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In situ Localization of *Rhizobium* mRNAs in Pea Root Nodules: *nif*A and *nif*H Localization

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Here we demonstrate that *Rhizobium* mRNAs can efficiently be detected in developing root nodules with the *in situ* hybridization technique. We have been able to localize the *Rhizobium nif*H mRNA as well as the transcript of the regulatory *nif*A gene. Therefore, we expect that the *in situ* hybridization technique can generally be applied to locate *Rhizobium* mRNAs in root nodules. In pea nodules, the *nif*A and *nif*H mRNAs are first detectable in the third to fourth cell layer of the late symbiotic zone. In these cell layers, these mRNAs are detectable immediately at maximal levels. In older parts of the late symbiotic zone, the level of *nif*H mRNA remains constant, whereas the level of *nif*A mRNA decreases. Finally, the pattern of *nif* mRNA accumulation was compared with that of nodulin mRNAs.

Additional keywords: nitrogen fixation, *Pisum sativum*, *Rhizobium leguminosarum*.

The formation of nodules on roots of *Leguminosae* by *Rhizobium* bacteria involves a series of successive steps that require the expression of plant as well as bacterial genes (Long 1989). The plant genes specifically expressed during nodule formation are the nodulin genes (Van Kammen 1984). A comprehensive list of nodulins has been published by Delauny and Verma (1988), and these genes have been reviewed extensively by Nap and Bisseling (1989).

In indeterminate nodules, like pea root nodules, a persistent meristem is present at the apex, which continuously generates cells that develop into different nodule tissues. As a consequence, the different tissues of a nodule are of graded age, and so the central tissue of indeterminate nodules has been divided in the following zones: the apical meristem; the invasion zone, in which the growing infection threads penetrate the meristem cells; the early symbiotic zone, where the bacteria proliferate and the plant cells elongate; and the late symbiotic zone, which harbours infected cells filled by nitrogen-fixing bacteroids. In old nodules also, a senescent zone is present containing degenerated rhizobia and plant cells (Newcomb 1976).

Recently, a new set of pea early nodulin cDNA clones was characterized, and the location of the corresponding transcripts in specific cells and tissues of infected roots and pea root nodules was determined by *in situ* hybridization (Van De Wiel *et al.* 1990; Scheres *et al.* 1990a,b). The ENOD2 mRNA was localized in the nodule parenchyma ("inner cortex") (Van De Wiel *et al.* 1990), whereas all other pea early nodulin mRNAs are present in the central tissue. Scheres *et al.* (1990a) showed that ENOD12 gene expression is restricted to the invasion zone. Expression of the ENOD5 gene starts in the invasion zone but reaches its maximal level in the early symbiotic zone. The ENOD3 and the homologous ENOD14 mRNAs are present at maximal levels in the early symbiotic zone and the first cell layers of the late symbiotic zone (Scheres *et al.* 1990b), whereas in older parts of the late symbiotic zone the level of these transcripts decreases. The mRNA of the late nodulin leghemoglobin (Lb) is first detectable in the early symbiotic zone, but its maximal level is first reached in the late symbiotic zone. These observations clearly showed that at different stages of root nodule development specific nodulin genes are induced.

Rhizobial genes involved in different steps of the plant-bacterium interaction are the nodulation (*nod*), nitrogen fixation (*nif* and *fix*) genes, and genes encoding for surface compounds of the bacteria. Examples of the latter group are genes involved in exopolysaccharide (*exo*), lipopolysaccharide (*lps*), and β-1,2-glucan (*ndv*) synthesis.

