GunA of Sinorhizobium (Ensifer) fredii HH103 is a T3SS-secreted cellulase that differentially affects symbiosis with cowpea and soybean

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Abstract

Aims

The symbiosis between *Sinorhizobium fredii* HH103 and its host legumes is influenced by the type 3 secretion system (T3SS), which delivers proteins (effectors) directly into the host cells to promote infection. GunA, one of the predicted HH103 effectors, potentially codes for a cellulase. In this work we tried to characterise GunA and elucidate its role in symbosis with soybean and cowpea.

Methods

A GunA::HA fusion protein was constructed to study T3SS-dependent secretion. Cellulase activity of GunA was measured and *gunA*::*uidA*-GFP and *gunA*::*cyA* fusions were constructed to monitor *gunA* expression in nodules and to study translocation to the host cells, respectively. Finally, the symbiotic performance of a *gunA* mutant was studied in soybean and cowpea.

Results

GunA from *S. fredii* HH103 shows cellulase activity and is secreted through the T3SS in response to the inducer flavonoid genistein. Interestingly, at the beginning of the symbiotic process, GunA was partially responsible for the induction of the expression of the soybean *GmPR1* gene, a gene used as a marker for plant defense responses. However, GunA was also detected in soybean and cowpea developed nodules. Finally, nodulation assays indicate that GunA is beneficial for symbiosis with soybean but detrimental with cowpea.

Conclusion

Secretion of GunA through the *S. fredii* HH103 T3SS clearly and differentially impacts the symbiotic performance of this strain with soybean and cowpea. GunA, or its cellulase activity, is recognised by soybean root cells very early in the symbiotic process but, curiously, its secretion can also be detected in mature nodules. This suggests different symbiotic roles at different symbiotic stages that need to be further elucidated.

1. Introduction

Legumes can establish a symbiotic relationship with nitrogen-fixing rhizobia. Thus, in response to the appropriate Rhizobium partner, plants form a specific symbiotic organ, called nodule, in which rhizobia fix atmospheric nitrogen (Poole et al. 2018). This process requires a specifically evolved signal exchange between both symbiotic partners. Certain plant flavonoids exuded by legume roots induce the synthesis of specific rhizobial lipochitooligosaccharides (also called Nod factors), whose recognition by the host plant triggers both rhizobial infection and initiation of nodule organogenesis (Gage 2004). Besides, there are other rhizobial molecules that play an important role in the establishment of a succesful symbiosis with the host plant, which includes polysaccharides and secreted proteins (Downie 2010; López-Baena et al. 2016).

The type 3 secretion system (T3SS) is a protein export pathway present in many plant and animal pathogenic Gram negative bacteria. This apparatus delivers proteins, called effectors, directly into the host cells in order to suppress basal defenses and alter the host metabolism to promote disease (**Galan and Collmer, 1999; Pallen *et al.*, 2003**). In the case of rhizobia, T3SS effectors manipulate also cellular processes in host cells to suppress plant defense responses against rhizobia and to promote symbiosis-related processes (**Deakin and Broughton, 2009; Staehelin and Krishnan, 2015**). In this sence, the T3SS of the broad host-range bacterium *S. fredii* HH103, hereafter HH103, is responsible of the suppression of early soybean defense responses to effectively nodulate this legume (**Jiménez-Guerrero *et al.*, 2015 a**). In the other hand, the uncommon T3SS of *Bradyrhizobium elkanii* USDA61 is implied in the promotion of the nodulation with soybean when infecting via crack-entry or intercellular infection even in the absence of Nod factors (**Okazaki *et al.*, 2013**).

The expression of the T3SS genes is controlled by the transcriptional regulator TtsI, whose expression is activated by NodD upon recognition of appropriate flavonoids. Then, TtsI binds to specific promoter sequences called *tts* boxes, inducing the transcription of the whole set of T3SS genes (Krause et al. 2002; López-Baena et al. 2008; Wassem et al. 2008; Zehner et al. 2008). Rhizobial T3SS secreted proteins are called Nodulation outer proteins (Nops). While certain Nops are external components of the T3SS apparatus (NopA, NopB, and NopX), other Nops (such as NopC, NopD, NopI, NopJ, NopL, NopM, and NopT) are considered to be effector proteins that are translocated into plant cells (Jiménez-Guerrero et al. 2015a, 2017; Rodrigues et al. 2007; Schechter et al. 2010; Staehelin and Krishnan 2015).

Analysis of the HH103 genome showed the presence of a conserved *tts* box upstream of *gunA* (SFHH103_04355; pSfHH103d - pSym), which is likely to encode an endoglucanase (Vinardell et al. 2015). Transcriptomic data revealed that its expression depends on NodD1, TtsI and flavonoids (PérezMontaño et al. 2016). A similar gene (*gunA2*) has been previously described in the symbiotic region of the *B. diazoefficiens* USDA110 chromosome (Caldelari Baumberger et al. 2003). The expression of *gunA2* in USDA110 is also induced by genistein and the protein exhibits cellulase activity (Caldelari Baumbergeret al. 2003). GunA2 has been reported to be secreted through the *B. diazoefficiens* USDA110 T3SS (Süß et al. 2006).

Plant cell wall degradation is an important process of microbial plant pathogenesis and plays a key role in the rhizobial infection process (Bellincampi et al. 2014; Murray 2011; Robledo et al. 2015). In some pathogenic plant-microbe interactions, cell wall degradation products, released as consequence of enzyme activity, have been found to act as infection reporters sensed by the plant as damage-associate molecular patterns (DAMP), which results in the activation of innate immune responses (Sinha et al. 2013).

