The Absence of Nops Secretion in *Sinorhizobium fredii* HH103 Increases *GmPR1* Expression in Williams Soybean

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Sinorhizobium fredii HH103 secretes through the type III secretion system at least eight nodulation outer proteins (Nops), including the effector NopP. These proteins are necessary for an effective nodulation of soybean. In this work, we show that expression of the *nopP* gene depended on flavonoids and on the transcriptional regulators NodD1 and TtsI. Inactivation of *nopP* led to an increase in the symbiotic capacity of *S. fredii* HH103 to nodulate Williams soybean. In addition, we studied whether Nops affect the expression of the pathogenesis-related genes *GmPR1*, *GmPR2*, and *GmPR3* in soybean roots and shoots. In the presence of *S. fredii* HH103, expression of pathogenesis-related (PR) gene *PR1* was induced in soybean roots 4 days after inoculation and it increased 8 days after inoculation. The absence of Nops provoked a higher induction of *PR1* in both soybean roots and shoots, suggesting that Nops function early, diminishing plant defense responses during rhizobial infection. However, the inactivation of *nopP* led to a decrease in *PR1* expression. Therefore, the absence of NopP or that of the complete set of Nops seems to have opposite effects on the symbiotic performance and on the elicitation of soybean defense responses.

Rhizobia are soil bacteria that induce the formation of nodules on the roots of many leguminous plants. During nodule development, rhizobia differentiate into bacteroids that reduce atmospheric nitrogen to ammonia, which is assimilated by the plant in the form of amino acids (Jones et al. 2007). This process requires the exchange of symbiotic signals between both members of the symbiosis. Thus, flavonoids exuded by legume roots induce the biosynthesis and secretion of bacterial signal molecules known as Nod factors or lipochitooligosaccharides, which trigger the initiation of nodule organogenesis.

Plants have evolved various sophisticated defense mechanisms to resist colonization by microbial pathogens and parasites. These defense responses include the production of reactive oxygen species (ROS) and nitric oxide (NO), alterations in the plant cell wall, induction of antimicrobial compounds (phytoalexins), and the synthesis of pathogenesis-related (PR)

proteins. PR proteins comprise a number of families that include enzymes (chitinase and β -1,3-glucanase) which can directly attack pathogen structures, antimicrobial peptides, and other proteins such as PR1, which is of unknown function (Thomma et al. 2001; Jones and Takemoto 2004).

In order to be successful, pathogenic bacteria must either suppress or fail to elicit basal defenses. Some plant-pathogenic bacteria such as *Pseudomonas* spp. or *Xanthomonas* spp. have developed a strategy to overcome plant defense responses. They have the capacity to deliver proteins known as effectors directly into the eukaryotic host cell via a specialized apparatus called the type III secretion system (T3SS) (Pallen et al. 2003). These effectors can suppress basal defenses and alter the host metabolism to promote disease. However, certain effectors can eventually be recognized by specific plant receptors, triggering the hypersensitive response (HR). This response is characterized by a collapse of the tissue in the area of bacterial inoculation, which eventually becomes necrotic. This programmed cell-death response serves to contain and eliminate the pathogen (Nümberger et al. 2004). All these responses are mounted in a localized fashion. Moreover, plants also exhibit systemic resistance responses, such as the systemic acquired resistance (SAR) and induced systemic resistance (ISR). In the SAR response, the plant phenolic salicylic acid (SA) plays a key role in signaling. The ISR response differs from SAR in that it does not require necrosis at the site of bacterial inoculation and is dependent on jasmonic acid (JA) and ethylene (ET) as signaling molecules. Both SAR and ISR defense responses are induced by certain molecules called elicitors. These molecules are produced either by pathogenic or nonpathogenic microorganisms, many of which are cell surface-derived components (He et al. 2007). It has been proposed that, during the initial steps of nodulation, rhizobia are recognized as pathogenic agents and, at some stages, the infection resembles a pathogenic interaction. Triggered defense responses are usually weak, local, and transitory, suggesting that the host modulates the defense response to help to establish the symbiosis (Mithöfer 2002). However, Vasse and associates (1993) showed that, in a compatible *Rhizobium*-legume interaction, the bacteria induce a localized HR that could play a key role in the plant regulation of nodule number.

The T3SS has been found in some symbiotic rhizobial strains such as *Rhizobium* strain NGR234, *Sinorhizobium fredii* HH103 and USDA257, *Mesorhizobium loti* MAFF303999, *Bradyrhizobium japonicum* USDA110, and *B. elkanii* (Viprey et al. 1998; Krause et al. 2002; Krishnan et al. 2003; de Lyra et al. 2006; Okazaki et al. 2009; Sánchez et al. 2009). Pro-

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teins secreted through the rhizobial T3SS are collectively known as nodulation outer proteins (Nops). Each rhizobial strain secretes a cocktail of Nops that will eventually be involved in host-range determination and symbiotic efficiency. The expression of genes coding for the type III secretion machinery and Nops is controlled by the transcriptional regulator TtsI, which binds to specific promoter sequences called *tts* boxes. TtsI is an intermediary in the regulatory cascade between NodD1, previously activated by flavonoids, and T3SS-related genes (Krause et al. 2002; López-Baena et al. 2008; Wasseem et al. 2008).

