

# The Auxin Transport Inhibitor *N*-(1-Naphthyl)phthalamic Acid Elicits Pseudonodules on Nonnodulating Mutants of White Sweetclover<sup>1</sup>

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The collection of symbiotic (*sym*) mutants of white sweetclover (*Melilotus alba* Desr.) provides a developmental sequence of mutants blocked early in infection or nodule organogenesis. Mutant phenotypes include non-nodulating mutants that exhibit root-hair deformations in response to *Rhizobium meliloti*, mutants that form ineffective nodules lacking infection threads, and mutants that form infection threads and ineffective nodules. Mutant alleles from both the *sym-1* and the *sym-3* loci exhibited a non-nodulating phenotype in response to *R. meliloti*, although one allele in the *sym-1* locus formed ineffective nodules at a low frequency. Spot-inoculation experiments on a non-nodulating allele in the *sym-3* locus indicated that this mutant lacked cortical cell divisions following inoculation with *R. meliloti*. The auxin transport inhibitor *N*-(1-naphthyl)phthalamic acid elicited development of pseudonodules at a high frequency on all of the sweetclover *sym* mutants, including the non-nodulating mutants, in which the early nodulin ENOD2 was expressed. This suggests that *N*-(1-naphthyl)phthalamic acid activates cortical cell divisions by circumventing a secondary signal transduction event that is lacking in the non-nodulating sweetclover mutants. The *sym-3* locus and possibly the *sym-1* locus appear to be essential to early host plant responses essential to nodule organogenesis.

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Establishment of the nitrogen-fixing symbiosis between bacteria of the genus *Rhizobium* and specific leguminous host plants requires an exchange of signal molecules leading to expression of both bacterial and host plant genes involved in infection and root nodule organogenesis (reviewed by Denarie and Roche, 1992; Hirsch, 1992; Long, 1992). Specific flavonoid signals secreted by the host plant roots elicit the expression of essential bacterial *nod* genes that in turn produce modified lipo-oligosaccharide signals called Nod factors (Peters et al., 1986; Kosslak et al., 1987;

Maxwell et al., 1989; reviewed by Carlson et al., 1994). In particular, purified NodRm-IV(S) from *Rhizobium meliloti* elicits early host plant responses in alfalfa, including root-hair deformation and cortical cell division, resulting in nodule organogenesis (Lerouge et al., 1990; Truchet et al., 1991). The recent report that Nod factors rescue a carrot embryogenesis mutant suggests that these factors may represent a class of chitin derivatives involved in initiation of meristematic cell divisions (De Jong et al., 1993).

Inoculation with a host-specific species of *Rhizobium* or *Bradyrhizobium* elicits cortical cell divisions within 24 h that develop into a nodule primordium (Turgeon and Bauer, 1982; Calvert et al., 1984; Dudley et al., 1987). Bacteria are released into cells of the emerging nodule from infection threads originating from tightly curled root-hair cells. The response of root-hair cells to Nod factors is very rapid (Ehrhardt et al., 1992; Heidstra et al., 1994) and more stringent in the structural requirements for the Nod factors than activation of cortical cell divisions (Ardourel et al., 1994). Other studies have shown that susceptibility of cortical cells to nodulation factors is not due to arrest of the cell cycle, and implicate the additional involvement of a factor released from the stele (Libbenga and Bogers, 1974; Yang et al., 1994). A mature indeterminate nodule infected by *Rhizobium* contains an apical meristematic zone, a prefixing zone in which differentiation of both symbionts occurs, a zone of cells containing nitrogen-fixing bacteroids, and a proximal zone in which senescence and degeneration of bacteroids takes place (nomenclature of Vasse et al., 1990).

Expression of nodule-specific genes of the host plant, termed nodulin genes, is correlated with early events in nodule development (Scheres et al., 1990b). The early nodulin ENOD2 is expressed in the nodule parenchyma of alfalfa, pea, and soybean nodules (Van de Wiel et al., 1990a, 1990b). ENOD2 is also expressed in bacteria-free nodules elicited by exopolysaccharide (*exo*) mutants of *R. meliloti* or by *Agrobacterium tumefaciens* transconjugants carrying the *R. meliloti nod* genes (Dickstein et al., 1988; Van de Wiel et al., 1990a; Allen et al., 1991), as well as in spontaneous nodules that occur in the absence of *Rhizobium* (Truchet et al., 1989). The early nodulin MtENOD12 is expressed within 3 to 6 h in root epidermal cells following inoculation in transgenic alfalfa (Pichon et al., 1992) and in cells just

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Abbreviations: ATI, auxin transport inhibitor; Hac<sup>+</sup>/Inf, root-hair curling with infection threads; Had, root-hair deformation; NPA, *N*-(1-naphthyl)phthalamic acid.

ahead of the infection zone in nodules of pea (PsENOD12, Scheres et al., 1990a) and *Medicago truncatula* (Pichon et al., 1992). Most recently, a *Rhizobium*-induced peroxidase expressed transiently prior to infection in *M. truncatula* was reported (Cook et al., 1995).

The formation of spontaneous nodules or nodulation in the absence of *Rhizobium* in some lines of alfalfa (Truchet et al., 1989; Caetano-Anollés et al., 1990) and white clover (Blauenfeldt et al., 1994) suggests that the plant regulates nodule morphogenesis. The development of spontaneous nodules must overlap at least partially with the developmental pathway leading to *Rhizobium*-induced nodulation, since nodulins such as ENOD2 (Truchet et al., 1989) and ENOD12 (Pichon et al., 1994) are expressed in these nodules. Spontaneous nodules also suppress nodulation of alfalfa by *R. meliloti*; likewise, nodules infected by *R. meliloti* suppress formation of spontaneous nodules (Caetano-Anollés et al., 1990). However, spontaneous nodules initiate primarily in the older portion of the root system, the pattern of ENOD12 expression in the nodule primordia is distinct from *Rhizobium*-induced nodulation, and systemic autoregulation does not occur in spontaneous nodulation, indicating that some differences in development from *Rhizobium*-induced nodulation exist (Pichon et al., 1994).