In this work, we studied the secretion of GunA by the HH103 T3SS and its expression in cowpea and soybean nodules. We analyzed the cellulase activity of GunA and its possible implication in the elicitation of an early defense reaction in soybean roots. Finally, we constructed a *gunA* mutant strain and tested its symbiotic behaviour with soybean and cowpea.

Materials and Methods

Microbial and molecular techniques

Bacterial strains and plasmids used in this work are listed in Supplementary Table 1. *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 and Russell 2001) at 37 °C. When required, media for *Sinorhizobium* were supplemented with the antibiotics rifampicin (Rif, 50 μg mL^{-1}), spectinomycin (Spc, 50 μg mL^{-1}) and kanamycin (Km, 60 μg mL^{-1}). For *E. coli*, 100 μg mL^{-1} (Spc) or 30 μg mL^{-1} (Km) were used. Genistein was dissolved in ethanol and used at 1 μg mL^{-1} to give a final concentration of 3.7 μM. Plasmids were transferred from *E. coli* to *Sinorhizobium* strains by conjugation as described by Simon (1984) using plasmid pRK2013 as helper.

Recombinant DNA techniques were performed according to the general protocols of Sambrook and Russell (2001). For hybridization, DNA was blotted to Hybond-N nylon membranes (Amersham, UK) and the DigDNA method of Roche (Switzerland) was applied following the manufacturer's instructions. PCR amplifications were performed as previously described (Jiménez-Guerrero et al. 2017). Primer pairs used for the amplification of the HH103 Rif^R genes are summarized in Supplementary Table 2.

The in-frame *gunA* gene deletion was constructed by overlap extension polymerase chain reaction (Ho et al. 1989) using the primer pairs gunA-1/gunA-2 and gunA-3/gunA-4 (Supplementary Table 2). The final DNA fragment was digested with HindIII/ BamHI and cloned into plasmid pK18*mobsacB* previously digested with the same enzymes, obtaining plasmid pMUS1340. This plasmid was then used for the homogenotization of the mutated version of *gunA* in HH103 Rif^R. The deletion event was confirmed by PCR and hybridization (data not shown). HH103 Rif^R with plasmid

pMUS1340 integrated downstream of the *gunA tts* box was used for complementation analysis.

For the expression of the GunA-Hemagglutinin (HA) tag fusion protein, a ~0.8-kb fragment containing the *gunA* gene without end codon was amplified using the primers gunA_EcoRI_F and gunA_HA_KpnI_R, which added the DNA sequence of HA epitope to the 3´ terminus of *gunA* gene. Then, this fragment was digested with *Eco*RI/*Kpn*I enzymes and cloned into pK18*mob*, previously digested with the same enzymes, obtaining plasmid pMUS1367. This plasmid was integrated into the symbiotic plasmid of HH103 Rif^R by a simple homologous recombination event.

For the integration of a transcriptional *gunA-uidA* fusion into the HH103 Rif^R genome, an internal DNA fragment of the *gunA* gene (~1 kb) was amplified using primers gunAint_SalI_F and gunAint_XbaI_R (Supplementary Table 2). This fragment was digested with the enzymes SalI and XbaI, and the resulting 427-bp fragment was subcloned into the vector pVO155-npt2-GFP, previously digested with the same enzymes. The resulting plasmid pMUS1384 was transferred to HH103 Rif^R by conjugation and a single homologous recombination event was selected.

For the construction of the translational fusion *gunA*::*cya*, primers attB1_gunA and attB2nostop_gunA (Supplementary Table 2) were used for the amplification of the *gunA* gene without stop codon. The amplified 800-bp fragment was then cloned into pDONR221 by Gateway (Invitrogen, USA) cloning, obtaining plasmid pMUS1332. This plasmid was used to subclone the *gunA* gene into plasmid pLMS150 (Schechter et al. 2010), which possesses recombination sites for clonase II upstream of the *cya* gene, rendering plasmid pMUS1337. The *gunA*::*cya* gene fusion was confirmed by sequencing. The *gunA*::*cya* fusion was then amplified using primers gunA_EcoRI and cya_XbaI (Supplementary Table 2). The amplified fragment was digested with the

enzymes EcoRI and XbaI and the resulting DNA fragment was cloned into pK18*mob*, previously digested with the same enzymes, obtaining plasmid pMUS1338. This plasmid was used for integration of the *gunA*::*cya* fusion into the genomes of HH103 Rif^R and in its *rhcJ*::Ω mutant derivative (de Lyra et al. 2006) by single recombination. The integration into the symbiotic plasmid resulting of a single recombination event was confirmed by antibiotic resistance and PCR amplification using primers described in Supplementary Table 2.

For heterologous expression of GunA in *E. coli*, *gunA* was amplified from total DNA of *S. fredii* HH103 Rif^R with Pfu DNA polymerase (Thermo Fisher Scientific, USA) using primers SfgunAf_NcoI and SfgunAr_EcoRI (Thermo Fisher Scientific, USA). The fragment and the vector pMal-c5X, which contains the MBP (maltose binding protein) tag, were restricted with NcoI and EcoRI and ligated. This resulted in plasmid pMal-c5X-SfgunA-1, which was used for expression of the MBP-GunA fusion protein. The nucleotide sequence of *gunA* and the fusion site were confirmed by sequencing.

