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# Regulation of Nodulation by *Rhizobium meliloti* 102F15 on Its Mutant Which Forms an Unusually High Number of Nodules on Alfalfa

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**A mutant (WL3A150) of *Rhizobium meliloti* 102F51 that elicits an unusually high number of nodules on its host, alfalfa (*Medicago sativa*), supports the idea that the host may rely on early bacteroid development in the nodule or on metabolites produced in the infection thread as one of the signals to control further nodulation. This mutant was initially isolated because of its Fix⁻ phenotype. It consistently formed many more nodules than all the other Fix⁻ mutants isolated from strain 102F51 (a total of 11 mutants). Nodules formed by this mutant were small and white and were indistinguishable in appearance from nodules formed by the other Fix⁻ mutants. An ultrastructural study of the nodules, however, showed that this mutant, although forming numerous infection threads, failed to develop into bacteroids. The ability of the mutant to form an unusually high number of nodules could be suppressed in a time-dependent manner by the presence of the wild type.**

The nodulation process of legumes by compatible *Rhizobium* spp. involves the establishment of a delicate balance between the symbionts to achieve a beneficial relationship. The total number of nodules formed on the roots of a legume species usually lies within a certain range if the host plants are grown under well-defined conditions and are inoculated in excess with compatible *Rhizobium* strains. Control of nodulation is essential in maintaining a symbiotic relationship, since the development and subsequent maintenance of the nodules is very energy requiring (4, 5). Excessive uncontrolled nodulation would overburden the host plant and could shift the balance towards a parasitic relationship.

Although it is generally believed that control of nodulation must be exerted by the host plant in response to certain signals produced by the bacteroid, direct evidence is scarce. One such signal may be the amount of fixed nitrogen available to the host. At or above certain critical concentrations, nitrogen sources such as nitrate and ammonium inhibit nodulation in many legumes. In clover, fixed nitrogen inhibits the synthesis (or mobilization) on the root surface of a lectin believed essential for the initiation of the nodulation process (1). In this communication, we present evidence which suggests that metabolites other than fixed nitrogen resulting from early infection events may be signals the host depends on to initially control nodulation.

Mutants defective in symbiotic nitrogen fixation (Fix⁻) were isolated from *Rhizobium meliloti* 102F51 after treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and extended growth (over 30 generations) in a minimal medium by direct selection on alfalfa plants. These mutants were either Nod⁻ or Fix⁻ and were isolated at a frequency of $10^{-3}$ for Nod⁻ mutants and $10^{-2}$ for Fix⁻ mutants. Three Nod⁻ mutants in this collection have been described previously (7). Nodules formed by some of the Fix⁻ mutants are white but still contain some leghemoglobin detectable with antisera prepared against purified alfalfa leghemoglobin species (2). All of the Fix⁻ mutants formed more nodules on alfalfa roots than did the wild type. However, WL3A150 formed more nodules than did any of the other Fix⁻ mutants. This mutant consistently formed up to 10 times more nodules than did the wild type (Fig. 1). Other Fix⁻ mutants formed a range of three to six times more nodules than did the wild type. This nodulation phenomenon (i.e., the host fails to control nodulation in response to infection by Fix⁻ mutants) pro-

TABLE 1. Nodulation and acetylene reduction activity of alfalfa seedlings inoculated with wild-type 102F51, Fix⁻ mutant WL3A150, and two partially effective mutants, WL4A56 and WL2B35

