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Investigation of Four Classes of Non-nodulating White Sweetclover (*Melilotus alba* annua Desr.) Mutants and Their Responses to Arbuscular-Mycorrhizal Fungi[1]

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SYNOPSIS. The nitrogen-fixing symbiosis between Rhizobiaceae and legumes is one of the best-studied interactions established between prokaryotes and eukaryotes. The plant develops root nodules in which the bacteria are housed, and atmospheric nitrogen is fixed into ammonia by the rhizobia and made available to the plant in exchange for carbon compounds. It has been hypothesized that this symbiosis evolved from the more ancient arbuscular mycorrhizal (AM) symbiosis, in which the fungus associates with roots and aids the plant in the absorption of mineral nutrients, particularly phosphate. Support comes from several fronts: 1) legume mutants where Nod$^-$ and Myc$^-$ co-segregate, and 2) the fact that various early nodulin (*ENOD*) genes are expressed in legume AM. Both strongly argue for the idea that the signal transduction pathways between the two symbioses are conserved. We have analyzed the responses of four classes of non-nodulating *Melilotus alba* (white sweetclover) mutants to *Glomus intraradices* (the mycorrhizal symbiont) to investigate how Nod$^-$ mutations affect the establishment of this symbiosis. We also re-examined the root hair responses of the non-nodulating mutants to *Sinorhizobium meliloti* (the nitrogen-fixing symbiont). Of the four classes, several sweetclover *sym* mutants are both Nod$^-$ and Myc$^-$. In an attempt to decipher the relationship between nodulation and mycorrhiza formation, we also performed co-inoculation experiments with mutant rhizobia and *Glomus intraradices* on *Medicago sativa*, a close relative of *M. alba*. Even though sulfated Nod factor was supplied by some of the bacterial mutants, the fungus did not complement symbiotically defective rhizobia for nodulation.

INTRODUCTION

Plants undergo two different symbioses to assist them in the acquisition of nutrients from soil solution: the mycorrhizal symbiosis between fungi and roots results in the assimilation of phosphorous and other nutrients, whereas the nitrogen-fixing symbiosis generates ammonia from atmospheric nitrogen by the action of rhizobial bacteria living within nodules, specialized structures formed on plant roots. The arbuscular-mycorrhizal (AM) symbiosis is ancient; it most likely evolved in the Early Devonian (398 million years ago). Fossils of *Aglaophyton major*, a leafless and rootless plant from the Rhynie chert, have been verified as containing arbuscules (Remy *et al.*, 1994). However, fungal spores resembling extant *Glomus* sp. were already present in the mid-Ordovician (476 million years ago) (Redecker *et al.*, 2000), which implies that the AM symbiosis may have evolved as early as the late Silurian, during which the initial diversification of vascular plants occurred (Kendrick and Crane, 1997). It has been proposed that the evolution of the mycorrhizal symbiosis led to the colonization of the land (Pirozynski and Malloch, 1975). In contrast, fossils of the earliest angiosperms have been found only in the Cretaceous (110 million years ago), making it unlikely that the legume-*Rhizobium* symbiosis would have been established earlier.

There are several common elements observed in the symbiosis between members of the Rhizobiaceae and species of the Fabaceae (legumes) and the association between AM fungi and more than 80% of green land plants (see reviews by Harrison, 1997; Hirsch and Kapulnik, 1998; Barker and Tagu, 2000; Peterson and Guinel, 2000). Flavonoids, molecules secreted by plant roots and seeds, induce rhizobial *nod* genes as well as AM fungal spore germination and hyphal colonization. Upon induction of their *nod* genes, rhizobia synthesize Nod factor, a lipochitooligosaccharide signal molecule that is more typical of fungal cell walls than of Gram-negative bacteria. These similarities, together with the ancient nature of the association between AM fungi and terrestrial plants, have led to the proposal that the *Rhizobium*-legume symbiosis is derived from the mycorrhizal interaction (LaRue and Weeden, 1994). In partial support of this hypothesis, van Rhijn *et al.* (1997) found that two early nodulin (*ENOD*) genes, *ENOD40* and *ENOD2*, which serve as markers of the plant's response to rhizobial inoculation, are also expressed in mycorrhizal roots of *Medicago sativa*, demonstrating that steps in the downstream signal transduction pathways are conserved. These genes are also induced by the plant hormone cytokinin and, as cytokinin levels are enhanced in mycorrhizal roots (van Rhijn *et al.*, 1997), it is likely that the downstream stages in mycorrhiza establishment include an increase in endogenous cytokinin concentrations. Additionally,

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the genes *ENOD5* and *ENOD12A* are expressed in *Pisum sativum* mycorrhizal roots (Albrecht *et al.*, 1998), and *VfLb29*, which encodes a leghemoglobin, is expressed in mycorrhizal roots of *Vicia faba* (Frühling *et al.*, 1997).