RNA isolation, cDNA synthesis and quantitative RT-PCR

Soybean qRT-PCR assays were carried out as described by Jiménez-Guerrero et al. (2015b). To quantify soybean cv. Williams 82 gene expression, specific primers were designed (Supplementary Table 2). Reactions were performed in a final volume of 10 μL containing 25 ng of cDNA, 0.6 pmol of each primer and 5 μL of FastStart SYBR Green Master Mix (Roche, Switzerland). PCR was conducted on a Light Cycler 480 II (Roche, Switzerland) with the following conditions: 95 °C, 10 min; 95 °C, 30 s; 54 °C, 30 s; 72 °C, 15 s; 45 cycles, followed by analysis of the melting curve profile from 65 to 95 °C to verify the specificity of the reaction. The threshold cycles (Ct) were determined with the Light Cycler 480 II software and the individual values for each sample were generated by averaging three technical replicates that varied less than 0.5

cycles. Plant gene expression was calculated relative to the uninoculated treatment. The UBI3 gene was used as an internal control to normalize gene expression. The expression data shown are the mean (± standard deviation of the mean) of two independent biological replicates performed at least with three technical repeats each. The fold-change in the target gene, normalized to UBI3 and relative to the gene expression in the control sample was calculated.

Purification and analysis of nodulation outer proteins

Extracellular proteins were recovered as described by Jiménez-Guerrero et al. (2015a). Extracellular proteins were separated by SDS-PAGE using the discontinuous buffer system of Laemmli (1970). The same volume of each protein extraction was loaded in each taking into consideration that protein extractions were carried out from the same volume of cultures at the same growth stage with similar cell number. Growth rate in the mutant and the complemented strains was not affected. Electrophoresis was performed on SDS 12% (w/v) polyacrylamide gels. After the electrophoresis, proteins were silver stained. Briefly, proteins were fixed with fixation solution (40% ethanol, 10% acetic acid) for 30 min with slight agitation. Then, the gel was submerged in oxidizing solution [30% ethanol, sodium acetate 0.8 M, 0.04% (v/v) sodium thiosulphate 5% (m/v)] for 30 min and washed three times with water for 5 min. The silver reagent was then added [silver nitrate 0.0025% (m/v)] and the gel was maintained in the dark for 20 min with slight agitation. Proteins were visualized using the developing solution (Na_2CO_3 0.22 M, 0.0002% formaldehyde (v/v)] until clear bands appeared. The reaction was stopped with Na_2-EDTA.$2H_2O$ 0.03 M.

For immunostaining, extracellular proteins were separated on SDS 15% (w/v) polyacrylamide gels and electroblotted to Immun-Blot polyvinylidenedifluoride (PVDF) membranes (Bio-Rad, USA) using a Mini Trans-Blot electrophoretic transfer cell (Bio-

Rad, USA). Membranes were blocked with TBS containing 2% (m/v) bovine serum albumin (BSA) and then incubated with antibodies raised against the HA epitope (Cell Signalling Technology, USA) diluted 1:1000 in the same solution. Anti-rabbit immunoglobulin APconjugated secondary antibody was used and reaction results were visualized using NBT-BCIP

Adenylate cyclase (cya) assay.

To determine whether the HH103 Rif^R T3SS was able to translocate the GunA-Cya fusion protein into soybean root cells, the protocol described by Jiménez-Guerrero et al. (2015a) was used. Briefly, 18 pre-germinated *Glycine max* cv. Williams 82 seeds were aseptically transferred to a recipient containing vermiculite and 150 mL of a Fåhraeus solution (Fåhraeus 1957) 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 system was inoculated at the time of transferring the pre-germinated seeds with bacterial cultures of about 108 cfu mL^{-1} of the *S. fredii* strains HH103 Rif^R or its HH103 Rif^R *rhcJ*::Ω mutant derivate, both containing a chromosomal integration of the *gunA*::*cya* fusion. Cyclic AMP (cAMP) accumulation was measured in nodules harvested at 18 days post-inoculation (dpi). Nodules were frozen in liquid nitrogen, ground to a fine powder and resuspended in a 0.1 M hydrochloric acid solution. The suspension was centrifuged, and the supernatant was used for cAMP measurement using the cyclic AMP (direct) EIA kit (Cayman Chemical Company, USA) according to the manufacturer's instructions. Each sample was diluted for quantification to measure cAMP concentration in the detection range of the assay. The HH103 Rif^R parental strain was used as a control for quantification.

Staining of nodule sections

Nodules of *V. unguiculata* cv. Red Caloona and *G. max* cv. Obelix were harvested, immediately frozen at −20 °C and stored until further use. Nodules were taken out one at a time and hand-sectioned with a razor blade. Slices were placed immediately in 100 mM sodium phosphate buffer pH 7.0. For staining, slices were incubated at 37 °C for 4–7 h in a buffer containing 100 mM sodium phosphate pH 7.0, 1 mM EDTA, 0.1% Tween 20, 2 mM X-Gluc (Panreac-Applichem, Germany), 0.5 mM $K_3Fe(CN)_6$ and 0.5 mM $K_4Fe(CN)_6$). Slices were fixed with 4% paraformaldehyde in 100 mM sodium phosphate buffer (pH 7.0). Results were analyzed and documented with a Leica MZ10 F stereo microscope (Leica, Germany) equipped with a ProgRes® SpeedXT core 5 microscope camera (Jenoptik, Germany).