| *R. meliloti* strain | Days after inoculation | No. (± SD) of nodules · plant$^{-1}$ | nmol (± SD) of acetylene reduced · plant$^{-1}$ · hour$^{-1}$ |
|---|---|---|---|
| 102F51 | 7 | 3.1 ± 1.7 | 29.6 ± 27.8 |
| | 14 | 5.5 ± 2.1 | 61.4 ± 28.5 |
| | 21 | 7.2 ± 3.2 | 54.0 ± 19.5 |
| | 28 | 6.7 ± 3.9 | 36.2 ± 13.3 |
| | 35 | 5.5 ± 2.6 | 30.2 ± 12.1 |
| WL4A56 | 7 | 3.4 ± 2.1 | 3.0 ± 3.9 |
| | 14 | 13.9 ± 4.4 | 10.5 ± 7.6 |
| | 21 | 21.1 ± 7.5 | 15.8 ± 9.3 |
| | 28 | 35.9 ± 10.7 | 7.0 ± 3.2 |
| | 35 | 36.9 ± 18.6 | 9.9 ± 7.9 |
| WL2B35 | 7 | 4.7 ± 2.0 | 1.2 ± 1.1 |
| | 14 | 12.2 ± 3.1 | 1.1 ± 0.8 |
| | 21 | 22.9 ± 8.6 | 2.5 ± 2.3 |
| | 28 | 33.2 ± 10.7 | 0.4 ± 0.6 |
| | 35 | 49.1 ± 26.5 | 1.9 ± 1.3 |
| WL3A150 | 7 | 3.2 ± 2.1 | <0.1 |
| | 14 | 14.7 ± 2.7 | <0.1 |
| | 21 | 33.0 ± 13.6 | <0.1 |
| | 28 | 53.3 ± 18.8 | <0.1 |
| | 35 | 69.5 ± 31.6 | <0.1 |

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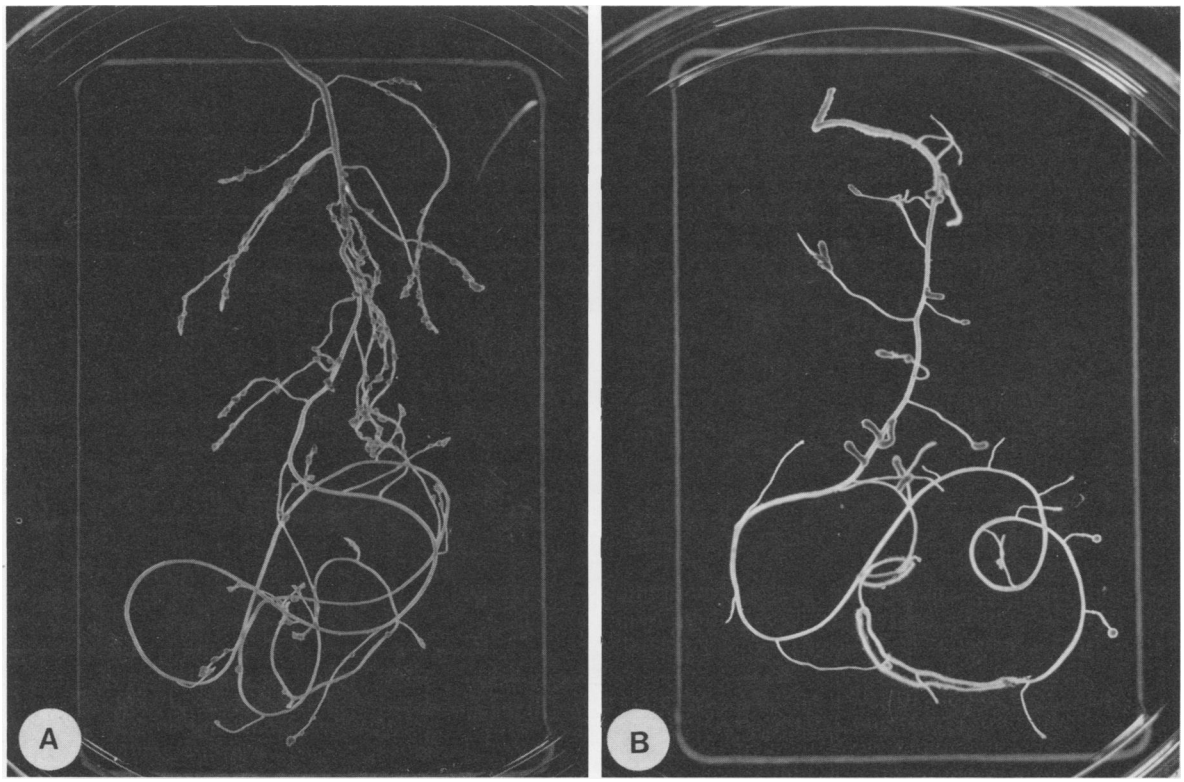



FIG. 1. Nodulation phenotypes of Fix$^-$ mutant WL3A150 (A) and a partially effective mutant, WL4A56 (B). The seedlings are of identical age. Note the unusually high number of nodules formed in panel A.

vides evidence that the host depends on signals provided directly or indirectly by infecting rhizobia. Presumably, at least one of the signals is a result of the nitrogen fixation process. This is indicated by the fact that other mutants in this collection with partial nitrogenase activity form, on the average, a nodule number intermediate between those of the wild type and the Fix$^-$ mutants (Table 1). However, data obtained with mutant WL3A150 indicate that metabolites from early infection events, probably that of early bacteroid development, may also play a role in controlling nodulation.