It has been almost 60 years since Thimann (1936) first suggested that plant hormones, specifically auxin, regulate nodule development, a hypothesis supported by accumulating evidence that auxin and/or cytokinins, or gradients of these hormones, are involved in nodule organogenesis (Libbenga et al., 1973; reviewed by Hirsch and Fang, 1994). Application of an ATI has been shown to induce formation of nodule-like structures on a number of legumes (Allen et al., 1953). The nodule-like structures, also called pseudonodules, elicited by the ATIs NPA and 2,3,5-triiodobenzoic acid, express the ENOD2 and ENOD12 genes (Hirsch et al., 1989; Van de Wiel et al., 1990a; Scheres et al., 1992). Evidence that cytokinins are involved in nodule morphogenesis is provided by reports of ENOD2 expression in cytokinin-treated *Sesbania* roots (Dehio and de Bruijn, 1992) and in nodules elicited by *R. meliloti nod* mutants carrying the trans-zeatin secretion gene from *A. tumefaciens* (Cooper and Long, 1994). Nodule-like structures are also formed on a non-nodulating line of soybean following application of 2,4-D (Akao et al., 1991).

We recently reported the symbiotic phenotypes of a group of *sym* mutants of white sweetclover (*Melilotus alba*), representing five genetic loci (Kneen and LaRue, 1988; Miller et al., 1991), that included mutants blocked at root-hair curling, mutants blocked at infection, and mutants that formed ineffective nodules in response to *R. meliloti* (Utrup et al., 1993). The single mutant allele characterized in the *sym-3* locus was of particular interest because it exhibited limited root-hair deformations in response to *R. meliloti* but formed no nodules, suggesting that a secondary signal leading to nodule organogenesis may be lacking in this mutant.

In this study, we have characterized the phenotype of additional nodulation mutants of sweetclover, including non-nodulating alleles in both the *sym-1* and the *sym-3* loci.

We found that the ATI NPA elicited nodule-like structures, or pseudonodules, on all of the non-nodulating mutants, as well as on the mutants that form ineffective nodules with *R. meliloti*, in which the ENOD2 gene was expressed. Our data suggest that the products of the *sym-1* and *sym-3* loci of sweetclover are essential components of the signal transduction pathway leading to nodule morphogenesis.

## MATERIALS AND METHODS

### Plant Material

Nine isogenic mutant lines of white sweetclover (*Melilotus alba* Desr. cv U389, PI 165554) derived from wild-type cv U389 by mutagenesis with ethyl methanesulfonic acid or neutron radiation (Kneen and LaRue, 1988), kindly provided by Dr. Tom LaRue (Boyce Thompson Institute for Plant Research, Ithaca, NY), have been maintained by self-pollination for more than five generations in our laboratory. Complementation analysis has indicated that four genetic loci are represented among the nine mutants analyzed in this study, with allelic mutants available in the *sym-1* and *sym-3* loci (Miller et al., 1991; listed in Table I).

Plants were grown in sterile vermiculite in 12-cm pots or 1-quart Mason jars with foam stoppers as previously described (Utrup et al., 1993). For nitrogen-fixing nodules, plants were inoculated with *Rhizobium meliloti* SU47 48 h after planting. Alternatively, seedlings were given a single application of 20  $\mu$ M NPA (generously provided by Uniroyal Chemical Co., Middlebury, CT) 1 week after germination as described by Hirsch et al. (1989). The NPA application was prepared in nitrate-free modified Jensen's nutrient medium (Vincent, 1970), which was used for all additional watering of plants. For evaluation of the effect of nitrate, 5 mM KNO<sub>3</sub> and 5 mM CaNO<sub>3</sub> were added to the Jensen's nutrient medium containing NPA. Nodules and pseudonodules were harvested at 4 weeks, fixed, embedded in Paraplast (Monoject Scientific, St. Louis, MO), sectioned, stained with safranin, and counterstained with fast green as previously described (Jensen, 1962; Utrup et al., 1993).

### Statistical Analysis

The data on the number of plants forming pseudonodules for all of the mutant lines and wild-type sweetclover were evaluated by the  $\chi^2$  test for larger frequency tables to assess whether the observed frequencies of forming pseudonodules deviated significantly from the null hypothesis (Finney, 1980). In this case, the hypothesis was that the probability of forming pseudonodules is the same for all lines, whether mutant or wild type. Table II (observed data) was compared with a table of expected data, using an expected frequency value of 0.606, obtained by summing all of the observed data, with 7 *df*. For  $\chi^2$  values greater than 18.47, the hypothesis is rejected and the observed values for frequency of formation of pseudonodules are significantly different at the 99% probability level (Finney, 1980).

### Spot-Inoculation Experiments

Seedlings were grown on nodulation plates and inoculated with a concentrated culture of *R. meliloti* SU47 using a micromanipulator according to the method of Dudley et al. (1987). Two types of black, nontoxic ink were used interchangeably to mark the spot of inoculation: Pelikan drawing ink (Gunther Wagner, Germany) and Speedball water-soluble ink for block printing (Hunt Manufacturing Co., Statesville, NC). Neither caused detectable damage to the root. Inoculated roots were harvested at times from 24 h to 21 d after inoculation, fixed, and serially sectioned as above. The sectioned tissue was stained with a commercial preparation of Gill No. 2 hematoxylin (Sigma) for 10 to 20 min before mounting with a permanent resin (Jensen, 1962). Serially sectioned roots of spot-inoculated wild-type and noninoculated wild-type and mutant roots (dye-spotted only) were compared with spot-inoculated mutant roots at each time.