Mutations that result in a non-nodulating (Nod^-) condition in several legumes often produce a non-mycorrhizal (Myc^-) phenotype as well (Duc *et al.*, 1989; Bradbury *et al.*, 1991; Sagan *et al.*, 1995; Peterson and Guinel, 2000). Resendes *et al.* (2001) described a low-nodulating pea mutant that shows a corresponding reduction in mycorrizal association. However, the *sym10* mutant of pea is Nod^-, but Myc^+ (Duc *et al.*, 1989), and the *Glycine max* non-nodulating mutants *nod49* and *nod139* are also Myc^+ (Wyss *et al.*, 1990). Likewise, the Nod^- *Phaseolus vulgaris* mutant R99 is Myc^+, but mutant R69, which forms uninfected nodules that lack peripheral vascular bundles, is Myc^- (Shirtliffe and Vessey, 1996). Senoo *et al.* (2000) found that several mycorrhizal mutants of *Lotus japonicus* (designated *Ljsym72, Ljsym71-1* and *Ljsym71-2*) co-segregate with Nod^-. Mutations in the *LjSym2, LjSym3,* and *LjSym4-1* genes, which result in a Nod^- phenotype, still allow the formation of a few arbuscules (Myc^+) (Wegel *et al.*, 1998), but *Ljsym4-2* mutants do not form arbuscules (Bonfante *et al.*, 2000). So far, the identity of only one gene, which when mutated gives rise to a non-nodulating phenotype, has been revealed (Schauser *et al.*, 1999). However, this *L. japonicus* non-nodulating mutant, described as *nin* (non-infective), exhibits root hair deformation in response to rhizobial inoculation and is also Myc^+ (J. Stougaard, personal communication).

Five different complementation groups, *sym1-sym5,* of non-nodulating white sweetclover (*Melilotus alba* annua Desr.) have already been described (Miller *et al.*, 1991; Utrup *et al.*, 1993). Of the five different Nod^- complementation groups, earlier studies showed that the *sym2* and *sym4* mutants underwent root hair curling (Hac) and infection thread formation (Inf) in response to *Sinorhizobium meliloti* inoculation, and some nodules developed (Utrup *et al.*, 1993). The *sym4* mutant (BT68) has been excluded from further studies because it is not a prolific seed producer. Although the *sym1* and *sym5* mutants exhibited marked root hair deformation (Had), but not Hac, following inoculation with *S. meliloti*, no infection threads were observed in the Utrup *et al.* (1993) study. However, one of the *sym1* mutant alleles, BT62, represents a weak allele because Wu *et al.* (1996) found that 12% of the plants formed ineffective nodules. Small, white ineffective nodules were also found on occasion on roots of the *sym5* mutant plants.

Of the five different complementation groups, *sym3* (represented by mutants BT61, BT69, and BT70) is the least responsive to *S. meliloti* inoculation. According to Utrup *et al.* (1993), *sym3* root hairs branched or became bulbous at their tips, but infection threads, cortical cell divisions, or nodule primordia were not initiated. In this report, we re-examined the root hair responses of the three different *sym3* mutant alleles to *S. meliloti* at earlier time points than that analyzed by Utrup *et al.* (1993). Our investigations indicate that the root hair deformation response for the *sym3* mutants is significantly less at these earlier time points than at 3 days post-inoculation (dpi), but can be augmented by inoculation with wild-type *S. meliloti*.

This report gives a first description of the symbiotic phenotypes of the different white sweetclover non-nodulating mutants in response to inoculation with *Glomus intraradices*. The complexity of responses among the various legumes to inoculation with either rhizobia or AM fungi also led us to examine whether functional complementation could occur between rhizobial Nod^- or Exo^- mutants and mycorrhizal fungi with the restoration of the bacteria's ability to nodulate or infect nodules, respectively.

MATERIALS AND METHODS

Plant material

The wild-type white sweetclover (*Melilotus alba* Desr.) U389 and its various non-nodulating mutants were utilized (Miller *et al.*, 1991). The mutants *sym2* (BT59), generated by EMS, and *sym5* (BT71), generated by neutron radiation, are represented by mutations at a single locus whereas four different mutant alleles exist for *sym1* and three for *sym3* (Miller *et al.*, 1991). For *sym3*, two different mutagens, EMS and neutron radiation, produced mutations at the same locus; BT61 is derived from EMS mutagenesis, and BT69 and BT70 from neutron radiation (Miller *et al.*, 1991). The four different *sym1* mutants result from EMS mutagenesis; in this report, we analyzed BT35, BT58, and BT62. Alfalfa (*Medicago sativa* L. cv. Iroquois) was used for the co-inoculation studies.

Sweetclover seeds were scarified with a scarifier or sandpaper. Both sweetclover and alfalfa seeds were surface-sterilized briefly in 95% ethanol, followed by immersion in commercial bleach for 45 min. The seeds were then washed five times with sterile water, placed on 0.8% Phytagar (GiboBRL), and grown in the dark for 48 hr.