The only observable difference between WL3A150 and the other mutant strains came from transmission electron microscopic studies. Nodules formed by WL3A150 contained infection threads but no recognizable bacteroids. Rhizobia released into the plant cytoplasm from these infection threads were quickly degraded (Fig. 2) and failed to develop into enlarged pleomorphic bacteroids. Plant cells in these nodules, especially those with infection threads, contained an abundance of endoplasmic reticulum. This was apparently in response to infection by WL3A150, since nodules formed by the wild type and the other Fix$^-$ mutants did not contain excessive amounts of endoplasmic reticulum. Previous studies suggest that, at least in alfalfa, rhizobia begin to differentiate into bacteroids as soon as they are in the infection thread (6). Mutant WL3A150 seems to be defective in this early differentiation step and may alert a host defense mechanism that quickly destroys rhizobia as they are released from the infection thread. The lack of bacteroid formation in these nodules is also confirmed by centrifugation studies of nodule homogenates in a step-wise sucrose gradient (8). Bacteroids from alfalfa nodules have previously been shown to accumulate at the 45/50% interface ($\rho$ = 1.224). No rhizobia from nodules formed by WL3A150 accumulated at this interface (data not shown). These observations, along with the ability of this mutant to form an unusually high number of nodules, suggest that, apart from the fixation process, the early infection event of bacteroid development itself may present a signal(s) to the host to begin to control nodulation and that this signal(s) is not expressed by mutant WL3A150. To test this hypothesis, we performed the following experiments.

Alfalfa seedlings were inoculated with the wild type or mutant WL3A150. At various intervals after the primary inoculation, seedlings were reinoculated with the other strain to challenge the initial inoculation. The results show that when the wild type and WL3A150 were applied together (data not shown) or the wild type was applied before the mutant (Table 2), the ability of the mutant to form an unusually high number of nodules was greatly suppressed. When the mutant was applied first, subsequent inoculation with the wild type resulted in partial suppression. The degree of suppression seemed dependent on the time elapsed between the primary and secondary inoculations.

These results may be explained by the hypothesis that the wild type, but not WL3A150, produces a regulatory nodulation signal early during bacteroid formation (perhaps before release from the infection thread). This signal may also repress (or avoid the induction of) a host defense mechanism, thus preventing bacteroid destruction after release from the infection thread. Primary inoculation with WL3A150 and subsequent challenge with the wild type would lead to partial suppression of nodulation as the wild type began to produce the regulatory signal. The earlier the challenge, the greater would be the degree of suppression.