Protein purification

Plasmid pMal-c5X-SfgunA-1 was transformed into *E. coli* BL21(DE3). The strain was grown in LB medium containing ampicillin at 150 μg mL^{-1}. At an OD600 of 0.6–0.8, heterologous expression of the MBP-GunA fusion protein was induced with IPTG (200 μM final concentration). Cultures were grown at 28 °C for 4 h. Crude extracts were obtained as described by Schirrmeister et al. (2011) using TKE buffer (50 mM Tris, 200 mM KCl, 10 mM EDTA, pH 8.0). The GunA-MBP fusion protein was eluted from a MBP Trap HP column (GE Healthcare, Sweden) by TKE containing maltose (10 mM) with the ÄKTAprime™ system (GE Healthcare, Sweden). Purified GunA protein was stored in TKE elution buffer at 4 °C until use. The GunA protein was cleaved from its fusion partner MBP with Factor Xa (New England BioLabs, USA) for 4 h at room temperature. To concentrate the purified protein and/or exchange the TKE elution buffer, the AMICON®Ultra15 Centrifugal Filter (Merck, USA) was used. Protein concentration for enzyme assays was determined by Roti® Nanoquant (Carl Roth, Germany) according to the manufacturer's protocol.

Cellulase activity assay

The activity assay was done similarly as described (Teather and Wood 1982). Ultra high viscosity carboxymethyl cellulose (CMC; 0.2% (w/v); Fluka, USA) and agar at 1% (w/v) were dissolved in sodium acetate buffer (0.1 M sodium acetate, pH 5.0) by stirring at 80 °C. Approximately 20 mL were poured into Petri dishes. After solidification, wells of approximately 6 mm were cut in the agar and 30 µL of protein solution was applied per well. Plates were subsequently incubated for 18–22 h at 28 °C. After incubation, the plates were rinsed with distilled water, flooded with an aqueous Congo red solution (5 mg mL^{-1}, Sigma-Aldrich, USA) and incubated for 30 min with agitation at room temperature. The plates were then rinsed with distilled water, washed with 1 M sodium chloride for 30 min with agitation and rinsed again with distilled water. Results were documented using digital photography.

As positive control, cellulase from Aspergillus niger (Fluka, USA) was dissolved at 1 mU μL^{-1} in sodium acetate and filter sterilised. As negative control, the fusion partner MBP was used.

Plant assays

Nodulation assays on *Glycine max* (L.) Merrill cv. Williams 82 (soybean) and *Vigna unguiculata* cv. Bisbee Red (cowpea) were performed as described by de Lyra et al. (2006). At least two independent nodulation assays (giving similar results) were performed. Each Leonard jar contained two plants. Each plant was inoculated with about 5×10^8 bacteria. Plants were grown for 42 days with a 16 h-photoperiod at 25 °C in the light and 18 °C in the dark. Plant shoots were dried at 70 °C for 48 h and weighed. For nodule staining, *G. max* cv. Obelix and *V. unguiculata* cv. Red Caloona plants were cultivated in a plant growth chamber SANYO MLR-H (Sanyo Electric Co., Japan) with controlled temperature and light cycle: 8 h dark at 22 °C, 16 h light at 26 °C

278 with light intensity of about 230 µmol s^{-1} m^{-1}. For surface sterilisation, seeds were
279 submerged in 96% ethanol for 3 min, washed two times with sterile water, submerged
280 in 0.6% sodium hypochlorite solution for 5 min and finally washed 6 times with sterile
281 water. Sterilised seeds were germinated on 0.8% agar plates in the dark at 28 °C or at
282 room temperature. Seedlings were transferred into sterile GA-7 Magenta boxes (Merck,
283 Germany) containing vermiculite (1– 2 mm), 100 mL of nitrogen free Jensen medium
284 (Vincent 1970) and 50 mL distilled water. Seedlings were inoculated with strain HH103
285 Rif^R::pMUS1384, as previously described (Krause et al. 2002) and watered with sterile
286 water. Nodules were harvested 21 or 28 dpi.
287

Results

Up-regulation of gunA is a flavonoid-, NodD1-, and TtsI-dependent process in S. fredii HH103

As commented in introduction, the *S. fredii* HH103 genome sequencing allowed the identification of the *gunA* gene, which is preceded by a conserved *tts* box, located 105 bp upstream *gunA* (**Vinardell *et al.*, 2015**). Therefore, is the expression of the *gunA* gene regulated in the same manner than the rest of T3SS genes? In order to confirm this question quantitative PCR assays were performance to quantify the expression of *gunA* in the *S. fredii* HH103 Rif^{R}, HH103 Rif^{R} *nodD1*Ω, and HH103 Rif^{R} *ttsI*Ω strains, in the absence and in the presence of the inducing flavonoid genistein. Results showed an increase of *gunA* expression (about 13-fold) in the parental strain when induced with genistein (**Figure X, A**). On the other hand, up-regulation of this gene was not detected in both *nodD1* and *ttsI* mutants, confirming that the expression of *gunA* depends on flavonoids and on the rhizobial transcriptional regulators NodD1 and TtsI. Finally, the *nodA* expression was analyzed as control, obtaining higher gene expression levels in the presence of genistein only in the parental strain and in the *ttsI* mutant (about 120-fold and 140-fold, respectively) (**Figure X, B**).

GunA is secreted through the Sinorhizobium fredii HH103 Rif^{R} T3SS.