Strains

The *S. meliloti* strains used in this study were Rm1021 (wild-type), Rm5612 (*nodC*::Tn*5*), Rm2212 (*nodH*::Tn*5*), RJW14 ((*nodF*)*nodL*::Tn*5*), Rm7225 (*exoH*::Tn5), and Rm7210 (*exoY*::Tn5). GFP-labeled Nod^- strains were generated by introducing the plasmid pHC60 (Cheng and Walker, 1998) into each strain by triparental mating using the helper plasmid pRK2013 (Ditta *et al.*, 1980). The Exo^- strains were a generous gift from G. C. Walker. Liquid cultures were grown shaking in an incubator at 30°C, washed once with sterile water, and re-suspended to approximately 10^8 cells per ml.

Aseptic spores of *G. intraradices* (Premier Tech, Quebec) were used for inoculating the roots.

Root hair assay

Germinated wild-type and mutant sweetclover seeds were transferred to Petri dishes containing 1/4 strength Hoagland's medium (Machlis and Torrey, 1956) minus nitrogen. Each plant was inoculated with 10 μl of Rm1021 (10^8 cells/ml). A qualitative assessment of root hairs along the entire length of the root was made at 5, 9.5 and 24 hr post-inoculation (hpi).

Mycorrhizal association

Wild-type and mutant sweetclover seedlings were transferred to autoclaved sand in enclosed Magenta jars (Magenta Corp., Chicago, IL) that were modified with holes in the bottom for drainage and holes in the top covered with Micropore™ tape (3M Health Care) to prevent ethylene accumulation. Each jar contained 9 plants and was watered with 1/4 strength Hoagland's medium containing limiting phosphate (0.2 mM KH_2PO_4). Thirty to 50 spores of *G. intraradices* were supplied to each plant by layering them 4 cm below the sand surface. Mycorrhizal association was assessed for intracellular hyphae and arbuscules 3 weeks after inoculation.

Co-inoculation studies

Alfalfa plants were grown as described for assessing mycorrhizal association except that the plants were watered with 1/4 strength Hoagland's medium minus nitrogen, or minus nitrogen and with limiting phosphate, or with limiting phosphate. Each plant was inoculated with 50 μl of rhizobia (10^8 cells/ml). For each rhizobial strain, treatments were set up for inoculation with rhizobia alone, co-inoculation with rhizobia and *G. intraradices* simultaneously, or inoculation of rhizobia one week after inoculation with *G. intraradices*. Roots were harvested and assessed for root hair deformation, infection thread formation, and nodule development 2 and 4 wk after inoculation with the bacteria.

Growth conditions

The plants were grown in a growth cabinet where they were maintained under a 16-hr light, 8-hr dark cycle at 24°C.

Staining and microscopy

Whole roots were harvested for the assessment of mycorrhizal association. Roots were fixed in FAA (formalin, acetic acid, alcohol) overnight, cleared in 10% KOH for 2 hr at 50°C, and then incubated overnight in a 10 μg/ml solution of wheat germ agglutinin-Alexa Fluor® 488 conjugate (Molecular Probes), which is a fluorescent lectin that binds to N-acetyl-glucosamine and will thus stain fungal structures. For confocal microscopy, counterstaining of the root was done by subsequently incubating the roots in 10 μg/ml acid fuchsin in phosphate-buffered saline for 5 minutes to take advantage of this stain's fluorescent properties. Some roots were cleared and stained with chlorazole black E (Brundrett *et al.*, 1984) or with 1% HCl followed by 0.01% acid fuchsin-lactic acid. The fungi were initially observed and assessed for mycorrhizal association with a Zeiss Axiophot light microscope using fluorescence and a 488 nm excitation filter. Confocal images were taken with a Bio-Rad MRC1024ES (krypton/argon) confocal laser scanning microscope associated with a Nikon Eclipse E800 light microscope using settings for FITC (488 nm) for observation of the fungus and TRITC (568 nm) for observation of the counterstained roots.

Of the three different staining techniques, chlorazole black E gave the best definition of fungal structures at the light microscope level. However, because it is not fluorescent, this stain cannot be used for confocal microscopy, which more clearly illustrates the relationship of the fungus to the inside or outside of the root. Acid fuchsin, which is commonly used as a stain for mycorrhizal fungi, can be used for confocal microscopy because it is fluorescent. However, the time required for destaining the roots to eliminate background fluorescence was inordinately long. Wheat-germ agglutinin conjugated to Alexa Fluor® 488 (Molecular Probes, Inc.) provided the best specific staining to the fungus with no background. Low concentrations of acid fuchsin provided an effective means for staining the root itself.

Sweetclover and alfalfa roots that had been inoculated by rhizobia were harvested and assessed using a Zeiss Axiophot light microscope under bright-field or Nomarski optics in conjunction with fluorescence microscopy for observation of GFP-labeled rhizobia.