S. fredii HH103 (hereafter HH103) secretes, in response to flavonoids, at least eight proteins through the T3SS: NopA, NopB, NopC, NopD, NopL, NopM, NopP, and NopX (Rodrigues et al. 2007). Three of these proteins, NopL, NopP, and NopM, have been described as putative effectors in related rhizobia (Bartsev et al. 2003, 2004; Ausmees et al. 2004; Skorpil et al. 2005; Kambara et al. 2008). In HH103, the total absence of Nops has different symbiotic effects that depend on the host plant but are independent of the type of nodule formed: detrimental with soybean (determinate nodules) and *Glycyrrhiza uralensis* (indeterminate nodules) but highly beneficial with *Erythrina variegata* (determinate nodules) (López-Baena et al. 2008).

To further understand the role of the T3SS in the symbiosis between HH103 and its host plants, it is important to know the function of each effector protein. In this work, we studied the transcriptional regulation and secretion of the *S. fredii* HH103 nodulation outer protein NopP. The symbiotic capacity of a *nopP* mutant to nodulate soybean cv. Williams and *E. variegata* was also analyzed. Moreover, to determine whether HH103 Nops are involved in controlling host defense responses similar to those triggered in a pathogen–plant interaction, we studied the expression of three plant *PR* genes, *PR1*, *PR2*, and *PR3*, in soybean roots and shoots following inoculation with the wild-type strain HH103, a mutant derivative defective in Nops synthesis and secretion, and a *nopP* mutant strain.

RESULTS

Isolation of the *S. fredii* HH103 *nopP* gene.

In order to isolate the HH103 *nopP* gene and the preceding *tts* box, primers yPF and yPR were designed from the sequence of the *nopP* gene (formerly y4yP) of *Rhizobium* strain NGR234 (GenBank accession number U00090). Using these primers and the HH103 genomic DNA as template, a 1,800-bp polymerase chain reaction (PCR) fragment was amplified. This fragment was sequenced (AY775562) and subcloned in the broad-host-range vector pMP92 and in the suicide plasmid pK18mob, generating plasmids pMUS983 and pMUS843, respectively.

The 1,800-bp fragment contained one complete open reading frame (ORF), the *nopP* gene, between positions 138 and 950. The HH103 *nopP* gene showed 97 and 96% of identity to the *nopP* genes of *S. fredii* USDA257 (AF229441) and *Rhizobium* strain NGR234 (U00090), respectively. The deduced protein sequence of the HH103 *nopP* gene (270 amino acids [aa], AAY33495) was 95, 94, and 44% identical to those of *Rhizobium* strain NGR234 (273 aa, AAB91955), *S. fredii* USDA257 (270 aa, AAL98702), and *B. japonicum* USDA110 (277 aa, AAG60738), respectively. The *tts* box located upstream of the *Rhizobium* strain NGR234 *nopP* was also present in HH103 (5'-TAGTCAGCTGAACGTCAGTTGTGCGGGTCTA), 62 bp upstream of the *nopP* gene.

As described below, a *nopP* mutant (SVQ559) was generated. Mutant SVQ559 harbors the *lacZ*-Gm^r cassette in the *nopP* coding sequence. The mutation had no major detrimental effect on bacterial growth and the mutant showed no alteration in either exopolysaccharide production or its lipopolysaccharide (LPS) and plasmid profiles when compared with the parental strain HH103 Rif^r (data not shown).

Expression of *nopP* is a flavonoid-, NodD1-, and TtsI-dependent process in *S. fredii* HH103.

As previously mentioned, TtsI regulates the expression of the *nop* genes by direct binding to the *tts* boxes. Because the *S.*

Fig. 1. Regulation of the expression of the *Sinorhizobium fredii* HH103 *nopP* gene. **A**, Semiquantitative reverse-transcriptase polymerase chain reaction (RT-PCR) analysis. +G: cultures induced with 3.7 μ M genistein. Sizes of the molecular marker are shown on the left. **B**, Quantitative RT-PCR analysis. Expression data shown are the mean ($\pm$ standard error of the mean) for two biological replicates. Expression was calculated relative to the parental strain not induced with genistein. Gray bars indicate induction with genistein (3.7 μ M).

fredii HH103 *nopP* gene is preceded by a *tts* box, we studied whether the expression of *nopP* was activated by flavonoids and controlled by the transcriptional regulators NodD1 and TtsI.