The TtsI- and genistein-dependent regulation of the *gunA* gene in HH103 (Pérez-Montaño et al. 2016) suggests that GunA is a secreted protein. However, the protein was not detected in the supernatant of a genistein-treated culture (Rodrigues et al. 2007). For a more sensitive assay, GunA was tagged with the HA epitope. The genetic construct was integrated into the symbiotic plasmid of strains HH103Rif^{R} (wild type), HH103 Rif^{R} *rhcJ*::Ω, and HH103 Rif^{R} *ttsI*::Ω. These two mutants are unable to secrete

proteins through the T3SS (de Lyra et al. 2006; López-Baena et al. 2008). Western-blots using an antibody against the HA epitope allowed the detection of GunA only in supernatants from the wild type strain induced with genistein (Fig. 1a). No clear specific signal was detected in the supernatants of the *ttsI* mutant and a slight signal was observed in the lane corresponding to the *rhcJ* mutant, most probably due to slight cell lysis in the bacterial culture or during centrifugation of the culture. These results indicate that GunA was secreted through the T3SS in a flavonoid dependent manner. To determinate whether the absence of GunA could affect the secretion of other Nops, we analyzed the extracellular protein profiles of the *S. fredii* strains HH103 Rif^R, HH103 Rif^R Δ*gunA* and HH103 Rif^R Δ*gunA* complemented with plasmid pMUS1340 by SDS-PAGE. The absence of GunA did not affect secretion of the rest of Nops (Fig. 1b).

GunA exhibits cellulase activity

Based on a database search, GunA belongs to the glycosyl hydrolase family 12. It was reported to be highly similar (93% identity) to GunA2 of *B. diazoefficiens* (Vinardell et al. 2015), for which cellulase activity was previously shown (Caldelari Baumberger et al. 2003). To test whether GunA also possesses cellulase activity, CMC agar plates were used. This assay allowed visualisation of enzyme activity as a diffusion zone of CMC hydrolysis product around an applied sample. A commercially available *Aspergillus niger* cellulase was used as a positive control. MBP isolated from *E. coli* containing the vector without insert served as a negative control. Data shown in Fig. 2 confirmed that the fusion protein GunA-MBP, as well as GunA alone, exhibited cellulase activity.

The gunA gene is expressed in soybean and cowpea nodules

In order to investigate the expression of *gunA* in nodules, cowpea and soybean seedlings were inoculated with strain HH103 Rif^{R}::pMUS1384, which contains a chromosomally integrated *gunA*::*uidA* transcriptional fusion. Expression of *gunA* was detected in cowpea and soybean nodules at 21 dpi and 28 dpi (Fig. 3), indicating that the gene is expressed in developed nodules.

Adenylate cyclase assays were used to determine whether GunA is translocated to the interior of the nodule host cells. This method is based on the utilization of the Bordetella pertussis calmodulin-dependent adenylate cyclase (Cya) toxin like a reporter protein The toxin is activated within eukaryotic cells and hence increase cAMP levels. However, no activation of this protein is detected in prokaryotic cells so it can be used to identify bacterial effector proteins (Sory and Cornelis 1994). Previous works of Jiménez-Guerrero et al. (2015a, 2017) demonstrated the translocation of NopC- and NopL-Cya fusions expressed in HH103 Rif^{R} into soybean nodules. No significant differences in the levels of cAMP were detected among HH103 Rif^{R}, HH103 Rif^{R} *gunA*::*cya* and HH103 RifR rhcJ::Ω gunA::cya at 18 dpi (Supplementary Fig. 1). Therefore, based on the results obtained under the used assay conditions, we cannot conclude that GunA was translocated into the host cells.

GunA induces the expression of the soybean GmPR1 gene at an early stage of symbiosis

To determine whether cell wall degradation products, potentially generated by the activity of GunA, could elicit the early defense response previously observed in soybean roots inoculated with HH103 Rif^{R} (LópezBaena et al. 2009), the expression of the pathogenesis related gene *GmPR1* (Mazarei et al. 2007) was measured by qRT-PCR. RNA from plant roots was isolated 8 h post inoculation with strains HH103 Rif^{R}, HH103 Rif^{R} Δ^{gunA}, HH103 Rif^{R} Δ^{gunA} complemented, HH103 Rif^{R} *rhcJ*::Ω and HH103

Rif^R *ttsI*::Ω. As shown in Fig. 4, the highest induction of *GmPR1* was observed in plants inoculated with the parental strain (about 3.9- fold) and the complemented *gunA* mutant strain (about 6-fold). By contrast, GmPR1 expression levels in plants inoculated with the *rhcJ* and *ttsI* mutants were similar to those obtained for uninoculated plants.

Interestingly, the *GmPR1* expression values in plants inoculated with the *gunA* mutant were reduced compared to the wild type. In summary, our results indicated that soybean was able to recognize the presence of HH103 Rif^R at least partially through the activity of GunA, activating an early defense response indicated by the transcriptional activation of *GmPR1*

GunA is detrimental for symbiosis with cowpea but beneficial for soybean

Nodulation assays were performed to study the effect of the deletion of *gunA* in the symbiosis with soybean and cowpea. Plants were inoculated with the parental strain HH103 Rif^R, the HH103 Rif^R Δg^{unA} mutant and the complemented strain (Table 1). The absence of GunA caused a significant reduction in the number of nodules formed on soybean roots (from 91.4 ± 21.2 to 51.7 ± 14.9). Nodule fresh weight and shoot dry weight were also reduced but these reductions were not significant either. Complementation of the mutation increased the number of nodules formed to a number similar to that formed by the parental strain (85.7 ± 13.9; Table 1). In the case of cowpea, the deletion of *gunA* was beneficial for symbiosis. Although the final number of nodules formed on cowpea roots when the plant was inoculated with the *gunA* mutant was similar to that formed by the wild type strain (40.1 ± 6.4 vs 26.8 ± 8.2), there were significant increases in nodule fresh weight (1.03 ± 0.24 vs 0.53 ± 0.22 g) and shoot dry weight (1.61 ± 0.68 vs 0.65 ± 0.28 g), indicating that this symbiosis is less effective when GunA is present.