RESULTS

Root hair deformation

We re-examined root hair curling in the sweetclover *sym* mutants because Utrup *et al.* (1993), who had studied root hair deformation for a number of *sym* mutants, did not report observations earlier than 3 dpi. In addition, they examined only one of four *sym1* mutant alleles (BT62), and only one of three *sym3* mutant alleles (BT70).

We detected root hair deformation in response to Rm1021 as early as 5 hpi (Fig. 1A). For example, the root hairs of wild-type line U389 and the *sym2* mutant (BT59) (Fig. 1C) showed significant deformation (Had^+) 5.5 hpi whereas the *sym1* (Fig. 1B) and *sym5* (data not shown) mutants did not show a significant response until 9 hpi, at which time *sym2* and wild-type sweetclover root hairs were extensively deformed (data not shown).

Utrup *et al.* (1993) described a ''rippley'' or ''arcing'' root hair response for the *sym3* mutant BT70 3 dpi. We did not observe such a response in the three different *sym3* mutant alleles when examined within hours of inoculation. At 5.5 hpi, we observed some swelling (Has^+; Catoira *et al.*, 2000) of the root hair tip (Fig. 1D), but a comparable amount of swelling was observed in uninoculated *sym3* mutant root tips

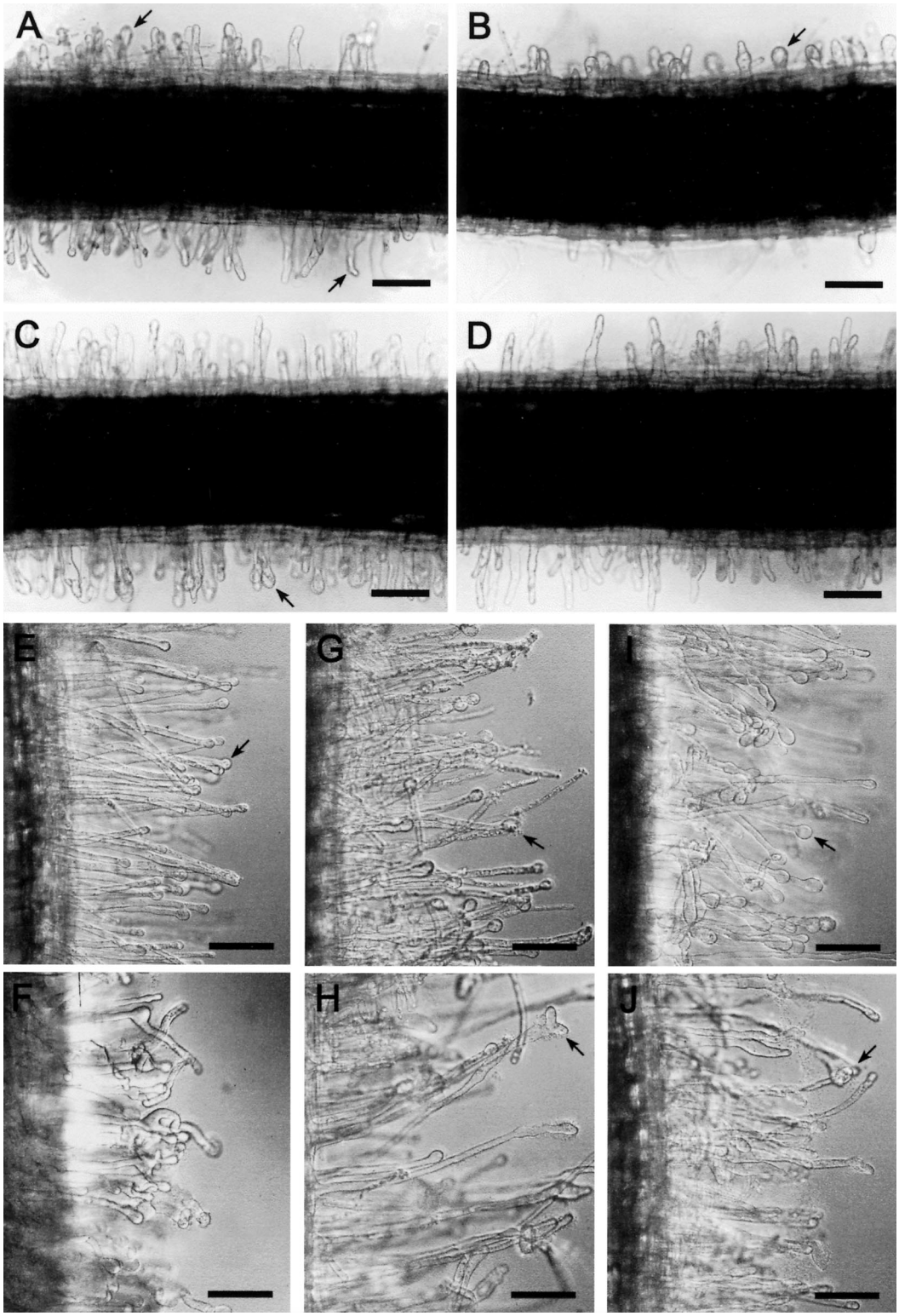

FIG. 1. Responses of root hairs from wild-type and *sym* mutant *M. alba* plants to inoculation with *S. meliloti*. Arrows point to representative deformed hairs. Bar = 100 μm. A. Wild-type *M. alba* U389, 5 hpi. B. BT62 (*sym1*), 9 hpi. C. BT59 (*sym2*), 5.5 hpi. D. BT61 (*sym3*), 5.5 hpi. E. Wild-type *M. alba* U389, uninoculated. F. Wild-type *M. alba* U389, 24 hpi. G. BT61 (*sym3*), uninoculated, 24 hpi. H. BT61 (*sym3*), 24 hpi. I. BT70 (*sym3*), uninoculated, 24 hpi. J. BT70 (*sym3*), 24 hpi.

(data not shown). By 24 hpi, uninoculated wild-type root hairs were slightly Has$^+$ (Fig. 1E) whereas inoculated U389 root hairs exhibited extensive Had (Fig. 1F) and the formation of shepherd's crooks. All *sym3* mutants at this time had root hairs that were bulging at the tips (Fig. 1G, I); the Has response was somewhat augmented in the Rm1021-inoculated roots (Fig. 1H, J). To test whether the *sym3* response was related to the presence of Nod factor, we inoculated *sym3* roots with Rm5612, a *nodC* mutant. We found no difference in root hair response between Rm5612-inoculated roots and the uninoculated control roots (data not shown), indicating that the augmented Has response was most likely due to the presence of Nod factor.

There was no obvious root hair response in any of the sweetclover roots following AM-fungal inoculation.

AM formation