### Northern Blot Analysis

Total RNA was isolated from roots and nodules by precipitation with LiCl (Govers et al., 1985). Whereas nitrogen-fixing nodules could be harvested cleanly, some attached root tissue was unavoidably harvested along with the smaller and more fragile NPA-elicited nodules. The concentration of the total RNA samples was estimated by spectrophotometric determination. Total RNA samples were also evaluated for equal intensity and integrity of rRNA bands by ethidium bromide staining. For blotting, 10  $\mu$ g of total RNA were loaded in each lane of a 1% agarose gel containing formaldehyde (Sambrook et al., 1989) and transferred to a positively charged nylon membrane following electrophoresis (Boehringer Mannheim). RNA molecular weight standards (Boehringer Mannheim) were also run on the gel and visualized by ethidium bromide staining before blotting. The blot was prehybridized in 15 mL of solution containing 5 $\times$  SSC (0.75 M NaCl, 75 mM sodium citrate, pH 7.0), 50% formamide, 0.02% SDS, 0.1% *N*-lauroylsarcosine, 2% blocking reagent (Genius kit, Boehringer Mannheim), 20 mM sodium maleate, pH 7.5, for 16 h at 42°C. Hybridization was carried out at 42°C for 16 h in fresh prehybridization solution containing the denatured 273-bp insert of pB1-A2ENOD2 from alfalfa (Dickstein et al., 1988) labeled with digoxigenin-dUTP (Boehringer Mannheim). The membrane was washed twice for 5 min each in 2 $\times$  SSC containing 0.1% SDS at 25°C, followed by two washes of 15 min each at 65°C in 0.5 $\times$  SSC containing 0.1% SDS. All hybridization procedures and subsequent chemiluminescent detection were carried out according to the instructions in the Genius kit manual (Boehringer Mannheim).

## RESULTS

### Mutant Alleles in Both the *sym*-1 and *sym*-3 Loci Are Non-Nodulating Following Inoculation with *R. meliloti*

Our previous characterization of the nodulation phenotype and root-hair-curling response of five sweetclover *sym*

mutants (Utrup et al., 1993) was limited to 100 to 300 plants of a single mutant allele from each *sym* locus. For this study, greater numbers of plants, as well as additional mutant alleles in the *sym*-1 and *sym*-3 loci, were tested for nodulation following inoculation with *R. meliloti* to determine whether nodules might be found on a small percentage of the mutant plants.

Non-nodulating alleles were found in two of the sweetclover *sym* loci analyzed in this study. As shown in Table I, three of the four *sym*-1 alleles formed no nodules following inoculation with *R. meliloti* SU47. Only BT62, the single *sym*-1 allele tested in our previous analysis (Utrup et al., 1993), formed white, non-nitrogen-fixing nodules. All three mutant alleles in the *sym*-3 locus formed no nodules on more than a total of 1600 inoculated plants.

Mutants in two additional sweetclover *sym* loci formed white, non-nitrogen-fixing nodules. The single mutant available in the *sym*-2 locus, BT59, formed white nodules on 25% of the plants inoculated with *R. meliloti* (Table I). Mutant BT71, the sole *sym*-5 mutant, exhibited white nodules on 13% of the inoculated plants. For all of the mutants that formed ineffective nodules in response to *R. meliloti*, the number of nodules per plant varied from 1 to 10, distributed both on the tap root and lateral roots, as previously shown (Utrup et al., 1993).

### *sym*-3 Allele BT70 Exhibits No Cortical Cell Divisions in Response to *R. meliloti*

The *sym*-3 alleles are of particular interest because they were all non-nodulating when inoculated with *R. meliloti* (Table I). *sym*-3 allele BT70 also exhibited only limited root-hair deformations in response to *R. meliloti* (Utrup et al., 1993). To elucidate the point at which nodule development is blocked in this *sym*-3 allele, we carried out spot-inoculation assays of mutant BT70 seedlings with *R. meliloti* according to the method of Dudley et al. (1987). A total of

**Table 1.** Frequency of formation of non-nitrogen-fixing nodules elicited by *R. meliloti* on symbiotic mutants of sweetclover

Seedlings of wild-type and mutant sweetclover were grown in sterile vermiculite and inoculated with a late log phase culture of *R. meliloti* SU47 on d 3. White nodules were collected from the mutants 4 weeks after inoculation.

Locus	Allele	No. of Plants Inoculated <sup>a</sup>	No. of Plants Nodulated	Percentage of Nodulation
<i>sym</i> -1	BT35	220	0	0
	BT58	90	0	0
	BT62	1060	123	12
	BT64	430	0	0
<i>sym</i> -2	BT59	1030	260	25
	<i>sym</i> -3	BT61	570	0
BT69		280	0	0
BT70		800	0	0
<i>sym</i> -5	BT71	1080	145	13
Wild type		100	100 <sup>b</sup>	100

<sup>a</sup> The number of plants inoculated varied because of availability of seeds. <sup>b</sup> Nodules on wild-type plants were pink and nitrogen fixing.