388 Complementation of the mutation restored all symbiotic phenotypes (Table 1).

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Discussion

The analysis of the HH103 genome (Vinardell et al. 2015) allowed the identification of *gunA*, a gene that codes for a glycosyl hydrolase which is highly similar (93% identity) to the genistein-inducible gene *gunA2* from *B. diazoefficiens* USDA110 (Caldelari Baumberger et al. 2003). The promoter region of the HH103 *gunA* gene, like the *B. diazoefficiens* USDA110 *gunA2*, contains a *tts* box and its expression is inducible by genistein and regulated by NodD1 and TtsI (PérezMontaño et al. 2016). All these data would suggest that the HH103 GunA could be secreted through the T3SS to the extracellular milieu in response to inducer flavonoids like previously reported for the *B. diazoefficiens* USDA110 GunA2 (Süß et al. 2006). In this work, a *gunA*::HA fusion was constructed and integrated into the parental strain and into mutants unable to secrete Nops to confirm this secretion. The results obtained showed that GunA was secreted by the wild type strain in response to flavonoids but not by the T3SS mutants. In addition, the inactivation of *gunA* did not affect secretion of the rest of Nops. These results indicate that GunA is a secreted Nop and not a component of the T3SS machinery.

The analysis of the predicted tertiary structure of the HH103 GunA indicated that it could be a glycosyl hydrolase. In addition, we tested the activity of a GunA-MBP fusion protein or GunA alone, and both could degrade cellulose in vitro. It seems possible that GunA, once secreted to the extracellular millieu, collaborates with plant cellulases or other rhizobial glycanases for the degradation of the infection thread wall to enter the host cell endocytotically (Verma et al. 1978). Alternatively, GunA could degrade the infection thread wall to bring rhizobia in closer contact with the host cell plasma membrane and help to insert the T3SS-pilus. Because infection threads can be still detected in soybean nodules at an intermediate stage (Tu 1974), GunA might collaborate in the degradation of the infection threads at different stages of nodule

development. This hypothesis is supported by the fact that the adenylate cyclase assay indicated that GunA was not translocated into the interior of the host cells, at least at 18 dpi, in contrast to other HH103 RifR effectors like NopC and NopL, which were translocated at this stage (Jiménez-Guerrero et al. 2015a, 2017). It could be possible that GunA::CyA was delivered into root cells at other time points during the infection process or translocated at levels below the range of detection of the assay. Determination of the exact moment in which each effector is delivered would help to determine whether it is translocated to the interior of the root cells. However, this would need very sensitive fluorescent reporters to monitor gene expression due to the low levels of secretion of Nops.

The HH103 Rif^R T3SS is expressed in young soybean nodules but expression declines in older nodules (Jiménez-Guerrero et al. 2015a). Similarly, Pessi et al. (2007) reported that for *B. diazoefficiens* USDA110, genes of the T3SS are expressed stronger in young soybean nodules (13 dpi) than in older nodules (21 or 31 dpi). Data obtained here with GUS staining of nodules suggest that *gunA* is active also at three or 4 weeks after infection. However, nodule staining is very sensitive and does not indicate the strength of the expression. Expression of type 3 secretion genes in determinate nodules at 4 weeks after inoculation was also reported for *Macroptilium* atroprupureum nodules infected with *B. diazoefficiens* (Zehner et al. 2008). Thus, the T3SS might still have a function in fully developed nodules.

An increase in the levels of the plant hormone salicylic acid (SA) and the induction of the expression of the SA-dependent *PR1* gene are important components of the defense response developed by plants in response to the recognition of biotrophic pathogens (Glazebrook 2005; Denoux et al. 2008). It has been reported that in the *S. fredii* HH103 Rif^R-soybean interaction, two different defense responses are elicited at

different stages of the nodulation process (Jiménez-Guerrero et al. 2015b). In the earlier stages (2 h post inoculation), an unknown molecule associated to the T3SS induces the expression of a weak defense response in soybean roots that is quickly suppressed. At later stages (2–4 dpi), the T3SS suppresses the defense responses induced by a still unknown microbial associated molecular pattern (MAMP). To determine whether the early peak of SA associated to the T3SS could be caused by the cellulolytic activity of GunA and the subsequent production of a possible DAMP, the expression of GmPR1 was measured at 8 hpi. In comparison to the wild-type strain, there was a complete absence of induction of *GmPR1* gene expression in plants inoculated with the *rhcJ* and *ttsI* mutants and a significant reduction due to the absence of GunA. Therefore, it could be possible that the plant recognizes the presence of HH103 Rif^R by the T3SS-dependent cellulase activity of GunA, which triggers a defense response that is then suppressed by a still unknown mechanism.