Induction with genistein doubled the expression of *nopP* (388 ± 10 Miller units) when compared with the control without flavonoids (169 ± 24 Miller units). Regulation of the expression of the *nopP* gene by NodD1 and TtsI was also analyzed. Thus, semiquantitative reverse-transcriptase (RT)-PCR and quantitative real-time (q)RT-PCR assays were carried out to study more accurately the expression of *nopP* in the parental strain and in a *nodD1::Ω* and a *ttsI::Ω* mutant background in the absence or presence of genistein. A PCR fragment of the predicted size could only be detected in the lane corresponding to the parental strain induced with genistein, whereas inactivation of *nodD1* or *ttsI* resulted in the absence of this PCR fragment (Fig. 1A). These results were confirmed using qRT-PCR. Thus, induction with genistein enhanced approximately 47-fold the level of transcription of *nopP* in the parental strain (Fig. 1B).

Inactivation of the *S. fredii* HH103 *nopP* gene did not affect secretion of the other Nops.

Ausmees and associates (2004) previously reported that inactivation of the *Rhizobium* strain NGR234 *nopP* gene did not affect secretion of the rest of the Nops. *S. fredii* HH103 *nopP* also codes for a putative effector protein; therefore, its inactivation would not be expected to change the pattern of

secreted proteins other than NopP. To study the effects of the inactivation of the *nopP* gene on Nops secretion, proteins found in the supernatants of cultures of HH103 Rif^r, HH103 Rif^r *rhcJ::Ω* (de Lyra et al. 2006), HH103 Rif^r *nopP::lacZ-Gm^r*, and the *nopP* mutant strain harboring plasmid pMUS983 (a pMP92 derivative carrying *nopP* and its *tts* box) were isolated and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2). HH103 secretes, in response to flavonoids, at least eight proteins through the T3SS: NopA (approximately 6.5 kDa), NopC (approximately 12 kDa), NopB (approximately 21 kDa), NopP (approximately 31 kDa), NopL (approximately 37 and 34 kDa), NopM (approximately 64 kDa), NopX (approximately 64 kDa), and NopD (approximately 180 kDa) (Rodrigues et al. 2007). NopD is not always detected in silver-stained polyacrylamide gels due to its low expression and its high size; NopC is sometimes not detected because it is hidden by the polysaccharide that migrates between 6 and 12 kDa (Fig. 2A; for example, lanes 2 and 8); and NopP migrates together with flagellins, which are apparently constitutively expressed in this strain (Rodrigues et al. 2007). Mutation of the HH103 *rhcJ* gene, which codes for a component of the type III secretion machinery, completely blocks secretion of Nops. Thus, although we found that inactivation of the *nopP* gene did not prevent secretion of the other Nops (Fig. 2A), it was not easy to clearly identify changes in NopP expression across the different strains in polyacrylamide gels, due to problems of resolution. Therefore, specific antibodies raised against NopP (Ausmees et al. 2004) were used to

Fig. 2. Extracellular proteins profiles of various *Sinorhizobium fredii* HH103 derivatives. **A**, Silver-stained gel of secreted extracellular proteins of HH103 Rif^r (lanes 1 and 2), HH103 Rif^r *rhcJ::Ω* (lanes 3 and 4), HH103 Rif^r *nopP::lacZ-Gm^r* (lanes 5 and 6), and HH103 Rif^r *nopP::lacZ-Gm^r* (pMUS983) (lanes 7 and 8). Lanes 2, 4, 6, and 8 show cultures induced with 3.7 μ M genistein. Proteins whose secretion depends on genistein and a type III secretion system are indicated on the right of the figure. Molecular masses (kDa) of the marker are shown on the left. Samples were separated by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). **B**, Immunodetection of nodulation outer protein NopP in extracellular protein extracts of noninduced cultures of *S. fredii* HH103 Rif^r (lane 2) and induced cultures (genistein 3.7 μ M) of HH103 Rif^r (lane 3), HH103 Rif^r *nopP::lacZ-Gm^r* (lane 4), and HH103 Rif^r *nopP::lacZ-Gm^r* (pMUS983) (lane 5). Molecular masses (kDa) of the marker (lane 1) are shown on the left. Samples were separated by 15% SDS-PAGE.

confirm the results (Fig. 2B). NopP was detected in the supernatants of genistein-induced cultures of the parental strain HH103 Rif^r and the *nopP* mutant strain complemented with plasmid pMUS983 but not in the lane corresponding to the *nopP* mutant strain induced with genistein (lane 4).

The absence of the effector protein NopP affects the symbiosis between *S. fredii* HH103 and soybean cv. Williams and *E. variegata*.

The *S. fredii* HH103 T3SS is involved in host-range determination and symbiotic effectiveness with soybean and *E. variegata* (López-Baena et al. 2008). To elucidate the role of NopP in the interaction between HH103 and *Glycine max* (soybean), the symbiotic capacities of the mutant strain HH103 Rif^r *nopP::lacZ-Gm^r* were determined in nodulation assays with the American soybean cv. Williams, which is effectively nodulated by the wild-type strain HH103. *E. variegata*, a tropical legume which is poorly nodulated by HH103, was also included in these studies.