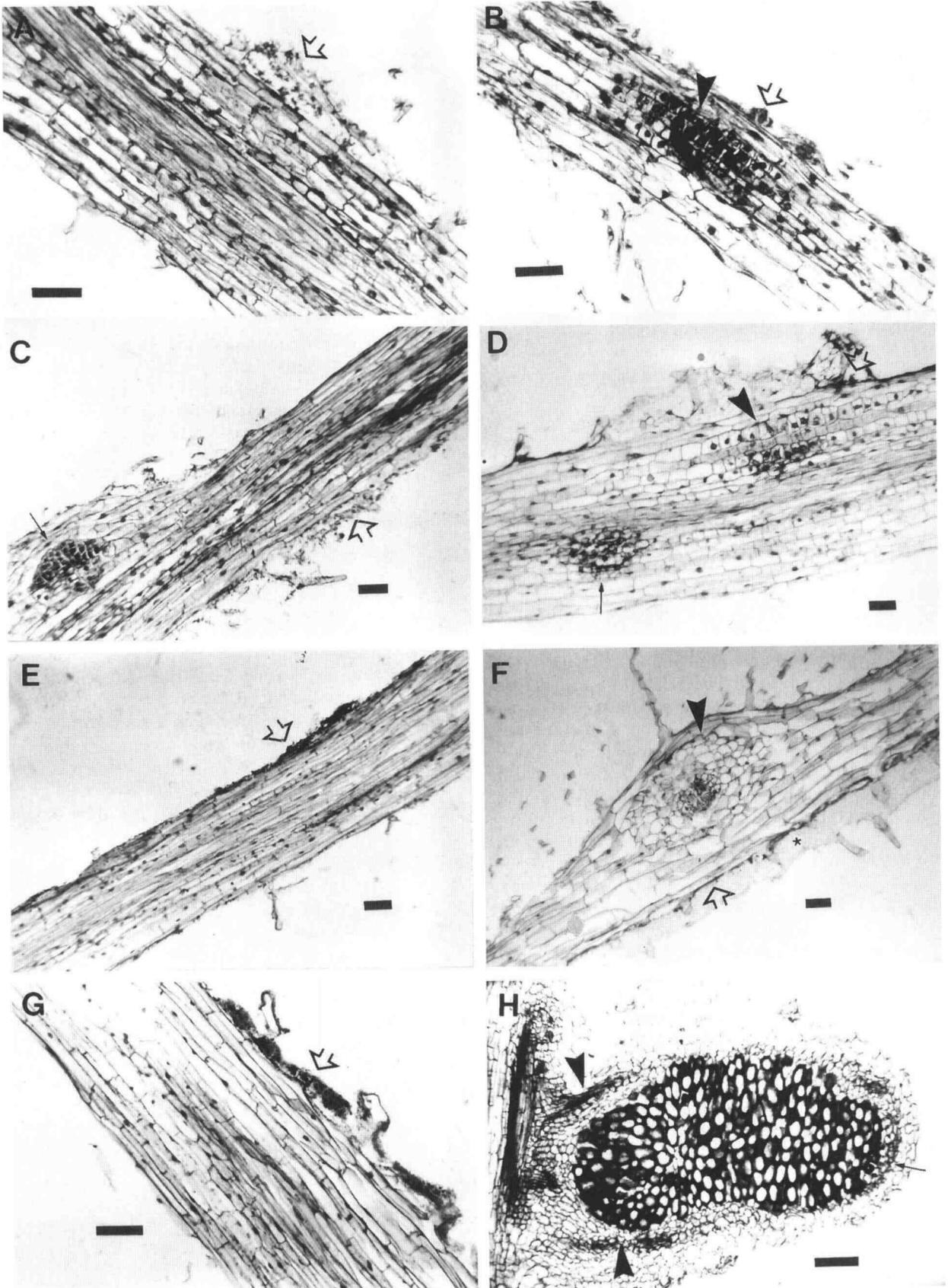


Figure 1. (Legend appears on facing page.)

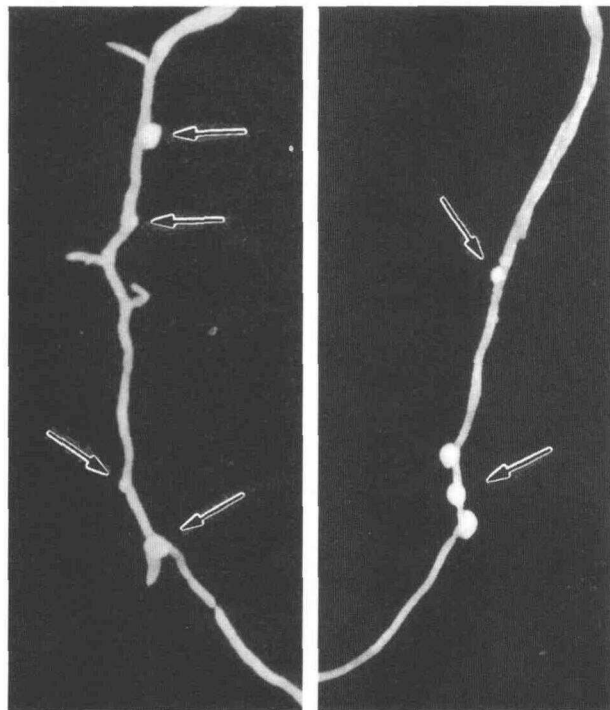
23 spot-inoculated seedlings of allele BT70 were examined by serial sectioning 48 h, 3 d, 4 d, 7 d, and 11 d after inoculation and compared with an equal number of serially sectioned, spot-inoculated wild-type sweetclover seedlings, noninoculated wild-type seedlings, and noninoculated mutant seedlings at all times.

No nodules were observed on spot-inoculated BT70 plants, including plants maintained up to 28 d after inoculation. No activated regions of cortical cell mitoses or nodule primordia were observed in serially sectioned, spot-inoculated BT70 plants, shown at 48 h, 3 d, 7 d, and 11 d after inoculation in Figure 1. In comparison, inner cortical cell mitoses were observed in about one-half of the spot-inoculated wild-type plants at 24 h and in almost all wild-type seedlings at 48 h after inoculation (Fig. 1B). An emerging nodule primordium, often with a darkly staining infection thread (not shown), was observed at the dye spot in wild-type plants 3 d after inoculation (Fig. 1D). Nodules were clearly visible in spot-inoculated wild-type plants 7 d after inoculation (Fig. 1F). Only lateral root primordia were observed in serial sections of both mutant and wild-type noninoculated control plants (not shown).

#### NPA Elicits Pseudonodules on All of the Sweetclover Nodulation Mutants

In preliminary testing on wild-type sweetclover and mutants from all of the *sym* loci, approximately 80% of 60 seedlings treated with 20  $\mu\text{M}$  NPA 7 d after germination formed white pseudonodules similar to those observed on alfalfa (Hirsch et al., 1989), in comparison to pseudonodules formed on approximately 55% of the seedlings when treated with 200  $\mu\text{M}$  NPA. Seedlings of the mutants treated with NPA 14 d after germination formed fewer pseudonodules; approximately 55% of the seedlings treated with 20  $\mu\text{M}$  NPA and 15% of the seedlings treated with 200  $\mu\text{M}$  NPA formed pseudonodules. Treatment with 20  $\mu\text{M}$  NPA 7 d after germination was chosen for further experiments.