We examined the responses of wild-type sweetclover roots and the non-nodulating *sym* mutants to inoculation with *G. intraradices*. Roots of U389, the wild-type sweetclover, formed well-established mycorrhizal associations within three weeks after inoculation. Appressoria were present, fungal hyphae penetrated the root cells, and both vesicles and arbuscules were detected within cortical cells (Fig. 2A). The *sym2* mutant exhibited a Myc$^+$ phenotype (Fig. 2B), appearing to have even more extensive mycorrhizal colonization than that of the U389 roots. We do not know the reason for this difference in response, particularly because U389 normally develops nitrogen-fixing nodules whereas the *sym2* mutant produces ineffective nodules (Utrup *et al.*, 1993).

The *sym1* mutant responses were somewhat variable, depending on the allele studied. BT62, described as ''leaky'' by Wu *et al.* (1996), was Myc$^+$ (Fig. 2D) whereas BT58 and BT35 were Myc$^-$. External hyphae ramified over the surface of the BT58 roots, but there was no evidence for hyphal penetration (Fig. 2C).

The single mutant at the *sym5* locus was also Myc$^-$ even though *sym5* mutants on occasion form very small, white, ineffective nodules. There was widespread hyphal proliferation over the surface of the root with no penetration of the root cells, although appressoria were observed (Fig. 2H).

Similarly, there was no indication of hyphal penetration, or arbuscule or vesicle development in any of the *sym3* mutant roots (Fig. 2E–G). The hyphae were extensively branched all over the root surface and appressoria were formed, but there was no penetration into the root tissue (Fig. 2F). These results are summarized in Table 1.

Complementation experiments

Complementation experiments were done using *M. sativa* because its response to various rhizobial mutants is well documented. Alfalfa seedlings were co-inoculated with *G. intraradices* spores and wild-type or *nodC, nodH, nodFL, exoH,* or *exoY* mutants of *S. meliloti*. Root systems were assessed for whether or not the mycorrhizal fungus was able to complement the mutants and allow an increased progression of the rhizobial symbiosis.

At 2 and 4 wk after inoculation, Had, Hac, Inf, and pink nodules were observed on plants that had been inoculated with Rm1021, whether or not plants had also been co-inoculated with *G. intraradices*. However, signs of nodule development could not be detected on plants inoculated with any of the Nod$^-$ mutants, with or without the fungus. The *nodC* mutant, which is completely deficient in Nod factor production, caused no visible root response with or without co-inoculation. The *nodH* mutant, which produces Nod factor lacking the sulfate at the reducing end, and the *nodFL* mutant, which produces sulfated but non-*O*-acetylated Nod factor, both triggered root hair deformation, similar to that reported previously (Debellé *et al.*, 1986; Ardourel *et al.*, 1994). However, co-inoculation with *G. intraradices* did not enhance the response. Cytological observations of roots aided by the observation of GFP-labeled bacteria did not reveal infection thread formation after co-inoculation of alfalfa with *G. intraradices* and any of the Nod$^-$ mutants.