The number and fresh weight of nodules formed by soybean cv. Williams plants inoculated with the *nopP* mutant derivative were significantly higher than those formed in plants inoculated with the parental strain (Fig. 3A). Complementation of the mutant strain with plasmid pMUS983 restored its capacity to nodulate soybean as the parental strain.

In marked contrast, in *E. variegata*, there was a significant decrease in the number and fresh weight of nodules when plants were inoculated with the mutant strain HH103 Rif^r *nopP::lacZ-Gm^r* compared with those inoculated with the parental strain HH103 Rif^r (Fig. 3B). The plant-top dry weight of plants inoculated with HH103 Rif^r *nopP::lacZ-Gm^r* was simi-

lar to that shown by noninoculated control plants, suggesting that, although the mutant strain has a reduced ability to form nodules, they could not effectively fix nitrogen, as demonstrated by acetylene reduction assays (ARA) measured 90 days postinoculation: data expressed as nmol ET plant⁻¹ h⁻¹ = 837 ± 124, 1,750 ± 354, 1,118 ± 167, and 12,525 ± 1,117 for non-inoculated plants and for plants inoculated with HH103 Rif^r, HH103 Rif^r *nopP::lacZ-Gm^r*, and HH103 Rif^r *rhcJ::Ω*, respectively.

S. fredii HH103 Nops induce changes in the expression of the *G. max* PR1 gene.

In order to investigate whether HH103 Nops are involved in the elicitation of defense responses in soybean, the expression of different defense-related genes was examined. Thus, three PR genes were selected: *GmPR1*, *GmPR2*, and *GmPR3*. *GmPR1* (accession number BU577813) codes for an acidic PR1 protein, *GmPR2* (M37753) encodes a β-1,3-endoglucanase, and *GmPR3* (AF202731) encodes a chitinase class I. The expression characteristics of these PR genes in soybean roots and shoots following treatment with SA, ET, and JA have been previously established. The expression of *GmPR1* increases in shoots but not roots following treatment with SA and is not affected by ET or JA. *GmPR2* is expressed in both roots and shoots following treatment with an ET precursor (1-aminocyclopropane-1-carboxylic-acid [ACC]) and its expression is not altered by JA and SA treatments. Finally, *GmPR3* mRNA abundance significantly increases by ET and JA in roots and shoots and is not affected by SA (Mazarei et al. 2007).

A preliminary study of the expression of the *GmPR1*, *GmPR2*, and *GmPR3* genes in roots and shoots of noninocu-

Fig. 3. Plant responses to inoculation of **A**, *Glycine max* cv. Williams and **B**, *Erythrina variegata* with *Sinorhizobium fredii* HH103 Rif^r (white bars), HH103 Rif^r *nopP::lacZ-Gm^r* (gray bars), and HH103 Rif^r *nopP::lacZ-Gm^r* complemented with pMUS983 (gray-striped bars). Data represent means ± standard deviations of six jars for soybean. Each jar contained two soybean plants. Data corresponding to noninoculated plants are represented by black bars. Determinations were made 6 weeks after inoculation. For *E. variegata* plants, data represent means ± standard deviations of five plants. Determinations were made 90 days after inoculation. Bacteria isolated from 20 nodules formed by each inoculant showed the expected resistance markers. Mutant HH103 Rif^r *nopP::lacZ-Gm^r* and the complemented strain were individually compared with the parental strain HH103 Rif^r by using the Mann-Whitney nonparametric test. Values topped by one or two asterisks are significantly different at the level $\alpha = 5$ and 10%, respectively.

lated *G. max* cv. Williams control plants was made by semi-quantitative RT-PCR (data not shown). *GmPR1* was not expressed in either soybean roots or shoots. The *GmPR2* gene was constitutively expressed in soybean roots and only a weak signal could be detected in shoots. Finally, *GmPR3* was not expressed in shoots and its expression was quite weak in roots.

qRT-PCR assays were then used to quantify the expression of *GmPR1*, *GmPR2*, and *GmPR3* in roots and shoots of soybean cv. Williams plants that were not inoculated and inoculated with the parental strain HH103 Rif^r and two mutant derivatives, HH103 Rif^r *ttsI*:: Ω and HH103 Rif^r *nopP*::*lacZ*-Gm^r. Whereas the former mutant strain does not secrete Nops upon induction with flavonoids, the latter secretes all the Nops except the effector protein NopP (López-Baena et al. 2008) (Fig. 2). Krishnan (2002) has previously shown that the *S. fredii* USDA257 NopX is localized in the infection threads of 8-day-old soybean nodules and is not present in mature nod-

ules, suggesting that Nops could be functioning during the development of infection threads. Therefore, soybean RNA samples were collected 8 days after inoculation, during the early stages of nodule development.

The expression of the *GmPR1* gene significantly increased in roots but not in shoots when plants were inoculated with the parental strain HH103 Rif^r (approximately 9-fold), HH103 Rif^r *ttsI*:: Ω (13-fold), and HH103 Rif^r *nopP*::*lacZ*-Gm^r (5-fold) compared with noninoculated control plants (Fig. 4A). In addition, only soybean plants that were inoculated with the mutant strain HH103 Rif^r *ttsI*:: Ω showed an increase of the expression of *GmPR1* in shoots (approximately fourfold) when compared with noninoculated plants (Fig. 4A).