No visible differences were apparent between the NPA-elicited pseudonodules found on wild-type sweetclover (U389) and the pseudonodules formed on all of the mutants in the *sym-1*, *sym-2*, *sym-3*, and *sym-5* loci; pseudonodules on *sym-3* allele BT70 are shown in Figure 2. The number of pseudonodules on individual plants ranged from 1 to 12 white pseudonodules 4 weeks after application of 20  $\mu\text{M}$  NPA. The size of pseudonodules on an individual plant also varied, with an average diameter of about 1 mm. Pseudonodules on wild type and all of the mutants were



**Figure 2.** Pseudonodules elicited on an allele in the *sym-3* locus of sweetclover following treatment with 20  $\mu\text{M}$  NPA. Small, spherical nodules of varying sizes are indicated by arrows along the primary root of two plants of allele BT70 4 weeks after NPA treatment. The lower portion of the root, containing no pseudonodules or lateral roots, was trimmed from each photograph.

found on the upper portion of the tap root, in the same region in which nitrogen-fixing nodules are formed on sweetclover plants inoculated with *R. meliloti* (Utrup et al., 1993), and were rarely found on lateral roots. The addition of 5 mM  $\text{KNO}_3$  and 5 mM  $\text{CaNO}_3$  did not inhibit formation of NPA-elicited pseudonodules on wild-type sweetclover but did block formation of nitrogen-fixing nodules on wild-type plants inoculated with *R. meliloti*.

All of the sweetclover mutants formed pseudonodules on 30 to 60% of the total number of plants treated with NPA, a rate slightly lower than observed for wild-type plants (Table II). All of the alleles in the *sym-1* and *sym-3* loci exhibiting a non-nodulating phenotype when inoculated with *R. meliloti* (Table I) formed large numbers of pseudonodules after NPA treatment. The number of plants that formed pseudonodules following NPA treatment of

**Figure 1.** (On facing page.) Spot inoculation of the *sym-3* allele BT70 with *R. meliloti*. Seedlings of mutant and wild-type plants grown on nodulation plates were spot inoculated with *R. meliloti* strain SU47 in the zone of emerging root hairs 48 h after germination. The inoculation spot was marked with dye, visible as particles marked by the open arrows (A–G). Fixed roots were serially sectioned and stained with hematoxylin and median longitudinal sections were compared with noninoculated controls (not shown) at each time. A, *sym-3* allele BT70 48 h after spot inoculation. B, Wild-type sweetclover 48 h after inoculation; arrowhead indicates a nodule primordium. C, *sym-3* allele BT70 3 d after inoculation; solid arrow indicates a lateral root primordium near the inoculation spot (open arrow). D, Wild type 3 d after inoculation; arrowhead indicates nodule primordium; arrow indicates lateral root primordium. E, *sym-3* allele BT70 7 d after inoculation. F, Oblique section of emerging nodule (arrowhead) on wild type 7 d after inoculation; star marks agar debris from nodulation plate. G, *sym-3* allele BT70 11 d after inoculation. H, Nitrogen-fixing nodule on wild-type sweetclover 21 d after inoculation; arrowheads indicate vascular tissue; arrow indicates nodule meristem. Bars = 50  $\mu\text{m}$  (A–G); 400  $\mu\text{m}$  (H).

**Table II.** Frequency of formation of pseudonodules elicited by NPA on symbiotic mutants of sweetclover

Seedlings of wild-type and mutant sweetclover were grown in sterile vermiculite and treated with 20  $\mu\text{M}$  NPA 1 week after germination (Hirsch et al., 1989). White, spherical pseudonodules were harvested 28 d later.

Locus	Allele	No. of Plants NPA Treated <sup>a</sup>	No. of Plants with Pseudonodules <sup>b</sup>	Percentage of Plants with Pseudonodules
<i>sym-1</i>	BT35	380	160	42
	BT58	660	420	64
	BT62	820	500	61
<i>sym-2</i>	BT59	2190	1380	63
<i>sym-3</i>	BT61	240	82	33
	BT70	1120	630	56
<i>sym-5</i>	BT71	990	620	63
Wild type		1390	930	67

<sup>a</sup> The number of plants treated varied because of availability of seeds. <sup>b</sup>  $\chi^2 = 65$ , using the  $\chi^2$  test for larger frequency tables (Finney, 1980), indicating that the observed differences in frequency of formation of pseudonodules are significant at the 99% probability level.

allele BT62 in the *sym-1* locus was also greater than the number of plants that form ineffective nodules when this allele is inoculated with *R. meliloti* (compare to Table I). This observation was also true for mutants in the *sym-2* and *sym-5* loci that form ineffective nodules with *R. meliloti* (cf. Tables I and II).

The structure of pseudonodules elicited on the sweetclover mutants and wild-type sweetclover by NPA treatment was analyzed by paraffin serial sectioning (Jensen, 1962). Like pseudonodules elicited by ATIs on alfalfa (Hirsch et al., 1989) and Afghanistan pea (Scheres et al., 1992), the sweetclover pseudonodules were usually spherical in shape and lacked a clearly defined apical meristem, as shown in Figure 3. Abundant vascular tissue was present in the proximal and central portion of the pseudonodules, surrounded by a layer of smaller cells often filled with amyloplasts (not shown). The outer cells of pseudonodules on *sym-1* alleles, the *sym-5* mutant, and wild-type sweetclover (Fig. 3, A, B, E, and 3F) were larger and highly vacuolate and often resembled a nodule cortex, except that this layer of cells sometimes completely surrounded the pseudonodule (see Fig. 3B, for example). These pseudonodules also contained a densely delineated endodermal layer, clearly outlined under dark field (not shown). Occasionally, multilobed pseudonodules were observed in this group (Fig. 3, B and E). In pseudonodules from the *sym-2* mutant and *sym-3* alleles (Fig. 3, C and D), the outer cells were generally smaller and the endodermis was less apparent. However, the structure of pseudonodules observed on each mutant varied considerably within a range encompassed by the examples shown in Figure 3.