The *exoH* mutant lacks succinyl transferase (Leigh *et al.*, 1987), which is required for the addition of the succinyl modification to the exopolysaccharide, and the *exoY* mutant lacks a galactosyl transferase (Leigh *et al.*, 1985), which is required for the first step of succinoglycan biosynthesis. Inoculation with either of these mutants resulted in aborted infection threads and uninfected nodules as reported previously (Cheng and Walker, 1998). As with the Nod$^-$ mutants, co-inoculation with *G. intraradices* did not promote further progression of the nodulation phenotype. These results are summarized in Table 2.

DISCUSSION

We found that several *sym* mutants of sweetclover are both Nod$^-$ and Myc$^-$: two mutant alleles of *sym1* (BT35 and BT58), all three mutant alleles of *sym3* (BT61, BG69, BT70), and the single mutant of *sym5* (BT71). There are two distinct Myc$^-$ phenotypes, Myc^{-1} and Myc^{-2}, which have been described (Gianinazzi-Pearson, 1996; Harrison, 1997; Peterson and Guinel, 2000). These designations refer to a breakdown in internal colonization of the plant (Myc^{-1}) or of arbuscule formation (Myc^{-2}). Of the two, the Myc^{-1} phenotype is the more commonly described. We did not observe arbuscule formation in response to *G. intraradices* in roots of two of the *sym1* mutants (BT35 and BT58), the *sym3* mutants, or of the single *sym5* (BT71) mutant of sweetclover; all are thus designated Myc^{-1}. Some preliminary experiments with *G. mosseae*, however, indicate that the *sym1* mutant (BT58) may be leaky with respect to mycorrhiza formation (unpublished data, Y.K.). The sweetclover mutants, *sym1* (BT62) and *sym2* (BT59) are colonized by *G. intraradices*, and arbuscules are formed within the root cells; thus, they are Myc$^+$. Preliminary results also in-