Inoculation with HH103 Rif^r, HH103 Rif^r *ttsI*:: Ω , and HH103 Rif^r *nopP*::*lacZ*-Gm^r induced a slight increase in the expression of *GmPR2* in soybean roots when compared with control plants (Fig. 4B). However, no clear changes with re-

Fig. 4. Analysis of the expression of pathogenesis-related (*PR*) genes in soybean cv. Williams roots and shoots using quantitative real-time polymerase chain reaction. **A**, *GmPR1*; **B**, *GmPR2*; and **C**, *GmPR3*. Soybean plants were inoculated with the wild-type strain *Sinorhizobium fredii* HH103 Rif^r (white bars), HH103 Rif^r *ttsI*:: Ω (dotted bars), and HH103 Rif^r *nopP*::*lacZ*-Gm^r (gray bars). Data shown are means $\pm$ standard deviations for two biological triplicates. Expression is calculated relative to noninoculated control plants (black bars).

spect to the level of transcription of *GmPR2* in noninoculated control plants were detected in shoots (Fig. 4B).

Finally, changes in *GmPR3* gene expression in shoots after inoculation with the rhizobial strains assayed could not be detected. In the case of the roots, the expression of *GmPR3* slightly increased upon inoculation with the parental strain and the two mutant derivatives (Fig. 4C).

Although it has been proposed that the T3SS is required only during soybean nodule initiation (Krishnan 2002; Chang et al. 2007), the exact stage or stages of the symbiotic process in which Nops participate is not already clear. Because differences in the expression of *PR1* were found when soybean plants were inoculated with different strains affected in the T3SS, we carried out a time-course experiment to analyze the expression of *PR1* in soybean roots at different time points, 4 h and 2, 4, and 8 days after inoculation (Fig. 5). In these experiments, the HH103 *nopP* and *ttsI* mutants complemented with plasmid pMUS983 (discussed below) and plasmid pMUS984 (a derivative of the broad-host-range vector pMP92 carrying the *ttsI* gene), respectively (López-Baena et al. 2008) were included. In any case, no differences were observed in the expression of *PR1* 4 h and 2 days after inoculation compared with noninoculated plants. In plants inoculated with the parental strain HH103 Rif^r with either the *ttsI* or *nopP* mutants, the expression of *PR1* started to increase 4 days postinoculation and reached its maximum value after 8 days. As in the previous experiments, the maximum value of *PR1* expression was

obtained in plants inoculated with the HH103 *ttsI* derivative. The presence of plasmid pMUS984 in the *ttsI* derivative significantly decreased *PR1* expression. Only at 8 days after inoculation was the expression of *PR1* in soybean roots inoculated with the HH103 *nopP* derivative significantly lower than that observed in soybean roots inoculated with the parental strain. However, introduction of plasmid pMUS983 (carrying the *nopP* gene) did not restore *PR1* expression to the values obtained upon inoculation with HH103 Rif^r.

DISCUSSION

S. fredii HH103 secretes a cocktail of Nops, including NopP, a putative effector protein of approximately 31 kDa, to the extracellular space upon induction with flavonoids (Rodrigues et al. 2007). This protein is encoded by the *nopP* gene, an ORF that is also present in the *tts* clusters of *Rhizobium* strain NGR234, *S. fredii* USDA257, *B. japonicum* USDA110, *B. elkanii*, and *M. loti* MAFF303099. In all these cases, *nopP* is preceded by a *tts* box. In this work, we show that inactivation of both *ttsI* and *nodD1* blocked transcription of *nopP* upon induction with genistein (Fig. 1), indicating that expression of *nopP* is a flavonoid-, NodD1-, and TtsI-dependent process in HH103. Inactivation of the HH103 *nopP* gene prevented secretion of NopP to the extracellular medium but did not affect secretion of the rest of the Nops (Fig. 2). Secretion of this protein could be restored when the mutation was complemented

Fig. 5. Time-course analysis of the expression of the pathogenesis-related (*PR*) gene *PR1* in soybean cv. Williams roots. Soybean plants were inoculated with the wild-type strain *Sinorhizobium fredii* HH103 Rif^r (white bars), HH103 Rif^r *ttsI*::Ω (dotted bars), HH103 Rif^r *ttsI*::Ω complemented with plasmid pMUS984 (black-stripped bars) (López-Baena et al. 2008), HH103 Rif^r *nopP*::*lacZ*-Gm^r (gray bars), and HH103 Rif^r *nopP*::*lacZ*-Gm^r complemented with pMUS983 (gray-stripped bars). Data shown are the means ± standard deviations for two biological triplicates. Expression, calculated relative to noninoculated control plants (black bars), was determined 4 h and 2, 4, and 8 days postinoculation, respectively.

with a broad-host-range plasmid carrying a wild copy of the HH103 *nopP* gene including the *tts* box (Fig. 2).