#### The NPA-Elicited Pseudonodules Express the ENOD2 Gene

To assess whether the pseudonodules elicited on the sweetclover mutants by NPA expressed nodulin genes, we

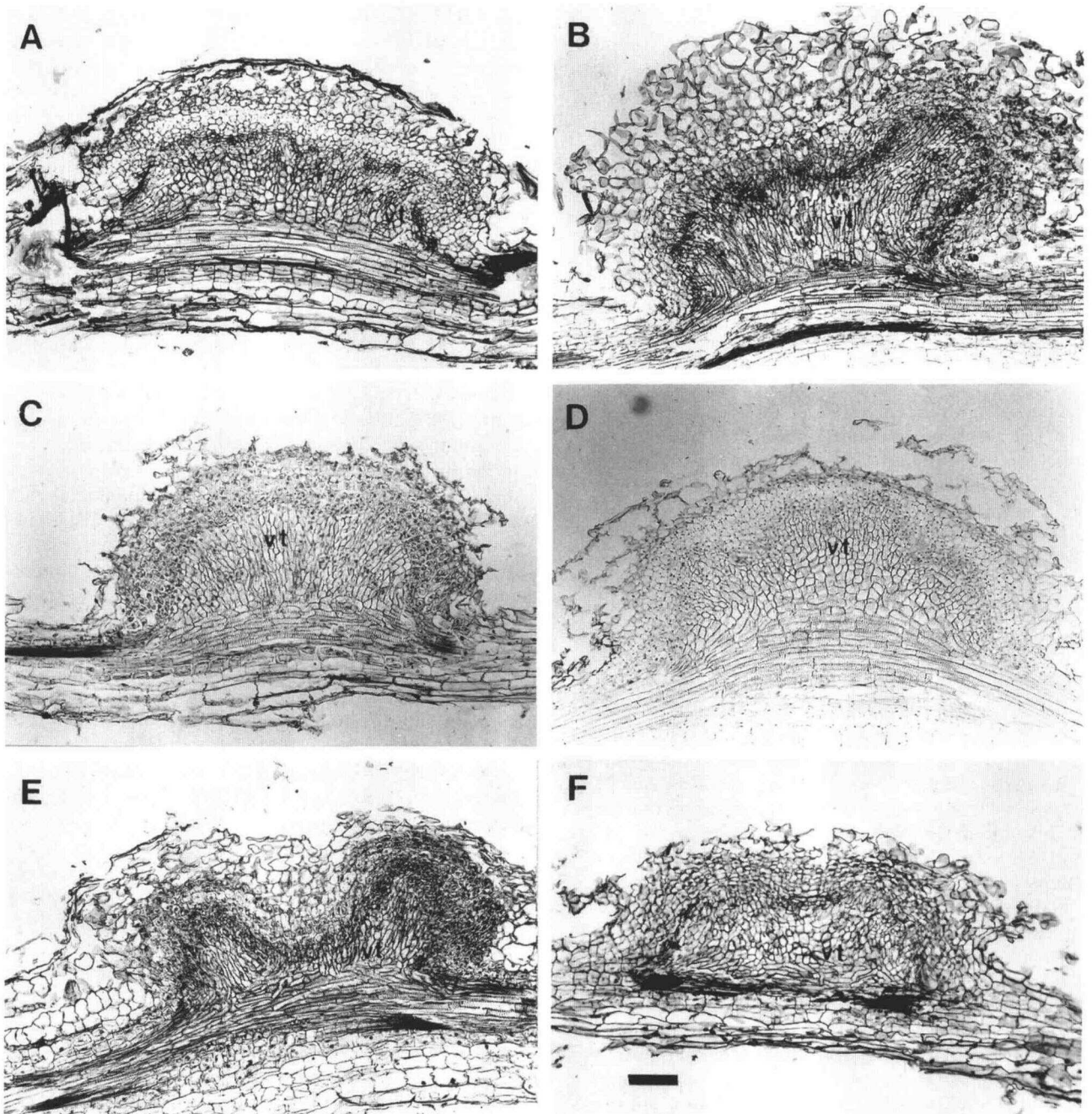
analyzed total RNA for expression of the ENOD2 gene by northern blot analysis using a heterologous probe from alfalfa (Dickstein et al., 1988). As shown in Figure 4, the ENOD2 probe hybridized to RNA from nitrogen-fixing wild-type nodules infected by *R. meliloti* (lane 1), as well as to RNA from NPA-elicited pseudonodules on the nodulating *sym-1* allele BT62, the *sym-2* mutant, *sym-3* allele BT70, and the *sym-5* mutant (lanes 2–5). No hybridization occurred in lane 6, which contained RNA from untreated wild-type sweetclover roots. The transcript hybridizing to the ENOD2 probe was determined to be 1.3 kb in size by comparison to RNA molecular weight standards (data not shown). Possibly because the tissue samples used for RNA isolation unavoidably contained attached root tissue, the level of hybridization observed for NPA-elicited pseudonodules on each mutant varied between samples; no quantitative conclusion regarding the level of hybridization should be drawn from the northern blot shown in Figure 4. However, ENOD2 expression was observed in all of the NPA-elicited pseudonodules from all of the mutants tested. No ENOD2 expression was detected in roots from mutant control plants that were not treated with NPA (not shown).

#### DISCUSSION

In this study, nodulation of wild-type and symbiotic mutants of sweetclover has been examined in detail using both *R. meliloti* and the ATI NPA to elicit nodules. Not unexpectedly, we found that NPA elicited nodule-like structures, or pseudonodules, on the roots of wild-type sweetclover similar to those observed on alfalfa treated with NPA (Hirsch et al., 1989). We also discovered that NPA elicited pseudonodules on the roots of seven sweetclover nodulation-defective mutants, representing four distinct genetic loci (Miller et al., 1991). This included an allele in the *sym-3* locus that was shown to be deficient in its ability to undergo cortical cell divisions following spot inoculation of roots with *R. meliloti*.