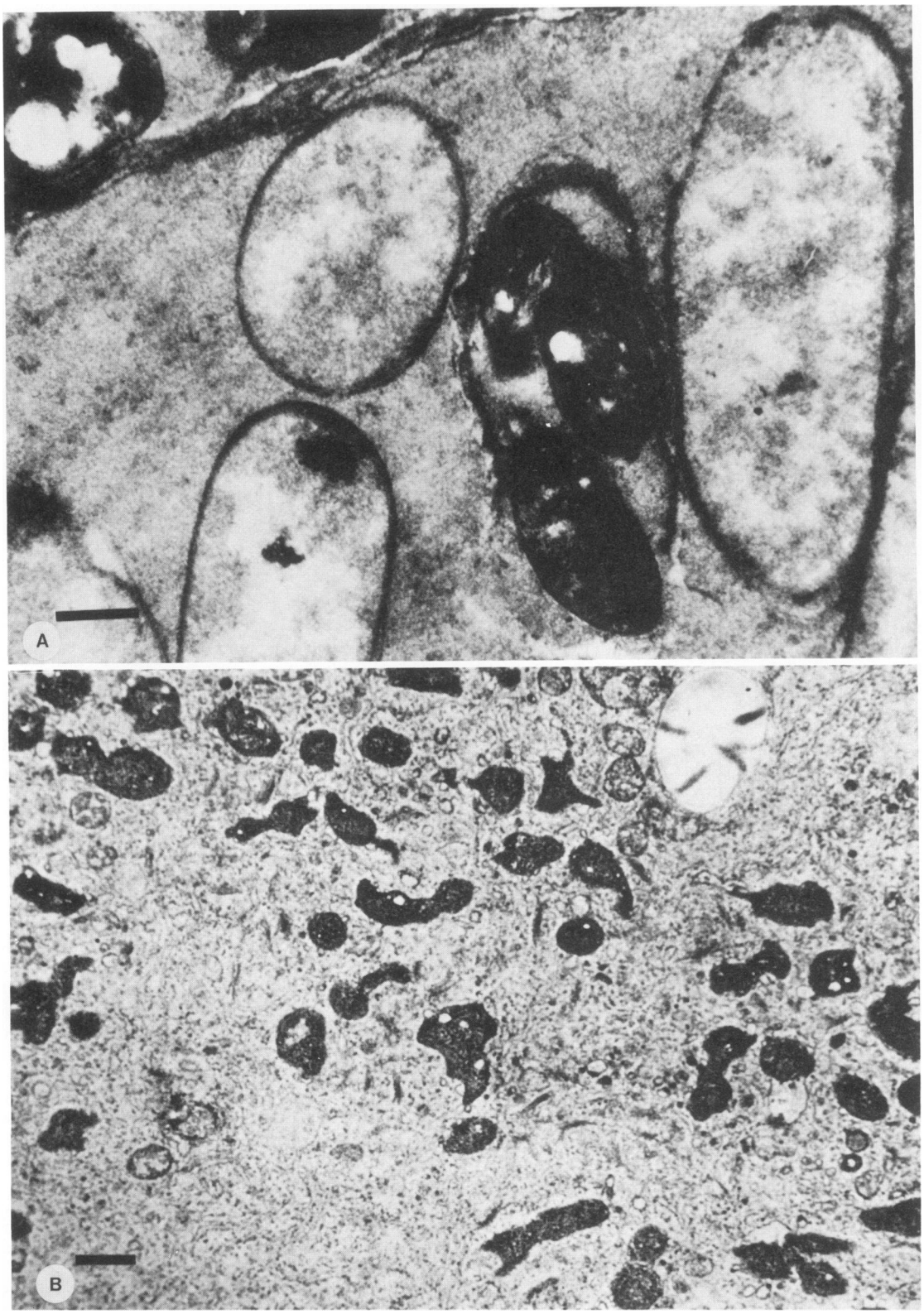