In *Rhizobium* strain NGR234, NopP is a secreted effector protein that can be phosphorylated by plant kinases (Skorpil et al. 2005). In *Pachyrhizus tuberosus*, a *Rhizobium* strain NGR234 *nopP* mutant shows a Nod⁻ phenotype, indicating that NopP is required for the formation of nodules. However, the inactivation of *nopP* increases the nodule number in the tropical legumes *Flemingia congesta* and *Tephrosia vogelii* (Ausmees et al. 2004). Our results show that, in HH103, the absence of the effector protein NopP caused symbiotic effects opposite to those provoked by the complete absence of Nops. Thus, inactivation of *nopP* led to a significant increase in the number and fresh weight of nodules formed in the American soybean cv. Williams. In *E. variegata*, however, NopP was necessary to induce the formation of a reduced number of nodules and its symbiotic behavior was even worse than that shown by the parental strain (Fig. 3). In contrast, HH103 *rhcI* or *ttsI* mutants, which fail to secrete Nops, are impaired in their symbiotic capacity with soybean and show an improved symbiotic behavior with *E. variegata* (de Lyra et al. 2006; López-Baena et al. 2008). Thus, whereas the complete set of Nops secreted by HH103 acts positively for soybean nodulation, NopP exerts a negative effect on symbiosis. However, in *E. variegata*, the cocktail of Nops secreted by HH103 is detrimental for nodulation but NopP was required to induce the formation of a reduced number of ineffective nodules (Fig. 6). Therefore, results shown in this work suggest that the final symbiotic effect of the Nops secreted by HH103 would be the result of a complex interaction between each effector secreted and the host plant.

Plant-pathogenic and rhizobial T3SSs are well conserved and have been proposed to play similar roles in pathogenicity and symbiosis, respectively (Deakin and Broughton 2009; Soto et al. 2009). Previous results have suggested a role of rhizobial Nops in the modulation of plant defense responses (Bartsev et al. 2004; Dai et al. 2008). In this work, we have investigated whether the HH103 Nops modulate the expression of three soybean *PR* genes, *GmPR1*, *GmPR2*, and *GmPR3*, in roots and shoots. Our results show that only *GmPR1* expression was clearly affected (Fig. 4A), because no significant differences in *GmPR2* and *GmPR3* transcript levels in roots and shoots were scored between noninoculated control plants and those inoculated with either HH103 and a *ttsI* or a *nopP* mutant derivative (Fig. 4B and C). These results are partially in agreement with those shown by Brechenmacher and associates (2008) that only detected slight differences in the expression of a chitinase-like protein in soybean plants 8 days after inoculation with *B. japonicum*. To our knowledge, this is the first time that an increase in the expression in roots of *PR1*, a known marker for SAR, has been observed in a compatible *Rhizobium*-legume interaction.

To date, it is not clear how rhizobial effectors act during symbiosis. It has been proposed that they could facilitate bacterial release from infection threads, initiate symbiosome development, or promote or maintain persistence of bacteroids within cortical cells (Deakin and Broughton 2009). Results obtained in this work suggest that, in the symbiotic relationship established between HH103 and Williams soybean, these proteins could have a double function during the early stages of infection: i) they could be involved in the elicitation of a localized and controlled defense response in roots that would not compromise nodulation and ii) they could block the induction, caused by unknown elicitors, of a stronger defense response in roots and the systemic expression of the *PR1* gene in noninfected organs. This stronger defense response would clearly affect nodulation.

It has been shown that genes of the T3SS are active in early infection stages and also in mature nodules of different host plants (Perret et al. 1999; Zehner et al. 2008). However, in soybean, NopX could be detected only in infection threads but not in mature nodules (Krishnan 2002). In addition, proteomic and transcriptomic approaches have revealed that type III-related genes are downregulated in soybean mature nodules (Sarma and Emerich 2005; Chang et al. 2007). We found that the expression of *PR1* started to increase in roots 4 days after inoculation with the three rhizobial strains mentioned above (Fig. 5). Taking into consideration that there must be a time lapse between the recognition of the possible elicitors and the synthesis of the *PR1* proteins, these results suggest that HH103 Nops are recognized in early infection stages of the symbiosis with soybean.

The expression of *PR1* in soybean roots was significantly reduced at 8 days after inoculation when plants were inoculated with the complemented *ttsI* derivative compared with the expression detected in the parental strain HH103. This fact could be explained as a gene-dosage effect because plasmid pMUS984 (which carries the *ttsI* gene) is present in more than one copy. However, the fact that the presence of plasmid pMUS983 (which carries the *nopP* gene) in the *nopP* derivative of HH103 did not restore *PR1* expression in soybean roots to the levels exhibited by plants inoculated with the parental strain indicates that not only the absence of NopP but also its overexpression diminished *PR1* expression. Perhaps, this could be due to the fact that, in both cases, the amount of NopP (nothing or too much) is different from that present in the wild-type set of Nops.