In alfalfa nodules, the expression of several *R. meliloti nif* and *nod* genes has been studied *in planta* by using the *gus*A gene (Sharma and Signer 1990) as a reporter gene. Because thick sections (100–250 μm) were used in this study, only an inaccurate picture of the spatial distribution of *Rhizobium* gene expression could be obtained. Furthermore, localization data obtained by using a reporter enzyme are obfuscated by the stability of this enzyme. To allow a more accurate localization of *Rhizobium* gene expression, we have used the *in situ* hybridization technique to examine whether bacterial mRNAs can be detected *in planta*. In this paper, we report how, by using this technique, transcripts of *R. leguminosarum* bv. *viciae nif*A and *nif*H genes can be localized in root nodules. The *nif*H gene encodes a subunit of the nitrogenase enzyme, and it is abundantly expressed in nodules, whereas the *nif*A gene is probably expressed at a relatively low level, as it is a regulatory gene required for the induction of expression of other *nif* and *fix* genes (Hennecke 1990).

MATERIALS AND METHODS

Plant materials. Pea (*Pisum sativum* L. 'Rondo') plants were cultured and inoculated with *R. leguminosarum* bv.

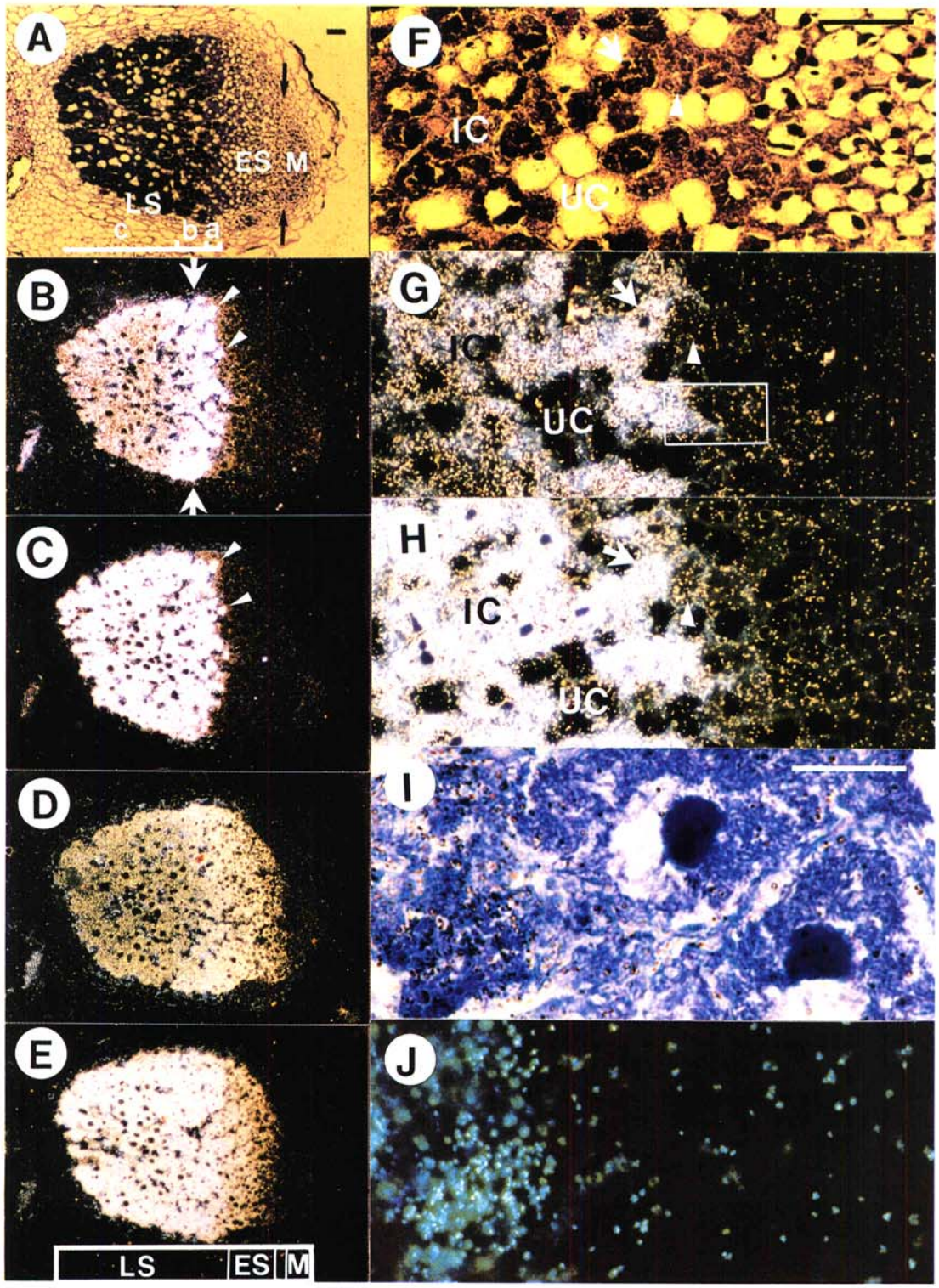

Fig. 1. Localization of *nif* and nodulin transcripts in longitudinal sections of a 16-day-old pea nodule. **A,F,I,** Bright field micrographs; **B,C,D,E,G,H,** dark field micrographs in which silver grains are visible as white dots; **J,** epipolarization micrograph. A,B,C,D, and E are adjacent sections. In all cases, antisense RNA probes were labeled with ^{35}S-UTP. **A,** Nodule meristem (M), invasion zone (arrows), early symbiotic zone (ES), and late symbiotic zone (LS) are indicated. The late symbiotic zone is divided into region a, b, and c. Bar = 100 μm. **B,** Section hybridized with antisense *nif*A RNA. Arrowheads indicate the same cells as in C. Arrow indicates where the *nif*A mRNA level decreases. Exposure time: 4 wk. **C,** Section hybridized with antisense *nif*H RNA. The same cells as