The absence of GunA caused differential symbiotic effects in soybean and cowpea. With soybean, a significant reduction in nodule number was observed. However, this reduction did not correlate with a reduction in shoot dry weight. By contrast, a significant increase in nodule fresh weight and shoot dry weight was observed in cowpea, indicating that GunA was detrimental in this symbiotic interaction. Differently, for the *B. diazoefficiens* USDA110 *gunA2* mutant, no symbiotic differences with soybean or cowpea were found (Caldelari Baumberger et al. 2003). Apart from GunA, cell-bound cellulases might influence symbiosis (Jimenéz-Zurdo et al. 1996). In *R. leguminosarum* bv. *trifolii*, the cell-bound cellulase CelC2 - which shares no similarity with GunA of HH103 Rif^R - is essential for the initial infection of clover (Robledo et al. 2015). This suggests very specific requirements for cellulase activity depending on the individual symbiont-host interaction. Results obtained in this work confirm that GunA

is a *S. fredii* HH103 Rif^{R} T3SS secreted protein. Type 3- secretion of most of the proteins predicted to be effectors (Pérez-Montaño et al. 2016; Vinardell et al. 2015) has been demonstrated (Jiménez-Guerrero et al. 2015a, 2017; López-Baena et al. 2009; Rodrigues et al. 2007), with the exception of NopT, whose secretion is still unknown. The cellulase activity of GunA would indicate a role of this protein in the degradation of cellulose of the plant cell wall at different stages of the symbiotic process: from early stages as indicated by the induction of *GmPR1* expression at 8 hpi to later stages, since the *gunA* gene is expressed in mature nodules. However, more efforts are necessary to determine the specific targets for GunA and the role of GunA in each stage of the infection process.

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593

Figure legends

Table X. Bacterial strains and plasmids.

Strain or plasmid	Relevant properties	Source or reference
Sinorhizobium fredii HH103		
HH103 Rif^R	Parental strain; Rif^R	**Madinabeitia *et al.*, 2002**
HH103 Rif^R *nodD1*Ω	HH103 Rif^R derivative containing the Ω interposon inserted into *nodD1*; Rif^R, Spc^R	**Vinardell *et al.*, 2004**
HH103 Rif^R *ttsI*Ω	HH103 Rif^R derivative containing the Ω interposon inserted into *ttsI*; Rif^R, Spc^R	**López-Baena *et al.*, 2009**
HH103 Rif^R *rhcJ*Ω	HH103 Rif^R derivative containing the Ω interposon inserted into *rhcJ*; Rif^R, Spc^R	**de Lyra *et al.*, 2006**
HH103 Rif^R Δ*gunA*	HH103 Rif^R derivative with a deletion of *gunA;* Rif^R	This work
HH103 Rif^R (pMUS1340)	HH103 Rif^R derivative with Δ*gunA* integrated in the genome; Rif^R, Km^R	This work
HH103 Rif^R (pMUS1384)	HH103 Rif^R derivative with plasmid pVO155-npt2-GFP integrated into *gunA*; Rif^R, Km^R, Spc^R	This work
HH103 Rif^R *ttsI*Ω (pMUS1384)	HH103 Rif^R *ttsI*Ω derivative with plasmid pVO155-npt2-GFP integrated into *gunA*; Rif^R, Km^R, Spc^R	This work
HH103 Rif^R (pMUS1367)	HH103 Rif^R with *gunA*::HA integrated in the genome; Rif^R, Km^R	This work
HH103 Rif^R *ttsI*Ω (pMUS1367)	HH103 Rif^R *ttsI*Ω with *gunA*::HA integrated in the genome; Rif^R, Km^R	This work
HH103 Rif^R *rhcJ*Ω (pMUS1367)	HH103 Rif^R *rhcJ*Ω with *gunA*::HA integrated in the genome; Rif^R, Km^R	This work
HH103 Rif^R (pMUS1338)	HH103 Rif^R with the *gunA-cya* fusion integrated in the genome; Rif^R, Km^R	This work

HH103 Rif^R *rhcJ*Ω (pMUS1338)	HH103 Rif^R *rhcJ* mutant with the *gunA-cya* fusion integrated in the genome, Rif^R, Km^R	This work
Escherichia coli		
DH5α	*supE44*, Δ*lacU169*, *hsdR17*, *recA1*, *endA1*, *gyrA96*, *thi-1*, *relA1*, Nx^R	**Sambrook *et al.*, 1989**
DB3.1	*F- gyrA462 endA1 glnV44 Δ(sr1-recA) mcrB mrr hsdS20(r_B^-, m_B^-) ara14 galK2 lacY1 proA2 rpsL20(Sm^r) xyl5 Δleu mtl1*; Nx^R	Invitrogen (USA)
Plasmids		
pK18*mob*	Suicide and narrow-broad-host vector; Km^R	**Schäfer *et al*., 1994**
pK18*mobsacB*	Suicide and narrow-broad-host vector; Km^R	**Schäfer *et al*., 1994**
pVO155-npt2-GFP	Plasmid for GusA- and GFP-tagged; Km^R, Spc^R	**Oke and Long, 1999**
pRK2013	Helper plasmid; Km^R	**Figurski and Helinski, 1979**
pDONR221	Entry vector in Gateway technology; Km^R	Invitrogen (USA)
pLMS150	Destination vector in Gateway technology. Gene-*cya* fusion; Tc^R	**Dr. Schechter**
pMUS1340	pK18*mobsacB* carrying a HH103 1-kb DNA fragment containing *gunA* deleted; Km^R	This work
pMUS1384	Plasmid pVO155-npt2-GFP carrying a *Sal*I/*Xba*I *gunA* fragment; Km^R, Spc^R	This work
pMUS1357	pK18*mob* carrying a HH103 1-kb DNA fragment for the expression of NopI fusioned to HA epitope; Km^R	This work
pMUS1332	pDONR221 carrying a HH103 0.8-kb DNA fragment containing *gunA*; Gm^R	This work
pMUS1337	pLMS150 carrying a 2-kb DNA fragment containing *gunA-cya* fusion; Tc^R	This work
pMUS1338	pK18*mob* carrying a 2-kb DNA fragment containing *gunA-cya* fusion; Km^R	This work

601 **Table X.** DNA oligonucleotide primers used in this study.