The fact that Nops modulate plant defense responses suggests that rhizobia may manipulate host metabolic responses using mechanisms common to pathogens. Defense responses in leg-

Fig. 6. Rates of nodulation in soybean cv. Williams and *Erythrina variegata*. Values are expressed in percentages with respect to the number of nodules induced in plants inoculated with the wild-type strain *Sinorhizobium fredii* HH103 = 100% (white bars), *S. fredii* HH103 *ttsI*::Ω (dotted bars), and *S. fredii* HH103 *nopP*::*lacZ*-Gm^r (gray bars). Data of number of nodules induced by the *ttsI* mutant derivative have been previously reported (López-Baena et al. 2008).

ume plants are not well characterized yet and further work is necessary to determine how these responses affect nodulation.

MATERIALS AND METHODS

Microbial and molecular techniques.

Sinorhizobium strains were grown at 28°C on tryptone yeast (TY) medium (Beringer 1974) or yeast extract mannitol (YM) medium (Vincent 1970). *Escherichia coli* strains were cultured on Luria-Bertani (LB) medium (Sambrook et al. 1989) at 37°C. When required, the media were supplemented with the appropriate antibiotics as described by Lamrabet and associates (1999). Plasmids were transferred from *E. coli* to *Sinorhizobium* strains by conjugation as described by Simon (1984) using plasmid pRK2013 (Figurski and Helinski 1979) as helper.

Assays for β -galactosidase activity in liquid bacterial cultures on YM media were carried out as described by Vinardell and associates (2004). Three independent β -galactosidase assays were carried out in triplicate.

Recombinant DNA techniques were performed according to the general protocols of Sambrook and associates (1989). For hybridization, DNA was blotted to Hybond-N nylon membranes (Amersham, Tokyo), and the DigDNA method of Roche was employed according to the manufacturer's instructions. PCR amplifications were performed as previously described (Vinardell et al. 2004). Primer pairs used for amplification of the *S. fredii* HH103 *nopP* gene were yPF (5'-AGCAG CAACAGAGCCAGTGA) and yPR (5'-CGCCGATGGAGCC AACTCCA). Plasmids pMUS983 and pMUS843 were obtained by cloning in the broad-host-range vector pMP92 (Spaink et al. 1987) and the suicide plasmid pk18mob (Schäfer et al. 1994), respectively, a 1.8-kb PCR fragment containing the *nopP* gene and its *tts* box.

The pk18mob derivative plasmid pMUS843 was digested with *SspI*, treated with the Klenow enzyme, and ligated to an *SmaI* 4-kb fragment that contains the *lacZ*-Gm^r cassette harbored in plasmid pAB2001 (Becker et al. 1995). The unique *SspI* site is located 259 bp downstream of the ATG start codon of *nopP*. The plasmid generated was used for the homogenization of the mutated version of the *nopP* gene in *S. fredii* HH103 Rif^r (Madinabeitia et al. 2002) by using the methodology previously described (Vinardell et al. 2004), generating the mutant strain SVQ559 (= HH103 Rif^r *nopP*::*lacZ*-Gm^r). The double recombination event was confirmed by hybridization (data not shown).

Plant assays.

Nodulation assays on *G. max* (L.) Merrill cv. Williams and on *Erythrina variegata* (L.) were performed as described by de Lyra and associates (2006). Each Leonard jar contained two plants (one plant in the case of *E. variegata*). Each plant was inoculated with approximately 5×10^8 bacteria. Plants were grown for 42 days (90 days in the case of *E. variegata*) with a 16-h photoperiod at 26°C in the light and 18°C in the dark. Plant tops were dried at 70°C for 48 h and weighed. Nitrogen fixation was determined by acetylene reduction assay (Crespo-Rivas et al. 2007).

Purification and analysis of secreted proteins.

Extracellular proteins from *S. fredii* strains were recovered according to López-Baena and associates (2008). Briefly, 50 ml of YM bacterial cultures grown on an orbital shaker for 40 h were centrifuged for 20 min at $10,000 \times g$ at 4°C. Then, supernatants were mixed with three volumes of cold acetone and maintained at -20°C for 24 h. The mixtures were centrifuged for 45 min at $22,000 \times g$ at 4°C and dried pellets were resuspended in 300 μ l of sample buffer. Extracellular proteins were

separated by SDS-PAGE using the discontinuous buffer system of Laemli (1970). Electrophoresis was performed on SDS 15% (wt/vol) polyacrylamide gels and proteins were visualized by silver staining.

For immunostaining, extracellular proteins were separated on SDS 15% (wt/vol) polyacrylamide gels and electroblotted to Immobilon-P polyvinylidene difluoride membranes (Bio-Rad) using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad). Membranes were blocked with Tris-buffered saline containing 2% (wt/vol) bovine serum albumen (BSA) and then incubated with antibodies raised against NopP (Ausmees et al. 2004) diluted 1:3000 in the same solution. Anti-rabbit immunoglobulin antibodies (AP-conjugate) were used as secondary antibodies. Reaction results were visualized using nitroblue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate (NBT-BCIP).