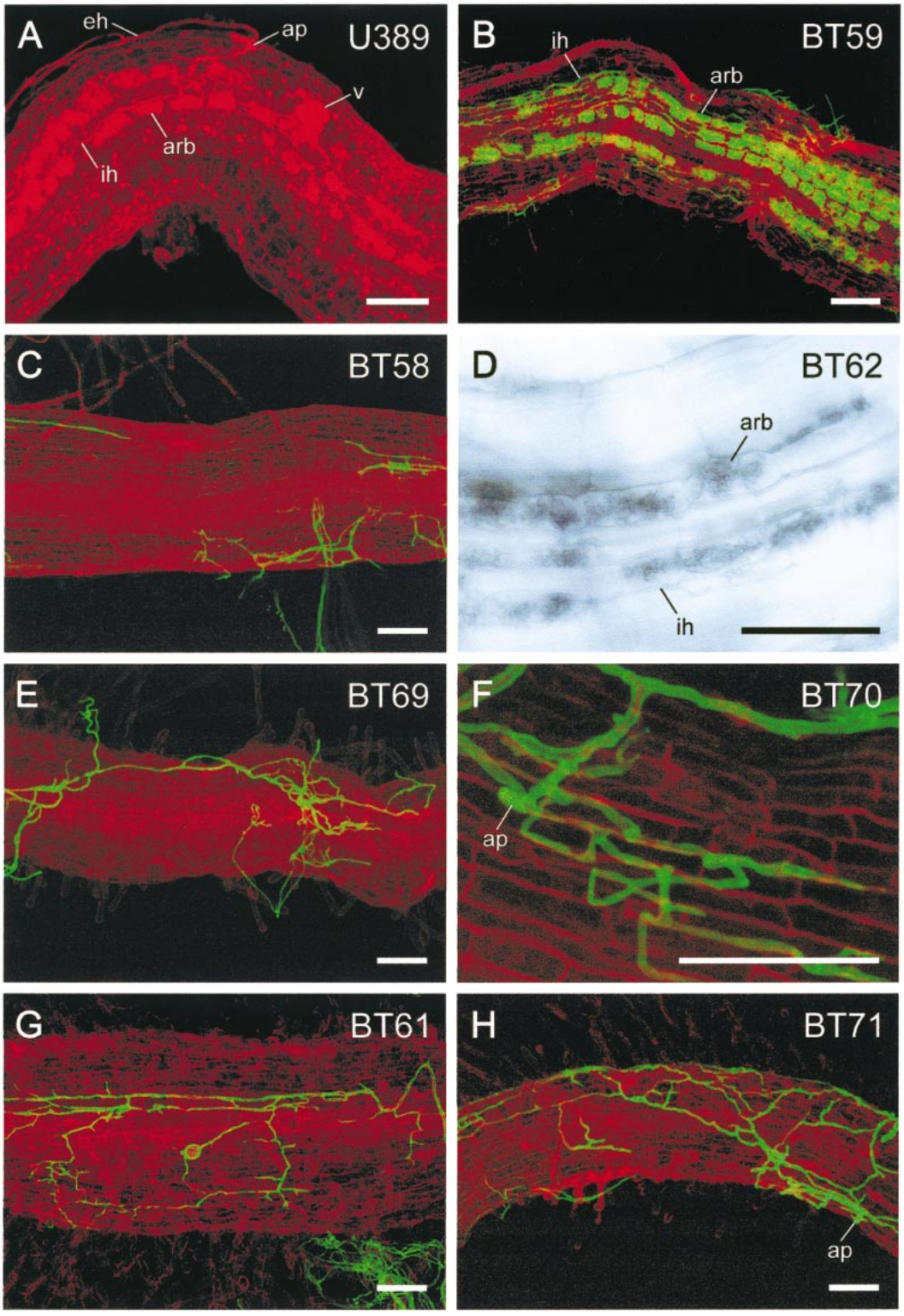

FIG. 2. Inoculation of wild-type and *sym* mutant *M. alba* with the arbuscular mycorrhizal (AM) fungus *G. intraradices*. Bar = 100 μm. A. Wild-type *M. alba* U389 shows external hyphae (eh) and appressoria (ap) and well-established intracellular colonization of the root cortex with internal hyphae (ih), arbuscules (arb) and vesicles (v). Stained with acid fuchsin alone. B. BT59 (*sym2*) shows well-established mycorrhizal colonization with both internal hyphae (ih) and arbuscules (arb). Stained with wheat germ agglutinin (WGA) conjugated to Alexa Fluor® 488 and counter-stained with acid fuchsin. C. The BT58 allele of *sym1* is Myc^-; the hyphae do not penetrate the root. Stained with WGA conjugated to Alexa Fluor® 488 and counter-stained with acid fuchsin. D. BT62 (*sym1*) is a weak allele; roots are occasionally colonized (ih) and contain arbuscules (arb). Stained with chlorazole black E. E.–G. None of the *sym3* roots were colonized internally by *G. intraradices*; the hyphae are associated only with the outside of the root and appressoria (ap) can be seen. Stained with WGA conjugated to Alexa Fluor® 488 and counter-stained with acid fuchsin. E. BT69. F. BT70. G. BT61. H. BT71 (*sym5*) has an extensive net of hyphae around the root, but no internal colonization has taken place.

TABLE 1. *Effects of* Sinorhizobium meliloti *and* Glomus intraradices *on wild-type and* sym *mutant sweetclover* (Melilotus alba *Desr.*)

Genotype/Mutant	Nodulation phenotype*	Mycorrhizal phenotype
Wild type/U389	Hac^{+a}, Inf^{+b}, Nod^{+c}	Pen^{+d}, Ves^{+e}, Arb^{+f}
sym1/BT62, BT58, and BT35	Had^{+g}, Inf^{-}; small, white nodules (12% of the plants) for BT62 but BT58 and BT35 are Nod^{-}	Pen^{+}, Ves^{+}, Arb$^{\pm}$ (BT62); Pen^{-}, Ves^{-}, Arb^{-} (BT35, BT58)
sym2/BT59	Hac^{+}, Inf^{+}, similar to U389. White, ineffective nodules (25%), but 1.7% of the studied plants formed effective nodules	Pen^{+}, Ves^{+}, Arb^{+}
sym3/BT61, BT69, and BT70	Has^{+h}, Had^{-}, Hac^{-}, Inf^{-}, Nod^{-}	Pen^{-}, Ves^{-}, Arb^{-}
sym5/BT71	Had^{+}, Inf^{-}; occasional small, white nodules on 13% of the plants	Pen^{-}, Ves^{-}, Arb^{-}

* Based on Utrup *et al.* (1993), Wu *et al.* (1996) and this report.
[a] Root hair curling, *i.e.*, shepherd's crook formation.
[b] Infection thread formation.
[c] Nodule development.
[d] Penetration of hyphae.
[e] Vesicle formation.
[f] Arbuscule formation.
[g] Root hair deformation.
[h] Root hair tip swelling.

dicate that there are differences in the expression of the early nodulin gene, *ENOD40,* in the various Myc^{-} roots *versus* those that are Myc^{+}(M. R. Lum and A. M. Hirsch, unpublished results). Hence, as observed for other non-nodulating legumes, there is a link between being blocked in the earliest stages of nodulation (Nod^{-}) and Myc^{-}.

