the overlap between symbiotic development and hormonal mechanisms regulating general plant growth and development.

MATERIALS AND METHODS

Mutant Screen

M_2 generation plants derived from two 0.15% (v/v) ethyl methanesulfonate-treated seed bulks of *Medicago truncatula* genotype A17 (bulks C and B; Penmetsa and Cook, 2000) were screened for nodulation mutants. Procedures for plant growth and germination were as previously reported (Cook et al., 1995; Penmetsa and Cook, 2000). In brief, seedlings were grown in aeroponic chambers for 5 d before inoculation with 5 mL of washed inoculum from an overnight culture of compatible *Sinorhizobium meliloti* strain ABS7M or ABS7M containing pXLGD4 (Leong et al., 1985). Seven to 10 d subsequent to inoculation, at which time nitrogen-fixing nodules could be observed on wild-type control plants, plantlets were visually assessed for gross changes in nodule number or morphology. Individuals scored as having altered nodulation properties were further examined with a dissecting microscope (model SZH10, Olympus, Tokyo) to provide a more precise description of the corresponding phenotype. Heritability of M_2 phenotypes was initially determined by examining nodulation phenotypes in multiple M_3 siblings for each putative mutant. Lines containing heritable phenotypes were used as pollen donors in crosses to a male-sterile mutant of *M. truncatula* genotype A17, previously described as a tool for efficient genetic analysis (Penmetsa and Cook, 2000).

Histochemical Staining for Analysis of *S. meliloti* Infections

Histochemical staining for *lacZ* activity was performed essentially as reported by Boivin et al. (1990). In brief, whole roots were vacuum infiltrated with 2.5% (v/v) glutaraldehyde in 0.1 M PIPES (pH 7.2) buffer and incubated for 1 h at room temperature before rinsing twice (15 min each rinse) in 0.1 M PIPES (pH 7.2). To visualize bacterial strains expressing *lacZ*, samples were incubated in staining buffer (50 mM potassium ferricyanide, 50 mM potassium ferrocyanide, and 0.08% [w/v] X-Gal in 0.1 M PIPES [pH 7.2] buffer) for 16 h in the dark at room temperature, rinsed twice in PIPES buffer, and stored at 4°C until further analysis. For microscopy, specimens were rinsed in deionized water, mounted on slides with coverslips, and viewed under an Olympus SZH10 dissection microscope and/or an Axioskop compound microscope (Zeiss, Jena, Germany). Transverse sections of the nodulation zone were obtained by embedding 0.5- to 1.0-cm root segments in 3.5% (w/v) washed agar dissolved in 0.1 M PIPES (pH 7.2). Embedded agar blocks were trimmed by hand and sections of 75 to 100 μm were obtained with a microslicer (Ted Pella, Inc., Redding, CA).

Light Micrographs of Nodules

Nodulation zones were dissected from aeroponically grown seedlings and dehydrated by immersion for 10 min each in a 0% to 100% (v/v) water:ethanol gradient, with a 10% increase in ethanol concentration per step followed by two cycles in 100% ethanol. Ethanol was replaced with LR White acrylic resin by immersion for 1 h each in 2:1, 1:1, and 1:2 (v/v) mixtures of ethanol:resin, followed by immersion in pure resin. Resin was replaced several times to obtain a total infiltration time in pure resin of more than 24 h, before polymerization at 55°C for 24 h. Transverse sections of nodules were obtained by semithin (1 μm) sectioning using an ultracut microtome (Leica Microsystems, Wetzlar, Germany). Semithin sections were stained with basic fuchsin and observed by bright-field optics.

Acetylene Reduction Assay

Nitrogenase activity was assayed on entire nodulation zones excised from seedlings at 16 d postinoculation. Excised tissue was blotted on filter paper and the nodulation zones from three seedlings were sealed in a 1.8-mL serum-stoppered glass vial. One hundred microliters of air was replaced with 100 μL of pure acetylene and after 60 to 120 min of incubation,

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the conversion of acetylene to ethylene was measured by gas chromatography using a Photovac 10S (Photovac Inc., Markham, ON, Canada) equipped with a flame-ionizing detector. Ethylene was quantified by comparison to a 5-ppm ethylene standard, and the values were normalized for injection volumes and incubation times. Ethylene production in the absence of acetylene was typically at or below the detection limit of this assay.