RNA isolation and cDNA synthesis.

For bacterial RNA extraction, *S. fredii* strains HH103 Rif^r, HH103 Rif^r *ttsI*:: Ω (López-Baena et al. 2008), and HH103 Rif^r *nodD1*:: Ω (Lamrabet et al. 1999) were incubated with shaking at 28°C in YM medium supplemented with 3.7 μ M genistein when necessary. When cultures reached an optical density at 660 nm of 0.5, cells were harvested and RNA was extracted using the RNeasy minikit following the manufacturer's instructions (Qiagen, Basel, Switzerland). Samples were treated with RNase-free DNase (Qiagen). Two independent RNA extractions were performed.

For soybean RNA extraction, five pregerminated *G. max* (L.) Merrill cv. Williams 82 seeds were aseptically transferred to a stainless-steel lattice placed in a glass cylinder containing 150 ml of a 1 M Fåhræus solution, pH 6.8, and grown in a controlled environment chamber with a 16-h day, 8-h night cycle and a relative humidity of 70%. Growth temperatures were set to 26°C during the day and 18°C during the night. The hydroponics system was inoculated at the time of transferring the pregerminated seed with approximately 10^6 bacteria. Eight days after inoculation, shoots and the upper third of the soybean roots were excised, fast-frozen in liquid nitrogen, and stored at -80°C until use. For the time-course experiments, root samples were obtained 4 h and 2, 4, and 8 days postinoculation. RNA was extracted using the RNeasy plant minikit following the manufacturer's instructions (Qiagen) and samples were treated with RNase-free DNase (Qiagen). For confirmation of sterile conditions, a sample of each root exudate was inoculated in TY and growth was assessed after overnight incubation at 28°C. Two independent RNA extractions were carried out.

RNA concentration was quantified using the fluorometer Qubit (Invitrogen, Carlsbad, CA, U.S.A.) and the integrity of the RNA was assayed on 1% agarose gels. cDNA was synthesized using the Quantitect kit according to the manufacturer's instructions (Qiagen).

RT-PCR analysis.

For RT-PCR analysis of the expression of the *S. fredii* HH103 *nopP* gene, an internal 359-bp fragment of *nopP* was amplified using primers yPintF (5'-TCAGGAGGGAGATCA GGA) and yPintR (5'-GTCCCTCCGAAGTTGGTA). As an internal control, primers of the rRNA *16S* gene were used (Crespo-Rivas et al. 2009).

The gene-specific primer pairs employed for the detection of transcripts of the soybean *GmPR1* (acidic PR1), *GmPR2* (basic β -1,3-endoglucanase), *GmPR3* (basic chitinase), and *UBI3* (ubiquitin-3, used as an internal control) genes in noninoculated control plants have been previously described (Mazarei et al. 2007).

To analyze gene expression, equal amounts (1 µg) of total RNA were used in cDNA synthesis and then equivalent amounts of cDNA were used as probes in the PCR reactions under the following conditions: 95°C, 5 min; 95°C, 30 s; 55°C (soybean *PR* genes) or 51°C (*S. fredii* HH103 *nopP*), 30 s; 72°C, 30 s for 25 cycles, and then, 72°C, 5 min. The resultant PCR products were visualized on a 1% agarose gel for comparison. RT-PCR assays were performed at least twice for each RNA sample.

qRT-PCR.

To quantify the *S. fredii* HH103 *nopP* gene expression using qPCR, primers rt-nopP-F (5'-GTTCAGGAGGGAGATCAGGAA) and rt-nopP-R (5'-CCAACACACTGGTTTCCCAA) amplifying an internal fragment of 189 bp were used. Expression was calculated relative to the parental strain not induced with genistein. The *S. fredii* HH103 *ARN 16S* gene was used as an internal control to normalize gene expression.

To quantify soybean gene expression, primers previously described for RT-PCR were used. Expression was calculated relative to noninoculated soybean control plants. The *UBI3* gene was used as an internal control to normalize gene expression across different samples.

The expression data shown are the mean (± standard error of the mean) for two biological replicates. The fold change in the target gene, normalized to RNA *16S* or *UBI3* and relative to the gene expression in the control sample, was calculated.

The reactions were performed in a 20-µl final volume containing 1 µl of cDNA, 0.6 pmol each primer, and 10 µl of Fast-Start SYBR Green Master Mix (Roche Diagnostics). PCR was conducted on the iCycler IQ (Bio-Rad Laboratories SA) with the following conditions: 95°C, 10 min; 95°C, 30 s; 55°C (soybean *PR* genes) or 63°C (*S. fredii* HH103 *nopP*), 30 s; 72°C 20 s; 40 cycles, followed by the melting curve profile from 60 to 95°C to verify the specificity of the reaction. The threshold cycles were determined with the iCycler software and the individual values for each sample were generated by averaging three technical replicates that varied less than 0.5 cycles.

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