If *Rhizobium's* ability to establish a symbiotic association with plants was derived from arbuscular mycorrhizal fungi, then co-inoculation of *G. intraradices* and Nod^{-} rhizobia might rescue the non-nodulation phenotype and moreover, co-inoculation of *G. intraradices* and Exo^{-} rhizobia might produce infected nodules. However, these results did not occur even when a *nodFL* mutant or Exo^{-} mutant, each of which makes sulfated Nod factors, was utilized. Nod factor sulfation appears to be absolutely required for *S. meliloti* hosts to respond to inoculation (van Rhijn *et al.*, 2001). The fact that AM-fungal co-inoculation does not rescue Nod^{-} *S. meliloti* contrasts with our earlier results whereby Exo^{-} and Nod^{-} *S. meliloti* functionally complemented each other resulting in infected nitrogen-fixing nodules (Klein *et al.*, 1988). However, there are many more profound differences between the potential signal molecule(s) produced by the fungal *versus* bacterial symbionts than there are between the two different types of rhizobial mutants. For one, *S. meliloti* Nod factor is both sulfated and acetylated whereas the likely comparable signal molecule in *G. intraradices* is not. In addition, the host may respond quite differently to the various microbial molecules it encounters. For example, some legume hosts such as alfalfa are known to respond by undergoing cortical cell divisions to treatment with Nod factor alone (Truchet *et al.*, 1991), but others do not. Moreover, there is little published evidence for plant hosts responding to a secreted molecule from AM-fungi although there are reports about a host-produced molecule affecting branching of the AM-fungus (see references in Hirsch and Kapulnik, 1998).

At least two possible mechanisms could be invoked to explain the overlap between the two symbioses: 1) genes were laterally transferred from the fungus to the bacteria; or 2) the plant's long history of establishing mycorrhizal associations with phosphate-acquiring fungi led to the convergence of signal molecules produced by rhizobial cells with those produced by AM fungi. Our data do not allow us to differentiate between these two possibilities. It does enable us to suggest that multiple steps in the symbiotic process are conserved, and not just a single step as evidenced by

TABLE 2. *Summary of the effect of co-inoculation of* Sinorhizobium meliloti *Nod⁻ mutants and* Glomus intraradices *on alfalfa plants.*

	Had[a]	Hac[b]	Infection threads	Nodules
Rm1021 (−N)	+	+	+	+
Rm1021*	+	+	+	+
Rm1021/*G. intraradices*	+	+	+	+
nodC (−N)	−	−	−	−
nodC	−	−	−	−
nodC/G. intraradices	−	−	−	−
nodH (−N)	+	−	−	−
nodH	+	−	−	−
nodH/G. intraradices	+	−	−	−
nodFL (−N)	+	+	−	−
nodFL	+	+	−	−
nodFL/G. intraradices	+	+	−	−
exoH (−N)	+	+	Aborted	Empty
exoH	+	+	Aborted	Empty
exoH/G. intraradices	+	+	Aborted	Empty
exoY (−N)	+	+	Aborted	Empty
exoY	+	+	Aborted	Empty
exoY/G. intraradices	+	+	Aborted	Empty
uninoc. (limit. P)	−	−	−	−
G. intraradices (limit. P)	−	−	−	−

* Unless indicated otherwise, plants were watered with 1/4 strength Hoagland's medium with limiting phosphate and minus nitrate.
[a] Root hair deformation.
[b] Root hair curling.

the fact that several loci (*Sym1*, *Sym3*, and *Sym5*) when mutated are both Nod$^-$ and Myc$^-$. However, mutations in the *Sym3* locus exhibit the tightest correspondence between Nod$^-$ and Myc$^-$ because *sym3* mutants show the least response to rhizobial inoculation, and all three mutant alleles show the same phenotype in response to *S. meliloti* and *G. intraradices*. This suggests that *Sym3* is upstream of *Sym1* and probably of *Sym5* also.

Even though it was originally classed as non-nodulating (Miller *et al.*, 1991), the sweetclover *sym2* mutant develops ineffective nodules approximately 25% of the time (Utrup *et al.*, 1993). This may explain why *sym2* is Myc$^+$, proceeding beyond the Myc^{-2} block. However, we do not know why *sym2* roots appear to be colonized even more efficiently than the wild-type U389 roots. At this time, only one allele of *sym2* is available for study, and thus, any conclusions about its position in a developmental pathway are premature.

To our knowledge, there are no examples of legumes that are both Myc$^-$ and capable of producing infected nodules. Those legume Myc$^-$ mutants that are Nod$^+$ are generally uninfected (Bradbury *et al.*, 1991; Shirtliffe and Vessey, 1996). The correlation between Myc$^-$ and the inability to produce infected nodules suggests that many of the initial stages in the interaction between *Rhizobium* and its legume host are upstream of those leading to mycorrhiza formation. The lack of functional complementation of rhizobial mutants by co-inoculation with a mycorrhizal fungus further supports this hypothesis.

How do we explain the overlap in gene products involved in both nodulation and mycorrhiza formation? Could these gene products represent upstream components in a common signal transduction pathway such as a receptor or signal transducers? The fact that a number of legume mutations exist, some of which are Nod$^-$ and Myc$^-$ and others which are Nod$^-$ and Myc$^+$, suggests that the two symbiotic pathways share a number of steps at the initial stages of the interaction, or alternatively, that several proteins are grouped together in a receptor complex, some of which are used for both plant-microbe associations. Elucidation of *Sym3* and equivalent genes in other legumes may help us sort out the relationship between these two important plant symbioses. The fact that *Sym3* is absolutely required for both symbiotic interactions points to the pivotal role of its gene product in serving as an initial ''gatekeeper.''

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