in B are indicated by arrowheads. Exposure time: 4 days. **D,** Localization of pea ENOD3 mRNA. Exposure time: 2 wk. **E,** Localization of pea leghemoglobin mRNA. Exposure time: 4 days. **F,** Arrows and arrowheads in F, G, and H indicate the same cells. IC = infected cell; UC = uninfected cell. Bar = 100 μm. **G,** Detail of B. Arrowhead indicates the cell without signal, arrow indicates the cells with signal. **H,** Detail of C. **I,** Detail of box region in G. Dark dots represent silver grains. Bar = 10 μm. **J,** Epipolarization micrograph of I. Bright dots are silver grains.

viciae (PRE) as described by Bisseling *et al.* (1978).

***In situ* hybridization.** Pea nodules were harvested 16 days after inoculation and fixed immediately with 4% paraformaldehyde and 0.25% glutaraldehyde in 50 mM sodium phosphate buffer (pH 7.2) for 4 hr. The nodules were dehydrated by passing through a routine ethanol series and were embedded in paraffin. Sections (7 μm thick) were cut with a Leitz microtome. They were adhered on poly-L-lysine-coated slides, and thereafter deparaffinized with graded xylene. The sections were hybridized by a method derived from the procedure described by Cox and Goldberg (1988) (Van De Wiel *et al.* 1990). In short, sections were dehydrated and dried under vacuum. The sections were hybridized with RNA probes as described by Van De Wiel *et al.* (1990). Slides were coated with Kodak NTB2 nuclear emulsion and exposed at 4° C. Afterwards, the slides were developed in Kodak D19 developer and fixed in Kodak fixer. Sections were stained with 0.25% toluidine blue and mounted with DPX (BDH). The sections were photographed with a Nikon microscope with dark field and epipolarization optics.

Labeling of antisense/sense RNAs. The 1.8-kb *Eco*RI-*Bam*HI fragment of pT7.BB containing the coding region of the *nif*A gene (Roelvink *et al.* 1989) was subcloned in the pT7-5 vector (kindly provided by S. Tabor). The pT7-5/*nif*A plasmid was cut with *Xba*I before antisense *nif*A RNA (from position 893-469 base pair [bp]) was made with T7 polymerase.