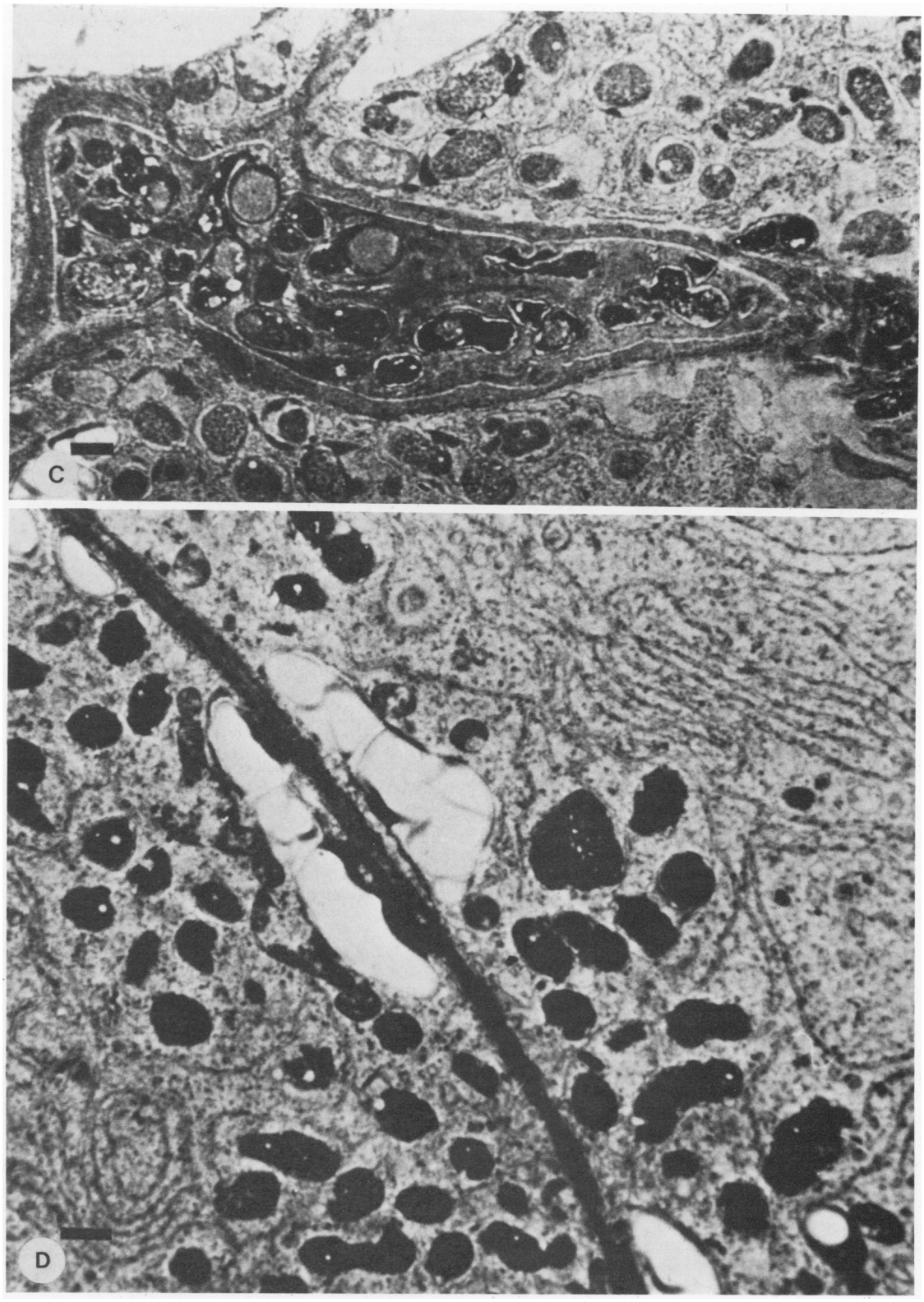



