MPMI Vol. 11, No. 2, 1998, pp. 153–155. Publication no. M-1997-1121-01N.

Research Note

Involvement of Salicylic Acid in the Establishment of the *Rhizobium meliloti*–Alfalfa Symbiosis

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Accepted 15 October 1997.

Inoculation of alfalfa plants with either incompatible *Rhizobium* or a *Rhizobium* mutant blocked in Nod factor synthesis led to an accumulation of salicylic acid in roots, in contrast to plants inoculated with a wild-type (compatible) *R. meliloti* strain. When salicylic acid was exogenously applied prior to inoculation of alfalfa plants with either purified Nod factor or compatible *Rhizobium* strains, a significant inhibition of nodule primordia formation and a reduction of the number of emerging nodules, respectively, as well as a delay in nodule visualization, were observed. These results suggest an involvement of *Rhizobium*-synthesized Nod factors in the inhibition of salicylic acid–mediated defense in legumes.

Additional keywords: hypersensitive response, plant defense, systemic acquired resistance.

Salicylic acid (SA) is an important endogenous molecule involved in plant defense. The link between SA production and systemic acquired resistance (SAR) has been well established (Klessig and Malamy 1994; Delaney 1997). Transgenic plants expressing the salicylate dehydrogenase (*nahG*) gene, which converts SA into inactive catechol, do not establish SAR (Gaffney et al. 1993). Furthermore, there is a correlation between an increase in SA levels and plant gene expression. Pathogenesis-related (PR) proteins show up a few hours after the SA level begins to rise (Yalpani et al. 1993). Exogenous SA can induce simultaneous PR expression and resistance to pathogens, even in the absence of pathogenic organisms (Ward et al. 1991).

Rhizobium, *Bradyrhizobium*, and *Azorhizobium* are bacteria that form nitrogen-fixing nodules on legume roots. Their association with legumes is a rather special plant-microbe interaction, in which the successful interaction does not induce a plant defense response. Several bacterial genes are essential for a successful interaction with the host. These are, for example, the nodulation (*nod*) genes, and the genes involved in exopolysaccharide (*exo*) and lipopolysaccharide (*lps*) synthesis (see Spaink 1995). Upon activation by plant flavonoids, the *nod* genes are transcribed and their protein products participate in the biosynthesis of Nod factors (lipochito-oligosaccharides, LCOs) (see Long 1996). These Nod factors induce at least the first steps of nodule formation. Although the function of *exo* and *lps* genes in nodulation is not completely clear, several studies strongly suggest a role in the control of the host defense response (Battisti et al. 1992; Niehaus et al. 1993; Perotto et al. 1994). In addition to lipopolysaccharide (LPS) and exopolysaccharide (EPS), Nod factors might also play a role in controlling defense, as suggested by the fact that Nod factors can act as elicitors of phytoalexins biosynthesis (Savouré et al. 1994, 1997). Furthermore, Vasse et al. (1993) showed that, in a compatible interaction, the bacteria induce a hypersensitive response (HR), and propose that this is part of the plant mechanism controlling the number of successful infections. During the recognition process, either the plant discriminates between the mutualistic and the pathogenic bacteria, or the mutualistic ones elude the host defense response. McKhann et al. (1997), studying the expression of genes of the PAL pathway, suggest that, somehow, rhizobia prevent the triggering of the host defense response. Several reports referring to the resemblance between *Rhizobium*-legume infection and pathogenic organism–plant interactions have been published (Baron and Zambriski 1995; Djordjevic et al. 1986; Long and Staskawicz 1993; Vance 1983).

To determine whether Nod factors might be involved in controlling host defense responses, we quantified SA accumulation in alfalfa plants inoculated with compatible (*R. meliloti* wild type) or incompatible (*R. leguminosarum* bv. *trifolii*) rhizobia as well as a *nod* mutant of *R. meliloti* defective in LCO synthesis.

Ten-day-old alfalfa plants (cv. Aragón), axenically grown in test tubes as previously described (Olivares et al. 1980), were inoculated with 10^8 cell · ml^{-1} of *R. meliloti* strain AK631 or the *nod* mutant AK1672 ($NodC^-$) (Kondorosi et al. 1984) taken from a 24-h culture grown in tryptone–yeast extract medium. Cells were washed twice to remove the culture medium. Fifty to 100 plant roots were harvested and frozen in liquid nitrogen 4, 8, 12, 24, and 48 h after inoculation, and SA content was analyzed according to Rasmussen et al. (1991).

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Figure 1 presents data from a representative experiment. It clearly shows a different pattern of SA accumulation in roots inoculated with *R. meliloti* wild type or the NodC⁻ mutant. SA content in roots inoculated with the wild-type strain remained close to basal levels, whereas in those inoculated with the NodC⁻ mutant it increased markedly and was already apparent 4 h after inoculation. Inoculation of alfalfa with *R. leguminosarum* bv. *trifolii*, strain Rt103 (this laboratory), also induces SA accumulation, compared with *R. meliloti* wild type. Thus, the production of specific Nod factors seems to suppress the accumulation of SA induced by the bacteria.