The *nif*H antisense RNA (from position 89-433 bp) was transcribed by T7 polymerase from a pTZ19 derivative containing a 518-bp *Acc*I-*Hpa*I fragment of pGBI (Schetgens *et al.* 1984). For sense *nif*H RNA production, a pTZ18 derivative carrying the same insert was used. The production of antisense PsENOD3 and Lb RNAs was carried out according to Scheres *et al.* (1990b). The antisense RNA probes were radioactively labeled with [^{35}S]-UTP (1,000–1,500 Ci/mmole, Amersham Corp., Arlington Heights, IL) as described previously (Van De Wiel *et al.* 1990) and were degraded to about 150 nucleotide-long fragments before hybridization, according to Van De Wiel *et al.* (1990).

RESULTS AND DISCUSSION

***Rhizobium* mRNAs can efficiently be detected *in situ*.** To localize *nif*H mRNA by *in situ* hybridization, nodules from 16-day-old pea plants were used. Longitudinal sections of nodules were hybridized to ^{35}S-labeled antisense *nif*H RNA. As shown in Figure 1C and H, the *nif*H mRNA was clearly detectable in the infected cells of the late symbiotic zone. No hybridization was obtained when a ^{35}S-labeled sense *nif*H RNA was used as a probe (data not shown), showing that the signal obtained after hybridization with antisense *nif*H RNA was due to the presence of *nif*H mRNA and not to that of *nif*H DNA of the Sym-plasmid. This was further supported by the absence of a hybridization signal in the cells of the youngest cell layers of the late symbiotic zone, though these cells are already fully packed with bacteria (Fig. 1H–J).

The signals obtained after hybridization with antisense *nif*H RNA were just as intense as the signals obtained if antisense Lb RNA was used (Fig. 1C,E). Because Lb accounts for about 10% of the total soluble nodule protein of the plant and nitrogenase for 10% of the total bacterial protein (Bisseling *et al.* 1978), we assumed that similar amounts of Lb and nitrogenase mRNA were present in the nodule. This indicates that the prokaryotic *nif*H mRNA and the eukaryotic Lb mRNA are detected with the same efficiency.

In situ hybridization with ^{35}S-labeled antisense *nif*A RNA as a probe was similarly carried out to sections of 16-day-old nodules. Like the *nif*H mRNA, the *nif*A transcript was detectable in the infected cells of the late symbiotic zone (Fig. 1B,G,I,J), but the intensity of the signal obtained after hybridization with the *nif*A probe was considerably lower than with the *nif*H probe. In most experiments, an exposure time of 2–4 wk was required for showing *nif*A mRNA localization, whereas a 2- to 4-day exposure was sufficient to visualize the *nif*H mRNA hybridization (see legend, Fig. 1). Because even the transcript of the regulatory *nif*A gene, which will be present in low concentrations, can be detected with *in situ* hybridization, it seems plausible that other *Rhizobium* mRNAs can similarly be localized with this method.

***nif*H and *nif*A mRNA accumulation during nodule development.** By definition, the late symbiotic zone consists of the cells of the central tissue that are fully packed with rhizobia and have already reached their maximal size (Newcomb 1976). Analysis of serial sections of pea nodules, hybridized with the two *nif* probes, showed that the *nif*H and *nif*A mRNAs (Fig. 1C and B, respectively) were detectable in almost all infected cells of the late symbiotic zone. However, in the first two to three cell layers of this zone (Fig. 1A), only a small number of silver grains were detectable (Fig. 1G,H,I,J). To determine whether the *nif* genes are expressed at a low level in these cell layers, we determined the number of silver grains in infected cells of the different zones of the nodule central tissue. The silver grains were counted in five areas of 400 μm^2 in each zone of the central tissue as well as in the nodule cortex, root cortex, and parts of the slide containing no section. The average values and standard deviations are given in Table 1. The data presented in this table show that on nodule sections hybridized with a *nif*A or *nif*H probe the number of silver grains in the nodule meristem, invasion zone, early symbiotic zone, and in the first two to three cell layers of the late symbiotic zone was not higher than the background level present in the nodule cortex, root cortex, or areas of the slides containing no section. In the third or fourth cell layer of the late symbiotic zone (Fig. 1A) both *nif*A and *nif*H mRNA were present at a maximal level (Fig. 1G,H–J; Table 1). In the proximal part of the late symbiotic zone (Fig. 1A), the level of *nif*H mRNA remained at a similar level, whereas the number of silver grains above this zone in a *nif*A-hybridized section decreased to about 35% of the maximal value. Hybridization with a sense *nif*A or *nif*H probe gave a signal that was similar to the background level obtained with antisense probes (results not shown). These observations show that both the *nif*A and *nif*H genes are first expressed in the third or fourth cell layer of the late symbiotic zone. Analyses of 1 μm thick sections of technovit-embedded pea nodules showed that