We previously characterized the phenotype of a single mutant from each of five genetic loci required for symbiosis in white sweetclover and found that each mutant was blocked at a distinct stage of infection or early nodule development (Utrup et al., 1993). An allele from the *sym-3* locus exhibited the most profound nodulation deficiency: only a limited Had<sup>+</sup> response was observed after inoculation of this mutant with *R. meliloti*. The single mutant in the *sym-5* locus and one allele from the *sym-1* locus were found to have phenotypes similar to that of wild-type alfalfa treated with purified NodRm-IV(S) (Truchet et al., 1991): root hairs were deformed or branched in response to *R. meliloti* and ineffective white nodules were formed. The single *sym-2* mutant had the most advanced phenotype, producing white, ineffective nodules containing infection threads after inoculation with *R. meliloti*. The single mutant available in the *sym-4* locus did not appear to be part of the same developmental sequence suggested by the phenotypes of the other mutants, because it exhibited infection threads but only rarely formed either ineffective or nitrogen-fixing nodules. This mutant was not further analyzed



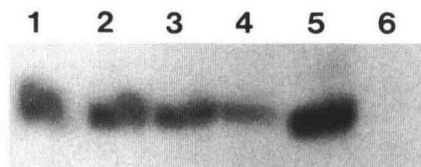


**Figure 3.** Longitudinal sections of NPA-elicited pseudonodules on sweetclover *sym* mutants. The pseudonodules were analyzed by paraffin serial sectioning and stained with safranin/fast green 4 weeks after treatment with 20  $\mu\text{M}$  NPA. vt, Vascular tissue; bar = 100  $\mu\text{m}$ . A, *sym-1* allele BT58 (non-nodulating with *R. meliloti*); B, *sym-1* allele BT62 (forms ineffective nodules with *R. meliloti*); C, *sym-2* mutant BT59; D, *sym-3* allele BT70; E, *sym-5* mutant BT71; F, wild-type sweetclover.

in the current study because of insufficient seed. Together, these data suggested that different plant genes are required for infection thread formation than for  $\text{Had}^+$  and nodule morphogenesis.

In the current study, we have extended our analysis of the *sym* loci of sweetclover to additional alleles of the *sym-1* and *sym-3* loci (Miller et al., 1991). Using large numbers of plants, we found that the three new *sym-1* alleles failed to

nodulate after inoculation with *R. meliloti* (Table I); we have also observed that two of these alleles, BT35 and BT58, exhibit a  $\text{Had}^+$  response to *R. meliloti* (A.J. Cary and J.H. Norris, unpublished data). The BT62 *sym-1* allele, previously characterized as  $\text{Had}^+$  with ineffective nodules (Utrup et al., 1993), nodulated at a low frequency (12%) in this study (Table I). Comparison of the non-nodulating phenotype of the three new *sym-1* alleles with the low



**Figure 4.** Expression of ENOD2 in NPA-elicited pseudonodules on sweetclover *sym* mutants. Approximately 10  $\mu$ g of total RNA were loaded in each lane of a gel probed with an ENOD2 clone from alfalfa labeled with digoxigenin-dUTP that hybridized to a 1.3-kb transcript. Lane 1, RNA from nitrogen-fixing nodules inoculated with *R. meliloti*; lanes 2 to 5, RNA from NPA-elicited nodules on mutants: lane 2, *sym-1* allele BT62; lane 3, *sym-2* mutant; lane 4, *sym-3* allele BT70; lane 5, *sym-5* mutant; lane 6, RNA from noninoculated, untreated wild-type roots.

frequency of nodulation observed in BT62 suggests that allele BT62 may produce a small amount of functional gene product or the mutated gene product may be partially functional. We note that the nodules elicited on BT62 are white and ineffective rather than nitrogen fixing. This could be interpreted to mean that *sym-1* is required for both nodule morphogenesis and infection, an explanation supported by the absence of infection threads in this mutant (Utrup et al., 1993).

All three mutant alleles in the *sym-3* locus, BT61, BT69, and BT70, failed to form visible nodules after inoculation with *R. meliloti* (Table I), confirming our earlier observations of the BT70 allele (Utrup et al., 1993). To closely examine the possibility that limited cortical cell divisions might occur that fail to develop into visible nodule primordia, we carried out spot-inoculation assays on the BT70 allele, followed by serial sectioning of roots at several times after inoculation, following the method of Dudley et al. (1987). We found that BT70 demonstrated no cortical cell divisions in response to *R. meliloti* (Fig. 1), similar to the non-nodulating alfalfa mutant MN-1008 (Dudley and Long, 1989). However, BT70 differs from MN-1008 in that BT70 displays a Had<sup>+</sup> response to *R. meliloti*, whereas MN-1008 shows no root-hair response (Dudley and Long, 1989). This suggests that the BT70 allele recognizes the bacterial NodRm-IV(S) signal in some limited fashion but lacks some secondary signal transduction event required to trigger cortical cell divisions.

All of the non-nodulating alleles in the *sym-1* and *sym-3* loci, including BT70, formed NPA-elicited pseudonodules at a high frequency. In addition, for all of the mutants that formed ineffective nodules in response to *R. meliloti*, *sym-1* allele BT62 and mutants in the *sym-2* and *sym-5* loci, the frequency of formation of NPA-elicited pseudonodules was much higher than the frequency of nodulation observed after inoculation with *R. meliloti*.

The anatomy and structural characteristics of the NPA-elicited pseudonodules on the sweetclover mutants appeared to be very similar to pseudonodules elicited by ATIs on alfalfa (Hirsch et al., 1989) and the intermediate-sized pseudonodules found on Afghanistan pea (Scheres et al., 1992). The sweetclover pseudonodules were spherical or multilobed in shape but lacked the distinct apical meristem normally found in nitrogen-fixing nodules (Fig. 3), similar to empty nodules

elicited by *exo* mutants of *R. meliloti* (Yang et al., 1992). Rather than the peripheral vascular bundles found in nitrogen-fixing nodules, the pseudonodules contained a broad, proximal region of vascular tissue. Although some structural differences were observed in the pseudonodules on different mutants, no conclusions are possible until tissues in the NPA-elicited pseudonodules are further characterized by *in situ* hybridization.