602

Name	Sequence	Usage
gunA-1	5'- ATTAAGCTTTGATTTTGGCCAAGTTGAAG -3´	*gunA* in-frame delection
gunA-2	5'- ACAGACCAGTTGTTTATCCGATTGGAAT -3´	
gunA-3	5'- ATTCCAATCGGATAAACAACTGGTCTGT -3´	
gunA-4	5'- ATAGGATCCTTGAAGTACGAGTCTTACGA -3´	
gunAint_SalI_F	5'- AAAGTCGACAAAGGATCCCCAACGGGGTA -3´	For expressing GusA and GFP tagged protein
gunAint_XbaI_R	5'- AAATCTAGATCCAGAGCATTATCTCATGC -3´	
gunA_EcoRI_F	5'- ATAGAATTCATGGATTCCAATCGGATA -3´	For expressing HA tagged protein
gunA_HA_KpnI_R	5'- ATAGGTACCAGCGTAATCTGGAACATCGTATGGGTACTTCGAGGTCACAGACCA -3´	
attB1_gunA	5'- GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGATTCCAATCGGATA -3´	Cloning in a Gateway vector
attB2nostop_gunA	5'- GGGGACCACTTTGTACAAGAAAGCTGGGTACTTCGAGGTCACAGACCA -3´	
gunA_EcoRI	5'- AAAGAATTCATGGATTCCAATCGGATA -3´	For expressing Cya tagged protein
cya_XbaI	5'- ATATCTAGATCAGCTGTCATAGCCGGAAT -3´	
gunAq_F	5'- GTGGCTTTCAATATCGGG -3´	*q*RT-PCR assays
gunAq_R	5'- CGGTGTAGTTTGTCCAGA -3´	
nodAq_F	5´- CGTCATGTATCCGGTGCTGCA -3´	*q*RT-PCR assays
nodAq_R	5´- CGTTGGCGGCAGGTTGAGA -3´	
16Sq_F	5´- TAAACCACATGCTCCACC -3´	*q*RT-PCR assays
16Sq_R	5´- GATACCCTGGTAGTCCAC -3´	
PR1_F	5´- AACTATGCTCCCCCTGGCAACTATATTG -3´	*q*RT-PCR assays

PR1_R	5´- TCTGAAGTGGTAGCTTCTACATCGAAACAA -3´	
UBI_F	5´- GTGTAATGTTGGATGTGTTCCC -3´	*q*RT-PCR assays
UBI_R	5´- ACACAATTGAGTTCAACACAAACCG -3´	

603

604

605 **Table X**. Plant responses to inoculation of *Vigna unguiculata* with different
606 *Sinorhizobium fredii* HH103 strains.
607

Inoculant	Number of nodules	Nodules fresh mass (g)	Plant-top dry mass (g)
None	0 ± 0 *	0 ± 0 *	0.74 ± 0.18 *
HH103 Rif^R	30.8 ± 6.9	0.55 ± 0.14	1.54 ± 0.65
HH103 Δ*gunA*	39.9 ± 4.4 **	0.99 ± 0.26 *	0.98 ± 0.32 *
HH103 Δ*gunA* (pMUS1340)	34.8 ± 8.4	0.73 ± 0.23	0.07 ± 0.05

608
609 Data represent averages of 6 jars that contained two cowpea plants. Determinations
610 were made 6 weeks after inoculation. For each legume tested, bacteria isolated from 20
611 nodules formed by each inoculant showed the expected resistance markers.
612
613 *S. fredii* HH103 mutants were individually compared to its parental strain HH103 Rif^R
614 by using the Mann-Whitney non-parametrical test. Numbers on the same column
615 followed by an asterisk are significantly different at the level α= 5%. Numbers on the
616 same column followed by two asterisk are significantly different at the level α= 10%.
617
618 **Table X**. Plant responses to inoculation of *Glycine max* cv. Williams with different
619 *Sinorhizobium fredii* HH103 strains.
620

Inoculant	Number of nodules	Nodules fresh mass (g)	Plant-top dry mass (g)
None	0 ± 0 *	0 ± 0 *	1.28 ± 0.27 *
HH103 Rif^R	72.5 ± 27.2	0.92 ± 0.24	1.04 ± 0.40
HH103 Δ*gunA*	47.4 ± 9.8 **	0.73 ± 0.21	1.15 ± 0.40

HH103 Δ*gunA* (pMUS1340)	79.2 ± 22.9	0.89 ± 0.30	0.16 ± 0.07

Data represent averages of 6 jars that contained two soybean plants. Determinations were made 6 weeks after inoculation. For each legume tested, bacteria isolated from 20 nodules formed by each inoculant showed the expected resistance markers.

S. fredii HH103 mutants were individually compared to its parental strain HH103 Rif^R by using the Mann-Whitney non-parametrical test. Numbers on the same column followed by an asterisk are significantly different at the level α= 5%. Numbers on the same column followed by two asterisk are significantly different at the level α= 10%.