the infected cells of the first two cell layers already contained bacteroids with the characteristic Y-shaped form (data not shown, see Van De Wiel *et al.* 1988). This implies that the development into pleiomorphic bacteroids precedes the stage where the *nif* genes are expressed and actual nitrogen fixation can occur.

Recently, Vasse *et al.* (1990) proposed a new nomenclature for the zones of the central tissue of alfalfa nodules. They named the meristem, zone I; the invasion zone and early symbiotic zone, zone II; the youngest part of the late symbiotic zone, interzone II-III; and the rest of the late symbiotic zone, zone III. The interzone II-III is characterized by the presence of prominent amyloplasts, and the bacteroids in this interzone have a typical morphology but most likely do not yet fix nitrogen. In zone III, the number of amyloplasts is strongly reduced. In pea nodules, the amyloplasts are not restricted to a specific zone of the late symbiotic zone, and bacteroid morphology has not extensively been studied. Therefore, these criteria cannot be used to indicate the interzone II-III in pea nodules. However, the absence of *nif*A and *nif*H mRNA in bacteroids of the youngest cell layers of the late symbiotic zone suggests that these cell layers correspond to the interzone II-III of alfalfa nodules. In Figure 1A, the part of the late symbiotic zone that could be the equivalent of the alfalfa interzone II-III is marked with LSa.

Both *nif*A and *nif*H mRNA have a striking accumulation pattern during nodule development. Both mRNAs were first found in the third or fourth cell layers of the late symbiotic zone. In these cell layers, these *nif* genes were immediately expressed at maximal levels (Table 1). Such a gene expression pattern of *nif*A suggests that a major change occurs in the third or fourth cell layer of the late symbiotic zone, causing *nif*A gene induction. In free-living *R. meliloti* bacteria, expression of the *nif*A gene is induced at microaerobic O_2 concentrations (Ditta *et al.* 1987). Because microaerobic O_2 concentrations prevail in root nodules, it has been postulated that *nif*A gene expression in nodules is also mediated by the O_2 concentration (Hennecke 1990). The microaerobic O_2 concentration in the nodule is thought to arise by respiratory activity of the bacteria and the presence of an O_2 diffusion barrier in the nodule parenchyma ("inner cortex") (Witty *et al.* 1986; Van De Wiel *et al.* 1990). If the O_2 concentration is the only factor controlling *nif*A gene expression in the nodule, a rapid drop in O_2 concentration must occur in the third or fourth cell layer of the late symbiotic zone. Although we cannot exclude the possibility of this sharp change of O_2 concentration, at this time there are no cytological or physiological studies that indicate that such a rapid drop occurs. Therefore, it will be essential to demonstrate that the *nif*A gene is regulated by a similar mechanism in free-living bacteria and in nodules. The level of the *nif*A mRNA decreased from about the eighth cell layer of the late symbiotic zone (Fig. 1B, arrow). Because the *nif*A gene is autoregulated (Hennecke 1990), we supposed that this decrease was caused by the accumulation of the *nif*A protein, but this needs to be checked by following the accumulation of the *nif*A protein with immunocytochemical localization methods.

Because *nif*A is a regulatory protein involved in the induction of other *nif* and *fix* genes, we expected that *nif*A gene expression would precede the expression of the *nif*HDK operon. We tried to test this assumption by determining the location of *nif*H and *nif*A mRNA in adjacent sections. To facilitate the comparison of the corresponding cell patterns in these sections, we have indicated a few cells with arrowheads (Fig. 1B,C) and arrows (Fig. 1G,H). These studies showed that cells containing *nif*A mRNA also harbour *nif*H transcripts. Apparently the induction of the *nif*A gene results in a prompt switching on of the *nif*HDK operon.