The early nodulin ENOD2, expressed in cells of the nodule parenchyma along the periphery of the nodule in pea, soybean, and alfalfa nodules (Van de Wiel et al., 1990a, 1990b), has been used as a molecular marker for the development of nodule parenchyma tissue in pseudonodules elicited by ATIs on alfalfa and Afghanistan pea (Scheres et al., 1992) and in nodules elicited by *R. meliloti* nodulation mutants carrying a *trans-zeatin* secretion gene (Cooper and Long, 1994). ENOD2 was expressed in the NPA-elicited pseudonodules on all of the sweetclover mutants, as detected by blot hybridization, indicating that these pseudonodules share at least some of the developmental characteristics of nodules elicited by *R. meliloti*. We also detected expression of ENOD8 (Dickstein et al., 1993) in NPA-elicited pseudonodules on all of the sweetclover mutants described here (C. Wu, R. Dickstein, A.J. Cary, and J.H. Norris, unpublished data).

The finding of formation of pseudonodules on allelic non-nodulating mutants from the *sym-1* and *sym-3* loci of sweetclover after treatment with the ATI NPA contributes to a growing body of evidence that the bacterial Nod factors may cause perturbations in endogenous plant hormone levels. We speculate, as was suggested previously by Hirsch et al. (1989), that the ATI NPA may be mimicking a secondary signal required for nodule organogenesis, resulting in the formation of pseudonodules on all of the mutants. The possibility that this signal involves an increase in the cytokinin-to-auxin ratio in the root cortex tissue is suggested by both pseudonodules elicited by ATIs (Hirsch et al., 1989; Scheres et al., 1992; this study) and nodules elicited by *R. meliloti* nodulation mutants carrying a *trans-zeatin* secretion gene (Cooper and Long, 1994), although some differences in the characteristics of these nodules remain to be resolved (Cooper and Long, 1994). The fact that alleles in both the *sym-1* and *sym-3* loci that are non-nodulating with *R. meliloti* form pseudonodules after NPA treatment indicates that at least two plant genes act upstream of this point in signal transduction leading to nodule organogenesis.

Recently, Ardourel et al. (1994) proposed a model for the role of *R. meliloti* Nod factors in which two root-hair receptors of differing stringency for recognition of Nod factors activate two signal transduction pathways, both required for nodule morphogenesis. The putative "signaling receptor" pathway, involved in cell-wall tip growth and cortical cell activation, is responsible for the Had<sup>+</sup> response to *nodF/nodL* mutants of *R. meliloti*, accompanied by cortical cell activation but no nodules. The proposed "entry receptor" pathway of greater stringency for the nonreducing end substitutions of NodRm factors, based on the symbiotic phenotype of *nodFE* and *nodL* mutants, is involved in for-



mation of infection sites and infection thread development (Ardourel et al., 1994). The phenotypic response of the sweetclover *sym* mutants to *R. meliloti*, characterized in this study and by Utrup et al. (1993), supports this model with the following interpretation and modification.

The Had<sup>+</sup> phenotype of the *sym-3* allele BT70, in which no cortical cell divisions were observed, would indicate that the signaling receptor pathway is intact in this mutant to the extent of allowing root-hair deformations, but the remainder of the pathway leading to cortical cell activation is blocked. But the mutant also fails to form a Hac<sup>+</sup>/Inf response through the entry receptor pathway, even though genetic analysis indicates that all of the sweetclover mutants are monogenic and recessive (Miller et al., 1991). Based on this observation, we propose that the signal receptor pathway must activate the entry receptor pathway for the more stringent response to Nod factor to occur, leading to root-hair curling and infection thread development. The *sym-3* BT70 allele and possibly the Had<sup>+</sup>, non-nodulating *sym-1* alleles must be blocked in the signaling receptor pathway prior to the point of activation of the entry receptor pathway. In comparison, the Hac<sup>+</sup>, non-nodulating *sym-4* mutant (Utrup et al., 1993) may be blocked late in the signaling receptor pathway, before cortical cell activation but after the entry receptor pathway leading to formation of infection threads has been activated. It will be of great interest to determine whether cortical cell activation occurs in the *sym-4* mutant and the non-nodulating *sym-1* alleles.

The Had<sup>+</sup> *sym-5* mutant, which forms ineffective nodules, appears to have an intact signaling receptor pathway, but may be blocked in the entry receptor pathway, so that root-hair curling and infection thread formation do not occur. The Hac<sup>+</sup> *sym-2* mutant, in which infection threads and ineffective nodules are observed, appears to be blocked late in the entry receptor pathway. These hypotheses regarding the relationship of the sweetclover *sym* genes to the two-receptor model of Ardourel et al. (1994) could be tested in part by construction of double mutants, such as a *sym-4/sym-5* mutant, in which an additive phenotype (Had<sup>+</sup> and non-nodulating) would support the model, and an epistatic effect would suggest a single signal transduction pathway. We are also investigating the hypothesis that a defense response occurs in the sweetclover mutants after inoculation with *R. meliloti*, as elaborated in the model recently proposed by Mellor and Collinge (1995).

We suggest that the ATI NPA may be circumventing a secondary signal of plant origin in the signaling receptor pathway (Ardourel et al., 1994) that is required for cortical cell activation and nodule organogenesis, resulting in the formation of pseudonodules. This signal is normally missing in the *sym-3* alleles and possibly in the non-nodulating *sym-1* alleles, which form pseudonodules only in the presence of NPA. The fact that these mutants form pseudonodules rather than simply exhibiting cortical cell activation, as did alfalfa plants inoculated with a *nodF/nodL* mutant of *R. meliloti* (Ardourel et al., 1994), suggests that the factor mimicked by NPA may be limiting in the plants inoculated with the *nodF/nodL* mutant, which do nodulate at a very low frequency (Ardourel et

al., 1994). Alternatively, the formation of ATI-elicited pseudonodules, as well as nodules elicited by bacteria carrying a *trans*-zeatin secretion gene (Cooper and Long, 1994), may indicate that cross-talk occurs between the signaling receptor pathway and the entry receptor pathway to trigger nodule morphogenesis following cortical cell activation. Based on the data presented here, we propose that the products of the *sym-1* and *sym-3* genes of sweetclover are required for plant-regulated steps of the signaling receptor transduction pathway (Ardourel et al., 1994). Further characterization of the sweetclover *sym* mutants should assist in elucidation of the signal transduction pathways leading to nodule organogenesis.

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