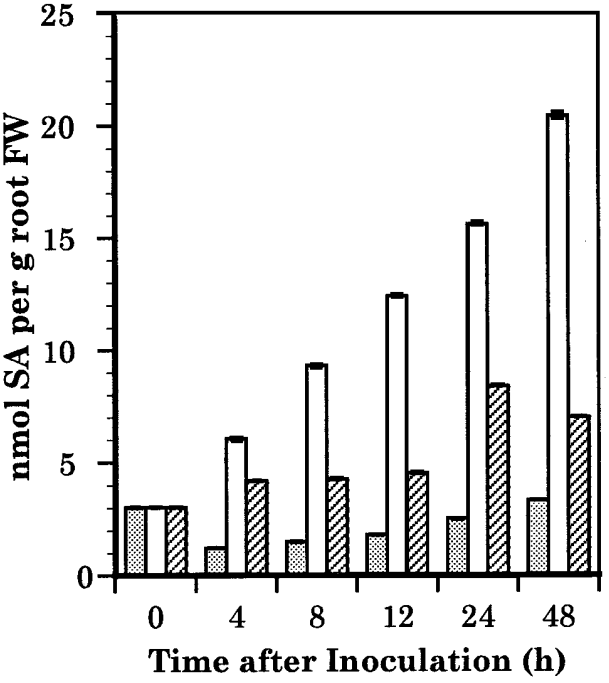

Fig. 1. Salicylic acid (SA) accumulation, expressed as nmol per g of root fresh weight (FW), in roots of alfalfa plants, noninoculated (0 h), or inoculated with *Rhizobium meliloti* wild type (dotted bars), NodC⁻ mutant (white bars), and *R. leguminosarum* bv. *trifolii* (hatched bars) at different times after inoculation. Each data point reported is the average of the amount of SA detected in three replicate samples from one representative experiment. Each experiment was performed at least three times. The value of each replicate is the mean of 5 fluorescent readings taken over 10 s. The detection limit of SA was 1 nmol ml^{-1}.

The effect of exogenously applied SA was analyzed on two parameters of the *R. meliloti*–alfalfa association: nodulation kinetics and the number of nodules formed. Ten-day-old plants, prepared as above, were inoculated with *R. meliloti* AK631 to a final concentration of 10^6 cell · ml^{-1}. SA (25 µM) in the form of sodium salt (Sigma, St. Louis, MO) was added to plant growth medium 24 h prior to bacterial inoculation. Previously, we found that 25 µM SA added to the nutrient solution did not affect shoot and root plant development and

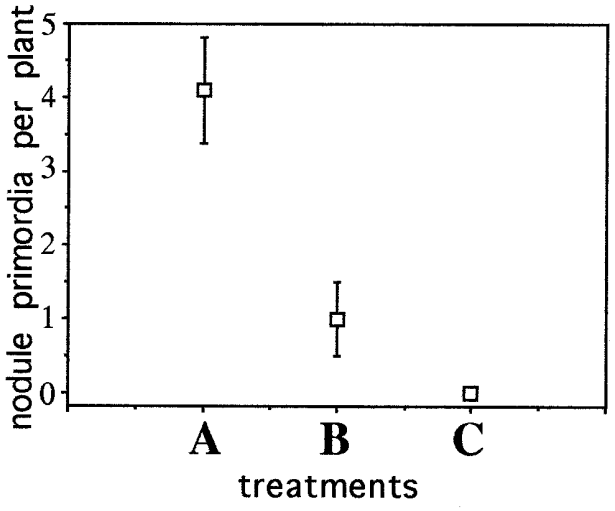

Fig. 3. Effect of exogenous application of salicylic acid (SA) on Nod factor–induced nodule primordia on alfalfa plants. **A,** 10^{-7} M NodRm-1. **B,** 10^{-7} M NodRm-1 plus 25 µM SA. **C,** 25 µM SA. Standard errors are represented by bars.