Our *nif*H mRNA localization studies might be consistent with the studies on *nif* gene expression in alfalfa nodules by Sharma and Signer (1990). They showed by using a *gus*A reporter gene that *nif*H gene expression "occurred throughout the nodule, except in the meristematic zone." It is possible that the meristematic zone in their studies includes the meristem, invasion zone, early symbiotic zone, and a few cell layers of the late symbiotic zone. However, because thick sections were used to detect *gus*A activity, the different zones could not be identified. Recently, Boivin *et al.* (1990) used thinner sections to follow *R. meliloti* gene expression with *Lac*Z as a reporter gene. In these studies, a more accurate localization was achieved. Sharma and Signer (1990) did not observe any difference in *nif*H and *nif*A gene expression in older parts of the late symbiotic zone. In their studies, this difference might have been masked by the stability of the *gus*A protein.

Comparison of accumulation patterns of nodulin and *nif* mRNAs. Clues on possible functions of nodulins and bacterial gene products can be obtained by determining at which stage of development specific gene products are made. For that purpose, we compared the spatial distribution of the ENOD3 early nodulin mRNA with that of *nif* mRNAs. Previously, we have shown that the amino acid sequence of the ENOD3 polypeptide contains four cysteine residues in relative positions characteristic for metal-binding proteins (Scheres *et al.* 1990b). We then proposed that this early nodulin might be involved in transport of molybdenum and/or iron ions into the bacteroids, because the bacteroids require high amounts of these metal ions for the synthesis of the nitrogenase enzyme (Shah and Brill 1977). As shown in Figure 1B–D, the maximal level of

Table 1. *Nif* gene expression in a 16-day-old pea nodule

Zone	Silver grains (400 μm^2)[z]	
	*Nif*A	*Nif*H
Nodule meristem	3.8 ± 1.2	10.2 ± 3.5
Invasion zone	4.2 ± 1.0	7.4 ± 0.8
Early symbiotic zone	4.8 ± 1.6	9.0 ± 2.1
Late symbiotic zone a	6.6 ± 3.6	9.4 ± 1.9
Late symbiotic zone b	141.4 ± 9.0	145.0 ± 18.1
Late symbiotic zone c	47.2 ± 7.2	144.0 ± 9.5
Nodule cortex	3.6 ± 2.2	3.4 ± 2.3
Root cortex	3.4 ± 1.3	3.5 ± 2.0
Area without tissue	3.2 ± 1.2	3.6 ± 0.8

[z] The number of silver grains are the average values of five areas of 400 μm^2 ± standard deviation. The nodule section hybridized with a *nif*A probe was exposed for 4 wk, and the section hybridized with a *nif*H probe was exposed for 1 wk.

ENOD3 gene expression coincided with the region of the late symbiotic zone where the expression of *nif*A and *nif*H genes starts. Therefore, we concluded that the mRNA localization studies are consistent with the postulated function of ENOD3 in transport of molybdenum and iron ions toward bacteroids.

Lb is a nodulin whose appearance during nodule development has been frequently compared with that of *nif* proteins (Bisseling *et al.* 1986). Because a more accurate comparison of the order of induction of genes can be made by *in situ* hybridization, we compared the pattern of Lb and *nif*H mRNA accumulation in longitudinal sections. As shown in Figure 1E, the Lb mRNA was first detectable in the early symbiotic zone and gradually reached a maximal level in the late symbiotic zone. Therefore, Lb gene expression markedly precedes *nif* gene expression in pea nodules. This is consistent with most of the previously published biochemical studies (Bisseling *et al.* 1986).

In this paper, we have demonstrated that rhizobial mRNAs can efficiently be detected in root nodules with the *in situ* hybridization technique. Therefore, *in situ* hybridization is a very powerful tool to study the sequential order of both plant and bacterial gene expression in plant-microbe interactions.

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