Northern-Blot Analysis

Northern-blot analysis was performed on RNA extracted from whole roots essentially as described by Cook et al. (1995), with the exception that total RNA was extracted using the RNeasy Plant kits (Qiagen USA, Valencia, CA) according to the manufacturer's instructions. General nucleic acid manipulations, including gel electrophoresis and transfer of RNA to nylon membranes, were performed as described by Sambrook and Russell (2001). DNA probes were obtained either as cloned plasmid DNAs, or by PCR amplification from genomic templates using gene-specific primers designed from characterized genes. The accession numbers for the respective sequences are as follows: *RIP1* (U1627), *ENOD40* (X80262). Radioactive probes were prepared with α-^{32}P-dCTP using the procedure of Feinberg and Vogelstein (1984). Hybridization was conducted at 60°C in a solution of 7% (w/v) SDS, 0.25 M NaH_2PO4 (pH 7.0), and 0.1 mM EDTA. After hybridization, filters were washed successively with solutions of 0.1% (w/v) SDS and 2× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate; 21°C for 15 min), 0.1% (w/v) SDS and 0.1× SSC (21°C for 15 min), and 0.1% (w/v) SDS and 0.1× SSC (65°C for 1 h).

Phytohormone Assays of Seedlings

Assays for ethylene sensitivity were performed in magenta boxes containing 50 mL of 1.5% (w/v) washed agar in water medium, poured at a slant, and supplemented with specified concentrations of phytohormones. Seedlings were germinated overnight at room temperature in the dark, transferred to agar-containing magenta boxes, and maintained at room temperature in the dark for 3 d before growth measurements. For assays employing ethylene gas, seedlings were grown on phytohormone-free washed agar medium. Magenta boxes were placed in a 3-L airtight plastic jar with a lid containing a serum septum, through which specified amounts of pure ethylene were injected.

The effect of exogenous ACC on nodulation was assessed using seedlings grown in growth pouches (Vaughn's Seed Company, Downers Grove, IL) containing 10 mL of nutrient solution devoid of nitrogen (Cook et al., 1995). Each pouch contained five to six pregerminated 1-d-old seedlings with roots approximately 1 cm in length. Seedlings were grown at 21°C in the dark for 36 to 48 h before being moved to light, with the root zone shielded from light by means of an aluminum foil covering. Pouches were weighed at 1-d intervals and sterile water was used to bring the weight of each pouch to the initial medium-filled pouch weight. One to 2 days after transfer to light, pouches were inoculated with 10 μL of washed inoculum from a late log phase culture of *S. meliloti*. ACC solutions in sterile water were added at 48 h after inoculation.

Root Graft Experiments

Root grafts were prepared essentially according to the protocol described by Journet et al. (2001). Details of the refined EMBO protocol can be obtained from http://www.isv.cnrs-gif.fr/embo01/manuels/index.html. In the present study, seedlings were germinated overnight in the dark at room temperature and transferred directly to 24-× 24-cm^2 sterile plastic trays containing buffered Nod medium (Erhardt et al., 1992) supplemented with 1 μM AVG medium in 1.5% (w/v) agar. Plantlets were grown for an additional 2 to 3 d before preparation of grafts. Seedlings with similar hypocotyl diameters were selected and cut transversely within the lower, chlorophyll-containing hypocotyl zone using a fresh razor blade. Roots were exchanged between plants, and the graft junction was sealed by application of 1.5% (w/v) purified water agar (Sigma, St. Louis). The agar trays were sealed with Parafilm and transferred in a near-vertical position to a growth chamber maintained at 24°C with a 16-h photoperiod (100 μE m^{-2} s^{-1}). Grafted plants were allowed to recover for at least 1 week before inoculation with *S. meliloti* strain 1021.

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