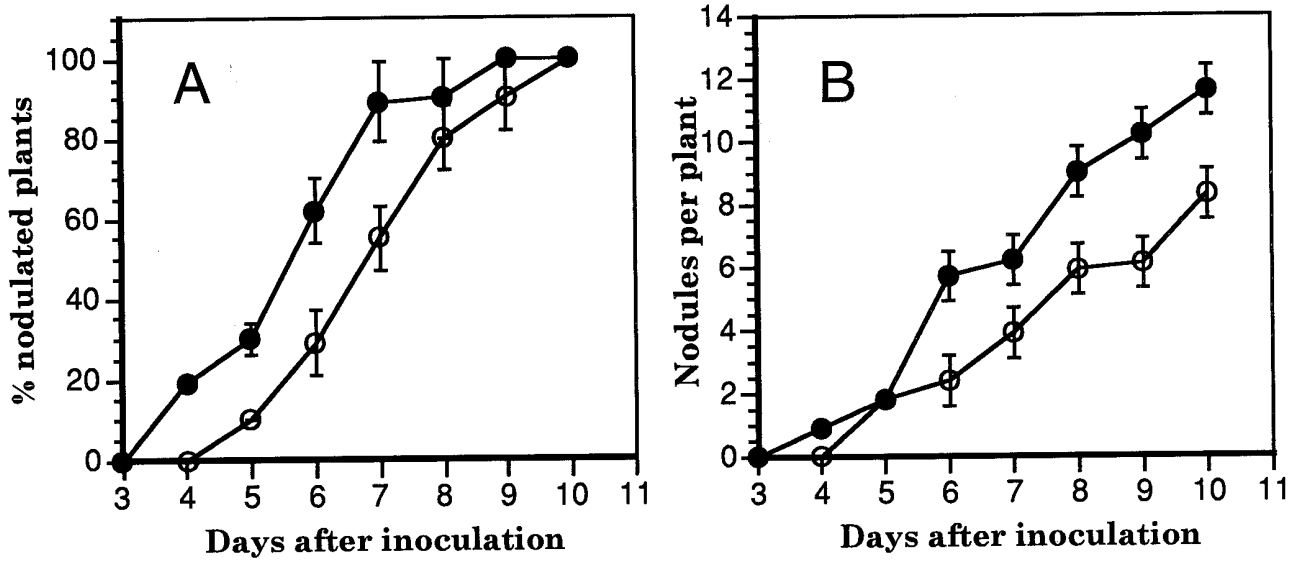

Fig. 2. Effect of the addition of 25 µM sodium salicylate to the plant growth mineral solution 24 h before the inoculation of alfalfa plants with *Rhizobium meliloti* GR4. **A,** Percentage of nodulated plants. **B,** Number of nodules per plant. Open circles correpond to the treatment with salicylic acid and filled circles to the control. Standard errors are represented by bars on each data point.

chlorophyll concentration. SA application caused a marked delay in nodule emergence (Fig. 2A) as well as a significant decrease in the number of nodules (Fig. 2B).

To understand which step of the nodulation process could be affected by the increased SA levels, we studied the effect of exogenous SA application on nodule primordia formation induced by Nod factors of *R. meliloti*. The nutrient solution of 5-day-old plants was supplemented with 10^{-7} M NodRm-1 (obtained according to Lerouge et al. 1990), alone or together with 25 µM SA. Eight days later, roots were collected and cleared with sodium hypochlorite (Truchet et al. 1989). The number of putative nodules was determined by visually scoring root deformations clearly differentiated from secondary roots. Results obtained (Fig. 3) showed that the addition of 25 mM SA significantly decreased the number of nodule primordia induced by Nod factors (down to 25% of the control). These results demonstrate that SA could interfere in a specific way with nodule organogenesis.

Twenty-five µM SA shows a low inhibitory effect (ca. 10%) on *R. meliloti* growth. One could think that the effect observed on the nodulation kinetics and nodule number formed when SA is added to plants could be due to this inhibitory effect. However, inocula density has no significant effect on the number of nodules formed (Olivares et al. 1980). Moreover, the inhibitory effect of exogenous SA on nodule primordia induction by Nod factor in alfalfa plants supports the conclusion that SA directly affects the nodulation process rather than bacterial fitness.

Considering the relationship between pathogenic-induced SA synthesis in plants and the expression of SAR (Klessig and Malamy 1994), it can be argued that a defense mechanism associated with SA accumulation takes place in the establishment of the *Rhizobium*-legume symbiosis. The suppression of the defense response only occurs when the plant recognizes its correct partner, producing a compatible Nod factor. Salzwedel and Dazzo (1993) reported an increased peroxidase activity in roots elicited by heterologous *Rhizobium* as a plant defense response that contributes to expression of host specificity during the infection process. Peroxidase induction could be related to the triggering of the defense response. This enzyme activity could be required for lignification and, possibly, for cell wall protein cross-linking.

Our results strongly suggest that Nod factors, in addition to their well-established role in root infection and nodulation, control the suppression of the SA accumulation. This is essential to facilitate the formation of nodule primordia. Our results also suggest that *Rhizobium* produces unidentified elicitors that induce SA accumulation in the host. Compatible Nod factor production would inhibit either their synthesis or their recognition by the plant.

ACKNOWLEDGMENTS

We thank A. Kondorosi for supplying strain AK631 and its derivative NodC^{-}. This work has been supported by the Plan Andaluz de Investigación (PAI).

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