FIG. 2. Electron micrographs showing the failure of mutant WL3A150 to develop into bacteroids and its destruction after release from the infection thread. (A) Bacteroids and infection thread formed by wild-type 102F51 in the plant nodule cytoplasm; (B) aborted bacteroids of Fix$^-$ mutant WL3A150 undergoing deterioration in the nodule cytoplasm; (C) infection thread formed by mutant WL3A150; (D) micrograph showing the abundance of endoplasmic reticulum in the nodule cytoplasm infected by mutant WL3A150. Bars, approximately 1 μm.




The regulatory signal may also prevent degradation of WL3A150 cells occupying the same nodule with the wild type, since WL3A150 could be isolated from either white or pink nodules in as high frequencies as the wild type from all doubly infected plants (see footnote *b* to Table 2). Primary inoculation with the wild type was expected to suppress excessive nodulation by WL3A150 no matter when the challenge was made, because the wild type would already be

TABLE 2. Time-dependent nodulation of alfalfa seedlings in response to primary inoculation and subsequent challenge by mutant WL3A150 and wild-type 102F51[a]

| Zero-time inoculant | Challenged | | 2 wk | | | 3 wk | | | 4 wk[b] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | By | At time (h) | No. of nodules | No. of pink nodules | Range of nodule no. | No. of nodules | No. of pink nodules | Range of nodule no. | No. of nodules | No. of pink nodules | Range of nodule no. |
| WL3A150 | | | 15.6 | 0 | 11–21 | 24.5 | 0 | 23–26 | 51.8 | 0 | 26–70 |
| WL3A150 | 102F51 | 4 | 6.5 | 2.6 | 2–11 | 19.4 | 3.6 | 8–25 | 18.7 | 4.3 | 13–20 |
| WL3A150 | 102F51 | 48 | 10.2 | 1.0 | 6–13 | 18.5 | 1.2 | 9–28 | 30.4 | 2.0 | 18–24 |
| WL3A150 | 102F51 | 144 | 8.5 | 0 | 4–13 | 30.6 | 0 | 17–38 | 33.0 | 0.2 | 16–57 |
| WL3A150 | 102F51 | 192 | 9.2 | 0 | 4–15 | 26.0 | 0 | 14–41 | 39.0 | 0 | 20–61 |
| None | 102F51 | 4 | 4.3 | 4.3 | 1–6 | 5.8 | 5.8 | 2–13 | 10.0 | 10.0 | 6–13 |
| | 102F51 | 48 | 7.0 | 5.0 | 5–11 | 7.4 | 7.4 | 4–12 | 12.2 | 12.2 | 3–17 |
| | 102F51 | 144 | 10.3 | 2.0 | 7–13 | 10.8 | 8.0 | 9–13 | 16.0 | 16.0 | 14–19 |
| | 102F51 | 192 | 9.0 | 0 | 7–11 | 7.3 | 5.0 | 6–8 | 9.6 | 9.6 | 7–14 |
| 102F51 | | | 5.0 | 3.0 | 3–8 | 5.8 | 5.8 | 4–8 | 5.5 | 5.5 | 3–6 |
| 102F51 | WL3A150 | 3 | 5.8 | 2.8 | 4–8 | 6.0 | 3.2 | 4–9 | 7.4 | 3.0 | 5–13 |
| 102F51 | WL3A150 | 48 | 5.8 | 4.2 | 5–7 | 7.0 | 6.2 | 5–12 | 7.0 | 7.0 | 5–13 |
| 102F51 | WL3A150 | 144 | 5.0 | 4.4 | 2–6 | 5.5 | 5.3 | 2–7 | 7.5 | 7.5 | 5–12 |
| 102F51 | WL3A150 | 192 | 3.8 | 3.4 | 3–5 | 4.0 | 4.0 | 3–5 | 6.6 | 6.6 | 5–8 |
| None | WL3A150 | 3 | 17.4 | 0 | 10–25 | 30.8 | 0 | 25–35 | 62.4 | 0 | 59–67 |
| | WL3A150 | 48 | 15.0 | 0 | 12–22 | 25.8 | 0 | 11–44 | 56.8 | 0 | 41–90 |
| | WL3A150 | 144 | 14.8 | 0 | 11–20 | 34.0 | 0 | 15–42 | 59.2 | 0 | 49–80 |
| | WL3A150 | 192 | 11.0 | 0 | 7–16 | 25.2 | 0 | 11–30 | 37.4 | 0 | 19–71 |

[a] Seeds were treated as described previously and then planted in serum vials filled with vermiculite and a plant nutrient solution and inoculated (3). The day the plants were inoculated was considered day zero and the time of inoculation was time zero. Nodule numbers are the averages of two experiments, each with five plants.

[b] Mutant WL3A150 could be reisolated from either white or pink nodules in all doubly inoculated vials after 4 weeks. The mutant was distinguished from the wild type by testing reisolated colonies individually on sterile seedlings for nodulation phenotypes. Mutants formed high numbers of white, inactive nodules, whereas the wild type formed low numbers of pink, active nodules after 4 weeks.

producing the regulatory signal. If only nitrogen fixation by mature bacteroids were required for suppression, it would be expected that suppression would occur later, since nitrogen fixation is detected after 5 days in our assay. Although detection of the actual initiation of nitrogen fixation depends on the sensitivity of the assay, a head start by the wild type for as short as 3 h already is long enough to significantly suppress excessive nodulation by WL3A150.

The results suggest that mutant WL3A150 is defective in some early bacteroid differentiation process which produces important signals for the host plant. These early signals may serve two functions. One is to repress (or avoid the induction of) a host defense mechanism, thus preventing destruction of rhizobia released from the infection thread. The second is to begin control of nodule number, probably through nodule development rather than nodule initiation or infection. Later signals, presumably from the fixation process, prevent further unnecessary nodulation and may act at the site of infection (1). Although mutant WL3A150 has not been genetically characterized and may have multiple mutations, the precisely located lesion of this mutant should make it valuable for understanding the molecular biology and biochemistry of the process of nodulation control.

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